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STUDIES ON BIOTRANSFORMATION OF ELASTASE

I. TRANSPORT OF ^{131}I-LABELED ELASTASE ACROSS RAT INTESTINE *IN VITRO*

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SUMMARY

Elastase, an elastolytic enzyme isolated from hog pancreas, was labeled with ^{131}I and its transport investigated using rat everted jejunum.

The everted sac of rat jejunum was incubated in Krebs–Ringer bicarbonate solution containing 0.01 or 0.1 % of ^{131}I-labeled elastase. The fact that ^{131}I-bound protein was transported from the mucosal to serosal side was confirmed by several methods such as precipitation by trichloroacetic acid, gel filtration and electrophoresis. It was also found that the transfer rate of ^{131}I-bound protein agreed approximately with that determined by the enzymatic and immunological methods.

INTRODUCTION

In recent years, numerous studies on the intestinal absorption of enzyme proteins have been carried out to elucidate the pharmacological effects of the orally administered enzymes. It has been suggested that a small amount of the enzyme proteins is selectively absorbed from the intestinal tract[1–5].

Elastase (EC 3.4.4.7), a proteolytic enzyme which is present in pancreatic juice and has the ability to digest elastin or the elastic fibrous protein of connective tissue, is distinct from the other pancreatic endopeptidases. For instance, in its pharmacological activity this enzyme is expected to have a potential antiatheromatous property[6]. The present study was undertaken to investigate *in vitro* transport of ^{131}I-labeled elastase as part of studies on the intestinal absorption and metabolic fate of exogenous elastase.

MATERIALS

Elastase

Elastase* was extracted from hog pancreas and purified by Loeven's method[7]. The specific activity analysed by Sachar's method[8] was 106–108 elastase units/mg

* Elastase was supplied by Dr K. Koide, Department of Organic Chemistry, Eisai Research Laboratories.

protein. Lewis[9] has reported that the molecular weight of elastase is 25000 and its isoelectric point is pH 9.5 ± 0.5.

^{131}I-labeled elastase (abbreviated as [^{131}I]elastase)

A modified Greenwood's[10] method was employed for iodination of elastase with radioiodine obtained from Daiichi Radioisotope Laboratory. Details of this method were reported previously[11]. The specific radioactivity of [^{131}I]elastase, which had an enzymatic activity identical with that of the starting material, was approximately 10 μCi/mg protein. The measurements for the radiochemical purity using electrophoresis and paper chromatography indicated 97.7 and 96.1 %, respectively.

Anti-elastase serum

Anti-elastase serum was prepared with purified elastase in rabbits by the protocol of McIvor and Moon[12].

METHODS

In vitro procedure with everted jejenum

The determination of the transportation of [^{131}I]elastase across the everted jejenum was based on Crane and Wilson's method[13].

Male rats of Wistar strain, weighing about 250 g, were fasted for 24 h and killed by decapitation. The removed jejunum was washed out with saline and everted on a glass rod. Then the jejunum was divided into 7-cm wide segments and each segment was tied to a glass cannula. The sac of jejunum, into which 1 ml of Krebs–Ringer bicarbonate solution (pH 7.4) was poured, was placed in a test tube containing 10 ml of 0.01 or 0.1 % of [^{131}I]elastase in Krebs–Ringer solution. For the control experiments, K^{131}I was added in the solution instead of [^{131}I]elastase. These test tubes were kept in an incubator at 37°C for 2 h, and a gas mixture consisting of O_2:CO_2 (95:5, v/v) was bubbled into the mucosal fluid. The serosal and mucosal fluid were then collected for radioactivity determination. In order to observe the time course of intestinal transport, approximately 0.15 ml serosal fluid was taken out every 30 min for 2.5 h.

Measurement of enzyme activity

The measurement of elastolytic activity of [^{131}I]elastase, using elastin–orcein (Nutritional Biochemical Co.) as a substrate, was based on Sachar's method[8].

Radioactivity determinations

All counting was done in a well-type scintillation counter (Aloka JDC-207).

Immunoassay

Each 0.1 ml of serosal or mucosal fluid, of saline solution of elastase (100 μg/ml) and of anti-elastase serum was poured in succession into a 7-ml conical centrifuge tube. This solution was incubated at 37 °C for 30 min and then cooled at 4 °C for 24 h, the precipitate was then collected by centrifugation at 3000 rev./min for 20 min. The precipitate was rinsed 3 times by suspension in 0.5 ml of saline and centrifugation. The radioactivity was measured after dissolving the precipitate in 2 ml of 0.1 M

NaOH. In this manner, the recovery of radioactivity of [^{131}I]elastase in the precipitate was 92–94 %.

