Purification of β-Acetylglucosaminase and β-Galactosidase from Ram Testis

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1. The presence of β-galactosidase (EC 3.2.1.23) in an acetic acid extract of ram testis is reported. Some properties of the crude enzyme preparation were studied. 2. The purification of β-acetylglucosaminase (EC 3.2.1.30) and of β-galactosidase from the ram-testis extract by ammonium sulphate precipitation and chromatography on a CM-cellulose column is described. 3. The final purifications of the separated enzymes achieved were for the β-acetylglucosaminase 35 times and for the β-galactosidase 99 times. 4. The possibility of using DEAE-cellulose and Sephadex G-200 to purify the enzymes was investigated.

Although the protein portion of ovomucoid has been degraded by proteolytic enzymes [pancreatic enzymes and trypsin (Tanaka, 1961*a*,*b*,*c*,*d*), pepsin (Hartley & Jevons, 1962), Pronase (Marks, Marshall, Neuberger & Papkoff, 1962) and papain (Montgomery & Wu, 1963)], little work on the enzymic degradation of the carbohydrate moiety has been reported. In a preliminary communication Caygill & Jevons (1964) reported the slow release of *N*-acetylglucosamine from the carbohydrate moiety of ovomucoid by a crude extract of ram testis. As the ram-testis extract was known to contain a β-acetylglucosaminase (Borooah, Leaback & Walker, 1961), further purification of this enzyme was considered desirable to investigate whether it was responsible for the release of *N*-acetylglucosamine from ovomucoid, and whether a purer preparation would change the quantity and rate of release. During the course of this purification, β-galactosidase, for which ovomucoid could be a substrate, was detected.

MATERIALS AND METHODS

p-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside was synthesized by the method of Leaback (1963) (m.p. 216–218°, $[\alpha]_D^{20}$ −17°, *c* 0·5% in water), or was bought from Koch–Light Laboratories Ltd., Colnbrook, Bucks. *o*-Nitrophenyl β-D-galactopyranoside was obtained from Koch–Light Laboratories Ltd., and *p*-nitrophenyl β-D-galactopyranoside from British Drug Houses Ltd., Poole, Dorset.

CM-cellulose, prepared by the method of Peterson & Sober (1956), was kindly supplied by Dr C. H. Wynn, and DEAE-cellulose was purchased from Eastman–Kodak Ltd., Kirkby, Liverpool. Sephadex G-200 (in the bead form) was obtained from Pharmacia, Uppsala, Sweden.

Bovine plasma albumin was obtained from Armour Pharmaceuticals Ltd., Eastbourne, Sussex, and *o*-nitrophenol was synthesized in this Department.

An extract of ram testis, prepared by mincing 500g. of the unskinned organs, and extracting with 2l. of acetic acid at 0–5°, was a gift from Fisons Pharmaceuticals Ltd., Holmes Chapel, Cheshire.

Estimation of enzymic activities. β-Acetamido-2-deoxy-D-glucoside acetamidoglucohydrolase (EC 3.2.1.30) (β-acetylglucosaminase) activity was estimated by the method of Woollen, Heyworth & Walker (1961) with *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (final concn. 3·6mM). Bovine plasma albumin (0·2%) was included in all assays, as recommended by the above-named authors.

β-Galactoside galactohydrolase (EC 3.2.1.23) (β-galactosidase) was estimated by a slight modification of the method of Lederberg (1950), with the synthetic substrates *o*- and *p*-nitrophenyl β-D-galactopyranoside. Duplicate mixtures containing, except where otherwise indicated, *o*-nitrophenyl galactoside (final concn. 4·0mM) or *p*-nitrophenyl galactoside (final concn. 2·0mM), and enzyme solution diluted as appropriate, in 0·05M-citric acid–sodium citrate buffer, pH4·5, to give final vol. 1·0ml., were incubated at 37°. The reaction was stopped by the addition of 0·2M-sodium tetraborate adjusted to pH9·8 with 0·2N-NaOH (2·0ml.), to give final pH9·2. The extinction at 400mμ was measured in a Unicam SP.500 spectrophotometer, and the quantity of aglycone liberated determined from the appropriate calibration curve. In those experiments where the assay was carried out in buffers of different pH values the final pH was adjusted to pH9·2 (where necessary) by the addition of 1 to 3 drops of N-NaOH or N-citric acid or N-acetic acid.

