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AN EXO-β-*N*-ACETYLGLUCOSAMINIDASE FROM *BACILLUS SUBTILIS* B; EXTRACTION AND PURIFICATION

J. M. ORTIZ*, J. B. GILLESPIE** AND R. C. W. BERKELEY
Department of Bacteriology, The Medical School, University Walk, Bristol BS8 1TD (Great Britain)

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SUMMARY

Exo-β-*N*-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) is produced by *Bacillus subtilis* B growing in a glucose–peptone–salts medium towards the end of growth. The enzyme is released into the growth medium and is mainly found in a sedimentable form. Attempts to solubilize the enzyme by freezing and thawing, by sonication and by treatment with organic solvents, detergents or enzymes were unsuccessful. More than 90% of the enzyme was released from cells and from material sedimented by centrifugation at 30 000 × *g* after treatment with high salt concentrations (*e.g.* 4 M NaCl).

Extraction of the enzyme with high salt concentrations was made use of as the first step in the purification procedure. Subsequent steps of ultrafiltration, chromatography on Sephadex G-200 equilibrated with 3 M NaCl, to remove the high molecular weight material causing the enzyme to sediment, dialysis, to remove the salt, and ion exchange chromatography on DEAE- and CM-Sephadex led to a product purified 7000 times as compared to the culture supernatant. The purified preparation gave a single peak on disc gel electrophoresis and moved as a single band of constant specific activity on a Sephadex G-100 column.

INTRODUCTION

Purified exo-β-*N*-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) preparations from mammalian sources have been widely used in structural studies on bacterial cell walls (Ghuysen[1]; Hughes[2]). But despite the common occurrence of this enzyme among Gram positive and Gram negative bacteria (Woollen *et al.*[3]; Berkeley, *et al.*[4]) there are no reports of highly purified preparations from bacteria although exo-β-*N*-acetylglucosaminidase from

* Present address: Department de Bioquimica, Faculty de Farmacia, Universidad de Madrid, Madrid, Spain.
** Present address: Department of Industrial Microbiology, University College, Stillorgan Road, Dublin, 4, Ireland.

Diplococcus pneumoniae (Hughes and Jeanloz[5]) and a group A *Streptococcus* (Ginsberg *et al.*[6]) have been partially purified. T. Wadström, L. Linder, C. E. Nord and A. A. Lindberg (personal communication) have recently purified this enzyme from *Streptococcus mitis*. We required a pure preparation of the exo-β-*N*-acetylglucosaminidase from *Bacillus subtilis* B in connection with a study of the role of the enzyme in this organism and we report here the procedure used to obtain such a preparation.

Studies of some of the factors influencing the formation of this enzyme by *B. subtilis* B are described in a previous paper (J. M. Ortiz, R. C. W. Berkeley and S. J. Brewer, unpublished). β-*N*-Acetylglucosaminidase occurs, in the late logarithmic phase and in the stationary phase of cultures grown in a complex medium, mainly in the supernatant but also to a small extent in association with the cells. The enzyme in the supernatant can be sedimented by high speed centrifugation and is assumed to be combined with particulate material. In this paper we describe a procedure which takes advantage of the particulate nature of the exo-β-*N*-acetylglucosaminidase and which involves its release by high salt concentrations from the material causing it to be sedimentable. The characteristics of this enzyme are to be described elsewhere (R. C. W. Berkeley, S. J. Brewer, J. M. Ortiz and J. B. Gillespie, unpublished).

MATERIALS AND METHODS

Organism

B. subtilis B was chosen for this work because it produced greater amounts of exo-β-*N*-acetylglucosaminidase under the conditions used than the other strains of this species that were tested (J. M. Ortiz, R. C. W. Berkeley and S. J. Brewer, unpublished).

