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A PROTEASE FROM RAT INTESTINE

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Summary

Partially purified ribonuclease H from rat liver nuclei can be inactivated by a soluble fraction from rat intestine; this inactivation is restored by adding trypsin inhibitor, suggesting that the factor is a protease. A preparation has been isolated and purified to homogeneity. The molecular weight of the enzyme was estimated as 28 000 with an optimum pH of 8.0 and an isoelectric point at pH 4.5—4.7. The inactivating and proteolytic activities were observed in parallel throughout the purification procedures. Diisopropylphosphorofluoridate inhibited the protease activity. The protease inactivates deoxyribonuclease I, pyruvate kinase, and aldolase. From experiments with protease modifiers, it seems to be a serine protease of a trypsin-like nature.

Introduction

In the course of studies on the distribution of ribonuclease H (RNAase H) activity in various rat tissues, we detected no RNAase H activity in the cytosol fraction, as well as in homogenized intestine preparations. Activity was detected when soybean trypsin inhibitor was added to the cytosol from intestine fraction. The present paper describes the purification and some properties of the inactivating protein to RNAase H from rat intestine and evidence that it can be classed of a neutral, trypsin-like protease.

Materials and Methods

Materials

Synthetic substrates for proteases, the reagents for chemical modification, casein, bovine hemoglobin, phenylmethylsulfonyl fluoride, *N*-ethylmaleimide,

and elastin-orcein were from Sigma Chemical Co. Azocoll was from Calbiochem. Ampholyte was purchased from LKB. Protease inhibitors from cultured broth of Actionomycetes were supplied by Research Resources Program for Cancer Research, Ministry of Education, Science and Culture, Japan. DEAE-cellulose, Sephadex G-100 and Sephadex G-75 were obtained from Pharmacia. The enzymes purchased commercially were: pyruvate kinase from rabbit muscle, aldolase from rabbit muscle, hexokinase from yeast all from Sigma, DNAase from bovine pancreas, glucose-6-phosphate dehydrogenase from yeast, lactate dehydrogenase from rabbit muscle, alcohol dehydrogenase from horse liver, catalase from beef liver, alkaline phosphatase from calf intestine all from Boehringer, and RNAase from bovine pancreas from Worthington. Trypsin inhibitor from soybean and chymotrypsinogen were purchased from Boehringer and Sigma. All other reagents were of the best grade available.

Analytical methods

Polyacrylamide gel electrophoresis was carried out by the method of Davis [1]. 7.5% acrylamide gel were used and stained with 0.5% Coomassie brilliant blue for 1 h and then destained. Protein was determined by the method of Lowry et al. [2] using bovine serum albumin as standard.

Determination of molecular weight

Molecular weight of protease was determined by the method of Weber and Osborn [3] using 10% SDS-polyacrylamide gels with bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 45 000), chymotrypsinogen (M_r = 25 500) and cytochrome *c* (M_r = 12 300) as marker proteins. After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue for 4 h and then destained.

Molecular weight of the protein was also determined by gel filtration using a Sephadex G-100 (1.75 × 140 cm) column. 1 mg purified protease together with marker proteins: bovine serum albumin (dimer M_r = 136 000, monomer M_r = 68 000), ovalbumin (M_r = 45 000), chymotrypsinogen (M_r = 25 500), soybean trypsin inhibitor (M_r = 21 000), and myoglobin (M_r = 17 200) was applied to the column in a total volume of 1.0 ml. The column was equilibrated with 0.1 M Tris-HCl (pH 8.1), 0.3 M KCl. Marker proteins were identified by absorbance at 280 nm for bovine serum albumin and ovalbumin and at 412 nm for myoglobin.

Assay of the protease activity

Protease activities were based on the hydrolysis of casein and acid-denatured hemoglobin, according to Kunitz [4]. Azocoll was assayed according to Jackson and Matsuda [5]. The protease activity, using *N*-α-benzoyl-L-arginine-*p*-nitroanilide as substrate, was measured by the change in absorbance at 410 nm by the method Erlanger et al. [6].

Preparation and assay of substrate enzymes

RNAase H was purifed from rat liver nuclei as described previously [7].

The assay of RNAase H was based on the release, into the acid-soluble fraction, of radioactive material from RNA hybridized to DNA by the method described previously [7]. Inhibitor activity to RNAase H was assayed by

estimating the reduction of RNAase H activity when a standard amount of partially purified RNAase H from rat liver nuclei [8], with Mg^{2+}, was first mixed with a sample of the inhibitor and then incubated with substrate.

