Substrate Binding and Catalytic Mechanism of a Barley \( \beta \)-\( \alpha \)-Glucosidase/(1,4)-\( \beta \)-\( \alpha \)-Glucan Exohydrolase* \\

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A \( \beta \)-glucosidase, designated isoenzyme \( \beta \)II, from germinated barley (Hordeum vulgare L.) hydrolyzes aryl-\( \beta \)-glucosides and shares a high level of amino acid sequence similarity with \( \beta \)-glucosidases of diverse origin. It releases glucose from the non-reducing termini of cellobextrins with catalytic efficiency factors, \( k_c/ \kappa \alpha \), that increase approximately 9-fold as the degree of polymerization of these substrates increases from 2 to 6. Thus, the enzyme has a specificity and action pattern characteristic of both \( \beta \)-glucosidases (EC 3.2.1.21) and the polysaccharide exohydrolases, (1,4)-\( \beta \)-glucan glucohydrolase (EC 3.2.1.74). At high concentrations (100 mM) of 4-nitrophenyl \( \beta \)-glucoside, \( \beta \)-glucosidase isoenzyme \( \beta \)II catalyzes glycosyl transfer reactions, which generate 4-nitrophenyl-\( \beta \)-laminaribioside, -cellobioside, and -gentiobioside. Subsite mapping with cellobioseglycosides indicates that the barley \( \beta \)-glucosidase isoenzyme \( \beta \)II has six substrate-binding subsites, each of which binds an individual \( \beta \)-glucosyl residue. Amino acid residues Glu181 and Glu391 are identified as the probable catalytic acid and catalytic nucleophile, respectively. The enzyme is a family I glycoside hydrolase that is likely to adopt a \( \alpha \)-barrel fold and in which the catalytic amino acid residues appear to be located at the bottom of a funnel-shaped pocket in the enzyme.

Two \( \beta \)-glucosidases of apparent molecular mass 62,000 have been purified from extracts of germinated barley grain (1–3) and can be classified in the family I group of glycosyl hydrolases and related enzymes (4). The two enzymes have been designated isoenzymes \( \beta \)I and \( \beta \)II, and have isoelectric points of 8.9 and 9.0, respectively. Amino acid sequence analyses reveal a single amino acid difference in the first 50 NH\(_2\)-terminal amino acid residues, and the complete amino acid sequence of isoenzyme \( \beta \)II has been deduced from the nucleotide sequence of a corresponding cDNA clone (2).

The function of the \( \beta \)-glucosidases in the germinated barley grain has not been defined unequivocally, but will clearly be related to their substrate specificities. It has been widely assumed that \( \beta \)-glucosidases prefer substrates of the type G-O-X, where G indicates the glycosyl residue and X can either be another glycosyl residue, for which the linkage position is not crucial, or a non-glycosyl aglycone group. As a result of the capacity of many \( \beta \)-glucosidases to hydrolyze glucosides with a range of glycosyl or non-glycosyl aglycone groups, non-physiological substrates such as 4-nitrophenyl \( \beta \)-glucopyranoside (4-NPG) have been synthesized to measure activity in convenient spectrophotometric assays; the barley enzymes have also been assayed in this way. It has further been assumed that the rate of hydrolysis of oligomeric substrates by \( \beta \)-glucosidases will remain approximately constant or decrease with increasing degree of polymerization (DP) of the substrate (5). However, the barley \( \beta \)-glucosidase isoenzyme \( \beta \)II hydrolyzes (1,4)-\( \beta \)-oligoglucosides much more efficiently than it hydrolyzes the aryl-\( \beta \)-glucoside 4-NPG (3). The increased hydrolytic rate with oligosaccharides is a characteristic often observed with polysaccharide exohydrolases (5), although the barley \( \beta \)-glucosidase is not able to hydrolyze polymeric (1,4)-\( \beta \)-glucans (2, 3).

These apparent anomalies led Hrmova et al. (3) to suggest that the barley enzyme could be classified either as a polysaccharide exohydrolase of the (1,4)-\( \beta \)-glucan glucohydrolase group (EC 3.2.1.74) or as a \( \beta \)-glucosidase of the EC 3.2.1.21 class. Because \( \beta \)-glucosidases are widely distributed in nature, a precise understanding of substrate specificity and the mechanisms of substrate binding and catalysis is essential for defining the functions and tracing the evolution of this important group of enzymes. Here, subsite binding energies of barley \( \beta \)-glucosidase isoenzyme \( \beta \)II have been calculated from kinetic data during the hydrolysis of a series of \( \beta \)-oligoglucoside substrates. The analyses indicate that the enzyme has at least six glucosyl-binding subsites. The catalytic amino acids have been defined, together with their disposition in the substrate-binding region. The substrate specificity, action pattern, putative catalytic residues, and subsite mapping data can all be reconciled with a three-dimensional model of the barley \( \beta \)-glucosidase, which is based on the x-ray crystal structure of an homologous cyanogenic \( \beta \)-glucosidase from white clover (6).

