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Characterization and identification of essential residues of the glycoside hydrolase family 64 laminaripentaose-producing-β-1, 3-glucanase

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Laminaripentaose-producing β-1,3-glucanase (LPHase) from *Streptomyces matensis* DIC-108 uniquely catalyzes the hydrolysis of β-1,3-glucan to release laminaripentaose as the predominant product. For studying this novel enzyme, the gene of LPHase was reconstructed with polymerase chain reaction and over-expressed in *Escherichia coli*. The recombinant wild-type enzyme and various mutants were further purified to >90% homogeneity on an ion-exchange chromatograph. The catalysis of the recombinant LPHase is confirmed to follow a one-step single-displacement mechanism with ^{1}H-NMR spectrometry. To determine the amino-acid residues essential for the catalysis, more than ten residues, including five highly conserved residues—Asp^{143}, Glu^{154}, Asp^{170}, Asp^{376} and Asp^{377}, were mutated. Among the mutants, E154Q, E154G, D174N and D174G significantly lost catalytic activity. Further investigation with chemical rescue using sodium azide on E154G and D174G confirmed that Glu^{154} functions as the general acid whereas Asp^{170} serves as the general base in a catalytic turnover. This work is the first report that provides direct information for the identification of the essential residues of GH-64 through kinetic examination.

Keywords: GH-64/Laminaripentaose-producing β-1,3-glucanase/catalytic mechanism/essential residue/site-directed mutagenesis/chemical rescue

Introduction

β-Glucans are polymers composed of glucose with polymerization to various degrees, branching and diverse glycosidic linkages, such as β-1,3-, β-1,4-, β-1,6-, β-1,3/1,4- and β-1,3/1,6-linkages (Martin *et al.*, 2007). They can be obtained from diverse sources, including fungi, yeasts, bacteria and plants. They serve as components of the cell wall of these organisms but might also be found in extracellular secretions of microbial origin. β-1,3-Glucanases, generally termed as laminarinases, including endo-β-1,3-glucanase (EC 3.2.1.39) and exo-β-1,3-glucanase (EC 3.2.1.58) are found simultaneously in several distinct families of glycoside hydrolases such as GH-5, GH-16, GH-17, GH-55, GH-64 and GH-81. These enzymes cleave the β-1,3-bond found in various β-1,3-glucans and pachyman (McGrath and Wilson, 2006; Blättel *et al.*, 2011) to release various oligosaccharides as products. The laminaripentaose-producing β-1,3-glucanase (LPHase), acting on the β-1,3-glycosidic bond of curdlan to release laminaripentaose from β-1,3-glucan as the predominant product, was first purified from *Streptomyces matensis* DIC-108 and the corresponding gene was further cloned (accession no. BAA34349 in GenBank) (Nakabayashi *et al.*,1998). The enzyme, composed of 401 amino acids with the first 35 amino acids as its signal peptide, was classified as a member of GH-64. The information on the catalytic essential residues of GH-64 has not yet been confirmed.

Enzymatic hydrolysis of glycosidic bonds occurs with two possible stereochemical outcomes—inversion or retention of the anomeric configuration at the site of cleavage (Sinnott, 1990; McCarter and Withers, 1994). Both mechanisms normally require amino acids containing side-chain carboxylic residues as essential groups. For the retaining glycosidases, enzymes catalyze the hydrolysis via a two-step double-displacement mechanism involving a covalent glycosyl-enzyme intermediate with one of the two essential amino-acid residues functioning as a nucleophile and the other as a general acid/base. In contrast, the inverting glycosidases follow a one-step single-displacement mechanism with the assistance of a general acid and a general base. The general base polarizes a water molecule to develop a stronger nucleophile to attack the anomeric carbon, whereas the general acid protonates the glycosidic oxygen to accelerate the reaction (Davies and Henrissat, 1995; Zechel and Withers, 2000). Kinetic and stereochemical tests of the enzymatic reaction provide information that allows one to draw conclusions about the catalytic mechanism (Koshland, 1953; Nishimura *et al.*, 2001). The molecular mechanism is conserved within the sequence-based families of glycoside hydrolases (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996, Cantarel *et al.*, 2009). For the six families containing β-1,3-glucanases, the GH-5 is classified as the retaining enzyme (Baker *et al.*, 2005; O'Connell *et al.*, 2011). GH-16 (Kawai *et al.*, 2006) and GH-17 (Chen *et al.*, 1995) containing various glycoside hydrolases, such as endo-β-1,3-D-glucanase, licheninase, agarase, xyloglucanase, were also found to be the retaining

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enzyme. Protein three-dimensional structures were available for β-1,3-glucanases from GH-5 (Dias *et al.*, 2004), GH-16 (Vasur *et al.*, 2006) and GH-17 (Receveur-Brechot *et al.*, 2006). Detailed enzymology and catalytic investigations, including binding model experiments of various oligosaccharide, inhibition, kinetic analysis, and essential group(s) identification were reported for the enzymes of families GH-5, GH-16 and GH-17 (Armand *et al.*, 1994; Varghese *et al.*, 1994; Chen *et al.*, 1995; Hrmova and Fincher, 2001; Johansson *et al.*, 2004; Baker *et al.*, 2005; Kawai *et al.*, 2006; O'Connell *et al.*, 2011). Family GH-55, GH-64 and GH-81 are classified as the inverting GH for β-1,3-glucanases. Among these families, the proteins from GH-55 were extensively studied including crystal structure and essential group identification (Ishida *et al.*, 2009), whereas studies on GH-64 and GH-81 were mainly focused on the subjects of protein purification (Bara *et al.*, 2003; Palumbo *et al.*, 2003), gene cloning (Shen *et al.*, 1991; Martin *et al.*, 2006) and limited studies on catalytic features of GH-64 (Nishimura *et al.*, 2001; Ferrer, 2006) and GH-81 (Fliegmann *et al.*, 2005; McGrath *et al.*, 2009). Laminaripentaose-producing β-1,3-glucanase, classified as a member of GH-64, was first confirmed to be an inverting enzyme by Nishimura *et al.* (Nishimura *et al.*, 2001). Recently, we resolved its crystal structure (3G0 in protein data bank) to 1.62 Å and performed computer simulations to model the enzyme-laminarihexaose structure (Wu *et al.*, 2009) (Fig. 1). The structure provides valuable information to explore the catalytic mechanism and the candidate of the essential residues in the active site and sugar-binding site of LPHase. The present work is continuing the structure study to confirm the function of the conserved residues in the active site by site-directed mutagenesis, chemical rescue and kinetic analysis.

