Amino Acid Sequence of a Unique Protease from the Crayfish *Astacus fluviatilis*†

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ABSTRACT: The amino acid sequence of a protease from the crayfish *Astacus fluviatilis* has been determined from overlapping sets of peptides derived largely by cleavage at Met, Lys, or Arg residues. The protein comprises 200 amino acid residues in a single polypeptide chain, corresponding to a molecular mass of 22 614 daltons. Two disulfide bonds link Cys-42 to Cys-198 and Cys-64 to Cys-84. The sequence of this invertebrate protease appears to be unique since it has no homologous relationship to any of the known protein sequences.

In 1967, Pfleiderer et al. (1967) isolated from the cardia of the decapod crayfish *Astacus fluviatilis* an endoprotease with novel properties. On the basis of more recently determined structural and catalytic properties, this protease appears to have no counterpart among recognized families of proteolytic enzymes (Zwilling et al., 1981). The enzyme exhibits an unusual type of cleavage specificity that proved to be useful for generating overlapping peptides for protein sequence analysis (Krauhs et al., 1982). The protein was originally described as a low-molecular-weight protease of approximate M_r 11 000. However, subsequent evidence, including the present data, indicates that the molecular mass of this protease is within the normal minimal range of mammalian proteases, i.e., 22 614. Under physiological conditions, the *Astacus* protease is remarkably stable and resistant against self-digestion, but at pH below 4 it is rapidly and irreversibly inactivated. The catalytic mechanism of the enzyme is unique and does not correspond to that of any known protease. No natural or synthetic protease inhibitor has been described to date, nor has any functionally important amino acid residue been identified. Partial amino-terminal sequence analysis has failed to demonstrate any similarity to any known protease (Zwilling et al., 1981).

These unique properties are in sharp contrast to those of two other proteolytic enzymes that have been isolated from crayfish cardia, namely, *Astacus* trypsin and *Astacus* carboxypeptidase [for a review, see Zwilling and Neurath (1981)]. The complete amino acid sequences of these two enzymes have been determined (Titani et al., 1983, 1984) and shown to be homologous to those of their mammalian pancreatic counterparts. It appeared therefore of particular interest and importance to determine the complete amino acid sequence of the *Astacus* protease and to search for any sequence homology to known proteases and other proteins. The results of these analyses are reported in this paper.

Materials and Methods

The protease was prepared from the digestive juice of the living crayfish *Astacus fluviatilis* by a combination of anion-exchange chromatography and gel filtration as described by Zwilling and Neurath (1981). The yield was approximately 1 mg of enzyme/mL of digestive fluid.

The protein was reduced with dithiothreitol and carboxymethylated with iodoacetic acid as previously described (Titani et al., 1984). Methionyl bonds were cleaved with cyanogen bromide (Titani et al., 1984) and lysyl bonds by incubation with *Achromobacter* protease I (a gift from Dr. T. Masaki, Ibaraki University, Ibaraki, Japan) in 50 mM Tris·HCl, pH 9.0, containing 2 M urea at 37 °C for 4 h (Masaki et al., 1981). Peptide K2 was subdigested with TPCK[1]–trypsin (Worthington) in 0.1 M NH_4HCO_3, pH 8.0, at 37 °C for 3 h, and the fragments were purified by HPLC on a Cosmosil C18 column. Asparaginyl–glycine bonds were cleaved by the following modification of the method of Bornstein and Balian (1969). Peptides were dissolved in 2 M hydroxylamine hydrochloride containing 6 M guanidine hydrochloride and maintained at pH 9.0 (at 45 °C) by the addition of 1.0 M NaOH in a pH stat for 6 h (Titani et al., 1978). The reaction mixture was then adjusted to pH 2.5 with formic acid and fractionated by reversed-phase HPLC on a SynChropak RP-8 column. Aspartyl bonds were cleaved with 2 M formic acid, pH 1.98, at 110 °C for 4 h (Titani et al., 1986).

