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PROTEOLYSIS OF HUMAN TRYPSINOGEN 1. PATHOGENIC IMPLICATION IN CHRONIC PANCREATITIS

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SDS electrophoresis on polyacrylamide gels of purified trypsinogen 1 has shown the occurence of a proteolysis in some molecules during long storage at -20°C. This proteolyzed trypsinogen gives a positive reaction with an antiserum directed against the precipitate protein, major protein of about 14 000 molecular weight extracted from precipitates present in the pancreatic juice of patients with chronic pancreatitis. The autoactivation of proteolyzed trypsinogen 1 liberates a polypeptide of 14 000 molecular weight which is immunologically identical to the precipitate protein. These results show that the major protein present in pancreatic precipitates (and pancreatic stones) of patients with chronic pancreatitis is a degradation product of trypsinogen 1 liberated by a proteolysis which necessarily requires a premature zymogen activation in the disease.

It is now well established that the formation of protein precipitates in pancreactic ducts play an important role in the pathogenesis of chronic calcifying pancreatitis (CCP) but the reasons why precipitation occurs are still unclear. A major protein with a low molecular weight (Ψ 14 000) has been isolated from these precipitates but the origin of this protein is also not known yet (1). Our previous biochemical studies on the proteins of pathological juice had shown a partial denaturation of some enzymes leading to lower specific activities and abnormal chromatographic behaviour (2). If protein denaturation may favor the precipitation phenomenon it remains to understand why and when partial denaturation occurs. As already noticed by us (3) and others (4, 5) human pancreatic juice is easily activated. This activation can be explained by the very fast conversion of the two human trypsinogens into trypsins and especially of the major form, trypsinogen 1 (6). This phenomenon is increased in the pancreatic juice of patients with CCP and we recently characterized a chymotrypsin- α1 proteinase inhibitor complex in these pathological juices apparently devoid of free proteolytic

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activity and still containing potential activity (7). These results gave support to the hypothesis of a premature activation of zymogens in the disease as suggested by others ten years ago (8) but the possibility that this activation occured after collection of pancreatic juice could not be eliminated. This paper brings arguments to demonstrate that the major protein present in the precipitates of patients with CCP (precipitate protein), is liberated by proteolysis of trypsinogen 1. This proteolysis necessarily requires an activation of zymogens which must be the key event in the pathogenesis of the disease.

MATERIAL

Human trypsinogens 1 and 2 purified by chromatography on Sephadex G-100 and DEAE-cellulose (9) and kept stored at -20°C for 3 to 6 years were used.

Precipitate protein was extracted by sodium citrate or EDTA from precipitates present in pancreatic juices of patients with chronic calcifying pancreatitis as explained elsewhere (1).

DFP-treated trypsin 1 (DP-trypsin 1) was prepared by chromatography of the proteins of human pancreatic juice on DEAE-cellulose at pH 8.0 (3), autoactivation of trypsinogen 1 in the presence of 20 mM $CaCl_2$ and affinity chromatography on PTI-Sepharose as described below. The eluted trypsin was subsequently inhibited by one hundred fold molar excess of DFP for 24h at 4°C and dialyzed against 0.001 M HCl.

Pancreatic trypsin inhibitor-Sepharose (PTI-Sepharose) was obtained by coupling activated Sepharose 4B Pharmacia with kallikrein trypsin inhibitor (R) (Bayer AG).

Antisera to pancreatic juice, DP-trypsin 1 and precipitate protein were prepared in rabbits by classical method.

METHODS

Preparation of trypsinogen samples : lyophilized trypsinogen (500µg) were dissolved in 0.5ml of distilled water containing 1 mM benzamidine at 4°C.

Protein concentration was determined by measuring the absorbance at 280 nm using the extinction coefficient $E_{1cm}^{1\%}$ = 20.0 for a mixture of protein and 15.0 for purified trypsinogens and trypsins.

Determination of trypsin activity : Trypsinogen in solution in 1 mM benzamidine was autoactivated by dilution 1:10 in a Tris-HCl pH 7.8 buffer containing 20 mM $CaCl_2$ and 40 mM Na Cl. Trypsin activity was measured on 10 mM p-tosyl-L-arginine methyl ester in a 5 mM Tris-HCl buffer containing 20 mM $CaCl_2$ and 100 mM KCl.

