

**Mutagenesis and mechanistic study of a family 54 α -L-arabinofuranosidase
from *Trichoderma koningii***

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Running Title: Study of a family 54 α -L-arabinofuranosidase

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ABSTRACT

A GH54 α -L-arabinofuranosidase (Abf) from *Trichoderma koningii* G-39 was successfully expressed in *Pichia pastoris* and purified to nearly homogeneity by cation-exchange chromatography. To determine amino acid residues essential for the catalytic activity of Abf, extensive mutagenesis of 24 conserved Glu and Asp residues was performed. Among the mutants, D221N, E223Q, and D299N were found to decrease catalytic activity significantly. The k_{cat} values of the D221N and D299N mutants were 7000 and 1300-fold lower, respectively, than that of the wild-type Abf. E223Q was nearly inactive. These results are consistent with implications obtained from the *Aspergillus kawachii* α -L-arabinofuranosidase three-dimensional structure. This structure indicates that D221 of *T. koningii* Abf is significant for substrate binding and E223 as well as D299 function as a nucleophile and a general acid/base catalyst for the enzymatic reaction, respectively. The catalytic mechanism of wild-type Abf was further investigated by NMR spectroscopy and kinetic analysis. The results showed that Abf is a retaining enzyme. It catalyzes the hydrolysis of various substrates via the formation of a common intermediate that is probably an arabinosyl-enzyme intermediate. A two-step, double-displacement mechanism involving first the formation, and then the breakdown, of an arabinosyl-enzyme intermediate was proposed. Based on the k_{cat} values of a series of aryl- α -L-arabinofuranosides catalytically hydrolyzed by wild-type Abf, a relatively small Brønsted constant, $\beta_{\text{lg}} = -0.18$, was obtained, suggesting that the rate-limiting step of the enzymatic reaction is the dearabinosylation step. Further kinetic studies with D299G mutant revealed that the catalytic activity of this mutant depended largely on the $\text{p}K_{\text{a}}$ values (> 6) of leaving phenols, with $\beta_{\text{lg}} = -1.3$, indicating that the rate-limiting step of the reaction becomes the arabinosylation step. This kinetic outcome supports the idea that D299 is the general acid/base residue. The pH activity profile of D299N provided further evidence strengthening this suggestion.

INTRODUCTION

α -L-Arabinofuranosidases (EC 3.2.1.55) are among key enzymes of the hemicellulase system, which is tremendously useful in biobleaching of paper pulp [1], bioconversion of lignocellulose material to fermentative products [2], and improvement of animal feedstock digestibility [3, 4]. α -L-Arabinofuranosidases catalyze the hydrolysis of α -1,2- and α -1,3- α -1,5-L-arabinofuranosidic bonds in hemicelluloses such as arabinoxylan, L-arabinan, and other L-arabinose-containing polysaccharides [4, 5]. These enzymes also hydrolyze the glycosides of monoterpenes, sesquiterpenes, and other alcohols, releasing the aromatic potential of wine. The enzymes have general applications in flavour improvement [6–8].

Owing to their industrial importance, a variety of α -L-arabinofuranosidases have been purified from various sources such as bacteria [9], fungi [10], and plants [11]. Many genes encoding Abf enzymes have been cloned and sequenced. On the basis of amino acid sequence similarities [12–13], α -L-arabinofuranosidases are grouped mainly in four glycoside hydrolase (GH) families, termed GH43, GH51, GH54, and GH62. GH43 and GH62 can be further classified into Clan GH-F, and GH51 is in Clan GH-A, whereas GH54 remains unique from all existing clans. Although many studies on the industrial applications of α -L-arabinofuranosidases from the above families have been reported, only a few papers have been published describing detailed catalytic mechanisms with reference to the specific enzyme residues involved. This lack of information reflects difficulties in obtaining synthetic substrates bearing different leaving groups [14, 15], as well as problems with effective recombinant protein expression.

There are two general mechanisms of glycoside hydrolase cleavage of glycosidic bonds. The original anomeric configuration is either inverted or retained. Both mechanisms require amino acids containing side-chain carboxylic residues as essential group(s). Inverting glycosidases catalyze the hydrolysis of the glycosidic bond via a single displacement reaction. One of the catalytic residues, acting as a general acid, provides protonic assistance to the departing glycosidic oxygen, while the second, acting as a general base, activates a water molecule that effects a direct displacement at the anomeric center [16]. For retaining glycoside hydrolases, two key active-site carboxylic acid residues are involved in the catalytic reaction. One serves as the nucleophile and the other serves as a general acid/base. The proposed mechanism is shown in Figure 1. In the first step (the glycosylation step), the nucleophile attacks the anomeric carbon of the glycoside, while the acid/base catalyst protonates the glycosidic oxygen, thereby assisting the leaving of the aglycon moiety. This leads to the

formation of a covalent glycosyl-enzyme intermediate. In the second step (the deglycosylation step), the breakdown of the glycosyl-enzyme intermediate proceeds through a general base-catalyzed attack of water at the anomeric center to release the glycoside. For kinetic analysis of a catalytic mechanism, a series of artificial substrates, each with a glycoside moiety attached to different leaving phenols, is commonly prepared. Typically, the extended Brønsted relationship is measured. As enzymes in the same family presumably possess a similar catalytic mechanism, solving the mechanistic action of a particular enzyme may help in the understanding of the reaction pattern of its family. Among the four α -L-arabinofuranosidase families, a detailed mechanistic study of family GH51 was recently reported [17], but mechanistic details for the other family members remain unknown. Very recently, the potential for mechanistic studies of members of the GH54 family has greatly increased, owing to the complete resolution of the three-dimensional structure of an α -L-arabinofuranosidase from *Aspergillus kawachii* IFO4308 [18]. On the basis of protein structure and very preliminary mutagenic studies, E221 and D297 were suggested as candidates for the nucleophile and the general acid/base catalytic residues, respectively. In this study, we confirm the catalytic functions of essential Abf residues by site-directed mutagenesis and kinetic analysis of mutant enzyme properties. Further, the enzymatic mechanism of a GH54 family member is described and discussed, for the first time, in this paper.

EXPERIMENTAL PROCEDURES

Materials

Plasmid pPICZ α B and *Pichia pastoris* strain GS115 were purchased from Invitrogen (Carlsbad, CA, USA). *Escherichia coli* strain JM109 (ECOS, Taipei, Taiwan) served as the host for recombinant plasmids. Oligonucleotides were synthesized by Integrated DNA Technologies (Mission Biotechnology, Taipei, Taiwan). *Vent* polymerase from New England Biolabs (Ipswich, MA, USA) was used in polymerase chain reactions (PCR). The antibiotic zeocin was obtained from Invitrogen (Carlsbad, CA, USA). Restriction endonucleases and T4 DNA ligase were obtained from Roche Applied Science (Mannheim, Germany). Low molecular-weight protein marker standards for electrophoresis were purchased from Amersham Biosciences (Piscataway, NJ, USA). Buffers and chemicals for synthesis were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

The media BMGY, BMMY, YPD, YPD plus zeocin, Luria-Bertani (LB), and low-salt LB plus zeocin are described in the EasySelect™ *Pichia* Expression Kit manual (version F) and the

pPICZ α A, B, C manual (Invitrogen). BMGY, BMMY, and YPD media were prepared according to the manufacturer's instructions. The medium used for 1 L fermentations was that described in the *Pichia* fermentation guidelines.

