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Note

Purification of the pancreatic stone protein by high-performance liquid chromatography

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In 1979, De Caro et al. [1] reported the isolation of a minute amount of a low-molecular-mass ($M_r = 13\,500$) protein from human pancreatic stones, and subsequently demonstrated the presence of the same protein in normal and pathological human pancreatic juices [1]. In 1983, Multigner et al. [2] indicated that this protein may act as an inhibitor of spontaneous calcium carbonate precipitation in supersaturated pancreatic juice and hence the protein was referred to as pancreatic stone protein (PSP).

In 1984, De Caro et al. [3] purified PSP by classical biochemical techniques such as Trisacryl gel permeation and DEAE-Trisacryl ion-exchange chromatography. Recently, Provansal-Cheylan et al. [4] prepared a monoclonal antibody against PSP.

This paper demonstrates that PSP can be easily purified from human pan-

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creatic stone by high-performance liquid chromatography (HPLC). However, there is a discrepancy between the values for the molecular mass obtained by different analytical methods.

EXPERIMENTAL

Materials

Five human pancreatic stones were obtained at surgery from three patients with chronic calcifying pancreatitis. Case 1, a 55-year-old male, was diagnosed as having chronic alcoholic pancreatitis. Case 2, a 35-year-old male, with hyperparathyroidism and alcoholism, was operated on for parathyroid adenoma before three months of pancreatic surgery. Case 3, a 65-year-old male, was diagnosed as having chronic alcoholic pancreatitis.

Size-exclusion HPLC

HPLC analyses were carried out on a Pharmacia (Uppsala, Sweden) automated FPLC system with Superose 12 HR column (30 cm × 1.0 cm I. D.). The system was equipped with an LCC-500 gradient programmer, two P-500 dual-piston pumps, MV-7 and MV-8 automated injection valves, a solvent mixer, a prefilter, a sample loop (0.2 ml), a UV-1 monitor with HR low-dead-volume flow-cell and a Rec-482 recorder.

Protein determination

Protein concentrations were determined by bicinchoninic acid reagent (Pierce, Rockford, IL U.S.A.), according to the method of Smith et al. [5]. Bovine serum albumin (BSA) was used as the standard.

Isolation and purification of PSP

The pancreatic stones were extensively washed with 150 m*M* NaCl under continuous magnetic stirring to remove contaminating superficial proteins. The dried stones were demineralized by 0.2 *M* disodium ethylenediaminetetraacetate (EDTA), according to the method as modified by De Caro et al. [3]. Then the extracted proteins were dialysed (VT-351, Nakarai Chemicals, Kyoto, Japan) against distilled water for five days at 4 °C, freeze-dried, and stored at −80 °C until analysis. The resulting materials were dissolved in 1.0 ml of 150 m*M* NaCl–50 m*M* phosphate buffer (pH 7.2) and subjected to HPLC on the Superose 12 column equilibrated with the same buffer at 4 °C.

Fractions (0.4 ml) were collected for 90 min at a flow-rate of 0.4 ml/min. The protein content in each fraction was continuously monitored by a UV monitor at 280 nm, while the PSP content was monitored by an enzyme-linked immunoassay (ELISA) method described below. For the determination of the molecular mass of PSP, the following standard proteins (Pharmacia) were used: thyroglobulin (M_r = 660 000), aldrase (M_r = 158 000), BSA (M_r = 67 000),

ovoalbumin (M_r=43 000), chymotrypsinogen A (M_r=25 000), ribonuclease A (M_r=13 700) and bovine insulin (M_r=6000).