Culture conditions

The organism was grown in the glucose–peptone–salts medium which has the following composition: K_2HPO_4, 0.4 g; $MgSO_4 \cdot 7\ H_2O$, 0.05 g; NaCl, 0.1 g; $FeCl_2 \cdot 6\ H_2O$, 0.017 g; $(NH_4)_2HPO_4$, 0.5 g; Bacto Peptone, 5.0 g; distilled water to 1000 ml. Glucose sterilized by filtration is added aseptically after autoclaving to give a concentration of 1.0 g/l. 20 l of medium in 40-l aspirators were inoculated with 1 l of a culture grown up in shake flasks at 37 °C overnight. Sterile air was bubbled (20 l/min) through the medium which was maintained at 37 °C in a water bath. After 20 h the level of enzyme activity increased no further and the enzyme was collected by pumping the culture through a Sharples 1PV turbine driven continuous flow centrifuge. Although β-*N*-acetylglucosaminidase is found principally in the culture supernatant, due to the particulate nature of the enzyme most of the activity sedimented in the Sharples. The sediment could be stored at −20 °C without loss of activity.

Enzyme assays

Enzyme solutions (1 ml) were incubated with 1 ml 2 mM *p*-nitrophenyl-2-acetamido-2-deoxyglucose in 1 ml 0.1 M sodium phosphate buffer (pH 5.9) at 37 °C. After 30 min or less, depending on the activity of the preparation, the reaction was stopped by addition of 4 ml 0.2 M borate buffer, pH 9.8 (Clark and Lubs[7]) *p*-Nitro phenol was measured at 400 nm (Woollen *et al.*[8]).

Enzyme units

A unit is defined as that amount which releases 1 μmole *p*-nitrophenol per min at pH 5.9 and 37 °C.

Protein content

This was determined by the method of Warburg and Christian[9].

Extraction of β-N-acetylglucosaminidase

Portions of the whole bacterial culture were added to solutions of the solubilising agents (Table IV) made up in 0.05 M sodium phosphate (pH 5.8) or in 0.05 M Tris–HCl (pH 8.0). After 10–20 min at room temperature the suspensions were centrifuged at 30 000 × *g* for 30 min. The supernatants were collected, the pellet resuspended in the same volume of 0.05 M buffer and the *β-N*-acetylglucosaminidase activity in the two fractions determined.

Ultrafiltration

Large volumes of protein solution were concentrated using the Amicon 402 ultrafiltration cell with PM-30 membranes in conjunction with the RS-4 reservoir (Amicon, 57, Queen's Road, High Wycombe, Bucks.) Smaller volumes were concentrated using collodion shells in a Membranefiltergesellschaft holder (V. A. Howe, Ltd, 88, Peterborough Road, London, S.W. 6.).

Preparation of columns

Sephadex G-200 was swollen, equilibrated and de-fined in 0.1 M Tris (pH 8.0) containing 3 M NaCl over a period of several days. It was then degassed and packed to give a bed of 2.5 cm × 80 cm. DEAE-Sephadex A-25 and CM-Sephadex C-25 were swollen, equilibrated, and de-fined in 50 mM Tris (pH 8.0) similarly, and packed to give beds 1.5 cm × 30 cm. The columns were run at 4 °C.

Polyacrylamide gel electrophoresis

This was carried out essentially according to Davies[10] in a Shandon disc gel electrophoresis apparatus (Shandon Scientific Co. Ltd, 65 Pound Lane, London N.W.10). Polyacrylamide gels, containing 3.75% (w/v) acrylamide and 0.057% (v/v) *N,N,N',N'*-tetra methylethylene diamine in 0.37 M Tris–HCl, pH 8.5, were cast in tubes 6 mm × 96 mm using riboflavin initiator and a Shandon Photopol fluorescent lamp. These gels were run with 0.05 M Tris–glycine buffer (pH 8.5) in the reservoirs. Alternatively gels containing 10% acrylamide in 0.01 M sodium phosphate (pH 7.0) were used with 0.1 M sodium phosphate buffer (pH 7.0) as the reservoir buffer. The protein bands were fixed with sulphosalicylic acid (20%) and stained with amido black or coomassie blue. (Weber and Osborn[11]). The gels were destained over a period of several days with 7% acetic acid. Duplicate gels were run for enzyme localization. These were frozen at −50 °C on completion of the process and cut into slices (1 mm thick) with a razor blade. The slices were then thawed and homogenized in 0.1 M sodium phosphate buffer, pH 5.9 (1 ml) and incubated with 2 mM *p*-nitrophenyl-2-acetamido-2-deoxy glucose (1 ml) in 0.1 M sodium phosphate buffer (pH 5.9) at 37 °C for 30 min when the reaction was stopped by addition of 0.2 M sodium borate

buffer, pH 9.8 (4 ml). The tubes were then centrifuged at 5000 × *g* for 10 min and the *p*-nitrophenol in the supernatant determined.

