PURIFICATION OF A FUNGAL ENDO- β -GLUCANASE WITH HIGH ACTIVITY ON BARLEY β -GLUCAN

by

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Keywords: Endo- β -glucanase, *Aspergillus niger*, enzyme isolation,
isoelectric focusing, amino acid composition, barley β -glucan hydrolysis

An endo- β -glucanase was purified from a commercial enzyme preparation of fungal origin by ammonium sulphate fractionation, ion exchange chromatography, and gel filtration followed by preparative isoelectric focusing. The enzyme was homogeneous in sedimentation equilibrium analysis from which the molecular weight was determined to be 23.500 in agreement with the value 23.653 calculated on the basis of the amino acid composition. The enzyme did not contain carbohydrate and a molecular weight of 24.000 estimated by polyacrylamide gel electrophoresis in dodecyl sulphate after 2-mercaptoethanol treatment, indicated that it consisted of a single polypeptide chain. It had an isoelectric point of 4.47.

The enzyme rapidly decreased the specific viscosity of barley β -glucan with a small concomitant increase in reducing sugar. Its activity toward carboxymethyl-cellulose, acid-swollen cellulose, and a mixture of cellodextrins was low. Laminarin, carboxymethyl-pachyman, cellobiose, and p-nitrophenyl- β -D-glucoside were not hydrolyzed. The K_m for hydrolysis of barley β -glucan and carboxymethyl-cellulose was 1.8 and 11 mg/ml, respectively, the corresponding molecular activities being 7750 and 750 equivalents of glucosidic bonds hydrolyzed per min per mole of enzyme. The products of exhaustive hydrolysis of barley β -glucan were 4.3% glucose, 4.2% disaccharide, 72.7% trisaccharide, and 18.8% tetrasaccharide, higher oligomers and polymers were absent. The enzymic activity was completely destroyed by treatment with N-bromosuccinimide but was insensitive to EDTA, sulphhydryl modifying reagents, and glucono-1,5-lactone.

1. INTRODUCTION

Endo-1,4- β -glucanases (cellulases, EC 3.2.1.4) from various fungi, *Streptomyces* (3, 34), *Aspergillus niger* (5, 22, 28) *Trichoderma viride* (23), *Penicillium notatum* (35), and

Trametes sanguina (23), some plants, pea (42) and barley (32), and the snail *Helix pomatia* (31) have been reported to hydrolyze both cellulose and the 1,3;1,4- β -glucans (mixed-linked β -glucans) from barley, oats or Iceland moss. Ex-

haustive hydrolysis of barley β -glucan catalyzed by the pea enzymes and some of the fungal enzymes yielded 4-O-laminaribiosyl-glucose as major product (7, 23, 42) whereas the main product obtained with the snail enzyme was 4-O-laminaribiosyl-cellobiose (30). Furthermore the activity of the pea enzyme (42) in contrast to the fungal (6, 21, 24a), barley (32), and snail enzymes (31) so far studied was sensitive to sulfhydryl group modifying reagents. Differences are thus found within this group of very closely related enzymes.

Aspergillus niger is known to produce a variety of enzymes which attack different types of β -glucosidic linkages (5, 24b, 25, 28). IKEDA et al. (24) and very recently HURST et al. (20) have isolated two different *A. niger* endo-1,4- β -glucanases in homogeneous form. The only other highly purified *A. niger* endo-1,4- β -glucanase tested on a mixed-linked β -glucan was described by CLARKE and STONE (7), but the physico-chemical and chemical properties of this enzyme preparation have not been examined.

The present study describes the purification to homogeneity of an *A. niger* endo- β -glucanase showing a very high activity on barley β -glucan compared with CM-cellulose and acid-swollen cellulose. Chemical evidence showed this enzyme to be different from those described by IKEDA et al. (24a) and HURST et al. (20). In addition the relative activities on various β -glucan substrates suggested the enzyme was different also from the one studied by CLARKE and STONE (7).