MATERIALS AND METHODS

Chemicals—The glucose diagnostic kit, 4-NPG, gentiobiose, laminarin, bovine serum albumin (BSA), diethiothreitol, glycine ethyl ester (GEE), and orcinol were purchased from Sigma. Condruritol B epoxide was from ICN (Costa Mesa, CA); [\(^{14}\)C]GEE was from American Radio labeled Chemicals (St. Louis, MO), t-1-tosylamido-2-phenylthyl chlomethyl ketone-treated trypsin was from Worthington, trifluoroacetic

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‡ The abbreviations used are: 4-NPG, 4-nitrophenyl \( \beta \)-glucopyranoside; BSA, bovine serum albumin; DP, degree of polymerization; EAC, 1-ethyl-3-(4-azoni-4,4-dimethylpentylcarboamide; GEE, glycine ethyl ester; G4G3Gred, 3-O-\( \beta \)-cellotriosyl-D-glucose; G4G4G3Gred, 3-O-\( \beta \)-cellotetraosyl-D-glucose; HCA, hydrophobic cluster analysis; 4-NP, 4-nitrophenyl; \( \beta \)-glucosidase, \( \beta \)-\( \alpha \)-glucoside glucohydrolase (EC 3.2.1.21); PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
Enzyme Purification—Barley β-D-glucosidase isoenzyme βII was purified from a homogenate of 8-day-old seedlings as described previously (3). Its purity was assessed by SDS-PAGE, where a single protein band was detected at high protein loadings. The purity was further confirmed by NH2-terminal amino acid sequence analysis; no secondary sequences were detected, and recoveries were very close to theoretical values (data not shown).

Protein determination during the purification process, SDS-PAGE, and amino acid sequence analyses were performed as described previously (3).

Enzyme Assays and Kinetic Analyses—Kinetic parameters on (1,4)-β- and (1,3)-β-oligoglucosides were measured at 37 °C by incubating 2–7 pmol of the purified β-glucosidase in 100 mM sodium acetate buffer, pH 5, containing 160 μg/ml BSA. Initial rates of hydrolysis were determined in triplicate at substrate concentrations ranging from 0.2 to 4 times the KM value. Enzymic reactions were stopped by heating to 100 °C for 2 min, and released glucose was measured by the glucose oxidase method (7). Standard deviations, which ranged between 1% and 7%, were calculated (8), and kinetic data were processed by a proportional weighted fit, using a nonlinear regression analysis program based on the Michaelis-Menten model equation (9). The initial enzyme concentration [E]0 was kept very much lower than the initial substrate concentration [S]0, and care was taken to measure initial reaction rates in all cases (10).

Initial hydrolysis rates of β-glucosidase isoenzyme βII with the synthesized aryl-glycosides 4-NP-glucosides (laminaridextrins) of DP 2–7 were from Seikagaku Kogyo Co. (Tokyo, Japan). 4-NP-glucoside glucosides were calculated using Michaelis constants (KM) synthesized aryl-glycosides 4-NP-glucosides (laminaridextrins) of DP 2–6 and (1,3)-β-oligoglucosides (cellotetraodextrins) of DP 2–7 were from Seikagaku Kogyo Co. (Tokyo, Japan).

Calculation of Subsite Affinities—Subsite affinities of β-glucosidase were calculated from Michaelis constants (KM) and catalytic rate constants (kcat) during the hydrolysis of (1,4)-β-oligoglucosides of DP 2–6. In addition, the affinities of subsites +2 and +3 for (1,3)-β-linked oligoglucosides were calculated using KM and kcat values obtained with (1,3)-β-oligoglucosides of DP 2–4.

Subsite affinities of β-glucosidase in 100 mM sodium acetate buffer, pH 5.0, were determined from the semi-logarithmic plots of residual activity as a function of time, using Equation 5.

\[
\frac{\ln (A_0/A_t)}{K_{n+1}} = -k_{\text{app}} t
\]

The equation uses the A0 and A values obtained for the (1,4)-β-oligoglucosides (Table I). The comparison of the experimental and theoretical hcat/KM values gave a maximum deviation of 0.4%, which confirms the validity of the model and the calculations of subsite affinities.

Glycosyl Transfer Reactions—Freshly prepared 4-NPG (100 mM) in 20 mM sodium acetate buffer, pH 5.0, was incubated with 2–3 pmol of the purified β-glucosidase for up to 72 h at 37 °C. The reaction was stopped by heating to 100 °C for 2 min. Aliquots of the reaction mixture were separated by thin layer chromatography on the paper plates and developed in ethyl acetate/acidic acid/H2O (5:2:1, by volume). Reducing sugars were detected using the orcinol reagent (12). Individual products were scraped off the plates, eluted from the Kieselgel with water, and their relative abundances determined spectrophotometrically at 300 nm using Δε of 1.104 μM −1 cm −1.

