Pergamon

PII: S0031-9422(97)00392-0

Phytochemistry, Vol. 46, No. 6, pp. 997–1003, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

IDENTIFICATION OF ESSENTIAL AMINO ACIDS FOR THE CATALYTIC ACTIVITY OF BARLEY β-GLUCOSIDASE

ANGELOS SKOUBAS and JOHN G. GEORGATSOS*

Laboratory of Biochemistry, School of Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece

(*Received in revised form* 1 *April* 1997)

Key Word Index—*Hordeum vulgare*; barley; β-glucosidase; essential histidyl and carboxyl residues.

Abstract—The active site of β-glucosidase of barley seeds was investigated by means of kinetic and chemical modification studies. DEPC (diethylpyro-carbonate) totally inactivates the enzyme but not in the presence of substrate. The inactivation reaction follows pseudo-first order kinetics with a second-order constant of 0.049 mM^{-1} min^{-1}. Reversal of inactivation in the presence of hydroxylamine leads to the inference that histidyl, tyrosyl and/or seryl residues are essential for catalysis. Treatment of the enzyme with pHMB (*p*-Hydroxymercuribenzoate), PMSF (phenylmethyl-sulphonyl fluoride) and NAI (*N*-acetyl-imidazole), does not influence activity, thus eliminating the possibility that cysteine, serine or tyrosine participate in catalysis. Kinetic analysis of DEPC inactivation indicates that one histidyl residue per mol of protein is essential for catalysis. EEDQ (2-ethoxy-1-ethoxy-1,2-dihydroquinoline) inactivates the enzyme, but not in the presence of substrate, following pseudo-first order kinetics with a second-order constant of 0.01 mM^{-1} min^{-1}. This is indicative of the involvement of residues with a carboxyl group in the catalytic activity. Further kinetic analysis of the inactivation caused by EEDQ, strongly implies that modification of a single residue of aspartate or glutamate inactivates the enzyme. The pH profile of enzyme velocity (V_{max}) and efficiency constant (V_{max}/K_m) gave apparent pK values of 3.51 and 7.56 for the enzyme-substrate complex and 3.88 and 6.38 for the free enzyme, further supporting the concept of histidyl and carboxyl residues engagement in the catalytic activity of barley β-glucosidase. © 1997 Elsevier Science Ltd

INTRODUCTION

β-Glucosidase of barley seeds catalyses the hydrolysis of cellobiose and aryl-β-glucosides and less favourably of β-galactosides [1]. According to the known sequence information [2] it belongs to family 1, of glycosyl hydrolases, also referred to as BGA enzyme family (β-glucosidase enzyme family A) [3, 4]. This large gene family of β-glycosidases includes β-glucosidases, β-galactosidases and phospho-β-glycosidases from as diverse organisms as archaebacteria, bacteria, plants and mammals.

There is a generally accepted mechanism of β-glycosidase action, based on acid/base catalysis involving two catalytic groups. One is generally an ionized carboxylate group, acting as a nucleophile centre, stabilizing the positively charged oxocarbonium-ion-like intermediate, or forming covalent glycosyl-enzyme intermediate. The other group is a protonated amino acid side chain with both acid catalytic function, protonating the leaving group as it departs, and general base function, deprotonating the incoming water molecule [5]. The incoming water molecule is not very nucleophilic and therefore cannot displace the leaving group on its own. The base function of the second catalytic group is to render the water more nucleophilic [6].

With respect to the amino acid residues responsible for catalysis of β-glycosidases, kinetic and chemical modification studies on the enzyme of *Botryodiplodia theobromae* [7, 8], almonds [9], *Brassica napus* [10], *Manihot esculenta* Crantz (Cassava) [11, 12] and *Trichoderma reesei* QM 9414 [13, 14] point to the involvement of both carboxylate and histidine at the active site. Similar studies indicate the essential role of only carboxyl residues in the active site of the enzyme of *Schizophyllum commune* [15], human acid β-glucosidase [16], human intestinal Lactase-phlorizin hydrolase [17], and *Agrobacterium faecalis* [18, 19]. The importance of thiol groups for β-glucosidase activity in the enzyme of the thermophile *Clostridium stercoarium* [20], *Trichoderma reesei* OM 9414 [13] and Maize (*Zea mays* L.) [21] has also been reported.

The present paper describes both kinetic and chemical modification studies that provide exper-

* Author to whom correspondence should be addressed.

imental evidence for the essential role of histidyl and carboxyl groups in the mechanism of action of barley β-glucosidase.