2. EXPERIMENTAL

2.1. Materials

The *A. niger* liquid concentrate marketed as Glucanase GV-L was a product of Grindstedværket, Brabrand, Denmark. Barley β -glucan isolated as described by PREECE and MACKENZIE (36) was a gift from Mrs. E. MEILING and J. A. SØRENSEN, M.Sc., Department of Brewing Chemistry, Carlsberg Research Laboratory. CM-cellulose sodium salt, high viscosity and degree of substitution 0.7-0.8 and glucono-1,5-lactone were from BDH Chemicals Ltd., Poole, England.

Lichenin ex. *Cetraria islandica*, pure, was obtained from Koch-Light Laboratories, Ltd., Colnbrook, England. Laminarin and N-bromosuccinimide, both purum were products of Fluka AG, Buchs, Switzerland. CM-pachyman, degree of substitution 0.32, was a gift from Dr. B. A. STONE, Biochemistry Department, La Trobe University, Victoria, Australia to Dr. B. S. ENEVOLDSEN, Department of Brewing Chemistry, Carlsberg Research Laboratory. Acid-swollen cellulose was prepared as described by WOOD (42) using MN-cellulose powder 300 without binder from Macherey, Nagel & Co, Düren, West-Germany. A mixture of celloextrins of DP 3-10 prepared by hydrochloric acid hydrolysis of the MN-cellulose was a gift from Dr. B. S. ENEVOLDSEN. Cellobiose was a product of FRANKEL and LANDAU, Berlin, *p*-nitrophenyl- β -D-glucoside and *p*-hydroxymercuribenzoate were bought from Sigma Chem. Comp., St. Louis, Mo. Iodoacetic acid p.a. was from Merck, Darmstadt, West-Germany. The methanesulfonic acid reagent was from Pierce Chem. Comp., Rockford, Ill. DEAE-Cellulose, DE 52 was obtained from Whatman Ltd., Springfield, England. Bio-Gel P-100, 100-200 mesh, and P-60, 100-200 mesh, were products of Bio-Rad Laboratories, Richmond, Calif. Sephadex-G50, fine, was from Pharmacia, Uppsala, Sweden.

2.2. Enzyme purification

The precipitate obtained from one liter of crude glucanase by fractionation at 40% to 65% saturation with ammonium sulphate was redissolved in deionized water and desalted either by continuous diafiltration on an Amicon TCF 10 laboratory unit using a Diaflo PM 10 membrane, or ultrafiltration by circulation through a Romicon PM 10 hollow fiber cartridge (1.8 Ft²). The desalted enzyme solution was adjusted to pH 5.0 and after appropriate dilution applied in a batchwise manner to 400 ml of DEAE-cellulose equilibrated with 0.01 M-sodium acetate pH 5.0. Elution was performed with a linear salt gradient from zero to 0.6 M-NaCl in the acetate buffer. Fractions having high activity on barley β -glucan were pooled,

adjusted to pH 8.0 and after appropriate dilution applied batch-wise on 150 ml of DEAE-cellulose equilibrated with 0.02 M-Tris-HCl pH 8.0. Elution was performed with a linear gradient from zero to 1.0 M-NaCl in the Tris-buffer and fractions with high activity on barley β -glucan were pooled and lyophilized. This material was dissolved in approx. 30 ml of deionized water and fractionated on Bio-Gel P-100 (50 $\times$ 900 mm) in 0.02 M-sodium acetate pH 5.0, 0.1 M in NaCl, at a flow rate of 31 ml/h. The fractions active on barley β -glucan were pooled, lyophilized, and redissolved in deionized water to a final volume of 80 ml. The solution was dialyzed for 6 $\times$ 45 min against 1 liter portions of 1% of glycine with changes to a fresh dialysis bag every 90 min to avoid bursting of the cellophane tubing caused by cellulolytic action. The dialyzed solution was fractionated by preparative flat bed isoelectric focusing on the LKB 2117 Multiphor using LKB Ultrodex and LKB Ampholines in the range pH 3.5-5.0 as described by WINTER et al. (40). The gel sections cut out from the flat bed were eluted with 2 $\times$ 2.5 ml of 0.05 M-sodium acetate pH 5.0, 1 M in NaCl. Ampholines were removed from the eluate of the section containing high activity on barley β -glucan by gel filtration on Sephadex G-50, fine, (26 $\times$ 400 mm), with 0.0025 M-sodium acetate, 0.5 M in NaCl, at a flow rate of 18 ml/h. The enzyme containing fractions were pooled, lyophilized and finally desalted by gel filtration in deionized water on Sephadex G-50, fine, (26 $\times$ 400 mm) at a flow rate of 18 ml/h.