Activities of DNAase I, RNAase, and alkaline phosphatase were determined as described previously [9]. Activities of pyruvate kinase, aldolase, hexokinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase, and catalase were assayed by the method of Beisenherz et al. [10], Racker [11], Crane and Sols [12], Kornberg and Horecker [13], Stolzenbach [14], Bonnichsen and Brink [15], and Beers and Sizer [16], respectively.

Purification of neutral protease from rat intestine

Preparation of crude intestine extract. Intestines (about 170 g) were quickly excised from 25 male Wistar rats (150—200 g) killed by decapitation. To wash out pancreatic protease present in the lumen, the intestines were individually washed with six times 500 ml 0.15 M NaCl. No inactivating activity for RNAase H could be detected in the washings after the fifth wash. The intestines were cut into small pieces, rinsed twice with saline, and homogenized at low speed in 3 vols. 0.25 M sucrose with a Teflon-glass homogenizer. The homogenate was centrifuged at 15 000 × *g* for 15 min. The supernatants were used as starting material for enzyme purification.

Heat treatment. $(NH_4)_2SO_4$ was added to the supernatant to a final saturation of 20% and the suspension stirred for 10 min at 50°C. The activity remained in the supernatant after centrifugation at 15 000 × *g* for 10 min.

$(NH_4)_2SO_4$ fractionation. Further $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 55%, and stirring was continued for 20 min. The precipitate after centrifugation was dissolved in 0.1 M potassium phosphate buffer (pH 7.0), and dialyzed against the same buffer for several hours.

Calcium phosphate gel treatment. Calcium phosphate gel (15 mg/ml; 1 : 3, v/v) was added to the dialyzed solution, stirred for 5 min and centrifuged. The precipitate was washed twice with 0.1 M potassium phosphate buffer (pH 7.0). To the combined supernatant solid $(NH_4)_2SO_4$ to 55% saturation was slowly added with stirring. After stirring for further 20 min the suspension was centrifuged. The precipitate was dissolved in 10 mM Tris-HCl (pH 8.1) and dialyzed.

DEAE-cellulose column chromatography. The dialyzed solution was applied to a column of DEAE-cellulose (2.1 × 21.5 cm) previously equilibrated with 0.01 M Tris-HCl (pH 8.1). The elution was done with a linear gradient 0—1.5 M NaCl in 0.01 M Tris-HCl (pH 8.1) (5.0-ml fractions). Fractions were assayed for protein by measuring absorbance at 280 nm and for enzyme activity (Fig. 1). Two peaks of protease activities were eluted at 0.15 M and 0.5 M KCl. The major casein-hydrolyzing activity was observed in the first peak. The fractions from second peak, containing mostly *N*-α-benzoyl-L-arginine-*p*-nitroanilide hydrolytic activity, as well as RNAase H-inactivating activity, were pooled and concentrated with Amicon ultrafiltration apparatus (UM-10 membrane).

Sephadex G-75 gel filtration. The concentrated enzyme solution was then placed on a Sephadex G-75 column (1.75 × 137 cm) equilibrated with the 0.01 M Tris-HCl (pH 8.1), 0.5 M NaCl and eluted with the same buffer (flow rate, 10—15 ml/h; 2.6-ml fractions). The enzyme activity was eluted as a sharp

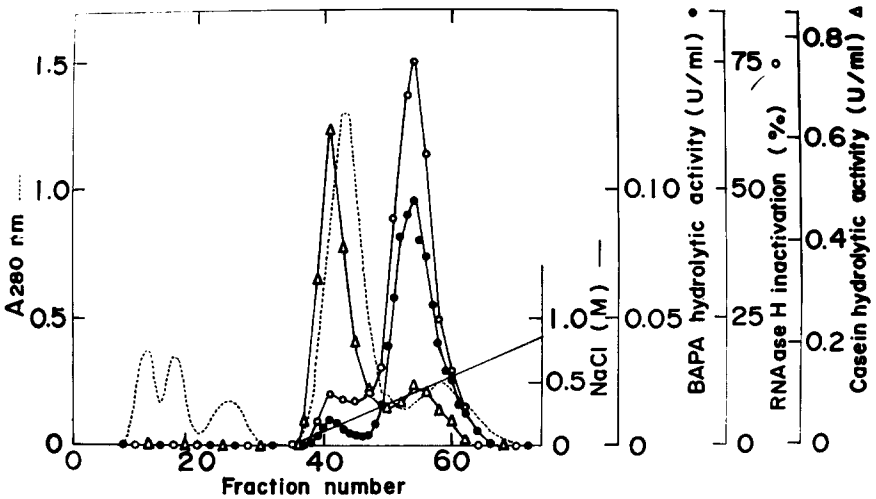

Fig. 1. DEAE-cellulose column chromatography of protease from rat intestine. The dialyzed solutions from calcium phosphate gel treatment were applied to the column of DEAE-cellulose, and eluted. Fractions were analyzed for protein at 280 nm (- - - - -), caseinolysis (△), protease activity by using *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BAPA) (●), and inhibitor activity to RNAase H (○).

peak in the retarded fraction (Fig. 2).

