ACTA CHEMICA SCANDINAVICA 22 (1968) 1924–1934

Purification and Characterisation of a Fungal β-Mannanase

KARL-ERIK ERIKSSON and MARGARETA WINELL*

Swedish Forest Products Research Laboratory, Paper Technology Department, Stockholm, Sweden

A mannanase from "Cellulase 36", a commercial preparation of *Aspergillus* enzymes, has been purified and characterised. The purified enzyme contains traces of cellulase and laminarinase activities. The purification factor is 61 and the enzyme yield 11 %. The molecular weight was determined and found to be 42 000 ± 2 000. The enzyme is rich in aromatic and acidic amino acids.

A solution of guaran is partly broken down to mannobiose and mannotriose by the purified enzyme.

The pH optima and stability as well as temperature optimum and stability have been determined.

The present work comprises the purification and characterisation of a β-mannanase from a commercial enzyme preparation, "Cellulase 36", supplied by Rohm and Haas Co., Philadelphia. The fungus producing the enzyme belongs to the *Aspergillus niger-oryzae* group. (More exact information concerning the type of organism was not available.) The crude preparation contains many different enzymes, such as cellulase, β-glucosidase, aryl-β-glucosidase, xylanase, xylosidase, mannanase, and mannosidase. These enzymes have been studied by Ahlgren *et al.*[1,2] with respect to their isoelectric points and other physico-chemical properties.

β-Mannanases have earlier been isolated from fungi, bacteria, higher plants, and animals. β-Mannanases of fungal origin have been studied by Lyr,[3] Ritter,[4] and by Reese and Shibata.[5] Lyr has cultivated ten different wood-rotting fungi on guaran and cellulose and measured the β-mannanase secretion of the fungi. Ritter has cultivated mycorrhiza fungi on cellulose and found that they produce mannanase. Reese and Shibata have separated a mannanase of fungal origin from β-glucosidase, α-galactosidase, and β-mannosidase.

A bacterial β-mannanase from human gastro-instestinal bacteria has been isolated and characterised by Innami.[6] Williams and Doetsch [7] isolated, from rumen streptococci, a mannanase which they purified 33-fold and characterised.

Mannanases from plants have been studied by Hylin and Sawai [8] and by Beaugirand and Percheron.[9] The former have isolated a galactomannan depolymerase in crystalline

* Present address: Institute of Biochemistry, University of Stockholm, Stockholm, Sweden.

form from the seeds of *Leucaena glauca* and the latter have purified a mannanase enzyme from the seeds of clover by fractional precipitation.

Mannanases of animal origin have been found in the digestive juice of *Astacus fluviatilis* and of *Homerus vulgaris* by Kooiman [10] and in the cockchafer by Courtois *et al.*[11]

As far as is known to the authors, no mannanase enzyme has been reported to have been obtained sufficiently pure to make molecular weight or amino acid composition determinations possible.

The aim of the present work was to obtain a mannanase enzyme pure enough to make a characterisation meaningful. The work has also been carried out to allow comparisons of data from other polysaccharases.[12]

The mannanase enzyme has been purified by ammonium sulphate precipitation, gel filtration, ion-exchange chromatography, and zone electrophoresis. The purification has been carried out on a quantitative basis and the carbohydrate content of the enzyme has been followed as the purification has proceeded.

The purified enzyme has been characterised with regard to its optimal pH and temperature as well as the range of stability for the enzymic activity. The molecular weight and amino acid composition have also been determined.

METHODS AND RESULTS

Enzyme assays. Cellulase and mannanase activities were determined viscometrically as described by Ahlgren *et al.*[1] The substrate for the mannanase assays in the present work was, however, a solution of guar powder supplied by General Mills, Minneapolis. The concentration of CMC 7HP was 3.2 g/l and the concentration of guar powder was 3.0 g/l.

Determinations of the relative mannanase units in the purification table were carried out by the viscometric method worked out by Almin *et al.*[13,14] The value of the empirical substrate specific constant, α, used in the calculations, was 1.68.

