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Characterization of Cellulases and Related Enzymes by Isoelectric Focusing, Gel Filtration and Zone Electrophoresis

I. Studies on *Aspergillus* Enzymes

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Physico-chemical characterization has been carried out on the enzymes cellulase, mannanase, xylanase, β-glucosidase, aryl-β-glucosidase, mannosidase, and xylosidase from "Cellulase 36", a commercial preparation of *Aspergillus* enzymes obtained from Rohm & Haas Co.

By the isoelectric focusing method the isoelectric points of the enzymes were found to be in the range 3.9–4.7.

By gel filtration the relationships between the molecular sizes of the enzymes were estimated. It was found that the extracellular endoenzymes cellulase, mannanase, and xylanase, *i.e.* those capable of hydrolyzing insoluble high molecular polysaccharides, are smaller molecules than their corresponding exoenzymes which bring about hydrolysis of soluble oligosaccharides.

The heterogeneities of the enzymes observed by zone electrophoresis were less pronounced than those observed by the isoelectric focusing method.

The reasons for the apparent heterogeneities of the enzymes as indicated by the three separation methods that were studied are discussed.

In this paper the physico-chemical properties of cellulases and related enzymes influencing their separability have been investigated. The studies have involved comparisons of molecular sizes by gel filtration, comparisons of isoelectric points by isoelectric focusing, and comparisons of mobilities in electrophoretic columns.

Separations on Sephadex columns [1–3] have shown that cellulases capable of hydrolyzing high molecular weight substrates are small molecules compared to the β-glucosidases that hydrolyze low molecular weight substrates. By

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further gel filtrations the behaviour of the cellulase—β-glucosidase system was compared to the systems xylanase—xylosidase and mannanase—mannosidase.

Very little information is available about the physico-chemical character of the enzyme group studied here. However, the isoelectric point of the cellulase from *Myrothecium verrucaria* has been reported (pI $\approx$ 4.7).[4]

In order to determine the isoelectric points of the enzymes, and to investigate their homogeneity and the possibility of separating them, the method of isoelectric focusing by electrolysis in stable pH gradients [5-7] was tried. The principle of this method is to focus proteins at their respective pI's (isoelectric points) in a stable pH gradient created by the electrolysis of suitable ampholytes, *viz.* carrier ampholytes.[8]

With this method it has been possible to separate proteins differing in pI by only 0.06 pH units. The resolving power of the method was mathematically calculated.[7] In addition to the separation thus obtained, the method permits a direct determination of the pI of proteins by measuring the pH at the point of focusing in the pH gradient. These pI values are easily obtained with a high degree of reproducibility, and are thus valuable for characterization and identification of proteins.

Zone electrophoresis has also been carried out and the results were compared with those obtained by the other separation methods.

MATERIALS AND METHODS

Enzyme preparation. The enzymes under investigation were obtained from a commercial preparation supplied by Rohm & Haas Co., Philadelphia, and designated "Cellulase 36". The fungus producing the enzymes belongs to the *Aspergillus niger-oryzae* group. (More exact information concerning the type of organism was not available).

The crude preparation contains, amongst others, the following enzymes which were studied: cellulase, mannanase, xylanase, β-glucosidase, aryl-β-glucosidase, mannosidase, and xylosidase.

The purification of "Cellulase 36" included the following steps:

1. Solution to 10 % in pyridine acetate buffer (pH 5.0; 0.05 M) and centrifugation.
2. Dialysis in acetylated cellophane membranes [9] against the same buffer.
3. Precipitation with $(NH_4)_2SO_4$ to 80 % saturation.
4. Solution of the precipitate and dialysis against pyridine acetate buffer.
5. Freeze-drying.
6. Gel filtration through a Sephadex G-75 column (60 $\times$ 1300 mm).
7. Concentration by the aid of Sephadex G-25.

The solution obtained was used for further studies. The dry weight of solids was 25 mg/ml, the protein content approximately 18 mg/ml and sugar, calculated as glucose, 5 mg/ml.