RESULTS

Incubation of *B. subtilis* B in an aerated complex medium of glucose and peptone in a salts base gave an average yield of 0.006 units β-*N*-acetylglucosaminidase per ml in the whole culture at the beginning of the stationary phase. Only very low levels of enzyme were found with poor aeration. The level of enzyme was also affected by the concentration of glucose and peptone and by substitution of other carbon sources for glucose (J. M. Ortiz, R. C. W. Berkeley and S. J. Brewer, unpublished). However, when this work was started, the best enzyme yields were obtained in glucose–peptone-salts medium and this was routinely used for enzyme production.

Distribution of β-N-acetylglucosaminidase between soluble and particulate forms in the culture supernatant

The enzyme was released into the growth medium during the growth of *B. subtilis* B and reached a maximum level in the stationary phase. However, the enzyme was not entirely soluble since most of it can be sedimented in a centrifuge. With increasing centrifugal force the proportion of the enzyme in the supernatant decreases (Table I). The relative proportion of soluble and particulate enzyme altered during

TABLE I

SEDIMENTATION OF EXO-β-*N*-ACETYLGLUCOSAMINIDASE ACTIVITY FROM CULTURES OF *B. subtilis* B

Overnight cultures, grown in 50 ml glucose–peptone–salts medium in 250-ml erlenmeyer flasks at 37 °C in an orbital incubator with a speed of 225 rev./min and a throw of 32 mm, were fractionated by centrifugation. The amounts of enzyme present initially and the amounts remaining after centrifugation were assayed as described in the text.

Treatment	*% of enzyme remaining in the supernatant*
1200 × *g* for 10 min	87
30 000 × *g* for 30 min	31
100 000 × *g* for 30 min	26

TABLE II

PROPORTION OF SEDIMENTABLE EXO-β-*N*-ACETYLGLUCOSAMINIDASE IN *B. subtilis* B CULTURES OF DIFFERENT AGES

Cultures were grown in glucose–peptone–salts medium and 5-ml portions harvested at the times after inoculation indicated in the table and centrifuged at 30 000 × *g* for 20 min. The pellet was resuspended in 5 ml 0.1 M sodium phosphate buffer (pH 5.9) and the enzyme activity in the pellet suspension and in the supernatant assayed as described in the methods section.

Time (h)	*% of total enzyme remaining in the supernatant*
8	35
11	29
12	27
13	16
15	10

growth and post-growth autolysis. With increasing culture age the proportion of soluble enzyme decreased and conversely that of the particulate material increased. After 15 h only 10–20% of the enzyme was in the soluble form (Table II). Centrifugation of the particulate enzyme in a sucrose gradient resulted in 60% of the enzyme being recovered from the pellet: the rest was distributed irregularly throughout the gradient suggesting that the enzyme–particle complex obtained by centrifugation at 30 000 × *g* after a low speed spin to remove cells and spores is not homogenous with respect to size.

Extraction of β-N-acetylglucosaminidase

A variety of approaches were used in an attempt to increase the amount of soluble enzyme in the 30 000 × *g* supernatant of whole stationary phase cultures. Sonication and freezing and thawing had no effect. A range of hydrolytic enzymes—lysozyme (EC 3.2.1.17), trypsin (EC 3.4.4.4), pronase, papain (EC 3.4.4.10), chymotrypsin (EC 3.4.4.5) and lipase (EC 3.1.1.3) (all at 200 μg/ml)—neither released any enzyme into the supernatant nor affected the total enzyme activity. Detergents also had no effect on the enzyme's sedimentation characteristics but certain detergents stimulated the total activity of the culture (Table III). Sodium lauryl sarcosinate and sodium deoxycholate stimulated the activity of the crude but not the solubilised enzyme.

TABLE III

THE EFFECT OF CERTAIN DETERGENTS ON THE ACTIVITY OF EXO-β-*N*-ACETYLGLUCOSAMINIDASE IN THE CULTURE SUPERNATANT OF *B. subtilis* B

Portions of culture supernatant were incubated with detergents at the final concentrations indicated below. After 10–20 min the solutions were centrifuged at 30 000 × *g* for 20 min and the activity remaining in the supernatant assayed as described in the methods section. The results are expressed as a percentage of the activity in a sample to which no detergent had been added.