Substrate synthesis

The synthesis of phenol-linked arabinofuranosides differs slightly from methods followed in the preparation of glycosides where the glucose has a six-membered ring. To obtain the arabinofuranoside as the major final product, methyl- α -L-arabinofuranoside was synthesized and used as the starting material for further reactions. The complete procedure has been published [19]. All substrates, including phenyl- α -L-arabinofuranoside (PAF), p-cyanophenyl- α -L-arabinofuranoside (pCPAF), m-nitrophenyl- α -L-arabinofuranoside (mNPAF), p-nitrophenyl- α -L-arabinofuranoside (pNPAF), 4-chloro-2-nitrophenyl- α -L-arabinofuranoside (CNPAF), and 2,5-dinitrophenyl- α -L-arabinofuranoside (2,5-DNPAF) were synthesized according to this published protocol. All substrates were purified by column chromatography and structures confirmed by NMR, which yielded the following data:

methyl- α -L-arabinofuranoside, 4.81 (d, 1H, C1-H, $J = 1.53$ Hz), 3.92 (m, 2H, C2-H, C4-H), 3.83 (m, 1H, C3-H), 3.70 (dd, 1H, $J = 3.36, 8.89$ Hz), 3.58 (dd, 1H, $J = 5.79, 6.51$ Hz), 3.30 (s, 3H); phenyl- α -L-arabinofuranoside, 7.33 (t, 2H), 7.05 (m, 3H), 5.64 (s, 1H, C1-H), 4.29 (m, 1H, C2-H), 4.11 (dd, 1H, C4-H, $J = 3.57, 5.49$ Hz), 4.02 (dd, 1H, C3-H, $J = 2.19, 3.56$ Hz), 3.75 (dd, 1H, $J = 3.57, 8.78$ Hz), 3.66 (dd, 1H, $J = 5.49, 11.25$ Hz); p-cyanophenyl- α -L-arabinofuranoside, 7.65 (d, 2H, $J = 8.91$ Hz), 7.12 (d, 2H, $J = 8.89$ Hz), 5.71 (s, 1H, C1-H), 4.30 (dd, 1H, C2-H, $J = 1.53, 1.55$ Hz), 4.07 (dd, 1H, C4-H, $J = 2.00, 3.38$ Hz), 4.00 (dd, 1H, C3-H, $J = 2.53, 3.26$ Hz), 3.73 (dd, 1H, $J = 3.42, 9.05$ Hz), 3.64 (dd, 1H, $J = 5.38, 6.94$ Hz); m-nitrophenyl- α -L-arabinofuranoside, 7.8 (d, 2H, $J = 8.78$ Hz), 7.48 - 7.37 (m, 2H), 5.68 (s, 1H, C1-H), 4.29 (dd, 1H, C2-H, $J = 1.54, 1.67$ Hz), 4.07 (m, 1H, C4-H), 3.99 (m, 1H, C3-H), 3.72 (m, 1H), 3.63 (m, 1H); p-nitrophenyl- α -L-arabinofuranoside, 8.28 (d, 2H, $J = 9.34$ Hz), 7.26 (d, 2H, $J = 9.06$ Hz), 5.88 (d, 1H, C1-H, $J = 1.1$ Hz), 4.46 (dd, 1H, C2-H, $J = 1.37, 1.67$ Hz), 4.20 (dd, 1H, C4-H, $J = 2.74, 3.33$ Hz), 4.14 (dd, 1H, C3-H, $J = 3.57, 9.06$ Hz), 3.77 (dd, 1H, $J = 5.67, 7.14$ Hz), 3.66 (dd, 1H, $J = 5.38, 5.45$ Hz); 4-chloro-2-nitrophenyl- α -L-arabinofuranoside, 7.92 (d, 1H, $J = 2.74$ Hz), 7.58 (dd, 1H, $J = 2.74, 6.32$ Hz), 7.32 (d, 1H, $J = 9.06$ Hz), 5.66 (d, 1H, C1-H, $J = 1.37$ Hz), 4.34 (dd, 1H, C2-H, $J = 1.65, 1.92$ Hz), 4.12 (m, 1H, C4-H), 3.98 (dd, 1H, C3-H, $J = 2.47, 3.57$ Hz), 3.73 (dd, 1H, $J = 3.57, 9.05$ Hz), 3.63 (dd, 1H, $J = 5.49, 6.86$ Hz); 2,5-dinitrophenyl- α -L-arabinofuranoside, 8.27 (s, 1H), 8.01 (d, 2H, $J = 1.1$ Hz), 5.78 (d, 1H, C1-H, $J = 1.37$ Hz), 4.32 (dd, 1H, C2-H, $J = 1.37, 2.47$ Hz), 4.09 (m, C4-H, 1H), 4.02 (dd, 1H, C3-H, $J = 2.74, 3.84$ Hz), 3.79 (dd, 1H, $J = 3.02, 9.05$ Hz), 3.68 (dd, 1H, $J = 4.66, 7.41$ Hz).

Vector construction for Pichia expression system

A vector containing the *Abf* gene from *Trichoderma koningii* G-39 (also known as *Hypocrea koningii* G39) was the generous gift of Prof. T.-H. Hseu from the National Tsing Hua University, Hsinchu, Taiwan. To insert the *abf* gene in an expression vector, an N-terminal primer (5'- TTAAGAAGGAGCTGCAGCAATGGGGCCTTG -3') and a C-terminal primer (5'- GCTCGAATTCGGTACCTTAAGCAAACC -3'), containing *Pst*I and *Kpn*I restriction enzyme sites, respectively, were used for PCR amplification of the *abf* gene. The gene was further cloned into the *Pst*I and *Kpn*I site of pPICZ α B. The recombinant plasmid, pPICZ α B-*abf*, was then amplified in *E. coli* JM109 and further linearized and introduced into *P. pastoris* strain GS115 by electroporation. The electroporation mixture was plated onto YPD-zeocin agar medium. After 72 h at 28 °C, colonies were picked for direct colony PCR using the 5'- and 3'-*AOX1* primers, followed by DNA sequence analysis of the resulting PCR products. Positive clones were then used to screen for Abf protein expression by initial growth, with shaking at 180 rpm, in 50 mL BMGY medium. Recombinant protein expression in cultures at OD₆₀₀ values of 7–8 was induced by transferring the cells to BMMY medium. At 24, 48, 72, 96, and 120 h post-induction, methanol, to a final concentration of 0.5% (v/v), was added to cultures. After 160 h of incubation, cultures were centrifuged and proteins in the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis. Clones producing the protein of interest were identified by the presence of an appropriately sized (50 kDa) supernatant protein band.

In vitro site-directed mutagenesis

The *abf* gene was mutagenized using the QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA, USA). The primers used to generate the mutations were as follows (mutations are underlined): D170N(+), 5'-CATGTATGCGGTTCTCAATGGTACACAC-3' and D170N(-), 5'-GTGTGTACCATTGAGAACCGCATAACATG-3'; D221N (+), 5'-GATCATGGCTAATCTCGAGAACGGCTTG-3' and E223Q(-), 5'-CAAGCCGTTCTGGAGATCAGCCATGAT C-3', E223G(+), 5'-GATCATGGCTGATCTTCGGCAAGGCTTG-3', and E223G(-), 5'-CAAGCCGTTGCCGAGATCAGCCATGAT C-3', D299N(+), 5'-CTCGGCATTGGCGGCAACAACAGC-3' and D299N(-), 5'-GCTGTTGTTGCCGCCAATGCCGAG-3', D299G(+), 5'-CATTGGCGGGCGGCAACAGCAACGGC-3' and D299G(-), 5'-GCCGTTGCTGTTGCCGCCGCCAATG-3', E310Q(+), 5'-TCTATCAGGGCATGACCTCTGGCTATC-3' and E310Q(-),

5'-GATAGCCAGAGGTCATGACGCCCTGATAGA-3'. Note that D221N (+) and E223Q(-) primers were used to performed both mutations. Primers creating other mutations that did not cause significant loss in enzyme activity are listed as supplementary data. All mutations were confirmed by DNA sequencing. For those mutations creating significant losses in enzyme activity, the entire mutated genes were sequenced to confirm that only the intended mutations had occurred.

