A New Systematic Strategy for the Isolation of Proteins, Illustrated by the Purification of a Mammalian Exo- β -N-acetyl-D-glucosaminidase

By HOWARD K. ROBINSON* and DAVID H. LEABACK†

* Enzyme Chemistry Section, Microbiological Research Establishment, Porton, Wilts., U.K. and

† Department of Biochemistry, Institute of Orthopaedics, Stanmore, Middx., U.K.

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The 'B' form of the exo- β -N-acetyl-D-glucosaminidase from pig epididymis was purified by a new systematic strategy to yield a preparation with a specific activity at least equal to that obtainable by an existing empirically derived procedure. The new strategy is essentially a sequence of three carefully chosen steps consisting of an initial fractionation of the constituent proteins according to molecular size, followed by an ion-exchange step designed to select out proteins with closely similar electric-charge properties to those of the protein of interest, and a final high-resolution step dependent on the isoelectric points of the residual proteins. Gel isoelectric focusing itself, or as an element in the technique described by Leaback & Robinson (1974) for the separate display of the molecular size and electric-charge characteristics of proteins, played an important part in the choice of the experimental conditions used in the new strategy, and also in monitoring the progress of the purification.

Schemes for the purification of particular proteins are still devised by what is typically an *ad hoc* empirical approach. Given the task of isolating a particular protein, there is usually little to guide the selection of individual techniques to make up the purification scheme or the choice of the associated experimental conditions. A number of relatively simple analytical methods have been developed which are applicable to proteins in crude biological extracts, and which offer considerable insight into important molecular properties of the component proteins (Morris, 1964; Awdeh *et al.*, 1968; Dale & Latner, 1968; Fawcett, 1968; Leaback & Rutter, 1968; Wrigley, 1968; Weber & Osborn, 1969; Margolis, 1973). Such methods were examined by the authors, and the outcome was a new technique in which the molecular size and electric-charge characteristics of native proteins in crude mixtures could be clearly and separately displayed (Leaback & Robinson, 1974). Since such characteristics are very important in certain methods for the purification of proteins, the possibilities arose that they might be made the basis of a systematic strategy for the isolation of proteins, and that the display technique might be of value in the choice of conditions to be used in the purification scheme.

The present paper describes work investigating these possibilities in connexion with the purification of proteins with exo- β -N-acetyl-D-glucosaminidase activity in preparations from pig epididymis. This enzyme (EC 3.2.1.30) was chosen partly because of the need for an extremely active, highly purified, preparation for use in other studies (Leaback, 1974);

partly as the result of our experience with the existing, empirically derived purification scheme (Findlay & Levvy, 1960; Leaback & Walker, 1967) and also because of the intense interest in almost all aspects of mammalian β -N-acetyl-D-glucosaminidases (Leaback, 1970). As to the origin of the enzyme, pig epididymis is the richest known source of exo- β -N-acetyl-D-glucosaminidase activity (Conchie *et al.*, 1959; Findlay & Levvy, 1960).

Materials and Methods

Protein preparations

Crude extracts of pig epididymis were prepared as described by Findlay & Levvy (1960), but were concentrated before use by vacuum dialysis at 5°C through Visking tubing. Solutions of human carbon-monoxynaemoglobin A were prepared freshly by the method of Weiss (1968).

Purification of pig epididymal β -N-acetyl-D-glucosaminidase

The crude, concentrated enzyme extract (1 ml) was applied to a column (40 cm $\times$ 2.5 cm diam.) of Sephadex G-200 [Superfine; Pharmacia (G.B.) Ltd., London W.5, U.K.] equilibrated at 5°C with 0.05 M-sodium phosphate buffer, pH 6.8, containing 0.5 M-NaCl, and eluted at about 3 ml/h with the same buffer by using a Mariotte constant-head device. The E_{254} of the effluent was monitored continuously by using a Uvicord (LKB Instruments Ltd., South Croydon,