For structural analysis of the transglycosylation products, the reaction mixtures were scaled up 5 times and products were separated by descending paper chromatography on Whatman no. 3MM paper in ethyl acetate/acidic acid/H2O (3:2:1, by volume) at ambient temperature. The products were excised from the chromatogram, eluted from the paper with water, concentrated under reduced pressure, and analyzed using a Perkin-Elmer Sciex PSS 300 electropray ionization triple quadrupole mass spectrometer (Perkin Elmer Sci Instruments, Thornhill, Ontario, Canada) and by 13C NMR spectroscopy.

13C NMR Spectroscopy—13C NMR spectra of oligosaccharides (3–8 μmol) were measured on a Varian Gemini 300 multinuclear spectrometer using 5 mm external diameter sample tubes at a probe temperature of 297 K. Transients (32) were collected into 16,000 data points using a spectral width of 4.5 KHz and a relaxation delay of 3 s with a 45° pulse width. No Gaussian weight factor or line broadening was applied to the data before Fourier transformation. Spectra were referenced and chemical shifts (ppm) were given using sodium 2,2-dimethyl-2-silapentane-5-sulfonic acid as an external standard.

Inactivation of β-Glucosidase by Conduritol B Epoxyide—Inactivation of β-glucosidase isoenzyme βII was monitored at 37 °C by incubating 57 pmol of purified enzyme in 100 mM sodium acetate buffer, pH 5.0, containing 160 μg/ml BSA, with 0–10 mM conduritol B epoxyide. To stop the inactivation and to determine the residual activity at different times, 5-μl aliquots of the reaction mixture were diluted into 250 μl of 0.01 M sodium acetate buffer, pH 5.0, containing 160 μg/ml BSA. The residual enzyme activity was monitored spectrophotometrically at 410 nm. First-order rate constants (kapp) were determined from the semi-logarithmic plots of residual activity as a function of time, using Equation 5.

\[
\ln (A_0/A_t) = -k_{\text{app}} t
\]

The parameters A0 and At are enzyme activities at time 0 and t, respectively. The K and kapp constants were determined and the order of the inactivation reaction was estimated (13) to yield an inhibitor/enzyme stoichiometry of 0.7 (± 0.1) (data not shown).

Identification of the Catalytic Nucleophile—β-Glucosidase isoenzyme βII (3 μg) was inactivated at 37 °C by incubating the purified enzyme in 100 mM sodium acetate buffer, pH 5.0, in the presence of 24 μM conduritol B epoxide; this corresponded to 4.4 times the KM value. The inactivated enzyme, which lost 99% of its activity over 5 h, was concentrated, and excess inhibitor was removed in a microconcentrator (exclusion limit 10,000).

Native and conduritol B epoxide-inactivated enzymes were denatured and digested with trypsin, and the resulting tryptic peptides purified by HPLC and sequenced essentially as described by Chen et al. (14). The tryptic peptide-inhibitor conjugate (20 pmol), which was unique to the conduritol B epoxide-inactivated enzyme, was treated with 20 μl of ammonium hydroxide at 50 °C for 20 min to remove covalently bound conduritol. Excess ammonium was removed under vacuum, and the peptide was acid-extracted to NH2-terminal amino acid sequence analysis.

Identification of the Catalytic Acid—Purified enzyme (57 pmol) was inactivated with 15 mM 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carboximid (EAC) in the presence of 125 mM 1HCl (GE), the inactivated enzyme was denatured and digested with trypsin, and the resultant tryptic peptides were purified by HPLC for amino acid sequence analysis.
Laminarioligosaccharides

In addition, the molecular mass of the HPLC-purified peptide-inhibitor conjugate was estimated on a Finnigan Lasermat 2000 matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Finnigan MAT, Hemel Hempstead, UK).

Hydrophobic Cluster Analysis—Hydrophobic cluster analysis (HCA) plots were obtained using standard computer software and interpreted as described by Gaboriaud et al. (15). The amino acid sequence of the barley β-glucosidase isoenzyme βII was taken from Leah et al. (2), and the sequence of the cyanogenic β-glucosidase from white clover (Trifolium repens) was obtained from the Brookhaven Protein Data Bank (entry 1cbg).

Protein Modeling—Co-ordinates for a three-dimensional model of the barley β-glucosidase were obtained using Swiss-Model, the automated protein modeling service of the ExPasy Molecular Biology Server (16–18). The barley enzyme sequence and co-ordinates of the white clover β-glucosidase (6) (Brookhaven Data Bank entry 1cbg) were supplied as inputs for the program. The three-dimensional model of cellobiose was constructed from the co-ordinates of cellobiose (19). Fitting the cellobiose model into the active site pocket of the barley enzyme sequence was performed on a Silicon Graphics Iris Indigo Elan 4000 work station using the GRASP (20) and O (21) software programs. Cocrystallization of cellooctaose with the barley enzyme sequence was constructed from the cocrystallized cellooctaose model into the active site pocket of the barley enzyme sequence as described by Gaboriaud et al. (22). Protein models and stereoview diagrams were generated with the RasMo software program (22).

