PURIFICATION AND AMINO ACID SEQUENCE DETERMINATION OF AN ENDO-1,3- β -GLUCANASE FROM BARLEY

by

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An endo-1,3- β -glucanase was purified from malted barley by ion exchange column chromatography. The purified enzyme protein was demonstrated to be free of other proteins by SDS-polyacrylamide gel electrophoresis and had a molecular weight of 32,000. The amino acid composition of the protein was determined and the complete amino acid sequence was constructed from overlapping peptides generated by enzymatic as well as chemical cleavages. The molecule contains 306 amino acids arranged in one peptide chain. The molecular weight calculated from the sequence is 32,286. The sequence of the 1,3- β -glucanase shows approximately 50% positional identity with two other β -glucanases from barley and tobacco, respectively.

1. INTRODUCTION

During germination the endosperm cell walls of cereal grains are degraded to facilitate the access of amylolytic and proteolytic enzymes to the cell contents. The cytolytic activities which change most significantly during this period are those of the endo-1,3;1,4- β -glucanase and endo-1,3- β -glucanase enzymes (4, 19). These enzymes are responsible for the degradation of the β -glucan constituents of the endosperm cell walls. Extensive attention has been given to the endo-1,3;1,4- β -glucanase because of the major role it plays in degrading mixed-linkage β -glucans (6, 12, 17, 26) which form the main constituent of

barley endosperm cell walls (2, 11). Although the barley endo-1,3- β -glucanase has long been recognized as a separate enzyme from the endo-1,3;1,4- β -glucanase (6, 16), its function in the kernel is still not fully understood. The enzyme is specific for 1,3- β -glucans and will not degrade the mixed-linkage β -glucan to oligosaccharides (20). It thus belongs to the enzyme category EC 3.2.1.39 as opposed to EC 3.2.1.6. A potential substrate in barley and wheat for this enzyme is the aniline blue stainable deposits localized in the aleurone-subaleurone junction region (1, 13). These deposits in barley have now been shown to be degradable by endo-1,3- β -glucanase

Abbreviations: CM = carboxymethyl; CNBR = cyanogen bromide; DEAE = diethylaminoethyl; DPCC = diphenylcarbamyldichloride; HPLC = high performance liquid chromatography; PTH = phenylthiohydantoin; SDS = sodium dodecyl sulphate; TFA = trifluoroacetic acid.

purified in this study (MACGREGOR, BALLANCE and DUSHNICKY, manuscript in preparation) adding further evidence of the 1,3- β -glucan nature of these deposits. However, the level of enzyme activity produced during germination would seem excessive if the degradation of these deposits was the enzyme's sole function. It has also been reported that endo-1,3- β -glucanase has an effect on the mixed-linkage β -glucans isolated from both barley and malt (7) and can cause the solubilization of insoluble mixed-linkage β -glucans (2, 22). As part of a project to study the regulation of the synthesis of these enzymes, the endo-1,3- β -glucanase enzyme was purified and sequenced.

2. MATERIALS AND METHODS

2.1. Purification of endo-1,3- β -glucanase

Commercial malt prepared from Havila barley was used as the source material for preparation of the endo-1,3- β -glucanase. The procedure followed for the extraction, concentration and ammonium sulphate fractionation was as described by BREDDAM et al. (9). The 38% ammonium sulphate precipitated fraction was found to be rich in endo-1,3- β -glucanase activity and was recovered by centrifugation and lyophilized. Extraction of this enriched fraction was carried out with distilled water (20 g/100 ml) by stirring for 2 h at 4 °C. The extract was clarified by centrifugation at 10,000 g for 20 min and the supernatant dialysed for 48 h against 10 mM Tris-HCl pH 8.5 (4×4 L) in the cold. The dialysed sample was centrifuged to remove precipitated material. The supernatant (150 ml) was adjusted to pH 8.5 and loaded onto a DEAE-cellulose column (5.5×11 cm) equilibrated with the above pH 8.5 buffer. The flow-through volume plus a 600 ml wash was collected as a single fraction as the activity is not retained by the column. This fraction (750 ml) was adjusted to pH 7.5 with dilute HCl and loaded onto a CM-cellulose column (2.6×35 cm) equilibrated with 10 mM Tris-HCl pH 7.5 buffer. The column was washed with equilibration buffer (200 ml) before being eluted with a 1300 ml salt gradient (0 to 0.24 M NaCl) in equilibration buffer while 15 ml fractions were collected. Fractions were tested for 1,3- β -glu-

canase activity and the absorbance measured at 280 nm. Selected fractions containing activity were pooled, concentrated and run through two additional CM-cellulose columns (2.6×35 cm) in 20 mM NaOAc equilibrated at pH 4.0 and 5.5, respectively. In each case the column fractions were analysed for glucanase activity and absorbance at 280 nm before selected fractions were pooled, concentrated by ultrafiltration and dialysed against the next column equilibration buffer. The two columns were eluted with linear gradients of NaCl (0–0.4 M, 1400 ml; 0–0.3 M, 1100 ml, respectively) in the equilibration buffer.