U.K.) and fractions (2 ml) were collected, assayed for β -*N*-acetylglucosaminidase activity, and peak fractions (i.e. those with not less than one-quarter of maximum enzyme activity) were combined, washed free of Na^+ ions at 20°C on a Diaflo membrane (UM10; Amicon Ltd., High Wycombe, Bucks., U.K.) with 0.02M-Tris-HCl buffer, pH 7.3, and then concentrated on the same filter to the original sample volume. The concentrate (1 ml) was passed through a column (4.5 cm $\times$ 1.1 cm diam.) of CM-cellulose (CM 52; Whatman Biochemicals, Maidstone, Kent, U.K.) carefully equilibrated with the same Tris-HCl buffer (pH 7.6) at 5°C, washed with four portions (2 ml each) of the buffer and eluted with 12 portions (1 ml each) of 4% (w/v) Ampholine carrier ampholyte mixture, pH 8-10 (LKB Instruments Ltd.). After analysis for β -*N*-acetylglucosaminidase activity, active fractions were combined and subjected to density-gradient stabilized isoelectric focusing in a pH 7-9 gradient for 24 h at 5°C.

Isoelectric focusing

This was carried out at 5°C in sucrose density gradients with mixtures of pH 3-10, 7-9 and 8-10 Ampholine to produce appropriate pH gradients in an LKB 8101 column (Vesterberg & Svensson, 1966). After 24 h at 600 V, the column contents were pumped at 2 ml/min through the flow cell of a spectrophotometer (SP.600; Pye-Unicam Ltd., Cambridge, U.K.) and fractions (1 ml) were collected, and their pH values measured immediately at about 5°C.

Gel isoelectric focusing was carried out as described by Robinson (1972) or as part of the display technique of Leaback & Robinson (1974). The latter was essentially an initial molecular-size fractionation on Sephadex G-200 followed by a 'second dimensional' electric-charge separation by gel isoelectric focusing. Proteins were detected on the gels by staining with Coomassie Brilliant Blue R250 by the method of Vesterberg (1972).

Enzyme assays

β -*N*-Acetyl-D-glucosaminidase activities were estimated in fractions from columns either by a manual fluorimetric method (Leaback & Walker, 1961) or by an automated colorimetric procedure (Robinson, 1973). At each stage in the purification the enzyme activities were determined by using the *p*-nitrophenyl substrate precisely as described by Findlay & Levvy (1960), but the enzyme units used here are given as μmol of substrate hydrolysed/min under the defined conditions, and specific activities as enzyme units/mg of protein. The coefficient for conversion into the units used by Findlay & Levvy (1960) is 8340. Hyaluronidase assays were as described by Aronson & Davidson (1967) and other glycosidases were assayed

by using the corresponding methylumbelliferyl substrate (0.5 mM), but otherwise as described by Leaback & Walker (1961).

Protein assays

The concentration of protein was determined by the automated method of Wade & Phillips (1971) with dried bovine serum albumin (fraction V, Armour Pharmaceuticals Ltd., Eastbourne, Sussex, U.K.) as the standard. In certain samples, prior removal of Ampholines was necessary, and this was carried out by gel filtration at 5°C through a column (100 cm $\times$ 0.8 cm diam.) of Sephadex G-50 (fine grade) (Vesterberg, 1969).

Small-scale ion-exchange experiments

Columns (4.5 cm $\times$ 0.4 cm diam.) of CM-cellulose (CM 52) were equilibrated carefully at 5°C and at the pH value indicated, and then samples (1 ml) of either carbonmonoxyhaemoglobin A or the epididymal enzyme in the appropriate buffer were added and washed through with two further 1 ml samples of the buffer. The combined eluates were assayed either for haemoglobin by measuring E_{550} or for the enzyme as indicated above.

Results

The molecular-size and isoelectric-point display technique has already been applied to a preliminary characterization of the enzyme (Leaback & Robinson, 1974) and indicated that the enzyme has a molecular size of 100 000-150 000 daltons, and that considerable purification would be likely on Sephadex G-200 gel filtration. Table 1 shows that a purification of about fivefold resulted from such a step carried out on the preparation from whole epididymis (a relatively poor source) (Findlay & Levvy, 1960) and about eightfold from the richer, most active sections of epididymis.

The display technique also indicated an isoelectric point for the enzyme of about pH 8.3 (Leaback & Robinson, 1974). This was confirmed by the results of the density stabilized isoelectric-focusing experiment shown in Figs. 1(a) and 1(b), which also demonstrated considerable heterogeneity of the enzyme protein with isoelectric points in the pH range 7.8-8.8. The almost complete absence of the acidic 'A' form of the enzyme in the preparations used here was especially notable.

