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

II. Studies on *Stereum sanguinolentum*, *Fomes annosus* and *Chrysosporium lignorum* Enzymes

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Some properties of the enzymes cellulase, mannanase, xylanase, β-glucosidase, aryl-β-glucosidase, mannosidase, and xylosidase obtained from culture filtrates of the fungi *Stereum sanguinolentum*, *Fomes annosus*, and *Chrysosporium lignorum* have been investigated by gel filtration, isoelectric focusing and zone electrophoresis.

With few exceptions, the isoelectric points of the enzymes were found to be in the pH range 3.6–4.7.

The extracellular endoenzymes were found to be of smaller molecular sizes than their corresponding exoenzymes, as previously reported.

The relation between the electrophoretic mobilities of two proteins could not be predicted even if the isoelectric points and the relation between the molecular sizes were known.

In the previous paper of this series [1] some properties influencing the separability of cellulases and related enzymes were investigated. The enzymes were obtained from a commercial *Aspergillus* enzyme preparation. The studies involved comparisons of molecular sizes by gel filtration, of isoelectric points by isoelectric focusing, and of mobilities in electrophoretic columns.

From the data obtained from gel filtration it was stated for this enzyme preparation that the extracellular endoenzymes cellulase, mannanase, and xylanase hydrolyzing insoluble high molecular polysaccharides are smaller molecules than the corresponding exoenzymes hydrolyzing soluble oligosaccharides. By the method of isoelectric focusing, it was possible to determine the isoelectric points of the different enzymes. They were all in the interval 3.9–4.7 pH units. The heterogeneities of the enzymes observed by this method were more pronounced than those observed by zone electrophoresis.

In this paper a similar study has been carried out on enzymes from three other fungal sources in order to investigate the general validity of the results presented in the first paper.

Two of the fungi, *Stereum sanguinolentum** and *Fomes annosus*,** belong to *Basidiomycetes* and the third one, *Chrysosporium lignorum*,** belongs to *Fungi imperfecti*, while the earlier studied *Aspergillus* fungus is an *Ascomycetes* species. They are all potent wood destroyers. The fungi used in the present study were cultivated in this laboratory.

The enzyme distribution patterns from all the gel filtrations were found to be very similar. The isoelectric focusing and zone electrophoresis distribution patterns, on the other hand, showed great dissimilarities.

MATERIALS AND METHODS

Enzyme preparations. The enzymes were obtained from culture filtrates of the fungi. The medium used was a slight modification of that used by Norkrans.[2] Spruce wood powder was used as the carbon source and 1.0 g of yeast extract was added per litre of the medium.

Nine litres of the medium were mixed in each culture container and sterilized in steam at 125° for 35 min. After cooling, each container was inoculated with approximately 5 g (dry weight) of washed mycelium suspended in distilled water. The mycelium was obtained from a culture grown on 1.5 % malt extract. After inoculation of the medium in the containers, the cultures were maintained at a temperature of 25° and aerated at a rate of 1.5 litres of air per litre of medium per minute. After incubation for 5 to 6 days, the cellulolytic activity had attained its maximum and the medium was made cellfree by centrifuging. The resulting culture filtrate was concentrated by partial freezing and batchwise by the aid of Sephadex G-25.

After freeze-drying, it was filtered through a Sephadex G-75 column (60 × 1300 mm). The enzyme part of the effluent was pooled and freeze-dried again. The weight of the obtained material was about 1 g, containing approximately 40 % of sugar calculated as glucose.

Gel filtration was carried out on two connected G-75 columns of different diameters as described in Ref. 1. All the enzyme material was filtered through these columns in order to obtain a purer material for the other separations.

Isoelectric separation was carried out according to a method essentially described by Vesterberg and Svensson.[3] The modifications are given in Ref. 1.

Zone electrophoresis was performed on a Sephadex G-25 Superfine column, Ref. 1. The electrophoretic studies of the enzymes from *Fomes annosus* were carried out in phosphate buffer, pH 7.05, ionic strength 0.1, in order to obtain mobility in the same direction for all its enzymes. In all other cases, pyridine acetate buffer, pH 5.4, 0.1 M, was used.

Enzyme assays were carried out as described in Ref. 1.

RESULTS

Investigation of differences in relative molecular sizes. The distribution of molecular sizes of the enzymes is shown in the gel filtration diagrams (Fig. 1). In agreement with the result reported in the earlier paper of this series,[1] the first eluted group of enzymes is excluded by the gel and contains the exo-enzymes β-glucosidases, mannosidase, and xylosidase. The retarded group

* Identified by Dr. M. K. Nobles at the Canadian Department of Agriculture, Canada, as being of a *Stereum sensu stricto* type, most probably *Stereum sanguinolentum*.