Gel filtration. The separation was carried out on two connected recycling columns * with the dimensions 30 $\times$ 900 mm and 70 $\times$ 1050 mm. The void volumes of the columns were 160 ml and 1010 ml, respectively. The gel filtering medium was Sephadex G-75. The buffer system used was ammonium acetate (pH 5.0; 0.1 M). The enzyme sample (5 ml containing 180 mg protein) was put on the smaller column. The top of the smaller column was connected with the bottom of the bigger one through a plastic tube of diameter 1 mm. The run was performed upwards with a flow speed of 15 ml/h when the material was being filtered through the small column but was increased to 50 ml/h when all the enzymes had passed over to the bigger column.

* Available from LKB-Produkter AB, Box 76, Stockholm-Bromma, Sweden.

Isoelectric separation. The separation was performed essentially as described by Vesterberg and Svensson.[7] An electrolysis column * of 110 ml capacity was used. It was cooled by water at +4° from a thermostated bath. The density gradient was made up of water solutions of ethanediol and the denser solution contained 75 % (v/v) of the latter component. The common compounds normally used for preparing density gradients, *viz.* sucrose and glycerol, could not be employed in this case since the enzyme preparation contained sucrase activity which disturbed the enzyme analyses. Glycerol was found to act as a strong inhibitor of some of the enzymes being studied. As a result of the search for another compound ethanediol was selected since it proved to be the best one available in spite of its inhibiting effect on some of the enzymes. The carrier ampholytes,* synthesized and isolated according to Vesterberg,[8] were chosen in order to give a pH gradient with its shallow part lying between 3.5 and 5.0. The ampholytes in the column amounted to 1 g. The anode compartment contained ethanediol (75 % [v/v]) to which 0.05 ml of sulfuric acid was added. The enzyme solution, containing 75 mg of protein, was dialyzed against distilled water to free it from electrolytes.

The run was performed with a maximum load of 1 W which implies a final voltage of approximately 600 V. After focusing for 48–60 h fractions of 2.3 ml were removed from the column.

The pH values of the fractions were measured by the aid of a Radiometer pH Meter, model 25 SE. A Radiometer combination electrode of the type GK 2026 C was used. It was standardized against KH-phthalate buffer of pH 4.01 at +4°. The pH-measurements of the fractions were carried out at +4° with an accuracy of ± 0.02 pH unit.

The inhibition of the enzymes due to the high concentration of ethanediol used in the electrolysis column was studied as follows:

Solutions of 10, 20, 30, 40, 50, and 60 % (v/v) of ethanediol in ammonium acetate buffer (pH 5.0; 0.1 M) were made. To 2 ml of these solutions 0.2 ml of enzyme solution were added. A buffer solution without ethanediol was used as reference. The enzymic activities were measured after one and three days.

In order to find out whether the observed inhibition was reversible or not the 50 % solution of ethanediol plus enzyme, and the reference solution were dialyzed overnight in collodium sacks against distilled water. The volumes of the dialyzed solutions were reduced to the initial volumes by evaporation in collodium sacks. The enzymic activities were then rechecked.

Zone electrophoresis was carried out in a column with the dimensions 25 × 800 mm, the carrier medium was Sephadex G-25 Superfine and the void volume was determined to be 135 ml. The buffer solution was pyridine acetate (pH 5.4; 0.1 M). The zone volume was 3 ml containing 180 mg of protein. The electrophoresis was run for 35 h at 600 V, giving a current of approximately 50 mA. Every sixth hour the buffer solutions of the electrode compartments were automatically mixed. The column was eluted with a speed of 4 ml/h and fractions of 2 ml were collected.

Enzyme assays. Cellulase and mannanase were determined viscometrically. The substrate for the cellulase determination was CMC (sodium carboxymethyl cellulose) 7 HP obtained from Hercules Powder Co. The substrate for the mannanase determination was guaran prepared from guar beans by a modification of the Heyne and Whistler method.[10] The substrates were incubated with the enzyme samples in the proportions 100:1 for 15 min. The temperature was 25° and the pH 5.0. The variation in the viscometric assay method was determined for 10 equal samples. The arithmetic mean was 1.04 and the standard deviation 0.03.

The enzymes β-glucosidase, mannosidase, xylosidase, and xylanase were determined by measuring the increase in reducing power produced by action on the substrate. The substrates were, respectively: cellobiose, mannobiose, xylobiose, and xylodextrins of DP 5–7. The method used for end group determination was that employing dinitrosalicylic acid as worked out by Sumner.[11] The enzymes were incubated with their substrates in the proportions 1:10 for 20 min at 37° and pH 5.0. The variation in this method was also measured for 10 equal samples. The mean value was 0.65 and the standard deviation 0.03.