RESULTS

Dependence of kinetic parameters on pH

Initial velocities for β-glucosidase-catalysed hydrolysis of cellobiose were determined and recorded as the average of at least three measurements at each substrate concentration and pH value. The dependence of initial velocity upon substrate concentration was hyperbolic at each pH value studied and all Lineweaver–Burk plots were linear. Values for V_{max} and K_m were obtained for each pH value and are presented graphically in Fig 1. These plots (Dixon-plots) [22] have limiting slopes of +0.96 and −0.98 in the low and high pH region, respectively, and indicate the dependence of catalytic activity on the ionization of at least two essential groups at the active site of the enzyme. The lines drawn through the data, are the theoretical curves generated using the equation:

$$V_{max} = \frac{(V_{max})m}{1 + \frac{[H^+]}{Kes1} + \frac{Kes2}{[H^+]}} \quad (1)$$

where Kes1 and Kes2 are the dissociation constants for essential ionizable groups in the enzyme-substrate complex and $(V_{max})m$ is the maximum rate when the enzyme is in its optimally ionized form. The plot of $\log(V_{max})$ vs pH [Fig. 1(a)] fits reasonably well with the curve calculated using Kes1 and Kes2 values of 2.98×10^{-4} and 9.77×10^{-8}, respectively, thus providing pKes values of 3.51 and 7.56 for groups in the enzyme-substrate complex. Values of pKe were calculated using the equation:

$$V_{max}/K_m = \frac{(V_{max}/K_m)m}{1 + \frac{[H^+]}{Ke1} + \frac{Ke2}{[H^+]}} \quad (2)$$

where Ke1 and Ke2 are the dissociation constants for essential ionizable groups in the free enzyme. $(V_{max}/K_m)m$ is the efficiency constant when the enzyme is in its optimally ionized form. The plot of $\log(V_{max}/K_m)$ vs pH [Fig. (1b)], fits quite well with a theoretical curve calculated using Ke1 and Ke2 values of 1.30×10^{-4} and 4.15×10^{-7}. The corresponding pKe values are 3.88 and 6.38, respectively, for the free enzyme and the limiting slopes at low and high pH region are +0.95 and −0.97, respectively.

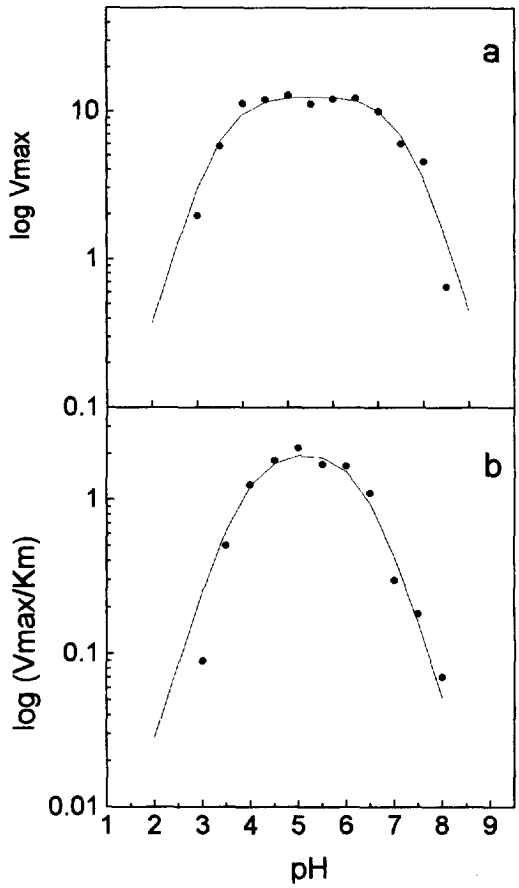

Fig. 1. Dependence of kinetic parameters on pH for the barley β-glucosidase-catalysed hydrolysis of cellobiose. Values of K_m and V_{max} were obtained from Lineweaver–Burk plots of initial rates of hydrolysis with twelve substrate concns (1.25–50 mM) and enzyme concn 0.9 mg ml^{-1}. Buffers used: 50 mM Na-citrate buffer for the 3.5–4.5 pH range, 50 mM NH_4OAc for the 4.5–6.5 pH range and 70 mM Na-Pi buffer for the 6.5–8.0 pH range. Total volume: 150 μl. (a) Effect of pH on $\log V_{max}$; (b) effect of pH on $\log(V_{max}/K_m)$. The solid lines represent the theoretical curve calculated using pKa values of 3.51 and 7.56 for (a) and 3.38 and 6.38 for (b), respectively.

