calf thymus H2B sequence. Both peptides contained carboxymethylcysteine (CM-Cys) or aspartic acid, which were indistinguishable in our amino acid analyzer system: peptide T13 probably contained CM-Cys since it was radioactive, while the non-radioactive peptide T14 probably contained aspartic acid. The amino acid analyses shown here clearly indicate the presence of structural differences between rat TH2B and rat H2B, which must be of genetic origin rather than due to post-translational modifications. Some of these differences, e.g. peptide H10, probably involve the highly variable N-terminal region. However, other differences, e.g. peptides H9/T9 and H18/T18 involve the C-terminal portion, which generally shows little variation in H2Bs of different species[15].

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Low-molecular-weight proteinase inhibitor in human plasma inhibiting papain and trypsin activity

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Summary. A low-molecular-weight proteinase inhibitor was isolated from human plasma on the basis of its papain-inhibiting capacity. The isolated fraction demonstrated inhibitory activity against papain and trypsin activity and the molecular weight was estimated to be approximately 3200 by gel-filtration.

Human plasma contains several proteinase inhibitors[1], but except for α_2-macroglobulin[2] and antithrombin III[3], there is no proteinase inhibitor which has inhibitory activity against plural proteinase groups such as cysteine, serine, carboxyl and metallo proteinases. We describe here the purification of a low-molecular-weight proteinase inhibitor from human plasma on the basis of its papain-inhibiting capacity using casein as a substrate. The isolated fraction demonstrated inhibitory activity not only against papain but also against trypsin.

Materials and methods. 45 ml of plasma obtained from a healthy human was eluted by diafiltration through an Amicon YM 5 membrane. The filtrate was then concentrated 3-fold by the use of an Amicon YM 2 membrane and chromatographed on a Sephadex G-25 column. Fractions number 90–99 with inhibitory activity against papain were combined and passed through 3 g of CM Cellulofine (Seikagaku Kogyo, Tokyo) which had been equilibrated with 28 mM phosphate buffer, pH 7.2. The eluate was then applied to DEAE Cellulofine (Seikagaku Kogyo) that had previously been equilibrated with the same buffer. The column (1 × 5 cm) was washed with equilibrating buffer and eluted with a linear gradient formed from 100 ml of equilibration buffer and 100 ml of 0.2 M NaCl containing same buffer, at a flow rate of 13.5 ml/h (3 ml per tube). The samples with inhibitory activity were combined and con-

