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Separation of Pancreatic Enzymes by Gel Filtration

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The use of gel filtration on Sephadex columns for fractionation of enzymes in pancreatic extracts has been studied. On gels with porous structures an adequate separation of lipase, proteolytic enzymes and amylase is obtained, indicating a simple and convenient method for analytical and preparative purposes. The behaviour of individual enzymes under various experimental conditions is discussed. Amylase is slightly retained on the column due to an interaction with the gel matrix, and pancreatic lipase is separated into two fractions, probably consisting of polymer and monomer forms. The molecular weight of the monomer is estimated to be approximately 35 000.

With the development of the gel filtration technique, based on the separation of solutes of different molecular size, a simple and rapid method for preparative and analytical work has become available. This technique has proved useful in desalting operations,[1] in fractionation of proteins, peptides, and amino acids,[2] and has given an almost complete separation of a homologous series of oligosaccharides.[3] The gels used in these procedures consisted of highly cross-linked dextran and were suitable for fractionation in the range of relatively small molecules only.

Later on, the availability of more porous dextran gels has greatly extended the fractionation range. Some publications have appeared about the fractionation of polysaccharides [4] and polynucleotides,[5] but the porous gels have found their greatest application in the field of protein chemistry.[6]

This article describes the use of porous dextran gels for separation of enzymes in pancreatic extracts. It also gives some examples of the applicability of gel filtration as a step in the purification of some of the enzymes. So far, only a few investigations have been made on gel filtration of pancreatic components. The isolation of insulin from pancreas of cat [7] and of beef [8] has been described, and Crestfield *et al.* used gel filtration for the separation of monomer and polymer ribonuclease.[9]

MATERIALS

The gel filtration experiments were made with cross-linked dextran, Sephadex, (Pharmacia, Uppsala, Sweden) as bed material. Five different types of this material were used: the compact gel Sephadex G-25 (water regain 2.5 g of water per g of dry gel; particle

size 200—400 mesh) and the porous gels Sephadex G-75 (water regain 7.6 g/g; particle size 100—200 mesh), Sephadex G-100 (water regain 10.6 g/g; particle size 200—270 mesh), and Sephadex G-200 (water regain 21.4 g/g; particle size 200-270 mesh). One experiment was also made on a dextran gel with water regain 16.0. The latter gel as well as Sephadex G-100 and G-200 were in bead form, while the gel particles of G-25 and G-75 had an irregular shape.

Pancreactic extracts were obtained by extracting defatted, powdered hog pancreas during 30 min at +5°C with the appropriate buffer. Extraction was made with stirring, and the extract was centrifuged 30 min at 3000 × *g* (5 ml buffer per g powder). When not stated otherwise, the same buffer as used for extraction was used as eluant in subsequent gel filtration experiments.

The individual pancreatic enzymes were partially purified by the following methods:

Amylase was obtained by precipitation of a pancreatic extract with ethanol and ether according to Caldwell *et al.*[10] The purification scheme was followed up until step 8. No crystallization was tried.

Trypsinogen, chymotrypsinogen a, chymotrypsinogen B, and *ribonuclease* were prepared from acidic extracts of pancreas by fractionated precipitation with ammonium sulphate according to Kunitz and Northrop.[11] Chymotrypsinogen B was precipitated between 0.2 and 0.4 saturation, trypsinogen and chymotrypsinogen *a* between 0.4 and 0.6 saturation, and ribonuclease between 0.6 and 0.8 saturation. No attempts to further purification or crystallization were made.

METHODS

The chromatographic columns were prepared and the gel filtration experiments made in accordance with earlier descriptions.[1,12] The experiments were performed either at room temperature or in a cold room at +5°C. Effluents were taken up in a fraction collector provided with a siphon (RadiRac, LKB-Produkter, Stockholm, Sweden). Gel filtration at room temperature did not show a significantly lower recovery of enzyme activity if the eluted fractions were placed in an icebath immediately after collection.

The absorbancy at 280 mμ was measured for every effluent fraction, and enzymatic activities were determined as follows:

Lipase according to Desnuelle *et al.*[13] with olive oil emulsion as substrate.

Proteolytic activity according to Anson[14] with the modification that spectrophotometric measurements were made at 280 mμ instead of using the phenol reagent.

Amylase was assayed with 3,5-dinitrosalicylic acid and colorimetric determination at 350 mμ as described by Stein and Fischer.[15]

Ribonuclease was determined with ribonucleic acid as substate.[16] For obtaining low blank va'ues, a high molecular weight ribonucleic acid was prepared as described by Crestfield *et al.*[17] and futher purified by gel filtration on Sephadex G-75.[5] All spectrophotometric measurements were made on a Unicam Spectrophotometer SP 500.

