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The essential acidic amino acid residues for catalytic activity of an exo-β-D-glucosaminidase from *Trichoderma reesei*

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Abstract

In order to investigate the residues important for the catalysis of an exo-β-D-glucosaminidase from *Trichoderma reesei* (Gls93), we performed site-directed mutagenesis study on this enzyme. Seven acidic amino acid residues in putative catalytic TIM domain, Glu409, Glu415, Asp464, Glu470, Asp513, Glu539 and Asp655, were selected for the mutational studies, each of which is located close to a C-terminus of the predicted β-strands and conserved within exo-β-D-glucosaminidase (GlcNase) subgroup of glycoside hydrolase family (GHF) 2 except for Glu470. The mutants of Gls93 at the residue Asp464 and/or Glu539, which showed a similar CD spectrum pattern to that of the wild-type enzyme, hardly have activity, indicating that Asp464 and Glu539 function as the catalytic residues of Gls93. Furthermore, the sterically conserved mutation of Glu409, Glu415 and Asp513 (the mutants E409Q, E415Q and D513N, respectively) gave rise to the change in its kinetic parameters, suggesting that these carboxylic residues could contribute to substrate recognition/binding or arrangement of ionic environment of the catalytic residues.
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Keywords: *Trichoderma reesei* (*Hypocrea jecorina*); Exo-β-D-glucosaminidase; Exochitosanase; Catalytic residue; Site-directed mutagenesis

1. Introduction

Chitin and chitosan consist of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN), respectively. Chitin is widely distributed in many organisms such as exoskeletons of arthropods, shells of crustaceans and cell walls of fungi, whereas chitosan is found in cell walls of a limited number of fungi. The degrading enzymes of these biopolymers are produced by a variety of organisms and may play important physiological roles such as moulting, cell-wall morphogenesis, and defensive actions against pathogenic fungi [1–3].

Two types of chitinolytic enzymes, endochitinase (E.C. 3.2.1.14) and *N*-acetylhexosaminidase (E.C. 3.2.1.52), are thought to be involved in chitin degradation. Similarly, there are two kinds of chitosan-degrading enzymes; endochitosanase (E.C. 3.2.1.132) randomly hydrolyzes chitosan to produce oligosaccharides, which are further hydrolyzed into GlcN by exo-β-D-glucosaminidase (GlcNase). Endochitosanases have been found in a variety of microorganisms and are classified into five glycosyl hydrolasae families, GHF 5, 8, 46, 75 and 80 (http://www.cazy.org/). Furthermore, 3D-structures of three endochitosanases from bacterial sources have been deciphered, and site-directed mutagenesis studies, especially in an endochitosanase from *Streptomyces* sp. N174, have also been reported, allowing more detailed discussion on the catalytic and substrate recognition mechanisms of endochitosanases [4–9]. However, the information on the GlcNase structure has been quite limited. Although several GlcNases have been purified from filamentous fungi and actinomycete, only five GlcNase-encoding genes have been reported thus far from a filamentous fungus *Trichoderma reesei* PC-3-7 [10], two actinomycete *Amycolatopsis orientalis* and *Streptomyces avermitilis* MA-4680 [11], and two archaea *Thermococcus kodakaraensis* KOD1 and *Pyrococcus horikoshii* OT3 [12,13]. Among these GlcNases, the fungal and actinomycetous ones belong to GHF2 and the others GHF35. Very recently, the catalytic residues of *A. orientalis* GlcNase have been identified [14]. Hence, the research on the structure–function relationship of GlcNases has just started.

In our previous paper [10], we reported the primary structure of the GlcNase from *T. reesei* (Gls93, GenPept accession

Abbreviations: GHF, glycoside hydrolase family; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; GlcNase, exo-β-D-glucosaminidase; Gls93, an exo-β-D-glucosaminidase from *Trichoderma reesei*.

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number: BAD99604), and proposed that this enzyme belongs to a new subgroup of GHF2, GlcNase. Furthermore, the heterologous expression of Gls93 in *Pichia pastoris* has been reported. In the current study, in order to provide fundamental data for the catalytic reaction of Gls93, we carried out site-directed mutagenesis of Gls93, identified the catalytic residues of this enzyme, and found several acidic amino acid residues that may contribute to this enzymatic reaction.