2.2. Enzyme activity

Endo-1,3- β -glucanase activity was routinely measured during purification by incubation of aliquots (20 μ l) with 0.2% laminarin (200 μ l) in 0.2 M NaOAc pH 5.0 buffer at 35 °C. Reducing sugars were measured colourimetrically by the Nelson-Somogyi method using the reagent formulations outlined by ROBYT and WHELAN (21).

2.3. Electrophoresis

The purity of the final enzyme preparation and the protein molecular weight were tested by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis using the buffer system of LAEMMLI (15) and a 12.5% separating gel. Gels were fixed in a solution of 5 parts methanol, 5 parts water, 1 part glacial acetic acid for 30 min before being stained with a 0.05% of Coomassie Brilliant Blue in fix solution for 1 h. Gels were destained in several changes of a solution consisting of 2 parts methanol, 1 part acetic acid and 7 parts water.

2.4. Amino acid analysis

Prior to hydrolysis the protein was reduced with dithiothreitol and alkylated with 2-vinyl pyridine following a procedure described by SVENSSON et al. (25). The pyridylethylated protein was hydrolyzed in 6 N HCl at 110 °C for 24, 48 and 72 hrs in evacuated and sealed tubes and analyzed on a Durrum 500 amino acid analyzer. Raw data were corrected to account for

the partial destruction of threonine and serine during hydrolysis by extrapolation to 0-time hydrolysis. Tryptophan was estimated by second derivative spectroscopy according to the procedure of SERVILLO et al. (1982) (23).

2.5. Protein cleavage and peptide isolation

Cleavage with cyanogen bromide: To 1 mg of protein in 0.5 ml 70% formic acid was added 10 mg CNBr. The reaction mixture was left overnight under nitrogen at 22 °C then flushed with nitrogen to reduce the volume and remove most of the CNBr. The peptides were separated by HPLC on a Vydac C₄ column using a 0 to 90% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). The eluate was monitored at 210 nm and appropriate peaks lyophilized and sequenced.

Cleavage with hydroxylamine was performed as described by BORNSTEIN and BALIAN (8). The resulting peptides were separated on a BioGel P-60 column in 30% acetic acid, monitored at 280 nm and relevant peaks pooled and lyophilized.

Cleavage with o-iodosobenzoic acid in the presence of tyramine was done in 80% acetic acid containing 4 M guanidinium chloride for 5 hrs in the dark at room temperature (18). The resulting peptides were desalted on a BioGel P-6 (0.9×60 cm) column equilibrated with 30% acetic acid. Further separation of peptides was done by HPLC on a Vydac C₄ column using a linear gradient of acetonitrile (10 to 60% in 0.1% TFA).

Tryptic peptides were prepared by digestion with DPCC-trypsin. Glucanase (1 mg) in 50 mM Tris-HCl pH 7.8 (800 μ l) was placed in a boiling water bath for 2 min to partly denature the protein then cooled on ice. To this sample was added 30 μ l of a trypsin solution (1.0 mg/ml), and the digest incubated at 40 °C for 20 h. The digest was then adjusted to be 30% acetic acid and separated on a BioGel P-30 column (0.9×20 cm) equilibrated with 30% acetic acid. The peak of included material eluting from the column was pooled, diluted with 20 ml of water and lyophilized. This fraction was redissolved in 0.1% TFA and separated by HPLC on a Ultrasphere-ODS C18-reverse phase column (Beck-

man Instruments, Inc.). The elution gradient was 0-80% 2-propanol in 0.1% TFA.

Cleavage with activated clostripain was made in 20 mM ammonium bicarbonate containing 3 M urea at 40 °C overnight. After acidification the volume was reduced in a vacuum centrifuge and then the reaction mixture was applied to a Vydac C₄ HPLC column and the peptides eluted with a linear gradient in acetonitrile (10 to 60% in 0.1% TFA).

Cleavage with endoproteinase Lys-C was made at pH 8 in 25 mM Tris buffer containing 5 M urea for 30 hrs. at 30 °C. HPLC-separation of peptides was conducted on a Vydac C₄ as described above. Cleavage with *S. aureus* proteinase V8 was done at pH 7.8 in 0.1 M ammonium bicarbonate containing 3 M urea at 39 °C overnight. HPLC-separation of the resulting peptides was made on a Vydac C₄ column as described above.