RESULTS

Kinetic Analysis—The relationship between the kinetic parameters (K_m, k_cat, and k_cat/K_m) and the DPs of oligosaccharide substrates of the barley β-glucosidase isoenzyme βII are shown in Table I. For cellobextrin substrates, k_cat values increase with increasing chain length of the substrate, while K_m values appear to be relatively independent of DP, except in the case of cellotriose and to a lesser extent cellotetraose, where K_m values are somewhat lower (Table I). Catalytic efficiency factors k_cat/K_m increase steadily with increasing DP of the (1,4)-β-oligoglucoside substrates, again with the exception of cellotriose. Indeed, the k_cat/K_m value for cellohexaose is 9-fold higher than the value for cellobiose.

In marked contrast to the hydrolysis of the cellobextrin series, the k_cat and k_cat/K_m values for laminaridextrins decreased by over 1000-fold and over 130-fold, respectively, as the DP increased from 2 to 4 (Table I). The rate of hydrolysis of laminarinpentose and longer (1,3)-β-oligoglucosides was too low to allow the precise measurement of kinetic parameters. The enzyme had no activity on the cyanogenic substrate, linamarin.

Subsite Mapping—Binding energies for the six β-glucosyl binding subsites in the barley β-glucosidase isoenzyme βII during hydrolysis of cellobextrins were compared in Fig. 1. Hrmova et al. (3) reported that the enzyme catalyzes the hydrolytic removal of glucose units from the non-reducing termini of oligosaccharide chains; this demonstrated that the enzyme is an exohydrolase. It is clear, therefore, that the catalytic amino acids are located between the non-reducing terminal glucosyl-binding subsite and the penultimate subsite; these are designated subsites −1 and +1, respectively (Fig. 1). Binding energies have been expressed in the past as positive or negative values (23–25), but we prefer to use positive values to indicate binding (10) (Fig. 1). The negative value observed at subsite +2 indicates that there is a degree of repulsion between the enzyme and the glucosyl residue at this subsite. The binding energies at subsites +3, +4, and +5 are positive, but decrease in magnitude as the distance from the catalytic site increases (Fig. 1). Although the barley β-glucosidase hydrolyzes laminaribiose at a slightly faster rate than cellobiose, the rate decreases steeply as the DP of the (1,3)-β-oligoglucoside substrates increases (Table I). Calculation of the apparent binding energies of these laminaridextrins to substrates +2 and +3 yielded values of

### Table I

| DP | K_m (mM) | k_cat (s⁻¹) | k_cat/K_m (10⁻³ s⁻¹ M⁻¹) |
|----|---------|------------|--------------------------|
| 2  | 2.67 ± 0.19 | 11.58 ± 0.63 | 4.34 ± 0.07 |
| 3  | 0.97 ± 0.06 | 1.95 ± 0.12 | 2.01 ± 0.01 |
| 4  | 0.89 ± 0.05 | 8.88 ± 0.58 | 9.89 ± 0.08 |
| 5  | 0.41 ± 0.02 | 11.66 ± 0.76 | 28.44 ± 0.44 |
| 6  | 0.29 ± 0.02 | 11.80 ± 0.77 | 40.69 ± 0.14 |

### Table II

| Compound | Carbon | Non-reducing residue | Residue carrying the aglycon |
|----------|--------|----------------------|-----------------------------|
| 4-NP-β-laminaribiose | 1 | 104.1 | 100.5 |
| 2 | 74.7 | 73.8 |
| 3 | 76.8 | 85.1 |
| 4 | 70.4 | 69.1 |
| 5 | 77.3 | 77.2 |
| 6 | 62.0 | 61.8 |
| 4-NP-β-celllobiose | 1 | 103.8 | 100.5 |
| 2 | 73.8 | 74.4 |
| 3 | 76.4 | 75.3 |
| 4 | 70.7 | 79.4 |
| 5 | 77.3 | 76.7 |
| 6 | 61.9 | 61.0 |
| 4-NP-β-gentiobiose | 1 | 104.0 | 100.5 |
| 2 | 73.8 | 74.3 |
| 3 | 76.7 | 76.5 |
| 4 | 70.9 | 70.4 |
| 5 | 77.1 | 76.9 |
| 6 | 61.9 | 69.6 |
−4.2 and −8.4 kJ·mol$^{-1}$, consistent with the low affinities and $k_{cat}$ values of the enzyme for laminaritriose and laminaritettraose (Table I).

The subsite mapping results (Fig. 1) offer an explanation for the relatively low rate of hydrolysis of cellotriose (Table I). Based on the binding energies of individual subsites, one might anticipate that “non-productive” binding of cellotriose across subsites +1 to +3 would be almost as likely to occur on thermodynamic grounds as would “productive” binding from subsites −1 to +2. Because the non-productive binding does not span the catalytic site, hydrolysis will not occur, but occupation of part of the substrate-binding region would be expected to lower the catalytic rate, and hence the catalytic efficiency (Table I). Non-productive binding of cellotetraose in subsites +1 to +4 might also occur, but to a lesser extent.