Inhibition of β-glucosidase by group specific reagents

Iodoacetamide inactivates β-glucosidase at pH values of 8.0 and 6.0. At pH values greater than 7.5, iodoacetamide modifies mainly the side group of cysteine, whereas at pH values between 5.0 and 7.0 the side group of histidine is principally affected [26]. At pH 8.0 and iodoacetamide concentrations higher than 50 mM, the enzyme loses 90% of its activity [Fig. 2(a)]. The presence of 100 mM cellobiose does not interfere with the inactivation. At pH 6.0 the same concentrations of reagent inactivate the enzyme by 40%, but the presence of 100 mM cellobiose in the reaction mixture protects enzyme activity very efficiently [Fig. 2(b)].

DEPC, at concentrations higher than 10 mM causes a 95% loss of enzyme activity. Cellobiose at 100 mM protects the enzyme from inactivation (80% residual activity when modified with 10 mM DEPC) (Fig. 3). The possibility that a reaction between DEPC and cellobiose was responsible for this protection by lowering the effective concentration of DEPC was ruled

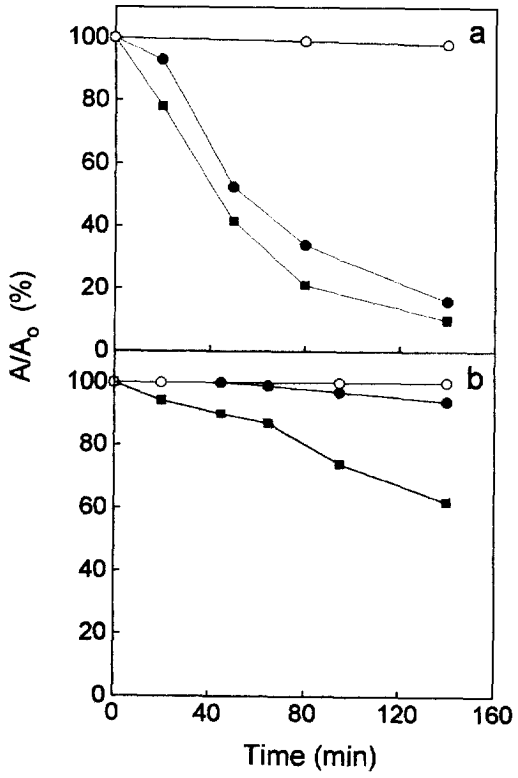

Fig. 2. Effect of iodoacetamide on barley β-glucosidase activity at pH 8.0 (a) and pH 6.0 (b), as assayed on *o*NPGlu under standard conditions. (○) Control, no iodoacetamide, no substrate; (■) in the presence of 50 mM iodoacetamide; (●) in the presence of 50 mM iodoacetamide plus 100 mM cellobiose. For details, see Experimental. *A/Ao* is fractional residual activity.

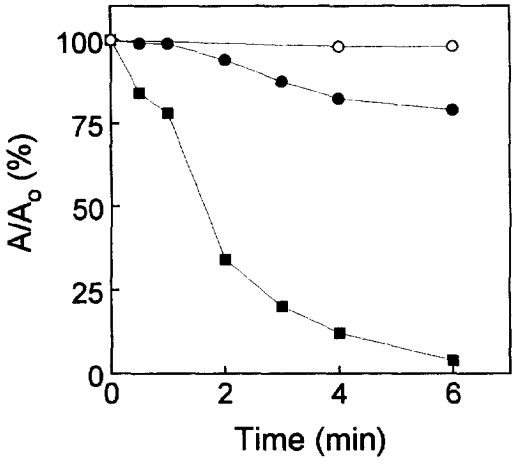

Fig. 3. Effect of DEPC on barley β-glucosidase activity as assayed on *o*NPGlu under standard conditions. (○) Control, no DEPC, no cellobiose; (■) in the presence of 10 mM DEPC; (●) in the presence of 10 mM DEPC plus 10 mM cellobiose. For details see Experimental. *A/Ao* is fractional residual activity.

out, since the rate of decomposition of the latter did not increase in the presence of cellobiose under identical experimental conditions.

