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LOW MOLECULAR WEIGHT PEPTIDE INHIBITORS OF MEDULLASIN: PURIFICATION AND STRUCTURE

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Two low molecular weight peptide inhibitors of medullasin were isolated from human bone marrow cells. Determination of their amino acid composition and amino acid sequence revealed that one inhibitor was composed of 36 amino acid residues and the other 34 amino acid residues which are identical with the C-terminal portions (Val^{111}----His^{146}, Val^{113}----His^{146}, respectively) of the β-chain of human hemoglobin. These two peptides when synthesized also showed the same degree of inhibitory effect on medullasin activity as the natural products. Neither the N-terminal portion of the inhibitor, composed of 21 amino residues, nor the C-terminal peptide, composed of 20 amino acids, inhibited medullasin activity. Medullasin was inhibited reversibly and non-competitively against by these inhibitors and was the most effectively inhibited serine protease among several tested.

KEY WORDS: medullasin, peptide inhibitors, hemoglobin

INTRODUCTION

Medullasin, a serine protease found in human bone marrow cells, resembles elastase, but has no elastinolytic activity.[1-4] We have succeeded in cloning both the cDNA and the gene of medullasin, and elucidated their structures.[5,6] Medullasin activity in granulocytes increases in patients with chronic inflammation in the active phase and decreases to normal levels in remission.[7,8] An injection of a small amount of medullasin into the an animal's skin caused inflammation characterized by the infiltration of a large number of macrophages.[9] An electron microscopic examination revealed that endothelial cells in small veins were characteristically degenerated by the injection.[9] Both DNA and RNA synthesis of human lymphocytes were stimulated when they were treated with a physiological amount of medullasin. Superoxide production of monocytes was stimulated and their chemotactic activity inhibited by treatment with medullasin.[9] From the results described above medullasin in granulocytes is considered to play an important role in the development of inflammation, especially chronic inflammation.

Certain protease inhibitors were shown to inhibit medullasin activity. Elastatinal,[10] a protease inhibitor obtained from the culture supernatant of Actinomycetes de-

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creased[2] protease activity by half at a concentration of 3.5 μg/ml. α_1-Antitrypsin inhibited medullasin activity in an equimolar ratio.[2] Both α_2-macroglobulin and ovomacroglobulin inhibited the protease activity at an almost 1:1 molar ratio.[11] α_2-Plasmin inhibitor also inhibited the protease activity (Aoki, Y., unpublished data). However, when protease inhibitors from microbial origin, including elastatinal, were administered to animals, several unexpected effects other than those derived from the inhibition of the specific protease usually develop.[12] Protease inhibitors previously reported from animal tissues have a molecular weight[13] above 6,000. Therefore, we attempted to isolate low molecular weight inhibitors of medullasin from human tissues in order to obtain stable and non-toxic potential drugs as anti-inflammatory agents.

MATERIALS AND METHODS

Materials

Crystallized trypsin from porcine pancreas (type IX) and porcine pancreas elastase (type III) were obtained from Sigma Chemical Co., USA. Bovine α-chymotrypsin was purchased from Boehringer Mannheim Co., West Germany. Crystallized human hemoglobin was obtained from Sigma Chemical Co.

Medullasin

Medullasin was purified, and further crystallized, from human bone marrow cells (approximately 10^{10} nucleated cells) according to the method described.[1,2] Medullasin activity was determined by measuring the inactivation of apo-ornithine transaminase by the method described.[14,15]

Purification of Inhibitors

Freeze-thawed bone marrow cells obtained from human resected ribs were suspended in 10 vols. of water, and centrifuged at 20,000 g for 10 min. The supernatant was dialyzed against 30 vols of water overnight at 4°C, and extracted with 0.1 M acetic acid for 3 h at room temperature. After dialysis of the extract against 10 vols. of water for 24 h at 4°C, the solution outside the dialysate was concentrated by a rotary evaporator, and lyophilized. The residue was dissolved in 20% acetonitrile containing 0.05% trifluoroacetic acid, applied to a reverse-phase HPLC (M & S Pack, C18-B, 20 × 250 mm), and eluted with a linear gradient solvent system of acetonitrile. The main peak was applied to the same HPLC and re-chromatographed. The main fraction containing the inhibitory activity to medullasin was further applied to a reverse-phase HPLC (TSK, phenyl-5PWRP, 4.6 × 75 mm), and eluted with a linear gradient solvent system of acetonitrile. Two main peaks were collected and lyophilized.

Amino acid analysis

Peptides (150 mg) dissolved in 0.1 ml of constant boiling hydrochloric acid (6 M) containing 4% thioglycolic acid were hydrolyzed under vacuum for 22 h at 110°C. The analyses were performed on a Hitachi # 835 amino acid analyzer.

