3
Enzyme Assays

In chapter 2 the fundamental requirements for enzyme assays have been described while in the current chapter protocols for various enzyme assays and general methods for dealing with enzymes – such as protein assays and concentration methods – are presented. The protocols can be treated like cookery recipes and reproduced exactly step by step, but it is understood that the rules discussed earlier should be kept in mind, because any description can mediate only a general pattern, and according to the particular system, modifications are often required. The discussed rules should enable the reader to independently develop an assay; for example, for a newly isolated enzyme. Conditions essential for any enzyme assay are summarized in Box 3.1 and in Box 3.2 general hints for concentration ranges of the assay components are given. Table 3.1 lists stock solutions frequently needed for enzyme assays, while buffers are listed in Table 2.2 and their preparation is described in Boxes 2.8 and 2.9.

3.1
Enzyme Nomenclature

The following enzyme assays are described in the order of the respective EC numbers. The significance of these numbers will be explained in this section. When first investigating enzymes, the discoverers named the new enzymes and many of these historical names are still in usage, such as *diaphorase*, *Zwischenferment*, and *old yellow enzyme;* however, in the course of time, many have lost their meaning. With an increasing number of enzymes being discovered, the need for a clear system of nomenclature became urgent. In 1956 the *International Union of Biochemistry* (IUB) established an *International Commission of Enzymes* under the leadership of Prof. M. Florkin; the commission prepared a classification and nomenclature system for all known enzymes. In 1961 the commission was substituted by a *Standing Committee on Enzymes* and from 1969, by an *Expert Committee on Enzymes*, which consistently updates the nomenclature and publishes supplements (see literature below). The present enzyme list contains over 3000 entries. Each enzyme is designated by a four-figure code and a systematic enzyme name.

Practical Enzymology, Second Edition. Hans Bisswanger.

Box 3.1: Essential Conditions for Enzyme Assays

Condition	Recommended range[a]
Solvent	Polar (aqueous)
Temperature	25–37 °C
pH	6.5–8.5
Ionic strength	0.05–0.2 M
Enzyme amount	As low as possible
Substrate amount	Saturating (~50-fold K_m)
Cofactors	Saturating (~50-fold K_d)

[a] For most assays, distinct enzymes require deviating conditions.

Box 3.2: Dependence of Enzyme Reaction on Concentrations of Assay Components

Component	Mode of dependency
Enzyme	Strictly linear, no saturation
Specific binding components, directly involved in the reaction (substrates, products, cofactors, essential metal ions)	Hyperbolic saturation behavior according to Michaelis–Menten equation (Eq. (2.13))
Nonspecific components, not directly involved in the reaction (ions, buffer, stabilizing and protecting substances) (cf. Table 2.3)	Weak influences, no saturating effects

The **systematic name** (SN) should characterize the reaction catalyzed. At first, the direction of the reaction must be defined. For a distinct class of enzymes the same direction is taken for all reactions, even for cases where this is not the physiological direction. The systematic name consists of two parts, the first denotes the substrate(s), separated by a colon (:), while the second describes the nature of the reaction, ending with *–ase;* for example, alcohol:NAD^+ oxidoreductase. The systematic names of oxidoreductases follow the general pattern donor:acceptor oxidoreductase.

It turned out that the systematic names are too circumstantial for practical usage and therefore, have been simplified into **recommended names** (RNs), which are also specified in the enzyme list. For instance, the recommended name for alcohol:NAD^+ oxidoreductase is alcohol dehydrogenase. It implies an oxidation of the hydroxyl group of the alcohol, but unlike the systematic name, gives no information about the acceptor. For the recommended names

Table 3.1 Frequently used solutions for enzyme assays. For the given substances, the most common forms are indicated but other forms are also available, for example with (or without) crystal water, as free acid or with different counterions.

Solution	Molecular mass (M_r)	Concentration (M)	Preparation	Remarks
NaOH, sodium hydroxide, caustic soda	40.0	1	4.0 g, resolve and adjust to 100 ml with H_2O	Etching! Vigorous warming up upon resolution Store in PE flasks
KOH, potassium hydroxide, potash lye	56.1	1	5.61 g, resolve and adjust to 100 ml with H_2O	Etching! Store in PE flasks
HCl, hydrochloric acid	34.5	1	82.8 ml concentrated HCl (37%, density 1.18) fill up with H_2O to 1 l	Etching liquid and vapors
$MgCl_2 \cdot 6H_2O$	203.3	0.1	203 mg in 10 ml	–
TCA, trichloroacetic acid, $C_2HCl_3O_2$	163.4	3	49 g, adjust to 100 ml with H_2O	Protein precipitating reagent for protein determination (biuret) and stopped enzyme tests
ADP, disodium salt	471.2	0.1	471 mg, adjust to 10 ml with H_2O	Keep frozen
ATP, disodium salt, trihydrate	605.2	0.1	605 mg, adjust to 10 ml with H_2O	Keep frozen
NAD^+, free acid	663.4	0.1	663 mg, adjust to 10 ml with H_2O	Keep frozen
NADH, disodium salt	709.4	0.01	71 mg, adjust to 10 ml with H_2O	Unstable, even in frozen state
$NADP^+$, sodium salt	765.4	0.1	765 mg, adjust to 10 ml with H_2O	Keep frozen
NADPH, tetrasodium salt	833.4	0.01	83 mg, adjust to 10 ml with H_2O	Unstable, even in frozen state
DTE, dithioerythritol, Cleland's reagent	154.2	0.1	154 mg, adjust to 10 ml with H_2O	Similar to DTT, dithiothreitol, both can be used alternatively

the term oxygenase is used if molecular oxygen is incorporated: monooxygenases incorporating one atom and dioxygenases both atoms of O_2. D and L, applied to define asymmetric C-atoms, e.g. in D-sugars and L-amino acids, are ignored as long as no ambiguity exists, otherwise they must be mentioned as in L-lactate dehydrogenase and D-lactate dehydrogenase. Anionic substrates should end with *–ate* (mal*ate* dehydrogenase) instead of *-ic* (malic enzyme). Directly annexing *–ase* to the substrate name indicates its hydrolysis (lactase). Recommended names should generally end in *–ase*, with the exception of some proteolytic enzymes where the ending *-in* (trypsin) is retained. In contrast to systematic names recommended names usually refer to the direction of the reaction that has been demonstrated. Fantasy names (reparase, caspase) should not be used.

If an enzyme catalyzes more than one reaction, for example if it is composed of various subunits performing successive partial reactions, it should be denoted as a *system*, but often a *complex* is preferred for such functional units (e.g., pyruvate dehydrogenase system (complex), fatty acid synthase system (complex)). Since enzymes are classified on the basis of the chemical reaction they catalyze, enzymes catalyzing the same reaction, for example those from different organisms or isoenzymes, are classified by one systematic name and one code number; but some mostly historically founded exceptions exist, such as acid and alkaline phosphatase or cholinesterase and acetylcholinesterase. Enzymes not performing a chemical reaction are not classified in the enzyme list.

The **code numbers** for enzyme classification (**EC numbers** from **E**nzyme **C**ommission) contain four figures, separated by points. The first figure indicates the number of one of the six main enzyme classes to which the particular enzyme belongs:

1) Oxidoreductases
2) Transferases
3) Hydrolases
4) Lyases
5) Isomerases
6) Ligases (synthetases)

The following two figures indicate subclasses and sub-subclasses, respectively. The fourth figure is the serial number of the individual enzyme within its sub-subclass. An excerpt of the enzyme list with the main, the sub, and the sub-subclasses including some characteristic examples of individual enzymes is presented at the end of the book.

References

Enzyme Nomenclature (1976) *Biochim. Biophys. Acta*, **429**, 1–45.
Webb, E.C. (ed.) (1992) *Enzyme Nomenclature*, Academic Press Inc., San Diego.
Schomburg, D. and Salzmann, M. (1990) *Enzyme Handbook*, Springer-Verlag, Berlin.

3.2 Practical Considerations for Enzyme Assays

The assay protocols are ordered according to the EC numbers and start with the recommended name and the EC number of the respective enzyme as headings, followed by the systematic name (SN), abbreviation, and common (trivial) names (if in use), and the reaction formula. A short description of enzyme features follows, especially of those that are of some significance for the assay, such as cofactors, molecular mass, Michaelis constants, and pH optimum. This information is given only for orientation. Enzymes of the same type but from different organisms often differ remarkably in their features and it is not the intention of this chapter to collect all the known data. For instance, Michaelis constants reported in separate publications, even for enzymes from the same organism, can differ by more than a factor of 10. Actually, it is not possible to determine such constants with too high an accuracy, so very precise values (e.g., 2.163 mM) must be regarded with caution. From the variety of organisms, mammalian (human) enzymes are preferentially considered; in some cases thoroughly investigated examples, for example from bacteria, are also considered.

It was not possible to consider all enzyme assays described so far. But from comparing distinct enzyme assays, general principles can be observed. For instance, various hydrolyzing enzymes are tested with substrates connected with a chromophoric residue, like the nitrophenyl or umbelliferyl groups. Upon cleavage a strongly absorbing or fluorescent compound is released, which can be detected by the appropriate method. Various assays of such types are described here, but combined substrates of related enzymes are also available and can be tested by adapting the assay conditions.

Originally, enzymes were isolated directly from the respective organisms by various purification procedures, but this approach became increasingly displaced by the advent of **recombinant enzymes**. With the methods of gene technology, the respective DNA region from any organism can be cloned and expressed, for example in a bacterial host with an *N*-terminal polyhistidine tag, consisting of six successive histidine residues (*6xHis-tag*). This tail enables easy purification using a nickel affinity column (Ni-Separose®, Ni-NTA-Agarose®) and specific elution with a 20 mM imidazole solution. The bound polyhistidine tag can change the features of the enzyme, but if this is not too extensive, the enzyme may be used in this form. Otherwise the polyhistidine tag can be removed with exoproteases. A further advantage of recombinant enzymes is the application of side-directed mutagenesis and the expression of mutant enzymes with various modifications.

A list of all required **assay solutions** with instructions for their preparation is given (for the preparation of buffers see Boxes 2.8 and 2.9). The respective substances are often available in various forms, as free acid or as salt with differing counterions, anhydrous or hydrated. Usually the most common form is indicated together with its molecular mass (M_r) and the amount needed (in grams) for preparation of the solution. For routine assays larger quantities may be prepared and stored, either in the cold, or for sensitive substances such as

substrates and cofactors, in the frozen state (~ −20 °C or less). Usually solid substances are dissolved in water (H_2O, principally understood as *distilled* or *deionized*) or buffer (especially for acid or alkaline substances). Enzymes must always be dissolved or diluted in buffer, the pH set according to their pH optimum. Water-insoluble substances must be dissolved in an appropriate solvent, for example dimethylsulfoxide (DMSO), acetone, ethanol or methanol. A small volume of such a solution is diluted with the aqueous assay mixture assuming that the substance may remain dissolved, but this condition must be controlled.

Each assay can be prepared separately by mixing the respective aliquots of the assay solutions directly in a test tube or a cuvette, but such a procedure is prone to errors and hence, more asssays may need to be performed. It is therefore recommended that one **assay mixture** be prepared to be used for all assays. This saves time, reduces errors, and establishes equal composition for all assays. In the following descriptions, the quantity is calculated for 10 assays, more or less can be prepared accordingly. The assay solutions should contain all required components, with the exception of one essential component with which the assay will be started. This is usually an enzyme; therefore, it is omitted from the assay mixture. However, if there is reason, one may start with another component, the substrate or a cofactor. In this case, the enzyme may be added directly to the assay mixture instead of the particular substance. It must be remembered that components of the assay mixture should not influence one another (e.g., by forming precipitates or inducing oxidative processes). This holds true especially for enzymes. As already discussed, they are not very stable in diluted solution, but on the other hand, cofactors and other additions of the assay mixture can act as stabilizing influences. Substrates or cofactors can induce slow activation processes with special enzymes (*hysteretic enzymes*) and preincubation with the respective components is required in such cases. However, conversion of the substrate by the enzyme must strictly be avoided (e.g., by omitting another essential component). The assay mixture is prepared only for the actual test series and should not be stored for a long time. It must be kept on ice and be warmed up before starting the assay.

For the assay **procedure**, the respective quantity of the assay mixture for a single assay is filled in a test tube or cuvette and the reaction is started by addition of the missing component from the assay mixture, usually the enzyme. The final assay volume depends on the respective detection method; here it is generally estimated for 1 ml. Upon addition, the progression of the reaction must be observed immediately. It is essential that it occurs within the observable range, that is not too slow and not too fast. This can be regulated by the amount of enzyme as discussed in the previous sections (2.2.3ff, Box 2.3). Because of the varying activities of enzyme preparations, suggestions for the quantity of enzyme are given only in exceptional cases. Usually 20 µl of diluted enzyme solution is considered and the appropriate activity should be tested out in preliminary assays. The suitable amount can be obtained by changing the dilution factor and/or the added volume. In the latter case, the buffer quantity must be modified to achieve the final assay volume (1 ml). An absorption change of 0.1 in 1 min is favorable for photometric assays (cf. Boxes 2.18 and 2.19).

Any enzyme assay requires a **blank** or reference, the type of blank depends on the type of assay. Stopped assays refer to the blank as zero value and it is obvious that any deviation of the blank will distort all data. Therefore, the blank must be measured with high accuracy and during longer series, repeated determinations of the blank should be carried out. Usually the assay mixture can be taken as blank, the starting component (enzyme sample) replaced by the buffer. It must, however, be made sure, that the starting component does not change the features of the assay mixture; for example, by contributing a distinct absorption in photometric assays. Otherwise, this contribution must be considered or a more indifferent component (e.g., substrate) is used as the starting component. It is also obvious that the blank must be stable, neither a spontaneous reaction nor an enzyme reaction dare occur. Usually, the photometer is adjusted to zero with the blank. This is convenient, because the sample values will be obtained directly, already reduced by the blank value. However, this is not really necessary and it is often advantageous to adjust the photometer to zero without blank and to afterwards measure the absorption, both of the blank and the samples. This appears circumstantial, because the blank must be subtracted from the sample values. However, examination of the blank absorption is a valuable control. Unusual deviations can be detected and if necessary, a new blank can be prepared for correction of the samples, while all sample values are of no use if the instrument was adjusted to zero with a wrong blank value. A further control for the blank is the fact that limiting values of a measured series must tend to zero (respectively the blank value); that is, a calibration curve must extrapolate to zero absorption for zero concentration of the component determined.

For continuous assays such as the progress curve of an enzyme reaction, a blank is not really necessary because the velocity is taken from the steepness of the curve, an absolute value does not have to be determined. Nevertheless, absorption of the assay mixture before starting the reaction is a control for correct composition. But for time-dependent measurements, it must be made sure that the assay solution shows no time-dependent change in the absence of the enzyme reaction. Sometimes spontaneous drifts are observed; for example, due to instability of a component or oxidative processes. If such effects cannot be suppressed, the drift must be subtracted from the velocity of the sample.

The **calculation** of enzyme activity is generally described in Section 2.2.3 and in Boxes 2.18 and 2.19: in special cases it is indicated at the end of the assay procedure.

The knowledge of hazardousness of chemicals is presupposed. Hints are given in special cases, but the reader is generally referred to the security rules indicated in the relevant literature; instructions provided together with the chemicals by the respective companies are recommended. All data and instructions presented in the following protocols have been presented carefully, but due to the large amount of data complete guarantee cannot be given.

Reference

Hengen, P. (1995) *Trends Biochem. Sci.*, **20**, 285–286.

3.3 Special Enzyme Assays

3.3.1 Oxidoreductases, EC 1

3.3.1.1 Optical Assay

NAD(P)-dependent dehydrogenases (oxidoreductases) belong to one of the most frequently tested enzyme classes, therefore some general aspects are mentioned beforehand. These enzymes differ in their substrate specificity; for example, for lactate or malate, but all share a similar cosubstrate, only differing in a phosphate group (NAD^+ or $NADP^+$). Because both forms possess the same spectroscopic feature of an additional absorption band at 340 nm appearing only in the reduced state, a general assay – the *optical assay* based on this feature – can be performed for all NAD(P)-dependent dehydrogenases. Not only the substrate must be adapted to the respective enzyme specificity, but also other features such as affinity (K_m), pH optimum, temperature behavior, and in particular, the state of equilibrium must be considered. The latter aspect determines whether the reaction proceeds in the reductive (absorption increase) or the oxidative (absorption decrease) direction. The assay can be carried out in any conventional UV or visible photometer and in a filter photometer; the respective wavelengths are indicated in Figure 2.27. As has been discussed earlier, it is not really necessary to measure just at the maximum wavelength; the absorption at 339 nm is nearly the same as that at 340 nm, but is more stable against temperature fluctuations.

The state of the equilibrium is not the only decisive factor for the direction of the assay reaction. A further aspect is the availability of the respective substrate and the fact that the absorption is considerably high if started from the reductive site; for example, an absorption of 0.63 for 0.1 mM NADH. Usually, enzyme assays should be performed at saturation concentrations of all substrates, practically at least 10-fold K_m. This cannot always be realized under such conditions. For the alcohol dehydrogenase reaction, the unfavored reductive reaction is enforced by trapping the products (acetaldehyde and H^+) with a chemical reagent and an alkaline pH (cf. Section 3.3.1.3).

Reference

Ziegenhorn, J., Senn, M., and Bücher, T. (1976) *Clin. Chem.*, **22**, 151–160.

3.3.1.2 Fluorimetric Assay

Fluorimetric assays have the advantage of high sensitivity and are recommended for detection of low activity levels. However, the method requires special equipment (fluorimeter), high accuracy, and purity of the solutions. Only the reduced form of NAD(P), when excited at 260 nm (or 340 nm), emits light at 470 nm with about 2% quantum yield and the fluorescence increase at this wavelength is a signal for the enzyme reaction. The fluorimetric assay is principally applicable to all

dehydrogenase reactions forming or oxidizing NAD(P)H. The same solutions and concentrations described for the respective photometric test can be used, but very high concentrations of NAD(P)H cause quenching effects, so that the saturation range is not completely accessible. Therefore, the direction of reduction is preferred for this assay.

3.3.1.3 Alcohol Dehydrogenase, EC 1.1.1.1

SN: alcohol:NAD oxidoreductase. aldehyde reductase, ADH

$$\text{aldehyde} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{alcohol} + \text{NAD}^+$$

The reaction is reversible and can be tested from either side. The equilibrium constant for the reaction with acetaldehyde is 8×10^{-12} M and favors the formation of ethanol. Alcohol dehydrogenase, a Zn enzyme, exists in different forms depending on the source. The commonly used ADHs are from yeast or horse liver, they differ in structure and specificity. ADH from yeast ($M_r = 148\,000$), homotetramer, is most active with ethanol. K_m (mM): 16.7 (ethanol), 2.8 (acetaldehyde), 0.12 (NAD), 0.06 (NADH), pH optimum: 8.3. Mammalian ADH ($M_r = 79\,000$), a homodimer, has a preference for higher alcohols. K_m (mM): 7.0 (ethanol), 3.4 (acetaldehyde), 0.25 (NAD), 0.01 (NADH), pH optimum: 7.5. Inhibitors: 1,10-phenanthroline (competitive with NAD, Zn-complexing agent); fluoroethanol ($K_i = 2.5$ mM), inhibitor of mammalian ADH, competitive with ethanol. ADH is not stable in dilute solution, addition of BSA and a thiol reagent (DTE, DTT) is recommended for stabilization.

A. Reduction Assay The reaction is tested in the physiological direction:

$$\text{aldehyde} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{ethanol} + \text{NAD}^+$$

A disadvantage is the volatility and toxicity of acetaldehyde.

Assay solutions

0.1 M potassium phosphate pH 7.5

BSA solution: 0.1 g BSA in 100 ml 0.1 M potassium phosphate pH 7.5

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg 10 ml^{-1} H_2O)

0.5 M acetaldehyde ($M_r = 44.0$, $d = 0.78$ g ml^{-1}; 2.2 g (2.82 ml) in 100 ml H_2O)

ADH diluted in BSA solution: commercially available preparations must be diluted according to their activity: 0.02 IU ADH convert 0.02 µmol NADH/min, corresponding to an absorption difference of 0.126; from a commercial ADH with 400 IU mg^{-1} prepare a stock solution of 10 mg ml^{-1} (~4000 IU ml^{-1}) in 0.1 M potassium phosphate pH 7.5. A 4000-fold dilution (2.5 µl in 10 ml BSA solution) corresponds to 1 IU ml^{-1}, 20 µl for the enzyme assay ~0.02 IU

Assay mixture

Components	*Concentration (mM)*
9.5 ml 0.1 M potassium phosphate pH 7.5	95
0.1 ml 10 mM NADH	0.1
0.2 ml 0.1 M acetaldehyde	2.0

Procedure

0.98 ml assay mixture

0.02 ml ADH (0.02 IU)

The absorption decrease at 340 nm is measured at 25 °C, absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3\,\mathrm{l\,mol^{-1}\,cm^{-1}}$.

B. Oxidation Assay To test the unfavored back reaction

$$\text{ethanol} + \text{NAD}^+ \rightleftharpoons \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

the products must be removed from equilibrium: acetaldehyde by semicarbazide and the protons by alkaline pH.

Assay solutions

75 mM glycine/sodium diphosphate pH 9.0 (10 g $Na_4P_2O_7 \cdot 10H_2O$ + 0.5 g glycine in 300 ml H_2O, adjust to pH 9.0 with 1 N HCl)

0.1 M potassium phosphate, pH 7.5

Ethanol p.A. ($M_r = 46.1, d = 0.81\,\mathrm{g\,ml^{-1}}$)

0.1 M NAD ($M_r = 663.4$; 0.663 g in 10 ml 0.1 M potassium phosphate pH 7.5)

0.1 M 1,4-dithioerythritol (DTE, $M_r = 154.3$; 154 mg in 10 ml 0.1 M potassium phosphate pH 7.5)

BSA solution: 0.1 g BSA in 100 ml 0.1 M potassium phosphate, pH 7.5

2.2 M semicarbazide (semicarbazide·HCl, $M_r = 111.5$; 2.5 g in 10 ml 2 N NAOH, adjust to pH 6.3–6.5 with 5 N NaOH)

ADH, for dilution see above

Assay mixture

Components	*Concentration (mM)*
8.8 ml 75 mM glycine/sodium diphosphate pH 9.0	66 mM
0.3 ml ethanol	0.53 M
0.2 ml 0.1 M NAD	2.0 mM
0.2 ml 0.1 M DTE	2.0 mM
0.3 ml 2.2 M semicarbazide	66 mM

Procedure

0.98 ml assay mixture

0.02 ml diluted ADH

The increase in the absorption at 340 nm is measured at 25 °C. The absorption coefficient for NADH is $\varepsilon_{340} = 6.3 \times 10^3\ \mathrm{l\,mol^{-1}\ cm^{-1}}$.

References

Andersson, L. and Mosbach, K. (1982) *Meth. Enzymol.*, **89**, 435–445.
Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, 3rd edn, vol. **2**, Verlag Chemie, Weinheim, pp. 139–141.
Dafeldecker, W.P., Meadow, P.E., Pares, X., and Vallee, B.L. (1981) *Biochemistry*, **20**, 6729–6734.
Pietruszko, R. (1982) *Meth. Enzymol.*, **89**, 429–435.

3.3.1.4 Alcohol Dehydrogenase (NADP$^+$), EC 1.1.1.2

SN: alcohol:NADP$^+$ oxidoreductase, aldehyde reductase (NADPH)

$$\text{aldehyde} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{alcohol} + \text{NADP}^+$$

From different sources, mammalian enzyme $M_r = 74\,000$, dimeric, K_m (acetaldehyde) 0.25 mM; identical with glucuronate dehydrogenase, EC 1.1.1.19, mevaldate reductase EC 1.1.1.33, lactaldehyde reductase, EC 1.1.1.55; can be tested with alcohol dehydrogenase assays, EC 1.1.1.1 (Section 3.3.1.3), replacing NAD (respectively NADH) by NADP (or NADPH).

References

Das, B. and Srivastava, S.K. (1985) *Biochim. Biophys. Acta*, **840**, 324–333.
Turner, A.J. and Hryszko, J. (1980) *Biochim. Biophys. Acta*, **613**, 256–265.
Wartburg, V.J.-P. and Wermuth, B. (1982) *Meth. Enzymol.*, **89**, 506–513.

3.3.1.5 Homoserine Dehydrogenase, EC 1.1.1.3

SN: homoserine:NAD(P)$^+$ oxidoreductase, aspartate kinase–homoserine dehydrogenase I, AK–HDH I

$$\text{L-homoserine} + \text{NAD(P)}^+ \rightleftharpoons \text{L-aspartate-4-semialdehyde} + \text{NAD(P)H} + \text{H}^+$$

Multifunctional allosteric enzyme (*Escherichia coli*), fused with aspartate kinase (cf. assay in Section 3.3.2.11), M_r: 360 000, homotetramer. ($4 \times 84\,000$), K_m (mM): 0.013 (homoserine), 0.17 (L-aspartate-4-semialdehyde), 0.073 (NADP), 0.09 (NADPH).

Assay solutions

0.1 M CHES/HCl, 0.4 M KCl pH 9.0 (2-(*N*-cyclohexylamino)ethanesulfonic acid, $M_r = 207.3$, 20.7 g, KCl, $M_r = 74.6$, 29.8 g, dissolve in 600 ml, adjust to pH 9.0 with 1 M HCl, fill up to 1 l)

0.25 M L-homoserine ($M_r = 119.1$, 298 mg in 10 ml H_2O)

0.1 M NADP (disodium salt, $M_r = 787.4$; 787 mg in 10 ml)

0.1 M DTT (dithiotreitol, $M_r = 154.2$, 154 mg in 10 ml H_2O)

Assay mixture

Components	*Concentration (mM)*
9.26 ml 0.1 M CHES/HCl, 0.4 M KCl pH 9.0	93/370
0.5 ml 0.25 M L-homoserine	12.5
0.03 ml 0.1 M NADP	0.3
0.01 ml 0.1 M DTT	0.1

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption increase at 340 nm is measured at 30 °C, absorption coefficient for NADPH: $\varepsilon_{340} = 6.3 \times 10^3\ \text{l mol}^{-1}\ \text{cm}^{-1}$.

References

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Wedler, F.C. and Ley, B.W. (1993) *J. Biol. Chem.*, **268**, 4880–4888.

3.3.1.6 Shikimate Dehydrogenase, EC 1.1.1.25

SN: shikimate:$NADP^+$ 3-oxidoreductase

$$3\text{-dehydroshikimate} + NADPH + H^+ \rightleftharpoons \text{shikimate} + NADP^+$$

This enzyme catalyzes a step of the biosynthetic pathway of aromatic amino acids. For the assay, the reverse reaction is observed. In *Escherichia coli* the enzyme exists as a monomer ($M_r = 29\,400$), in other organisms as a dimer. K_m : 1.25×10^{-5} M (NAD), pH optimum 8.5.

Assay solutions

0.1 M sodium carbonate pH 10.6 (Na_2CO_3, $M_r = 106$; 10.6 g in 1 l, adjust to pH 10.6 with a 0.1 M (8.4 g l^{-1}) $NaHCO_3$ solution)

0.1 M shikimic acid ($M_r = 174.2$; 174 mg in 10 ml)

0.1 M NADP (disodium salt, $M_r = 787.4$; 787 mg in 10 ml)

Assay mixture

Components	*Concentration (mM)*
9.2 ml 0.1 M sodium carbonate pH 10.6	92
0.4 ml 0.1 M shikimic acid	4
0.2 ml 0.1 mM NADP	2

Procedure

0.98 ml assay mixture

0.02 ml diluted enzyme

The increase in the absorption at 340 nm is measured at 25 °C. The absorption coefficient for NADPH is $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

Chaudhuri, S. and Coggins, J.R. (1985) *Biochem. J.*, **226**, 217–223.
Coggins, J.R., Bookock, M.R., Chaudhuri, S., Lambert, J.M., Lumsden, J., Nimmo, G.A., and Smith, D.D.S. (1987) *Meth. Enzymol.*, **143**, 325–341.
Lumsden, J. and Coggins, J.R. (1977) *Biochem. J.*, **161**, 599–607.

3.3.1.7 L-Lactate Dehydrogenase, EC 1.1.1.27

SN: *S*-lactate:NAD^+ oxidoreductate, L-lactic dehydrogenase, LDH

$$\text{L-lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

The reaction can be tested in both directions, but the equilibrium favors the reduction of pyruvate, although high absorption of NADH must be taken into account. For routine assays, LDH from pig heart is suitable. In mammals (bovine, pig, rabbit) a homotetramer, $M_r = 140\,000$, two isoenzymes, M_4 type prevailing in anaerobic tissue (skeleton muscle), H_4 type predominant in aerobic tissues (heart muscle, liver, kidney); hybrids composed of subunits from both types (e.g., H_3M) exist depending upon the oxygen supply. LDH from bacteria is specific for NAD, but oxidizes besides *S*-lactate other *S*-2-hydroxymonocarboxylic acids; mammalian LDH accepts NADP also, but with reduced efficiency. K_m (mM, pig, mammalian): 6.7 (lactate), 0.16 (pyruvate, strong substrate inhibition at pyruvate concentrations > 0.2 mM), 0.25 (NAD), 0.011 (NADH); inhibitors: oxalate ($K_i = 0.2$ mM), competitive with lactate, noncompetitive with pyruvate, malonate, tartrate; pH optimum 7.0.

A. Spectrophotometric Reduction Assay

$$\text{pyruvate} + NADH + H^+ \rightleftharpoons \text{L-lactate} + NAD^+$$

Assay solutions

0.1 M potassium phosphate pH 7.0

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg 10 ml^{-1} H_2O)

0.1 M pyruvate (sodium salt, $M_r = 110$; 110 mg in 10 ml H_2O)

LDH solution (e.g., 10 mg ml^{-1}, 550 U ml^{-1}; dilute to 1 IU ml^{-1} with 0.1 M potassium phosphate pH 7.0)

Assay mixture

Components	*Concentration (mM)*
9.4 ml 0.1 M potassium phosphate pH 7.0	94
0.2 ml 0.01 M NADH	0.2
0.2 ml 0.1 M pyruvate	2.0

Procedure

0.98 ml assay mixture

0.02 ml diluted LDH

The absorption decrease at 340 nm is measured at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3\ \mathrm{l\,mol^{-1}\,cm^{-1}}$.

B. Fluorimetric Reduction Assay The fluorimetric assay for LDH is described representatively for all reactions where NAD(P)H is formed from or converted to NAD(P). The fluorimetric assay is about 100-fold more sensitive than the spectrophotometric assay, depending on the sensitivity of the used fluorimeter.

Assay solutions

0.1 M potassium phosphate pH 7.0

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg 10 ml^{-1} H_2O)

0.1 M pyruvate (sodium salt, $M_r = 110$; 110 mg in 10 ml H_2O)

LDH solution (e.g., 10 mg ml^{-1}, 550 U ml^{-1}; dilute to 1 IU ml^{-1} with 0.1 M potassium phosphate pH 7.0)

Assay mixture

Components	*Concentration (mM)*
19.35 ml 0.1 M potassium phosphate pH 7.0	97
0.05 ml 0.01 M NADH	0.025
0.4 ml 0.1 M pyruvate	2.0

Procedure

1.98 ml assay mixture

0.02 ml LDH dilution

The relative fluorescence intensity per minute, excited at 260 nm and emitted at 470 nm ($\Delta\mathrm{Fl} \cdot \mathrm{min}^{-1}$) and 25 °C is determined. A fluorescence standard curve with NADH in the respective concentration range is performed for quantification.

Calculation

Volume activity

$$\mathrm{IU\ ml^{-1}} = \frac{\Delta\mathrm{Fl\,min^{-1}} \cdot \text{proportionality factor} \cdot 2 \cdot \text{dilution factor}}{0.02}$$

Specific activity

$$\text{IU mg}^{-1} = \frac{\Delta\text{Fl min}^{-1} \cdot \text{proportionality factor} \cdot 2 \cdot \text{dilution factor}}{0.02 \text{ mg protein/ml}}$$

C. Oxidation Assay

Assay solutions

0.1 M potassium phosphate pH 7.6

0.1 M NAD ($M_r = 663.4$; 0.663 g in 10 ml 0.1 M potassium phosphate pH 7.5)

0.5 M L-lactate (lithium salt, $M_r = 96$; 480 mg in 10 ml 0.1 M potassium phosphate pH 7.6)

LDH solution (e.g., 10 mg ml^{-1}, 550 IU ml^{-1}; dilute to 1 IU ml^{-1} with 0.1 M potassium phosphate pH 7.0)

Assay mixture

Components	*Concentration (mM)*
8.2 ml 0.1 M potassium phosphate pH 7.6	82
0.2 ml 0.1 M NAD	2.0
1.4 ml 0.5 M sodium lactate	70

Procedure

0.98 ml assay mixture

0.02 ml diluted LDH

The absorption increase at 340 nm is measured at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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Lee, C.Y., Yuan, J.H., and Goldberg, E. (1982) *Meth. Enzymol.*, **89**, 351–358.

3.3.1.8 Malate Dehydrogenase, EC 1.1.1.37

SN: (*S*)-malate:NAD oxidoreductase, malic dehydrogenase, MDH

$$(S)\text{-malate} + NAD^+ \rightleftharpoons \text{oxalacetate} + NADH + H^+$$

Mammalian enzyme (pig heart, cytosol) M_r 67 000, homodimer (2 × 33 000), K_m (mM): 0.14 (NAD), 0.021 (NADH), 0.0083 (oxalacetate); bacterial enzyme (*Escherichia coli*): homotetramer (4 × 33 500), K_m (mM): 2.5 (L-malate), 0.26 (NAD), 0.061 (NADH), 0.026 (oxalacetate), pH optimum 7.5.

For the assay the back reaction is preferred.

Assay solutions

0.1 M potassium phosphate pH 7.5

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg 10 ml^{-1} H_2O)

0.1 M oxaloacetic acid ($M_r = 132.1$; 132 mg in 10 ml 0.1 M potassium phosphate pH 7.5)

MDH solution (dilute to 1 IU ml^{-1} with 0.1 M potassium phosphate pH 7.5)

Assay mixture

Components	*Concentration (mM)*
9.1 ml 0.1 M potassium phosphate pH 7.5	91
0.2 ml 0.01 M NADH	0.2
0.5 ml 0.1 M oxaloacetic acid	5.0

Procedure

0.98 ml assay mixture

0.02 ml diluted MDH

The absorption decrease at 340 nm is measured at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

Banaszak, L.J. and Bradshaw, R.A. (1975) in *The Enzymes*, 3rd edn, vol. 12 (ed. P.D. Boyer), Academic Press, New York, pp. 369–396.
Lin, J.J. *et al.* (2002) *J. Mol. Evol.*, **54**, 107–117.
Muslin, E.H. (1995) *Biophys. J.*, **68**, 2218–2223.
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Yin, Y. and Kirsch, J.F. (2007) *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 17353–17357.

3.3.1.9 Malate Dehydrogenase (Oxaloacetate-Decarboxylating) (NAD^+), EC 1.1.1.38, and Malate Dehydrogenase (Decarboxylating), EC 1.1.1.39

SN: (*S*)-malate:NAD^+ oxidoreductase (oxalacetate-decarboxylating), NAD malic enzyme, ME.

$$(S)\text{-malate} + NAD^+ \rightleftharpoons CO_2 + \text{pyruvate} + NADH + H^+$$

Enzyme from *Escherichia coli*, $M_r = 260\,000$, tetramer ($4 \times 65\,000$); K_m (mM): 0.046 (NAD), 0.025 (NADH), 0.19 (L-malate), 2.1 (oxalacetate), pH optimum 7.2; decarboxylates also oxalacetate, in contrast to malate dehydrogenase (decarboxylating) EC 1.1.1.39, SN: (*S*)-malate:NAD^+ oxidoreductase (decarboxylating), mitochondrial enzyme (*Arabidopsis thaliana*): M_r 120 000, homodimer ($2 \times 58\,000$), K_m (mM): 3.0 (L-malate), 0.5 (NAD), pH optimum 6.6.

Assay solutions

50 mM Tris/HCl, 20 mM imidazole/HCl pH 6.4 (tris(hydroxymethyl)aminomethane, $M_r = 121.1$; 6.06 g, dissolve in 600 ml H_2O together with 1.36 g imidazole ($M_r = 68.1$), adjust to pH 6.4 with 1 M HCl and bring to 1 l)

0.2 M malate solution (L-malic acid, disodium salt, $M_r = 178.1$; 356 mg in 10 ml H_2O)

0.1 M NAD ($M_r = 663.4$; 3.31 g in 50 ml Tris/imidazole/HCl pH 6.4)

0.1 M $MnCl_2$ ($MnCl_2 \cdot 2H_2O$, $M_r = 161.9$; 162 mg in 10 ml H_2O)

Assay mixture

Components	*Concentration (mM)*
8.95 ml 50 mM Tris/imidazole/HCl pH 6.4	45/18
0.5 ml 0.2 M malate solution	10
1.0 ml 0.1 M NAD	10
0.1 ml 0.1 M $MnCl_2$	1

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption increase at 340 nm is measured at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3\ l\,mol^{-1}\ cm^{-1}$.

References

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Tronconi, M. *et al.* (2008) *Plant Physiol.*, **146**, 1540–1552.
Yamaguchi, M. *et al.* (1974) *J. Biochem.*, **76**, 1259–1268.

3.3.1.10 Malate Dehydrogenase (Oxaloacetate-Decarboxylating) ($NADP^+$), EC 1.1.1.40

SN: (*S*)-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), $NADP^+$ dependent malic enzyme

$$(S)\text{-malate} + NADP^+ \rightleftharpoons CO_2 + \text{pyruvate} + NADPH + H^+$$

The enzyme exists in the liver extramitochondrial (90%) and mitochondrial (10%); human enzyme: M_r 257 000, homotetramer (4 × 63 000); K_m (mM): 0.0053 (NADPH), 0.0092 (NADP), 0.12 (L-malate), 4.8 (NAD), 5.9 (pyruvate), pH optimum 7.25.

Assay solutions

50 mM Tris/HCl pH 7.8

0.2 M malate solution (L-malic acid, disodium salt, $M_r = 178.1$; 356 mg in 10 ml H_2O)

0.01 M NADP ($M_r = 787.4$; 79 mg in 10 ml H_2O)

0.1 M $MnCl_2$ ($MnCl_2 2\,H_2O$, $M_r = 161.9$; 162 mg in 10 ml H_2O)

Assay mixture

Components	*Concentration (mM)*
8.95 ml 50 mM Tris/HCl pH 7.8	45
0.5 ml 0.2 M malate solution	10
0.25 ml 0.01 M NADP	0.25
0.1 ml 0.1 M $MnCl_2$	1.0

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption increase at 340 nm is measured at 25 °C; absorption coefficient for NADPH: $\varepsilon_{340} = 6.3 \times 10^3\ l\,mol^{-1}\,cm^{-1}$.

Reference

Zelewsky, M. and Swierczynski, J. (1991) *Eur. J. Biochem.*, **201**, 339–345.

3.3.1.11 Isocitrate Dehydrogenase (NAD^+), EC 1.1.1.41

SN: isocitrate:NAD^+ oxidoreductase (decarboxylating), IDH

$$\text{isocitrate} + NAD^+ \rightleftharpoons CO_2 + \text{2-oxoglutarate} + NADH + H^+$$

Mitochondrial enzyme from the citric acid cycle, cofactors: Mg^{2+} or Mn^{2+}, oxalosuccinate is not accepted as substrate. Human enzyme: M_r 315 000, heterotetramer ($\alpha_2\beta\gamma$), K_m (mM): 0.32 (isocitrate), 0.004 (NAD), 0.22 (Mn^{2+}), pH optimum 7.2.