Glycosyl Transfer Reactions—When the barley β-glucosidase was incubated with 100 mM 4-NPG, the major products were glucose and 4-NP, as expected. However, significant amounts of other, higher molecular weight 4-NP derivatives were visible on thin layer chromatograms (data not shown). When the three most abundant of these were eluted from the plates and analyzed, $m/z$ values of 486.2 or 486.3 were obtained by electrospray mass spectrometry (data not shown). These values correspond to disaccharide derivatives of 4-NP. Chemical shifts obtained for $^{13}$C NMR spectroscopy indicated that the disaccharide derivatives were 4-NP-β-laminaribioside, 4-NP-β-cellobioside, and 4-NP-β-gentiobioside (Table II).

The relative proportions of individual transglycosylation products are compared in Table III. The 4-NP-β-laminaribioside was the most abundant, but significant levels of 4-NP-β-cellobioside and 4-NP-β-gentiobioside were also detected. This suggests that there is some flexibility in the binding of a glucosyl residue to subsite +1, because the 4-NPG is able to move sufficiently in the active site to present hydroxyls on C atoms 3, 4, or 6 to the bound glycone prior to transfer of that bound glucosyl residue to the 4-NPG (Scheme I). The purified transglycosylation products were subsequently used as substrates under standard conditions for hydrolysis. The specific activities of hydrolysis were found to reflect, approximately at least, their relative rates of synthesis under conditions that promote glycosyl transfer reactions (Table III, column 3; cf. column 2).

**Identification of Catalytic Amino Acid Residues—Conduritol B epoxide, or 1,2-anhydro-myo-inositol, has been used extensively as a mechanism-based inhibitor to label the catalytic nucleophile of β-glucosidases (26). This compound inhibited the barley β-glucosidase with a $k_{cat}/K_m$ value of 0.2 s$^{-1}$ M$^{-1}$, and when tryptic peptides of the inhibited enzyme were separated by HPLC, one new peptide appeared (Fig. 2, peak 1). Amino acid sequence analyses of peaks 1 and 2 of the inhibited and native enzymes showed that their sequences were identical, except for a gap in the sequence of the inhibited enzyme at cycle 10. Following aminolysis, a glutamic acid residue appeared in this position during subsequent sequence analysis. It could be concluded that the inhibitor bound to amino acid residue Glu$^{391}$ in the native enzyme and that Glu$^{391}$ is likely to be the catalytic nucleophile. It should be noted that peak 3 derived from the native, uninhibited enzyme was not present in digests of the inhibited enzyme (Fig. 2). This peptide has a single glutamic acid residue which corresponds to Glu$^{212}$ in the native enzyme; the peak was not detected after inhibition, and subse-
Catalytic Mechanism of Barley β-D-Glucosidase

**DISCUSSION**

Kinetic analyses of β-glucosidase isoenzyme βII from barley have shown that the enzyme rapidly hydrolyzes (1,4)-β-oligoglucosides and that the catalytic efficiency factor, $k_{cat}/K_m$, increases as the DP of the substrate increases (Table I). This preference for longer (1,4)-β-oligoglucosides is not observed with (1,3)-β-oligoglucosides. Although laminaribiose can be hydrolyzed with relatively high $k_{cat}$ and $k_{cat}/K_m$ values, catalytic rates and efficiencies decrease dramatically as the DP of this group of substrates increases; the catalytic rate for laminaritetrose is extremely low (Table I), and activity on longer (1,3)-

**FIG. 2. Comparative peptide mapping of tryptic fragments of native and conduritol B epoxide-inactivated β-glucosidase isoenzyme βII.** Tryptic digests of native (continuous line) and conduritol B epoxide-inactivated (dotted line) enzymes were separated on a Waters C18 reverse-phase column and eluted from the column at a flow rate of 0.15 ml/min, using the gradient of 0.05% (v/v) trifluoroacetic acid (solvent A) and 70% (v/v) CH$_3$CN with 0.035% (v/v) trifluoroacetic acid (solvent B). The eluent program at 0–5 min was 1% (v/v) solvent B and 99% (v/v) solvent A (isocratic); 5–75 min, 35% A and 65% B (linear); 75–85 min, 100% B (linear). The peptide fractions 1–3, which are indicated by full and dashed arrows and are unique to the digests of the native and inactivated enzymes, respectively, were subjected to amino acid sequence analysis.

In attempts to identify the catalytic acid, the barley β-glucosidase was labeled using the carbodiimide-nucleophile displacement procedure (27). After labeling, enzymic activity was reduced by 93% and several tryptic peptides with altered mobilities were isolated and sequenced. Only one peptide had a blocked amino acid residue, and this corresponded to Asp$_{362}$ (data not shown). Mass spectrometric analysis of the peak revealed an $m/z$ of 1874, which is very close to the value expected for the glycine ethyl ester adduct of that particular tryptic peptide (data not shown).