All operations took place at 4°C. The enzyme was stored as a lyophilized powder at -20°C.

2.3. Analytical procedures

The enzymic activity on the various polysaccharide substrates was measured by determination of the amount of reducing sugar produced. The substrate (350 μ l), 0.57% barley β -glucan, 0.26% CM-cellulose, or 0.17% laminarin all in 0.025 M-sodium acetate pH 5.0, was preincubated for 5 min at 30°C. The hydrolysis was initiated by addition of enzyme (50 μ l) and stopped after 30 min at 30°C by addition of the SOMOGYI low-alkalinity copper

reagent (400 μ l). The colorimetric assay was completed as described (19), the extinction being read at 546 nm. One unit of β -glucanase catalyzes the increase in reducing power equivalent to 1 μ mole of glucose per min under the conditions stated above. β -Glucosidase was assayed with *p*-nitrophenyl- β -D-glucoside, in principle as described by OKADA (33). Endo- β -glucanase activity was measured on barley β -glucan (0.5% in 0.05 M-sodium acetate pH 5.0) by viscometry at 30°C using an Ostwald viscometer. Exhaustive hydrolysis of barley β -glucan (1.25% in 0.005 M-sodium acetate pH 5.0) was performed at 30°C for a period of 10 days. The products were separated by gel filtration chromatography and analysed as described by ENEVOLDSEN & SCHMIDT (12).

The Michaelis-Menten parameters of the hydrolysis of barley β -glucan and CM-cellulose in 0.025 M-sodium acetate pH 5.0 at 30°C were obtained from Lineweaver-Burk plots of initial rates of hydrolysis determined at 8 substrate concentrations within the ranges 0.025-0.75% and 0.04-0.35% for barley β -glucan and CM-cellulose, respectively, the corresponding enzyme concentrations were 2.7×10^{-9} M and 2.3×10^{-7} M. Aliquots were removed for reducing sugar determination at intervals of three minutes.

Protein contents were estimated by the Lowry procedure (27) using crystalline bovine serum albumin as standard. Carbohydrate was determined by the phenol-sulphuric acid procedure of DUBOIS et al. (10).

Prior to amino acid analysis the protein samples were subjected to gel filtration on Bio-Gel P-60 in 5% CH₃COOH to remove traces of salt and carbohydrate. The protein containing fractions were pooled and lyophilized. The samples were hydrolyzed in 6 M-HCl in sealed, evacuated tubes for 24, 48 and 72 hours at 110°C. The amino acid contents were measured on a Durrum D-500 amino acid analyzer. Half-cystine was determined as cysteic acid after performic acid oxidation (18). Tryptophan contents were measured both by the spectroscopic procedure of EDELHOCH (11) and by amino acid analysis after hydrolysis in methanesulfonic acid (26) at 125°C for 20 hours.

The progress of the endo- β -glucanase purification was followed by disc gel electrophoresis in 7.5% polyacrylamide gels in 0.37 M-Tris-HCl pH 8.8 (9). Polyacrylamide gel electrophoresis in dodecyl sulphate was performed after reduction of the protein sample by 2-mercaptoethanol (38). Bovine serum albumin, chymotrypsinogen, and bovine ribonuclease were included as reference proteins for estimation of the molecular weight of the endo- β -glucanase. The gels were stained with Coomassie brilliant blue (38) or the periodate-Schiff reagent (17).

Isoelectric focusing of endo- β -glucanase pooled after Bio-Gel P-100 gel filtration and dialyzed against 1% glycine as described above was performed at 4°C in a LKB 8101 column using the Ampholine gradient from pH 3.5 to 5.0 and a sucrose gradient from zero to 40% for stabilization (39). After 24 hours the contents of the column were pumped out in 1.8 ml fractions. The pH was measured at 4°C using a Radiometer PHM-63 pH meter and a Radiometer GK 2321 C combination electrode standardized with Radiometer buffer solutions type S 1326 and S 1316. Aliquots were removed from the fractions for activity measurements on barley β -glucan.