Assay solutions

33 mM Tris/acetate pH 7.2

0.1 M NAD (M_r = 663.4; 3.31 g in 50 ml 33 mM Tris/acetate pH 7.2)

0.1 M $MnSO_4$ ($MnSO_4 \cdot H_2O$, M_r = 169; 169 mg in 10 ml H_2O)

0.1 M isocitrate solution (D,L-isocitric acid, trisodium salt, M_r = 258.1; 258 mg in 10 ml H_2O)

isocitrate dehydrogenase solution

Assay mixture

Components	*Concentration (mM)*
9.1 ml 33 mM Tris/acetate pH 7.2	30
0.1 ml 0.1 M NAD	1
0.1 ml 0.1 M $MnSO_4$	1
0.5 ml 0.1 M isocitrate solution	5

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption increase at 340 nm is measured at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3\ l\,mol^{-1}\ cm^{-1}$.

Reference

Soundar, S., Park, J.H., Huh, T.L., and Colman, R.F. (2003) *J. Biol. Chem.*, **278**, 52146–52153.

3.3.1.12 Isocitrate Dehydrogenase ($NADP^+$), EC 1.1.1.42

SN: isocitrate:$NADP^+$ oxidoreductase (decarboxylating), ICDH

$$\text{D-isocitrate} + NADP^+ \rightleftharpoons CO_2 + \text{2-oxoglutarate} + NADPH + H^+$$

Mitochondrial enzyme (porcine): M_r 104 000, dimer (2 × 47 000), K_m (mM): 0.00046 (NADP), 0.0075 (isocitrate), 0.001 (Mn^{2+}), pH optimum 7.4.

Assay solutions

0.1 M imidazole/HCl pH 8.0

0.01 M NADP (M_r = 787.4; 79 mg in 10 ml)

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, M_r = 203.3; 203 mg in 10 ml)

0.025 M isocitrate solution (D,L-isocitric acid, trisodium salt, M_r = 258.1; 65 mg in 10 ml)

Isocitrate dehydrogenase solution (10 mg ml^{-1}, dilute 100-fold before usage)

Assay mixture

Components	*Concentration (mM)*
8.8 ml 0.1 M imidazole·HCl pH 8.0	88
0.4 ml 0.01 M NADP	0.4
0.4 ml 0.1 M $MgCl_2$	4.0
0.2 ml 0.025 M isocitrate solution	0.5

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption increase at 340 nm is measured at 25 °C; absorption coefficient for NADPH is $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, 3rd edn, vol. 2, Verlag Chemie, Weinheim, pp. 230–231.
Kim, T.K., Lee, P., and Coleman, R.F. (2003) *J. Biol. Chem.*, **278**, 49323–49331.
Plaut, G.E.W. (1963) in *The Enzymes*, 2nd edn, vol. 7 (eds P.D. Boyer, H. Lardy, and K. Myrbäck), Academic Press, New York, pp. 105–126.

3.3.1.13 Glucose-6-phosphate Dehydrogenase, EC 1.1.1.49

SN: D-glucose 6-phosphate:NADP oxidoreductase, G6P-DH, *Zwischenferment*

$$\text{D-glucose-6-phosphate} + \text{NADP}^+ \rightleftharpoons$$
$$\text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

Enzyme from human erythrocytes (human): M_r: 220 000, homotetramer ($4 \times 57\,000$); K_m (mM): 0.01 (glucose-6-phosphate), 0.8 (6-phosphogluconate), 0.006 (NADP), 0.025 (NADPH), pH optimum 9. ATP inhibits competitively with glucose-6-phosphate; control enzyme of the pentose phosphate pathway, exists as isoenzyme I and II.

Assay solutions

0.1 M triethanolamine/NaOH pH 7.6 (triethanolamine·HCl, $M_r = 185.7$; 18.6 g adjusted with 1 M NaOH, fill up to 1 l)

0.1 M D-glucose-6-phosphate (sodium salt, $M_r = 282.1$, 282 mg in 10 ml)

0.1 M $MgCl_2$ (hexahydrate, $M_r = 203.3$, 203 mg in 10 ml)

0.04 M NADP (disodium salt, $M_r = 787.4$, 315 mg in 10 ml)

Assay mixture

Components	*Concentration (mM)*
9.1 ml 0.1 M triethanolamine·NaOH pH 7.6	91
0.5 ml 0.1 M $MgCl_2$	5.0
0.1 ml 0.1 M D-glucose-6-phosphate	1.0
0.1 ml 40 mM NADP	0.4

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption increase at 340 nm is measured at 25 °C; absorption coefficient for NADPH: $\varepsilon_{340} = 6.3 \times 10^3\ \text{l mol}^{-1}\ \text{cm}^{-1}$.

References

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Cho, S.W. and Joshi, J.G. (1990) *Neuroscience*, **38**, 819–828.

3.3.1.14 Glucose Oxidase, EC 1.1.3.4

SN: β-D-glucose:oxygen 1-oxidoreductase, GOD.

$$\beta\text{-D-glucose} + H_2O + O_2 \rightleftharpoons \delta\text{-D-glucono-1,5-lactone} + H_2O_2$$

Enzyme from *Aspergillus niger*: $M_r = 150\,000$, homodimer; cofactors: FAD and Fe K_m: 1.5 mM (β-D-glucose), pH optimum 5.5. The assay is coupled with peroxidase(POD), *o*-dianisidine acts as the donor:

$$H_2O_2 + \text{donor} \xrightarrow{\text{POD}} 2H_2O + \text{oxidized donor}$$

Assay solutions

0.1 M potassium phosphate pH 7.0

o-Dianisidine solution (*o*-dianisidine dihydrochloride, $M_r = 317.2$, *carcinogenic* in solid form, prepare 1 ml 25 mM solution (7.9 mg in 1 ml H_2O) and dissolve in 100 ml 0.1 M potassium phosphate pH 7.0, saturate with O_2 for 10 min)

0.5 M D-glucose ($M_r = 180.2$; 9 g in 100 ml 0.1 M potassium phosphate pH 7.0)

Peroxidase (POD) from horseradish (dilute to 120 IU ml^{-1} with 0.1 M potassium phosphate pH 7.0 before use)

Assay mixture

Components	*Concentration*
7.7 ml dianisidine solution in	0.2 mM
0.1 M potassium phosphate pH 7.0	77 mM
2.0 ml 0.5 M D-glucose	0.1 M
0.1 ml POD	1.2 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Record the absorption at 436 nm, 25 °C, $\varepsilon_{436} = 8300\ l\,mol^{-1}\,cm^{-1}$.

Calculation

Volume activity

$$\text{IU ml}^{-1} = \frac{\Delta A \text{ min}^{-1} \cdot \text{dilution factor}}{8.3 \times 0.02}$$

Specific activity

$$\text{IU mg}^{-1} = \frac{\Delta A \text{ min}^{-1} \cdot \text{dilution factor}}{8.3 \times 0.02 \text{ mg protein/ml}}$$

References

Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, 3rd edn, vol. 2, Verlag Chemie, Weinheim, pp. 201–202.
Bright, H.B. and Porter, D.J.T. (1975) in *The Enzymes*, 3rd edn, vol. 12B (ed. P.D. Boyer), Academic Press, New York, pp. 421–505.
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Pazur, J.H. (1966) *Meth. Enzymol*, **9**, 82–87.

3.3.1.15 Formate Dehydrogenase, EC 1.2.1.2

SN: formate:NAD^+ oxidoreductase, FDH

$$\text{formate} + NAD^+ \rightleftharpoons CO_2 + NADH + H^+$$

Reported from various bacteria and plants, for example *Arabidopsis thaliana*. M_r 82 000, dimer, cofactors: FMN and non-heme iron, K_m (mM): 0.011 (formate), 0.075 (NAD), pH optimum 7.5.

Assay solutions

0.05 M sodium phosphate pH 7.5

1 M sodium formate (M_r = 68.0, 0.68 g in 10 ml)

0.1 M NAD (M_r = 663.4; 663 mg in 10 ml)

0.5 mM FMN (flavin mononucleotide, riboflavin 5′-phosphate, sodium salt, M_r = 478.3, 2.4 mg in 10 ml)

Assay mixture

Components	*Concentration*
9.5 ml 0.05 M sodium phosphate pH 7.5	95 mM
0.2 ml 1 M sodium formate	20 mM
0.05 ml 0.1 M NAD	0.5 mM
0.05 ml FMN	2.5 µM

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption increase at 340 nm at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3\ \mathrm{l\,mol^{-1}\,cm^{-1}}$.

References

Baack, R.D. (2003) *J. Plant Physiol.*, **160**, 445–450.
Yoch, D.C. *et al.* (1990) *J. Bacteriol.*, **172**, 4456–4463.

3.3.1.16 Glyceraldehyde-3-phosphate Dehydrogenase, EC 1.2.1.12

SN: D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), GAPDH, triosephosphate dehydrogenase

$$\text{D-glyceraldehyde-3-phosphate} + NAD^+ + P_i \rightleftharpoons \text{1,3-bisphosphoglycerate} + NADH + H^+$$

Mammalian enzyme (human): $M_r = 142\,000$, homotetramer, K_m (mM): 0.01 (diphosphoglyceric acid), 0.07 (glyceraldehyde-3-phosphate), 0.01 (NADH), 0.05 (NAD), pH optimum 8. The enzyme can be tested either in the forward reaction or in a coupled test in the reverse reaction. For the forward reaction the unstable D,L-glyceraldehyde-3-phosphate is needed.

A. Oxidation Assay

Assay solutions

0.1 M triethanolamine/NaOH pH 7.6 (triethanolamine·HCl, $M_r = 185.7$; dissolve 18.6 g in 800 ml H_2O, adjust with 1 M NaOH to pH 7.6, fill up to 1 l)

0.3 M D,L-glyceraldehyde-3-phosphate ($M_r = 170.1$, unstable, aqueous solution 50 mg ml^{-1})

0.1 M NAD ($M_r = 663.4$; 663 mg in 10 ml)

0.1 M potassium dihydrogen arsenate ($M_r = 180.0$; 180 mg in 10 ml)

Assay mixture

Components	*Concentration (mM)*
9.37 ml 0.1 M triethanolamine/NaOH, pH 7.6	94
0.03 ml 0.3 M glycerate-3-phosphate	0.9
0.3 ml 0.1 M potassium dihydrogen arsenate	3.0
0.1 ml 0.1 M NAD	1.0

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption increase at 340 nm at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

B. Reduction Assay Coupled with 3-Phosphoglycerate Kinase (PGK)

$$\text{3-phosphoglycerate} + \text{ATP} \overset{\text{PGK}}{\rightleftharpoons} \text{1,3-diphosphoglycerate} + \text{ADP}$$

$$\text{1,3-bisphosphoglycerate} + \text{NADH} + \text{H}^+ \overset{\text{GAPDH}}{\rightleftharpoons} \text{D-glyceraldehyde-3-phosphate} + \text{NAD}^+ + \text{P}_\text{i}$$

Assay solutions

0.1 M triethanolamine/NaOH pH 7.6 (triethanolamine· HCl, $M_r = 185.7$; dissolve 18.6 g in 800 ml H_2O, adjust to pH 7.6 with 1 M NaOH, fill up to 1 l)

0.1 M glycerate-3-phosphate (3-phosphoglyceric acid, disodium salt, $M_r = 230.0$; 230 mg in 10 ml)

0.1 M ATP (disodium salt, trihydrate, $M_r = 605.2$; 605 mg in 10 ml)

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg in 10 ml)

0.1 M EDTA (ethylenediaminetetraacetic acid, $M_r = 292.2$; 292 mg in10 ml)

0.1 M magnesium sulfate ($MgSO_4^{\cdot}$ 7 H_2O, $M_r = 246.5$; 247 mg in 10 ml)

3-phosphoglycerate kinase, from yeast (~2000 IU mg^{-1})

Assay mixture

Components	*Concentration*
8.6 ml 0.1 M triethanolamine/NaOH pH 7.6	86 mM
0.5 ml 0.1 M glycerate-3-phosphate	5.0 mM
0.1 ml 0.1 M ATP	1.0 mM
0.2 ml 0.01 M NADH	0.2 mM
0.1 ml 0.1 M EDTA	1.0 mM
0.2 ml 0.1 M magnesium sulfate	2.0 mM
0.1 ml 3-phosphoglycerate kinase	10 IU

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption decrease at 340 nm at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3\ \mathrm{l\,mol^{-1}\,cm^{-1}}$.

References

Beisenherz, G., Boltze, H.J., Bücher, T., Czok, R., Garbade, K.H., Meyer-Arendt, E., and Pfleiderer, G. (1953) *Z Naturforsch.*, **8b**, 555–577.
Heinz, F. and Freimüller, B. (1982) *Meth. Enzymol.*, **89**, 301–305.
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Scheek, R.M. and Slater, E.C. (1982) *Meth. Enzymol.*, **89**, 305–309.

3.3.1.17 Pyruvate Dehydrogenase (Acetyl-Transferring), EC 1.2.4.1

SN: pyruvate:[dihydrolipoyllysine-residue acetyltransferase]-lipoyllysine 2-oxidoreductase (decarboxylating, acceptor acetylating), PDH, E1p

$$\text{pyruvate} + \text{ThDP-E1p} \rightarrow \text{hydroxyethyl-ThDP-E1p} + CO_2$$

$$\text{hydroxyethyl-ThDP-E1p} + \text{electron acceptor} \rightarrow$$

$$\text{acetate} + \text{ThDP-E1p} + \text{reduced electron acceptor}$$

The enzyme exists in the cell only as the E1p component of the pyruvate dehydrogenase complex (see Section 3.3.7.1); cofactors: ThDP and Mg^{2+}; mammalian enzyme (bovine heart) M_r: 154 000 tetramer ($2 \times 41\,000, 2 \times 35\,000$); K_m (mM): 0.027 mM (pyruvate), 0.0034 (thiamin diphosphate); enzyme from *Escherichia coli*, $M_r = 200\,000$, homodimer; K_m: 0.3 mM (pyruvate); strong inhibition by fluoropyruvate. Two photometric assays for the partial reaction of the complex-bound

E1p component are described. Alternatively, the release of CO_2 can be measured manometrically, with a CO_2 electrode or by determining the radioactivity, released as $^{14}CO_2$ when ^{14}C1-pyruvate is the substrate.

A. Ferricyanide as Electron Acceptor The assay is less susceptible against disturbances but not very sensitive; larger amounts of enzyme are required.

Assay solutions

0.05 M Tris/HCl pH 7.6

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6\ H_2O$, $M_r = 203.3$; 203 mg in 10 ml H_2O)

0.1 M pyruvate (sodium salt, $M_r = 110.0$; 110 mg in10 ml H_2O)

0.01 M ThDP (thiamin diphosphate, cocarboxylase, $M_r = 460.8$; 46.1 mg in 10 ml 0.05 M Tris/HCl pH 7.6)

0.1 M $K_3[Fe(CN)_6]$ ($M_r = 329.2$; 330 mg in 10 ml H_2O)

Assay mixture

Components	*Concentration (mM)*
8.9 ml 0.05 M Tris/HCl pH 7.6	45
0.1 ml 0.1 M $MgCl_2$	1.0
0.1 ml 0.01 M ThDP	0.1
0.5 ml 0.1 M pyruvate	5.0
0.2 ml 0.1 M $K_3[Fe(CN)_6]$	2.0

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption decrease is measured at 436 nm, 30 °C, $\varepsilon_{436} = 755\ l\ mol^{-1}\ cm^{-1}$.

B. Dichlorophenolindophenol as Electron Acceptor

Assay solutions

0.05 M triethanolamine/HCl pH 7.8

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6\ H_2O$, $M_r = 203.3$; 203 mg in 10 ml)

0.1 M pyruvate (sodium salt, $M_r = 110.0$; 110 mg in 10 ml)

0.01 M thiamin diphosphate (ThDP, cocarboxylase, $M_r = 460.8$; 46.1 mg in 10 ml)

0.01 M 2,6-dichlorophenolindophenol ($M_r = 290.1$; 87 mg in 30 ml *n*-propanol)

Assay mixture

Components	*Concentration (mM)*
8.7 ml 0.05 M triethanolamine/HCl pH 7.8	87
0.2 ml 0.1 M $MgCl_2$	2.0
0.2 ml 0.01 M ThDP	0.2
0.5 ml 0.1 M pyruvate	5.0
0.2 ml 0.01 M 2,6-dichlorophenolindophenol	0.2

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption change is pursued at 600 nm, 30 °C.

References

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Schwartz, E.R., Old, L.O., and Reed, L.J. (1968) *Biochem. Biophys. Res. Commun.*, **31**, 495–500.

3.3.1.18 Oxoglutarate Dehydrogenase (Succinyl-Transferring), EC 1.2.4.2

SN: 2-oxoglutarate:[dihydrolipoyllysine-residue succinyltransferase]-lipoyllysine 2-oxidoreductase (decarboxylating, acceptor succinylating), OGDH, E1o

2-oxoglutarate + ThDP-E1o → 2-hydroxy-γ-carboxypropyl-ThDP-E1o + CO_2

2-hydroxy-γ-carboxypropyl-ThDP-E1o + electron acceptor →

succinate + reduced electron acceptor + ThDP-E1o

In the cell, the enzyme is exists only as the E1o component of the α-oxoglutarate dehydrogenase complex (see Section 3.3.7.2); cofactors: thiamin diphosphate (ThDP) and Mg^{2+}; $M_r = 210\,000$, homodimer, K_m: 0.1 mM (α-oxoglutarate), pH optimum 8.0. A photometric assay for the partial reaction of the complex-bound E1o component applying $K_3[Fe(CN)_6]$ as artificial electron acceptor is described. Alternatively, the release of CO_2 can be measured manometrically, or with a CO_2 electrode.

Assay solutions

0.1 M potassium phosphate pH 6.5

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6\ H_2O$, $M_r = 203.3$; 203 mg in 10 ml)

0.5 M 2-oxoglutarate (α-ketoglutarate, 2-oxopentanedioic acid, $M_r = 190.1$, disodium salt, 0.95g in 10 ml)

0.01 M ThDP (thiamin diphosaphte, cocarboxylase, $M_r = 460.8$; 46.1 mg in 10 ml)

0.1 M $K_3[Fe(CN)_6]$ ($M_r = 329.2$; 330 mg in 10 ml H_2O)

1% bovine serum albumine (BSA, 100 mg in 10 ml 0.1 M potassium phosphate pH 6.5)

Assay mixture

Components	*Concentration*
8.1 ml 0.1 M potassium phosphate pH 6.5	81 mM
0.2 ml 0.1 M $MgCl_2$	2.0 mM
0.2 ml 0.01 M ThDP	0.2 mM
1.0 ml 1% BSA	0.1%
0.1 ml 0.5 M α-oxoglutarate	5.0 mM
0.2 ml 0.1 M $K_3[Fe(CN)_6]$	2.0 mM

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Absorption decrease at 436 nm, 30 °C, $\varepsilon_{436} = 755\ l \cdot mol^{-1}\ cm^{-1}$

Reference

Hager, L.P. and Gunsalus, I.C. (1953) *J. Am. Chem. Soc.*, **75**, 5767–5768.

3.3.1.19 Pyruvate Ferredoxin Oxidoreductase, EC 1.2.7.1

SN: pyruvate:ferredoxin 2-oxidoreductase (CoA-acetylating), pyruvate synthase

$$\text{pyruvate} + \text{CoA} + 2\ \text{oxidized ferredoxin} \rightleftharpoons$$
$$\text{acetylCoA} + CO_2 + 2\ \text{reduced ferredoxin} + 2H^+$$

Iron-sulfur and thiamin diphosphate as cofactors, heterodimer, $M_r = 103\,000$ (69 000 and 34 000) K_m: 0.28 mM (pyruvate), pH optimum 7 (*Sulfolobus solfataricus*), K_m: 0.32 mM (pyruvate), 3.7 µM (CoA) pH optimum 7.5 (*Clostridium acetobutylicum*).

Assay with Cytochrome *c* as Electron Acceptor

$$\text{pyruvate} + \text{CoA} + \text{cyt } c_{ox} \rightleftharpoons \text{acetyl CoA} + CO_2 + \text{cyt } c_{red}$$

Assay solutions

0.05 M Tris/HCl pH 7.3

0.01 M cytochrome *c* (from horse heart, $M_r = 12380$, 620 mg in 5 ml 0.05 M Tris/HCl pH 7.3)

0.10 M pyruvate (sodium pyruvate, $M_r = 110.0$; 110 mg in 10 ml H_2O)

0.01 M CoA (free acid: $M_r = 767.5$, CoA· Li_3, $M_r = 785.4$; 23 mg in 3 ml 0.05 M Tris/HCl pH 7.3)

Assay mixture

Components	*Concentration (mM)*
9.1 ml 0.05 M Tris/HCl pH 7.3	93
0.1 ml 0.01 M cytochrome *c*	0.1
0.5 ml 0.10 M pyruvate	5.0
0.1 ml 0.01 M CoA	0.1

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption increase at 550 nm, 25 °C. The difference absorption coefficient (reduced–oxidized) for cytochrome *c* is $\varepsilon_{550} = 21 \times 10^3\ \mathrm{l\,mol^{-1}\ cm^{-1}}$.

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Zhang, Q., Iwasaki, T., Wakagi, T., and Oshima, T. (1996) *J. Biochem.*, **120**, 587–599.

3.3.1.20 Alanine Dehydrogenase, EC 1.4.1.1

SN: L-alanine:NAD^+ oxidoreductase (deaminating)

$$\text{L-alanine} + NAD^+ \rightleftharpoons \text{pyruvate} + NH_4^+ + NADH$$

Bacterial enzyme (*Bacillus subtilis*) homohexamer, M_r 220 000, K_m (mM): 1.7 (L-alanine), 0.65 (pyruvate), 38 (NH_4^+), 0.18 (NAD), 0.023 (NADH), pH optimum 10.0.

A. Oxidation of Alanine

Assay solutions

0.05 M $Na_2CO_3/NaHCO_3$ buffer pH 10.0

0.1 M NAD (M_r = 663.4; 0.663 g in 10 ml H_2O)

0.1 M L-alanine (M_r = 89.1; 89.1 mg in 10 ml H_2O)

Assay mixture

Components	*Concentration (mM)*
8.7 ml 0.05 M $Na_2CO_3/NaHCO_3$ buffer pH 10.0	43.5
0.1 ml 0.1 M NAD	1
1 ml 0.1 M L-alanine	10

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption increase at 340 nm at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

B. Reduction of Pyruvate

Assay solutions

0.05 M Tris/HCl pH 8.0

0.1 M pyruvate (sodium salt, $M_r = 110$; 110 mg in 10 ml H_2O)

2 M NH_4Cl ($M_r = 53.5$; 10.7 g 100 ml)

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg in 10 ml)

Assay mixture

Components	*Concentration*
8 ml 0.05 M Tris/HCl pH 8.0	40 mM
1 ml 2 M NH_4Cl	0.2 M
0.6 ml 0.1 M pyruvate	6 mM
0.2 ml 0.01 M NADH	0.2 mM

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution (~30 µg)

Follow the decrease in the absorption at 340 nm at 25 °C. The absorption coefficient for NADH is $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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3.3.1.21 Glutamate Dehydrogenase, EC 1.4.1.3

SN: L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating); glutamic dehydrogenase

$$\text{L-glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \rightleftharpoons \text{2-oxoglutarate} + \text{NAD(P)H} + \text{NH}_4^+$$

Mammalian enzyme: $M_r = 330\,000$, homohexamer ($6 \times 56\,000$), K_m (mM): 0.076 (NADH), 1.25 (2-oxoglutarate), pH optimum 7.5–8.0, ADP activates, ATP inhibits. A similar NAD-dependent glutamate:NAD^+ oxidoreductase (deaminating), EC 1.4.1.2 has been found in bacteria and plants, an NADP-dependent (EC 1.4.1.4) in bacteria.

The back reaction is used for the assay.

Assay solutions

0.1 M imidazole buffer pH 7.9 (imidazole, $M_r = 68.1$; dilute 0.68 g in 80 ml H_2O, adjust with 1 M HCl to pH 7.9)

0.2 M 2-oxoglutarate (2-oxoglutaric acid, monosodium salt, $M_r = 168.1$, 336 mg in 10 ml)

10 M ammonium acetate ($M_r = 77.1$; 0.77 g in 10 ml)

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg 10 ml^{-1})

0.1 M EDTA ($M_r = 292.2$; 292 mg 10 ml^{-1})

0.1 M ADP (disodium salt, $M_r = 471.2$; 471 mg 10 ml^{-1})

Assay mixture

Components	*Concentration*
8.4 ml 0.1 M imidazole buffer pH 7.9	84 mM
0.7 ml 0.2 M oxoglutarate	14 mM
0.2 ml 10 M ammonium acetate	0.2 M
0.2 ml 0.01 M NADH	0.2 mM
0.1 ml 0.1 M EDTA	1.0 mM
0.2 ml 0.1 M ADP	2.0 mM

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption decrease at 340 nm at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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3.3.1.22 Leucine Dehydrogenase, EC 1.4.1.9

SN: L-leucine:NAD^+oxidoreductase (deaminating), LeuDH

$$\text{L-leucine} + H_2O + NAD^+ \rightarrow \text{4-methyl-2-oxopentanoate} + NH_4^+ + NADH$$

Bacterial enzyme (*Bacillus* sp.), homohexamer, $M_r = 245\,000$ ($6 \times 41\,000$), reacts also with isoleucine, valine, norvaline, and norleucine; K_m (mM): 1.0 (L-leucine), 1.8 (L-isoleucine), 1.7, (L-valine), 0.31 (4-methyl-2-oxopentanoate), 1.5 (NAD), 0.12 (NADH); inhibitors: Cu^{2+}, Co^{2+}. The reaction amino acid $\rightarrow$ α-oxo-acid is also catalyzed by the branched-chain amino acid transaminase (EC 2.6.1.42).

Assay solutions

0.05 M sodium carbonate/1 mM EDTA, pH 10.0 (Na_2CO_3, $M_r = 106.0$, 5.3 g; EDTA, $M_r = 292.2$, 0.29 g in 1 l and adjust to pH 10.0 with 1 M HCl)

0.2 M L-leucine ($M_r = 131.2$, 0.26 g in 10 ml)

0.1 M NAD ($M_r = 663.4$; 0.663 g in 10 ml)

Enzyme sample (~1 IU ml^{-1})

Assay mixture

Components	*Concentration (mM)*
8.5 ml 0.05 M sodium carbonate/1 mM EDTA, pH 10.0	42.5/0.85
1.0 ml 0.2 M L-leucine	20
0.3 ml 0.1 M NAD	3.0

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Follow the absorption increase at 340 nm at 25 °C; absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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Katoh, R. *et al.* (2003) *J. Mol. Catal.*, **B23**, 239–247.
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3.3.1.23 L-Amino Acid Oxidase, EC 1.4.3.2

SN: L-amino acid:oxygen oxidoreductase (deaminating), ophio-amino-acid oxidase

$$\text{L-amino acid} + H_2O + O_2 \rightarrow \text{2-oxo acid} + NH_3 + H_2O_2$$

Lysosomal glycoprotein, involved in the amino acid catabolism; mammalian enzyme (*Mus musculus*): M_r = 113 000, homodimer; K_m (mM): 6.5 (phenylalanine). pH optimum 4.0; *Rhodococcus opacus*: M_r = 104 000, homodimer, cofactor: FAD, K_m (mM) 0.3 (L-alanine), 0.028 (L-leucine), 0.022 (L-phenylalanine), pH optimum 8.

Coupled assay with peroxidase (POD):

$$H_2O_2 + \text{donor} \xrightarrow{\text{POD}} 2H_2O + \text{oxidized donor}$$

Assay solutions

0.2 M triethanolamine solution (dissolve 3.7 g triethanolamine hydrochloride, M_r = 185.7, and adjust to pH 7.6 in 100 ml, add 0.1 g L-leucine and 6.5 mg *o*-dianisidine)

peroxidase (POD, from horseradish, dilute to 120 IU ml^{-1} with 0.1 M potassium phosphate pH 7.0 before use)

Procedure

0.97 ml triethanolamine solution

0.01 ml peroxidase

Start with 0.02 ml enzyme solution (or H_2O for the blank) and follow the absorption decrease at 436 nm, 25 °C for about 5 min, ε_{436} = 8300 l mol^{-1} cm^{-1}.

References

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Sun, Y. *et al.* (2002) *J. Biol. Chem.*, **277**, 19080–19086.

3.3.1.24 D-Amino Acid Oxidase, EC 1.4.3.3

SN: D-amino acid:oxygen oxidoreductase (deaminating), ophio-amino acid oxidase, new yellow enzyme.

$$\text{D-amino acid} + H_2O + O_2 \rightarrow \text{2-oxo acid} + NH_3 + H_2O_2$$

Mammalian enzyme (human, porcine) in peroxisomes; M_r 38 000, monomer, cofactor: FAD, K_m (mM) 0.77 (D-alanine), 1.4 (D-phenylalanine), pH optimum 9.0. *Neurospora crassa* in mitochondria, M_r 118000, K_m (mM) 0.28 (D-leucine), 0.24 (D-methionine), pH optimum 9. The coupled assay in Section 3.3.1.23 with peroxidase can be used, replacing L-leucine by D-leucine:

$$H_2O_2 + \text{donor} \xrightarrow{\text{POD}} 2H_2O + \text{oxidized donor}$$

References

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Setoyama, C. *et al.* (2006) *J. Biochem.*, **139**, 873–879.

3.3.1.25 Monoamine Oxidase, EC 1.4.3.4

SN: amine:oxygen oxidoreductase (deaminating)(flavin-containing), adrenaline oxidase, amine oxidase, tyraminase, tyramine oxidase

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$$

Catalyzes oxidative deamination of neurotransmitter and biogenic amines

Mammalian liver enzyme (bovine); K_m (mM) 0.19 (phenylethylamine), 0.51 (benzylamine), 2.0 (dimethylaminobenzylamine), pH optimum 8.0, *Aspergillus niger*: K_m (mM): 0.56 (benzylamine), 20 (ethylamine).

Assay solutions

0.05 M sodium phosphate buffer pH 7.2

Triton X-100

0.02 M kynuramine solution (3-(2-aminophenyl-3-oxopropanamine) dihydrobromide, $M_r = 326$; 65.2 mg in 10 ml H_2O)

Assay mixture

Components	*Concentration*
9.28 ml 0.05 M sodium phosphate buffer pH 7.2	46 mM
0.02 mM Triton X-100	0.2%
0.5 ml kynuramine solution	1 mM

For provision of oxygen the mixture must be air-equilibrated.

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption change at 314 nm, 25 °C, $\varepsilon_{314} = 12.3 \times 10^3$ l mol^{-1} cm^{-1}.

Modification

Kynuramine in the assay mixture can be replaced by 0.5 ml 60 mM D-dimethylaminobenzylamine 2HCl ($M_r = 223.1$, 134 mg in 10 ml, assay concentration 3 mM). The absorption is measured at 355 nm, $\varepsilon_{355} = 27.7 \times 10^3$ l mol^{-1} cm^{-1}.

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Suzuki, H., Ogura, Y., and Yamada, H. (1972) *J. Biochem.*, **72**, 703–712.

3.3.1.26 Primary Amine Oxidase, EC 1.4.3.21

SN: primary amine:oxygen oxidoreductase (deaminating), amine oxidase (copper-containing), benzymamine oxidase

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$$

Closely related to 1.4.3.22, but not to 1.4.3.4, preferentially oxidizes primary monoamines, but neither diamines such as histamine nor secondary and tertiary amines. In some mammalian tissues also functions as vascular-adhesion protein (VAP-1). Microbial enzyme (yeast): M_r 136 000, dimer; *Candida boidinii*): K_m (mM): 0.01 (O_2), 0.2 (methylamide), pH optimum 7.0.

A. Spectrophotometric Assay H_2O_2 formed with methylamine is coupled to the oxidation of ABTS with peroxidase:

$$\text{ABTS} + H_2O_2 \rightarrow \text{oxidized ABTS} + 2H_2O$$

Assay solutions

0.05 M potassium phosphate pH 7.0

0.1 M methylamine (hydrochloride, $M_r = 67.5$; 0.67 g in 100 ml 0.05 M potassium phosphate pH 7.0)

0.02 M ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, diammonium salt, $M_r = 548.7$; 1.1 g in 0.1 M potassium phosphate pH 7.0)

peroxidase (POD) from horseradish (prepare a solution of 150 IU ml^{-1} in 0.1 M potassium phosphate pH 7.0 before usage)

Assay mixture

Components	*Concentration*
8.8 ml 0.05 M potassium phosphate pH 7.0	50 mM
0.3 ml 0.1 M methylamine	3 mM
0.5 ml 0.02 M ABTS	1 mM
0.2 ml peroxidase	3 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Follow absorption increase at 414 nm, 25 °C, $\varepsilon_{414} = 24.6 \times 10^3$ l mol^{-1} cm^{-1}; 1 mol H_2O_2 oxidizes 2 mol ABTS.

B. Polarographic Assay of O_2 Uptake with O_2 Electrode

Asaay Solutions

0.05 M potassium phosphate pH 7.0

0.1 M methylamine (hydrochloride, $M_r = 67.5$; 0.67 g in 100 ml 0.05 M potassium phosphate pH 7.0)

Assay mixture

Components	*Concentration (mM)*
9.5 ml 0.05 M potassium phosphate pH 7.0	50
0.3 ml 0.1 M methylamine	3

Procedure

The O_2 consumption is measured at 25 °C with an O_2 electrode. One enzyme unit is defined as the consumption of 0.5 µmol min^{-1} (corresponding to the spectrophotometric assay)

C. Assays for Benzylamine Oxidase Activity The assays A and B can be similarly used, replacing methylamine by

- 0.01 M benzylamine (hydrochloride, $M_r = 67.5$; 0.67 g in 100 ml 0.05 M potassium phosphate pH 7.0; assay concentration = 0.3 mM)

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Saysell, C.G. *et al.* (2002) *Biochem. J.*, **365**, 809–816.

3.3.1.27 Diamine Oxidase, EC 1.4.3.22

SN: histamine:oxygen oxidoreductase (deaminating), amine oxidase (copper containing), copper amine oxidase (CAO), histamine deaminase, histaminase, semicarbazide-sensitive amine oxidase (SSAO)

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$$

$$\text{histamine} + H_2O + O_2 \rightarrow \text{(imidazol4-yl)acetaldehyde} + NH_3 + H_2O_2$$

Copper quinoproteins (2,4,5-trihydroxyphenylalanine quinone and copper as cofactors), oxidizes diamines such as histamine and primary monoamines, but not secondary and tertiary amines; sensitive to inhibition by carbonyl group reagents such as semicarbazide. Mammalian (human, porcine): M_r 200 000, homodimer, K_m (mM) 0.0028 (histamine), 0.03 (cadaverine), 0.02 (putrescine), pH optimum 7.0.

Assay solutions

0.1 M potassium phosphate pH 7.6

[β ^{3}H]-Putrescine ($M_r = 88.2$, 1.5×10^6 cpm, ~3 nmol ml^{-1} in 0.1 M potassium phosphate pH 7.6)

Scintillation liquid (Instabray)

Procedure

0.1 ml [β ^{3}H]-putrescine

0.1 ml enzyme solution

Incubate together for 1 h at 37 °C in sealed cups

Extract the deaminated product in two successive steps with 1 ml toluene, each

Add 0.2 ml of the toluene phase to 10 ml scintillation liquid and determine the radioactivity

Histaminase activity can be determined in a similar procedure, replacing [β ^{3}H]-putrescine by [β ^{3}H]-histamine (0.5×10^6 cpm, ~0.15 nmol ml^{-1} in 0.1 M potassium phosphate pH 6.8).

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3.3.1.28 Urate Oxidase, EC 1.7.3.3

SN: urate:oxygen oxidoreductase, uricase

$$\text{urate} + H_2O + O_2 \rightarrow \text{5-hydroxyisourate} + H_2O_2$$

$$(\text{urate} + 2H_2O + O_2 \rightarrow \text{allantoin} + H_2O_2 + CO_2)$$

Enzyme from *Bacillus subtilis*: M_r 150 000, dimer, Cu^{2+} as cofactor, K_m (mM) 0.0034 (urate), inhibitor: nitroxanthine K_i (mM) 0.0021 (competitive with urate)

Assay solutions

0.1 M sodium borate pH 8.5 ($Na_2B_4O_7 \cdot 10H_2O$, $M_r = 381.4$; 3.8 g in 100 ml, adjust to pH 8.5 with 0.1 M boric acid, saturated with gaseous oxygen for 15 min prior to use)

1.0 mM urate ($M_r = 168.1$, dissolve 17 mg in 100 ml borate buffer pH 8.5, heat to ~50 °C if necessary)

Assay mixture

Components	*Concentration (mM)*
9.4 ml 0.1 M sodium borate pH 8.5	94
0.4 ml 1.0 mM urate	0.04

Procedure

0.98 assay mixture

0.02 ml enzyme solution

Follow the absorption change at 295 nm, 25 °C; $\varepsilon_{295} = 12.6 \times 10^3$ l mol^{-1} cm^{-1}.

References

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3.3.1.29 Dihydrolipoamide Dehydrogenase, EC 1.8.1.4

SN: NADH:lipoamide oxidoreductase, diaphorase, E3

$$\text{lipoamide} + \text{NADH} + \text{H}^+ \overset{\text{E3}}{\rightleftharpoons} \text{dihydrolipoamide} + \text{NAD}^+$$

In the cell, the enzyme exists in a free form as a homodimer ($M_r = 112\,000$), and as the E3 component bound to the pyruvate dehydrogenase complex, the α-oxoglutarate dehydrogenase complex and the branched chain α-oxoacid dehydrogenase complex (see Section 3.3.7). The designation *diaphorase* refers to enzymes that can catalyze the oxidation of NAD(P)H by artificial electron acceptors such as dichlorophenolindophenol, ferrricyanide, and quinones.

The reaction can be tested from both sides. Lipoic acid and its amide, which is about five times more active, are commercially available in the oxidized form and can be reduced by a simple procedure (see Section 3.3.2.1).

A. Oxidation of Dihydrolipoamide

Assay solutions

0.1 M potassium phosphate pH 7.5

0.1 M NAD (free acid, $M_r = 663.4$; 663 mg in 10 ml)

0.1 M DTE (dithioerythritol, $M_r = 154.2$; 154 mg in10 ml)

0.2 M D,L-dihydrolipoamide (M_r 207.3; 52 mg in 1.25 ml ethanol; alcohol dehydrogenase if present, for example in crude extracts, forms NADH from ethanol and NAD. In this case, acetone must be taken as solvent)

Assay mixture

Components	*Concentration (mM)*
9.46 ml 0.1 M potassium phosphate pH 7.5	95
0.1 ml 0.1 M NAD	1.0
0.04 ml 0.1 M DTE	0.4

Procedure

0.96 ml assay mixture	–
0.02 ml 0.2 M D,L-dihydrolipoamide	4.0
0.02 ml enzyme solution	–

The absorption increase is measured at 340 nm, 30 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

B. Reduction of Lipoamide To avoid overreduction of the enzyme into the inactive state, NAD must be present in addition to NADH.

Assay solutions

0.1 M potassium phosphate pH 7.5

0.1 M NAD (free acid, $M_r = 663.4$; 663 mg in10 ml)

0.01 M NADH (disodium salt, $M_r = 709.4$, 71 mg in10 ml)

0.1 M DTE (dithioerythritol, $M_r = 154.2$; 154 mg in 10 ml)

0.1 M EDTA (ethylenediaminetetraacetic acid, M_r = 292.2; 292 mg in 10 ml)

0.2 M D,L-lipoamide (6,8-thioctic acid amide, M_r 205.3; 51 mg in 1.25 ml ethanol or acetone, see Section 3.3.1.29.A)

Assay mixture

Components	*Concentration (mM)*
9.06 ml 0.1 M potassium phosphate pH 7.5	91
0.1 ml 0.1 M NAD	1.0
0.02 ml 0.01 M NADH	0.02
0.1 ml 0.1 M EDTA	1.0
0.02 ml 0.1 M DTE	0.2

Procedure

0.93 ml assay mixture	–
0.05 ml 0.2 M D,L-lipoamide	10 mM
0.02 ml enzyme solution	–

The absorption decrease is measured at 340 nm, 30 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

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3.3.1.30 Glutathione Disulfide Reductase, EC 1.8.1.7

SN: glutathione:NADP$^+$ oxidoreductase, glutathione reductase

$$2\ \text{glutathione} + \text{NADP}^+ \rightarrow 2\ \text{glutathione disulfide} + \text{NADPH} + \text{H}^+$$

Mammalian enzyme (bovine), M_r 118 000, homodimer, coenzyme FAD, K_m (mM): 0.008 (NADPH), 0.04 (glutathione), 0.065 (glutathione disulfide), pH optimum 7.3.