The details of the purification of β-acetylglucosaminase

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and β-galactosidase by precipitation with $(NH_4)_2SO_4$ and column chromatography on CM-cellulose are given with the relevant results.

RESULTS

As no report of the properties of ram-testis β-galactosidase has been found, some properties were studied to ascertain the best conditions in which to assay the enzyme. Linear reaction rates were recorded over a 10-fold range of enzyme concentrations, with times of incubation up to 90min.

Effect of pH and substrate concentration on β-galactosidase activity. Fig. 1 shows the effect of pH on β-galactosidase activity with *o*-nitrophenyl β-D-galactoside as substrate. Similar results were obtained with 2mM-*p*-nitrophenyl β-D-galactoside, 0·1ml. of a fourfold-diluted extract, incubated for 15min. in 0·05M-citrate or 0·05M-acetate buffer, being used.

Fig. 2 shows the variation of β-galactosidase activity with concentration of *o*-nitrophenyl β-D-galactoside. It will be noted that the enzyme was inhibited by excess of substrate. The double-reciprocal plot attributed to Lineweaver & Burk (1934) gives the apparent K_m 0·67mM. A similar shaped curve, showing slight inhibition by excess of substrate, was obtained in 0·05M-acetic acid–sodium acetate buffer, pH4·5, when *p*-nitrophenyl β-D-galactoside was used as substrate.

The apparent K_m with *o*-nitrophenyl β-D-galactoside as substrate was also 0·67mM when measured in 0·1M-citric acid–sodium phosphate buffers of pH6·95, 6·7, 6·3, 5·9, 5·0 and 4·05, or in 0·05M-acetic acid–sodium acetate buffers of pH6·7, 5·5, 5·05, 4·5, 4·3 and 3·5. At pH2·7, in the former buffer, K_m 0·15mM was obtained. A value for the apparent K_m 0·15mM was found with *p*-nitrophenyl β-D-galactoside as substrate in 0·1M-citric acid–sodium phosphate buffers of pH5·5, 4·45 and 3·4.

Purification of β-acetylglucosaminase and β-galactosidase. (*a*) Ammonium sulphate fractionation. Ram-testis extract was fractionated by the addition of solid ammonium sulphate, and the results are shown in Table 1. The fraction precipitating between 30 and 40% saturation with ammonium sulphate (fraction P_{40}), which had specific activities of 7·1 times (β-acetylglucosaminase) and 6·2 times (β-galactosidase) that of the ram-testis extract, was used for the next stage of the purification.

(*b*) Chromatography on a CM-cellulose column. CM-cellulose (5g.) was added to 150ml. of 0·05N-sodium hydroxide–0·05M-sodium chloride (1:1, v/v). This was poured into a Buchner funnel, and washed without suction with 2l. of this mixture followed by 2l. of 0·1N-hydrochloric acid and 0·05M-citrate buffer, pH4·5, until no Cl^- ions were detected in the washings. A slurry of the CM-cellulose in the citrate buffer was poured into a column (40cm. by 1·5cm. diam.). Dialysed fraction

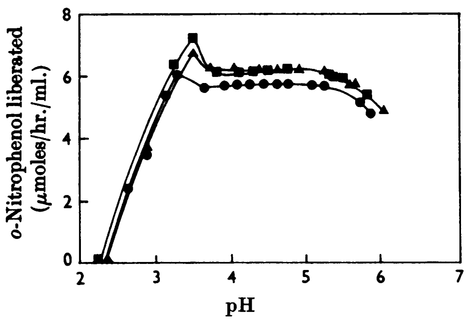

Fig. 1. pH–activity curve of β-galactosidase. Incubation mixtures (1·0ml.), incubated for 30min. at 37°, contained 0·25ml. of diluted ram-testis extract (1:2), 4·0 mM-*o*-nitrophenyl β-D-galactopyranoside in 0·05M-buffers of various pH values: ■, citric acid–sodium citrate; ●, acetic acid–sodium acetate; ▲, citric acid–disodium hydrogen phosphate.