2. Experimental

2.1. Strains and plasmids

Escherichia coli MV1184 (sup^0) and the plasmid vector pKF19 (Takara, Kyoto, Japan) were used for site-directed mutagenesis. *P. pastoris* X-33 (Invitrogen, Carlsbad, CA, USA) was used as a host for heterologous expression of *gls93* gene. The plasmids, pBK93c, which contain full-length of *gls93* cDNA, and pPIC93, for heterologous expression of wild-type Gls93 in *P. pastoris*, were previously constructed [10].

2.2. DNA manipulations

All DNA manipulations were performed by standard techniques [15] or manufacturer's recommendations. Dideoxy DNA sequencing reactions were performed with a "CEQ^{TM}DTCS Quick Start Kit" (Beckman Coulter, Brea, CA, USA) as specified by the manufacturer and the DNA fragments were analyzed on CEQ2000XL (Beckman Coulter).

2.3. Bioinformatic analysis

InterProScan Sequence Search program was used for the analysis of modular structure of Gls93 [16]. The secondary structure of Gls93 was predicted with PSIPRED Program [17]. Among the GlcNases and hypothetical proteins belonging to GHF2 GlcNase subgroup [10], the protein sequences of putative catalytic TIM domain were aligned with the program Clustal W.

2.4. Site-directed mutagenesis in gls93

Construction of the plasmids for the mutants is summarized in Fig. 1. The PstI-EcoRI fragment of pBK93c, a part of *gls93* cDNA, was inserted into the same sites of pKF19 (pKF93PE). First, to easily confirm that the desired mutation was incorporated and no PCR error had occurred, we introduced NruI and Tth111I restriction sites, which cause no amino acid changes in the Gls93 primary structure, to pKF93PE by site-directed mutagenesis using "Mutan-Super Express Km" (Takara) (pKF93PE′). We then exchanged the PstI-XhoI fragment between pPIC93 and pKF93PE′ to give pPIC93′. The PstI-EcoRI fragment from pKF93PE′ was reintroduced into pKF19, and the resulting plasmid was subjected to site-directed mutagenesis using the synthetic oligonucleotide primers listed in Table 1. After confirmation of DNA sequence, we exchanged the mutated fragments for the corresponding ones of pPIC93′ to construct mut.pPIC93; (i) PstI-NruI fragment for the mutants of E409 and E415, (ii) NruI-KpnI fragment for the mutants of D464, E470, D513 and E539, or (iii) KpnI-Tth111I fragment for the mutant of D655. The mutated plasmids were linearized with PmeI, and then were introduced into *P. pastoris* X-33 cells by electroporation.