The display technique, and the isoelectric-focusing gel in Plate 1, indicated that the majority of the proteins in the active fraction (II) from the gel-filtration had isoelectric points more acidic than those of the enzyme, and suggested that, if the removal of these contaminating proteins could be effected by ion-exchange methods, considerable purification would

EXPLANATION OF PLATE I

Polyacrylamide-gel isoelectric focusing of stages in the purification of β -N-acetyl-D-glucosaminidase from the 'best section' of pig epididymis

Photographs of isoelectric-focusing gel (with associated pH gradient) carried out and stained for proteins as described by Leaback & Robinson (1974). Samples: (a), crude epididymal concentrate; (b), effluent from Sephadex G-200 (fraction II); (c), proteins from fraction (II) not adsorbed on CM 52 column at pH 7.6; (d), proteins eluted from CM 52 resin column by carrier ampholytes and (e), pooled enzymically active fractions from isoelectric-focusing column. (b'), (c') and (d') were higher contrast photographs of samples (b), (c) and (d) respectively. Samples (a)–(e) were applied to the gel at concentrations corresponding to those at which they occurred in the original samples and therefore give a visual impression of the progress of the purification. The pH scale on the right of the Plate is not an ordinate but should be used in conjunction with the broken line (----) to indicate the pH gradient measured along the length of the gel. ▲ indicates presence of enzyme activity; △, pH of CM-cellulose adsorption.

Table 1. *Purification of β -N-acetyl-D-glucosaminidase from pig epididymis*

The enzyme was estimated by using the *p*-nitrophenyl substrate by the method of Findlay & Levvy (1960) but the enzyme units are expressed as μ mol of substrate hydrolysed/min under the defined conditions and specific activities are expressed as enzyme units/mg of protein. The coefficient for conversion into the units used by Findlay & Levvy (1960) is 8340. Pooled fractions under stage 5(b) of the purification were (for the whole epididymis) 1, pH range 8.0–8.5, and (for the most active sections of epididymis) (I), pH 8.5–8.6; (II), 8.3–8.5; (III), 8.25–8.35; (IV), 7.6–8.25. The results presented are typical of several experiments.

		Whole epididymis			Most active section of epididymis			
Stage	Description	Total enzyme units	Specific activity	Enzyme recovery (%)	Fractions	Total enzyme units	Specific activity	Enzyme recovery (%)
1	Crude concentrated extract	64.1	1.73	100		1512	16.3	100
2	Effluents from Sephadex G-200							
	(I)	2.4	—	4	(I)	60	—	4
	(II)	50.0	8.92	78	(II)	1104	—	73
	(III)	9.2	—	15	(III)	132	—	9
3	Ultrafiltered (UM10) concentrate of above fraction (II)	43.6	8.35	68		1128	75.1	75
4	CM 52 effluent:							
	(a) At pH7.6	6.8	—	11	(a)	84	5.9	6
	(b) Ampholine eluted	35.0	128	55	(b)	756	175	50
5(a)	Total active fractions from isoelectric focusing column	31.8	—	49	(a)	648	—	43
(b)	Pooled fractions	14.8	244	23	(I)	120	420	8
					(II)	96	324	7
					(III)	96	288	7
					(IV)	120	396	8

Fig. 1. *Sucrose-density-gradient isoelectric focusing of crude and partially purified β -N-acetyl-D-glucosaminidase from the 'best sections' of pig epididymis*

Isoelectric focusing at 5°C for 24 h and 600 V, (a) of crude pig epididymal extract (4.7 mg of protein) in a pH 3–10 gradient; (b) of an enzyme preparation (0.15 mg of protein) eluted from a column (4.5 cm $\times$ 0.4 cm) of CM-cellulose. —, pH gradient; ●, β -N-acetyl-D-glucosaminidase activity; —, E_{1cm}^{280} .

result. Experiments with small columns of CM-cellulose showed that the enzyme activities were largely adsorbed at 1 pH unit lower than the indicated isoelectric points (Plate 1 and Fig. 2).

