Purification and characterization of a high- M_r proteinase inhibitor of pro-phenol oxidase activation from crayfish plasma

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Crayfish plasma was found to contain a proteinase inhibitor, which was purified to apparent homogeneity by acid precipitation, affinity chromatography on concanavalin A–Sepharose and hydrophobic-interaction chromatography. The inhibitor is a monomeric protein with an M_r of about 155000 and a pI in the range 4.6–4.8. It is heat-stable and tolerant to low pH. It inhibits the serine proteinases trypsin and chymotrypsin, but not thrombin or subtilisin. Furthermore, it is efficient in decreasing the activity of a proteinase from crayfish haemolymph that is involved in the activation cascade of pro-phenol oxidase and can also block pro-phenol oxidase activation by this serine proteinase. This cascade is believed to play a central role in the recognition mechanism of non-self material in crustaceans and insects. The data presented give some evidence that the new proteinase inhibitor is involved in the regulation of this system.

INTRODUCTION

Although arthropods lack immunoglobulins they are able to protect themselves against parasites and to remove non-self particles from the circulation. This is achieved by the activities of the blood cells that can phagocytose or encapsulate foreign material. The mechanism by which an arthropod can discriminate self from non-self is far from clear, but a likely candidate is the so-called ‘pro-phenol oxidase-activating system’ (Durlat, 1985; Ratcliffe, 1985; Ratcliffe *et al.*, 1985; Söderhäll, 1982; Söderhäll & Smith, 1986a,b). This system is specifically activated by lipopolysaccharides or β -1,3-glucans. It consists of at least two serine proteinases, whose activation leads to the formation of the active phenol oxidase from the zymogen proPO.

The components of the proPO-activating system are sequestered in an inactive form in vesicles in the granular and semigranular cells, and are released from these cells by exocytosis (Johansson & Söderhäll, 1985). This process can be induced by lipopolysaccharides, β -1,3-glucans or a degranulation factor (Johansson & Söderhäll, 1985; Söderhäll *et al.*, 1986). The degranulation factor appears to be produced upon activation of the proPO-activating system, as well as a cell-adhesive protein (M. W. Johansson & K. Söderhäll, unpublished work). This means that activation of the proPO-activating system and subsequent production of the degranulation factor amplifies further release and activation of this system. Hence it should be necessary to regulate activation of the proPO-activating system by inhibiting the proteinases of the system and limiting the enzymic reactions where they would be unnecessary or detrimental for the host. A first candidate for such a role was an α_2 -macroglobulin-like protein that has been detected in crayfish plasma (Hergenhahn & Söderhäll,

1985), as well as in other crustaceans (Armstrong *et al.*, 1985). However, we have not yet succeeded in demonstrating that this macromolecular inhibitor can affect activation of proPO, whereas a different high- M_r proteinase inhibitor is able to prevent activation of the proPO-activating system. In the present paper we describe the purification and some of the biochemical properties of this inhibitor.

MATERIALS AND METHODS

Animals

Crayfish (*Pacifastacus leniusculus*) were maintained and housed as previously described (Söderhäll & Häll, 1984).

Materials

Concanavalin A–Sepharose 4B, phenyl-Sepharose CL-4B, heparin–Sepharose CL-6B and PD-10 columns with Sephadex G-25 (medium grade) were obtained from Pharmacia. Trypsin (bovine pancreas, type III s), thrombin (bovine plasma, 250 NIH units/mg), plasmin (pig blood), elastase (pig pancreas, type IV) and subtilisin (type VIII) as well as methyl α -D-mannopyranoside were from Sigma Chemical Co. Suc-Ala-Ala-Ala-NH-Np, the chemicals for electrophoresis and electrofocusing and the proteins for calibration were obtained from Serva and Pharmacia. The chromogenic substrates S-2222 [1:1 mixture of Bz-Ile-Glu-Gly-Arg-NH-Np and Bz-Ile-Glu(Me)-Gly-Arg-NH-Np] and S-2586 (MeO-Suc-Arg-Pro-Tyr-NH-Np) were from Kabi AB. Amino acid standards and HCl for amino acid analysis were from Pierce Chemical Co. Human α_2 -macroglobulin was prepared in accordance with Swenson & Howard (1979). All the other chemicals were obtained from commercial sources.

