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Presence of a Thiol Protease in Regenerating Rat-Liver Nuclei

Partial Purification and Some Properties

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1. Nuclei of regenerating rat liver washed with Triton X-100 were found to contain a new protease. Since the enzymatic activity for degrading ribosomal proteins was inhibited *in vivo* by administration of E-64, a thiol protease inhibitor, the enzyme may participate in the degradation of newly synthesized ribosomal proteins and histones in regenerating rat liver nuclei as reported previously by us [*Biochem. Biophys. Res. Commun. 75*, 525–531 (1977)]. The optimum pH was 5.5.

2. The enzyme was extracted from washed nuclei and partially purified by gel filtration through Sepharose 6B. Its molecular weight was about 40000. A maximal activity of partially purified enzyme was observed in the presence of 1 mM EDTA and 2 mM dithiothreitol at pH 5.5. It was inhibited by thiol reagents, E-64, leupeptin and heavy metal ions. The enzyme degraded ribosomal proteins endoproteolytically and degraded most proteins tested as substrates, although liver cell sap proteins and serum albumin were less degraded than ribosomal proteins and histones. α-*N*-Benzoylarginine-β-naphthylamide and benzoylarginine amide were not hydrolyzed.

When the synthesis of rRNA in regenerating rat liver was selectively inhibited by administration of a low dose of actinomycin D *in vivo*, the synthesis of ribosomal proteins decreased only slightly [1]. Therefore, ribosomal proteins are synthesized in excess over rRNA. It was found that newly synthesized ribosomal proteins are degraded rapidly with a half-life of 20–40 min [1] in regenerating rat liver treated with actinomycin D. Recently we showed that the newly synthesized ribosomal proteins accumulated in liver nuclei after injection of E-64, a thiol protease inhibitor [2, 3], into actinomycin-D-treated rat liver [4]. Newly synthesized histones also accumulated in nuclei after E-64 treatment, regardless of actinomycin D pretreatment [4]. From these results we concluded that these two kinds of proteins which have not associated with the nascent rRNA or DNA are degraded post-translationally by thiol protease(s) in regenerating rat liver nuclei. A few studies on chromatin-bound protease(s) have been reported in rat liver; Bonner and colleagues isolated a 200000-M_r protease [5, 6] and Carter and Chae reported a protease which is active in 2 M NaCl and 5 M urea [7, 8]. Since these proteases are inhibited by diisopropylfluorophosphate (iPr_2P-F), a specific inhibitor of serine protease, they are different from the protease which were inhibited by administration of E-64 *in vivo* as described previously [4].

In this paper we describe the presence of a thiol protease in nuclei of regenerating rat liver which may participate in the degradation of newly synthesized ribosomal proteins and histones as reported previously [4]. Partial purification of the enzyme and some properties of it are also reported.

Abbreviations. iPr_2P-F, diisopropylfluorophosphate; BzArg-Nap, α-*N*-benzoylarginine-β-naphthylamide; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; $BzArgNH_2$, α-*N*-benzoylarginine amide; E-64, mixture of the optical isomers *N*-[*N*-(DL-3-*trans*carboxyiran-carbonyl)-L-leucyl]-agmatine; Leupeptin, *N*-acetylleucyl-leucyl-arginal.

Enzymes. Cathepsin B1 (EC 3.4.22.1); rhodanese (EC 2.8.1.1); glucose-6-phosphatase (EC 3.1.3.9); acid phosphatase (EC 3.1.3.2); deoxyribonuclease (EC 3.1.21.1).

MATERIALS AND METHODS

Chemicals

[^{3}H]Formaldehyde (85.0 Ci/mol) was obtained from New England Nuclear (Boston, MA). E-64, a thiol protease inhibitor, originally isolated from a mold *Aspergillus japonicus*, is a mixture of the optical isomers *N*-[*N*-(L and D-3-*trans*carboxyoxiran-carbonyl)-L-leucyl]-agmatine synthesized chemically by Hanada et al. (Taisho Pharmaceutical Co. Ltd, Japan). 3.0 mg E-64/100 g body weight was injected intraperitoneally into a partially hepatectomized rat.

