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# THE TWO HUMAN TRYPSINOGENS

## EVIDENCE OF COMPLEX FORMATION WITH BASIC PANCREATIC TRYPSIN INHIBITOR-PROTEOLYTIC ACTIVITY

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## Summary

The formation of complexes between human trypsinogens and the basic pancreatic trypsin inhibitor is demonstrated by using affinity chromatography on Sepharose coupled to basic pancreatic trypsin inhibitor. This interaction indicates the pre-existence of the active site in human trypsinogens. This active site induces the proteolytic activity of the two zymogens which activate spontaneously at pH 5.6 and pH 8.0 before and after affinity chromatography. The effect of affinity-chromatography on trypsinogen spontaneous activation is not the same on trypsinogens 1 and 2. A striking difference appears between the activation of the two trypsinogens. In all cases, trypsinogen 1 autoactivates more rapidly than trypsinogen 2, except at pH 5.6 in the presence of 10 mM $Ca^{2+}$, which inhibits the autoactivation of trypsinogen 1. The effect of inherent proteolytic activity of human trypsinogens is discussed in relation to pathological conditions of enterokinase deficiency and acute pancreatitis.

## Introduction

Comparative studies of interactions of substrates and pseudo-substrates with bovine cationic trypsinogen and trypsin have demonstrated that the same functional binding site was involved in zymogen and enzyme [1—6]. The pre-existence of the active site in bovine trypsinogen was further demonstrated by the ability of the zymogen to form a complex with the basic pancreatic trypsin inhibitor [7]. However the dissociation constant of this complex was found to

Abbreviation: MES, 4-morpholinoethanesulfonic acid.

be higher than the dissociation constant of the enzyme-inhibitor complex [8]. This fact, associated with the weak proteolytic activity of the zymogen [9], indicates the importance of the rearrangement of the active site after the cleavage of the activating peptide bond for the full activity of the enzyme.

These results led us to study similar properties in the two human trypsinogens purified from pancreatic juice. These two trypsinogens are known by their amino acid composition and their activation peptides [10,11]. The catalytic properties of the two derived trypsins have been determined [12]. The demonstration of a pre-existing active site in human trypsinogens was particularly important since activation of trypsinogens 'in situ' seems to be associated with the development of acute pancreatitis in man [26].

In this paper, we describe the interaction of each human trypsinogen with basic trypsin inhibitor, and we compare the kinetics of trypsinogen activation before and after affinity chromatography on basic trypsin inhibitor coupled to Sepharose.

## Material and Methods

### *Material*

Human trypsinogens 1 and 2 were prepared from human pancreatic juice according to the method previously described [10]. The potential specific activities of the two zymogens were maximal and equal to 175 for trypsinogen 1 and 225 for trypsinogen 2 (measured on 10 mM Tos-Arg-OMe as substrate).

Human trypsins were prepared by autocatalytic activation of trypsinogens at 4° C for 3 h in a 5 mM Tris-HCl/40 mM NaCl/20 mM $CaCl_2$ buffer, pH 8.0.

Trypsin inhibitor-Sepharose was obtained by activating Sepharose 4B with cyanogen bromide and by coupling this activated Sepharose with kallikrein trypsin inhibitor® (Bayer AG) according to the method of Cuatrecasas [13]. Bovine trypsinogen was a commercial product (Worthington). Porcine enterokinase was the gift of S. Maroux.

### *Methods*

*I. Routine assays.* (a) Trypsinogen concentration was determined by measuring the absorbance at 280 nm, using the extinction coefficient $E^{1\%}_{1\,cm}$ of 15.2 for trypsinogen 1 and 15.1 for trypsinogen 2 [10].

(b) Activation of trypsinogen in chromatographic fractions was carried out with porcine enterokinase (1% by weight) in a 5 mM Tris-HCl/40 mM NaCl/20 mM $CaCl_2$ buffer, pH 8.0, at 4° C for 3 h.

(c) Trypsin activity was measured at 25° C, pH 7.9, with a pH-stat (Radiometer) using 100 mM NaOH. The assay was carried out in 5 mM Tris-HCl/20 mM $CaCl_2$/100 mM KCl in the presence of 10 mM Tos-Arg-OMe as substate. Trypsin units are given by the number of $\mu$mol of substrate hydrolyzed per min and specific activity is the number of trypsin units per mg of protein.

