

TABLE II. Comparison of Elution pHs of Oxytocin and Vasopressin from immobilized NPs Columns and pK Values for the Hormone-binding to Free NPs

		For oxytocin		For vasopressin	
		Acidic side	Basic side	Acidic side	Basic side
NP-I	Free ^{a)}	4.65	7.15	4.6	6.4
	Immobilized ^{b)}	3.6	8.7	4.2	9.0
NP-II	Free ^{a)}	4.05	6.75	4.75	6.4
	Immobilized ^{b)}	3.3	8.35	3.2	8.35

a) apparent pK values given by Camier *et al.*^{3e)}

b) pHs of the peaks in the elution profiles.

is an essential element in hormone recognition by NPs, and this is the reason why Sepharose-NPs retain the ability to bind the hormones. [1-Desamino]-oxytocin, which lacks the α -amino group in the cysteine residue, does not bind to agarose-NP.^{4c)} This result also supports the existence of electrostatic interaction between NPs and hormones.

Another interesting finding can be seen in Fig. 1, *i.e.*, that oxytocin and vasopressin can be separated on a Sepharose-NP-I column. The attempts of Robinson and Walker to elute oxytocin and vasopressin separately from a column of agarose-NP by the use of shallow pH gradients in either direction from pH 5.8 were unsuccessful.^{4c)} They used a mixture of NP-I and NP-II to prepare their immobilized protein column. Since oxytocin and vasopressin are hardly separated on a Sepharose-NP-II column (Fig. 2), their use of the mixture may account for their lack of success. On the other hand, Sepharose bound NP-I does have the ability to separate the two hormones (Fig. 1) and thus may represent a useful analytical tool for studies on these hormones.

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A Simple Purification Procedure for Rat Pancreatic Elastase and Radioimmunoassay of the Enzyme

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Rat pancreatic elastase was purified on a column of lima bean trypsin inhibitor-Sepharose after inactivating trypsin and chymotrypsin in the pancreatic homogenate by treatment with N^α-*p*-tosyl-L-lysylchloromethyl ketone and N-tosyl-L-phenylalanylchloromethyl ketone. The molecular weight of the enzyme was 25000, as determined by polyacrylamide gel electrophoresis. A radioimmunoassay method was established for rat elastase; the enzyme level could be determined over the range of 2.5—300 ng of the enzyme per ml.

Keywords—rat pancreatic elastase; purification; characterization; radioimmunoassay; tissue level of immunoreactive elastase

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Elastase, a proteolytic enzyme which is present in pancreatic secretion, has the ability to hydrolyze elastin or the elastic fibrous protein of connective tissue. This action of elastase suggests that it may play an important role in the metabolism of elastin in several tissues.²⁾

In our previous papers,³⁾ the metabolic fate of hog pancreatic elastase in rats was described. There is also much interest in studies of the physiological function and metabolic fate of rat elastase in homogeneous systems, as described by Genell *et al.*,⁴⁾ who purified the enzyme from rat pancreatic juice by column chromatography on SP-Sephadex, Trasyol-Sephadex and then SP-Sephadex. Elastase in pancreatic tissue is generally purified by acetate extraction, fractional ammonium sulfate precipitation, column chromatography with diethylaminoethyl (DEAE)-Sephadex and crystallization.⁵⁾ The yields of elastase by these methods^{4,5)} were low, however, and the latter method was complicated. It is, therefore, desirable to establish a simpler method to purify elastase in high yield.

This paper deals with a simple method to purify rat pancreatic elastase, and presents a radioimmunoassay of the enzyme.

Materials and Methods

The following chemicals were used: N-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide and N^α-benzoyl-L-arginine *p*-nitroanilide (Protein Research Foundation, Osaka), N-succinyl-L-phenylalanine *p*-nitroanilide, diisopropyl fluorophosphate and N^α-*p*-tosyl-L-lysylchloromethyl ketone (Sigma), N-tosyl-L-phenylalanylchloromethyl ketone (Seikagaku Kogyo, Tokyo), elastin (Nutritional Biochemicals) and lima bean trypsin inhibitor (Worthington Biochemicals).

