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ENDOGENOUS THIOL PROTEASE INHIBITOR FROM RAT LIVER

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SUMMARY: A thiol protease inhibitor was purified from rat liver by a rapid procedure involving heat treatment of the post-lysosomal fraction, affinity chromatography on papain-Sepharose 4B and Sephadex G-75. The purified inhibitor appeared homogeneous on sodium dodecyl sulfate electrophoresis. The inhibitor had a molecular weight of about 11,500 and consisted of three forms (pI 4.9, 5.2 and 5.6). The preparation inhibited thiol proteases, such as papain, cathepsin H, cathepsin B and cathepsin L, but not serine proteases (trypsin, chymotrypsin, mast cell protease and cathepsin A) or cathepsin D.

Three distinct thiol endopeptidases have been found in lysosomes of several tissues and designated as cathepsin B, cathepsin H and cathepsin L (1-6). Available information on lysosomal sulfhydryl proteases was recently reviewed by Barrett (7), Kirschke et al. (8) and Katunuma et al. (9).

There have been several reports on endogenous inhibitors of tissue thiol proteases. Inhibitors have been extracted and partially characterized from human skin (10), peritoneal monocytes (11), epidermis of new-born rats (12,13), rat muscle (14), rabbit lung (15) and bovine nasal cartlage (16). Recently, Lenney et al. (17) made extensive studies on thermostable inhibitors of cathepsin B and H in rat and human tissues and found separate inhibitors of cathepsin B and H with molecular weights of 14,000. However, these inhibitors have been only partially purified.

BANA; α-N-Benzoyl-DL-arginine-2-naphthylamide

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Lysosomal thiol proteases in rat liver have been studied the most, and so purification and characterization of the endogenous inhibitor from this organ may provide information on the control of intracellular proteolysis.

This paper reports a rapid method for purification of thiol protease inhibitor from rat liver by papain-affinity column chromatography and some properties of the purified inhibitor.

MATERIALS AND METHODS

Materials: α-N-Benzoyl-DL-arginine-2-naphthylamide, N-acetyl tyrosine ethyl ester, ovalbumin, trypsinogen (Type I), horse heart cytochrome c (Type VI), pancreatic trypsin inhibitor, glucagon, papain (Type III), trypsin (Type IV) and chymotrypsin were obtained from SIGMA. CNBr-activated Sepharose 4B and Sephadex G-75 were from Pharmacia Fine Chemicals. Diaflo PM 10 and YM 5 ultrafiltration membranes were from Amicon.

Preparation of the Papain Affinity Column: Activated CNBr Sepharose 4B (1 g) was swollen and washed as described in the pamphlet of the manufacturer. Papain was dissolved in 0.1 M sodium bicarbonate, pH 8.3, containing 0.5 M NaCl at a protein concentration of 4 mg/ml. The washed gel was suspended in 4 ml of ligand solution and shaken for 2 h at room temperature. About 90% of the ligand was bound to the gel, as judged from enzymatic determination of the remaining papain concentration in the reaction solution. To block excess active groups, the gel was reacted with 0.2 M glycine-NaOH buffer, pH 8.0, for 2 h. The gel was washed sucessively with 0.5 M NaCl in sodium bicarbonate, pH 8.3, 0.5 M sodium chloride in 0.1 M sodium acetate buffer, pH 4.0, and 20 mM potassium phosphate buffer, pH 6.0.

Gel Electrophoresis and Electrofocusing: Dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (18) in 15% gel. Gel electrofocusing was carried out by the method of Wrigley (19). Polyacrylamide gel (7.5%) containing 1% carrier ampholyte, pH 3 to 8, was used. After electrofocusing (300 V, 3 h), each gel was cut into 2-mm slices; each slice was extracted with 1.0 ml of water, and the pH and papain inhibitory activity of the extracts were measured.

Preparation of Proteases: Homogeneous preparations of cathepsin B and L from rat liver were obtained by the methods of Towatari et al. (5,6). Cathepsin H was prepared from rat liver by a modification of the method of Kirschke et al. (3).

Cathepsin D was partially purified from rat liver by the method of Yamamoto (20). Cathepsin A from rat liver was prepared by the method of Taylor and Tappel (21). Mast cell protease was obtained from rat small intestine by the method of Katunuma et al. (22).

