Purification and characterization of an α_2 -macroglobulin-like proteinase inhibitor from plasma of the crayfish *Pacifastacus leniusculus*

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An α_2 -macroglobulin (α_2 M)-like proteinase inhibitor from plasma of the crayfish *Pacifastacus leniusculus* was purified to apparent homogeneity by acid precipitation, hydrophobic interaction chromatography, affinity chromatography on concanavalin A-Sepharose and anion-exchange chromatography. The subunit M_r is about 190 000. Pore-size-limit electrophoresis proved the native protein to be a dimer. The purified protein resembled vertebrate α_2 Ms in that it protected trypsin from inhibition by soyabean trypsin inhibitor, and in its sensitivity to methylamine treatment. Methylamine also prevented the protein from being autolytically cleaved into M_r 60 000 and 140 000 fragments when subjected to heat treatment. The amino acid composition showed similarities with both human α_2 M and an α_2 M-like protein from the arthropod *Limulus polyphemus*. These data indicate that this *Pacifastacus* α_2 M-like protein (P α_2 M) may be a distantly related homologue of vertebrate α_2 Ms.

INTRODUCTION

α_2 -Macroglobulin (α_2 M), a high-molecular-mass proteinase inhibitor, has received considerable interest in recent years owing to its unique inhibitory properties (for reviews see Starkey & Barrett, 1977; Starkey, 1979; van Leuven, 1982). It was first detected in human blood (Jacobsson, 1953, 1955), but similar molecules were subsequently found in several mammals (Starkey & Barrett, 1977), as well as in other vertebrates (Starkey & Barrett, 1982). The 'trap hypothesis' for the mechanism by which α_2 M inhibits a proteinase (Barrett & Starkey, 1973) does not involve the inhibitor binding to or destroying the catalytic site of the proteinase. Instead, the proteinase is 'trapped' by the α_2 M after a conformational change induced in the proteolytically-cleaved inhibitor molecule. As a result of this 'trapping' only proteolysis of high-molecular-mass substrates is inhibited by the proteinase- α_2 M complex, whereas substrates with low molecular mass, e.g. synthetic peptides, still have access to the active site of the proteinase and are therefore proteolytically cleaved.

α_2 M contains an internal thiolester between the side-chains of a Cys and a Glx residue (Howard, 1981), which is vital for the inhibitor activity. When subjected to heat treatment, the subunit (180 kDa) is autolytically cleaved at this thiol ester site, which results in two heat fragments of 60 kDa and 120 kDa. Methylamine treatment, however, causes a loss of inhibitor activity and prevents this heat fragmentation (Eccleston & Howard, 1985; Harpel *et al.*, 1979).

In vertebrates α_2 M sequesters several proteinases and, owing to its broad inhibitory spectrum, it can regulate

several proteolytic processes in blood and also prevent harmful effects of proteinases from parasitic organisms. Recently, an α_2 M-like inhibitor has been reported to occur in the blood of some arthropods, namely *Limulus polyphemus* (Quigley & Armstrong, 1983a,b), as well as in both marine (Armstrong *et al.*, 1985) and freshwater crustaceans (Hergenhahn & Söderhäll, 1985; Stöcker & Zwilling, 1986). These observations are interesting because they indicate that at least some arthropods may be able to regulate and reduce the activity of endogenous as well as foreign proteinases in their haemolymph. However, until now only one α_2 M-like protein from an invertebrate (*L. polyphemus*; Quigley & Armstrong, 1985) has been purified. In this paper we describe the purification and some properties of an α_2 M-like inhibitor from a crustacean species, the crayfish *Pacifastacus leniusculus*, and point out some of the similarities and differences it has with the vertebrate α_2 Ms and with the *Limulus* α_2 M-like protein.

MATERIALS AND METHODS

Animals

Crayfish, *Pacifastacus leniusculus*, were maintained and housed in aquaria in aerated running tap water.

Materials

Concanavalin A-Sepharose 4B, phenyl-Sepharose CL-4B, Q-Sepharose Fast Flow and PD-10 columns with Sephadex G-25 medium were obtained from Pharmacia. Trypsin (bovine pancreas, type III S), soya-

Abbreviations used: α_2 M, α_2 -macroglobulin; P α_2 M, *Pacifastacus* α_2 M-like protein; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; proPO, pro-phenol oxidase.