Purification of Enzyme

Nuclei were prepared from regenerating rat liver at 18 h after the partial hepatectomy with a modification [9] of the method of Chauveau et al. [10]. For further purification of nuclei, the nuclear pellets were suspended in 0.25 M sucrose (about 4 mg protein/ml) and Triton X-100 was added at a final concentration of 0.1%. Nuclei were sedimented by centrifugation at $700 \times g$ for 5 min and the resulting pellet was washed twice with 0.25 M sucrose. The chromatin was purified as described previously [9]. To extract the enzyme, the chromatin was homogenized in 0.25 M sucrose by sonication with a Branson Sonifier for 15 s. After addition of 2.5 M NaCl, to give the final concentration of 0.7 M, the mixture was stirred at 4 °C for 3 h and then centrifuged at $59000 \times g$ for 30 min. The supernatant was applied to a column (3.6 × 35 cm) of Sepharose 6B equilibrated with 1 mM EDTA, 40 mM sodium acetate buffer, pH 5.5 and the column was eluted with the same buffer; 6-ml fractions were collected and a 0.5-ml sample of each second fraction was assayed for the enzyme activity. The elution of protein was monitered by measuring the turbidity in 15% trichloroacetic acid at 400 nm.

Enzyme Assay

To assay the activity of the partially purified protease, the reaction mixture contained in a final volume of 0.5 ml, 20 µg protease protein in 1 mM EDTA and 40 mM acetate buffer (pH 5.5), 2 mM dithiothreitol and 1.0 µg of ^{3}H-labeled ribosomal proteins (24000 counts/min). After the mixture was incubated at 37 °C for 16 h, 10 µl of rat liver cell sap (about 0.25 mg protein) as a carrier and 0.5 ml of 10% trichloroacetic acid were added. After centrifugation at $700 \times g$ for 10 min, the radioactivity of the supernatant was measured as described later.

Protease activities of nuclei and of subnuclear fractions were assayed in the same reaction mixture as described above. Incubation was carried out at 37 °C for 8 h. Buffers used are acetate buffers for pH 4–5.5, phosphate buffers for pH 6.0–7.5 and glycine buffers for pH 8.0–9.0. As the pH in the reaction mixture was decreased 0.5–1 by the addition of nuclei or the chromatin, it was readjusted with 0.5 M NaOH. The activity is expressed as the amount of ^{3}H radioactivity released into the trichloroacetic-acid-soluble fraction for a specified time period.

Assay of Marker Enzymes

To determine activities of marker enzymes, homogenate of regenerating rat livers in 0.25 M sucrose was fractionated into nuclei, light and heavy mitochondria, microsomes and cell sap fractions by a modification [11] of the method of De Duve et al. [12]. Total activities were determined by summing up those of all subcellular fractions. Rhodanese and glucose-6-phosphatase were assayed according to De Duve et al. [12] and acid phosphatase was according to Gianetto and De Duve [13]. Hydrolyzing activity for α-*N*-benzoyl-arginine-β-naphthylamide (BzArgNap) was assayed by the method of Barrett [14]. One unit of enzymes is that amount decomposing 1 µmol substrate/min at 37 °C.

Estimation of Molecular Weights of Hydrolyzed Ribosomal Proteins

^{3}H-labelled ribosomal proteins (2×10^4 counts min^{-1} μg^{-1}) were hydrolyzed in 0.5 ml of the incubation mixture containing 50 µg protein of partially purified protease, 40 mM acetate buffer, pH 5.5, 2 mM dithiothreitol and 1 mM EDTA at 37 °C for specified time periods. DNase (25 µg) was then added to the reaction mixture and the mixture was incubated for 10 min to degrade the chromatin structure. After incubation an equal volume (0.5 ml) of 8 M urea, 4 M LiCl solution was added and insoluble materials were sedimented by low-speed centrifugation. The resulting supernatant was applied to a column (1.4 × 25 cm) of Sephadex G-75 equilibrated with 6 M urea, 20 mM acetate buffer, pH 4.2 and the column was eluted with the same buffer. 1.5-ml fractions were collected and 0.5 ml of each fraction was used to measure radioactivity. Approximate molecular weights were estimated from the elution positions of cytochrome *c* (M_r = 11000), insulin (M_r = 6450) and its subunits (M_r = 3700 and 2750).