*II. Conditions of trypsinogen autocatalytic activation.* All the kinetics studies of trypsinogen activation were carried out at 4° C. Aliquots (0.2 ml) of incubation mixture were taken off periodically and trypsin activity was measured on Tos-Arg-OMe. Autocatalytic activations of trypsinogens 1 and 2 were studied under the conditions described for optimal activation of bovine

trypsinogen at pH 5.6 [14] and pH 8.0 [15]. Protein concentrations were in the range 17.5—35 μg/ml. Half-times of activation were determined on the kinetic graphs. These points correspond to 50% of the maximal activity.

## Results

*I. Interactions of human trypsinogens with the basic pancreatic trypsin inhibitor*

The interactions of human trypsinogens with the basic pancreatic trypsin inhibitor were studied by submitting pancreatic juice and purified trypsinogens to affinity chromatography on basic pancreatic trypsin inhibitor-Sepharose.

*(a) Chromatography of protein of pancreatic juice.* Fig. 1 shows the diagram of affinity chromatography of a sample of human pancreatic juice. A first peak of protein was eluted. It was devoid of direct and potential trypsin activity. The application of a 200 mM NaCl solution adjusted to pH 3.0 released a second peak of protein containing the two human trypsinogens eluted with a potential specific activity equal to 200, which is the average specific activity of trypsinogens 1 and 2.

*(b) Chromatography of purified trypsinogens.* At pH 8.0 in the column buffer (10 mM Tris-HCl/100 mM NaCl), both trypsinogens interacted with trypsin inhibitor and were adsorbed on the column. A pH gradient eluted each trypsinogen at pH 4.0. Fig. 2A shows the diagram of affinity chromatography of a sample of trypsinogen 1. The homogeneity of this sample was checked by polyacrylamide gel electrophoresis at pH 5.0 which showed one single band, and by dansylation which revealed one single N-terminal residue of alanine. However, the specific activity of this sample was low (75). As shown in Fig.

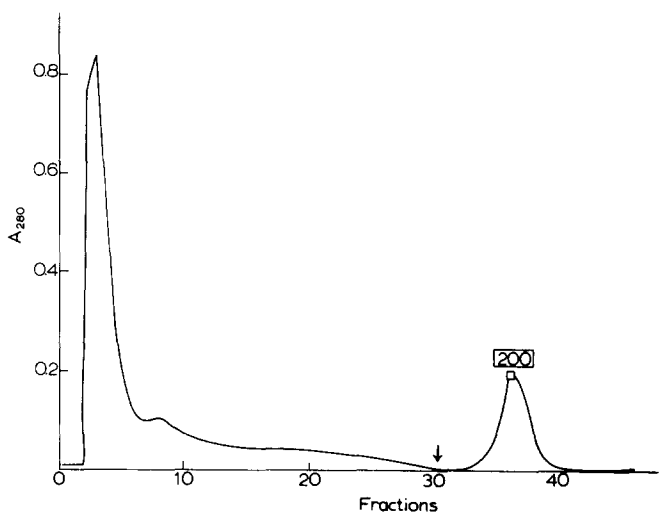


Fig. 1. Affinity chromatography on basic pancreatic trypsin inhibitor-Sepharose of proteins of pancreatic juice. The column (0.6 × 18 cm) was equilibrated with a 10 mM MES/100 mM NaCl buffer, pH 6.5. 1.6 ml of pancreatic juice containing 3 mg of proteins were loaded. At the arrow a 200 mM NaCl solution adjusted to pH 3 was applied. Volume of fractions, 1.2 ml. Number on the diagram indicates specific activity.