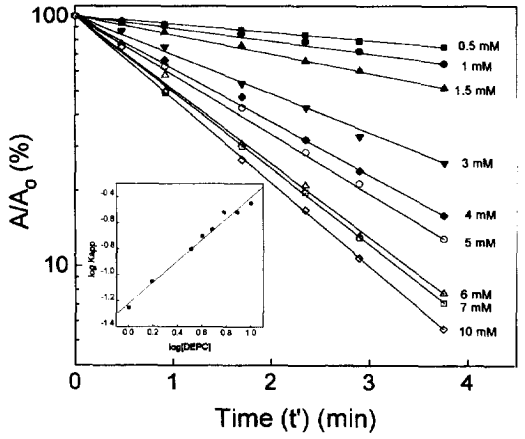

Fig. 4. Kinetics of inactivation of barley β-glucosidase by DEPC. The conditions of inactivation were the same as in Fig. 3, except that the enzyme was incubated with the indicated concns of DEPC. The definition of time (*t'*) is given in equation (4) in text under Results. Inset: Replot of log of the observed pseudo-first order K_{app} constants against log DEPC concns. *A/Ao* is fractional residual activity.

The kinetics of enzyme inactivation by DEPC were investigated at initial concentrations of inhibitor (0.5–10 mM) much larger than the enzyme concentration (Fig. 4). DEPC is unstable in aqueous solutions. Decomposition of the inhibitor followed first-order kinetics with a rate of 1.7 min^{-1}. To allow for hydrolysis of DEPC the inactivation data were plotted as log (*A/Ao* %) vs *t'* [23]. *A/Ao* is the fractional residual activity at time *t*, and *t'* is used to correct for the hydrolysis of the inhibitor according to:

$$t' = \frac{1 - e^{k't}}{k'} \qquad (3)$$

where *k'* is the first-order rate constant for DEPC hydrolysis. The plots of inactivation were linear (Fig. 4), indicating that the inactivation of β-glucosidase follows pseudo-first order kinetics. Second-order rate constants (*k*) were calculated as described by Levy *et al.* [24] from the replots of $\log k_{app}$ vs log [DEPC]:

$$\log k_{app} = \log k + n * \log [\text{DEPC}] \qquad (4)$$

where k_{app} is the pseudo-first order rate constant at each DEPC concentration. From the intercept of such a replot (Fig 4, inset) a rate constant *k* of 0.049 mM^{-1} min^{-1} was obtained. The slope of the replot (*n*) gave a reaction order of 0.84 ± 0.05, indicating that reaction with approximately one residue resulted in inactivation. The reaction of DEPC with proteins is specific for histidyl residues at neutral or near neutral pH [25], but in weakly alkaline media DEPC can also react with lysyl, tyrosyl and cysteinyl residues [26].

Hydroxylamine has been shown to remove carboethoxy groups from modified histidyl, seryl and tyrosyl residues but not from modified lysyl and sulphydryl residues. Treatment of enzyme carboethoxylated with various concentrations of DEPC,

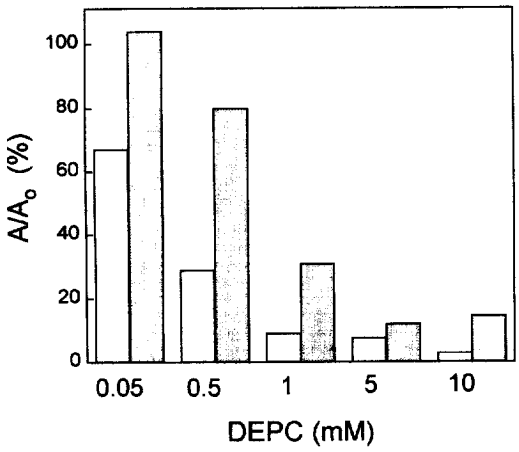

Fig. 5. Treatment of carboethoxy-β-glucosidase with hydroxylamine. Experimental conditions are described in Results. Columns represent percent of residual activity (A/Ao) of β-glucosidase modified by DEPC before (white columns), and after (grey columns) treatment with 0.1 M hydroxylamine. The residual activity of β-glucosidase that was not modified by DEPC but was treated with 0.1 M hydroxylamine for 1 hr at 37° was considered as 100% (in fact it was 85% of activity of the untreated enzyme).

with 0.1 m NH_2OH at pH 7.0 resulted in restoration of enzyme activity to a considerable extent (Fig 5).