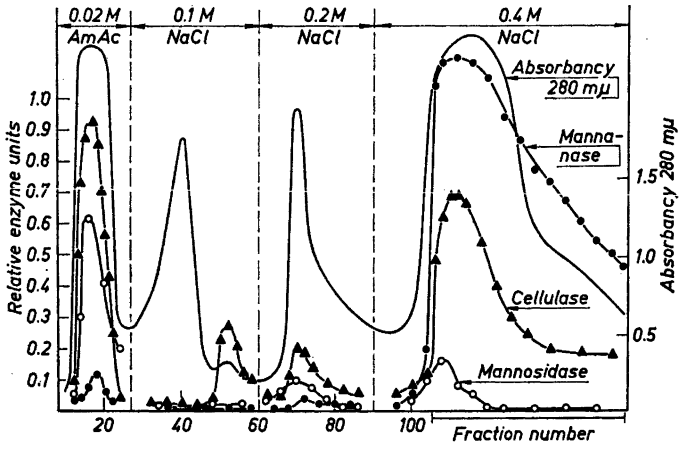

Fig. 1. Distribution of protein and enzymic activities after chromatography of $(NH_4)_2SO_4$ precipitated enzyme on DEAE-Sephadex A-25. The ion strength of the ammonium acetate buffer (pH 5.0; 0.02 M) was raised by adding sodium chloride to the concentrations 0.1 M, 0.2 M and 0.4 M at the points indicated. The fraction volume was 50 ml.

Mannanase activity was also determined by measuring the increase in reducing end groups with guaran as substrate. Mannosidase activity was determined with mannobiose as substrate.[1] End group determination was carried out by the method of Sumner [15] using dinitrosalicylic acid. The enzyme substrate ratio was 1:10 (v/v) and the incubations were carried out for 20 min at 40°C and pH 5.0.

Enzyme purification. A 10 % suspension of "Cellulase 36" in pyridine acetate buffer (pH 5.0; 0.5 M) was stirred for 3 h at 4°C and centrifuged. The supernatant was precipitated with ammonium sulphate at 80 % saturation and the precipitate dissolved in ammonium acetate buffer (pH 5.0; 0.02 M). The solution was desalted on a Sephadex G-25 column (80 × 400 mm). The pooled eluant was freeze-dried and further purified by fractionation on a DEAE-Sephadex A-25 column with an initial sedimentation height of 510 mm and a diameter of 80 mm. The buffer system used was ammonium acetate (pH 5.0; 0.02 M). The column was eluted with buffer solutions by a step-wise increase in ionic strength, as is seen in Fig. 1, where a diagram of the fractionation result is given. The mannanase enzyme is seen to appear in the last peak together with cellulase and a small amount of mannosidase. Other polysaccharases like xylanase and β-glucosidase are not recorded in the figure but have been shown in earlier experiments to be eluted in the first three peaks. The enzymes from the last peak were pooled as indicated in the figure, freeze-dried, desalted on the Sephadex G-25 column mentioned above and freeze-dried again.

As has been shown,[1] the mannosidase molecule is of greater molecular size than the mannanase and cellulase molecules. In order to separate the mannosidase enzyme from the two latter enzymes, a gel filtration was made on a biogel P-150 column (70 × 1100 mm, void volume 1190 ml). All gel filtrations were carried out in ammonium acetate buffer (pH 5.0; 0.1 M). To prevent bacterial growth the buffer was saturated with chloroform. Packing of all columns was carried out as described by Widén and Eriksson.[16] The result of the gel filtration experiment can be seen in Fig. 2. The almost coinciding mannanase and cellulase peaks were pooled, freeze-dried and divided in two equal parts, each of which was run on a Sephadex G-75 column (35 × 940 mm, void volume 160 ml). Fig. 3 shows that a partial separation of the mannanase and cellulase enzymes was achieved in this step. The mannanase peaks from both runs were pooled, concentrated by freeze-drying and dialyzed in collodion tubes against distilled water.