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bean trypsin inhibitor and methyl α -D-mannopyranoside were from Sigma. The chemicals for electrophoresis and for calibration were obtained from Serva or Pharmacia. The chromogenic substrate S-2222 (benzoyl-Ile-Glu-(OR)-Gly-Arg-4-nitroanilide, R is 50% -H and 50% -CH₃) was from Kabi Vitrum AB. Amino acid standards and HCl for amino acid analysis were from Pierce. Human α_2 -macroglobulin was prepared according to Swenson & Howard (1979). All other chemicals were of analytical grade from Merck.

Preparation of plasma

Plasma was prepared as previously described (Hergenhahn & Söderhäll, 1985).

Purification of *Pacifastacus* α_2 -macroglobulin-like protein ($P\alpha_2M$)

The lyophilized powder of 500 ml of plasma was suspended in 500 ml of 0.1 M-Tris buffer containing 3 M-NaCl, pH 8.0, and stored overnight and then centrifuged at 20000 *g* for 30 min at 2 °C. The supernatant was adjusted to pH 5.5 and centrifuged as above. The resulting supernatant was adjusted to pH 8.0, centrifuged again and applied to a column (2.2 cm $\times$ 12 cm) with about 45 ml of phenyl-Sepharose CL-4B, equilibrated with 0.1 M-Tris buffer, pH 8.0, containing 3 M-NaCl. The column was washed with about 500 ml of this buffer and the $P\alpha_2M$ was eluted with 0.1 M-Tris/1 M-NaCl, pH 8.0. The flow rate was 36 ml/h, and 6 ml fractions were collected. The fractions containing the $P\alpha_2M$ were combined and applied to a 0.9 cm $\times$ 8 cm column with about 5 ml of concanavalin A-Sepharose 4B, previously equilibrated with 0.1 M-Tris buffer, pH 8.0. The column was washed with 100 ml of the same buffer and then 5 ml of the elution buffer (0.1 M-Tris/2 M-NaCl/0.5 M-methyl α -D-mannopyranoside, pH 8.0) were applied and the column was incubated overnight. The $P\alpha_2M$ was eluted with 40 ml of the elution buffer. This procedure was repeated twice. The flow rate was 24 ml/h, and 4 ml fractions were collected. The fractions containing the $P\alpha_2M$ were combined, concentrated on an Amicon PM10 membrane and transferred to PD-10 columns, equilibrated with 50 mM-piperazine buffer, pH 6.0. The desalted material was then applied on a 2 cm $\times$ 8 cm column with about 25 ml of Q-Sepharose Fast Flow, previously equilibrated with the piperazine buffer. The column was washed with 150 ml of the buffer and eluted with a 0–400 mM-NaCl gradient dissolved in the same buffer. The flow rate was 30 ml/h and 5 ml fractions were collected. The fractions containing the $P\alpha_2M$ were combined, concentrated on an Amicon PM 10 membrane and transferred to a PD-10 column equilibrated with 50 mM-sodium phosphate buffer, pH 7.0.

Determination of α_2 -macroglobulin-like activity

The samples (100 μ l) were incubated with 500 μ l of a 0.1 M-Tris buffer, pH 8.0, containing 10 μ g of trypsin, at 30 °C. After 10 min, 100 μ l of a 0.1 M-Tris buffer, pH 8.0, containing 100 μ g of soyabean trypsin inhibitor, was added. The protease assay was initiated by addition of 100 μ l of 1 mM-S-2222 and the reaction was terminated after 30 min with 200 μ l of 50% (v/v) acetic acid. Absorbance was read at 405 nm. One unit of α_2 -macroglobulin-like activity was defined as that amount which inhibits 1 unit of trypsin, defined as the release of 1 μ mol of 4-nitroaniline/min.

Methylamine-treatment experiments

The pH of the sample containing the $P\alpha_2M$ was adjusted to pH 8.3 using 1 M-Tris base. Methylamine (2 M in 0.05 M-Tris buffer, pH 8.0), was added to a final concentration of 0.2 M-methylamine. The controls received only 0.05 M-Tris buffer, pH 8.0. The mixtures were incubated for 9–24 h at room temperature, and then assayed for α_2 -macroglobulin-like activity as described above, or boiled in sample buffer for 15 min, then reduced by adding dithiothreitol, and boiled again for 5 min, and analysed by SDS/polyacrylamide-gel electrophoresis (PAGE).