EEDQ treatment of β-glucosidase at concentrations higher than 15 mM, results in total loss of activity. However, the enzyme is protected by substrate (100 mM cellobiose) retaining 80% activity when modified with 15 mM EEDQ (Fig. 6). Semilogarithmic plots of residual activity vs time of inactivation at several EEDQ concentrations are linear (Fig. 7), indicating that inactivation follows pseudo-first order kinetics. From the slope of the line resulting from plotting the log pseudo-first order rate constants (k_{app}) vs log [EEDQ], it is deduced that the order of the reaction

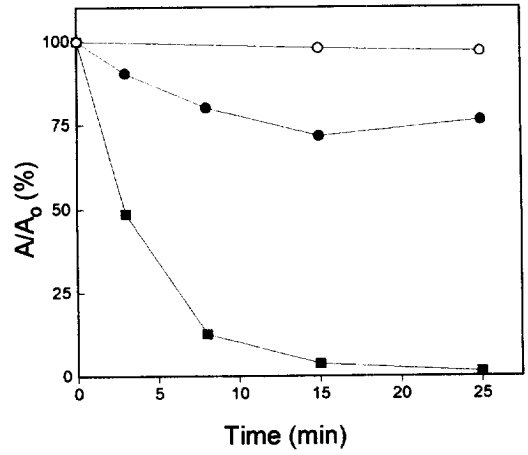

Fig. 6. Effect of EEDQ on barley β-glucosidase activity as assayed on oNPGlu under standard conditions. (○) Control, no EEDQ, no cellobiose; (■) in the presence of 15 mM EEDQ; (●) in the presence of 15 mM EEDQ plus 100 mM cellobiose. For details see Experimental. A/Ao is fractional residual activity.

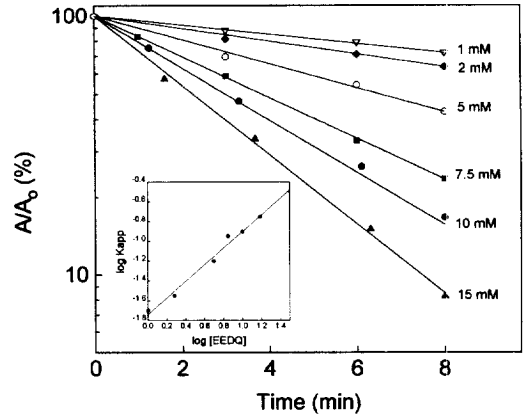

Fig. 7. Kinetics of activation of barley β-glucosidase by EEDQ. The conditions of inactivation were the same as in Fig. 6, except that the enzyme was incubated with the indicated concns of EEDQ. Inset: Replot of log of the observed pseudo-first order K_{app} constants against log EEDQ concns. A/Ao is fractional residual activity.

[n in equation (4)] is 0.93 ± 0.06 (Fig. 7, inset) leading to the inference that at least one carboxyl group per mol of enzyme is essential for activity. From the intercept of the replot, the second-order rate constant (k) of the inactivation is 0.01 mM^{-1} min^{-1}.

Enzyme activity was unaffected by treatment with 5 mM pHMB [27], 20 mM NAI [28] and 5 mM PMSF [29], thus ruling out the probable participation in catalysis of sulphydryl, tyrosyl and seryl groups, respectively (data not shown).

DISCUSSION

The nature of the catalytically essential ionizable groups in barley β-glucosidase was delineated by kinetic and chemical modification studies.

Inactivation of the enzyme by iodoacetamide at pH 6.0 is due to the modification of histidyl residues. Participation of the latter in the catalytic activity is further corroborated by the protection offered to the enzyme by the substrate cellobiose.

Modification of β-glucosidase by DEPC leads to inactivation due to the reaction of histidine side chains with the reagent, as inferred by the following findings: (i) inactivation by several concentrations of DEPC follows pseudo-first order kinetics, which indicates a direct correlation between inactivation and modification of the enzyme; (ii) substrate protects the enzyme from inactivation, and (iii) reversal of inactivation by treatment of the modified enzyme with 0.1 M hydroxylamine rules out the participation of lysyl and cysteinyl residues in the catalysis. Nevertheless the absence of total recovery of enzyme activity after hydroxylamine treatment might be due to the reaction of two DEPC molecules per histidyl residue with the formation of a dicarbethoxyhistidyl derivative, followed a ring-opening reaction that destroys the imidazole group [26].

The absence of protection against inactivation by

substrate when the enzyme is modified by iodoacetamide at pH 8.0 and the persistence of catalytic activity when treated with *p*HMB, further excludes the involvement of cysteinyl residues in catalysis. However, the loss of activity observed in enzyme preparations treated with iodoacetamide, probably reflects conformational changes in the enzyme induced by acylation of other than cysteinyl residues that are expected to occur during the 2.5 hr incubation period. It should be stressed that iodoacetamide is not absolutely specific for cysteine under these conditions. Incubation of β-glucosidase with PMSF and NAI did not cause inhibition. This observation makes it highly unlikely that either seryl or tyrosyl residues are important for catalysis.

