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PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASE AND NEUTRAL PROTEASE FROM CHROMATIN OF RATS

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It was previously reported that, in addition to a known chymotrypsin-like protease capable of hydrolyzing histones with an optimum pH of 8 (neutral protease), another protease is bound to the chromatin of various rat tissues and in situ hydrolyzes casein more quickly than histones with an optimum pH of 10 (alkaline protease). In the present study, the alkaline protease was purified 14 00-fold to approx. 75% purity from the chromatin of Rhodamine sarcoma. This tumor contains both proteases at higher levels than normal tissues. For purification, affinity columns of Sepharose with bound soybean trypsin inhibitor, casein and histones were successively used. Also, the neutral protease was purified 920-fold to an apparently homogeneous state by affinity chromatography on a Sepharose column with bound soybean trypsin inhibitor under conditions, in which an excess amount of the enzyme was applied on the column so that part of the enzyme would pass through the column without adsorption and the enzyme thus adsorbed was then eluted. The purified alkaline and neutral proteases had molecular weights of approx. 18 000 and 27 000, respectively, and isoelectric points of approx. 11. The former enzyme hydrolyzed casein (100) in preference to histones (18) with an optimum pH of 9.5, whereas the latter enzyme preferred histones (100) to casein (32) with an optimum pH of 8. Their actions against other proteins and synthetic substrates were also studied.

Introduction

Since Maver and Greco [1] reported that chromatin fractions from calf thymus show a cathepsin-like protease activity, many studies have been made on the proteases present in fractions of chromatin and nuclei [2–12]. These proteases are classified into three groups, which have optimum pH values of 4.0–4.5 (acid protease) [2,3,12], 7.0–8.5 (neutral protease) [3–12] and approx. 10 (alkaline protease) [12].

It is considered that non-histone chromosomal proteins play important roles in gene expression in eukaryote cells [13]. It has been reported that, in vivo, non-histone chromosomal proteins are metabolized at significantly faster rates than histones [14,15]. Recently, Hagiwara et al. [12] found that, in sonicated nuclei, non-histone chromosomal proteins and histone H1 are more rapidly hydrolyzed at pH 10 than at pH 8, whereas the other histones (H2A, H2B, H3 and H4) are hydrolyzed at pH 8, but hardly ever at pH 10.

The present study deals with the purification and characterization of alkaline and neutral proteases from the chromatin of Rhodamine sarcoma, a tumor specific to rats.

Materials and Methods

Materials. The molecular weight markers used were blue dextran 2000 (M_r $2 \cdot 10^6$), phosphorylase *a* (94 000), serum albumin (68 000), ovalbumin (43 000), chymotrypsinogen A (26 000), myoglobin (17 000) and cytochrome *c* (12 000). Soybean

trypsin inhibitor, diisopropylfluorophosphate and calf thymus histones (type II-S, type V-S and type VIII-S) were purchased from Sigma Chemical Co., St. Louis, MO; casein (Hammarsten grade) was from Merck, Darmstadt; collodion bag (SM 13200) was from Sartorious Membrane Filter Co., Göttingen; acetyl-L-phenylalanine ethyl ester, acetyl-L-tyrosine ethyl ester, benzoyl-L-arginine ethyl ester and other synthetic substrates were from Protein Research Foundation, Osaka. [1-^{3}H(n)]diisopropylfluorophosphate (specific activity, 1 Ci/mmol; 1 Ci/l) was obtained from New England Nuclear, Boston.

Animals and tumor. Adult male albino rats of the Donryu strain were used. Rhodamine sarcoma cells were transplanted into the subcutaneous region in the backs of rats, as described previously [16].

Preparation of chromatin. Nuclei were prepared from Rhodamine sarcoma cells by the method described previously [17] with some modifications. The tumor tissue was homogenized in 10 vol. (v/w) 10 mM Tris-HCl/0.25 M sucrose/3 mM $CaCl_2$ (pH 7.5) and then filtered through two layers of cheesecloth. The resulting filtrate was centrifuged at 1500 × *g* for 10 min. The precipitate thus obtained was used as the starting material for preparation of crude and pure chromatin.

For preparation of crude chromatin, the precipitate was washed once with 5 vol. (v/w) 10 mM Tris-HCl/1% Triton X-100/0.25 M sucrose/3 mM $CaCl_2$ (pH 7.5) and then twice with 5 vol. (v/w) 0.15 M NaCl, followed by centrifugation. The precipitate was suspended in water at approx. 2 mg DNA/ml and sonicated in a Sonifier (model 350, Branson Sonic Power Co., Dunbury, CT) for 1 min at 0–4°C. The sonicated suspension was cooled to 0°C, mixed with 4 vol. (v/v) cold acetone (–20°C) and stirred at about 0°C for 1 min, followed by filtration through filter paper. The resulting material was washed with 1 vol. (v/v) cold acetone and then dried at 4°C under vacuum (crude chromatin).