Sedimentation equilibrium analysis according to a modification of the meniscus depletion technique of YPHANTIS described by CHERVENKA (4) was performed at 29,500 rev./min at 20°C in sodium acetate pH 5.0, $I=0.1$. The partial specific volume of endo- β -

glucanase was calculated from the amino acid composition (8) assuming 24 carboxyl amides per molecule.

Iodoacetate treatment was performed in principle as described by GURD (15). Endo- β -glucanase (400 μ g) was incubated for 4 h at 22°C in 0.1 M-iodoacetate, 0.2 M in sodium phosphate pH 6.5 (1 ml). At appropriate time intervals aliquots (200 μ l) were removed and the enzyme freed from excess of reagent by gel filtration on Sephadex G 50, fine, (9 $\times$ 100 mm) in the same phosphate buffer. The eluted protein was checked for carboxymethylation and its specific activity on barley β -glucan determined.

Treatment with N-bromosuccinimide a.m. SPANDE and WITKOP (37) was carried out as follows. Endo- β -glucanase (400 μ g) was dissolved in water (100 μ l) and mixed with 0.01 M-N-bromosuccinimide in 0.01 M-sodium acetate (100 μ l). Aliquots (20 μ l) were removed at appropriate time intervals, diluted 10-fold in the acetate buffer, subjected to gel filtration as above using 0.01 M-sodium acetate for elution and the specific activity of the purified enzyme derivative determined.

3. RESULTS

3.1. Purification of an endo- β -glucanase

The results of the purification procedure of the endo- β -glucanase described in section 2.2. are given in Table I. Precipitation at 80% saturated $(\text{NH}_4)_2\text{SO}_4$ improves the final yield of

Table I

Purification of endo- β -glucanase

Fraction	Volume (ml)	Recovery of activity (%)	Total ^{a)} protein (mg)	Specific activity (units/mg)	Purification factor
Glucanase GV	1000	100	59100	0.55	1
40-65% satd. $(\text{NH}_4)_2\text{SO}_4$ ppt.	730	76	19000	1.30	2.4
Desalted, redissolved $(\text{NH}_4)_2\text{SO}_4$ ppt.	1620	49	10300	1.54	2.8
DEAE-cellulose, pH 5.0, pooled eluate	388	26	2540	3.35	6.1
DEAE-cellulose, pH 8.0, pooled eluate	32	15	443	10.7	19.5
Bio-Gel P-100, pooled eluate	50	8.2	33.7	78.6	143
Isoelectric focusing eluate ^{b)}	Lyophilized powder	4.8	17.3	90.2	164

a) Determined by the Lowry procedure

b) After removal of ampholines and desalting, see section 2.2.

active enzyme, but also leads to a contamination with a carbohydrate containing pigment which is not removed in the subsequent steps of the procedure. The ion exchange chromatography on DEAE-cellulose at pH 5.0 separated several enzymes hydrolyzing both barley β -glucan and CM-cellulose (Figure 1). Most of the pigment remained on the DEAE-cellulose and only small amounts were eluted at an ionic strength below 0.2. Further pigment and unidentified protein were removed by chromatography on DEAE-cellulose at pH 8.0. Most of the proteins remaining at this state of purification including β -glucoside and 1,3- β -glucan hydrolyzing enzymes, were separated from the endo- β -glucanase by gel filtration (Figure 2). Finally, small amounts of contaminating proteins, very acidic pigments, and some minor barley β -glucan hydrolyzing com-

ponents were completely removed from the major endo- β -glucanase component by preparative flat bed isoelectric focusing (Figure 3). Amino acid analysis of the homogeneous protein later demonstrated its colour yield in the Lowry reaction to be 205% relative to that of bovine serum albumin. Thus the actual amount of the endo- β -glucanase isolated from 1 liter of Glucanase GV is 8.4 mg instead of 17.3 mg (Table I) calculated on the basis of the bovine serum albumin standard. The 164-fold purification of the endo- β -glucanase (Table I) is actually too low a value because several enzymes in the crude Glucanase GV contribute to the formation of reducing sugar from barley β -glucan. The isolated endo- β -glucanase must therefore have been obtained in higher yield and with a higher degree of purification than reported.