EEDQ causes inactivation of the enzyme, but not in the presence of substrate. This is indicative of the involvement in the catalytic activity of residues with a carboxyl side chain (i.e. aspartate and/or glutamate). With this specific reagent a discrimination of the species of the modified residue cannot be done [30]. Inhibition of β-glucosidase by EEDQ follows pseudo-first order kinetics, as in the case of DEPC inactivation. In both cases, the replots of $\log k_{app}$ vs log [inhibitor] are linear with slopes of 0.84 ± 0.05 and 0.93 ± 0.06 for DEPC and EEDQ, respectively. These values are close to unity and suggest that the modification of a single residue of histidine and a single residue of aspartate or glutamate inactivates the enzyme.

Studies of the pH dependence of kinetic parameters, showed that the slope of the line resulting from plotting $\log (V_{max}/K_m)$ for cellobiose as a function of pH changes markedly at both high and low pH values [Fig 1(b)]. The change at low pH corresponds to a pK value of 3.88 and has a limiting slope close to $+1$, demonstrating that the change in the parameter value is a result of the alteration in protonation state of a single amino acid chain. Consequently a group in the substrate or in the enzyme must be unprotonated for activity. As cellobiose has no ionizable groups, it is highly likely that such a group in the enzyme is essential for activity, since carboxyl groups exhibit such pK values. It is very probable that this group is the side chain of an aspartate or a glutamate residue. The pK value of this potent carboxyl group is closer to that of aspartate ($pK_{COOH} = 3.65$). The pK values of the essential carboxylate was found to be 3.3 in β-glucosidase of *Schizophyllum commune* [15], 4.3 of *Botryodiplodia theobromae*, [7, 8], 4.6 of *Manihot esculenta* [11] and 5.64 of *Trichoderma reesei* QM 9414 [13].

The change in slope in the V_{max}/K_m profile at higher pH values [Fig. 1(b)] corresponds to a pK value of 6.38 and has a limiting slope close to -1. As the substrate contains no ionizable group with this pK value, a single group on the enzyme that must be protonated is essential for activity. A histidine side chain is a prime candidate for this protonated group. This pK value correlates quite well with the values previously suggested for essential histidines in various β-glycosidases. Thus the essential histidine of the enzyme of *Manihot esculenta* had a pK value of 7.0 [11], of *Trichoderma reesei* QM 9414, 5.2 [13], and of *Botryodiplodia theobromae*, 6.0 [7, 8].

In the case of *Agrobacterium* β-glucosidase, two groups were found essential for catalysis. The unprotonated group had a pK value lower than 5.0 and the protonated between 7.2 and 8.1. The former is attributed to the carboxyl side chain of glutamate 358 [31, 32].

These data are consistent with the presence of at least one histidyl residue and one carboxyl group at the active site of β-glucosidase, which are responsible for substrate binding, for catalysis or for both. The histidyl side chain probably plays the role of acid/base catalyst participating in the departure of the aglycone moiety of the substrate; the carboxyl residue stabilizes the oxycarbonium-ion intermediate or participates in the formation of the glucosyl-enzyme intermediate.

In the literature one finds a number of papers on the catalytic mechanism of β-glucosidase, some of which are mentioned in the Introduction. Still, the location of the two potent catalytic residues is the subject of debate. Sequence comparisons to β-glycosidases belonging to the BGA enzyme family (by hydrophobic cluster analysis) shows that all BGA enzymes share a well-conserved region, which is homologous to the catalytic domain of the widely distributed cellulase family A. A distinctive feature of this domain is the sequence motif His-...-Asn-Glu-Pro present within the first 130 N-terminal amino acid residues where the presumed catalytic residues His and Glu are separated by 35–55 amino acids [4].

A different hypothesis suggests that highly conserved sequences located towards the C-termini are responsible for catalysis. This hypothesis is supported by structure and sequence comparison of β-glycosidases, β-glucanases, xylanases and related enzymes. The catalytic domain contains the motif Asn-Glu-$X_{(100)}$-Glu, where the two glutamates are the essential residues for catalysis. The Glu in the asparagine-glutamate sequence has the role of acid/base catalyst, while the second glutamate some 100 residues further down the chain may be the nucleophile [33, 34].

Recently the complete sequence of barley β-glucosidase cDNA has been published [35]. The derived amino acid primary structure revealed that the enzyme is composed of 485 residues and contains the sequence ^{388}Ile-Leu-Ser-Glu-Asn-Gly, where the glutamate is considered the nucleophile. On the other hand it also embodies the motif ^{113}His and ^{121}His-...-^{158}Asn-Glu-Pro which may also include the catalytic site [4]; if the latter is the case, then it agrees well with the findings reported in the present paper.

