ELSEVIER

Carbohydrate Research 277 (1995) 145–159

CARBOHYDRATE RESEARCH

Characterization of high pI α-glucosidase from germinated barley seeds: substrate specificity, subsite affinities and active-site residues ☆

Hana Im, Cynthia A. Henson *

Department of Agronomy, University of Wisconsin (H.I., C.A.H.); and U.S. Department of Agriculture [1], Agricultural Research Service, Cereal Crops Research Unit (C.A.H.), Madison, WI 53706, USA

Received 18 January 1995; accepted 1 May 1995

Abstract

Substrate specificity and subsite affinities of high pI α-glucosidase from germinated barley (*Hordeum vulgare* L.) seeds were investigated by kinetics. The enzyme has only one maltose binding site per molecule and shows high activity on small maltooligosaccharides and nigerose. Hydrolysis of isomaltose and *p*-nitrophenyl α-glucoside is moderate. Trehalose is not hydrolyzed at detectable rates. The ratios of the maximum velocities for maltose, nigerose, isomaltose, *p*-nitrophenyl α-glucoside and malto-triose, -tetraose, -pentaose, -hexaose, -heptaose are 100:95:21:9:111:116:119:104:111. The K_m values for these substrates are 1.91, 1.29, 5.32, 1.04, 1.11, 2.37, 2.92, 5.44 and 7.89 mM, respectively. Based on the rate parameters for maltooligosaccharides, the subsite affinities (A_is) in the active site of the enzyme were evaluated according to subsite theory. Subsites 1, 2 and 3, having positive A_i values (A_1, A_2 and A_3; 1.34, 5.37 and 0.27 kcal/mol, respectively), were considered to be effective for the binding of substrate to the active site. The different arrangement of subsite affinities among α-glucosidases, glucoamylases and amylases was used to explain their substrate specificities.

☆ This material is based upon work supported by the U.S. Department of Agriculture–Agricultural Research Service, the University of Wisconsin–Madison, Anheuser-Busch Companies, Inc., American Malting Barley Association, Inc. and the U.S. Department of Agriculture-Cooperative State Research Service under Agreement No. 593-0120-07.

* Corresponding author.

[1] Mention of a proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other suitable products.

Elsevier Science Ltd.
SSDI 0008-6215(95)00212-X

Chemical modification with conduritol B epoxide and *N*-bromosuccinimide in the presence and absence of ligands revealed carboxylate and tryptophanyl groups, respectively, as essential functional groups in the active site of the enzyme. Chemical modification with phenylglyoxal and pyridoxal 5′-phosphate suggests that arginine and lysine, respectively, also have significant roles in enzyme function.

Keywords: Barley α-glucosidase; Substrate specificity; Subsite mapping; Active-site residues; Chemical modification

1. Introduction

Degradation of starch during seed germination is critical to the viability of cereal seeds because starch, which constitutes as much as 70% of seed dry weight, is the primary source of respirable substrates and carbon skeletons until seedlings become fully autotropic. Degradation of cereal starch by endogenous seed enzymes is also critical in many industrial fermentation systems (e.g., production of alcohol fuels and beverages). Starch degradation in germinating cereal seeds results from the concerted action of α-amylase, β-amylase, debranching enzyme and α-glucosidase. Path coefficient analysis revealed α-glucosidase, the least studied of the above four carbohydrases, to be the second most important enzyme (α-amylase is the most important) in the pathway of seed starch degradation [1]. Using a reconstituted barley starch hydrolyzing system, synergism was demonstrated between α-glucosidase and α-amylase in the hydrolysis of native seed starch [2]. Konishi et al. [3] demonstrated that the specific inhibition of wheat seed α-glucosidase during early stages of germination reduced starch degradation by 48%, and inhibited subsequent seedling growth by 89% as compared to control seedlings. This inhibition of α-glucosidase with miglitol resulted in increased levels of maltose, decreased glucose, and a reduction in sucrose biosynthesis, which is needed for seedling growth [3]. It has, therefore, been demonstrated that α-glucosidase is critical for starch degradation and the subsequent growth and development of cereal seeds.

α-Glucosidases [EC 3.2.1.20, α-D-glucoside glucohydrolase] catalyze the exolytic splitting of a glucose residue from the nonreducing terminus of the substrate. Various α-glucosidases from mammals, insects, fungi and bacteria have been well studied, yet those from plants have not been examined in great detail. Plant α-glucosidases have been reported to have broad substrate specificity, as do α-glucosidases from other organisms, but few detailed kinetic studies of plant α-glucosidases have been made. In this paper, we explore the details of substrate specificity, the subsite structure of the active site, and the identity of amino acids important for enzyme function, of high pI α-glucosidase from barley seeds. These studies are part of ongoing work directed to develop seed α-glucosidases with improved catalytic properties (e.g., altered affinities for substrates with non-α-(1 → 4)-glucosidic linkages, altered preference for short or long maltooligosaccharides, etc.) for potential industrial application. The information obtained is important not only in understanding the roles of this enzyme in glucan degradation in higher plants, but also in designing and evaluating future mutagenesis experiments.