Electrophoretic techniques

SDS/PAGE was performed according to Laemmli (1970). Polyacrylamide gels (7.5%) were used. About 5 μ g of each of the purified $P\alpha_2M$ and of the standards (human α_2M , 180000; phosphorylase *b*, 94000; albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; trypsin inhibitor, 20100; and α -lactalbumin, 14400) were used for electrophoresis after reduction in a solution containing dithiothreitol for 5 min at 100 °C. The gels were stained with Serva Blue G-250.

Gradient electrophoresis

The purified $P\alpha_2M$ was run on a native, non-denaturing 3–15% polyacrylamide-gradient gel, in the presence of 0.1% Triton at pH 8.0, essentially as described in Kuonen *et al.* (1986). The standard proteins were thyroglobulin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin and were from Pharmacia.

Amino acid analysis

The amino acid composition was determined as described previously (Hergenhahn *et al.*, 1984). For each analysis 4 μ g was employed. Cysteine was determined after oxidation with performic acid.

Protein determination

Protein was determined according to Lowry *et al.* (1951) and bovine serum albumin was used as a standard.

RESULTS

Purification

As summarized in Table 1, an α_2 -macroglobulin-like protein was purified from plasma of the crayfish *Pacifastacus leniusculus* by means of acid precipitation, hydrophobic interaction chromatography (Fig. 1), affinity chromatography on concanavalin A-Sepharose (Fig. 2) and ion-exchange chromatography (Fig. 3). The binding of the $P\alpha_2M$ to concanavalin A-Sepharose was strong and, therefore, a higher yield was obtained if the column was incubated overnight with the elution buffer containing α -D-mannopyranoside before the elution of the $P\alpha_2M$. Although the protein was homogeneous as judged by SDS/PAGE (Fig. 4), the apparent purification factor was only 12.7 and the recovery of the inhibitor was rather low (4.6%). The final yield was 25.2 mg from 500 ml of the plasma preparation, corresponding to about 250 ml of crayfish haemolymph.

The purified protein had an α_2 -macroglobulin-like activity since it protected trypsin from being inhibited by soya-bean trypsin inhibitor and methylamine treatment reduced this inhibitory activity to about 20% compared

Table 1. Purification of α_2 -macroglobulin-like protein from plasma of the crayfish *Pacifastacus leniusculus*

Step	Total α_2 -M activity (units)	Total protein (mg)	Spec. act. (units/mg)	Purification (-fold)	Recovery (%)
Crude plasma	75.29	7011	0.0107	1	100
Acid precipitation	69.96	3276	0.0214	2	92.9
Phenyl-Sepharose	15.18	441	0.0344	3.21	20.2
Concanavalin A-Sepharose	7.92	70.9	0.117	10.43	10.5
Q-Sepharose Fast Flow	3.43	25.2	0.1361	12.7	4.6

Fig. 1. Elution profile of $P\alpha_2M$ from a phenyl-Sepharose column

The column (2.2 cm $\times$ 12 cm) was equilibrated with 0.1 M-Tris buffer, pH 8.0, containing 3 M-NaCl. After application of the plasma (fractions 1–93) and washing the column with the equilibrating buffer (fractions 93–173), the $P\alpha_2M$ was eluted with a 0.1 M-Tris buffer, pH 8.0, containing 1 M-NaCl (fractions 173–197). $\circ$, A_{280} ; $\bullet$, α_2M activity (munits/ml).

Fig. 2. Elution profile of $P\alpha_2M$ from a concanavalin A-Sepharose column

The column (0.9 cm $\times$ 8 cm) was equilibrated with 0.1 M-Tris buffer, pH 8.0. After application of the fractions containing α_2M activity from the phenyl-Sepharose run (fractions 1–9), the column was washed with the equilibrating buffer (fractions 10–35). After incubation overnight with the elution buffer (0.1 M-Tris/2 M-NaCl/0.5 M-methyl α -D-mannopyranoside, pH 8.0, fractions 36–37), the $P\alpha_2M$ was eluted on 3 consecutive days (fractions 38–47, 48–57 and 58–61). $\circ$, A_{280} ; $\bullet$, α_2M activity (munits/ml).

