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RADIOIMMUNOASSAY OF HUMAN PLASMA TRYPSIN

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Summary

A radioimmunoassay has been developed for the determination of human trypsin (3.4.21.4) in plasma. It allows the measurement of trypsin concentration in spite of the presence of plasma or pancreatic inhibitors.

The human trypsin used as a standard and for labelling was isolated from pancreatic tissue and purified by affinity chromatography. The antiserum was obtained from guinea-pigs immunized with partially purified human trypsin.

In the radioimmunoassay, the values of trypsin in serial dilutions of plasma were parallel to those of the standard curves. The assay was shown to be reproducible, sensitive and specific. However, the two antisera used did not distinguish between the enzyme and its proenzyme.

In normal subjects, plasma values were found to be around 400 ng/ml. They were 10—40 times higher in patients with acute pancreatitis. The method appears to be much more specific for the diagnosis of acute pancreatitis than the current determinations of amylase and lipase activity.

Introduction

The introduction of a great variety of synthetic substrates for proteolytic enzymes has allowed the development of several methods for measuring these enzymes. However, the presence of trypsin (EC 3.4.21.4) in plasma is still difficult to demonstrate by any of the enzymatic methods available on account of the very high inhibitory capacity of three different circulatory trypsin inhibitors, α_1-antitrypsin, α-macroglobulin, and inter-α-trypsin.

Measurement of trypsin in plasma made on the basis of the observation that

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trypsin, although bound as a complex together with a plasma inhibitor, retains some proteolytic activity towards specific substrates [1,2] has given rise to controversy on the tryptic activity of plasma in normal subjects and in patients with acute pancreatitis [3—5].

In a previous study on radioimmunoassay of enzymes from the exocrine pancreas, Temler and Felber [6] have demonstrated that measurement of porcine trypsin is not inhibited by the presence of diisopropylfluorophosphate iPr_2P-f, beef pancreas trypsin inhibitor (Trasylol) or bovine plasma.

The purpose of the present study was to develop a similar assay for measuring human trypsin in plasma in view of the need for such a measurement in pancreatitis because of the lack of specificity of the enzymatic assays for amylase and lipase generally used for the diagnosis of this disease.

Material and Methods

Enzymes

Human trypsin was isolated from human pancreatic tissue obtained from autopsy and immediately frozen. It was purified by affinity chromatography, using bovine pancreatic inhibitor (Kunitz) coupled to activated CNBr-Sepharose. Human trypsin was readily bound to the insoluble adsorbent at pH 4.0 and released at pH 1.5. The isolated material gave a single band on polyacrylamide gel electrophoresis. It was free of chymotryptic activity, and the value of its specific activity was slightly higher than the bovine 3 × crystallized commercial trypsin (Miles-Seravac). An enzyme solution (0.2 mg/ml in 0.001 M HCl) was divided into microtubes (5 μg/tube) and stored at −20° C until use for labelling or standard. When stored this way, trypsin remains stable for at least one year.

Enzyme inhibitor

Beef pancreas trypsin inhibitor (Trasylol) was a gift from Bayer, Pharma AG, Basel, Switzerland.

Enzymatic assay

The assay for enzymatic trypsin was performed by using benzoyl arginine ethyl ester from Fluka, Buchs, Switzerland, as a substrate according to the method of Schwert and Takenata [7]. Values were read on a Beckman DB spectrophotometer at room temperature. When necessary ΔA/min was converted into NF units, 1 NF unit being the activity which causes an increase of 0.003 in absorbance under the conditions used for the assay.

Radioimmunoassay

Human trypsin was labelled according to the method of Greenwood, Hunter and Glover [8], using Chloramin T for oxidation of $Na^{131}I$ or $Na^{125}I$. $Na^{131}I$ was purchased from the Eidg. Institut für Reaktorforschung (5303 Würenlingen, Switzerland), $Na^{125}I$ from Philips Duphar, Eindhoven, The Netherlands.

Aliquots of 5 μg of human trypsin (one tube from the stock) were thawed just before use and kept in crushed ice during labelling. The quality of the labelled material was checked by chromatoelectrophoresis according to the method of Yalow and Berson [9]. The specific activity was approx. 150—180

μCi/μg of trypsin. Further details on labelling, separation of the labelled hormone from the damaged fraction and verification of the purity of the labelled enzyme were reported in a previous paper dealing with radioimmunoassays of enzymes from beef, pork and rat exocrine pancreas [6]. Antisera against human trypsin were produced as previously described, from bovine, porcine and rat trypsin antisera [6,10].