For preparation of pure chromatin, the precipitate was homogeneized in 10 vol. (v/w) 10 mM Tris-HCl/2.2 M sucrose/3 mM $CaCl_2$ (pH 7.5) and centrifuged at 50000 × *g* for 1 h. The resulting precipitate was washed as above. It was always found under microscopic observation that the washed precipitate was composed of pure nuclei. It was then suspended in water at approx. 2 mg DNA/ml and stored at –20°C until use. The frozen suspension was thawed and sonicated as described above. The sonicated suspension was supplemented with 1 vol. (v/v) 0.3 M NaCl, followed by centrifugation at 10000 × *g* for 10 min. The resulting precipitate was suspended in water at approx. 2 mg DNA/ml (pure chromatin).

Preparation of adsorbents for affinity chromatography. Sepharose 4B with bound soybean trypsin inhibitor, casein and histones were prepared by the method of Axèn et al. [18], using CNBr-activated Sepharose 4B. After the coupling reactions, the gel beads were washed with water, suspended in 0.1 M Tris-HCl (pH 8.0) and stirred overnight at 4°C, followed by filtration. These Sepharose-ligand preparations contained 2 mg soybean trypsin inhibitor, 5 mg casein and 7 mg histones per ml of packed gel beads.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out at 20°C in a temperature-controlled apparatus (model E-IE 12–15) (Koike Precision Instruments, Kawasaki) by the method of Laemmli [19], using 12.5% acrylamide and glass columns (0.5 × 13 cm). The staining and destaining were carried out by the method of Miyazaki et al. [17]. In some cases, the gels were cut into 1.4-mm slices and the slices were measured for radioactivity.

Affinity labeling of proteases with [^{3}H]diisopropylfluorophosphate. Labeling of proteases with [^{3}H]diisopropylfluorophosphate was carried out by the method of Carter et al. [10]. 1 ml sample in 20 mM Tris-HCl (pH 8.0) was mixed with 50 μl [^{3}H]diisopropylfluorophosphate. The resulting mixture contained 50 μM (50 μCi) radioactive reagent. It was then incubated at 25°C for 16 h and dialyzed against 62.5 mM Tris-HCl/1% SDS/1% 2-mercaptoethanol/5 M urea (pH 6.8) at 25°C for 16 h. The resulting solution (150 μl) was subjected to SDS-polyacrylamide gel electrophoresis. Radioactivity was measured by the method described previously [12], using a Packard liquid scintillation spectrometer (model 2002).

Isoelectric electrophoresis. Isoelectric electrophoresis (fractionation) with Ampholine carrier-ampholytes was carried out by the method of Vesterberg and Svensson [20], using a 110-ml isoelectric focusing column.

Activity assay of proteases. Protease activities with casein, histones and other polypeptides were

measured by the method described previously [12]. With crude enzyme samples, the hydrolytic activity of casein (Hammarsten grade) at pH 9.5–10 and that of histones (type II-S) at pH 8 were regarded as mostly due to alkaline protease and neutral protease, respectively. Esterase activities with synthetic substrates were measured by the method of Jusic et al. [21].

Determination of protein and DNA. Protein contents were determined by the method of Lowry et al. [22], using human serum albumin as standard, and DNA contents by the method of Schneider [23], using calf thymus DNA as standard.

Measurement of absorbance. The absorbances of solutions were measured by a Cary spectrophotometer (model 17) and those at 550 nm of stained protein bands in gels by a Shimadzu dual-wavelength thin layer chromatography scanner (model CS-910).

Purification of alkaline protease. Step 1. Solubilization: In each experiment, the crude chromatin preparation obtained from 200 g wet weight of Rhodamine sarcoma was suspended at 2 mg DNA in 10 mM Tris-HCl (pH 7.5). Usually, approx. 200 ml of the buffer was required. The resulting suspension was sonicated at 0–4°C for 2 min. The sonicated suspension was supplemented with 0.25 vol. 2.5 M NaCl and stirred at 4°C for 1 h. The stirred suspension was then mixed with 0.25 vol. 1 M H_3PO_4 to bring the pH to 1.5 and stirred at 4°C for 15 min, followed by centrifugation at 10000 $\times g$ for 15 min. The resulting supernatant was immediately adjusted to pH 8 by adding 2 M NaOH (acid extract).