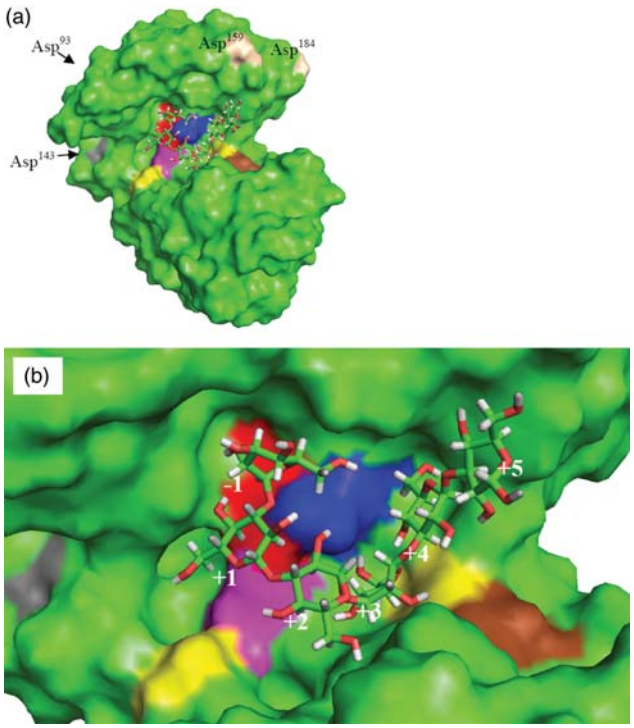


Fig. 1 Docking model of LPHase·laminarihexaose complex. The docked laminarihexaose is drawn as sticks with carbon (green), oxygen (red) and proton (gray) atoms. Two catalytic residues—Glu^{154} and Asp^{170}—are shown in blue and magenta, respectively, in the structure shown with a surface model. Other residues at the substrate binding site such as Arg^{115} (red), Tyr^{232} (yellow), Tyr^{371} (yellow) and Asp^{377} (brown) are shown. Other highly conserved residues (Asp^{93}, Asp^{143} and Asp^{376}) and partially conserved residues (Asp^{159}, Asp^{184} and Asp^{221}) are shown in figure (**a**). Note that Asp^{93} and Asp^{221} are located on the back side of the cleft and Asp^{376} is buried inside the structure. The close view of the active site with the docked laminarihexaose was shown in figure (**b**).

Experiments

Materials

Curdlan (a laminarin β-1,3-glucan, Wako Chemical, Osaka, Japan), various polysaccharide substrates (TCI, Tokyo Kasei Kogyo Co. Ltd), oligonucleotides (synthesized by Integrated DNA Technologies, Mission Biotechnology, Taipei, Taiwan), *Vent* polymerase (New England Biolabs, Ipswich, MA, USA) used in polymerase chain reaction (PCR) reactions, restriction endonucleases and T4 DNA ligase (Roche Applied Science, Basel, Switzerland), buffers and chemicals for synthesis (Sigma-Aldrich, St Louis, MO, USA or E. Merck Co., Gibbstown, NJ, USA) and columns and gels for protein purification and protein marker for electrophoresis (GE Healthcare, Piscataway, NJ, USA) were obtained from the indicated sources. Synthetic substrates were characterized with ^{1}H-NMR spectra (Bruker Avance spectrometer 300 MHz) and mass spectra (Q-TOF, Micromass, in the positive ESI mode).

Gene construction, protein expression and purification

Polymerase chain reaction was employed to reconstruct the full-length gene of LPHase (accession number BAA34349) using 24 primers (sequence not shown) including 12 of sense and 12 of anti-sense primers. The PCR-amplified LPHase gene was inserted in the yT&A cloning vector. The DNA sequence was fully confirmed with cycle sequencing (ABI 3100 DNA sequencer, Perkin-Elmer Applied Biosystems). For the construction of the expression vector, the LPHase gene was inserted at the NdeI and Eco*R*I sites of pRSET_A.

The purifications of recombinant LPHase and mutants were identical. Complete procedures involve three steps of ion-exchange chromatographic separation. All purification steps were performed near 25°C. The detailed procedures have been described earlier (Wu *et al.*, 2009). Protein concentration was determined by means of the BCA (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 weight of the purified enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli, 1970) and by mass spectrometry.

CD Spectra of recombinant LPHase

Spectra were recorded at 25°C on a spectropolarimeter (JASCO J-815; cell length 1 mm). The concentration of proteins used for CD measurement was 1 mg/ml. All spectra, with correction for the buffer background, were recorded from 200 to 255 nm. Ten spectra were recorded and averaged.

Site-directed mutagenesis in vitro

Site-directed mutagenesis for mutations was performed according to the QuikChange® method (Stratagene). The

Table I. Primer sequences for various mutations[a]

Mutation site	Primer sequence[b]
D93G	(+): 5'- GGATGGAGGC**GCC**TGGAGCTGGAGTAGG-3'
	(−): 5'- CCTACTCCAGCTCCA**GGC**GCCTCCATCC-3'
R115Q	(+): 5'- CAAGTTCTCTGG**CCA**GATCTACTTCTCGTAC-3'
	(−): 5'- GTACGAGAAGTAGATC**TGG**CCAGAGAACTTG-3'
D143G	(+): 5'- CAGAACCCGACT**GGC**CCGAACCGCGAC-3'
D143N	(−): 5'- GTCGCGGTTCGG**GTT**AGTCGGGTTCTG-3'
E154G	(+): 5'- CTTCAACTGGTCC**GGC**TACACGCTCAACG-3'
E154Q	(−): 5'- CGTTGAGCGTGTA**CTG**GGACCAGTTGAAG-3'
E154D	(+): 5'- CTTCAACTGGTCC**GAC**TACACGCTCAACG-3'
D159G	(+): 5'- GTACACGCTCAAC**GGC**TCCGGGCTCTG-3'
D159N	(−): 5'- CAGAGCCCGCA**GTT**GTTGAGCGTGTAC-3'
D170G	(+): 5'- CAGTACGCAGGTC**GGC**ATGTTCTCAGCTC-3'
D170N	(−): 5'- GAGCTGAGAACAT**GTT**GACCTGCGTACTG-3'
D170E	(+): 5'- CAGTACGCAGGTC**GAG**ATGTTCTCAGCTC-3'
D170C	(+): 5'- CAGTACGCAGGTC**TGC**ATGTTCTCAGATC-3'
D184G	(+): 5'- GTGCGGCGCGGC**GGC**GGCACCACACTGAGC-3'
D184N	(−): 5'- GCTCAGTGTGGTGCC**GTT**GCCGCGCCGCAC-3'
D221G	(+): 5'- CAGACGCGATCC**GGC**GGTACCGTGCTTCG-3'
D221N	(−): 5'- CGAAGCACGGTACC**GTT**GGATCGCGTCTG-3'
Y232A	(+): 5'- CGCTCTCTCCACTC**GCT**GGTGTCGAGACAGG-3'
Y232F	(−): 5'- CCTGTCTCGACACC**GAA**GAGTGGAGAGAGCG-3'
D376G	(+): 5'- CGGCTTCGCATTC**GGC**GACGTGGGACATCAC-3'
	(−): 5'- GTGATGTCCCACGTC**GCC**GAATGCGAAGCCG-3'
D377G	(+): 5'- GTGATGTCCCAC**GCC**GTCGAATGCGAAGCCG-3'
	(−): 5'- CGGCTTCGCATTCGAC**GGC**GTGGGACATCAC-3'