2. Experimental

Chemicals.—Malto-triose, -tetraose, -pentaose, -hexaose, and -heptaose were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Acarbose was a generous gift from Dr Joseph Vasselli (Miles Pharmaceuticals, West Haven, CT). $CuCl_2$, $ZnCl_2$, and iodine were purchased from Mallinckrodt, Inc., St. Louis, MO. The Pantrak Amylase Test kit was purchased from Behring Diagnostics, La Jolla, CA. All other biochemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Plant materials and preparation of enzyme.—Seeds of barley (*Hordeum vulgare* L. cv. Morex) were imbibed, germinated, lyophilized, ground to a fine flour, and extracted with 50 mM sodium phosphate (pH 9), 1 M NaCl, and 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) as previously described [2]. After the removal of insoluble materials by centrifugation, the crude extract was precipitated by step gradients of ammonium sulfate (25% intervals). Pellets were collected by centrifugation at 12,000 × *g* for 15 min, resuspended in a small volume of 50 mM sodium succinate, pH 4.5, and dialyzed overnight against the same buffer at 4°C. The 25–50% ammonium sulfate cut, which had the most α-glucosidase activity, was chromatographed on CM MemSep [2] 1500 (Millipore Corporation, Bedford, MA) equilibrated with 50 mM sodium succinate, pH 4.5. The low pI α-glucosidase was eluted with equilibration buffer, and the high pI isoform, which accounted for the majority of the α-glucosidase activity, was eluted with a 0–1 M NaCl gradient. The high pI isoform was then passed through a DEAE-Sepharose CL-6B column equilibrated with 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), pH 8. Fractions containing high pI α-glucosidase activity were pooled, then dialyzed against 5 mM $ZnCl_2$, 1 mM $CuCl_2$, 20 mM sodium succinate, pH 4.5. α-Glucosidase was then stored at 4°C. Activity was stable under these conditions for several months.

The protein profile of the α-glucosidase preparation was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). When α-glucosidase was eluted from a castanospermine affinity column with 1.7 M Tris–Cl, pH 7.6, the 33 kDa protein band was detected on SDS-PAGE. The presence of additional band(s) in the preparation eluted from DEAE-Sepharose was not considered problematic for the following three reasons. First, barley high pI α-glucosidase activity was eluted as a single symmetrical peak from all chromatographic resins tested. Second, high pI α-glucosidase preparations resulting from different combinations of chromatographic separations had the same K_m for maltose and relative activities on disaccharides and linear maltooligosaccharides. Third, the absence of contaminating carbohydrase activities which might interfere with this study was confirmed as follows. Neither α-amylase nor β-amylase activity was detected when this preparation was incubated with 1.67% boiled starch azure or a mixture of *p*-nitrophenyl maltooligosaccharides (purchased as the Pantrak Amylase Test kit), respectively, as the substrate [1,4]. The α-glucosidase preparation did not show any contaminating carbohydrase activity when the reaction products of maltoheptaose hydrolysis were separated on an HPLC CarboPac PA1 anion exchange column and detected/quantitated by pulsed amperometry.

[2] MemSep is a registered trademark of Millipore Corporation.

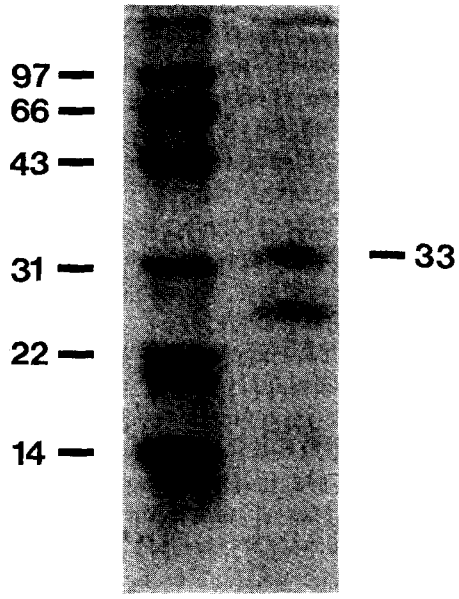

Fig. 1. SDS-polyacrylamide gel electrophoresis of barley high pI α-glucosidase preparation. Left lane, molecular mass standards (in order of decreasing M_r; phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme); right lane, barley high pI α-glucosidase preparation. Molecular mass of each band is expressed in kDa.

α-Glucosidase assays.—α-Glucosidase hydrolytic activities were measured by release of glucose from all substrates except for *p*-nitrophenyl α-glucoside, from which release of *p*-nitrophenolate was determined. Assays in which glucose release was determined were conducted in 50 mM sodium succinate, pH 4.5, in 0.2 mL reactions. After incubation (30°C, 15 min), the reactions were terminated by boiling for 5 min. Glucose released was quantified by determining the reduction of NAD^+ by the coupled reaction of hexokinase and glucose-6-phosphate dehydrogenase [5]. Activities are expressed as μmol glucose cleaved from the nonreducing end of substrates per min per mg active α-glucosidase. Assays in which *p*-nitrophenolate release was measured were conducted in the above buffer in 0.9 mL reactions. After incubation (37°C, 30 min), the assays were terminated by bringing the reaction to 0.1 N NaOH, and released *p*-nitrophenolate was immediately quantified at 420 nm. Activities are expressed as μmol *p*-nitrophenolate released per min per mg active α-glucosidase, using a standard curve made with *p*-nitrophenolate in the same buffer. The effects of glucose on α-glucosidase activity were measured with various concentrations of *p*-nitrophenyl α-glucoside as the substrate in the absence and presence of 3.3 mM or 5 mM glucose. Protein concentration was determined by the method of Bradford [6] using bovine serum albumin (BSA) as the standard.

Determination of α-glucosidase concentration.—Only the concentration of catalytically functional or active α-glucosidase was considered important in the determination of enzyme concentrations used in the kinetic studies. Hence, Barrett and Kirschke's procedure [7] for quantifying only the functional enzyme was used with the following

modifications for α-glucosidase. Castanospermine was used as the competitive inhibitor of α-glucosidase, and preincubation was for 4.5 h at 30°C in 50 mM sodium succinate, pH 4.5. The remaining activities were measured with 25 mM maltose in 50 mM sodium succinate, pH 4.5 for 30 min at 30°C.

It was necessary to determine first the number of castanospermine binding sites per molecule of α-glucosidase. This was done by using the following inhibition kinetics equation,

$$\log[\nu_i/(\nu_o - \nu_i)] = -n \log[\mathrm{I}] + \log K'$$

where ν_i is the initial rate of enzyme action at a given concentration of inhibitor, [I], ν_o is the initial rate of enzyme action in the absence of inhibitor, and n is the number of bound inhibitor molecules per enzyme molecule. The inhibitor concentration that gives 50% inhibition of the initial activity, $[\mathrm{I}]_{0.5}$, raised to the nth power is equal to K' [8]. The slope of the plot in coordinates $\log[\nu_i/(\nu_o - \nu_i)]$ and $\log[\mathrm{I}]$ gives $-n$. Competition assays for α-glucosidase activities were done with 20 mM maltose in 50 mM sodium succinate, pH 4.5, for 30 min at 30°C in the presence of various concentrations of castanospermine. Released glucose was quantified as described in α-glucosidase assays.