Since xylosidase also attacks xylodextrins of DP 5–7 and thus gives rise to an apparent xylanase activity it was necessary to determine if xylanase activity was also

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present. The investigation was carried out as follows: xyloheptaitol, obtained by borohydride reduction of xyloheptaose, was incubated with the enzyme sample from the xylosidase peak as described above. The time of hydrolysis had to be chosen so that complete hydrolysis was not obtained. Since xylosidase does not attack xyloheptaitol from the reduced end it was possible to find out by paper chromatography if xylanase was also present.[12]

The substrate for the determination of aryl-β-glucosidase was p-nitrophenyl-β-D-glucoside.[13] A substrate volume of 2 ml was incubated with 0.1 ml of enzyme for 20 min at 37° and pH 5.0. Released p-nitrophenol was measured spectrophotometrically. The variation was determined for 10 equal samples. The mean value was 0.778 and the standard deviation 0.008.

RESULTS

Investigation of differences in relative molecular sizes. Differences in molecular sizes between the investigated enzymes were determined by gel filtration on two connected Sephadex G-75 columns of different diameter (*cf.* experimental section). The diagram of the fractionation is shown in Fig. 1.

It is possible to distinguish two groups of molecular sizes. The first group contains enzymes of such large molecular sizes that they are not retarded by the gel. The enzymes in this group are β-glucosidase, aryl-β-glucosidase, mannosidase, and xylosidase. The enzymes mannanase, cellulase, and xylanase are of smaller molecular sizes and are considerably retarded by the gel. It can be seen in the figure that two peaks containing cellulase activity have been obtained.

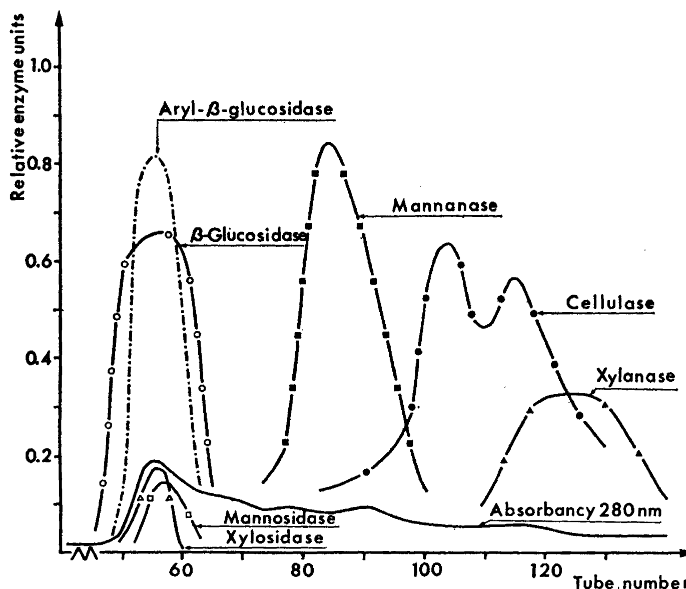

Fig. 1. Distribution of protein and enzymic activities after gel filtration of a partly purified commercial enzyme powder (Cellulase 36, Rohm & Haas Co., Philadelphia) on two connected Sephadex G-75 columns. The void volume of the system was 1170 ml. The fraction volume was 27 ml.

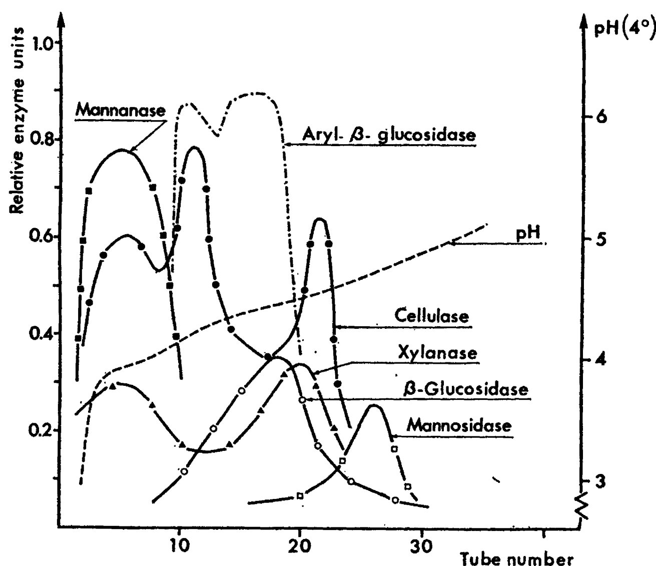

Fig. 2. pH diagram with distribution of enzymic activities from isoelectric separation. The fraction volume was 2.3 ml.

