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NOVEL PROTEASE BOUND WITH CHROMATINS IN NORMAL AND TUMOROUS TISSUES OF RATS

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SUMMARY: A protease capable of hydrolyzing casein with optimum pH 10 (alkaline protease), perhaps functional in hydrolysis of non-histone proteins and H1 histone, was found to exist at the state bound with chromatins of various normal and tumorous tissues of rats, in addition to the protease capable of hydrolyzing histone with optimum pH 8 (neutral protease). Alkaline protease was not observed in other subcellular fractions than nuclear fraction. It had approximately 18,000 daltons, and was chymotrypsin-like as inhibited by diisopropyl fluorophosphate, soybean trypsin inhibitor and Chymostatin. Its contents were significantly high in rapidly proliferating cells; Yoshida sarcoma>> Rhodamine sarcoma≥ AH 130≥ thymus> spleen>> kidney≥ liver>> brain.

Several researches have been carried out on a histone-hydrolyzing protease in nuclei or chromatins (1-7). In addition to the protease with optimum pH 8, we found a novel protease capable of hydrolyzing casein with optimum pH 10, which existed at the state bound with chromatins of various normal and tumorous tissues of rats. The present paper reports the properties of the protease present in nuclei, compared with those of the histone-hydrolyzing protease.

MATERIALS AND METHODS

Nuclei were prepared from various normal and tumorous tissues of adult, male albino rats of the Donryu strain (150-200 g) by the method of Chauveau *et al.*(8) as modified by Miyazaki *et al.*(9), and suspended in 0.2 mM EDTA (pH 7.0). An aliquot of the suspension was sonicated at 0-4°C for 1 min, and used as nuclear fraction. The remaining aliquot was supplemented with 0.15 M NaCl, followed by centrifugation at 10,000 x g for 30 min. The precipitate was suspended in 0.2 mM EDTA (pH 7.0) and used as chromatin fraction.

Rhodamine sarcoma, Yoshida sarcoma and ascites hepatoma AH 130 were used as tumors. These tumors were transplanded on the back of rats as described previously (9).

The other subcellular fractions (lysosomes, mitochondria, microsomes and supernatant) were centrifugally prepared by the method of Hogeboom (10) and Reid (11). Acid protease was measured as a marker enzyme of lysosomes with the use of denatured bovine hemoglobin as a substrate by the method of Mycek (12), cytochrome oxidase as a marker enzyme of mitochondria by the method of Wharton and Tzagoloff (13), and NADH-cytochrome reductase as a marker enzyme of microsome by the method of Phillips and Langdon (14).

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Protease activities were measured, using casein (Hammarsten grade, Merk) and calf thymus histone (Type II-S, Sigma) as substrates. The reaction mixture (1.0 ml) contained a substrate (5 mg), an appropreate buffer and an enzyme sample (0.1-0.3 mg protein in the cases with fractions of lysosomes, mitochondria, microsomes and supernatant, and 0.05-0.1 mg DNA in the cases with fractions of nuclei and chromatins). The buffers used were 20 mM GTA buffer (an equimolar mixture of 3,3-dimethyl glutaric acid, Tris and 2-amino-2-methyl-1,3-propanediol) (pH 3-10) and 0.1 M borate buffer (pH 7.5-12). The reaction was carried out at 37°C for 18 h, stopped by adding 1.0 ml of 10% trichloroacetic acid, and centrifuged at 40,000 x g for 30 min. The resulting supernatant was determined of protein content. One unit of protease was defined as the enzyme amount that could hydrolyze 1 mg protein/h.

Labeling of protease with [^{3}H]DFP (diisopropyl fluorophosphate) was performed by the method of Carter *et al.*(6). One milliliter of each subcellular fraction (2.5 mg protein) was incubated with 50 µl of 1 mM [^{3}H]DFP (1 mCi/ml, NEN) in 20 mM Tris-HCl (pH 8.0) or 20 mM borate buffer (pH 10) at 25°C for 18 h. The incubated solution was dialyzed overnight against 62.5 mM Tris-HCl (pH 6.8) containing 1% SDS (sodium dodecyl sulfate), 1% 2-mercaptoethanol and 5 M urea, and the dialyzed solution (150 µl) was then subjected to SDS-polyacrylamide gel disc electrophoresis by the method of Laemmli (15), in which 12.5% of acrylamide was used. After electrophoresis, the gel was sliced every 1.4 mm, and the gel slices were incubated in toluene scintillation cocktail containing 0.5% Omniflour (NEN) and 5% Protosol (NEN) at 37°C for 24 h. The molecular weight markers used were serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen A (26,000), myoglobin (17,000) and cytochrome *c* (12,000).

Protein and DNA contents were determined by the methods of Lowry *et al.* (16) and Schneider (17), using human serum albumin and calf thymus DNA as standards, respectively.