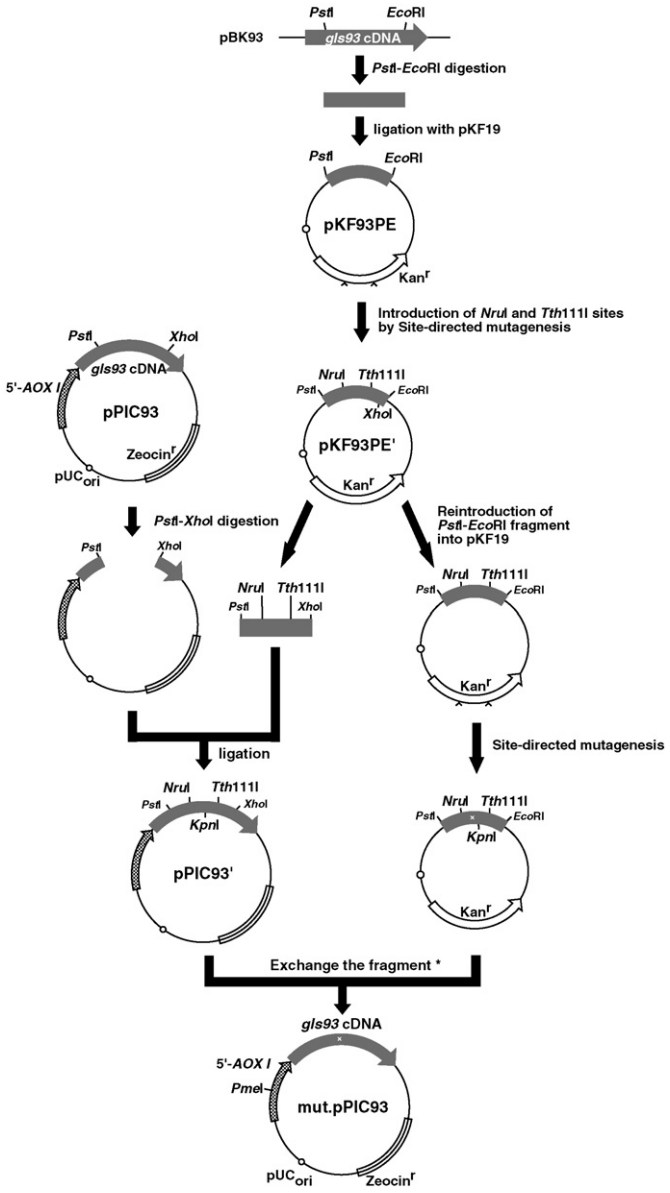

Fig. 1. Construction of the plasmids for mutated Gls93 expression in *Pichia pastoris*. At the final step, the exchanged fragments are (i) PstI-NruI fragment for the mutants of E409 and E415, (ii) NruI-KpnI fragment for the mutants of D464, E470, D513 and E539, and (iii) KpnI-Tth111I fragment for the D655 mutant.

2.5. Production and purification of mutated enzymes

The transformed cells were grown in 5 ml of BMGY medium at 30 °C for 16 h with constant shaking. The cells were collected by centrifugation (4000 × *g* for 5 min) and resuspended in 50 ml of BMMY medium (in 500-ml shake flask). Cultures were supplemented daily with 0.5% methanol, and cultivation was

Table 1
Synthetic oligonucleotide primers used for site-directed mutagenesis

Mutation[a]	Oligonucleotide sequence (5′–3′)[b]
Introduction of Tth111I site (silent mutation)	CTACCAGACGGG**G**TCGCGGAACATCAAGG
Introduction of NruI site (silent mutation)	CGACGAACTCGC**G**ATTTTCCCCCCACCC
Glu409 → Gln (E409Q)	GGCTGG**C**AATGCTGCGATAAGTGGGAGGC
Glu415 → Gln (E415Q)	GCGATAAGTGG**C**AGGCTTGGGCTTACAACG
Asp464 → Ala (D464A)	CCTTTCTCGTTGGAAGCG**C**TTTCTGGCCC
Asp464 → Glu (D464E)	CCTTTCTCGTTGGAAGCGA**G**TTCTGGCCC
Asp464 → Asn (D464N)	CCTTTCTCGTTGGAAGC**A**ATTTCTGGCCC
Glu470 → Gln (E470Q)	GGCCCAACGAC**C**AGGCCGTTG
Asp513 → Asn (D513N)	GGACGGTCCGTAT**A**ACTGGGTGCCACC
Glu539 → Ala (E539A)	GGCTCTG**C**GCTGGGAGCTGGTGTTGG
Glu539 → Asp (E539D)	GGCTCTGA**C**CTGGGAGCTGGTGTTGG
Glu539 → Gln (E539Q)	GGCTCT**C**AGCTGGGAGCTGGTGTTGG
Asp655 → Asn (D655N)	GGAACCAATTT**A**ATTACTATCTTCACCCTGCCGGC

[a] All of the mutational sites are conserved within GlcNase subgroup of GHF2 except for Glu470.
[b] The nucleotides indicated in bold and underlined are the mutation sites and restriction sites, respectively.

continued at 30 °C for 96 h with constant shaking. The mutant proteins were purified by the same procedure as described previously [10]. The recombinant Gls93 was secreted as two forms, named F1 and F2, whose discrepancy could be explained by the difference of *N*-glycosylation pattern [10]. Purified F2 form of Gls93-mutants was used in the following experiments because the expression amount of F1 form was inconsistent for each mutant.

2.6. Enzyme assay and protein determination

GlcNase activity was usually determined by measuring the concentration of reducing sugar liberated during the hydrolysis of Chitosan 10B using a modified version of the Schales method [18]. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar from substrate per minute with GlcN as the standard. The kinetic parameters and Michaelis constant of the mutated enzymes were calculated by Hanes-Woolf plot. The protein concentration was determined using the Lowry method with bovine serum albumin as a standard [19].