The rabbit anti-guinea pig γ-globulin serum which served in this study as a precipitating antiserum was obtained by immunization of rabbits with guinea-pig γ-globulin according to the method of Hales and Randle [11].

Assay conditions

The method used in this study is derived from the double-antibody technique developed by Hales and Randle [11] for insulin determination. All the dilutions of human plasma trypsin and antisera were carried out with stock buffer (0.004 M phosphate, pH 7.4) containing 0.25 mg/l merthiolate (Tiomersal, BDH) as a preservative.

The following solutions were left to incubate for 3 days at 4°C: (a) 0.1 ml antiserum diluted 1/8000 in stock buffer containing 20 ml/l bovine serum, (b) 0.1 ml of successive dilutions of human trypsin in stock buffer containing 3.5 mg/ml of human serum albumin (Swiss Red Cross) or 0.1 ml of the human plasma to be tested diluted solely in stock buffer, (c) 0.1 ml of labelled human trypsin (approximately 100 pg) diluted in the same buffer as the standard. At the end of this first incubation, 0.2 ml of anti-γ-globulin serum diluted 1/10 in stock buffer was added to each tube in order to precipitate the enzyme-antibody complex. Incubation was continued for another 16—24 h. At the end of this second incubation, free and antibody-bound antigens were separated by centrifugation at 5000 $\times$ *g* for 15 min. Each tube was then counted for 2 min in a Packard auto-gamma counter.

The percent binding of the tracer antigen was expressed as the logit of the ratio of the radioactivity of the bound fraction to the radioactivity of the same fraction in the absence of unlabelled antigen (B/B_0) multiplied by a hundred. Calculation of the standard curve and quality control of the assay were carried out according to the method of Rodbard et al. [12,13].

Study of the specificity of the antisera

Radioimmunological determinations of trypsin and trypsinogen were performed in parallel in human pancreas extracts prepared as described by Travis and Roberts [14]. The extracts were divided into two equal parts; one part was submitted to activation by overnight incubation at +4°C with a trace of enterokinase in 0.1 M phosphate buffer pH 5.8, whereas the second part was protected from activation by addition of a beef pancreas trypsin inhibitor at a concentration of approximately 1/10 that of trypsin. Both extracts were tested for trypsin activity. The activity of the activated extract had reached its plateau, whereas the activity of the extract protected from activation was undetectable. The active and inactive extracts were prepared in serial dilutions corresponding to the range of the radioimmunoassay standard curve, and concentrations of the enzyme and proenzyme were determined by radioimmunoassay in both solutions (Fig. 2).

Recovery studies

4 samples containing a stock solution of 50 μg/ml of human trypsin were preincubated overnight at +4°C with the following solutions: the first sample with the incubation buffer alone, the second with addition of an equimolar concentration of iPr_2P-f, the third with an equimolar concentration of beef pancreas trypsin inhibitor, and the fourth with bovine plasma in a 1/10 dilution. All the dilutions were made in 0.04 M phosphate buffer, pH 6.

Following incubation, the samples were diluted so as to reach a concentration of 100 ng/ml, and each sample was added to aliquots of normal human plasma that contained 420 ng/ml of trypsin. This plasma was prepared in four successive dilutions, as shown in Table IV. The concentration of immunoreactive human trypsin was measured in the four series of combined solutions, as well as in a series of dilutions of the same normal human plasma without any trypsin added. The endogenous concentration of the plasma was subtracted from the total value for measuring the concentration of immunoreactive trypsin recovered.

Cross-reactivity

Purified preparations of human, bovine and porcine trypsin (Miles Seravac) and rat trypsinogen II (prepared according to the method of Vandermeers and Christophe [15]) were tested for cross-reactivity with two human trypsin antisera. The results were calculated as the ratio (in percent) between the amounts of human trypsin and of the other enzymes required for a 50% displacement (B/B_0 = 50%) on a standard curve, as described above.