Detergent	*Concentration (%)*		
	0.01	*0.1*	*1.0*
Sodium lauryl sarcosinate	100	115	130
Sodium deoxycholate	97	118	129
Sodium lauryl sulphate	103	94	45
Nonidet P40	100	93	92

The results in Table IV illustrate the effect of a range of compounds on the release and inhibition or activation of the enzyme. The salts of calcium and magnesium were potent inhibitors and even after removal by dialysis no activity could be recovered from either the supernatant or the sediment. The most effective releasing agents are high concentrations of the salts of sodium, potassium and ammonium. The release of β-*N*-acetylglucosaminidase from particulate material as a result of treatment by different NaCl concentrations is shown in Fig. 1. 4 M NaCl was routinely used to solubilize the enzyme in the first stages of the purification process.

TABLE IV

THE SOLUBILIZATION OF EXO-β-*N*-ACETYLGLUCOSAMINIDASE FROM MATERIAL NORMALLY SEDIMENTED FROM WHOLE CULTURES BY CENTRIFUGATION AT 30 000 × *g*

Portions of whole culture were added to solutions of compound to be tested. The compounds were dissolved either in 0.05 M sodium phosphate (pH 5.8) or in 0.05 M Tris–HCl (pH 8.0). After 10–20 min at room temperature the suspensions were centrifuged at 30 000 × *g* for 30 min. The supernatants were collected, the pellet resuspended in the same volume of 0.05 M buffer and the enzyme activity in the two fractions assayed as described in the methods section.

Compound added	*Final concn*	*% inhibition*	*% of residual enzyme activity in supernatant*
In 0.05 M Tris–HCl, pH 8.0			
Water	—	0	8
Acetone	16.7% (v/v)	0	7
Chloroform	16.7% (v/v)	0	7
Ethanol	16.7% (v/v)	0	10
Propan-1-ol	16.7% (v/v)	0	6
Propan-2-ol	16.7% (v/v)	0	6
Butan-2-ol	16.7% (v/v)	0	5
Pentan-1-ol	16.7% (v/v)	0	3
$MgCl_2$	0.5 M	78	66
$MgCl_2$	1.0 M	82	68
$MgCl_2$	2.0 M	100	—
$MgCl_2$	3.0 M	100	—
$Ca(NO_3)_2$	1.0 M	100	—
$Ca(NO_3)_2$	3.0 M	100	—
LiCl	1.0 M	0	8
LiCl	3.0 M	0	8
Lithium acetate	1.0 M	0	10
Lithium acetate	2.0 M	0	7
EDTA	0.25 M	0	4
In 0.05 M sodium phosphate, pH 5.8			
Water	—	0	8
Urea	2.0 M	1	8
Urea	4.0 M	19	11
Urea	6.0 M	33	12
Urea	8.0 M	40	13
KNO_3	1 M	0	60
Sodium citrate	1 M	0	83
$(NH_4)_2SO_4$	0.5 M	7	40
NH_4Cl	1.0 M	20	47
NH_4Cl	2.0 M	5	75
NaCl	2.0 M	0	82

PURIFICATION PROCEDURE

Extraction

Cells and particulate enzyme (400 g wet weight) were harvested by centrifugation and stirred with 0.1 M Tris buffer (pH 8.0) containing 3 or 4 M NaCl (300 ml) until completely resuspended (4 h). The suspension was then centrifuged at 23 000 × *g* for 2 h, the supernatant collected and the pellet extracted twice more in the same way. The pooled material from the three extractions was then centrifuged again to obtain a clear supernatant. During the extraction process only a small proportion of the cells lysed and the clarified supernatant finally obtained contained β-*N*-acetylglucosaminidase whose specific activity was only slightly reduced due to the presence of protein of intracellular origin.