Step 2. $(NH_4)_2SO_4$ fractionation: The acid extract was supplemented with $(NH_4)_2SO_4$ up to 80% saturation, followed by centrifugation at 10000 $\times g$ for 15 min to collect the precipitate. The resulting precipitate was dissolved in a small volume of 30 mM sodium citrate-HCl/50 mM NaCl (pH 6.5), and then dialyzed against an excess volume of the same buffer containing 50 mM NaCl at 4°C for 6 h, followed by centrifugation at 30000 $\times g$ for 15 min. The resulting supernatant was collected ($(NH_4)_2SO_4$ preparation).

Step 3. Affinity chromatography on soybean trypsin inhibitor-Sepharose column: The $(NH_4)_2SO_4$ preparation was applied on a soybean trypsin inhibitor-Sepharose column, previously equilibrated with 30 mM sodium citrate-HCl/50 mM NaCl (pH 5.0) (Fig. 1). Both the alkaline and neutral proteases were adsorbed on the column and eluted in the same fractions with 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 1.5) (soybean trypsin inhibitor-Sepharose eluate).

Step 4. Affinity chromatography on casein-Sepharose column: The soybean trypsin inhibitor-Sepharose eluate showing alkaline and neutral protease activities was adjusted to pH 5.5 with 2 M NaOH and applied on a casein-Sepharose column, previously equilibrated with 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 5.5) (Fig. 2). Alkaline protease was adsorbed to a slightly higher extent than neutral protease and both proteases thus adsorbed were eluted by 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 4.0) (casein-Sepharose eluate).

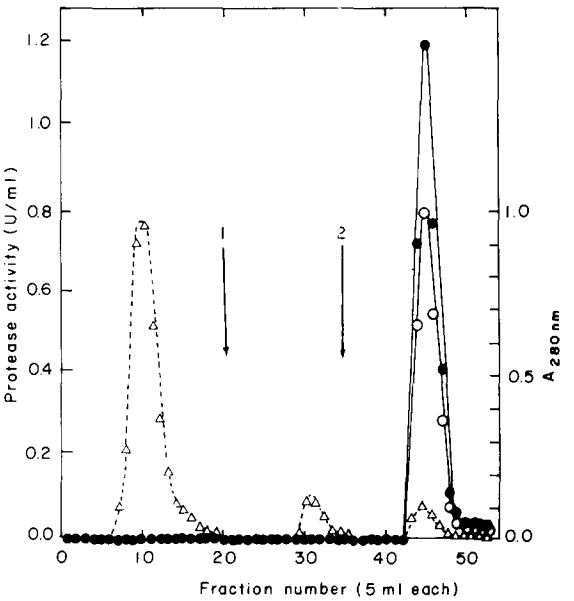

Fig. 1. Affinity chromatography of acid extract on soybean trypsin inhibitor-Sepharose column. The $(NH_4)_2SO_4$ preparation (7 ml), which contained 43.8 mg protein, was applied on a soybean trypsin inhibitor-Sepharose column (1.6 X 20 cm). The charged column was developed successively with 50 mM sodium acetate-HCl/50 mM NaCl (pH 1.5) (arrow 1) and with 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 1.5) (arrow 2), at a flow rate of 1 ml/min. Of the resulting fractions, those from No. 43 to No. 46 were immediately mixed, adjusted to pH 5.5 with 2 M NaOH, and stored at −90°C until use (soybean trypsin inhibitor-Sepharose eluate). ●———●, casein-hydrolysis at pH 10; ○———○, histone-hydrolysis at pH 8; △———△, A_{280nm}.

