Fractionation of the β -Glucosidases from *Aspergillus niger*

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In common with many saprophytic moulds *Aspergillus niger* produces in its culture medium a complex mixture of polysaccharide- and protein-depolymerizing enzymes. The commercial preparation (E19) of the extracellular enzymes of *A. niger* has been shown to hydrolyse cellulosic substrates, yeast glucan, laminarin, xylan, starch, polygalacturonic acid and gelatin (Walseth, 1952*a, b*; Holden & Tracey, 1950; Whistler & Smart, 1953; Stone, 1957*a*; Wolf, Jurkovich & MacMasters, 1959). The β -glucosidases of *A. niger* active on β -linked glucose disaccharides were investigated by Crook & Stone (1957). It was shown that during the hydrolysis of cellobiose, a possible intermediate in the enzymic hydrolysis of cellulose, the transient formation of oligosaccharides by transglucosylation occurred. Before attempting any study of the action patterns of the β -glucosidases from *A. niger* that hydrolyse cellulose it was therefore desirable to distinguish and if possible to separate them from the β -glucosidases studied earlier. Preliminary experiments, which have been briefly reported (Stone, 1957*a*), showed that calcium phosphate could reversibly adsorb the *A. niger* enzymes, and, subsequently, synthetic ion-exchange resins were also found satisfactory as adsorbents for elution chromatography. Details of the fractionation experiments are now reported together with some data on the specificity of the fractionated components.

MATERIALS AND METHODS

Substrates. Salicin and cellobiose were from L. Light and Co. Ltd. Cellopentaose was prepared and donated by Dr D. R. Whitaker, National Research Laboratories, Ottawa, Canada. Cellodextrin was prepared by ethanol fractionation of a cellulose hydrolysate. The average degree of polymerization (av. D.P.) as determined by iodometric titration (Martin, Smith, Whistler & Harris, 1941) was 80. Sodium carboxymethylcellulose, Edifas 'B' (controlled metals content, normal viscosity, 0.45–0.55 degree of substitution) was a product of Imperial Chemical Industries Ltd., Stevenon, Ayrshire, Scotland. Phosphoric

acid-swollen cellulose was prepared according to Walseth (1952*a*) from cotton lint (American card sliver) obtained from the Shirley Institute, Didsbury, Manchester; the D.P. determined by the British Rayon Research Association from the intrinsic viscosity of the nitrate according to Immergut, Rånby & Mark (1953) was 2059. Soluble laminarin from *Laminaria digitata* was purchased from the Institute of Seaweed Research, Inveresk, Scotland. Lichenin was extracted from the dried fronds of *Cetraria icelandica* (Iceland moss) purchased from Brome and Schimmer (7, Leather Market, London, S.E. 1). The method used was that of Hess & Lauridsen (1940) described by Meyer & Gürtler (1947); the final stages were as described by Drake (1942). The light-brown product, dried at 60° *in vacuo*, contained 96.2% of polyglucose.

Enzyme preparation. The commercial preparation E19 (Rohm and Haas Co., Philadelphia 5, Penn., U.S.A.), an ethanol-precipitated culture filtrate of *Aspergillus niger*, was supplied as a stable powder. Stock batches of the enzyme were prepared by allowing a 10% (w/v) suspension of the powder in water to stand for several hours in a refrigerator with occasional stirring. The insoluble material was spun off and the dark-brown supernatant dialysed against distilled water in cellulose nitrate sacs (Stone, 1957*b*) until free of reducing sugars and inorganic salts. The solutions were then freeze-dried and the powders stored in a desiccator under vacuum. These powders were readily soluble in water and will be referred to as the crude enzyme. The carbohydrate content determined as glucose by the orcinol method (Pirie, 1936) was 45.5% and by the anthrone method (Trevelyan & Harrison, 1952) was 19.5%. The protein content, determined by the Folin biuret method with bovine serum albumin as standard (Lowry, Rosebrough, Farr & Randall, 1951), was 63.5% and based on Kjeldahl-nitrogen determination was 47.3%.

Enzyme assays. Enzyme activity on all substrates was measured by determining reducing sugar liberated under standard conditions. In all cases the pH of the reaction mixture was 5.0 and the incubation temperature 40°. Reactions were stopped by mixing 1 ml. of the reaction mixture with 1 ml. of ZnSO₄ soln. in a 15 ml. centrifuge tube and rapidly adding 1 ml. of Ba(OH)₂ (Somogyi, 1945). After centrifuging, samples (1 ml.) of the supernatant were removed to 25 ml. Folin-Wu tubes and the reducing sugar was determined as glucose by the Nelson (1944) colorimetric method with the Somogyi (1952) alkaline copper reagent. Light-absorption was measured in a colorimeter (A. Gallenkamp and Co. Ltd., London) with an Ilford 204 filter.

