

## Activation of pro-astacin

### Immunological and model peptide studies on the processing of immature astacin, a zinc-endopeptidase from the crayfish *Astacus astacus*

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To contribute knowledge of the processing and activation of invertebrate proteolytic enzymes, we studied the metalloprotease astacin, a digestive enzyme from the freshwater crayfish *Astacus astacus* (decapod crustacean). It is the prototype of the protein family of astacins, members of which occur in organisms from bacteria to man and are involved in a variety of physiological reactions. According to its genomic structure, astacin is produced as a zymogen [Geier, G., Jacob, E., Stöcker, W. & Zwillig, R. (1997) *Arch. Biochem. Biophys.* **337**, 300–307]. To localize and follow the processing of pro-astacin in different parts of the digestive tract, we synthesized two peptides covering the pro part of pro-astacin and raised antibodies against them. In addition, antiserum against the whole active astacin was produced. Using immunohistochemical investigation, we detected pro-astacin in the F cells of the hepatopancreas and all the way into the tubular lumen and the collecting ducts of this gland. Immunoblot assays revealed only active astacin, and never pro-astacin, present in the cardiac

stomach. We conclude from these studies that astacin is secreted into the lumen of the hepatopancreatic tubules in its pro form and is activated on its way to the stomach. To investigate which of the two endopeptidases found in the digestive tract of crayfish, astacin or trypsin, is responsible for cleaving the propeptide from pro-astacin, we synthesized different peptides that mimic the activation site. MS analysis of the cleavage products of astacin and trypsin showed that astacin is capable of catalyzing its own activation. Any contribution of trypsin would require the successive action of an aminopeptidase. Substituting glycine for arginine at position –1 of the activation site does not prevent astacin activity. As most members of the astacin protein family have basic amino-acid residues in this position, in these cases also astacin-specific cleavage would be possible.

**Keywords:** activation; astacin family; *Astacus astacus*; immunohistochemistry; pro-astacin.

Although the physiological and biochemical events that occur during activation of mammalian proteases served as a model for the emerging subject of protein chemistry 30 years ago [1], it is still not known whether phylogenetically more primitive organisms had evolved similar mechanisms. Mammalian digestive serine proteases, such as

trypsin, are synthesized in the exocrine pancreas as inactive precursors characterized by N-terminal sequence extensions (prepro parts). The prepeptide (signal peptide) is already split off during the passage of the newly synthesized protein from the ribosome into the endoplasmic reticulum. From there the proenzyme is transferred through the Golgi apparatus and then densely packed into zymogen granules [1].

On the appropriate hormonal or neuronal stimulus, the contents of zymogen granules are released at the microvillous border of the pancreatic acinus cells and transported via the pancreatic duct into the duodenum. There, the active enzyme is formed by removal of the propeptide. In mammals, trypsin is the most important activator of the pancreatic digestive enzymes. It acts on chymotrypsinogen, pro-elastase and pro-carboxypeptidase, and also acts autocatalytically. Initial activation of trypsinogen normally occurs in the duodenum by the action of enterokinase, a brush-border enzyme found in enterocytes [2,3]. For some time it was thought that, in the case of bovine trypsin, liberation of the 23-amino-acid residues that represent the propeptide would give access to the catalytic site, leading to activation. However, X-ray crystallographic studies have shown that it is the interaction between the new N-terminal Ile24 and Asp194 in the neighbourhood of the catalytic Ser195 that stabilizes the catalytic site and hence activates the enzyme [4,5].

The purpose of this study was to elucidate the probability of such mechanisms at the organizational level of

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**Abbreviation:** MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

**Enzymes:** astacin from *Astacus astacus* (EC 3.4.24.21); trypsin from human and *A. astacus* (EC 3.4.21.4); bone morphogenetic protein BMP-1 from human and *Xenopus laevis* (EC 3.4.24.19); mepirin A and B from human (EC 3.4.24.18); choriolysins LCE and HCE from *Oryzias latipes* (EC 3.4.24.66 and EC 3.4.24.67); flavastacin from *Flavobacterium meningosepticum* (EC 3.4.24.76); alveolin from *Oryzias latipes*, *Astacus* egg astacin AEA from *A. astacus*, blastula proteins BP-10 from *Paracentrotus lividus*, embryonic protein UVS.2 from *X. laevis*, early span protein SpAN from *Strongylocentrotus purpuratus*, hatching protein hch-1 from *Caenorhabditis elegans*, metalloproteinase PMP-1 from *Podocoryne carnea*, metalloproteinase HMP-1 from *Hydra vulgaris*, nephrosin from *Cyprinus carpio*, tolloid and xolloid from *Drosophila melanogaster* (all: EC 3.4.24.-).