It is possible that with the methods applied in the present work, not all residues essential for catalysis have been revealed In this event the necessity for the presence of one of them (histidine presumably), may lie in modulating the charge state of other unexpected catalytic residues [33, 36].

EXPERIMENTAL

Enzyme source. β-Glucosidase of barley (*Hordeum vulgare* cv *Sofia*) was used throughout this work. The purification protocol and assay conditions with cellobiose or *o*NPGlu as substrate, were published previously [1].

Dependence of kinetic parameters on pH. Maximal velocity (V_{max}) and Michaelis (K_m) constants for the hydrolysis of cellobiose were determined as described in the legend to Fig. 1. Kinetic parameters were calcd by nonlinear fitting of the classic Michaelis–Menten rate equation to the experimental data. Molecular acid dissociation constants of the enzyme molecule (Ke1, Ke2) and the enzyme-substrate complexes (Kes1, Kes2) were determined on the basis of diprotic enzyme model [22].

Chemical Modification Reactions. All experiments were carried out in triplicate and mean values are recorded in the figures.

Iodoacetamide. The reaction of β-glucosidase (10 μg) with CH_2ICONH_2 (5–50 mM) took place in the presence of 20 mM Tris–HCl pH 8.0 or 50 mM NH_4OAc pH 6.0, at 37° in a total vol. of 80 μl. At various time intervals, 10 μl aliquots were withdrawn and the residual enzyme activity was measured under the standard assay conditions. Substrate protection assays were performed under identical conditions as above, except that the reaction mixts contained 100 mM cellobiose each, and the residual activity was measured with 3 mM *o*NPGlu (*o*-nitrophenyl glucoside) as substrate.

DEPC. β-Glucosidase (25 μg) in 150 μl of 50 mM Na–Pi buffer, PH 7, was incubated at 37° for 10 min with 7.5 μl of DEPC–EtOH soln containing the appropriate amount of the reagent (0.05–10 mM). The concn of EtOH in the mixt. did not exceed 5% and was found to have no noticeable effect on the stability or activity of the enzyme. At various time intervals, aliquots of 15 μl were withdrawn and the reaction was stopped by adding 2 μl of 0.2 M imidazole–HCl buffer, pH 7.4 and cooling on ice. Residual enzyme activity was measured after addition of 100 mM cellobiose under the standard assay conditions.

Reactivation of DEPC-modified enzyme was achieved by adding 0.1 M NH_2OH at pH 7.2. To DEPC-treated reaction mixts (*cf* above), 8.5 μl 2 M NH_2OH pH 7.2 were added and the mixts were incubated at 37°. Excess NH_2OH was removed by exhaustive dialysis against deionised H_2O. Residual enzyme activity was measured under standard assay conditions. The rate of DEPC decomposition under the experimental conditions described above, was determined as described in [25].

pHMB. β-Glucosidase (18 μg) in 60 μl of 50 mM Na–Pi buffer, pH 8 was incubated at 37° for 30 min with sufficient amount of *p*HMB to render the solns 1–5 mM. Aliquots of 8 μl were withdrawn at various time intervals, mixed with 2.5 μl 0.4 M Na-citrate buffer pH 4.8 and placed on ice. Residual enzyme activity was assayed under standard conditions.

PMSF. β-Glucosidase (25 μg) in 150 μl of 50 mM Na-Pi buffer, pH 7 was incubated for 60 min at 37° with varying amounts of PMSF (1–5 mM final concns) dissolved in EtOH. Aliquots of 15 μl were withdrawn at various time intervals and residual enzyme activity was measured under standard conditions.

NAI. β-Glucosidase (25 μg) in 150 μl of 50 mM Na-Pi buffer, pH 7 was incubated for 1 hr at 37° with varying amounts of NAI (1–20 mM final concns). Aliquots of 15 μl were withdrawn at various time intervals and residual enzyme activity was measured under standard conditions.

EEDQ. β-Glucosidase (20 μg) in 60 μl of 50 mM MES buffer, pH 5 was incubated for 30 min at 37° with varying amounts of EEDQ (1–15 mM final concns). At various time intervals, aliquots of 8 μl were transferred to tubes, each containing 2.5 μl 0.4 M Na-citrate buffer pH 4.8 and placed on ice. Residual enzyme activity was measured under standard conditions. Substrate (cellobiose) protection assays were carried out as described under CH_2ICONH_2 modification.