The dialyzed enzyme solution was then run on a DEAE-Sephadex A-50 column with an initial sedimentation height of 350 mm and a diameter of 50 mm. The result of the discontinuous salt gradient elution in ammonium acetate buffer (pH 5.0; 0.02 M) is shown in Fig. 4; a mannanase fraction almost free from cellulase was obtained. The

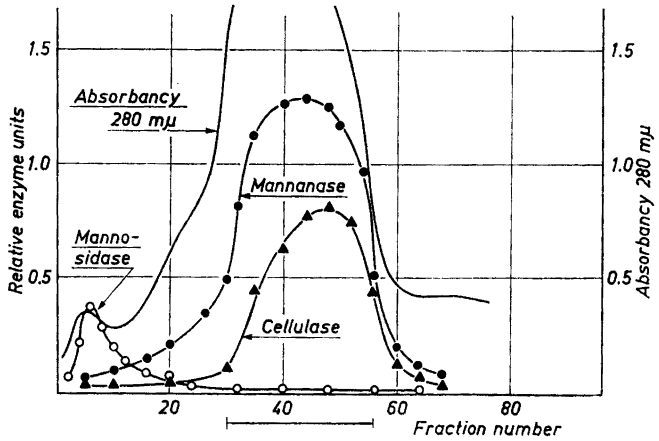

Fig. 2. Distribution of protein and enzymic activities after gel filtration on a polyacrylamide gel, P-150 column, of the mannanase peak from the chromatography on DEAE-Sephadex A-25 (*cf.* Fig. 1). The fraction volume was 35 ml.

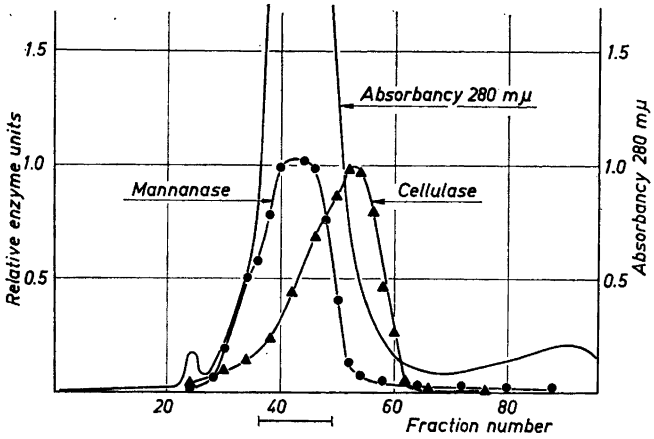

Fig. 3. Distribution of protein and distribution of cellulase and mannanase activities after gel filtration on Sephadex G-75 of the mannanase peak from gel filtration on P-150 (*cf.* Fig. 2). The fraction volume was 8 ml.

mannanase peak was pooled, dialyzed in cellulose acetate sacs against distilled water and freeze-dried.

Zone electrophoresis of the material obtained was carried out on the column as described by Eriksson and Pettersson.[17] The electrophoresis was run for 70 h at 60 mA, giving rise to a potential difference of approximately 400 V. Since the weight of the freeze-dried material was about 100 mg, only one fourth of it could be run on the column at the same time. The first electrophoresis showed that the enzyme preparation was not homogeneous, since two protein peaks were obtained (*cf.* Fig. 5). Peak two, containing the mannanase, was pooled as indicated in the figure, freeze-dried and again run on the electrophoresis column. Fig. 6 shows the result of the second electrophoresis. Only one protein peak coincident with the mannanase activity can be seen. The mannanase fractions were pooled and analyzed for carbohydrate content. As the carbohydrate

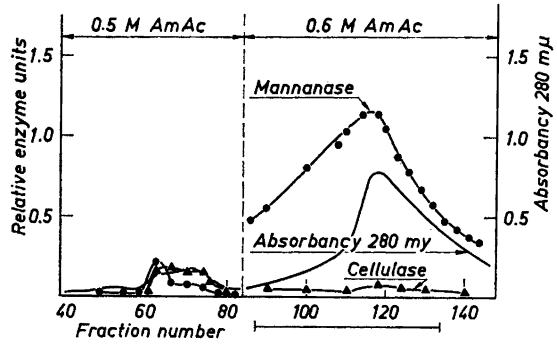

Fig. 4. Distribution of protein and distribution of cellulase and mannanase activities after chromatography on DEAE-Sephadex A-50 of the mannanase peak from gel filtration on Sephadex G-75 (*cf.* Fig. 3). The buffer was changed from 0.1 M ammonium acetate to 0.4 M, 0.5 M, and 0.6 M ammonium acetate, respectively, at the points indicated. The pH was 5.0 and the fraction volume was 10 ml.