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invertebrate animals or determine whether alternative solutions may have been developed. We therefore investigated the fate and activation status of the zinc-endopeptidase astacin throughout the digestive tract of the freshwater crayfish *Astacus astacus* (Crustacea, Decapoda) by immunohistochemistry and immunoblotting with antibodies against the active astacin and the pro sequence of the pro-astacin. Furthermore, synthetic peptides that mimic the activation site of the pro-astacin were cleaved with astacin and trypsin from crayfish and analysed by MS in order to understand the role of these enzymes in the activation process.

Crayfish astacin is the prototype of a family of zinc-endopeptidases, the astacins. Members of this family occur in organisms from bacteria to man and serve in a wide variety of physiological processes (reviewed in [6]). Different structural and functional aspects of crayfish astacin are well known, such as its amino-acid sequence [7], 3D structure [8–10], genomic organization [11], cleavage specificity [12,13], and site of synthesis [14].

Because of the importance of astacin as a family prototype, our investigations into its activation not only complete our earlier studies on this enzyme, but should also contribute to our understanding of the processing of a variety of other family members.

## MATERIALS AND METHODS

### Animals

Adult crayfish, *A. astacus*, were obtained from a commercial supplier (Keller, Augsburg, Germany) and kept as previously described [14].

### Preparation of tissues and protein extracts

To locate the sites of biosynthesis and activation of astacin by immunoassay, 18 crayfish were starved for 1 week. The production of digestive enzymes was then stimulated by artificial emptying of the stomach by inserting a curved glass capillary through the oesophagus. Groups of six specimens were killed either immediately or after 3 or 7 h. Parts of the hepatopancreases from each group were processed for routine light microscopy and immunohistochemistry as described below. Other parts of the same hepatopancreases were used to prepare protein extracts for immunoblotting. In addition, gastric fluid was collected from three animals per group at 0, 3 and 7 h. All samples for immunoblot analysis were directly transferred to SDS gel-loading buffer (50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) which contained 50 mM EDTA and 50 mM EGTA added as metalloprotease inhibitors. The samples were heated to 100 °C, homogenized using 50 strokes of the Microfuge pestle and centrifuged at 13 000 *g* for 5 min. The supernatant was then decanted and kept at –20 °C until use.

### Peptides

Peptide syntheses were carried out on a modified Applied Biosystems 430A peptide synthesizer using the *in situ* neutralization protocol for Boc chemistry solid-phase

peptide synthesis as described in [15]. Peptides were deprotected and cleaved from the resin with HF, purified by RP-HPLC, and analysed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. The sequence of the peptides (ProAst-N, ProAst-C) used for preparation of antibodies and the peptides (AstG, AstR, AstR\*) used for cleavage assays is given in Fig. 1 and Fig. 4b.

### Antibodies

For immunohistochemistry and immunoblotting, antisera against astacin and pro-astacin were used. The astacin antiserum was prepared as previously described [14]. In addition, two different pro-astacin antisera were raised in rabbits, which were immunized with the synthetic peptides ProAst-N and ProAst-C (Fig. 1) conjugated via succinimidyl maleimidobenzoate to keyhole limpet haemocyanin. Booster injections were performed 14, 28, and 56 days after the first immunization. The antisera anti-(ProAst-N) and anti-(ProAst-C) from the final bleed, 5 days after the last boost, yielded the best reactivity and were therefore used in this study. The immunization for the pro-astacin antiserum was carried out by Pineda Antibody Service, Hamburg, Germany. For immunohistochemistry and immunoblotting, fluorescein isothiocyanate-conjugated goat anti-(rabbit IgG) Ig (Dianova, Hamburg, Germany) and alkaline phosphatase-conjugated goat anti-(rabbit IgG) Ig (Sigma, Steinheim, Germany) were used as secondary antibodies, respectively.