^{3}H-Labelling of Proteins in vitro

Protein substrates were labelled *in vitro* with [^{3}H]formaldehyde by reductive methylation [15], dialyzed overnight at 0 °C against 20 mM Tris-HCl buffer, pH 7.6 and then precipitated by adding 10 vol. acetone. After washing precipitates with acetone twice, they were dissolved in 6 M urea at a concentration of about 1 mg protein/ml. Specific radioactivities of the substrates ranged over 5200–20000 counts min^{-1} (µg protein)$^{-1}$.

Determination of Radioactivity and Protein

Radioactivity was measured using a toluene-based scintillater containing 30% Triton X-100 as described previously [4] and protein was determined by the method of Lowry et al. [16].

RESULTS

Purification of Nuclei

Since the proteolytic activity of regenerating rat liver nuclei prepared with 2.2 M sucrose as measured

by hydrolysis of ribosomal proteins is low, it is important to remove cytoplasmic contamination, especially lysosomes. For this purpose, washing with 0.5–1% Triton X-100 which removes the outer nuclear envelope is usually used. However such high concentrations of Triton were found to inhibit nuclear protease (see below) and nuclei were therefore washed with 0.1% Triton X-100. The purity of washed nuclei was examined by measuring activities of marker enzymes of major cellular organelles: rhodanese for mitochondria, glucose-6-phosphatase for microsome and acid phosphatase for lysosomes. Furthermore, to examine the extent of contamination by lysosomal cathepsins, BzArgNap-hydrolyzing activity which is mainly due to cathepsin B1 [14] and BzArgNap amidohydrolase [17], was measured.

Table 1 shows that washing of nuclei with 0.1% Triton X-100 removes lysosomal enzymes, acid phosphatase and BzArgNap-hydrolyzing enzymes almost completely, whereas it does not entirely eliminate mitochondrial and microsomal contaminations. However, since the extent of the contamination with these two organelles were very small and these organelles do not contain so many proteases as lysosomes, it can be concluded that, after Triton X-100 washing, nuclei are pure enough for investigation of nuclear proteases. They are designated 'washed nuclei'.

It must be added that the low BzArgNap-hydrolyzing activity detected in nuclei before Triton X-100 treatment was present in the nuclear sap fraction and may be due to contaminating lysosomal cathepsin B1, as judged from its optimal pH (6.2), the molecular weight (25000) estimated by filtration through Sepharose 6B and sensitivities to various protease inhibitors (data not shown).

A Thiol Protease Activity in Nuclei

To examine whether nuclei of regenerating rat liver contain any thiol protease, washed nuclei were incubated with ^{3}H-labeled ribosomal proteins at various pH (4–9) in the presence of dithiothreitol. In these experiments we used a minimal amount of highly labeled ribosomal proteins because of their low solubility in the absence of high concentrations of urea. Incubation was carried out at 37 °C in the presence or absence of phenylmethylsulfonyl fluoride ($PhMeSO_2F$), a serine-protease inhibitor. As shown in Fig. 1 A, ribosomal protein-degrading activity was

Table 1. *Activities of marker enzymes and BzArgNap-hydrolyzing activity in nuclei before and after washing with Triton X-100*
A portion of a homogenate of liver of partially hepatectomized rats was used to prepare nuclei with 2.2 M sucrose and enzyme activities were determined before and after washing with 0.1% Triton X-100. Another portion was used for subcellular fractionation and the total activities of marker enzymes in the liver were determined as described in Materials and Methods. Enzyme activities of nuclei were expressed as μmol substrate decomposed/min by nuclei equivalent to 1 g wer weight of the liver

Enzyme	Total activity	Activity in nuclei	
		−Triton	+Triton
	units/g liver (% total)		
Rhodanese	158.7	0.57 (0.36)	0.28 (0.18)
Acid phosphatase	4.34	0.013 (0.30)	0.003 (0.07)
Glucose-6-phosphatase	15.4	0.159 (1.1)	0.038 (0.25)
BzArgNap-hydrolyzing activity	0.172	0.0009 (0.52)	0.0 (0)
	mg		
Protein	136	7.2	4.2

