Vol. 87, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 27, 1979 Pages 1176-1182

# CHARACTERIZATION OF A PROTEIN ISOLATED FROM PANCREATIC CALCULI OF MEN SUFFERING FROM CHRONIC CALCIFYING PANCREATITIS

Alain De Caro, Jürgen Lohse and Henri Sarles

Unité de Recherches de Pathologie Digestive
U 31 INSERM - 46 ,chemin de la Gaye
13009 Marseille, France

Received February 28,1979

SUMMARY : A major protein was extracted by 0.15 M sodium chloride from human pancreatic calculi. It was found immunologically identical in ten different calculi and was present in different layers of the same stone. Its molecular weight was estimated to 13,500 daltons and its isoelectric point was found to be 5.5. This protein is present in normal and pathological human pancreatic juices as demonstrated by gel filtration of pancreatic juice on Sephadex G 100.

INTRODUCTION : Several works of our laboratory have shown that during the course of alcoholic pancreatitis the first lesion visible at the optical level was the formation in the pancreatic ducts of protein precipitates (1). These plugs obstruct the ducts and later calcify, forming stones. Chronic consumption of alcohol favors in man (2), in rats (3) and in dogs (4) the precipitation of proteins in the pancreatic juice and the formation of chronic pancreatitis. It has been proved in animals (1) and very recently in man (5, 6) that the consumption of ethanol was associated with increased concentration of proteins in pancreatic juice.

It was further demonstrated the presence of lactoferrin in increased amounts in the juice of men suffering from chronic calcifying pancreatitis (7, 8). This protein is known to associate strongly with acidic macromolecules to form complexes and may favour the phenomenon of precipitation (9).

All these data led us to the working hypothesis that the first lesion of alcoholic pancreatitis was the obstruction of the ducts by precipitates of normal secretory proteins within a hyperconcentrated pancreatic juice (1).

In this paper we afford a strong support to this hypothesis by demonstrating the presence of a major protein in human pancreatic stones that represents a normal constituent of pancreatic juice.

0006-291X/79/081176-07$01.00/0

A preliminary report of this work has been previously given (10).

MATERIALS AND METHODS : Human pancreatic stones were obtained at surgery from patients suffering from chronic calcifying pancreatitis.

Antisera to normal and pathological human pure pancreatic juice proteins were prepared by immunization of rabbits as previously described (11). Other antisera were used and included antisera to human serum, lysozyme and lactoferrin. Pancreatic proteins of low molecular weight were obtained by gel filtration of pancreatic juice through a Sephadex G 100 column (12).

Protein concentration was routinely determined from absorbance measurements using $A^{1\%}_{1cm}$ = 20.0 at 280 nm.

Method of protein isolation : All washing and extraction procedures were carried out in 0.15 M sodium chloride containing 0.05% sodium azide. In a first step the stones were extensively washed during about eight days under magnetic stirring to remove blood and contaminating superficial proteins, then oven dried. The dried stones were ground in a mortar and the resulting powder suspended in the minimum volume of the chloride solution. The suspension was stirred for 4-5 days then centrifuged (3000 g, 10 min). After filtration through a 0.45 µ Sartorius membrane filter the supernatant containing protein was dialyzed against distilled water using semi-permeable tubings (molecular weight cut off 3500, Spectrum Medical Industries, INC). After filtration the dialysate is concentrated by lyophilization.

Gel electrophoresis : Dodecyl sulfate polyacrylamide gel electrophoresis was performed in 15% gels according to the method of Weber et al. (13) with the previously described modifications (14). Apparent molecular weights were derived after calibration of the gels with the following purified proteins : Kunitz pancreatic inhibitor (molecular weight : 6500), cytochrome c (12 300), porcine prophospholipase $A_2$ (14 500), soybean trypsin inhibitor (21 500), trypsinogen (24 000) and yeast alcohol dehydrogenase (37 000).

Isoelectric focusing was performed in polyacrylamide gel according to Desharnais et al. (15). The pH gradient was made with ampholine carrier ampholytes in the pH range 5-7 and focusing was performed at 300 volts for 8 h at 4°C.

Immunological techniques : 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 and destained with the mixture acetic acid/ethanol/water (10:45:45).

RESULTS :

Existence of a major protein in pancreatic stones :

In a first set of experiments the stones from ten patients were submitted to the extraction procedure described above. Analysis of the protein content by Ouchterlony double diffusion against