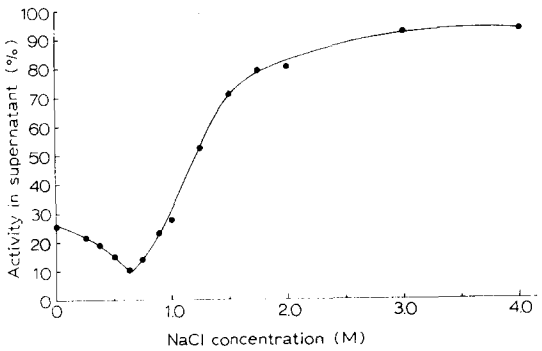


Fig. 1. The influence of salt concentration on the release of exo-β-*N*-acetylglucosaminidase into the 30 000 $\times$ *g* supernatant. Overnight cultures (>16 h) were used. To 2 ml of culture were added 6 ml of NaCl made up in 0.05 M Tris buffer (pH 8.0). The suspension was kept at room temperature for 20 min and then centrifuged at 30 000 $\times$ *g* for 30 min. The amount of enzyme in the supernatant and in the resuspended pellet were then assayed. The amount of enzyme in the supernatant is expressed as a percentage of the total amount found.

Concentration

The extract thus obtained was concentrated up to 10-fold by filtration through Amicon PM-30 membranes. No activity was lost in this step and a further 33-fold purification was achieved presumably because of the removal of contaminating high molecular weight protein. The concentrate (14 ml) was centrifuged at 140 000 $\times$ *g* for 2 h to obtain a clear, though viscous solution.

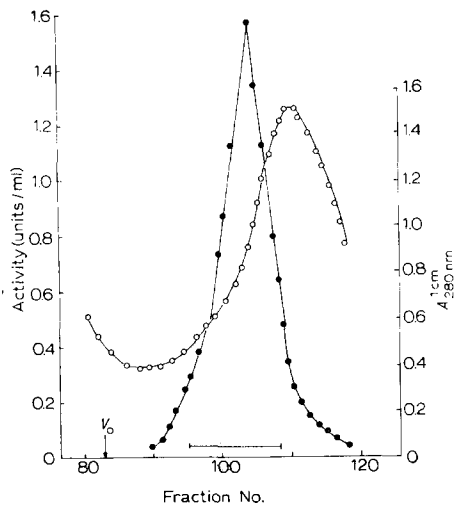
At this stage dialysis against solutions of low ionic strength led to the formation of a heavy precipitate in the dialysis bag. The precipitate, following collection by centrifugation, could be redissolved in 0.1 M Tris (pH 8.0) containing 3 or 4 M NaCl and was found to contain 50–90% of the activity before dialysis.

Sephadex G-200 chromatography

The product of the concentration step was applied to a Sephadex G-200 column equilibrated with 0.1 M Tris (pH 8.0) containing 3 M NaCl and the enzyme eluted, with good recoveries (80–100%), by the same buffer (Fig. 2). When buffers of lower ionic strength were used either to equilibrate or to elute the columns, little or no enzyme was recovered. On these occasions, however, activity could be detected associated with the dextran beads although it could not be recovered even by washing the Sephadex with solutions of high salt concentration, suggesting that the enzyme interacts very strongly with the Sephadex at low ionic strength. Similar results were obtained with Biogel P-200.

Dialysis

Material from the pooled fractions (Nos 94–108) indicated in Fig. 2 was dialysed for 16 h against 10 l 50 mM Tris (pH 8.0). During this period a precipitate formed and was removed by centrifugation at 140 000 $\times$ *g* for 1 h. Less than 1% of enzyme was associated with the precipitate in this case. If the enzyme obtained from this step was mixed with material eluted in the void volume from the Sephadex G-200 columns it sedimented suggesting that during the gel-filtration step the material responsible



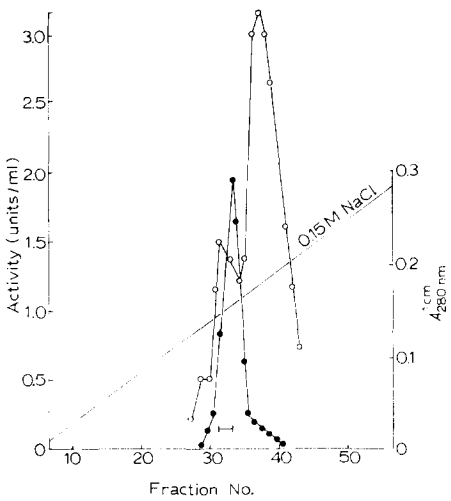


Fig. 2. Chromatography on Sephadex G-200. A 9-ml portion of enzyme from the ultrafiltration concentration step was applied to a column of Sephadex (100 cm × 3.5 cm) equilibrated in 0.1 M Tris buffer (pH 8.0) containing 3 M NaCl. The size of the effluent fractions collected was 3.3 ml. Samples were assayed for enzyme activity (●—●) and $A^{1\,cm}_{280\,nm}$ was measured (○—○). The fractions (94–108) indicated by the bar were pooled.