Primary mixtures of peptides were separated on tandem TSK columns in 6 M guanidine hydrochloride and 10 mM sodium phosphate, pH 6.0 (Titani et al., 1986). Unresolved peptide mixtures were then separated by reversed-phase HPLC on SynChropak RP-8, Cosmosil C18, or Altex Ultrapore RPSC columns with gradients of acetonitrile into dilute aqueous trifluoroacetic acid (Mahoney & Hermodson, 1980). Amino acid compositions were determined with a Waters Picotag system (Bidlingmeyer et al., 1984). Sequence analyses were carried out in a Beckman 890C spinning cup sequencer with 1–2 nmol of peptide (Takio et al., 1983) or in an Applied Biosystems sequencer with smaller amounts (Hunkapiller et al., 1983). Phenylthiohydantoins were identified by complementary HPLC systems (Glajch et al., 1985; Ericsson et al., 1977). Analyses for homology were performed according to the SEARCH, ALIGN, and RELATE programs described by Dayhoff et al. (1983).

Results

The general strategy for the determination of the amino acid sequence of the *Astacus* protease is summarized in Figure 1. A major portion of the proof was obtained by Edman degradation of the S-carboxymethylated (S-CM) protein and of

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[1] Abbreviations: S-CM, *S*-carboxymethyl; M_r, molecular weight; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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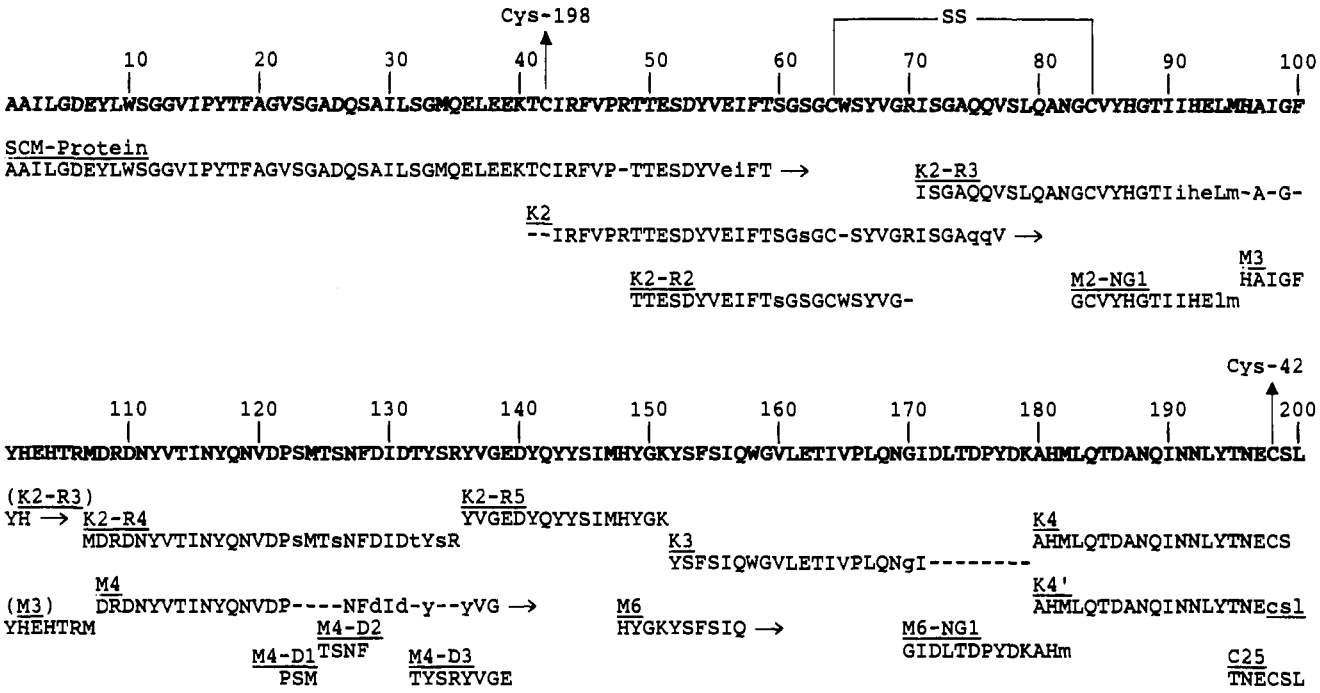