** Obtained from the Department of Forest Products, Royal College of Forestry, Sweden.

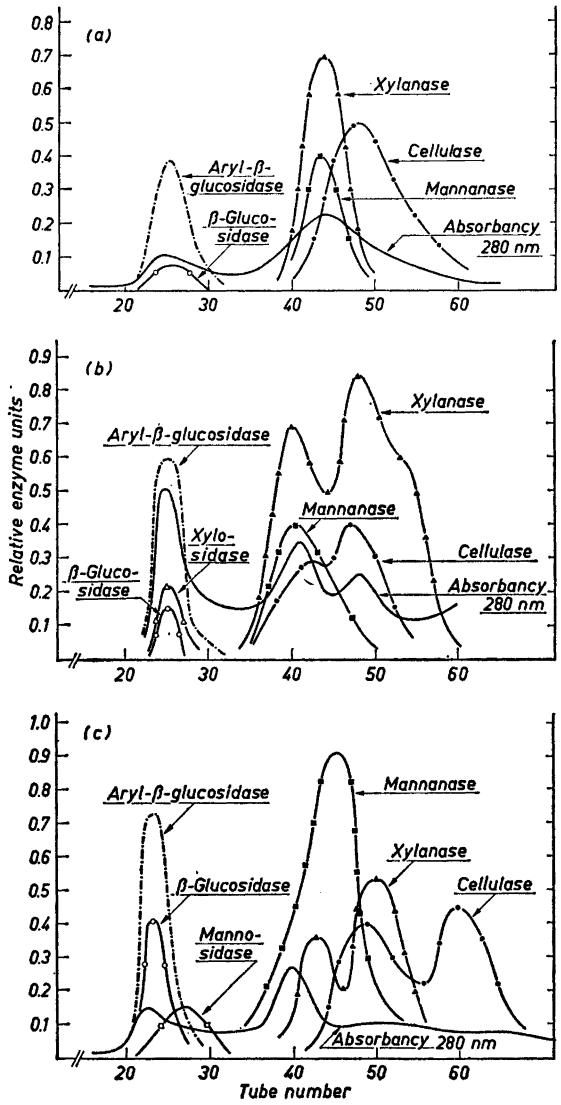

Fig. 1. Distribution of protein and enzymic activities after gel filtration of culture filtrates from the fungi *Stereum sanguinolentum* (a), *Fomes annosus* (b), and *Chrysosporium lignorum* (c) on two connected Sephadex G-75 columns. The void volume of the system was 1170 ml. The fraction volume was 50 ml.

contains their corresponding endoenzymes. No recurrent sequence can be observed for the endoenzymes.

For all the studied fungi, mannanase appears as a single peak and has a relatively large molecular size. The xylanase enzyme appears as a single peak for the *Stereum* and *Aspergillus* fungi (Ref. 1, Fig. 1), but as two peaks for

the two other fungi. The cellulase appears as a single peak for the *Stereum* fungus and as two peaks for the three other fungi.

Since the exoenzymes are excluded by the gel, neither differences in molecular sizes between the enzymes nor their heterogeneities can be observed.

No attempt was made to fractionate the exoenzymes on a gel retarding molecules of their sizes.

Isoelectric focusing. The enzyme distributions obtained by isoelectric focusing are shown in Fig. 2. The differences in the distribution patterns are striking. The sequence of the enzymes is not the same for the different fungi and the number of peaks for one enzyme varies from fungus to fungus.

The isoelectric points, which can be read off from the diagrams in Fig. 2, are given in Table 1 together with the pI values of the *Aspergillus* enzymes. With few exceptions, the isoelectric points fall into the narrow pH interval 3.6—4.7. The exceptions are two cellulases of *Chrysosporium lignorum* (pI 5.0 and 5.5), one of its xylanases (pI 6.0) and the xylanase of *Fomes annosus* (pI $\approx$ 7).

Zone electrophoresis. Fig. 3 shows the enzyme distributions obtained by zone electrophoresis. It can be seen that the sequence of the migration rates of the enzymes varies from fungus to fungus even by this separation. The sequence differs from that obtained by isoelectric focusing.

DISCUSSION

The striking similarities of the patterns obtained by gel filtration of cellulases and related enzymes from the studied fungi from different classes indicate that it is generally valid that the exoenzymes are of greater molecular size than the endoenzymes; *cf.* Ref. 1 for discussion.

In some of the diagrams, mannosidase and xylosidase are absent. This is most probably due to the fact that the exoenzymes, possibly with the exception of aryl-β-glucosidase, are intracellular or located in the cell wall.[4] Their presence in the culture solution is limited and may be due to lysis of old cells.