[a]Note that the forward and reverse primers designed for different mutants at the same position were used for site-directed mutagenesis. For example, D143G(+) and D143N(−) were used as primers to produce both D143G and D143N. Other cases such as E154Q(−), E154D(+), D159G(+), D159N(−), D170N(−), D170E(+), D170C(+), D184G(+), D184N(−), D221G(+), D221N (−), Y232A(+) and Y232F(−) primers were used to perform all mutations at the corresponding sites. DNA sequencing was employed to identify and confirm the mutations achieved.
[b]Mutations are underlined.

basic procedure involved PCR amplification with pRSET_A-*lphase* plasmid DNA as the template and two synthetic oligonucleotides containing the desired mutation as the primer. The primers used to generate the mutations are listed in Table I. *Escherichia coli* JM109 strain cells were transformed with mutation plasmid DNA. The desired mutations were confirmed with DNA sequencing. Plasmids were further transformed into *E. coli* BL21 (DE3) for protein overexpression. For those mutants with significant activity loss, the entire genes were sequenced to confirm that only the intended mutations had occurred.

Protein analysis with a mass spectrometer

The purity and molecular weight of the enzyme were tested by means of SDS-PAGE. The mass spectrometer (Q-TOF; Micromass, Manchester, UK) was set to scan with a ratio of mass to charge in a range $m/z = 100-2500$ units, with a scan 2 s/step and an interscan duration 0.1 s/step. In all electrospray ionization mass spectrometry (ESI-MS) experiments, the quadrupole scan mode was used under a capillary needle at 3 kV, a source block temperature 80°C and a dessolvation temperature 150°C. Purified protein (100 μL, ~25–30 μg) was precipitated with trifluoroacetic acid (25%) at 4°C for 30 min. After centrifugation, the precipitate was washed twice with acetone. The precipitant was redissolved in 0.1% formic acid solution for mass analysis.

Substrate preparation and enzymatic assays

Curdlan (5 g) was washed with aqueous ethanol (20 % by volume) and then suspended in phosphate buffer (100 ml, 50 mM, pH 10.5) with stirring near 25°C for 30 min. The reaction solution was neutralized with HCl (0.1 M) to obtain gel-like curdlan. After centrifugation, the precipitant was washed with ddH_2O to remove salt and re-suspended in ddH_2O at 70–75°C for 2 h. The supernatant was obtained after centrifugation to remove the insoluble portion and evacuated to dryness. The resulting powder was redissolved (final concentration 5% w/v) in phosphate buffer (50 mM, pH 7.5) for enzymatic assay and application. The activity of LPHase was analyzed on estimating the amount of the reducing ends of sugars using the dinitrosalicylic acid (DNS) method (Miller, 1959). The standard assay mixture (0.5 ml) contained enzyme solution properly diluted, curdlan substrate (2% w/v) in phosphate buffer (50 mM, pH 7.5). The reactions were performed for 2 h at 37°C; DNS reagent (0.5 ml) was then added, and the resulting mixture was boiled for 15 min, chilled and centrifuged to isolate the insoluble curdlan. The resulting adducts of reducing sugars were analyzed and measured spectrophotometrically at 540 nm. The absorption coefficient of the resulting adducts was determined to be 788 M^{-1} cm^{-1} when D-glucose served as control sample. One unit of LPHase activity is defined as the amount of enzyme required to release 1 μmol of detectable reducing sugars at 37°C in 1 min. The products of enzymatic reaction were analyzed with a mass spectrometer (triple quadrupole, Quattro Micro; Micromass).

Enzyme characterization

The optimum pH for LPHase activity was determined on incubating the purified enzyme with substrate curdlan (2%), in buffers with pH in a range 2.0–11.5. Buffers used were glycine (pH 1.8–3.5 and pH 9.0–10.0), sodium acetate (pH 4.0–5.5), morpholinoethanesulfonic acid (pH 5.5–6.5), phosphate (pH 6.5–7.5), Tris (pH 7.5–8.5) and Caps (10.5–11.5). The optimum temperature was determined on assaying the enzyme activity (at pH 7.0) at temperatures from 4 to 95°C. All activities were measured following a standard protocol as described above. For tests of pH stability, LPHase was pre-incubated in buffers (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) at 25°C and was further assayed at varied intervals using a standard protocol. For tests of thermal stability, LPHase was pre-incubated at pH 7.0 and temperatures 25, 40, 50, 60, 65, 75 and 85°C, and the residual activity of the enzyme was measured after various periods.

The effects of various metal ions and reagents (Cu^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Hg^{2+}, EDTA and DTT) on LPHase activity were investigated by incorporating 1–10 mM ion (or reagent) to the standard assay system.

Preparation of laminaripentaose, synthesis of p-nitrophenyl-β-1,3-D-laminaripentaoside and kinetic assay

To prepare laminaripentaose, we poured the reaction mixture containing curdlan (100 mL, 5 % w/v), prepared in Caps buffer (50 mM, pH 10.5) and purified LPHase (5 ml, 10 mg/

ml) into tubular regenerated-cellulose membrane (molecular weight cut-off 3000), which was kept in ddH_2O (250 mL), 45°C for 12 h. The enzymatic reaction was performed in the dialysis system and ddH_2O (250 mL) was changed every 4 h. The reaction was stopped on adding ethanol to final of 85 %. The resulting solution was kept at −20°C for 4 h. The precipitated protein was removed via centrifugation and the supernatant ethanol solution containing laminaripentaose was concentrated. The product was analyzed with a mass spectrometer with electrospray ionization. *p*-Nitrophenyl-β-1,3-D-laminaripentaoside (*p*-NPLP) was synthesized according to the literature (Smits *et al.*, 1996).