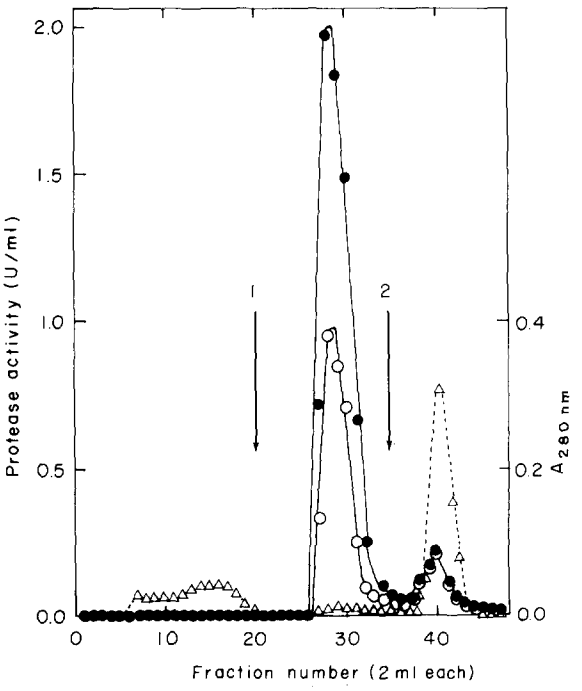

Fig. 2. Affinity chromatography of soybean trypsin inhibitor-Sepharose eluate on casein-Sepharose column. The soybean trypsin inhibitor-Sepharose eluate (20 ml), which contained 4 mg protein, was applied on a casein-Sepharose column (1.7 X 4 cm). The charged column was developed successively with 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 4.0) (arrow 1) and with 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 1.5) (arrow 2), at a flow rate of 1 ml/min. Of the resulting fractions, those from No. 27 to No. 31 were immediately mixed and adjusted to pH 5.5 with 2 M NaOH. The resulting mixture was concentrated by using a collodion bag, and stored at −90°C until use (casein-Sepharose eluate). ●——●, casein-hydrolysis at pH 10; ○——○, histone-hydrolysis at pH 8; △——△, A_{280nm}.

Step 5. Affinity chromatography on histone-Sepharose column: The casein-Sepharose eluate was applied on a histone-Sepharose column, previously equilibrated with 25 mM sodium acetate-HCl/25 mM NaCl/1 M urea (pH 5.5) (Fig. 3). Alkaline protease passed directly through the column (purified alkaline protease), while neutral protease was adsorbed.

Results

Solubilization of alkaline and neutral proteases

When Rhodamine sarcoma chromatin was suspended at pH 7.5, alkaline protease was solubilized

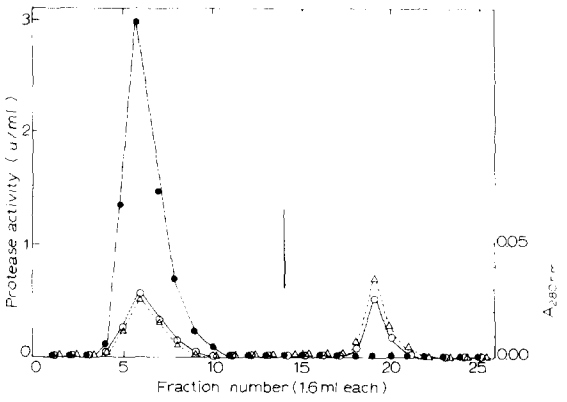

Fig. 3. Affinity chromatography of casein-Sepharose eluate on histone-Sepharose column. The casein-Sepharose eluate (2 ml), which contained 0.4 mg protein, was diluted with 1 vol. water and then applied on a histone-Sepharose column (1.0 X 8 cm). At the arrow, the column was further developed with 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 3.0). The flow rate was 1 ml/min. Of the resulting fractions, those from No. 5 to No. 7 were mixed and used as purified alkaline protease. ●——●, casein-hydrolysis at pH 10; ○——○, histone-hydrolysis at pH 8; △——△, A_{280nm}.

by NaCl to a significantly lower extent than neutral protease; the proteases were solubilized by 5 and 48% at 0.7 M, and by 26 and 53% at 2 M, respectively. However, they were not solubilized by 1% Triton X-100, whether the detergent was used in either preparing or washing chromatin, although it was slightly effective for removal of other proteins. These results indicate that alkaline protease was more tightly bound with chromatin than neutral protease. On the other hand, both proteases were effectively solubilized at pH 1.5. Of the acidic conditions tested, the most effective solubilization was achieved by 0.2 M H_3PO_4 plus 0.5 M NaCl (acid extract). The acid extract was subjected to affinity-labeling with [^{3}H]di-isopropylfluorophosphate at pH 8.0 and then dialyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). It was found that two major protein species were labeled with the radioactive reagent; one had a molecular weight of approx. 27000 and the other that of approx. 18000. This result was highly reproducible, except for variations in extent of the minor peak at approx. 16500 daltons. This indicates that the acid extract contained comparable amounts of two different kinds of proteins, which could be