RESULTS AND DISCUSSION

Effect of pH on protease activities in chromatin fraction Histone and casein, if added, were hydrolyzed by the chromatin fraction from Rhodamine sarcoma (Fig. 1). The histone- and casein-hydrolyzing activities were optimum at pH 8 and 10, respectively, indicating the existence of two kinds of protease, neutral protease and alkaline protease. The nuclear fraction from the tumor showed practically the same pH-specific activity curves for histone hydrolysis and casein hydrolysis as the chromatin fraction, except for minor difference at acidic pH range. In nuclear fractions from various normal tissues and other tumorous tissues, pH-specific activity curves for histone hydrolysis and those for casein hydrolysis were essentially the same as in the figure, except that the histone- and casein-hydrolyzing activities varied independently from one kind of tissues to another (Fig. 2). Alkaline protease was more abundant than neutral protease in the nuclear fractions from all kinds of tissues examined.

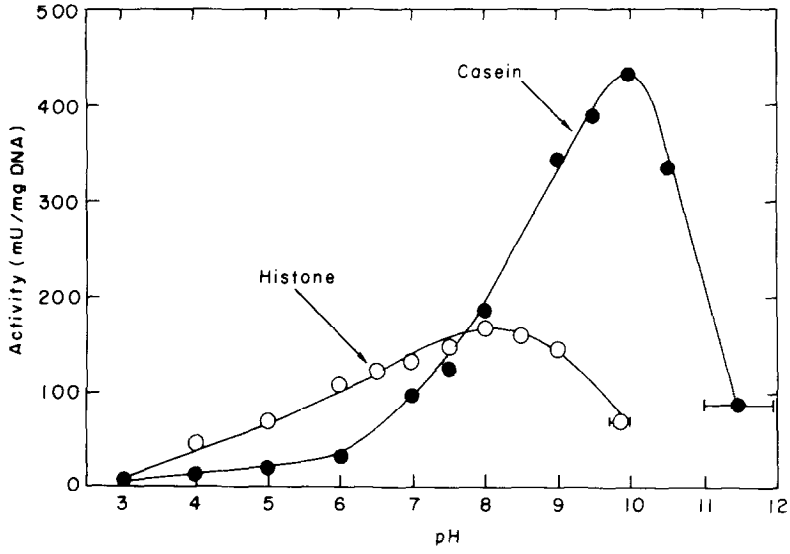

Fig. 1. Effect of pH on histone- and casein-hydrolyzing activities in chromatin fraction from Rhodamine sarcoma. The pH shifts during reactions are shown by the horizontal bars.

In addition, its specific activity was remarkably high in nuclear fractions from rapidly proliferating tissues in the order, Yoshida sarcoma (Ys)>> Rhodamine sarcoma (Rs)≥ AH 130≥ thymus (T)> spleen (S)>> kidney (K)≥ liver (L)>> brain (B).

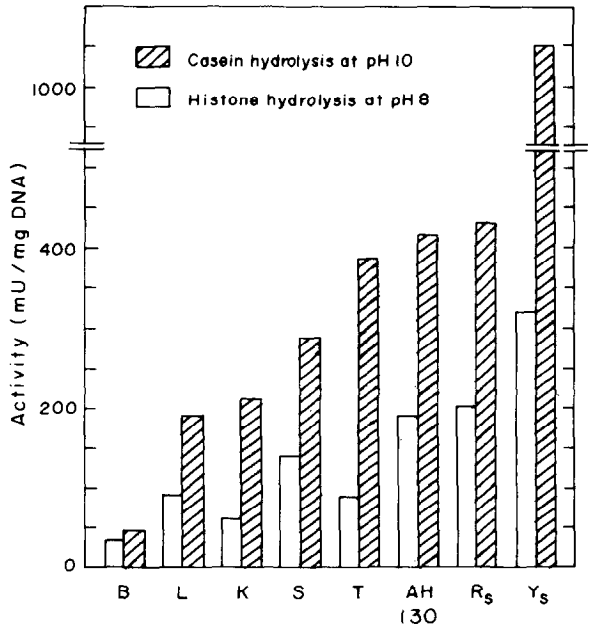

Fig. 2. Comparison of histone-hydrolyzing activity at pH 8 and casein-hydrolyzing activity at pH 10 in nuclear fractions from various tissues of rats.