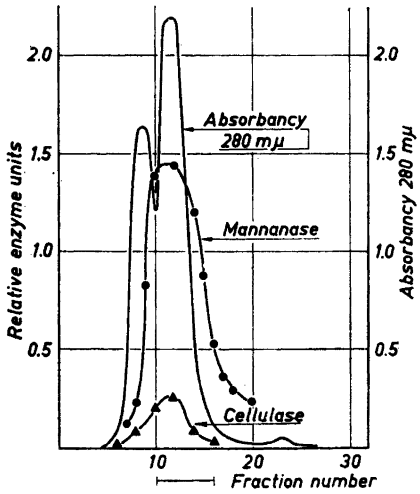

Fig. 5. Distribution of protein and distribution of cellulase and mannanase activities after zone electrophoresis I for 70 h of the mannanase peak from chromatography on DEAE-Sephadex A-50 (*cf.* Fig. 4). Bed dimensions: 20 × 670 mm, buffer system: ammonium acetate, pH 5.4, 0.1 M, current: 50 mA. The fraction volume was 3 ml.

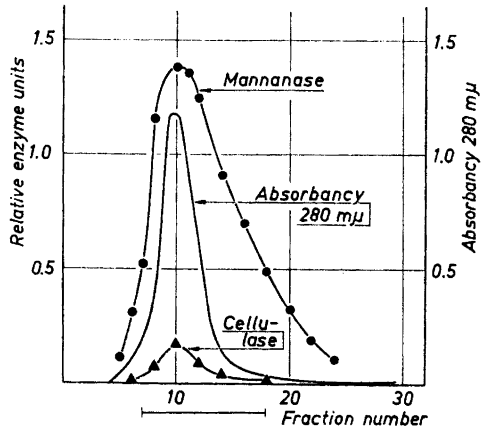

Fig. 6. Distribution of protein and distribution of cellulase and mannanase activities after zone electrophoresis II for 70 h of the mannanase peak from zone electrophoresis I (*cf.* Fig. 5). Conditions as described in Fig. 5. The fraction volume was 2 ml.

content, determined by the orcinol method according to Vasseur,[18] was found to be 20 % of the dissolved material, the enzyme solution was dialyzed in a cellulose acetate membrane against distilled water. During the dialysis samples were taken and carbohydrate tests were made. When the carbohydrate content had decreased to less than 1 %, the solution was freeze-dried. The dried material was stored at −30°C until needed for the characterisation experiments described below.

In Table 1 a quantitative presentation of the purification obtained in the different steps is given. It can be seen here that the purification factor is 61 and the enzyme yield 11 %.

Ultracentrifugation studies

Ultracentrifugation studies were performed in a Spinco Model E ultracentrifuge equipped with phase plate schlieren optics. Sedimentation velocity experiments were performed at 59 780 rev/min at 20° in 12 mm, 4° sector cells with standard and wedge windows. The enzyme was dissolved in 0.05 M sodium acetate buffer at pH 5.0 containing 0.1 M sodium chloride. The sedimentation coefficients were extrapolated to infinite dilution.

Ultracentrifugation of the purified enzyme gave rise to one symmetrical peak. The sedimentation coefficient of the enzyme was found to be 3.8 S.

The molecular weight was determined by the Archibald method for various concentrations by approach to equilibrium experiments which were evaluated by the method of Ehrenberg[19] and also by the method of Trautman and

Table 1. Purification table for the mannanase from "Cellulase 36".