### Immunohistochemistry

For immunohistochemistry and routine light microscopy, the hepatopancreases were fixed in Susa fixative and Bouin's solution without acetic acid [14], respectively, and then dehydrated in a graded series of ethanol. Finally, the tissues were embedded in paraffin using methylbenzoate as intermedium. Sections ( $\approx$  6- $\mu$ m thick) were deparaffinized and either stained with Goldner's stain for routine microscopy or used for immunohistochemistry. In the latter technique, the sections were incubated for 2 h with the first antibody, anti-astacin, anti-(ProAst-N) or anti-(ProAst-C), diluted 1 : 10 with 0.15 M NaCl/P<sub>i</sub> (pH 7.2). After three 15-min washes in NaCl/P<sub>i</sub>, the sections were incubated for 1 h with fluorescein isothiocyanate-conjugated anti-rabbit IgG from goat diluted 1 : 40 in NaCl/P<sub>i</sub>. The sections were then rinsed with NaCl/P<sub>i</sub>, stained with 0.1% Evans blue for



**Fig. 1. Amino-acid sequence of the complete prepropeptide and the N-terminal partial sequence of astacin.** The sequence of the prepropeptide was deduced from the c-DNA sequence established previously by us [11]. The signal peptide cleavage site (position –35) was predicted by the program SIGNALSEQ. The propeptide sequence is represented in bold letters. The N-terminus of the mature astacin (position 1) is known from Edman sequencing of the mature protease [7]. The position and structure of the synthetic peptides ProAst-N and ProAst-C used for the production of antibodies are indicated by bold lines.

5 min, and mounted with NaCl/P<sub>i</sub> containing 25 mg·mL<sup>-1</sup> diazabicyclo[2,2,2]octane to stabilize the fluorescence. Control screenings were carried out by performing the procedure without the first antibodies (anti-astacin and anti-pro-astacin) or by using anti-(*A. astacus* haemocyanin) serum (obtained from W. Stöcker, University of Münster, Germany) as first antibody. The results were documented with a Leitz Aristoplan microscope using various Kodak film types.

### Immunoblotting

SDS/PAGE was performed according to standard protocols [16,17] using a polyacrylamide concentration of 15% in the separation gel. For immunoblot determinations, 50 µg total protein and 2 µg purified astacin were loaded, and gels were electroblotted to poly(vinylidene difluoride) membranes (Macherey & Nagel, Düren, Germany) using a semidry blotting apparatus. The protein bands of the size markers were stained with Coomassie Brilliant Blue. The membranes were blocked using 1% blocking powder® (Schleicher & Schuell, Dassel, Germany) dissolved in NaCl/P<sub>i</sub> (0.05 M sodium phosphate, pH 7.4, 0.15 M NaCl) overnight at 4 °C and incubated with anti-astacin or anti-(pro-astacin) serum in 0.5% blocking powder in NaCl/P<sub>i</sub> for 2 h at room temperature. The blot was washed with NaCl/P<sub>i</sub>/0.1% Tween 20. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-(rabbit IgG) Ig for 1 h. After a wash with NaCl/P<sub>i</sub>/0.1% Tween 20, staining was carried out using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

### Cleavage assays

Crayfish trypsin and astacin were prepared as described by Zwilling & Neurath [18], and the lyophilized enzymes were dissolved in 0.05 M Tris/HCl buffer, pH 8.0. Astacin was further purified on an affinity column with Pro-Leu-Gly-hydroxamate as ligand (Bachem, Heidelberg, Germany) coupled to CH-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's recommendations. Affinity chromatography was performed as described in [19]. The cleavage assays were conducted in 0.5-mL plastic tubes containing a total volume of 20 µL, buffered by 0.05 M Tris/HCl/0.01 M CaCl<sub>2</sub>, pH 8.0, for trypsin and 0.05 M Tris/HCl, pH 8.0, for astacin. Then 5 µg of the peptides AstG, AstR or AstR\* (Fig. 4b) were incubated with 0.5 µg affinity-purified astacin or crayfish trypsin for 2 h at 30 °C. Quantification of the enzymes was based on A<sub>280</sub> measurements, and substrate concentrations were determined by weight. Release of cleavage products was monitored by MALDI-TOF MS.

### MALDI-TOF MS

MALDI-TOF mass spectra were recorded in the positive-ion mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with a SCOUT multiprobe inlet and a 337-nm nitrogen laser. Ion-acceleration voltage was set to 20.0 kV, the reflector voltage was set to 21.5 kV, and the

first extraction plate was set to 15.4 kV. Mass spectra were obtained by averaging 10–100 individual laser shots. Calibration of the spectra was performed externally by a two-point linear fit using angiotensin I (*m/z* 1269.69) and oxidized insulin β chain (*m/z* 3495.65). Samples were prepared by the thin-film protocol of Jensen *et al.* [20]. Aliquots (0.3 µL) of a nitrocellulose-containing saturated solution of α-cyano-4-hydroxycinnamic acid in acetone were deposited on to individual spots on the target. Subsequently, 0.5 µL of the peptide sample was loaded on top of the thin-film spots and allowed to dry slowly at room temperature. Peptides digested with either trypsin or astacin were diluted 10-fold with 10% formic acid before analysis.