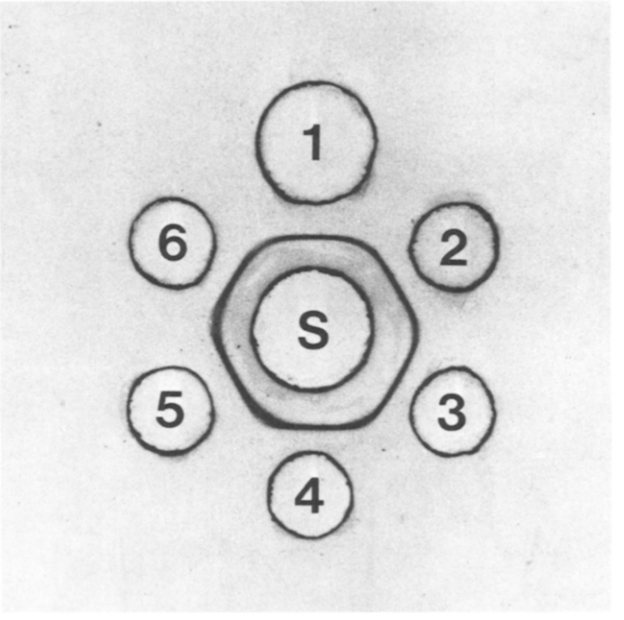


Figure 1 : Cross-reactions between the protein isolated from different calculi. Protein content of six different pancreatic stones were located in the outer wells and antiserum to human pancreatic juice was located in the center well.

antiserum to human pancreatic juice show in most cases only one line of precipitation. But in some stones one or two minor additional components were visualized. Ouchterlony's analysis show no immunological difference between the major protein extracted from the different calculi as shown in figure 1.

In a second set of experiments washed stones from one patient were partially dissolved by 0.15 M sodium chloride under magnetic stirring. Analysis by Ouchterlony double diffusion of the protein component liberated in the medium gave as previously only one line of precipitation. When the same stone was carefully ground, submitted to the same extraction procedure and the supernatant analyzed by immunodiffusion again only one line of precipitation was visualized. Moreover Ouchterlony's analysis did not discriminate between these two protein samples extracted from different layers of the same stone as well as with the major protein present in the other stones since a complete cross-reaction toward antiserum to human pancreatic juice was observed.

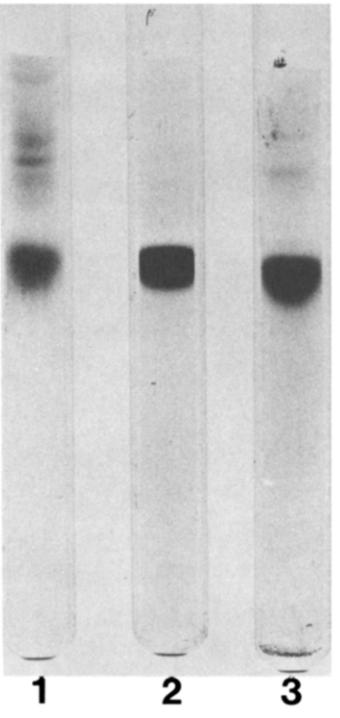


Figure 2 : Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein content isolated from three different pancreatic stones.

Characterization of the protein : Immunoelectrophoresis of the protein content of the stones at pH 8.6 gave after diffusion against antiserum to whole human pancreatic juice a weakly cationic line of precipitation. In each case the protein presented the same electrophoretic mobility. The molecular weight of the calculi protein determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis was estimated to 13,500 ± 500 daltons. As can be seen from figure 2 in some cases we observed two other proteins with a molecular weight around 30 000.

Electrofocusing of the protein carried out in the pH range 5-7 gave three or four bands corresponding to an isoelectric point slightly acidic estimated to 5.5.

This protein is present in normal and pathological pancreatic juices since the same immunological reaction is observed using different antisera either to normal or to pathological pancreatic juice. Figure 3 shows that the protein is present in the fractions

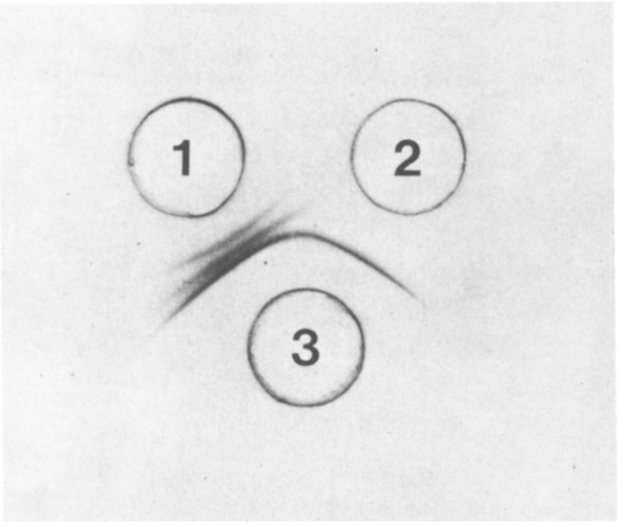


Figure 3 : Cross-reactions between pancreatic proteins of low molecular weight and protein isolated from pancreatic stones.
Pancreatic proteins (1), calculi protein (2), antiserum to human pancreatic proteins (3).

of low molecular weight (15,000 to 30,000) obtained by gel filtration of human pancreatic juice at high ionic strength as previously described (12).

The protein is immunologically different from proteins of the same molecular weight as lysozyme and prophospholipase $A_2$ as demonstrated by double diffusion technique against specific antisera. The protein is not present in serum since there is no line of precipitation when tested with antiserum to human serum. Moreover it is immunologically different from hemoglobin, and dodecyl sulfate polyacrylamide gel electrophoresis of these two proteins show different electrophoretic mobilities.