Abbreviations used: proPO, pro-phenol oxidase; Bz, benzoyl; NH-Np, 4-nitroanilide; Suc, 3-carboxypropionyl; MeO-Suc, 3-methoxycarbonylpropionyl.

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Preparation of haemocyte lysate

Haemocyte lysate was prepared as described previously (Söderhäll, 1981).

Preparation of plasma

The 800 g supernatant obtained when preparing the haemocyte lysate was employed.

Purification of trypsin inhibitor

The freeze-dried powder of 500 ml of plasma was suspended in 500 ml of 0.1 M-Tris/HCl buffer, pH 8.0, the suspension was centrifuged at 20 000 g for 40 min and the supernatant was adjusted to pH 3.0 and centrifuged as above. The supernatant was re-adjusted to pH 8.0, centrifuged again and applied to a column (2.2 cm × 21 cm) containing about 80 ml of concanavalin A-Sepharose 4B equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0. The column was washed with 300 ml of this buffer. Then 70 ml of elution buffer (0.1 M-Tris/HCl buffer, pH 8.0, containing 2 M-NaCl and 0.5 M-methyl α -D-mannopyranoside) was applied and the column was incubated at room temperature overnight. The inhibitor was then eluted with 100 ml of the elution buffer. The flow rate was 36 ml/h, and 6 ml fractions were collected.

The fractions containing the inhibitor were combined and concentrated on an Amicon PM 10 membrane. This material was applied to a 0.9 cm × 14 cm column containing about 9 ml of phenyl-Sepharose CL-4B equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0, containing 2 M-NaCl. The column was washed with 50 ml of this buffer and subsequently with 100 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl. The inhibitor was eluted with 80 ml of 0.1 M-Tris/HCl buffer, pH 8.0, and the residual material with the same buffer but containing additionally 50% (v/v) ethylene glycol. The flow rate was 24 ml/h, and 4 ml fractions were collected. The fractions containing the inhibitor were concentrated, transferred to 20 mM-ammonium acetate buffer pH 7.0, and used directly for experiments or freeze-dried.

Purification of serine proteinases from haemocyte lysate

Haemocyte lysate was diluted 2:1 with 10 mM-sodium cacodylate buffer, pH 7.0, and applied to a heparin-Sepharose column (1.0 cm × 10 cm) equilibrated in 10 mM-sodium cacodylate buffer, at a flow rate of 7.5 ml/h (1 ml fractions). The column was eluted with a step gradient consisting of 50 ml each of 0.15 M-, 0.5 M- and 1.5 M-NaCl in the above buffer. The enzyme was eluted in a sharp peak after the protein peak at 0.5 M-NaCl. The enzyme-containing fractions were pooled and dialysed against 2 × 1 litre of 10 mM-sodium cacodylate buffer, pH 7.0. This proteinase preparation had an activity corresponding to an increase in A_{405} of 6/min per mg of protein. No proPO or phenol oxidase activity was detectable in this preparation.

Isolation of proPO free of activating enzyme

Haemocyte lysate was applied to a heparin-Sepharose column (1.0 cm × 10 cm) equilibrated in 10 mM-sodium cacodylate buffer, pH 7.0, containing 0.1 M-CaCl₂ and washed with the same buffer at a flow rate of 7.5 ml/h

(1 ml fractions). The protein-containing fractions were pooled, and used as proteinase-free proPO.

Assay of proteinase inhibitor

Routinely the ability of the inhibitor to inhibit trypsin was tested as follows. A 100 μ l portion of the sample was preincubated with 600 μ l of 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 μ g of trypsin at 30 °C for 3–5 min. The assay was started by addition of 100 μ l of 1 mM-S-2222 and terminated after 10 min of incubation with 200 μ l of 50% (v/v) acetic acid. The absorbance was read at 405 nm. The activities of thrombin and trypsin (substrate S-2222), subtilisin (substrate S-2586) and elastase (substrate Suc-Ala-Ala-Ala-NH-Np) were assayed in an analogous manner.