Measurement of immunoreactive trypsin in human plasma or serum

Trypsin was measured either in plasma or in serum. In the latter case, blood was withdrawn from an antecubital vein, left standing for half an hour at room temperature to allow it to clot, and the serum was separated by centrifugation. Serum or plasma was kept frozen at −20°C until use. In sera from 15 normal subjects, normal values were found to range between 300 and 460 ng/ml with a mean of 380 ± 100 (S.D.). No difference was observed between measurements in plasma and serum.

In patients with pancreatic diseases, the concentration of plasma trypsin, measured by radioimmunoassay was compared with the amylase activity in the same plasma and in urine taken on the same day and measured according to the method of Street and Close [16].

Results

The mean standard curve (Fig. 1) resulting from 6 separate experiments was performed within a period of 4 months. In each case, fresh ^{125}I- or ^{131}I-labelled trypsin was used as a marker, all the other conditions of the assay remaining unchanged.

The mean levels were calculated with their standard error means for each group of experimental values (namely 6 per group), corresponding to successive dilutions of the standard. A linear relationship was found between logit $B/B_0 \cdot 100$ and log of human trypsin concentration.

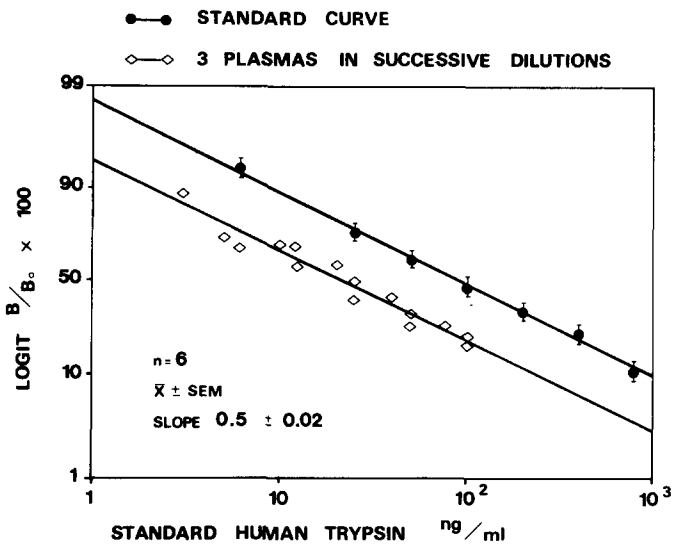

Fig. 1. Radioimmunoassay curves for human trypsin. ●———●, mean points of standard human trypsin; ◇———◇, serial dilutions of human plasma.

A dose-response curve for sera from 3 normal subjects prepared in 5 successive dilutions was found to be parallel to the standard curve.

Comparison of serial dilutions of human pancreas extract before and after activation of the proenzyme by enterokinase showed identity of results (Fig. 2). The common dilution curve ran parallel to the standard curve.

Assay statistics (Table I) were obtained from 4 separate experiments. The

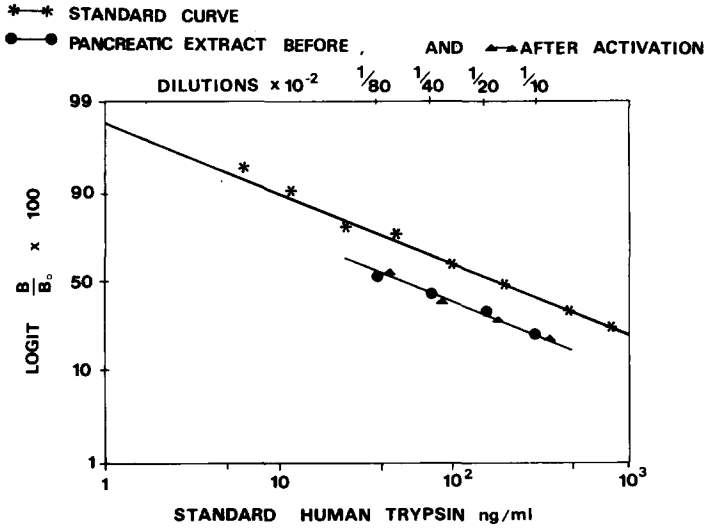

Fig. 2. The experiment was performed simultaneously with that shown in Fig. 1. *———*, radioimmunoassay standard curve for human trypsin. Serial dilutions of the human pancreatic extract before (●———●) and after activation (▲———▲) of the proenzyme.