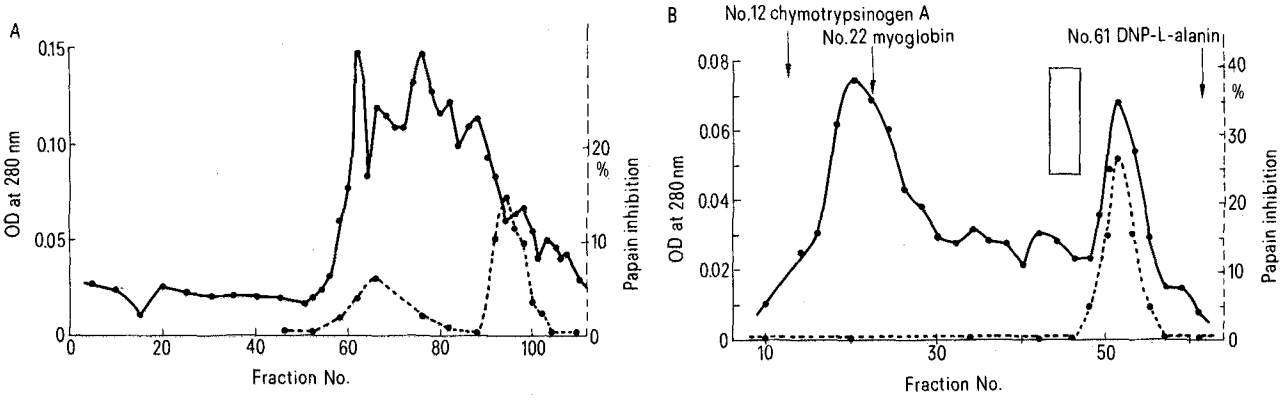

Figure 1. Gelfiltration of the low-molecular-weight proteinase inhibitor. *A* Plasma diafiltrate which was concentrated was applied on a column (2.64 × 90 cm) of Sephadex G-25 (Pharmacia Fine Chemicals) which had been equilibrated with 28 mM phosphate buffer, pH 7.2 and eluted with the same buffer at a flow rate of 13.5 ml/h. Fractions (6 ml) were collected and assayed for the inhibition against papain using casein as a substrate. *B* The sample that was partially purified by diafiltration, gelfiltration, CM Cellulofine and DEAE Cellulofine was applied on a column (1.9 × 85 cm) of Sephadex G-25 that had been equilibrated with the same buffer and eluted with equilibrating buffer at a flow rate of 13.5 ml/h. Fractions (3 ml) were collected and assayed.

	Total volume (ml)	Total protein (mg)	ID_{50} (μg)	Total units	S A	Yield (%)	Purity
Diafiltration (Amicon YM 5)	45	19.4	75.0	258.1	13.3	100.0	1.0
Gelfiltration (Sephadex G-25)	60	3.0	34.0	88.2	29.4	34.3	2.2
CM Cellulofine	60	1.9	27.0	70.4	37.0	27.3	2.8
DEAE Cellulofine	18	0.6	9.5	63.2	105.3	24.5	7.9
Gelfiltration (Sephadex G-25)	26	0.2	5.3	37.7	188.7	14.6	14.2

centrated to 2.5 ml using an Amicon YM 2 membrane, and again applied to a Sephadex G-25 column. The active fractions were combined and concentrated by diafiltration. The molecular weight of the inhibitor was estimated by gel filtration on Sephadex G-25. Chymotrypsinogen A from bovine pancreas (Serva), equine myoglobin from skeletal muscle (Serva) and DNP-L-alanine (Serva) were used as standards. Slab gel electrophoresis was carried out on a 15% polyacrylamide gel at pH 9.4 for 120 min at 8 mA[4]. Micro slab gel electrophoresis equipment (Marysol Ind., Tokyo) was used. 2 μg of the isolated fraction was applied onto the gel in a 0.3-cm slot. Silver staining (BIO-Rad) was used for staining protein band.