Assay solutions

0.1 M potassium phosphate, 1 mM EDTA pH 7.0 (dissolve 0.29 g EDTA in 1 l 0.1 M potassium phosphate pH 7.0)

0.01 M NADPH (M_r = 833.4, Na_4 salt, 83 mg in 10 ml 0.1 M potassium phosphate, 1 mM EDTA pH 7.0, freshly prepared)

0.01 M GSSG (oxidized glutathione, M_r = 612.6; 61 mg in 10 ml 0.1 M potassium phosphate, 1 mM EDTA pH 7.0)

Assay mixture

Components	*Concentration (mM)*
8.6 ml 0.1 M potassium phosphate, 1 mM EDTA pH 7.0	86/0.86
0.2 ml 0.01 M NADPH	0.2
1.0 ml 0.01 M GSSG	1.0

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

The absorption decrease at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

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3.3.1.31 Catalase, EC 1.11.1.6

SN: hydrogenperoxide: hydrogenperoxide oxidoreductase

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

The green enzyme ($M_r = 240\,000$, bovine liver) consists of four identical subunits each carrying a protohematin IX group in a high-spin state with a central trivalent iron. Four coordination sites of the iron ion are occupied by the porphyrin ring structure, the fifth by a histidine residue of the protein. The sixth position remains free and may be occupied by anions like cyanide or fluoride, and block the enzyme reaction. Binding of cyanide induces the iron to move into the plane of the porphyrin ring, the high spin changes to the low spin state, accompanied by a remarkable spectral shift. Catalase possesses a strong tendency to crystallize, so the crystal suspension is the preferred storage form. At higher concentrations hydrogen peroxide damages the enzyme, so it cannot be tested at a saturating substrate. The enzyme has an absorption coefficient of 380×10^3 l mol^{-1} cm^{-1} at 405 nm. *Escherichia coli*: K_m (mM): 3.5 (H_2O_2), pH optimum 10.5; human, K_m (mM): 80 (H_2O_2), pH optimum 6.8.

Assay solutions

0.05 M potassium phosphate pH 7.0

H_2O_2 solution (~10 mM), dilute 0.06 ml 30% H_2O_2 in 50 ml 0.05 M potassium phosphate pH 7.0, the absorption against buffer at 240 nm should be 0.50 ± 0.01; otherwise, adjust to this value with buffer or H_2O_2

Catalase solution (20 mg ml^{-1}, dilute in 0.05 M potassium phosphate, pH 7.0 before use, according to the specific activity of the sample, for example 2000-fold)

Procedure

0.98 ml H_2O_2 solution

0.02 ml catalase solution

Follow the absorption decrease at 240 nm, 25 °C in quartz cuvettes; $\varepsilon_{240} = 40 \times 10^3$ l mol^{-1} cm^{-1}. An absorption change of 0.04/min corresponds to a decomposition of 1 µmol H_2O_2/min ~1 IU (16.67 nkat).

References

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3.3.1.32 Peroxidase, EC 1.11.1.7

SN: donor:hydrogenperoxide oxidoreductase, lactoperoxidase, myeloperoxidase

$$\text{donor} + H_2O_2 \rightarrow \text{oxidized donor} + 2H_2O$$

Glycoprotein, $M_r = 44\,000$ (horseradish), related to catalase with protohematin IX as prosthetic group. It is specific for the acceptor H_2O_2, but reacts with various donor substrates, all applicable for the enzyme assay: 5-amino-2,3-dihydro-1,4-phthalazinedion (luminol), 3-amino-9-ethylcarbazole (AEC), 5-aminosalicylic acid, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 4-chloro-1-naphthol, 3,3′-diaminobenzidine, *o*-dianisidine, guaiacol, *o*-phenylenediamine, ascorbic acid, resorcinol, pyrogallol, hydroquinon, 3,3′,5,5′-tetramethylbenzidine. Peroxidase is applied in various conjugates; for example, with antibodies in Western blots and ELISA, or colloidal gold in cell biology. K_m (mM): 10.5 (guajacol), 0.48 (dianisidin), 0.21 (ABTS); inhibitors: azide, 1,10-phenathrolin, pH optimum 4.5.

A. Assay with 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS)

Assay solutions

0.1 M potassium phosphate pH 6.0

0.02 M ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, diammonium salt, $M_r = 548.7$; 1.1 g in 0.1 M potassium phosphate pH 6.0)

H_2O_2 solution (~ 10 mM), dilute 0.06 ml 30% H_2O_2 in 50 ml 0.05 M potassium phosphate pH 7.0, the absorption against buffer at 240 nm should be 0.50 ± 0.01. Otherwise, adjust to this value with buffer or H_2O_2

Assay mixture and procedure

	Concentration (mM)
0.78 ml 0.1 M potassium phosphate pH 6.0	98
0.1 ml 0.02 M ABTS	2.0
0.1 ml H_2O_2 solution	1.0
0.02 ml enzyme solution	–

Follow absorption change at 414 nm, 25 °C, $\varepsilon_{414} = 24.6 \times 10^3$ l mol^{-1} cm^{-1}; 1 mol H_2O_2 oxidizes 2 mol ABTS.

B. Assay with Guaiacol

Assay solutions

0.1 M potassium phosphate pH 7.0

0.02 M guaiacol (2-methoxyphenol, catechol monomethylether, M_r = 124.1; 24.8 mg in 10 ml)

H_2O_2 solution (~10 mM), dilute 0.06 ml 30% H_2O_2 in 50 ml 0.05 M potassium phosphate pH 7.0; the absorption against buffer at 240 nm should be 0.50 ± 0.01, otherwise adjust to this value with buffer or H_2O_2

peroxidase solution (dilute concentrated peroxidase sample about 2000-fold in 0.1 M potassium phosphate pH 7.0)

Assay mixture

Components	*Concentration (mM)*
9.55 ml 0.1 M potassium phosphate pH 7.0	98
0.15 ml 0.02 M guaiacol	0.3

Procedure

0.97 ml assay mixture

0.02 ml peroxidase solution

Start by addition of

0.01 ml H_2O_2 solution	0.1 mM

Follow the absorption change by 436 nm, 25 °C, ε_{436}= 25501 · mol^{-1} mm^{-1}.

C. Assay with Dianisidine

Assay solutions

0.1 M potassium phosphate pH 6.0

0.03 M *o*-dianisidine (3,3′-dimethoxybenzidine dihydrochloride, M_r = 317.2; 95 mg in 10 ml methanol, *carcinogenic!*)

H_2O_2 solution (~10 mM), dilute 0.06 ml 30% H_2O_2 in 50 ml 0.05 M potassium phosphate pH 7.0; the absorption against buffer at 240 nm should be 0.50 ± 0.01, otherwise adjust to this value with buffer or H_2O_2

peroxidase solution (dilute concentrated peroxidase sample about 2000-fold in 0.1 M potassium phosphate pH 7.0)

Assay mixture

Components	*Concentration (mM)*
9.6 ml 0.1 M potassium phosphate pH 6.0	98
0.1 ml 0.03 M *o*-dianisidine	0.3

Procedure

0.97 ml assay mixture

0.02 ml peroxidase solution

Start by addition of

0.01 ml H_2O_2 solution 0.1 mM

Record the absorption at 436 nm, 25 °C, $\varepsilon_{436} = 8.3 \times 10^3$ l mol^{-1} cm^{-1}.

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3.3.1.33 Luciferase, EC 1.13.12.7

SN: Photinus-luciferin:oxygen oxidoreductase (decarboxylating, ATP-hydrolyzing), Photinus-luciferin 4-monooxygenase, firefly luciferase

$$\text{Photinus-luciferin} + \text{ATP} + O_2 \rightarrow$$
$$\text{oxidized photinus-luciferin} + CO_2 + \text{AMP} + PP_i + H_2O + \text{light}$$

Enzyme from *Photinus pyralis*: M_r 100 000, dimer; K_m (mM): 0.004 (ATP), 0.008 (luciferin), pH optimum 7.8.

The light intensity I is related to the Michaelis–Menten equation:

$$I = \frac{d(h\nu)}{dt} = \frac{V[\text{ATP}]}{K_m + [\text{ATP}]}$$

h is Planck's constant and ν the frequency of emitted light. At very low concentrations of ATP ($[\text{ATP}] \ll K_m$) the Michaelis–Menten equation reduces to

$$I = \text{const} \times [\text{ATP}];$$

const $= V K_m^{-1}$, the light intensity becomes proportional to [ATP].

Assay solutions

0.1 M Tris/acetate pH 7.75

10 µM ATP (disodium salt, trihydrate, $M_r = 605.2$; prepare a 10 mM solution with 60.5 mg in 10 ml, and dilute 10 µl in 10 ml 0.1 M Tris/acetate pH 7.75)

0.1 M $MgSO_4$ ($MgSO_4 \cdot 7H_2O$, $M_r = 246.5$; 246 mg in 10 ml H_2O)

1 mM D-luciferin (4,5-dihydro-[6-hydroxy-2-benzothiazolyl]-4-thiazole carboxylic acid, $M_r = 280.3$; sodium salt, $M_r = 302.3$; 3 mg in 10 ml 0.1 M Tris/acetate pH 7.75, store at −20 °C)

Luciferase (1 µg ml^{-1} in 0.1 M Tris/acetate pH 7.75)

Assay mixture and procedure

	Concentration
0.34 ml 0.1 M Tris/acetate pH 7.75	68 mM
0.05 ml 0.1 M $MgSO_4$	10 mM
0.05 ml 1 mM D-luciferin	0.1 mM
0.05 ml luciferase	50 ng
Start with addition of	
0.01 ml 10 µM ATP	0.2 µM

Read total light intensity (emission maximum at 562 nm) after 10 s (25 °C). Prepare a standard curve with ATP between 0.05 and 0.5 µM.

One enzyme unit is defined as the light output from a 50-µl assay mixture containing 5 pmol ATP and 7.5 nmol luciferin in glycine-Tris pH 7.6 at 25 °C. One light unit produces a biometer peak height equivalent to 0.02 µCi of ^{14}C in PPO/POPOP (2,5-diphenyloxazole/2,2′-p-phenylene-bis[5-phenyloxazole]) cocktail (before 1991, 1 unit was the amount of luciferase producing 1 nmol pyrophosphate/min at pH 7.7, 25 °C in the presence of 0.6 mM ATP and 0.1 mM D-luciferin).

References

DeLuca, M. and McElroy, W.D. (1978) *Meth. Enzymol.*, **57**, 3–15.
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3.3.2 Transferases, EC 2

3.3.2.1 Dihydrolipoamide Acetyltransferase, EC 2.3.1.12

SN: acetyl-CoA :enzyme N6-(dihydrolipoyl)lysine *S*-acetyltransferase, E2p

$$\text{hydroxyethyl ThDP-E1p} + \text{lipoyl E2p} \rightarrow \text{acetyl dihydrolipoyl-E2p} + \text{ThDP-E1p}$$

$$\text{acetyl dihydrolipoyl-E2p} + \text{CoA} \rightarrow \text{acetyl CoA} + \text{dihydrolipoyl-E2p}$$

In the cell, the enzyme exists only as E2p component of the pyruvate dehydrogenase complex (E1p, pyruvate dehydrogenase, EC 1.2.4.1, see Section 3.3.7.1); lipoic acid is a covalent bound cofactor; enzyme from *Escherichia coli*: $M_r = 86\,400$, *Azotobacter vinelandii*: K_m (mM) 0.022 (acetyl-CoA), 1.1 (dihydrolipoamide), pH optimum 8.

A. Spectrophotometric Assay

$$\text{CoA} + \text{acetylphosphate} \overset{\text{PTA}}{\rightleftharpoons} \text{acetyl-CoA} + P_i$$

$$\text{acetyl CoA} + \text{D,L-dihydrolipoamide} \overset{\text{E1p}}{\rightleftharpoons} \text{acetyl dihydrolipoamide} + \text{CoA}$$

Assay solutions

0.1 M Tris/HCl pH 7.6

0.01 M CoA (trilithium salt, $M_r = 785.4$; 24 mg in 3 ml)

1.0 M acetylphosphate (LiK salt, $M_r = 184.1$; 1.84 g in 10 ml)

phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8, 4000 IU ml^{-1})

0.2 M D,L-dihydrolipoamide (50 mg in 1.25 ml ethanol). The available oxidized form of D,L-lipoamide (D,L-6.8-thioctic acid amide, $M_r = 205.3$) can be reduced according to Reed, Leach, and Koike (1958)

- Suspend 2 g D,L-lipoamide at 0 °C in 40 ml methanol and 10 ml H_2O
- Add 2 g $NaBH_4$ successively during 2 h; the solution must become colorless, if necessary add more $NaBH_4$
- Acidify the solution is with 5 N HCl
- Extract the reagent three times with 20 ml chloroform
- Wash the collected chloroform phases three times with water
- Remove the solvent in the vacuum
- Dissolve the remaining dry powder in 5 ml hot benzene (or toluene)
- Add petroleum ether dropwise until a faint turbidity remains, crystals will be formed overnight at 4 °C. The crystallization procedure should be repeated two times, and the final product must be completely white

Assay mixture

Components	*Concentration*
9.38 ml 0.1 M Tris/HCl pH 7.6	94 mM
0.2 ml 0.2 M D,L-dihydrolipoamide	4.0 mM
0.1 ml 0.01 M CoA	0.1 mM
0.1 ml 1 M acetylphosphate	10 mM
0.02 ml phosphotransacetylase	4 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Follow the absorption change at 240 nm, 30 °C, in quartz cuvettes.

B. Stopped Assay

Assay solutions

0.1 M Tris/HCl pH 7.6

0.01 M CoA (trilithium salt, $M_r = 785.4$; 24 mg in 3 ml)

1.0 M acetylphosphate (LiK salt, $M_r = 184.1$; 1.84 g in 10 ml)

phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8, 4000 IU ml^{-1})

0.2 M D,L-dihydrolipoamide

1 N HCl

2 M hydroxylamine (mix equal volumes of 4 M hydroxylamine hydrochloride, $M_r = 69.49$, and 4 M KOH, $M_r = 56.11$)

$FeCl_3$ reagent:

- 100 ml 5% $FeCl_3$ (8.33 g $FeCl_3 \cdot 6H_2O$, $M_r = 270.3$, bring to 100 ml with 0.1 N HCl)
- 100 ml 12% TCA (trichloroacetic acid, $M_r = 163.4$)
- 100 ml 3 M HCl (mix 1 part 37% HCl with 3 parts H_2O)

Mix the three solutions together

Assay mixture

Components	*Concentration*
8.98 ml 0.1 M Tris/HCl pH 7.6	90 mM
0.2 ml 0.01 M CoA	0.2 mM
0.1 ml 1 M acetylphosphate	10 mM
0.5 ml 0.2 M D,L-dihydrolipoamide	10 mM
0.02 ml phosphotransacetylase	4 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Incubate for 15 min at 30 °C

0.1 ml 1 N HCl

5 min in a boiling water bath

Remove 0.4 ml and mix with

0.2 ml 2 M hydroxylamine

0.6 ml $FeCl_3$ reagent.

10 min at room temperature

Centrifuge for 5 min

Measure the absorption at 546 nm.

Calculation

The specific activity is expressed in special units, referring to 1 h of reaction time:

$$\text{specificactivity} = \frac{A_{546} \times 20 \times \text{assay volume (1.1 ml)} \times \text{dilution factor}}{\text{enzyme volume (ml)} \times \text{protein (mg ml}^{-1})}$$

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3.3.2.2 Fatty Acid Synthase, EC 2.3.1.85

SN: acyl-CoA:malonyl-CoA C-acyltransferase (decarboxylating, oxoacyl- and enoyl-reducing and thioester-hydrolyzing)

$$\text{acetyl CoA} + n\ \text{malonyl-CoA} + 2n\ \text{NADPH} + 2n\ \text{H}^+ \rightarrow$$
$$\text{CH}_3\text{-(CH}_2\text{-CH}_2)_n\text{-CO-CoA} + n\ \text{CoA} + n\text{CO}_2 + 2n\ \text{NADP}^+ + n\ \text{H}_2\text{O}$$
$$n = 6–8$$

Assay solutions

0.1 M potassium phosphate pH 6.5

0.1 M EDTA (ethylenediamine tetraacetic acid, $M_r = 292.2$; 292 mg in 10 ml)

0.1 M DTE (dithioerythritol, $M_r = 154.2$; 154 mg in 10 ml)

0.01 M acetyl-CoA (Li_3 salt, $M_r = 827.4$; 8.3 mg in 1 ml)

7 mM malonyl-CoA ($M_r = 877.3$, Li_4 salt; 6.1 mg ml^{-1})

0.01 M NADPH (Na_4 salt, $M_r = 833.4$, 83 mg in 10 ml)

2.4% BSA (240 mg in 10 ml)

Assay mixture

Components	*Concentration*
8.64 ml 0.1 M potassium phosphate pH 6.5	86 mM
0.25 ml 0.1 M EDTA	2.5 mM
0.25 ml 0.1 M DTE	2.5 mM
0.25 ml 2.4% BSA	0.06%
0.06 ml 0.01 M acetyl-CoA	0.06 mM
0.15 ml 0.01 NADPH	0.15 mM

Procedure

0.96 ml assay mixture

0.02 ml enzyme solution

Observe absorption decrease at 340 nm, 25 °C, for 3–4 min

Start the reaction by addition of

0.02 ml 7 mM malonyl-CoA	0.14 mM

Follow the absorption decrease at 340 nm, 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

Reference

Lynen, F. (1969) *Meth. Enzymol.*, **14**, 17–33.

3.3.2.3 Phosphorylase a, EC 2.4.1.1

SN: 1.4-α-D-glucan:orthophosphate-α-D-glucosytransferase, glycogenphosphorylase

The enzyme splits α-1,4-glucosidic bonds in polysaccharides:

$$(\text{glucose})_n + P_i \rightarrow (\text{glucose})_{n-1} + \text{glucose-1-P}$$

Enzyme from *Zea mays*, homodimer, M_r 2 × 112 000, K_m (mM): 0.25 (glycogen), 0.028 (amylopectine) 1.5 (glucose 1-phosphate), pH optimum 5.5–6.5.

For the assay, the reaction is coupled to phosphoglucomutase (EC 5.4.2.2)

$$\text{glucose-1-P} \rightarrow \text{glucose-6-P}$$

and glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

$$\text{glucose-6-P} + \text{NADP}^+ \rightarrow \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

Assay solutions

0.05 M potassium phosphate pH 6.8

0.01 M EDTA (ethylenediaminetetraacetic acid, $M_r = 292.3$, 29.2 mg in 10 ml)

glycogen (60 mg in 10 ml)

1 mM glucose 1,6-bisphosphate (tetracyclohexylammonium salt, $M_r = 736.8$; 7.4 mg in 10 ml)

0.01 M NADP ($M_r = 787.4$; 79 mg in 10 ml)

1 M $MgCl_2$ (hexahydrate, $M_r = 203.3$; 2g in 10 ml)

phosphoglucomutase 100 IU mg^{-1} (solution of 100 IU ml^{-1})

Glucose 6-phosphate dehydrogenase, ~400 IU mg^{-1} (solution of 300 IU ml^{-1})

Both enzyme preparations must be free of ammonium salt. If they are present as ammonium sulfate suspensions, they must be dialyzed against 0.05 M potassium phosphate pH 6.8 before use.

Assay mixture

Components	*Concentration*
8.5 ml 0.05 M potassium phosphate pH 6.8	42.5 mM
0.1 ml 0.01 M EDTA	0.1 mM
0.3 ml glycogen	5.0 mM
0.05 ml 1 mM glucose-1,6-bisphosphate	5 µM
0.4 ml 0.01 M NADP	0.4 mM
0.15 ml 1 M $MgCl_2$	15 mM
0.1 ml phosphoglucomutase	1 U/ml
0.2 ml glucose-6-phosphate dehydrogenase	6 U/ml

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

The absorption increase at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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3.3.2.4 Aspartate Transaminase, EC 2.6.1.1

SN: L-aspartate:2-oxoglutarate aminotransferase, AAT, aspartate amidotransferase, glutamic-aspartic transaminase, glutamate-oxalacetate transaminase, transaminase A, 2-oxoglutarate-glutamate aminotransferase.

$$\text{L-aspartate} + \text{2-oxoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{L-glutamate}$$

Mammalian enzyme (rat, cytoplasm,) M_r 44 600, monomer; cofactor pyridoxal 5-phosphate; K_m (mM): 0.35 (L-aspartate), 1.25 (2-oxoglutarate); enzyme from *Escherichia coli*: M_r 82 000, dimer, K_m (mM): 4.4 (L-aspartate), 0.07 (2-oxoglutarate), 0.37 (2-oxaloacetate), 0.00025 (pyridoxalphosphate), pH optimum 8.0.

Assay reaction coupled with malate dehydrogenase (EC 1.1.1.37):

$$\text{oxaloactetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{malate} + \text{NAD}^+$$

Assay solutions

0.1 M Tris/HCl pH 7.4

0.2 M 2-oxoglutarate (sodium salt, $M_r = 168.1$; 3.4 g in 10 ml H_2O)

0.2 M L-aspartate (sodium salt, hydrate, $M_r = 155.1$, 3.1 g in 10 ml H_2O)

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg in 10 ml H_2O)

Malate dehydrogenase (1000 IU mg^{-1})

Assay mixture

Components	*Concentration*
9.0 ml 0.1 M Tris/HCl pH 7.4	90 mM
0.35 ml 0.2 M 2-oxoglutarate	7 mM
0.35 ml 0.2 M L-aspartate	7 mM
0.03 ml 0.01 M NADH	0.03 mM
0.07 ml MDH	7 IU

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

The absorption decrease at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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Mavrides, C. and Orr, W. (1975) *J. Biol. Chem.*, **250**, 4128–4133.
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3.3.2.5 Alanine Transaminase, EC 2.6.1.2

SN: L-alanine:2-oxoglutarate aminotransferase, alanine aminotransferase, glutamic:alanine transaminase, glutamine:pyruvate transaminase

$$\text{L-alanine} + \text{2-oxoglutarate} \rightleftharpoons \text{pyruvate} + \text{L-glutamate}$$

Mammalian enzyme (rat liver): M_r 18 300, monomer, cofactor pyridoxal 5-phosphate, K_m (mM): 0.12 (2-oxoglutarate), 0.5 (L-alanine), 0.4 (pyruvate), pH optimum 8.5. *Clamydomonas reinhardtii*: M_r 105 000, homodimer; K_m (mM) 0.05 (2-oxoglutarate), 0.52 (L-glutamate), 0.24 (pyruvate), 2.7 (L-alanine), pH optimum 7.3. The assay is coupled with the lactate dehydrogenase reaction:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{L-lactate} + \text{NAD}^+$$

Assay solutions

0.1 M Tris/HCl, pH 7.3

0.5 M L-alanine (M_r 89.1, 4.46 g in 100 ml 0.1 M Tris/HCl, pH 7.3)

0.01 M NADH (disodium salt, M_r = 709.4; 71 mg in 10 ml H_2O)

4 mM PLP (pyridoxal 5-phosphate, $M_r = 247.1$; 10 mg in 10 ml H_2O)

0.2 M 2-oxoglutarate (sodium salt, $M_r = 168.1$; 3.4 g in 10 ml H_2O)

LDH (lactate dehydrogenase, commercial products contain about 500 IU mg^{-1}, prepare a solution with 120 IU ml^{-1} in 0.1 M Tris/HCl, pH 7.3)

Assay mixture

Components	*Concentration*
8.5 ml 0.5 M L-alanine	0.425 M
0.2 ml 0.01 M NADH	0.2 mM
0.25 ml 4 mM PLP	0.1 mM
0.75 ml 0.2 M 2-oxoglutarate	15 mM
0.1 ml LDH	1.2 IU

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

The absorption decrease at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

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3.3.2.6 Tyrosine Transaminase, EC 2.6.1.5, Tryptophan Transaminase, EC 2.6.1.27, Phenylalanine Transaminase, EC 2.6.1.58

The following assay is similar for the three transaminases, only the respective amino acid must be exchanged

Tyrosine transaminase, SN: L-tyrosine:2-oxoglutarate aminotransferase; TAT, tyrosine aminotransferase

L-tyrosine + 2-oxoglutarate ⇌ 4-hydroxyphenylpyruvate + L-glutamate

Mammalian enzyme (rat): M_r 110 500, dimer (2 × 50 000), cofactor: pyridoxal 5-phosphate; K_m (mM): 0.4 (2-oxoglutarate), 0.5 (pyruvate), 1.2 (L-tyrosine), 2.2 (L-glutamate); pH optimum 7.7.

Tryptophan transaminase, SN: L-tryptophan:2-oxoglutarate aminotransferase, Tam 1

$$\text{L-tryptophan} + \text{2-oxoglutarate} \rightleftharpoons \text{(indol-3-yl)pyruvate} + \text{L-glutamate}$$

Mammalian enzyme (porcine): M_r 55 000, cofactor: pyridoxal 5-phosphate; K_m (mM): 0.74 (2-oxoglutarate), 15 (tryptophan): pH optimum 8.

Phenylalanine (histidine) transaminase, SN: L-phenylalanine:pyruvate aminotransferase, histidine:pyruvate transaminase

$$\text{L-phenylalanine} + \text{pyruvate} \rightleftharpoons \text{phenylpyruvate} + \text{L-alanine}$$

Mammalian enzyme (mouse): M_r 75 000, dimer, cofactor: pyridoxal 5-phosphate; K_m (mM): 6.5 (histidine), 21 (pyruvate); pH optimum 9.

Assay solutions

0.2 M potassium phosphate pH 7.3

40 mM L-tyrosine ($M_r = 181.2$, 72.4 mg in 10 ml 0.1 N HCl)

40 mM tryptophan ($M_r = 204.2$, 81.6 mg in 10 ml H_2O)

40 mM L-phenylalanine ($M_r = 165.2$; 66 mg in 10 ml H_2O)

0.2 M 2-oxoglutarate (sodium salt, $M_r = 168.1$; 3.4 g in 10 ml 0.2 M potassium phosphate pH 7.3)

4 mM PLP (pyridoxal 5-phosphate, $M_r = 247.1$; 10 mg in 10 ml H_2O)

10 N NaOH

Assay mixture

Components	*Concentration (mM)*
7.7 ml 0.2 M potassium phosphate pH 7.3	164
1.5 ml 40 mM L-tyrosine (respectively L-phenylalanine L-tryptophan)	6
0.5 ml 0.2 M 2-oxoglutarate	10
0.1 ml 5 mM PLP	0.05

Procedure

1 ml assay mixture

0.02 ml enzyme sample

10 min, 37 °C

0.2 ml 10 N NaOH

30 min, room temperature

Measure the absorption at the respective wavelength:

Tyrosine transaminase: 331 nm, $\varepsilon_{331} = 19.9 \times 10^3\ \mathrm{l\ mol^{-1}\ cm^{-1}}$

Tryptophan transaminase: 335 nm, $\varepsilon_{335} = 10 \times 10^3\ \mathrm{l\ mol^{-1}\ cm^{-1}}$

Tyrosine transaminase: 315 nm, $\varepsilon_{315} = 17.5 \times 10^3\ \mathrm{l\ mol^{-1}\ cm^{-1}}$

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3.3.2.7 Hexokinase, EC 2.7.1.1

SN: ATP:D-hexose-6-phosphotransferase, HK

$$\text{D-hexose} + \text{ATP} \rightarrow \text{D-hexose-6-phosphate} + \text{ADP}$$

Hexokinase exists in two forms: in most cells it shows high affinity for glucose (K_m 0.057 mM), while the liver enzyme (*glucokinase*) has low affinity (K_m 5.5 mM); human enzyme: M_r 100 000, homodimer, Mg^{2+} as cofactor, K_m (mM) 0.56 (ATP), pH optimum 7.8.

There exists no simple photometric assay for this reaction. Instead, the phosphorylation of glucose may be followed by different methods, like phosphate determination or labeling with ATP^{32}. For these assays, glucose-6-phosphate (G6P) must be completely separated from ATP and ADP by chromatographic methods, especially the HPLC technique. Photometric detection can be achieved by coupling with glucose-6-phosphate dehydrogenase (G6PDH):

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\underline{\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+}$$

$$\text{D-glucose} + \text{ATP} + \text{NADP}^+ \rightarrow \text{6-phosphogluconate} + \text{ADP} + \text{NADPH} + \text{H}^+$$

When first performing this assay the G6PDH reaction should be tested separately (cf. assay in Section 3.3.1.13) and thereafter, G6P should be replaced by glucose and ATP to follow the complete reaction sequence in the presence of hexokinase.

Assay solutions

100 mM triethanolamine · HCl/NaOH pH 7.6 (triethanolamine · HCl, M_r = 185.7; 18.6 g adjusted with 1 M NaOH, fill up to 1 l)

1.0 M D-glucose (M_r = 180.2; 18 g in 100 ml)

0.1 M ATP (disodium salt, trihydrate, M_r = 605.2; 605 mg in 10 ml)

0.1 M $MgCl_2$ (hexahydrate, M_r = 203.3; 203 mg in 10 ml)

0.01 M NADP (disodium salt, M_r = 787.4; 79 mg in 10 ml)

Glucose-6-phosphate dehydrogenase (commercial products have 100–500 IU mg^{-1}, prepare a dilution ~5 IU 0.1 ml^{-1})

Assay mixture

Components	*Concentration*
5.8 ml 0.1 mM triethanolamine · HCl/NaOH pH 7.6	58 mM
0.6 ml 0.1 mM $MgCl_2$	6.0 mM
2.0 ml 1.0 M D-glucose	200 mM
1.0 ml 10 mM NADP	1.0 mM
0.3 ml 0.1 M ATP	3.0 mM
0.1 ml glucose-6-phosphate dehydrogenase	0.5 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

The absorption increase at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

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Vandercammen, A. and Van Schaftingen, E. (1991) *Eur. J. Biochem*, **200**, 545–551.

3.3.2.8 Pyruvate Kinase, EC 2.7.1.40

SN: ATP:pyruvate 2-O-phosphotransferase, PK, phosphoenolpyruvate kinase

$$\text{phosphoenolpyruvate} + \text{ADP} \overset{\text{PK}}{\rightleftharpoons} \text{pyruvate} + \text{ATP}$$

Mammalian enzyme (porcine), M_r 230 000, homotetramer (4 × 60 000), cofactor: Mg^{2+}, K_m (mM): 0.4 (ADP), 0.3 (phosphoenolpyruvate), pH optimum 7.4; exists in different isoenzymes: L (liver), M (muscle), A (other tissues). According to other kinases, the reaction can directly be tested with P^{32}; to avoid radioactivity, a coupled photometric assay with the LDH is described:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{L-lactate} + \text{NAD}^+$$

Assay solutions

0.1 M triethanolamine · HCl/KOH pH 7.6 (triethanolamine · HCl, M_r = 185.7; dissolve 18.6 g in 800 ml H_2O, adjust to pH 7.6 with 1 N KOH and fill up to1 l)

0.5 M KCl (M_r = 74.6; 373 mg in 10 ml)

0.25 M $MgCl_2$ (hexahydrate, M_r = 203.3; 508 mg in 10 ml)

0.01 M phosphoenolpyruvate (PEP, tricyclohexylammonium salt, M_r = 465.6; 46.6 mg in 10 ml)

0.1 M ADP (disodium salt, M_r = 471.2; 471 mg in 10 ml)

0.01 M NADH (disodium salt, M_r = 709.4; 71 mg in 10 ml)

LDH (commercial products contain about 500 IU mg^{-1}, prepare a solution with 150 IU 0.1 ml^{-1})

Assay mixture

Components	*Concentration*
8.2 ml 0.1 M triethanolamine/KOH pH 7.6	82 mM
0.2 ml 0.5 M KOH	10 mM
0.1 ml 0.25 M $MgCl_2$	2.5 mM
0.5 ml 0.01 M PEP	0.5 mM
0.5 ml 0.1 M ADP	5.0 mM
0.2 ml 0.01 M NADH	0.2 mM
0.1 ml LDH	15 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

The absorption decrease at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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3.3.2.9 Acetate Kinase, EC 2.7.2.1

SN: ATP:acetate phosphotransferase, AK

$$\text{acetate} + \text{ATP} \rightarrow \text{acetyl phosphate} + \text{ADP}$$

Enzyme from *Escherichia coli*: dimer, M_r 70 000, K_m (mM): 7 (acetate), 0.16 (acetyl-phosphate), 0.07 (ATP), 0.05 (ADP), pH optimum 7.5. For the assay, the reaction is coupled with pyruvate kinase (PK) and lactate dehydrogenase (LDH):

$$\text{phosphoenolpyruvate} + \text{ADP} \overset{\text{PK}}{\rightleftharpoons} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{L-lactate} + \text{NAD}^+$$

Assay solutions

0.1 M triethanolamine · HCl/NaOH pH 7.6 (triethanolamine · HCl, $M_r = 185.7$; 18.6 g adjusted with 1 M NaOH, fill up to 1 l)

2 M sodium acetate ($M_r = 82.0$; 16.4 g in 100 ml)

0.1 M ATP (disodium salt, trihydrate, $M_r = 605.2$; 605 mg in 10 ml)

0.1 M $MgCl_2$ (hexahydrate, $M_r = 203.3$; 203 mg in 10 ml)

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg in 10 ml)

0.01 M phosphoenol pyruvate (PEP, tricyclohexylammonium salt, $M_r = 465.6$; 46.6 mg in 10 ml)

LDH, 20 mg ml^{-1}, about 500 IU mg^{-1}

PK, 10 mg ml^{-1}, 500 IU mg^{-1}

Assay mixture

Components	*Concentration*
6.3 ml 0.1 M triethanolamine/NaOH pH 7.6	63 mM
1.5 ml 2.0 M sodium acetate	0.3 M
0.5 ml 0.1 M ATP	5.0 mM
0.15 ml 0.1 M $MgCl_2$	1.5 mM
1.0 ml 0.01 M PEP	1.0 mM
0.3 ml 0.01 M NADP	0.3 mM
0.025 ml LDH	25 IU ml^{-1}
0.015 ml PK	7.5 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

The absorption decrease at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, vol. 2, Verlag Chemie, Weinheim, pp. 127–128.

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3.3.2.10 Phosphoglycerate Kinase, EC 2.7.2.3

ATP:3-phospho-D-glycerate 1-phosphotransferase, PGK

$$\text{3-phospho-D-glycerate} + \text{ATP} \overset{\text{PGK}}{\rightleftharpoons} \text{1,3-diphosphoglycerate} + \text{ADP}$$

Mammalian enzyme (human): M_r 50 000, Mg^{2+} as cofactor, K_m (mM): 0.1 (3-phospho-D-glycerate), 0.005 (1,3-diphosphoglycerate), 1.2 (ADP), 1.1 (ATP), pH optimum 7.5. For the assay the reaction is coupled with glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

$$\text{1,3-diphoshoglycerate} + \text{NADH} + \text{H}^+ \overset{\text{GAPDH}}{\rightleftharpoons} \text{D-glyceraldehyde-3-phosphate} + \text{NAD}^+$$

Assay solutions

0.1 M triethanolamine/NaOH pH 7.6 (triethanolamine · HCl, M_r = 185.7; dissolve 18.6 g in 800 ml H_2O, adjust to pH 7.6 with 1 M NaOH, replenish to 1 l)

0.1 M glycerate-3-phosphate (3-phosphoglyceric acid, disodium salt, M_r = 230.0; 230 mg in 10 ml)

0.1 M ATP (disodium salt, trihydrate, M_r = 605.2; 605 mg in 10 ml)

0.01 M NADH (disodium salt, M_r = 709.4; 71 mg in 10 ml)

0.1 M EDTA (ethylenediaminetetraacetic acid, M_r = 292.2; 292 mg in 10 ml)

0.1 M magnesium sulfate ($MgSO_4$ $7H_2O$, M_r = 246.5; 247 mg in10 ml)

3-phosphoglycerate kinase, from yeast (~2 000 IU ml^{-1})

GAPDH (glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, 800 IU ml^{-1})

Assay mixture

Components	*Concentration*
8.67 ml 0.1 M triethanolamine/NaOH pH 7.6	87 mM
0.5 ml 0.1 M glycerate-3-phosphate	5.0 mM
0.1 ml 0.1 M ATP	1.0 mM
0.2 ml 0.01 M NADH	0.2 mM
0.1 ml 0.1 M EDTA	1.0 mM
0.2 ml 0.1 M magnesium sulfate	2.0 mM
0.03 ml GAPDH	2.4 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme solution

Follow the absorption decrease at 340 nm at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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3.3.2.11 Aspartokinase, EC 2.7.2.4

SN : ATP: aspartate 4-phosphotransferase, AK

$$\text{L-aspartate} + \text{ATP} \rightarrow \text{4-phospho-L-aspartate} + \text{ADP}$$

Allosteric enzyme in *Escherichia coli*, fused mitochondrial homoserine dehydrogenase (cf. assay in Section 3.3.1.5) to aspartokinase–homoserine dehydrogenase I, M_r 360 000, homotetramer (4 × 84 000, dimer if separate from homoserine dehydrogenase), requires Mg^{2+}, K_m (mM): 0.18 (ATP), 0.51 (L-aspartate), 16 (L-asparagine), pH optimum 7. For the assay the reaction is coupled to pyruvate kinase (PK) and lactate dehydrogenase (LDH)

$$\text{ADP} + \text{phosphoenolpyruvate} \overset{\text{PK}}{\rightleftharpoons} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{lactate} + \text{NAD}^+$$

Assay solutions

0.1 M HEPES/NaOH, 0.1 M KCl pH 8.0 (*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, HEPES, M_r = 238.3; dissolve 23.8 g and 7.6 g KCl (M_r = 74.6) in 600 ml H_2O, adjust to pH 8.0 with 0.1 M NaOH and bring to 1 l with H_2O)

0.36 M magnesium acetate ($Mg^{2+}C_4H_6O_4 \cdot 4H_2O$, M_r = 214.5, 772.2 mg in 10 ml H_2O)

0.01 M PEP (phosphoenolpyruvate, tricyclohexylammonium salt, M_r = 465.6; 46.6 mg in 10 ml)

0.1 M ATP (disodium salt, trihydrate, M_r = 605.2; 605 mg in 10 ml H_2O)

0.01 M NADH (disodium salt, M_r = 709.4; 71 mg in 10 ml H_2O)

LDH (commercial products contain about 500 IU mg^{-1}, prepare a solution with 400 IU in 0.2 ml buffer)

pyruvate kinase (commercially available, prepare a solution with 200 IU in 0.2 ml buffer)

0.1 M sodium aspartate (L-aspartic acid, sodium salt, $NaC_4H_5NO_4 \cdot H_2O$, M_r = 173.1, 173 mg in 10 ml H_2O)

Assay mixture

Components	*Concentration*
9 ml 0.1 M HEPES/NaOH, 0.1 M KCl pH 8.0	90 mM
0.1 ml 0.36 M magnesium acetate	3.6 mM
0.7 ml 0.01 M PEP	0.7 mM
0.18 ml 0.1 M ATP	1.8 mM
0.1 ml 0.01 M NADH	0.1 mM
1.0 ml 0.1 M sodium aspartate	10 mM
0.2 ml LDH	40 IU ml^{-1}
0.2 ml PK	20 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample (about 10 µg)

Follow the absorption decrease at 340 nm at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

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3.3.3 Hydrolases, EC 3

3.3.3.1 Lipase, EC 3.1.1.3

SN: triacylglycerol acyl-hydrolase, triacylglycerol lipase

$$\text{triacylglycerol} + H_2O \rightarrow \text{diacylglycerol} + \text{long-chain fatty acid}$$

Pancreatic enzyme (human, porcine): $M_r = 44\,000$, monomer, K_m (mM): 0.86 (1-olein), 0.36 (triolein), 1,2 (1-caprin); pH optimum 8–9. Two different assays are described. The first one is based on the formation of free carboxyl groups due to the cleavage of the triglyceride, which causes a decrease in the pH. This is detected by a pH-stat connected to an automatic burette (see Section 2.3.2.2), which keeps the pH constant. If a pH-stat is not available, a pH meter may be used but care must be taken that the pH remains constant

during the assay. The fluorometric assay observes the release of the fluorescent residue from a synthetic substrate.