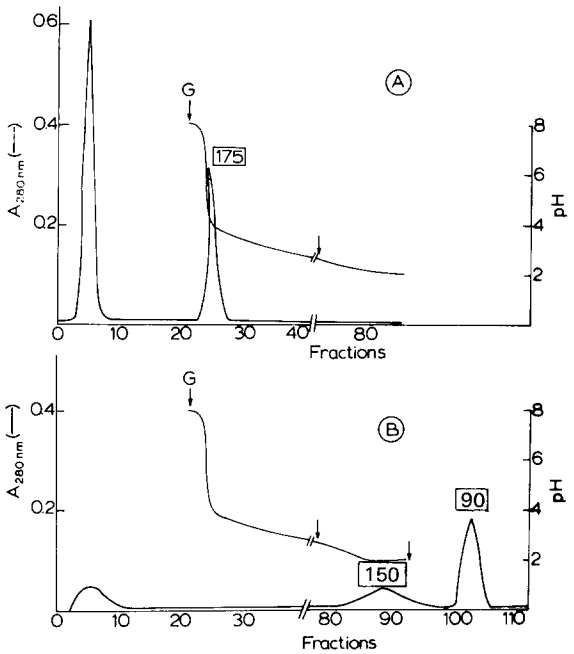


Fig. 2. Affinity chromatography on trypsin inhibitor-Sepharose of human trypsinogen 1 and trypsin 1. The columns (0.6 × 18 cm) were equilibrated in a 10 mM Tris-HCl/100 mM NaCl buffer, pH 8.0. Volume of fractions, 1.2 ml. The first arrow (G) indicates the beginning of the pH gradient (pH 4.0 to pH 2.5 in a glycine-HCl 0.2 M buffer; volume of each gradient chamber, 100 ml). The second arrow indicates the elution with a 0.2 M glycine-HCl buffer pH 2.0. The last arrow (in B) indicates the elution with a 6.5 M urea solution at pH 2.0. Numbers on the diagram are specific activities. (A) Chromatography of trypsinogen 1, 1.6 mg; (B) chromatography of trypsin 1, 0.6 mg.

2A, the first peak of protein eluted at pH 8.0 was devoid of direct and potential trypsin activity and contained 70% of the absorbance. A subsequent filtration on Sephadex G25 demonstrated that this absorbance was constituted by an equal amount of denatured trypsinogen and benzamidine, a competitive trypsin inhibitor added to all purification buffers (except that used for affinity chromatography). Trypsinogen 1 was eluted at pH 4.0 by the pH gradient with a unit recovery of 90%. Its potential specific activity increased 2.3 times to attain the value of 175, close to the maximal potential activity of trypsinogen 1. No direct trypsin activity was detectable.

The affinity chromatography of human trypsins under the same conditions showed that the behaviour of the active enzymes on trypsin inhibitor-Sepharose was quite different.

Fig. 2B represents the chromatography of a sample of trypsin 1. The breakthrough peak contained inert proteins devoid of trypsin activity (10% of loaded protein). The pH gradient was not able to remove any protein. When a 0.2 M glycine-HCl buffer, pH 2.0, was applied to the column, a small peak of trypsin activity (10%) was eluted with a specific activity of 150, and the remaining trypsin, strongly adsorbed on the column, was eluted with 6.5 M urea solution adjusted to pH 2.0. The specific activity of the enzyme partly denatured by urea and acidic pH was about 90.

The affinity chromatographies demonstrated the interaction of the two human trypsinogens with the basic pancreatic inhibitor at pH 8.0. Moreover, they allowed purification in a single step of the two human trypsinogens from the total proteins of pancreatic juice and of native trypsinogen from purified zymogen. This seemed to be a good method to separate the native precursor from contaminants such as denatured precursor, benzamidine and traces of trypsin.

*II. Autocatalytic activation of human trypsinogens 1 and 2 at pH 8.0 and 5.6. Effect of $Ca^{2+}$ concentration*

Human trypsinogens 1 and 2 activated spontaneously at pH 8.0 and the rates of activation increased with $Ca^{2+}$ concentration (Fig. 3). In the presence of 20 mM $Ca^{2+}$, the half-times of activation were 58 min for trypsinogen 1 and 98 min for trypsinogen 2.