Precipitation by trichloroacetic acid

To 0.1 ml of serosal or mucosal fluid, 0.4 ml of 1 mM KI aqueous solution and 0.5 ml of 10 % trichloroacetic acid solution were added. The protein precipitate collected by centrifugation was washed 3 times with 5 % trichloroacetic acid. The radioactivities of supernatant and precipitate dissolved in 2 ml of 1 M NaOH were then counted. The trichloroacetic acid precipitable radioactivity was expressed as a percentage of the total radioactivity.

Dialysis

0.1 ml of serosal or mucosal fluid and 0.4 ml of 1 mM KI solution placed in a cellophane tube were dialysed in 1 l of distilled water under continuous stirring for 24 h. During dialysis, the external phase was changed 4 times. The radioactivity remaining in the tube was counted and represented as a percentage of the initial radioactivity.

Electrophoresis

0.01 ml of serosal or mucosal fluid was applied on a 5 cm × 9 cm cellulose acetate strip and electrophoresis was conducted in 0.1 M glycine buffer (pH 10.0) with a potential gradient of 0.7 mA/cm for 2 h using a Fujiox Model AS-D IV cellulose acetate paper electrophoresis apparatus. After electrophoresis, the strip was split into 5-mm wide segments and the radioactivity counted in each segment was expressed as a percentage to the radioactivity applied on a strip.

Gel filtration

Approximately 0.4 ml of pooled serosal fluid charged on a Sephadex G-25 column (1.5 cm × 30 cm) was eluted with 0.05 M phosphate buffer (pH 7.4). The radioactivity and absorbance at 280 nm in each fraction were measured.

Paper chromatography

Paper chromatography was performed on Toyo filter paper (No. 51) using 95 % ethanol–2 M ammonia (9:1, v/v) as a solvent.

RESULTS

Time course of the concentration of macromolecule-bound ^{131}I in serosal fluid

The data on the time course of the concentration determined by immunoassay and the other analyses are presented in Fig. 1 and Table I. The results obtained by these methods were in good agreement with each other. The transport of immunoprecipitable and protein-bound radioactivity was already observed 30 min after the initiation of incubation and thereafter the concentration in serosal fluid increased with time lapse. The transport rate of immunoprecipitable radioactivity, which was measured every 30 min, increased with incubation time, showing a value of 10 μg elastase equiv per 30 min at the final period. As shown in Fig. 1, the concentration of immunoprecipitable radioactivity in mucosal fluid decreased slowly.

Transport rate of [^{131}I]elastase-like substance

The transport rate calculated from the radioactivities in serosal and mucosal fluid before or after 2 h incubation is shown in Table II. The amount of transported radioactivity across the intestine incubated in 0.01 and 0.1 % of [^{131}I]elastase on mucosal side was 1.58 and 2.26 % of initial mucosal radioactivity, respectively. The initial concentration of radioactivity in the mucosal fluids was only decreased

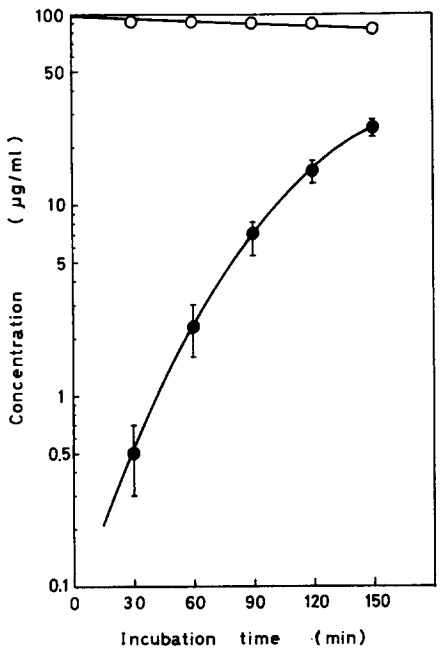

Fig. 1. Change of the concentration of immunoprecipitable radioactivity as elastase equiv in serosal and mucosal fluid during incubation. The initial concentration of [^{131}I]elastase in mucosal side was 0.01%. Each value indicates the mean with S.E. of 4 experiments. ●—●, concentration in serosal fluid; ○—○, concentration in mucosal fluid.