Second Sephadex G-75 gel filtration. Fractions containing protease activity were pooled and concentrated using an Amicon ultrafiltration apparatus (UM-10 membrane) and loaded onto a Sephadex G-75 column with the same conditions as above. The most active fractions were pooled and dialyzed against 70% satd. $(NH_4)_2SO_4$ in 0.1 M Tris-HCl (pH 8.1) at 0°C for overnight, and the

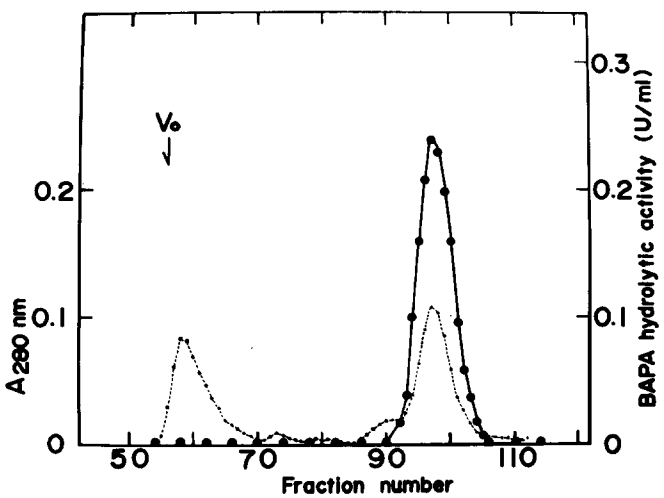

Fig. 2. Sephadex G-75 gel filtration of protease from rat intestine. The dialyzed preparations from DEAE-cellulose purification step were applied to the column of Sephadex G-75, and eluted. Fractions were analyzed for protein at 280 nm (- - - - -) and protease activity by using *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BAPA) (●).

precipitated protein was collected by centrifugation at 15 000 × *g* for 30 min. The precipitate was suspended in a small amount of 0.1 M Tris-HCl (pH 8.1), 0.15 M NaCl and then dialyzed against the same buffer for 3 h. The enzyme preparation could be stored at 0°C for more than 1 month without loss of activity.

Results

Protein to inactive RNAase H activity in intestine

Table I shows the distribution of RNAase H activity in various rat tissues. Activity was high in thymus, bone marrow and spleen, which undergo high levels of cell replication. In intestine, no activity of RNAase H was detectable in the enzyme preparations from the whole homogenate, because of the inhibitor activity present in this fraction. Certainly, addition of the enzyme fraction from intestine to partially purified RNAase H resulted in marked inhibition of RNAase H activity. No detectable activity was observed in the purified RNAase H, from other organs except intestine, to interfere with the RNAase H reaction, because mixtures of both fractions provided almost the expected values from the sum of the activities in the two fractions. When soy-bean trypsin inhibitor was added to the enzyme fraction from intestine, RNAase H activity was detected and showed maximal activity at 12 μg/ml trypsin inhibitor. Trypsin inhibitor did not stimulate RNAase H activity in other tissues. These results indicated that trypsin-like protease in rat intestine might be inactivating the RNAase H activity.

Table II shows the extent of purification and yields at different stages in the preparation. The purified protein had a specific activity of approx. 5.7 units activity/mg protein. Protease was purified 1325-fold with a 15% yield.

TABLE I

DISTRIBUTION OF RNAase H IN VARIOUS TISSUES FROM RAT

Rats were killed by decapitation and every tissue was rinsed with cold 0.25 M sucrose and freshly excised rat tissues were homogenized with 10 vols. of 0.25 M sucrose, 0.1 M Tris-HCl (pH 8.1), 3 mM $CaCl_2$, 0.5 M KCl and 5 mM 2-mcercaptoethanol and centrifuged at 105 000 × *g* for 1 h. All enzyme preparations were used after dialysis against 0.025 M Tris-HCl (pH 7.7), 5 mM 2-mercaptoethanol for 3 h. In each tissue, 100—200 μg protein was used as the enzyme source. Each preparation was prepared from the pooled tissues of 3—4 rats.