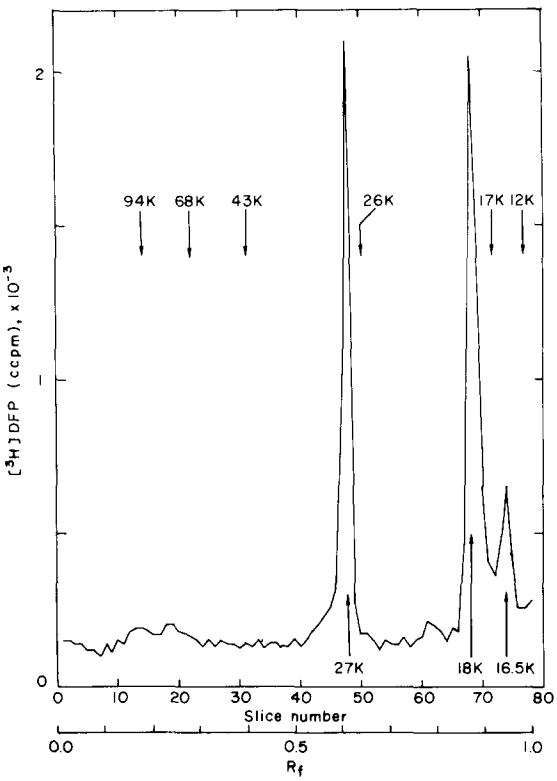

Fig. 4. Radioactivity profile of [^{3}H]diisopropylfluorophosphate-treated acid extract in SDS-polyacrylamide gel electrophoresis. The pure chromatin preparation was extracted at pH 1.5 by 0.2 M H_3PO_4 with 0.5 M NaCl. The acid extract contained 0.3 U/ml of neutral protease and 0.46 U/ml of alkaline protease. An aliquot (1 ml) of the extract was treated with [^{3}H]diisopropylfluorophosphate, and then subjected to SDS-polyacrylamide gel electrophoresis. The positions of the molecular weight markers are indicated by downward arrows.

combined with diisopropylfluorophosphate. It has been proved that the proteins of 18 000 and 27 000 daltons are the alkaline and neutral proteases, respectively [12].

Practically the same results were obtained with crude and pure chromatin preparations if the protease activities were estimated per mg DNA. This indicates that major proteases capable of binding with diisopropylfluorophosphate were the alkaline and neutral proteases, both in crude and pure chromatin preparations. In addition, the crude chromatin preparation was 10-times as high as the pure one with regard to the yield of the protease activities from the tumor tissue, although the former preparation was one-third as low as the latter with regard to the protease activities per mg protein.

Behavior of proteases on isoelectric electrophoresis and molecular-sieve chromatography

In isoelectric electrophoresis of the acid extract, both the alkaline and neutral protease activities were focused at approx. pH 11. In molecular-sieve chromatography of the acid extract on an Ultrogel AcA 54 column with 0.1 M sodium acetate-HCl/0.2 M NaCl/2 M urea (pH 6.0), both the alkaline and neutral protease activities were eluted in almost the same fractions centered at approx. 27 000 daltons. These results imply that the conventional ion-exchange and molecular-sieve chromatographies might not be advantageous for separation of these two proteases.

Essentially the same results were obtained with the acid extracts from crude and pure chromatin preparations.

Purification of alkaline protease

By the procedure described, alkaline protease was purified 1 400-fold with a yield of 4% from the crude chromatin preparation. The purified enzyme had a ratio in activity of the hydrolysis of casein at pH 10 to the hydrolysis of histones at pH 8 of 5.6. When the pure chromatin preparation was used, the same enzyme was obtained with a comparable purity, but with lower than half the yield with the crude preparation, if estimated from the amount of the tissue used.

The results of the purification are summarized in Table I.

Alternative procedure for purification of neutral protease

When the histone-Sepharose column at the last step in the purification procedure described above was eluted with 50 mM sodium acetate-HCl/50 mM NaCl/2 M urea (pH 3.0), the eluate showed the histone-hydrolyzing activity to be significantly higher than the casein-hydrolyzing activity. This indicates that the eluate contained neutral protease, but hardly any alkaline protease. However, the yield of neutral protease was too low. With an attempt to obtain neutral protease preparations free of alkaline protease with a higher yield, neutral protease was alternatively purified as follows. An excess amount of the acid

TABLE I

SUMMARY FOR PURIFICATION OF ALKALINE PROTEASE AND NEUTRAL PROTEASE FROM CHROMATIN OF RHODAMINE SARCOMA

Steps	Volume (ml)	Total protein (mg)	Hydrolysis of histones at pH 8 (A)				Hydrolysis of casein at pH 10 (B)				
			Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity	B/A
Chromatin suspension	240	3 940	155	0.040	(100)	(1)	233	0.059	(100)	(1)	1.5
Acetone-dried powder	300	3 690	165	0.045	110	1.1	238	0.067	106	1.1	1.5
Acid extract	375	525	113	0.215	73	5.4	172	0.328	74	5.6	1.5
Ammonium sulfate preparation	32	200	61.0	0.305	40	7.6	91.2	0.456	40	7.7	1.5
Soybean trypsin inhibitor-Sepharose eluate	63	12.6	44.0	3.50	28	88	66.2	5.25	28	90	1.5
Casein-Sepharose eluate	33	0.40	10	26	7	650	22	55	10	940	2.1
Non-adsorbed on histone-Sepharose	8	0.11	1.6	15	1	360	9.0	82	4	1 400	5.6
Histone-Sepharose eluate	8	0.10	1.3	13	0.8	330	–	–	–	–	–