2.7. Circular dichroism (CD) spectroscopy

CD spectra of the wild-type and mutant of Gls93 solution were recorded using a Jasco J-720 spectropolarimeter (Jasco, Tokyo, Japan). The spectra were obtained at a protein concentration of 0.15 mg/ml in 10 mM sodium acetate buffer (pH 4.0) using a 0.1 cm path length quartz cuvette.

3. Results and discussion

3.1. Selection for the mutation sites and site-directed mutagenesis in Gls93

As previously described, Gls93 belongs to the GlcNase subgroup of GHF2 and the primary structure of the enzyme had low similarity (around 10–15% overall identity) to those of other subgroups, β-galactosidase, β-glucuronidase and β-mannosidase subgroup of GHF2 [10]. In order to select the mutation point in Gls93, we first analyzed the primary structure of Gls93 to identify the modular structure. Three modules were found in Gls93 primary structure; GHF2 sugar-binding domain, immunoglobulin-like β-sandwich domain and GHF2 TIM barrel domain that can function as the catalytic domain (Fig. 2A). Next, we predicted the secondary structure of TIM domain using the PSIPRED program, and aligned the primary structure of the TIM barrel sequence of GlcNase subgroup enzymes. According to CAZY database (http://www.cazy.org/), GHF2 is a member of the clan-A, where two catalytic acidic residues acting as catalytic acid/base and nucleophile are located close to C-terminus of the fourth and seventh β-strand, respectively. As shown in Fig. 2B, the residues Asp464 and Glu539 in Gls93, which are conserved within GlcNase subgroup of GHF2, appeared to be located at the suitable positions as catalytic residues of the clan-A. Thus, we selected these two residues for site-directed mutagenesis study as candidates for the catalytic residues. Furthermore, we focused on the acidic residues that are conserved within the GlcNase subgroup of GHF2 and are located at the end of the β-strand or at the loops between β-strand and α-helix due to the following reasons: (i) for an endochitosanase from

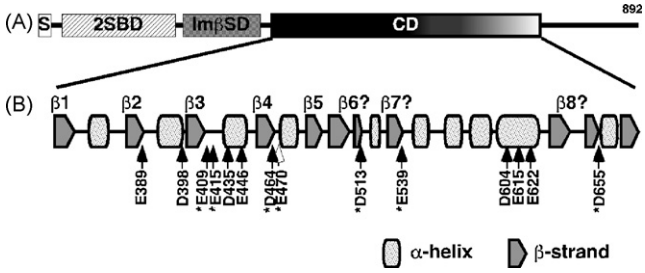

Fig. 2. Location of the acidic amino acids selected for site-directed mutagenesis. (A) Domain structure of Gls93. S: signal peptide; 2SBD: GHF2 sugar-binding domain; ImβSD: immunoglobulin-like β-sandwich domain; CD: GHF2 catalytic TIM barrel domain, the latter part of which shows low similarity to other GHF2 proteins. (B) Secondary structure of catalytic TIM domain predicted by PSIPRED program. The positions of glutamic and aspartic acid residues conserved in GlcNase subgroup of GHF2 and 470th glutamic acid (not conserved) are shown by the black and white arrow, respectively, and residue number of Gls93 primary structure is pointed. The residues chosen for site-directed mutagenesis study are also indicated by asterisk.

Streptomyces sp. N174, some carboxylic amino acid residues are contributing in substrate recognition and/or binding [8,9] and (ii) from the 3D-structures of glycosyl hydrolases in GH clan-A, the residues sterically situated in the catalytic cleft/pocket are located at the end of the β-strand or at the loops between β-strand and next α-helix of its catalytic TIM domain. Thirteen acidic residues conserved within GlcNase subgroup (including Asp464 and Glu539) were found in the primary structure of Gls93 TIM domain. Among these 13 residues, we further chose four conserved acidic residues, Glu409, Glu415, Asp513 and Asp655, which are located at the end of the β-strand (Fig. 2B). Finally, seven acidic residues including Glu470, which is located close to Asp464 but not conserved in GlcNase subgroup, were chosen. Site-directed mutagenesis was carried out by the strategy shown in Fig. 1, and all mutants were expressed in *P. pastoris* and purified as described in Section 2. All of the Gls93-mutants produced were separately purified according to apparent homogeneity in SDS-PAGE.