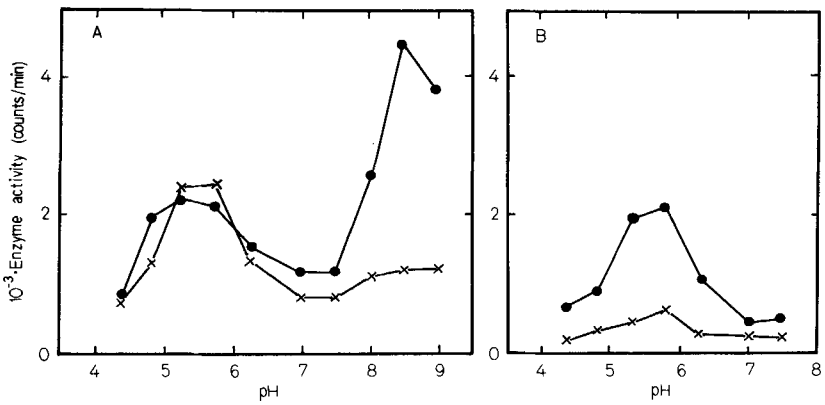

Fig. 1. *Ribosomal protein-degrading activity of regenerating rat liver nuclei at various pH and effects of $PhMeSO_2F$* in vitro *(A) and of E-64* in vivo *(B)*. (A) Triton-washed nuclei (0.2 mg protein) from regenerating rat liver were incubated with ^{3}H-labeled ribosomal proteins (2×10^4 counts min^{-1} μg^{-1}) at various pH with and without 4 mM $PhMeSO_2F$ at 37 °C for 8 h and protease activity was measured as described in Materials and Methods. (●) Activities in the absence of $PhMeSO_2F$; (×) activities in the presence of $PhMeSO_2F$. (B) Partially hepatectomized rats were injected intraperitoneally with 3 mg E-64/100 g body weight and killed 1 h after the injection. Protease activities of washed nuclei from E-64-treated and control rat livers were determined at various pH. (●) Activity of control nuclei without E-64 treatment; (×) activity of nuclei with E-64 treatment

observed at pH above 4.5, with two activity peaks at pH 5.5 (acetate buffer) and pH 8.5 (glycine buffer). Since only the former activity was resistant to $PhMeSO_2F$, the activity at pH 5.5 was thought to be due to a thiol protease. Complete absence of this activity in washed nuclei from partially hepatectomized rats pretreated with E-64 *in vivo* for 1 h (Fig. 1 B) confirms this deduction. Thus, it is very likely that nuclei of regenerating rat liver contain a thiol protease which may participate in degradation of newly synthesized ribosomal proteins and histones as reported in the previous report [4].

Intracellular and Intranuclear Distribution of Protease Activity for Hydrolyzing Ribosomal Proteins at pH 5.5

To examine the distribution of the protease activity, an aliquot of liver homogenate from partially hepatectomized rats was fractionated into subcellular fractions according to the method of De Duve et al. [12] and another aliquot was used for the preparation of purified nuclei. The acid phosphatase activity of these subcellular fractions and their capacity to hydrolyze ribosomal proteins at pH 5.5 were assayed. As shown in Fig. 2 A, B a significant amount of protease activity (2% of total activity) is found in washed nuclei which contain only traces of acid phosphatase activity.

The intracellular localization of this protease activity was then examined by using nuclear sap, extranucleolar chromatin and nucleoli. As shown in Fig. 2 C the protease was found mainly in the extranucleolar chromatin.

Partial Purification of the Nuclear Thiol Protease

About 60% of the enzyme activity for hydrolyzing ribosomal proteins at pH 5.5 was extracted from the chromatin fraction including nucleoli by 0.7 M NaCl at pH 7. This extract was then applied to a column of Sepharose 6B equilibrated with 1 mM EDTA, 40 mM sodium acetate buffer, pH 5.5 and eluted with the same buffer. As shown in Fig. 3, 80% of the enzyme activity was eluted a little behind the major protein peak and the remaining portion of the activity in the void volume probably associated with the fragmented chromatin.