TABLE II

SUBSTRATE SPECIFICITY OF PURIFIED ALKALINE PROTEASE FROM RHODAMINE SARCOMA

Substrate	Alkaline protease activity at pH 9.5		Neutral protease activity at pH 8.0	
	(U/mg protein)	(%)	(U/mg protein)	(%)
Natural				
Casein (Hammarsten)	91	(100)	12	32
Histones (Type II-S)	16	18	37	(100)
Arginin-rich histone (Type VIII-S)	7	8	25	68
Lysine-rich histone (Type V-S)	4	5	17	46
Synthetic				
Acetyl-L-phenylalanine ethyl ester	1.6		19	
Acetyl-L-tyrosine ethyl ester	1.5		31	
Benzoyl-L-arginine ethyl ester	0.5		4.0	

extract was applied on a soybean trypsin inhibitor-Sepharose column until the passed solution showed the histone-hydrolyzing activity to an appreciable extent (Fig. 5). When the charged column was washed with 0.1 M sodium acetate-HCl/0.2 M NaCl (pH 1.5), the adsorbed neutral protease was eluted in a small part. When the washed column was then developed with 0.1 M sodium acetate-HCl/0.2 M NaCl/2 M urea (pH 1.5), the eluate showed the histone-hydrolyzing activity, but little casein-hydrolyzing activity. By this procedure, neutral protease was purified 920-fold with a yield of 12% (purified neutral protease). When the pure chromatin was used, the same enzyme was obtained with a comparable purity, but with a significantly lower yield, if estimated from the amount of the tissue used.

Properties of the purified enzymes

Both the purified alkaline and neutral proteases were inhibited by various reagents including diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor and chymostatin almost to the same extents as those at the chromatin-bound state [12].

The purified alkaline protease hydrolyzed casein in preference to histones with an optimum pH of 9.5, whereas the purified neutral protease preferred histones to casein with an optimum pH of 8 (Fig. 6).

In SDS-polyacrylamide gel electrophoresis after affinity-labeling with [^{3}H]diisopropylfluorophosphate, the purified alkaline and neutral proteases individually gave a single major peak of radioactivity (Figs. 7 and 8). The radioactivity profiles were coincident with the main protein bands stained with Coomassie brillant blue R-250. These results indicate that the purified alkaline protease had a purity of approx. 75% and was contaminated with a negligible amount of neutral protease and that the purified neutral protease was nearly homogeneous and was not contaminated with alkaline protease. When the purified alkaline protease was incubated with [^{3}H]diisopropylfluorophosphate at pH 8 for prolonged periods of time, the radioactivity at 18 000 daltons decreased, while that at 16 500 daltons increased. The increase of radioactivity at 16 500 daltons was not observed with the purified neutral protease.

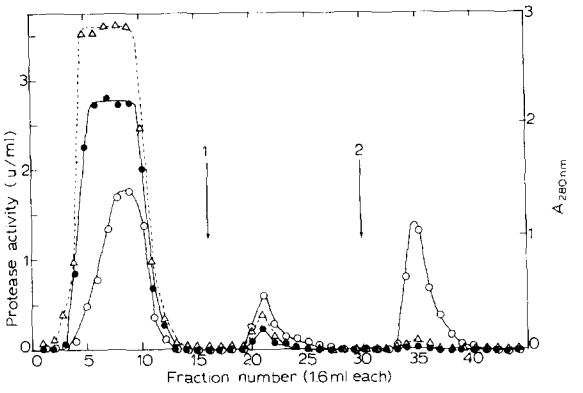

Fig. 5. Alternative affinity chromatography of acid extract on soybean trypsin inhibitor-Sepharose column. The precipitate by 80% saturated $(NH_4)_2SO_4$ at the step 2 for purification of alkaline protease was dissolved in a small volume of 30 mM sodium citrate-HCl/0.2 M NaCl (pH 6.5) and then dialyzed against an excess volume of the same buffer containing 0.2 M NaCl. The dialyzed solution (10 ml), which contained 50 mg protein, was applied on a soybean trypsin inhibitor-Sepharose column (1.7 X 2.0 cm). The charged column was developed successively with 0.1 M sodium acetate-HCl/0.2 M NaCl (pH 1.5) (arrow 1) and with 0.1 M sodium acetate-HCl/0.2 M NaCl, 2 M urea (arrow 2), at a flow rate of 0.8 ml/min. Of the resulting fractions, those from No. 34 to No. 38 were immediately mixed, adjusted to pH 5.5 with 2 M NaOH, and used as purified neutral protease. ●———●, casein-hydrolysis at pH 10; ○———○, histone-hydrolysis at pH 8; △———△, A_{280nm}.