The adsorption of the enzyme from the washed, ultrafiltered concentrate was about 90% of the total, and the isoelectric spectrum of the proteins not adsorbed at this pH value (7.6) showed acidic isoelectric

points below about pH 7.5–7.0 (Plate 1, tracks c or c'). The enzyme activity was eluted from the CM-cellulose in good yield by using 4% (w/v) pH 8–10 carrier ampholyte mixture and the isoelectric spectrum of the eluted proteins indicated isoelectric points complementary with those of the unadsorbed proteins (Plate 1, tracks c, d or c', d'). Density-gradient stabilized isoelectric focusing in a pH gra-

Fig. 2. Effect of pH on the elution of the epididymal enzyme and human haemoglobin A from small columns of CM-cellulose

Columns (4.5 cm $\times$ 0.4 cm diam.) of CM-cellulose (CM52) were equilibrated at 5°C with sodium phosphate or Tris-HCl buffers (0.02M) in the range pH 6.0–9.0. Crude epididymal enzyme (about 1.5 mg of protein in 1 ml of appropriate buffer) was added to columns of CM52, washed with the same buffer (2 ml) and the enzyme (●) was assayed fluorimetrically in the effluents. Freshly prepared human carbonmonoxyhaemoglobin A (about 0.5 mg in 1 ml of 0.02M-sodium phosphate buffers) was treated similarly on columns of CM52 and the effluent estimated spectrophotometrically at 550 nm (▲).

dient designed to separate these proteins (Fig. 1b), confirmed the heterogeneity of the 'B' form of the enzyme hinted at in the earlier experiment (Fig. 1a). Estimations showed that the specific activities of the separated 'isoenzymes' (Table 1) were at least equal to those reported by Findlay & Levvy (1960) and were obtained in greater yield. However, the enzyme was found to be only moderately unstable above about pH 8.3 and at temperatures approaching 20°C, so that to obtain good enzyme recoveries at certain stages in the new purification scheme (i.e. in some fractions from the CM-cellulose and isoelectric-focusing columns), it proved important that the enzyme should not remain longer than necessary at such pH values.

Discussion

Like many other proteins, β -N-acetylglucosaminidases are inactivated unless kept concentrated in solution or unless a protecting protein is added. In our experience proteins are diluted at least 15–20-fold, even in the best conducted gel-filtration experiments with Sephadex G-200, so it seemed important to conduct such a fractionation step while the enzyme was still protected by contaminating proteins. Further reasons for the choice of the gel filtration as the initial step in the purification scheme were to take advantage of the information gained from the display technique (Leaback & Robinson, 1974), and also to reserve the higher resolution steps (based on electric-charge

properties) until later in the purification scheme. Further, the high salt concentration which is necessary for the successful gel-filtration of proteins, but undesirable for subsequent fractionation procedures that depend on electric charge, can be conveniently decreased during the subsequent ultrafiltration-concentration process.

The results both of the two-dimensional display technique described earlier (Leaback & Robinson, 1974) and of the isoelectric-focusing gel represented in Plate 1 suggested a strategy for the further purification of the enzyme based on the use of ion-exchange adsorbents. Unfortunately, the relationship between the adsorption of proteins on to such materials and the isoelectric points of proteins determined by isoelectric focusing does not seem to have been studied. Experiments with carbonmonoxyhaemoglobin A [a readily available, readily assayable, fairly homogeneous protein with an established isoelectric point of 6.95 (Drysdale *et al.*, 1971)] and the epididymal enzyme on small columns of CM-cellulose showed that the active proteins were largely unadsorbed on CM-cellulose at the indicated isoelectric points and adsorbed at 1 pH unit lower (Fig. 2). The shallower curve for the enzyme adsorption in Fig. 2 is explicable in terms of the electric charge heterogeneity of the enzymically active proteins (see Fig. 1b).

Although no general principle can be inferred from the results in Fig. 2 alone, the complementarity of the isoelectric spectra of the many adsorbed and unadsorbed proteins shown in Plate 1 (tracks *c'* and *d'*), and the relationship of these spectra to the pH of the ion-exchange process, are consistent with the principle that proteins with little or no net electric charge are unlikely to be strongly adsorbed on to ion-exchange materials. Lampson & Tytell (1965) studied the pH values at which a number of proteins were eluted from CM-Sephadex in relation to isoelectric points determined by classical methods, and their findings were broadly in accord with the above principle.