## RESULTS AND DISCUSSION

### Immunohistochemistry

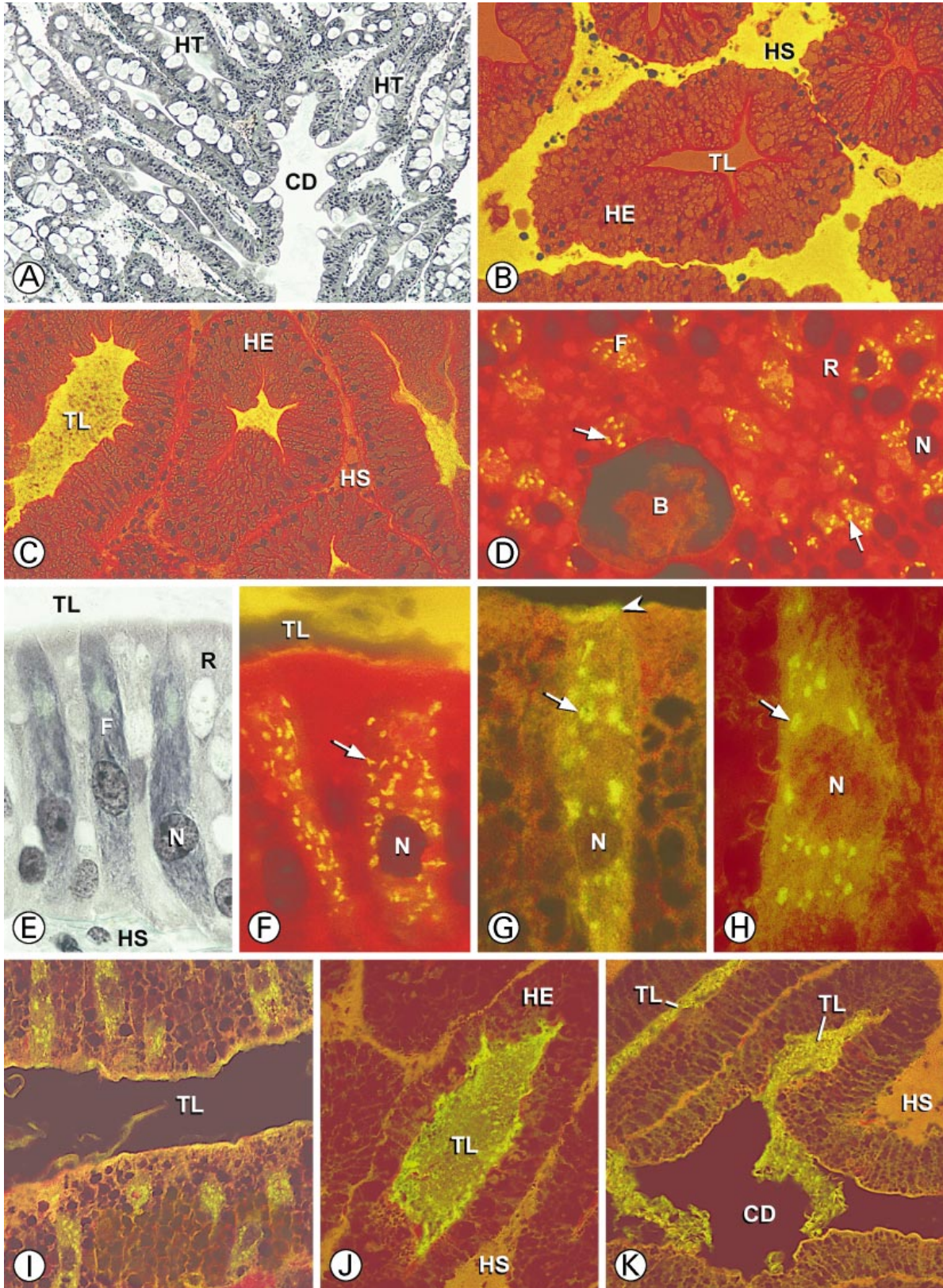
The hepatopancreas of crayfish is composed of a system of blindly ending hepatopancreatic tubules and adjoining collecting ducts (Fig. 2a), which finally terminate in the stomach [14]. The anterior part of the stomach, the cardia, normally includes a brownish gastric fluid rich in digestive enzymes [18]. The hepatopancreatic tubules are composed of a single-layered epithelium, which directly contacts the tubule lumen and the haemolymph space (Fig. 2b). Apart from the embryonic E cells located at the blind tips of the tubules, four cell types are present in the hepatopancreatic epithelium: R (resorptive) cells; F (fibrillar) cells (Fig. 2e); B (blister-like) cells; M (midgut) cells. R cells are responsible for absorption of the nutrients [21], F cells synthesize digestive enzymes [14], and M cells are apparently regulatory [21]. The function of B cells is still not known.