Enzyme activity and kinetic parameters were determined at pH 7.0 on monitoring the hydrolysis of *p*-NPLP to release *p*-nitrophenol (or *p*-nitrophenolate); the absorption coefficient ($\triangle\varepsilon$) was 7280 M^{-1} cm^{-1} (400 nm, pH 7.0). A spectrophotometer (Agilent 8453) equipped with a circulating water bath at 40°C was used. All kinetic data were calculated as the average of at least three experiments.

Chemical rescue and ^{1}H-NMR spectral analysis

The rescue of the activities of E154G and D170G was performed on adding sodium azide or sodium formate (0.01–2.0 M) to the assay reagent that contained enzyme (1.6 mg/ml) and curdlan (2%) in phosphate buffer (50 mM, pH 7.5). The reaction took place at 37°C for 4 h. The activity was estimated from the amount of the reducing-end sugar. The products derived from the catalysis were determined from ^{1}H-NMR spectra measured at 27°C, as described previously (Nishimura *et al.*, 2001). In preparing for a temporal ^{1}H-NMR experiment, the wild-type and mutated LPHases and curdlan were deuterated on repeated lyophilization and solvent exchange with ^2H_2O (99.8 %). The final condition of the reaction mixture contained wild-type LPHase (1.6 mg/ml for D170G) and substrate (2 %) in ^2H_2O (99.96 %). The spectra were recorded at various intervals after the addition of enzyme. The conditions for the temporal ^{1}H-NMR spectral tests of chemical rescue are identical to the above description except for D170G (1.6 mg/ml) and azide (1.5 M) being employed.

Chemical modification of D170C

The labeling reactions were performed at 25°C for 12 h in Tris buffer (50 mM, pH 8.0) containing D170C (1 mM) and labeling reagents (5 mM, iodoethanoic, dithiodiglycolic acid). The excess labeling reagent was removed by means of ultra-filtration. The efficiency of the labeling reaction was evaluated with a mass spectrometer (ESI-MS). The catalytic activities of the resulting mutants were determined with curdlan (2 %, w/v) in phosphate buffer (50 mM, pH 7.5). *p*-Nitrophenyl-β-1,3-D-laminaripentaoside served as the substrate for determination of the kinetic parameters.

Results and discussion

Protein expression, purification and characterization

β-1,3-D-glucanase promotes the hydrolysis of the β-1,3-glycosidic linkage of a linear biopolymer of β-1,3-linked polysaccharides. Among β-1,3-D-glucanases, LPHase, releasing laminaripentaose as the predominant product (Nakabayashi *et al.*, 1998), is of particular interest. The gene of LPHase was reconstructed with primers and PCR. The mature LPHase gene (without the first 35-aa signal peptide) fused with a methionine at the N-terminus of the mature protein was inserted into pRSET_A. This new clone, pRSET_A-*lphase*, was transformed into *E. coli* BL21 (DE3) for expression. The recombinant LPHase was expressed as a soluble form. After three consecutive steps of purification, the purity of LPHase was enhanced 26-fold; the recovery yield was 34 %. The purity (>95 % homogeneity) and the Mr of the purified LPHase were confirmed with SDS-PAGE and mass spectrometry Mr 39424 ± 3 (calculated Mr 39421). The apparent Mr of LPHase was estimated to be about 38,000 with a size-exclusion chromatograph, indicating LPHase to have a monomeric form in solution, and is consistent with the observation in crystal form.

The catalytic activity of recombinant LPHase was evaluated on several polysaccharides containing the β-1,3-glucosidic bond such as curdlan, laminarin, lentinan, schizophyllan and pachyman. The reactions were carried out with 2% (w/v) substrate in phosphate buffer (50 mM, pH 7.5) for 2 h at 37°C. Of those, only curdlan and laminarin (~60 % activity of that with curdlan) were effective substrates. The specific activity of LPHase with curdlan was determined to be 7.1 μmol/min/mg. Other soluble β-glucans (highly branched β-1,3/1,6 glucans) were digested mildly by LPHase (<10 % activity of that with curdlan). No significant activity was detected when polysaccharides containing the α or β-1,4-glucosidic bond, such as lichenan, xylan, cellulose, starch, chitin and chitosan were employed as the substrate for LPHase. The LPHase specifically catalyzes the hydrolysis of the β-1,3-glucosidic bond.

The thermal (25–85°C) and acid (pH 2–11) stability of LPHase was investigated with curdlan as the substrate. In general, the recombinant LPHase was stable up to 60°C with incubation for 4 h at pH 7.0 and rapidly lost its activity at temperature > 65°C; the optimum temperature for LPHase is ~55°C. The recombinant protein was stable in pH range 5.0–9.0 (at 25°C for at least 4 h) and maintained ~80% activity for pH between 9.5–11.0. The enzyme dramatically lost its catalytic activity in solution with pH ≥ 11.5 or < 3.0. Tests of the pH-dependent activity showed that the recombinant enzyme had optimum activity in pH range 7.5–8.5.

To investigate the effect of metal ion, we added various divalent metal cations (Cu^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and Hg^{2+}) and dithiothreitol (DTT) up to 10 mM in enzymatic assay. No significant loss on activity was observed except for Hg^{2+} (>20 μM) and DTT (>100 μM), for which 90–95% inhibition were observed.

β-1,3-D-laminaripentaose on a large scale was prepared from curdlan by enzymatic catalysis using recombinant LPHase. Laminaripentaose was further used to synthesize *p*-NPLP, which was employed for kinetic tests on LPHase.

Mutagenic study of LPHase

As the molecular mechanism is believed to be conserved within the same family of glycosyl hydrolases (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996; Cantarel *et al.*, 2009), a successful investigation of one particular enzyme is valuable to characterize general features of the family. The residues in the catalytic domain are