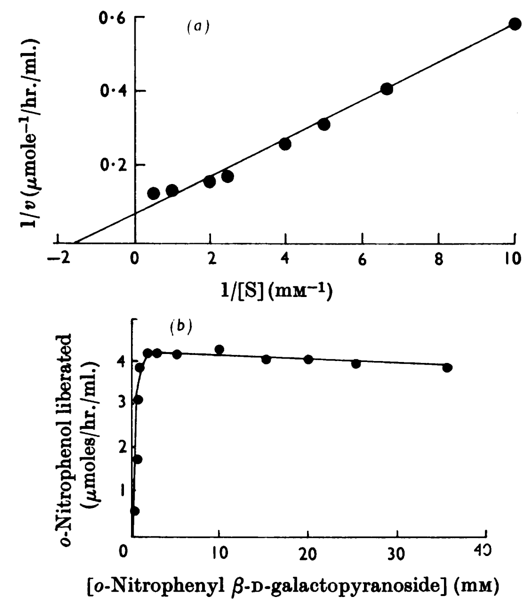

Fig. 2. Effect on β-galactosidase activity of varying substrate concentration. The β-galactosidase activity of 0·25ml. of diluted ram-testis extract (1:2) was determined by incubation in 0·05M-citric acid–sodium citrate buffer, pH4·5, for 30min., with *o*-nitrophenyl β-D-galactopyranoside of the concentrations shown: (*a*) as a double reciprocal plot of 1/[substrate] against 1/activity; (*b*) as a plot of activity against substrate concentration.

Table 1. *Ammonium sulphate fractionation of ram-testis extract*

Solid $(NH_4)_2SO_4$ was added at 4° to the ram-testis extract, and stirred for 40 min. Precipitates were redissolved in water and dialysed against four changes (20 vol.) of distilled water, followed by two changes (10 vol.) of 0·05 M-citrate buffer, pH 4·5, for 12 hr. in each case. Enzyme activities were estimated as described in the text. Protein was estimated by measuring the extinction at 280 mμ. Enzyme activity/ml. is expressed as μmoles of aglycone liberated/hr./ml. and specific activity as activity/ml./E_{280} unit.

	β-Acetylglucosaminase			β-Galactosidase			
Fraction	Activity/ ml.	Specific activity	Recovery of enzyme activity (%)	Activity/ ml.	Specific activity	Recovery of enzyme activity (%)	Recovery of protein (%)
Ram-testis extract	216	14·1	100	7·26	0·47	100	100
0–20% satn. $(NH_4)_2SO_4$ ppt.	70	7·8	3	3·77	0·42	5	6
20–30% satn. $(NH_4)_2SO_4$ ppt.	107	21·2	5	3·59	0·69	5	4
30–40% satn. $(NH_4)_2SO_4$ ppt.	1761	100·0	81	48·40	2·75	63	17
40–45% satn. $(NH_4)_2SO_4$ ppt.	57	8·4	3	0·92	0·13	1	11

Table 2. *Chromatography on carboxymethyl-cellulose*

Recovery, expressed as % of material applied to the column, of β-acetylglucosaminase and β-galactosidase activities, and of protein, from a CM-cellulose column eluted as described in Fig. 3.

	Recovery (%)		
Fraction (tube nos.)	β-Acetyl-glucosaminase	β-Galacto-sidase	Protein
5– 8	0	65·5	8·4
35–37	56·2	5·5	13·8
63–65	30·6	0	24·8
Total	94·5	79·0	90·0

P_{40} (5·0 ml.) was washed into the column with 0·05 M-citrate buffer, pH 4·5. The column was then eluted with a series of buffers of increasing pH as indicated in Fig. 3. Table 2 shows the recovery of the two enzymes and protein in the tubes containing most enzyme, as a percentage of that applied to the column. The highest purification factors obtained were for β-galactoside, 15·9 times (tube 6), and for β-acetylglucosaminase, 4·9 times (tube 36).