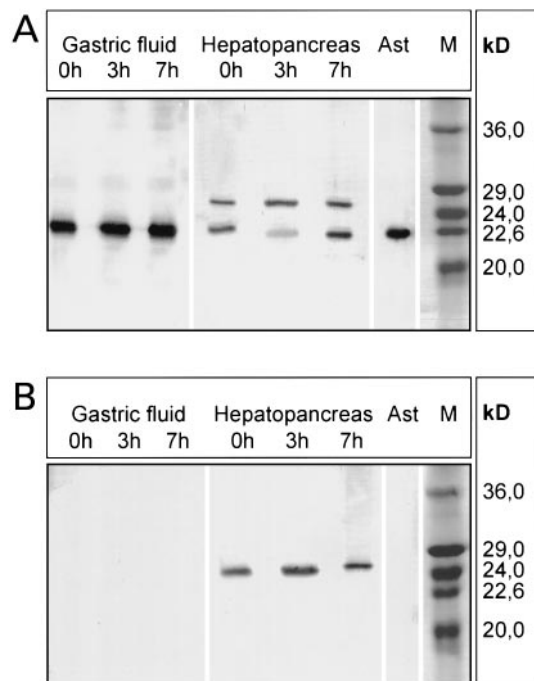
Immunohistochemistry with antibodies against the mature astacin which also recognize the pro-astacin (Fig. 3a) corroborated our earlier findings [14] that this enzyme is synthesized in the F cells only (Fig. 2d,f). After stimulation of enzyme synthesis by withdrawal of the gastric fluid [14], all F cells stained positive for astacin (Fig. 2d). Extracellular astacin was found in the lumen of all parts of the hepatopancreatic tubule system (Fig. 2c) except where it had been washed out during processing of the tissues. In controls in which anti-haemocyanin was used as first antibody instead of anti-astacin, only the haemolymph space was positive and not the hepatopancreatic epithelium or the tubule lumen (Fig. 2b). Immunohistochemistry with antibodies against ProAst-N or ProAst-C, which specifically recognize the N-terminal or C-terminal moiety of the pro-peptide produced principally the same results. The immunofluorescence was particularly noticeable in the Golgi bodies (Fig. 2g,h) and also along the microvillous border (Fig. 2g) where these enzymes are released into the tubular lumen. The fluorescing compartments of the F cells have previously been identified by electron microscopy as Golgi bodies [14]. In the period after stimulation of enzyme synthesis, all F cells stained positive with the pro-astacin antibodies (Fig. 2i). Interestingly, pro-astacin-related fluorescence was also found in the lumina of the hepatopancreatic tubules (Fig. 2j,k) and even in the collecting ducts (Fig. 2k). This implies that pro-astacin is activated in the more proximal parts of the hepatopancreatic tubule system on its way to the stomach, where it is exclusively found in the active form (see immunoblotting, Fig. 3a).

### Immunoblotting

Immunoblotting demonstrated that the gastric fluid taken from the cardiac stomach strongly reacts with astacin antibodies, indicating the presence of active astacin in the

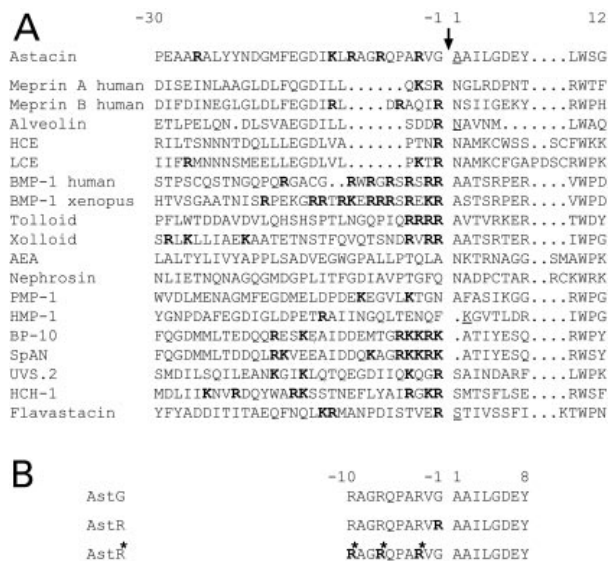
stomach (Fig. 3a). In contrast, no trace of pro-astacin was detectable in the gastric fluid when anti-(ProAst-N) serum was applied (Fig. 3b). In the hepatopancreas homogenates, astacin antibodies recognized both (Fig. 3a) astacin and its pro form, but anti-(ProAst-N) reacted specifically with the