FIGURE 1: Detailed summary of the proof of sequence of *Astacus* protease. Sequences derived by Edman degradation of specific peptides (designations are underlined) are given below the summary sequence (bold type). Prefixed K and M denote peptides derived by cleavage at lysyl and methionyl residues, respectively. Peptide C25 is a product of chymotryptic digestion of performic acid oxidized protease. Subpeptides are identified by hyphenated suffixes, where R, NG, and D refer to tryptic cleavage at Arg and chemical cleavage at Asn-Gly or aspartyl residues, respectively. Within the specified sequences, lower case letters denote tentative identification of phenylthiohydantoins, whereas underlined sequences are deduced from amino acid compositions. Those not identified are indicated by dashes or, for long unidentified sequences, by arrows. Disulfide bonds link Cys-42 to Cys-198 and Cys-64 to Cys-84.

three of the nonoverlapping fragments resulting from cleavage at the three lysyl residues in the molecule. The remainder of the sequence and overlaps of the lysyl fragments were obtained largely by analysis of peptides generated by cleavage of methionyl bonds.

Edman degradation of intact S-CM-protein (4 nmol) provided the sequence of 58 residues at the amino terminus. Treatment of the S-CM-protein with *Achromobacter* protease I yielded a mixture that was eventually resolved into four major fragments (K1–K4) by a combination of molecular exclusion and reversed-phase HPLC techniques (Figure 2). Sequenator analysis of K1 (not shown in Figure 1) identified it as the amino-terminal 40-residue segment.

Analysis of fragment K2 yielded a 39-residue sequence that overlapped the amino-terminal region by 17 residues, beginning at Ile-43 and extending the overall sequence to Val-77. The first two residues of K2 were not identified by Edman degradation due to interference by contaminants (from dialysis tubing) that were extracted before the third cycle. Fragments K4 and K4′ were both placed at the carboxyl terminus on the basis of their lack of lysine and their identical amino-terminal sequences. Approximately one-third of the protein preparation contained a carboxyl-terminal leucine residue that was absent in the remainder (cf. the ratio of K4′ and K4 in Figure 2C). The carboxyl-terminal leucine was also placed in a chymotryptic peptide (designated C25 in Figure 1) isolated from performic acid oxidized protein.

The composition and amino-terminal sequence of fragment K2′ (Figure 2A) corresponded to a peptide having the same amino-terminal sequence as K2, but which extended beyond the carboxyl terminus of K2 by virtue of an uncleaved lysyl bond. The remaining lysyl fragment K3 can therefore tentatively be placed between fragments K2 and K4 (Figure 1).

Subdigestion of fragment K2 with trypsin yielded five peptides (K2-R1 through K2-R5). K2-R1 corresponded to

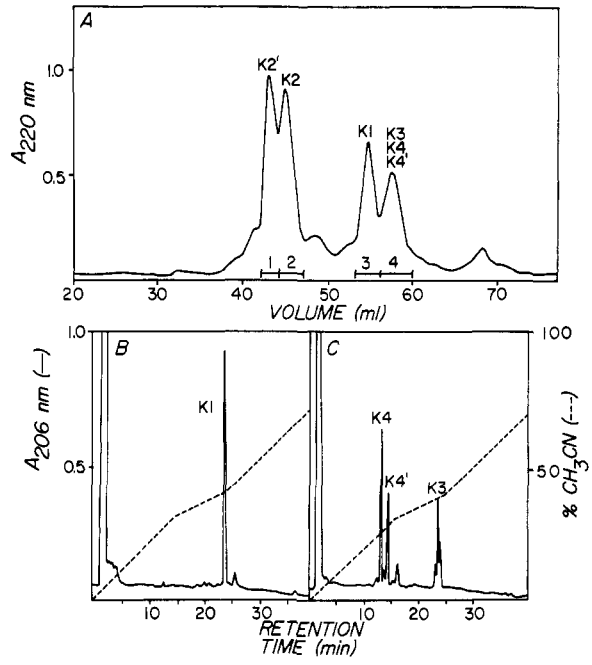

FIGURE 2: Separation of peptides in a digest of S-CM-protein (1.0 mg) by *Achromobacter* protease I. Panel A illustrates the primary separation of the digest on three columns of TSK G3000SW–G2000SW–G2000SW (each 7.5 × 600 mm) connected in a series and eluted with 6 M guanidine hydrochloride and 10 mM sodium phosphate, pH 6.0, at a flow rate of 0.5 mL/min. Panels B and C illustrate the subsequent purification of pooled fractions 3 and 4 on a SynChropak RP-8 column (4.1 × 250 mm) by reversed-phase HPLC.