In all experiments described below the elution volume, V_e, of the different components was determined as the effluent volume from the moment of sample application to the moment, when maximum concentration of the component appeared in the effluent. From the expression

$$V_e = V_o + K_d \cdot V_i$$

where V_o is the void volume of the bed, and V_i is the total internal liquid volume of the swollen gel particles, the coefficient, K_d, is calculated. K_d values are used throughout when describing and discussing the experimental results. The coefficient K_d for a certain component is equivalent to the volume fraction of liquid inside the gel particles, which is available as solvent for the component.

V_o was determined for every column as the elution volume of a suitable high molecular weight substance. For columns with Sephadex G-25 a 0.5 % solution of hemoglobin was used with 0.05 M phosphate buffer, pH 7.0 as eluant. For the porous gels G-75, G-100, and G-200 India ink ("Pelikan", Günther Wagner, Germany), diluted 1:200 with distilled water and eluted with distilled water, was tried. On some occasions, however, the carbon particles were irreversibly precipitated on the column. Therefore, fibrinogen or a serum sample, using tris buffer, pH 8.0 containing 0.2 M NaCl as eluant, was preferred

Table 1. K_d values of pancreatic enzymes in gel filtration on Sephadex using 0.05 M sodium acetate + 0.005 M calcium acetate, pH 5.3, as eluant.

Enzyme	K_d			
	Sephadex G-75	Sephadex G-100	Gel with water regain 16.0	Sephadex G-200
Lipase	0.12	0.31	0.46	0.50
Proteolytic enzymes	0.31	0.50	0.66	0.70
Ribonuclease	0.20	0.24	0.46	0.50
Amylase	0.45	0.79	0.86	0.90

for the porous gels. The serum proteins are resolved into two fractions on Sephadex G-100 and into three fractions on Sephadex G-200,[18] but the first eluted fraction, containing fibrinogen and macroglobulins, will give a rather accurate measure of the void volume.

The internal volume, V_i, was calculated from the dry weight of the bed material, a, and the water regain value of the gel, W_r, as

$$V_i = a \cdot W_r$$

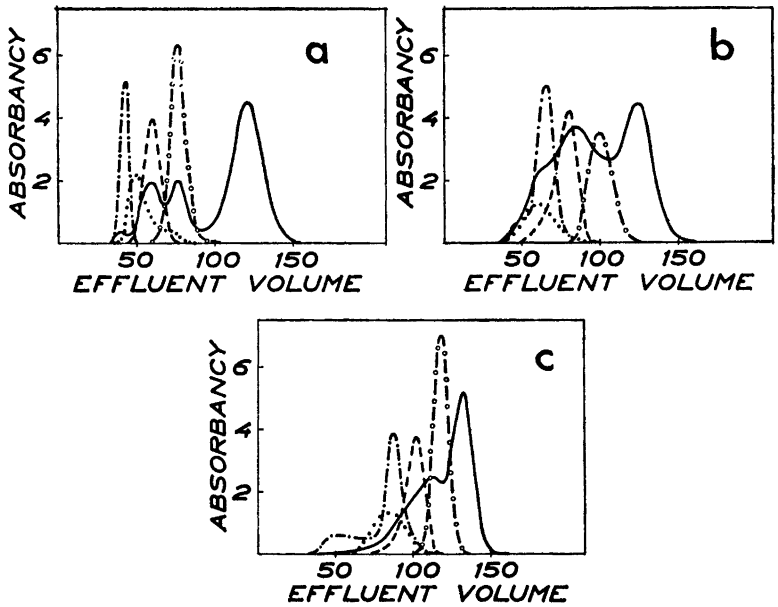

Fig. 1. Gel filtration of 3 ml extract of hog pancreas powder on 130 ml (2 × 42 cm) columns of a) Sephadex G-75, b) Sephadex G-100, and c) Sephadex G-200. Elution was made with 0.05 M sodium acetate buffer pH 5.3 containing 0.005 M calcium acetate. — absorbancy at 280 mμ, —·— lipase activity, ··· ribonuclease activity, — — — proteolytic activity, —O— amylase activity.

RESULTS

As could be expected, no resolution of the various pancreatic enzymes was achieved by gel filtration on Sephadex G-25. On a column of Sephadex G-75, a partial separation was obtained. Consequently Sephadex G-100 and Sephadex G-200 ought to give at least the same mutual separation of the enzymatic activities. Fig. 1 shows three comparable experiments on the porous Sephadex types, where elution was made with 0.05 M sodium acetate buffer, pH 5.3, containing 0.005 M calcium acetate. Table 1 gives the K_d values calculated from these diagrams. It also includes the data obtained in a similar experiment made on a dextran gel with water regain 16.0.