A. Assay with pH Stat (Autotitrator)

Assay solutions

3.0 M sodium chloride (NaCl, $M_r = 58.4$; 17.5 g in 100 ml)

75 mM calcium chloride ($CaCl_2 \cdot 6H_2O$; $M_r = 219.1$; 1.62 g in 100 ml)

5 mM calcium chloride (dilute the 75 mM calcium chloride solution 15-fold)

0.5% BSA (bovine serum albumine, 0.5 g in 100 ml H_2O)

27 mM sodium taurocholate ($M_r = 537.7$; 1.45 g in 100 ml)

Olive oil–gum arabic emulsion: Dissolve 16.5 g gum arabic in 130 ml H_2O and fill up to 165 ml. Add 20 ml olive oil and 15 g crushed ice, treat in a blender at low speed until an emulsion is formed. Filter through glass wool

Lipase (dissolve to 1 mg ml^{-1} in 5 mM calcium chloride, dilute further for assay in 5 mM calcium chloride)

Use 0.01 M NaOH standard solution as titrant for the autotitrator

Assay mixture and procedure

5 ml olive oil–gum arabic emulsion

5 ml H_2O

2 ml 3.0 M NaCl

1 ml 75 mM calcium chloride

2 ml 0.5% BSA for yeast lipase or 27 mM sodium taurocholate for porcine lipase

---

15 ml, adjust to pH 8.0

Blank: Record the volume per unit time of the titrant added by the auto-titrator for about 3 min

Sample: Add an appropriate amount of diluted enzyme (according to the activity) to the assay mixture and record the volume per unit time of the titrant added by the autotitrator for about 5 min

Calculation

One unit releases 1 µmol fatty acid/min from emulsified olive oil at 25 °C and pH 8.0.

B. Fluorimetric Assay This assay is also applicable for chymotrypsin, cholinesterase, and acylase. In case of acetylcholinesterase, diacetylfluorescein instead of dibutyrylfluorescein should be used as substrate.

$$\underset{\text{nonfluorescent}}{\text{dibutyrylfluorescein}} + 2H_2O \rightarrow \underset{\text{fluorescent}}{\text{fluorescein}} + 2\,\text{butyric acid}$$

Assay solutions

0.1 M Tris/HCl pH 8.0

0.05 mM substrate solution (dibutyrylfluorescein, $M_r = 472.5$; dissolved 2.36 g in 5 ml ethyleneglycol–monomethylether (methylcellosolve) and 95 ml 0.1 M Tris/HCl pH 8.0)

Procedure

To 2.9 ml of the substrate solution, 0.1 ml of the enzyme sample is added and the fluorescence, excited at 470 nm, is measured at 510 nm.

References

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3.3.3.2 Phospholipase A_2, EC 3.1.1.4

SN: phosphatidylcholine 2-acylhydrolase, lecithinase A

$$\text{phosphatidylcholine} + H_2O \rightarrow \text{1-acylglycerophosphocholine} + \text{carboxylate}$$

Mammalian enzyme (human, porcine), cofactor Ca^{2+}, monomer M_r 14 000, K_m (mM): 8.3 (lecithin), 0.1 (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine), 2(1,2-dipalmitoyl-phosphatidylcholine), EDTA inhibits, pH optimum 8. For the following pH-stat assay preparation of vesicles is required.

Assay solutions and reagents

1,2-dipalmitoylphosphatitylcholine (DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine, $M_r = 734.0$)

50 mM KCl ($M_r = 74.5$; 0.37 g in 100 ml H_2O)

0.1 M $CaCl_2$ ($M_r = 111$; 1.11 g in 100 ml H_2O)

Enzyme solution (~0.2 mg ml^{-1}, pH 8.0)

Preparation of vesicles

Dilute individual phospholipids or mixtures in chloroform

Lyophilize to dryness

Suspend in 2–8 ml 50 mM KCl

Sonicate under nitrogen in a glass tube with flat bottom (the clearance to the tube walls should not exceed 0.5 cm) together for 30 min, after every 3 min of sonication interrupt for 1 min for cooling

Centrifuge at 100 000 × *g* for 30 min in an ultracetrifuge

Centrifuge the supernatant again at 159 000 × *g* for 3 h to obtain a homogeneous vesicle preparation

Procedure

1.75 ml of the vesicle preparation

0.2 ml 0.1 M $CaCl_2$ solution

Adjust to pH 8.0

0.05 ml enzyme solution

Record the volume per unit time of the titrant added by the autotitrator.

References

Barenholz, Y. *et al.* (1977) *Biochemistry*, **16**, 2806–2810.
Dennis, E.A. (1983) in *The Enzymes*, 3rd edn, vol. 16 (ed. P.D. Boyer), Academic Press, New York, pp. 307–353.
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3.3.3.3 Acetylcholine Esterase, EC 3.1.1.7

SN: acetylcholine acetylhydrolase, AChE

$$\text{acetylcholine} + H_2O \rightarrow \text{choline} + \text{acetate}$$

Mammalian enzyme (mouse): M_r 66 000, monomer, K_m (mM): 0.046 (acetylcholine), pH optimum 8.0. For assays, see also choline esterase (assay in Section 3.3.3.2) and lipase (assay in Section 3.3.3.1). An assay for a pH-stat is described, the amount of base required to neutralize the acetic acid should be determined. If a pH-stat is not available, a pH meter may be used but care must be taken that the pH remains constant during the assay.

Assay solutions

0.02 M potassium phosphate pH 7.0

5 mM acetylcholine chloride ($M_r = 181.7$; 91 mg in 100 ml)

0.01% gelatin, containing 0.2 M sodium chloride and 0.04 M magnesium chloride (0.1 g gelatin, 11.7 g NaCl, 8.1 g $MgCl_2 \cdot 6H_2O$ in 1 l H_2O)

0.01 M sodium hydroxide, standard solution as titrant for the autotitrator

Acetylcholinesterase, dilute in 0.02 M potassium phosphate pH 7.0 to about 20 IU ml^{-1}

Assay mixture and procedure

	Concentration
7.5 ml 5 mM acetylcholine	2.5 mM
7.5 ml 0.01% gelatin-salt solution	0.005%
15 ml, adjust to pH 7.0; 25 °C	

Blank: record for some minutes the volume of 0.01 M sodium hydroxide needed per unit time to maintain a pH of 7.0

Sample: add enzyme solution (0.1–1 ml) and record for about five min, the volume of 0.01 M sodium hydroxide needed per unit time to maintain a pH of 7.0

Calculation

One unit hydrolyzes 1 µmol acetylcholine/min at pH 7.0, 25 °C.

$$\text{unit mg}^{-1} = \frac{\text{mL base min}^{-1} \times \text{base normality} \times 1000}{\text{mg enzyme in reaction mixture}}$$

References

Boyd, A.E. *et al.* (2000) *J. Biol. Chem.*, **275**, 22401–22408.
Froede, H.C. and Wilson, I.B. (1971) in *The Enzymes*, 3rd edn, vol. 5 (ed. P.D. Boyer), Academic Press, New York, pp. 87–114.
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Reed, D.L., Goto, K., and Wang, C.H. (1966) *Anal. Biochem.*, **16**, 59–64.

3.3.3.4 Choline Esterase, EC 3.1.1.8

SN: acylcholine acylhydrolase, pseudocholine esterase, butyrylcholine esterase, ButChE

$$\text{acylcholine} + H_2O \rightarrow \text{choline} + \text{carboxylate}$$

Mammalian enzyme (human): 85 000, monomer; K_m (mM) 0.018 (butyrylthiocholine), 0.05 (acetylthiocholine), 0.003 (benzoylcholine), 0.08 (acetylcholine), pH optimum 8.0. The enzyme hydrolyzes butyrylcholine 2.5 times faster than acetylcholine. The assay is performed preferentially with a pH-stat or a pH meter determining the amount of base required to neutralize the acetic acid. Besides, a colorimetric assay is described. Assays for acetylcholine esterase (assay in Section 3.3.3.3) and lipase (assay in Section 3.3.3.1) are principally applicable.

A. pH-Stat Assay

Assay solutions

0.02 M Tris/HCl pH 7.4

2.2 M acetylcholine chloride ($M_r = 181.7$; 40 g in 100 ml^{-1}, or butyrylcholine iodide, $M_r = 301.2$; 66 g in 100 ml^{-1})

0.2 M magnesium chloride ($MgCl \cdot 6H_2O$; $M_r = 203.3$; 4.1 g in 100 ml H_2O)

0.01 M sodium hydroxide, standard solution as titrant to fill the burette of the autotitrator

Cholinesterase, dissolve 1 mg in 1 ml^{-1} H_2O

Assay mixture and procedure

	Concentration
7.0 ml H_2O	–
3.0 ml 0.2 M magnesium chloride	43 mM
3.0 ml 0.02 M Tris/HCl pH 7.4	4.3 mM
1 ml 2.2 M acetylcholine chloride	157 mM
14 ml, adjust to pH 7.4; 25 °C	–

Blank: record for some minutes the volume of 0.01 M sodium hydroxide needed per unit time to maintain a pH of 7.4

Sample: add enzyme solution (e.g., 0.1–1 ml) and record for about 5 min the volume of 0.01 M sodium hydroxide needed per unit time to maintain a pH of 7.4

Calculation

One unit hydrolyzes 1 µmol acetylcholine/min at pH 7.0, 25 °C.

$$\text{unit mg}^{-1} = \frac{\text{ml base min}^{-1} \times \text{base normality} \times 1000}{\text{mg enzyme in reaction mixture}}$$

B. Colorimetric Assay

indophenyl acetate → indophenol + acetic acid

The color changes from red to blue.

Assay solutions

0.1 M potassium phosphate pH 8.0

0.62 mM indophenyl acetate (M_r = 241.2; 0.375 g indophenyl acetate in 25 ml ethanol)

Procedure

	Concentration
0.68 ml 0.1 M potassium phosphate pH 8.0	68 mM
0.3 ml indophenyl acetate solution	0.19 mM
0.02 ml enzyme solution	–

Determine the absorption at 625 nm in comparison to a calibration curve at 25 °C.

References

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Masson, P. *et al.* (2004) *Eur. J. Biochem.*, **271**, 220–234.
Mehrani, H. (2004) *Proc. Biochem.*, **39**, 877–882.
Nachmanson, D.W. and Wilson, J.B. (1951) *Adv. Enzymol.*, **12**, 259–339.

3.3.3.5 S-Formylglutathione Hydrolase, EC 3.1.2.12

SN: *S*-Formylglutathione hydrolase

$$S\text{-formylglutathione} + H_2O \rightarrow \text{glutathione} + \text{formate}$$

Enzyme from human liver: M_r 58 200, dimer K_m (mM): 0.29 (*S*-formylglutathione). 0.12 (*S*-acetylglutathione), pH optimum 6.9

Assay solutions

0.1 M Tris/HCl 7.4

10 mM DTNB (5,5′-dithio-bis(2-nitrobenzoic acid), Ellman's reagent, M_r = 396.3; 39.6 mg in 10 ml)

10 mM *S*-formylglutathione, (M_r = 335.3, 33.5 mg in 10 ml)

Assay mixture

Components	*Concentration (mM)*
9.45 ml 0.1 M Tris/HCl 7.4	95
0.15 ml 10 mM DTNB	0.15
0.2 ml *S*-formylglutathione	0.2

Procedure

0.98 assay mixture

0.02 enzyme sample

Follow the absorption increase at 412 nm, 25 °C, ε_{412} = 14 150 l mol^{-1} cm^{-1}.

References

Baylin, S.B. and Margolis, S. (1975) *Biochim. Biophys. Acta*, **397**, 294–306.
Uolila, L. and Koivusola, M. (1974) *J. Biol. Chem.*, **249**, 7664–7672.

3.3.3.6 Alkaline Phosphatase, EC 3.1.3.1

SN: phosphate monoester phosphohydrolase (alkaline optimum)

$$\text{phosphate monoester} + H_2O \rightarrow \text{alcohol} + P_i$$

Mammalian enzyme (human) dimer, M_r 158 000 (2 × 75 000), cofactor: Zn^{2+}, Mg^{2+}, K_m (mM): 2.5 (2′-AMP), 0.8 (*p*-nitrophenylphosphate, human), 0.0078 (*Escherichia coli*), phosphate inhibits, pH optimum 10.5. Alkaline phosphatase is frequently applied as a conjugate to antibodies, for example in Western blots and ELISA. Apart from the assays presented below, the cleavage of *o*-carboxyphenyl phosphate described for the acid phosphatase (assay in Section 3.3.3.7) can be observed in an alkaline medium.

A. Mammalian Alkaline Phosphatase

$$p\text{-nitrophenylphosphate} + H_2O \rightarrow p\text{-nitrophenol} + P_i$$

Assay solutions

0.1 M glycine/KOH pH 10.5, 1 mM Mg^{2+}, 0.1 mM Zn^{2+} (dissolve 7.5 g glycine, 203 mg $MgCl_2$ $6H_2O$, and 14 mg $ZnCl_2$ in 750 ml H_2O; adjust to pH 10.5 with 1 N KOH and fill up to 1 l with H_2O)

0.5 M *p*-nitrophenylphosphate (disodium salt, hexahydrate, $M_r = 371.1$; 1.86 g in 10 ml)

Procedure

	Concentration
0.97 ml 0.1 M glycine/KOH pH 10.5	97 mM
0.01 ml 0.5 M nitrophenylphosphate	5.0 mM
0.02 ml enzyme solution	–

Follow the absorption increase at 405 nm, 25 °C, $\varepsilon_{405} = 18\,500\,\text{l mol}^{-1}\,\text{cm}^{-1}$.

B. Bacterial Alkaline Phosphatase

Assay solutions

0.5 M Tris/HCl, pH 8.2

6.6 mM nitrophenylphosphate (disodium salt, hexahydrate, $M_r = 371.1$; dissolve 49 mg in 20 ml 0.5 M Tris/HCl pH 8.2)

Procedure

	Concentration
0.98 ml 6.6 mM nitrophenylphosphate	6.5 mM
0.02 ml enzyme solution	–

Follow the absorption increase at 405 nm, 25 °C, $\varepsilon_{405} = 18.5\,\text{l mmol}^{-1}\,\text{cm}^{-1}$.

References

Bessey, O.A., Lowry, H.O., and Broch, M.J. (1946) *J. Biol. Chem.*, **164**, 321–329.
Fernley, K.N. (1971) in *The Enzymes*, vol. 4 (ed. P.D. Boyer), Academic Press, New York, pp. 417–447.
Garattini, E. *et al.* (1986) *Arch. Biochem. Biophys.*, **245**, 331–337.
Garen, A. and Levinthal, C. (1960) *Biochim. Biophys. Acta*, **38**, 470–483.
Harkness, D.R. (1968) *Arch. Biochem. Biophys.*, **126**, 513–523.
Morton, R.K. (1965) *Compr. Biochem.*, **16**, 55–84.
Orhanovic, S. *et al.* (2006) *Int. J. Biol. Macromol.*, **40**, 54–58.
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Torriani, A. (1968) *Meth. Enzymol.*, **12B**, 212–218.

3.3.3.7 Acid Phosphatase, EC 3.1.3.2

SN: phosphate monoester phosphohydrolase (acid optimum)

$$\text{phosphate monoester} + H_2O \rightarrow \text{alcohol} + P_i$$

Mammalian enzyme (human); M_r 30 000, monomer, cofactor Fe^{2+}, K_m (mM):1 (*p*-nitrophenylphosphate), 0.2 (*o*-carboxyphenyl phosphate), 0.27 (ATP), 0.33 (ADP), pH optimum 5.7. The assay system described for alkaline phosphates with *o*-nitrophenylphosphate (assay in Section 3.3.3.6) can be used, applying acid conditions.

Assay Reaction

o-carboxyphenyl phosphate + H_2O → salicylic acid + P_i

Assay solutions

0.15 M acetate buffer pH 5.0 (8.55 ml acetic acid, 100%, M_r = 60, in 800 ml H_2O, adjust to pH 4.0 with 1 N NaOH, fill up to 1 l)

3.65 mM *o*-carboxyphenyl phosphate (salicylic acid phosphate, fosfosal, M_r = 218.1; 80 mg in 100 ml)

Procedure

	Concentration
0.7 ml 0.15 M acetate buffer pH 5.0	105 mM
0.2 ml 3.65 mM *o*-carboxyphenyl phosphate	0.73 mM
Incubate for 3 min at 25 °C	
0.1 ml enzyme solution	

Record the absorbance at 300 nm, 25 °C, molar absorption coefficient for salicylic acid $\varepsilon_{300} = 3500\ l\ mol^{-1}\ cm^{-1}$.

References

Belfield, A. *et al.* (1972) *Enzymologia*, **42**, 91–106.
Bessey, O.A., Lowry, H.O., and Broch, M.J. (1946) *J. Biol. Chem.*, **164**, 321–329.
Brouillard, J. and Quellet, L. (1965) *Can. J. Biochem.*, **43**, 1899–1905.
Hayman, A.R. *et al.* (1989) *Biochem. J.*, **261**, 601–609.
Hofstee, B.H.J. (1954) *Arch. Biochem. Biophys.*, **51**, 139–146.
Hollander, V.P. (1971) in *The Enzymes*, vol. 4 (ed. P.D. Boyer), Academic Press, New York, pp. 449–498.

3.3.3.8 Ribonuclease (Pancreatic), EC 3.1.27.5

RNase I Cleaves RNA to 3′-phospho-mononucleotides and 3′-phospho-oligonucleotides ending in Cp or Up forming 2′,3′-cyclic phosphate intermediates. Human enzyme: M_r 14 000 monomer, K_m (mM) 0.063 (poly(A)poly(U)), 0.1 (poly(C)), pH optimum 7.7.

Assay solutions

0.1 M acetate buffer pH 5.0 (5.7 ml acetic acid, 100%, $M_r = 60$, in 800 ml H_2O, adjust to pH 4.0 with 1 N NaOH, fill up to 1 l)

Uranylacetate-perchloric solution (0.75% uranyl acetate in 25% perchloric acid)

RNA (yeast) solution (50 mg 10 ml^{-1} in 0.1 M sodium acetate buffer pH 5.0)

RNase (1 mg ml^{-1}, dilute 100-fold in 0.1 M sodium acetate buffer pH 5.0 prior to use)

Procedure

0.6 ml 1% RNA solution

0.1 ml enzyme (1.0 ml 0.1 M respectively of acetate buffer pH 5.0 for the blank).

Incubate for 4 min at 37 °C and stop the reaction by adding

0.3 ml uranylacetate-perchloric acid solution.

Five min on ice, centrifuge for five min, dilute

0.1 ml supernatant with 0.9 ml H_2O

Measure the absorption increase at 260 nm.

Calculation

One unit is defined as the amount of enzyme causing a change in absorbance of 1.0 at 37 °C and pH 5.0 under the described conditions.

References

Dalaly, B.K. *et al.* (1980) *Biochim. Biophys. Acta*, **615**, 381–391.
Dey, P. *et al.* (2007) *Biochem. Biophys. Res. Commun.*, **350**, 809–814.
Kalnitsky, G., Hummel, J.P., and Dierks, C. (1959) *J. Biol. Chem.*, **234**, 1512–1516.
Kunitz, M. (1946) *J. Biol. Chem.*, **164**, 563–568.

3.3.3.9 α-Amylase, EC 3.2.1.1

SN: 1,4-α-D-glucan glucanohydrolase

Cleaves starch into reducing fragments and maltose; bacterial enzyme (*Bacillus subtilis*) M_r 41 000, monomer, K_m 1.2 mM (soluble starch), pH optimum 6.9.

Assay solutions

0.1 M potassium phosphate pH 7.0

10 mM maltose solution (maltose monohydrate, $M_r = 360.3$; 36 mg in 10 ml)

Starch solution (dissolve 0.5 g soluble starch and 17.5 mg NaCl in 50 ml 0.1 M potassium phosphate pH 7.0, boil for dissolving)

Dinitrosalicylate reagent (3,5-dinitrosalicylic acid, 2-hydroxy-3,5 dinitrobenzoic acid, $M_r = 228.1$; dissolve 1 g in 20 ml 2 N NaOH and 50 ml H_2O, dissolve 30 g K-Na-tartrate and fill up to 100 ml with H_2O)

Procedure

0.05 ml starch solution

0.05 ml enzyme sample (diluted in 0.1 M potassium phosphate pH 7.0).

Incubate for 5 min at 25 °C

0.1 ml dinitrosalicylate reagent

Mix and incubate 10 min a 100 °C

1 ml H_2O

Measure the absorption at 546 nm

The enzyme activity is determined with the aid of a calibration curve:

Fill up different amounts of the maltose solution in the range between 4 and 30 µl with H_2O to 0.1 ml

Add 0.1 ml of the dinitrosalicylate reagent

10 min at 100 °C

Add 1 ml H_2O

Measure the absorption at 546 nm.

Calculation

One unit is defined as the enzyme amount releasing 1 µmol reducing groups (calculated as maltose)/min from soluble starch at 25 °C, pH 7.0.

References

Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, 3rd edn, vol. 2, Verlag Chemie, Weinheim, pp. 151–152.
Bernfeld, P. (1951) *Adv. Enzymol.*, **12**, 379–428.
Das, K., Doley, R., and Mukherjee, A.K. (2004) *Biotechnol. Appl. Biochem.*, **40**, 291–298.
Fischer, E.H. and Stein, E.A. (1960) in *The Enzymes*, 2nd edn, vol. 4, Academic Press, New York, pp. 313–343.
Takagi, T., Toda, H., and Isemura, T. (1971) in *The Enzymes*, 3rd edn, vol. 5 (ed. P.D. Boyer), Academic Press, New York, pp. 235–271.

3.3.3.10 Amyloglucosidase, EC 3.2.1.3

SN: 1,4-α-D-glucan glucohydrolase, glucan 1,4-α-glucosidase, glucoamylase.

$$\text{starch} + H_2O \rightarrow \beta\text{-D-glucose}$$

Releases β-D-glucose by hydrolyzing terminal 1,4-and 1,6-linked α-1,6-glucoside bonds successively from the nonreducing ends of the chains. Human enzyme: M_r 105 360, K_m (mM) 7.7 (maltose), 0.05 (maltotetraose), inhibition by salanicol (K_i 0.19 mM), pH optimum 6.5, *Aspergillus awamori*, K_m (mM): 3.4 (4-nitrophenyl-α-glucoside), 3 (maltose), 0.18 (maltotetraose), pH optimum 5.5; shrimps (*Penaeus japonicus*), M_r 105 000, monomer, K_m (mM) 0.125 (4-methylumbelliferyl-α-D-glucoside), 0.2 (soluble starch, 2.3 (amylose), 4.2 (amylopectin), pH optimum 5.

A. Coupled Assay with HK and G6PDH The assay is similar to that described for α-glucosidase (assay in Section 3.3.3.14), except that glycogen is used instead of maltose as substrate. After a certain time (e.g., 5 min) the reaction is stopped and glucose determined by the coupled hexokinase/glucose-6-phosphate dehydrogenase assay:

$$\text{glycogen} + n - 1\text{H}_2\text{O} \xrightarrow{\text{Amyloglucosidase}} n\ \text{D-glucose}$$

$$n\ \text{D-glucose} + n\ \text{ATP} \xrightarrow{\text{HK}} n\ \text{glucose-6-phosphate} + n\ \text{ADP}$$

$$n\ \text{glucose-6-phosphate} + n\ \text{NADP}^+ \xrightarrow{\text{G6PDH}} n\ \text{6-phosphogluconat} + n\ \text{NADPH} + n\ \text{H}^+$$

Assay solutions

0.1 M acetate buffer pH 4.0 (5.7 ml acetic acid, 100%, $M_r = 60$, in 800 ml H_2O, adjust to pH 4.0 with 1 N NaOH, fill up to 1 l)

Glycogen solution (80 mg glycogen from oysters in 10 ml)

0.3 M tris(hydroxymethyl)aminomethan ($M_r = 121.1$; 363 mg in 10 ml)

Procedure

0.54 ml 0.1 M acetate buffer pH 4.0

0.25 ml glycogen solution

0.01 ml enzyme sample

5 min at 25 °C

0.2 ml 0.3 M tris(hydroxymethyl)aminomethane

Determine the glucose content (against a blank without enzyme sample) as described in Section 3.3.3.14 for α-glucosidase.

B. Photometric Assay with 4-Nitrophenyl-D-Glucose

$$\text{4-nitrophenyl-}\alpha\text{-D-glucopyranoside} \rightarrow \text{4-nitrophenol} + \text{D-glucose}$$

The assay is described for 96 well microplates for serial measurements, but can be performed in a conventional spectrophotometer, thereby increasing the reaction volume (RV) tenfold. The reaction can be followed continuously at 405 nm.

Assay solutions

100 mM MES buffer pH 6.5 (2-[*N*-morpholino]ethanosulfonic acid, $M_r = 213.2$, 21.3 g l^{-1}, adjust to pH 6.5 with 1 M NaOH)

33.3 mM *p*NP-glucose (4-nitrophenyl-α-D-glucopyranoside, $M_r = 301.3$; 100 mg in 10 ml MES buffer pH 6.5)

0.5 M Na_2CO_3 solution ($M_r = 106$; 5.3 g in 100 ml H_2O)

Procedure

	Concentration (mM)
45 µl 33.3 mM *p*NP-glucose	30
5 µl enzyme sample	–
Incubate for 30 min at 37 °C	
50 µl 0.5 M Na_2CO_3 solution	–

Measure the absorption at 405 nm with a microplate reader. ε_{405} = 18 500 l mol^{-1} cm^{-1}.

C. Fluorimetric Assay with 4-Methylumbelliferyl-α-D-Glucoside

Assay solutions

0.1 M sodium citrate buffer pH 5.5

1 mM substrate solution (4-methylumbelliferyl-α-D-glucoside, $M_r = 338.3$; 33.8 mg in 100 ml 0.1 M sodium citrate buffer pH 5.5)

50 mM glycine/NaOH pH 9.0

10 mM 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, $M_r = 176.2$, 17.6 mg in 10 ml), for the standard curve

Procedure

0.2 ml 1 mM substrate solution

0.02 ml enzyme solution

30 min, 37 °C

1.78 ml 50 mM glycine/NaOH pH 9.0

Measure the fluorescence, excitation at 358 nm, emission at 448 nm. For quantification, prepare a standard curve with various concentrations of 4-methylumbelliferone.

References

Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, 3rd edn, vol. 2, Verlag Chemie, Weinheim, pp. 154–155.
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3.3.3.11 Cellulases, β-1,4-Glucanase, EC 3.2.1.4, and β-Glucosidase, EC 3.2.1.21

The designation "cellulase" is used for a series of enzymes degradating cellulose from its ends (exocellulases) or within the chain (endocellulases); two examples are mentioned.

β-1,4-glucanase, EC 3.2.1.4, SN: 4-β-D-glucan 4-glucano-hydrolase, cellulase, avicelase.

The enzyme catalyzes the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin, and cereal β-D-glucans. Bacterial enzyme (*Trichoderma viride*): M_r 64 000, monomer; K_m (mM): 0.04 (cellotetraose), 0.15 (cellopentaose), 0.33 (cellohexaose), 1.63 (cellotriose), pH optimum 5.5.

β-glucosidase, EC 3.2.1.21, SN: 4-β-D-glucoside glucano-hydrolase, β-1,6-glucosidase, cellobiase.

The enzyme catalyzes the hydrolysis of terminal nonreducing β-D-glucose residues with release of β-D-glucose. Mammalian enzyme (human): M_r 53 000, monomer, K_m (mM): 4.1 (methylumbelliferyl-β-D-glucoside), pH optimum 5–6.5.

An orcinol assay determining the release of reducing units (glucose) from cellulose and an activity staining to detect active enzyme bands in electrophoresis gels are described.

A. Oricinol Assay

Assay solutions

0.1 M sodium phosphate/citric acid pH 6.1

Cellulose suspension, (microcrystalline cellulose, Avicel®, 2.5% (w/v) suspended in 0.1 M sodium phosphate/citric acid pH 6.1)

0.2% orcinol (5-methylresorcinol, 3,5-dihydroxytoluene, M_r = 142.2) in 70% sulfuric acid

1 M glucose solution (M_r = 180.2, 1.8 g in 10 ml) for a standard curve

Assay mixture and procedure

0.4 ml cellulose suspension

0.1 ml enzyme solution.
- 60 min 37 °C with permanent shaking (temperature depends on the source of the enzyme)
- Chill on ice
- Centrifuge 3 min, 5000 rpm

0.1 ml supernatant

0.9 ml 0.2% orcinol in 70% sulfuric acid.
- 20 min at 100 °C
- Cool to room temperature

Measure absorption at 550 nm, prepare a standard curve with glucose solution for quantification.

B. Activity Staining This staining can be performed in SDS polyacrylamide gels. To avoid inactivation, the enzyme sample should not be heated before applying it onto the electrophoresis gel. It is recommended to run two identical gels: one for activity staining, another one for staining with Coomassie Blue according to the standard procedure of SDS gel electrophoresis (cf. Laemmli, 1970).

Assay solutions

50 mM Na_2HPO_4/12.5 mM citric acid, pH 6.3

50 mM Na_2HPO_4/12.5 mM citric acid, pH 6.3, 25% isopropanol

Agar (agar-agar)

Carboxymethylcellulose, sodium salt

0.1% Congo red solution ($M_r = 696.7$)

1 M NaCl ($M_r = 58.44$, 5.84 g in 100 ml H_2O)

5% acetic acid

2 glass plates (~6 × 10 cm, ~2 mm thickness)

Preparation of the substrate gel

Heat 2% agar, 0.1% carboxymethylcellulose in 50 mM Na_2HPO_4/12.5 mM citric acid, pH 6.3 in a boiling water bath

Warm up two glass plates (size comparable to the polyacrylamide gel) in an incubator for 10 min at 60 °C

Immediately pour the hot agar solution on one glass plate and cover with the second one, maintaining a distant of 2 mm between both; this is achieved by mounting stoppers between both (avoid enclosure of air)

Store the gel in the refrigerator before usage

Preparation of the polyacrylamide gel

After running, wash the electrophoresis gel two times for 30 min with 50 mM Na_2HPO_4/12.5 mM citric acid, pH 6.3, 25% isopropanol

Wash two times for 30 min with 50 mM Na_2HPO_4/12.5 mM citric acid, pH 6.3 (omitting isopropanol)

Remove remaining fluid by rolling a glass tube over the gel surface

Performing the activity staining

Warm both, the substrate gel on the glass plate and the polyacrylamide gel, for 10 min at 60 °C in an incubator and remove eventually remaining fluid by rolling a glass tube over the gel surface

Layer the polyacrylamide gel onto the substrate gel, avoiding enclosure of air bubbles

Wrap the gels together with a parafilm foil and incubate for 60 °C at the assay temperature (e.g., 37 °C) in an incubator

Cool for 15 min in the refrigerator

Remove the polyacrylamide gel, stain with Coomassie Brilliant Blue according the to standard electrophoresis procedure (cf. Laemmli, 1970)

Immerse the substrate gel in 0.1% Congo red solution for 30 min at room temperature

Wash the substrate gel with 1 M NaCl until unbound dye is completely removed

Wash with 5% acetic acid; the hydrolyzed zones indicating enzyme activity should become visible

References

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Daniels, L.B. *et al.* (1981) *J. Biol. Chem.*, **256**, 13004–13013.
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3.3.3.12 Lysozyme, EC 3.2.1.17

SN : peptidoglycan *N*-acetylmuramoylhydrolase, muramidase

Hydrolysis of 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycans and between *N*-acetyl-D-glucosamine residues in chitodextrins. Enzyme from *Micrococcus lysodeikticus*: M_r 12 000, K_m (mM): 3.0 (4-nitrophenyl *N*,*N*′,*N*″,*N*‴,*N*⁗ pentaacetylchitopentanose), pH optimum 5.2. The enzyme is tested following the turbidity decrease due to the lysis of the bacterium *Micrococcus lysodeikticus (M. luteus)*.

Assay solutions

0.1 M potassium phosphate pH 7.0

Suspension of 10 mg of lyophilized cells of *Micrococcus lysodeikticus* in 50 ml 0.1 M potassium phosphate pH 7.0

Procedure

0.98 ml *Micrococcus lysodeikticus* suspension

0.02 ml enzyme sample (about 500 IU ml^{-1})

Record the absorption (turbidity) decrease at 450 nm, 25 °C, for 5 min.

Calculation

$$\text{units mg}^{-1} = \frac{\text{absorbance change min}^{-1} \times 1000}{\text{mg enzyme in reaction mixture}}$$

References

Bergmeyer, H.U. (1984) *Methods of Enzymatic Analysis*, 3rd edn, vol. 4, Verlag Chemie, Weinheim, pp. 189–195.
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Shugar, D. (1952) *Biochim. Biophys. Acta*, **8**, 302–309.

3.3.3.13 Sialidase, EC 3.2.1.18

SN: acetylneuraminyl hydrolase, exo-α-sialidase, α-neuraminidase

Hydrolyzes $\alpha(2\rightarrow3)$-, $\alpha(2\rightarrow6)$-, $\alpha(2\rightarrow8)$-glycosidic linkages of terminal sialic acid residues in oligosaccharides, glycoproteins, and glycolipids. Mammalian enzyme (human): monomer, M_r 48 000, requires Ca^{2+}; K_m (mM): 5.6 (*N*-acetyl-neuraminic acid-α-2,6-lactose, 0.028 (2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid); pH optimum 4.6.

A. Fluorimetric Assay

2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid + H_2O →
4-methylumbelliferone + α-D-*N*-acetylneuraminic acid

Assay solutions

0.05 M acetic acid/sodium acetate pH 4.6

1 M $CaCl_2$ (M_r = 111, 1.11 g in 10 ml H_2O)

BSA (bovine serum albumine,200 mg in 10 ml H_2O)

10 mM Mumana (2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, sodium salt, M_r = 489.4; 4.89 mg ml^{-1} H_2O)

10 mM 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, M_r = 176.2, 17.6 mg in 10 ml), for the standard curve

Assay mixture

Components	*Concentration*
9.7 ml 0.05 M acetic acid/sodium acetate pH 4.6	48.5 mM
0.05 ml BSA	0.1 mg/ml
0.05 ml 10 mM Mumana	0.05 mM

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Assay volume 1 ml for reduced fluorescence cuvettes, for standard cuvettes take double the amount (2 ml assay volume).

Determine the fluorescence intensity (excitation 358 nm, emission 448 nm); for quantification, prepare a standard curve with various concentrations of 4-methylumbelliferone.

B. Activity Staining Specific method for staining active enzyme bands in electrophoresis gels.

Solutions and substances

0.1 M potassium phosphate buffer pH 6.0.

MPN (2-(3′-methoxyphenyl)-*N*-acetyl-α-neuramininc acid, $M_r = 415.4$)

Black K salt (diazonium salt of 4-amino-2,5-dimethoxy-4-nitroazobenzene, $M_r = 835.8$)

7% acetic acid

Staining solution

1 mg MPN

1 mg Black K salt.

Dissolve in 1 ml 0.1 M potassium phosphate buffer pH 6.0

Procedure

Cover the gel with sufficient volume (usually 1–2 ml) of the staining solution

Incubate at 37 °C for about 1 h (the staining solution can be exchanged every 20 min); active enzyme bands develop a deep red color

Wash with 7% acetic acid to intensify the bands

References

Groome, N.P. and Belyavin, G. (1975) *Anal. Biochem.*, **63**, 249–254.
Schauer, R., Wember, M., and Tschesche, H. (1984) *Hoppe-Seyler's Z. Physiol. Chem.*, **370**, 419–426.
Tuppy, H. and Palese, P. (1969) *FEBS Lett.*, **3**, 72–75.
Wang, Z.M. *et al.* (2001) *J. Virol. Methods*, **98**, 53–61.

3.3.3.14 α-Glucosidase, EC 3.2.1.20

SN: α-D-glucoside glucohydrolase, maltase.

Cleaves α-D-glucose from terminal, nonreducing 1,4-linked α-D-glucose residues. Human enzyme: M_r 110 000, K_m (mM) 2.1 (4-methylumbelliferyl-α-D-glucoside) pH optimum 4. *Aspergillus niger*, glycoprotein, M_r 120 000, K_m (mM): 0.62 (4-nitrophenyl-α-D-glucoside), 1.26 (maltose), pH optimum 4.5.

In the assay maltose is split into two glucoses, which are determined separately in the hexokinase reaction (HK), coupled with glucose-6-phosphate dehydrogenase (G6PDH).

$$\text{maltose} + H_2O \xrightarrow{\alpha\text{-glucosidase}} 2\ \text{D-glucose}$$

$$2\ \text{D-glucose} + 2\ \text{ATP} \xrightarrow{\text{HK}} 2\ \text{glucose-6-phosphate} + 2\ \text{ADP}$$

$$2\ \text{glucose-6-phosphate} + 2\ \text{NADP}^+ \xrightarrow{\text{G6P-DH}} 2,6\text{-phosphogluconate} + 2\ \text{NADPH} + 2\ \text{H}^+$$

A. α-Glucosidase Assay

Assay solutions

0.1 M acetate buffer pH 6.0, containing 1.5 mM EDTA (5.7 ml acetic acid, 100%, $M_r = 60$, in 800 ml H_2O, add 560 mg EDTA · Na_2 · $2H_2O$, $M_r = 372.2$, adjust to pH 6.0 with 1 N NaOH, fill up to 1 l)

0.5 M maltose solution (monohydrate, $M_r = 360$, 18 g in 100 ml H_2O)

Procedure

	Concentration (mM)
0.74 ml 0.1 M acetate buffer pH 6.0	74
0.25 ml 0.5 M maltose solution	125
0.01 ml enzyme solution	

5 min at 25 °C

Transfer the reaction tube into a 100 °C water bath or heating block

Glucose is determined in 0.2 ml aliquots.

B. Glucose Determination

Assay solutions

100 mM triethanolamine · HCl/NaOH pH 7.6 (triethanolamine · HCl, adjusted with NaOH)

1.0 M D-glucose ($M_r = 180.2$; 18 g in 100 ml)

0.1 M ATP (disodium salt, trihydrate, $M_r = 605.2$; 605 mg in 10 ml)

0.1 M $MgCl_2$ (hexahydrate; $M_r = 203.3$; 203 mg in 10 ml)

0.01 M NADP (disodium salt, $M_r = 787.4$; 79 mg in 10 ml)

Glucose-6-phosphate dehydrogenase (G6PDH, commercial products are 100–500 IU mg^{-1}, dilute to ~5 IU 0.1 ml^{-1})

Hexokinase (HK, ~200 IU ml^{-1})

Assay mixture

Components	*Concentration*
5.8 ml 0.1 mM triethanolamine/NaOH pH 7.6	58 mM
0.6 ml 0.1 mM $MgCl_2$	6.0 mM
1.0 ml 10 mM NADP	1.0 mM
0.3 ml 0.1 M ATP	3.0 mM
0.1 ml G6PDH	0.5 IU ml^{-1}

Procedure

0.78 ml assay mixture

0.2 ml D-glucose sample (1 M for the reference 200 mM)

0.01 ml HK

The absorption increase at 340 nm is measured at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l · mol^{-1} cm^{-1}.

Calculation

$$\text{glucose concentration} = \frac{\Delta A \times \text{RV glucosidase} \times \text{RV glucose}}{\varepsilon_{340} \times 1 \times 0.1} = \frac{\Delta A \times 1 \times 1}{6.3}$$

= µmol glucose in 1 ml incubation mix (the blank must be subtracted), RV = reaction volume (ml).