Fig. 3. Chromatography on DEAE-Sephadex. A 30-ml sample of enzyme from the dialysis step was applied to a column of DEAE-Sephadex A-25 (30 cm × 2.5 cm) equilibrated in 50 mM Tris buffer (pH 8.0). The column was washed with 50 ml of the same buffer and eluted by application of a linear NaCl gradient also in 50 mM Tris buffer (pH 8.0). The size of the effluent fractions collected was 3.3 ml. Samples were assayed for enzyme activity (●—●) and $A^{1\,cm}_{280\,nm}$ (○—○) was measured. The fractions (32–34) indicated by the bar were pooled.

for the particulate behaviour of the enzyme in the earlier purification stages had been removed.

DEAE-Sephadex chromatography

The dialysed enzyme (30 ml) was applied to a column of DEAE-Sephadex A-25 (30 cm × 2.5 cm) equilibrated in 50 mM Tris (pH 8.0) and eluted with a linear NaCl gradient (0–0.3 M) in the same buffer. The column effluent was collected in 3.3-ml fractions and this enzyme was found to be in a single peak corresponding to a NaCl concentration of 0.1 M (Fig. 3).

CM-Sephadex chromatography

The fractions (Nos 32–34) collected from the DEAE-Sephadex column, indicated in Fig. 3 were pooled and 9 ml applied to a column of CM-Sephadex (28 cm × 1.5 cm) equilibrated in 50 mM Tris (pH 8.0) containing 0.1 M NaCl and a linear NaCl gradient applied; the enzyme was eluted in a single peak at a concentration of 1 M NaCl. The specific activity was constant in most of the peak fractions (Nos 41–44) and these were pooled (Fig. 4.).

The final purification achieved was 7000-fold with a specific activity of 23.3 units/mg and an overall yield of 2.1%. The values obtained in the intermediate stages of the process are summarised in Table V.

TABLE V

THE PURIFICATION OF EXO-β-N-ACETYLGLUCOSAMINIDASE FROM *B. subtilis* B

Material	*Vol.* (*ml*)	*Enzyme Concn* (*munits/ml*)	*Total enzyme* (*munits* $\times 10^{-3}$)	*Protein* (*mg/ml*)	*Spec. act.* (*munits/mg*)	*Yield* (%)	*Purification factor*
Culture	40000	6.67	266.67	2	3.33	100	1
NaCl extract	275	367	100	6.5	57	37.5	17
Concentrate from ultrafiltration	13.6	7167	75	43	167	36.5	50
Eluate from Sephadex G-200	50	583	29.17	0.98	600	10.9	180
Dialysate	55	492	27.00	0.88	558	10.1	167
DEAE-Sephadex eluate	9	1500	13.50	0.30	5000	5.1	1500
CM-Sephadex eluate	12	458	5.50	0.02	23300	2.1	7000