Figure 1. DEAE-cellulose ion exchange chromatography of $(\text{NH}_4)_2\text{SO}_4$ -fractionated and desalted glucanase GV in 0.01 M-sodium acetate pH 5.0. Activities on barley β -glucan (—), CM-cellulose (CMC) (— · —), laminarin (---), and p-nitrophenyl- β -glucoside (PNPG) (— — —) are expressed in absorbancy units multiplied by the dilution factor of the enzyme sample. Protein (—) was determined by the Lowry procedure. The salt gradient (0.06 M-NaCl) is indicated, after the gradient was finished, elution was performed with 2M-NaCl added to the buffer. The bar indicates fractions pooled for the second DEAE-cellulose chromatography.

Figure 2. Bio-Gel P-100 gel filtration in 0.02 M-sodium acetate pH 5.0, 0.1 M in NaCl. For explanation of symbols see legend of Figure 1. The bar indicates fractions pooled for isoelectric focusing.

Figure 3. Activity on barley β -glucan (—) and protein contents (---) in eluates from gel sections of the preparative flat bed isoelectric focusing. The activity is expressed in μ equiv. glucose formed/min per ml of eluate. Material from section 20 was taken as the pure endo- β -glucanase.

3.2. Physical and chemical characterization of endo- β -glucanase

In polyacrylamide gel electrophoresis at pH 8.8 or in dodecyl sulphate/polyacrylamide gel electrophoresis using 1-50 μg of the endo- β -glucanase a single protein band appeared (Figure 4). A further indication of the homogeneity was obtained by sedimentation equilibrium analysis for which a plot of the logarithm of the fringe displacement versus the square of the radial distance gave a straight line (Figure 5). Using the sedimentation equilibrium data in combination with the partial specific volume, calculated to be $0.717 \text{ cm}^3 \times \text{g}^{-1}$, a molecular weight of 23,500 was found for the endo- β -glucanase. Estimation of the molecular weight of the endo- β -glucanase by its elution position from the Bio-Gel P-100 (marker

Figure 4. Polyacrylamide gel electrophoresis of 50 μg of the purified endo- β -glucanase stained for protein. The arrow indicates the position of the front.

Figure 5. Sedimentation equilibrium of the endo- β -glucanase. Plot of the logarithm of fringe displacement versus the radial distance squared (cm^2).

proteins: bovine serum albumin, chymotrypsinogen, and ribonuclease) under non-denaturing conditions and of the reduced, denatured enzyme by polyacrylamide electrophoresis in dodecyl sulphate gave values of 22,500 and 24,000, respectively. The molecular weights determined by different techniques thus agree well, and are consistent with the enzyme possessing a single polypeptide chain.

Isoelectric focusing in the LKB 8101 column of the endo- β -glucanase pool from Bio-Gel P-100 chromatography (see section 2.2.) revealed one major enzyme component of isoelectric point 4.47 at 4°C . The amino acid composition showing excess of free carboxylate groups over basic groups (Table II) was consistent with the acidic isoelectric point. Most probably the two half-cystine groups of the endo- β -glucanase, formed a disulfide bridge or were deeply buried sulfhydryls as iodoacetate treatment of the enzyme at non-denaturing conditions did not result in incorporation of carboxymethyl groups. The enzyme preparation contained less than 0.2 weight per cent carbohydrate as determined by the phenol-sulphuric acid procedure and showed no carbohydrate when tested by the periodate-Schiff staining after gel electro-

phoresis. The molecular weight calculated on the basis of the amino acid composition was 23.653 in agreement with the values obtained by other procedures.

3.3. Enzymic properties

The *A.niger* β -glucanase showed the highest activity on the mixed-linked substrates, barley β -glucan and lichenin (Table III). A much lower rate of hydrolysis was found for the 1,4- β -glucans CM-cellulose, acid-swollen cellulose, and cellodextrins. The 1,3- β -glucans, laminarin and CM-pachyman, seemed not degraded to

any significant extent. It cannot be excluded though that the negligible amounts of reducing sugar formed from the 1,3- β -glucans were due to a contaminating enzyme, but this seems unlikely because the hydrolysis stopped after a rather short period of incubation in contrast to previous findings on 1,3- β -glucan cleavage by *A. niger* enzymes (5, 22). The endo-mode of action of the present enzyme was demonstrated by viscometry (Figure 6). Exhaustive digestion of barley β -glucan gave a mixture of oligosaccharides with the composition shown in Table IV. The products were not structurally characterized.