C. Assay with 4-Nitrophenylglucopyranoside

Assay solutions

50 mM acetate buffer pH 4.5

1.25 mM 4-nitrophenyl-α-D-glucopyranoside solution ($M_r = 301.3$; 37.7 mg in 100 ml 50 mM acetate buffer pH 4.5)

0.5 M Na_2CO_3 solution ($M_r = 106$, dissolve 5.3 g in 100 ml H_2O)

Procedure

0.8 ml 1.25 mM 4-nitrophenyl-α-D-glucopyranoside solution

0.2 ml enzyme sample

10 min 37 °C

0.1 ml 0.5 M Na_2CO_3 solution

Measure absorption at 405 nm; $\varepsilon_{405} = 18\,500\,l\,mol^{-1}cm^{-1}$.

References

Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, 3rd edn, vol. 2, Verlag Chemie, Weinheim, pp. 205–206.
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Sorensen, S.H., Nòren, O., Sjöström, H., and Danielsen, E.M. (1982) *Eur. J. Biochem.*, **126**, 559–568.

3.3.3.15 β-Galactosidase, EC 3.2.1.23

SN: β-D-Galactoside galactohydrolase

Hydrolysis of terminal nonreducing β-D-galactose residues in β-D-galactosides:

$$\text{lactose} + H_2O \rightarrow \beta\text{-D-galactose} + \text{D-glucose}$$

Bacterial enzyme (*Escherichia coli*): M_r 516 000, homotetramer (4 × 116 000), K_m (mM): 0.03 (4-nitrophenyl-β-D-galactose).

Assay solutions

0.1 M potassium phosphate pH 7.6

0.05 M *o*-nitrophenyl β-D-galactopyranoside (ONPG, M_r = 301.3; 150 mg in 10 ml 0.1 M potassium phosphate pH 7.6)

β-Galactosidase from *E. coli* (~300 IU mg^{-1})

Procedure

	Concentration
0.88 ml 0.1 M potassium phosphate pH 7.6	88 mM
0.1 ml 0.05 M ONPG	5.0 mM
0.02 ml enzyme solution	–

Follow the absorption increase at 405 nm at 25 °C, $\varepsilon_{405} = 18\,500\,l\,mol^{-1}\,cm^{-1}$.

References

Bergmeyer, H.U. (1983) *Methods of Enzymatic Analysis*, 3rd edn, vol. 2, Verlag Chemie, Weinheim, pp. 197–198.
Craven, R.G., Steers, E., and Anfinsen, C.B. (1965) *J. Biol. Chem.*, **240**, 2468–2477.
Rotman, B., Zderic, J.A., and Edelstein, M. (1963) *Proc. Natl. Acad. Sci.*, **50**, 1–6.
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Woolen, J.W. and Turner, P. (1965) *Clin Chim. Acta*, **12**, 647–658.

3.3.3.16 β-Fructosidase, EC 3.2.1.26

SN: β-D-fructosefuranoside fructohydrolase, β-fructofuranosidase, invertase, invertin, saccharase, sucrase.

The enzyme hydrolyzes the terminal nonreducing β-D-fructofuranoside residues in β-D-fructofuranosides, especially saccharose. This is one of the most intensely studied enzymes in the early epoch of biochemical research; the Michaelis–Menten equation was originally derived from the invertase reaction. The assay utilizes the fact that the deflection of the plane of polarized light becomes inversed ("invertase") when saccharose is cleaved into an equimolar mixture of glucose and fructose. The reaction can be continuously followed with a polarimeter (ORD spectrometer). The assay is described for 10 ml samples for a 10 cm cuvette and may be performed accordingly if smaller cuvettes and a more sensitive instrument are used. Enzyme from *Aspergillus niger*: M_r 95 000, homodimer (2 × 47 000), K_m (mM): 0.06625 (saccharose), pH optimum 5.

Assay solutions

0.05 M acetate buffer pH 4.62 (2.85 ml acetic acid, 100%, $M_r = 60$, in 800 ml H_2O, adjust to pH 4.62 with 1 N NaOH, fill up to 1 l)

0.3 M D-(+)-saccharose, ($M_r = 342.3$; 10.3 g in 100 ml H_2O)

1 M sodium carbonate ($Na_2CO_3 \cdot 10H_2O$, $M_r = 286.1$; 28.6 g in 100 ml H_2O)

Invertase from yeast (~300 IU mg^{-1})

Procedure

	Concentration (mM)
1.9 ml 0.05 M sodium acetate buffer pH 4.62	24
2.0 ml 0.3 M saccharose	150

Bring to 30 °C

0.1 ml invertase.

Incubate for different times (1–10 min) and add

6 ml 1 M Na_2CO_3

Measure the polarization in a 10 cm cell of a polarimeter. 1 unit of the enzyme splits 1 µmol substrate/min.

References

Myrbäck, K. (1960) *The Enzymes*, 2nd edn, vol. 4 (eds P.D. Boyer, H. Lardy, and K. Myrbäck), Academic Press, New York, pp. 379–396.
Rubio, M.C. and Maldonado, M.C. (1995) *Curr. Microbiol.*, **31**, 80–83.
Sumner, J.B. and Howell, S.F. (1935) *J. Biol. Chem.*, **108**, 51–54.

3.3.3.17 β-Glucuronidase EC 3.2.1.31

SN: β-D-glucuronoside glucuronosohydrolase

$$\beta\text{-D-glucuronoside} + H_2O \rightarrow \text{D-glucuronate} + \text{alcohol}$$

Glycoprotein, human enzyme: M_r 390 000, tetramer (4 × 83 000), K_m (mM): 1.3 (4-umbelliferyl-β-D-glucuronide), 0.13 (*p*-nitrophenyl-β-D-glucuronide), 0.018 (phenolphthalein-β-D-glucuronide), pH optimum 4.5.

A. Fluorimeteric Assay

Assay solutions

0.2 M sodium acetate pH 4.8

10 mM 4-umbelliferyl-β-D-glucuronide (7-hydroxycoumarin-β-D-glucuronide, sodium salt, M_r = 361.3; 36.1 mg in 10 ml 0.2 M sodium acetate pH 4.8)

Procedure

0.1 ml 10 mM 4-umbelliferyl-β-D-glucuronide

0.01 ml enzyme sample.

Incubate for 30 min at 37 °C

Measure the fluorescence, excitation at 358 nm, emission at 448 nm. For quantification, prepare a standard curve with various concentrations of 4-methylumbelliferone.

References

Brot, F.E., Bell, E., and Sly, W.S. (1978) *Biochemistry*, **17**, 385–391.
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3.3.3.18 Proteases, EC 3.4, General Assays

Due to the different kinds and specificities of the proteases, many different protease assays have been described. Proteases are classified according to distinct criteria, with respect to (i) the catalysis mechanism as serine proteases, cysteine proteases, and metalloproteases; (ii) the mode of attacking the substrate as exo- and endoproteases; (iii) the substrate specificities, for example as trypsin- and chymotrypsin-like proteases. These criteria must be considered for performing a protease assay. Principally, two types of protease assays exist: (i) general assays demonstrating only the peptide-cleaving capacity and (ii) specific protease assays. Usually, in the former case, proteins are used and their digestion into fragments is detected, while in the latter case, synthetic substrates or defined peptides are applied.

Reference

Cunningham, L. (1965) *Compr. Biochem.*, **16**, 85–188.

A. Anson Assay Proteins in their native structure often resist proteolytic attack. Therefore, hemoglobin, denatured by urea, is applied for this assay. The digested fragments are separated from nonhydrolyzed protein and detected in the supernatant with the Folin–Ciocalteau reagent, which preferentially recognizes tryptophan and tyrosine and to a lesser extent, cysteine and histidine. According to the respective protease assay, temperatures from room temperature (25 °C) to 50 °C are chosen.

Assay solutions

1 N NaOH ($M_r = 40.0$; 4 g NaOH in 100 ml H_2O, store in PE flasks)

0.5 N NaOH (1 part 1 N NaOH, 1 part H_2O)

1 M KH_2PO_4 (1 $M_r = 136.1$; 13.61 g in 100 ml H_2O)

0.3 M trichloroacetic acid ($M_r = 163.4$; 4.9 g in H_2O)

Folin–Ciocalteau phenol reagent (commercially available, keep at 4 °C; dilute 1 volume with 2 volumes H_2O before use)

0.2 M HCl (bring 3.31 ml HCl (37%) to 200 ml with H_2O)

1 mM L-tyrosine ($M_r = 181$; 18.1 mg in 100 ml 0.2 M HCl)

Hemoglobin substrate: place a 100 ml beaker, containing 10 ml in a water bath and keep at 25 °C. The components listed below are added one after the other and dissolved by stirring

- 11.54 g urea
- 2.4 g 1 N NaOH
- 0.635 g lyophilized hemoglobin

Stir for 45 min

- Dissolve 3.16 g 1 M KH_2PO_4 and add. H_2O to give a total weight of 32.6 g
- Adjust the pH to 7.5 with 1 N HCl

The solution should be stored in the cold (5 °C) and used within 14 days. For every new preparation of the hemoglobin substrate, a distinct standard curve is needed.

Procedure

	Sample	**Blank**
Enzyme solution	250 µl	250 µl
Hemoglobin substrate	250 µl	–
15 min incubation at assay temperature (25 °C)		
0.3 M trichloroacetic acid	1000 µl	1000 µl
Hemoglobin substrate	–	250 µl
5 min centrifugation		
Supernatant	500 µl	500 µl
0.5 N NaOH	1000 µl	1000 µl
Folin–Ciocalteau phenol reagent (1 : 2 diluted)	250 µl	250 µl

Measure absorption difference sample – blank at 750 nm

Calibration curve

A calibration curve between 0 and 250 nmol L-tyrosine is prepared. Increasing amounts (~12 different samples) from 0 to 250 µl 1 mM L-tyrosine (1 µl to 1 nmol) are filled up with 0.2 M HCl to a final volume of 0.5 ml and 1 ml 0.5 N NaOH and 0.250 ml Folin–Ciocalteau phenol reagent (1 : 2 diluted) are added to each sample. Absorption difference at 750 nm against the blank (without tyrosine) is determined. A linear relationship of absorbance with tyrosine concentration should be obtained, the slope of the resulting line is absorbance per nmol Tyr.

Calculation of Enzyme Activity

Since determination of molarity of product formation from hemoglobin is difficult, special Anson Units (AU) are defined: 1 AU is the amount of enzyme producing a color intensity corresponding to 1 mEq of tyrosine (1 nmol tyrosine ~1 µAU)/min under assay conditions.

The absorbance at 750 nm divided by the slope of the calibration curve gives the amount of product formed, equivalent to nanomole Tyr. To obtain μAU, the value must be divided by the incubation time (min) and multiplied by a factor of 3 (since only 0.5 ml out of the total assay volume of 1.5 ml is taken for the color reaction) and by the dilution factor.

Enzyme activity in the assay:

$$\mu\text{AU} = \frac{A_{750} \times 3 \times \text{dilution factor}}{\text{slope} \times \text{time(min)}}$$

With reference to the total enzyme solution, the value obtained must be multiplied by the total volume of the enzyme solution and divided by the enzyme amount used for the assay and a factor for converting to AU:

Total enzyme activity:

$$\text{AU} = \frac{A_{750} \times 3 \times \text{dilution factor} \times \text{total enzyme volume (ml)}}{\text{slope} \times \text{time (min)} \times \text{test enzyme volume (ml)} \times 10^6}$$

References

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B. Casein Assay This is a simple, but not very sensitive assay. The absorption of the amino acids cleaved from casein by protease action is measured at 280 nm in the supernatant after separation from the remaining protein by TCA precipitation. For quantification, a tyrosine calibration curve must be prepared.

Assay solutions

0.01 M Tris/HCl pH 8.0, containing 10 mM $CaCl_2$ (1.47 g $CaCl_2 \cdot 2H_2O$ in 1 l 0.01 M Tris/HCl pH 8.0)

0.2 M NaOH ($M_r = 40.0$; 0.8 g in 100 ml H_2O)

0.2 M acetic acid ($M_r = 60.05$; 12 g in 1 l)

1.2 M trichloroacetic acid (TCA, $M_r = 163.4$; 19.6 g in 100 ml H_2O)

1 mM L-tyrosine ($M_r = 181$; 18.1 mg in 100 ml 0.2 M HCl)

Casein substrate: dissolve 2 g casein in 90 ml 0.01 M Tris/HCl pH 8.0, 10 mM $CaCl_2$ under stirring, add 0.2 M NaOH until a clear solution is obtained, adjust the pH to 8.0 with 0.2 M acetic acid and bring the solution to 100 ml with H_2O. Divide it in aliquots and store at −20 °C

Procedure

Warm up 0.4 ml casein substrate to 35 °C

Add 0.2 ml protease solution in 0.01 M Tris/HCl pH 8.0, 10 mM $CaCl_2$

Incubate at 35 °C for 10 min

Add 1 ml 1.2 M TCA to stop the reaction

Treat the blank accordingly, except that the protease solution is given to the casein substrate after addition of TCA

Centrifuge the samples for 5 min

Measure the absorption of the supernatant at 275 nm

One unit is defined as the amount of protease, producing an absorption corresponding to 1 µmol tyrosine of the calibration curve after 1 min at 35 °C. The calibration curve is prepared in the micromolar range with the 1 mM tyrosine solution.

Reference

Kunitz, M. (1947) *J. Gen. Physiol.*, **30**, 291–310.

C. Azocasein Assay The sensitivity of the casein assay is essentially improved by azo groups covalently bound to casein. Upon liberation by proteolytic digestion, an intense color is developed in the supernatant.

Assay solutions

0.1 M potassium phosphate pH 8.0

0.2% azocasein (sulfanilamide azocasein) in 0.1 M potassium phosphate pH 8.0

10% trichloroacetic acid (TCA, w/v)

Procedure

0.5 ml azocasein solution

0.2 ml protease solution

Incubate at 25 °C for 30 min

Add 0.2 ml 10% trichloroacetic acid

Centrifugate for 5 min

Measure the absorption in the supernatant at 340 nm, 1 unit is defined as the protease amount producing an absorption change of 1 within 1 h

References

Brock, F.M., Frosberg, C.W., and Buchanan-Smith, J.G. (1982) *Appl. Environ. Microbiol.*, **44**, 561–569.

Peek, K., Daniel, R.M., Monk, C., Parker, L., and Coolbear, T. (1992) *Eur. J. Biochem.*, **207**, 1035–1044.

Tomarelli, R.M., Charney, J., and Harding, M.L. (1949) *J. Lab. Clin. Med.*, **34**, 428–433.

D. Ninhydrin Assay Ninhydrin develops a very intense color with free amino acids and the high sensitivity compensates for the relatively laborious method. Casein is used as protease substrate. After precipitation of nonhydrolyzed protein with trichloroacetic acid the ninhydrin reaction is performed in the supernatant.

Assay solutions

0.01 M Tris/HCl pH 8.0, containing 10 mM $CaCl_2$ (1.47 g $CaCl_2$ $2H_2O$ in 1 l 0.01 M Tris/HCl pH 8.0)

0.2 M NaOH ($M_r = 40.0$; 0.8 g in 100 ml H_2O)

0.2 M acetic acid ($M_r = 60.05$; 12 g in 1 l)

1.2 M trichloroacetic acid (TCA, $M_r = 163.4$; 19.6 g in 100 ml H_2O)

Casein substrate: dissolve 2 g casein in 90 ml 0.01 M Tris/HCl pH 8.0, 10 mM $CaCl_2$ under stirring, add 0.2 M NaOH until a clear solution is obtained, adjust the pH to 8.0 with 0.2 M acetic acid and bring the solution to 100 ml with H_2O. Divide it in aliquots and store at −20 °C

Acetate buffer pH 5.4 (dissolve 270 g sodium acetate trihydrate, $C_2H_3O_2Na \cdot 3H_2O$, $M_r = 136.1$, in 200 ml H_2O, add 50 ml glacial acetic acid and fill up to 750 ml)

10 mM KCN ($M_r = 65.1$; 65 mg in 100 ml H_2O, *volatile, even the vapor is extremely poisonous!)*

0.2 mM KCN in acetate buffer (0.2 ml 10 mM KCN + 9.8 ml acetate buffer, prepare fresh)

Ninhydrin reagent (2,2-dihydroxy-1,3-indanedione, $M_r = 178.1$, commercially available)

50% ethanol

Standard BSA solution, 40 µg ml^{-1}, prepared by 100-fold dilution of a stock solution, 100 mg in 25 ml

10 mM leucine (D,L-leucine, $M_r = 131.2$; 131 mg in 100 ml H_2O)

Protease solution

Procedure for casein degradation

Warm up 0.4 ml casein substrate to 35 °C

Add 0.2 ml protease solution in 0.01 M Tris/HCl pH 8.0, 10 mM $CaCl_2$

Incubate at 35 °C for 10 min

Stop the reaction by addition of 1 ml 1.2 M TCA

Treat the blank accordingly, except that the protease solution is given to the casein substrate after addition of TCA

Centrifuge the samples for 5 min to get the TCA supernatant

Ninhydrin reaction

0.2 ml TCA supernatant

0.1 ml 0.2 mM KCN in acetate buffer

0.1 ml ninhydrin reagent

10 min in a boiling water bath or a heating block at 100 °C

Chill in ice for 1 min and add 0.5 ml 50% ethanol

Measure the absorbance at 570 nm against a blank where the hydrolysate is displaced by water

Calibration curve

For quantification, a calibration curve is generated with 12 aliquots (1–50 µl, fill up to 200 µl) of the 10 mM leucine solution.

Reference

Rosen, H. (1957) *Arch. Biochem. Biophys.*, **67**, 10–15.

3.3.3.19 Leucine Aminopeptidase, EC 3.4.11.1

Leucyl aminopeptidase, LAP, peptidase S, cathepsin III; bacterial leucyl aminopeptidase, EC 3.4.11.10.

Zn-containing exopeptidase, splits *N*-terminal of an amino acid Xaa-/-Yaa, in which Xaa is preferably leucine, but also other amino acids, including proline, but

not arginine and lysine; Yaa may be proline. Amides and methylesters from amino acids are hydrolyzed with considerably lower rates. Human enzyme: M_r 120 000, K_m (mM) 1.28 (L-leucine-4-nitroanilide), pH optimum 7.5.

A. Assay with Leucineamide

Assay solutions

0.5 M Tris/HCl pH 8.5

0.025 manganese chloride ($MnCl_2 \cdot 2H_2O$, $M_r = 161.9$; 40.5 mg in 10 ml)

0.1 M magnesium chloride ($MgCl_2 \cdot 6H_2O$, $M_r = 203.3$; 203 mg in 10 ml)

0.0625 M L-leucine ($M_r = 131.2$; 820 mg in 100 ml, adjust to pH 8.5)

0.125 M L-leucinamide (hydrochloride, $M_r = 166.7$; 2.08 g in 100 ml, adjust to pH 8.5)

LAP solution (0.1 ml, corresponding to 0.4 mg, activate prior to use by incubating at 37 °C for 2 h in a mixture of 0.05 ml 0.025 M $MnCl_2$, 0.05 ml 0.5 M Tris/HCl pH 8.5, 0.8 ml water)

Assay mixture and procedure

	Concentration (mM)
0.05 ml 0.5 M Tris/HCl pH 8.5	25
0.4 ml 0.125 M leucinamide	50
0.05 ml 0.1 M magnesium chloride	5
0.4 ml H_2O	–
0.1 ml LAP solution	–

Follow the decrease of absorption at 238 nm, 25 °C.

For the blank, replace leucinamide and H_2O by 0.8 ml 0.0625 M leucine.

Calculation

Prepare a calibration curve with different concentrations of leucinamide at 238 nm (α_m = absorption/[leucinamide]):

$$\text{unit mg}^{-1} = \frac{A_{238}\text{min}^{-1} \times 1000 \times \text{ml reaction mixture}}{\alpha_m \times \text{mg enzyme}}$$

B. Assay with Leucine-*p*-nitroanilide

L-leucine-4-nitroanilide → L-leucine + *p*-nitroaniline

Assay solutions

0.05 M Tricine pH 8.0 (*N*-tris[hydroxymethyl]methylglycine, M_r = 179.2, dissolve 8.96 g in 0.8 l H_2O, adjust with 1 N NaOH to pH 8.0 and bring to 1 l)

1 mM L-leucine-*p*-nitroanilide (hydrochloride, M_r = 287.7; 28.8 mg in 100 ml 0.05 M tricine, pH 8.0)

Assay mixture

Components	*Concentration (mM)*
7.8 ml 0.05 M tricine pH 8.0	50
2 ml 0.01 M L-leucine-*p*-nitroanilide	0.2

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Measure the absorption increase at 405 nm ($\varepsilon_{405,\ p\text{-nitroaniline}}$ = 10 800 $l \cdot mol^{-1}\ cm^{-1}$), 25 °C.

References

Delange, J.R. and Smith, E.L. (1971) in *The Enzymes*, 3rd edn, vol. 3 (ed. P.D. Boyer), Academic Press, New York, pp. 81–118.
Hattori, A. *et al.* (2000) *J. Biochem.*, **128**, 755–762.
Himmelhoch, R. (1970) *Meth. Enzymol.*, **19**, 508–513.
Mitz, M.A. and Schlueter, R.J. (1958) *Biochim. Biophys. Acta*, **27**, 168–172.
Spungnin, A. and Blumberg, S. (1989) *Eur. J. Biochem.*, **183**, 471–477.

3.3.3.20 α-Chymotrypsin, EC 3.4.21.1

Protease preferentially cleaving peptides behind tyrosine, tryptophan, phenylalanine, and leucine. Mammalian enzyme (bovine): M_r 25 210, K_m (mM): 0.32 (*N*-glutaryl-L-phenylalanine-4-nitroanilide, GLUPHEPA), pH optimum 8. A fluorimetric assay is described under lipase (assay in Section 3.3.3.1.B).

A. Assay with SUPHEPA

Assay solutions

Buffer I: 0.2 M TEA/NaOH pH 7.8 (triethanolamine · HCl, M_r = 185.7, 37.13 g, 2.2 g calcium chloride in 800 ml H_2O, adjust with 2 N NaOH to pH 7.8, fill up to 1 l)

Buffer II: 0.2 M TEA/NaOH pH 7.8 (37.13 g triethanolamine · HCl in 800 ml H_2O, adjust with 2 N NaOH to pH 7.8, fill up to 1 l)

60 µM α-chymotrypsin (15.2 mg in 10 ml 1 mM HCl)

0.1 M SUPHEPA (*N*-succinyl-L-phenylalanine-*p*-nitroanilide, M_r = 385.4; 385 mg in 10 ml buffer II)

Assay mixture and procedure

	Concentration (mM)
0.95 ml buffer I	190
0.04 ml 0.1 M SUPHEPA	4.0
0.01 ml α-chymotrypsin solution	–

Follow the increase of absorbance at 405 nm, 25 °C. Absorption coefficient of *p*-nitroanilide $\varepsilon_{405} = 10.2 \times 10^3$ l mol^{-1} cm^{-1}.

B. Assay with GLUPHEPA

Assay solutions

0.1 M potassium phosphate pH 7.6

10 mM GLUPHEPA (*N*-glutaryl-L-phenylalanin-4-nitroanilide, M_r = 399.4; 40 mg in 10 ml 0.1 M potassium phosphate pH 7.6)

Assay mixture and procedure

	Concentration (mM)
0.98 ml GLUPHEPA	10
0.02 ml α-chymotrypsin solution	–

Measure the absorption increase at 405 nm, 25 °C. Absorption coefficient of *p*-nitroanilide $\varepsilon_{405} = 10.2 \times 10^3$ l mol^{-1} cm^{-1}.

References

Bergmeyer, H.U. (1984) *Methods of Enzymatic Analysis*, 3rd edn, vol. 5, Verlag Chemie, Weinheim, pp. 99–104.

Erlanger, B.F., Cooper, A.G., and Bendich, H.J. (1964) *Biochemistry*, **3**, 1880–1883.

Fiedler, F., Geiger, R., Hirschauer, C., and Leysath, G. (1978) *Hoppe Seyler's Z. Physiol. Chem.*, **259**, 1667–1673.
Nagel, W., Willig, F., Peschke, W., and Schmid, F.H. (1965) *Hoppe Seyler's Z. Physiol. Chem.*, **340**, 1–10.
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Schwert, G.W. and Takenaka, Y. (1955) *Biochim. Biophys. Acta*, **16**, 570–575.
Spreti, N. *et al.* (2001) *Eur. J. Biochem.*, **268**, 6491–6497.

3.3.3.21 Pancreatic Elastase, EC 3.4.21.35 (Previous EC 3.4.4.7)

Hydrolysis of proteins, including elastin, preferential cleavage at Ala.

Mammalian pancreatic enzyme (porcine): M_r 25 900, monomer; enzyme from salmon: K_m (mM): 1.47 (succinyl-Ala-Ala-Ala-*p*-nitroanilide); 0.3 (NBA, *p*-nitrophenyl-*N*-*tetr*-butyloxycarbonyl-L-alaninate), pH optimum 8.7.

A. Assay with Succinyl-Ala-Ala-Ala-*p*-Nitroanilide

Assay solutions

0.1 M Tris/HCl pH 8.5

2.5 mM succinyl-Ala-Ala-Ala-*p*-nitroanilide (M_r = 451.4, 11.3 mg in 10 ml)

Procedure

	Concentration (mM)
0.88 ml 0.1 M Tris/HCl pH 8.5	88
0.1 ml 2.5 mM succinyl-Ala-Ala-Ala-*p*-nitroanilide	0.25
0.02 enzyme sample	–

Measure the absorption increase at 405 nm ($\varepsilon_{405,\ p\text{-nitroaniline}}$ = 10 200 l · mol^{-1} cm^{-1}), 25 °C.

B. Esterase Activity of Elastase

Assay solutions

0.05 M potassium phosphate pH 6.5

0.01 M NBA (*p*-nitrophenyl-*N*-*tetr*-butyloxycarbonyl-L-alaninate, *N*-tert-BOC-L-alanine *p*-nitrophenylester, M_r = 310.3, 31 mg in 10 ml acetonitrile or methanol)

Assay mixture

Components	*Concentration (mM)*
9.5 ml 0.05 M potassium phosphate pH 6.5	47.5
0.3 ml 0.01 M NBA	0.3

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample (~5 µg)

Measure the absorption increase at 347.5 nm ($\varepsilon_{374.5} = 5500\,M^{-1}\,cm^{-1}$, the absorption of *p*-nitrophenol at this wavelength is independent of pH), 25 °C.

References

Berglund, G.I. *et al.* (1998) *Mol. Marine Biol. Biotechnol.*, **7**, 105–114.
Hilpert, K. *et al.* (2003) *J. Biol. Chem.*, **278**, 24986–24993.
Visser, L. and Blout, E.R. (1972) *Biochim. Biophys. Acta*, **268**, 257–266.

3.3.3.22 Pepsin, EC 3.4.23.1

Pepsin A Endopeptidase in the gastric juice; cleaves hydrophobic, preferentially aromatic amino acid residues. Mammalian enzyme (human): M_r 35 000, monomer, activated by Ca^{2+}; K_m (mM, *Gallus gallus*): 0.1 (hemoglobin), 1.05 (casein), pH optimum 2.

Assay solutions

0.3 M HCl (24.8 ml 37% HCl, density 1.18, fill up with H_2O to 1 l)

2% hemoglobin (dissolve 2.5 g bovine hemoglobin in 100 ml H_2O, mix for 5 min in a Waring blender, filter through glass wool and add to 80 ml filtrate 20 ml 0.3 M HCl)

0.3 M trichloroacetic acid ($M_r = 163.4$, 4.9 g in 100 ml H_2O)

0.01 M HCl (dilute 0.3 M HCl 30-fold)

Pepsin solution (5 mg in 10 ml 0.01 M HCl, dilute for the assay 50-fold in 0.01 M HCl)

Procedure

	Sample	Blank
2% hemoglobin substrate	0.3 ml	0.3 ml
Incubation at 37 °C (water bath)		
0.3 M trichloroacetic acid	–	0.6 ml
Pepsin solution	0.1 ml	0.1 ml
10 min incubation at 37 °C		
0.3 M trichloroacetic acid	0.6 ml	–
5 min, 37 °C		
5 min centrifugation		
Record absorption at 280 nm		

Calculation

$$\text{units mg}^{-1} = \frac{A_{280,\text{sample}} - A_{280,\text{blank}} \times 1000}{10\ \text{min} \times \text{mg enzyme}}$$

References

Althauda, S.B.P. *et al.* (1989) *J. Biochem.*, **106**, 920–927.
Bergmeyer, H.U. (1984) *Methods in Enzymatic Analysis*, 3rd edn, vol. 5, Verlag Chemie, Weinheim, pp. 232–233.
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Kassell, B. and Meitner, P.A. (1970) *Meth. Enzymol.*, **19**, 337–347.
Klomklao, S. *et al.* (2007) *Comp. Biochem. Physiol., B*, **147B**, 682–689.

3.3.3.23 Trypsin, EC 3.4.21.4

Cleaves preferentially behind Arg- and Lys-; pancreatic enzyme (*Engraulis japonicus*, human): M_r 24 000, monomer, strong inhibition by the soybean inhibitor ($IC50$ = 0.009 µM), K_m (mM): 0.033 (benzoylarginine-4-nitroanilide), 0.014 (benzoylarginine-ethylester), 0.1 (leupeptin); pH optimum 9.5.

Assay solutions

0.3 M potassium phosphate pH 8.0

20 mM *N*′-benzoyl-L-arginine-*p*-nitroanilide (BAPNA, M_r= 434.9; 174 mg in 20 ml DMSO)

1 mM HCl (83 µl 37% HCl in 1 l H_2O)

1 mM trypsin, 23.4 mg in 1 ml 1 mM HCl (dilute 50-fold in 0.05 M potassium phosphate pH 8.0 immediately before testing)

Assay mixture and procedure

	Concentration
0.70 ml H_2O	–
0.18 ml 20 mM BAPNA	3.0 mM
0.10 ml 0.3 M potassium phosphate pH 8.0	30 mM
0.02 ml trypsin solution	9 µg ml^{-1}

Record the absorbance at 405 nm 25 °C, $\varepsilon_{405} = 9.62 \times 10^3$ l mol^{-1} cm^{-1}.

References

Ahsan, M.N. and Watabe, S. (2001) *J. Protein Chem.*, **20**, 49–58.
Gaertner, H.F. and Puigserver, A.J. (1992) *Enzyme Microb. Technol.*, **14**, 150–155.
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3.3.3.24 Asparaginase, EC 3.5.1.1

SN: L-Asparagine amidohydrolase, ASNase,

$$\text{L-asparagine} + H_2O \rightarrow \text{L-aspartate} + NH_3$$

Bacterial enzyme (*Escherichia coli*) M_r 150 000, homotetramer, (4 × 37 000), K_m (mM): 0.0035 (L-glutamine), 0.015 (L-asparagine): pH optimum 8.6.

The released ammonia is detected with Nessler's reagent.

Assay solutions

0.05 M Tris/HCl pH 8.6

0.01 M L-asparagine solution (asparagine monohydrate, M_r =150.1; 150 mg in 100 ml 0.05 M Tris/HCl pH 8.6)

1.5 M trichloroacetic acid (TCA, M_r = 163.4; 24.5 g in 100 ml)

Nessler's reagent

Procedure

	Sample	Blank
0.05 M Tris/HCl pH 8.6	0.05 ml	–
0.01 M L-asparagine solution	0.85 ml	0.85 ml
1.5 M TCA	–	0.05 ml
Enzyme sample	0.05 ml	0.05 ml
10 min incubation at 37 °C (water bath)		
1.5 M TCA	0.05 ml	–
0.05 M Tris/HCl pH 8.6	–	0.05 ml
5 min centrifugation		
0.5 ml of the clear supernatant		
7.0 ml H_2O		
1.0 ml Nessler's Reagent		
10 min at room temperature		
Record absorption at 480 nm		

One unit is defined as the enzyme amount releasing 1 µmol NH_3/min at 37 °C, pH 8.6. Prepare an ammonium sulfate standard curve for quantification of the ammonia concentration.

Calculation

$$\text{units mg}^{-1} = \frac{\mu\text{mol NH}_3}{10\text{ min} \times \text{mg enzyme}}$$

References

Derst, C., Henseling, J., and Röhm, K.H. (2000) *Protein Sci.*, **9**, 2009–2017.
Kushoo, A. *et al.* (2004) *Protein Expr. Purif.*, **38**, 29–36.
Sult, H.M. and Herbut, P.A. (1970) *J. Biol. Chem.*, **240**, 2234–2242.
Wriston, J.C. and Yellin, T.O. (1973) *Adv. Enzymol.*, **39**, 185–248.

3.3.3.25 Glutaminase, EC 3.5.1.2

SN: L-glutamine amidohydrolase

$$\text{L-glutamine} + H_2O \rightarrow \text{L-glutamate} + NH_3$$

Enzyme from *Escherichia coli*: M_r 90 000, K_m (mM): 0.42 (L-glutamine), 2.9 (L-glutamate), enzyme from rat mitochondria: M_r 260 000, homotetramer (4 × 63 200), K_m (mM): 2.0 (L-glutamine), pH optimum 8.6.

A. Determination of Ammonia with Nessler's Reagent

Assay solutions

0.1 M acetate buffer pH 4.9 (sodium acetate, $M_r = 82.0$, dissolve 8.2 g in 800 ml H_2O, adjust to pH 4.9 with concentrated acetic acid, fill up to 1 l)

Substrate/acetate pH 4.9 (dissolve 0.82 g sodium acetate and 1.17 g L-glutamine, $M_r = 146.2$, in 80 ml H_2O, adjust to pH 4.9 with concentrated acetic acid, bring to 100 ml with H_2O)

15% TCA (trichloroacetic acid, $M_r = 163.4$)

Nessler's reagent

1 M NH_4Cl ($M_r = 53.5$, 0.53 g in 10 ml H_2O), for standard curve

Procedure

	Concentration
0.25 ml substrate/acetate pH 4.9	40/50 mM

0.25 ml enzyme sample.

Incubate 30 min, 37 °C

0.5 ml 15% TCA

Centrifuge, 5000 rpm, 5 min

0.5 ml supernatant

7.0 ml H_2O

1.0 ml Nessler's reagent

Read absorption at 480 nm against a blank with 0.1 M acetate buffer instead of the enzyme sample. Quantification with a standard curve prepared with different amounts of NH_4Cl.

B. pH-Stat Assay

Assay solutions

0.1 M L-glutamine ($M_r = 146.2$, 14.6 gl^{-1})

0.33 M KCl (M_r = 74.6, 24.6 g in 1 l H_2O)

0.01 M EDTA (ethylenediaminetetraacetic acid, M_r = 292.2; 29.2 mg in 10 ml)

1% BSA (bovine serum albumin, 100 mg in 10 ml)

5 mM HCl, as titrant

Assay mixture

Components	*Concentration*
6.1 ml 0.33 M KCl	200 mM
3.0 ml 0.1 M L-glutamine	30 mM
0.2 ml 0.1 M EDTA	0.2 mM
0.5 ml 1% BSA	0.05%

Procedure

Fill the automatic burette with 5 mM HCl

Add 0.98 ml assay mixture and

0.02 ml enzyme solution into the sample compartment of the pH stat

Keep the pH at 5.0 by the pH stat, 25 °C

Record the volume per unit time of the titrant added by the autotitrator for about 10 min

References

Arwadi, M.S.M. and Newsholme, E.A. (1984) *Biochem. J.*, **217**, 289–296.
Hartman, S.C. (1968) *J. Biol. Chem.*, **243**, 853–863.
Hartman, S.C. (1971) in *The Enzymes*, 3rd edn, vol. 4 (ed. P.D. Boyer), Academic Press, New York, London, pp. 79–100.

3.3.3.26 Urease, EC 3.5.1.5

SN: Urea amidohydrolase

$$\text{urea} + H_2O + 2H^+ \rightarrow CO_2 + 2NH_4^+$$

Nickel-containing enzyme; mammalian enzyme (bovine): M_r 130 000, K_m 0.83 mM (urea), pH optimum 8: *Klebsiella aerogenes*, K_m (mM):2.9 (urea), pH optimum 7.8. The release of ammonia can be determined either directly with the aid of a pH stat or by a photometric assay system coupled with the glutamate dehydrogenase reaction.

A. pH Stat Assay

Assay solutions

0.2 M urea ($M_r = 60.0$; 1.2 g in 100 ml H_2O, adjust to pH 6.1)

0.1 M HCl, standard solution

Urease solution (1 mg ml^{-1})

Procedure

Fill the automatic burette with 0.1 N HCl

Give 9 ml 0.2 M urea and

0.2 ml urease into the sample compartment of the pH stat

Keep the pH at 6.1 by the pH stat

Record the volume per unit time of the titrant added by the autotitrator for about 10 min

B. Photometric Assay

$$\text{urea} + H_2O + 2H^+ \rightarrow CO_2 + 2NH_4^+$$

$$2NH_4^+ + 2\alpha\text{-oxoglutarate} + 2\text{NADH} \xrightarrow{\text{GluDH}} 2\ \text{glutamate} + 2\ NAD^+ + 2H_2O$$

Assay solutions

0.1 M potassium phosphate pH 7.6

2.0 M urea ($M_r = 60.0$; 12 g in 100 ml 0.1 M potassium phosphate pH 7.6)

25 mM ADP (disodium salt, $M_r = 471.2$; 118 mg in 10 ml 0.1 M potassium phosphate pH 7.6)

10 mM NADH (disodium salt, $M_r = 709.4$; 71 mg in 10 ml 0.1 M potassium phosphate pH 7.6)

0.1 M α-oxoglutarate (monosodium salt, $M_r = 168.1$; 168 mg in 10 ml 0.1 M potassium phosphate pH 7.6)

Glutamate dehydrogenase from bovine liver (GluDH, 500 IU ml^{-1} in 0.1 M potassium phosphate pH 7.6)

Urease (1 mg ml^{-1}, dilute to 0.1 U/ml in 0.1 M potassium phosphate pH 7.6 prior to use)

Assay mixture

Components	*Concentration*
8.6 ml 0.1 M potassium phosphate pH 7.6	86 mM
0.3 ml 25 mM ADP	0.75 mM
0.2 ml 10 mM NADH	0.2 mM
0.1 ml 0.1 M α-oxoglutarate	1.0 mM
0.3 ml 2.0 M urea	60 mM
0.3 ml GluDH	15 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml urease

Record the absorbance decrease at 340 nm, 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

Mahadevan, S., Sauer, F.D., and Erfle, J.D. (1977) *Biochem. J.*, **163**, 495–501.
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3.3.3.27 Adenosinetriphosphatase, EC 3.6.1.3

SN: ATP phosphohydrolase, NTPase/helicase, ATPase

$$ATP + H_2O \rightarrow ADP + P_i$$

ATPase activities are connected with membrane components, such as the oxidative phosphorylation or transport systems. The nonsoluble activity must be measured in cell homogenates or membrane suspensions. Requirement for Mg^{2+}, M_r 67 000 (*Escherichia coli*), K_m 0.1 mM (ATP, hepatitis C virus), pH optimum 7 (human).

Assay solutions

0.1 M TES/Tris pH 7.5 (TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, $M_r = 229.3$; 3.44 g in 100 ml, adjust to pH 7.5 with 0.2 M TRIS {tris(hydroxymethyl)aminomethane, M_r 121.1; 4.84 g in 200 ml} and fill up to 200 ml)

0.1 M ATP (disodium salt trihydrate, $M_r = 605.2$; 605 mg in 10 ml)

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, $M_r = 203.3$; 203 mg in 10 ml)

BSA solution, 100 mg in 100 ml

10% (w/v) sodium dodecylsulfate (SDS)

Assay mixture

Components	*Concentration*
7.5 ml 0.1 M TES/Tris pH 7.5	75 mM
1 ml 0.1 M $MgCl_2$	10 mM
0.5 ml 0.1 M ATP	5.0 mM
1 ml BSA	0.1 mg ml^{-1}

Procedure

Incubate 1 ml assay mixture with samples of 5–20 µl of the membrane suspension under gentle shaking at 37 °C

Add 1 ml SDS solution after a distinct time (1, 5, 10 min, depending on the enzyme activity) to stop the reaction

Measure inorganic phosphate formed by the phosphate determination method (cf. Section 3.4.2)

The *enzyme activity* is defined as 1 µmol inorganic phosphate formed per minute.