Hog elastase, human elastase-1 and their antibodies were the preparations described previously.⁶⁾

Isolation of Rat Pancreatic Elastase—A homogenate of 57 g of rat pancreas in 120 ml of water was incubated with 2 g of mucosal scraps of rat duodenum at room temperature for 1 hr. The homogenate was then stirred with 40 ml of 0.5 M acetate buffer (pH 4.5) at 4° for 3 days and centrifuged at 15000 rpm for 20 min. The resulting supernatant was used as a starting extract. The extract was adjusted to pH 7.0 and incubated with 80 mg of N^α-*p*-tosyl-L-lysylchloromethyl ketone and 114 mg of N-tosyl-L-phenylalanylchloromethyl ketone at room temperature for 4 hr, then overnight at 4° in order to inactivate trypsin and chymotrypsin, respectively. The extract was applied to a column of lima bean trypsin inhibitor-Sepharose 4B (0.84 g of lima bean trypsin inhibitor was coupled to 98 ml of the gel) and then further purified on a column of SP-Sephadex C-25 as described by Feinstein *et al.*⁷⁾ An active fraction from the SP-Sephadex column was rechromatographed on the column of lima bean inhibitor-Sepharose.

Radioimmunoassay—An antiserum (rabbit serum) against rat pancreatic elastase and ¹²⁵I-labeled elastase were prepared by methods similar to those used in the case of hog pancreatic elastase.^{6a)} Radioimmunoassay of rat elastase was performed in 0.05 M phosphate buffer (pH 7.4) containing 1 mM diisopropyl fluorophosphate, 0.2% bovine serum albumin and 0.15 M NaCl as a diluent. An aliquot (0.1 ml) of the antiserum diluted 1/30000, ¹²⁵I-labeled elastase (8000 cpm) and a standard antigen (or sample) were mixed with 0.4 ml of the diluent and the mixture was incubated at 4° for 2 days. One-tenth ml of anti-rabbit γ -globulin goat serum and 0.1 ml of normal rabbit serum diluted 1/900 were added to the mixture, and the whole was incubated overnight at 4°. The tube was centrifuged at 3000 rpm for 30 min and the radioactivity in the precipitates was counted with a well-type scintillation counter.

Radioimmunoassay of hog pancreatic elastase or human elastase-1 was carried out as described previously.^{6b,c)}

Measurements of Immunoreactive Elastase in Tissues—Tissues from a Wistar rat were homogenized in 4 volumes of 0.15 M NaCl in a Potter-Elvehjem type homogenizer with a Teflon pestle for 2 min, and the

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homogenates were centrifuged at 10000 rpm for 30 min. Immunoreactive elastase in the supernatant was assayed by the radioimmunoassay method for rat elastase described above.

Enzyme Assays—Esterolytic activity of elastase was measured at 25° with 1 mM N-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide⁸⁾ in 0.05 M Tris-HCl (pH 9.0). Elastolytic activity was determined with elastin as a substrate.⁹⁾ Tryptic and chymotryptic activities were measured using N^α-benzoyl-L-arginine *p*-nitroanilide¹⁰⁾ and N-succinyl-L-phenylalanine *p*-nitroanilide,¹¹⁾ respectively.

Results and Discussions

Purification of Rat Pancreatic Elastase

Proelastase in the pancreatic homogenate was fully activated by incubating the homogenate with the mucosal scraps of rat duodenum at room temperature for 1 hr. The acetate

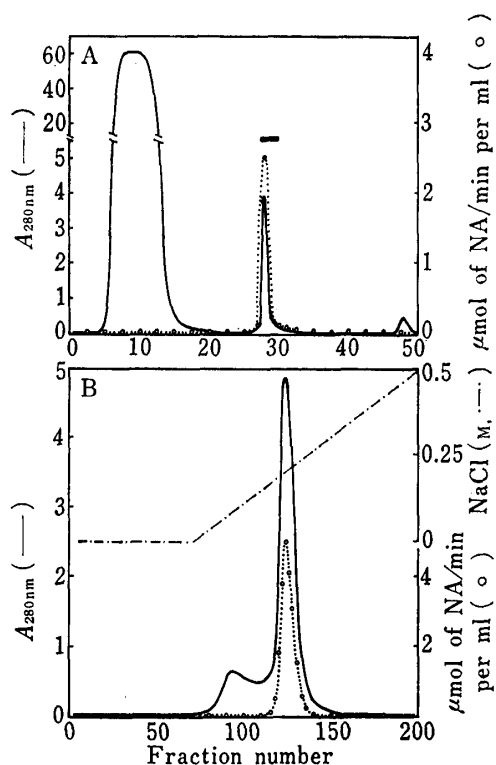


Fig. 1. Chromatography of Rat Pancreatic Elastase on Lima Bean Trypsin Inhibitor-Sepharose (A) and on SP-Sephadex (B)