Table I. Effects of various inhibitors on histone-hydrolyzing activity at pH 8 and casein-hydrolyzing activity at pH 10 in chromatin fraction from Rhodamine sarcoma

Inhibitor	Concentration	Relative hydrolyzing activity(%)	
		Histone at pH 8	Casein at pH 10
No addition		(100)	(100)
DFP	1 mM	14	43
	5 mM	0	15
Phenylmethylsulfonyl fluoride	1 mM	40	80
	5 mM	9	70
Soybean trypsin inhibitor	100 µg/ml	41	44
Chymostatin	1 µg/ml	20	32
	10 µg/ml	9	6
Leupeptin	10 µg/ml	91	100
Antipain	10 µg/ml	81	100
Pepstatin	10 µg/ml	60	84
p-Chloromercuri-benzoate	1 mM	69	87
	5 mM	16	63
EDTA	5 mM	84	84

When the nuclear fractions from Rhodamine sarcoma were incubated at pH 8 and 10 without external addition of substrate for various lengths of time and then the proteins present in the incubated nuclear fractions were analyzed by SDS-polyacrylamide gel disc electrophoresis, it was found that all kinds of the non-histone proteins and H1 histone, but not the other kinds of histones, were hydrolyzed at pH 10 two to three times as fast as at pH 8, although the latter histones were hydrolyzed at pH 8 (data not shown).

Effects of inhibitors on protease activities in chromatin fraction Effects of several inhibitors on histone-hydrolyzing activity at pH 8 and casein-hydrolyzing activity at pH 10 in the chromatin fraction from Rhodamine sarcoma are summarized in Table I. Both activities were effectively inhibited by DFP,

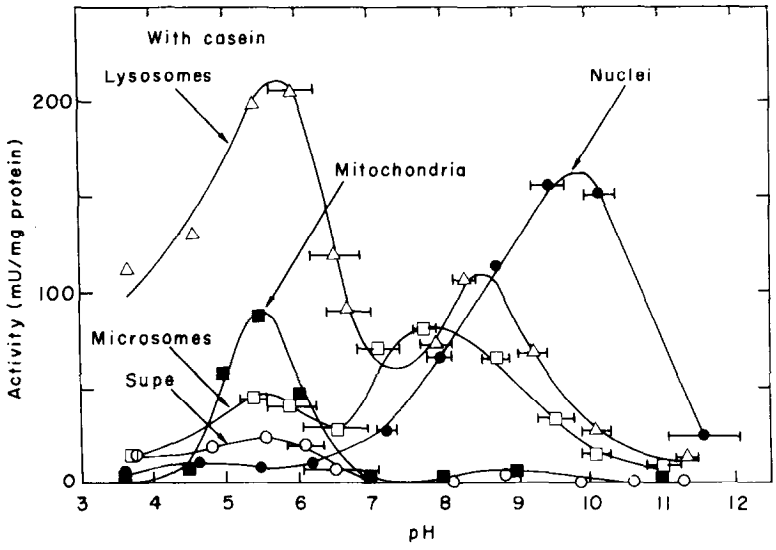

Fig. 3. Effect of pH on casein-hydrolyzing activities in various subcellular fractions from Rhodamine sarcoma.

soybean trypsin inhibitor and Chymostatin (18)(for chymotrypsin). Phenylmethylsulfonyl fluoride and *p*-chloromercuribenzoate were more effective to the histone-hydrolyzing activity than to the casein-hydrolyzing activity. Leupeptin and Antipain (18)(for trypsin and papain), Pepstatin (18)(for pepsin and cathepsin D) and EDTA were not effective to both activities. These results indicate that both neutral and alkaline proteases were chymotrypsin-like.

Intracellular distribution of histone- and casein-hydrolyzing activities

Heinrich *et al.*(19) detected histone-hydrolyzing activity in mitochondrial fraction from rat liver, whereas Paik and Lee (20) in microsomal fraction from rat kidney. Haas *et al.*(21) reported that the histone-hydrolyzing activity in mitochondrial inner membrane is originated from mast cells. Using several subcellular fractions from Rhodamine sarcoma, pH-activity curve for casein hydrolysis, histone-hydrolyzing activity at pH 8 and casein-hydrolyzing activity at pH 10 were measured. The casein-hydrolyzing activity with optimum pH 10 was hardly detectable in the other fractions than the nuclear fraction (Fig. 3). On the other hand, the histone-hydrolyzing activity around pH 8 was detected in the nuclear, lysosomal and microsomal fractions, but hardly in the

Table II. Distribution of marker enzyme activities, histone-hydrolyzing activity at pH 8 and casein-hydrolyzing activity at pH 10 to various subcellular fractions from Rhodamine sarcoma

Fraction	Acid Protease (mU/mg protein)	Cytochrome oxidase (mU/mg protein)	NADH-cytochrome reductase (mU/mg protein)	Hydrolysis (mU/mg protein)	
				Histone at pH 8	Casein at pH 10
Nuclei	37	N.D.[5]	N.D.	72	160
Lysosomes[1]	877	68	N.D.	60	29
Mitochondria[2]	616	847	N.D.	N.D.	N.D.
Microsomes[3]	336	34	29	27	13
Supernatant[4]	61	N.D.	N.D.	N.D.	N.D.