Assay of phenol oxidase activity

Phenol oxidase activity was assayed by the method of Söderhäll & Häll (1984) with slight modifications. A 50 μ l portion of trypsin or chymotrypsin (1 mg/ml) was added to 50 μ l of sample together with 50 μ l of L-3,4-dihydroxyphenylalanine (3 g/l). After 2.5 min of incubation at room temperature, 450 μ l of buffer was added and the absorbance was measured at 490 nm. Activity is expressed as the difference in absorbance at 490 nm/min per ml.

Assay of serine proteinase activity

Serine proteinase activity was measured as described by Söderhäll (1983).

To check the influence of the inhibitor on the proteinases of the proPO-activating system, the usual proteinase assay system (Söderhäll, 1983) was used, with the exception that 100 μ l of the buffer was replaced by the purified inhibitor.

The influence of the inhibitor on the activation of proPO was assayed by preincubation of the partly purified serine proteinases with laminarin or lipopolysaccharide in the presence of trypsin inhibitor. After 15 min partly purified proPO and L-3,4-dihydroxyphenylalanine were added together with Tris/HCl buffer, pH 8.0, and the reaction was monitored for 30 min at 490 nm. As controls proPO and 3,4-dihydroxyphenylalanine were added to a pre-activated serine proteinase fraction.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed in accordance with Laemmli (1970) and 7.5% gels were used. About 20 μ g of the inhibitor was applied. Human α_2 -macroglobulin (M_r 180 000), phosphorylase b (M_r 94 000), bovine serum albumin (M_r 67 000) and egg albumin (M_r 43 000) were used as standards. The electrophoresis was performed on 0.75 cm × 14 cm × 16 cm gels. The gels were stained with Serva Blue G-250.

Isoelectric focusing

Isoelectric focusing was performed in an LKB 2117 Multiphor apparatus with an LKB 2103 power supply. The experiments were performed at 4 °C. Servalyt Precotes pH 3–6 were used in accordance with the

Table 1. Purification of a proteinase inhibitor from crayfish plasma

For experimental details of the purification procedure see the Materials and methods section. The amount of inhibitor was determined according to the calibration curve shown in Fig. 4; the amount of inhibitor was not determined in the crude extract, because of the presence of α_2 -macroglobulin.

Step	Total amount of inhibitor (mg)	Total amount of protein (mg)	Specific activity (mg/mg of protein)	Purification (fold)	Recovery (%)
Crude plasma	—	11985	—	—	—
Acid precipitation	25	725	0.034	1	100
Concanavalin A-Sepharose chromatography	8	36.8	0.217	6.38	32
Phenyl-Sepharose chromatography	5.4	5.4	1	29.4	21.6

Fig. 1. Elution profile of the crayfish plasma proteinase inhibitor from a concanavalin A-Sepharose column (2.2 cm × 21 cm)

After a washing with equilibrating buffer (0.1 M-Tris buffer, pH 8.0) (I), the column was incubated at room temperature overnight with 0.1 M-Tris buffer, pH 8.0, containing 2 M-NaCl and 0.5 M-methyl α -D-mannopyranoside (IIa). The inhibitor was eluted as described in the Materials and methods section (IIb). ●, A_{280} ; ○, inhibitor.

Fig. 2. Elution profile of crayfish plasma proteinase inhibitor from a phenyl-Sepharose column equilibrated with 0.1 M-Tris buffer, pH 8.0, containing 2 M-NaCl

The pooled fractions from the concanavalin A-Sepharose step were applied (Pool) and the column was washed with the equilibrating buffer (0.1 M-Tris buffer, pH 8.0, containing 2 M-NaCl) (III). After a further wash with 0.1 M-Tris buffer, pH 8.0, containing 0.5 M-NaCl (IV), a considerable amount of the inhibitor was eluted with 0.1 M-Tris buffer, pH 8.0, without NaCl (I). The residual amount was eluted with 0.1 M-Tris buffer, pH 8.0, containing 50 % ethylene glycol (V). ●, A_{280} ; ○, inhibitor.

manufacturer's instructions. About 10 μ g of the purified inhibitor was applied, and the pH gradient was determined by using the standards amyloglucosidase (pI 3.5), ovalbumin (pI 4.6), bovine serum albumin (pI 4.9) and lactoglobulin (pI 5.3). The gel was stained with Serva Blue G-250.