Table 1. Isoelectric points at 4° of cellulolytic and related enzymes from a commercial *Aspergillus* preparation and from the culture filtrates of *Stereum sanguinolentum*, *Fomes annosus*, and *Chrysosporium lignorum*.

Enzymes	Isoelectric points at 4°			
	Aspergillus	*Stereum*	*Fomes*	*Chrysosporium*
Cellulases	3.90, 4.22 4.52	3.58	3.9—4.2	4.24, 4.57 5.00, 5.5
Mannanases	3.95	3.58	3.9—4.2	4.11
Xylanases	3.90, 4.50	3.62 4.30	4.1—4.6 ≈7	4.44, 6.0
β-Glucosidases	4.46	—	—	4.38
Aryl-β-glucosidases	4.21, 4.43	4.00 4.08	4.08 4.17	4.22, 4.38 4.44
Mannosidases	4.68	—	—	—

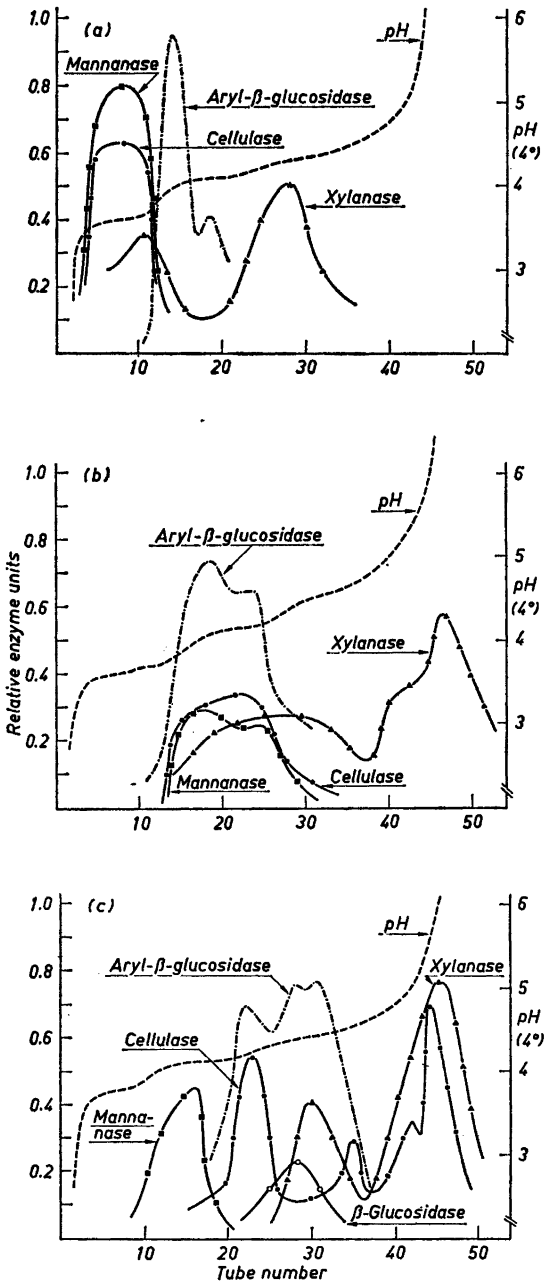

Fig. 2. pH diagrams with distribution of enzymic activities from isoelectric separation of culture filtrates from the fungi *Stereum sanguinolentum* (a), *Fomes annosus* (b), and *Chrysosporium lignorum* (c). The fraction volume was 2 ml.