**Fig. 3.** Immunodetection of astacin and pro-astacin in the gastric fluid and hepatopancreas homogenate by immunoblotting. (a) Antiserum, anti-astacin (raised against purified native astacin from the gastric fluid); antigens, gastric fluid, hepatopancreas homogenate, and native astacin from the gastric fluid (Ast). M, marker proteins. 0 h, 3 h, 7 h = hours after stimulation of enzyme synthesis. (b) Antiserum, anti-(pro-astacin) (raised against the synthetic peptide ProAst-N); antigens, as in (a).

proenzyme (Fig. 3b). Purified active astacin was detected in a control reaction only by anti-astacin, and not by anti-pro-astacin (Fig. 3a,b, lane 'Ast'). We also noticed that 3 h after stimulation of enzyme synthesis by withdrawal of the gastric fluid, the amount of pro-astacin had considerably increased compared with active astacin (Fig. 3a). This indicates considerable new synthesis of the zymogen, which is in accordance with earlier stimulation experiments [14]. Finally, it must be emphasized that the immunoblots of hepatopancreas homogenates detected no traces of free propeptide of astacin (not shown), while, under the same conditions, pro-astacin can clearly be seen. To prove that the limited size of the propeptide would not prevent its detection by immunoblotting, in preliminary experiments we applied the peptides ProAst-N and ProAst-C to



**Fig. 4.** Activation site of members of the astacin protein family and synthetic sequences of peptides mimicking this cleavage site. (a) Alignment of amino-acid sequences around the activation site of astacin and other astacins. The putative N-terminus of the mature protein is indicated by an arrow. Experimentally proven N-termini of processed enzymes are underlined. Basic residues representing possible recognition and binding sites for processing enzymes are emphasized by bold letters. Residues are numbered according to the astacin numbering system. (b) Amino-acid sequences of synthetic peptides mimicking the processing site of pro-astacin. AstG, unmodified sequence with glycine in position -1; AstR, modified sequence with arginine in position -1; AstR\*, modified sequence with D(-)-arginine residues (R\*) in positions -3, -7, and -10.

poly(vinylidene difluoride) membranes and showed immunopositive reactions under these conditions. These observations should also rule out any interference of free propeptide with our pro-astacin localization in the hepatopancreas lumen by immunofluorescence (Fig. 2j,k). Furthermore, it is reasonable to assume that intact propeptide is rapidly degraded by trypsin or astacin activity which is present in certain amounts in the hepatopancreas (Fig. 3a).

### Processing of astacins

The alignment of a variety of propeptides from members of the astacin protein family shows that, in most cases, there is a basic amino-acid residue in position -1 where the

**Fig. 2.** Immunohistochemical localization of astacin and pro-astacin in the hepatopancreas of crayfish. (a) Longitudinal section through hepatopancreatic tubules (HT) and adjoining collecting duct (CD). Goldner staining, bright field microscopy.  $\times 29$ . (b) Cross-section of hepatopancreatic tubule stained with anti-haemocyanin serum (control). Haemocyanin-related fluorescence (yellow) is restricted to the haemolymph space (HS). HE, hepatopancreatic epithelium; TL, tubule lumen.  $\times 75$ . (c) Tubule lumina filled with astacin (yellow) as shown with anti-astacin serum.  $\times 68$ . (d) Oblique section of hepatopancreatic epithelium showing intracellular astacin immunofluorescence in F cells only (arrows). B cells (B) and R cells (R) are negative. N, cell nucleus.  $\times 320$ . (e) Longitudinal section of R cells and F cells alternating in the hepatopancreatic epithelium. Goldner staining, bright field microscopy.  $\times 420$ . (f-h) Higher magnification of F cells after incubation with antiastacin (f), anti-(ProAst-N) (g) and anti-ProAst-C (h) sera. In all treatments intracellular immunofluorescence is particularly noticeable in Golgi bodies (arrows). Arrowhead in (g) indicates presence of pro-astacin along microvillous border, the release site of the enzyme into the tubule lumen.  $\times 410$  (f),  $\times 470$  (g),  $\times 700$  (h). (i) Section of hepatopancreatic tubule showing ProAst-N-related immunofluorescence in all F cells.  $\times 150$ . (j-k) Presence of pro-astacin in lumina of hepatopancreatic tubules and collecting duct as shown with anti-(ProAst-N). (j) and anti-ProAst-C (k) sera.  $\times 69$ .

