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## Abstract

Sulfated acidic mucopolysaccharides have been found to be significant components of “protein plugs” in patients with chronic pancreatitis. The precise identification of the mucopolysaccharides and their distribution within the protein plugs may clarify the pathogenesis of the plugs. Pure pancreatic juice from five patients with chronic pancreatitis was obtained by endoscopic retrograde catheterization of the papilla of Vater. Enzymes for digestion of the plugs included hyaluronidase of the bovine testes and streptomyces hyalurolyticus, chondroitinase ABC and AC, and sialidase (neuraminidase). Our study indicated that: 1) Sialic acid is distributed throughout the plugs and may be a major component, followed by a lesser amount of chondroitin sulfate B. 2) Chondroitin sulfate A, C, D and E and chondroitin may be minor components. 3) Hyaluronic acid is negligible in the plugs.

**KEYWORDS:** chronic pancreatitis, pure pancreatic juice, protein plugs, enzyme-histochemistry

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## HISTOCHEMICAL STUDIES ON ENZYME-DIGESTED PROTEIN PLUGS OF PATIENTS WITH CHRONIC PANCREATITIS: A PRELIMINARY REPORT

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*Abstract.* Sulfated acidic mucopolysaccharides have been found to be significant components of "protein plugs" in patients with chronic pancreatitis. The precise identification of the mucopolysaccharides and their distribution within the protein plugs may clarify the pathogenesis of the plugs. Pure pancreatic juice from five patients with chronic pancreatitis was obtained by endoscopic retrograde catheterization of the papilla of Vater. Enzymes for digestion of the plugs included hyaluronidase of the bovine testes and *streptomyces hyalurolyticus*, chondroitinase ABC and AC, and sialidase (neuraminidase). Our study indicated that: 1) Sialic acid is distributed throughout the plugs and may be a major component, followed by a lesser amount of chondroitin sulfate B. 2) Chondroitin sulfate A, C, D and E and chondroitin may be minor components. 3) Hyaluronic acid is negligible in the plugs.

*Key words:* chronic pancreatitis, pure pancreatic juice, protein plugs, enzyme-histochemistry.

The pathogenesis of chronic pancreatitis, in particular alcoholic pancreatitis, is most likely due to the formation of "protein plugs" (abbreviated as plugs) in the pancreatic duct (1, 2). We have made histochemical studies on plugs obtained through endoscopic retrograde catheterization of the papilla of Vater, and described possible mechanisms of plug formation elsewhere (3); in brief, numerous clusters of desquamated epithelial cells were entrapped in a mixture consisting mainly of highly concentrated sulfated acidic mucopolysaccharides and glycoproteins. This communication describes the identification of the acidic mucopolysaccharides, the major components of plugs, using several enzymes to digest the plugs. An effort was made to minimize the amount of enzymes used because of the costliness of these enzymes.

### MATERIALS AND METHODS

The same five patients with chronic pancreatitis, including one non-alcoholic and four

heavy alcoholics, reported previously (3) were used. The diagnosis was made on past and present histories, clinical findings, pancreozymin secretin test, an endoscopic retrograde pancreatogram and computerized tomogram.

The gross appearance of the plugs in pure pancreatic juice (PPJ) was illustrated in Fig. 1 of the previous report (3). In the present study, PPJ together with protein plugs in the pancreatic duct was aspirated with an Olympus JF-B3 or -B4 (4).

The specimen was centrifuged at 3,000 rpm for 10 min which resulted in three layers consisting of a sediment, mucous layer and overlying clear layer. The sediment, the major part of the plugs, was fixed with 10% formaldehyde, embedded in paraffin, and serially sectioned to an approximate thickness of  $6\mu$  for enzyme study. Umbilical cord, aorta, cartilage

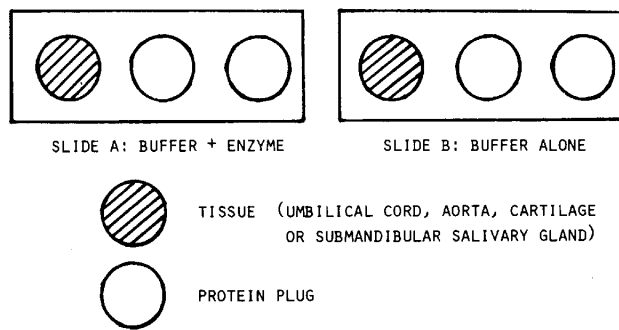


Fig. 1. Enzyme-histochemical studies: slide glass A and B.

TABLE 1. ENZYMES FOR DIGESTION OF PROTEIN PLUGS

Enzyme tested	Enzyme concentration	Buffer solution	Temperature for exposure (°C)	Time for exposure <sup>a</sup> (h)		Tissue <sup>b</sup>
				Exp I	Exp II	
Hyaluronidase (bovine testes)	10 mg/20 ml	0.1 M phosphate (pH 5.5)	37	2		Umbilical cord
Hyaluronidase (streptomyces)	1 ampule/1 ml	0.1 M phosphate (pH 5.0)	60	2	4	Umbilical cord
Chondroitinase ABC	1 ampule/2.5 ml	0.1 M tris-HCl (pH 8.1)	37	2		Aorta
Chondroitinase AC	1 ampule/2.5 ml	0.1 M tris-HCl (pH 7.4)	37	2		Cartilage
Sialidase (Neuraminidase)	100 u/ml	0.1 M acetic acid containing 0.04 M CaCl <sub>2</sub> (pH 5.2)	40	17	22	Submandibular salivary gland

<sup>a</sup>: After exposing specimens for a certain time, *e.g.*, the umbilical cord and protein plug specimens for 2 h, they were rinsed with distilled water and stained with alcian blue (pH 2.5)

<sup>b</sup>: To test the effectiveness of each enzyme, tissue containing a certain substance was chosen for the appropriate enzyme, *e.g.*, the umbilical cord containing hyaluronic acid for hyaluronidase derived from the bovine testes.

and salivary gland tissues as controls were also sectioned.

