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Amino acid sequence around the thiolester of α_2-macroglobulin from plasma of the crayfish, *Pacifastacus leniusculus*†

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α_2-Macroglobulin (α_2M) was isolated from plasma of the freshwater crayfish, *Pacifastacus leniusculus*, using ultracentrifugation, ion-exchange chromatography and gel filtration techniques. The *Pacifastacus* α_2M molecule (Pα_2M) was radioactively labeled in the thiol ester structure with iodo [^{14}C]acetic acid in the presence of methylamine. After reduction and carboxymethylation of the protein, it was digested with trypsin. A ^{14}C-labeled tryptic peptide was sequenced and contained an amino acid sequence very similar to other known thiol ester sequences from human α_2M and related proteins. The N-terminal sequence of Pα_2M was related to that recently determined for lobster α_2M [(1987) J. Biol. Chem. 262, 14606–14611].

Macroglobulin, α_2-; Protease inhibitor; Plasma protein; (Crustacea)

1. INTRODUCTION

α_2-Macroglobulin (α_2M) is a high-M_r proteinase inhibitor, a tetramer with identical subunits of M_r 180 000, which is present in human blood; similar molecules have been detected in all major vertebrate [1] and some invertebrate [1–6] taxa (for review on α_2M and related proteins, see [7]). α_2M-like inhibitors bind proteinases by a so-called 'trapping' mechanism [8]. The inhibitor has a stretch in the polypeptide chain termed the 'bait region' [8], which contains specific cleavage sites for different proteinases [9]. If a proteinase cleaves the bait region, a conformational change is induced in the α_2M molecule which traps the proteinase [10]. The trapped proteinase, retains its proteolytic activity towards small substrates, while the activity towards larger substrates is greatly reduced due to steric hindrance [8].

Another characteristic structure in α_2M is an internal β-cysteinyl-γ-glutamyl thiol ester, formed from the side chains of a Cys and a Glx residue [11,12]. Following bait region cleavage, the thiol ester is cleaved and mediates covalent binding of the proteinase to α_2M. Methylamine cleaves the thiolester, forming a residue of γ-glutamyl methylamide [11,12]. As a result of this, the SH-group of the Cys residue becomes available for reaction with alkylating agents such as iodoacetic acid. If the thiolester of α_2M is cleaved by treatment with methylamine, the inhibitory activity of the molecule is greatly reduced [13]. Also, methylamine treatment prevents the molecule from being autolytically cleaved in the thiol ester region by heat-treatment into fragments of M_r 60 000 and 120 000 [14]. Earlier studies have shown that

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Abbreviations: α_2M, α_2-macroglobulin; DFP, diisopropyl fluorophosphate; DTE, dithioerythreitol; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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crayfish plasma contains an α_2M-like molecule (Pα_2M), which is a dimer with subunits of M_r 190 000 [15]. This Pα_2M-molecule possesses characteristics typical for α_2M-like proteinase inhibitors since it can trap proteinases and is methylamine sensitive. In this study we have determined the sequence of the first 11 N-terminal residues of intact Pα_2M, and furthermore we report the sequence of a 21-residue stretch containing its internal thiolester.

2. MATERIALS AND METHODS

2.1 *Animals*

Freshwater crayfish, *Pacifastacus leniusculus*, were maintained and housed in aquaria with aerated running tap water.

2.2. *Assay for α_2M-like activity*

25 μl of the sample to be tested were incubated with 525 μl of 0.1 M Tris-HCl, pH 8.0, containing 0.25 μg of trypsin (bovine pancreatic, type III S, Sigma) for 10 min at 30°C. Then 25 μl of 0.1 M Tris-HCl, pH 8.0, containing 2.5 μg of soya bean trypsin inhibitor (Sigma) were added. After 5 min at 30°C, the proteinase activity assay was started by the addition of 50 μl of 2 mM S-2222 (chromogenic peptide, Bz-Ile-Glu-(OR)-Gly-Arg-pNa, R is 50% -H and 50% -CH_3, Kabi Vitrum) dissolved in distilled H_2O. The mixture was incubated for 5 min at 30°C and the reaction stopped by addition of 200 μl of 50% acetic acid. The absorbance at 405 nm was measured. 1 unit of Pα_2M is defined as the amount that in this assay gives an absorbance of 0.1 at 405 nm.

2.3. *Preparation of crayfish plasma*

Haemolymph was bled from the abdominal haemocoel of the crayfish by penetrating the cuticle with a sterile injection needle. The haemolymph was collected in an equal volume of 0.01 M sodium cacodylate, 0.1 M $CaCl_2$, 0.25 M sucrose, pH 7.0, on ice. The haemocytes were pelleted by centrifugation at 800 × *g*, 10 min, 4°C, and the supernatant, termed crayfish plasma, was used in the experiments.

