Studies on Porcine Pancreatic Elastase Activity. I. Radioimmunoassay of Porcine Pancreatic Elastase

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NAKAJIMA, Y., MATSUNO, S., NOTO, N., SAITOH, Y. and SATO, T. *Studies on Porcine Pancreatic Elastase Activity. I. Radioimmunoassay of Porcine Pancreatic Elastase.* Tohoku J. exp. Med., 1980, 131 (2), 119-126 — An improved method of radioimmunoassay was devised to offer a successful formula for determining blood concentration of elastase. With porcine pancreatic elastase as the antigen, rabbits were immunized to obtain antiserum. Iodinated elastase labeled by the chloramine-T procedure using ^{131}I (or ^{125}I) had a specific activity of 200-300 mCi/mg. The double antibody method was used for BF separation. While the usual method of radioimmunoassay was not always successful in obtaining accurate serum concentration of elastase, the use of diisopropyl fluorophosphate (DFP) was able to eliminate the disturbing influence of intra-serous inhibitors, α_1 -AT and α_2 -M, eventually producing satisfactory results. With the use of DFP, the elastase standard curve and the porcine serum dilution curve had a statistically significant correlation; precision and recovery were both satisfactory; cross-reactivity of the antiserum with trypsin and chymotrypsin was less than 0.001%. The minimal detectable concentration of elastase was 5 ng/ml, and the range of normal fasting porcine serum level was 70-100 ng/ml. ——— porcine pancreatic elastase; radioimmunoassay; diisopropyl fluorophosphate

Elastase is clinically known to have a close relation to arteriosclerosis (Loeven 1969) and acute pancreatitis (Geokas et al. 1968a) for its two functions of proteolysis and elastolysis. Especially in acute pancreatitis, elastase is suspected to destroy elastic fiber on the wall of the pancreatic blood vessel, aggravating the disease into hemorrhagic or necrotic pancreatitis (Satake et al. 1974). For the quantitation of elastase, a variety of methods have been reported: using Congo red-elastin (Hall 1966), DNS-elastin (Rinderknecht et al. 1968) or other elastin substrates (Geokas et al. 1968b), or employing various synthetic substrates specific to elastase, being joined recently by radioimmunoassay (Caraballo et al. 1974). But these methods are only applicable for elastase in pancreatic juice and tissue. In the blood, the intervention of α_1 -antitrypsin (α_1 -AT) and α_2 -macroglobulin (α_2 -M) results in inhibition of the activity of the elastase, and therefore, no reliable method has so far been established to determine serum elastase. Now we have found that the influence of such inhibitors could be eliminated by employing diisopropyl fluorophosphate (DEP) and developed a fairly satisfactory method of radio-

immunoassay (R.I.). This report offers a summary of our improved method of R.I.

MATERIALS AND METHODS

^{131}I (or ^{125}I)-labeled elastase. Elastase was labeled with $\text{Na-}^{131}\text{I}$ (or $\text{Na-}^{125}\text{I}$) obtained from the New England Nuclear Co., Boston, by the method of Greenwood et al. (1963), and the labeled preparation was refined by gel filtration with a column of Sephadex G-50 (1.5×45 cm) equilibrated with 0.05 M phosphate buffer, pH 7.5.

Anti-porcine pancreatic elastase serum. The rabbit antielastase antibody was produced in rabbits by successive injections at 1 week intervals for 5 weeks with 2 mg of three times crystallized porcine pancreatic elastase which was produced by Eizai Pharm. Co., Tokyo, emulsified in complete Freund's adjuvant obtained from Sanko Junyaku, Tokyo. After five times of immunization, the blood was drawn from the ear vein and centrifuged to obtain antiserum which was stored at -20°C .

Reagents for radioimmunoassay. Gout anti-rabbit globulin serum and normal rabbit serum were obtained from Eiken Kagaku Co., Tokyo. Diisopropyl fluorophosphate (DFP) was obtained from Koch-Light Lab., London; bovine pancreatic trypsin was from Sigma Co., St. Louis; bovine pancreatic alpha chymotrypsin was from P-L Biochemicals, Milwaukee; enterokinase was from Sanko Junyaku, Tokyo.

Methods of radioimmunoassay (R.I.). R.I. 1. All reagents were diluted with standard radioimmunoassay buffer containing 0.5 M phosphate buffer, pH 7.5, and 0.2% (W/V) bovine serum albumin. After addition of standard buffer to make final volume of 0.7 ml, the following reagents were added, in the order indicated, 0 to 100 ng/0.1 ml of standard elastase or serum sample, 0.1 ml of elastase antiserum diluted 1:50,000 with standard buffer, and 0.1 ml of ^{131}I -(or ^{125}I) labeled elastase (10,000 cpm). After incubation for 2 days at 4°C , bound and free labeled antigen were separated by the double antibody procedure. An aliquot of 0.1 ml of gout anti-rabbit globulin serum and normal rabbit serum were added to each assay tube, followed by incubation for 24 hr at 24°C . Bound fraction was then isolated by centrifugation for 30 min at 3,000 rpm which was counted by α -scintillation system.