The reaction mixtures incorporating the individual substrates had the following compositions. (a) Salicin: 0.5 ml. of 0.5% salicin, 0.4 ml. of 0.1 M-acetate buffer, 0.2 ml. of enzyme solution; incubation time 1 hr. (b) Cellopentaose: 0.8 ml. of 0.06% cellopentaose in 0.1 M-acetate buffer, 0.2 ml. of enzyme solution; incubation time 1 hr. (c) Cello-

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dextrin: 0.8 ml. of 0.25% celloidextrin in 0.1M-acetate buffer, 0.2 ml. of enzyme solution; incubation time 2 hr. (d) Sodium carboxymethylcellulose (SCMC): 0.7 ml. of 0.75% SCMC, 0.2 ml. of 0.2M-acetate buffer, 0.1 ml. of enzyme solution; incubation time 2 hr. Since partially swollen fibres could be seen with the microscope in simple aqueous solutions of the SCMC used, complete dispersion of the SCMC for use in enzyme assays was obtained by the following procedure; 500 ml. of an approx. 5% (w/v) solution was prepared by swelling SCMC in 200 ml. of water overnight and stirring in the remaining 300 ml. of water with a high-speed stirrer. Enough 40% NaOH was added to bring the NaOH concentration to 5%; the mobile solution was neutralized with 0.5N-HCl and the NaCl removed by dialysis against distilled water. The final SCMC concentration was determined by dry weight and the SCMC substrate solution prepared by dilution. (e) Phosphoric acid-swollen cellulose: 12.5 mg. of swollen cellulose, 2 ml. of 0.2M-acetate buffer, 2 ml. of enzyme solution; incubation in 25 ml. conical flasks for 5 hr. with shaking at 60 strokes/min. (f) Laminarin: 0.6 ml. of 0.25% laminarin in 0.1M-acetate buffer, 0.4 ml. of enzyme solution; incubation time 2 hr. (g) Lichenin: 0.5 ml. of 2% (w/v) lichenin dispersion in 0.2M-acetate buffer made by heating to 60°, 2 ml. of enzyme solution; incubation time 5 hr.

Calcium phosphate columns. Calcium phosphate (Brushite modification) was prepared according to Tiselius (1954). A total precipitation time of 1.5 hr. gave a product with suitable packing properties. After washing by decantation six times with 5 mM- Na_2HPO_4 the precipitate was stored under the same solution.

Columns were packed by pouring a suspension of calcium phosphate on to a glass-wool pad and allowing the calcium phosphate to settle under gravity, excess of suspending solution then being run off. When sufficient calcium phosphate had been added the bed was packed down under slight positive pressure of air (about 3 lb./in.²). The column was then washed until equilibrated with phosphate buffer of desired pH.

Dowex-1 columns. Dowex-1 X4, 200-400 mesh, a quaternary-ammonium anion-exchange resin, was used in all experiments. Before packing a column the resin was converted into the hydroxyl form with 2N-NaOH and then into the chloride form with N-HCl and was washed on a Büchner funnel to remove excess of chloride. The suspended resin was deaerated (cf. Boman & Westland, 1956) in a Büchner flask and the column packed by pouring the suspension on to a glass-wool pad. After washing with water until the chloride test was negative the column was equilibrated with the desired buffer.

Elution procedure. All chromatographic experiments reported were conducted at room temperature. The enzyme was applied to the column as a solution in a small volume of the same buffer as was used to equilibrate the column. The enzyme was eluted from the calcium phosphate columns with a linearly increasing concentration of phosphate at a constant pH and from Dowex-1 columns by a linearly increasing concentration of chloride in 2-amino-2-hydroxy-methylpropane-1:3-diol-HCl (tris-HCl) buffer at constant pH. The linear gradients were obtained with the mixing device described by Parr (1954). The flow rate from the columns was controlled by a tungsten-needle valve. Fractions were collected automatically in equal volumes (Crook & Datta, 1951). The linearity of the gradients was checked

by phosphate (Allen, 1940) or chloride (Kolthoff & Sandell, 1952) analyses of the fractions. The protein content of the fractions was estimated by measuring E at 280 m μ in a Uvispek spectrophotometer. In some experiments carbohydrate concentrations of the fractions were measured by the orcinol method.

RESULTS

Elution analysis of Aspergillus niger preparation on Dowex-1 columns

A sample (15 mg.) of the crude enzyme dissolved in 5 mM-tris-HCl, pH 7.0, was applied to a column (67 cm. $\times$ 0.7 cm.) of Dowex-1 equilibrated with 5 mM-tris-HCl, pH 7.0. Elution was effected with 90 ml. of 5 mM-tris-HCl, pH 7.0, followed by a concentration gradient of chloride (mixing vessel: 250 ml. of 5 mM-tris-HCl, pH 7.0; reservoir: 250 ml. of 0.75M-NaCl in 5 mM-tris-HCl, pH 7.0). Fractions (7.5 ml.) were collected at a flow rate of 0.4 ml./min. and stored at 5°.