These results establish that β-glucosidase is of greater size than the cellulases and that xylosidase and mannosidase are of greater sizes than xylanase and mannanase, respectively. For the enzyme systems of this fungus that have been studied it is thus settled that enzymes hydrolyzing high molecular weight substrates are smaller molecules than those hydrolyzing low molecular weight substrates.

Isoelectric focusing. In Fig. 2 a diagram of one representative isoelectric separation is shown. The pI values of the enzymes can be read off from this figure. At least three of the enzymes seem to be heterogeneous. Thus three peaks with cellulase activity, two peaks with xylanase activity, and two peaks with aryl-β-glucosidase activity were obtained. In Table 1 the pI values of the main peaks obtained as average values from three experiments are given.

The enzymes were found to be stable at their isoelectric points since no decrease in activity could be observed when the enzymes were kept in buffer solution at the respective pI values for six days at 4°.

The enzymes cellulase, mannanase, and aryl-β-glucosidase showed no significant decrease in activity as determined in the presence of up to 60 % of ethanediol (*cf.* experimental section). Xylanase, β-glucosidase, and mannosidase lost approximately half of their initial activity at 60 % of ethanediol while xylosidase lost most of its activity. However, the xylosidase activity was initially already low. There was no significant difference in the activity determined after one and three days. The inhibition caused by ethanediol was found to be reversible.

Table 1. Isoelectric points of the enzymes at 4°. Average values of three runs. Approximate accuracy ± 0.05 pH unit.

Enzymes	pI's (isoelectric points)
Cellulases	3.90; 4.22; 4.52
Mannanase	3.95
Xylanases	3.90; 4.50
β-Glucosidase	4.46
Aryl-β-glucosidases	4.21; 4.43
Mannosidase	4.68

Zone electrophoresis. The diagram in Fig. 3 shows the result of a separation by zone electrophoresis. It can be seen in the figure that only the cellulase gave two activity peaks, whereas all the other enzymes showed only one peak. The broad peak obtained for the aryl-β-glucosidase activity shows, however, that the protein is heterogeneous.

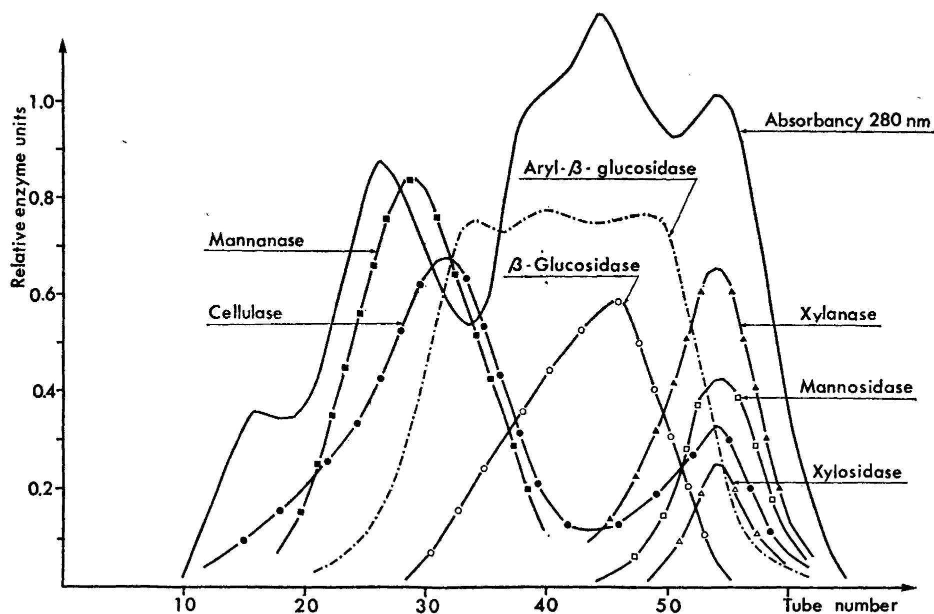

Fig. 3. Distribution of protein and enzymic activities after zone electrophoresis in pyridine acetate buffer at pH 5.4; 0.1 M. Carrier medium Sephadex G-25 Superfine. Void volume 135 ml. Fraction volume 2 ml.