Fig. 3 also shows that the β-acetylglucosaminase activity is eluted in two peaks, and the intervening tubes contain little enzyme activity. Rechromatography of the second peak (which had been eluted by buffer pH 5·5) showed that no activity was eluted by buffer pH 4·9, the pH at which the major portion (67%) of the activity was eluted from the first column, but was again eluted by buffer pH 5·5. It would thus appear that the β-acetylglucosaminase activity present can be separated into two fractions, which are eluted from CM-cellulose by buffers of different pH.

(*c*) Investigation of alternative purification procedures. β-Acetylglucosaminase was adsorbed by DEAE-cellulose from 0·02 M-sodium dihydrogen phosphate–disodium hydrogen phosphate buffer, pH 7·5, and could be eluted by 0·05 M-citrate buffer, pH 3·0, containing a final concentration of 0·67 M-sodium chloride. When an attempt was made to elute β-acetylglucosaminase from a DEAE-cellulose column, no activity was recovered, despite the recovery of 102% of the added protein, as estimated by the extinction at 280 mμ. However, the enzyme would be expected to be inactivated at pH 3·0, in the time taken to elute it from a column. Fraction P_{40} (0·2 ml.) was incubated at 20° in 0·05 M-citrate buffer (1·8 ml.) of various pH values from 2·0 to 9·0, and 0·1 ml. samples were withdrawn at intervals, diluted with 0·9 ml. of 0·05 M-citrate buffer, pH 4·5, and 0·1 ml. of this was used to estimate residual β-acetylglucosaminase activity. Between pH 4·0 and pH 9·0 the β-acetylglucosaminase was relatively stable for up to 2 hr., but activity was rapidly lost in more acid solutions. Findlay & Levvy (1960) reported that pig-epididymal β-acetylglucosaminase was stable in the range pH 4·0–8·0 for 1 hr. at 37°, but lost all its activity in this time at pH 2·2.

β-Acetylglucosaminase in 0·05 M-citrate buffer, pH 4·5, was absorbed at room temperature by sand previously washed with 2 N-hydrochloric acid followed by 0·1 N-sodium hydroxide and distilled water, and 75% of the absorbed enzyme could be eluted with 0·02 M-phosphate buffer, pH 7·5.

The use of Sephadex G-200 was investigated. Sephadex G-200 (5 g.) was equilibrated for at least 4 days with 0·05 M-citrate buffer, pH 4·5, and poured into a column (40 cm. by 3 cm. diam.), Fraction P_{40} (5·0 ml.) was applied to this column. and then eluted with 0·05 M-citrate buffer, pH 4·5. The acetylglucosaminase activity appeared in the

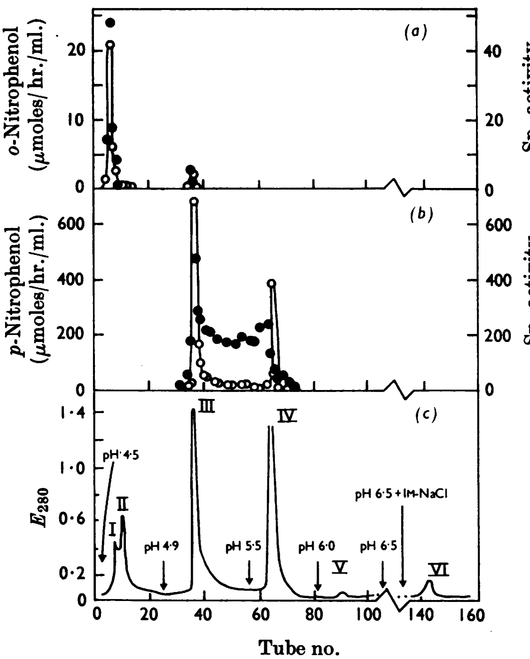

Fig. 3. Chromatography of fraction P_{40} on CM-cellulose. Fraction P_{40} was applied to a CM-cellulose column and eluted with a series of 0·05M-citric acid–sodium citrate buffers, of increasing pH as indicated. The initial pH was 4·5 and the pH was changed as shown by arrows. Fractions (5ml.) were collected. (*a*) Activity/ml. (○) and specific activity (●) of β-galactosidase. (*b*) Activity/ml. (○), and specific activity (●) of β-acetylglucosaminase. (*c*) Protein, estimated by extinction at 280mμ, plotted against tube no. (5·0ml. fractions). No protein was detected in tubes 100–130.