Fig. 3. Elution profile of $P\alpha_2M$ from a Q-Sepharose Fast Flow column

The column (2 cm $\times$ 8 cm) was equilibrated with 50 mM-piperazine buffer, pH 6.0. After application of fractions containing α_2M activity from the concanavalin A-Sepharose run which had been transferred to the piperazine buffer (fractions 1–4), the column was washed with this buffer (fractions 5–24) and the $P\alpha_2M$ was eluted with a 0–400 mM-NaCl gradient in the same buffer (fractions 25–123). $\circ$, A_{280} ; $\bullet$, α_2M activity (munits/ml).

with the untreated $P\alpha_2M$. The protein was also autolytically cleaved into two fragments of M_r about 60000 and 140000, when subjected to heat treatment, and this cleavage was prevented by prior treatment of the protein with methylamine (Fig. 5). However, the preparation of $P\alpha_2M$ was probably partly inactivated, since a relatively small portion of the protein was heat fragmented.

Molecular mass determination

The M_r of the subunits as revealed by SDS/PAGE after reduction was about 190000 (Fig. 4). Pore-limit electrophoresis gave an M_r of approximately 340000 for the native molecule (Fig. 6).

Amino acid composition

The amino acid composition of $P\alpha_2M$ is shown in Table 2 and compared with the compositions of human α_2 -macroglobulin as obtained from sequencing data (Sottrup-Jensen *et al.*, 1984) and that of *Limulus* α_2 -macroglobulin (Quigley & Armstrong, 1985). The α_2M -like protein from *Pacifastacus* has an amino acid composition nearly identical to that of *Limulus* and to a lesser extent to that of human α_2M . The content of valine

Fig. 4. Proof of electrophoretic purity of the purified $P\alpha_2M$ and molecular-mass estimation of the subunit on SDS/PAGE under reduced conditions

$P\alpha_2M$ (5 μ g; lane 1) as well as 5 μ g of the standard proteins (lane 2, see the Materials and methods section) was applied on a 7.5% polyacrylamide gel. The gel was stained with Serva Blue G-250.

Fig. 5. SDS/PAGE showing autolytic cleavage of $P\alpha_2M$, prevented by methylamine treatment

The samples (15 μ g of $P\alpha_2M$) were run on a 7.5% SDS/polyacrylamide gel under reducing conditions. Lane 1, heat-treated methylamine- $P\alpha_2M$; lane 2, heat-treated $P\alpha_2M$; lane 3, protein standards; lane 4, human α_2M .

and isoleucine in crayfish and *Limulus* is different from that of human α_2M .

DISCUSSION

In this paper we describe a purification procedure for an α_2 -macroglobulin-like proteinase inhibitor from plasma of the crayfish *Pacifastacus leniusculus*, as well as some of its properties. The purification procedure includes hydrophobic-interaction chromatography, affinity chromatography on concanavalin A-Sepharose and anion-exchange chromatography. The first step was efficient in removing another high- M_r proteinase inhibitor present in the crayfish plasma (Hergenhahn *et al.*, 1987). The second step was chosen since vertebrate α_2M is a glycoprotein and glycoproteins bind to concanavalin A-Sepharose. The $P\alpha_2M$ was also bound to this matrix and could be eluted with mannose at high ionic strength.

The physical and chemical properties of the molecule suggest homology with α_2M from vertebrates. The M_r of the subunits, 190 000, is close to vertebrate α_2M (M_r ,

180 000), and the M_r of the native inhibitor, as revealed by pore-size electrophoresis, was about 340 000. This suggests that the active molecule is a dimer. Starkey & Barrett (1982) have observed that α_2M -like proteins from all mammals, birds, reptiles and amphibians investigated so far have an M_r of about 725 000, comprising four subunits, whereas in fish, including cyclostomes, α_2M has only half the M_r , about 360 000. In a close relative of *P. leniusculus*, namely the crayfish *Astacus astacus*, Stöcker & Zwilling (1986) reported the M_r of an α_2M -like protein to be about 360 000, and therefore, the molecule also appears to be a dimer in this crayfish. However, the α_2M -like protein from *Limulus polyphemus* had an apparent M_r of 500 000–550 000 and the authors proposed that the protein is a homotrimer (Quigley & Armstrong, 1985). Furthermore, the activity of the crude α_2M -like protein (Hergenhahn & Söderhäll, 1985), as well as of the purified protein from crayfish plasma, was destroyed by treatment with methylamine. This inactivation of the $P\alpha_2M$ by methylamine may indicate that an internal thiol ester bond is required for the α_2M -like activity of this protein,

Fig. 6. Native gradient/PAGE of $P\alpha_2M$

Lanes 2 and 3 were loaded with purified $P\alpha_2M$, 10 μ g and 5 μ g, respectively. Standard proteins (see the Materials and methods section) were run on lane 1. The gel was stained with Serva Blue G-250.

as is the case for mammalian α_2M (Starkey & Barrett, 1977; Starkey, 1979). The fact that $P\alpha_2M$ is autolytically cleaved into fragments of 60000 and 140000 when subjected to heat treatment, and that this cleavage is prevented by prior treatment with methylamine, is also characteristic of α_2M molecules containing a thiol ester bond.