Autoactivation of both trypsinogens occurred also at pH 5.6, but the effects of $Ca^{2+}$ were different on trypsinogens 1 and 2. For trypsinogen 1 the autoactivation was rapid in the absence of $Ca^{2+}$ and markedly retarded in the presence of 10 mM $Ca^{2+}$; no activity was detectable after 4.5 h (Fig. 4), but the same sample was fully activated after 18 h (data not shown). For trypsinogen 2 the autoactivation was not retarded by the presence of 10 mM $Ca^{2+}$ (Fig. 5). The initial rates of activation obtained in the absence and in the presence of 10 mM $Ca^{2+}$ were similar, but a difference was observed in the levels of maximal activity. The decrease of the maximal activity obtained in the absence of $Ca^{2+}$ was due to the fast autolysis of trypsin 2, as described elsewhere [12]. Bovine cationic trypsinogen studied under the same conditions showed, on the one hand a much slower rate of activation than the two human trypsinogens, and on the other hand no inhibitory effect of $Ca^{2+}$ at pH 5.6.

*III. Effect of affinity chromatography on autocatalytic activation of human trypsinogens*

In order to eliminate any traces of trypsin which would provoke the autocatalytic activation of human trypsinogens, each trypsinogen was submitted to

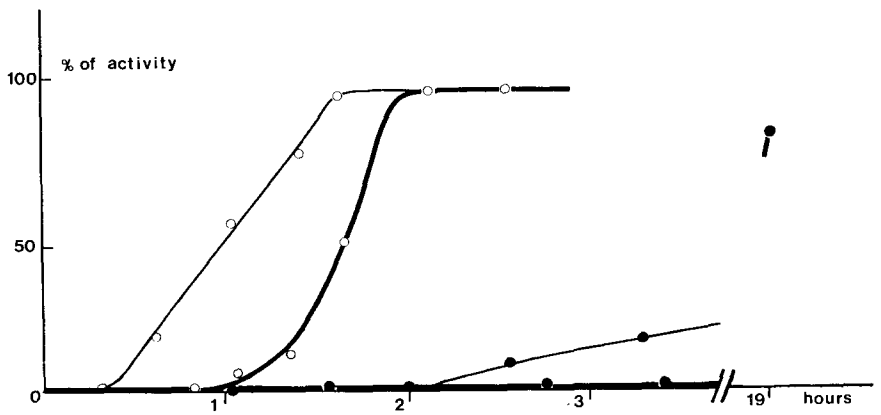


Fig. 3. Autocatalytic activation of trypsinogens 1 and 2 at pH 8.0 in the presence of 1 mM and 20 mM $Ca^{2+}$. Trypsinogen 1 (—) and trypsinogen 2 (—) in the concentration of 35 μg/ml were incubated at 4° C in a 5 mM Tris-HCl/100 mM NaCl buffer, pH 8.0, in the presence of 1 mM (●) and 20 mM (○) $Ca^{2+}$.

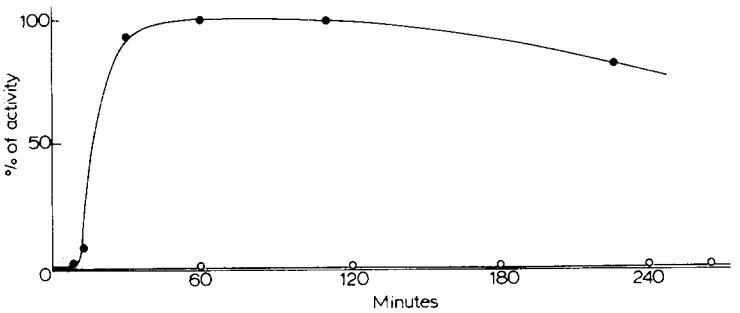


Fig. 4. Autocatalytic activation of trypsinogen 1 at pH 5.6. 40 μg/ml trypsinogen 1 was incubated at 4°C in a 28 mM succinate buffer, pH 5.6, in the absence of $Ca^{2+}$ (●) and in the presence of 10 mM $Ca^{2+}$ (○).