References

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3.3.4 Lyases, EC 4

3.3.4.1 Pyruvate Decarboxylase, EC 4.1.1.1

SN: 2-oxo-acid carboxy-lyase (aldehyde-forming). PDC

$$\text{pyruvate} \rightarrow \text{acetaldehyde} + CO_2$$

Thiamine diphosphate and Mg^{2+} as cofactors; bacterial enzyme (*Zymomonas mobilis*): M_r 240 000, homotetramer (4 × 59 000), K_m 0.3 mM (pyruvate), pH optimum 6. The carbon dioxide released can be determined manometrically or with $^{14}C1$-pyruvate as substrate. Here, an assay coupled to ADH (EC 1.1.1.1) is described:

$$\text{acetaldehyde} + \text{NADH} + \text{H}^+ \xrightarrow{\text{ADH}} \text{ethanol} + \text{NAD}^+$$

Assay solutions

0.2 M citrate buffer pH 6.0 (prepare 0.2 M citric acid, $M_r = 192.1$, 38.4 g in 1 l, and 0.2 M trisodium citrate, $M_r = 258.1$, 51.6 g in 1 l, measure pH in the salt solution, and adjust to pH 6.0 with the citric acid solution)

1 M pyruvate (sodium salt, $M_r = 110.0$; 1.1 g in 10 ml H_2O)

0.01 M thiamin diphosphate (ThDP, cocarboxylase, $M_r = 460.8$; 46.1 mg in 10 ml H_2O)

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, $M_r = 203.3$; 203 mg in 10 ml)

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg in 10 ml)

ADH from yeast (20 mg ml^{-1})

Assay mixture

Components	*Concentration (mM)*
8.9 ml 0.2 M citrate buffer pH 6.0	178
0.3 ml 0.01 M NADH	0.3
0.3 ml 1 M pyruvate	30
0.20 ml 0.01 M ThDP	0.2
0.10 ml 0.1 M $MgCl_2$	1.0
0.01 ml ADH	–

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Record the absorbance decrease at 340 nm, 25 °C, $\varepsilon_{340} = 6300\,l\,mol^{-1}\,cm^{-1}$.

References

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3.3.4.2 Glutamate Decarboxylase, EC 4.1.1.15

SN: L-glutamate 1-carboxy-lyase (4-aminobutanoate-forming), GAD, aspartate 1-decarboxylase

L-glutamate → 4-aminobutanoate + CO_2

Mammalian enzyme (human): M_r 140 000, dimer (2 × 67 000), cofactor: pyridoxal 5-phosphate, K_m (mM): 1.28 (L-glutamate), pH optimum 7.4. The following assay measures the release of $^{14}CO_2$ from L-[1-^{14}C]glutamic acid.

Assay solutions

0.1 M potassium phosphate pH 7.0

L-glutamic acid solution, containing 50 mM L-glutamate (83.6 mg monosodium salt, M_r = 169.1) and 1 µCi L-[1-^{14}C]glutamic acid in 10 ml 0.1 M potassium phosphate pH 7.0

0.02 M AET (2-aminoethylisothiouronium bromide hydrobromide, M_r = 281.0, 54.2 mg in 10 ml)

0.02 M EDTA (ethylenediaminetetraacetic acid, M_r = 292.2; 58.4 mg in 10 ml)

5 mM PLP (pyridoxal 5-phosphate, M_r = 247.1; 12 mg in 10 ml potassium phosphate pH 6.8)

0.02 M DTT (dithiothreitol, Cleland's reagent, M_r = 154.2, 31 mg in 10 ml)

Hyamine hydroxide

Scintillation fluid

0.25 M H_2SO_4

Assay mixture

Components	*Concentration*
0.6 ml L-glutamic acid solution	30 mM, 0.06 µCi ml^{-1}
0.1 ml 0.02 M AET	2 mM
0.05 ml 0.02 M EDTA	1 mM
0.1 ml PLP	0.5 mM
0.05 ml 0.02 M DTT	1 mM

Procedure

The reaction is carried out in disposable tubes (~12 × 80 mm), tightly closed by a rubber stopper. CO_2 released is adsorbed either on a scintillation pad,

containing 0.25 mmol KOH, fixed ~6 cm above the reaction mixture, or by 0.2 ml hyamine hydroxide, placed in a separate, open central well in the reaction tube.

0.09 ml reaction mixture

0.01 ml enzyme sample

Incubate 60 min at 37 °C in a shaking temperature bath

Stop the reaction by injecting 0.2 ml 0.25 M H_2SO_4 through the stopper into the reaction mixture

60 min 37 °C to establish complete CO_2 release and adsorption

Transfer the scintillation pad, respectively the central well with hyamine into 5 ml scintillation fluid, and count radioactivity in a scintillation counter

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3.3.4.3 Aldolase, EC 4.1.2.13

SN: D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase (glycerone-phosphate-forming), fructose-bisphosphate aldolase (ALDC).

D-fructose-1, 6-bisphosphate ⇌

glyceronephosphate + D-glyceraldehyde-3-phosphate

Enzyme from human liver: M_r 158 000, dimer, K_m (mM): 0.0027 (D-fructose-1,6-bisphosphate). 0.88 (D-fructose-1-phosphate). D-Glyceraldehyde-3-phosphate forms a hydrazone with hydrazine, absorbing at 240 nm.

Assay solutions

3.5 mM hydrazine sulfate in 1 mM EDTA, pH 7.5 (dissolve 455 mg hydrazine sulfate, M_r = 130.1, and 372 mg EDTA · Na_2 · $2H_2O$, M_r = 372.2, in 1 l H_2O, adjust to pH 7.5 with 1 N NaOH)

12 mM fructose-1,6-bisphosphate (FDP-Na_3H, M_r = 406.1; 487 mg in 100 ml H_2O, adjust to pH 7.5)

Aldolase solution, about 1 IU ml^{-1} (prepare just before use)

10 mM D,L-glyceraldehyde-3-phosphate (M_r = 170.1, 17 mg in 10 ml)

Assay mixture

Components	*Concentration (mM)*
3.0 ml 12 mM fructose-1,6-bisphosphate	3.6
6.0 ml 3.5 mM hydrazine sulfate solution	2.1

Procedure

0.9 ml assay mixture

0.1 ml aldolase solution

Record the absorption change at 240 nm, 25 °C for about 10 min. For the blank fructose-1,6-bisphosphate is substituted by water

For quantification, prepare a standard curve with aliquots of 0.6 ml 3.5 mM hydrazine sulfate solution and varying concentrations of D,L-glyceraldehyde-3-phosphate in 0.4 ml

References

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3.3.4.4 Anthranilate Synthase, EC 4.1.3.27

SN: chorismate pyruvate-lyase (amino-accepting, anthranilate-forming), chorismate lyase

$$\text{chorismate} + \text{L-glutamine} \rightarrow \text{anthranilate} + \text{pyruvate} + \text{L-glutamate}$$

Key enzyme in the biosynthesis of aromatic amino acids. Bacterial enzyme (*Serratia marcescens*): M_r 141 000, tetramer (2 × 21 000, 2 × 60 000), K_m (mM): 0.015 (chorismate), 0.5 (glutamine), inhibited by L-tryptophan, pH optimum 8.5. Chorismate is unstable and should be stored at −70 °C. A procedure for isolation of chorismate is described in Gibson (1964).

Assay solutions

10 mM chorismate (M_r = 361.5, barium salt, 18.1 mg in 5 ml)

0.2 M L-glutamine (M_r = 146.2, 292 mg in 10 ml)

0.1 M $MgCl_2$ (hexahydrate, M_r = 203.3; 203 mg in 10 ml)

0.1 M dithioerythritol (DTE, M_r = 154.2, 154 mg in 10 ml)

0.01 M anthranilic acid (M_r = 137.1, 13.7 mg in 10 ml)

Assay mixture and procedure

	Concentration (mM)
1.7 ml 50 mM potassium phosphate pH 7.4	42.5
0.1 ml 10 mM magnesium chloride	5.0
0.1 ml 0.2 M L-glutamine	10
0.02 ml 10 mM chorismate	0.1
0.03 ml 0.1 M DTE	1.5
0.05 ml enzyme sample	–

Measure the fluorescence emission at 400 nm, excitation is at 325 nm, 37 °C. For quantification, determine the fluorescence of various dilutions of the anthranilic acid solution within the concentration range 1–100 µM.

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3.3.4.5 Carbonic Anhydrase, EC 4.2.1.1

SN: carbonate hydro-lyase (carbon dioxide-forming), carbonate dehydratase, CA

$$H_2CO_3 \rightarrow CO_2 + H_2O$$

Zn-containing glycoprotein; mammalian enzyme (human, bovine stomach): M_r 44 000, K_m (mM) 2.9 (4-nitrophenylacetate), 2.3 (CO_2), pH optimum 7.6 (9.5 for hydrolysis of 4-nitrophenylacetate). An assay for a pH meter or pH stat and an assay to test the esterase activity of carbonic anhydrase with 4-nitrophenylacetate are described.

A. pH-Stat Assay

Assay solutions

0.02 M Tris–HCl, pH 8.0

CO_2-saturated H_2O (pass CO_2 gas through 200 ml of ice-cold water for 30 min)

Procedure

All solutions must be chilled to 0–4 °C

	Sample	Blank
0.02 M Tris-HCl pH 8.0	6 ml	6 ml
CO_2 water (ice cold)	4 ml	4 ml
	–	Measure pH and determine time T_0 required for the pH to drop from 8.3 to 6.3
Enzyme sample	0.1 ml	–
	Measure pH and determine time T required for the pH to drop from 8.3 to 6.3	–

Calculation

A unit is defined as $2 \times (T_0 - T)/T$:

$$\text{units mg}^{-1} = \frac{2 \times (T_0 - T)}{T \times \text{mg enzyme}}$$

B. Esterase Assay with 4-Nitrophenylacetate

$$\text{4-nitrophenylacetate} \rightarrow \text{4-nitrophenol} + \text{acetate}$$

Assay solutions

0.1 M HEPES pH 7.2

3 mM 4-nitrophenylacetate (M_r = 181.1, dissolve 27.2 mg in 1 ml acetone, dilute rapidly under vigorous shaking with H_2O to 50 ml)

Procedure

	Concentration (mM)
0.5 ml 0.1 M HEPES pH 7.2	50
0.5 ml 3 mM 4-nitrophenylacetate	1.5

Measure the absorption increase at 405 nm, $\varepsilon_{405} = 18\,500\,\text{l mol}^{-1}\,\text{cm}^{-1}$. The reaction can also be followed at 348 nm, the isobestic point of *p*-nitrophenol and the conjugated nitrophenolate ion, difference absorption coefficient: $\varepsilon_{348} = 5000\,\text{l mol}^{-1}\,\text{cm}^{-1}$.

References

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3.3.4.6 Fumarase, EC 4.2.1.2

S-malate hydro-lyase (fumarate-forming), fumarate hydratase

$$\text{L-malate} \rightleftharpoons \text{fumarate} + H_2O$$

Fe enzyme, exists in *Escherichia coli* in three forms, FUMA, FUMB, FUMC dependent on the oxygen level. FUMA : M_r 120 000, dimer, 2 × 61 000, K_m (mM): 1.1 (malate), 0.15 (fumarate), FUMC, M_r 200 000, tetramer (4 × 50 000), K_m (mM): 2.9 (malate) 0.39 (fumarate), pH optimum 8.5.

Assay solutions

0.1 M potassium phosphate pH 7.5

0.05 M L-malate solution (L-malic acid, $M_r = 134.1$; suspend 134 mg in 10 ml 0.1 M potassium phosphate pH 7.5, neutralize with 3.5 ml 1 N NaOH and fill up to 20 ml with 0.1 M potassium phosphate pH 7.5)

0.1% serum albumin (w/v) in 0.1 M potassium phosphate pH 7.5

Procedure

0.98 ml 0.05 M L-malate solution

0.02 ml fumarase solution (diluted with 0.1% serum albumin)

Follow the absorption increase at 240 nm at 25 °C, $\varepsilon_{240} = 2.44 \times 10^3\,\text{l mol}^{-1}\,\text{cm}^{-1}$.

References

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3.3.5 Isomerases, EC 5

3.3.5.1 Glucose/Xylose Isomerase EC 5.3.1.5

SN: D-xylose aldose–ketose isomerase, D-xylose ketol isomerase

D-xylose ⇌ D-xylulose

D-glucose ⇌ D-fructose

Glucose isomerase is an intensely technical applied enzyme, due to its capacity to convert glucose to the more valuable fructose, although its natural function is the isomerization of xylose, an important nutrient for bacteria. Xylose, produced by digestion of hemicelluloses, xylans, and arabans, is introduced into the pentose phosphate pathway after isomerization to xylulose. Because of its analogous conformation, glucose is isomerized by the same enzyme to fructose but with five times lower efficiency. Bacterial enzyme, dependent on divalent cations (Mn^{2+}, Co^{2+}, Mg^{2+}), inhibited by EDTA, *Thermus aquaticus*, M_r 196 000 homotetramer (4 × 50 000), K_m (mM): 8 (D-xylulose), 15, (D-xylose), 93 (D-glucose), K_d (mM): 0.0009 (Mn^{2+}), 0.001 (Co^{2+}), pH optimum 5.5; *Bacillus* sp. M_r 120 000, homodimer (2 × 58 000), K_m (mM): 0.076 (D-xylose), pH optimum 7; *Arthrobacter* sp.: K_m (mM): 3.3 (D-xylose), 110 (D-fructose), 230 (D-glucose).

A. D-Xylose Isomerase Assay

Assay solutions

0.2 M TES/NaOH pH 8.0 (*N*-tris[hydroxymethyl]-methyl-2-aminomethane sulfonic acid, M_r = 229.3; 9.2 g in 100 ml, adjust with 1 M NaOH to pH 8.0, fill up to 200 ml with H_2O)

0.4 M D-xylose (M_r = 150.1; 1.2 g in 20 ml)

0.03 M $MnSO_4$ (monohydrate, M_r = 169.0; 57 mg in 10 ml)

1.5% cysteine · HCl solution (monohydrate M_r = 175.6; dissolve 1.5 g in 100 ml H_2O)

0.12% carbazole solution (M_r = 167.2; 120 mg in 100 ml in ethanol)

70% sulfuric acid

10 mM D-xylulose (M_r = 150.1, 7.5 mg in 5 ml) for a standard curve

Assay mixture

Components	*Concentration (mM)*
0.24 ml 0.2 M TES/NaOH pH 8.0	48
0.25 ml 0.4 M D-xylose	100
0.01 ml 0.03 M $MnSO_4$	0.3

Procedure

20 µl assay mixture

20 µl enzyme solution (or 20 µl TES buffer for the blank)

- Incubate at the assay temperature (25 or 50 °C, dependent on the enzyme sample) for 15 min in tightly closed reaction vessels
- Stop by chilling on ice

40 µl cysteine · HCl

40 µl of carbazole solution

1.2 ml 70% sulfuric acid starts the development of the color

10 min, room temperature

Measure the absorption against the blank at 546 nm.

Remark: The color intensity is not constant, therefore the 10 min interval for color development must be observed carefully and all samples must be read exactly after the same time interval. The absorption of the blank should be about 0.2, if it exceeds an absorption of 0.3 the assay reagents should be prepared afresh.

For quantification, prepare a calibration curve with D-xylulose between 0 and 10 µM.

B. D-Xylose Isomerase Microplate Assay

Assay solutions

0.2 M TES/NaOH pH 8.0 (*N*-tris[hydroxymethyl]-methyl-2-aminomethane sulfonic acid, $M_r = 229.3$; 9.2 g in 100 ml, adjust with 1 M NaOH to pH 8.0, fill up to 200 ml with H_2O)

0.4 M D-xylose ($M_r = 150.1$; 1.2 g in 20 ml)

0.03 M $MnSO_4$ (monohydrate, $M_r = 169.0$; 57 mg in 10 ml)

Solution A: 0.05% resorcinol (M_r = 110.1; 50 mg in 100 ml ethanol)

Solution B: $FeNH_4(SO_4)_2 \cdot 12H_2O$ (M_r = 482.2; 216 mg in 1 l 37%. HCl)

Assay mixture

Components	*Concentrations (mM)*
0.24 ml 0.2 M TES/NaOH pH 8.0	48
0.25 ml 0.4 M D-xylose	100
0.01 ml 0.03 M $MnSO_4$	0.3

Procedure

Place 20 µl assay mixture and

20 µl enzyme solution (or 20 µl TES buffer for the blank) in a 96-well microplate

Incubate for 15 min, 50 °C

0.15 ml freshly prepared mixture (1 : 1, v/v) of solution A and solution B

Incubate at 80 °C for 40 min

Measure the absorption with a microplate reader at 630 nm

C. D-Glucose Isomerase Assay

Assay solutions

0.2 M TES/NaOH pH 8.0 (*N*-tris[hydroxymethyl]-methyl-2-aminomethane-sulfonic acid, M_r = 229.3; 9.2 g in 100 ml, adjust with 1 M NaOH to pH 8.0, fill up to 200 ml with H_2O)

2.0 M D-glucose (M_r = 180.2; 7.2 g in 20 ml)

0.03 M $CoCl_2$ (hexahydrate, M_r = 237.9; 71 mg in 10 ml)

1.5% cysteine · HCl (solution cysteine · HCl monohydrate M_r = 175.6; dissolve 1.5 g in 100 ml H_2O)

0.12% carbazole solution (M_r = 167.2; 120 mg in 100 ml in ethanol)

70% sulfuric acid

0.01 M D-fructose (M_r = 180.2, 18.2 mg in 10 ml), for calibration curve

Assay mixture and procedure

	Concentration (mM)
0.24 ml 0.2 M TES/NaOH pH 8.0	48
0.25 ml 2.0 M D-glucose	500
0.01 ml 0.03 M $CoCl_2$	0.3

The assay procedure is the same as for xylose isomerase (assay A), except that – after the enzyme reaction and the addition of cysteine hydrochloride, carbazole, and sulfuric acid – the mixture must remain for 30 min for color development; the absorption is measured at 560 nm. The calibration curve must be prepared with D-fructose.

D. D-Glucose Isomerase Microplate Assay

Assay solutions

0.2 M TES/NaOH pH 8.0 (*N*-tris[hydroxymethyl]-methyl-2-aminomethane sulfonic acid, $M_r = 229.3$; 9.2 g in 100 ml, adjust with 1 M NaOH to pH 8.0, fill up to 200 ml with H_2O)

2.0 M D-glucose ($M_r = 180.2$; 7.2 g in 20 ml)

0.03 M $CoCl_2$ (hexahydrate, $M_r = 237.9$; 71 mg in 10 ml)

Solution A: 0.05% resorcinol ($M_r = 110.1$; 50 mg in 100 ml ethanol)

Solution B: $FeNH_4(SO_4)_2 \cdot 12H_2O$ ($M_r = 482.2$), 216 mg in 1 l 37% HCl

Assay mixture

Components	*Concentration (mM)*
0.24 ml 0.2 M TES/NaOH pH 8.0	48
0.25 ml 2.0 M D-glucose	500
0.01 ml 0.03 M $CoCl_2$	0.3

The assay procedure is the same as for xylose isomerase (assay B), with the exception that the absorption is measured at 490 nm.

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3.3.5.2 Phosphoglucomutase, EC 5.4.2.2

SN: α-D-glucose-1,6-phosphoglucomutase, PGM

D-glucose-1-phosphate ⇌ D-glucose-6-phosphate

Bacterial enzyme (*Bacillus subtilis*): M_r 130 000, dimer (2 × 60 000), K_m (mM): 0.012 (α-D-glucose), pH optimum 8.3. Fluorimetric assay, coupled with glucose 6-phosphate dehydrogenase (EC 1.1.1.49)

D-glucose-6-phosphate + $NADP^+$ ⇌ 6-phosphogluconate + NADPH + H^+

Assay solutions

50 mM MOPS buffer pH 7.4, 1 mM DTT (MOPS, 3-*N*-morpholinopropanesulfonic acid, M_r = 209.3, dissolve 10.5 g in 0.8 l H_2O, add 154 mg DTT (dithiothreitol, Cleland's reagent, M_r = 154.2), adjust to pH 7.4 with 1 N NaOH, fill up to 1 l with H_2O)

0.1 M NAD (M_r = 663.4; 0.663 g in 10 ml 50 mM MOPS buffer pH 7.4)

0.1 M $MgSO_4$ (M_r = 120.4, 120 mg in 10 ml H_2O)

0.1 M D-glucose-1-phosphate (disodium salt, hydrate, M_r = 304.1, 304 mg in 10 ml H_2O)

0.5 mM D-glucose-1,6-bisphosphate (potassium salt, hydrate, M_r = 340.1, 1.7 mg in 10 ml H_2O)

0.01 M NADH (disodium salt, M_r = 709.4; 71 mg 10 ml^{-1} H_2O), for standard curve

Assay mixture

Components	*Concentration*
9.52 ml 50 mM MOPS buffer pH 7.4	48 mM
0.1 ml 0.1 M NAD	1.0 mM
0.02 ml 0.1 M D-glucose-1-phosphate	0.2 mM
0.01 ml 0.5 mM D-glucose-1,6-bisphosphate	0.5 µM
0.15 ml 0.1 M $MgSO_4$	1.5 mM

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Follow the fluorescence increase at 430 nm (excitation 340 nM), for quantification prepare a standard curve with NADH.

References

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3.3.6 Ligases (Synthetases) EC 6

3.3.6.1 Tyrosine-tRNA Ligase, EC 6.1.1.1

SN: L-tyrosine:tRNA Tyr–ligase (AMP-forming)

$$\text{ATP} + \text{L-tyrosine} \rightarrow \text{AMP} + \text{PP}_i + \text{L-tyrosyl-tRNA-Tyr}$$

Assays for this enzyme are described exemplarily for the group of amino acid tRNA ligases. Mammalian enzyme (human): M_r 130 000, dimer; K_m (mM) 3 (ATP), 0.034 (tyrosine), pH optimum 7.5; *Escherichia coli*: M_r 90 000, dimer, K_m 0.5 mM (ATP), 0.012 mM (tyrosine), 0.52 µM (tRNA–Tyr), pH optimum 7.6.

A. Fluorimetric Assay This assay depends on the decrease of intrinsic tyrosine fluorescence due to the reaction and is thus, specific for tyrosine–tRNA ligase. Pyrophosphatase, cleaving PP_i serves to support the reaction for the product side.

Assay solutions

0.15 M Tris/HCl, 0.15 M KCl buffer pH 7.5 (tris(hydroxymethyl)amino-methane, Tris, $M_r = 121.1$; KCl, $M_r = 74.6$; dissolve 18.2 g Tris and 11.2 g KCl in 600 ml H_2O, adjust the pH with 1 M HCl to 7.5 and fill up with H_2O to 1 l)

0.1 M ATP (disodium salt, trihydrate, $M_r = 605.2$; 605 mg in 10 ml buffer)

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, $M_r = 203.3$; 203 mg in 10 ml buffer)

2 mM L-tyrosine ($M_r = 181.2$; 36 mg in 100 ml buffer)

0.1 M DTE (1,4-dithioerythritol, M_r =154.3; 154 mg in 10 ml buffer)

Inorganic pyrophosphatase (dilute to 200 unit ml^{-1} with buffer)

Assay mixture

Components	*Concentration*
6.6 ml 0.15 M Tris/HCl/KCl pH 7.5	0.15 M
1 ml 0.1 M ATP	10 mM
1 ml 0.1 M $MgCl_2$	10 mM
0.5 ml 2 mM L-tyrosine	0.1 mM
0.2 ml 0.1 M DTE	2 mM
0.5 ml inorganic pyrophosphatase	10 units

Procedure

1.95 ml assay mixture

0.4 ml enzyme sample

Measure the decrease of the fluorescence intensity (excitation 295, emission $\geq$ 320 nm) in quartz fluorescence cuvettes at 25 °C; for quantification, prepare a standard curve with various tyrosine concentrations.

B. ATP – ^{32}PP-Exchange

Assay solutions and substances

0.1 M Tris/HCl, pH 7.0

0.1 M ATP (disodium salt, trihydrate, M_r = 605.2; 605 mg in 10 ml 0.1 M Tris/HCl, pH 7.0)

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, M_r = 203.3; 203 mg in 10 ml 0.1 M Tris/HCl, pH 7.0)

2 mM L-tyrosine (M_r = 181.2; 36 mg in 100 ml 0.1 M Tris/HCl, pH 7.0)

0.4 M sodium diphosphate (sodium pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$, M_r = 446; 17.84 g in 100 ml 15% perchloric acid)

0.1 M radiolabeled diphosphate ($^{32}PP_i$, 10^4–10^5 cpm/mol, adjust the concentration with cold sodium diphosphate, if necessary)

0.1 M DTE (1,4-dithioerythritol, M_r =154.3; 154 mg in 10 ml 0.1 M Tris/HCl, pH 7.0)

BSA (bovine serum albumine, 10 mg ml^{-1})

Charcoal (15% suspension)

Assay mixture

Components	*Concentration*
7.6 ml 0.1 M Tris/HCl pH 7.0	~0.1 M
0.2 ml 0.1 M ATP	2 mM
0.5 ml 0.1 M $MgCl_2$	5 mM
0.2 ml 0.1 M ^{32}PPi	2 mM
1.0 ml 2 mM L-tyrosine	0.2 mM
0.2 ml 0.1 M DTE	2 mM
0.1 ml BSA	0.1 mg ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample (~0.5 IU)

15 min, 37 °C

0.7 ml cold 0.4 M sodium pyrophosphate in 15% perchloric acid
0.1 ml 15% charcoal suspension

Filter through a glass microfiber filter (GF/A), wash five times with 5 ml portions of cold H_2O, and dry the filter.

Immerse in an appropriate scintillation liquid and count in a scintillation spectrometer.

An enzyme unit is defined as the incorporation of 1 µmol ^{32}PP into ATP in 1 min.

References

Austin, J. and First, E.A. (2002) *J. Biol. Chem.*, **277**, 14812–14820.
Austin, J. and First, E.A. (2002) *J. Biol. Chem.*, **277**, 28394–28399.
Buonocore, V. and Schlesinger, S. (1972) *J. Biol. Chem.*, **24**, 1343–1348.
Buonocore, V., Harris, M.H., and Schlesinger, S. (1972) *J. Biol. Chem.*, **247**, 4843–4849.

3.3.6.2 Glutamine Synthetase EC 6.3.1.2

SN: L-glutamate:ammonia ligase (ADP-forming)

$$\text{L-glutamate} + \text{ATP} + \text{NH}_3 \rightarrow \text{L-glutamine} + \text{ADP} + \text{P}_i$$

Enzyme from *Escherichia coli*: dodecamer (12 × 51 800), K_m (mM): 3.3 (L-glutamate), 0.2 (ATP), pH optimum 8.0. The assay is coupled with pyruvate kinase (PK) and lactate dehydrogenase (LDH)

$$\text{ADP} + \text{phosphoenolpyruvate} \overset{\text{PK}}{\rightleftharpoons} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{L-lactate} + \text{NAD}^+$$

Assay solutions

0.05 M buffer (HEPES or imidazol/HCl) pH 7.1

1 M L-glutamate (monosodium salt, $M_r = 169.1$, 1.69 g in 10 ml buffer)

0.1 M ATP (disodium salt trihydrate, $M_r = 605.2$; 605 mg in 10 ml buffer)

0.1 M PEP (phosphoenolpyruvate, tricyclohexylammonium salt, $M_r = 465.6$; 466 mg in 10 ml buffer)

0.01 M NADH (disodium salt, $M_r = 709.4$; 71 mg in 10 ml buffer)

1 M KCl ($M_r = 74.6$, 7.5 g in 100 ml H_2O)

1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, $M_r = 203.3$; 2.03 g in 10 ml buffer)

1 M NH_4Cl ($M_r = 53.5$, 0.53 g in 10 ml H_2O)

PK (pyruvate kinase, commercially available for various activities, prepare a solution with 300 IU 1 ml^{-1} in buffer)

LDH (lactate dehydrogenase, commercial products contain about 500 IU mg^{-1}, prepare a solution with 1500 IU ml^{-1} in buffer)

Assay mixture

Components	*Concentration*
6.8 ml 0.05 M buffer pH 7.1	45 mM
0.5 ml 0.1 M ATP	5.0 mM
0.1 ml 0.1 M PEP	1.0 mM
0.1 ml 1 M KCl	10 mM
0.5 ml 1 M $MgCl_2$	50 mM
0.4 ml 1 M NH_4Cl	40 mM
0.1 ml 10 mM NADH	0.1 mM
1.0 ml 1 M L-glutamate	100 mM
0.1 ml PK	3 IU ml^{-1}
0.2 ml LDH	30 IU ml^{-1}

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

Follow the absorption decrease at 340 nm at 25 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

References

Alibhai, M. and Villafranca, J.J. (1994) *Biochemistry*, **33**, 682–686.
Ginsburg, A. *et al.* (1970) *Biochemistry*, **9**, 633–649.
Kingdon, H.S., Hubbard, J.S., and Stadtman, E.R. (1968) *Biochemistry*, **7**, 2136–2142.
Pearson, J.T. (2005) *Arch. Biochem. Biophys.*, **436**, 397–405.

3.3.7 Assays for Multienzyme Complexes

3.3.7.1 Pyruvate Dehydrogenase Complex (PDHC)

This large enzyme complex ($M_r = \sim 5 \times 10^6$ from bacterial sources, $\sim 8 \times 10^6$ from mammalian sources) combines three different enzyme activities catalyzing consecutive reactions: pyruvate dehydrogenase (E1p, EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2p, EC 2.3.1.12), and dihydrolipoamide dehydrogenase (E3p, EC 1.8.1.4):

$$\text{pyruvate} + \text{ThDP-E1p} \rightarrow \text{hydroxyethyl ThDP-E1p} + CO_2$$
$$\text{hydroxyethyl ThDP-E1p} + \text{lipoyl E2p} \rightarrow \text{acetyl dihydrolipoyl-E2p} + \text{ThDP-E1p}$$
$$\text{acetyl dihydrolipoyl-E2p} + \text{CoA} \rightarrow \text{acetyl CoA} + \text{dihydrolipoyl-E2p}$$
$$\text{dihydrolipoyl E2p} + FAD_{ox}\text{-E3p} \rightarrow \text{lipoyl E2p} + FAD_{red}\text{-E3p}$$
$$FAD_{red}\text{-E3p} + NAD^+ \rightarrow FAD_{ox}\text{-E3p} + \text{NADH} + H^+$$

$$\text{pyruvate} + NAD^+ + \text{CoA} \rightarrow \text{acetyl CoA} + CO_2 + \text{NADH} + H^+$$

ThDP is the thiamin diphosphate cofactor of the E1p component, FAD_{ox} and FAD_{red} are the oxidized and the reduced cofactors, respectively, of E3p.

Assays for the three enzymes are described separately, while here assays for the overall activity are presented. Pyruvate dehydrogenase complexes from higher organisms possess two regulatory enzymes, the pyruvate dehydrogenase kinase which inactivates the overall activity by phosphorylating serine residues at the E1p component, and a pyruvate dehydrogenase phosphatase that reverses phosphorylation and reactivates the enzyme. Two assays for the overall reaction are described. The photometric assay observing the formation of NADH is convenient, but is disturbed in crude extracts by the LDH reaction which forms lactate and NAD from pyruvate and NADH and thus, counteracts the assay. The more laborious and less sensitive dismutation assay circumvents this problem. Alternatively, the

overall activity of the pyruvate dehydrogenase complex can be determined by observing the production of CO_2 either by manometric methods, using a CO_2 electrode, or by measuring the radioactivity released from [1-^{14}C]pyruvate as a substrate.

A. Overall Activity of PDHC by NAD^+ Reduction

Assay solutions

0.1 M potassium phosphate pH 7.6

0.1 M NAD^+ ($M_r = 663.4$, free acid; 663 mg in 10 ml)

0.01 M thiamin diphosphate (ThDP, cocarboxylase, $M_r = 460.8$; 46.1 mg in 10 ml)

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, $M_r = 203.3$; 203 mg in 10 ml)

0.1 M pyruvate (sodium pyruvate, $M_r = 110.0$; 110 mg in 10 ml)

0.1 M dithioerythritol (DTE, $M_r = 154.2$; 154 mg in 10 ml)

0.01 M coenzyme A (free acid, $M_r = 767.5$, $CoA \cdot Li_3$ $M_r = 785.4$; 23 mg in 3 ml)

Assay mixture

Components	*Concentration (mM)*
8.80 ml 0.1 M potassium phosphate pH 7.6	88
0.25 ml 0.1 M NAD^+	2.5
0.20 ml 0.01 M ThDP	0.2
0.10 ml 0.1 M $MgCl_2$	1.0
0.25 ml 0.1 M pyruvate	2.5
0.10 ml 0.1 M DTE	1.0
0.10 ml 0.01 M CoA	0.1

Procedure

0.98 ml assay mixture

0.02 ml enzyme sample

The absorption increase at 340 nm is measured at 37 °C. Absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

Reference

Schwartz, E.R., Old, L.O., and Reed, L.J. (1968) *Biochem. Biophys. Res. Commun.*, **31**, 495–500.

B. Overall Activity of PDHC by Dismutation Assay Stopped assay, using LDH to regenerate NAD. Acetylphosphate is formed from acetyl CoA by the phosphotransacetylase (PTA) and is converted into hydroxamic acid, which forms a colored complex with Fe^{3+}.

$$\text{pyruvate} + \text{NAD}^+ + \text{CoA} \overset{\text{PDHC}}{\rightleftharpoons} \text{acetyl CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

$$\text{acetyl CoA} + \text{P}_\text{i} \overset{\text{PTA}}{\rightleftharpoons} \text{acetyl P} + \text{CoA}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{lactate} + \text{NAD}^+$$

Assay solutions

0.1 M potassium phosphate pH 7.6

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, $M_r = 203.3$; 203 mg in 10 ml)

0.1 M pyruvate (sodium pyruvate, $M_r = 110.0$; 110 mg in 10 ml H_2O)

0.01 M thiamin diphosphate (cocarboxylase, $M_r = 460.8$; 46.1 mg in 10 ml)

0.1 M dithioerythritol (DTE, $M_r = 154.2$; 154 mg in 10 ml)

0.01 M coenzyme A (free acid, $M_r = 767.5$, $CoA \cdot Li_3$, $M_r = 785.4$; 23 mg in 3 ml)

0.1 M NAD^+ (free acid, $M_r = 663.4$; 663 mg in 10 ml)

phosphotransacetylase (4000 IU ml^{-1})

lactate dehydrogenase (10 000 U ml^{-1})

2 M hydroxylamine (mix equal volumes of 4 M hydroxylamine hydrochloride, $M_r = 69.49$, and 4 M KOH, $M_r = 56.11$)

$FeCl_3$ reagent:

- 100 ml 5% $FeCl_3$ (8.33 g $FeCl_3 \cdot 6H_2O$, $M_r = 270.3$, replenish to 100 ml with 0.1 N HCl)
- 100 ml 12% TCA (trichloroacetic acid, $M_r = 163.4$)
- 100 ml 3 M HCl (mix one part 37% HCl with three parts H_2O)

Mix the three solutions together.

Assay mixture

Components	*Concentrations*
4.0 ml 0.1 M potassium phosphate pH 7.6	80 mM
0.05 ml 0.1 M $MgCl_2$	1.0 mM
0.23 ml 0.1 M pyruvate	4.6 mM
0.1 ml 0.01 M ThDP	0.2 mM
0.1 ml 0.1 M DTE	2.0 mM
0.05 ml 0.01 M coenzyme A	0.1 mM
0.05 ml 0.1 M NAD^+	1.0 mM
0.01 ml phosphotransacetylase	4 IU ml^{-1}
0.01 ml LDH	10 IU ml^{-1}

Procedure

0.48 ml assay mixture

0.02 ml enzyme sample

Incubate at 30 °C for 15 min

Put the samples thereafter on ice

0.2 ml 2 M hydroxylamine.

10 min at room temperature

0.6 ml $FeCl_3$ reagent.

Centrifuge for 5 min, 5000 rpm

Measure the absorption at 546 nm against a blank with the enzyme sample substituted by buffer.

Calculation

The specific activity is expressed in special units, with reference to 1 h of reaction time.

$$\text{specific activity} = \frac{A_{546} \times 20 \times \text{assay volume (1.3 ml)} \times \text{dilution factor}}{\text{enzyme volume (ml)} \times \text{protein (mg ml}^{-1}\text{)}}$$

References

Korkes, S.A., Campillo, A., Gunsalus, I.C., and Ochoa, S. (1951) *J. Biol. Chem.*, **193**, 721–735.
Reed, L.J., Leach, F.R., and Koike, M. (1958) *J. Biol. Chem.*, **232**, 123–142.
Reed, L.J. and Willms, C.R. (1966) *Meth. Enzymol.*, **9**, 247–269.

3.3.7.2 α-Oxoglutarate Dehydrogenase Complex (OGDHC)

This enzyme complex resembles in structure and reactions the pyruvate dehydrogenase complex (Section 3.3.7.1). It also consists of several (24) identical copies of three enzyme components, α-oxoglutarate dehydrogenase (E1o, 1.2.4.2), dihydrolipoamide succinyltransferase (E2o, EC 2.3.1.61), and dihydrolipoamide dehydrogenase (E3o, EC 1.8.1.4) catalyzing the following reaction sequence:

α-oxoglutarate + ThDP-E1o → α-hydroxy-γ-carboxypropyl-ThDP-E1o + CO_2
α-hydroxy-γ-carboxypropyl-ThDP-E1o + lipoyl-E2o
→ succinyl-dihydro-lipoyl-E2o + ThDP-E1o
succinyl-dihydrolipoyl-E2o + CoA → succinyl-CoA + dihydrolipoyl-E2o
dihydrolipoyl-E2o + FAD_{ox}-E3o → lipoyl-E2o + FAD_{red}-E3o
FAD_{red}-E3o + NAD^+ → FAD_{ox}-E3o + NADH + H^+

α-oxoglutarate + NAD^+ + CoA → succinyl-CoA + CO_2 + NADH + H^+

Assays for the partial reactions of the three enzyme components are described separately; here, a photometric assay for the overall reaction is presented. The assays described for the pyruvate dehydrogenase complex and its enzyme components can be applied, substituting pyruvate by α-oxoglutarate (i.e., the acetyl group by a succinyl residue). E3o and E3p are identical enzymes (assay in Section 3.3.1.29).

Overall Activity by NAD^+ Reduction

Assay solutions

0.1 M potassium phosphate pH 7.6

0.1 M NAD^+ (free acid M_r = 663.4; 663 mg in 10 ml)

0.01 M thiamin diphosphate (ThDP, cocarboxylase, M_r = 460.8, 46.1 mg in 10 ml)

0.1 M $MgCl_2$ ($MgCl_2 \cdot 6H_2O$, M_r = 203.3, 203 mg in 10 ml)

0.5 M α-oxoglutarate (α-ketoglutarate, 2-oxopentanedioic acid, disodium salt, M_r = 190.1, 0.95 g in 10 ml)

0.1 M dithioerythritol, DTE, M_r = 154.2; 154 mg in 10 ml)

0.01 M coenzyme A (free acid, M_r = 767.5, $CoA \cdot Li_3$, M_r = 785.4; 23 mg in 3 ml)

Assay mixture

Components	*Concentration (mM)*
7.95 ml 0.1 M potassium phosphate pH 7.6	80
0.25 ml 0.1 M NAD^+	2.5
0.20 ml 0.01 M ThDP	0.2
0.10 ml 0.1 M $MgCl_2$	1.0
0.10 ml 0.5 M α-oxoglutarate	5.0
0.10 ml 0.1 M DTE	1.0
0.10 ml 0.01 M CoA	0.1

Procedure

0.98 ml assay mixture

0.20 ml enzyme solution

The absorption increase at 340 nm is measured at 37 °C, $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}.