residues 41–48 (not shown); sequence analysis of K2-R2 (residues 49–70) confirmed Ser-62 and Trp-65 (Figure 1). K2-R3 overlapped the amino-terminal sequence of K2 by seven residues and extended the overall sequence to His-102, except

Table I: Amino Acid Compositions of Peptides from *Astacus* Protease[a]

peptide residues		M1 1–34	M2 35–95	M3 96–107	M4 108–147	M6 148–182	M7 183–200	K4′ 180–200	S-CM-protein 1–200
Asx	D/N	1.1 (2)	3.0 (2)		8.7 (10)	3.7 (4)	3.8 (5)	4.6 (5)	18.2 (23)
Glx	E/Q	2.0 (2)	7.4 (10)	0.9 (1)	3.5 (3)	4.1 (3)	2.9 (3)	3.2 (3)	21.3 (22)
CM-Cys	C		1.2[b] (3)				*b* (1)	0.4 (1)	4.5 (4)
Ser	S	4.0 (4)	5.1 (6)		3.8 (4)	2.8 (2)	1.0 (1)	1.8 (1)	15.0 (17)
Gly	G	6.8 (6)	6.4 (6)	1.2 (1)	2.0 (1)	4.3 (3)		1.2 (0)	17.4 (17)
His	H		2.1 (2)	2.6 (3)		1.7 (2)		1.0 (1)	6.3 (7)
Arg	R		2.4 (3)	1.0 (1)	2.0 (2)			0.4 (0)	6.0 (6)
Thr	T	1.1 (1)	3.8 (5)	1.0 (1)	2.8 (3)	1.9 (2)	1.6 (2)	1.8 (2)	12.0 (14)
Ala	A	5.0 (5)	2.7 (2)	1.0 (1)		2.3 (1)	1.0 (1)	2.0 (2)	10.8 (10)
Pro	P	1.3 (1)	1.4 (1)		1.0 (1)	2.2 (2)		0.6 (0)	5.4 (5)
Tyr	Y	1.9 (2)	3.0 (3)	0.7 (1)	6.3 (7)	2.5 (3)	0.6 (1)	1.0 (1)	13.5 (17)
Val	V	2.0 (2)	4.0 (5)		2.7 (3)	1.9 (2)			12.0 (12)
Met	M[c]	0.9 (1)	0.7 (1)	0.7 (1)	0.9 (2)	0.6 (1)		0.6 (1)	4.8 (6)
Ile	I	3.0 (3)	4.3 (5)	1.2 (1)	3.2 (3)	2.7 (3)	1.5 (1)	1.2 (1)	14.1 (16)
Leu	L	3.5 (3)	3.2 (3)			3.0 (3)	2.8 (3)	2.7 (3)	11.4 (12)
Phe	F	1.2 (1)	1.8 (2)	1.0 (1)	1.0 (1)	0.9 (1)			6.3 (6)
Lys	K		1.0 (1)			1.3 (2)			3.0 (3)
Trp	W	ND[d] (1)	ND (1)			ND (1)			ND (3)

[a] Residues per molecule by amino acid analysis or (in parentheses) from the sequence. [b] Recoveries of S-CM-Cys were very low. [c] Recovered as homoserine (except for the S-CM-protein analysis). [d] Not determined.

for seven tentative or missing assignments. Since the peptide K2-R5 contained lysine but lacked arginine, it was placed at the carboxyl terminus of K2. Thus K2-R4 was tentatively placed between K2-R3 and K2-R5.

Cleavage of methionyl residues with cyanogen bromide (Figure 3) provided four principal fragments, M2, M3, M4, and M6, which overlapped the various lysyl peptides. The largest fragment M2 (residues 35–95), isolated from S-CM-protein (Figure 3C), contained three S-CM-Cys residues. However, when the protein was treated with cyanogen bromide *without* prior reduction and alkylation, a new fragment, X-X, (Figure 3B) was found. Upon purification, amino acid analysis, and Edman degradation, fragment M-X was found to comprise M2 (residues 35–95) in disulfide linkage with M7 (residues 183–199). In contrast, peptides M1, M3, M4, and M6 lacked Cys and hence could be isolated from either reduced or nonreduced protein. M4 contained two homoseryl residues, one as expected at the carboxyl terminus and an internal one at residue 124 due to the known resistance of the Met–Thr bond to CNBr cleavage. Fragment M6 overlapped K2-R5 and K3 in the region of residue 151 (Figure 1). Cleavage of the Asn–Gly bond of M6 with hydroxylamine yielded M6-NG1, which overlapped K3 and K4, thus completing the sequence from Tyr-136 to the carboxyl terminus.