Protein production and purification

Recombinant cultures of *P. pastoris* (pPICZ α B-*abf*) were incubated overnight at 28 °C, shaking at 180 rpm, in 250 mL BMGY medium contained in 1 L Erlenmeyer flasks, until OD₆₀₀ values of 7–8 were attained. The cells were then transferred to 100 ml of BMMY medium to induce the expression of Abf or Abf mutant proteins. After 120 h of incubation, the cells were pelleted by centrifugation at 4200 xg for 30 min, and supernatant containing secreted enzyme was clarified by filtering through 0.5 μ m filters. The proteins in the supernatant were precipitated by the addition of ammonium sulfate to 80–85% saturation. The precipitate was resuspended in 3 mL of sodium acetate buffer (20 mM, pH 4.5). The solution was then desalted using a 5 mL HiTrap desalting column (Pharmacia, Uppsala, Sweden). The filtrate (4 mL) was loaded onto three 5 mL cation-exchange HiTrap SP columns (Pharmacia) preequilibrated with 20 mM sodium acetate buffer, pH 4.5. The columns were eluted with a 150 mL linear gradient of NaCl (0–300 mM) at a flow rate of 1 mL/min. The fractions containing enzyme activity were collected and stored at 4 °C. All purification steps were performed at ambient temperature (approx. 25 °C). To minimize enzyme cross-contamination during purification, fresh HiTrap SP columns were used in the purification of each mutant protein. Protein concentrations were determined using the bicinchoninic acid method, as described in the manufacturer's protocol (Sigma; BCA-1 kit), followed by measurement of chromophore absorption at 280 nm. The molecular weights of purified enzymes were estimated by SDS-PAGE according to Laemmli [20] in comparison to the molecular weights of standard proteins (14–97 kDa).

Kinetic studies

Enzyme activity was determined at pH 4.1 by monitoring the hydrolysis of pNPAF (0.2 mM) to release p-nitrophenol (or p-nitrophenolate). An enzyme unit was defined as the amount of enzyme required for releasing 1 μ mol of p-nitrophenol from substrate in 1 min. A Hewlett-Packard model 8452A spectrophotometer equipped with a circulating water bath at 25 °C was used. The pH-dependence of enzyme activity in both wild-type and mutant enzymes

was assessed using pNPAF as substrate with assay pH values in the range 1.9–6.5. Reactions were monitored at the isosbestic point (348 nm) of p-nitrophenol and p-nitrophenolate and kinetic constants calculated on the basis that the molar absorption coefficient ($\Delta\epsilon$) was $2426 \text{ M}^{-1}\text{cm}^{-1}$. Buffers used in this study were glycine (pH 1.8 to 3.5), sodium acetate (pH 4.0 to 5.5), morpholinoethanesulfonic acid (pH 5.5 to 6.5), phosphate (pH 6.5 to 7.5), and Bicine (pH 7.5 to 9.5). Enzyme concentration used for kinetic measurement was $0.04 \mu\text{M}$ for wild-type and E223G and $2\text{--}4 \mu\text{M}$ for the rest of other mutants. The Michaelis constant was determined for each synthetic substrate from the Michaelis–Menten equation, using a double reciprocal plot. Reaction rates were determined, in triplicate, at pH 6.5 (in morpholinoethanesulfonic acid buffer, 100 mM) at each of seven different substrate concentrations ranging from 0.1–1 mM. The wavelengths employed and the $\Delta\epsilon$ ($\text{M}^{-1}\text{cm}^{-1}$) values obtained at those wavelengths for each arabinofuranoside substrate were as follows: 2,5-DNPAF, 440 nm, 3616; CNPAF, 400 nm, 2603; pNPAF, 400 nm, 3077; mNPAF, 380 nm, 330; pCPAF, 290 nm, 672; PAF, 278 nm, 754.

RESULTS AND DISCUSSION

Enzyme expression and purification

The *abf* gene from *T. koningii*, encoding α -L-arabinofuranosidase, was first inserted into plasmid pET22. The recombinant protein was highly expressed but formed an inclusion body. Although much effort was expended to refold this recombinant Abf, attempts were unsuccessful. The *abf* gene was next cloned into the pPICZ α B vector and expressed within *P. pastoris* GS115. The resulting recombinant protein was fused with three extra amino acids (Ala-Ala-Met) at the N-terminus of the mature Abf. The recombinant enzyme was expressed and secreted into the culture medium. In the case of purification of wild-type enzyme, 21 units of enzyme activity were detected in 100 ml of the culture filtrate. After three consecutive steps of purification, involving ammonium sulfate precipitation, desalting, and HiTrap SP (cation exchange) chromatography, 11.6 units (55% yields) of enzyme activity were recovered. The purified enzyme was further analyzed by SDS-PAGE (Figure 2) to confirm its purity (> 90% homogeneity) and molecular weight (50 kDa).

Characteristics of recombinant Abf

To analyze the catalytic activity and the substrate specificity of the recombinant enzyme, a variety of glycosides, including *p*-nitrophenyl- α -L-arabinopyranoside, *p*-nitrophenyl- β -D-glucopyranoside, *p*-nitrophenyl- β -D-galactopyranoside,

p-nitrophenyl- β -D-*N*-acetylglucosamine, *p*-nitrophenyl- β -D-mannopyranoside, and pNPAF were tested. Of the substrates investigated, only pNPAF was hydrolyzed. These results confirmed that the protein was indeed α -L-arabinofuranosidase.

The pH stability of the enzyme was investigated. In general, the enzyme was stable for at least 2 h at pH values between 2 and 8.3, at room temperature. Enzyme activity decreased dramatically when the pH exceeded 9.5 or dropped below 1.5. The pH-dependent activity assay showed that the purified enzyme had optimum activity at pH 2.8–3.2 (Figure 3). The pH-dependent activity curve of recombinant Abf exhibited a bell shape, with two apparent pK_a values of 1.8 ± 0.3 and 4.2 ± 0.1 . It is of interest that both pK_a values were about 2 pK_a units lower than those found for other glycoside hydrolases.