The specific activity of the enzyme fraction thus obtained was about 15 times higher than that of the original chromatin fraction. A molecular weight of about 40000 was estimated from the results in Fig. 3. Dodecyl sulfate gel electrophoresis of this enzyme fraction showed two major protein bands and three minor bands with molecular weights between 25000 and 40000 (data not shown).

Effects of −SH Compounds, EDTA and Metal Ions

As shown in Table 2, the partially purified enzyme was activated by dithiothreitol and a maximal stimulation was observed at 2 mM in the presence of 1 mM EDTA. Cysteine showed similar effects but 2-mercaptoethanol was less effective (data not shown). All these were slightly inhibitory to the enzyme at concentrations higher than 5 mM. EDTA was not so stimulatory as dithiothreitol but a maximal stimulation was found at 1 and 2 mM EDTA in the presence of 2 mM dithiothreitol. The enzyme activity was markedly inhibited by heavy metal ions (Table 4).

Effects of Various Protease Inhibitors and Triton X-100

As shown in Table 3, the enzyme is very sensitive to the non-ionic detergent Triton X-100 and is inhibited by −SH reagents and thiol-protease inhibitors but

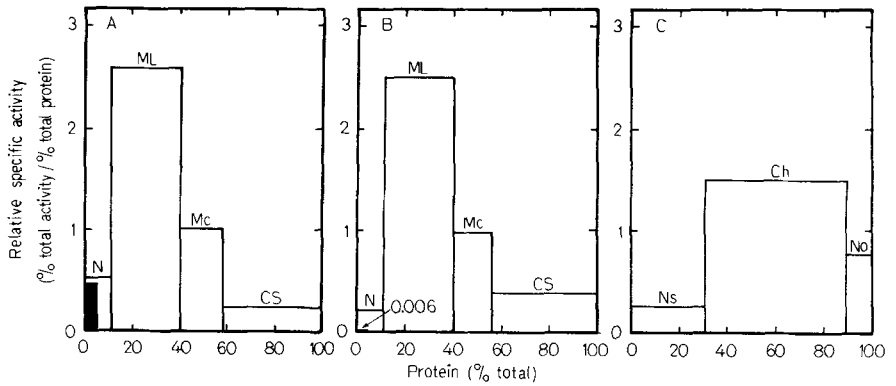

Fig. 2. *Intracellular distributions of protease activity (at pH 5.5) (A) and of acid phosphatase (B), and intranuclear distribution of the protease activity (C).* (A, B) Subcellular fractions were obtained by the method of De Duve et al. [12], and their protease activity at pH 5.5 (A) as well as acid phosphatase activity (B) were measured. N, nuclear fraction; ML, light and heavy mitochondrial fraction; Mc, microsomal fraction and CS, cell sap fraction. The solid bar in the nuclear fraction is the enzyme activity found in the washed nuclei. (C) Subnuclear fractions were obtained from Triton-washed nuclei and their protease activities at pH 5.5 were measured. Ns, nuclear sap fraction; Ch, extranucleolar chromatin fraction and No, nucleolar fraction