TABLE I

CHANGE OF CONCENTRATION OF RADIOACTIVITY FRACTIONATED BY SEVERAL METHODS IN SEROSAL FLUID

Experimental conditions as described under Methods.

Time of incubation (min)	*Methods*		
	Trichloroacetic acid precipitation	*Paper chromatography*	*Electrophoresis*
30	0.8*	0.1	0.8
60	3.2	3.1	4.6
90	8.5	8.8	9.0
120	16.3	17.1	18.8
150	26.8	27.9	29.2

* Each value was reprecented as elastase equiv concentration (μg/ml) = (radioactivity × % of radioactivity fractionated as macromolecule-bound ^{131}I) / 100.

TABLE II

TRANSPORT RATE OF RADIOACTIVITY ACROSS INTESTINAL WALL FOR 2 h INCUBATION

Values indicate the mean with S.E. of 5 experiments.

Materials and concentration	*Mucosal fluid*		*Serosal fluid*			
	Initial amount in 10 ml (A) ($10^3 \times cpm$)	*Final concentration (B)* ($10^3 \times cpm/ml$)	*Final amount (C)* ($10^3 \times cpm$)	*Final concentration (D)* ($10^3 \times cpm/ml$)	*Concentration ratio of both sides (D/B)*	*Rate of transport (%) (C/A) × 100*
[^{131}I]Elastase 0.01%	766.7	72.0 ± 1.3	12.1 ± 1.9	30.1 ± 4.2	0.42 ± 0.06	1.58 ± 0.25
K^{131}I *	1333.9	115.3 ± 1.4	56.5 ± 7.3	101.2 ± 2.1	0.88 ± 0.02	4.24 ± 0.55
[^{131}I]Elastase 0.1%	4105.5	379.0 ± 2.4	92.6 ± 13.5	200.1 ± 20.9	0.53 ± 0.06	2.26 ± 0.33
K^{131}I **	4896.2	419.0 ± 4.4	175.9 ± 10.3	401.6 ± 21.0	0.96 ± 0.05	3.59 ± 0.21

* 2.6 μg/ml as iodine.
** 10 μg/ml as iodine.

TABLE III

PERCENTAGE OF TRICHLOROACETIC ACID-PRECIPITABLE AND NON-DIALYSABLE RADIOACTIVITY TO THE TOTAL RADIOACTIVITY AFTER 2 h INCUBATION

Each value indicates the mean of percentages with S.E. of 5 experiments. Experiments were performed with the same samples as in Table II.

Materials in mucosal fluid	*% of trichloroacetic acid precipitable*		*% of non-dialysable*	
	Serosal fluid	*Mucosal fluid*	*Serosal fluid*	*Mucosal fluid*
[^{131}I]Elastase 0.01%	39.7 ± 4.4	70.0 ± 0.2	39.7 ± 2.0	71.1 ± 6.3
K^{131}I *	0.11 ± 0.02	***	***	***
[^{131}I]Elastase 0.1%	41.2 ± 2.7	65.9 ± 0.2	36.3 ± 1.5	61.3 ± 2.6
K^{131}I **	0.18 ± 0.01	***	0.38 ± 0.10	***

*, ** The same as Table II.
*** Not examined.

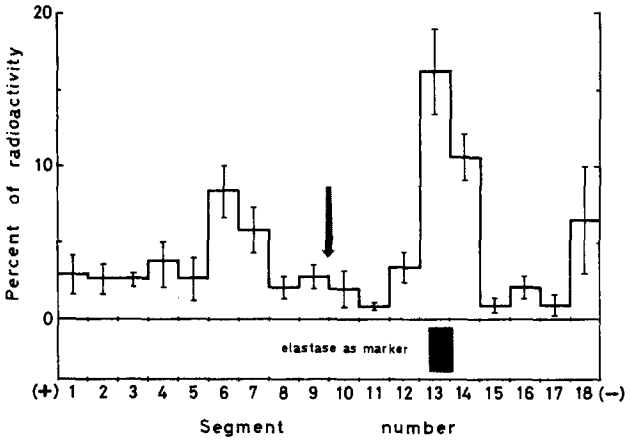

Fig. 2. Radioactivity distribution of serosal fluid after 2 h incubation shown by an electrophoretic diagram using cellulose acetate strips. The initial concentration of [^{131}I]elastase in mucosal side was 0.01%. The arrow indicates the site of application of sample. Visulalized spot of elastase as a marker was stained with light green. Each value represents mean percentage with S.E. of 5 experiments.