Fig. 1 (a-c) shows the elution pattern of hydrolytic activity towards cellobiose, cellopentaose,

Fig. 1. Chromatography of 15 mg. of crude enzyme (7 mg. of protein) on a Dowex-1 column (67 cm. $\times$ 0.7 cm.) by gradient elution with sodium chloride, 0.005-0.75M, in 5 mM-tris-HCl buffer, pH 7.0. Activity towards cellobiose (●), cellopentaose (□), salicin (▽), celloidextrin (■), SCMC (○), swollen cellulose (▼), lichenin (▲) and laminarin (△), protein concentration (not shown) and chloride concentration (continuous line) were determined on each fraction as described in the text. The enzyme activities are expressed as μ g. of reducing sugar (as glucose) liberated under standard conditions/ml. of deproteinized assay solution.

cellodextrin, SCMC, alkali-swollen cellulose, laminarin, lichenin and salicin in the same experiment. Protein was eluted in the initial 5 mM-tris-HCl fractions and was accompanied by a very low activity towards laminarin but no activity towards the other substrates. Protein was eluted throughout the chloride gradient but little correlation between protein content and the enzymic activities of the fractions was apparent. Approximately 50 % of the orcinol-reacting carbohydrate was eluted by the 5 mM-tris-HCl fractions; the remainder was eluted in the chloride gradient. There was no correlation between carbohydrate content of the fractions and their enzymic activities.

Inspection of the elution patterns of hydrolytic activity towards the polymeric series of β -1:4-glucosidic substrates suggests the presence of two different groups of components, a group eluted by chloride concentrations in the range 0.1–0.3 M and a group eluted in the range 0.3–0.6 M. The hydrolysis of cellobiose and cellopentaose is high in the first group and low in the second group, whereas the reverse is true of the hydrolysis of cellodextrin, SCMC and swollen cellulose; in fact there was no detectable hydrolysis of swollen cellulose by the 0.1–0.3 M-chloride components and the activity towards SCMC was very weak.

Fig. 1 (a) shows that salicin hydrolysis was confined to those fractions which hydrolyse cellobiose and further that not all the fractions which hydrolyse cellobiose were equally active in hydrolysing salicin. Only very weak hydrolysis of laminarin was shown by the fractions eluted in the 0.3–0.6 M-chloride region. Almost all the activity towards soluble laminarin was eluted by chloride below 0.22 M (Fig. 1c) and thus at least part of this activity was due to components different from those which hydrolyse cellobiose and cellopentaose. Fractions which hydrolyse lichenin appeared in both the 0.1–0.3 M- and the 0.3–0.6 M-chloride region although the activity was higher in the latter.

Elution analysis of Aspergillus niger enzymes on calcium phosphate columns

Preliminary experiments under the conditions reported earlier (Stone, 1957a) showed that as the pH was lowered the molarity of phosphate required to elute completely all the activity towards SCMC was raised. Thus 0.045 M-phosphate was required at pH 7.4, 0.055 M at 6.8, and 0.09 M at 5.8. There was a corresponding improvement in the resolution of the components as the pH was lowered. Resolution was further improved by using columns at least three times as long as those used in earlier experiments (Stone, 1957a).

A sample (15 mg.) of the crude enzyme dissolved in 5 mM-phosphate buffer, pH 5.8, was applied to a column 65 cm. $\times$ 0.7 cm. equilibrated with 5 mM-

phosphate buffer, pH 5.8. Elution was effected under slight positive pressure with 120 ml. of 5 mM-phosphate buffer, pH 5.8, followed by a phosphate-concentration gradient (mixing vessel: 450 ml. of 5 mM-phosphate, pH 5.8; reservoir: 450 ml. of 0.15 M-phosphate, pH 5.8). Fractions (7.5 ml.) were collected at a flow rate of 0.4 ml./min. and stored at 5°.