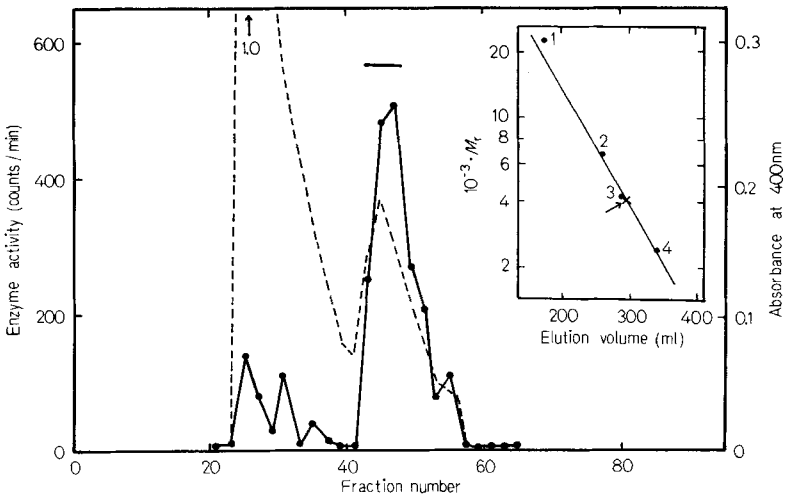

Fig. 3. *Partial purification of a nuclear protease with Sepharose 6B.* About 5 mg protein extracted from regenerating rat liver chromatin with 0.7 M NaCl was applied to a Sepharose 6B column equilibrated with 40 mM acetate buffer, pH 5.5, containing 1 mM EDTA and eluted with the same buffer. Proteolytic activity for ribosomal proteins was measured as described in Materials and Methods. Fractions shown with a bar were pooled and used as a partially purified protease. (----) Absorbance at 400 nm; (●) proteolytic activity. The insertion is the figure for calculation of the molecular weight of the partially purified protease. Standard proteins used are catalse (1), bovine serum albumin (2), ovalbumin (3) and insulin tetramer (4)

Table 2. *Effects of EDTA and dithiothreitol on the partially purified nuclear protease*
Before the assay the partially purified protease fraction was dialysed against 40 mM acetate buffer, pH 5.5, at 0 °C overnight to remove EDTA. The incubation was carried out as described in Materials and Methods

Concentration of		Enzyme activity	
Dithiothreitol	EDTA	measured	cf. maximum
mM		units/g	%
0	1	1542	41
1	1	3484	93
2	1	3733	100
5	1	3494	94
10	1	2570	69
2	0	3188	82
2	1	3894	100
2	2	3813	98
2	5	2645	68
2	10	3104	80

not by serine-protease inhibitors. The latter results suggest that it is a thiol protease.

Substrate Specificity

As shown in Table 4, the enzyme has a broad specificity for proteins although cell sap proteins and serum albumin were less degraded than ribosomal proteins, histones, hemoglobin and casein. Synthetic small-molecular-weight substrates, i.e. BzArgNap and $BzArgNH_2$ which are widely used as substrates for thiol proteases including some cathepsins, were not hydrolyzed by this enzyme.

To determine whether this enzyme is an endopeptidase or not, the size distribution of products of the proteolysis of ^3H-labeled ribosomal proteins was determined on gel filtration through Sephadex G-75. As shown in Fig. 4, fragments of molecular weight from 3000 to 8000 accumulated, depending on the incubation time periods. On the other hand, the radioactivity was very low in the region (fractions 30–32) in which free amino acid should be eluted. This result shows that the enzyme is an endopeptidase.

DISCUSSION

In the previous report we suggested a presence of an E-64-sensitive thiol protease in regenerating rat liver nuclei which participates in the degradation of newly synthesized ribosomal proteins and histones [4]. As the next step we attempted to show the presence of a thiol protease in rat liver nuclei. For this purpose it is important to exclude the contamination of nuclei with lysosomal proteases. Purified rat liver nuclei were further washed with 0.1% Triton X-100. Washed nuclei thus obtained were free from lysosomal enzymes, acid phosphatase and cathepsin B1, but contain a protease activity with an optimal pH at 5.5 and sensitive to E-64 *in vivo*. This result suggests that the protease is a true nuclear enzyme.

Since most of the protease activity was present in the chromatin fraction, we next attempted to purify this thiol enzyme from the chromatin fraction. The partially purified enzyme showed a maximal activity in the presence of 2 mM dithiothreitol and 1 mM EDTA. The enzyme is a thiol protease, because it is

Table 3. *Effect of various protease inhibitors, metal ions and Triton X-100 on the partially purified protease*
20 μg protein of partially purified nuclear protease was incubated with ^{3}H-labelled ribosomal proteins as described in Materials and Methods