**Hydrophobic Cluster Analysis**—HCA of the barley β-glucosidase is compared with that of a cyanogenic β-glucosidase from white clover (6) in Fig. 3; comparisons were also made with other β-glucosidases (data not shown). Similarities in the positions of hydrophobic clusters suggest that α-helices and β-strands of the barley enzyme are likely to be located in positions similar to those determined by x-ray crystallography for the white clover β-glucosidase, as indicated in Fig. 3.

Conserved acidic amino acid residues, in similar environments with respect to clusters of hydrophobic amino acids, are detected at Glu$_{77}$, Glu$_{181}$, Glu$_{391}$, and Glu$_{445}$ for the barley β-glucosidase; corresponding residues in the white clover β-glucosidase are Glu$_{77}$, Glu$_{181}$, Glu$_{391}$, and Glu$_{445}$, respectively. Amino acid residue Glu$_{391}$ of the barley enzyme was labeled with conduritol B epoxide (Fig. 2), but the Asp$_{362}$ that was labeled during the EAC/GEE modification is not highly conserved in the sequences of β-glucosidases examined here. Similarly, Glu residues in positions equivalent to Glu$_{445}$ of the barley enzyme were conserved only in about 50% of the 30 β-glucosidase sequences extracted from the data bases for alignment purposes in the present work, and Glu residues equivalent to Glu$_{77}$ are not highly conserved either (data not shown). It might be concluded on this basis that the catalytic acid of the barley β-glucosidase is likely to be the more highly conserved Glu$_{181}$, but there are also several highly conserved Asp residues in the enzymes that might be involved (data not shown).

**Protein Modeling**—The 3D-1D score (Fig. 4) gives a measure of the compatibility of the predicted three-dimensional structure of the barley β-glucosidase, which was modeled on the white clover enzyme by the ExPasy service, with the known primary sequence of the barley β-glucosidase isoenzyme βII. Here, the score varies between 0.2 and 1.0, with an average value of 0.65 (Fig. 4). The white clover β-glucosidase (6) shows 49% sequence identity with the barley enzyme (Fig. 3). Scores below zero are obtained for incorrectly folded models, although correctly folded models can still have average scores as low as 0.4 (28). In a separate technique for evaluating the similarity in protein folds (29), z-scores greater than 7 have been shown to indicate correctly identified folds. Using this procedure, a z-score of 65 was obtained here for the prediction of barley β-glucosidase structure, again based on the white clover enzyme. Similarly, under PredictProtein a z-score of 7.3 was obtained and may be compared with the value of 4.5, which is found to give correct predictions in 88% of test cases (30, 31). Based on these high reliability values, the model of the barley β-glucosidase was also considered reliable and is shown in stereoview in Fig. 5.
b-oligoglucosides in the series could not be measured. These kinetic and substrate specificity data confirm the suggestion by Hrmova et al. (3) that the barley enzyme could be classified as a polysaccharide exohydrolase of the (1,4)-b-glucan glucohydro-lase group (EC 3.2.1.74), despite the fact that it does not hy-
drolyze cellulosic substrates. The enzyme might be better de-
scribed as a cellodextrin glucohydrolase than as a (1,4)-
b-glucan glucohydrolase. Similar substrate specificities have
been observed for the celD gene product from Pseudomonas
fluorescens (32) and a b-glucosidase from the thermophilic bac-
Catalytic Mechanism of Barley β-D-Glucosidase

Kinetic parameters will be dependent on the DP of the substrate via tandemly arranged subsites, it follows that and glycosidases (10, 24, 34). If enzymes bind their polysaccharides of increasing chain length (Table I) suggested that it and subsequently confirmed by subsite mapping (Fig. 1). According to Hiromi and co-workers (11, 24), the substrate-binding region of polysaccharide hydrolases consists of an array of tandemly arranged subsites, where each subsite binds a single glycosyl residue of the polymer. Such an array of subsites has now been demonstrated for endo- and exo-acting hydrolases and glycosidases (10, 24, 34). If enzymes bind their polysaccharide substrates via tandemly arranged subsites, it follows that kinetic parameters will be dependent on the DP of the substrates. The $k_{cat}/K_m$ and $k_{cat}$ values for oligosaccharides of increasing chain length were therefore used to calculate binding affinities for individual β-glucosyl-binding subsites on the barley β-glucosidase (11).

At least six glucosyl-binding subsites were detected on barley β-glucosidase isoenzyme βII (Fig. 1). Binding energies are highest at subsites −1 and +1 (Fig. 1), and although six subsites are shown in Fig. 1, it is formally possible that additional subsites exist. The insolubility of (1,4)-β-oligoglucosides of DP greater than 6 precluded the kinetic analyses required to test this possibility. The significance of the negative energy term at subsite +2, which indicates that glucosyl binding at this position is thermodynamically unfavorable, is not yet clear. However, similar negative binding energies have been observed at certain subsites in polysaccharide endohydrolases (10, 24).