Finally the protein has no common antigenic determinant with lactoferrin as demonstrated by double diffusion technique.

DISCUSSION : Previous chemical studies on pancreatic stones have shown the prevalence of calcium carbonate (95.5% by weight) (16). X-Ray diffraction studies have confirmed the presence of $CaCO_3$ in the form of calcite (17). Up to this study the protein content of the stones was not examined althought it was known that intraductal protein plugs are at the origin of calcified stones

and that the presence of an amino acid material was previously observed in the stones (Dr. Vérine, personal communication).

In this paper we demonstrate the presence in the human pancreatic stones of a major protein, immunologically identical in ten different cases. This protein of 13,500 M.W. does exist in normal and pathological pancreatic juice since it has been found present in low molecular weight proteins of both juices and since antibodies raised with normal and pathological juices give strictly the same immunological reaction. The homogeneity of the protein and the reproducibility of our results argue in favour of a peculiar protein different from the known enzymatic proteins but further studies will be necessary to confirm this hypothesis. The existence of multiple bands given by electrofocusing of the protein may be due to a heterogeneity in amide content or eventually some amino acid substitution.

Except one or two minor additional proteins found in four cases this protein is the major protein constituent of pancreatic calculi.

Previous disc electrophoresis studies on different samples of pancreatic juice collected from patients suffering from chronic calcifying pancreatitis had shown the random disappearance of normal secretory enzymes (18). The presence of these normal proteins was further demonstrated in redissolved protein precipitates obtained from the same patients (Dr. Guy, personal communication). In addition we could recently demonstrate the presence of the calculi protein in these precipitates using double diffusion technique. All these data strongly support the hypothesis that the first lesion of alcoholic pancreatitis is due to the precipitation within the pancreatic ducts of normal secretory proteins.

The disappearance of most of the secretory protein in the calcified stones as well as the persistance of the calculi protein would be explained by the delay of many years that does exist between the onset of lesions and the surgical removal of the stones leading to a modification of their protein content. It is probable that the protein we have characterized in the calcified stones participate in the initiation of calcification of protein plugs. Studies on its $Ca^{++}$ interaction properties as well as its relationship with the other proteins in pancreatic juice would be necessary to shed light on its physiological and pathological role.

ACKNOWLEDGEMENTS : The authors wish to thank Pr. J.C. Sarles for his gift of human pancreatic calculi and Dr. C. Figarella for helpful discussion and her interest all along this work. J. Lohse is a recipient of a Fellowship from the Deutsche Forschungsgemeinschaft (West Germany). The assistance of Miss D. Richaud for the careful preparation of the manuscript is gratefully acknowledged.

## REFERENCES

1 - Sarles, H., Figarella, C., and Tiscornia, O.M. (1975) Mount Sinaï J Med, 42, 540-551
2 - Sarles, H., Sarles, J.C., Camatte, R., Muratore, R., Gaini, M., Guien, C., Pastor, J., and Le Roy, F. (1965) Gut, 6, 545-559
3 - Sarles, H., Lebreuil, G., Tasso, F., Figarella, C., Clemente, F., Devaux, M.A., Fagonde, B., and Payan, H. (1971) Gut, 12, 377-388
4 - Sarles, H., Tiscornia, O.M., and Palasciano, G. (1977) Gastroenterology, 72, 238-243
5 - Sahel, J., and Sarles, H. Submitted to Gastroenterology
6 - Palasciano, G., and Sarles, H. Submitted to Gut
7 - Colomb, E., Pianetta, C., Estevenon, J.P., Guy, O., Figarella, C., and Sarles, H. (1976) Digestion, 14, 242-249
8 - Figarella, C., Estevenon, J.P., and Sarles, H. (1978) Lancet, I, 8073, 1107-1108
9 - Hekman, A.M. (1971) Biochim. Biophys. Acta, 251, 380-387
10 - Lohse, J., De Caro, A., and Sarles, H. Gastroenterol. Clin. Biol. (in press)
11 - Clemente, F., Ribeiro, T., Colomb, E., Figarella, C., and Sarles, H. (1971) Biochim. Biophys. Acta, 251, 456-466
12 - Guy, O., Lombardo, D., Bartelt, D.C., Amic, J. and Figarella, C. (1978) Biochemistry, 17, 1669-1675
13 - Weber, K., and Osborn, M. (1969) J. Biol. Chem, 244, 4406-4412
14 - De Caro, A., Figarella, C., and Guy, O. (1975) Biochim. Biophys. Acta, 379, 431-443
15 - Desharnais, M., Paradis, D., Morisset, J., Beaudoin, A.,and Dunnigan, J. (1975) Rev. Can. Biol , 34, 59-64
16 - Périnet, G., Vérine, H., Lafont, R., and Haladjian, J. (1972) C. R. Acad. Sc. Paris, 274, 3303-3305
17 - Mallet-Guy, P., Perrin, M., Thozet, A., and Vérine, H. (1969) C. R. Soc. Biol , 163, 1838-1841
18 - Figarella, C., Souville, C., and Sarles, H. (1968) Bull. Soc. Chim. Biol , 50, 675-677