activation occurs (Fig. 4a). In addition, an accumulation or clustering of arginine or lysine residues N-terminally to position -1 is obvious in many cases (BMP-1, Tolloid, Xolloid, BP-10, SpAN, HCH-1, HMP-1). The conservation of these residues over long periods of evolution suggests a common function, the significance of which is still not known. On the other hand, in several cases, because of the lack of basic residues at position -1, tryptic cleavage is not possible. This is true for nephrosin, PMP-1 and HMP-1, and also for astacin and AEA. For astacin, a basic residue next to the activation site is at position -3, but in AEA there is no basic side chain present at all in the whole propeptide. In HMP-1, the experimentally established N-terminus of the core enzyme is a lysine residue, which also cannot be due to tryptic cleavage (Fig. 4a). These different observations suggest that tryptic activation may play a major role in the processing of the astacins. For instance, it has been shown that human pro-meprin can be activated by the action of trypsin [22]. However, in several other cases in which a basic residue is missing at the activation site, other enzymes must also be involved. Such differences are not surprising given the wide range of occurrence, functional divergence, and tissue specificity of the astacins.

In the digestive tract of the crayfish, two endopeptidases have been described that could be responsible for the activation of pro-astacin: crayfish trypsin and astacin [18]. The different cleavage specificities of these two proteases must be decisive for correct processing of pro-astacin (Fig. 5).

**MS analysis**

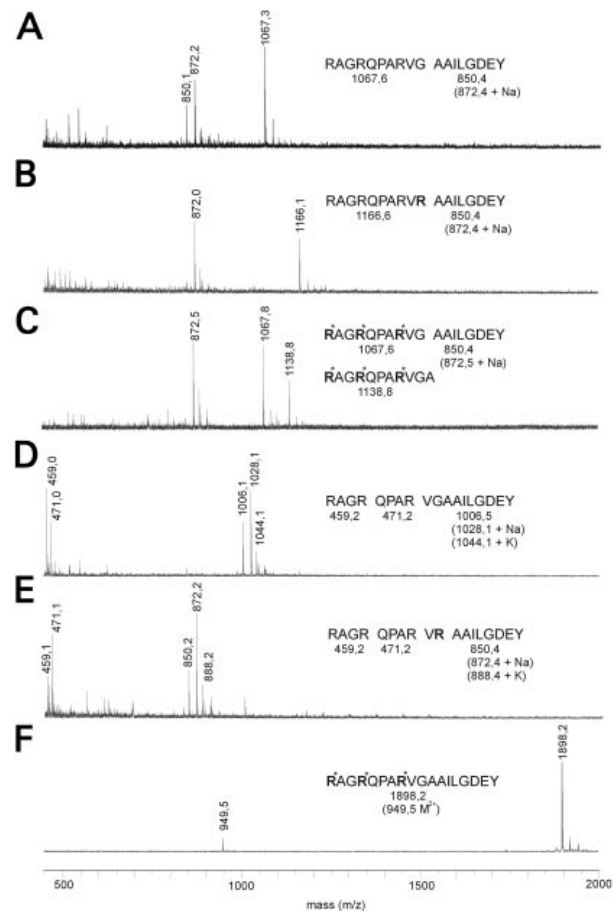
We investigated the effect of crayfish trypsin and astacin on synthetic peptides overlapping the activation site (Fig. 4b) by MS analysis. The amino-acid sequence of the propeptide of pro-astacin was deduced from the cDNA structure [11]. The propeptide extends from position -34 to -1 and is preceded by a presequence or signal sequence, and it thus comprises 34 amino-acid residues. The demarcation of the propeptide was accomplished N-terminally by prediction with the program SIGNALSEQ (German Cancer Research Centre, Heidelberg, Germany) and C-terminally by direct Edman amino-acid sequencing of active astacin [7] (Fig. 1).