Fraction	Total volume ml	Mannanase activity, relative units/ml	Mannanase activity, total relative units	Enzyme yield %	A_{280}	Total A_{280}	Specific activity units/mg	Purification factor	Carbohydrate mg/ml	Total carbohydrate mg
Crude enzyme solution	1000	3.8	3.8×10^3	—	42.0	4.2×10^4	0.074	1	120	1.2×10^5
Redissolved $(NH_4)_2SO_4$ precipitation	160	19	3.0×10^3	97	145	2.3×10^4	0.13	1.8	110	1.8×10^4
Eluate from DEAE-Sephadex A-25 column	2500	0.79	2.0×10^3	65	1.68	4.2×10^3	0.48	6.5	0.76	1.9×10^3
Eluate from polyacrylamide P-150 column	890	1.6	1.4×10^3	45	1.76	1.6×10^3	0.88	12	0.32	2.8×10^2
Eluate from Sephadex G-75 column	204	3.5	7.1×10^2	23	4.08	8.3×10^2	0.88	12	0.74	1.5×10^2
Eluate from DEAE-Sephadex A-50 column	520	0.88	4.6×10^2	15	0.391	2.0×10^2	2.3	31	0.08	42
Eluate from zone electrophoresis I	4×22	4.5	4.0×10^2	13	1.60	1.4×10^2	2.9	39	0.06	5
Eluate from zone electrophoresis II	4×22	3.7	3.3×10^2	11	0.84	74	4.5	61	0.007	0.6

Crampton.[20] The purified mannanase enzyme had a molecular weight of 42 000±2 000. The partial specific volume calculated from the amino acid composition was 0.72 cm^3/g.

Amino acid analysis

The analysis was carried out with a Spinco Model 120 B automatic amino acid analyser. The enzyme sample, 3.0 mg, was hydrolyzed for 24 and 72 h with 6 M HCl at 110°C.

Average values were used for all amino acids except threonine, serine, methionine, and tyrosine, where "zero time" values were calculated by first order extrapolation, and valine and isoleucine where only the higher, 72 h, values were used. Amide ammonia was estimated by extrapolation to zero time after correction of the 24 and 72 h values for exogenous ammonia. For glucosamine a corrected 24 h value was used.

The content of tryptophan was determined spectrophotometrically by the method of Bencze and Schmid.[21]

The amino acid composition obtained is given in Table 2. The molecular weight calculated from the results of the amino acid analysis and the Archibald method is 42 000.

Table 2. Amino acid composition of mannanase from "Cellulase 36" as determined by amino acid analysis and the molecular weight estimated by ultracentrifugation.

Amino acid	Number of residues	Closest integer
Try	24.24	24
Lys	8.79	9
His	5.30	5
Arg	5.57	6
Asp	43.60	44
Thr	33.79	34
Ser	38.15	38
Glu	28.87	29
Pro	8.68	9
Gly	34.10	34
Ala	27.88	28
Half-Cys	5.19	5
Val	20.45	20
Met	4.21	4
Ileu	16.72	17
Leu	20.56	21
Tyr	19.39	19
Phe	11.44	11
Glucosamine	5.96	6
Amide NH_3	31.17	
	Total number of residues	363

Molecular weight: 42 000.

Characterisation of the purified enzyme towards different substrates

The action of the enzyme towards different substrates was investigated as follows:

All enzymic hydrolysis studies were carried out in ammonium acetate buffer at pH 5.0 and 40°C. The incubation time was 24 h. The hydrolysis products were developed by paper chromatography in the solvent system ethyl acetate—acetic acid—water (3:1:1).

A solution of guaran was partly broken down to mannobiose and mannotriose by the purified enzyme. Difficulties to get the guaran powder into solution may account for the slowness of the reaction. The enzyme showed no activity towards mannobiose. Traces of activity could be detected towards cellodextrins and laminarin but not towards xylodextrins or pullulan.

Determination of pH optimum and pH stability

Attempts were made to study the optimal pH of the enzyme viscometrically, but these attempts were not successful since alterations of the pH in the guaran solution gave rise to marked changes of the viscosity of the solution. The optimal pH was instead determined by measuring the increase in reducing power. The method of Sumner,[15] employing dinitrosalicylic acid, was used. The incubation temperature was 60°C. The results are plotted in Fig. 7 and indicate two pH optima, namely at pH 3.0 and pH 3.8, respectively.

The pH stability was determined by mixing the enzyme solution with an equal amount of 0.1 M buffer solutions of different pH values. The mixed solutions were allowed to stand for 3 h at room temperature after which the enzymic activity was measured viscometrically. The enzyme was found to be pH stable in the pH range 2.0—9.2 as shown in Fig. 8.