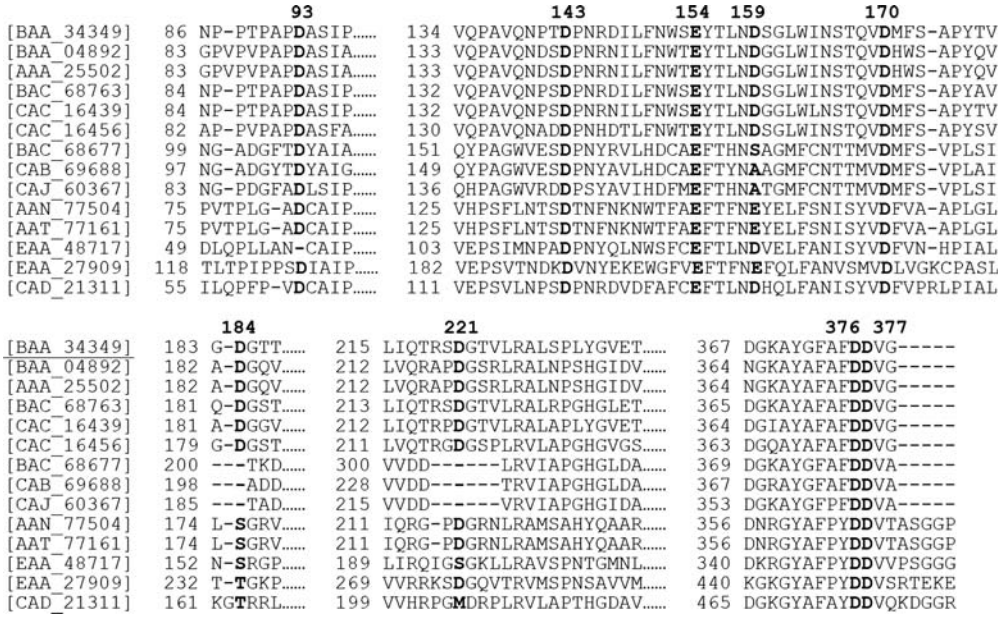


Fig. 2 Partial gene multi-alignment of GH-64 β-1,3-glucanase members. Data from a multi-alignment exercise, using partial sequences of family GH64 β-1,3-glucanases. Biology WorkBench 3.2 CLUSTALW (San Diego Supercomputer Center, CA, USA) software was used. All enzyme sequences were derived from published gene sequences. NCBI Genbank accession details are: Eukaryota: EAA_48717(*Magnaporthe grisea70–15*), CAD_21311 and EAA_27909 (*Neurospora crassa OR74A*), others was belong to bacteria : BAA_04892 (*Arthrobacter sp. YCWD3*), AAA_25502 (*Cellulosimicrobium cellulans*), CAJ_60367 (*Frankia alni ACN14a*), AAT_77161 (*Lysobacter enzymogenes C3*), AAN_77504 (*L. enzymogenes N4–7*), BAC_68763 and BAC_68677 (*Streptomyces avermitilis MA-4680*), CAB_69688, CAC_16456 and CAC_16439 (*Streptomyces coelicolor A3*), BAA_34349 (*S. matensis DIC-108*). Only partial sequences are shown. The conserved Glu and Asp are marked and numbered based on the *S. matensis* LPHase sequence.

Table II. Relative activities of LPHase and mutants with curdlan as the substrate[c]

Mutation sites[a]	Relative activity (%)[b]	Mutation sites[a]	Relative activity (%)[b]
Wild type	100	D170G	~0
D93G	90	D170N	~0
R115Q	91	D170E	81
D143G	93	D184G	92
D143N	95	D184N	89
E154G	~0	D221G	96
E154Q	~0	D221N	94
E154D	19	Y232A	38
D159G	93	D376G	91
D159N	94	D377G	85

[a]All mutational sites are conserved in family 64 except for Asp^{93}, Arg^{115}, Asp^{159}, Asp^{184} Asp^{221} and Tyr^{232}.
[b]The assay of enzymatic activity was performed at 37°C with curdlan as the substrate (2 %) in sodium phosphate buffer (50 mM, pH 7.5).
[c]Note that the specific activity of wild type is 7.1 μmol/min/mg.

Table III. Michaelis–Menten parameters of LPHase and mutants with *p*-NPL as the substrate[a]

Enzyme	k_{cat} (s^{-1})	K_m (mM^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Wild-type	8.1 ± 0.1	1.60 ± 0.02	5063
R115Q	6.8 ± 0.3	1.68 ± 0.01	4023
D143G	7.8 ± 0.2	1.59 ± 0.03	4906
D184G	7.9 ± 0.1	1.57 ± 0.01	5031
D376G	7.7 ± 0.1	1.58 ± 0.01	4873
D377G	7.3 ± 0.2	1.66 ± 0.01	4397
E154G	0.0083 ± 0.0004	2.35 ± 0.03	3.53
E154Q	0.009 ± 0.0001	2.33 ± 0.01	3.86
E154D	0.34 ± 0.02	1.82 ± 0.02	187
D170N	0.0014 ± 0.0005	3.06 ± 0.02	0.46
D170E	5.36 ± 0.01	1.72 ± 0.02	3081
D170C[b]	ND	ND	
D170Css[c]	2.28 ± 0.02	1.67 ± 0.03	1365
D170Cds[d]	0.035 ± 0.003	1.75 ± 0.02	20
D170G[b]	ND	ND	
Sodium azide[e]	0.19 ± 0.02	1.96 ± 0.02	96
Sodium formate[f]	0.057 ± 0.003	2.04 ± 0.03	28

[a]Enzymatic assays were performed at 40°C on monitoring the released *p*-nitrophenolate at 400 nm in sodium phosphate buffer (50 mM, pH 7.1).
[b]ND: the activity was undetectable even with enzyme up to 0.2 mg/ml in the assay.
[c]Resulting protein of D170C labeled by iodoethanoate.
[d]Resulting protein of D170C labeled by dithiodiglycolate.
[e]Chemical rescue of D170G was performed at 40°C in sodium phosphate buffer (50 mM, pH 6.8) containing sodium azide (2.5 M) with D170G.
[f]Chemical rescue of D170G was performed at 40°C in sodium acetate buffer (50 mM, pH 5.5) with sodium formate (0.5 M) with D170G mutant.