The acetate extract was applied to a column (2.5×19 cm) of lima bean inhibitor-Sepharose equilibrated with 0.05 M Tris-HCl (pH 8.0). The column was washed with the same buffer and then eluted with 0.1 M acetate buffer (pH 3.0). An active fraction (indicated as a solid bar in Fig. 1-A) was applied to a column (1.5×22 cm) of SP-Sephadex C-25. The column was eluted with 0.02 M acetate buffer (pH 4.0), 0.02 M acetate buffer (pH 5.0) and a linear NaCl gradient in 0.02 M acetate buffer (pH 5.0). The esterolytic activity is represented in terms of *p*-nitroaniline (NA) released from N-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide.

extract contained not only elastase but also trypsin and chymotrypsin, which might be adsorbed on the lima bean inhibitor-Sepharose. To prevent the adsorption of trypsin and chymotrypsin on the Sepharose, these enzymes were inactivated with N^α-*p*-tosyl-L-lysylchloromethyl ketone and N-tosyl-L-phenylalanylchloromethyl ketone. The acetate extract was then applied to a column of lima bean inhibitor-Sepharose and eluted as shown in Fig. 1-A. An active fraction eluted with 0.1 M acetate buffer (pH 3.0) was purified on a column of SP-Sephadex as described in Fig. 1-B.

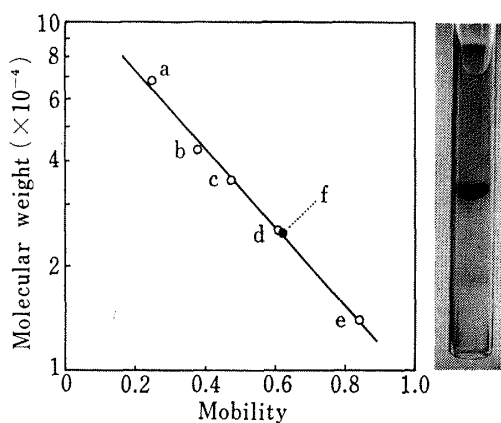


Fig. 2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Rat Pancreatic Elastase and Estimation of the Molecular Weight of the Protein

Electrophoresis was carried out in 10% polyacrylamide gels at 8 mA/tube for 4 hr. The standard proteins were bovine serum albumin (a), ovalbumin (b), pepsin (c), hog elastase (d), egg white lysozyme (e) and rat elastase (f).

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TABLE I. Summary of the Purification of Rat Pancreatic Elastase

	I $A_{280\text{nm}}^a$	II Activity ^{b)}	Ratio II/I	Purification index
Extract from 57 g of rat pancreas	25721 (100%)	307 (100%)	0.0119	1
Lima bean inhibitor- Sephadex column	247 (9.6%)	205 (66.8%)	0.830	70
SP-Sephadex column	146 (5.7%)	146 (47.6%)	1.000	84
Lima bean inhibitor- Sephadex column	134 (5.2%)	157 (51.1%)	1.172	98

a) $(A_{280\text{nm}}) \times (\text{total volume, ml})$.

b) $\mu\text{mol of } p\text{-nitroaniline released/min per total volume}$.

The specific activity of the elastase increased 98-fold over that of the starting extract, and the yield of the esterolytic activity was 51% as shown in Table I.

Characterization of Rat Pancreatic Elastase

The purity and molecular weight of the elastase were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.¹²⁾ As shown in Fig. 2, a single component was identified

TABLE II. Cross Immunoreactivities of Elastases

Immunoassay system	% of replacement by the enzymes ^{a)}		
	Rat elastase	Human Elastase-1	Hog elastase
Rat elastase	100	12.6	<0.1
Human elastase-1	0.1	100	<0.1
Hog elastase	<0.1	<0.1	100

a) Each elastase was assayed at concentrations of 25 and 50 ng/ml.