Figs. 2 (a, b) and 4a show the distribution among the fractions of activity toward cellobiose, cellopentaose, cellodextrin, SCMC, swollen cellulose and laminarin. Solutions of the crude enzyme preparation are dark brown. The pigment responsible was adsorbed at the top of the column and was not eluted by 0.15 M-phosphate buffer, so that with the exception of some of the first 5 mM-phosphate fractions the eluate was water-clear. In preliminary experiments carbohydrate (orcinol) determination showed that 60 % of the total carbohydrate was eluted in the 5 mM-phosphate fractions. The level of carbohydrate in the fractions eluted by the phosphate gradient was on the limits of sensitivity of the method. Protein (17 % of the total recovery) was eluted in the 5 mM-phosphate fraction and these fractions showed a low activity towards all

Fig. 2. Chromatography of 15 mg. of crude enzyme (7 mg. of protein) on a 67 cm. $\times$ 0.7 cm. calcium phosphate column by gradient elution with phosphate buffer, 5–150 mM, pH 5.8. Activity towards cellobiose (●), cellopentaose (□), cellodextrin (■), SCMC (○), swollen cellulose (▽), protein concentration (not shown) and phosphate concentration (continuous line) were determined on each fraction as described in the text.

substrates except cellobiose and cellodextrin. Protein was eluted throughout the phosphate gradient but little correlation between the protein content of the fractions and their enzymic activity was apparent. As in the chromatography on Dowex-1, the elution pattern of activity towards the polymeric series of β -1:4-glucosidic substrates suggests the presence of two different groups of components, a group eluted by phosphate concentrations in the range 0.04–0.09M and a second in the range 0.09–0.14M. The first group shows high activity towards the cellulosic substrates and some activity towards cellopentaose but does not hydrolyse cellobiose. The second group shows high activity towards cellobiose and cellopentaose, a low activity towards cellodextrin but no activity towards SCMC or swollen cellulose. Soluble laminarin (Fig. 4a) was hydrolysed by fractions eluted by 0.02–0.06M-phosphate and also by fractions eluted by 0.10–0.12M-phosphate.

It is significant that the two groups of components hydrolysing the β -1:4-glucosidic link appear to have retained their identities, as judged by their relative activities on the cellosaccharides and cellulosic substrates, under the different conditions prevailing on the two adsorbents. These differences in conditions are reflected in the reversal of the elution order of the two groups in going from one adsorbent to the other. The components hydrolysing laminarin are eluted in the low-molarity chloride fractions on Dowex-1 and partially in the low and partially in the high-molarity phosphate fractions from calcium phosphate. These differences in elution order on the two adsorbents were exploited to obtain a separation of the two groups of components hydrolysing β -1:4-glucosidic linkages from one another and partially from components hydrolysing β -1:3-glucosidic linkages.

Preparative chromatography on Dowex-1

In order to accumulate sufficient material for rechromatography several preparative-scale experiments were run with Dowex-1. In these experiments 1 g. of crude enzyme, dissolved in 5 mM-tris-HCl, pH 7.0, was applied to a column (73 cm. $\times$ 3.8 cm.) of Dowex-1 equilibrated with the same buffer. Elution was effected with 750 ml. of 5 mM-tris-HCl followed by a concentration gradient of chloride (mixing vessel: 1500 ml. of 5 mM-tris-HCl, pH 7.0; reservoir: 1500 ml. of 0.75-M-NaCl in tris-HCl, pH 7.0). Fractions (20 ml.) were collected and assayed for activity towards cellobiose, SCMC and laminarin. The elution patterns closely resembled those found for the analytical columns (Fig. 3a). The fractions were pooled to give two solutions, one containing the components eluted by 0.1–0.3M-chloride and the other the components eluted by 0.3–0.6M-chloride. These two solutions were

freeze-dried and reconstituted in a small volume of water and dialysed in cellulose nitrate sacs for 48 hr. against frequently changed distilled water. The small amount of material precipitated during dialysis was centrifuged off and the clear solutions were stored in the frozen condition.

Rechromatography of fractions separated on Dowex-1

With Dowex-1. A portion of the solution containing the 0.1–0.3M-chloride components was dialysed overnight against 5 mM-tris-HCl. A sample (0.3 ml.; 10.7 mg. of protein) of the equilibrated solution was chromatographed under the same conditions as previously described for the crude enzyme. The elution pattern of the activity against cellobiose, SCMC and soluble laminarin is shown in Fig. 3 (b). The result of a similar experiment with 0.6 ml. (7.6 mg. of protein) of the 0.3–0.6M-chloride fraction is shown in Fig. 3 (c).

Fig. 3. Fractions from preparative chromatography on Dowex-1 rechromatographed on Dowex-1 columns (67 cm. $\times$ 0.7 cm) by gradient elution with tris-HCl buffer, 0.005–0.75M, pH 7.0. Activity towards cellobiose ($\bullet$), laminarin (Δ), protein concentration (not shown) and SCMC ($\circ$), chloride concentration (continuous lines) were determined on each fraction. (a) Elution diagram of 15 mg. of crude enzyme (7 mg. of protein) included for comparison. (b) Elution diagram of 0.3 ml. of concentrated 0.1–0.3M-chloride fractions (10.7 mg. of protein). (c) Elution diagram of 0.6 ml. of concentrated 0.3–0.6M-chloride fractions (7.6 mg. of protein).