TABLE I

ASSAY STATISTICS

Assay (No.)	Slope of standard curve	Slope of plasma dilution curve	Concentration of trypsin (ng/ml at 50% displacement)	Minimum detected enzyme (ng/ml)	Correlation coefficient standard curve	Correlation coefficient plasma dilution curve
1	0.48	0.43	150	10	—0.995	—0.990
2	0.42	0.42	180	5	—0.991	—0.991
3	0.53	0.51	185	2	—0.984	—0.995
4	0.55	0.54	190	5	—0.994	—0.997

slopes of the plasma dilution curves were closely related to the corresponding slopes of the standard curve. Correlation coefficients of the standard curves and of the plasma dilution curves varied between —0.984 and —0.997, and the minimum detected enzyme concentration from 2 to 10 ng/ml.

Reproducibility of the method was tested by measuring variances within and between assays, using two plasmas at different dilutions (Table II).

A study of the cross-reactivities of human chymotrypsin, bovine and porcine trypsin and rat trypsinogen II with two human trypsin antisera (655 B and A 1) is shown in Table III. No significant cross-reactivity was shown between human trypsin and chymotrypsin or trypsin and trypsinogen from the other animal species.

Trypsin concentration determined on many occasions in human plasma in parallel to human serum always displayed identical results.

Table IV represents a series of experiments on recovery. The values of human trypsin do not appear to be significantly modified by the presence of the various plasma dilutions to which it was added, nor by the presence of various enzymatic inhibitors (iPr_2P-f, beef pancreas trypsin inhibitor, bovine plasma).

Table V shows the preliminary results of plasma trypsin concentration in 5 cases of acute pancreatitis, 3 cases of suspected pancreatitis and one case of parotiditis with meningitis. The elevated plasma immunoreactive trypsin concentration agrees well with the plasma and urine activities in acute pancreatitis, whereas, in parotiditis, the plasma immunoreactive trypsin concentration remains within the normal range in spite of a high amylase activity.

TABLE II

WITHIN AND BETWEEN ASSAY VARIANCE

	Plasma dilution	Plasma No.	Mean trypsin value ng/ml	Standard deviation	S.E.M.	C.V.
Within-assay variance $n = 15$ for each plasma	1/5	1	53.6	2.7	1.2	55.0
		2	93.5	13.8	6.9	13.8
	1/10	1	26.1	1.1	0.5	4.3
		2	44.0	5.2	3.0	12.0
Between-assay variance $n = 3$ for each plasma	1/2	2	233	57.0	33	24.7
	1/4		100	5.0	2.8	5.0

TABLE III

PERCENTAGE OF CROSS-REACTION WITH HUMAN TRYPSIN ANTISERUM

Antiserum (No.)	Human trypsin	Human chymotrypsin	Bovine trypsin	Porcine trypsin	Rat trypsinogen II
655 B	100.0	6.2	0.05	0.8	2.5
A 1	100.0	0.7	0.01	0.6	2.0

TABLE IV

PERCENTAGE OF EXOGENOUS TRYPSIN RECOVERED IN SUCCESSIVE HUMAN PLASMA DILUTIONS

Plasma dilution	Added exogenous trypsin	Added exogenous trypsin iPr_2P-F complex	Added exogenous trypsin bovine pancreatic trypsin inhibitor	Added exogenous trypsin in bovine plasma diluted 1/10
1/2	100.0	90.0	89.5	98.2
1/4	105.0	98.0	95.2	99.2
1/8	110.5	97.0	110.4	102.4
1/16	115.4	98.5	102.2	101.5

TABLE V

PLASMA IMMUNOREACTIVE TRYPSIN AND AMYLASE ACTIVITY IN CLINICAL CASES

No.	Date	Trypsin in plasma (ng/ml)	Amylase in plasma (units/ml)	Amylase in urine (units/ml)	Clinical diagnosis
1	26.11.74	8 500	15 000	45 000	acute pancreatitis
2	4. 1.75	12 000	22 000	49 000	acute pancreatitis
3	13. 1.75	12 000	10 400	—	acute pancreatitis
4	4. 3.75	20 000	11 800	14 600	acute pancreatitis
5	16. 4.75	5 000	8 130	15 000	acute pancreatitis
6	7. 1.75	750	1 000	—	episode of acute pancreatitis
7	9.12.74	2 000	2 300	—	suspicion of pancreatitis
8	13. 1.75	875	915	—	suspicion of pancreatitis
9	1. 5.75	400	9 400	—	parotiditis with meningitis
		<500	<2 700	<10 000	Normal values

Discussion

A radioimmunoassay for the determination of human pancreatic trypsin in plasma is reported here for the first time. Other immunological methods have previously been reported. Hureau et al. [17] were able to demonstrate the presence of trypsin in plasma in cases of haemorrhagic pancreatitis, using an agar gel plate, and Shapira et al. [18] have developed an immunoassay for the quantitative determination of human trypsin in the intestine content. These methods, however, were not intended for quantitative measurement of trypsin in plasma as the present radioimmunoassay is.