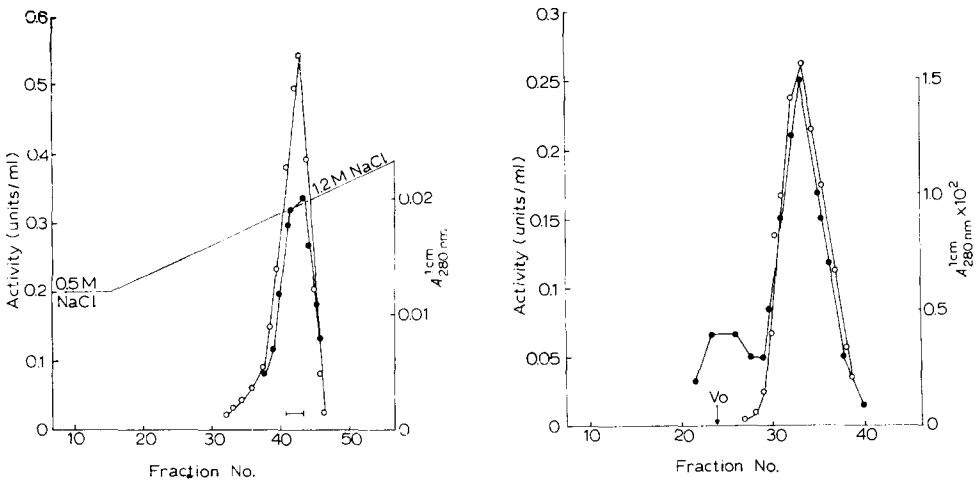


Fig. 4. Chromatography on CM-Sephadex. A 9-ml sample of enzyme preparation from the DEAE-Sephadex step was applied to a column of CM-Sephadex (28 cm × 1.5 cm) equilibrated in 50 mM Tris buffer (pH 8.0) containing 0.1 M NaCl. The column was washed with 50 ml of the same buffer also containing 0.1 M NaCl and then with 50 ml containing 0.5 M NaCl. The enzyme was eluted by applying a linear gradient of the NaCl in the same buffer. The size of the effluent fractions was 3.3 ml. Samples were assayed for enzyme activity (○—○) and $A^{1\,cm}_{280\,nm}$ (●—●) was measured. The fractions indicated by the bar (41–44) were pooled.

Fig. 5. Chromatography on Sephadex G-100. Enzyme preparation from the CM-Sephadex step was dialysed against 0.1 M Tris buffer (pH 8.0) containing 3 M NaCl and applied to a Sephadex G-100 column (145 cm × 1.5 cm) equilibrated in the same buffer and salt concentration. The size of the effluent fractions was 3.0 ml. These were assayed for enzyme activity (○—○) and $A^{1\,cm}_{280\,nm}$ (●—●) was measured.

Criteria of purity

Sephadex G-100 chromatography

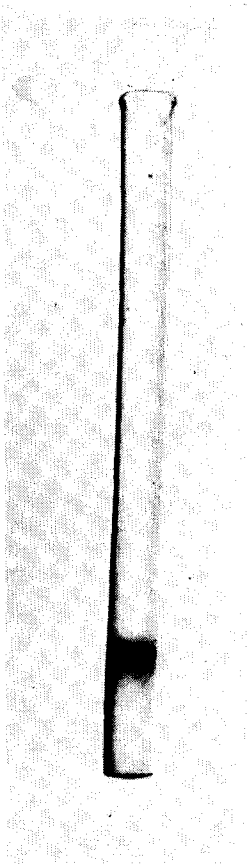
Enzyme from the final purification step was concentrated by ultrafiltration through a collodion sac and dialysed against 50 mM Tris (pH 8.0) containing 3 M NaCl for 5 h and applied to a column of Sephadex G-100 equilibrated in the same buffer. Nearly 100% of the enzyme was eluted in a single peak which corresponds to the single protein peak found by $A^{1\,cm}_{280\,nm}$ measurements (Fig. 5).

Polyacrylamide gel electrophoresis

Enzyme concentrated in the same way but dialysed against 50 mM Tris (pH 8.0) was subjected to electrophoresis. Samples (20–50 μg) were applied to gels made up in Tris-glycine buffer (pH 8.5) (discontinuous system) or in phsphate buffer, (pH 7.0) (continuous system). In both instances a single protein band corresponding to the band of enzyme activity was found. The band formed using the continuous buffer system (Fig. 6) was rather broader than that found using the discontinuous one. Duplicate gels were frozen, sliced and the activity in the slices assayed. Only one peak was found in the whole length of the gel (Fig. 7).

DISCUSSION

The procedures used to obtain exo-β-*N*-acetylglucosaminidase preparations from *Diplococcus pneumoniae* (Hughes and Jeanloz[5]), an anaerobic caeriogenic



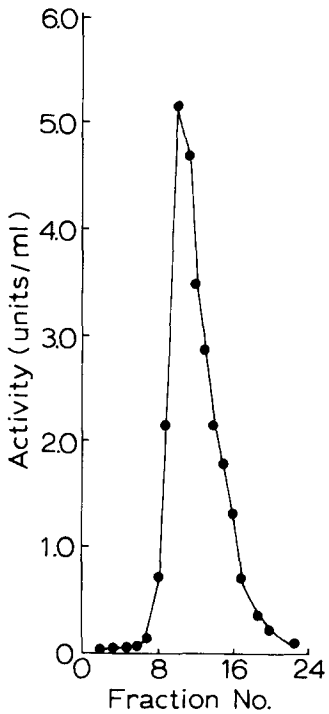


Fig. 6. Polyacrylamide disc gel electrophoresis of purified exo-β-*N*-acetylglucosaminidase. 30 μg of enzyme preparation from the CM-Sephadex step were applied to a polyacrylamide gel made up in 0.01 M sodium phosphate buffer (pH 7.0) and developed by applying a potential of 5 mV per gel for 4 h. The gel was removed and stained with amido black.