It is apparent that the fractionated components are eluted at approximately the same point in the chloride gradient as in the chromatography of the unfractionated enzyme. The 0.1–0.3 M-chloride fraction is free from all activity towards SCMC but by comparison with the elution pattern of the whole enzyme it appears to lack some of the components which hydrolyse cellobiose. The 0.3–0.6 M-chloride fraction contains the SCMC-hydrolysing activity together with a small amount of activity towards cellobiose and laminarin, some of which appears in the low chloride concentration region. This contamination is most probably due to 'tailing' of the cellobiose- and laminarin-hydrolysing components normally eluted in the 0.1–0.3 M-chloride fractions into the later fractions on the preparative columns.

Fig. 4. Fractions from preparative chromatography on Dowex-1 rechromatographed on calcium phosphate columns by gradient elution with phosphate buffer, 0.005–0.15 M, pH 5.8. Activity towards cellobiose (●), SCMC (○), laminarin (Δ), protein concentration (not shown) and phosphate concentration (continuous lines) were determined on each fraction. (a) Elution diagram of 15 mg. of crude enzyme (7 mg. of protein). (b) Elution diagram of 3.5 ml. of concentrated 0.1–0.3 M-chloride fractions (12.5 mg. of protein). (c) Elution diagram of 0.6 ml. of concentrated 0.3–0.6 M-chloride fractions (7.6 mg. of protein).

With calcium phosphate. A portion of the solution containing the 0.1–0.3 M-chloride components was dialysed overnight against 5 mM-phosphate buffer, pH 5.8, and 0.35 ml. (12.5 mg. of protein) of the equilibrated solution was applied to a column 65 cm. $\times$ 0.7 cm. and chromatographed under the same conditions as previously described for the crude enzyme. The results of this experiment and a similar one with 0.6 ml. (7.6 mg. of protein) of 0.3–0.6 M-chloride fraction is shown in Fig. 4 (b, c). On rechromatography on calcium phosphate the 0.1–0.3 M-chloride fraction showed only components hydrolysing laminarin and cellobiose, in agreement with the results obtained on Dowex-1 rechromatography. However, in this case there has been a complete resolution of the laminarin- and cellobiose-hydrolysing components. The rechromatographed 0.1–0.3 M-chloride fraction lacks the activity towards laminarin that was eluted in the 0.09–0.12 M-region in the chromatography of the crude enzyme. The 0.3–0.6 M-chloride fraction showed only SCMC-hydrolysing activity together with a small amount of activity towards laminarin and cellobiose. The second major SCMC-hydrolysing component present in the 0.07–0.09 M-phosphate fractions of the crude enzyme was absent in the rechromatographed 0.3–0.6 M-chloride fraction.

From these experiments it is clear that the appearance of activity towards cellobiose, laminarin and SCMC in different fractions on Dowex-1 chromatography is not caused by the chromatographic procedure itself, and further that the active fractions obtained by calcium phosphate chromatography can be identified with those obtained by chromatography on Dowex-1.

Hydrolysis of lichenin

Chromatography on Dowex-1 (Fig. 1c) shows clearly that those fractions eluted by 0.3–0.6 M-chloride with activity towards the cellulosic substrates also hydrolysed lichenin. However, it is not possible to associate the lichenin-hydrolysing activity in the 0.1–0.3 M-chloride fractions exclusively with either the laminarin- or cellodextrin-hydrolysing components since the activity towards lichenin extends to fractions active towards both of these substrates.

DISCUSSION

Comparison of components hydrolysing β -1:4-glucosidic linkages

The results of the elution analyses of the *Aspergillus niger* preparation indicate the presence of two different groups of components hydrolysing β -1:4-glucosidic linkages. The difference in specificity between these two groups at first sight would appear to depend on whether the β -1:4-glucosidic

linkage was available in a substrate of a low or a high degree of polymerization (D.P.). Two fractions with similar apparent specificities have been obtained by Grassman, Zechmeister, Tóth & Stadler (1933) by treating an *Aspergillus oryzae* (Luitzym) preparation with aluminium metahydroxide at pH 3.5. Two successive treatments gave a preparation, which was free of activity towards cellobiose, that slightly hydrolysed cellotetraose, but which actively hydrolysed cellohexaose, cellodextrin (av. D.P. 9) and sulphuric acid-treated cellulose ('hydrat-cellulose'). Treatment of the aluminium metahydroxide with 0.04M-sodium bicarbonate eluted material which actively hydrolysed cello-biose, -triose, -tetraose and -hexaose, whereas the cellodextrin and cellulose were only weakly attacked. Grassman *et al.* (1933) called the unadsorbed fraction a 'polysaccharase' and the eluted fraction an 'oligosaccharase'. The two groups of components now identified in the *A. niger* preparation would fit this classification, although the overlap in activity in the cellosaccharide range is different.