Once the number of castanospermine binding sites per molecule of α-glucosidase was determined, the concentration of functional α-glucosidase was determined by the titration of the active site with castanospermine following the procedure of Barrett and Kirschke [7] as described above. After plotting the activity remaining after inhibition vs. the concentration of castanospermine, the extrapolated concentration of the inhibitor at which no activity remained was estimated to be the concentration of active enzyme.

Kinetic analysis.—α-Glucosidase activities on various substrates were measured using at least six different concentrations of substrates ranging from 0.2 to 5 times the K_m value. Four replications were done for all enzyme assays. Four replications were also conducted for all blanks, which contained boiled enzyme. No assays gave four replicate values greater than ±2% of the mean value. The effect of substrate concentration on the initial rate of enzyme action was examined by use of Lineweaver–Burk plots. Rate parameters are expressed as means ± standard errors (Tables 1 and 2).

Subsite theory.—In hydrolase-catalyzed degradation of polymeric substrates, it has often been observed that the Michaelis constant (K_m) and the maximal velocity (V_{max})

Table 1
Michaelis constants (K_m) and maximal rates (V_{max}) for the hydrolysis of various substrates by barley high pI α-glucosidase

Substrate	K_m (mM)	V_{max} [a]
Maltose	1.89 ± 0.07	12.6 (100) ± 0.37
Nigerose	1.29 ± 0.09	12.1 (95) ± 0.50
Isomaltose	5.32 ± 0.19	2.64 (21) ± 0.16
p-Nitrophenyl α-glucoside	1.04 ± 0.04	1.19 (9) ± 0.05

[a] The V_{max} values are μmol of glucose cleaved from the nonreducing end of substrates per min per mg protein. The values in parentheses are the relative V_{max} for the hydrolysis of the α-glucosidic linkage.

Table 2
Rate parameters for the hydrolysis of maltooligosaccharides by barley high pI α-glucosidase

Substrate	K_m (mM)	V_{max} [a]	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Maltose	1.91 ± 0.01	12.8 (100) ± 0.12	6.02	3.15
Maltotriose	1.11 ± 0.07	14.2 (111) ± 1.02	6.65	5.98
Maltotetraose	2.37 ± 0.03	14.9 (116) ± 0.76	6.98	2.94
Maltopentaose	2.92 ± 0.03	15.2 (119) ± 1.15	7.14	2.45
Maltohexaose	5.44 ± 0.01	13.4 (104) ± 0.69	6.26	1.15
Maltoheptaose	7.89 ± 0.09	14.2 (111) ± 0.92	6.67	0.85

[a] The V_{max} values are μmol of glucose cleaved from the nonreducing end of substrates per min per mg protein. The values in parentheses are the relative V_{max} for the hydrolysis of the α-glucosidic linkage.

are intimately related to the degree of polymerization (dp) of substrates. According to the subsite theory [9,10], the active site of these enzymes consists of a definite number of subsites, each of which has its own affinity and interacts with a substrate residue. Additionally, this theory proposes that the intrinsic hydrolysis rate of each substrate in a productive substrate–enzyme complex is constant regardless of dp of the substrate. The apparent dependency of V_{max} on dp arises merely from the probability of productive complex formation, which is in turn determined by the arrangement of subsite affinities (A_i; where i = subsite number) in the active site of the enzyme. Observed K_m values are lower than the dissociation constant of the productive enzyme–substrate complex (K_p) values because of the existence of nonproductive binding modes. The dissociation constant of productive and nonproductive binding complexes can be attributed to the sum of the affinities of the occupied subsites in each binding mode for each substrate. The value, k_{cat}/K_m, can be used to determine the subsite affinities by using linear substrates with different dp.

Evaluation of subsite affinities of barley high pI α-glucosidase.—Subsite affinities of several carbohydrases including α-amylaes, β-amylases, glucoamylases, and glucosidases have been evaluated [11] according to the subsite theory. The active site of α-glucosidase is considered to be composed of a definite number of subsites, each of which interacts with a glucose moiety of a substrate [12]. In this study, subsite affinities of barley high pI α-glucosidase were evaluated from the Michaelis constant (K_m) and the turnover number (k_{cat}) on maltooligosaccharides of different chain length using equations for subsite analysis of exolytic enzymes [10]. A brief outline of this kinetic treatment is given below. The subsites are numbered from the binding site for the nonreducing end of substrates, and A_i for $i > 2$ can be obtained from $A_{n+1} = RT\{\ln(k_{cat}/K_m)_{n+1} - \ln(k_{cat}/K_m)_n\}$. Since the k_{cat} is proportional to the fraction of productive binding complex out of total (productive and nonproductive) binding complexes, A_1 and k_2 can be calculated from the equation, $\exp(A_{n+1}/RT) = \exp(A_1/RT)k_2/(k_{cat})_n - \exp(A_1/RT)$; a plot of $\exp(A_{n+1}/RT)$ against $(1/k_{cat})_n$, gives vertical and horizontal intercepts of $-\exp(A_1/RT)$ and $1/k_2$, respectively. A_2 is then calculated by $(k_{cat}/K_m)_n = k_2\ 0.018\ \exp\{(A_1 + A_2 + ... + A_n)RT\}$. The validity of a nonchanging, true rate constant (k_2) and subsite affinities were evaluated by comparing experimentally obtained rate parameters (K_m, k_{cat}) to those predicted by the theory.