The remaining proof (from Ile-90 to Tyr-136) was derived as follows. Cleavage of the Asn–Gly bond in M2 yielded M2-NG1 and extended the sequence to Met-95. The partial sequence of K2-R3 overlapped M3. The carboxyl-terminal Met in M3 must correspond to the amino-terminal methionine of K2-R4 because the peptide was generated by arginyl cleavage and the only scissile bond is Arg–Met at the carboxyl terminus of M3. The sequence of K2-R4 extends the structure to Arg-135, except for tentative identifications of four hydroxyamino acids. These assignments were confirmed. An overlap to the carboxyl-terminal segment was provided by three small peptides generated from M4 by partial acid hydrolysis at Asp bonds (and at the previously uncleaved homoseryl residue 124).

The amino acid composition determined in hydrolysates of the methionyl peptides and in the intact S-CM-protein is compared with that calculated from the sequence (Table I). Values of Asp and Ile are lower than predicted by the sequence. Our determinations of aspartic acid by the Picotag method were inferior at that time, and the Ile–Ile bond at

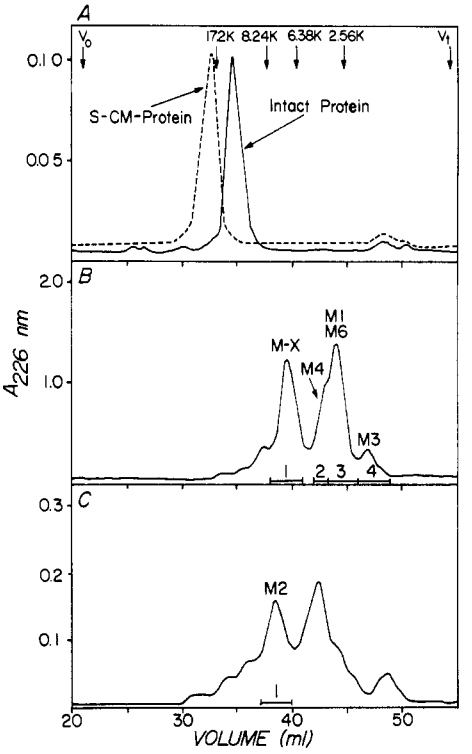

FIGURE 3: Molecular-exclusion HPLC of *Astacus* protease (10 μg) before and after reduction and carboxymethylation (panel A) and of cyanogen bromide digests of the protein before (1.5 mg, panel B) and after (0.5 mg, panel C) reduction and carboxymethylation. In each case, two TSK G3000SW columns (each 7.5 × 600 mm) were connected in series and eluted with 6 M guanidine hydrochloride and 10 mM sodium phosphate (pH 6) at 0.5 mL/min. Each of the pooled fractions in panel B (except M-X) was further fractionated to yield homogeneous peptides on a SynChropak RP-8 column. The pooled fractions labeled M2 in panel C and M-X in panel B were further fractionated to purity on an Altex Ultrapore RPSC column. Amino acid compositions of the peptides are given in Table I.

residues 90–91 is resistant to hydrolysis under our conditions. Hence the analyses are considered to be satisfactory.

The disulfide bonds were assigned in a subdigest of the large peptide M-X (Figure 3B) that was only found in unreduced protein. Tryptic digestion of M-X was carried out in 2 M urea and 0.1 M NH_4HCO_3 at 37 °C for 3 h. As mentioned earlier, amino acid composition and amino-terminal analysis indicated that it contained both M2 and M7, apparently linked by a

disulfide bond. The single Cys in M7 (residue 198) was shown to be linked to Cys-42 in M2 by subdigesting pure M-X with trypsin and purifying two disulfide-containing peptides. The digest was first separated on two tandem TSK G3000SW columns and then by reversed-phase HPLC. One pure peptide corresponded by composition and sequence analysis to Thr-Cys-Ile-Arg (residues 41–44) linked to residues 183–199. Another contained residues 49–70 linked to residues 71–95, indicating that Cys-64 was in a disulfide bond with Cys-84.