Determination of amino acid composition

Amino acid composition was determined as described previously (Hergenbahn *et al.* 1984). A 0.3–6 μ g portion per analysis was employed. Cysteine was determined after oxidation with performic acid. Tryptophan was not determined.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard protein.

RESULTS

Purification of proteinase inhibitor

As summarized in Table 1, a proteinase inhibitor was purified from crayfish plasma by means of acid precipitation, affinity chromatography on concanavalin A-Sepharose (Fig. 1) and hydrophobic-interaction chromatography (Fig. 2) to apparent electrophoretic homogeneity (Fig. 3). Since the binding to concanavalin A-Sepharose was very strong, a better yield was obtained if the column was incubated with elution buffer at room temperature overnight (IIa in Fig. 1). As shown in Fig. 2, a considerable amount of the inhibitor could be eluted from phenyl-Sepharose with Tris buffer containing no additional salt. However, a further, much sharper, peak could be obtained if 50 % (v/v) ethylene glycol was

included. As Table 1 reveals, 25 mg of inhibitor was obtained after acid precipitation. So the inhibitor content of the native plasma should be at least in the region of 0.1 mg/ml. The final yield of pure inhibitor was 5.4 mg.

Biochemical characterization of proteinase inhibitor

The M_r as obtained by SDS/polyacrylamide-gel electrophoresis was calculated to be 140000 (Fig. 3) in the absence and 155000 in the presence of 2-mercaptoethanol, which suggests that the proteinase inhibitor is monomeric. In isoelectric focusing, in the range of pH 4.6–4.8 four bands could be observed. Amino acid analysis revealed a relatively high content of acidic and hydrophobic amino acids (Table 2).

Studies on proteinase inhibition

The inhibitor was highly specific, since only the serine proteinases trypsin and chymotrypsin were efficiently inhibited, whereas elastase was inhibited only to a limited extent (Table 3). Hence trypsin was used throughout the experiments to quantify the amount of

inhibitor. The correlation between the amount of the inhibitor and trypsin inhibition is shown in Fig. 4. The purified trypsin inhibitor from crayfish plasma could also inhibit the purified proPO-activating enzyme, a serine proteinase, which converts proPO into the active form, phenol oxidase (Fig. 4). When the purified inhibitor (at a concentration that inhibits proPO-activating enzyme by 50%) was incubated with this serine proteinase preparation and proPO from crayfish blood cells phenol oxidase activity was decreased to 55%.

DISCUSSION

A proteinase inhibitor was isolated and purified to apparent homogeneity from crayfish plasma. The purified inhibitor was a monomeric protein with an M_r of 155000 and a pI of 4.6–4.8. It was specific in its inhibitory capacity, since only trypsin and chymotrypsin were efficiently inhibited, whereas elastase was only slightly and thrombin or subtilisin not at all affected. Further-

Fig. 3. Proof of electrophoretic purity of the purified proteinase inhibitor and determination of the M_r by SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed in the presence (a) and in the absence (b) of reducing agent.

Table 2. Amino acid composition of the proteinase inhibitor

Amino acid	Composition (mol of residue/mol)
Asx*	11.57
Thr*	7.20
Ser*	10.17
Glx†	10.10
Pro†	3.25
Gly†	7.17
Ala†	6.74
Cys‡	2.95
Val§	5.67
Met‡	0.88
Ile§	3.62
Leu§	7.66
Tyr*	3.80
Phe†	3.67
His†	6.55
Lys†	4.06
Arg†	5.08

* Values from 24 h hydrolysis.

† Average values from 24 h, 48 h and 72 h hydrolysis.

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

§ Values from 72 h hydrolysis.

Table 3. Inhibition of some proteinases by the inhibitor

The enzymes were assayed with chromogenic substrates; for trypsin and thrombin S-2222 was employed, for chymotrypsin and subtilisin S-2586 was used, and Suc-Ala-Ala-Ala-NH-Np was used for elastase. The activity was monitored as the release of *p*-nitroaniline at 405 nm. The amount of purified inhibitor was 100 μ g for thrombin, 10 μ g for elastase and subtilisin and 1 μ g for trypsin and chymotrypsin. The experiments were performed in triplicate.