Chemical modification.—Enzyme was incubated with each amino acid-modifying chemical at 30°C for 1 h, except in the case of *N*-bromosuccinimide (NBS), which was incubated for 5 min. Reactions containing NBS or conduritol B epoxide (CBE) were conducted in 30 mM sodium succinate buffer at pH 4.5. Reactions containing pyridoxal 5′-phosphate were run in 30 mM potassium phosphate buffer at pH 6.8. Diethylpyrocarbonate was diluted in ethanol and immediately added to reactions in 30 mM potassium phosphate buffer, pH 6. The final concentration of ethanol was less than 0.5%. Iodine, phenylmethylsulfonyl fluoride, iodoacetate, or phenylglyoxal treatments were conducted in 30 mM HEPES buffer, pH 7.5. For most cases, remaining α-glucosidase activities were measured with 25 mM maltooligosaccharides in 50 mM sodium succinate buffer at pH 4.5 as described in enzyme assays. In the case of diethyl pyrocarbonate treatments, remaining α-glucosidase activities were measured with 0.089% *p*-nitrophenyl α-glucoside as the substrate because diethyl pyrocarbonate inactivated the hexokinase and/or glucose-6-phosphate dehydrogenase used to quantify glucose released from maltooligosaccharides. Controls consisted of enzyme samples treated the same way except no modifying chemicals were used. Blanks consisted of enzyme samples treated the same way, then boiled for 5 min.

Substrate protection.—Enzyme was preincubated with 0.2 M maltose, 0.2 M maltohexaose, 0.2 M trehalose, or 0.2 mM acarbose in 30 mM sodium succinate buffer, pH 4.5, for 30 min at 30°C. NBS and CBE treatments were done as described. The ligands and modifying chemicals were then removed by extensive dialysis in 30 mM sodium succinate, pH 4.5, and subsequent overnight dialysis in 1.7 M Tris–Cl, pH 7.6, to remove the tightly bound acarbose from the enzyme as described by Clarke and Svensson [13]. The enzyme was then dialyzed again in 30 mM sodium succinate buffer at pH 4.5, and the remaining activities were measured with 25 mM maltose as the substrate. Enzyme samples treated the same way minus modifying chemicals were used as controls, and enzyme samples treated the same way and boiled for 5 min were included as blanks.

3. Results

Determination of the enzyme concentration.—Since kinetic calculations dependent upon protein concentration are most valid when based only upon the concentrations of enzyme capable of performing catalysis, the concentration of active α-glucosidase in the preparation was determined by titrating the active site with a substrate analog inhibitor, castanospermine, instead of measuring total protein amount, which would include both active and inactive forms of α-glucosidase. Titration of the active site was done by preincubation of α-glucosidase with castanospermine following Barrett and Kirschke [7] as described in Experimental.

Castanospermine, an active-site specific inhibitor for various glucosidases [14], was previously shown in this laboratory to competitively bind to the active site of high pI α-glucosidase from germinated barley seeds with a K_i value of 0.11 μM [15]. For determination of K_i, enzyme was exposed to both inhibitor and substrate simultaneously, and activity was assayed over a short time period. In contrast, the experiment

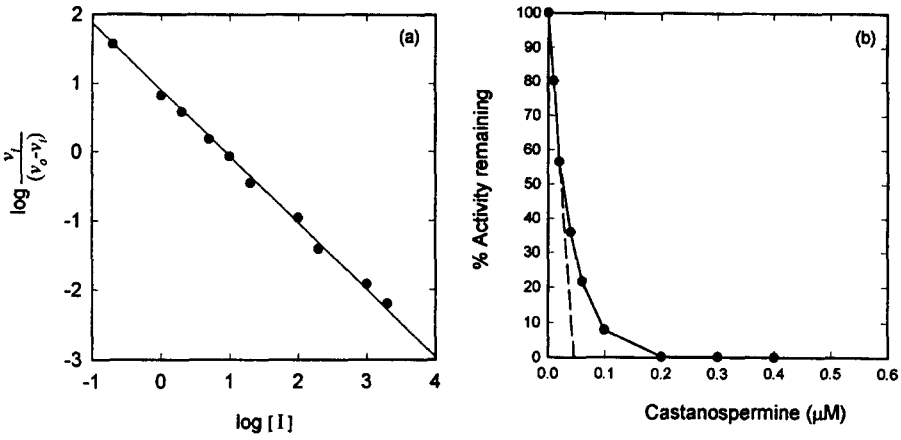

Fig. 2. Determination of barley high pI α-glucosidase concentration. (a) Determination of the number of castanospermine-binding sites per enzyme molecule; a Hill plot showing the kinetics of inhibition by castanospermine. α-Glucosidase activities were measured with 20 mM maltose in the presence of various concentrations of castanospermine in 0.2 mL reactions. The concentrations of inhibitor, castanospermine, are shown in μM. (b) Active-site titration of barley high pI α-glucosidase using castanospermine. Preincubation with castanospermine and α-glucosidase assays were done as described in Experimental. The initial rate of maltose hydrolysis was 11.76 μmol/min mg protein. —; extrapolated values. The extrapolated concentration of castanospermine for the complete inhibition of α-glucosidase activity was 0.046 μM.

reported here used long preincubations of enzyme and inhibitor, in the absence of substrate, to take advantage of the difference between the relatively slow rate (k_{on}) of EI formation and the extremely slow rate (k_{off}) of EI decomposition. Due to the extremely slow k_{off}, castanospermine behaves similar to a slow-binding, active-site specific irreversible inhibitor in our active-site titration experiments, i.e., no detectable reactivation occurred during the assay time, when castanospermine-treated enzyme was diluted to 1/10 K_i. Only barely detectable reactivation (< 4%) was obtained when the treated enzyme was diluted and incubated for 8 h, which is 16 times longer than the assay time used here. Enzyme diluted and incubated the same way was stable during this incubation (data not shown). We used a Hill plot of the kinetics of castanospermine inhibition to determine the number of binding sites per α-glucosidase molecule (Fig. 2a). The n value for castanospermine was unity, indicating that this inhibitor has only one competitive binding site per α-glucosidase molecule.