The experiments in Fig. 1 were made under the most favourable conditions for enzyme stability and resolution. Changes in pH or ionic strength do not influence the experimental results to any considerable extent, but some variations in the elution pattern have been observed and will be described in the following. To make the description easier and more clear, the various enzymes will be treated separately.

Lipase. Preliminary experiments to study the extractability of lipase from dry, powdered pancreas gave the highest yield at pH 6—8. At pH 4.5—5 the yield was reduced to about half, and at pH 3 or less it was not possible to extract any lipase activity at all (Fig. 2).

In the gel filtration experiments on Sephadex G-75 the lipase activity showed a low K_d value, which means that lipase is almost completely excluded from Sephadex G-75. Nevertheless it is sometimes possible to observe a differentiation of the lipase activity into two peaks. The occurrence of two lipase fractions was further confirmed in experiments on Sephadex G-100. Fig. 3 is the elution diagram from an experiment on Sephadex G-100 in 0.05 M tris buffer, pH 7.15, where one lipase fraction is completely excluded ($K_d = 0$) and the other one is retained. The same behaviour is obtained with Sephadex G-200. The extent of retention of the second peak increases when the degree of cross-linkage of the gel decreases.

The two lipase fractions appeared in different ratios depending on the composition of the buffer used for extraction. At pH values above 6 the first fraction predominated, and at lower pH values most of the lipase was obtained as the slowly moving component. There was also another possibility to regulate the distribution of lipase. By addition of small amounts of calcium ions to the

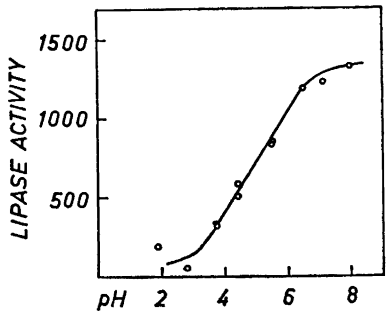

Fig. 2. Extractability of lipase activity at various pH-values. 2 g of dry pancreas powder were extracted 30 min at +5°C with 10 ml of buffer (ionic strength 0.1).

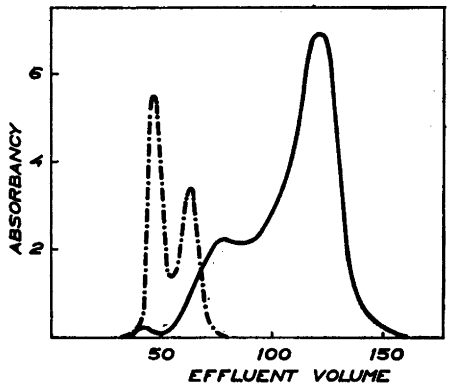

Fig. 3. Gel filtration of 3 ml extract of hog pancreas powder on a 130 ml (2 × 42 cm) column of Sephadex G-100. Elution was made with 0.05 M tris-HCl buffer pH 7.15. — absorbancy at 280 mμ, —·— lipase activity.

buffer, the greater part of the activity appeared in the second fraction even at high pH values, and almost all activity was obtained in the second fraction when more acidic buffers were used. Furthermore, a stabilization of the lipase was observed in the presence of calcium ions. Table 2 illustrates the effect of pH and calcium ions on the recovery and distribution of lipase activity. The experimental data indicate a pH-dependent transition into one molecular form or other. On the other hand, it was not possible to get a reversible transformation of the lipase by mere pH adjustment. If the first lipase fraction from a pH 7.2 extract was isolated and immediately adjusted to pH 5.3 with simultaneous addition of calcium ions and submitted to a new gel filtration experiment at the lower pH, the lipase activity was eluted in the same way as before. Likewise, if the second lipase fraction was isolated at pH 5.3 and rerun in tris buffer of pH 7.2, it was retained on the column even without calcium ions present. In both experiments, however, there was a great decrease in activity.

Table 2. Recovery and distribution of lipase activity on gel filtration.