Mutagenic study

The first protein structure of a family 54 α -L-arabinofuranosidase, from *A. kawachii*, was recently resolved [18]. This structure is valuable for structural simulation of enzymes in the same family. Based on sequence comparisons, our enzyme has 73% identity and > 85 % homology to the *A. kawachii* α -L-arabinofuranosidase. By inspection of the protein structure (Figure 4), residues appearing in the active site of *A. kawachii* α -L-arabinofuranosidase and *T. koningii* (shown in parentheses) may be listed: C176 (C178), D219 (D221), E221 (E223), D297 (D299), and S299 (S301). All these residues are highly conserved in 12 enzymes of the GH54 family. The carboxyl groups of E221 (E223 in *T. koningii* Abf) and D297 (D299 in Abf) are located on either side of the anomeric C-1 carbon of enzyme-bound arabinofuranose, strongly suggesting that these residues serve as nucleophile and general acid/base residue, respectively. To confirm the catalytic roles of these residues, an extensive mutagenesis study was performed on E223, D299, and 22 other Asp or Glu residues (D95, D120, D160, E163, D170, D181, E186, D191, E198, D221, D238, E289, E310, D320, E323, E407, D410, D429, E436, D437, D482, and D490), which are highly conserved in GH54 family members. We changed the putative catalytic residues, aspartate (D) and glutamate (E), to asparagine (N), glutamine (Q) or glycine (G) by site-directed mutagenesis. All crude mutant enzymes with mutations in D170, D221, E223, D299 or E310 lost virtually all activity with pNPAF as substrates. Some of these mutant enzymes were further purified and studied. Kinetic parameters are summarized in Table 1. The K_m values of mutant enzymes, ranging from 0.22–0.32 mM, are quite similar to that of wild-type Abf, except for the mutants D299N, and E310G, where the K_m values were 0.05 mM, and 3.0 mM, respectively. The k_{cat} values of the mutant enzymes D170N, D221N, D299N, D299G, and E310G decreased (with respect to the wild-type Abf) by factors of 31, 7000, 1300, 262, and 70,

respectively, while E223Q was completely inactive. The reduction in the catalytic activities of the mutant enzymes E223Q and D299N was consistent with the suggested roles of the altered amino acids as catalytic residues, as indicated by the structure of the *A. kawachii* IFO4308 enzyme. Though residue E223, conserved in the DXE (Glu-X-Asp) sequences of glycoside hydrolase family 54 members, has been postulated to act as a nucleophile, this study uses site-directed mutagenesis for direct confirmation of this function. Residue D221 (D219 in the *A. kawachii* enzyme) engages in strong hydrogen bonding with the C3 and C5 sugar hydroxyl groups [18]. The large decrease in activity observed when this residue is mutated presumably occurs because the substrate is now incorrectly oriented in the active site. The catalytic activities (k_{cat}/K_m) of D170N and E130G were about 30- and 700-fold weaker than that of wild-type Abf. Since the residues D170 and E130 are more than 20 Å distant from the active site, perturbations of general protein structure by these mutations should perhaps be considered.

One unexpected finding is that the E223G mutant still possesses extremely high activity. Its k_{cat} (22 s^{-1}) and k_{cat}/K_m ($59460 \text{ M}^{-1}\text{s}^{-1}$) values are nearly identical to those of wild type Abf. After careful inspection of the active-site structure of *A. kawachii* enzyme, the Asp-189 (Asp-191 in our Abf) was found to locate closely to the nucleophile, E221 (E223 in our Abf). The orientation and distance (4–5 Å) between Asp-189 and the C-1 position of the substrate is perfect for an inverting-type of catalysis to take place. It is very likely that when the side-chain of the nucleophile is replaced by a proton (ie, in the E223G mutant), more space will be gained and therefore will allow water molecule to diffuse deeply into the active site. However, there would be no space around the nucleophile if the Glu is mutated into Gln. This hypothesis has been tested by analyzing the activity of double mutant (D191N/E223G) and the stereochemistry of E223G catalysis. The results from this study will be published elsewhere.

In many glycoside hydrolases, exogenous nucleophiles such as azide, formate, and other anions have been shown to enhance the catalytic activity of enzymes mutated in residues that serve as nucleophiles or provide general acid/base functions [21-24]. Activity enhancement of a mutant by addition of a nucleophile (for example, azide) and formation of a stereospecific product (α - or β -glycosyl azide) offers a useful technique for identifying essential residues of glycosidases. In this study, however, addition of high concentrations of azide (up to 2 M) did not rescue the activities of the D299G, D299N, and E223Q mutants. The lack of azide rescue of the activity of the mutants cannot be easily explained. It is possible that structural constraints in Abf mean that azide ion can not access the catalytic centre, as proposed for *A. kawachii* IFO4308 enzyme [18]. Alternatively, considering that at the pH of the reaction (4.1) azide ion ($\text{p}K_a =$

4.72) is mainly protonated to form HN_3 , this may complicate the kinetic analysis and affect azide ion rescue. Nevertheless, comparing the pH activity profiles (k_{cat} vs pH) of the wild-type Abf and the D299N mutant (Figure 3) may provide an insight into the essential function of D299. As shown in Figure 3, the activity of the D299N mutant was nearly unchanged in the buffer conditions with $\text{pH} > 3$, indicating the absence of the second pK_a , which is generally related to the general acid/base residue. Also, the low K_m value (0.05 mM) of the D299N mutant suggests accumulation of the glycosyl-enzyme intermediate, whose hydrolysis is then accelerated by the general acid/base catalytic action of the enzyme. In sum, the results of the site-directed mutagenesis and kinetic studies, in combination with structure analysis, point to E223 and D299 as the essential nucleophile and general acid/base residues of Abf, respectively. The function of D299 will be further discussed below.

Transglycosylation and product partition

A time-course ^1H NMR study is commonly employed to examine anomeric preference in the catalytic action of a glycoside hydrolase. Though such work provides unequivocal stereochemical data, the process is tedious, as equilibration with a deuterated buffer system is required and enzyme concentration must be carefully monitored. Also, a significant limitation of this technique is that product mutarotation rate must be relatively slow so that NMR detection of authentic product is feasible. In the case of arabinofuranosidases, an arabinofuranoside product may be expected to undergo fast mutarotation to form four arabinose tautomers. These are α - and β -L-arabinofuranosides and α - and β -L-arabinopyranosides [25]. To overcome this limitation, a method involving transglycosylation of the Abf using methanol as glycosyl acceptor was employed. The advantage of this strategy is the formation of a methyl glycoside that cannot mutarotate. For most retaining enzymes, although the formation of a covalent enzyme intermediate is expected, it is difficult to detect as the lifetime is short. In the past, useful 2-fluoroglycoside products have been obtained using a specific glycosyl-enzyme trapping technique [26–29]. However, perhaps because 2-fluoroarabinofuranoside is difficult to synthesize, this strategy has not yet been used in any study of α -L-arabinofuranosidase. We used an alternative, indirect, method. If a constant chemical bias may be noted in different enzyme products, the formation of a common intermediate in an enzymatic reaction may be inferred. Our study showed that the Abf exhibited strong transglycosylation activity when methanol was used as an arabinofuranosyl acceptor. Here, a suitable amount of pNPAF, pCPAF and CNPAF were enzymatically hydrolyzed in acetate buffer (pH 4.1) containing 12% (v/v) methanol. The solutions were then dried and exchanged with D_2O several times before

$^1\text{H-NMR}$ analysis. For all three reactions, the $^1\text{H-NMR}$ spectra (measured at 25 °C) of the sugar moieties (in the range of 3–6 ppm) were nearly identical. In principle, five different end products with arabinosyl ring structures should be observed (Figure 5a). According to the literature [30] and our study, the C1 protons of each sugar ring were assigned as follows: methyl- α -arabinofuranoside (4.86 ppm, $J = 1.03$ Hz), α -arabinofuranose (5.17, ppm), β -arabinofuranose (5.22 ppm, $J = 3.8$ Hz), α -arabinopyranose (4.43 ppm, $J = 5.9$ Hz), and β -arabinopyranose (5.16 ppm, $J = 3.24$ Hz) (Figure 5b). Based on peak assignment and the integration of the C1 proton on the sugar ring, the ratio of methyl- α -arabinofuranoside/arabinose was calculated from each spectrum. Regardless of the substrates, these ratios were nearly constant, and averaged 1.04 ± 0.02 (1.04 for pCPAF, 1.06 for pNPAF, and 1.03 for CNPAF). This suggests that a common intermediate, most likely an arabinosyl-enzyme structure, occurs in the reaction pathways. As the product of transglycosylation in this experiment is methyl- α -arabinofuranoside, we can confirm that the Abf is indeed a retaining enzyme.