Table 1

Purification table for Pα_2M from crayfish plasma

Purification step	Protein (mg)	Total act. (U)	Spec. act. (U/mg)	Purification	Yield (%)
Plasma	3 800	36 000	9.5	1	100
Ultracentr. supernatant	160	16 000	100	10.5	44
Q Sepharose Fast Flow	8	7 800	975	102.6	22
Superose 6	2	3 400	1 700	178.9	9

2.4. *Purification of Pα_2M*

200 ml of fresh plasma was dialyzed extensively against distilled water at 4°C, and then ultracentrifuged at 200 000 × *g*, 2.5 h, 4°C, to remove the majority of the hemocyanin. The supernatant was then applied to a Q Sepharose Fast Flow (Pharmacia) column (16 × 170 mm), and the column was washed with 50 mM piperazine buffer, pH 6.0. The column was eluted with a gradient from 0 to 450 mM NaCl in the piperazine buffer (flow rate: 12 ml/h; fraction size: 2.4 ml). The Pα_2M-containing fractions were pooled and concentrated on an Amicon Cell using a PM 10 membrane. Aliquots (200 μl) of the concentrated pool were separated by FPLC on a Superose 6 gel filtration column (10 × 300 mm, Pharmacia, flow rate: 0.3 ml/min; fraction size: 0.5 ml) in 0.1 M Na-phosphate buffer, pH 8.3, containing 0.15 M NaCl. Fractions from the different FPLC runs containing pure Pα_2M, as judged by SDS—PAGE, were pooled and used in the experiments.

2.5. *Isolation of ^{14}C-labeled, thiolester-containing peptide*

10 mg of Pα_2M in 5 ml of 0.1 M Na-phosphate, 0.15 M NaCl, pH 8.3, were incubated with 200 μCi of ^{14}C-labeled iodoacetic acid (Amersham, 56 Ci/mol) and 570 μl of 2 M methylamine in 0.1 M Na-phosphate, 0.15 M NaCl, pH 8.3, at room temperature, 5 h. Then 0.1 M iodoacetic acid was added to a

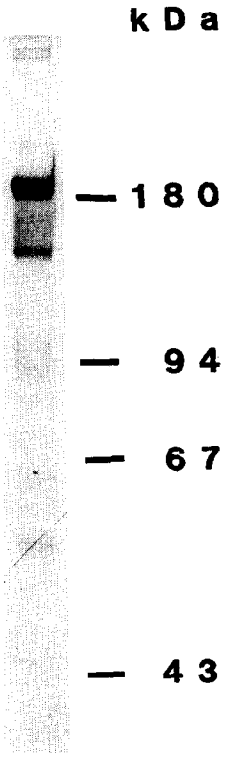

Fig.1. SDS-PAGE. Pure Pα_2M (6 μg) reduced with DTE and applied to a 7.5% gel. The gel was stained with Serva blue G-250. Besides the Pα_2M subunit (M_r 190 000) heat cleavage fragments (M_r 140 000 and 60 000) are also seen.

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                                  1 . . . 5 . . . . 10 .

Crayfish Pα2M                     Ser-Tyr-Val-Ile-Thr-Thr-Pro-Lys-Met-Trp-Val-...
Lobster α2M (6)                   Ser-Tyr-Ile-Ile-Thr-Thr-Pro-Arg-Met-Trp-Val-...
Human α2M (16)  Ser-Val-Ser-Lys-Pro-Gln-Tyr-Met-Val-Leu-Val-Pro-Ser-Leu-Leu-His-...
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Fig.2. N-terminal amino acid sequence of crayfish Pα_2M-like molecule. Comparison with human and lobster α_2M.

final concentration of 10 mM. The mixture was then dialyzed extensively against 0.1 M Tris-HCl, pH 8.5. The sample was concentrated on an Amicon Cell, PM 10 membrane, and to the solution (2.7 ml) was added 1.75 g guanidinium hydrochloride and 35 μl of 0.1 M DTE. After 40 min incubation at room temperature, 235 μl of 0.1 M iodoacetic acid was added to block all free SH-groups. The sample was then desalted and transferred to 100 mM NH_4HCO_3, pH 8.5, by gel chromatography on a G25 SF column. 8.4 mg of protein in a volume of 9.5 ml was recovered. 84 μg of TPCK-treated trypsin was added, and after 2 h at 37°C, tryptic digestion was stopped by the addition of 20 μl of 1 M DFP dissolved in propanol. The material was lyophilized, redissolved in 3 ml of 10 mM NH_4HCO_3, pH 8.5, and separated, first on a Mono Q anion-exchange column (5 × 50 mm, Pharmacia), and then further by reverse-phase HPLC on a Nucleosil C18 column (8 × 250 mm) as described by Sottrup-Jensen et al. [16]. Selected fractions from the HPLC separation containing radioactivity were vacuum dried. After dissolving in 0.1% trifluoroacetic acid, samples were taken for amino acid analysis (Hewlett-Packard AminoQuant), and the sequence of the major radioactive pools were determined using an Applied Biosystem 470 protein sequencer. PTH-residues were analyzed by HPLC on a Hewlett-Packard 1084B liquid chromatograph, using a Spherosorb ODS 2, 3 μm column, with isocratic elution in 33% CH_3CN, 67% of 15 mM Na-acetate, pH 5.3. Intact Pα_2M was desalted into 50% formic acid by Superose 6 gel chromatography and 50–100 pmol analyzed on an Applied Biosystem model 477A sequencer using 'on-line' determination of PTH-residues in a model 120A chromatograph. SDS-PAGE was performed according to the method of Laemmli [17].