Table II

Amino acid composition of endo- β -glucanases from *Aspergillus niger*

Amino acid	Residues per molecule			
	Experimental values (Leu=10)	Nearest integer (this paper)	HURST et al. (20)	IKEDA et al. (24a)
Aspartic acid	22.0	22	28	53
Threonine	21.8a)	22	24	32
Serine	31.0a)	31	26	36
Glutamic acid	20.6	21	24	30
Proline	7.8	8	10	7
Glycine	20.8	21	28	47
Alanine	15.6	16	20	32
Valine	14.0b)	14	16	22
Methionine	1.9	2	3	6
Isoleucine	6.8b)	7	9	22
Leucine	10.0	10	7	23
Tyrosine	10.7	11	15	27
Phenylalanine	8.9	9	11	16
Histidine	2.7	3	4	4
Lysine	7.9	8	8	8
Arginine	0.95	1	2	5
Ammonia	24.1c)	24	—	—
Half-cystine	2.0d)	2	0-1	2
Tryptophan	10.0e), 10.3f)	10	6	7
Total number of residues		218	241	379
Molecular weight		23.653	25.900	44.232g)

a) Extrapolated to zero-time of hydrolysis

b) Value of 72 h hydrolysis

c) Corrected for NH_3 formed by destruction of threonine and serine (16)

d) As cysteic acid

e) After methanesulfonic acid hydrolysis

f) Determined spectrophotometrically

g) Includes 12 glucosamine and 10 hexose residues per molecule

Table III

Substrate specificity of endo- β -glucanase

Substrate	Substrate concentration (%, w/v)	Enzyme concentration (M $\times 10^6$)	Formation of reducing sugar ^{a)} Time of incubation (h)			
			0.25	3	24	48
Barley β -glucan	0.7	1.3	2500	-	2700	-
Lichenin	0.5	1.3	1800	-	1900	-
CM-cellulose	0.7	1.3	200	390	655	750
Acid-swollen cellulose	0.7	1.3	25	55	83	110
Cellodextrins (DP 3-10)	0.1	4.4	160	210	-	245
Laminarin	0.7	1.3	10	12	10	-
CM-pachyman	0.7	1.3	4	6	6	-
Cellobiose	0.1	1.3	0	0	0	0
p-Nitrophenyl- β -D-glucoside ^{b)}	0.1	4.4	0	-	0	-

a) The reactions were performed in 0.025 M-sodium acetate pH 5.0 at 40°C and aliquots were removed at the indicated time intervals. The amount of reducing sugar formed per ml of reaction mixture is expressed by the amount of glucose (in μ g) of identical reducing power.

b) Determined a.m. OKADA (33).

The pH-dependence of the activity on barley β -glucan was determined in the range pH 3.0-7.2 using McIlvaine citrate-phosphate buffers. The pH optimum was rather sharp around pH 4.8, in contrast to the *A. niger* endo-1,4- β -glucanase described by CLARKE and STONE (6). The enzyme described by HURST et al. (20, 22) showed a sharp pH optimum at about pH 4.0 on CM-cellulose and barley β -glucan in acetate buffers.

Figure 6. Hydrolysis of barley β -glucan by 4.9×10^{-8} M endo- β -glucanase as followed by the decrease in specific viscosity (○) and release of reducing sugars (●) expressed as $100 \times (\text{reducing sugars}/\text{total carbohydrate})$ using glucose as standard.

Table IV

Oligosaccharides produced in exhaustive hydrolysis of barley β -glucan by endo- β -glucanase

Product	Amount ^{a)} (%)
Glucose	4.3
Disaccharide	4.2
Trisaccharide	72.7
Tetrasaccharide	18.8

The enzyme concentration was 2.1×10^{-8} M

a) Expressed in per cent of the total amount of eluted sugar.