Using a plot of the remaining activity vs. the concentration of inhibitor, the concentration of active α-glucosidase in our enzyme preparation was determined by extrapolating the linear portion of the curve as done by Barrett et al. [16]. The concentration of active α-glucosidase was 4.6×10^{-8} M (Fig. 2b). The slope of the linear portion was not changed by longer preincubation with castanospermine.

Hydrolytic activity on various substrates.—The hydrolytic activities at various concentrations of maltose, nigerose, isomaltose, and *p*-nitrophenyl α-glucoside were investigated. Lineweaver–Burk plots of the initial velocity (v) and the substrate concentration (s) of each substrate were linear. The Michaelis constant (K_m) and the

maximal rate (V_{max}) values are summarized in Table 1, together with the relative V_{max}, which represents the relative values of the cleavage rates of the different α-glucosidic bonds in each substrate. High pI α-glucosidase from germinated barley seeds hydrolyzed nigerose (α-(1 → 3)-linkage) and maltose (α-(1 → 4)-linkage) rapidly, and hydrolyzed isomaltose (α-(1 → 6)-linkage) and *p*-nitrophenyl α-glucoside at moderate rates. The enzyme did not hydrolyze trehalose (α-(1 → 1)-linkage) at any measurable rate with the assay method used here (data not shown).

Hydrolytic activity on maltooligosaccharides and evaluation of subsite affinities.— Lineweaver–Burk plots of the initial velocity (v) and the substrate concentration (s) of each maltooligosaccharide were linear. The amount of glucose produced by the hydrolysis of maltooligosaccharides was proportional to the reaction time up to 45 min, which was three times longer than the reaction time used in these studies, implying that the actual initial velocity was being measured. The rate parameters obtained are summarized in Table 2. The k_{cat} values are relatively constant upon increasing dp. However, the K_m values reach a minimum for maltotriose (dp = 3) and increase when dp ≥ 4.

Action patterns of α-glucosidases have been reasonably explained by subsite theory [9,10]. In this study, the measurable rate parameters, the Michaelis constant (K_m) and the turnover number (k_{cat}) of barley high pI α-glucosidase are expressed in terms of the subsite affinities (A_i, where i = subsite number) and the intrinsic rate constant (k_2) for the hydrolysis of substrate in a productive enzyme–substrate complex, using the equations of subsite theory as shown in Experimental. A histogram of the subsite affinities is given in Fig. 3, together with the A_i values and k_2 value calculated from the rate parameters according to subsite theory. The increase in K_m for maltooligosaccharides of dp ≥ 4 is due to the negative values of A_4–A_7 (Fig. 3). Subsites 1, 2, and 3, having positive A_i values, are probably effective in binding substrate to the active site of the enzyme.

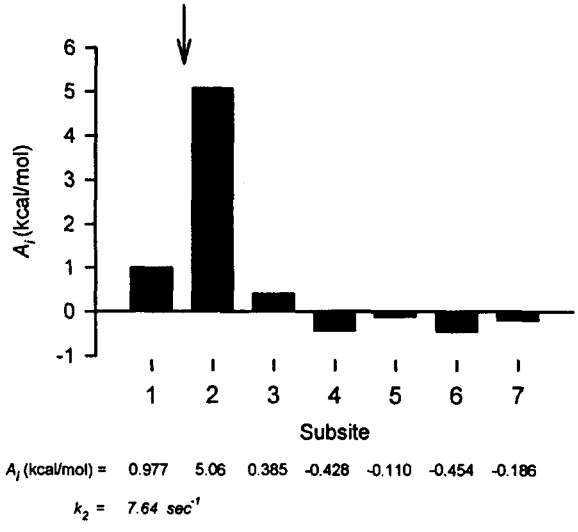

Fig. 3. Histograms showing subsite affinities of barley high pI α-glucosidase. The arrow indicates the position of the catalytic site.

Table 3
Camparison between observed and calculated rate parameters for barley high pI α-glucosidase

Substrate	K_m (mM)			k_{cat} (s^{-1})			k_{cat}/K_m (s^{-1} mM^{-1})		
	Obs [a]	Cal [b]	O/C [c]	Obs [a]	Cal [b]	O/C [c]	Obs [a]	Cal [b]	O/C [c]
Maltose	1.91	1.78	1.07	6.02	5.56	1.08	3.15	3.12	1.01
Maltotriose	1.11	1.18	0.94	6.65	6.96	0.95	5.98	5.92	1.01
Maltotetraose	2.37	2.26	1.05	6.98	6.56	1.06	2.94	2.91	1.01
Maltopentaose	2.92	2.89	1.01	7.14	6.99	1.02	2.45	2.42	1.01
Maltohexaose	5.44	5.86	0.93	6.26	6.67	0.94	1.15	1.14	1.01
Maltoheptaose	7.89	7.63	1.03	6.67	6.38	1.05	0.85	0.84	1.01

[a] Obs: experimentally obtained values.
[b] Cal: calculated using A_i and k_2 values obtained by subsite theory [12].
[c] O/C: Obs/Cal.

The rate parameters (K_m, k_{cat}, and k_{cat}/K_m) were calculated conversely by using the A_i and k_2 values listed in Fig. 3. The calculated values are summarized in Table 3, together with the ratios between the observed and calculated rate parameters. There was close agreement between the two values with a maximum deviation of about 8% for a series of maltooligosaccharides.

Effect of glucose on enzyme action.—Since end products and substrates for alternative reactions can modulate enzyme activity, the impact of glucose on barley high pI α-glucosidase was examined using *p*-nitrophenyl α-glucoside as the substrate. As shown in Fig. 4, the presence of glucose inhibits the hydrolysis of *p*-nitrophenyl α-glucoside with the K_i value of 2.1 mM.