3. RESULTS AND DISCUSSION

Since Pα_2M prepared by the method of Hergenhahn et al. [15] was partially inactive, a new purification procedure was developed using fresh crayfish plasma instead of lyophilized. About 2 mg of crayfish Pα_2M (a yield of approximately 9%), was obtained from 200 ml of crayfish plasma (table 1). Fig. 1 shows the result of SDS-PAGE of the purified protein.

While the N-terminal sequence of Pα_2M (11 residues determined) is strongly related to that of lobster α_2M [6] the similarity of both these sequences with human α_2M [16] is modest (fig.2). The isolated ^{14}C-labeled peptide (21 residues determined) revealed a striking sequence similarity with corresponding sequences from other α_2M-related proteins (fig. 3).

The common characteristics of the different α_2M-like molecules from distantly related species, having a highly conserved amino acid sequence around the thiolester, suggests that the molecule appeared early in evolution, and that its potential for formation of covalent complexes with proteinases is of physiological significance. Notably,

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                          1 . . . 5 . . . . 10 . . . . 15 . . . . 20 .
                                    *       #
Crayfish Pα2M     (Lys/Arg-)Met-Pro-Tyr-Gly-Cys-Gly-Glu-Glx-Asn-Met-Val-Asn-Phe-Ala-Pro-Asn-Ile-Phe-Ile-Met-Glu-...
Lobster α2M (6)   (Lys/Arg-)Met-Pro-Tyr-Gly-Cys-Gly-Glu-Glx-Asn-Met-Val-Asn-Phe-Ala-Pro-Asn-...
Human α2M (16)      (Gln-)Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Val-Leu-Phe-Ala-Pro-Asn-Ile-Tyr-Val-Leu-Asp-...
Human PZP (19)            Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-...
Bovine α2M (20)     (Gln-)Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Ala-Arg-...
Rat α2M (21)        (Lys-)Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Val-Leu-Phe-Ala-Pro-Asn-Ile-Tyr-Val-Leu-Asp-...
Rat α1I3 (22)       (Gln-)Met-Pro-Tyr-Gly-Cys-Gly-Glu-Gln-Asn-Met-Val-Leu-Phe-Ala-Pro-Asn-Ile-Tyr-Val-Leu-Lys-...
Human C3 (23)       (Val-)Thr-Pro-Ser-Gly-Cys-Gly-Glu-Gln-Asn-Met-Ile-Gly-Met-Thr-Pro-Thr-Val-Ile-Ala-Val-His-...
Human C4 (24)       (Arg-)Leu-Pro-Arg-Gly-Cys-Gly-Glu-Gln-Thr-Met-Ile-Tyr-Leu-Ala-Pro-Thr-Leu-Ala-Ala-Ser-Arg-...
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Fig.3. Amino acid sequence of the isolated, ^{14}C-labeled peptide from crayfish Pα_2M. Comparison with thiolester sequences from other α_2M-related proteins. (*) ^{14}C-labeled residue, determined as PTH-carboxymethylated-Cys; (#) determined as PTH-γ-glutamylmethylamide [18].

the group of proteins related to α_2M includes not only proteinase inhibitors, but also the thiolester-containing complement proteins C3 and C4 [25]. In blood, α_2M plays a major role in the clearance of proteinases from the circulation [26]. In crayfish, the physiological role of this molecule is not known, but perhaps it participates in the regulation of clotting reactions in the hemolymph and/or in the regulation of the activation of the internal defense system in crayfish, the so-called prophenol oxidase (proPO) activating system (for a review on the proPO activating system, see [27]). Another high-M_r (155 000) proteinase inhibitor from crayfish plasma has already been found to inhibit the proteinase activity that is associated with the activation of the proPO system [28]. The crayfish Pα_2M may also serve as an inhibitor of proteinases released from invading pathogens and other microorganisms.

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