A nomenclature for these enzymes based on their action patterns (cf. Jermyn, 1957) would be more significant but the fractionation experiments themselves do not allow any direct conclusions in this respect. It is very likely, however, that the polysaccharase components of both *A. oryzae* and *A. niger* resemble the cellulose-hydrolysing enzymes of *Myrothecium verrucaria* (Whitaker, 1957) and of *Stachybotrys atra* (Thomas, 1956) in having an endoglucosidase action pattern. This is supported by the evidence of Husemann & Lotterle (1950), who have shown by physical methods that the *A. oryzae* (Luitzym) preparation attacks cellulose in a random fashion characteristic of an endoglucosidase, and the appearance of a series of oligosaccharide intermediates during the hydrolysis of cellodextrins by the *A. niger* preparation suggests that it also possesses an endo- β -1:4-glucosidase (Stone, 1954). The striking correlation in the present study between the elution positions of the components hydrolysing lichenin and those active against cellulosic substrates suggests that the same enzymes are responsible. Moreover, the ability of these β -1:4-glucosidases to hydrolyse lichenin also suggests their endoglucosidase nature. The most recent work on the structure of lichenin shows that it is a β -glucan with about 70% 1:4-linkages and 30% 1:3-linkages (Chanda, Hirst & Manners, 1957; Peat, Whelan & Roberts, 1957), and that these are arranged in the repeating sequence $-G\beta-4G\beta-4G\beta-3-$ (Peat *et al.* 1957). The molecule is believed to be entirely linear and to have an av. D.P. of 63 (Manners, 1959). If this lichenin structure is valid then an exo- β -glucosidase hydrolysing only 1:4-glucosidic linkages and detaching glucose units singly or in pairs from the chains might be blocked by the 1:3-links. An

endo- β -1:4-glucosidase, on the other hand, would be able to hydrolyse at least one of the two β -1:4-glucosidic linkages in the cellotriosyl residues of the chain.

The identity of this fraction of the 'lichenase' activity with the 'cellulase' activity of *A. niger* is in agreement with the findings of Otto (1929), who was unable to separate the two activities from barley malt with a series of adsorbents. However, other workers have concluded that 'lichenase' and 'cellulase' are different enzymes (Ullman, 1932; Freudenberg & Ploetz, 1939; Ploetz, 1939).

The substrate range of the cellosaccharide-hydrolysing components is comparable with that of the intracellular exo- β -glucosidase of *S. atra* described by Youatt (1958), which removed single glucose residues from cellosaccharides with D.P. values at least up to 11, and to the exo- β -glucosidase of almond emulsin (Peat, Thomas & Whelan, 1952) reported by Grassman *et al.* (1933) to have the same substrate range as the *A. oryzae* (Luitzym) 'oligosaccharase'.

The apparent chain-length specificities of exo- and endo-glucosidases can be regarded as a consequence of their action patterns. Exoglucosidase action, other things being equal, will depend on the concentration of terminal glucosidic linkages; thus for equal concentrations of substrate the low D.P. cellosaccharides would provide the highest concentration of hydrolysable β -glucosidic linkages.

It is not known if there is an upper D.P. limit for the exoglucosidases hydrolysing cellosaccharides, i.e. if their action is independent of chain length as with β -amylase (Manners, 1954) and gluca-amylase (Phillips & Caldwell, 1951), or if as the substrate D.P. increases the affinity for the enzyme decreases and becomes effectively zero at a certain D.P. [cf. *Saccharomyces cerevisiae* maltase (Whelan, 1953; Phillips, 1959)]. The action of endoglucosidases is generally independent of substrate length above a certain D.P., the value of which varies with the type and source of the enzyme, e.g. above 4 for the cellulose-hydrolysing enzymes of *Myrothecium verrucaria* (Whitaker, 1954), above 3 for salivary α -amylase (Manners, 1954), above 7 for *Lactobacillus bifidus* dextranase (Bailey & Clarke, 1959). The relatively low activity of the *A. niger* 'polysaccharase' components on the lower D.P. cellosaccharides is thus consistent with an endoglucosidase action pattern.

Linkage specificity of the β -glucosidase components

Ploetz & Pogacar (1949), using a crude and a fractionated *A. oryzae* (Luitzym) preparation, studied the kinetics of the hydrolysis of three β -glucans, cellulose, yeast glucan and luteose, in which the main inter-glucose linkages are of the β -1:4-, β -1:3- and β -1:6-glucosidic type respectively.