3.2. Characterization of the mutants of Gls93

Initially, we successfully produced seven Gls93-mutants with sterically conserved mutations (Glu → Gln or Asp → Asn). The specific activity of the purified mutant enzymes for Chitosan 10B, more than 98% deacetylated chitosan, is shown in Table 2. The five Gls93-mutants, E409Q, E415Q, D464N, D513N and E539Q, showed lower or undetectable activity compared with the wild-type enzyme, indicating that these five residues participate in the catalysis of Gls93, and Glu470 and Asp655 are not essential. Thus, we further investigated the mutants at the position of Glu409, Glu415, Asp464, Asp513 and Glu539.

3.2.1. Asp464 and Glu539 are the catalytic residues of Gls93

As shown in Table 2, the activities of two mutants, D464N and E539Q, remarkably reduced to 1.9% and undetectable levels as compared with wild-type Gls93, respectively. We produced a further five mutants of these residues, D464E and E539D (functionary conserved mutants), D464A and E539A (mutants replaced with Ala), and D464E/E539D (double mutant), and measured their activities. All mutants, except for D464A whose relative activity was 0.44% compared with wild-type enzyme, did not show any significant activity for Chitosan 10B. To ascertain that decrease in activity at the mutants of Asp464 and Glu539 were not caused by an incorrect folding of the mutant protein, CD spectra were collected for the wild-type and these mutants. As shown in Fig. 3, the spectrum of D464N and E539Q were identical within the experimental error, and those of five other mutants were almost the same as the wild-type (data not shown), indicating that the secondary structure of the mutant enzymes did not change by site-directed mutagenesis. These results clearly indicate that Asp464 and Glu539 are the essential residues for catalytic function of Gls93. Two mutants of Asp464 (D464A and D464N) were retained their weak activity. In some cases of retaining glycoside hydrolases, mutation on the catalytic acid/base shows the slight activity, whereas the nucleophile mutation causes almost complete loss of its activity [20,21]. From practically and complete loss of the Glu539 mutants' activities, secondary structure prediction of Gls93, and the primary structure alignment to other GHF2 enzymes, Asp464 and Glu539 may function as the catalytic acid/base residue and catalytic nucleophile residue, respectively.

As mentioned above, GHF2 also contains β-galactosidases, β-glucuronidases and β-mannosidases. There are a few reports on the catalytic residues of these enzymes [20,22,23]. Among these enzymes, combination of an aspartic acid and a glutamic acid as the catalytic residue is not found, and a pair of glutamic acid residues acts as the catalytic acid/base and nucleophile. Fukamizo et al. also reported that the catalytic residues of GlcNase from *A. orientalis*, a member of GHF2 GlcNase subgroup, are Asp469 and Glu541, which correspond to Asp464 and Glu539 in Gls93, respectively [14]. Thus, we concluded that the catalytic residues of GHF2 GlcNases are the aspartic acid and

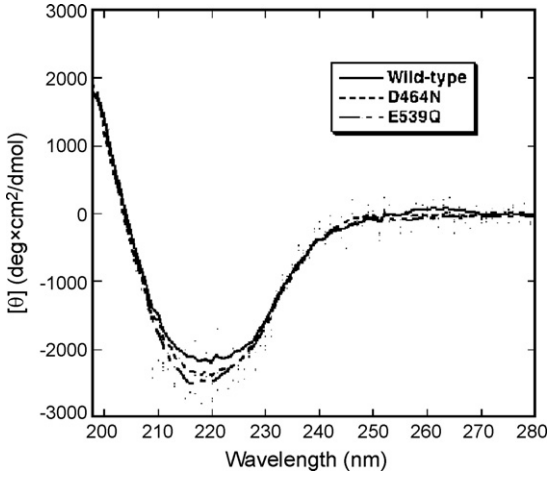

Fig. 3. Circular dichroism spectra of wild-type and mutants of Gls93. Each spectrum was obtained at concentrations of 0.15 mg/ml for the each protein. Sodium acetate buffer (10 mM, pH 4.0) was used as the solvent. Only the spectrum of wild-type, mutants D464N and E539Q are presented. Those of other mutants at Asp464 and/or Glu539 are almost the same as the wild-type.