ELISA

The immunoreactive PSP was detected by a non-competitive ELISA employing the FAST system (Beckton Dickinson Labware, Oxnard, CA, U.S.A.) consisting of a screening plate with 96-beads, trough and 96-well microplate. Briefly, the procedure was as follows: 0.1 ml of each fraction separated by HPLC was put into the well of microplate. Then, the plate with 96 beads was placed onto the microplate and incubated to achieve sufficient protein adsorption for 18 h at 4°C. After adsorption, this plate was removed and placed in a trough containing 16 ml of 1% BSA in 150 m*M* NaCl–60 m*M* phosphate buffer (pH 7.2) (phosphate buffered saline, PBS) to remove unbound protein and to block the remaining adsorption sites on the beads. The plate was incubated for 10 min at room temperature. The unbound material was washed off with 0.05% Tween-20 in PBS (T-PBS), and the plate was placed in the trough containing 16 ml (1 μg/ml in T-PBS) of mouse anti-PSP monoclonal antibody (Immunotech, Marseille, France) for 2 h at room temperature. Washed as above, the plate was placed in the peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako, Glostrup, Denmark) solution (0.1% in T-PBS) for 2 h at room temperature. The second antibody was washed off with T-PBS, and the plate was placed on the 96-well microplate containing 0.1 ml of ABTS [2,2′-azino-di(3-ethylbenzthiazoline sulphonate)] peroxidase substrate solution (KPL Laboratories, Gaithersburge, MD, U.S.A.) and incubated for 30 min at room temperature. The reaction was stopped simultaneously in all 96 wells when the the plate was removed. Absorbances were read in a Titertech Multiskan (Flow Laboratories, Helsinki, Finland) at 405 nm.

Immunoblotting

For the determination of the molecular mass of PSP, 10–20% sodium dodecyl sulphate (SDS) gradient slab gel (Daiichi Pure Chemicals, Tokyo, Japan) electrophoresis was performed according to the method of Laemmli [6]. The gels were stained with 0.25% Commassie brilliant blue R-250 in 45% methanol–10% acetic acid. Immunoblotting was carried out according to the method of Towbin et al. [7]. Proteins were transferred electrophoretically to a nitrocellulose strip (GV, 0.22 μm, Millipore, Bedford, MA, U.S.A.) in a Trans Blot Cell (Bio-Rad, Richmond, CA, U.S.A.) for 2 h at 250 mA. After the strips were blocked with T-PBS, the strips were incubated in mouse anti-PSP monoclonal antibody (4 μg/ml in T-PBS) for 18 h at 4°C. Bound antibodies were visualized by avidin–biotin–peroxidase complex reagents (Vector Labs., Burlingame, CA, U.S.A.) as described by Hsu et al. [8]. The following standard markers (BDH, Poole, U.K.) were used: ovotransferrin (M_r= 77 000), BSA

(M_r= 67 000), ovoalbumin (M_r=45 000), carbonic anhydrase (M_r=30 000), myoglobulin (M_r=17 200) and cytochrome c (M_r=12 300).

RESULTS

The dry masses of the pancreatic stones from the three patients ranged from 210 to 1010 mg. The protein content extracted by EDTA demineralization was 0.81–0.86% of the total dry mass of the stones. The typical elution pattern of the proteins on HPLC with Superose 12 column is shown in Fig. 1. Three major peaks (I, II and III) were noted: their retention times were 36.9, 43.6 and 55.8 min and the molecular masses were 20 000, 6500 and 2000, respectively. In addition, several minor peaks were observed for all extracts, but no further investigations were carried out on them.

A sensitive ELISA was developed for the purpose of monitoring the PSP content of each fraction. The detection limit was determined as the smallest amount of PSP producing an absorbance of two standard deviations over the mean of the background: it was found to be 2 ng of PSP, corresponding to 0.1 ml of the solution containing 20 ng PSP per ml. Based on the analysis of ELISA it was confirmed that peak I corresponded to immunoreactive PSP, and its molecular mass was estimated to be 20 000.