R.I. 2. After incubation of 0.1 ml of ^{131}I -(or ^{125}I) labeled elastase and 0.1 ml of elastase antiserum diluted 1:50,000 with 0.4 ml of standard buffer for 24 hr at 4°C , 0.1 ml of standard elastase or serum sample was added followed by incubation for 72 hr at 4°C . Then BF separation was performed by double antibody procedure in the same way as described in R.I. 1.

R.I. 3. All procedures were performed in the same manner as described in R.I. 1, except addition of 10^{-3} M DFP in the standard buffer.

Esterolytic activity of elastase. Esterolytic activity of elastase was determined by a pH-stat autotitrator (Radiometer Co.) at room temperature using 20 mM N-benzoyl-L-alanine methylester (BAME) obtained from Aldrich Chem. Co., Milwaukee, dissolved in 0.01 M Tris-HCl buffer, pH 8.0, as substrate. The unit of esterolytic activity was expressed in BAME $\mu\text{mole/min/mg}$. Inhibition of esterolytic activity of elastase by DFP was studied with different concentrations of DFP.

RESULTS

Gel filtration. The elution pattern of radioactivity showed two peaks, of which the first peak (A) was confirmed as ^{131}I -labeled elastase and the second peak (B) as free ^{131}I by determination of reaction of A or B and antielastase serum. The radioactive labeled elastase obtained had a specific activity of 200–300 mCi/mg (Fig. 1).

Anti-elastase serum. Titer of anti-elastase serum was confirmed by Ouch-

Fig. 1. Fractionation of ^{131}I -elastase on sephadex G-50.

The elution pattern of radioactivity showed two peaks. First peak, A, was confirmed as ^{131}I -labeled elastase and second peak, B, as free ^{131}I by the reaction of anti-elastase serum.

terlony's method (1948), and the final dilution was 350,000 fold in this radioimmunoassay system.

R.I. 1. The reaction of ^{131}I -labeled elastase and different concentration of anti-elastase serum showed a parabola with a peak at 1,000-fold dilution of anti-elastase serum, and the standard curve also showed a parabola with a peak at 1 ng/ml of standard elastase. Therefore, this method of radioimmunoassay was proved inadequate.

R.I. 2. The standard curve and porcine serum dilution curve showed almost parallel and seemed to be an adequate assay. However, the coefficient of variations of inter and intra assay precision was around 30%, and recovery rate was only around 10%. Therefore, this method was also proved still inadequate.

R.I. 3. Incubation at room temperature resulted in less precipitation of ^{131}I -labeled elastase than incubation at 4°C , and incubation at pH 7.5 resulted in most precipitation of ^{131}I -labeled elastase. Effect of incubation time of the first and second reaction resulted in a plateau after 48 hr and 24 hr. The standard curve and porcine serum dilution curve were parallel, which indicated statistically significant correlation between the theoretical value X and the measured value Y of elastase by $Y=1.015X-3.603$, $r=0.988$, $p<0.01$ (Fig. 2). The minimal detectable concentration of elastase was 5 ng/ml. Cross-reactions with other pancreatic enzymes were also studied with this method. No cross-reactivity has been observed with trypsin, chymotrypsin or enterokinase (Fig. 3). Inter-assay precision and intra-assay precision were studied in this assay. The coefficients of variations of the both precisions were mostly less than 10% (Tables 1 and 2). Recovery rate was 83% to 95% (Table 3). By contrast, when the exogenous elastase dissolved in standard buffer without DFP was added to this assay system, average recovery rate obtained was around 20% which suggested that exogenous elastase is bound to serum inhibitors α_1 -antitrypsin and α_2 -macroglobulin.

Fig. 2. Elastase standard curve and pig serum dilution curve.

The method of radioimmunoassay was described under "MATERIALS AND METHODS".
 ×-×, porcine pancreatic standard elastase; o-o, pooled normal porcine serum diluted until 1:32 with standard buffer.

Fig. 3. Cross-reactivity of various proteases with elastase antiserum. No cross-reaction was seen with trypsin, chymotrypsin, or enterokinase within usual assay concentration of elastase.

TABLE 1. Inter-assay precision obtained by R.I. 3

No.	Serum elastase (ng/ml)		
	A	B	C
1	15	38	74
2	16	38	80
3	18	41	83
Mean±s.d.	16.3±1.2	39.0±1.4	79.0±3.7
Coefficient of variation (%)	7.4	3.6	4.7

TABLE 2. *Intra-assay precision obtained by R.I. 3*

No.	Serum elastase (ng/ml)			
	A	B	C	D
1	16	38	76	150
2	13	41	74	185
3	15	37	76	186
4	16	38	76	180
5	15	39	90	150
6	19	35	73	140
7	18	41	70	160
Mean $\pm$ S.D.	16.1 $\pm$ 1.8	38.4 $\pm$ 1.9	76.4 $\pm$ 5.9	164.5 $\pm$ 17.2
Coefficient of variation (%)	11.1	4.9	7.7	10.5

TABLE 3. *Accuracy and recovery obtained by R.I. 3*

Sample	Mean (ng/ml)	Elastase added (ng/ml)	Mean $\pm$ S.D. (ng/ml)	Recovery (%)	Mean recovery (%)
A	12.0	50	53.0 $\pm$ 5.5	82.0	83.1
		100	103.5 $\pm$ 15.8	91.5	
		200	163.8 $\pm$ 17.8	75.9	
B	26.3	50	78.8 $\pm$ 7.4	105.5	94.4
		100	120.0 $\pm$ 9.4	93.7	
		200	195.0 $\pm$ 3.5	84.4	
C	76.0	50	123.7 $\pm$ 5.4	95.6	95.4
		100	183.8 $\pm$ 11.4	107.8	
		200	241.3 $\pm$ 11.4	82.7

Fig. 4. Inhibition by DFP of esterolytic activity of elastase.