Solvent	pH	Sephadex type	Recovery %	K_d	Approx. distribution %
0.05 M tris	7.2	G-75	44	0.00	90
				0.11	10
0.05 M phosphate	7.0	G-75	74	0.00	75
				0.11	25
0.05 M Na-acetate	5.5	G-75	66	0.02	15
				0.15	85
0.05 M tris + 0.005 M $CaCl_2$	8.0	G-75	54	0.09	100
0.05 M Na-acetate+ 0.005 M Ca-acetate	5.3	G-75	80	0.15	100
0.05 M tris	7.2	G-100	51	0.00	66
				0.26	34
0.05 M Na-acetate + 0.005 M Ca-acetate	5.3	G-100	60	0.00	7
				0.31	93

Ribonuclease. As illustrated in Fig. 1 the ribonuclease activity was eluted in a relatively large effluent volume and with a K_d value, which seems too low with respect to its molecular weight (13 700) as compared to the other enzymes. Also the RNase peak is unsymmetric and tends in some cases to separate into two maxima (K_d 0.2 and 0.4 on Sephadex G-75). A decrease in pH, particulary using acetic acid as eluant, resulted in a slightly increased retention on the column. This effect was not so pronounced in gel filtration of a pancreatic extract but more evident in experiments with partially purified RNase. Table 3 shows the K_d values obtained with Sephadex G-75 in different solvents. It should also be pointed out that in all experiments with the purified enzyme, the activity was always eluted as a symmetric peak. The recovery of RNase activity was between 80 and 90 %.

Proteolytic activity. The proteolytic activity of a pancreatic extract was always obtained as one single peak. Its position in the elution diagram was not influenced by changes in pH or ionic strength. Separate experiments with trypsin, the chymotrypsins, or their zymogens gave the same result as was obtained with the extracts. The recovery of proteolytic activity, measured after activation, ranged between 80 and 100 %.

Amylase. As was the case with the proteolytic enzymes, the elution of amylase was not affected by variation in pH from 8 to 5. Neither was any difference observed when, at pH 5, the ionic strength was shifted from 0.05 to 0.5. On the other hand it was noted that the elution of amylase was strongly dependent on the flow rate in the experiment. Below a certain flow rate limit, which seemed to be determined by the actual temperature, a maximum retention on the column was obtained, and the amylase was eluted in a symmetrical peak. Above this limit the enzyme was less retained and gave always an unsymmetrical peak with trailing. The recovery of enzymatic activity was, however, always in the neighbourhood of 100 %. Table 4 illustrates the behaviour of a partially purified amylase in gel filtration on different types of Sephadex at various flow rates and temperatures. Experiments at 5°C were

Table 3. Gel filtration of RNase on Sephadex G-75 in various solvents.

	Solvent	pH	K_d
Pancreas extracts			
	0.05 M tris + 0.005 M $CaCl_2$	7.5	0.10
	0.05 M Na-acetate + 0.005 M Ca-acetate	5.3	0.20 (0.41)
	0.1 M acetic acid	2.9	0.27 (0.41)
Partially purified RNase			
	0.02 M tris	7.0	0.30
	0.02 M Na-acetate	5.0	0.35
	0.2 M acetic acid	2.7	0.43
	0.02 M HCl	1.7	0.30

Table 4. Gel filtration of partially purified pancreatic amylase in 0.02 M tris buffer, pH 7.0.

Sephadex type	Experimental temperature (centigrade)	Flow rate ml/cm² hour	K_d
G-25	22	34.0	0.0
G-25	22	2.6	0.0
G-75	22	33.6	0.22
G-75	22	9.2	0.41
G-75	22	5.0	0.48
G-75	22	2.2	0.55
G-75	5	5.7	0.86
G-75	5	5.3	0.80
G-75	5	2.0	0.85

possible in a limited range only, due to a strong decrease in flow rate at low temperatures. It should be observed that no retention of amylase was obtained on Sephadex G-25.

Finally one experiment was made with an acidic extract of pancreas powder. Four grams of the powder were extracted at room temperature for 30 min with 20 ml of 0.25 M sulphuric acid. After centrifugation, the pH of the solution was adjusted to 3 by addition of 2 M sodium hydroxide. A sample of 5 ml was applied on a 400 ml column of Sephadex G-75 and eluted with 0.1 M acetic acid. Only ribonuclease and proteolytic activities were found in the effluent and the elution diagram is given in Fig. 4. A rather good separation of RNase and proteolytic enzymes is obtained, indicating a simple preparative technique for isolating these enzymes.