involved in the physical or chemical steps to orient the enzyme appropriately and to break or to form chemical bonds. Insight into the catalytic residues is derived from the X-ray structure, but to confirm the function of the essential residues demands extensive kinetic tests coupling with various strategies, including mechanism-based inhibition, the combination of amino-acid sequence analysis and mutagenesis, and chemical rescue. For most retaining enzymes, the formation of a covalent enzyme intermediate is expected. In such a case, mechanism-based or active-site-directed irreversible inhibition is a common strategy to assist the identification of key residues; 2-fluoroglycosides were employed to trap the glycosyl-enzyme intermediate and to facilitate the identification of the nucleophile through peptide sequencing with LC/MS (Hart *et al.*, 2000; Li *et al.*, 2002; Ferrer *et al.*, 2005). As LPHase is an inverting enzyme, its molecular mechanism involves no formation of a covalent

glycosyl-enzyme intermediate. Although many GH-64 β-1,3-D-glucanases were cloned, the extensive test for functional characterization and the essential residues involved in catalysis are less experimentally identified. Most glycohydrolases require two carboxylic acids for their catalytic reactions. The conserved glutamate and aspartate of GH-64 glycohydrolases, derived from the multi-alignment of 14 sequences of β-1,3-D-glucanase, were selected as potential candidates (Fig. 2). Five (glutamate and aspartate) residues—Asp^{143}, Glu^{154}, Asp^{170}, Asp^{366} and Asp^{367}—were highly conserved. An extensive mutagenesis test on all possible candidates was performed. Other residues (partially conserved)—Asp93, Asp159, Asp184 and Asp221—were also chosen for testing. We varied the putative catalytic residues, glutamate and aspartate to glutamine, asparagines or glycine by site-directed mutagenesis. All mutants were overexpressed in *E. coli.* The procedures of protein purification were similar to that of wild-type LPHase, as described in experiments. The relative activities of hydrolyzing curdlan and kinetic parameters of *p*-NPLP substrate for mutants are summarized in Tables II and III. The activity assay showed that D93G, D143G/N, D159G/N, D184G/N, D221G/N, D376G and D377G retained activity (>85%) relative to that of the wild-type enzyme. In contrast, E154Q, E154G, D170N, D170C and D170G lost catalytic activity (<0.1%). The measurement of kinetic parameters using *p*-NPLP as the substrate revealed that mutation at Glu^{154} and Asp^{170} caused a large effect on activity perturbation. For instance, the activities (k_{cat}/K_m) of D170N and E154Q (or E154G) decreased at least 10000-fold and 1300-fold, respectively. The K_m values of mutants were slightly perturbed with a range 1.6–3.06 mM. The structural alteration of E154G and D170G seemed to be minor as their CD spectra were nearly identical to that of wild-type or active mutants (Fig. 3). The kinetic outcome indicates that Glu^{154} and Asp^{170} play important roles for the catalytic activity of LPHase.

Chemical rescue

Activity rescue with exogenous nucleophiles, such as azide, formate and other anions, enhances the catalytic activity of enzymes mutated at residues that are essential for catalysis of glycoside hydrolases (Bravman *et al.*, 2001; Zechel and Withers, 2001; Cobucci-Ponzano *et al.*, 2005; Shallom *et al.*, 2005). For retaining glycosidases, an exogenous nucleophile such as azide ion might rescue the activity of mutant with mutation at the nucleophile residue (or the acid-base residue) to yield the glycosyl azide with inverting configuration (or retaining configuration) as the product. For inverting glycosidases, the catalytic hydrolysis proceeds via a single-displacement mechanism in which the general acid residue promotes the catalysis via protonation of the glycosidic oxygen, and the general base is employed to polarize a water molecule in the active site to attack the anomeric carbon of the substrate. The addition of exogenous nucleophile might activate a mutant with dysfunction on general-base residue but not on general-acid residue (MacLeod *et al.*, 1994; Zechel *et al.*, 2003).

We employed chemical rescue of the activities of E154G and D170G to elucidate the functions for both residues. Replacing the essential group with a glycine residue presumably results in obtaining sufficient space in the active site to accommodate a small nucleophile (or base) in the catalytic cavity. The activities of such mutated enzymes might be partially recovered if the small ion functions as a nucleophile or a general base. We employed sodium azide and formate to reactivate E154G and D170G. With azide or formate at various concentrations (0.05–2 M), the catalytic activity of D170G mutant was enhanced. The specific activity of the D170G mutant increased 52- and 31-fold with the addition of sodium azide (2 M) and sodium formate (1 M), respectively, whereas the catalysis of E154G remained unaltered (Fig. 4 and Table III). In summary, the kinetic outcome derived from site-directed mutagensis and chemical rescue indicates that Glu^{154} functions as the general acid and Asp^{170} serves as the general base in a catalytic turnover.

Two possible products are obtainable from the chemical rescue; one product is the α-anomeric azide-sugar derived

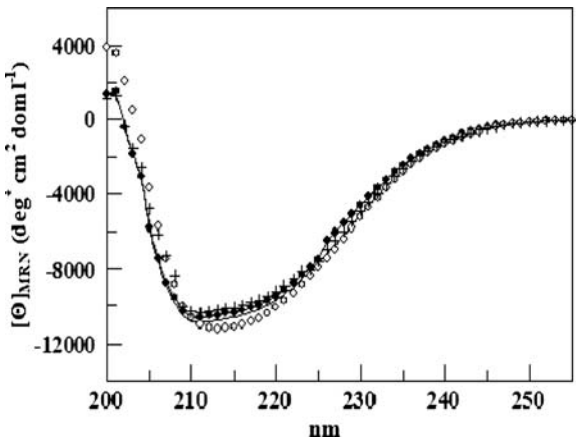


Fig. 3 CD spectra of recombinant LPHase and mutants. E154G (○) and D170G (+) exhibit CD spectra similar to those of wild-type LPHase (solid line) and other mutants with significant activity, such as D184G (•).