Digestion with carboxypeptidase Y was carried out at pH 4.5 in NaOAc buffer at room temperature with an enzyme to substrate ratio of 1:50. Aliquots were removed at suitable time intervals for determination of released amino acids on a Durrum 500 amino acid analyzer.

2.6. Amino acid sequence analysis

Amino acid sequence analyses were performed on a gas-phase sequencer (Applied Biosystems model 470A) equipped with an on line HPLC detection system (Applied Biosystems model 120A PTH analyser) according to the manufacturer's instructions.

3. RESULTS AND DISCUSSION

Previous work had shown that the cereal 1,3- β -glucanases are basic proteins (3, 5) and this has recently been confirmed for a barley 1,3- β -glucanase (14). This feature was used to selectively remove acidic and neutral proteins on DEAE-cellulose at pH 8.5 while allowing the 1,3- β -glucanase to pass through unbound. This preliminary clean-up also removes most of the coloured material which is very tightly bound to the column. The 1,3- β -glucanase enzyme was subsequently separated from the other basic proteins on CM-cellulose at pH values of 7.5,

Figure 1. Purification of endo-1,3- β -glucanase from malted barley. The unbound fraction from the DEAE-cellulose column was separated on CM-cellulose at pH 7.5 using a linear NaCl gradient (---). Absorbance at 280 nm (---) and the change in absorbance at 600 nm (—) were used to monitor protein elution and the enzyme activity, respectively. The solid bar indicates the fractions which were pooled for further purification.

Figure 3. Rechromatography on CM-cellulose at pH 5.5 of the pooled fractions from the pH 4.0 column. The line designations are as described in Figure 1.

4.0 and 5.5 (Figs. 1-3), respectively. Fractions were pooled from the first (Fig. 1) and second columns (Fig. 2) to exclude major peaks of contaminating protein. This also resulted in the exclusion of significant amounts of 1,3- β -glucanase activity. The final column (Fig. 3) separated a peak of protein which was roughly equivalent to the glucanase protein peak. The final protein fraction pooled was free of contam-

Figure 2. Rechromatography on CM-cellulose at pH 4.0 of those fractions pooled from the first CM-cellulose column. The line designations are the same as in Figure 1.

Figure 4. SDS-polyacrylamide gel of purified barley endo-1,3- β -glucanase. Lane 1 shows standard protein molecular weight markers (92 k, 68 k, 45 k, 30 k, 20.1 k, 14.4 k) and lanes 2 and 3 the purified enzyme (5 and 2 μ g, respectively).

Table I. Amino acid composition of barley endo-1,3- β -glucanase with comparison data for two related glucanases.

Amino Acid	Number of Residues			
	1,3-β-Glucanase (Barley)		1,3;1,4-β-Glucanase (Barley) ^a	1,3-β-Glucanase (Tobacco) ^a
	From A.A.A.	From sequence		
Asp	–	12	6	13
Asn	–	27	24	26
Asx	39.0	39	30	39
Thr	15.3	15	15	12
Ser	24.4	25	23	26
Glu	–	8	6	12
Gln	–	11	10	10
Glx	20.7	19	16	22
Pro	15.7	15	18	21
Gly	30.8	30	32	29
Ala	42.9	43	43	22
Val	20.6	21	30	21
Met	4.7	5	11	7
Ile	16.5	17	11	20
Leu	22.4	22	17	25
Tyr	15.4	16	15	17
Phe	12.7	13	14	14
His	1.2	1	7	6
Lys	9.6	10	9	15
Arg	14.4	12	8	13
Trp	3.0 ^b	2	4	6
Cys	2.0 ^c	1	2	1
Residues		306	305	316
Mwt		32,286	32,061	34,891

^a Based on the proposed mature protein sequence (24) of the polymorph represented in cDNA clone pGL43 (12, 24).^b Estimated by second derivative spectroscopy (23).^c Determined as 2-pyridylethylcysteine.

inating proteins as judged by SDS-polyacrylamide gel electrophoresis (Fig. 4). On the basis of comparison to standard molecular weight markers, the 1,3- β -glucanase has a molecular weight of approximately 32,000.

