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AMINO ACID SEQUENCE OF RAT EPIDERMAL THIOL PROTEINASE INHIBITOR

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SUMMARY: The complete amino acid sequence of rat epidermal thiol proteinase inhibitor was determined. The unique 103-residue sequence was derived by analysis of two peptides generated by limited proteolysis of the native inhibitor with Staphylococcus aureus V8 protease and of three cyanogen bromide fragments. The protein has a high degree of sequence homology to either rat liver or human leucocyte inhibitor but is not identical and may represent a new type of low molecular weight thiol proteinase inhibitors.

In recent years, many reports have been published on the presence of endogenous protein inhibitors of thiol proteinases (TPI) in a variety of mammalian tissues, as reviewed by Lenney (1). These inhibitors are classified into two groups. One group of TPI is mainly present in various tissues and has molecular weights in the range of 10,000 to 12,000 daltons. The other group is present in plasma and has higher molecular weights (greater than 60,000). Recently we purified a low molecular weight TPI from rat liver (2) and reported both the amino acid sequence of its 89 residues (3, 4), and differences from TPI of plasma origin (5). Turk and co-workers isolated a different TPI of low molecular weight from human polymorphonuclear granulocytes (6, 7), and showed that its primary structure (6, 8) is homologous with rat liver TPI. They also isolated inhibitors from chicken egg white and human sera of patients with autoimmune diseases and demonstrated that this type of low molecular weight TPI which they named "cystatin" is distantly related to tissue TPI (6, 9, 10).

A low molecular weight TPI was also found in rat epidermis (11, 12) and was recently shown to be different in composition from rat liver TPI

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(13). In this communication, we report the unique 103-residue sequence of this TPI, which is homologous but not identical to either rat liver or human leucocyte TPI, and may represent a new type of low molecular weight TPI.

MATERIALS AND METHODS

Rat epidermal TPI was prepared essentially as described by Takeda et al. (13). After extraction from newborn rat epidermis, it was purified by Sephadex G-75 chromatography and DEAE-cellulose chromatography. As described by Takeda et al. (13), two fractions with inhibitory activity were obtained on DEAE-cellulose chromatography. The second fraction, which was predominant and corresponds to thiol proteinase inhibitor-1 of Takeda et al. (13), was pooled and used for the present study. TPCK-trypsin was obtained from Worthington. Staphylococcus aureus V8 protease and proline-specific endopeptidase were purchased from Miles. Carboxypeptidase Y was a gift from Dr. M. Ottesen (Carlsberg Laboratories).

Peptides were purified by reversed phase high performance liquid chromatography (HPLC), which was performed with a Varian 5000 Liquid Chromatograph on a column of either SynChropak RP-P (4.1 x 250 mm)(SynChrom) or Ultrapore RPSC (4.6 x 7.5 mm)(Altex) using a trifluoroacetic acid (Pierce)/acetonitrile (Burdick & Jackson) system (14).

Amino acid analysis was performed with a Dionex D500 Amino Acid Analyzer. Automated sequence analysis was performed with an Applied Biosystems 470A Protein Sequencer using a program adapted from Hunkapiller et al. (15). PTH-derivatives were identified in a semi-quantitative manner by two HPLC systems (16, 17).

RESULTS AND DISCUSSION

Automated sequence analysis of the intact protein (2 nmol) indicated that the amino-terminus is blocked. Epidermal TPI is highly resistant to heat and extreme pH (13). It is also resistant to proteolysis by trypsin. Trypsin (2.5% by weight) generated no subpeptide after incubation at 21°C in 0.1 M NH_4HCO_3, pH 8.0 for 3 h and the undigested protein was quantitatively recovered by reversed phase HPLC. S. aureus V8 protease (2.5% by weight) cleaved native TPI (9 nmol) at a single glutamyl bond at residues 14-15 during incubation at 37°C in 0.05 M sodium phosphate, pH 7.8 for 6 h. The two subpeptides, E1 and E2, were separated by reversed phase HPLC (Table 1). Sequencer analysis demonstrated that only E1 was blocked, thus it must be derived from the amino-terminus. E2 (1.1 nmol) was analyzed by Sequencer almost to the carboxyl-terminus, providing the sequence of residues 15-81 and placing methionine at residues 61 and 73 (Fig. 1).