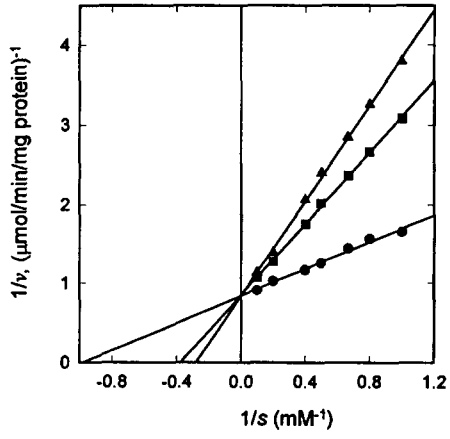

Fig. 4. Effect of glucose on cleavage of *p*-nitrophenyl α-glucoside by barley high pI α-glucosidase. 0.30 μg of active α-glucosidase was incubated with various concentrations of *p*-nitrophenyl α-glucoside in the absence and presence of glucose as described in Experimental. The initial rate, v, is expressed as μmol of *p*-nitrophenol liberated per min per mg protein. Glucose concentration: ●, 0 mM; ■, 3.3 mM; ▲, 5 mM.

Table 4
Effect of various reagents on barley high pI α-glucosidase

Reagent [a]	Concentration (mM)	pH	Remaining activity (%) [b]
N-Bromosuccinimide (NBS)	0.05	4.5	11.3 ± 0.2
Conduritol B epoxide (CBE)	2	4.5	12.6 ± 1.8
Diethyl pyrocarbonate	10	6.0	99.1 ± 5.8
Phenylmethylsulfonyl fluoride	2	7.5	98.2 ± 1.0
Iodine	2	7.5	99.8 ± 1.1
Iodoacetate	10	7.5	101.1 ± 0.4
Phenylglyoxal	10	7.5	72.7 ± 0.6
Pyridoxal 5′-phosphate	1	6.8	83.9 ± 1.3

[a] The enzyme was incubated with each reagent for 1 h at 30°C, except for NBS where treatment was for 5 min at 30°C.
[b] The remaining activities were measured with 25 mM maltose or 0.089% *p*-nitrophenyl α-glucoside as the substrate. The activity of the enzyme incubated in each reaction buffer minus the modifying reagent for the same length of time was referred to as 100%.

Chemical modification of active-site amino acids.—Several group-specific chemical reagents were used to determine the important amino acids at or near the active site of barley high pI α-glucosidase. The results of these studies are summarized in Table 4. Diethyl pyrocarbonate, phenylmethylsulfonyl fluoride, iodine, and iodoacetate did not inactivate this enzyme at pHs favorable for the modification of histidine, serine, tyrosine, and cysteine residues, respectively. Partial inactivation was observed with the arginine- and lysine-specific reagents, phenylglyoxal and pyridoxal 5′-phosphate, respectively. CBE, a glucosidase-specific substrate analogue [14], and NBS inactivated α-glucosidase, indicating the involvement of carboxylate group(s) and tryptophan residue(s) in the catalytic site. CBE inactivated the enzyme slowly, but NBS inactivated the enzyme very quickly even at the lowest concentration used. Higher concentrations of NBS (≥ 1 mM) caused precipitation of protein, as was observed with *Aspergillus niger* glucoamylase I and II [13], and thus were not used in further experiments.

Table 5
Ligand protection of barley high pI α-glucosidase against chemical modification

Preincubation [a]	Remaining activity (%) [b] after CBE treatment	Remaining activity (%) [b] after NBS treatment
Buffer	12.8 ± 0.2	11.8 ± 0.5
0.2 M Trehalose	12.9 ± 0.2	11.5 ± 0.2
0.2 M Maltose	90.7 ± 1.5	72.7 ± 2.9
0.2 mM Acarbose	78.5 ± 0.8	63.0 ± 1.4
0.2 M Maltohexaose	78.9 ± 0.3	65.3 ± 0.2

[a] The enzyme was preincubated with various ligands at 30°C for 30 min before chemical modification.
[b] Chemical treatments were done as in Table 4, and ligands were removed by dialysis. The remaining activities were then measured with 25 mM maltose as the substrate. The activity of the enzyme treated the same way except with no modifying reagent was referred to as 100%.

Substrate protection against chemical modification.—Preincubation with substrates (maltose or maltohexaose) protected the enzyme from inactivation by CBE and NBS (Table 5), suggesting that carboxylate and tryptophan groups are at the active site of the enzyme. Acarbose, a pseudotetrasaccharide substrate analogue and a strong competitive inhibitor of α-glucosidases [17], also protected the enzyme even at a very low concentration (0.2 mM). Trehalose (α-(1 → 1)-linked glucose dimer), which is not a substrate of high pI α-glucosidase, failed to protect the enzyme.

All substrates tested (maltose, maltotetraose, and maltohexaose) and a nonsubstrate (trehalose) failed to protect the enzyme against the partial inactivation by phenylglyoxal and pyridoxal 5′-phosphate (data not shown), probably due to weaker hydrogen bond formation between substrates and active-site amino acids at the higher reaction pH used for these reagents (pH 7.5 and 6.8 vs. pH 4.5).

4. Discussion

Barley high pI α-glucosidase's specificity toward small maltooligosaccharides supports its major role in the hydrolysis of small α-glucans released from starch granules during seed germination. This role is especially important because α-glucosidase is the only enzyme present in barley seeds that can degrade maltose, the major product of starch degradation. This enzyme's broad substrate specificity also suggests an additional role in the hydrolysis of various α-linkages besides α-(1 → 4)-bonds.

Transglycosylation and condensation reactions, if present, would interfere with the kinetics of the forward reactions. However, the absence of both types of reactions during the assay time was confirmed by the following criteria described by Meagher et al. [18]. The hydrolysis of maltooligosaccharides during the given reaction time did not yield any evidence for the production of oligosaccharides larger than the substrates when the reaction products were separated on an HPLC column and detected by pulsed amperometry which can detect a minimum of 3×10^{-11} mol glucose. As the hydrolytic rates of maltooligosaccharides larger than maltotriose become slower (Table 2), measurable amounts of these substances should accumulate if they had been produced. Also, hydrolyses of the substrates followed Michaelis – Menten kinetics, rather than having a sigmoidal dependence of rate on substrate concentration. Furthermore, no lags at the beginning of hydrolytic reactions at concentrations up to several times of their K_m values were observed.