Even with highly purified astacin or crayfish trypsin, minor traces of contaminating proteolytic activity were still

Astacin	<b>P<sup>4</sup></b>	<b>P<sup>3</sup></b>	<b>P<sup>2</sup></b>	<b>P<sup>1</sup></b>	<b>P<sup>1'</sup></b>	<b>P<sup>2'</sup></b>	<b>P<sup>3'</sup></b>	<b>P<sup>4'</sup></b>
	apolar		basic		A	small	apolar	
					T			
					S			
					G			
Trypsin	<b>P<sup>4</sup></b>	<b>P<sup>3</sup></b>	<b>P<sup>2</sup></b>	<b>P<sup>1</sup></b>	<b>P<sup>1'</sup></b>	<b>P<sup>2'</sup></b>	<b>P<sup>3'</sup></b>	<b>P<sup>4'</sup></b>
	X	X	X	R	X	X	X	X
								K

**Fig. 5. Preferential cleavage specificity of crayfish astacin and trypsin.** The substrate specificity of astacin was elucidated by extensive studies on the degradation of the  $\alpha$  and  $\beta$  chain of tubulin [12] and by its action on synthetic substrates [13,23], whereas that of crayfish trypsin was verified using the  $\beta$  chain of insulin and synthetic substrates [18]. Small aliphatic amino-acid side chains in the P1' position primarily determine the cleavage specificity of astacin, and basic residues in the P1 position that of trypsin.

detected by MS analysis, which could give rise to misleading interpretations. We therefore suppressed remaining traces of tryptic activity in astacin preparations by the addition of soybean trypsin inhibitor and/or 100 mM phenylmethanesulfonyl fluoride, and remaining traces of astacin activity in trypsin preparations by the addition of Pro-Leu-Gly-hydroxamate [19]. However, even in the presence of these inhibitors, a minor amount of tryptic activity was occasionally observed in astacin preparations, and we had to rule out the possibility that this was the result of intrinsic astacin activity. This problem was solved by shifting the specificity to the substrate itself, substituting L(-)-arginine for D(-)-arginine, which precluded any trypsin activity and allowed us to unambiguously distinguish between astacin and trypsin activity. There was no spontaneous hydrolysis of the peptide substrates under the incubation conditions used. The monoisotopic protonated masses for the intact peptides were 1899.0 Da for AstG and AstR\*, and 1998.1 Da for AstR. In the unmodified peptide AstG, astacin hydrolyzes only the bond G-1/A+1, which represents the natural activation site (Fig. 6a). Astacin also cleaves at the same position, if Gly-1 is substituted for an arginine residue



**Fig. 6. Identification of the peptide cleavage products by MALDI-TOF MS.** Astacin cleaves the synthetic peptides AstG (a) and AstR (b) exclusively between positions -1/+1. Crayfish trypsin cleaves the substrates AstG (d) and AstR (e) at all basic residues (i.e. R, arginine). After replacement of L-arginine by D-arginine (R\*) in AstR\*, astacin still cleaves between positions -1/+1 (c), but trypsin activity is completely prevented (f).

(Fig. 6b). Crayfish trypsin cleaves as expected at all available basic residues. Unmodified AstG is degraded into three peptides, but the naturally occurring N-terminus of active astacin is not produced in this way (Fig. 6d). Only when an arginine residue is introduced at position -1 (a modification that does not occur in pro-astacin) is this bond -1/+1 also hydrolysed by crayfish trypsin (Fig. 6e). With the modified peptide AstR\*, in which all arginine residues are replaced by the isostereomeric D(-)-arginine to preclude any tryptic activity, astacin still produces the correct N-terminus (Fig. 6c). At the same time, it can be demonstrated that this substrate is not altered at all by crayfish trypsin. (Fig. 6f). These results suggest that crayfish astacin is capable of catalysing its own activation. Any additional activation by trypsin would require the successive action of an aminopeptidase. We have shown that astacin is also capable of hydrolysing an Arg-Ala bond at the activation site. This suggests that for the majority of other astacin family members also, which have a basic amino-acid residue at position -1, this is not a hindrance to auto-catalytic activation.

From our studies we conclude that pro-astacin is auto-catalytically activated during its progress from the tubular lumen of the hepatopancreas to the cardiac stomach. Any additional contribution from crayfish trypsin would require the subsequent action of an aminopeptidase. Whether the small initial activation can be ascribed to an unknown membrane protease or intrinsic activity of pro-astacin remains to be established.

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