Nine nmol of TPI was treated with cyanogen bromide in 70% formic acid at 21°C for 5 h, and separated by reversed phase HPLC to yield three frag-

Table 1

Amino Acid Compositions of Rat Epidermal TPI and Its Fragments Used in the Sequence Analysis

Protein/Peptide Residue No.		TPI 1-103	M2 2-61	M3 62-73	M4 74-103	E1 1-14	E2 15-103	E1-P1 1-3	E1-P2 4-14
Aps/Asn	(D/N)	11.3(13)	3.0(3)	1.8(2)	7.5(8)	1.1(1)	10.9(12)	0.9(1)	
Thr	(T)	6.4(7)	3.4(4)	0.2	2.8(3)	1.9(2)	4.5(5)		1.8(2)
Ser	(S)	3.9(3)	1.8(2)		1.4(1)	1.0(1)	2.0(2)		1.1(1)
Glu/Gln	(E/Q)	13.8(14)	10.7(12)	0.3	2.4(2)	1.3(1)	13.2(13)		1.2(1)
Pro	(P)	4.1(3)	2.7(3)			1.2(1)	2.1(2)	1.0(1)	
Gly	(G)	9.4(9)	5.0(5)	2.1(2)	2.9(2)	4.0(4)	5.3(5)		4.0(4)
Ala	(A)	5.0(4)	4.0(4)	0.2			4.1(4)		
Val	(V)	8.7(11)	6.8(8)	2.0(2)	0.6(1)	1.7(2)	8.0(9)		1.7(2)
Met	(M)	2.7(3)				1.1(1)	2.2(2)	1.0(1)	
Ile	(I)	2.9(3)	2.6(3)			0.7(1)	2.0(2)		0.6(1)
Leu	(L)	8.8(9)	2.3(2)	1.0(1)	6.0(6)		9.0(9)		
Tyr	(Y)	2.9(3)	1.7(2)		0.9(1)		2.3(3)		
Phe	(F)	4.0(4)	1.9(2)	1.0(1)	1.2(1)		3.9(4)		
His	(H)	1.3(1)	0.4	1.0(1)			1.1(1)		
Lys	(K)	11.6(13)	6.9(8)	1.1(1)	3.9(4)		12.1(13)		
Arg	(R)	3.3(3)	1.3(1)	1.1(1)	1.1(1)		3.1(3)		
Hse			0.7(1)	0.7(1)					
Total Residue		103	60	12	30	14	89	3	11
Yield (%)			82	63	20	56	40	50	63

Data are expressed as residues per molecule. One-letter amino acid abbreviations are indicated in parentheses. Cys was determined as CM-Cys. Numbers in parentheses indicate the residues found in sequence. Hse signifies homoserine (derived from methionine).

ments, M2, M3 and M4 (Table 1). None were blocked, and the N-blocked homoserine must have been overlooked in the breakthrough peak of the HPLC separation. Analysis of M2 (1.1 nmol) yielded the sequence of residues 2 through 41, overlapping E2 by 27 residues. Analysis of M3 (1.1 nmol) confirmed the sequence of residues 62 through 73 already identified in E2.

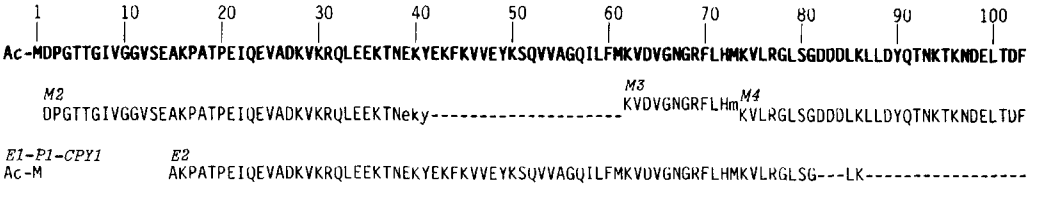

Fig. 1. Summary proof of the sequence of rat epidermal TPI. The one-letter codes (see Table 1) designate amino acid residues identified by Edman degradation unambiguously (capital letters) or tentatively (lower-case letters). Those not identified in each peptide are shown by dashes. Prefixes *E*, *M*, *P*, or *CPY* designate digestion (staphylococcal protease, cyanogen bromide, proline specific endopeptidase or carboxypeptidase Y, respectively) used to generate the fragment. The final sequence derived is shown at the top in bold face letters.