Substrate reactivity and Brønsted plot

Kinetic assessment of substrates bearing different leaving phenols is a common strategy in studying mechanistic actions of glycoside hydrolases [31]. For glycoside hydrolases with two-step mechanisms (formation of a glycosyl-enzyme intermediate in a glycosylation step followed by breakdown of the intermediate in a deglycosylation step), the aglycon moiety is cleaved in the glycosylation step of the reaction (Figure 1). Therefore, the reaction rate of the first step dictates the ease by which the leaving group may be released from the substrate. A strong correlation between the $\text{p}K_a$ of the phenol leaving group and the activity of the enzyme should be observed if the first step is rate-limiting. To determine the rate-limiting step of hydrolysis catalyzed by the recombinant Abf, we worked to prepare aryl- α -L-arabinofuranosides bearing different leaving phenols ($\text{p}K_a$ values 5.15 to 9.99) and to perform steady-state kinetic analysis using these substrates. The reaction temperature was held at 25 °C to reduce spontaneous substrate hydrolysis. Although Abf is specific with regard to the glycon moiety of the substrate, the enzyme shows a broad specificity for the aglycon portion. Kinetic parameters for reactions using the aryl α -L-arabinofuranoside substrates are summarized in Table 2. The data showed that both the K_m (0.28–0.54 mM) and the k_{cat} (1.23 – 8.54 s^{-1}) values were approximately the same for all substrates. These data permit calculation of the Brønsted relationship, which has been shown to be useful in understanding the mechanism of enzyme action [16, 32, 33]. Based on the k_{cat} values, an extended Brønsted plot was constructed by plotting the logarithm of k_{cat} against the $\text{p}K_a$ of the leaving phenol (Figure 6a). A plot with a

slightly downward trend was obtained, with a slope (β_{lg} value) of -0.18 . That the detailed structures of the leaving phenols do not affect the kinetic parameters of the reaction may indicate that the dearabinosylation step (breakdown of the arabinosyl-enzyme intermediate) is the rate-limiting step. Alternatively, although it may be considered unlikely, the low Brønsted constant could indicate that the arabinosylation step is the slow step and that an early transition state is attained. However, the very weak reaction inhibition shown by MAF ($K_i > 16$ mM) minimized this possibility. If the transition state was substrate-like, strong inhibition would be expected. We further studied substrate reactivity using the D299G mutant enzyme. A new plot with $\beta_{lg} = -1.3$ was obtained (Figure 6a). For substrates with $pK_a > 6$, the absence of general acid/base catalysis resulted in a strong correlation between the pK_a of the phenol leaving group and enzyme activity. Clearly, formation of the arabinosyl-enzyme intermediate is now the rate-limiting step. However, with 2,5-DNPAF (a good substrate, with $pK_a = 5.15$), the $\log k_{cat}$ value is clearly lower than expected if the enzyme reaction were to proceed as outlined above. This indicates that the dearabinosylation step becomes at least partially rate-limiting when 2,5-DNPAF breakdown is catalyzed by the D299G enzyme. In addition to alteration of catalytic properties, the D299G mutation also affects enzyme K_m values with different substrates (Table 2). The K_m values decrease as the pK_a values of the leaving phenols change in the substrates. For the wild-type enzyme, such changes in K_m values are not obvious. These data showed that as the ability of the aglycon moiety to depart the enzyme increased, the more glycosyl-enzyme intermediate accumulated. This may be appreciated if it is noted that K_m can be represented as $[E][S]/\sum[ES]$. Thus, the more the glycosyl-enzyme intermediate accumulates, the lower the K_m value. Such behavior may be expected in mutants affected in the general acid/base residue, as a good leaving group elevates the rate of the first catalytic step. The rate of the second step remains slow as the basic residue, which (in unmutated enzymes) activates the water molecule, is missing.

The k_{cat}/K_m values are informative with respect to the first irreversible step. For retaining glycoside hydrolases, the Brønsted relationship obtained by plotting k_{cat}/K_m values of substrates against the pK_a values of the leaving phenols provides information about the glycosylation step [15, 34]. As may be seen (Figure 6b), the Brønsted constants (β_{lg}) are -0.19 and -1.2 for wild-type Abf and the D299G mutant, respectively. This suggests that the arabinosylation step catalyzed by the wild-type Abf enzyme is not sensitive to the leaving abilities of different phenols, as the general acid/base residue protonates the oxygen of the glycosidic bond, thus imparting constant leaving abilities to different phenols. When the general acid/base residue is absent, however, the catalytic efficiency (k_{cat}/K_m) for the tested substrate became highly sensitive

to the leaving ability of phenol groups ($\beta_{lg} = -1.2$). A typical kinetic consequence of the general acid/base mutation is that for substrates requiring strong acid assistance (such as pCPAF and mNPAF), the first reaction step is much slower (12000–14000-fold decreases were noted in this study) than is the case with substrates that need less acid assistance (such as 2,5-DNPAF). The kinetic behavior displayed by the D299G mutant and the pH activity profile of D299N, when compared with data from the wild-type Abf, elegantly confirm the catalytic role of D299 and may be extended to corresponding residues in the other arabinofuranosidases of the GH54 family.

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Footnote

Chin-Feng Wan and Wei-Hung Chen made equal contributions to this study.

Keywords

α -L-arabinofuranosidase; site-directed mutagenesis; catalytic mechanism; common intermediate; Brønsted plot; rate-limiting step

Abbreviations

GH, glycoside hydrolase; 2,5-DNPAF, 2,5-dinitrophenyl- α -L-arabinofuranoside; CNPAF, 4-chloro-2-nitrophenyl- α -L-arabinofuranoside; pNPAF, p-nitrophenyl- α -L-arabinofuranoside; mNPAF, m-nitrophenyl- α -L-arabinofuranoside; pCPAF, p-cyanophenyl- α -L-arabinofuranoside; PAF, phenyl- α -L-arabinofuranoside; MAF, methyl- α -L-arabinofuranoside

FIGURE LEGEND

Figure 1. The proposed mechanism of a retaining α -L-arabinofuranosidase. A two-step, double displacement mechanism is proposed in which two essential amino acid residues, one functioning as the nucleophile and the other as the general acid/base, were involved.

Figure 2. Analysis of molecular weight of the recombinant Abf by SDS-PAGE. Lanes: M, markers; 1, the purified wild-type Abf ($\sim 4 \mu\text{g}$).

Figure 3. pH activity profiles of wild-type Abf (\circ) and the D299N mutant enzyme (\bullet). The k_{cat} values of wild-type and D299N mutant were measured at the final pH values: 1.9, 2.0, 2.4, 3.3, 3.9, 4.2, 5.5, 6.5. Data were fit by nonlinear regression analysis [35].

Figure 4. The active site of the GH54-family enzyme from *Aspergillus kawachii* IFO4308 (1WD4) with arabinofuranose in place. The corresponding amino acids in the *Trichoderma koningii* Abf are labeled in parentheses.

Figure 5. Stereochemical properties and common intermediates of Abf catalysis. (a) Enzymatic reactions, using various substrates, in the presence of methanol. (b) A partial NMR spectrum (chemical shift 3.4–5.4 ppm) of the end-products. Peak assignment is given in the text. The integrations of the signals of the C1 protons on each sugar ring were used to calculate the end-product ratio (methyl- α -arabinofuranoside/arabinose).