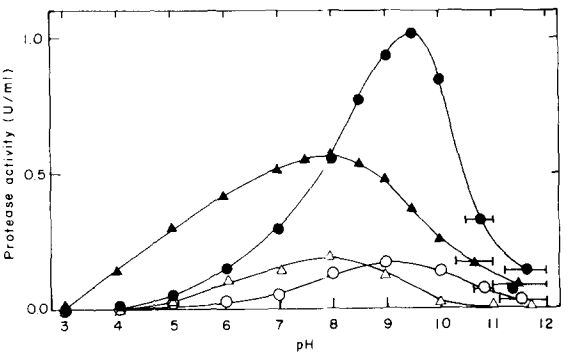

Fig. 6. Effect of pH on activities of purified alkaline and neutral proteases. The buffers used were mixtures of 20 mM 3,3-dimethylglutaric acid, 20 mM Tris and 20 mM 2-amino-2-methyl-1,3-propanediol, the pH values of which were adjusted from 3 to 11 with HCl or NaOH, and 0.1 M sodium borate/NaOH of pH values from 8 to 12. These two kinds of buffers gave essentially the same activities at the overlapped pH values. Horizontal bars represent pH shifts from the beginning to the end of reactions. ●———●, casein with purified alkaline protease; ○———○, histones with purified alkaline protease; △———△, casein with purified neutral protease; ▲———▲, histones with purified neutral protease.

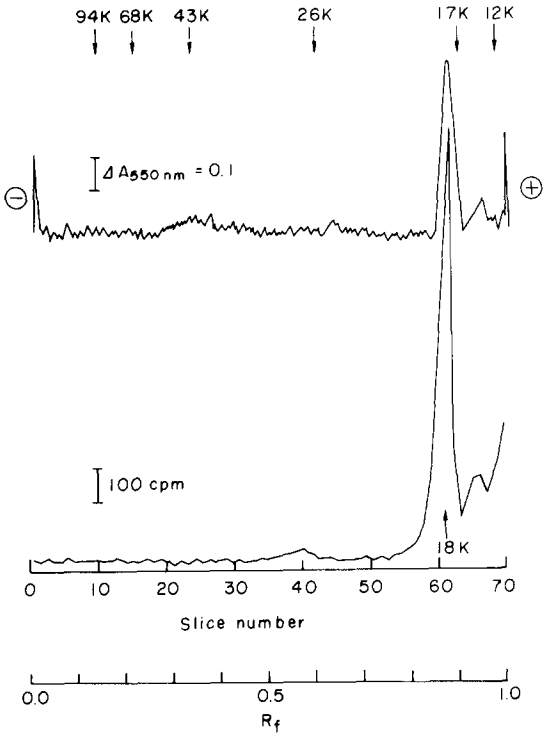

Fig. 7. Profiles of protein and radioactivity in SDS-polyacrylamide gel electrophoresis of purified alkaline protease treated with [^{3}H]diisopropylfluorophosphate. A solution of the purified alkaline protease was concentrated by using a collodion bag. The concentrated enzyme solution (0.2 ml) was mixed with 0.8 ml 20 mM Tris-HCl (pH 8) and 50 μl [^{3}H]diisopropylfluorophosphate. The resulting mixture (150 μl) was subjected to SDS-polyacrylamide gel electrophoresis.

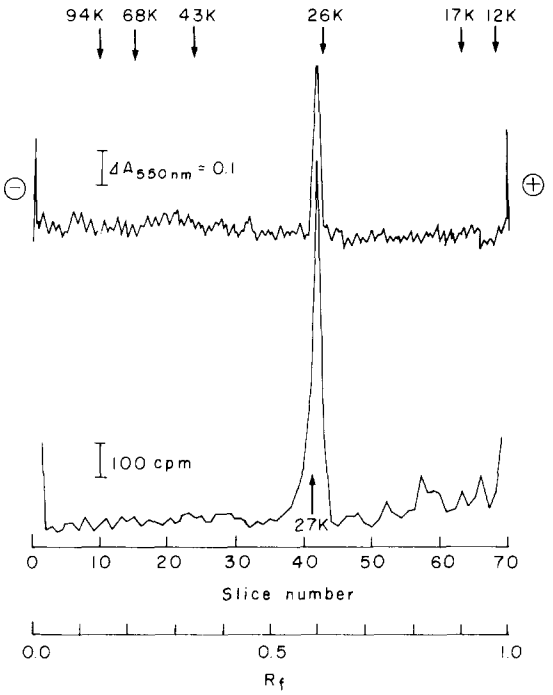

Fig. 8. Profiles of protein and radioactivity in SDS-polyacrylamide gel electrophoresis of purified neutral protease treated with [^{3}H]diisopropylfluorophosphate. Experimental conditions are the same as those in Fig. 7, except that the purified neutral protease was used.