Immunoelectrophoresis and Ouchterlony double diffusion were performed on glass slides using 1.5% agarose in 25 mM barbital-acetate buffer, pH 8.6 and 0.02% sodium azide. The slides were stained with Coomassie brillant blue R 250 and destained with the mixture acetic acid/methanol/water (10/40/50).

SDS gel electrophoresis was performed in 15%polyacrylamide in tubes and in slab according to the method of Laemli (10). Apparent molecular weights were derived after calibration with the following purified proteins : serumalbumin (M_r 66 000), ovalbumin (M_r 45 000), bovine trypsinogen (M_r 24 000), β-lactoglobulin (18 400) and lysozyme (14 300).

Affinity chromatography of trypsin 1 : One thousand to two thousand trypsin units obtained by autoactivation of trypsinogen 1 at pH 7.8 in the

presence of 20 mM Ca^{++} were applied to a PTI-Sepharose column of 1.1 x 5cm equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 20 mM $CaCl_2$ and 200 mM NaCl. After the elution of inactive material and washing with a solution of 20 mM $CaCl_2$ and 200 mM NaCl, trypsin 1 was eluted by the same solution adjusted to pH 2.2. Purified trypsin was either dialyzed against solution at pH 2.5 or inhibited by DFP at pH 7.8 and dialyzed in the same conditions.

RESULTS

Demonstration of limited proteolysis in stored samples of trypsinogen 1 and in DP-trypsin 1.

Figure 1 shows the electrophoresis on polyacrylamide gels in the presence of SDS of a sample of trypsinogen 1 stored for four years at -20°C. Its potential specific activity is 100 instead of 230 for trypsinogen 1 freshly prepared (9). Before reduction of disulfide bridges, the protein is present as a major band of 23 500 molecular weight as previously determined for this zymogen after purification (9). After reduction the same electrophoresis separates three bands corresponding to proteins of 24 000, 14 000 and 11 000 M_r. These results demonstrate that a limited proteolysis has occured in the polypeptide chain of some molecules of trypsinogen during storage at -20°C. Similar results were obtained for 10 different samples of purified trypsinogen 1 but could not be observed for the two samples of trypsinogen 2 stored in the same conditions.

The same proteolysis was observed with a sample of DP-trypsin 1 proteolysis which probably occured during the slow inhibition by DFP (11) As

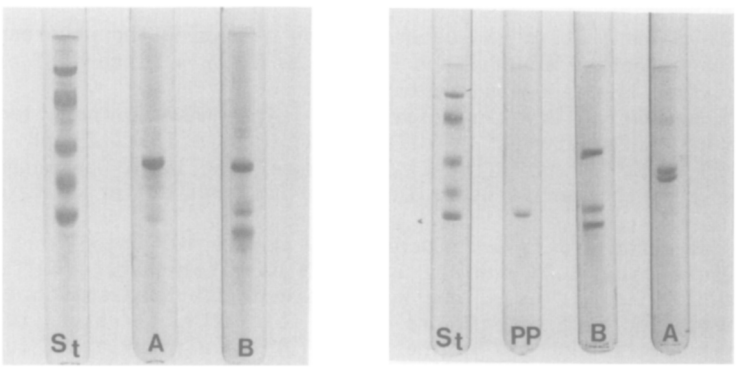

Figure 1 : Electrophoresis on SDS polyacrylamide gels of proteolyzed trypsinogen 1 (left side) and DP-trypsin 1 (right side)). 20-30 µg of protein were submitted to electrophoresis before reduction of disulfide bridges (A) and after reduction (B). St, reference proteins ; PP, precipitate protein (≃ 5 µg).

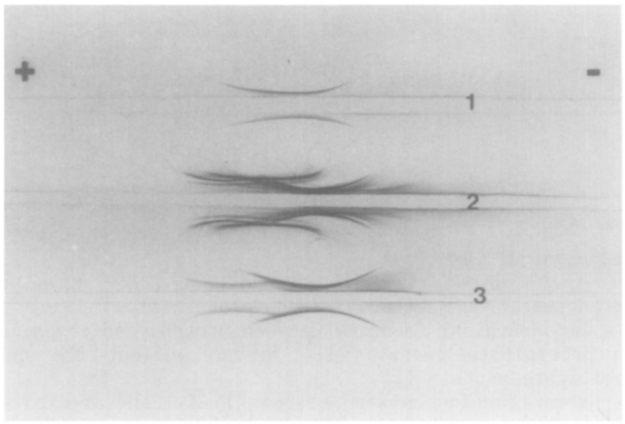

Figure 2 : Immunoelectrophoresis of human pancreatic juice.
The wells contain 120 μg of proteins of normal pancreatic juice in solution in 20 μl of 1 mM benzamidine and in the throughs are located 100 μl of antisera to precipitate protein (1), pancreatic juice (2) and DP-trypsin 1 (3).