Determination of temperature optimum and temperature stability

In order to determine the temperature optimum for the enzyme, solutions of guaran (3.0 g/l) in sodium acetate buffer (pH 5.0; 0.05 M) were thermostated

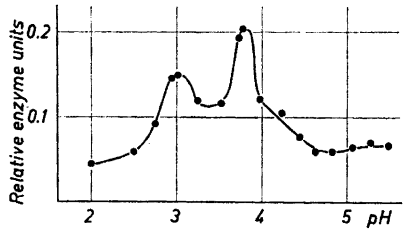

Fig. 7. Effect of pH on the activity of the purified mannanase towards guar powder.

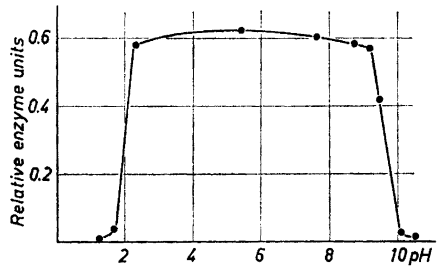

Fig. 8. pH stability of the purified mannanase at room temperature.

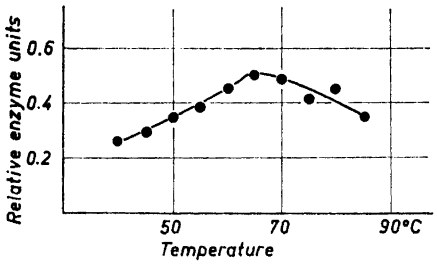

Fig. 9. Effect of temperature on the activity of the purified mannanase towards guar powder.

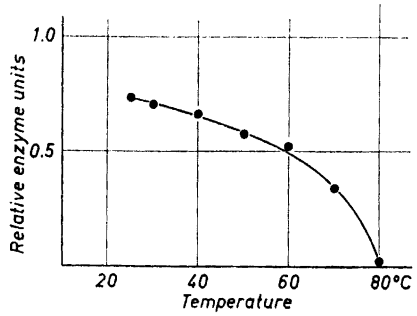

Fig. 10. Heat stability of the purified mannanase (ammonium acetate buffer, pH 5.0; 0.1 M).

at the investigated temperature (*cf.* Fig. 9) and incubated with the enzyme for 15 min. The guaran solutions were then rapidly cooled to 25°C and the decrease in viscosity was measured. The optimal temperature of the enzyme was found to be 65°C.

The heat stability of the enzyme was determined by keeping enzyme solutions in ammonium acetate buffer (pH 5.0; 0.05 M) at different temperatures. After 3 h a sample was taken from each solution and tested viscometrically for mannanase activity. The enzyme was found to be stable up to a temperature of about 70°C (*cf.* Fig. 10).

DISCUSSION

The viscometric method, worked out by Almin *et al.*,[13,14] for the determination of enzymic activity in absolute terms towards polymer substrates has been applied to the mannanase action on guaran. The mannanase activity in Table 1 is given as B (=relative enzyme units). The size of B is, however, proportional to the number of substrate bonds broken by the enzyme (=A). The relationship between A and B consists merely of substrate specific constants which still remain to be determined for the guar powder.

It is obvious from the investigation of activity of the purified enzyme towards different substrates that the mannanase enzyme is slightly contaminated by other enzymes. The main difficulty in the purification work was to separate the mannanase activity from the cellulase activity. At first sight the fractionation on Sephadex G-75 seemed to be a successful one, since a partial separation of these two enzymes was achieved according to Fig. 3. The purification table shows, however, that this fractionation step could as well have been excluded. The specific activity of the enzyme turned out to be the same before and after the fractionation. The result of the G-75 fractionation was merely a loss of enzyme material, about half of the mannanase

was retained by the gel. Eriksson and Pettersson [17] have reported a similar loss of cellulase activity by fractionation on Sephadex gels.

