

Caldecrin proform requires trypsin activation for the acquisition of serum calcium-decreasing activity

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Received 14 October 1993

Proform serum calcium-decreasing factor (procaldecrin) was purified from porcine pancreas acetone powder. Procaldecrin showed chymotrypsin activity after trypsin treatment in a time- and dose-dependent manner. Procaldecrin did not possess serum calcium-decreasing activity but acquired serum calcium-decreasing activity as well as protease activity after trypsin treatment. However, PMSF treatment after activation of procaldecrin by trypsin did not affect the serum calcium-decreasing activity, even though protease activity was nullified by treatment with PMSF. These findings suggest that the serum calcium-decreasing activity acquired by procaldecrin requires conformational change caused by trypsin treatment.

Chymotrypsin; Calcium; Proform

1. INTRODUCTION

Acute pancreatitis causes hypocalcemia, which suggests that pancreas tissue contains the serum calcium-decreasing factor [1]. Yoneda et al. reported the hypocalcemic factor in porcine pancreas [2]. We have reported the isolation and characterization of the serum calcium-decreasing factor (caldecrin) from porcine pancreas and that it is chymotrypsin-type serine protease [3].

The mammalian exocrine pancreas synthesizes, stores, and secretes proteins, in which the dominant fraction is the family of serine proteases [4]. The serine proteases are synthesized initially as precursor proteins (preproenzyme) for secretory proteins [5,6] and are secreted as proenzymes (zymogens) that require enzymatic cleavage of an amino terminus for conversion to active enzymes.

Thus, it is suggested that caldecrin exists in pancreas tissue as a proform. If so, it is of interest to know whether proform caldecrin possesses serum calcium-decreasing activity, and whether the mechanism for activation of the proform concerns the regulation of serum calcium-decreasing activity.

Activation of procaldecrin to caldecrin is essential for the acquisition of serum calcium-decreasing activity, but protease activity after activation is not necessary.

2. EXPERIMENTAL

2.1. Materials

Suc-Ala-Ala-Pro-Phe-pNA was purchased from the Peptide Institute, Inc. (Osaka, Japan). DFP, PMSF, APMSF and trypsin were obtained from Sigma Chemical Co. (ST. Louis, USA). Porcine pancreas acetone powder was prepared from fresh pancreas tissues [7].

2.2. Assay of procaldecrin

Procaldecrin was detected by enzyme-linked immuno assay (ELISA) using anti-caldecrin antibody as described previously [3] and by the acquisition of chymotrypsin activity after trypsin activation done as described below. Chymotrypsin activity was measured using a synthetic substrate, Suc-Ala-Ala-Pro-Phe-pNA [8].

2.3. Purification of proform caldecrin from porcine pancreas acetone powder

Acetone powder of porcine pancreas was extracted with 10 mM Tris-acetate buffer (pH 6.0) containing 1 mM DFP at 4°C for 1 h. After centrifugation, the supernatant was diluted with homogenizing buffer, and combined with DE-23 ion-exchange cellulose (Pharmacia). After gentle mixing for 2 h, the cellulose was packed on the column (2.7 × 8.7 cm), washed with the same buffer, and then the absorbed materials were eluted by a gradient of NaCl from 0 to 0.4 M in the starting buffer at a flow rate of 4.9 ml/min, and 9.2 ml fraction was collected. The fractions of proform caldecrin were combined and received saturated ammonium sulfate to 20%, and were then applied to a Toyo-pearl hydrophobic column (Tosoh, Japan). The absorbed materials were eluted by a gradient of ammonium sulfate from 20% to 0 in 30 mM sodium citrate buffer (pH 6.0) at a flow rate of 1.85 ml/min, and 3 ml fraction was collected. The third peak was collected, received ammonium sulfate and reapplied to a small hydrophobic column. Proform caldecrin was eluted with a small volume of sodium citrate buffer to concentrate, and then applied to a Sephadex G-100 (Pharma-

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Abbreviations: caldecrin, serum calcium-decreasing factor; DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; APMSF, (4-amidino-phenyl)methane-sulfonyl fluoride; Suc-Ala-Ala-Pro-Phe-pNA, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide.

cia) gel filtration column (3 × 95 cm) equilibrated with 30 mM sodium citrate buffer (pH 6.0). The fractions (5.7 ml per tube) were collected at a flow rate of 46 ml/h. The second peak was collected for proform caldecrin.

2.4. Time course of activation of procaldecrin by trypsin activation

Proform caldecrin (1.5 µg) was activated by incubation with trypsin at varied procaldecrin-trypsin ratios (mol:mol) for varied times at 4°C and the reaction was stopped by addition of 1 mM APMSF. Activated caldecrin was detected by acquisition of chymotrypsin activity for 0.1 mM Suc-Ala-Ala-Pro-Phe-pNA as a substrate.