The mobility of the S-CM-protein in 6 M guanidine hydrochloride on HPLC in our TSK molecular sizing columns (Figure 3A) indicates a molecular weight of ca. 18000. The present sequence analysis corresponds to a value of 22614.

Discussion

The proof of sequence shown in Figure 1 is largely based upon the alignment of peptides produced by cleavage at lysyl residues of the 200-residue protein. The largest peptide, K2, overlaps the amino-terminal sequence of the whole protein and is aligned with K3 and the carboxyl-terminal peptide K4 by segments of the overlapping cyanogen bromide fragment M6. The internal sequence of peptide K2 (residues 75–136) was determined in appropriate subdigests. Although the overlaps in the region of residues 106–110 (Arg-Met-Asp-Arg-Asp) are of marginal length, no other alignment appears to be compatible with the experimental data. The seemingly marginal overlap at residues 170–171 is considered quite reliable because of the unique specificity of hydroxylamine cleavage of the Asn–Gly bond that generated M6-NG1. Identification of the carboxyl terminus of the protein was ambiguous, as indicated by the two peptides denoted K4 and K4′, of which the latter possessed one extra leucine residue. It is not uncommon, however, to find such "ragged ends" in protein structures as a result of prior action by exopeptidases or endopeptidases.

The validity of the analysis is enhanced by the redundancy of the independent identifications of phenylthiohydantoins in the two complementary HPLC systems. All residues except His-96 were thus identified twice, and many residues were, in addition, identified in more than one overlapping peptide (Figure 1).

Estimates of the molecular weight of *Astacus* protease have varied over a wide range. The earliest molecular weight determination, based on Sephadex G-75 gel filtration in the presence of 1 M NaCl and calibrated against proteins of known molecular weight, indicated a M_r of 11000 for the native enzyme (Pfleiderer et al., 1967). More recent estimates by SDS–PAGE indicate a M_r of 20300 for the reduced protein. However, with increasing pore size lower values were obtained for the unreduced protein (Dörsam, 1983). In the present study, the mobility of the S-CM-protein in 6 M guanidine hydrochloride on a TSK molecular sizing column on HPLC indicated a M_r of ca. 18000. The present sequence analysis corresponds to a M_r of 22614.

There is evidence that *Astacus* protease displays unique retention characteristics on gel filtration, which may be related to the interaction of aromatic side chains (π-electron system) of the protein with different types of matrices, i.e., Sephadex (dextran) and Bio-Gel (polyacrylamide). High ionic strength enhances the retention (A. Feige and R. Zwilling, unpublished results). In contrast, crayfish trypsin and carboxypeptidase, found in the same digestive juice, elute as predicted by their molecular weights. Anomalous retention is displayed by *Astacus* protease-like enzymes of several other decapod crustacea.

The amino acid sequence of the *Astacus* protease was compared with those available in the National Biomedical Research Foundation databank of Feb 1986. No homologous relationship was found, nor was there any evidence of internal homology, as would result from divergence after partial gene duplication. In contrast to all known chordate proteases, this invertebrate protease does not fit into any of the established protease families. The unique sequence of the *Astacus* protease is therefore intriguing. As suggested previously (Zwilling et al., 1981), the enzyme could be the product of an evolutionary path that did not lead to homologous counterparts in vertebrates. This is not an uncommon observation in comparing vertebrates and invertebrates. For example, oxygen is transported by hemocyanin in crayfish and by hemoglobin in vertebrates, two proteins that are quite unrelated structurally. Alternatively, it is possible that a homologous enzyme may exist in higher organisms but remains unrecognized due to its low concentration, or perhaps because it serves a specific metabolic role rather than a digestive function.

Although the unusual specificity of this enzyme is well documented (Zwilling et al., 1981; Krauhs et al., 1982), nothing is known of its mechanism of action. Conventional site-directed inhibitors of the established families of proteases have no effect on its enzymatic activity. Studies of its three-dimensional structure, together with its amino acid sequence and kinetic analyses, may suggest features of its active site and may lead to the design of inhibitors that could be useful in finding putative homologues in higher organisms.