Sequence Analysis

Automated amino acid sequence analysis of peptides was performed using an Applied Biosystems sequencer (model 470A), and PTH-amino acids were identified by a PTH-analyzer (Applied Biosystems, model 120A).

Synthesis of Peptides

Peptides were synthesized on the basis of the proposed sequence for the inhibitors by the solid phase method employing a peptide synthesizer (Applied Biosystems, model 430A). The peptide consisting of 36 amino acid residues was purified by dissolving in 6 M guanidine-HCl and applying to a gel filtration column of Asahipack GS-320 (Asahi Chemical Ind., 9 μm, 7.6 $\times$ 500 mm). The peptide consisting of 34 amino acids was purified by reverse-phase HPLC (Waters, μBondasphere 5 μm 100 A, 3.9 $\times$ 150 mm, C_{18}). The peptides corresponding to the 20 amino acid residues of the C-terminal of the inhibitor and to the 21 amino acids of the N-terminal of inhibitor - (which consists of 34 amino acids) were synthesized, and purified by reverse-phase HPLC (Senshu, ODS 215 H 5 μm 100 A, 6 $\times$ 150 mm, C_{18}). The purity of the peptides was confirmed by the determination of both amino acid compositions and amino acid sequences.

Determination of the Effect of Inhibitors on Medullasin Activity

The incubation mixture (0.13 ml) containing medullasin (0.32 μg), the inhibitor dissolved in water (0.05 ml), apo-ornithine transaminase (7.5 μg) and potassium phosphate buffer (1 M, pH 8.5, 0.04 ml) was incubated for 10 min at 37°C. Control tubes contained water (0.05 ml) instead of the inhibitor. After incubation the ornithine transaminase activity remaining was measured to determine modullasin activity according to the method described.[14,15]

Digestion of the Inhibitors and Related Peptides by Medullasin

Degradation of the inhibitors and related peptides by medullasin was examined by analyzing the incubation mixture with HPLC. The incubation mixture (0.2 ml) which contains the inhibitor or related peptides (0.8 mg), medullasin (1.6–16 μg) and potassium phosphate buffer (pH 8.5) was incubated at 37°C for 20 min. After the addition of 10% acetonitrile, it was applied to a reverse-phase HPLC (M & S Pack, C18-B, 20 $\times$ 250 mm), and eluted with a linear gradient system of acetonitrile.

Effect of the Inhibitors on the Caseinolytic Activity of Medullasin

The incubation mixture containing heat denatured casein (1%, 0.05 ml), potassium phosphate buffer (1 M, pH 8.0, 0.02 ml), medullasin (40 μg/ml of 0.05 M acetate buffer, pH 5.0; 0.02 ml) and inhibitor solution (0.03 ml) was incubated at 37°C for 20 min to determine the effect of the inhibitors on the caseinolytic activity of medullasin. The reaction was stopped by adding trichloroacetic acid (2%, 0.6 ml), and the amount of ninhydrin-positive substance in the supernatant was measured.

RESULTS

Isolation and Structure of Inhibitors

Figure 1 shows the elution profile of the inhibitor from the first reverse-phase HPLC. The peak portions containing the inhibitory activity were collected and applied to the same chromatograph. The main fraction containing the inhibitory activity was lyophilized and dissolved in water containing trifluoroacetic acid (0.1%) and applied to another reverse-phase HPLC. As shown in Figure 2, the inhibitory activity was detected in two peaks which were separately collected and lyophilized.

As shown in Table I, the amino acid composition of peak 2 (inhibitor 2) was almost identical with that of peak 1 (inhibitor 1) except that inhibitor 2 has an additional half-cysteine and valine residues than inhibitor 1.

Both inhibitor 1 and inhibitor 2 were applied to a gas phase sequencer, and PTH-amino acids were analyzed by a PTH-analyzer as described in Methods. The amino acid sequence of inhibitor 2 is shown in Figure 3. Inhibitor 1 had the same amino acid sequence as that of inhibitor 2 except that inhibitor 1 was devoid of two amino acids (Val-Cys) from the N-terminal of inhibitor 2. The calculated molecular weights of inhibitors 1 and 2 were 3,707, and 3,905 respectively. The amino acid sequence of these two inhibitors was identical with that of the C-terminal portion (inhibitor 1, Val^{113}-His^{146}; inhibitor 2, Val^{111}-His^{146}) of the β-chain of human hemoglobin.

Effect of Inhibitors on Medullasin Activity

Figure 4 shows the relationship between the amount of inhibitor 1 added and the inhibitory effect on medullasin activity using apo-ornithine transaminase as subtrate.