The enzymes for digestion included hyaluronidase derived from the bovine testes and *Streptomyces hyalurolyticus* (Seikagaku Kogyo Co. Ltd., Tokyo), chondroitinase ABC and AC (Seikagaku Kohyo Co. Ltd., Tokyo), and sialidase (neuraminidase) (Sigma Chemical Co. Ltd., USA). These enzymes were dissolved with an appropriate buffer solution (Table 1); the buffer solution alone was used as controls.

For hyaluronidase (bovine testes), as shown in Fig. 1, umbilical cord tissue and two pieces of the plug sediment were placed side by side on slide glasses A and B. A drop of a solution (approximately 0.1 ml) containing 10 mg hyaluronidase of the bovine testes dissolved in 20 ml of 0.1 M phosphate buffer solution (pH 5.5) was placed on slide glass A. On slide glass B, a drop (likewise, approximately 0.1 ml) of the buffer solution only was placed. After a coverglass was placed on the slides, they were kept in a paraffin stretcher, Type PS-SB (Sakura Seiki Co. Ltd., Tokyo). The plastic paraffin stretcher compartment was 2 (height) × 27 (width) × 52.5 (length) cm, and its bottom was made of a plastic-coated aluminium plate by which the temperature was kept constant at 37°C. The bottom surface was covered by several pieces of wet gauze to keep the moisture constant. Two h later, specimens were rinsed with distilled water and stained with alcian blue (AB) (pH 2.5). Stainability of the material with AB was then compared between slide glass A and B.

As to hyaluronidase (streptomyces), chondroitinase ABC, chondroitinase AC and sialidase, the procedures were essentially the same as above, though two different exposure times to hyaluronidase (streptomyces) and sialidase were attempted (Table 1).

## RESULTS

The use of a paraffin stretcher made it feasible to maintain the temperature and moisture stable. In addition, the application of slide glasses helped in minimizing the amount of enzymes used. The time required for exposure to sialidase was 17 to 22 h before complete digestion of sialic acid of the submandibular salivary gland. During that period, a drop of solution containing sialidase was added to the glass slide, twice in experiment I (17 h) and three times in experiment II (22 h); this amounted to only 0.3 and 0.4 ml of solution, respectively. Through this procedure, the umbilical cord, aorta, cartilage and salivary gland tissue on slide glass A were completely digested with enzymes and not stained with AB (pH 2.5), whereas no digestion occurred on slide glass B to which only buffer solution was added.

As to hyaluronidase (bovine testes), chondroitinase ABC and sialidase, we found a prominent difference in the stainability of plugs with AB between slide glass A and B. That is, plug sediment on slide glass B of the hyaluronidase (bovine testes) and chondroitinase ABC experiments were stained blue in a lamellar pattern, whereas plug sediment on slide glass A of both experiments were stained mostly light blue with a few areas being very lightly stained. Plug sediment on slide glass A of the sialidase experiment appeared rather faint except for a minor area stained light blue. On the other hand, in the hyaluronidase (streptomyces) and chondroitinase AC experiments there was no appreciable difference in the stainability between slide glass A and B.

## DISCUSSION

TABLE 2. ENZYMES FOR DIGESTION OF MUCOPOLYSACCHARIDES

	Enzymes.				
	Hyaluronidase (bovine testes)	Hyaluronidase (streptomyces)	Chondroi- tinase ABC	Chondroi- tinase AC	Sialidase (Neuraminidase)
Mucopolysaccharides					
Hyaluronic acid	(+)	(+)	(+)	(+)	
Chondroitin sulfate A	(+)		(+)	(+)	
Chondroitin sulfate B			(+)		
Chondroitin sulfate C	(+)		(+)	(+)	
Chondroitin sulfate D			(+)	(+)	
Chondroitin sulfate E			(+)	(+)	
Chondroitin	(+)		(+)	(+)	
Sialic acid					(+)

(+): indicates enzymes reaction with a mucopolysaccharide.

As indicated in our previous study (3), sulfated acidic mucopolysaccharides form a significant part of protein plugs in patients with chronic pancreatitis. The precise identification of the mucopolysaccharides and their distribution within the plugs may clarify the pathogenesis of the plugs.

The use of several enzymes for digesting the plugs was satisfactory since tissues, including that of the umbilical cord, aorta, cartilage and submandibular salivary gland, were completely digested with the appropriate enzyme. Furthermore, we have succeeded in minimizing the amount of these enzymes required using a paraffin stretcher and glass slides.

The digestion scheme is shown in Table 2. Sialic acid may form 70-80 % of the plugs and be distributed throughout the plugs since plug sediment was digested almost completely with sialidase. A small amount of chondroitin sulfate B is also present in the plugs since hyaluronidase (bovine testes) and chondroitinase ABC were effective in digesting plug sediment, while hyaluronidase (streptomyces) and chondroitinase AC were not. Chondroitin sulfate A, C, D as well as E and chondroitin may be minor components distributed diffusely throughout the plugs. Hyaluronic acid is probably negligible in the plugs. Concerning non-digestible parts of the plugs, the minor presence of other mucopolysaccharides, such as heparin and keratan sulphate, or glycoproteins with sulfate groups can be considered.

Biochemical analysis of the plugs is currently in progress to quantitate the various constituents of the plugs.

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