Figure 6. Brønsted plots of wild-type Abf (\circ) and D299G (\bullet) mutant enzyme. (a) Plots of $\log k_{\text{cat}}$ against $\text{p}K_a$ of the leaving phenol. (b) Plots of $\log k_{\text{cat}}/K_m$ against $\text{p}K_a$ of the leaving phenol.

Table 1. Michaelis–Menten parameters for the hydrolysis of pNPAF by wild-type and mutant enzymes at pH 4.1 and 25 °C.

| Enzyme | k_{cat} (s^{-1}) | K_{m} (mM) | $k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$) |
|---------------|--------------------------------------|---------------------|--|
| wild-type | 21 ± 2 | 0.32 ± 0.04 | 65625 |
| D170N | 0.68 ± 0.15 | 0.30 ± 0.10 | 2250 |
| D221N | 0.003 ± 0.001 | 0.22 ± 0.10 | 14 |
| E223Q | 0.0005 ± 0.0003 | 0.88 ± 0.42 | 0.57 |
| E223G | 22 ± 2 | 0.37 ± 0.05 | 59460 |
| D299N | 0.016 ± 0.006 | 0.05 ± 0.03 | 320 |
| D299G | 0.08 ± 0.02 | 0.21 ± 0.07 | 400 |
| E310G | 0.30 ± 0.06 | 3.0 ± 0.7 | 100 |

Table 2. K_m and k_{cat} values of wild-type Abf using various aryl- α -L-arabinofuranosides at pH 6.5

| Substrate | pK_a | K_m , mM | | k_{cat} | | k_{cat} / K_m | | $\log k_{cat}$ | | $\log (k_{cat} / K_m)$ | |
|-----------|--------|-----------------|-----------------|-----------------|---------------------|-----------------|-------|----------------|-------|------------------------|-------|
| | | WT | D299G | WT | D299G | WT | D299G | WT | D299G | WT | D299G |
| 2,5-DNPAF | 5.15 | 0.28 \pm 0.03 | 0.10 \pm 0.05 | 8.25 \pm 0.80 | 0.69 \pm 0.10 | 29542 | 6761 | 0.92 | -0.16 | 4.47 | 3.83 |
| CNPAF | 6.45 | 0.42 \pm 0.05 | 0.20 \pm 0.08 | 6.52 \pm 0.54 | 0.12 \pm 0.04 | 15633 | 572 | 0.81 | -0.94 | 4.19 | 2.76 |
| pNPAF | 7.18 | 0.31 \pm 0.05 | 0.21 \pm 0.15 | 2.84 \pm 0.32 | 0.014 \pm 0.01 | 9215 | 64 | 0.45 | -1.86 | 3.96 | 1.81 |
| mNPAF | 8.39 | 0.54 \pm 0.10 | 0.65 \pm 0.28 | 2.02 \pm 0.24 | 0.0003 \pm 0.0002 | 7369 | 0.46 | 0.31 | -3.52 | 3.87 | -0.33 |
| pCPAF | 8.49 | 0.30 \pm 0.09 | 0.69 \pm 0.30 | 1.70 \pm 0.30 | 0.0004 \pm 0.0002 | 5592 | 0.58 | 0.23 | -3.41 | 3.75 | -0.25 |
| PAF | 9.99 | 0.28 \pm 0.10 | – | 1.23 \pm 0.22 | – | 4334 | – | 0.09 | – | 3.64 | – |

Unit: k_{cat} / K_m ($M^{-1}s^{-1}$), k_{cat} (s^{-1})

*: The D299G mutant enzyme is almost inactive towards PAF.