Tissues	RNAase H activity (pmol/mg protein)
Thymus	463
Bone marrow	371
Spleen	203
Testis	194
Lung	193
Liver	133
Cerebellum	105
Non-cerebellar part	90
Kidney	90
Heart	88
Small intestine	0
Plus trypsin inhibitor (12 μg/ml)	150

TABLE II

PURIFICATION OF PROTEASE FROM INTESTINE

One unit of enzyme activity was defined as being equivalent to the release of 1 μmol/min *p*-nitroaniline of incubation mixture. Specific activity is defined as units/mg. The incubation mixture containing 0.1 M Tris-HCl (pH 8.1), 1 mM *N*-α-benzoyl-L-arginine-*p*-nitroaniline and the enzyme preparations in a final volume of 1.0 ml was incubated at 37°C for 15 min. The reaction was stopped by the addition of 0.5 ml soy-bean trypsin inhibitor solution (0.1 mg/ml).

Fractions	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
1. Crude extract	8372	35.8	0.0043	100
2. Heat treatment with 20% $(NH_4)_2SO_4$ saturation	4550	31.8	0.0070	88.8
3. 55% $(NH_4)_2SO_4$ saturation	907	25.9	0.0286	72.3
4. Calcium phosphate treatment	294	20.6	0.07	57.5
5. DEAE-cellulose	9	10.4	1.16	29.1
6. 1st Sephadex G-75	2.2	7.5	3.41	20.9
7. 2nd Sephadex G-75	1.0	5.7	5.70	15.9

Characterization of protease from intestine

Molecular weight. The molecular weight was shown to be 28 000 when the enzymes from second Sephadex G-75 were passed through Sephadex G-100. When the peak eluted from second Sephadex G-75 chromatography was subjected to SDS electrophoresis, a single band was obtained, the calculated molecular weight of which was 28 000. Protease activity coincided with the band, when measured after cutting the gel into slices and extracting with 0.1 M potassium phosphate buffer (pH 8.0).

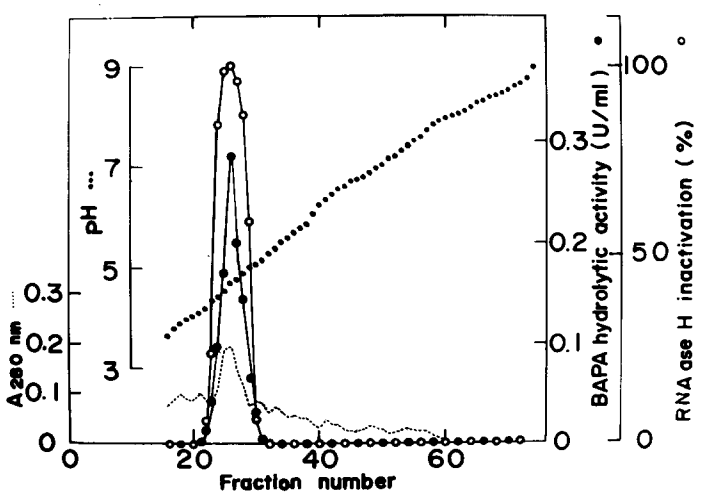

Fig. 3. Isoelectric focusing of trypsin-like protease from rat intestine. Concentrated enzyme preparations (20 mg protein) from second Sephadex G-75 gel filtration was applied onto a 110 cm LKB electrofocusing column in 2% (w/v) ampholines (pH 3—10). The voltage was maintained at 500 V for 32 h at 4°C (1.5-ml fraction; flow rate 32 ml/h). Activity and pH determination were performed immediately. ·····, pH; ○, inactivation of RNAase H activity, ●, protease activity assaying with *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BAPA) as substrate, - - - - - -, A_{280}.

TABLE III

SUBSTRATE SPECIFICITY OF PROTEASE FROM RAT INTESTINE

Various enzymes and synthetic substrates were incubated with the protease, and degree of inactivation or hydrolysis were compared. Values express inactivations or hydrolysis of these enzymes or synthetic substrates as percentages of that of RNAase H. Protease activity using these enzymes or synthetic substrates as substrate was measured.