They chose to interpret their results in terms of the presence of a non-specific oligosaccharase and polysaccharase rather than in terms of specific β -1:4-, β -1:3- and β -1:6-glucosidases. More recent experiments have suggested that specific β -1:3- and β -1:4-glucosidases do exist. Duncan, Manners & Ross (1956) showed that the ability of the extract of the alga *Cladophora rupestris* to hydrolyse laminarin, a predominantly β -1:3-linked glucan, was lost on heating it at 60° for 15 min. whereas the cellobextrin-hydrolysing activity was retained. Aitken, Eddy, Ingram & Weurman (1956) made similar observations on the hydrolysis of laminarin and cellulosic substrates by *M. verrucaria* filtrates before and after heating at 60° for 10 min. Their observations are, however, at variance with those of Kooiman (1957), who observed considerable laminarin hydrolysis by *M. verrucaria* filtrates after heating for 3 min. at 100°. Finally, Reese & Mandels (1959) have observed laminarin hydrolysis by culture filtrates of a number of fungi in the absence of detectable hydrolysis of cellulosic substrates. Furthermore there is evidence based on the paper-chromatographic identification of the end products of prolonged enzymic action that hydrolytic activity towards laminarin may be either the result of endo- β -1:3-glucosidase activity (algal enzymes: Manners, 1955; fungal enzymes: Reese & Mandels, 1959) or by exo- β -glucosidase action (almond emulsin: Peat *et al.* 1952; culture filtrates of basidiomycete QM 806 and *Sporotrichum pruinosum*: Reese & Mandels, 1959). MacWilliam & Harris (1957) fractionated barley-malt enzymes on alumina and concluded from the identity of the products of hydrolysis of laminarin by the fractions that both endo- and exo-glucosidases hydrolysing β -1:3-glucosidic links were present. The exo-glucosidase activity was eluted in the same fractions as some of the cellobiose-hydrolysing activity but none of these fractions hydrolysed salicin.

The elution patterns of the *A. niger* preparation chromatographed on Dowex-1 and calcium phosphate show directly that the fractions with activity towards the β -1:3-glucosidic linkages in laminarin are distinct from those with activity towards cellulosic substrates. As reported earlier (Stone, 1957*a*), the activity towards β -1:3-glucosidic linkages can be resolved into two distinct groups of components by chromatography on calcium phosphate. At present no statement can be made about the action patterns of these two groups of components, but it is relevant that the more strongly adsorbed components appear in fractions which also hydrolyse cellobiose. Coincidence of laminarin- and cellobiose-hydrolysing activity is also apparent in the Dowex-1 elution experiments. Whether this coincidence in elution position is due to the presence in these fractions of linkage-specific β -glucosidases

with similar physical properties or to the presence of exo- β -glucosidases whose specificities are not directed towards the position of the glucosidic linkage in the substrate remains to be determined. It will be noted, however, that all the cellobiose-hydrolysing activity is not associated with activity towards laminarin.

Salicin- and cellobiose-hydrolysing activity appear in the same fractions in Dowex-1 experiments but the relative activities of the fractions on these two substrates is variable. Again this may reflect the presence of several β -glucosidases with different affinities for salicin and cellobiose or separate specific enzymes with similar elution characteristics. Zechmeister, Tóth & Fürth (1940) have separated the cellobiose- and salicin-hydrolysing components from almond emulsin. A distinction between aryl β -glucoside- and cellobiose-hydrolysing enzymes has been made by other workers (Jermyn, 1957; Hash & King, 1958).

Nature of the components within each group

The elution patterns for both Dowex-1 and calcium phosphate show that for each of the β -glucosidases the activity is distributed among a number of components which may or may not be completely resolved under the experimental conditions. Among the components apparent in the two groups of fractions hydrolysing β -1:4-glucosidic linkages there generally appears to be a consistent ratio of activities towards the polymer homologous series of substrates. This is not, however, true of the component eluted at 0.06–0.07M-phosphate (Fig. 2*b*), which has apparently a much higher activity towards swollen cellulose than towards the lower D.P. cellulosic substrates (Halliwell, 1957).