[1] fraction with d=1.13 in sucrose density gradient centrifugation; [2] fraction with d=1.23 in sucrose density gradient centrifugation; [3] precipitate in centrifugation at 100,000xg for 60 min; [4] supernatant in centrifugation at 100,000xg for 60 min; [5] not detectable.

mitochondrial or supernatant fraction. As judged from the specific activities of acid protease, cytochrome oxidase and NADH-cytochrome reductase in the nuclear fraction (Table II), it seems likely that the nuclear fraction was contaminated with lysosomes to a small extent (approximately 4% of the specific activity of acid protease in the lysosomal fraction), but not with mitochondria or microsomes. This indicates that the casein-hydrolyzing activity at the neutral and alkaline pH ranges were mostly attributable to nuclei themselves.

Labeling of protease in subcellular fractions with [^{3}H]DFP The various subcellular fractions from Rhodamine sarcoma were individually incubated with 50 μM [^{3}H]DFP at pH 8 and 10, and the radioactive proteins thus formed were analyzed by SDS-polyacrylamide gel disc electrophoresis (Fig. 4). In the nuclear fraction (N) incubated at pH 8, the major radioactive protein species had a molecular weight of approximately 27,000 (27,000 d-protein), in accordance with Carter *et al.*(6), who reported that the major [^{3}H]DFP-binding protein species in the chromatins from rat liver has a molecular weight of approximately 25,000, and that the protease activity of the protein is measurable

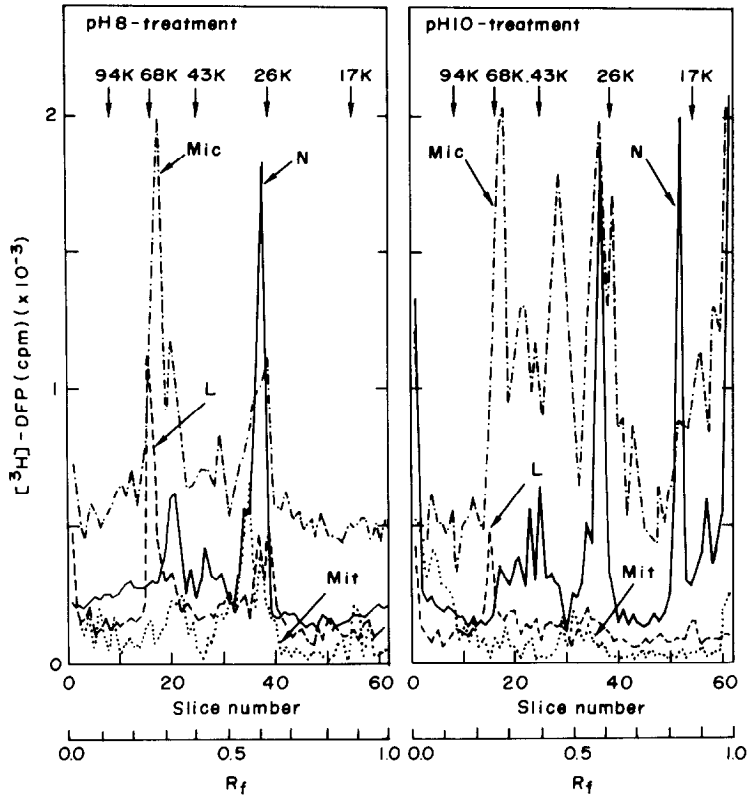

Fig. 4. Radioactivity profiles in SDS-polyacrylamide gel disc electrophoresis of proteins present in various subcellular fractions previously treated with [^{3}H]DFP. In order to attain a high specific radioactivity, the concentration of DFP in this experiment was remarkably lower than those in Table I.

with the use of histone as a substrate. On the other hand, when the nuclear fraction was incubated with 50 μM [^{3}H]DFP at pH 10, the other protein of approximately 18,000 daltons was also labeled, its total radioactivity comparable to that of 27,000 d-protein either at pH 8 or at pH 10. 18,000 d-protein was detected only in the nuclear fraction, whereas 27,000 d-protein was detected in the nuclear fraction and microsomal fraction (Mic), but not in the mitochondrial fraction (Mit) or lysosomal fraction (L).

The results described above imply that alkaline protease had a molecular weight of approximately 18,000 as protein species and was specifically localized in chromatins, whereas neutral protease was distributed to both chromatins and microsomes. It seems likely that in nuclei, neutral protease could function in the hydrolysis of H2A, H2B, H3 and H4 histones, whereas alkaline protease

could play a role in the hydrolysis of H1 histone and all of the non-histone proteins.

Recently, we have solubilized by 0.2 M H_3PO_4 and purified both alakline and neutral proteases to a nearly homogenous purity by the procedure involving successive affinity chromatographies on columns of Sepharoses bound with soybean trypsin inhibitor, casein and histone, and characterized their properties. These results have been presented (22) and will be published.

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