An indirect consequence of the above principle is that the complete resolution of the enzyme from the other proteins of closely similar isoelectric point is unlikely by ion-exchange chromatography as used here. Better resolution could perhaps be accomplished by gradient elution, but this would probably entail undesirable dilution of the purified enzyme.

Table 1 shows that whether applied to the very poorly active extract of whole epididymis or to the much more active extracts from the best sections of the organ, the purification scheme produced enzyme preparations in better yield and with specific activities comparable with those reported by Findlay & Levvy (1960). As to the purified enzyme preparations themselves, Table 2 shows that the relatively low activities of other glycosidases present in the crude extracts were further decreased by the purification procedure, and

Table 2. *Some relative glycosidase activities in various stages of the purification of β -N-acetyl-D-glucosaminidase preparations from the 'best sections of pig epididymis'*

Hyaluronidase activities [derived after Aronson & Davidson (1967)] were related in molar terms to activities of other glycosidases determined fluorimetrically under conditions described in the text.

	Relative enzyme activities				
	β -N-Acetyl-D-glucosaminidase (EC 3.2.1.30)	$10^2 \times \beta$ -D-Glucuronidase (EC 3.2.1.31)	$10^2 \times \beta$ -D-Galactosidase (EC 3.2.1.23)	$10^2 \times \alpha$ -D-Mannosidase (EC 3.2.1.24)	$10^2 \times$ Hyaluronidase (EC 4.2.99.1)
Crude enzyme concentrate	100	1.2	3.7	1.3	3
Fraction (II) from Sephadex G-200 effluent	100	2.0	2.3	1.7	<0.003
Pooled fractions from iso-electric-focusing column	100	0.02	<0.001	<0.001	—

this has been of considerable value in other applications of the enzyme requiring highly purified preparations of β -N-acetyl-D-glucosaminidase with extremely high activities (Leaback, 1974). The results of the density-gradient stabilized isoelectric focusing represented in Fig. 1(b) indicated a 'B' form of the enzyme of considerable polydispersity which must be due to differences in electric charge between enzymically active proteins of comparable molecular size.

The success of the above experimental strategy in the separation of the epididymal proteins prompted the formulation of the following proposals for a generalized approach to protein purification: (1) explore the pH stability of the protein of interest by some assay dependent on its three-dimensional structure (e.g. enzyme activity). (2) Subject the crude protein mixture to the two-dimensional display technique and apply available methods for the detection of the protein of interest. The results of this experiment should indicate the approximate positions of this protein in the isoelectric and molecular-size spectra of the proteins present, and should therefore suggest experimental strategy for purification on the basis of these molecular properties. (3) Estimate the range of isoelectric points of the protein of interest after density-gradient stabilized isoelectric focusing. (4) After appropriate small-scale trial experiments, adsorb the protein of interest on to an appropriate ion-exchange cellulose by using established isoelectric-point information. After washing free from salts used in the gel-filtration step, elute the adsorbed proteins with carrier ampholytes of suitable basicity. (5) Subject the proteins eluted from the ion-exchange material to isoelectric focusing in a density-stabilized column and collect fractions containing the protein of interest. If necessary, separate the protein from sucrose and carrier ampholytes by gel filtration (Vesterberg, 1969), or salt fractionation (Nilsson *et al.*, 1970).

As to the larger scale operation of the proposed scheme, Janson (1971) showed that substantial

amounts of proteins can be separated by gel filtration on Sephadex G-200. The ion-exchange step is also capable of handling large quantities of proteins and whereas the column isoelectric-focusing techniques can accommodate only moderate amounts of protein, the quantities involved at this final stage in the preparation should be considerably decreased.

The techniques used are of progressively increasing resolving power, and protein concentrations can be maintained or even increased towards the end of the purification. The use of carrier ampholytes for the elution of the required proteins from the ion-exchange material eliminates the introduction of unwanted salts for this purpose and, at the same time, elegantly provides the means for generating a pH gradient ideal for the subsequent separation of residual proteins. The carrier ampholytes probably also give added protection against protein denaturation (Wadstrom, 1970), until the protein of interest is concentrated as an isoelectric-focusing equilibrium is achieved.

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