Different forms of proteins with similar biological activity have been encountered by other workers during chromatography, e.g.: insulin (Dickinson, 1956); ribonuclease (Tanford & Hauenstein, 1956); phosphodiesterase (Boman, 1955); lysozyme (Tallan & Stein, 1953); enolase (Malmström, 1957); horse-radish acid phosphatase (Boman & Westland, 1956); pea phosphatase and metaphosphatase (Pierpoint, 1957). Several explanations for these components are possible. They may represent polymeric forms of a single protein. Tiselius, Hjertén & Levin (1956) have shown that R- and C-phycoerythrin yield two components in chromatography on calcium phosphate at pH 6.3. These phycocyanins are known to dissociate into half molecules in the range pH 6.2–6.8. Changes in the protein molecules due to hydrolysis of peptide or amide bonds have been shown to occur without loss of biological activity [e.g. insulin (Harfenist, 1953); ribonuclease (Tanford & Hauenstein, 1956); lysozyme (Tallan & Stein, 1953), papain (Finkle & Smith, 1958); phosphodiesterase (Björk & Boman,

1959; Razzell & Khorana, 1959)]. These alterations in the protein molecule are sufficient to allow separation of the different molecular species on the highly selective adsorbents now available. Auto-digestion of the enzymes in solution is responsible for the appearance of new active components with papain and phosphodiesterase, and the hydrolytic action of resins themselves cannot be neglected as a possible contributing factor where strong adsorption occurs (Boardman, 1959).

Finally, it is possible that part of a protein component is associated with other material in the crude mixture and that this may be responsible for the appearance of several components. Thus Hjertén (1959) has attempted to explain the chromatographic heterogeneity of serum albumin on the basis of its capacity to bind small molecules, and Åquist & Anfinsen (1959) have suggested that the binding of polyanions may be responsible for the chromatographic differences among the various sheep ribonucleases.

It is possible that one or more of the factors listed here may explain the appearance of the chromatographically different forms of the β -glucosidases of *A. niger* and may also provide an explanation for the change in the numbers of components on re-chromatography of the separated fractions.

SUMMARY

1. The β -glucosidases in an enzyme preparation from *Aspergillus niger* have been fractionated by gradient-elution chromatography on calcium phosphate and the anion-exchange resin Dowex-1. The activity of the fractions was tested against cello-saccharides (cellobiose, cellopentaose), cellulosic substrates (celloextrin, sodium carboxymethyl-cellulose, phosphoric acid-swollen cellulose), laminarin, lichenin and salicin.

2. The activity towards the β -1:4-glucosidic substrates was obtained in two well-separated groups of components on both calcium phosphate and Dowex-1. The differences between these two groups of components are discussed.

3. The presence of β -glucosidase components hydrolysing laminarin but not cellulosic substrates was indicated by the results of Dowex-1 and calcium phosphate chromatography. From each adsorbent some fractions hydrolysing laminarin also hydrolysed cellosaccharides. On calcium phosphate those fractions with both activities were completely resolved from the remainder of the fractions hydrolysing laminarin.

4. Lichenin was hydrolysed chiefly by those fractions from Dowex-1 which hydrolysed cellulosic substrates.

5. Salicin was hydrolysed only by those fractions from Dowex-1 which hydrolysed cellosac-

charides, but not all such fractions hydrolysed salicin.

6. For each of the β -glucosidases the activity is distributed among a number of components. The possible nature of these components is discussed.

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The Determination of Haptoglobins in Normal Human Serum

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Haptoglobins are proteins which combine with haemoglobin or certain derivatives of haemoglobin. Under certain conditions, combination of haptoglobin with haemoglobin or methaemoglobin enhances the peroxidase activities of the latter substances (Polonovski & Jayle, 1940; Guinaud, Tonnelat, Boussier & Jayle, 1956). This finding has been made the basis of methods for the determination of haptoglobins. Jayle (1951) estimated haptoglobins in serum by measuring the effect of the haemoglobin-haptoglobin complex on the reaction between ethyl hydroperoxide and iodide ions; liberated iodine was determined by titration with thiosulphate. More recently Connell & Smithies (1959) have utilized the reaction between guaiacol and hydrogen peroxide to determine the peroxidase activity of methaemoglobin-haptoglobin complex, measuring the rate of oxidation of guaiacol colorimetrically.

This paper presents observations on the use of the guaiacol-hydrogen peroxide reaction in the determination of haptoglobins and on the concentration of haptoglobins in normal human serum. A preliminary account of certain aspects of the work has already appeared (Owen, Better & Hoban, 1960).

MATERIALS

Guaiacol reagent. Standard reagent (Connell & Smithies, 1959) was prepared as described by Owen, Better & Hoban (1960). In experiments to test the effect of pH, similar reagents were prepared in which the guaiacol concentration was kept constant (3.72 g./l.) and the pH varied with appropriate additions of 0.1 M-sodium acetate (pH 3.2-5.0) or sodium phosphate (pH 6.0-7.0) buffers.

Hydrogen peroxide. This was 0.05 M as described by Owen *et al.* (1960).

Methaemoglobin solution. To 10 vol. of human erythrocytes, previously washed three times with 0.15 M-NaCl, were added 10 vol. of water and 1 vol. of peroxide-free ether. The mixture was shaken for 1 min. and centrifuged,

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