Reference

Stepp, L.R., Bleile, D.M., McRorie, D.K., Pettit, F.H., and Reed, L.J. (1981) *Biochemistry*, **20**, 4555–4560.

3.3.8 Substrate Determination

The principle of substrate determination by enzymatic assays has already been discussed (cf. Section 2.5). If the enzyme reaction proceeds in an irreversible manner, substrate becomes quantitatively converted to product and the amount of product corresponding to the initial substrate concentration can be determined if the reaction is allowed to reach to its conclusion (Figure 2.31). Please refer to the respective enzyme assays described in the previous sections. In this section, the assays for substrate determination using the principle of enzymatic cycling are presented using the examples of NAD(H) and NADP(H), which allow detection of very low concentrations.

3.3.8.1 Determination of NADP(H) by Enzymatic Cycling

For the determination of NADP and NADPH, a coupled reaction of glutamate dehydrogenase (GluDH, EC 1.4.1.3) and glucose-6-phosphate dehydrogenase (G6PDH,

EC 1.1.1.49) is used:

$$\alpha\text{-oxoglutarate} + \text{NADPH} + \text{NH}_4^+ \xrightarrow{\text{GluDH}} \text{glutamate} + \text{H}_2\text{O} + \text{NADP}^+$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

Each NADP molecule catalyzes the formation of 5000–10 000 molecules of 6-phosphogluconate under the specified conditions. Concentrations of 10^{-9} M NADP (respectively 10^{-15} moles in 1 µl) can be detected. ADP functions as an activator of GluDH.

The amount of 6-phosphogluconate formed is determined by an independent reaction with 6-phosphogluconate dehydrogenase (PGDH, 6-phospho-D-gluconate: $NADP^+$ 2-oxidoreducatse, EC 1.1.1.44) after stopping the enzymatic cycling reaction with a fluorimetric assay:

$$\text{6-phosphogluconate} + \text{NADP}^+ \overset{\text{PGDH}}{\rightleftharpoons} \text{D-ribulose-5-phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

Assay solutions

0.1 M Tris/HCl pH 8.0

0.1 M α-oxoglutaric acid (disodium salt, $M_r = 190.1$; 190 mg in 10 ml)

0.1 M D-glucose-6-phosphate (disodium salt, hydrate, $M_r = 304.1$; 304 mg in 10 ml)

0.01 M ADP (disodium salt, $M_r = 471.2$; 47.1 mg in 10 ml)

1.0 M ammonium acetate ($M_r = 77.1$; 771 mg in 10 ml)

BSA (20 mg ml^{-1})

Glutamate dehydrogenase solution (GluDH, from bovine liver, 40 U mg^{-1}, prepare a solution of 40 IU in 0.1 ml 0.1 M Tris/HCl pH 8.0)

Glucose-6-phosphate dehydrogenase solution (G6PDH, from yeast, 300 IU mg^{-1}, prepare a solution of 60 IU in 0.1 ml 0.1 M Tris/HCl pH 8.0)

0.01 M 6-phosphogluconic acid (trisodium salt, $M_r = 342.1$; 34 mg in 10 ml)

0.01 M NADP (M_r =787.4; 79 mg in 10 ml)

0.1 M EDTA (ethylenediaminetetraacetic acid, $M_r = 292.2$; 292 mg in 10 ml)

6-phosphogluconate dehydrogenase solution (GPDH, from the yeast *Torula*, 20 IU mg^{-1}, prepare a solution of 0.1 IU in 0.1 ml 0.1 M Tris/HCl pH 8.0)

Cycling mixture

Components	*Concentration*
1.7 ml 0.1 M Tris/HCl pH 8.0	85 mM
0.10 ml 0.1 M α-oxoglutaric acid	5.0 mM
0.02 ml 0.1 M D-glucose-6-phosphate	1.0 mM
0.02 ml 0.01 M ADP	0.1 mM
0.06 ml 1 M ammonium acetate	30 mM
0.02 ml BSA	0.2 mg/ml
0.04 ml GluDH solution	8 IU ml^{-1}
0.04 ml G6PDH solution	12 U ml^{-1}

Procedure

0.1 ml cycling mixture

x µl NADP or NADPH solution to be determined, (x between 1 and 20; final concentration between 3 and 50×10^{-9} M)

$20-x$ µl 0.1 M Tris/HCl pH 8.0

Incubate for 30 min at 37 °C

Stop the reaction by transferring the sample for 2 min to 100 °C

Fluorimeteric assay of 6-phosphogluconate	*Concentration*
2 ml 0.02 M Tris/HCl pH 8.0	20 mM
0.004 ml 10 mM NADP	0.02 mM
0.002 ml 0.1 M EDTA	0.1 mM
0.05 ml sample (6-phosphogluconate)	–
0.02 ml 6-phosphogluconate dehydrogenase	0.01 IU

Measure fluorescence light excited at 260 nm and emitted at 470 nm. For quantification a standard curve with NADPH must be prepared.

3.3.8.2 Determination of NAD(H)

The procedure is similar to the determination of NADP(H) described in Section 3.3.8.1. NAD formed by the glutamate dehydrogenase reaction is converted to

NADH by the LDH reaction. In a second step, NADH is added to determine pyruvate by the LDH reaction.

$$\alpha\text{-oxoglutarate} + \text{NADH} + \text{NH}_4^+ \xrightarrow{\text{GluDH}} \text{glutamate} + \text{H}_2\text{O} + \text{NAD}^+$$
$$\text{lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

The excess of NADH is destroyed by acid treatment. After alkaline treatment of NAD the fluorescence is measured.

Assay solutions

0.2 M Tris/HCl pH 8.4

0.1 M α-oxoglutaric acid (disodium salt, $M_r = 190.1$; 190 mg in 10 ml)

1 M lactate (sodium salt, $M_r = 112.1$; 1.1 g in 10 ml)

0.01 M ADP (disodium salt, $M_r = 471.2$; 47.1 mg $10\,ml^{-1}$)

1.0 M ammonium acetate ($M_r = 77.1$; 771 mg in 10 ml)

Glutamate dehydrogenase (GluDH, from bovine liver, 40 IU mg^{-1}, prepare a solution of 40 IU in 0.1 ml 0.1 M Tris/HCl pH 8.0)

lactate dehydrogenase (LDH, from pig heart, circa 550 IU mg^{-1}, 10 mg ml^{-1}, prepare a solution of 55 IU in 0.1 ml 0.1 M Tris/HCl pH 8.0)

9 M NaOH (36 g in 100 ml)

Sodium potassium phosphate buffer: dissolve 8.97 g NaH_2PO_4 (monohydrate, $M_r = 138.0$) and 2.61 g K_2HPO_4 ($M_r = 174.2$) in 100 ml H_2O

Cycling mixture (Solution I)

Components	*Concentration*
1.7 ml 0.2 M Tris/HCl pH 8.4	85 mM
0.10 ml 0.1 M α-oxoglutaric acid	5.0 mM
0.20 ml 1 M lactate	100 mM
0.06 ml 0.01 M ADP	0.3 mM
0.20 ml 1 M ammonium acetate	100 mM
0.04 ml GluDH	8 U/ml
0.04 ml LDH	11 IU/ml

Solution II

Add freshly to 1 ml of the sodium potassium phosphate buffer:

20 µl 10 mM NADH

LDH from stock solution according to 1.5 µg (dilute stock solution 100-fold and add 15 µl)

Procedure

0.1 ml cycling mixture

x µl NAD or NADH solution to be determined (x between 1 and 20; final concentration between 3 and 50×10^{-9} M)

$20 - x$ µl 0.2 M Tris/HCl pH 8.4

Incubate for 30 min at 37 °C

Transfer the sample for 2 min to 100 °C

Transfer to an ice bath

0.1 ml solution II

Incubate at 25 °C for 15 min

Put in an ice bath, add

25 µl 5 N HCl

Add 0.1 ml of each sample to

0.2 ml 9 M NaOH

Incubate at 60 °C for 10 min

1 ml H_2O

Measure the fluorescence emission at 470 nm, excitation is at 365 nm.

Reference

Lowry, O.H., Passonneau, J.V., Schultz, D.W., and Rock, M.K. (1961) *J. Biol. Chem.*, **236**, 2746–2753.

3.4 Assays for Enzyme Characterization

3.4.1 Protein Determination

In the following section, some frequently used protein assays are described together with some special examples regarding immobilized, membrane-bound,

and carrier-fixed proteins. Box 3.3 summarizes the essential evidence such as the approximate detection limits, whereby the lower limit is more important than the higher, which can be attained by dilution.

3.4.1.1 Biuret Assay

The biuret reagent reacts with the peptide bond and is therefore specific for proteins and relatively insensitive against perturbations, with the exception of urea, Tris and Good buffers (cf. Section 2.2.7.4), which also show a positive reaction. The biuret assay is not very sensitive and requires larger amounts of protein (0.1–0.8 mg). It is suited for crude extracts and large-scale purifications but cannot be recommended for valuable enzyme preparations. Since for purification procedures the same protein assay should be used throughout, it must be decided from the beginning whether this or a more sensitive test should be applied.

Box 3.3: Protein Assays: Advantages and Disadvantages

Method	Sensitivity range	Advantages	Disadvantages
Biuret	100–800 µg	Specific for peptide bonds Minor disturbances	Low sensitivity
BCA	5–100 µg	Specific for peptide bonds Few disturbances Suited for immobilized and membrane enzymes Considerable sensitivity	Expensive reagent
Lowry	4–40 µg	High sensitivity	Limited linearity Many disturbances Relatively laborious
Bradford	10–100 µg (2 µg for microassay)	Fast and simple procedure Moderate sensitivity	Blue coloring of cuvettes
Absorption	20–1000 µg	Fast and easy procedure No modification of the protein Recovery of the sample	Low sensitivity Strong disturbance especially of nucleic acids
Fluorescence	0.5–50 µg	High sensitivity	Various disturbances (quenching) Expensive instrumentation

Reagents and solutions

Biuret solution: dissolve in 400 ml H_2O, one after the other

- 9.0 g sodium potassium tartrate (Rochelle salt, $C_4H_4KNaO_6 \cdot 4H_2O$; M_r = 282.2)
- 3.0 g cupric sulfate ($CuSO_4 \cdot 5H_2O$, M_r = 249.7)
- 8.0 g sodium hydroxide (NaOH, M_r = 40.0)
- 5.0 g potassium iodide (KI, M_r 166.0)
- Adjust to 500 ml with H_2O

3 M TCA (trichloroacetic acid, $C_2HCl_3O_2$, M_r = 163.4; 49 g, adjust to 100 ml with H_2O)

BSA solution (20 mg ml^{-1} BSA in H_2O)

Biuret and TCA solutions are stable for months at room temperature, freeze the BSA solution at −20 °C.

Procedure

Different aliquots of the protein solution (0.01–0.1 ml, containing about 10–50 mg protein ml^{-1}) are filled up with H_2O to 1 ml. The blank contains only 1 ml water

Add 0.15 ml 3 M TCA

Centrifuge for 3 min at 5000 rpm

Discard the supernatant and dissolve the precipitate quantitatively in 1 ml solution A

Incubate 30 min at room temperature

Measure the absorption at 546 nm against the blank

The protein concentration can be calculated from a calibration curve prepared with the BSA solution in the respective concentration range or according to the equation:

$$\frac{\text{mg}}{\text{ml}}\text{protein} = \frac{A_{546} \times 3.01}{\text{volume protein sample (ml)}}$$

References

Beisenherz, G., Boltze, H.J., Bücher, T., Czok, R., Garbade, K.H., Meyer-Arendt, E., and Pfleiderer, G. (1953) *Z. Naturforsch.*, **8B**, 555–577.

Itzhaki, R.F. and Gill, D.M. (1964) *Anal. Biochem.*, **9**, 401–410.

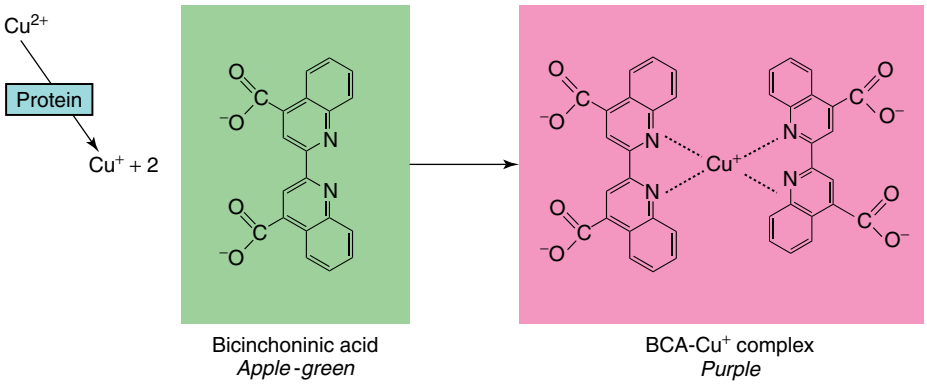

Figure 3.1 Structure of the BCA-Cu^{+} complex.

3.4.1.2 BCA Assay

The BCA test is based on the biuret reaction. Peptide bonds and oxidizable amino acids like tyrosine, tryptophan, and cysteine reduce Cu^{2+} ions in an alkaline solution to Cu^{+}, forming a purple complex with two molecules of the bicinchoninic acid (BCA, Figure 3.1). The BCA assay is significantly more sensitive (<10 µg) than the biuret method and also not very susceptible against disturbances and high detergent concentrations. Complex forming and reducing substances, like EDTA (>100 mM), DTT (>1 mM), Tris (>0.25 M), ammonium sulfate (>20%), and glucose disturb the assay. A further advantage of the BCA assay is that the color reaction develops with Cu^{+} ions in the solution and not directly on the protein, so that precipitation of the protein (e.g., with TCA) is not necessary and the assay is also suited for determination of immobilized and membrane-bound proteins.

A. Assay for Soluble Proteins

Reagents and solutions

BCA solution: dissolve the following substances, one after the other, in 50 ml of H_2O and adjust pH to 11.25 with 1 M NaOH (4 g in 100 ml H_2O) or $NaHCO_3$ (8.4 g in 100 ml H_2O)

- 1.0 g BCA (bicinchoninic acid, 4,4′-dicarboxy-2,2′-biquinoline, disodium salt, $M_r = 388.3$)
- 2.0 g $Na_2CO_3 \cdot H_2O$ ($M_r = 124.0$)
- 0.16 g sodium tartrate ($C_4H_4Na_2O_6 \cdot 2H_2O$, $M_r = 230.1$)
- 0.4 g NaOH ($M_r = 40.0$)
- 0.95 g $NaHCO_3$ ($M_r = 84.0$)

Bring to 100 ml in a volumetric flask, store at room temperature

$CuSO_4$ solution: 1.0 g $CuSO_4 \cdot 5H_2O$, $M_r = 249.7$, fill up to 25 ml with H_2O, store at room temperature

BSA solution: 10 mg BSA in 10 ml H_2O, store frozen at –20 °C

Procedure

Prepare a BCA/$CuSO_4$ mixture: 50 parts BCA solution, 1 part $CuSO_4$ solution (v/v)

Add 50 µl protein solution (5–50 µg protein) or x µl protein solution + 50 – x µl H_2O to

950 µg of the BCA/$CuSO_4$ mixture

Incubate for 30 min at 37 °C

Measure the absorption at 562 nm against a blank without protein.

Calibration Curve

Treat in the same manner 12 samples of BSA solution from 0 to 50 µl.

B. Modification for Immobilized Proteins

Reagents and solutions as with assay A.

Procedure

Place a defined amount of the immobilized protein, such as a distinct piece[1)] of the matrix, into 1 ml of the BCA/$CuSO_4$ mixture

Incubate for 30 min at 37 °C with gentle agitation

Remove the matrix

Measure the absorption at 562 nm against a blank without protein

Use the BSA calibration curve described for assay A. The value obtained for the sample must be related to the size of the matrix. Alternatively, a calibration curve can be prepared with a known amount of immobilized protein.

References

Redinbaugh, M.C. and Turtley, R.B. (1986) *Anal. Biochem.*, **153**, 267–271.
Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) *Anal. Biochem.*, **150**, 76–85.

1) The protein determined by this method depends on the size of the matrix applied to the assay. This must be clearly defined, for example as mg matrix (dry weight), or mm^2 matrix surface.

3.4.1.3 Lowry Assay

This is a very sensitive method but it is strongly susceptible to various disturbances, even in lower concentrations, such as EDTA, sucrose, glycine, Tris, detergents (SDS, Triton X-100, Lubrol, Brij 35, Chaps), and inorganic salts (ammonium sulfate >28 mM, sodium phosphate >0.1 M, sodium acetate >0.2 M). A further disadvantage is the limited linearity of absorption upon increasing amounts of protein. Care must be taken that the values obtained with the unknown samples range within the linear part of the calibration curve, which is usually performed with BSA.

Reagents and solutions

Carbonate buffer: dissolve

- 0.4 g sodium tartrate ($C_4H_4Na_2O_6 \cdot 2H_2O$, $M_r = 230.1$), and
- 20 g Na_2CO_3

in 100 ml 1 N NaOH, dilute with H_2O to 200 ml, store at room temperature

Alkaline copper tartrate solution: dissolve

- 2 g sodium potassium tartrate (Rochelle salt, $C_4H_4KNaO_6 \cdot 4H_2O$; $M_r = 282.2$) and
- 1 g $CuSO_4 \cdot 5H_2O$ ($M_r = 249.7$)

in 90 ml H_2O

Add 10 ml 1 N NaOH, store at room temperature

Diluted Folin–Ciocalteau reagent: prepare fresh

- 1 part Folin–Ciocalteau reagent
- 15 parts H_2O

BSA solution: 10 mg BSA in 10 ml H_2O, store frozen at −20 °C

Procedure

0.3 ml protein solution containing 4–40 µg protein

0.3 ml carbonate buffer

- Incubate for 10 min at 50 °C
- Bring to room temperature

33 µl alkaline copper tartrate solution

- 10 min at room temperature

1 ml diluted Folin–Ciocalteau reagent, mix immediately

- 10 min at 50 °C
- Bring to room temperature

Measure the absorption at 650 nm.

Calibration curve

Treat in the same manner 12 samples of BSA solution from 0 to 40 µl.

References

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265–275.
Peterson, G.L. (1979) *Anal. Biochem.*, **100**, 201–220.

3.4.1.4 Coomassie Binding Assay (Bradford Assay)

This rapid and simple assay was adapted from protein staining in electrophoresis gels. Upon binding to proteins in acidic solution, the Coomassie® Brilliant G-250 dye shifts its absorption maximum from 465 to 595 nm. The assay has relatively few perturbations (detergents like Triton X-100 and sodium dodecyl sulfate). Strength of interaction and thus staining intensity depend on the special protein. Therefore, the BSA calibration curve yields only relative values, variations of more than 50% are possible. For higher accuracy the calibration curve must be prepared with a known solution of the protein under study. The sensitivity is within 10–100 µg, with micro assays even 2 µg protein may be detected. A disadvantage is the blue coloring of the cuvettes; instead, disposable plastic cuvettes may be used. Glass cuvettes can be cleaned with acetone or by incubating in 0.1 M HCl for a few hours.

Reagents and solutions

Coomassie reagent: dissolve
- 100 mg Coomassie® Brilliant G-250 in 50 ml ethanol
- Add 100 ml 85% phosphoric acid (88%)
- Bring to 1 l with H_2O

Filter and store for several weeks at room temperature.

BSA solution: 10 mg BSA in 10 ml H_2O, store frozen at −20 °C

Procedure

Samples from 0 (for the blank) to 50 µl (2–40 µg protein), adjust with H_2O to 50 µl

0.95 ml of the Coomassie reagent, mix immediately

Measure after at least 2 min (but not more than 1 h) the absorption at 595 nm against a blank without protein. The protein content is determined from a standard curve prepared from the BSA solution by the same procedure.

Modification for immobilized proteins

The Coomassie binding assay can principally be applied for immobilized proteins, but essential modifications are necessary. The absorption must be measured directly at the matrix surface, after washing out the soluble dye. This requires a special photometric device. For calibration, immobilized samples of known protein content must be determined. Alternatively, an approximate calibration curve can be determined from protein immobilized to filter paper pieces of a defined size.

References

Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
Friedenauer, S. and Berlet, H.H. (1989) *Anal. Biochem.*, **178**, 263–268.

3.4.1.5 Absorption Method

Proteins, although differing considerably in their primary sequence, possess nearly identical absorption spectra (cf. Section 2.3.1.1), consisting of two characteristic peaks in the UV region; a more intense one at about 205–210 nm and a less intense one at 280 nm (Figure 3.2c), which are described in detail in Section 4.3.1. The far UV peak is composed of various contributions, while the near UV peak contains contributions of the aromatic amino acids alone, tryptophan clearly dominating the graph (Figure 3.2a). The absorption maximum at 280 nm is a clear indication of the presence of this amino acid. In its absence, the maximum shifts to shorter wavelengths. Since the relative tryptophan content is similar for most large proteins, the absorption intensity at 280 nm serves as a measure of protein concentration.

The absorption method is simple. It requires only the determination of the absorption of the sample solution at 280 nm with quartz cuvettes. There is no loss of the sample. As a crude estimate, protein solutions of 1 mg ml^{-1} show absorptions of about 1 at 280 nm. Solutions with higher absorptions must be diluted, concentrations lower than 0.02 mg ml^{-1} can hardly be measured with sufficient accuracy; thus the sensitivity of the method is not very high. Substances absorbing within this UV range disturb this method. Low molecular components can be removed by dialysis before the assay, but there is a strong interference with the absorption of nucleic acids. Although their maximum absorption is at 260 nm, due to high intensity, this overlaps considerably with the protein absorption at 280 nm (Figure 3.2b).

The method of Warburg and Christian (1941) is based on the absorption ratio between 280 and 260 nm and enables the simultaneous determination of proteins

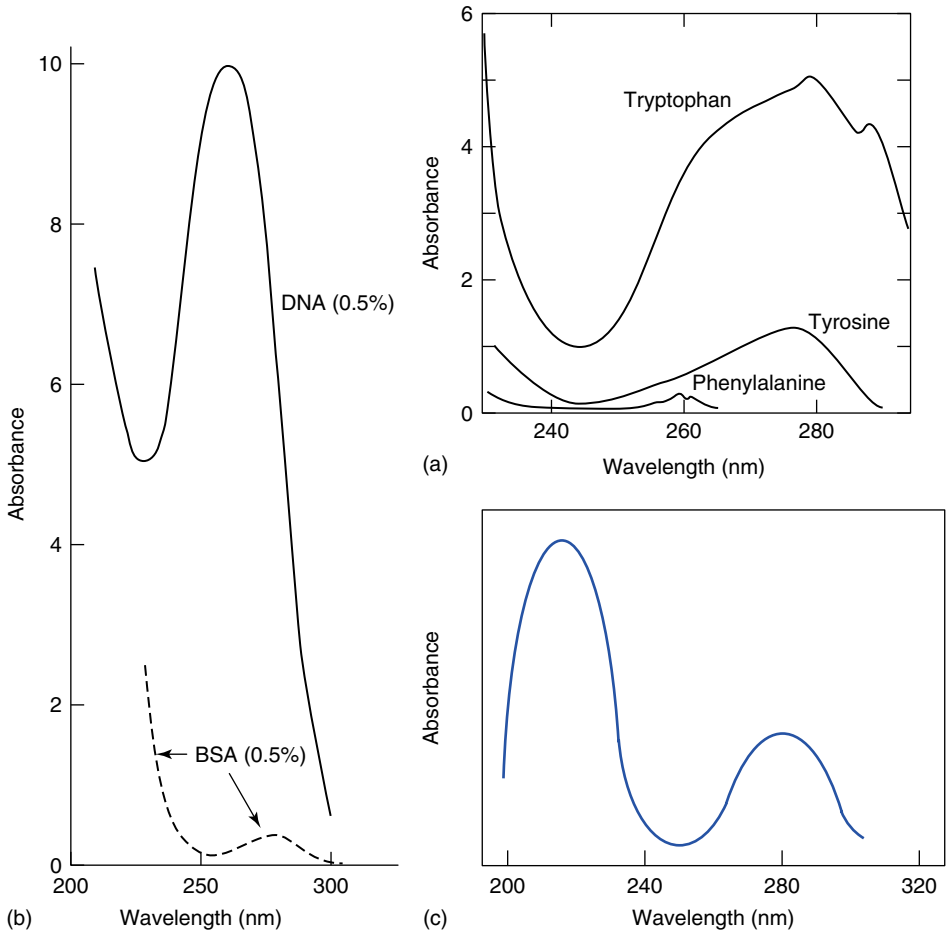

Figure 3.2 UV absorption spectra. (a) Absorption of the three aromatic amino acids in the near-UV range. (b) Comparison of absorption intensities of proteins (BSA), and of DNA in equivalent concentration ranges (0.5%) (K. L. Manchester (1997) *Biochemical Education* **25**, 214–215; with permission from *The American Society for Biochemistry & Molecular Biology*). (c) Typical protein spectrum.

and nucleic acids in the same solution. The absorption of the sample solution is measured (in quartz cuvettes) at both wavelengths against a blank. From the ratio a factor is obtained (Table 3.2), which allows the estimation of the protein concentration. Alternatively the formula

$$\text{protein (mg ml}^{-1}) = 1.55\ A_{280} - 0.76\ A_{260}$$

can be applied. In the presence of large nucleic acid concentrations, protein determination becomes difficult.

Table 3.2 Determination of the amount of protein and nucleic acids by the absorption method of Warburg and Christian (1941). The factor corresponding to the absorption ratio 280/260 nm multiplied with the absorption at 280 nm yields the protein concentration: $A_{280} \times \text{fp} = \text{mg protein/ml}$.

Absorption ratio 280/260 nm	Nucleic acids (%)	Factor (fp)
1.75	0	1.10
1.60	0.25	1.07
1.50	0.50	1.05
1.40	0.75	1.02
1.30	1.00	0.99
1.25	1.25	0.97
1.20	1.50	0.95
1.15	2.00	0.91
1.10	2.50	0.87
1.05	3.00	0.83
1.00	3.50	0.80
0.96	3.75	0.78
0.92	4.25	0.75
0.88	5.00	0.71
0.86	5.25	0.70
0.84	5.50	0.69
0.82	6.00	0.67
0.80	6.50	0.64
0.78	7.25	0.62
0.76	8.00	0.59
0.74	8.75	0.56
0.72	9.50	0.54
0.70	10.75	0.51
0.68	12.00	0.48
0.66	13.50	0.45
0.65	14.50	0.43
0.64	15.25	0.41
0.62	17.50	0.38
0.60	20.00	0.35
0.49	100.00	–

References

Layne, E. (1957) *Meth. Enzymol.*, **3**, 447–454.
Warburg, O. and Christian, W. (1941) *Biochem. Z.*, **310**, 384–421.

3.4.1.6 Fluorimetric Assay

For protein determination, the intrinsic fluorescence of tryptophan can be used (excitation 280 nm, emission 340 nm). Quantification, however, is difficult because the tryptophan fluorescence changes considerably upon integration of the amino

acid into the protein structure, both with respect to intensity and wavelength (blue shift) of the fluorescence maximum. Here, a very sensitive assay with fluorescamine, applicable down to 0.5 µg protein, is described. A fluorimeter is required (filter fluorimeter is sufficient). The sensitivity can be further increased by removal of unbound dye, applying dialysis or gel filtration, for example with Sephadex G-25. This, however, complicates routine assays and should be integrated into an automatic procedure.

Reagents and solutions

0.05 M sodium phosphate pH 8.0

Fluorescamine solution (30 mg in 100 ml dioxane)

BSA solution (1 mg ml^{-1})

Procedure

Bring the protein solution (10–250 µl) to 1.5 ml with 0.05 M sodium phosphate pH 8.0

Add 0.5 ml fluorescamine solution rapidly under vigorous shaking

Measure the fluorescence at 475 nm at an excitation of 390 nm.

Prepare a standard calibration curve for quantitative determination with the BSA solution.

Reference

Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) *Arch. Biochem. Biophys.*, **155**, 213–220.

3.4.1.7 Ninhydrin Assay

The ninhydrin reaction detects free amino acids with high sensitivity. This method is especially suited for immobilized proteins by determining the released amino acids after hydrolyzing the proteins from the matrix.

Reagents and solutions

6 N HCl (49.7 ml 37% hydrochloric acid bring to 100 ml with H_2O)

6 N NaOH ($M_r = 40.0$; 24 g in 100 ml H_2O, store in a PE flask)

Acetate buffer pH 5.4 (dissolve 270 g sodium acetate trihydrate, $C_2H_3O_2Na \cdot 3H_2O$, $M_r = 136.1$, in 200 ml H_2O, add 50 ml glacial acetic acid, fill up to 750 ml)

10 mM KCN ($M_r = 65.1$; 65 mg in 100 ml H_2O)

0.2 mM KCN in acetate buffer (0.2 ml 10 mM KCN + 9.8 ml acetate buffer, prepare fresh)

Ninhydrin reagent (2,2-dihydroxy-1,3-indanedione, $M_r = 178.1$, commercially available)

50% ethanol

Standard BSA solution, 40 µg ml^{-1}, prepared by 100-fold dilution of a stock solution of 40 mg in 10 ml H_2O

10 mM leucine (D,L-leucine, $M_r = 131.2$; 131 mg in 100 ml H_2O)

Hydrolysis

Incubate defined amounts of the protein sample and an exactly determined piece of the matrix for immobilized enzymes, overnight (16 h) at 90 °C in 1 ml 6 N HCl in closely sealed cups (screw caps are preferable to avoid dehiscence of the cap and splashing of concentrated acid)

Open the cups carefully (use goggles!), remove 0.5 ml and neutralize with 0.5 ml 6 M NaOH (control the pH)

Procedure

0.2 ml hydrolysate (sample aliquots filled up with H_2O to 200 µl)

0.1 ml 0.2 mM KCN in acetate buffer

0.1 ml ninhydrin reagent

10 min incubation at 100 °C (boiling water bath or heating block)

Chill in ice for 1 min and add 0.5 ml 50% ethanol

Measure the absorbance at 570 nm against a blank where the hydrolysate is displaced by water.

Calibration curve

Generate a calibration curve with 12 aliquots (1 and 50 µl, filled up to 200 µl) of the 10 mM leucine solution.

Reference

Rosen, H. (1957) *Arch. Biochem. Biophys.*, **67**, 10–15.

3.4.1.8 Modified Ninhydrin Assay without Hydrolysis

This method is especially recommended for proteins immobilized to acid-labile matrices. The samples must be free of salt and solvents. For preparation, wash with

H_2O and dry. Matrices in organic solvents should be washed with dichloromethane and dried before use.

Reagents and solutions

Amberlite MB-3

Potassium cyanide (KCN) (*Caution!* extremely toxic)

Ninhydrin (2,3-dihydroxy-1,3-indanedione, $M_r = 178.1$)

Phenol, p.A.

Pyridine

Absolute ethanol, p.A.

0.5 M TEA chloride (tetraethylammonium chloride, $M_r = 165.7$, 0.83 g in 10 ml dichloromethane)

BSA solution (1 mg ml^{-1} in H_2O)

Solution A: dissolve

- 40 g phenol in 10 ml by gentle warming and add
- 4 g Amberlite MB-3
- Stir for 45 min and filtrate thereafter

Solution B: dissolve

- 65 mg KCN ($M_r = 65.1$) in 100 ml H_2O
- give 2 ml of this solution to 98 ml pyridine and add
- 4 g Amberlite MB-3
- Stir the mixture and filtrate

Solution C:

- Mix equal parts of solutions A and B

Solution D:

- 2.5 g ninhydrin, dissolve in 50 ml ethanol. Store in a nitrogen-filled atmosphere

Procedure

Add 200 µl of solution C and 50 µl of solution D to an aliquot of the matrix with the immobilized enzyme, corresponding to about 2–5 mg and seal the samples closely. For the blank matrix without immobilized enzyme no aliquot was used

10 min at 100 °C in a heating block

Chill on ice, add

1 ml 60% ethanol and mix carefully

Filter or centrifuge to remove the matrix

Separate the supernatant

Wash the matrix two times with 0.2 ml 0.5 M TEA chloride and give the washing solution to the supernatant

Bring the samples to 2 ml with 60% ethanol

Measure against the blank at 570 nm

Calculation

Prepare a standard curve with BSA solution. Use an absorption coefficient of $\varepsilon_{570} = 1.5 \times 10^4$ l mol^{-1}cm^{-1} for free amino groups.

Reference

Sarin, V.K., Kent, S.B.H., Tam, J.P., and Merrifield, R.B. (1981) *Anal. Biochem.*, **117**, 147–157.

3.4.1.9 Protein Assay with 2-Hydroxy-1-naphthaldehyde

This time-consuming assay is suitable for immobilized proteins. The aldehyde forms a Schiff's base with amino residues of the protein, which are substituted by benzylamine. The absorption of the soluble Schiff's base is determined in the supernatant.

Reagents and solutions

1.2 M 2-hydroxy-1-naphthaldehyde (2-naphthol-1-carboxyaldehyde, M_r = 172.18; 2.07 g in 10 ml)

Dimethylformamide

0.4 M benzylamine (hydrochloride, M_r = 143.6; 0.57 g in 10 ml ethanol)

BSA solution (1 mg ml^{-1} in H_2O)

Ethanol p.A.

Procedure

Wash the sample with immobilized enzyme thoroughly with dimethylformamide

Overlay with 0.5 ml 1.2 M 2-hydroxy-1-naphthaldehyde

Shake overnight (~14 h) at room temperature

Remove the immobilized sample and wash it thoroughly, 5–10 times with dimethylformamide and 5 times with ethanol, until no more absorption can be detected at 280 nm

Add 1 ml 0.4 M benzylamine and shake for 15 h at room temperature

Centrifuge, 5 min, 5000 rpm

Measure the absorption in the supernatant at 420 nm ($\varepsilon_{420} = 1.09 \cdot 10^4$ l mol^{-1} cm^{-1})

Calculate the protein content using a calibration curve prepared with BSA solution.

Reference

Bisswanger, H., Figura, R., Möschel, K., and Nouaimi, M. (2001) *Enzymkinetik, Ligandenbindung und Enzymtechnologie*, 2nd edn, Shaker Verlag, Aachen, p. 71.

General References for Protein Assays

Kresse, G.B. (1983) in *Methods of Enzymatic Analysis* (ed. H.U. Bergmeyer), Verlag Chemie, Weinheim, New York, pp. 86–99.
Scopes, R.K. (1987) *Protein Purification*, Springer, New York, p. 280.
Stoscheck, C.A. (1990) *Meth. Enzymol.*, **182**, 50–68.
Thorne, C.J.R. (1978) in *Techniques in Protein and Enzyme Biochemistry*, vol. B104 (ed. H.L. Kornberg), Elsevier, Amsterdam, pp. 1–18.

3.4.2
Phosphate Determination

Various enzyme assays (e.g., ATPase) need the determination of inorganic phosphate. A micromethod with a sensitivity of 2–40 µg inorganic phosphate is described.

Reagents and solutions

Sodium phosphate standard solution (dissolve 0.5853 g KH_2PO_4 in 1 l water, the solution contains 133.3 µg phosphorus per ml)

12% (w/v) TCA (trichloroacetic acid, $M_r = 163.4$; 120 g/l)

10 N H_2SO_4 (add 278 ml concentrated sulfuric acid into 0.7 l of water, slowly and carefully, *use goggles*! Bring to 1 l after cooling)

10% ammonium molybdate solution (take 50 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in a beaker and add 400 ml 10 N H_2SO_4 with constant stirring. Transfer the

dissolved solution quantitatively into a volumetric flask and bring to 500 ml with 10 N H_2SO_4, store in the dark)

Ferrous sulfate–ammonium molybdate reagent (prepare fresh before use)

- Dilute 10 ml 10% ammonium molybdate solution to 70 ml with H_2O
- Dissolve 5 g of $FeSO_4 \cdot 7H_2O$
- Bring to 100 ml

Procedure

1.8 ml 12% TCA

0.1 ml sample, mix vigorously

- 10 min at room temperature
- Centrifuge at 1500 rpm for 10 min

Give 1.5 ml of the supernatant and 1 ml ferrous sulfate–ammonium molybdate reagent into a cuvette

Measure the absorption at 820 nm within 2 h.

Prepare a calibration curve with the sodium phosphate standard solution, each sample containing 4–20 µg P_i.

Modification

A modification of the above method uses an ascorbic–molybdate mixture:

One part 10% ascorbic acid

Six parts 0.42% ammonium molybdate $\times$ $4H_2O$ in 1 N H_2SO_3

Procedure

0.3 ml sample (supernatant, see above)

0.7 ml ascorbic–molybdate mixture

Incubate for 20 min at 45 °C

Measure at 820 nm; 0.01 µmol phosphate yields an absorption of 0.24

References

Ames, B.N. and Dubin, D.J. (1960) *J. Biol. Chem.*, **235**, 769–775.
Chen, P.S., Toribara, T.Y., and Warner, H. (1956) *Anal. Chem.*, **28**, 1756–1758.
Roufogalis, B.D. (1971) *Anal. Biochem.*, **44**, 325–328.
Taussky, H.H. and Shorr, E. (1953) *J. Biol. Chem.*, **202**, 675–685.

3.4.3 Glycoprotein Assays

From the various assays for determination of glycoproteins reported so far, a qualitative detection method in electrophoresis gels with Schiff's reagent and a quantitative method to determine protein-bound hexoses are described.

A. Detection in Electrophoresis Gels

Assay solutions

7% acetic acid (v/v) in H_2O

40% methanol

1% periodo acid in 7% acetic acid

1% sodium disulfite (sodium metabisulfite, $Na_2S_2O_5$, $M_r = 190.1$) in 0.1 N HCl

Schiff's reagent: though commercially available, steps for preparation are given below

- Dissolve 1 g pararosaniline (basic parafuchsin) in 500 ml H_2O
- Add 20 ml saturated sodium disulfite
- 10 ml concentrated HCl
- Make up to 1 l with H_2O

Procedure

Wash the electrophoresis gel overnight, with a large amount (>1 l) of 40% methanol and 7% acetic acid

Oxidize the gels for 1 h at 4 °C with 1% periodo acid in 7% acetic acid in the dark

Wash the gels with 7% acetic acid for 24 h, change the solution two times

Incubate the gel with Schiff's reagent for 1 h at 4 °C in the dark

Wash the gel four times with 1% sodium disulfite in 0.1 N HCl. The positive bands show a violet–purple coloring

B. Determination of Protein-Bound Hexoses

Assay solutions

Solution A:

- 60 ml concentrated H_2SO_4
- 40 ml H_2O

Solution B: 1.6 g orcinol (3,5-dihydroxytoluene, monohydrate, $M_r = 142.2$, in 100 ml H_2O)

Orcinol/H_2SO_4 reagent (freshly prepared):

- 7.5 parts solution A
- 1 part solution B

Galactose/mannose standard (20 mg D-galactose, $M_r = 180.2$, and 20 mg D-mannose, $M_r = 180.2$, in 100 ml H_2O)

95% ethanol

Procedure

0.1 ml protein sample

5 ml 95% ethanol

Mix and centrifuge for 15 min at 5000 rpm

Decant and suspend the precipitate in 5 ml 95% ethanol

Centrifuge 15 min at 5000 rpm

Dissolve the precipitate in 1 ml 0.1 N NaOH

Prepare for a calibration curve 10 aliquots with increasing volumes, from 0 to 1 ml, of the galactose/mannose standard; bring all samples to 1 ml with H_2O

Add 8.5 ml orcinol/H_2SO_4 reagent to each protein and standard sample and close the tubes with a sealing film (aluminum foil or parafilm)

Incubate at 80 °C for 15 min

Cool the tubes in tap water

Measure the absorption at 540 nm; for quantification, use the calibration curve

References

Glossmann, H. and Neville, D.M. (1971) *J. Biol. Chem.*, **246**, 6339–6346.
Winzler, R.J. (1955) *Meth. Biochem. Anal.*, **2**, 279–311.