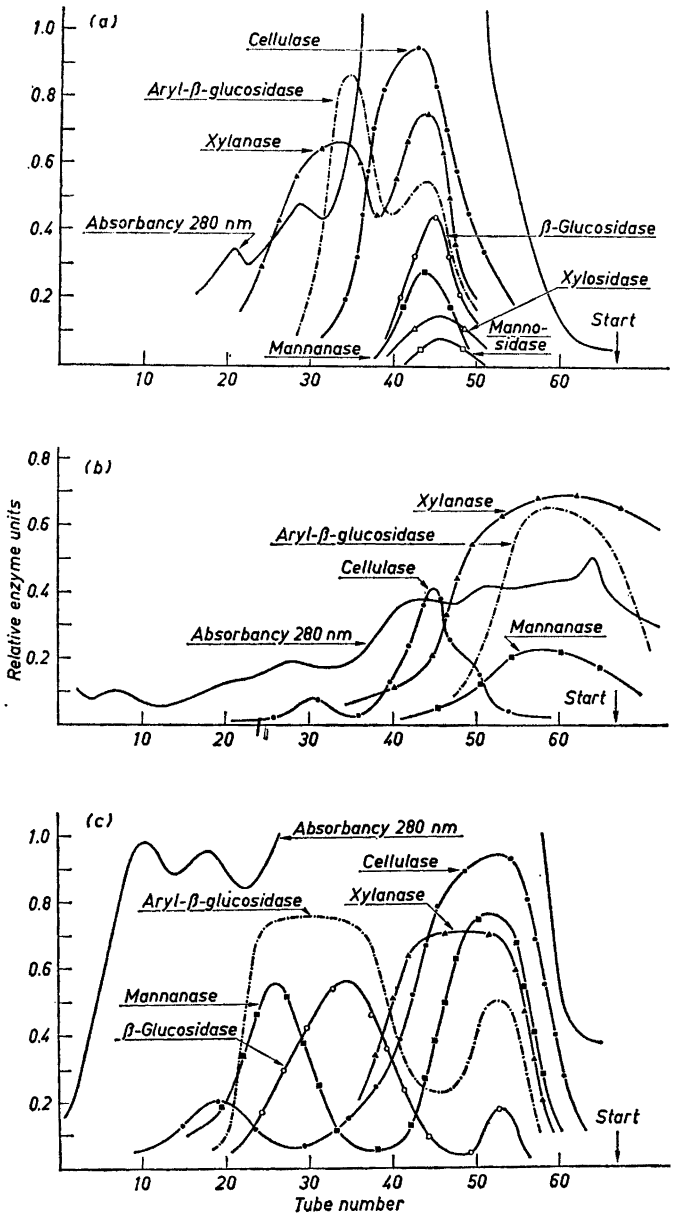

Fig. 3. Distribution of protein and enzymic activities after zone electrophoresis of culture filtrates from the fungi *Stereum sanguinolentum* (a), *Fomes annosus* (b), and *Chrysosporium lignorum* (c).

For (a) and (c) pyridine acetate buffer at pH 5.4, 0.1 M was used. For (b) phosphate buffer at pH 7.05, μ 0.1 was used. Carrier medium Sephadex G-25 Superfine. The void volume was 135 ml. The fraction volume was 2 ml.

The results obtained on isoelectric separation show that most of the investigated enzymes are proteins of acidic character (pI values close to 4). An investigation of the pH optima of the mannanase and mannosidase of *Aspergillus*, of the cellulase, aryl-β-glucosidase, and β-glucosidase of *Stereum* and of three cellulases of *Chrysosporium* showed that the pH optima are also close to 4. The studied enzymes, with the exception of those with high pI values mentioned above, probably attain their most active configurations in their neutral states.

It can be seen in the isoelectric focusing diagrams that the peaks of enzymic activity are broader for *Fomes annosus* than for the other fungi studied. The heterogeneities indicated by the broad peaks may be due to differences in genetic origin but may also be due to changes in molecular structure caused during the extremely long period of growth required for this fungus.

If the second explanation is valid, it is obvious that the enzyme proteins can undergo changes in their structure without loosing all their activity.

The absence of exoenzymes in the diagrams representing isoelectric separations, besides those already absent on gel filtration, is due to the reversible inhibition caused by the ethanediol building up the density gradient. The restoring of enzymic activity by the dialysis of each fraction was found to be extremely time-consuming.

A comparison of the gel filtration and the isoelectric focusing diagrams on the one hand and the zone electrophoresis diagrams on the other shows that in some cases the electrophoretic mobilities correspond to those expected from the molecular sizes and the pI values of the enzymes. One example is the cellulase of *Fomes annosus*, which is as acidic as the aryl-β-glucosidase but of smaller molecular size. Of the two enzymes, the cellulase shows the greater mobility.

However, there also appear enzymes possessing unexpected electrophoretic mobilities. For example, the main peak of the most acidic enzyme of *Chrysosporium lignorum*, the mannanase, migrates slower than its β-glucosidase, which is less acidic and of considerably larger molecular size.

In conclusion, the relation between the electrophoretic mobilities of two proteins cannot be predicted even if the isoelectric points and the relationship between the molecular sizes are known.

The resolving power of the different methods shows such variations from one fungus to another that none of the methods can be specially recommended for the purification of the enzymes. However, in order to discover charge heterogeneities of a protein, the isoelectric focusing method seems to be the most convenient as it separates entirely according to the electric charge possessed by the protein. In the cases of gel filtration and zone electrophoresis, several factors influence the behaviour of the enzymes; *cf.* Ref. 1.

In those cases where two or more activity peaks have been obtained with the same substrate, it has not been investigated whether they differ in substrate specificity.

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