2.5. Serum calcium-decreasing activity of procaldecrin

Procaldecrin was activated at 4°C for 60 min by trypsin at ratios of 1:100 and 2:100. Caldecrin and activated procaldecrin were incubated with and without 1 mM PMSF for 10 min at room temperature, and then the buffer was changed by the centrifuged-column procedure [9] with Sephadex G-50 column equilibrated with phosphate buffered saline (PBS). The eluted samples were diluted to a final concentration of 80 µg/ml with PBS. One hundred µl of the final sample per 20 g body weight of mouse was injected via mouse tail vein and serum calcium was assayed as described before [3].

2.6. Other methods

SDS-PAGE and Western blotting analysis were performed as described previously [3].

3. RESULTS AND DISCUSSION

Proform of porcine pancreas caldecrin (procaldecrin) was purified from homogenate of porcine pancreas acetone powder with 1 mM DFP by a combination of anion-exchange, hydrophobic interaction, and gel filtration chromatography with a yield of 5.7 mg of protein per 25 g of pancreas acetone powder.

Fractions containing procaldecrin were detected by ELISA and by the chymotrypsin assay after trypsin activation (see below). Fractions from each step of the purification were analyzed by SDS-PAGE (Fig. 1A) followed by Western blot analysis using antiserum for porcine caldecrin (Fig. 1B) as described previously [3].

Procaldecrin with a molecular weight of 30 kDa was immunoreacted with the anti-caldecrin antibody.

Next, we performed activation of purified procaldecrin by trypsin at varied ratios of trypsin to procaldecrin (Fig. 2A). The higher mol ratio of trypsin to procaldecrin caused rapid activation of procaldecrin and gave rise to maximum chymotrypsin activity within 5 min but later caused a slight decrease in the activity. Maximum activation was obtained by 60 min incubation at all ratios except the lowest ratio at 1:100. Procaldecrin activated by trypsin at the varied ratios for 60 min was analyzed by SDS-PAGE (Fig. 2B). The low treatment ratio (trypsin-procaldecrin ratio 1:100) caused partial conversion of pro to active caldecrin as indicated by double bands around 30 kDa, which corresponds to chymotrypsin activity (Fig. 2A and 2B, see 1:100 treatment ratio). The higher treatment-ratio of trypsin to procaldecrin showed complete conversion of proform to activated caldecrin, which was also indicated by chymotrypsin activity (Fig. 2A and 2B). These results suggest an apparently direct or one-step conversion of procaldecrin into activated caldecrin by trypsin treatment, and also suggest that the difference in molecular weight between procaldecrin (about 30 kDa) and activated caldecrin (about 28 kDa) was consistent with the removal of the activation peptide segment from the zymogen to its mature form.

We next investigated whether the activation of procaldecrin is related to the serum calcium-decreasing activity. As shown in Fig. 3, chymotrypsin activities of procaldecrin and procaldecrin activated by treatment with trypsin (1:100 and 2:100) were compared with serum calcium-decreasing activity. Procaldecrin possessed very low chymotrypsin activity and did not show serum calcium-decreasing activity. Trypsin treatment at 1:100 partially activated the chymotrypsin activity of procaldecrin to 7 times over basal activity, and serum

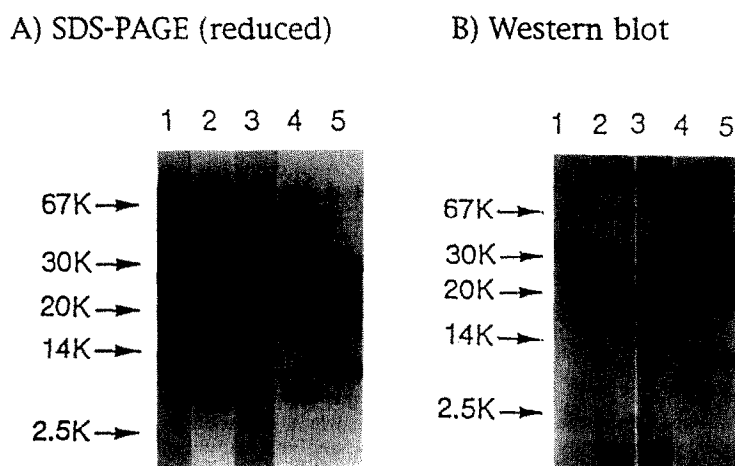


Fig. 1. (A) SDS-PAGE and (B) Western blotting analysis of procaldecrin during its purification steps for homogenate (lane 1, 32 µg), ion-exchange column eluate (lane 2, 25 µg), hydrophobic column eluate (lane 3, 19 µg), gel filtration column (lane 4, 9.4 µg) and porcine purified caldecrin (lane 5, 6.9 µg). The protein content of each lane of Western blotting analysis was half of that of SDS-PAGE.