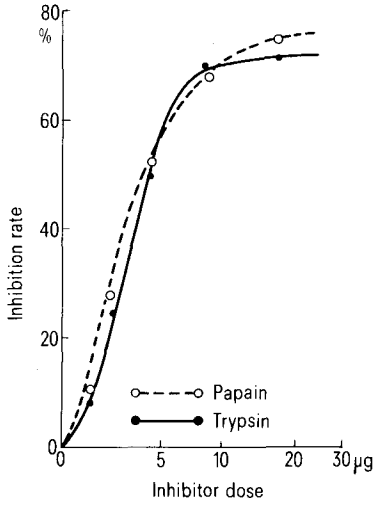

Figure 2. Effect of inhibitor concentration on papain and trypsin activity. 5 μg of papain (Sigma, type 4) in 20 μl of water and 5 μg of trypsin (Difco, 1:250) in 20 μl of 1 mM HCl were incubated with 200 μl of serially diluted isolated inhibitor at room temperature for 30 min respectively. After the incubation, enzyme activity was measured using synthetic substrates as follows. Papain activity was detected by a slight modification of the method described by Barrett[6]. Briefly, after the 30-min incubation, the mixture was incubated for 5 min at 37 °C with 400 μg of Nα-benzoyl-DL-arginine-β-naphtylamide HCl (Aldrich Chemical Company) in 100 μl of 0.1 M Tris-HCl buffer, pH 7.2 containing 4 mM dithiothreitol (Sigma) and 2 mM ethylenediamine tetraacetic acid disodium salt (Sigma) Next, color reagent[6] was added and after standing for 10 min, the mixture was centrifuged at 3000 rpm. The absorption of the supernatant was read at 520 nm. Trypsin activity was measured by the method of Erlanger[7]. Briefly, after a 30-min incubation, the mixture was incubated for 45 min at 37 °C with 800μg of benzoyl-DL-arginine-p-nitroanilid HCl (Boehringer Mannheim) in 200 μl of 0.1 M Tris-HCl buffer, pH 8.0 containing 10 mM $CaCl_2$. The reaction was stopped by the addition of 1.3 ml of 30% acetic acid and absorbance of the products were read at 410 nm. Blanks were prepared by adding the enzyme and inhibitor solution just after the colour reagent and acetic acid.

For assay of papain inhibition, 12.5 μg of papain and 300 μl of inhibitor solution were incubated at room temperature for 30 min. After the incubation, 0.5 mg of Hammarsten casein (Nutritional Biochemicals) in 400 μl of 28 mM phosphate buffer, pH 7.2 was added. The mixture was incubated at 37 °C for 30 min in a shaking water bath. The reaction was stopped by the addition of 200 μl of 20% trichloro acetic acid. After standing in ice water for 15 min, the reaction mixture was centrifuged at 3000 rpm for 15 min. The resulting supernatants were read at 750 nm after carrying out a Lowry reaction[8]. Control determinations were carried out in the same way, substituting a suitable buffer for the inhibitor solution; boiled papain was used as a blank. The percent inhibition of papain activity was calculated as:

$$\% \text{ inhibition} = 100 - \frac{\text{inhibition activity} - \text{blank}}{\text{control activity} - \text{blank}} \times 100.$$

The 50% inhibition dose (ID_{50}) was determined according to the method of Aoyagi and Mizuno[9]. The protein concentration (mg) for ID_{50} was defined as 1 inhibition unit. Specific activity was calculated as inhibition unit/mg of protein. Protein was measured by the method of Lowry et al.[8] with bovine serum albumin as a standard.

Results. Purification of the low-molecular-weight proteinase inhibitor from human plasma is summarized in the table. The inhibitor was eluted by diafiltration through an Amicon YM 5 membrane (exclusion limit < 5000 mol.wt) and concentrated by an Amicon YM 2 membrane (exclusion limit < 2000). Figure A shows the chromatographic pattern obtained when the concentrated plasma diafiltrate was chromatographed on a Sephadex G-25 column. Under our conditions, the inhibitor was not bound on CM Cellulofine. Approximately 24.5% of the activity appeared as a single peak that was eluted from DEAE Cellulofine between 0.11 and 0.12 M NaCl. The chromatographic profile of the gel filtration is shown in figure 1B. The molecular weight of the inhibitor was estimated to be approximately 3200 by gel filtration on Sephadex G-25 using 3 protein markers (fig. 1B). The polyacrylamide gel electrophoresis pattern of the isolated inhibitor is shown in figure 1B. A single peptide chain was stained in the gel. The inhibition of papain and trypsin activity at different dilutions of the inhibitor is shown in figure 3; however, the equilibrium point does not correspond to complete inactivation.

Discussion. Proteinases are divided into 4 classes (serine, cysteine, carboxyl and metallo proteinases) on the basis of their catalytic mechanisms[5]. As shown in figure 2, the isolated fraction possesses inhibitory activity not only against papain which is a cysteine proteinase but also against trysin, a serine proteinase. The isolated fraction showed a single band in polyacrylamide gel electrophoresis; however, the fraction may possess plural proteinase inhibitors against papain and trypsin respectively. This is the first report on a fraction from a human source with an approximate mol.wt of 3200 which possesses inhibitory activity against papain and trypsin. These results may indicte that the isolated inhibitor is a new kind of inhibitor present in human plasma.

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