Inhibitor Assay: Inhibitor was assayed under the conditions used for assay of protease activities, but with inhibitor in place of some of the buffer. The enzyme was incubated with the inhibitor for 5 min at 37°C and then the substrate. Inhibitor activities

with papain, cathepsin B, cathepsin H and trypsin were assayed by measuring BANA hydrolysis. For papain, cathepsin H or cathepsin B, an appropriate concentration of inhibitor was mixed with 0.1 N potassium phosphate buffer, pH 6.0, containing 4 mM cysteine, 2 mM EDTA and enzyme (1-2 μg/ml). For trypsin, it was mixed with 0.1 M Tris-HCl (pH 8.0) containing 0.01 M $CaCl_2$ and trypsin. BANA hydrolyzing activity was measured by the method of Barrett (23). Inhibition of cathepsin D was assayed by the method of Matsuda and Misaka (24) using acid-danatured bovine hemoglobin as a substrate. The inhibitions of chymotrypsin and mast cell protease from rat small intestine by the inhibitor were measured with acetyl tyrosine ethyl ester as substrate by the procedure of Jusic et al. (25). The method of Taylor and Tappel et al. (21) was used for assay of cathepsin A. One unit of inhibitor was defined as the amount which decreased the enzyme activity by one unit.

Protein Measurement: Protein was measured by the method of Lowry et al. (26) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification of Endogenous Thiol Protease Inhibitors from Rat Liver: Rat liver of male Wistar strain rats (280 g) was homogenized in a Waring blendor (National mixer MX140S) with an equal volume of 0.25 M sucrose, pH 7.0, for 30 s at top speed with ice-cooling. The homogenate was mixed with one volume of sucrose solution to give a 33% homogenate. The mixture was centrifuged at 12,000 xg for 20 min, and the post-lysosomal supernatant was adjusted to pH 3.0 with 6N HCl and heated at 80°C for 10 min. The mixture was cooled to 10°C and adjusted to pH 6.0, and the resulting precipitate was removed by centrifugation at 105,000 xg for 60 min. The supernatant was directly applied to papain-bound Sepharose 4B (1.8 x 2.8 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.0. The column was washed with 20 mM potassium phosphate buffer, pH 6.0, until all of non-adsorbed solvent was eluted. Then it was washed with 20 mM potassium phosphate buffer, pH 6.0 containing 1M NaCl. Inhibitors were eluted with 10 mM HCl. The fractions containing inhibitor were pooled, and adjusted to pH 5 with 1N NaOH and the resulting precipitate was removed by centrifugation at 12,000 xg for 10 min.

TABLE I
Purification of endogenous thiol protease inhibitor from rat liver. Inhibitory activity was assayed with papain and cathepsin B and H. Inhibitory activities on papain are shown.

Step	Protein	Total activity	Specific activity	Yield	Purification
	mg	U	U/mg protein	%	(-fold)
Post-lysosomal fraction	5,390	37.6	0.007	100	1
Heat treatment	783	25.4	0.033	68	4.6
Papain-Sepharose 4B	0.55	9.50	17.3	25	2,471
Sephadex G-75	0.24	5.25	21.7	14	3,100

The supernatant was concentrated by ultrafiltration on a YM 5 membrane. About 500-fold increase in specific activity was achieved in this step. The average recovery of inhibitors from the column was 25-40%. The solution was then chromatographed on a Sephadex G-75 column (1.6 x 95 cm) in 20 mM potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl. The small amount of high-molecular- weight inhibitor obtained was discarded and the pooled fractions from the major peak were concentrated by ultrafiltration on a YM 5 membrane. The yield of purified inhibitors was about 15% with 3100-fold increase of specific activity. A typical purification is summarized in Table I. The ratio of the specific activities of the inhibitor to papain, cathepsin H and cathepsin B remained constant throughout the purification, indicating that the same inhibitor in the liver extract inhibits papain, cathepsin B and cathepsin H.