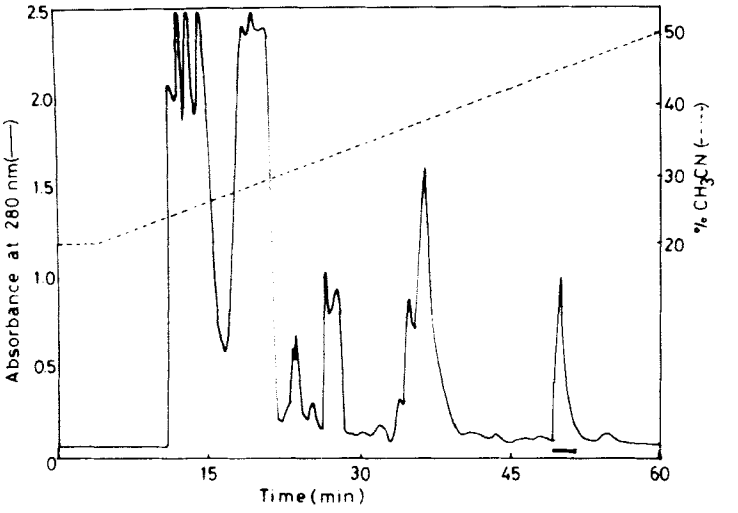

FIGURE 1 The first reverse-phase HPLC of the inhibitor. The sample laoded consisted of the concentrated outer solutions of the dialysate extracted from human bone marrow cells. Column: M & S Pack, C 18-B, 20 × 250 mm. Solvent system: A linear gradient from (A) to (B): (A) 0.05% TFA-20% CH_3CN, (B) 0.05% TFA-50% CH_3CN. Flow rate: 5 ml/min. Fractions containing the main inhibitory activity are marked with a bar.

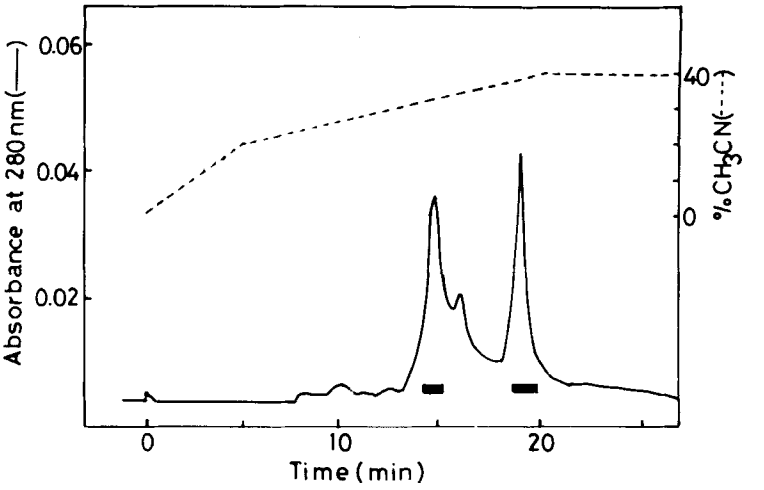

FIGURE 2 Reverse-phase HPLC of partially purified inhibitor. The sample loaded consisted of the lyophilized main fractions obtained by applying the marked fractions in Figure 1 on the same column. Column: TSK, phenyl-5PWRP, 4.6 × 75 mm. Solvent system: A linear gradient from (A) to (B). (A) 0.1% TFA-H_2O, (B) 0.1% TFA-CH_3CN. Flow rate: 1 ml/min. Fractions containing the inhibitory activity are marked with black bars. The first peak is referred to as inhibitor 1 and the second peak as inhibitor 2.

TABLE I
Amino acid composition of inhibitors

Amino acid	Inhibitor 1		Inhibitor 2	
	Molecular percent	No. of residues/mol[a]	Molecular percent	No. of residues/mol[a]
Aspartic acid	2.9	1	2.8	1
Threonine	2.9	1	2.8	1
Serine	0.0	0	0.0	0
Glutamic acid	8.8	3	8.3	3
Glycine	5.9	2	5.6	2
Alanine	20	7	19	7
Valine	15	5	17	6
Half-cysteine	0.0	0	2.9	1
Methionine	0.0	0	0.0	0
Isoleucine	0.0	0	0.0	0
Leucine	5.9	2	5.6	2
Tyrosine	5.9	2	5.6	2
Phenylalanine	5.9	2	5.6	2
Lysine	8.8	3	8.3	3
Histidine	12	4	11	4
Arginine	0.0	0	0.0	0
Proline	5.9	2	5.6	2
Tryptophan	0.0	0	0.0	0
Total	100	34	100	36

[a]Determined also by considering results of sequence analysis.