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Purification of Sarcotoxin II, Antibacterial Proteins of *Sarcophaga peregrina* (Flesh Fly) Larvae†

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ABSTRACT: Three antibacterial proteins with almost identical primary structures termed sarcotoxin IIA, IIB, and IIC were purified to homogeneity from the hemolymph of third instar larvae of *Sarcophaga peregrina*. The molecular masses of these proteins were about 24 000. These proteins were found to have common antigenicity, and antibody against sarcotoxin IIA cross-reacted with sarcotoxin IIB and IIC. Radioimmunoassay using this antibody showed that these proteins are induced in the hemolymph in response to injury of the larval body wall.

Humoral antibacterial substances are known to be induced in some insects on injection of dead or living bacteria (Whitcomb et al., 1974; Boman, 1981; Chadwick, 1982). These substance are thought to participate in the defense system of insects, which have no immune network. However, the molecular properties of most of these antibacterial substances have not well been characterized. The proteins studied most extensively are cecropins and attacins of *Hyalophora cecropia* (giant silk moth), which are groups of proteins induced in the hemolymph of pupae of *H. cecropia* by immunization (Steiner et al., 1981; Hultmark et al., 1982, 1983; Lee et al., 1983; Engström et al., 1984; Kockum et al., 1984; von Hofsten et al., 1984).

Previously, we reported the induction of bactericidal proteins in the hemolymph of third instar larvae of *Sarcophaga peregrina* (flesh fly) by injection of a light suspension of *Escherichia coli* (Natori, 1977). Subsequently, we found that mere pricking of the larval body wall with a hypodermic needle is sufficient for induction of antibacterial proteins. We purified one of these proteins, termed sarcotoxin I, to homogeneity (Okada & Natori, 1983) and found that it is a mixture of three proteins (sarcotoxin IA, IB, and IC) with almost identical primary structures. We showed that these proteins consisted of 39 amino acid residues and differed only in 2–3 amino acid residues (Okada & Natori, 1985a). The primary target of sarcotoxin I was shown to be the bacterial membrane (Okada & Natori, 1984). Treatment of *E. coli* with sarcotoxin I resulted in almost instantaneous inhibition of active transport and in a rapid decrease in ATP levels (Okada & Natori, 1985b).

This paper describes the purification of another group of antibacterial proteins termed sarcotoxin II. The molecular mass of sarcotoxin II is much higher than that of sarcotoxin I, being about 24 000. Sarcotoxin II was found to consist of three proteins (sarcotoxin IIA, IIB, and IIC) with almost identical primary structures. It is possible that the cooperative actions of multiple antibacterial proteins with different antibacterial spectra form a potent defense system protecting insects from bacterial infection.

Materials and Methods

Animals and Collection of Hemolymph. Third instar larvae of *Sarcophaga peregrina* were used throughout. For induction of antibacterial activity, larvae were pricked with a hypodermic needle. The injured larvae were kept in contact with water for 24–48 h at room temperature in plastic containers.

Hemolymph was collected by cutting off the anterior tip of the larvae with fine scissors and collecting the drop of hemolymph that exuded in a Petri dish on ice. The resulting hemolymph was centrifuged for 5 min at 200*g* to remove hemocytes, and the clear supernatant was stored at −20 °C (Okada & Natori, 1983).

Fractionation of Hemolymph. Previously, we found that the antibacterial activity in the hemolymph could be separated into three fractions (GI, CI, and CII). We purified and characterized a group of antibacterial proteins named sarcotoxin I from fraction CII. This paper describes purification of another group of antibacterial proteins, termed sarcotoxin II, from fraction GI. The GI fraction was obtained as described before (Okada & Natori, 1983).

Assay of Antibacterial Activity. The following method was used for determination of antibacterial activity. *E. coli* K-12 594 (streptomycin resistant) was grown in antibiotic medium (Difco). Cells in the exponential phase of growth were collected and suspended in 10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 6.0) containing 130 mM NaCl at an A_{650} of 0.3 (2.5×10^8 cells/mL) determined in a Shimazu 150-02 spectrometer. The sample (200 μL), antibiotic medium (190 μL), and *E. coli* suspension (10 μL) were mixed in a test tube and incubated at 37 °C for 140 min with shaking. Then the mixture was rapidly chilled, and the A_{650} was measured.

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