Having confirmed the extended nature of the substrate binding region of the barley β-glucosidase by subsite mapping, attention was shifted toward the identification of the amino acid residues that mediate the hydrolysis of the bound substrate. The catalytic residues are clearly located between subsite −1, which binds the non-reducing terminal glucosyl residue, and subsite +1, which binds the penultimate glucosyl unit of the substrate, because it has previously been shown that the enzyme releases glucose units from the non-reducing terminus of oligomeric substrates (Fig. 1) (3). To identify the catalytic nucleophile, the mechanism-based inhibitor conduritol B epoxide was used (26, 35). This led to a dramatic reduction in activity and sequence analysis of tryptic peptides from the inhibited enzyme showed that the conduritol B epoxide had bound to amino acid residue Glu391 (Fig. 2). To identify the catalytic acid, carbodiimide-mediated labeling of the enzyme with GEE (27) was attempted. The β-glucosidase was inhibited by the treatment and, although some difficulty was experienced in tagging the enzyme, a tryptic peptide was eventually isolated in which Asp362 was labeled. Thus, the inhibitor studies indicated that Glu391 and Asp362 represented the catalytic nucleophile and the catalytic acid, respectively, of barley β-glucosidase isoenzyme βII.

Given the difficulties that have been experienced in using any single procedure to unequivocally identify amino acid residues that participate in catalysis in polysaccharide hydrolases, hydrophobic cluster analyses and sequence alignments were subsequently used to search for highly conserved acidic amino acids in β-glucosidases generally. Although the probable role of Glu391 in catalysis was confirmed by these analyses, they suggested that Glu181 was more likely than Asp362 to be the catalytic acid (Fig. 3) and that the carbodiimide procedure might not have correctly labeled the catalytic acid.

The revised identification of Glu181 as the catalytic acid in the barley β-glucosidase was further investigated by molecular modeling. The primary structure of barley β-glucosidase isoenzyme βII (2, 3) was analyzed using the Swiss-Model molecular modeling software program (16–18), in the expectation that the dispositions of specific amino acid residues in the three-dimensional structure of the enzyme would indicate whether or not

**Fig. 4.** 3D-1D plot. Structure-sequence compatibility plot for the barley β-glucosidase model built on the x-ray crystal structure of the β-glucosidase from white clover (6).

**Fig. 5.** Stereo diagram of barley β-glucosidase space filling model illustrating catalytic residues and ten putative residues involved in substrate binding. The blue, black, purple, and green color indicate Tyr (Tyr252, Tyr252, Tyr252, Tyr252), Trp (Trp251, Trp251, Trp251, Trp251), Phe (Phe256, Phe256), and His (His272) side chains, respectively. Asp362 is colored yellow. The likely catalytic residues Glu181 and Glu391 are in red. The drawing was generated with RasMol.
they might participate in catalysis. While acknowledging the limitations and constraints associated with modeling, we obtained very high z-scores when the barley β-glucosidase sequence was “fitted” to the structure of the cyanogenic β-glucosidase from white clover (6) (Fig. 4) and, on the basis of these scores, the models shown in Figs. 5 and 7 were considered to be reliable. The most noteworthy feature of the three-dimensional model of the barley β-glucosidase is the presence of a deep slot, or pocket, in the surface of the enzyme. Close to the bottom of the pocket are the probable catalytic amino acid residues Glu181 and Glu391 (shown in red in Fig. 5). The distance of 5.6 Å between their closest O atoms is widely accepted as typical for retaining polysaccharide hydrolases (36, 37). The Asp288 residue, which was tagged with GEE during carbodiimide-mediated labeling, is located on the surface of the model, near the entrance to the pocket, but some 20 Å from the catalytic nucleophile (Fig. 6). This modeling result confirmed that the carbodiimide procedure did not correctly label the catalytic acid of the barley β-glucosidase. It also indicated that other conserved Glu and Asp residues, such as Glu77, which is also found in a conserved “hydrophobic” environment (Fig. 3), are located too far from the catalytic nucleophile to be direct participants in catalysis. Thus, the molecular modeling programs proved to be a useful tool for the identification of amino acid residues that are likely to be involved in catalysis.

As with most glycoside hydrolases, a number of highly conserved residues are clustered near the catalytic amino acid residues. In Fig. 6, conserved residues or their functional equivalents in the active sites of retaining glycoside hydrolases from family 1 (barley β-glucosidase), family 5 (endo-cellulase from C. thermocellum) (38), and family 17 (endo-cellulase from C. thermocellum) are compared. These families are members of the so-called 4/7 superfamily of β-glucosidases, all of which have their putative catalytic acid located in an Asn-Glu pair near the end of β-strand 4 and their catalytic nucleophile near the end of β-strand 7 (36, 40, 41). Of particular interest is the presence of a conserved Glu residue about 8 Å from the catalytic nucleophile. This is Glu445 in the barley β-glucosidase and Glu288 in the barley (1,3;1,4)-β-glucan endohydrolase. The exceptions are His198 of family 5 (B) and Trp33 of family 17 (C), which are colored in red and green, respectively, indicating structural replacements of the Asn and His residues at these positions. The drawing was generated with RasMol, and one-letter amino acid codes are used.