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 1      a)            5                        10
Val-(Cys)-Val-Leu-Ala-His-His-Phe-Gly-Lys-
11                   15                       20
Glu-Phe-Thr-Pro-Pro-Val-Gln-Ala-Ala-Tyr-
21                   25                       30
Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-
31                   35      b)
Leu-Ala-His-Lys-Tyr-(His)
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FIGURE 3 Amino acid sequence of inhibitor 2. [a]No peak was detected in this position, therefore, Cys was deduced from amino acid composition. [b]Amino acid was not determined in this position by sequencer analysis. His was deduced in this position from amino acid composition. Amino acid sequence of inhibitor 1 was the same as that for inhibitor 2, except that inhibitor 1 was devoid of the two N-terminal amino acids (Val-Cys) of inhibitor 2.

As shown in this Figure, the inhibition constant (K_i) for inhibitor 1 was 4 μM. The inhibition constant of inhibitor 2 was the same as that for inhibitor 1. The inhibition was completely reversible since it was removed after 100-fold dilution with water of a solution of medullasin and inhibitor stored for 10 min at room temperature. The inhibition was non-competitive with substrate (data not shown).

When casein was employed as substrate for medullasin, the same degree of inhibition was obtained as that using apo-ornithine transaminase as substrate (data not shown). Addition of crystallized human hemoglobin to the incubation mixture caused no inhibitory effect on medullasin activity up to a concentration of 7 mg/ml.

Synthetic peptides corresponding to inhibitor 1 and inhibitor 2 were tested to confirm that they inhibited medullasin activity. The Ki value for both synthetic peptides to medullasin was the same as that of the natural peptides. The peptide with 21 amino acid residues corresponding to the N-terminal portion of inhibitor 1 showed

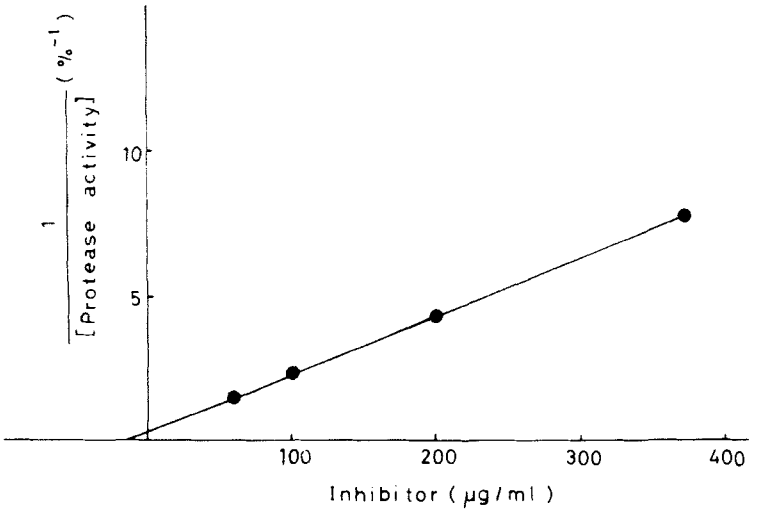

FIGURE 4 Relationship between the amount of the inhibitor (inhibitor 1) added and the inhibitory effect on medullasin activity. Apo-ornithine transaminase was used as substrate. The effect of the inhibitor was determined as described in Methods.

no inhibitory effect on medullasin activity up to concentration of 2 mg/ml. Also, the peptide with 20 amino acid residues corresponding to the C-terminal portion of inhibitor 1 showed negligible inhibitory effect on the protease activity up to a concentration of 1.5 mg/ml.

Susceptibility of the Inhibitors and Related Peptides to Medullasin

When the synthetic peptide consisting of 21 amino acid residues of the N-terminal portion of inhibitor 1 or the peptide consisting of 20 amino acid residues of the C-terminal portion were incubated with medullasin (1.6 μg) as described in Methods, more than 90% of both peptides was digested by medullasin as judged by the elution profile from HPLC. Figure 5 shows the HPLC-elution profile for the synthetic peptide corresponding to the 20 amino acids of the C-terminal of inhibitor 1 incubated with medullasin. On the contrary, both the HPLC-elution profile and the inhibitory activity remained unchanged when inhibitor 1 or inhibitor 2 was incubated with medullasin under the same conditions as described above. However, when inhibitor 1 or inhibitor 2 was incubated at 37°C for 20 min in the presence of a large amount of medullasin (16 μg), approximately half of the peptides were degraded and the inhibitory activity decreased.