Fig. 7. Polyacrylamide disc gel electrophoresis of purified exo-β-*N*-acetylglucosaminidase. The experimental procedure was as described in the legend to Fig. 6. After electrophoresis the gel was removed, frozen, cut into 1-mm slices and the enzyme activity in the slices assayed.

Streptococcus (T. Wadström, L. Linder, C. E. Nord and A. A. Lindberg, personal communication) and Jack Bean meal (Li and Li[12]) all involve $(NH_4)_2SO_4$ purification steps early in the purification protocol. The first approach to the purification of this enzyme was also to attempt a fractional precipitation using $(NH_4)_2SO_4$ (Berkeley *et al.*[4]). Further work along these lines led to the discovery that although precipitation of the culture supernatant normally gave two principal peaks of activity this was not consistently found and also that considerable amounts of activity were found in other fractions. In retrospect it is probable that these findings were due to the association of the enzyme with the sedimentable material and the changing proportion of the soluble enzyme during the stationary phase of growth. Brown *et al.*[13] report similar anomalous behaviour with respect to $(NH_4)_2SO_4$ precipitation of the autolytic *N*-acylmuramyl-L-alanine amidase from *B. subtilis*. They attributed the results to the interaction of the

enzyme with teichoic acid. We have no evidence as to the nature of the material making the *B. subtilis* B enzyme sedimentable but, because lysozyme treatment causes no release of the enzyme from the pellet material, cell wall mucopeptide may possibly be ruled out as the binding material. Since the alkaline phosphatase (EC 3.1.3.1) of *B. subtilis* is localized in the cytoplasmic membrane and can be released by treatment with high salt concentrations (Wood and Tristam[14]) membrane material and teichoic acid remain as possibilities.

Whatever the nature of the material, it seems clear that the exo-β-*N*-acetylglucosaminidase is not covalently bound to it because the enzyme can be removed from the particulate material and from cell walls merely by treatment with salt solutions, albeit, unusually concentrated ones and because the isolated enzyme will reassociate with the void volume material from the Sephadex G-200 column and subsequently behave identically to the crude enzyme preparation. Its interaction is, therefore, similar to the autolysin of *Streptococcus faecalis* (Pooley *et al.*[15]) and possibly even to the autolysin of *B. subtilis* which may in fact be bound to the cell by electrostatic interaction in spite of the suggestion of Brown *et al.*[13], who did not try to elute the enzyme with solutions of high salt concentration, that it is covalently bound.

Following the release of the enzyme from the particulate material during the purification procedure it has to be kept in high salt concentrations or it will reassociate and sediment. If this occurs in Sephadex or ion-exchange Sephadex columns the sedimentation is irreversible. Similar behaviour occurs in Bio-Gel P200 which indicates that the irreversibility is not attributable to a high affinity of the enzyme for polysaccharide material (Andrews[16]).

Another indication of the unusual binding behaviour of this enzyme is the fact that, although as judged by isoelectric focussing experiments the isoelectric point is about pH 4 (R. C. W. Berkeley, S. J. Brewer, J. M. Ortiz and J. B. Gillespie, unpublished), the enzyme binds strongly at pH 8 both to DEAE-Sephadex and to CM-Sephadex. A great increase in specific activity is achieved during the purification using these ion-exchange steps and it has been found that reversing the order makes no difference to the net result.

The significance of the binding of the *B. subtilis* autolysin has been discussed by Brown *et al.*[13] and the conclusions that the complex may serve to protect the enzyme from inactivation by proteases and to localize the enzyme for maximum utilization of substrate are equally attractive with respect to the exo-β-*N*-acetylglucosaminidase of *B. subtilis* B.

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