Multiple attack upon a single encounter of the substrate with the enzyme is very unlikely, because end product analysis of the hydrolysis of maltooligosaccharides by HPLC yielded only glucose and a maltooligosaccharide smaller by one glucose residue, not by two or more glucose units. This result indicates that a single catalytic event occurs for each productive enzyme–substrate encounter.

All α-glucosidases studied for subsite affinities, such as barley high pI α-glucosidase (this work), buckwheat α-glucosidase [19], *A. niger* α-glucosidase [20], honey bee α-glucosidase I [21], and rabbit liver acid α-glucosidase [22], have similar patterns of subsite affinities. The highest affinity is found at the second subsite, the second highest at the first subsite, and the third highest at the third subsite. The affinities of subsites $i \geq 4$ are either negative or near-zero. Comparisons of the characteristic arrangements of

subsite affinities of various amylases and α-glucosidases are of interest. As mentioned by Hiromi et al. [11], α-amylases and β-amylases have negative or near-zero values for the sum of A_is at the two essential subsites on either side of the bond-cleavage site, and they have significant positive values of A_i at the next subsites on both sides of the essential subsites. Therefore, they show low affinities with substrates smaller than maltotetraose (dp = 4), and essentially the same high affinities with larger maltooligosaccharides regardless of the degree of polymerization. Both α-glucosidases and glucoamylases split off glucose from nonreducing ends of substrates and they have the highest affinity at the second subsite from the substrate's nonreducing end. However, in the subsite affinities of the first and the third subsites, A_1 and A_3, there was a pronounced difference between these enzymes. Among the subsite affinities of the α-glucosidases, the value of A_1 is much bigger than that of A_3, but in the case of glucoamylases the value of A_1 is near zero or negative and that of A_3 is significantly positive [11,19]. The above observation explains the differences in their substrate specificities. In the α-glucosidase–substrate complex, the smallest substrate, maltose, preferentially occupies the first and second subsites (productive binding mode) rather than the second and third subsites (nonproductive binding mode). On the other hand, maltose may be preferentially bound in a nonproductive form to glucoamylase, and therefore is hydrolyzed slowly compared to larger maltooligosaccharides.

Comparisons of the observed and calculated (based on subsite theory) rate parameters for the above α-glucosidases show better agreement than those for other carbohydrases with preferences to large substrates, such as α-, β- or gluco-amylases. For α-glucosidases, which prefer small oligosaccharides as the substrates, the maximal deviations of the calculated values from the observed values (8% in this study) is in the range of the maximal experimental errors (also, 8% in this work). These data suggest the validity of the application of subsite theory to α-glucosidases, i.e., k_2 is independent of dp for α-glucosidases, and that additional binding of a glucose residue on a remote, noncatalytic subsite ($i \geq 3$) results in negligible additional strain on the productively bound-catalytic subsite(s) ($i = 1$, 2, or both) when the substrates are linear maltooligosaccharides. For amylases, the maximal deviation of the observed and calculated rate parameters are typically much larger than the maximal experimental errors, and the binding of substrates of different chain length is suggested to induce somewhat variable strains [23], resulting in variable k_2 on substrates of different dp. The additional strain of binding longer substrates as suggested by subsite analysis is supported by X-ray crystallography showing transmission of additional strain upon binding of acarbose (a pseudotetrasaccharide inhibitor) at the active site of *Aspergillus* glucoamylase [24], when compared to the binding of 1-deoxynojirimycin (a glucose analog inhibitor) [25].

At least one carboxylate group is involved in the catalytic reactions in all α-glucosidases [14,26,27] and glucoamylases [28–31] studied. Glucoamylases and the acidic α-glucosidases (optimal activity at pH 4–5), which include mammalian acid enzymes and plant enzymes not present in chloroplasts, have been demonstrated to possess two essential carboxyl groups. In contrast, the neutral α-glucosidases (optimal activity at pH 6–7), which include mammalian neutral enzymes, yeast enzymes and higher plant chloroplastic enzymes, possess one carboxyl group and one imidazole group as the essential ionizable amino acids [32]. In this study, a carboxylate has been implicated by

affinity labeling as the essential catalytic base of barley α-glucosidase. Another carboxyl amino acid might function as the catalytic acid, since this enzyme belongs to the acidic α-glucosidase family and modification of histidine did not show any significant effect on the catalytic reaction by this enzyme (Table 4).

Protein–carbohydrate interactions are characterized by common features such as extensive hydrogen-bonding involving all hydroxyl groups, interaction with mostly planar side-chains, and stacking of sugar residues by aromatic residues to confer affinity and specificity to the interaction [12,18,33,34]. Modification of the involved amino acids would cause a steric hindrance, an absence of hydrogen-bonding residues at the right orientation, and/or an unfavorable nonpolar environment [35]. Tryptophan was required for activity of *A. niger* glucoamylase [31], *A. awamori* glucoamylase [30], *Saccharomycopsis fibuligera* glucoamylase [28], yeast invertase [36], and human lysosomal α-glucosidase [26]. Chemical modification was used in yeast invertase and *A. niger* glucoamylase, and site-directed mutagenesis was used in the other three studies to demonstrate the requirement of a functional tryptophan. Even though tryptophan(s) in these carbohydrases are not the catalytic acid or base, they are essential components of the functional active center due to their binding to the glucose residues and/or their structural roles. Chemical modification of barley α-glucosidase shows that one or more tryptophan residues are required for a catalytically capable active center (Table 4). The required tryptophan was protected by even the smallest substrate tested, maltose, suggesting its location at either subsite 1 or 2. In chemical modification studies of *A. niger* glucoamylase, the Trp-120 (which was required for catalysis) was protected by acarbose but not by maltose or maltotriose, suggesting its location at subsite 4 [37]. However, recent X-ray crystallography of closely related *A. awamori* glucoamylase revealed its location at subsite 3 [25]. This discrepancy might be due to the lower concentration of maltose used to protect *A. niger* glucoamylase. When a lower concentration of maltose was used to protect barley α-glucosidase, the prevention against inactivation was greatly diminished (data not shown).