Substrate specificities of the purified enzymes

Substrate specificities of the purified alkaline and neutral proteases were examined with various kinds of polypeptides and synthetic substrates (Table II).

The purified alkaline protease hydrolyzed histones at 18%, arginine-rich histone at 8% and lysine-rich histone at 5% of the rate of hydrolysis of casein at pH 9.5. On the other hand, the purified neutral protease hydrolyzed casein at 32%, arginine-rich histone at 68% and lysine-rich histone at 48% of the rate of hydrolysis of histones at pH 8. Both proteases hydrolyzed acetyl-L-phenylalanine ethyl ester, acetyl-L-tyrosine ethyl ester and benzoyl-L-arginine ethyl ester, suggesting that they were chymotrypsin-like. However, the rates of hydrolysis of these synthetic substrates were significantly lower with the purified alkaline protease than with the purified neutral protease. Neither purified alkaline protease nor purified neutral protease hydrolyzed tosyl-L-arginine methyl ester, poly-L-lysine, poly-L-arginine, bovine serum albumin, denatured hemoglobin or γ-globulin.

Discussion

The purification of alkaline and neutral proteases from Rhodamine sarcoma chromatin was carried out by the procedure involving affinity chromatography on Sepharose columns with bound soybean trypsin inhibitor, casein and histones. The principle of the purification procedure was based on the previous findings that both proteases are inhibited by soybean trypsin inhibitor, whereas alkaline protease hydrolyzes casein in preference to histones and neutral protease prefers histones to casein [12]. In particular, histone-Sepharose was remarkably effective in separation of these two proteases from each

other, because the isoelectric points of histones and both proteases were similarly high so that electrostatic affinities would not be invoked between histones and the proteases, and the specific affinity to histones was significantly higher with neutral protease than with alkaline protease.

Earlier, Carter et al. [10] supposed that with the chromatin of rat liver, neutral protease of 25 000 daltons was converted to a smaller protease of 20 000 daltons during the time of acid extraction with 0.25 M HCl, on the basis of their findings that in chromatin, only the former protein can combine with [^{3}H]diisopropylfluorophosphate, whereas in acid extracts, both proteins can combine with the radioactive reagent. It was previously found, using tumorous tissues of rats, that both proteases of 27 000 daltons (neutral protease) and 18 000 daltons (alkaline protease) are detectable to a comparable extent when the chromatin is incubated with [^{3}H]diisopropylfluorophosphate at pH 10, whereas only the former protease is detectable when the chromatin is incubated with the radioactive reagent at pH 8 [12]. In the present study, it was found that the ratio of the alkaline protease to the neutral protease detectable when the acid extract was incubated with [^{3}H]diisopropylfluorophosphate at pH 8 was similar to that when the chromatin was incubated with the radioactive reagent at pH 10 (cf. Fig. 1 in the present paper and Fig. 4 in Ref 12), in spite of the fact that these two proteases were solubilized in the acid extract to a comparable extent. It seems unlikely, therefore, that neutral protease could be converted to alkaline protease. However, it is probable that during autodigestion, alkaline protease was hydrolyzed to a polypeptide of 16 500 daltons. Nevertheless, the results described above do not necessarily suggest that in chromatin at pH 8, alkaline protease was so bound with chromatin that it could not exhibit the activity as well as it could not combine with diisopropylfluorophosphate, because the molar concentration of [^{3}H]diisopropylfluorophosphate used in affinity-labeling (0.05 mM) was significantly lower than that usually used in inhibitory experiments (1–5 mM) [12].

It was previously reported that alkaline protease is specifically localized in chromatin, whereas neutral protease is distributed to both chromatin and microsomes, suggesting that in sonicated nuclei, the former protease hydrolyzes non-histone proteins and histone H1, whereas the latter protease hydrolyzes other histones [12]. However, it is known that in vivo, the turnover rate of histones is remarkably slow or negligible, while that of non-histone chromosomal proteins is appreciable [14,15]. Recently, Nagao et al. [24] reported that of the five kinds of histones, only histone H1 is phosphorylated when nuclei are incubated with [γ-^{32}P]ATP. Their findings suggest the possibility that in nuclei, histones H2A, H2B, H3 and H4 are buried in the nucleosomes [25] so as not to be attacked by the neutral protease. However, the functions of alkaline and neutral proteases in intact nuclei should be solved by advanced studies.

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