3.4.4
Cross-Linking of Proteins with Dimethylsuberimidate

The following method is one of the most intensely studied cross-linking methods and is described representatively for a large variety of cross-linking reactions. Intramolecular bridges within a protein and also between different proteins are

formed by this method. The extent of cross-linking depends on the distance of the reactive groups and their reactivity. Variation of the incubation time of the cross-linking reaction, of the concentration, and of the chain length of the cross-linking reagent gives insight into the spatial arrangement of complex protein structures, such as subunit composition and distances of distinct domains. A similar method is described in Section 3.5.4.7 with glutaraldehyde. The cross-linked proteins can be analyzed by SDS electrophoresis, where combined subunits migrate, corresponding to their aggregation state.

Reagents and solutions

0.2 M triethanolamine/HCl, pH 8.5

Dimethylsuberimidate (dihydrochloride, $M_r = 273.2$, prepare a solution in 0.2 M triethanolamine/HCl, pH 8.5, e.g. 3 mg ml^{-1}, just before use according to the procedure below)

Protein solution (required concentration see procedure below)

0.1 M potassium phosphate pH 7.0

Procedure

Mix dimethyl suberimidate solution (to give a final concentration 1–12 mg ml^{-1}) with protein (final concentration 0.4–5 mg ml^{-1}) in 0.2 M triethanolamine/HCl pH 8.5

Incubate 3 h at room temperature

Dialyze against 0.1 M potassium phosphate pH 7.0

References

Carpenter, F.H. and Harrington, K.T. (1972) *J. Biol. Chem.*, **247**, 5580–5586.
Davies, G.E. and Stark, G.R. (1970) *Proc. Natl. Acad. Sci.*, **66**, 651–656.
Hunter, M.J. and Ludwig, M.L. (1970) *Meth. Enzymol.*, **25**, 585–596.

3.4.5 Concentration of Enzyme Solutions

Concentration of protein solutions and especially of sensitive enzyme solutions is sometimes a difficult task and often an appropriate method, which concentrates without serious loss of activity can hardly be found. The numerous methods described so far reflect these difficulties and a generally applicable method is still missing. Besides some miscellaneous techniques, the essential methods are based on the principles of precipitation, ultrafiltration, ultracentrifugation, and lyophilization. Here, advantages and disadvantages are discussed to facilitate the choice but general rules for a special system cannot be given (Box 3.4).

Box 3.4: Concentration Methods: Advantages, and Disadvantages

Method	Advantages	Disadvantages
Precipitation with		
• $(NH_4)_2SO_4$, PEG	Reversible procedure Partial purification	Partial activity loss (facultative)
• Urea, guanidinium chloride	Reversible procedure Dissociation of subunits	Partial activity loss (facultative)
• TCA, perchloric acid	Total removal of protein, collection for protein determination	Irreversible procedure Complete inactivation
Ultrafiltration	Protein remains in solution Purification effected by an appropriate pore size	Losses due to adherence to the filter membrane
Dialysis	No special device necessary	Large losses due to adherence of protein to the large inner membrane surface
Ultracentrifugation	Purification effect, removal of small proteins and components	Only for large proteins Losses due to incomplete resolution of the pelleted protein
Lyophilization	Dry protein samples, suited for long-term storage and shipping	Considerable activity losses with distinct enzymes
Crystallization	For long-term storage	Only for proteins easy to crystallize Pure enzyme preparations

3.4.5.1 Precipitation

Precipitation, still widely applied, is the oldest concentration principle for enzymes. It combines two advantages: concentration and purification. However, precipitation can distort the native state of the enzyme even after removing the precipitating agent. On the other hand, even such fragile enzymes such as multienzyme complexes are treated by this method without discernible alterations. The method may be applied as long as no impairment is detectable. In the early periods of enzyme research, especially for purification, various principles were used: precipitation by heat, acid pH, ethanol, or acetone. However, such methods are rather denaturating and have largely been displaced by modern methods. An exception with respect to pH is precipitation at the *isoelectric point*, where the protein has its lowest solubility (cf. Section 2.2.7.3). Precipitation with perchloric acid and trichloroacetic acid (TCA) causes irreversible denaturation and is applied only to remove proteins quantitatively from solutions or to collect them for protein determination.

For concentration conserving the native structure, *chaotropic* substances are used. Urea (up to 6–8 M) and guanidinium chloride (4–6 M) are used especially for dissociating protein aggregates and separating subunits under more or less

native conditions (in contrast, for e.g., to sodium dodecyl sulfate). $MgCl_2$ in higher concentrations is also sometimes used for enzyme concentration, but mostly ammonium sulfate and polyethylene glycol (PEG) are applied. These reagents destabilize hydrogen bonds and destroy the hydrate shell around the protein surface, causing protein aggregation and subsequently, precipitation. It is assumed that removal of the reagent will reverse completely these effects and restore the native structure.

Each protein precipitates at a characteristic concentration of ammonium sulfate or polyethylene glycol. This feature is used to separate the desired protein from foreign proteins. For each special protein, the concentration limits of the reagent, at which the precipitation occurs, must be tested out. Generally with ammonium sulfate, precipitation happens between 20 and 100% saturation, with polyethylene glycol between 0 and 15% saturation (w/v).

Two different modes for addition of ammonium sulfate are used: solid form or saturated solution. The first procedure is simpler and has the further advantage of not enlarging the final volume severely in contrast to the second method, where especially for higher saturation degrees, exceptional volume increase must be accepted and 100% saturation cannot be reached. On the other hand, this is the more gentle procedure. Upon dissolving solid ammonium sulfate, air bubbles enclosed in the salt crystals become released. Their strong surface tension is harmful for proteins. Beyond that, zones of high concentrations are generated around the dissolving salt crystals and proteins precipitate in such a microenvironment even if the final concentration of the solution remains below the precipitation limit. The protein suffers repeated precipitation and dissolving cycles. To reduce such unfavorable effects, the calculated amount of salt crystals should be added very slowly within about 1 h. Special devices are described for slow additions of salts (Beisenherz *et al.*, 1953). Saturated ammonium sulfate solutions and fast stirring minimize such effects. Temperature is important for solubility of both the salt and the protein, and it must be decided beforehand whether the procedure should be performed at room temperature or (preferably) in the cold (4 °C). For concentration of the protein, a surplus of the salt may be added and the precipitate collected by centrifugation. If the method should be used for purification as well, the respective precipitation limits must be regarded. In a first step, salt is added to the lower limit, where the respective protein or enzyme remains just in solution, while some foreign proteins precipitate and can be removed by centrifugation. In a second step, ammonium sulfate is added to reach the upper limit so that the protein of interest precipitates completely, leaving foreign proteins in solution. The equation

$$S_2 = \frac{(S_2 - S_1) \times 515}{100 - 0.27 \times S_2} \times V \tag{3.1}$$

allows the calculation of the amount of ammonium sulfate (in grams) to be added to a protein solution to obtain the desired saturation degree (S_2) at 0 °C. S_1 is the initial saturation degree and V the volume (in milliliters) of the protein solution. Tables both for addition of solid ammonium sulfate and of saturated solutions are available (Table 3.3 Green and Hughes, 1955; Brewer, Pesce, and Ashworth,

Table 3.3 Precipitation of protein with ammonium sulfate. The values indicate the amount of solid ammonium sulfate to be added to 1 l of the protein solution to give the desired saturation degree S2 (upper line) at 0 °C. The initial saturation degree of the protein solution (S1) is indicated at the left column (after Green & Hughes, 1955, Brewer et al., 1977).

S1/S2	10	15	20	25	30	33	35	40	45	50	55	60	65	67	70	75	80	85	90	95	100
0	53	80	106	134	164	187	194	226	258	291	326	361	398	421	436	476	516	559	603	650	697
5	27	56	79	108	137	162	166	197	229	262	296	331	368	390	405	444	484	526	570	615	662
10		28	53	81	109	133	139	169	200	233	266	301	337	358	374	412	452	493	536	581	627
15			26	54	82	87	111	141	172	204	237	271	316	327	343	381	420	460	503	547	592
20				27	55	75	83	113	143	175	207	241	276	296	312	349	387	427	469	512	557
25					27	46	56	84	115	146	179	211	245	264	280	317	355	395	436	488	522
30						17	28	56	86	117	148	181	214	233	249	285	323	362	402	445	488
33							11	40	70	101	133	166	200	214	235	271	309	347	387	429	472
35								28	57	87	118	151	184	201	218	254	291	329	369	410	453
40									29	58	89	120	153	170	182	212	258	296	335	376	418
45										29	59	90	123	138	156	190	226	263	302	342	383
50											30	60	92	107	125	159	194	230	268	308	348
55												30	61	76	93	127	161	197	235	273	313
60													31	44	62	95	129	164	201	239	279
65														13	31	63	97	132	168	205	244
67															19	52	85	120	156	194	233
70																32	65	99	134	171	209
75																	32	66	101	137	174
80																		33	67	103	139
85																			34	68	105
90																				34	70
95																					35

1974). After precipitation, the pellet is collected by centrifugation and resolved in an appropriate buffer. The smallest possible volume should be used by adding low amounts of the respective buffer, until the precipitate is just dissolved. To avoid addition of larger amounts of buffer than necessary, a special device has been constructed: a glass or plastic cylinder with a fitting pestle, where the precipitate can be treated thoroughly with small amounts of buffer (Beisenherz *et al.*, 1953). Since a considerable amount of ammonium sulfate remains in the protein solution after resolution, extensive dialysis must follow the precipitation.

3.4.5.2 Ultrafiltration and Dialysis

Ultrafiltration is the most frequently used concentration method. It is easy in manipulation, needs only simple devices, and causes no undesirable effects like structural changes of the protein or increase in salt concentration the way precipitation does. Low molecular substances such as buffer ions pass the ultrafiltration membrane and are removed. This effect is used for changing buffer systems, for example during purification procedures. A severe disadvantage, however, is accumulation of the concentrated protein on the membrane surface, forming a closed layer and blocking the flow through the membrane. Accumulation of the protein on the membrane favors aggregation and often, the protein cannot be resolved, causing considerable losses in enzyme activity. In such cases, some of the protein may be recovered by treatment of the membrane with detergents, like Triton X-100.

Ultrafiltration membranes prepared from different materials such as cellulose, nitrocellulose, and synthetic polymers are available. For protein concentration, the membrane should possess a hydrophilic surface. According to pore size, membranes with distinct retention limits can be obtained; for example, for 3, 10, 30, and 100 KDa. This feature can be used to separate components of different sizes. However, pores of ultrafiltration membranes of any type are not homogeneous in their diameter like the meshes of a sieve and the retention limit must be considered more as a preference than as a strict cutoff.

To avoid membrane blocking, the solution near the ultrafiltration membrane should be continuously stirred. However, if the stirrer touches the fragile membrane, it may damage it and destroy the small pores. Therefore, a perpendicular axis fixed to the top of the ultrafiltration cell keeps the magnetic stirrer above the membrane. Ultrafiltration cells are available in distinct sizes for volumes ranging from a few milliliters up to several liters. The solution is forced through the membrane by compressed air or better, by nitrogen gas, and the ultrafiltrate is collected below the membrane. The blocking of the membrane by the protein progressively reduces the flow rate. Increase in the air pressure causes more blocking than the flow rate does. To continue, the filter must be replaced. Tangential flow devices counteract this blocking with a permanent flow of the solution over the membrane.

Various ultrafiltration systems have been developed. Instead of air pressure, some use the centrifugal force, while others suck the solution through the ultrafiltration membrane with the aid of a vacuum. With filter devices mounted on the tip of a syringe, the solution can be manually concentrated.

Dialysis can be regarded as a special type of ultrafiltration. A dialysis bag containing the protein solution is placed in an outer, usually buffered, solution. Only small compounds, but not the protein, penetrate the dialysis membrane and are diluted in the large outer volume. Dialysis is used to remove low molecular weight components, for example from crude homogenates, or reduce high salt after gradients or ammonium sulfate precipitation. For complete removal of the low molecular weight components, the outer solution must be repeatedly exchanged.

To concentrate proteins, the dialysis bag containing the protein solution is embedded in a hygroscopic powder, polyethylene glycol or Sephadex G-25, which withdraws water together with low molecular weight components from the dialysis bag and, thus concentrates the protein solution inside. No special device is required, but relatively high losses in protein and enzyme activity can occur due to interaction of the protein with the large inner surface of the bag. The hygroscopic medium adhering to the outside of the dialysis membrane renders the recovery of proteins attached to the inside difficult.

3.4.5.3 Ultracentrifugation

Ultracentrifugation is especially suited for very large proteins, such as multienzyme complexes, which sediment at a high speed within a reasonable time, while most proteins require very long centrifugation times. Given the presence of a preparative ultracentrifuge, this method is easy but when compared to the membrane effect of ultrafiltration, the concentrated protein forms a dense pellet at the bottom of the centrifugation tube, which may agglutinate to insoluble aggregates. A cushion of 30% sucrose solution layered at the tube bottom can reduce this effect. The time required for concentration depends, besides the protein size, on the rotor dimension and the centrifugation speed. Several hours are needed with a preparative centrifuge, but the time can be considerably reduced by applying a bench-top ultracentrifuge or an air-driven ultracentrifuge (Airfuge®) with a small rotor. The ultracentrifugation method is also useful to segregate smaller proteins and components that remain in the supernatant.

3.4.5.4 Lyophilization

In contrast to the methods described hitherto, which yield concentrated protein solutions, lyophilization produces dry protein, a convenient storage form. However, this technique often causes considerable loss of enzyme activity. Therefore, some precautions must be regarded for successful performance.

Unlike other methods like ultrafiltration or ultracentrifugation, lyophilization removes only the solvent, while all other components are concentrated together with the protein. This holds for the buffer as well, resulting in high salt concentrations. Volatile buffers containing acetate, carbonate, triethylamine, or triethanolamine ions should be used, but both buffer components, the acidic and the basic one, must be equally volatile; otherwise the pH changes severely. Additives like glycerol or BSA can protect the protein from denaturation. After lyophilization, the dry protein sample possesses a fluffy consistency and even faint drafts can

blow away the valuable material. Vacuum centrifuges are specially suited for the concentration of small samples, but cooling is often a problem.

In some cases, vacuum concentration of enzyme solutions in a rotatory evaporator has been reported, although this method cannot be recommended. Elevated temperature is necessary for evaporation and foaming may occur; this is harmful for proteins due to the high surface tension.

3.4.5.5 Other Concentration Methods

Crystallization can be regarded as one of the best methods for concentration and is also an excellent storage form. However, only few enzymes, like catalase or urease, show a particular tendency to form crystals. The method requires relatively pure preparations but can also be used to improve the purity of the enzyme. A variety of elaborate methods exist to obtain crystals suited for X-ray crystallography, but for normal crystallization, such efforts are not necessary. Unlike chemical substances, proteins do not crystallize from saturated solutions; rather, an additive is required to induce crystallization, a chaotropic agent such as ammonium sulfate. Its concentration should be slightly below the precipitation limit of the respective protein, which must be present in high concentrations ($\geq$10 mg ml^{-1}). Crystals will be formed within some days in the cold. Slow vaporizing during this time and thus, a continuous concentration increase, promotes the crystallization process. The crystals should be kept in the crystallization solution. Upon dilution or dialysis, the crystals dissolve again.

Column chromatography usually causes dilution of the applied protein solution, but a certain concentration can be achieved by ion exchange and hydroxylapatite chromatography. This effect is useful for collection of proteins from large volumes of highly diluted solutions, where other concentration methods are too laborious. A considerable concentration effect ($\sim$5 mg ml^{-1}) resulting in high yield from very diluted solutions has been attained with aminohexyl agarose which, in this case, acts as a gentle ion exchanger (Schmincke-Ott and Bisswanger, 1980). The gel bed must be so small that it becomes nearly overloaded with protein. Most proteins bind to the material at low ionic strength (10 mM potassium phosphate pH 7.6). Elution is achieved in one step with high ionic strength (0.1 M or higher potassium phosphate pH 7.6). The concentrated protein solution appears immediately thereafter and must be collected in one single fraction. Preliminary studies should be undertaken to find out the optimum binding and elution conditions.

References

Beisenherz, G., Boltze, H.J., Bücher, T., Czok, R., Garbade, K.H., Meyer-Arendt, E., and Pfleiderer, G. (1953) *Z. Naturforsch.*, **8B**, 555–577.

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Schmincke-Ott, E. and Bisswanger, H. (1980) *Prep. Biochem.*, **10**, 69–75.

Scopes, R.K. (1993) *Protein Purification, Principles and Practice*, 3rd edn, Springer-Verlag, New York.

3.5 Enzyme Immunoassays

3.5.1 Radioimmunoassays

Radioimmunoassays (RIA) are highly sensitive methods used to determine an ^{131}I-radiolabeled antigen with a specific, often monoclonal, antibody (or conversely, a radiolabeled antibody with an antigen). Small, not immunogenic compounds, such as drugs or metabolites, can also be measured by coupling as *hapten* to an immunogenic protein. The assay is often performed in a competitive manner, where a known amount of radiolabeled antigen is given to the unknown sample of the same, but not labeled, antigen (Figure 3.3a). A limiting amount of a first antibody specific for the antigen traps part of the antigen and is, together with its bound antigen, precipitated by a second antibody, directed against the first one. In this way, the unbound antigen remains in solution and becomes separated from the bound one. Radioactivity is determined in the precipitate and from this value, the amount of unlabeled antibody can be derived: the lower the radioactivity found, the higher the amount of unlabeled antigen.

Notwithstanding its high sensitivity, working with radioactivity is troublesome and needs experience, specialized laboratory facilities, and expensive instruments. Alternative targets with high sensitivity are chemiluminescence and fluorescence labels. Comparably as sensitive as RIA and easier to perform, are **enzyme immunoassays (EIA)**, namely **enzyme-linked immunoadsorbent assays (ELISA)**. They need only the common equipment found in biochemical laboratories, such as UV/Vis photometers and centrifuges. These assays are based on the reaction of the antibody with its antigen and make use of the high catalytic efficiency of enzymes, which are labeled as indicators to the reactants. In the **solid-phase EIA**, one of the immunoreactants, the antigen or the antibody, is immobilized on a solid support. The immobilized reactant captures in the following *immunoextraction* step the complementary reactant from the sample. With EIA two alternative strategies can be pursued, *activity amplification* (AA), or *activity modulation* (AM). In the AA procedure, the antibody is present in large excess to obtain an intense maximum signal for the assayed compound. Thus, the antigen is complexed proportional to its concentration. In the AM method, the modulation of the enzyme signal by competition of the test molecule for the same immunoreactant is observed. Here, the sensitivity increases with lower immunoreactant concentrations. AM-type EIA are more specific than AA assays.

Sensitivity is determined by the **dose-response curve** dR/dC, corresponding to the change in response (dR) per unit amount of reactant (dC). The response plotted against the reactant concentration should result in a straight line. Its steepness reflects the sensitivity: the steeper the curves, the more sensitively the

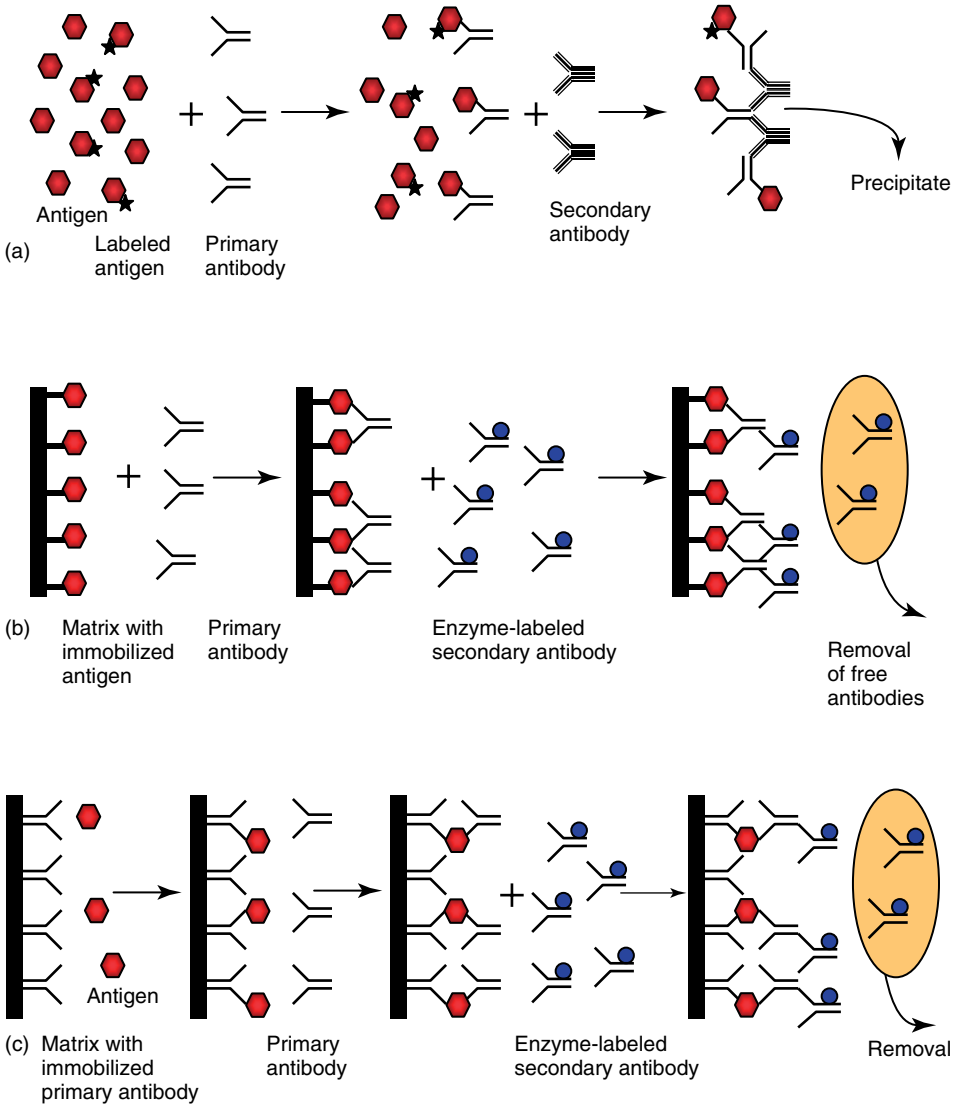

Figure 3.3 Schemes of different immunoassays: (a) competitive radioimmonoassay; (b) noncompetitive solid-phase immunoassay; (c) sandwich method; (d) noncompetitive homogeneous enzyme immunoassay (S_1, substrate of enzyme 1; P_1, product of enzyme 1 = substrate of enzyme 2; P_2, product of enzyme 2); and (e) competitive solid-phase enzyme immunoassay.

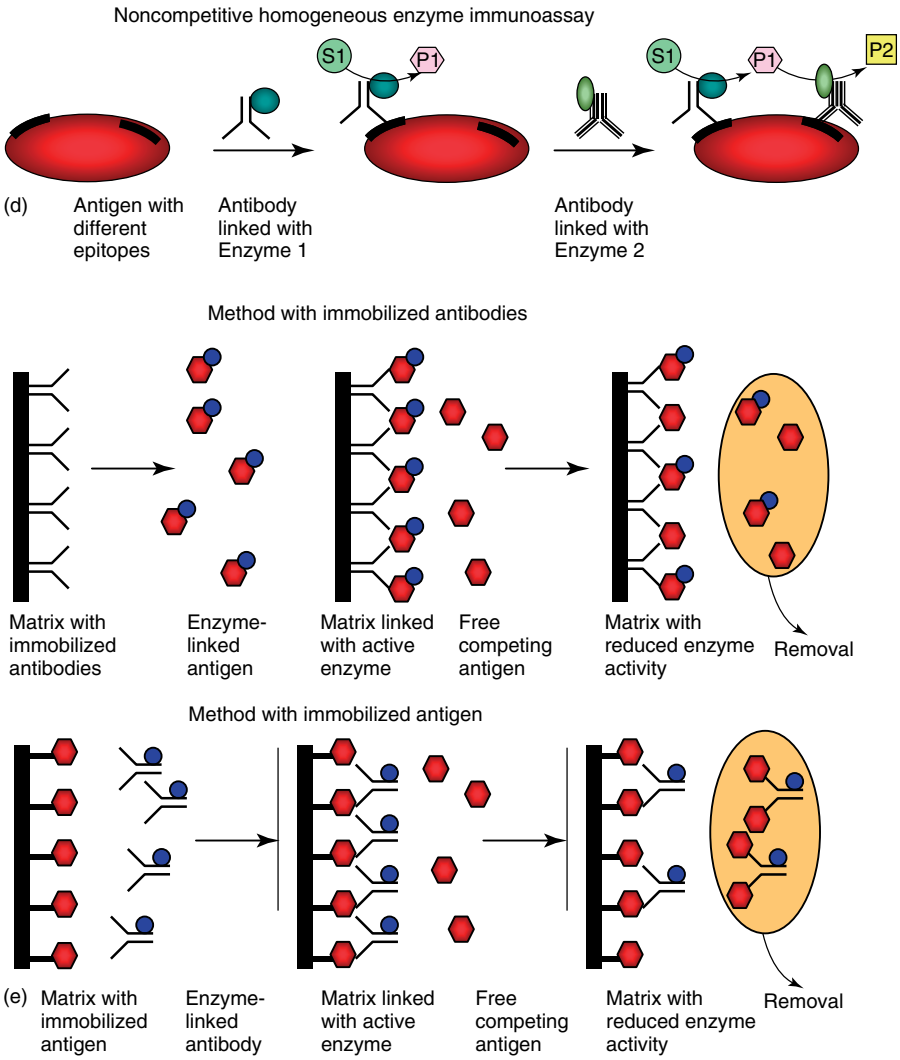

Figure 3.3 (Continued)

system responds to small changes in reactant concentration. Small amounts can be detected more easily by a less steep dependency (detection limit).

3.5.2 Noncompetitive Solid-Phase Enzyme Immunoassay

An immunoreactant, either antigen or antibody, is immobilized to a solid surface (Figure 3.3b). This captures the opposed (antibody or antigen) free immunoreagent from the sample, forming a second layer on the surface. Different methods can be

applied to detect quantitatively the opposed immunoreagent. If it is an antibody, an enzyme-labeled secondary antibody directed against it can be used. For instance, if the primary antibody is a mouse-IgG, anti-mouse-IgG from rabbit or goat may be used. This secondary antibody should exclusively react with the mouse-IgG fraction, but not with the antigen fixed to the support. The enzyme activity fixed to the secondary antibody is measured after removing the fraction of the unbound antibody. For the *bridge method*, nonimmunological recognition systems are used, for example biotin–avidin or protein A, a polypeptide from *Staphylococcus aureus*, binding to the Fc region of IgG molecules.

Instead of the antigen, the primary antibody may be directly fixed to the support, capturing antigens as the opposed free immunoreactants from the sample. In a second step, the unbound primary antibody attaches to the captured antigen (*sandwich method* Figure 3.3c) and finally, in a third step, the secondary enzyme-labeled antibody is bound.

A **noncompetitive homogeneous enzyme immunoassay** without need of a solid phase is achieved by the reactions of two types of monoclonal antibodies directed against different epitopes of the same antigen (Figure 3.3d). The antibodies are labeled with two different enzymes, selected in a manner such that the product of the first enzyme (e.g., hydrogen peroxide from glucose oxidase) serves as a substrate for the second (peroxidase). Its product will be formed in considerable amounts only if both enzymes are brought together in direct contact by binding to the same antigen.

3.5.3 Competitive Solid-Phase Enzyme Immunoassay

In this assay, the antibody is immobilized to a solid support and the free, enzyme-labeled antigen is bound to it. In a second step, the nonlabeled antigen from the sample competes with the bound enzyme-labeled antigen and displaces it. Thus, support-fixed enzyme activity will be reduced and after washing out the displaced enzyme-labeled antigen, the amount of product formed is inversely proportional to the amount of free antigen (Figure 3.3e).

In the opposite procedure, the antigen is immobilized to the support, to which the free, enzyme-labeled antibody will be bound. Free antigen in the sample competes with the enzyme-labeled antibody, thus reducing the amount of enzyme activity to be fixed to the support.

3.5.4 Methods for Enzyme Immunoassays and Immobilization Techniques

3.5.4.1 Protein Coupling to Cyanogen Bromide–Activated Agarose

This is a very useful method for covalent immobilization of enzymes or proteins such as peroxidase and antibodies to agarose or dextrans, for example Sepharose® or Sephadex®. Due to the high toxicity of cyanogen bromide, commercially prepared material (e.g., CNBr-activated Sepharose®) is emphatically recommended. The

procedure must be performed quickly because the strong alkaline condition needed for activation destabilizes the structure of the gel.

Reagents and solution

Sepharose 4B, suspension, 6.6 g in 20 ml H_2O

3 M NaOH ($M_r = 40.0$; 12 g in 100 ml)

Cyanogen bromide (CNBr, $M_r = 105.9$)

$NaHCO_3$/NaCl solution: 0.1 M $NaHCO_3$ ($M_r = 84.0$; 0.84 g in 100 ml) pH 8.2, containing 0.5 M NaCl ($M_r = 58.4$; 2.92 g in 100 ml)

1 M glycine ($M_r = 75.1$; 1.5 g in 20 ml H_2O)

0.1 M sodium acetate/acetic acid pH 4.0

0.1 M potassium phosphate pH 7.6

Preparation of CNBr-activated Sepharose 4B

The following procedure must be done in a fume hood with the utmost care.

Adjust the Sepharose 4B suspension to pH 11.2 with 3 M NaOH, using a pH-meter equipped with a pH-electrode

A dropping funnel is mounted on a balance and tared; add 0.67 g solid CNBr and dissolve in a small volume of water; close the funnel tightly with a stopper

Drop the CNBr solution slowly to the gel suspension under stirring (use a KPG or propeller stirrer, not a magnetic stirrer); keep the pH constant at pH 11.2 by adjusting with 3 M NaOH at the pH meter. If the pH remains constant for about 6 min, wash the gel thoroughly on a Buchner funnel or a frit with about 1 l of water

Coupling of protein

Transfer the CNBr-activated Sepharose into 20 ml $NaHCO_3$/NaCl solution and add 0.2 g of the protein

Shake the suspension gently for 3 h at room temperature or overnight at 4 °C

Determine the remaining protein content in the supernatant with a protein assay; the difference to the total protein is supposed to be immobilized

Add 5 ml 1 M glycine to block free reactive groups

Wash the gel with 0.1 M sodium acetate/acetic acid pH 4.0 and thereafter, with 0.1 M potassium phosphate pH 7.6

For storage add sodium azide (NaN_3) to a final concentration of 0.1 M to the gel

3.5.4.2 Coupling of Diaminohexyl Spacer

Instead of direct coupling of the protein to the matrix, a spacer can be used as a connecting bridge between matrix and protein.

Reagents and solutions

0.1 M potassium phosphate pH 7.6

1.6-hexane diamine (1.6 diaminohexane, M_r 116.2)

0.5 M NaCl ($M_r = 58.4$; 2.92 g in 100 ml)

EDC · HCl (*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride, $M_r = 191.7$)

1 N HCl

Procedure

The procedure for coupling the spacer is the same as for the protein (see above), instead of protein take the following:

1) 1 mg ml^{-1} (20 mg in 20 ml for the described procedure) of 1.6 hexane diamine
2) Wash the resulting aminohexyl derivative (AH-Sepharose) with 0.5 M NaCl
3) For coupling of the protein to the spacer, adjust the pH to 4.5 with 1 N HCl
4) Add 10 mg ml^{-1} gel EDC · HCl and
5) 10 mg ml^{-1} gel of the protein, keep the final pH at 4.5
6) Control the pH after 1 h and shake the mixture overnight at 4 °C
7) Wash the gel with 0.1 M potassium phosphate pH 7.6

3.5.4.3 Periodate Activation of Cellulose

This procedure is applicable for materials with vicinal glycols, like cellulose, agarose, dextrans (Separose, Sephadex, Sephacryl). Aldehyde groups are formed by periodate oxidation. They react with amino groups to Schiff's bases, which can be stabilized by borohydride reduction.

Reagents and solutions

Cellulose powder

0.1 M $NaHCO_3$/0.06 M $NaIO_4$ ($NaHCO_3$, $M_r = 84.0$; $NaIO_4$, $M_r = 213.9$; 8.4 g $NaHCO_3$, 12.8 g $NaIO_4$ in 100 ml H_2O)

0.1 M sodium carbonate buffer ($NaHCO_3/NaCO_3$) pH 9.0

$NaBH_4$ solution ($NaBH_4$, M_r = 37.8; 0.1 g in 10 ml 0.1 M NaOH, freshly prepared)

0.1 M sodium acetate/acetic acid pH 4.0

PBS (phosphate buffered saline: 10 mM sodium/potassium phosphate pH 7.2, 0.8% NaCl, 0.02% KCl)

0.05% Tween 20 in PBS

Procedure

Suspend 2 g cellulose in 40 ml 0.1 M $NaHCO_3$/0.06 M $NaIO_4$ and keep for 2 h in the dark

Wash the cellulose on a frit with 0.1 M sodium carbonate buffer pH 9.0 and suspend in 40 ml of this buffer

Add 40 mg of the protein (final concentration: 1 mg ml^{-1}) and shake overnight at room temperature

Add 2 ml $NaBH_4$ solution to reduce the Schiff's base and shake for 30 min at room temperature

Add another 2 ml $NaBH_4$ solution and shake for a further 30 min

Wash the solid material with 0.1 M sodium acetate/acetic acid pH 4.0, thereafter with PBS, and finally with 0.05% Tween 20 in PBS

3.5.4.4 Introduction of Thiol Groups into Proteins (Antibodies)

To link enzyme activities to proteins such as antibodies, free thiol groups should be available. They can be introduced with specific reagents, for example AMSA, which is described here.

Reagents and solutions

AMSA-solution (*S*-acetylmercaptosuccinic anhydride; M_r = 174.2, 60 mg in 1 ml) *N*,*N*′-dimethylformamide

0.1 M potassium phosphate pH 6.0

0.1 M Tris/HCl pH 7.0

Protein (antibody) solution (10 mg ml^{-1} in 0.1 M potassium phosphate pH 7.0)

Nitrogen gas

0.5 M hydroxylamine/0.01 M EDTA (3.46 g $NH_2OH \cdot HCl$, M_r = 69.5; 0.29 g EDTA, M_r = 292.3, in 100 ml 0.1 M Tris/HCl pH 7.0)

Sephadex G-25-column, 1.5 × 30 cm, equilibrated with 0.1 M potassium phosphate pH 6.0, 5 mM EDTA

Procedure

Add 0.1 ml AMSA solution to 1 ml protein solution under nitrogen gas, seal the tube, and stir gently for 0.5 h at room temperature

Add 0.2 ml 0.5 M hydroxylamine/0.01 M EDTA and adjust to pH 7.0

Incubate at 30 °C for 4 min

Apply the mixture to the Sephadex column and elute with 0.1 M potassium phosphate pH 6.0, 5 mM EDTA

Measure absorption at 280 nm to detect the protein fractions, collect the strongly absorbing fractions, and concentrate to 1 ml by ultrafiltration

Reference

Klotz, I.M. and Heiney, R.E. (1962) *Arch. Biochem. Biophys.*, **96**, 605–612.

3.5.4.5 Conjugation of a Protein (Antibody) with an Enzyme (Peroxidase)

Reagents and solutions

Peroxidase (horse radish)

SMCC solution (succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester; $M_r = 334.3$; dissolve 5 mg in 60 µl *N,N′*-dimethylformamide, at 30 °C)

0.1 M potassium phosphate pH 7.0

0.1 M potassium phosphate pH 6.0

Sephadex G-25-column, about 1.5 × 30 cm, equilibrated with 0.1 M potassium phosphate pH 6.0

0.1 M cysteamine ($M_r = 77.2$; 77.2 mg in 10 ml)

Procedure

Dissolve 10 mg peroxidase in 1 ml 0.1 M potassium phosphate pH 7.0

Warm up to 30 °C and add 60 µl SMCC solution (final concentration 15 mM)

Stir gently for 1 h

Centrifuge to separate the precipitate

Apply the supernatant onto the Sephadex-G25 column and elute with 0.1 M potassium phosphate pH 6.0

Peroxidase elutes first, determine the enzyme activity (cf. assay in Section 3.3.1.32), or absorption at 280 nm, concentrate the active fractions to about 1 ml by ultrafiltration

Add 10 mg of thiolated protein (antibody) in 1 ml and incubate 1 h at 30 °C

Add 0.15 ml 0.1 M cysteamine to block the unreacted maleimide groups

The reaction products can be further purified by gel filtration on the Sephadex-G25 column using the conditions described above.

Reference

Yoshitake, S., Imagawa, M., Ishikawa, E., Niitsu, Y., Urushizaki, I., Nishiura, M., Kanazawa, R., Kurosaki, H., Tachibana, S., Nakazawa, N., and Ogawa, H. (1982) *J. Biochem.*, **92**, 1413–1424.

3.5.4.6 Conjugation of β-Galactosidase to Proteins (Antibodies) by MBS

Reagents and solutions

MBS solution (3-maleimidobenzoyl-*N*-hydroxysuccinimide ester, $M_r = 314.3$; 20 mg/ml dioxane)

0.1 M potassium phosphate pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl (2 g $MgCl_2 \cdot 6H_2O$, 2.9 g NaCl per liter of buffer)

Protein (antibody) solution (1 mg ml^{-1} in 0.1 M potassium phosphate pH 7.0, 50 mM NaCl)

β-Galactosidase (from *Escherichia coli*)

Sephadex G-25-column, 1 × 30 cm, equilibrated with 0.1 M potassium phosphate pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl

1 M 2-mercaptoethanol ($M_r = 78.1$; 0.78 g in 10 ml)

0.1 M dithioerythritol, (DTE, $M_r = 154.2$, 154 mg in 10 ml)

Procedure

Add 1 µl MBS solution to 1 ml protein solution

Incubate 25 °C for 1 h

Apply to the Sephadex G-25 column, elute with 0.1 M potassium phosphate pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl

Collect protein fractions (absorption at 280 nm) and add immediately β-galactosidase (sample containing 2 mg)

Incubated at 30 °C for 1 h

Stop the reaction by addition of 50 µl ml^{-1} 0.1 M dithioerythritol

Reference

Kitagawa, T. and Aikawa, T. (1976) *J. Biochem.*, **79**, 233–236.

3.5.4.7 Conjugation of Alkaline Phosphatase to Antibodies by Glutaraldehyde

This method is generally applicable for cross-linking of proteins. A similar technique with dimethylsuberimidate, also suitable for enzyme immunoassays is described in Section 3.4.4.

Reagents and solvents

Glutaraldehyde (glutardialdehyde, pentane-1,5-dial, $M_r = 100.1$; 50% aqueous solution, commercially available)

Alkaline phosphatase (from bovine intestinal mucosa, 10 mg ml^{-1})

0.5 M Tris/HCl pH 8.0

Antibody solution (10 mg ml^{-1} in buffer, PBS, or 0.5 M Tris/HCl pH 8.0)

PBS (phosphate buffered saline: 10 mM sodium/potassium phosphate pH 7.2, 0.8% NaCl, 0.02% KCl)

BSA, bovine serum albumine

Dialysis tubes (Visking)

Procedure

Mix appropriate volumes of alkaline phosphatase solution (containing 25 mg) and antibody solution (10 mg) and dialyze overnight at 4 °C against PBS, changing the PBS solution several times

Add glutaraldehyde to the dialyzed solution, to a final concentration of 2% (v/v)

Stir gently for 2 h at 4 °C

Dialyze against PBS for 6 h and finally against 0.5 M Tris/HCl, pH 8.0, overnight at 4 °C. For storage of the solution, add 1% BSA

General References

Tijssen, P. (1985) *Practice and Theory of Enzyme Immunoassays*, Elsevier, Amsterdam.
Kemeny, D.M. and Challacombe, S.J. (1988) *ELISA and Other Solid Phase Immunoassays*, John Wiley & Sons, Ltd, Chichester.
Nowotny, A. (1979) *Basic Exercises in Immunochemistry*, Springer-Verlag, Berlin.