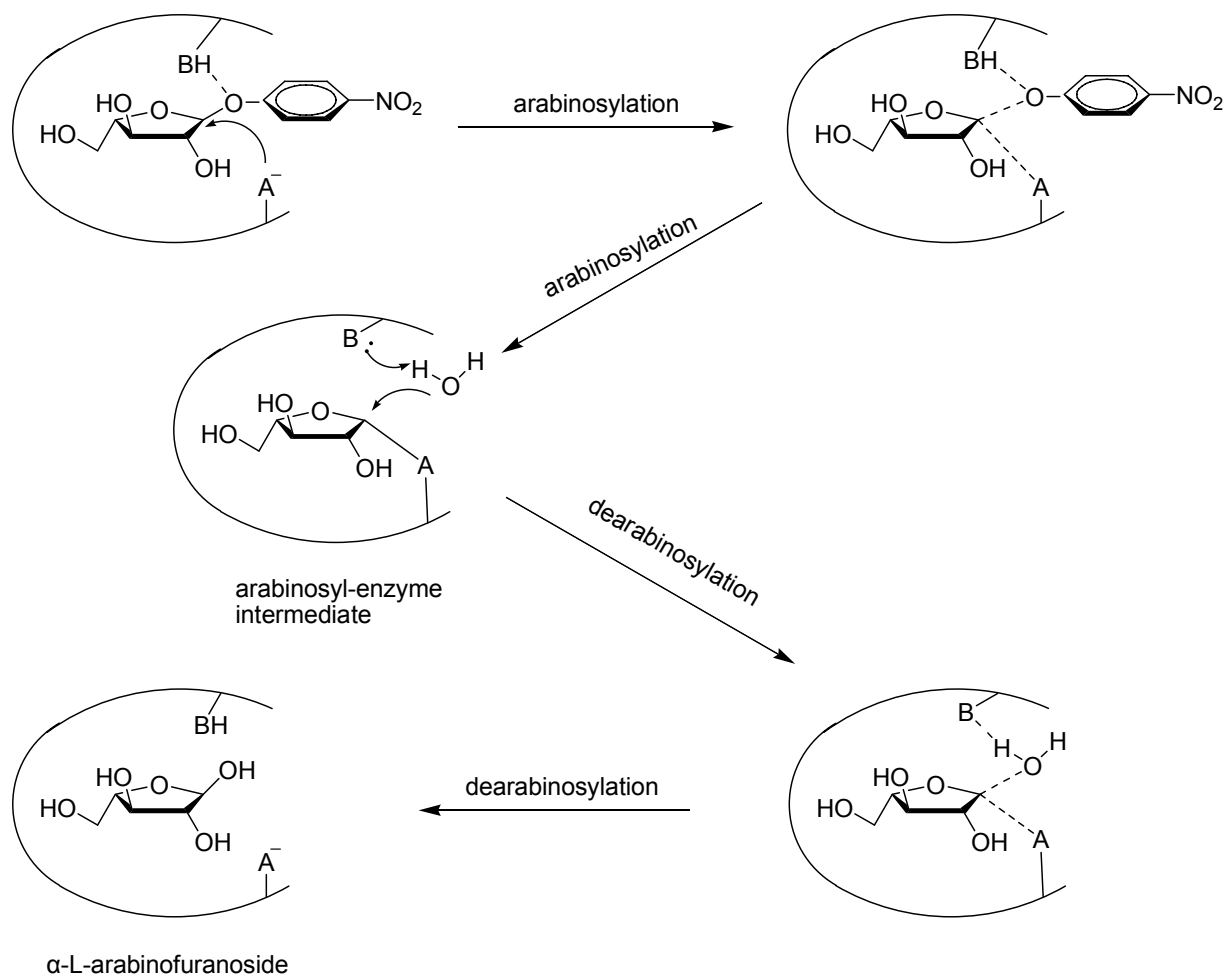


Figure 1

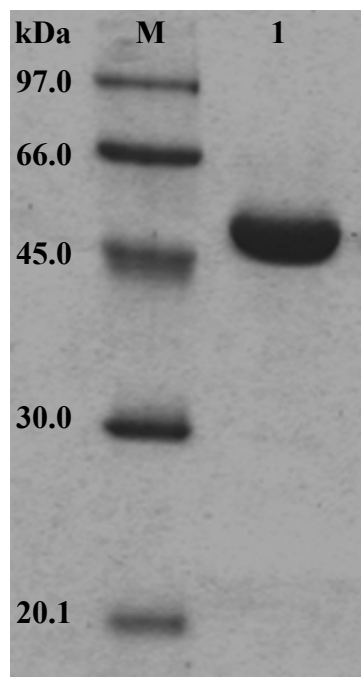


Figure 2

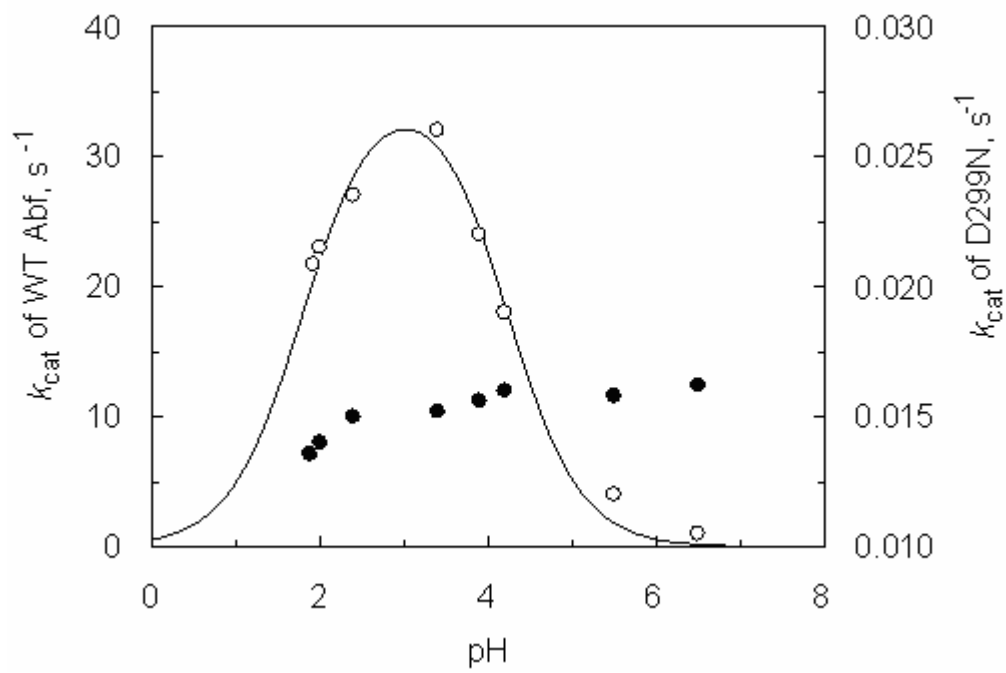


Figure 3

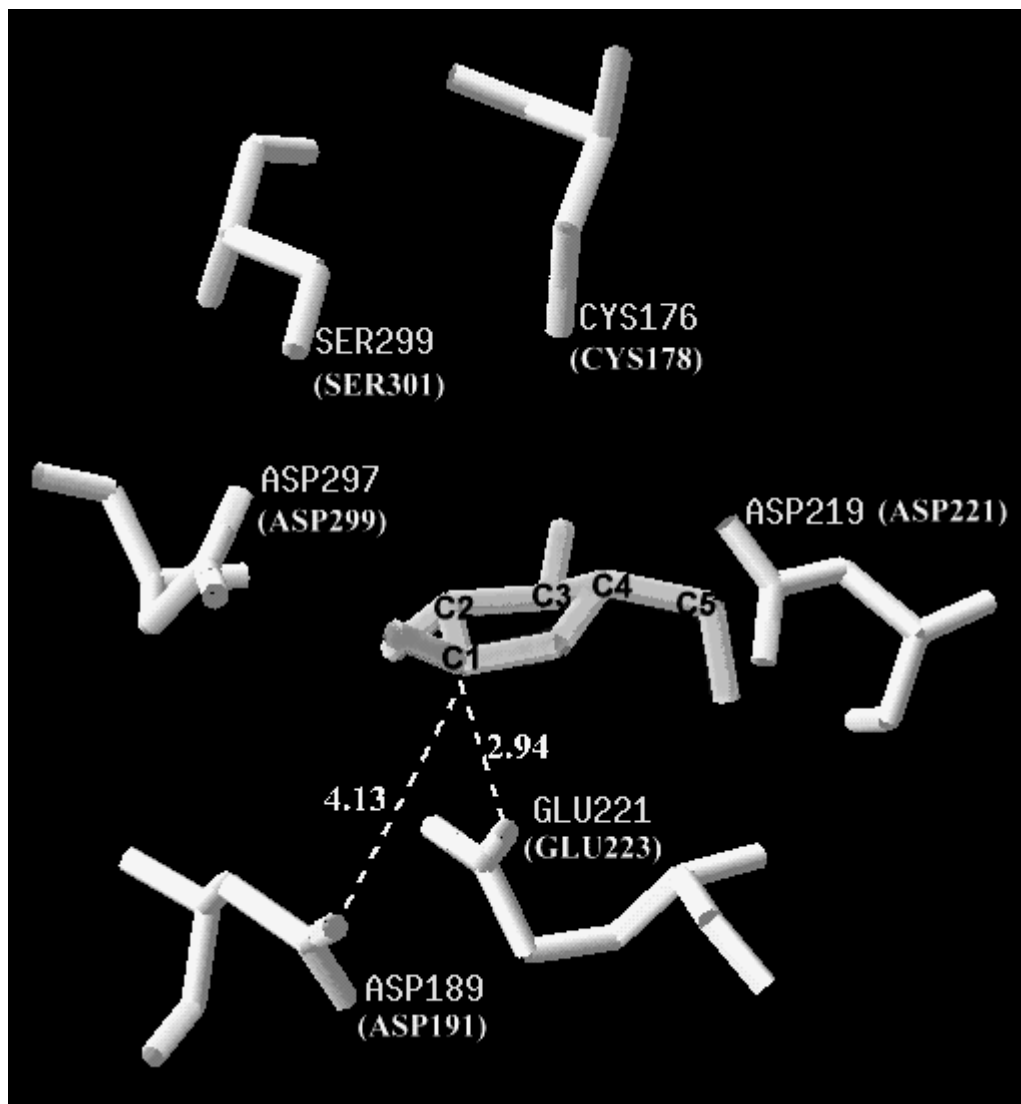