Proteinase	A_{405} without the inhibitor	A_{405} with the inhibitor	Inhibition (%)
Trypsin (0.1 μ g/assay)	0.26	0.04	85
Chymotrypsin (0.2 μ g/assay)	0.46	0.03	93
Thrombin (50 μ g/assay)	0.70	0.70	0
Elastase (5 μ g/assay)	0.58	0.47	19
Subtilisin (2 μ g/assay)	0.65	0.67	0

Fig. 4. Inhibition of trypsin (●) and the proPO-activating enzyme (○) by the purified inhibitor

The assay was carried out with the chromogenic substrates as described in the Materials and methods section.

more, it could inhibit the proPO-activating enzyme as well as the conversion of proPO into phenol oxidase by the proPO-activating enzyme. Since the proPO-activating system appears to be involved in the defence reactions arthropods mount towards parasites (Söderhäll, 1982; Söderhäll & Smith, 1986a,b), this system needs to be regulated to avoid activation of this system in places where it would be inappropriate or detrimental to the host. Therefore this trypsin inhibitor in crayfish plasma may constitute an important regulator of the activation of the proPO-activating system. Sugumaran and colleagues (Saul & Sugumaran, 1986; Sugumaran *et al.* 1985) have reported the presence of proteinase inhibitors in the hemolymph of the insects *Manduca sexta* and *Sarcophaga bullata*. These proteinase inhibitors were involved in preventing proPO activation, but as yet no purification or properties of these inhibitors have been reported. Several proteinase inhibitors have been isolated from invertebrates (Kang & Fuchs, 1974, 1980; Sasaki, 1978, 1984; Sasaki & Kobayashi, 1984; Armstrong *et al.*, 1984; Armstrong & Quigley, 1985; Eguchi & Shomoto, 1985; Suzuki & Natori, 1985, 1986). These inhibitors are all in the low- M_r range of 5000–15000, except in silkworm haemolymph, from which a chymotrypsin inhibitor (M_r 43000) (Sasaki & Kobayashi, 1984; Eguchi & Shomoto, 1985) and a trypsin inhibitor (M_r 42000) (Sasaki & Kobayashi, 1984) have been isolated. Most of these inhibitors are serine-proteinase inhibitors, with the exception of a cysteine-proteinase inhibitor from *Sarcophaga peregrina* (Suzuki & Natori, 1986). Although no clear biological function has been shown for most of these inhibitors, an acid-labile proteinase inhibitor from *Limulus polyphemus* haemolymph could inhibit the clotting enzyme of this animal (Armstrong *et al.*, 1984), and we have now shown that the plasma proteinase inhibitor of crayfish inhibits the proPO-activating enzyme, a serine proteinase. Interestingly, a subtilisin-specific proteinase inhibitor purified from crayfish blood cells (Häll & Söderhäll, 1982) with an M_r of 23000 could not inhibit the proPO-activating enzyme (results not shown) and may therefore be aimed at proteinase from parasitic micro-organisms.

Other high- M_r proteinase inhibitors have been isolated from invertebrate plasma. In *L. polyphemus* an α_2 -macroglobulin-like molecule, with a subunit M_r of

approx. 180000, has been isolated (Quigley & Armstrong, 1983, 1985), and a similar molecule has been reported to be present in crayfish plasma (Hergenhahn & Söderhäll, 1985) and plasma of some other crustaceans (Armstrong *et al.*, 1985). However, the 155000- M_r proteinase inhibitor purified from crayfish plasma had no α_2 -macroglobulin-like activity, e.g. to protect proteinase from inhibition of soya-bean trypsin inhibitor as determined with chromogenic peptides as proteolytic substrates. Furthermore, in contrast with α_2 -macroglobulin the activity of this inhibitor was not influenced by methylamine. Therefore it can be excluded that it is α_2 -macroglobulin or a part of it. In vertebrates several proteinase inhibitors of high M_r have been found. The only one with a similar M_r and inhibitor specificity to the proPO inhibitor is inter- α -trypsin inhibitor (Steinbuch, 1976); but inter- α -trypsin inhibitor has been reported to be highly unstable, which is not the case with the proteinase inhibitor of crayfish. Furthermore, they differ substantially in their amino acid compositions. We therefore consider that these two proteins may not be related, but certainly more detailed chemical information is required before we can compare this inhibitor with other known high- M_r proteinase inhibitors from vertebrates and invertebrates.

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