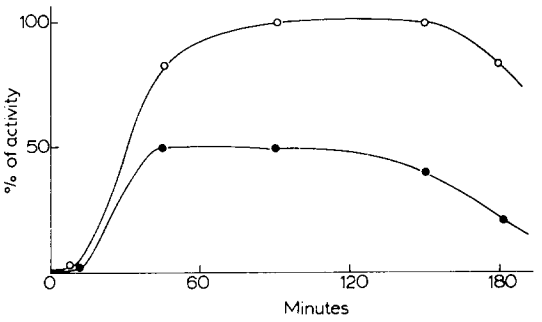


Fig. 5. Autocatalytic activation of trypsinogen 2 at pH 5.6. Trypsinogen 2 in the concentration of 30 μg/ml was incubated at 25°C under the same conditions as in Fig. 4.

affinity chromatography on trypsin inhibitor-Sepharose. The kinetics of the autoactivation of each zymogen obtained before and after chromatography are represented in Fig. 6. The kinetics of trypsinogen 2 were identical before and after chromatography. In contrast, trypsinogen 1 autoactivated more rapidly after affinity chromatography than before. Different samples of trypsinogen 1 and 2 were studied under the same conditions and some variability in the half-times of activation was observed. However, in all cases trypsinogen 1 autoactivated more rapidly after trypsin inhibitor-Sepharose chromatography than

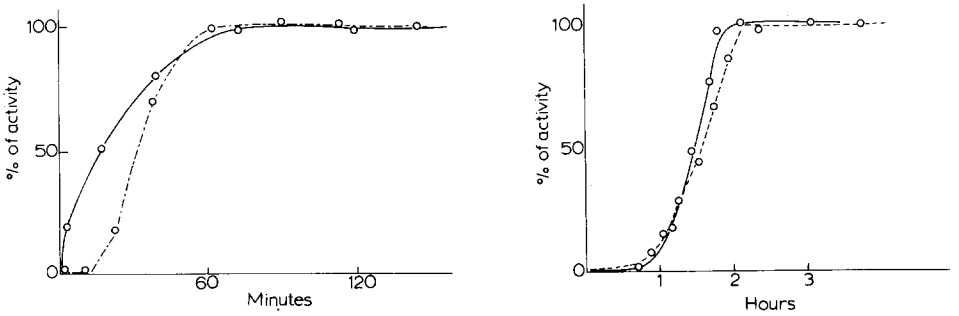


Fig. 6. Autocatalytic activation of trypsinogens 1 and 2 at pH 8.0 after affinity chromatography on trypsin inhibitor-Sepharose. Trypsinogen 1 (left, 17.5 μg/ml) and trypsinogen 2 (right, 35 μg/ml) were incubated at 4°C in a 5 mM Tris-HCl/100 mM NaCl buffer, pH 8.0, with 20 mM $Ca^{2+}$. (- - - - -), autocatalytic activation before affinity chromatography.

before. In some samples of trypsinogen 2 the elimination of some contaminant trypsin by this method led to an increase of the half-time of activation after chromatography. In these latter cases, after a second passage on trypsin inhibitor-Sepharose we found the same kinetics of autoactivation as after the first affinity chromatography. These results point to the reproducibility of the kinetics of autoactivation which characterizes trypsinogen 2. Moreover they demonstrate the inherent proteolytic activity of both human trypsinogens.

## Discussion

The purpose of this work was to answer the questions: is the active site preformed in the two human trypsinogens as in bovine trypsinogen, and is the catalytic apparatus efficient in human trypsinogens?

Our results of affinity chromatography show that pancreatic trypsin inhibitor forms a complex with human trypsinogens at pH 8.0 as is the case with human trypsins [16] and that therefore the binding site of the catalytic centre exists in human trypsinogens. The striking difference between the conditions of complex dissociation (pH 4.0 for trypsinogen-inhibitor and pH 2.0 with 6.5 M urea for trypsin-inhibitor) points to the weak binding of the zymogen with the inhibitor in comparison with the strong association of the enzyme-inhibitor complex. This result suggests that the active site is incompletely formed in the two human trypsinogens, as in bovine trypsinogen [7,8]. Our experiments show that the association of trypsinogen with the inhibitor is strong enough to separate denatured trypsinogen from native zymogen (Fig. 2A) and to isolate specifically trypsinogens from crude pancreatic juice in a single chromatography step. A similar procedure was recently described by Brodrick et al. using lima bean inhibitor-agarose for affinity chromatography [17]. However the elution profile obtained by these authors shows that in addition to trypsinogens other proteins interact with lima bean inhibitor-agarose, and that the association with the inhibitor in these conditions is not specific.