In the figure three groups can be distinguished containing enzymes of similar mobility in the electrophoretic column. The group with the highest mobility contains mannanase and one of the cellulases. The next group contains aryl-β-glucosidase and β-glucosidase. Xylanase, mannosidase, xylosidase, and the other cellulase peak are located in the slowest moving group.

DISCUSSION

The fact that the typically extracellular endoenzymes cellulase, mannanase, and xylanase isolated from this fungus are found to be smaller molecules than the corresponding exoenzymes β-glucosidase, mannosidase, and xylosidase seems logical. For the former group insoluble polysaccharides are the natural substrates. An efficient penetration of these polymers in the plant fibres requires a small enzyme molecule. The second group acts preferably on the soluble oligosaccharides released by the endoenzymes.

Fractionation on a molecular size basis is thus suggested for the separation of an endoenzyme from its corresponding exoenzyme.

From Fig. 1 it can also be seen that the cellulase and mannanase activities are almost completely separated and that the cellulase and xylanase activities are partially separated. There are several reports in the literature [14] concerning the difficulties encountered in separating these enzymes. The good separation obtained by gel filtration must be partly due to the technique used, *i.e.* connecting a column with a small diameter to one with a larger diameter (*cf.* experimental section). The increase in diameter causes a sharpening of the zones thus improving the separation. This effect cannot be achieved by using a longer column of the smaller diameter or by recycling the material on the same column. In the latter case the fastest moving enzymes would merely overtake the slowest moving ones.

It is not possible to judge merely from the fact that two cellulase peaks are obtained on gel filtration if these constitute two different enzymes. Several peaks have often been obtained on gel filtration of cellulases and related enzymes. Further investigation has, however, shown that only one enzyme is present.[2,3] The reasons for the heterogeneous appearance of the enzymes on gel filtration can be manifold. Jermyn [15] found that an aryl-β-glucosidase combined with polysaccharides so that several peaks of enzymic activity were obtained on fractionation on DEAE-cellulose ion exchanger. The existence of a polysaccharide — protein complex may also be the reason for the two peaks obtained in our case. However, the possibility of the existence of isoenzymes must be taken into consideration. Reviews of multiple forms of enzymes catalyzing the same function are given by Kaplan [16] and by Wilkinson.[17]

The method of isoelectric separation has been shown to possess a high focusing character and resolving power.[5,7] The shallowness of the pH gradient is one of the factors determining the degree of focusing. The results obtained here do not give enough information for a direct determination of the resolving power. However, in our separations it can be estimated that most of the enzyme content of one peak, if representing one homogeneous enzyme, should fall within three fractions each of 2.3 ml. In Fig. 2 this can be seen to be the case for some of the peaks but many of

them are broader. The reason for the appearance of the broad peaks may be due to a microheterogeneity in the protein molecule. By using a narrower pH interval it is possible to create an even shallower pH gradient which will permit the resolution of proteins whose pI differ by only some hundredths of a pH unit.[18] By applying such a pH gradient a resolution of the broad peaks would possibly take place.

As can be seen in the zone electrophoresis diagram (Fig. 3) rather broad peaks of enzymic activity are obtained for some of the enzymes indicating their heterogeneous nature. Only in the case of cellulase are two distinctly different peaks resolved. In the isoelectric focusing method the various fractions of the proteins are better resolved and more than one peak is found for several of the enzymes.

With the isoelectric method separation is obtained due solely to differences in the electrical charge of the molecules. This is not the case in an electrophoretic column where the mobility, in addition to the charge, most likely also depends upon the size and shape of the protein molecule, the nature of the carrier medium, and the buffer solution.

In a forthcoming paper the studies will be extended to include the same type of enzymes from three other fungal sources.

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