FIG. 6. Conserved amino acid residues in the active site region of barley β-glucosidase, C. thermocellum endoglucanase, and barley (1,3;1,4)-β-glucan endohydrolase. Figure shows the conserved residues in the family 1 (A), family 5 (B), and family 17 (C). The red residues are the catalytic residues and non-catalytic Glu residues, the yellow residues are aromatic residues, the green are His and Arg residues, and Asn is in purple. The exceptions are His198 of family 5 (B) and Trp33 of family 17 (C), which are colored in purple and green, respectively, indicating structural replacements of the Asn and His residues at these positions. The drawing was generated with RasMol, and one-letter amino acid codes are used.

If the non-reducing terminus of the substrate is pushed to the bottom of the pocket, where Glu445 and Trp446 are located, the catalytic amino acids (Glu181 and Glu391) are arranged close to the O atom of the first glycosidic linkage. Thus, there is room for subsite −1 (Fig. 1) between the catalytic residues and the bottom of the pocket. The pocket broadens out above the catalytic residues, and this would explain not only why a range of disaccharides can be hydrolyzed by the enzyme (2, 3), but the broadening above the catalytic residues would also allow the limited movement of an incoming 4-NPG molecule that would be necessary for the formation of (1,3)-, (1,4)-, and (1,6)-linked transglycosylation products (Table III). It is not clear why the non-reducing rather than the reducing end of the substrate is oriented at the bottom of the pocket, but this is presumably related to interactions of the enzyme with key hydroxyl groups of the non-reducing terminal residue (43). Again, the Glu445 residue at the very bottom of the pocket might be involved in...
this “orientation” aspect of specificity, as might residues near the entrance to the pocket.

There does not appear to be sufficient space for the non-reducing end glucose unit to diffuse out from subsite –1 after hydrolysis, if the substrate remains bound at subsite +1. Neither is there a “rear exit” for the departure of released glucose units from the bottom of the pocket, as there is for the release of the reaction product, cellobiose, through the rear of the tunnel found in the family 6 exohydrolase, cellobiohydrolase II from Trichoderma reesei (37, 44, 45). In the case of the barley β-glucosidase, it seems likely that the substrate is at least partially released from the enzyme after each hydrolytic event, although the oligosaccharide product could remain inserted in the pocket for rapid re-binding prior to the next hydrolytic event.

We conclude from these analyses that the three-dimensional model of the barley β-glucosidase provides valuable clues as to the identity of the catalytic amino acids, it can be reconciled with the presence of at least six glucosyl-binding subsites, it offers an explanation not only for the enzyme’s ability to hydrolyze a broad range of small, dimeric substrates but also for its preference for the relatively straight (1,4)-β-oligoglucoside substrates, and it is consistent with the observed transglycosylation reactions. The model also provides some indications as to the natural substrates and hence to the possible functions of the β-glucosidases in germinated barley grain. Barley β-glucosidases are synthesized in the starchy endosperm of developing grain, but amounts of the enzyme do not increase substantially after germination (1, 2). Leah et al. (2) have discussed in detail some of the possible functions of β-glucosidases in germinated barley grain. Perhaps the most likely function of the barley β-glucosidases is in the complete hydrolysis of cell wall (1,3; 1,4)-β-glucans in germinated grain. These polysaccharides comprise about 70% of walls in the starchy endosperm and constituent glucose residues account for as much as 18% of total glucose in the grain (46). The β-glucosidases will not hydrolyze the parent polysaccharide (3), and this almost certainly results from the irregularly spaced but relatively abundant (approximately 30%) (1,3)-β-glucosyl linkages that are distributed along the polysaccharide chain. The (1,3)-β-linkages introduce kinks into the chain (47) that would prevent it fitting into the active site pocket of the enzyme (Figs. 5 and 7). Initial hydrolysis of the (1,3,1,4)-β-glucan therefore relies on endohydrolases, which release as major products the trisaccharide 3-O-β-cellobiosyl-α-glucose (G4G3G red) and the tetrasaccharide 3-O-β-cellobiosyl-α-glucose (G4G4G3G red) (48). In addition, up to 10% of the (1,3,1,4)-β-glucan is released by the endohydrolases as longer chain (G4),G4G3G red oligosaccharides, where n can be 2–10 (49). These oligosaccharides, which are essentially (1,4)-β-oligosaccharidase with a single (1,3)-linkage at their reducing ends, have structures very similar to cellobextrins and would be expected to fit into the active site pocket of the β-glucosidase (Figs. 5 and 7). The β-glucosidases in germinated barley grain could therefore play a major role in the reclamation of cell wall-bound glucose as an energy source to support seedling growth.

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