Data in this study suggest arginine(s) and lysine(s) are required for full activity of barley high pI α-glucosidase. These residues may participate in the catalytic cleavage of the glycosidic linkage by donating a proton and/or in substrate binding. Butanedione, another arginine-specific chemical, also decreases enzyme activity of barley α-glucosidase (data not shown). Lys-108 of *A. awamori* glucoamylase is located at subsite 2 by X-ray crystallography, and implicated to play a specific role in this enzyme's strong selectivity toward α-(1 → 4)-linked substrates [33]. It is of interest to see whether the modification of lysine(s) of barley α-glucosidase would affect the affinity and specificity of substrates having other than α-(1 → 4)-linkages.

Acknowledgements

We thank Agnes M. Kanikula and Charles B. Karpelenia for their expert technical assistance.

References

[1] Z. Sun and C.A. Henson, *Arch. Biochem. Biophys.*, 284 (1991) 298–305.
[2] Z. Sun and C.A. Henson, *Plant Physiol.*, 94 (1990) 320–327.
[3] Y. Konishi, A. Okamoto, J. Takahashi, M. Aitani, and N. Nakatani, *Biosci. Biotech. Biochem.*, 58 (1994) 135–139.
[4] D.C. Doehlert and S.H. Duke, *Plant Physiol.*, 71 (1983) 229–234.
[5] C.A. Henson and M. Collins, *Crop Sci.*, 24 (1984) 727–732.
[6] M.M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
[7] A.J. Barrett and H. Kirschke, *Methods Enzymol.*, 80 (1981) 535–561.
[8] I.H. Segel, *Enzyme Kinetics*, Wiley, New York, 1975, pp 465–473.
[9] K. Hiromi, *Biochem. Biophys. Res. Commun.*, 40 (1970) 1–6.
[10] K. Hiromi, Y. Nitta, C. Nimata, and S. Ono, *Biochim. Biophys. Acta*, 302 (1973) 362–375.
[11] K. Hiromi, M. Ohnishi, and A. Tanaka, *Mol. Cell. Biochem.*, 51 (1983) 79–95.
[12] H. Matsui and S. Chiba, *Agric. Biol. Chem.*, 47 (1978) 715–722.
[13] A.J. Clarke and B. Svensson, *Carlsberg Res. Commun.*, 49 (1984) 111–122.
[14] G. Legler, *Adv. Carbohydr. Chem. Biochem.*, 48 (1990) 319–384.
[15] Z. Sun, Ph.D. Thesis, The University of Wisconsin, Madison, Wisconsin, 1991.
[16] A.J. Barrett, A.A. Kembhavi, M.A. Brown, H. Kirschke, C.G. Knight, M. Tamai, and K. Hanada, *Biochem. J.*, 201 (1982) 189–198.
[17] E. Trusheit, W. Frommer, B. Junge, L. Muler, D.D. Schmidt, and W. Wingender, *Angew. Chem. Int. Ed. Engl.*, 20 (1981) 744–761.
[18] M.M. Meagher, B.Y. Tao, J.M. Chow, and P.J. Reilly, *Carbohydr. Res.*, 173 (1988) 273–283.
[19] S. Chiba, K. Kanaya, K. Hiromi, and T. Shimomura, *Agric. Biol. Chem.*, 43 (1979) 237–242.
[20] A. Kita, H. Matsui, A. Kimura, M. Takata, and S. Chiba, *Agric. Biol. Chem.*, 55 (1991) 2327–2335.
[21] A. Kimura, S. Takewaki, H. Matsui, M. Kubota, and S. Chiba, *J. Biochem.*, 107 (1990) 762–768.
[22] S. Onodera, H. Matsui, and S. Chiba, *J. Biochem.*, 116 (1994) 7–11.
[23] J.A. Thoma, G.V.K. Rao, C. Brothers, and J. Spradlin, *J. Biol. Chem.*, 246 (1971) 5621–5635.
[24] A.E. Aleshin, L.M. Firsov, and R.B. Honzatko, *J. Biol. Chem.*, 269 (1994) 15631–15639.
[25] E.M.S. Harris, A.E. Aleshin, L.M. Firsov, and R.B. Honzatko, *Biochemistry*, 32 (1993) 1618–1626.
[26] M.M.P. Hermans, M.A. Kroos, J. Vanbeeumen, B.A. Oostra, and A.J.J. Reuser, *J. Biol. Chem.*, 266 (1991) 13507–13512.
[27] S.G. Withers, K. Rupitz, and I.P. Street, *J. Biol. Chem.*, 263 (1988) 7929–7932.
[28] I. Itoh, Y. Sakata, R. Akada, O. Nimi, and I. Yamashita, *Agric. Biol. Chem.*, 53 (1989) 3159–3169.
[29] M.D. Jolley and C.J. Gray, *Carbohydr. Res.*, 49 (1976) 361–370.
[30] M.R. Sierks, C. Ford, P.J. Reilly, and B. Svensson, *Protein Eng.*, 3 (1990) 193–198.
[31] B. Svensson, A.J. Clarke, and I. Svendsen, *Carlsberg Res. Commun.*, 51 (1986) 61–73.
[32] Y. Someya, H. Matsui, and S. Chiba, *Agric. Biol. Chem.*, 48 (1984) 873–879.
[33] P.M. Coutinho and P.J. Reilly, *Protein Eng.*, 7 (1994) 393–400.
[34] F. Quiocho, *Pure Appl. Chem.*, 61 (1989) 1293–1306.
[35] F. Quiocho, *Ann. Rev. Biochem.*, 55 (1986) 287–315.
[36] V.A. Reddy and F. Maley, *J. Biol. Chem.*, 265 (1990) 10817–10820.
[37] B. Svensson, A.J. Clarke, I. Svendsen, and H. Møller, *Eur. J. Biochem.*, 188 (1990) 29–38.