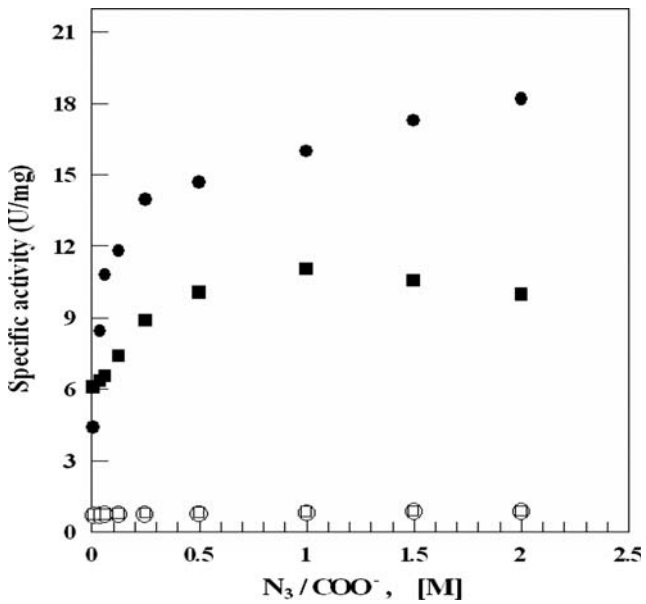
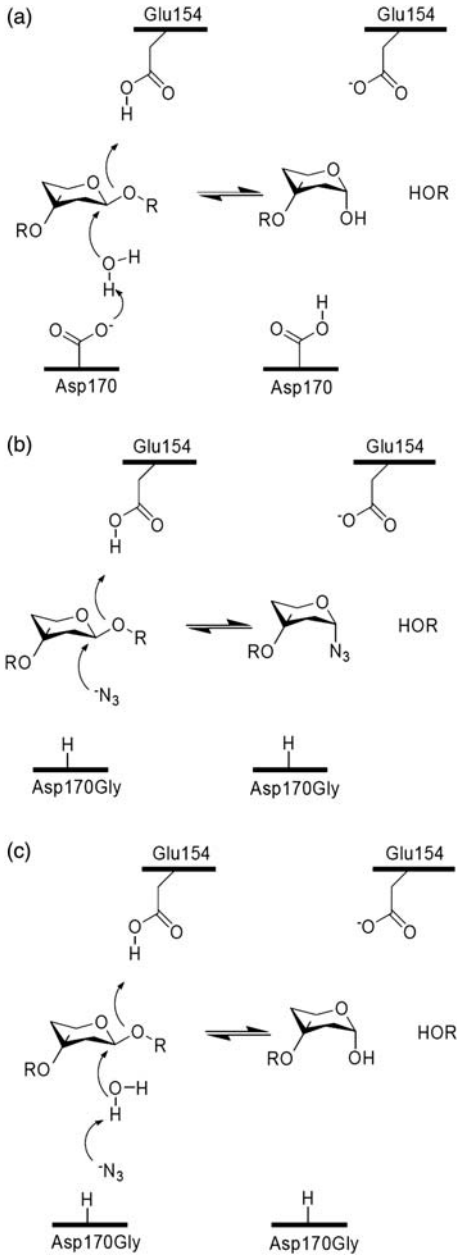


Fig. 4 Activity enhancement of E154G and D170G mutant by exogenous nucleophiles. The activity of E154G (○) and D170G (•) in the presence of sodium azide at various concentrations; E154G (□) and D170G (■) with sodium formate. The assays were performed with curdlan (2%, w/v) as the substrate at 37°C for 4 h in phosphate buffer (50 mM, pH 7.5).



Scheme 1 Proposed mechanism of LPHase for inverting catalysis and activity rescue of general base mutant by azide. (**a**) The inverting catalysis of LPHase. (**b**) Azide ion replaces the nucleophilic water molecule and attacks the anomeric carbon directly, forming the glycosylazide product with inverted anomeric configuration. (**c**) Azide ion functions as the general-base residue to activate the nucleophilic water molecule that subsequently attacks the anomeric carbon, forming free sugar with inverted anomeric configuration. Note that the glycosidic bond is β-1,3 linkage and the laminaripentaose is the predominant product (ROH) if curdlan is used as the substrate. In this study, p-NPL was used as the substrate for azide rescue, the ROH being *p*-nitrophenol.

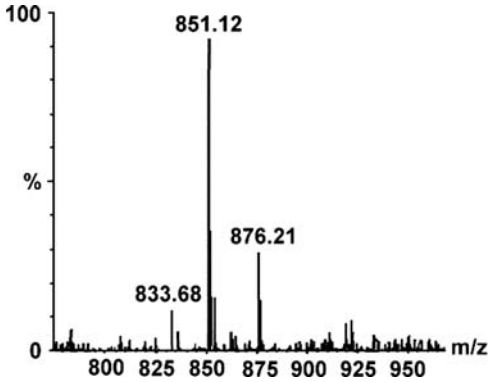


Fig. 5 Mass spectrum of products through azide rescue of D170G. The D170G reaction with curdlan (2 %, w/v) in sodium phosphate (50 mM) with sodium azide (2.5 M), pH 7.5, at 37°C for 4 h. Centrifugation, ESI-MS analyses of supernatant product. The laminaripentaose and laminaripentaose azide of the hydrolysis product have $m/z = 851.12$ and 876.21, respectively.

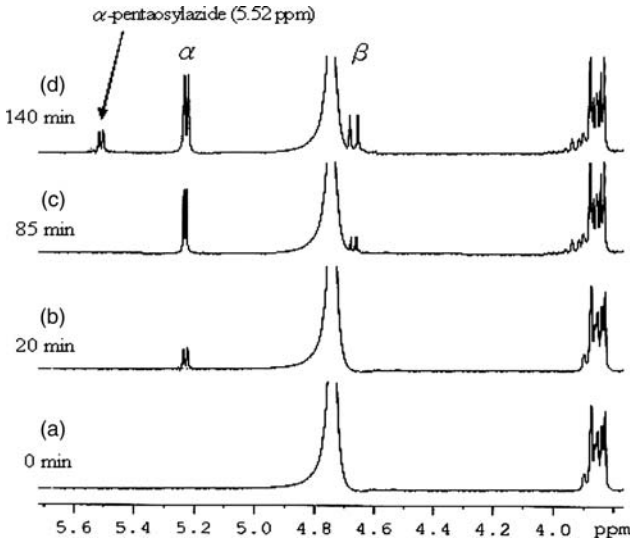


Fig. 6 ^{1}H-NMR spectra during hydrolysis of curdlan catalyzed by D170G in the presence of sodium azide. (**a**) spectrum of the substrate containing sodium azide (1.5 M) without D170G; (**b**–**d**) spectra of a sample after addition of D170G for 20, 85 and 140 min, respectively. The signals corresponding to the C1-H of α- anomer, β-anomer of laminaripentaose and α-laminaripentaosylazide are indicated.

from the direct nucleophilic attack of azide at the anomeric carbon (shown in Scheme 1a), and the other is the hydrolytic product released from the substrate through the attack of a water molecule that may be polarized with azide (shown in Scheme 1b). The products derived from the azide rescue of D170G catalysis were laminaripentaose and laminaripentaosyl azide, which were analyzed using a mass spectrometer (ESI-MS), $m/z = 851.1$ ($M+Na^+$) and 876.2 ($M+Na^+$), respectively (Fig. 5). An enzymatic temporal ^{1}H-NMR test was performed with curdlan as the substrate to examine both anomeric preference and production of azide-sugar in the chemical rescue of D170G. Weak signals assignable to a free hemiacetal H-1 were observed at 4.67 ppm (β-anomer) and 5.24 ppm (α-anomer) consistent with the polymeric structure of the substrate (Nishimura *et al.*, 2001). The temporal course of the reaction is shown in (Fig. 6). The spectrum corresponding to 20 min of hydrolysis (Fig. 6b) showed a new signal centered at 5.24 ppm ($J_{1,\ 2} = 3.6$ Hz) assigned to C1-H of a reducing oligosaccharide in an α-configuration, indicating that LPHase performs the catalysis with the inverting mechanism. For the reaction at 85 min (Fig. 6c), a new signal appeared at 4.67 ppm ($J_{1,\ 2} = 8.1$ Hz), assigned to C1-H of the reducing-end sugar in β-configuration. The β-form sugar was derived from the spontaneous mutarotation of the α-anomer. When the reaction approached 140 min, another

line appeared, centered at 5.52 ppm ($J_{1,2} = 4.3$ Hz), corresponding to the reducing end C1-H of the pentaosylazide in the α-configuration (Viladot *et al.*, 1998) (Fig. 6d). This outcome indicates that azide acts as a base to polarize a water molecule to promote the inverting catalysis and to function as a nucleophile to attack the anomeric center to give α-pentaosylazide. The test of azide rescue on D170G catalysis confirmed that Asp^{170} function as the general base and, thus, Glu^{154} serves as the general acid of LPHase.