first protein fraction (75–100ml. of eluate), but the extinction at 280mμ indicated that some protein was retarded. The recovery of β-acetylglucosaminase from the column was 79%, and of protein 99%, and the tube having the highest specific activity showed a purification factor of only 2·4. However, when a mixture from the tubes from the CM-cellulose column containing the β-acetylglucosaminase with the highest specific activity (tubes 36 and 37, 2·5ml. of each, adjusted to pH 4·5 with 0·05M-citric acid) was applied to an identical column of Sephadex G-200, only a small amount of protein devoid of β-acetylglucosaminase activity was eluted with 75–100ml. of eluent. The major protein-containing fraction was eluted with 140–200ml. of buffer, and this fraction included the only β-acetylglucosaminase activity eluted from the column. This fraction accounted for 105% of the applied protein, but only 25% of the applied enzyme activity, and had a lower specific activity than the applied material.

DISCUSSION

β-Galactosidase activity with *o*- or *p*-nitrophenyl galactoside as substrate, when plotted against pH, showed a sharp peak at approximately pH 3·5, followed by a plateau of uniform activity from pH 4·0 to 5·2. A double peak, showing maximum activity at approximately pH 2·9 and pH 3·5, was obtained by Conchie & Hay (1959) with phenyl β-D-galactoside in the assay of rat-epididymal β-galactosidase. With *o*-nitrophenyl β-D-galactoside they obtained single peaks at pH 2·9 or at pH 3·5 in acetate or citrate–phosphate buffers respectively. Levvy & McAllan (1963) found maximum activity at pH 2·9 for the rat-epididymal enzyme with *p*-nitrophenyl β-D-galactoside in citrate–phosphate buffer. For the experiments reported here pH 4·5 was selected, as at this pH the enzyme in the crude extract showed no variation in activity with minor variations of pH.

Inhibition by concentrations of substrate above 2mM (*o*-nitrophenyl β-D-galactoside) and 4mM (*p*-nitrophenyl β-D-galactoside) is reported. Slight inhibition of rat-epididymal β-galactosidase by excess of substrate was found at concentrations above 5mM (*o*-nitrophenyl β-D-galactoside) or 15mM (*p*-nitrophenyl β-D-galactoside) by Conchie & Hay (1959). The values for the apparent Michaelis–Menten constant, K_m, obtained were 0·67mM with *o*-nitrophenyl β-D-galactoside and 0·15mM with *p*-nitrophenyl β-D-galactoside, compared with values for the rat-epididymal enzyme of 0·38mM and 0·27mM respectively (Levvy & McAllan, 1963).

Ammonium sulphate fractionation appears to be a suitable first step in the purification from ram-testis extract of β-acetylglucosaminase (81% recovery) and β-galactosidase (63% recovery). Chromatography on CM-cellulose then gave good separations, so that the two stages of purification gave enzymes having specific activities 35 times (β-acetylglucosaminase) and 99 times (β-galactosidase) that of the original ram-testis extract.

The β-acetylglucosaminase activity was absorbed at pH 4·5; most was eluted at pH 4·9, and a little at pH 5·5. As on rechromatography of this second peak the β-acetylglucosaminase activity was eluted, not at pH 4·9, but again at pH 5·5, it is possible that the enzymic activity is associated with two different proteins, or one protein existing in two forms which behave differently on CM-cellulose.

Two peaks (at pH 4·5 and pH 4·9 respectively) containing β-galactosidase activity were eluted. As no activity is present in the intervening tubes, in spite of an intervening protein peak, it is possible that these activities are also caused by two different proteins. If this is so the pH optimum curve obtained with the ram-testis extract could be the result of two separate curves. On the other hand, the finding of the same K_m between pH 6·9 and pH 3·5 for *o*-nitrophenyl β-D-galactoside favours the alternative hypothesis that the activity is due to two forms of the same protein, which behave differently on CM-cellulose. It is unlikely that the same protein in peak III (pH 4·9) is responsible for both the β-galactosidase and β-acetylglucosaminase activities, as the ratio of the two enzymic activities in tubes 36, 37 and 38 (Fig. 3) is not constant.

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