shown on figure 1, before reduction of disulfide bridges, DP-trypsin 1 behaves like a mixture of two proteins with very close molecular weight (two forms of autolyzed trypsins) and after reduction three protein bands are separated with the same molecular weights as those found for reduced trypsinogen 1 (24 000, 14 000 and 11 000). On the same figure, the electrophoretic behaviour of the precipitate protein is represented for comparison. It migrates like a protein of roughly 14 000 M_r.

Immunological cross-reaction between trypsinogen 1 and the precipitate protein.

Fig.2 shows the specificity of the antiserum raised against the precipitate protein. By immunoelectrophoresis the antiserum gives one single line of precipitation against the proteins of pancreatic juice which by contrast exhibit many precipitating lines when tested with the antiserum directed against all these proteins. As shown on the same figure, the antiserum to DP-trypsin 1 tested against pancreatic juice gives two precipitating lines which correspond to the two trypsinogens which have commun antigenic determinants (9).

The analysis of proteolyzed trypsinogen 1 by Ouchterlony double diffusion gave a positive reaction with the antiserum directed against the

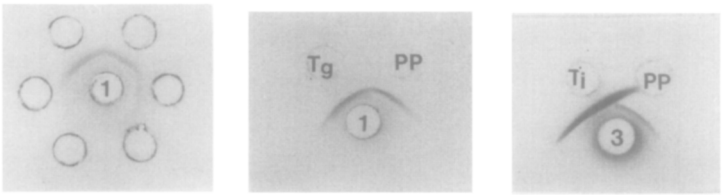

Figure 3 : Immunological reactions.
a - Immunological reaction between proteolyzed trypsinogen 1 and the antiserum against precipitate protein (1).
b - Immunological identity between proteolyzed trypsinogen 1 (Tg) and precipitate protein (PP) tested against the antiserum to precipitate protein (1).
c - Cross reaction between trypsin 1 (Ti) and precipitate protein (PP) tested against the antiserum to DP-trypsin 1 (3).

precipitate protein (Fig. 3a). Moreover a complete immunological identity does exist between trypsinogen 1 and the precipitate protein (Fig. 3b). In addition Figure 3c shows a positive reaction of the precipitate protein with the antiserum directed against DP-trypsin 1 and a cross reaction between the precipitate protein and trypsin 1. As expected, trypsin 1 contains additional antigenic determinants compared to the precipitate protein, which suggests that the precipitate protein is only one part of the polypeptide chain of trypsin 1 and consequently of trypsinogen 1.

Liberation of a polypeptide immunologically identical to the precipitate protein by autoactivation of proteolyzed trypsinogen 1.

A sample of proteolyzed trypsinogen 1 was autoactivated at pH 7.8 in the presence of 20 mM $CaCl_2$ and the active trypsin (specific activity, 130) was submitted to an affinity-chromatography on PTI-Sepharose (Fig.4) . A first peak of protein devoid of trypsin activity was eluted with the first buffer (peak A) and the active trypsin was eluted at pH 2.2. (peak B) with a mean specific activity equal to 180. The increase of specific activity corresponds to the separation of inactive material which is eluted with peak A. As shown on the SDS slab gel presented in the same figure peak A contains a polypeptide with 14 000 M_r, while peak B (purified trypsin) and trypsinogen 1 before activation do not contain any low molecular weight protein. Moreover a positive reaction with the antiserum against the precipitate protein and similar to that shown in fig. 1a was obtained with trypsinogen before