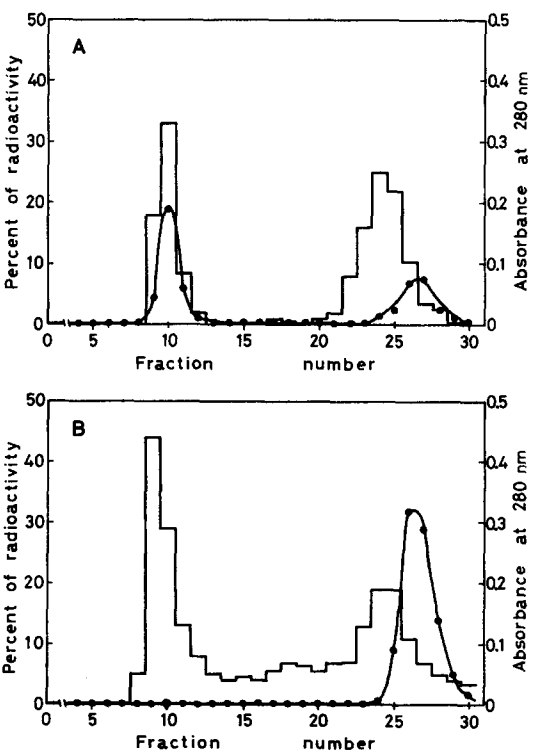

Fig. 3. Gel filtration of serosal fluid on Sephadex G-25. Sample was the serosal fluid after 2 h incubation with the initial mucosal fluid containing: (a) 0.01% of [^{131}I]elastase, (b) 2.6 μg/ml as iodine of K^{131}I. ●—●, radioactivity in each fraction as percentage of total applied radioactivity; ———, absorbance at 280 nm.

6–7 % after incubation. On the other hand, the amount of transported radioactivity was 4.24 and 2.26 %, when incubated in 2.6 μg iodine/ml and 10 μg iodine/ml as controls. The initial concentration of radioactivity in the control mucosal fluid was decreased 14 % by incubation.

The ratio of the serosal radioactivity concentration to that of mucosal fluid was 0.4–0.5 for [^{131}I]elastase, compared with 0.9–1.0 for K^{131}I. The ratio for K^{131}I indicated that transport of iodine was in equilibrium between serosal and mucosal fluid.

The radioactivity in the serosal side, which appeared to originate from [^{131}I]-elastase, was determined by trichloroacetic acid precipitation, dialysis, electrophoresis and gel filtration.

The results obtained by trichloroacetic acid precipitation and dialysis are shown in Table III. The percentages of trichloroacetic acid-precipitable and non-dialysable radioactivity of the total radioactivity in serosal fluid were found to be quite low (0.11–0.38 %) on K^{131}I incubation. In contrast, trichloroacetic acid-precipitable and non-dialysable radioactivity was approximately 40 % in serosal fluids and 60–70 % in mucosal fluids after incubation in 0.01 and 0.1 % of [^{131}I]elastase.

Fig. 2 presents the electrophoretic pattern of serosal fluid after 2 h incubation in the mucosal concentration of 0.01 % [^{131}I]elastase. The largest of the radioactive peaks migrates electrophoretically in the same area as that of elastase. In addition, the presence of a substance similar to [^{131}I]elastase in the serosal fluid was confirmed by the fact that ^{131}I-bound macromolecule was eluted at a void volume in gel filtration of the same sample (Fig. 3a). As shown in Table IV, the percentages of the total radioactivity corresponding to elastase were almost the same (approximately 30 %) in both procedures of electrophoresis and gel filtration.

TABLE IV

PERCENTAGE OF TOTAL RADIOACTIVITY APPLIED IN ASSAY CORRESPONDING TO [^{131}I]ELASTASE BY ELECTROPHORESIS AND GEL FILTRATION

Experiments were performed with the same samples as in Table II. Experimental conditions of electrophoresis and gel filtration were as in Methods. Values indicate the mean percentage with S.E. of 5 experiments.

Materials in initial mucosal fluid	*% of radioactivity at elastase position after electrophoresis (segment No. 12–14 of Fig. 2)*		*% of radioactivity eluted at void volume by gel filtration (fraction No. 9–12 of Fig. 3)*
	Serosal fluid	*Mucosal fluid*	*Serosal fluid*
[^{131}I]Elastase 0.01 %	30.1 ± 1.8	75.1 ± 1.1	30.4
K^{131}I*	0.0	***	1.2
[^{131}I]Elastase 0.1 %	32.1 ± 2.0	55.1 ± 3.5	34.1
K^{131}I**	0.0	***	

*, ** The same as Table II.
*** Not examined.