Human trypsinogens convert themselves into active trypsins, as demonstrated for bovine cationic trypsinogen by Kunitz [15]. However, the active sites of the human zymogens seem to be more efficient than the active site of the bovine cationic trypsinogen, since the half-times of activation are shorter for the two human zymogens than for bovine trypsinogen. These results are in agreement with the comparison made by Allan et al. [18] between bovine and one of the two human trypsinogens. Since the autocatalytic activation of purified trypsinogens may be impaired by the presence of a minute amount of active trypsin or benzamidine, the kinetics must be examined after affinity-chromatography of the two trypsinogens on inhibitor-Sepharose. Under these conditions remaining traces of trypsin and benzamidine are eliminated and our results show that all samples of trypsinogen retain the property of autoactivation. Moreover we have observed that trypsinogens purified from pancreatic juice by affinity chromatography also activate spontaneously. We know that this activation can in no case be due to a trypsin contamination, since, if present, free trypsin is strongly associated with the secretory trypsin inhibitor present in pancreatic juice. Therefore, the phenomenon of autoactivation must really be due to the inherent proteolytic activity

of the human zymogens, which are always in a dynamic system since they are at the same time both enzyme and substrate.

Whatever the conditions used (pH 5.6 or pH 8.0, 1 mM or 20 mM $Ca^{2+}$) a striking difference appears between the two trypsinogens. Before or after affinity chromatography, the activation of trypsinogen 1 is always faster than the activation of trypsinogen 2. Moreover, trypsinogen 1 autoactivates at pH 8.0 more quickly after chromatography than before. This phenomenon is not observed for trypsinogen 2, which in some cases shows an increase in half-time of activation after chromatography. The difference between the autoactivation of the two human trypsinogens is more obvious in presence of $Ca^{2+}$. There does exist a marked inhibition of the autoactivation of human trypsinogen 1 at pH 5.6 in the presence of 10 mM $Ca^{2+}$ while human trypsinogen 2, like bovine trypsinogen under the same conditions, is not inhibited. A more detailed study of the influence of calcium upon the activation of the two human trypsinogens will be reported in a further paper. The different behaviour of the two human zymogens can be explained by a difference in the conformation of the active sites of the two human trypsinogens and/or of the activating domain.

Crystallographic structural studies have shown that bovine trypsinogen in basic pancreatic trypsin inhibitor complex has a trypsin-like conformation [19,20]. In this case the Ile 16 binding pocket is formed but filled with some water molecules, as opposed to the Ile 16 N-terminus found in trypsin and in trypsin-trypsin inhibitor complex. In contrast, the chain segments forming the specificity pocket are disordered in the free zymogen [21]. Therefore, human trypsinogens must very probably undergo the same conformational changes upon interaction with basic pancreatic trypsin inhibitor. After dissociation of the complex, the return to the native conformation would be different for trypsinogens 1 and 2, which would explain the difference in the kinetics of autoactivation. This hypothesis is supported by our previous observation of the difference in the flexibility of the active site regions of the two human trypsins, which must pre-exist in trypsinogens [12]. In conclusion, an active site does pre-exist in human trypsinogens and is efficient to convert zymogens into active trypsins 'in vitro'.

The physiological significance of the inherent proteolytic activity of trypsinogens has to be questionned. It is reasonable to think that the two human trypsinogens may associate with the secretory trypsin inhibtor present in zymogen granules [22] and this complex would be one way of protection of the pancreas against zymogen-activation and autolysis. In the juice at alkaline pH, the low concentration of calcium, less than 3 mM (unpublished results) and the presence of all the other pancreatic zymogens which compete with trypsinogens, added to the presence of secretory trypsin inhibitor, could explain the absence of trypsinogen autoactivation. In the pathological case of enterokinase deficiency, human trypsinogens are not activated in duodenal contents [23,24]. However, the same trypsinogens are able to activate spontaneously 'in vitro'. Indeed, we have observed a complete activation of all zymogens in a sample of duodenal juice, after freezing and defreezing, from a patient suffering from congenital enterokinase deficiency (the sample was kindly supplied by Dr. B. Hadorn). Similar results have already been described by Polonovski [25] and the explanation is still not clear. These observations, as

well as our results on autoactivation of purified trypsinogens, may help us to understand why in other pathological conditions the autoactivation of trypsinogens may occur 'in vivo', leading to acute pancreatitis in man [26].

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