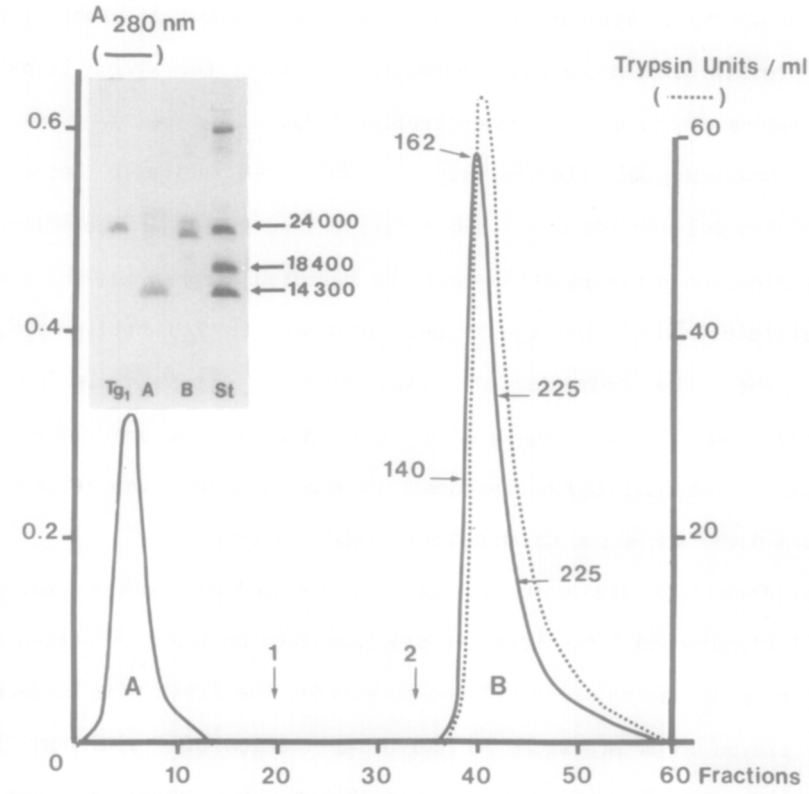

Figure 4 : Chromatography of the activation mixture of trypsinogen 1 on PTI-Sepharose.
The column (1.1x5cm) was equilibrated with a 10 mM Tris-HCl buffer pH 7.0 containing 20 mM $CaCl_2$ and 200 mM NaCl.
After the elution of the breakthrough peak the column was succesively washed with a solution of 20 mM $CaCl_2$, 200 mM NaCl (arrow 1) and the same solution adjusted at pH 2.2 (arrow 2).
Numbers along peak B represent specific activities of trypsin. SDS polyacrylamide gels of trypsinogen 1 before activation, peak A and peak B without treatment by β-mercaptoethanol are given on the diagram.

activation and with peak A and no precipitating line was obtained with peak B. This result demonstrates that the 14 000 M_r polypeptide is liberated from proteolyzed trypsinogen 1 during activation and consequently belongs to the polypeptide chain of trypsinogen 1.

DISCUSSION

The data presented here show that human trypsinogen 1 can liberate by proteolysis and/or autolysis a polypeptide immunologically identical to the protein with the same molecular weight found in pancreatic precipitates (1) and in stones (12) of patients with CCP. These results strongly suggest that the precipitate protein is a degradation product of a well-known pancreatic

secretory protein, trypsinogen 1. This degradation product is probably a form of inert protein similar to that described by Kunitz for bovine trypsinogen in the presence of low calcium concentration (13).

Our immunological studies suggest that the antigenic determinants carried by the polypeptide of 14 000 M_r are buried in the intact molecule of 24 000 M_r since no cross-reaction could be detected between the antiserum to the precipitate protein and the trypsin molecule in its native state. By contrast, when the molecule of trypsinogen or trypsin is partially proteolyzed there is an unmasking of the antigenic determinants of the polypeptide as demonstrated by the positive reactions observed between these molecules and the antiserum to the precipitate protein.

The fact that the protein found in precipitates was a proteolysis product of trypsinogen 1 confirms our previous data on the easy activation of pathological pancreatic juices (7) and permits for the first time to ascertain the role played by a premature "in vivo" activation of trypsinogen in the pathogeny of chronic calcifying pancreatitis. The conditions of this premature activation remain to be precised but a good probability would be an activation by lysosomal enzymes. Two arguments favor this hypothesis : an increase of the specific activities of lysosomal enzymes has been found in the pancreatic juice of patients with CCP (14) and an activation of bovine trypsinogen by cathepsin B has been reported (15). Experiments in progress in the laboratory must permit to confirm this hypothesis.

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