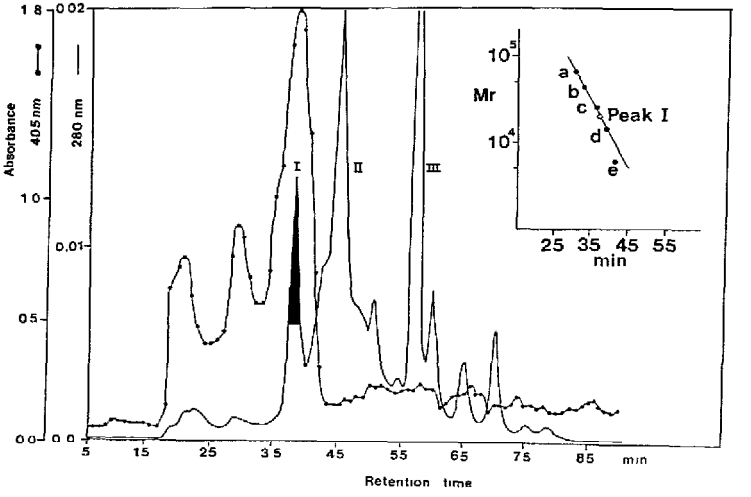

Fig. 1. Superose HPLC elution pattern of protein components of human pancreatic stone (case 1, 1010 mg as dry mass) extracted by EDTA demineralization: 0.25 mg of protein in 150 m*M* NaCl–50 m*M* phosphate buffer (pH 7.2) was injected and eluted with the same buffer. Protein (—) was monitored at 280 nm and immunoreactive PSP (—●—) was monitored at 405 nm. The shaded peak (peak 1), with an estimated M_r of 20 000, indicates the PSP peak. The calibration curve (upper right) was established using the following standard proteins: (a) BSA (M_r= 67 000), (b) ovoalbumin (M_r= 43 000), (c) chymotrypsinogen A (M_r= 25 000), (d) ribonuclease A (M_r= 13 700) and (e) bovine insulin (M_r= 6000).

On SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, the crude extracts were generally separated into a major band with M_r= 13 500 and a minor band with M_r= 30 000, and the peak with M_r= 20 000 obtained from HPLC with Superose 12 column was observed as a single band, thus agreeing with the M_r= 13 500 band (Fig. 2). Both the M_r=13 500 bands were strongly stained, but the M_r= 30 000 band of the crude extract was faintly stained.

The M_r= 20 000 fractions from the Superose 12 HPLC column were pooled and freeze-dried, and the purified PSP was found to range from 6.4 to 9.6% of the crude extracts, and 0.07% of the stones.

DISCUSSION

De Caro et al. [3] reported that PSP can be purified by Trisacryl GF 05 gel permeation and DEAE-Trisacryl M ion-exchange chromatography. In this HPLC study with a Superose 12 column we succeeded in purifying PSP from pancreatic stones.

The molecular mass of PSP was found to be 13 500, by the method of SDS-PAGE and immunoblotting, which confirmed the value reported by De Caro et at. [3]. On the Superose 12 HPLC column, its elution position indicated that the apparent molecular mass was ca. 20 000.

The amino acid composition of PSP has been reported by De Caro et al. [3]. The total number of residues was estimated to be 126 and the molecular mass was calculated to be 14 017. Its molecular mass determined by SDS-PAGE was

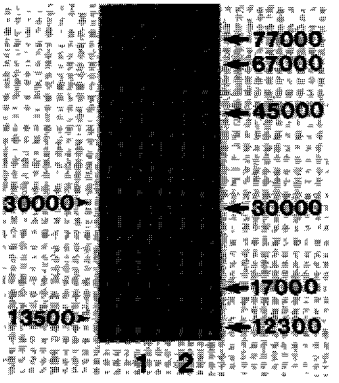

Fig. 2. Immunoblotting patterns of EDTA extract and the M_r= 20 000 peak prepared by HPLC on the Superose 12 column: 3 μg of protein were applied in each lane. Immunoreactive PSP was visualized using mouse PSP monoclonal antibody and avidin-biotin-peroxidase complex reagents. Lane 1= EDTA extract; lane 2= M_r= 20 000 peak prepared by on the Superose 12 HPLC column. Molecular mass markers: ovotransferrin (M_r= 77 000), BSA (M_r= 67 000), ovoalbumin (M_r= 45 000), carbonic anhydrase (M_r= 30 000), myglobulin (M_r= 17 200) and cytochrome c (M_r= 12 300).

close to the value calculated from the amino acid composition. Such elution behaviour on the Superose 12 column can be explained by the unusual protein conformation, as reported by De Caro et al. [3].

At present, detailed information on the protein components of pancreatic stones have not been reported. In the present study three major peaks were observed, and the PSP peak was found to have the highest molecular mass. However, the existence of the high-molecular-mass immunoreactive PSP was recognized by a sensitive ELISA.

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