M4 (1.3 nmol) was analyzed through the carboxyl-terminal end to provide the sequence of residues 74 through 103 (Fig. 1).

To identify the N-blocking group and to complete the sequence analysis, E1 (residues 1-14)(8 nmol) was further digested with proline-specific endopeptidase (10% by weight) at 37°C in 0.1 M NH_4HCO_3, pH 8.0, for 4 h. Two peptides were recovered (E1-P1 and E1-P2, Table 1). Of these only E1-P1 was blocked. It contained three amino acids, two of which must correspond to the amino-terminal Asp-Pro in M2. Thus a blocked methionyl residue must be at the amino-terminus of E1-P1. This was confirmed by digestion of the peptide (3 nmol) with carboxypeptidase Y at 22°C in 5 mM sodium citrate, pH 5.2 for 6 h, which yielded an acetyl-Met (2.8 nmol), with a retention time identical to synthetic N-acetyl-Met on reversed phase HPLC (4). Only Met was observed on amino acid analysis. In this chromatogram, the breakthrough fraction contained 2.0 nmol of aspartic acid and 2.1 nmol of proline.

The molecular weight of rat epidermal TPI is calculated to be 11,603 from the sequence shown in Fig. 1. Although Takeda et al. (13) have reported that the β-structure is predominant in this TPI and may play an important role in its interaction with thiol proteinases, secondary structure prediction by the method of Chou and Fasman (18, 19) indicated the presence of 40% α-helix, 10% β-strand and 7% β-turns in the TPI.

As shown in Fig. 2, the sequence of rat epidermal TPI is similar (49.5% identity) to that of rat liver TPI (3, 4). It is also homologous (58.8% identity) to human leucocyte TPI (6, 7). It cannot be ascertained whether rat epidermal TPI is identical to that from rat leucocytes. These three thiol proteinase inhibitors appear to belong to a single protein family, which have presumably diverged from a common ancester with preservation of function. These proteins bear no apparent relation to protein inhibitors of serine proteases or metallopeptidases. Homology between these three TPI is so extensive that it is difficult to point out the essential part(s) for the protease-inhibitor interaction site(s). The single histidyl residue

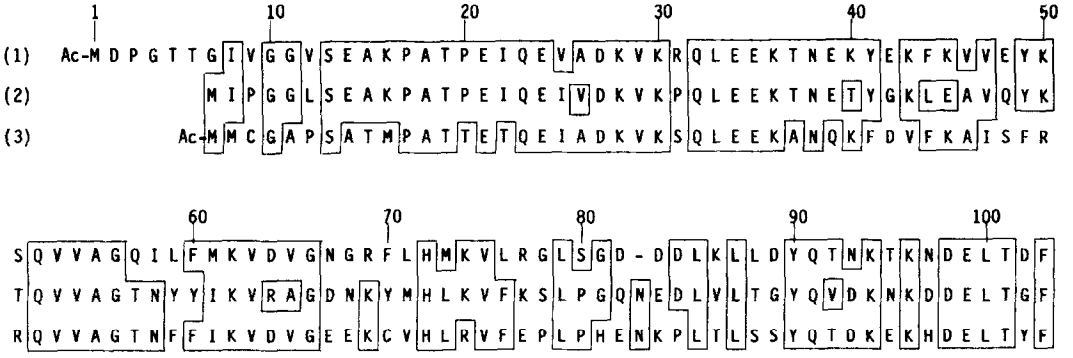

Fig. 2. Comparison of the three TPI sequences, (1) rat epidermal, (2) human leucocyte and (3) rat liver. Residue numbers are those of rat epidermal TPI. Ac- designates N-acetyl group. Common residues are enclosed in boxes.

in epidermal TPI is preserved in leucocyte and liver TPI. It is interesting to know whether this residue has some role in the function.

Out of three TPI shown in Fig. 2, only the rat liver TPI contains half-cysteinyl residues, suggesting no direct involvement of these residues in the inhibitory activity. In fact, it has been shown that the rat liver TPI is likely to be regulated through modification of a single cysteinyl residue (Cys-3)(3).

This study extends our knowledge of the ubiquitous finding of protease inhibitors in the immediate vicinity of proteases.

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