DISCUSSION

The results presented in Fig. 1 show the possibility to separate the main enzymatic activities in a pancreatic extract by gel filtration on porous dextran gels. An adequate resolution is obtained above a certain degree of cross-linkage, where the lower limit approximately corresponds to that of Sephadex G-75. A more porous gel structure does not increase the mutual separation but offers a larger space inside the swollen gel particles for penetration of the

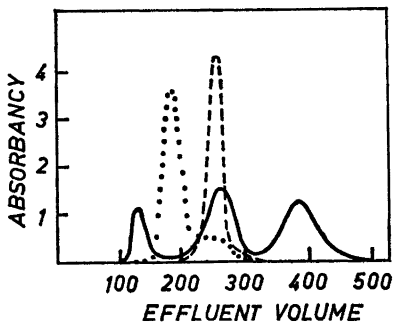

Fig. 4. Gel filtration of 5 ml of a 0.25 M sulphuric acid extract of hog pancreas powder on a 400 ml (4 × 32 cm) column of Sephadex G-75, 100–200 mesh. Elution was made with 0.1 M acetic acid. Before application to the column the sample was adjusted to pH 3. — absorbancy at 280 mμ, ··· ribonuclease activity, — — proteolytic activity.

proteins. Consequently, such a gel should be partially accessible to even larger molecules.

Earlier publications have shown the possibility to use porous dextran gels for fractionation of polysaccharides according to molecular weight.[4] A similar result was obtained in fractionation of barley proteins on Sephadex G-75 and of serum proteins on Sephadex G-200. Ultracentrifugal analyses of eluted fractions showed a successive decrease in sedimentation coefficients.[19,18] These studies indicate that also in the porous dextran gels the molecular sieving property is the predominating factor for separation. A detailed study of the various components in a heterogeneous mixture shows, however, as in the present investigation that distorting effects might obscure the results. This is particularly true when dealing with more complicated compounds, such as proteins. It was, for instance, found that the ribonuclease activity was eluted earlier than the proteolytic enzymes, which should indicate that the RNase molecule is the larger one. The discrepancies in behaviour of a partially purified RNase and RNase in the extract can be explained by supposing that RNase in the crude extract is present as a high molecular weight complex with inert proteins or in polymer form. This idea is also supported by the finding that RNase has a maximum retention in acetic acid. Similar effects are often found in gel filtration of proteins and peptides. In the same manner cytochrome c can be isolated from a high molecular weight complex by gel filtration on Sephadex G-75 in 0.2 M acetic acid (unpublished observation), and the hormones oxytocin and vasopressin have been purified by a similar procedure.[20] The strange behaviour of amylase is more difficult to explain. With a molecular weight of 50 000,[21] pancreatic amylase should have a very low K_d value on Sephadex G-75. The strong retardation on the column at low flow rates, the variation in K_d with the flow rate, and the unsymmetrical shape of the elution curves at high flow rates point to a specific adsorption of amylase on the polysaccharide framework in the gels. No such retention was, however, observed on Sephadex G-25.

Similar results have recently been published by Wilding from gel filtration experiments on Sephadex G-100 with human amylase and amylase in an extract of pig pancreas.[22] The retention of amylase on a bed of dextran gel must be caused by weak complex formation between the enzyme and the gel matrix. The interaction may be of a nature similar to an enzyme-substrate complex.

The proteolytic and lipolytic activities do not seem to be affected by a variaion in flow rate or in the composition of the eluant, except for the appearance of two peaks with lipolytic activity under certain conditions. One lipase fraction is always eluted with the void volume even on the most porous gel type, which indicates a high molecular weight. The lipase molecules in the second fraction must be of a considerably smaller size because they are able to penetrate into the gel to some extent. It seems reasonable to suppose that the second fraction consists of lipase in a monomer form and that the first eluted fraction contains a polymer of lipase. The first fraction can of course be a complex of lipase and other proteins from the extract, but according to the results listed in Table 2 it seems more likely that we are dealing with a lipase polymer. The relative distribution of lipase between the two fractions varies with pH. If there should be a mere complex between lipase and some

other protein in the extract, it would indicate a reversible equilibrium between complex and dissociated enzyme. A dissociation of the complex would then be effected by changing the pH. This is, however, not possible. Therefore, the two lipase fractions probably consist of polymer and monomer forms of the enzyme.

Judging from the gel filtration experiments and supposing that no interaction occurs between the monomer lipase and the gel, the molecular weight of pancreatic lipase monomer can be estimated to approximately 35 000. The idea of using the gel filtration technique for estimation of the molecular weight of proteins was first studied by Andrews [23] with agar gels. In some recent publications [24-26] Sephadex has been used for the same purpose. In all cases a good correlation was obtained for many proteins between elution volume and molecular weight, and the method is considered as good as many others now in use. There are, however, some proteins, which show an anomalous behaviour either due to interaction between solute and gel or due to a structural change of the solute molecule. A critical evaluation of the results obtained in a gel filtration experiment is therefore necessary before any conclusions can be drawn about the size of a molecule. This fact is pointed out by the authors and further exemplified by the results of the present investigation.

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