1×10^{-4} M DFP (C in the figure) inhibited the esterolytic activity of elastase within 20 min and 1×10^{-3} M DFP (D) and 1×10^{-2} M DFP (E) inhibited the esterolytic activity of elastase within several min.

Inhibition by DFP of esterolytic activity of elastase. Inhibition of esterolytic activity of elastase was studied with different concentrations of DFP. DFP of 10^{-4} M inhibited the esterolytic activity of elastase completely within 20 min, and DFP of more than 10^{-3} M inhibited the activity completely within several min (Fig. 4).

Furthermore, incubation of ^{131}I -labeled elastase and anti-elastase serum with different concentrations of DFP in the standard buffer resulted in a plateau of precipitation of ^{131}I -labeled elastase at DFP concentrations of more than 10^{-4} M. These results suggest that elastase which was bound with DFP holds immuno-reactivity despite that the enzyme activity was completely inhibited.

DISCUSSION

Elastase, like trypsin and chymotrypsin, belongs to serine proteinase, and is an endopeptidase with a molecular weight of 25,900 (Shotton and Hartley 1970). This enzyme is secreted from the pancreas as proelastase and activated by trypsin to become elastase. The activity of elastase has so far been determined using a variety of elastin (Hall 1966; Rinderknecht et al. 1968), and a synthetic substrate as substrate (Bieth and Wermuth 1973). Although these procedures are fairly useful for determining the activity of elastase in pancreatic juice, duodenal juice, and pancreatic tissue (Geokas and Rinderknecht 1974), they cannot be used for elastase in the blood, because elastase is conjugated with an abundance of elastase-inhibitors α_1 -AT and α_2 -M in the blood, losing its enzymic activity (Turino et al. 1974). Carballo et al. (1974) reported radioimmunoassay for the first time to determine the blood concentration of elastase in the canine pancreas. But their procedure remains doubtful, because they did not consider the inhibitor's influence and did not make the statistical analysis. Geokas et al. (1977) recently extracted elastase from the human pancreas, and eliminated the elastase-inhibitor's influence by using phenylmethane sulfonyl fluoride, and performed radioimmunoassay with satisfactory results.

In our experiments, both of elastase standard curves and antibody dilution curves obtained without use of DFP revealed to be unreasonable and inadequate to be used in determination of elastase concentration in the blood, suggesting interference with intra-serous inhibitors. Although Ooyama et al. (1977) reported that they have been able to eliminate the influence of inhibitors by incubation of the tracer and antibody for 24 hr following addition of standard elastase or serum sample, satisfactory result was not obtained in our own experiment.

Since Kaplan and Dugas (1969) reported BAME as a specific synthetic substrate of elastase, this substance has been used as a substrate for determining esterolytic activity of elastase. It is also known that a serine enzyme inhibitor DFP can inhibit esterolysis of BAME (Naughton and Sanger 1961). Our experiment has shown that DFP of more than 10^{-3} M can inhibit esterolytic activity of elastase completely within several min without any interference with immunoreactivity of elastase. On the basis of the result that elastase bound with DFP still holds

immunoreactivity despite showing no enzymatic activity, we performed radioimmunoassay with satisfactory result of inter-assay precision, intra-assay precision and recovery rate. The usual concentration of elastase in porcine serum obtained by our R.I. ranged 70~100 ng/ml; these values are almost equivalent to the level in human serum recently identified by Geokas et al. (1977) and only 1/10 of the values so far reported (Satake et al. 1974; Rinderknecht et al. 1968). However, there are reports that serum elastase is conjugated with α_1 -AT and α_2 -M, and only α_1 -AT-elastase shows a cross-reaction with the antibody (Geokas et al. 1977; Katayama and Fujita 1974), implying insufficiency in clinical applicability of this method for the determination of the concentration of total serum elastase, because the ratio of α_2 -M-elastase relative to α_1 -AT-elastase in the serum is unknown. Nevertheless, Baumstark (1967) reported that about 90% of elastase in serum are conjugated with α_1 -AT, and, therefore, the determination of α_1 -AT-elastase may give a reliable information for a general trend of total serum elastase. Furthermore, Geokas et al. (1977) reported that no cross-reaction was seen in either porcine pancreatic elastase antibody with human pancreatic elastase or human pancreatic elastase antibody with porcine pancreatic elastase. So, we are now making efforts to study the concentration of human serum elastase.

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