Chemicals	Concentration	Activity	Metal ions	Concentration	Activity
	mM	% control		mM	% control
Iodoacetate	1	20	Fe^{2+}	2	4
Iodoacetamide	1	8	Hg^{2+}	2	18
N-Ethylmaleimide	1	28	Cu^{2+}	2	24
p-Chloromercuribenzoate	1	0			
	μg/ml				
Leupeptin	1	18			
E-64	1	20			
	mM				
$PhMeSO_2F$	3	86	Zn^{2+}	2	53
iPr_2P-F	3	98	Mn^{2+}	2	63
	%				
Triton X-100	0.3	70	Mg^{2+}	2	86
Triton X-100	0.5	28	Ca^{2+}	2	101

Table 4. *Substrate specificity of the partially purified nuclear protease*
All protein substrates were ^{3}H-labelled by reductive methylation and 1.5 μg of each substrate was incubated with partially purified nuclear protease as described in Materials and Methods. As the specific radioactivities of substrates were different from each other, enzyme activities were expressed as the weight of substrate released into the trichloroacetic-acid-soluble fraction during the incubation (i.e. ^{3}H in the trichloroacetic-acid-soluble fraction were divided by the specific radioactivity of the substrate)

Substrate	Enzyme activity	Relative activity
	μg	%
Ribosomal proteins	0.217	100
Serum albumin (bovine)	0.089	41
Casein	0.275	127
Cell sap protein	0.065	30
Hemoglobin	0.202	93
Histones	0.263	121

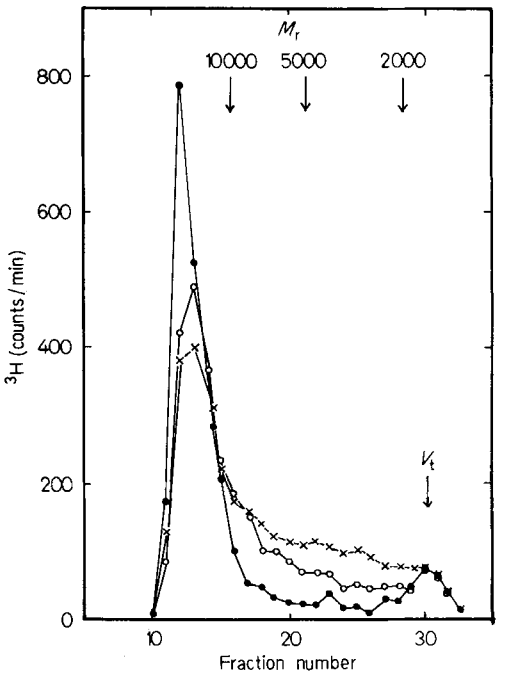

Fig. 4. *Sephadex G-75 elution pattern of the products of hydrolysis of ribosomal proteins by the partially purified enzyme.* The conditions for the hydrolysis and Sephadex G-75 column chromatography are described in Materials and Methods. V_t; the total volume of the column bed. (●) Ribosomal proteins before hydrolysis; (○) hydrolysis products after incubation for 3 h; (×) hydrolysis products after incubation for 6 h

inhibited by various thiol-protease inhibitors, but not by serine-protease inhibitors. This nuclear protease is different from any known cathepsins in its optimal pH, substrate specificity and molecular weight. The protease is most similar to cathepsin L [18] but it has a higher molecular weight than the latter (M_r = 25000).

Protease activity in calf thymus nuclei was first studied by several investigaters, using histones and chromatins as substrates [19–22]. Furlan and Jericijo [19] found two chromatin-bound proteases with pH optima at 4.4 and 7.8. They postulated that the neutral protease is a true nuclear enzyme but that the acid protease is a contaminant of cytoplasmic enzyme because cytoplasm has an acid protease with the same pH optimum, although they did not characterize these acid proteases. So far, there have not been any reports on the presence of thiol proteases in rat liver nuclei, although some studies on nuclear serine proteases were reported [5–8].

During the course of the preparation of this paper, we found a chromatin-bound acid protease from normal rat liver and partially purified it by gel filtration. The partially purified enzyme was also sensitive to −SH reagents and had almost the same molecular weight as the enzyme from regenerating rat liver described above. The results suggest the presence of the same thiol protease in normal rat liver nuclei. We are now undertaking the further purification and characterization of this enzyme.

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