REFERENCES

1. Simos, G. and Georgatsos, J. G., *Biochimica et Biophysica Acta*, 1988, **967**, 17.
2. Simos, G., Panagiotidis, C. A., Skoubas, A., Choli, D., Ouzounis, C. and Georgatsos, J. G., *Biochimica et Biophysica Acta*, 1994, **1199**, 52.
3. Henrissat, B., *Biochemical Journal*, 1991, **280**, 309.
4. Grabnitz, F., Seiss, M., Rucknagel, K. P. and Staudenbauer, W. L., *European Journal of Biochemistry*, 1991, **200**, 301.
5. Sinnott, M. L., *Chemical Review*, 1990, **90**, 1171.
6. Damude, G. H., Withers, G. S., Kilburn, G. D., Miller, C. R. Jr. and Warren, J. R. A., *Biochemistry*, 1995, **34**, 2220.
7. Umezurike, M. G., *Biochemical Journal*, 1979, **179**, 503.
8. Umezurike, M. G., *Biochemical Journal*, 1987, **241**, 455.
9. Hsuanyu, Y. and Laiker, K. J., *Canadian Journal of Biochemical and Cell Biology Sciences*, 1985, **63**, 167.
10. Lenman, M., Falk, A., Xue, J. and Rask, L., *Plant Molecular Biology*, 1993, **21**, 463.
11. Keresztessy, Z., Kiss, L. and Hughes, A. M., *Archives of Biochemistry and Biophysics*, 1994, **314**(1), 142.
12. Keresztessy, Z., Kiss, L. and Hughes, A. M., *Archives of Biochemistry and Biophysics*, 1994, **315**(2), 323.
13. De la Mata, I., Castillôn, M. P., Dominguez, J. M., Marcarrôn, R. and Acebal, C., *Journal of Biochemistry*, 1993, **114**, 754.
14. De la Mata, I., Estrada, P., Macarrôn, R., Dominguez, J. M. and Castillôn, M. P., *Biochemical Journal*, 1992, **283**, 679.

15. Clarke, A. J., *Biochimica et Biophysica Acta*, 1990, **1040**, 145.
16. Dinur, T., Osiecki, K. M., Legler, G., Gatt, S., Desnick, R. J. and Grabowski, G. A., *Proceedings of the National Academy of the Sciences of the U.S.A.*, 1986, **83**, 1660.
17. Wacker, H., Keller, P., Falchetto, R., Legler, G. and Semenza, G., *Journal of Biological Chemistry*, 1992, **267**(26), 18744.
18. Trimbur, E. D., Warren, J. R. A. and Withers, G. S., *Journal of Biological Chemistry*, 1992, **267**, 10248.
19. Withers, G. S., Rupitz, K., Trimbur, D. and Warren, J. R. A., *Biochemistry*, 1992, **31**, 9979.
20. Bronnenmeier, K. and Staudenbauer, W. L., *Applied Microbiology and Biotechnology*, 1988, **28**, 380.
21. Esen, A., *Plant Physiology*, 1992, **98**, 174.
22. Segel, I. H., *Enzyme Kinetics*. Wiley, New York, 1975, p. 884.
23. Topham, C. M., *Biochemical Journal*, 1985, **227**, 1025.
24. Levy, H. M., Leber, P. D. and Ryan, E. M., *Journal of Biological Chemistry*, 1963, **238**, 3654.
25. Miles, E. W., *Methods in Enzymology*, 1977, **47**, 431.
26. Means, G. E. and Feeney, R. E., *Chemical Modifications of Proteins*, Holden-Day, San Francisco, 1971.
27. Boyer, P. D., *Journal of the American Chemical Society*, 1954, **76**, 4331.
28. Ohta, Y., Nakamura, H. and Samajima, J., *Journal of Biochemistry*, 1972, **72**, 521.
29. Fahrney, E. D. and Gold, M. A., *Journal of the American Chemical Society*, 1963, **85**, 997.
30. Dugas, H and Penney, C., in *Biorganic Chemistry*. Springer, New York, 1981, p. 75.
31. Kempton, J. B. and Withers, S. G., *Biochemistry*, 1992, **31**, 9961.
32. Street, I. P., Kempton, J. B. and Withers, S. G., *Biochemistry*, 1992, **31**, 9970.
33. McCarter, J. D. and Withers, S. G., *Current Opinion in Structural Biology*, 1994, **4**, 885.
34. Jenkins, J., Leggio, L. L., Harris, G. and Pickersgill, R., *FEBS Letters*, 1995, **362**, 281.
35. Leah, R., Kigel, J., Svendsen, I. and Mundy, J., *Journal of Biological Chemistry*, 1995, **270**(26), 15789.
36. Warren, R. A. J., *Current Opinion in Biotechnology*, 1993, **4**, 469.