As shown in Fig. 1, the inhibitor appeared homogeneous on sodium dodecyl sulfate gel electrophoresis. The molecular weight of the inhibitor was estimated as 11,500 with ovalbumin, trypsinogen, cytochrome c, pancreas trypsin inhibitor and glucagon as markers. The purified inhibitor showed multiple molecular forms when subjected to isoelectric focusing on poly-

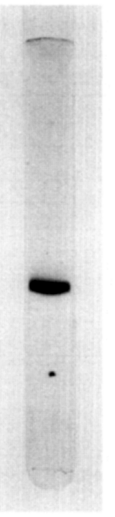

Fig. 1. Sodium dodecylsulfate polyacrylamide gel electrophoresis of endogenous thiol protease inhibitor from rat liver. 20 μg of inhibitor was denatured by heating at 90°C for 3 min in buffer with 1% sodium dodecyl sulfate. Electrophoresis was performed as described by Weber and Osborn (22) with 15% polyacrylamide gel. Gels were stained with Coomassie brilliant blue.

acrylamide gels. Three distinct peaks of inhibitor activity with isoelectric points of 4.9, 5.2 and 5.6 were resolved. The material in these protein bands inhibited not only papain but also cathepsin B and cathepsin H. The presence of multiple forms of thermostable inhibitors of cathepsin B and H from hog kidney and rat lung was described by Lenney et al. (17). They extracted the inhibitors from homogenates with water and so tissue organelles must have been disrupted under their conditions. In the present study, special attention was paid to the conditions for isolation of the inhibitor: we isolated the inhibitor from the post-lysosomal fraction by a rapid purification method, and we examined inhibitor purified without heat treatment. The post-lysosomal supernatant of rat liver was adjusted to pH 5.0 with HCl and centrifuged at 105,000 xg for 60 min, and then the inhibitor was purified from the supernatant by papain-affinity

TABLE II

Inhibition spectrum of endogenous thiol protease inhibitor from rat liver. Chymotrypsin and mast cell protease were assayed at pH 8.0 with N-acetyl-L-tyrosine ethyl ester as substrate. Cathepsin L and cathepsin D were assayed with azocasein and denatured bovine hemoglobin respectively, as substrates. Cathepsin A was assayed with Z-Glu-Phe as substrate (25). Other enzymes were assayed by the procedure of Barrett (27) with BANA as substrate. A concentration of 0.4 mg/ml of purified inhibitor was used.

Protease	Source	Inhibitor U/ml
Cathepsin H	rat liver	5.25
Cathepsin B	rat liver	1.70
Cathepsin L	rat liver	1.00
Papain	papaya fruit	7.70
Chymotrypsin	bovine pancreas	>0.1
Trypsin	bovine pancreas	>0.1
Mast cell protease	rat small intestine	>0.1
Cathepsin A	rat liver	>0.1
Cathepsin D	rat liver	>0.1

column and Sephadex G-75 column chromatographies as described above. The resulting preparation also separated into three forms (pI_3' of about 4.9, 5.2, 5.6) on polyacrylamide gel electrofocusing, indicating that these multiple forms of inhibitor are not artifacts formed by heat treatment. It is possible that these multiple forms of inhibitor are present in different cells of the liver.

Inhibition Spectrum: The effect of the highly purified inhibitor was tested on different proteases, and results are shown in Table II. All the proteases tested except cathepsin D and cathepsin A were homogeneous. The inhibitor had no activity on serine proteases, namely trypsin, chymotrypsin, mast cell protease, cathepsin A and cathepsin D, but inhibited the thiol proteases tested. It was most inhibitory on papain followed by cathepsin H. Titration of the activity showed that 1 mol of inhibitor reacted stoichiometrically with 1 mol of papain.

The kinetics of inhibition of papain by the inhibitor were studied using BANA as substrate, and results indicated that the inhibition was non-competitive and pseudo-irreversible.

Endogenous thiol protease inhibitors have been found in various tissues of rats, humans, and rabbits (10-17), and many of them are thermostable and have molecular weights of 11,000-14,000. It is difficult to compare these inhibitors with the present inhibitor because the enzymes used were not clearly defined and because the inhibitors were not completely purified. Recently, Hibino et al. (18) obtained a homogeneous inhibitor from epidermis of new-born rats which inhibited not only papain and ficin, but also BANA hydrolysing activity and cathepsin L from rat liver (31). Difference between this inhibitor and the present inhibitor is unclear at present. The production of antibody against two inhibitors will be helpful in answering this question.

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