Pure human trypsin is essential for the development of this radioimmunoassay. Commercially available bovine or porcine trypsin cannot be used for specific human trypsin antiserum (Table III) on account of its lack of cross-reactivity with the human enzyme. The human trypsin used in the present study corresponds to the cationic trypsin described by Mallory and Travis [19]. These authors showed anionic trypsin to be unstable at acid pH and to present only a weak cross-reaction with antibodies prepared to the cationic enzyme. In the present case, it was most probably lost during acidic extraction. It is not excluded, however, that traces of anionic trypsin could be included in the measurement of cationic trypsin.

The present study shows that the radioimmunoassay for human trypsin may be applied to the measurement of pancreatic trypsin in the bloodstream. It seems to be specific and very sensitive. Its specificity, of immunological order, depends on the antigenic properties of the enzymes, but not on the affinity of the enzyme for its substrate. For this reason, the radioimmunoassay is capable of measuring trypsin and trypsinogen in spite of the presence of enzymatic inhibitors. It does not depend on the numerous factors that increase or decrease the catalytic activity of the enzyme. It also allows determination of the enzyme in the presence of other proteases such as plasmin and thrombin. However, the present antisera do not distinguish between the enzyme and its proenzyme. Moreover, as previously described [10,20], the assay measures the enzyme in terms of concentration rather than of activity, as is the case with the enzymatic method.

The preliminary results reported in this paper suggest that this radioimmunoassay might be of great value in the diagnosis of acute pancreatitis, as it does not present the lack of specificity shown in enzymatic assays for amylase and lipase. The total amylolytic activity measured in the blood is known to result from the sum of the activities of several alpha amylases of various origins, such as the salivary glands, the pancreas and the liver [21]. It is interesting to note here that, in the case of a child suffering from parotiditis with meningitis (Table V; case 9), trypsin remained normal despite an elevated amylase value, thus indicating that the pancreas was probably not involved in the disease. Lipase, used in association with amylase [22] was reported to be useful, although not in all instances, in cases of acute pancreatitis.

It is hoped that the value of the present radioimmunoassay will be assessed not only in a larger number of cases of acute pancreatitis, but also in chronic pancreatitis and more particularly in acute exacerbation of chronic pancreatitis.

In Table V, cases 6, 7 and 8 presented a slight elevation of plasma immunoreactive levels, whereas amylase activity remained within the normal range. This could suggest that trypsin measurement might be more sensitive for detecting pancreatitis.

The normal values of immunoreactive trypsin in plasma (in the order of 400 ng/ml), seem surprisingly high considering that this enzyme is supposed to act at the duodenal level, and that only traces of trypsin could possibly leak into the bloodstream without having any physiological importance. However, several authors have recently been interested in this problem, suggesting hypotheses concerning the presence of trypsin in the blood. Webster [23] mentioned three routes of entry for the pancreatic enzymes into the peripheral circula-

tion: venous, lymphatic and peritoneal absorption. Constant diffusion of a certain amount of pancreatic enzymes into the bloodstream suggests exocrine-endocrine partition of the pancreatic enzymes [24]. This might be only a fortuitous escape of the enzyme in the blood or, perhaps, some feedback mechanism of control.

Liebow and Rothman [25] have recently reported that intact digestive enzymes, particularly chymotrypsin and amylase, can be absorbed by the intestine and, through the bloodstream, reach the pancreas where they would be resecreted, thus describing the existence of an enteropancreatic circulation for at least some of the digestive enzymes.

The present radioimmunoassay for human trypsin, together with the radioimmunoassay for pancreatic carboxypeptides B in human serum [26], seems to provide a new valuable tool for the diagnosis of acute pancreatitis as well as for the study of the physiology and physiopathology of the pancreatic function.

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