Substrates	Sources	Inactivating or hydrolyzing activity (%)
RNAase H	Rat liver	100
DNAase I	Bovine pancreas	100
Pyruvate kinase	Rabbit muscle	93
Aldolase	Rabbit muscle	75
Hexokinase	Yeast	73
Glucose-6-phosphate dehydrogenase	Yeast	20
α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide [a]		5.22
α-*N*-Benzoyl-L-arginine amide [a]		1.27
Casein [b]		8.75
Hemoglobin [b]		9.62
Azocoll [c]		52.0
Elastin-orcein [d]		0

[a] μmol/min per mg protein.
[b] A_{280}/min per mg protein.
[c] A_{520}/min per mg protein.
[d] A_{578}/min per mg protein.

pH optimum. The pH dependence was examined by the use of *N*-α-benzoyl-L-arginine-*p*-nitroanilide as the substrate. The enzyme was shown to possess an optimum of pH around 8.0.

Isoelectric point. Subjecting the enzyme to isoelectric focusing, following by its location in the fractionated gradient, resulted in the activities being recorded within the pH range 4.5—4.7 (Fig. 3).

Substrate specificity. The protease rapidly inactivated RNAase H as well as DNAase I. Inactivation of other enzymes tested was shown in Table III. Some

TABLE IV

EFFECT OF SEVERAL PROTEASE INHIBITORS AND CHEMICAL REAGENTS OF THE ACTIVITY OF PROTEASE FROM INTESTINE

The protease was preincubated with the reagents at the concentrations listed.

Inhibitors and chemical reagents	Concentration	Activity remaining
Leupeptin	0.2 μg/ml	0 (I_{50}:0.04 μg/ml)
Antipain	0.2 μg/ml	0 (I_{50}:0.03 μg/ml)
Chymostatin	50 μg/ml	65.9
Pepstatin	50 μg/ml	100
Soy-bean trypsin inhibitor	0.5 μg/ml	0 (I_{50}:0.10 μg/ml)
N-Tosyl-L-lysinechloromethyl ketone	0.1 mM	0
Diisopropyl phosphorofluoridate	1.0 mM	6.8
Phenylmethylsulfonylfluoride	1.0 mM	8.4
Tosyl-L-phenylalaninechloromethyl ketone	0.1 mM	100
N-Ethylmaleimide	2.0 mM	100
EDTA	2.0 mM	100
Dithiothreitol	2.0 mM	100

enzymes tested, lactate dehydrogenase, alcohol dehydrogenase, catalse, alkaline phosphatase and RNAase, were not inactivated by the protease from intestine. The specificity for synthetic substrates was also examined. As shown in Table III, only substrates containing arginine were hydrolyzed. Some reagents for chemical modification tested, *N*-acetyl-L-tyrosine ethyl ester, *N*-acetyl-L-tryptophan ethyl ester, L-leucine-*p*-nitroanilide and succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide, were not hydrolyzed. The ability of this enzyme to digest general protein substrates, e.g. azocoll, casein and hemoglobin, is very similar to that of trypsin.

Effects of inhibitor and chemical reagents of the protease. The effects of several protease inhibitors obtained from culture broths of Actinomycetes on this protease were examined. As shown in Table IV, the protease was inhibited by these protease inhibitors, except pepstatin, according to increasing concentration of these inhibitors. The protease was inhibited by diisopropylphosphorofluoridate, and, thus, could be identified as serine protease. The inhibition of *N*-tosyl-L-lysinechloromethyl ketone indicates that this protease is trypsin like in nature.

Discussion

After the extraction of the inactivating protein to RNAase H from rat intestine, it was purified about 1300-fold with the yield of 15.9%. The protein moved as a single band in SDS-polyacrylamide gel electrophoresis with a molecular weight of 28 000. The parallel behavior of both the inactivating activity of RNAase H and the proteolytic activity clearly indicates that a single enzyme with properties of the inactivating protein was present in the crude extract.

These results demonstrate that the enzyme is active toward casein, hemoglobin and azocoll. This enzyme hydrolyzes synthetic amide substrates containing arginyl residue such as trypsin; it does not hydrolyze synthetic amide substrates for elastase, and shows no elastolytic activity. From its susceptibility of known modifiers of proteolytic enzymes, it appears to be a serine protease of trypsin-like nature. However, the protease from rat intestine is different from trypsin in the following points: trypsin has relatively low molecular weight (M_r = 23 300) and an alkaline isoelectric point, and is stabilized by Ca^{2+} at pH 2.5—3.0.

During the course of the purification of this protease, Beynon and Kay [17] reported that a neutral protease, which inactivate some endogenous enzymes, was solubilized from the intestinal smooth muscle of rats, and it appeared to be a trypsin-like protease. Its molecular weight is about 33 000 and it is stable over a narrow pH range. It seems likely that the enzyme described here may be similar to their enzyme, on the basis of the trypsin-like characteristics. The function of this intestine enzyme in living cells is now under investigation.

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