On the other hand, there was no area of radioactivity corresponding to elastase on the radiodiagram of the serosal fluid analyzed by electrophoresis and gel filtration after incubation in K^{131}I. All of the radioactivity was also found to be inorganic ^{131}I on paper chromatography.

DISCUSSION

The data obtained from the present study are summarized in Table V. The amounts of transported radioactivity from the mucosal to the serosal side after incubation with 0.01 and 0.1 % of [^{131}I]elastase were 1.58 and 2.26 % of the total incubated radioactivity, respectively.

TABLE V

TRANSPORT RATE OF [^{131}I]ELASTASE ACROSS RAT EVERTED JEJUNUM

Transport rate (%) of ^{131}I]elastase was calculated as follows; (transport rate of radioactivity*) × (% of radioactivity fractionated macromolecule-bound ^{131}I)/100. Each value indicates the mean with S.E. of 5 experiments.

Method of analyses	*Concentration of [^{131}I]elastase in initial mucosal*			
	0.01%		*0.1%*	
	Fraction %	*Transport rate*	*Fraction %*	*Transport rate*
Radioactivity*		1.58 ± 0.25		2.26 ± 0.33
Trichloroacetic acid precipitation	39.7 ± 4.4	0.63 ± 0.10	41.2 ± 2.7	0.96 ± 0.18
Electrophoresis	30.1 ± 1.8	0.46 ± 0.07	32.1 ± 2.0	0.75 ± 0.13
Dialysis	39.7 ± 2.0	0.63 ± 0.11	36.3 ± 1.5	0.83 ± 0.14
Gel filtration	30.4	0.48	34.1	0.77
Elastase activity**		not detected		1.87 ± 0.37

* Percentage of the amount of radioactivity transferred to the total radioactivity in the initial mucosal fluid (see Table II).

** Determined using elastin–orcein as a substrate.

The transport rate of the substance which is regarded as [^{131}I]elastase was obtained from multiplying the amounts of transported radioactivity by the fractionation percentages of protein-bound radioactivity. The transport rate of ^{131}I-bound macromolecule at the serosal side, 2 h after incubation in the mucosal concentration of 0.01 and 0.1 % [^{131}I]elastase, was 0.5–0.6 and 0.7–0.9 % of the initial total radioactivity, respectively. In the latter case, 1.87 % of the total enzymatic activity added to the mucosal side is also detected in the serosal fluid.

The results reported here suggest that it is possible for [^{131}I]elastase to be transported across the intestinal wall *in vitro*; comparing with the experimental results using K^{131}I, ^{131}I-bound protein in the serosal fluid which was detected by several analytical methods might be the protein originated from the [^{131}I]elastase added in the mucosal side before incubation. Furthermore this fact was confirmed by the presence of enzymatic activity and immunoreactivity to anti-elastase antibody in the serosal fluid,

From the preliminary experiment performed using fluorescein isothiocyanate-labeled elastase instead of [^{131}I]elastase, we obtained the same results as those with [^{131}I]elastase. Namely, in the enzymatic activity determined by a hydrolysis activity of casein at pH 8.8, 1.8 % of the total activity at the initial mucosal side were transfered to the serosal side and this value coincided with the transfer rate of [^{131}I]-elastase analyzed using elastin–orcein as a substrate. Comparing with the reports of

Moriya *et al.*[4] and Ogawa *et al.*[14], it is suggested that the permeability of elastase across intestinal membrane is similar to that of α-chymotrypsin and cytochrome *c*.

Since the viability of isolated intestine is important for *in vitro* experiment, the viability of everted sacs following the experiments was ascertained by the effects of elastase on the transport of glucose and phenol red, and microscopic examination. Elastase showed no effect on the transport of glucose and phenol red in everted sac. However, the morphological examination of everted sacs revealed that the findings resulting from the addition of elastase to the mucosal fluid were comparable to those of the controls without the addition of elastase and the partial desquamation of mucosal epithelia was found in all cases. Similar results with the viability of the everted sac have been reported by Kimura[5].

It is suggested from the results obtained herein that the possibility exists that elastase is transported across rat intestine *in vivo*.

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