Figure 4

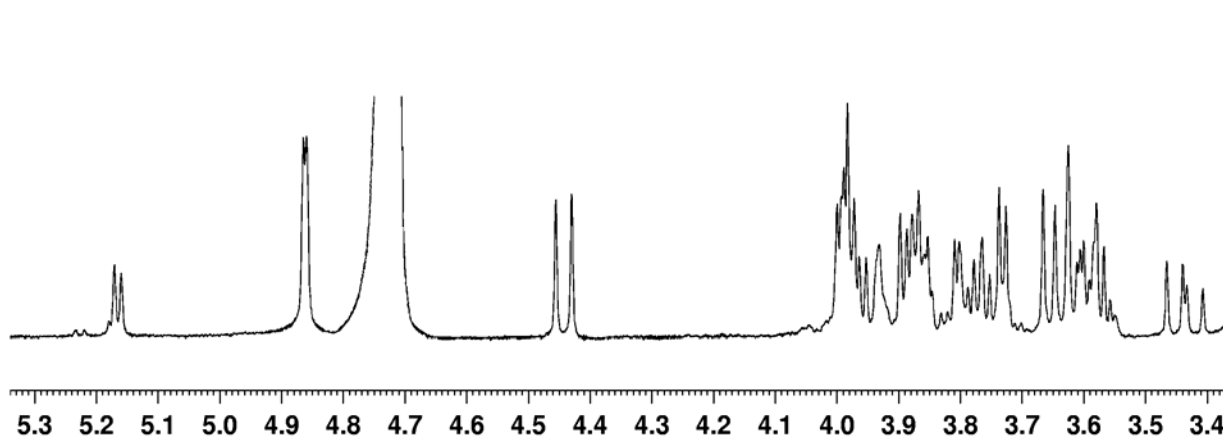
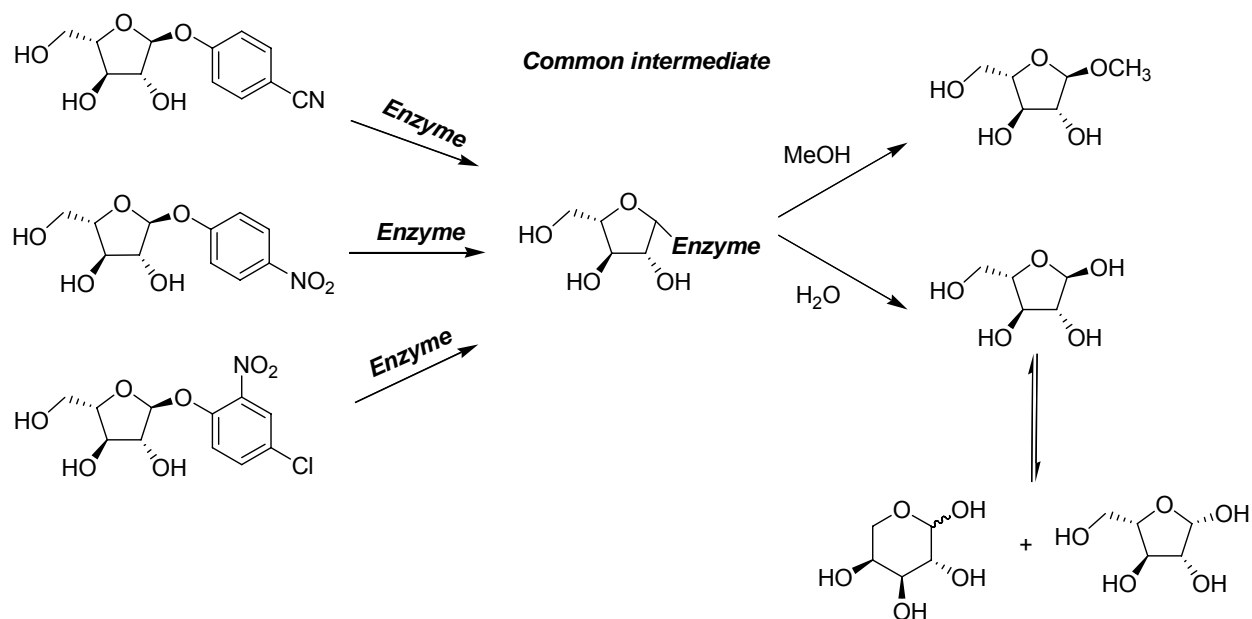


Figure 5 (a) & (b)

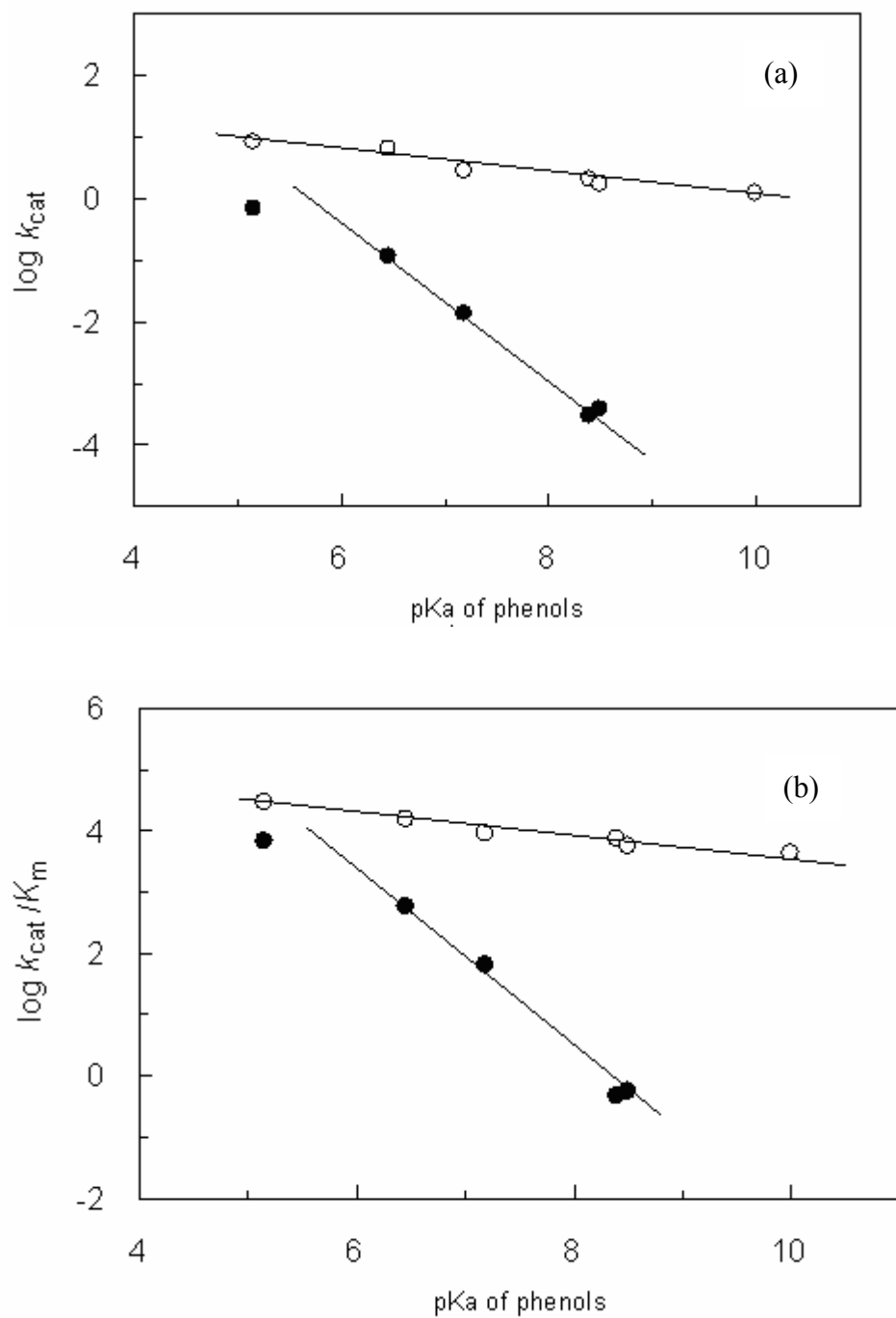


Figure 6