The data for the amino acid composition (Table 2) of human α_2M (Sottrup-Jensen *et al.*, 1984), α_2M -like protein from *Limulus* (Quigley & Armstrong, 1985) and that from *Pacifastacus* show obvious similarities, suggesting a possible relationship. The most striking difference between human α_2M and *Pacifastacus* α_2M -like protein on the one hand, and *Limulus* α_2M -like protein on the other hand, is the much higher glycine content of the latter, which may have resulted from an analytical error. To show further the apparent homology between $P\alpha_2M$ and human α_2M , we have calculated the SDQ values according to the method of Cornish-Bowden (1983) for the amino acid composition of *Limulus*, crayfish and human α_2M , as well as by the method of Marchalonis & Weltman (1971). With both methods a strong indication of homology between human α_2M and crayfish $P\alpha_2M$ was evident. However, to obtain more conclusive proof of homology, it will be necessary to sequence the protein and compare these sequences with that of human α_2M .

The function of this protein in crayfish remains to be

Table 2. Amino acid composition of the purified *Pacifastacus leniusculus* α_2 -macroglobulin-like protein is compared with those of human and *Limulus* α_2 -macroglobulin (Sottrup-Jensen *et al.*, 1984; Quigley & Armstrong, 1985)

Amino acid	Composition (mol %)		
	<i>Pacifastacus</i>	<i>Limulus</i>	Human
Asx	8.4	9.12	7.86
Thr†	8.22	7.48	6.8
Ser†	6.95	6.84	8.3
Glx	12.22	9.59	12.09
Pro	6.0	7.05	5.19
Gly	5.15	12.06	6.43
Ala	5.53	7.61	6.29
Cys*	2.84		1.64
Val§	5.95	6.73	9.36
Met‡	1.57	1.23	1.71
Ile§	6.23	5.84	3.9
Leu§	9.28	9.27	9.16
Tyr†	3.06	3.27	3.83
Phe	4.9	4.04	4.23
Trp†	1.35		0.75
His	3.25	1.24	2.67
Lys	5.63	5.73	5.95
Arg	3.6	2.95	3.14

* Determined after oxidation with performic acid and 24 h hydrolysis.

† Determined after 24 h hydrolysis with methanesulphonic acid.

‡ Extrapolated to zero time of hydrolysis.

§ Values of 72 h hydrolysis.

|| Average values of 24, 48 and 72 h hydrolysis.

shown, but it is possible that it serves as an important factor in regulating the pro-phenol oxidase (proPO)-activating system (Söderhäll & Smith, 1986a,b; Ratcliffe *et al.*, 1985) and/or the clotting of the haemolymph. The crayfish proPO and the proPO-activating enzyme, a serine proteinase, are enclosed in vesicles in the blood cells. Upon release of the proPO system from the blood cells and subsequent specific activation by microbial polysaccharides, factors are produced which participate in the internal defence that these animals mount towards parasites (Söderhäll & Smith, 1986a,b). It is therefore plausible that the crayfish α_2 -macroglobulin-like protein may serve to restrict activation of this proPO system where it is inappropriate or detrimental to the crayfish. The proPO-activating system has been likened to the alternative pathway of complement activation (Söderhäll, 1982), and since in mammalian systems, the complement proteins C3, C4 and α_2M have common evolutionary origin (Sottrup-Jensen *et al.*, 1985), an obvious relationship between $P\alpha_2M$ and human α_2M would be an indirect indication that crayfish might possess a distantly related, complement-like system.

We are very grateful to Dr. G. Kegel for performing the amino acid analysis and Mrs. M. Langhammer and Mr. R. Ajaxon for skilful technical assistance. This work was supported by grants from the Swedish Natural Science Research Council to K.S.

Note added in proof (received 8 August 1988)

After submission of this paper the purification and characterization of an α_2 M-like proteinase inhibitor from lobster was reported (Spycher *et al.*, 1987)

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Received 16 September 1987/14 March 1988; accepted 23 June 1988