Catalytic feature of E154 and D170 mutants

Although the laminaritetraose-complex protein structure was resolved (Wu *et al.*, 2009), the catalytic role of Glu^{154} and Asp^{170} is not confirmed. For an improved understanding of the catalytic mechanism and the substrate binding feature, a computer simulation to model an enzyme-laminarihexaose structure was performed. The LPhase-laminaritetraose complex shows that the glucose moieties from the reducing end of tetraose bound to the +5 to +2 subsites of the substrate binding site. The best model of the LPHase-laminarihexaose simulation was obtained by placing the first three glucose moieties from the reducing end of hexaose at the +5 to +3 subsites in the LPhase-laminarite traose complex (Wu *et al.*, 2009). The result shows that Glu^{154} is within hydrogen-bonding distance of the glycosidic oxygen (~1.9 Å), indicating that it may function as the proton donor. Asp^{170} is ~6 Å from the anomeric carbon; in this interval a water molecule can be accommodated between Asp^{170} and the sugar. Asp^{170} is predicted to serve as the general base for the catalysis. Of mutational tests on nine conserved carboxylic acid residues, only E154 and D170 mutants showed a large loss of activity without significant structural alteration. When these mutants were converted into the one with the other carboxylic residue, i.e. E154D and D170E, the activity was partially recovered. For example, with curdlan as the substrate, E154D retains 19% and D170E retains 81% of the activity of the wild-type enzyme. When *p*-NPLP served as the substrate, the catalytic activity (k_{cat}/K_m) of E154D and D170E was recovered to 4 and 61%, respectively (Table III). The weak activity of E154D is likely due to the insufficient distance of the general acid to protonate effectively the glycosidic oxygen for rate acceleration. In D170E mutant, the carboxyl group of Glu^{170} can provide a suitable orientation to enhance the reaction. The temporal ^{1}H-NMR tests (data not shown) confirmed that D170E also catalyzed the hydrolysis of curdlan with inversion.

An inspection of the protein structure from various GH families revealed that the distance between the two essential carboxylates is ~5.5 Å for retaining GH (Sinnott, 1990; McCarter and Withers, 1994) and ~10 Å for the inverting GH (Sinnott, 1990; Davies and Henrissat, 1995). To extend the carboxylic side-chain at the general base might result in converting LPHase into a retaining enzyme. For this approach, the D170C mutant was prepared; the mutant was inactive. Two labeling reagents—iodoethanoic acid and dithiodiglycolic acid—were used to modify the residue of Cys of D170C mutant, separately. Only a stoichiometric label was observed on D170C. Yet, no labeling reaction occurred with the wild-type LPHase, D170G and E154G mutants. Although Cys^{306} and Cys^{326} are present in LPHase, the labeling reaction indicated that both residues seem to be inaccessible for chemical modification. The resulting proteins derived from the labeling

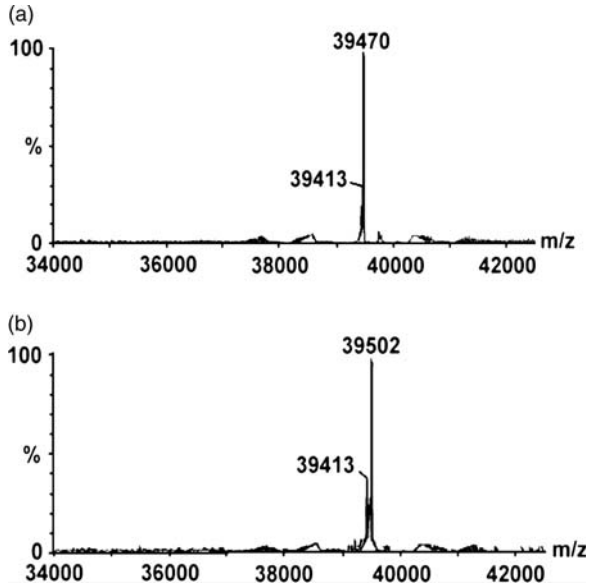


Fig. 7 ESI-MS analysis of D170Css (**a**) and D170Cds (**b**). The Mr of D170C is 39413.

reaction of D170C were analyzed using a mass spectrometer. The relative molar mass (Mr) of the modified D170C mutants increased by 57 and 89, corresponding to the addition of residue $-CH_2COO^-$ (designated as D170Css) and $-SCH_2COO^-$ (designated as D170Cds), respectively (Fig. 7). D170Css retained 27% of the activity (k_{cat}/K_m) relative to wild-type LPHase. Laminaripentaose is the predominant catalytic product. The catalytic activity of D170Cds was recovered only 0.4% (Table III) and a mixture of laminaribi-, tri-, tetra- and pentaose was produced. The temporal ^{1}H-NMR experiments demonstrated both D170Css and D170Cds still catalyzed the hydrolysis of curdlan with the inversion mechanism (data not shown). Although the attempt was unsuccessful in converting LPHase into a retaining enzyme by genetically or chemically re-placing a carboxylic side-chain back to the position corresponding to the Asp^{170}, the restore of the enzymatic activity of the mutants (D170Css and D170Cds) suggested that Asp^{170} of LPHase indeed function as the general base in the enzymatic reaction.

Conclusion

Our recent study (Wu *et al.*, 2009) described the X-ray structure of LPHase from *S. matensis* belonging to family GH64 and strongly suggested that Glu^{154} and Asp^{170} function as the general acid and the general base, respectively, of this inverting enzyme. In this study, by using a combination of site-directed mutagenesis, kinetic analysis, chemical rescue and product analysis, the catalytic roles of Glu^{154} and Asp^{170} were experimentally confirmed. This study appears to be the first direct identification of these essential residues in GH64.

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