The amino acid composition of the barley 1,3- β -glucanase was calculated using the number of Gly, Ala and Leu determined by sequencing and the amino acid analysis data (Table I). It is seen that a fair agreement exists between number of residues determined from amino acid analysis and sequencing. A major discrepancy is found in the number of Cys residues. Although 2 Cys were determined as the

pyridylethylated derivative only one Cys was found by sequencing. 1,3; 1,4- β -glucanase from barley contains 2 Cys residues one of which is in the same position as the one in 1,3- β -glucanase. Due to shortage of material no attempt has been made to determine whether the single Cys was free or blocked. Included is also data for two other β -glucanases. For certain amino acids (Val, Met, Ile, and Arg) the 1,3- β -glucanase from barley is very similar in its composition to that of the corresponding enzyme from tobacco while for other amino acids (Ala and Lys) it shows a strong correspondance to the 1,3;1,4- β -glucanase. It is noteworthy that 1,3- β -glucanase

Figure 7. Comparison of the amino acid sequence of 1,3- β -glucanase from barley with those of 1,3;1,4- β -glucanase from barley (12) and 1,3- β -glucanase from tobacco (24). The tobacco glucanase is polymorphous in four positions (160, 265, 268 and 316), only one of the amino acids is included in this comparison. Gaps are introduced in order to obtain maximal homology. Identical amino acids in barley 1,3- β -glucanase and either of the other two enzymes are boxed.

24). This homology also extends to the 1,3- β -glucanase from barley for the 240-248 region and the 282-289 region.

That both of these regions have been so well conserved would suggest they are essential to some function shared by all three enzymes. This is unlikely to be the catalytic site as unlike the two 1,3- β -glucanases which cleave a 1,3-linkage, the 1,3;1,4- β -glucanase cleaves a 1,4-linkage (17). A more likely location is the binding site adjacent to the catalytic site. All three enzymes require a 1,3- β -linked glucose disaccharide on the non-reducing side of the substrate cleavage site for hydrolysis to occur.

For the His residues, of the 6 in the tobacco enzyme and 7 in the 1,3;1,4- β -glucanase only the His at position 269 (Fig. 7) coincides. The single His residue in the barley 1,3- β -glucanase is also conserved in this position. This may again argue for some essential role for the residue at this position in these enzymes.

An N-terminal sequence of another barley

1,3- β -glucanase has recently been reported by Høj et al. (14). That sequence shows a 54% positional identity with the 1,3;1,4- β -glucanase and a 65% positional identity with the sequence reported here. The two 1,3- β -glucanase sequences are thus likely derived from two different β -glucanase encoding genes. The presence of several isoenzymic forms of 1,3- β -glucanase in rye has been reported previously (3). In the present study the purification procedure used excluded significant amounts of activity during the pooling of fractions. This may have resulted in the isolation of a particular isozyme while the procedure used by Høj et al. (14) may have yielded a different isozyme. It is interesting if not surprising, however, that the N-terminal sequences of these isozymes are so different (16 of 46 amino acids) relative to the number of differences observed (7 of 46 and 3 of 40, respectively) in the N-terminal regions of mature isozymic forms of α -amylase (10) and barley 1,3;1,4- β -glucanases (27).