The K_m for the hydrolysis of barley β -glucan and CM-cellulose was 1.8 and 11 mg/ml, respectively, the corresponding molecular activities being 7750 and 750 μ equiv. of glucose released per min per mole of enzyme. The kinetic parameters thus clearly demonstrate that the enzyme has higher affinity and turnover number for the 1,3;1,4- as compared with the 1,4- β -glucans.

The enzyme had no requirement for divalent metal ions as it was fully active in 0.001 M-EDTA. The β -glucosidase inhibitor, glucono-1,5-lactone, did not affect the activity at a concentration of 0.0025 M. The enzyme seemed not

to contain any essential sulfhydryl group since the activity towards barley β -glucan did not change in the presence of 1×10^{-4} M-*p*-hydroxy-mercuribenzoate or by treatment with iodoacetate. In contrast chemical modification with N-bromosuccinimide for 3 and 8 min destroyed 97.3% and 99.6%, respectively, of the initial activity on barley β -glucan, indicating one or more tryptophan residues to be essential for the enzymic activity. HURST et al. (21) recently showed that an endo-1,4- β -glucanase from *A.niger* lost activity completely following N-bromosuccinimide oxidation of a single tryptophan per enzyme molecule.

4. DISCUSSION

In terms of its properties as a protein the endo- β -glucanase isolated in the present study differs from other purified *A. niger* enzymes. It has a molecular weight of 23.700 which is similar to, but lower than, the molecular weights of 25.900 and 44.200 found for the endo-1,4- β -glucanases isolated by HURST et al. (20) and IKEDA et al. (24), respectively. The amino acid compositions of these three enzyme preparations closely resemble each other (Table II), but significant differences do exist (20, 24a). The enzyme described in the present study is the smallest of the three, but cannot be a degraded form of either of the other enzymes, since it contains more tryptophan than either of them, more leucine and half-cystine than the 25.900 molecular weight enzyme, and slightly more proline than the largest (compare Table II). Thus based on chemical properties the present endo- β -glucanase represents a different protein, but shows only subtle differences from the endo-1,4- β -glucanase described by HURST et al. (20).

Comparison of the enzymic properties of the present and some earlier studied *A. niger* endo- β -glucanases reveals many similarities and a few differences. The Glucanase GV enzyme mixture appears to contain two types of endo- β -glucanases as judged from the chromatographic behaviour on DEAE-cellulose. One type with essentially equal activity on CM-cellulose and barley β -glucan has high affinity for DEAE-cellulose and the second type with very high ac-

tivity on barley β -glucan and low activity on CM-cellulose has a lower affinity (compare Figure 1). The high affinity of cellulases from various sources for DEAE-cellulose has previously been demonstrated by MARSHALL and found useful in their purification (29).

The fractions containing the second type of endo- β -glucanase, which was later used for the purification of the enzyme, had about 60 times higher activity on barley β -glucan than on CM-cellulose under the assay conditions employed. HURST et al. (22) also found that the mixed-linked β -glucans were the most amenable to hydrolysis by their purified endo-1,4- β -glucanase. Thus at a substrate concentration of 0.1% the production of reducing end groups could be detected from the action of the enzyme at a tenth of the concentration needed to produce detectable hydrolysis of 1% CM-cellulose (22). However, as HURST and coworkers (22) point out, the apparent preference for the mixed-linked glucan substrate compared with the cellulosic substrate is likely to be somewhat exaggerated, when CM-cellulose of a relatively high degree of substitution, 0.7, is used, since there will be fewer glucosyl linkages involving unsubstituted glucose than in CM-cellulose with a degree of substitution of 0.45-0.55 used for example by KRISHNA MURTI and STONE (25).

The Glucanase GV endo- β -glucanase(s) of high affinity for DEAE-cellulose appear(s) comparable with a highly purified endo-1,4- β -glucanase isolated by CLARKE and STONE (5). This enzyme degraded barley β -glucan, lichenin (7), and CM-cellulose (5), but had in contrast to the Glucanase GV enzyme with low affinity for DEAE-cellulose little activity on phosphoric acid-swollen cellulose (5). A crude enzyme fraction corresponding to the fraction used by CLARKE and STONE (5) in the purification of this endo-1,4- β -glucanase had previously been found by KRISHNA MURTI and STONE (25) to hydrolyze lichenin less readily than CM-cellulose. It is clear however, that *A. niger* can synthesize more than one endo- β -glucanase hydrolyzing mixed-linked β -glucans and the preparations described by CLARKE and STONE (5-7), HURST et al. (20-22), and in the present study, respectively, are closely related in

specificity, but do not represent identical enzymes.