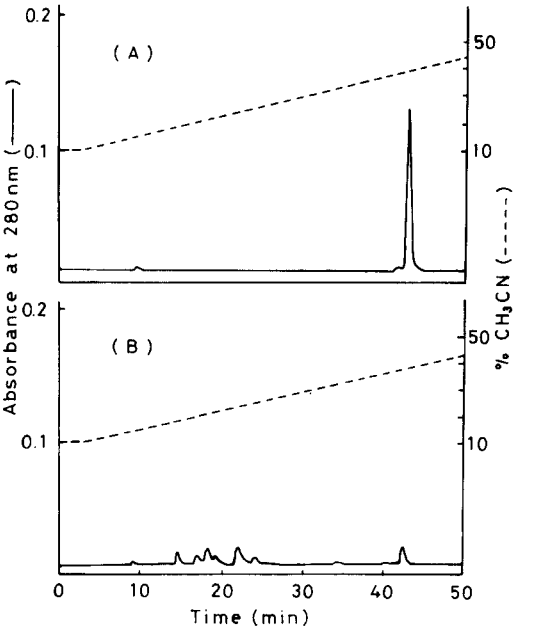

FIGURE 5 Reverse-phase HPLC of the synthetic peptide incubated with medullasin. The sample loaded consisted of 800 μg of the peptide corresponding to 20 amino acid residues of the C-terminal portion of inhibitor 1, incubated in the absence (A) or presence (B) of 1.6 μg of medullasin at 37°C for 20 min in potassium phosphate buffer (pH 8.5, 0.05 M, 0.2 ml). Column: M & S Pack, C18-B, 20 × 250 mm. Solvent system: A linear gradient from (A) to (B). (A) 0.05% TFA-10% CH_3CN, (B) 0.05% TFA-50% CH_3CN. Flow rate: 5 ml/min.

TABLE II
Effect of the inhibitor on the activity of certain serine proteases

Proteases	Inhibitor constants (K_i*, μM)
Medullasin	4.0
Trypsin	410
α-Chymotrypsin	43
Elastase	460

Inhibitor 1 was employed for the determination of K_i. K_i values were determined by the method of Dixon[18].

Effect of the Inhibitor 1 on the Activity of Other Serine Proteases

Effect of inhibitor 1 on the activity of some serine proteases other than medullasin was studied. As shown in Table II, the inhibitor revealed certain degrees of inhibitory effect on serine proteases other than medullasin e.g. trypsin, α-chymotrypsin and elastase, but its most striking inhibitory effect was on medullasin activity.

DISCUSSION

Peptides, corresponding to the C-terminal 34 (inhibitor) and 36 (inhibitor 2) amino acid residues of the β-chain of human hemoglobin, inhibited medullasin activity in a dose-dependent manner. The inhibition was relatively specific to medullasin among serine proteases. Synthetic peptides corresponding to these inhibitors also showed the same degree of inhibitory effect on medullasin activity as that of the natural peptides. The peptides inhibited medullasin activity in a non-competitively manner with substrate. They are not considered to act on apo-ornithine transaminase so causing a decrease in susceptibility to medullasin, since the protease activity was inhibited by them to the same degree whether apo-ornithine transaminase or casein was employed as substrate. The peptides corresponding to the 20 amino acid residues of the C-terminal of inhibitor 1 and the 21 amino acid residues of the N-terminal portion showed negligible inhibitory effect on medullasin activity. Whilst these synthetic peptides were easily digested by medullasin under the same conditions. From these results it is speculated that a certain conformation(s) is required for peptide inhibitors to inhibit medullasin activity and prevent the digestion by the protease. Despite the presence of cysteine in inhibitor 2, it is unlikely that it exists as a dimer because both inhibitor 1 and inhibitor 2 were eluted in the same position when subjected to gelfiltration chromatography (data not shown).

The inhibition by these inhibitors was reversible, and their inhibitory potency not so high as has been reported for other peptide protease inhibitors.[16,17] The possibility remains that the present inhibitors are artificial products formed during the procedure of extracting them from human bone marrow cells. Whether they are artificial products or not, the molecular weight of the present inhibitors is very low among peptide protease inhibitors from animal tissues.[13] Furthermore, they are the components of normal human tissues. Medullasin in granulocytes is considered to play an important role in the development of inflammation, especially chornic inflammation as described preivously. Therefore, we speculate that the inhibitors of medullasin could serve as anti-inflammatory drugs, with little side effect when used clinically.

The fact that the C-terminal portion of β-chain of human hemoglobin serves as a protease inhibitor is of interest and suggests another function for hemoglobin.

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