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REFERENCES

1. BACIC, A. & B.A. STONE: Isolation and ultrastructure of aleurone cell walls from wheat and barley. *Aust. J. Plant Physiol.* 8, 453-474 (1981)
2. BALLANCE, G.M. & D.J. MANNERS: Structural analysis and enzymic solubilization of barley endosperm cell walls. *Carbohydrate Res.* 61, 107-118 (1978)
3. BALLANCE, G.M. & D.J. MANNERS: Partial purification and properties of an endo-1,3- β -glucanase from germinated rye. *Phytochem.* 17, 1539-1543 (1978)
4. BALLANCE, G.M. & W.O.S. MEREDITH: Development of cytolytic enzyme activities during germination of different barleys. *Can. J. Plant Sci.* 54, 223-229 (1974)
5. BALLANCE, G.M. & W.O.S. MEREDITH: Purification and partial characterization of an endo- β 1,3-glucanase from green malt. *J. Inst. Brew.* 82, 64-67 (1976)
6. BASS, E.J. & W.O.S. MEREDITH: Enzymes that degrade barley gums. VII. Fractionation by column chromatography. *Am. Soc. Brewing Chemists, Proc.* 33-47 (1960)
7. BATHGATE, G.N., G.H. PALMER & G. WILSON: The action of endo 1,3- β -glucanases on barley and malt β -glucans. *J. Inst. Brew.* 80, 278-285 (1974)
8. BORNSTEIN, P. & G. BALIAN: Cleavage of Asn-Gly bonds with hydroxylamine. *Meth. Enzymol.* 47, 132-145 (1977)
9. BREDDAM, K., S.B. SØRENSEN & M. OTTESEN: Isolation of carboxypeptidase II from malted barley by affinity chromatography. *Carlsberg Res. Commun.* 50, 199-209 (1985)
10. CHANDLER, P.M., J.A. ZWAR, J.V. JACOBSEN, T.J.V. HIGGINS & A.S. INGLIS: The effect of gibberellic acid and abscisic acid on the α -amylase mRNA levels in barley aleurone studies using an α -amylase cDNA clone. *Plant Mol. Biol.* 3, 407-418 (1984)
11. FINCHER, G.B.: Morphology and chemical composition of barley endosperm cell walls. *J. Inst. Brew.* 81, 116-122 (1975)
12. FINCHER, G.B., P.A. LOCK, M.M. MORGAN, K. LINGELBACH, R.H.E. WETTENHALL, J.F.B. MERCER, A. BRANDT & K.K. THOMSEN: Primary structure of the (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- β -D-glucan 4-glucanohydrolase from barley aleurone. *Proc. Natl. Acad. Sci. USA* 83, 2081-2085 (1986)
13. FULCHER, R.G., G. SETTERFIELD, M.E. MCCULLY & P.J. WOOD: Observations on the aleurone layer. II. Fluorescence microscopy of the aleurone sub-aleurone junction with emphasis on the possible β -1,3 glucan deposits in barley. *Aust. J. Plant Physiol.* 4, 917-928 (1977)
14. HØJ, P.B., A.M. SLADE, R.E.H. WETTENHALL & G.B. FINCHER: Isolation and characterization of a (1,3)- β -glucan endohydrolase from germinating barley (*Hordeum vulgare*): Amino acid sequence similarity with barley (1,3;1,4) β -glucanase. *FEBS Lett.* 230, 67-71 (1988)
15. LAEMMLI, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685 (1970)
16. LUCHSINGER, W.W., W.J. FERRELL & G.L. SCHNEBERGER: The mechanism of action of malt beta-glucanases. IV. The preparation and properties of laminarinase from germinated barley. *Cereal Chem.* 40, 554-563 (1963)
17. LUCHSINGER, W.W., C. SHIH-CHIEH & A.W. RICHARDS: Mechanism of action of malt β -glucanases. 8. Structure of products formed during hydrolysis of barley β -D-glucan by AII-endo- β -glucanase. *Arch. Biochem. Biophys.* 112, 524-530 (1965)
18. MAHONEY, W.C. & M.A. HERMODSON: High-yield cleavage of tryptophanyl peptide bonds by *o*-iodosobenzoic acid. *Biochemistry* 18, 3810-3814 (1979)
19. MANNERS, D.J. & J.J. MARSHALL: Studies on carbohydrate metabolizing enzymes. Part XII. The β -glucanase system of malted barley. *J. Inst. Brew.* 75, 550-561 (1969)
20. MANNERS, D.J. & G. WILSON: Purification and properties of an endo-(1-3)- β -D-glucanase from malted barley. *Carbohydr. Res.* 37, 9-22 (1974)
21. ROBYT, J.F. & W.J. WHELAN: The α -amylases. In: *Starch and Its Derivatives*. 4th ed., J.A. Radley, Ed., Chapman and Hall Ltd., London, 430-476 (1968)
22. SCOTT, R.W.: Solubilization of β -glucan during

- mashing. J. Inst. Brew. 78, 411-412 (1972)
23. SERVILLO, L., G. COLONNA, C. BALESTRIERI, R. RAGONE & G. IRACE: Simultaneous determination of tyrosine and tryptophan residues in proteins by second derivative spectroscopy. Anal. Biochem. 126, 251-257 (1982)
24. SHINSHI, H., H. WENZLER, J.-M. NEUHAUS, G. FELIX, J. HOFSTEENGE & F. MEINS JR.: Evidence for N- and C-terminal processing of a plant defence-related enzyme: The primary structure of tobacco pre-pro- β -1,3-glucanase. Proc. Natl. Acad. Sci. vol. 85, p. 5541-5545 (1988)
25. SVENSSON, B., K. LARSEN & I. SVENDSEN: Amino acid sequence of tryptic fragments of glucoamylase G1 from *Aspergillus niger*. Carlsberg Res. Commun. 48, 517-527 (1983)
26. WOODWARD, J.R. & G.B. FINCHER: Substrate specificity and kinetic properties of two (1-3),(1-4)- β -glucan endohydrolases from germinating barley (*Hordeum vulgare*). Carbohyd. Res. 106, 111-122 (1982)
27. WOODWARD, J.R., F.J. MORGAN & G.B. FINCHER: Amino acid sequence homology in two 1,3;1,4- β -glucan endohydrolases from germinating barley (*Hordeum vulgare*). FEBS Lett. 138, 198-200 (1982)

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