Kinetic parameters for the hydrolysis of mixed-linked β -glucans by other fungal enzymes are not available. However, data exist for endo-1,4- β -glucanases from snail and pea; the K_m for lichenin and CM-cellulose for the snail enzyme (31) and CM-cellulose for the pea enzyme (42) being similar to the K_m of the *A. niger* enzyme acting on barley β -glucan. The turnover numbers of the pea enzyme on barley β -glucan and CM-cellulose are identical and are an order of magnitude higher than the value obtained for the *A. niger* enzyme using barley β -glucan as substrate. The molecular activity of the *A. niger* enzyme on the mixed-linked substrate is similar to the values found for some of the *Sporotrichum pulverulentum* endo-1,4- β -glucanases acting on CM-cellulose (2).

The ratios of the activities on mixed-linked β -glucans and CM-cellulose can be compared for the pea (42), the snail (31), and the present *A. niger* enzyme without the reservation concerning the degree of substitution of the CM-cellulose substrate, since it was about 0.7 in all cases. Unlike the *A. niger* enzyme both the endo-1,4- β -glucanase from pea and snail were found to be almost equally active on both types of substrate (42, 31).

The isoelectric points (1), pH-optima (6, 20, 24b), sensitivity to chemical modification (3, 21, 24a), and ability to degrade mixed-linked β -glucans (5, 22, 23, 28, 34, 35) of fungal endo-1,4- β -glucanases are comparable with the corresponding properties of the *A. niger* enzyme reported in this paper. However, although its substrate specificity and the endo-mode of action may justify its classification as an endo-1,4- β -glucanase (cellulase, EC 3.1.2.4), such a classification has not been made because of the preference of the enzyme for mixed-linked glucan substrates as compared with cellulosic substrates and also because the identity of the hydrolysis products of the barley β -glucan has not been determined and therefore the point of cleavage not clearly decided.

In the case of the *A. niger* endo-1,4- β -glucanase of CLARKE and STONE (7) the hydrolysis products were all terminated at the reducing end by 1,4-linkages, in line with the

observation made by PARRISH et al. for the *Streptomyces* enzyme products (34). From the amounts of tri- and tetrasaccharide formed after exhaustive hydrolysis of barley β -glucan with the purified Glucanase GV endo- β -glucanase, it seems likely that this enzyme acts in a similar manner. However, in contrast to CLARKE and STONE (7), neither HURST et al. (22) nor ourselves find oligosaccharides larger than a tetrasaccharide after exhaustive hydrolysis of lichenin and barley β -glucan, respectively. The interpretation of these findings depends on a knowledge of the fine structure of the mixed-linked β -glucans, which is not available for the preparations used. In barley β -glucan runs of 1,3-linkages have been demonstrated by chemical analysis, although in some preparations the numbers are quite low (13, 14). The absence of higher oligosaccharides after hydrolysis thus suggests either the absence of contiguous 1,3-linkages in the substrates or that such structures are present, but the corresponding oligosaccharides produced during the enzymic degradation are further degraded by contaminating exo-1,3- β -glucanase, as demonstrated by CLARKE and STONE (7). A definitive classification of the purified *A. niger* endo- β -glucanase thus awaits additional studies of its action on fully characterized oligo- and polysaccharides.

ACKNOWLEDGEMENTS

Professor M. OTTESEN is thanked for his encouragement during the course of this work. I am indebted to professor B. A. STONE for criticism and many helpful suggestions during the preparation of the manuscript. I would also like to thank Drs. B. S. ENEVOLDSEN and K. ERDAL for inspiring discussions, gifts of several substrates and gel filtration analysis on barley β -glucan hydrolysates. Dr. T. GRAVES PEDERSEN performed the sedimentation equilibrium analysis. Finally the technical assistance of Mss. A. BAYNE, B. CORNELIUSSEN and E. FLØISTRUP is gratefully acknowledged.

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