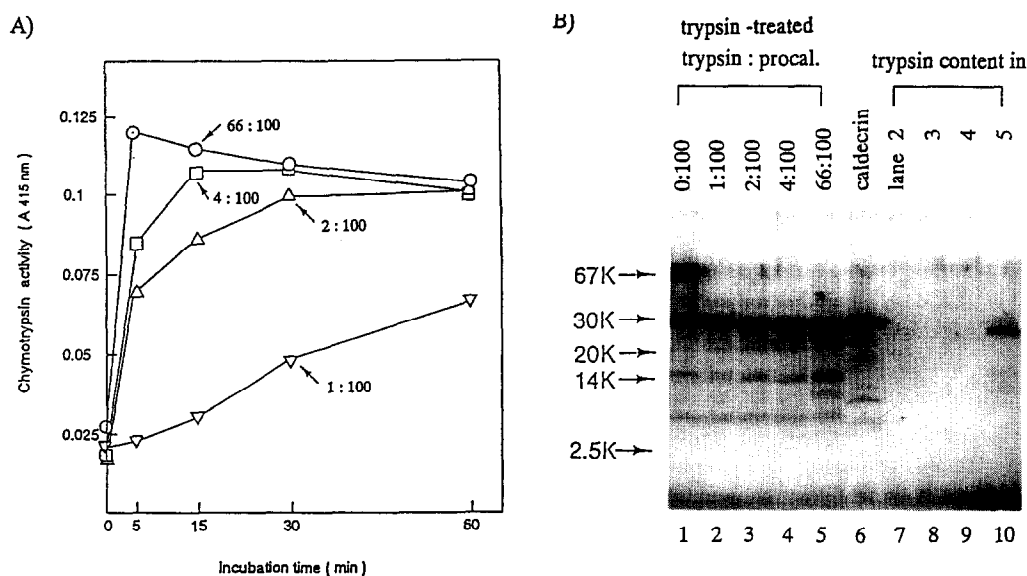


Fig. 2. Time course of activation of procaldecrin by trypsin. (A) chymotrypsin activity was determined by hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA. Procaldecrin was incubated with various ratios of trypsin for the indicated time at 4°C. Values are means of duplicate measurements. (B) SDS-PAGE (2.5 µg) per lane was used from the sample used for chymotrypsin activity at 60 min incubation.

calcium-decreasing activity also appeared (5.8% decrease from control serum calcium level). Treatment of PMSF after trypsin-activated procaldecrin destroyed chymotrypsin activity but retained the serum calcium-decreasing activity. In this assay, the trypsin used for procaldecrin activation was contaminated with procaldecrin. However, trypsin at the same dose as caldecrin did not show any serum calcium-decreasing activity [3]. Trypsin treatment of procaldecrin (2:100) caused full activation of chymotrypsin activity and serum calcium-decreasing activity comparable with the activities of purified active caldecrin. PMSF treatment to the fully activated procaldecrin by trypsin (2:100) and purified active caldecrin diminished the chymotrypsin activity but had no effect on the serum calcium-decreasing activity. These results suggest that the activation of the chymo-

trypsin activity of procaldecrin by trypsin is necessary for the acquisition of the serum calcium-decreasing activity, but that the chymotrypsin activity after activation of procaldecrin is not necessary for the serum calcium-decreasing activity. Conformational change of procaldecrin by trypsin treatment may be essential for the serum calcium-decreasing activity.

It is of great interest to know the mechanism of expression of serum calcium-decreasing activity from inactive procaldecrin. Recently, Arita et al. reported that mammalian secretory pancreatic phospholipase A₂ not only catalyzes the hydrolysis of phospholipids but also elicits a variety of biological responses in mammalian cells which have the specific receptor [10]. The enzymatically inactive precursor of phospholipase A₂ could not have receptor-binding capacity even though the structure of the precursor is almost the same as that of the mature form and the receptor-binding capacity of the mature form is separable from its enzyme activity [11,12].

Therefore, our present results showing that pancreatic procaldecrin acquires the serum calcium-decreasing activity by trypsin activation are highly homologous to the activation and bioactivity of pancreas phospholipase A₂.

Acknowledgements: We thank M. Gore for critical reading of the manuscript and M. Fujii for secretarial assistance. This work was partly supported by the Kodama Memorial Fund for Medical Science Research and the Tokyo Institute for Immunopharmacology.

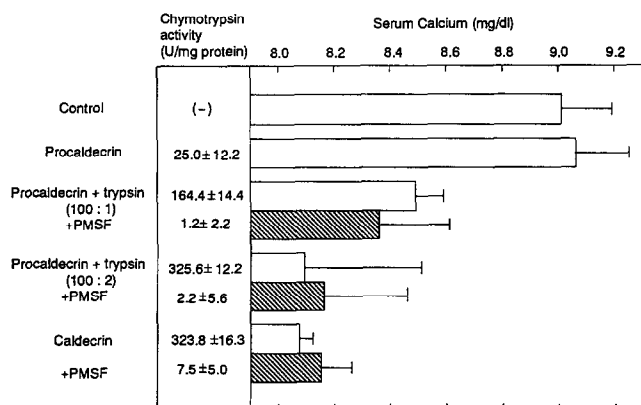


Fig. 3. Comparison of the chymotrypsin activity and the serum calcium-decreasing activity of procaldecrin and trypsin activated procaldecrin. One unit (U) of chymotrypsin activity indicates an increase of absorbance at 415 nm by 5 min incubation with substrate at 25°C. The chymotrypsin activity and serum calcium-decreasing activity are means ± S.D. of triplicate measurements and of five mice, respectively.

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