Table 2
GlcNase activity of wild-type and mutants of Gls93

Enzyme[a]	Specific activity[b] (U/mg)	Relative activity (%)
Wild-type	23.8	100
D464N	0.451	1.9
D464A	0.104	0.44
D464E	N.D.[c]	N.D.
E539Q	N.D.	N.D.
E539A	N.D.	N.D.
E539D	N.D.	N.D.
D464E/E539D	N.D.	N.D.
E409Q	18.4	77
E415Q	12.5	53
E470Q	23.5	99
D513N	17.1	72
D655N	24.6	103

[a] All of the mutational sites are conserved within GlcNase subgroup of GHF2 except for Glu^{470}.
[b] Chitosan 10B (0.2% (w/v), pH 4.0) was used as a substrate.
[c] N.D.: not detected.

Table 3
Kinetic parameters of wild-type and mutants of Gls93

Enzyme	V_{max}[a,b] (U/mg)	K_m[b] (mg/ml)	V_{max}/K_m	Relative V_{max}/K_m (%)
Wild-type	25.1	0.104	2.41×10^2	100.0
E409Q	25.2	0.221	1.14×10^2	047.2
E415Q	13.1	0.140	9.36×10	038.8
D513N	20.0	0.352	5.68×10	023.5

[a] Chitosan 10B (0.2% (w/v), pH 4.0) was used as a substrate.
[b] Kinetic parameters were calculated by Hanes-Woolf plot.

glutamic acid located close to the C-terminus of the fourth and seventh β-strands and that these function as a catalytic acid/base and nucleophile, respectively.

3.2.2. Glu409, Glu415 and Asp513 may indirectly participate in the enzymatic reaction

Three mutants E409Q, E415Q and D513N retained 58–77% specific activity as compared with wild-type enzyme (Table 2), indicating that these carboxyl residues are not essential for catalysis, but possibly have some role as full catalyst in this enzyme. For these mutants, we measured kinetic parameters (Table 3). Compared to wild-type enzyme, the K_m values of the mutants E409Q and D513N were nearly twice and 3.5-times higher, with no effect on and causing a slight reduction on the V_{max} value, respectively. We previously reported that Gls93 is specific to chitosan and does not have β-galactosidase, β-glucuronidase and β-mannosidase activities [10], implying that this enzyme can specifically recognize the chitosan chain. From the alignment of GlcNase subgroup enzymes to those of other GHF2 subgroup, these three acidic amino acid residues were not conserved with other subgroups (data not shown). For an endochitosanase from *Streptomyces* sp. N174, Asp57 that is located in the catalytic cleft of this enzyme is reportedly important for chitosan recognition and/or binding of this enzyme through both electrostatic and hydrogen bonding interactions with the sugar amine and the hydroxyl oxygen at C3 of the pyranose ring of the chitosan chain [4,8]. Moreover, Katsumi et al. reported that two other acidic amino acid residues (Glu197 and Asp201) of the endochitosanase are responsible for chitosan oligosaccharide binding [9]. Based on all this information including our results, Glu409 and Asp513 could participate in the recognition and/or binding of the chitosan chain. In contrast, the mutation of Glu415 to Gln decreased the V_{max} value to almost half that of the wild-type enzyme, with a slight change in the K_m value, suggesting that this residue may interact to the catalytic residue and play some role in the catalysis of this enzyme, e.g. determination of the orientation or arrangement of ionic environment of the catalytic residues, rather than substrate recognition and/or binding. To date, the 3D-structures of each subgroup of GHF2 except GlcNase subgroup, β-galactosidase from *E. coli*, β-glucuronidase from human and β-mannosidase from *Bacteroides thetaiotaomicron*, have been solved [24–26]. The roles of three carboxyl residues will become clearer if 3D-structure of Gls93 is solved. Determination of 3D-structure of Gls93 as well as further mutagenesis studies will make it possible to discuss on structure–function relationship of GlcNases and the difference with other GHF2 enzymes in detail.

4. Conclusions

In this paper, we performed site-directed mutagenesis study on an exo-β-D-glucosaminidase from *T. reesei* and showed that Asp464 and Glu539 function as the catalytic residues of this enzyme. Furthermore, we proposed that three acidic residues, Glu409, Glu415 and Asp513, indirectly participate in the enzymatic reaction, where Glu409 and Asp513 could contribute to substrate recognition/binding and Glu415 may play some role in the catalysis of this enzyme, e.g. determination of the orientation or arrangement of ionic environment of the catalytic residues.

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