The separation of mannanase and cellulase enzymes was mainly achieved on the DEAE-Sephadex A-50 column (*cf.* Fig. 4). The mannanase fraction from this column contained other proteins, which were separated by zone electrophoresis (*cf.* Fig. 5). In addition to the above experiments, attempts have been made to fractionate the enzyme on Dowex 2-X8 and Dowex 1-X2. Bucht and Eriksson [22] have reported good separations of aryl-β- and α-glycosidases and β-glycosidases on these types of ion exchangers. However, no separation of mannanase from other protein fractions was obtained on either of these ion exchangers.

The molecular weight, 42 000, calculated from the Archibald experiment, of the enzyme is in accordance with the sedimentation coefficient 3.8 S.

The amino acid composition (not exact, due to the presence of traces of contaminating enzymic activities) shows the same pattern as for other related enzymes, *i.e.* a high content of aromatic and acidic amino acids.[12] The acidity of the enzyme is also verified by the value of its isoelectric point, 4.1.[2]

The shape of the optimal pH curve is strange, showing two sharp peaks (Fig. 7). A possible explanation of the unusual curve is that substrate changes due to different buffer systems in the broad pH range might have influenced the determination of pH optimum.

The enzyme seems to be rather insensitive to pH changes (*cf.* Fig. 9). The pH range is somewhat broader than the ranges found by Reese and Shibata [5] for two fungal β-mannanases.

The heat stability and optimal temperature of the enzyme (*cf.* Figs. 7 and 8) are in agreement with similar investigations made by Reese.

Acknowledgements. The authors are indebted to Professor Börje Steenberg for his kind interest in the work and to Dr. P. Fredricks for revising the English text.

REFERENCES

1. Ahlgren, E., Eriksson, K.-E. and Vesterberg, O. *Acta Chem. Scand.* **21** (1967) 937.
2. Ahlgren, E. and Eriksson, K.-E. *Acta Chem. Scand.* **21** (1967) 1193.
3. Lyr, H. *Z. Allgem. Mikrobiol.* **3** (1963) 25.
4. Ritter, G. *Z. Allgem. Mikrobiol.* **4** (1964) 295.
5. Reese, E. T. and Shibata, Y. *Can. J. Microbiol.* **11** (1965) 167.
6. Innami, S. *Agr. Biol. Chem.* **25** (1961) 155.
7. Williams, P. P. and Doetsch, R. N. *J. Gen. Mikrobiol.* **22** (1960) 635.
8. Hylin, J. W. and Sawai, K. *J. Biol. Chem.* **239** (1964) 990.
9. Beaugirand, S. and Percheron, F. *Compt. Rend.* **259** (1964) 3879.
10. Kooiman, P. *J. Cellular Comp. Physiol.* **63** (1964) 197.
11. Courtois, J. E., Petek, F. and Kolahi Zansuzi, M. A. *Compt. Rend. Soc. Biol.* **156** (1962) 565.
12. Eriksson, K.-E. *Svensk Kem. Tidskr.* **79** (1967) 660.
13. Almin, K. E. and Eriksson, K.-E. *Biochim. Biophys. Acta* **139** (1967) 238.
14. Almin, K. E., Eriksson, K.-E. and Jansson, C. *Biochim. Biophys. Acta* **139** (1967) 248.
15. Sumner, J. B. *J. Biol. Chem.* **62** (1924–5) 287.
16. Widén, R. and Eriksson, K.-E. *J. Chromatog.* **15** (1964) 429.
17. Eriksson, K.-E. and Pettersson, B. *Arch. Biochem.* **124** (1968) 142.

18. Vasseur, E. *Acta Chem. Scand.* **2** (1948) 693.
19. Ehrenberg, A. *Acta Chem. Scand.* **11** (1957) 1257.
20. Trautman, R. and Crampton, C. F. *J. Am. Chem. Soc.* **81** (1959) 4036.
21. Bencze, W. L. and Schmid, K. *Anal. Chem.* **29** (1957) 1193.
22. Bucht, B. and Eriksson, K.-E. *Arch. Biochem. Biophys.* **124** (1968) 135.
23. Björndal, H. and Eriksson, K.-E. *Arch. Biochem. Biophys.* **124** (1968) 149.

Received January 20, 1968.