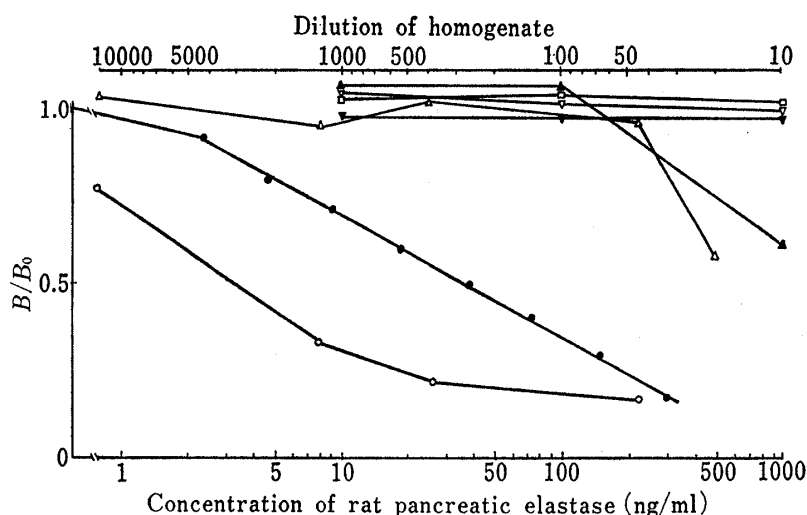


Fig. 3. A Standard Curve for the Measurement of Rat Pancreatic Elastase and Immunoreactive Elastase in Tissues

The ordinate, B/B_0 , represents the ratio of ^{125}I -labeled elastase bound to the antibody in the presence of unlabeled elastase to that in the absence of unlabeled elastase. The supernatants of 20% homogenates were diluted as shown in this figure and immunoreactive elastase was estimated by the radioimmunoassay method for rat elastase.

●: purified elastase, ○: pancreas, △: aorta, ▲: spleen, ▽: liver, ▼: kidney, □: lung.

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with a molecular weight of 25000 as determined by comparison with protein standards. The molecular weight of the enzyme was in good agreement with that reported by Genell *et al.*⁴⁾

One mg of the purified elastase hydrolyzed 1.53 μ mol of N-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide and solubilized 1.45 mg of elastin per min.

The immunological properties of the rat elastase were analyzed by the three radioimmunoassay methods for rat elastase, human elastase-1 and hog elastase. As shown in Table II, the rat elastase did not react with the antibodies against human and hog elastase, but the antibody against rat elastase showed slight cross-reactivity with human elastase-1.

Immunoreactive Elastase in Tissues

A representative standard curve for the radioimmunoassay of rat elastase is shown in Fig. 3, and indicates that 50% of the total ¹²⁵I-labeled elastase bound to the antibody can be replaced by 35 ng of elastase per ml.

Immunoreactive elastase was estimated in several tissues, and the results are shown in Fig. 3. The aorta and spleen homogenates contained a detectable amount of immunoreactive elastase, but the lung, kidney and liver did not. The immunoreactive elastase detected in the aorta, which was partly characterized by Miyake *et al.*,¹³⁾ and that in the spleen both require further characterization.

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Adsorption of Benzothiadiazines by Carbon Black from Aqueous Solution, and Related Phenomena^{1,2)}

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The adsorption of benzothiadiazines by carbon black from aqueous solution was investigated in detail. The adsorption isotherms obtained were well described by the Langmuir equation. It was shown that the adsorbability was positively related to the relative diuretic activity. The adsorption was found to be reversible with change of temperature in a perturbation experiment, suggesting a physical adsorption mechanism. The pH of the buffer solution had no clear effect on the adsorption of benzothiadiazines near the neutral pH region. It was shown qualitatively by thin-layer chromatography that the hydrolysis of benzothiadiazines was accelerated by carbon black.

Keywords—adsorption from aqueous solution; benzothiadiazine; carbon black; Langmuir equation; pH dependence; temperature dependence; hydrolysis

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2) A part of this work was presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1974.
3) Location: *Ebara 2-4-41, Shinagawa-Ku, Tokyo, 142, Japan.*