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Trp17 and Glu20 Residues in Conserved WMN(D/E)PN Motif Are Essential for *Aspergillus ficuum* Endoinulinase (EC 3.2.1.7) Activity

Seockyu Park, Yeonsoo Han, Heeun Kim, Songyi Song, Tai-Boong Uhm, and Keon-Sang Chae*

Division of Biological Sciences and Research Center for Industrial Development of Biofood Materials, Chonbuk National University, Chonju, Chonbuk 561-756, Republic of Korea; fax: +82 63 270 3345; E-mail: chaeks@moak.chonbuk.ac.kr

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Abstract—The importance of the WMN(D/E)PN motif, which is well conserved among β-fructofuranosidases grouped in the glycosylhydrolase family 32, in *Aspergillus ficuum* endoinulinase was accessed. Each mutant enzyme generated by site-directed mutagenesis of Trp17 in the conserved motif to Gln, Leu, Ser, Pro, Thr, or Met had an activity of less than 1% of the wild type. Another mutant enzyme obtained by mutation of Glu20 in the motif to Ser, Leu, Thr, Gln, Ala, or Val had an enzyme activity of less than 1% of the wild type. Furthermore, the E20D mutant enzyme, in which Glu20 in the conserved motif was replaced with Asp, had 1.1% of the wild type activity. These results clearly indicated that Trp17 and Glu20 are essential for the enzyme activity.

Key words: *Aspergillus ficuum*, endoinulinase, glutamic acid, *inuB*, tryptophan

β-Fructofuranosidases grouped in the glycosylhydrolases family 32 including invertase, levanase, exoinulinase, endoinulinase, and fructosyltransferase share well-conserved motifs in their amino acid sequences [1-5]. They are the WMN(D/E)PN motif, the RDP motif, and the EC(P) motif. Therefore, it has been proposed that these well-conserved motifs may be important for catalysis. To access the importance of residues in these conserved motifs for catalysis, chemical modification and site-directed mutagenesis approaches have been addressed on these enzymes. The catalytic mechanism of most glycosylhydrolases has been proposed to involve two acidic residues, one acting as a nucleophile and the other as a proton donor [6-8]. Later, the two acidic residues acting in the catalytic mechanism have been identified to be Asp23 (D23) in the WMN(D/E)PN motif and Glu204 (E203) in the EC(P) of yeast invertase, as analyzed by chemical modifications and site-directed mutagenesis [1, 9]. Based on results of chemical modifications and site-directed mutagenesis together with previous results, Reddy and Maley [1] proposed a reaction mechanism of sucrose hydrolysis by yeast invertase. The mechanism involves D23 and E204 in the conserved motifs of yeast invertase as a nucleophile and as an acid/base catalyst, respectively. The initial step in their mechanism is the nucleophilic attack of a carboxylate of D23 to the C-2 of the fructose moiety, forming a covalently bound fructosyl–enzyme complex. Separately, oxidation of 5-6 tryptophan residues/molecule of yeast invertase with N-bromosuccinimide, which appears to be specific for indole chromophore, has been proportional to the complete inactivation of the enzyme [10]. Furthermore, the *Penicillium* endoinulinase activity was also completely inhibited by chemical modification with N-bromosuccinimide [11]. These results suggested that at least one Trp residue may be involved in the active center of these enzymes. Although not conserved well, two other residues in the WMN(D/E)PN motif, Trp17 and Met18, are also conserved in several eukaryotic fructosylhydrolases including *Aspergillus ficuum* endoinulinase Afi-Endo [1, 3]. Therefore, it is expected that Trp17 in the WMN(D/E)PN motif can be the best candidate for the Trp residue responsible for the inactivation upon oxidation with N-bromosuccinimide. Furthermore, it should be noted that Afi-Endo as well as *Penicillium* endoinulinase have Glu in the WMN(D/E)PN motif, while other

Abbreviations: Afi-Endo) *Aspergillus ficuum* endoinulinase; PCR) polymerase chain reaction.

* To whom correspondence should be addressed.

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fructosylhydrolases have Asp [4, 11]. Therefore, in this study, the importance of Trp17 and Glu20 in the WMN(D/E)PN motif was analyzed by site-directed mutagenesis of each of these two residues to understand whether these two residues are important for Afi-Endo activity.

MATERIALS AND METHODS

Strains, DNAs, and medium. *Saccharomyces cerevisiae* YSH 2.64-2C (*MATa trp1-92 his4 ura3-52 Δsuc2::URA3 mal10*) used as a host for analysis of the endoinulinase activity was described previously [12]. The expression vector of the *inuB* gene, pYESINU2, constructed previously, has the *inuB* gene under the promoter of the *INU1* gene for *Kluyveromyces marxianus* exoinulinase [12]. All strains were cultured on YPD medium supplemented with 2% glucose as a carbon source [13].

Site-directed mutagenesis. To identify the importance of the tryptophan residue in the WMN(D/E)PN motif for the Afi-Endo activity, it was mutated to leucine (L), serine (S), proline (P), glutamine (Q), threonine (T), or methionine (M) using the primer UPINUWX in which an NNG codon was present in place of the W codon (table). Furthermore, in the Afi-Endo, Glu (E) is present instead of Asp (D) in the well-conserved WMN(D/E)PN motif, while many fructosylhydrolases have the D residue [4, 11]. It was of interest whether the Glu residue could be changed to Asp without loss of the enzyme activity. This was accomplished by using the primer UPINUED, which has a GAC (D) codon in place of the GAG (E) codon (table), being capable of generating E20D mutant *inuB* gene. Also, it was interesting whether the Glu residue is necessary for the Afi-Endo activity. The Glu residue was changed to Ser, Leu, Thr, Gln, Ala, or Val using the primer UPINUEX (table).

Site-directed mutagenesis of the *inuB* gene was achieved by inverse PCR according to the procedure of the ExSite™ PCR-Based Site-Directed Mutagenesis Kit (Stratagene, USA). The amino acid residues to be mutated, expected amino acids to be obtained, and primer pairs for mutagenesis are shown in the table. The PCR mixture contained 300 pg of the template pYESINU2 purified from *Escherichia coli* DH5α [12], 1 μl of 2.5 mM dNTP, 1 μl (5 pmol) of each primer, and 1 U of *Pfu* polymerase in a 20 μl volume. PCR consisted of incubation at 95°C for 3 min, and 35 cycles of incubation at 94°C for 30 sec, at 65°C for 15 sec, and at 72°C for 10 min, followed by the final extension at 72°C for 15 min. After PCR, a DNA fragment about 8 kb was eluted from agarose gel, phosphorylated with T4 polynucleotide kinase, and ligated with T4 DNA ligase. The template pYESINU2 in the PCR mixture was digested with *Dpn*I before transformation of *E. coli* DH5α. To eliminate side mutations in the coding regions, the 765 bp *Sal*I *Nde*I-digested fragment in each mutant plasmid that had been amplified and had a desired mutation was isolated from *E. coli*, replacing the corresponding wild type fragment and generating the mutant genes. The nucleotide sequence of the 765 bp fragment containing each mutation was determined, showing no mutations other than the desired one.

Measurement of endoinulinase activity. Each mutated expression vector was introduced into *S. cerevisiae* YSH 2.64-2C according to the procedure described previously [14]. The endoinulinase activity of each transformed *S. cerevisiae* having the mutated *inuB* gene was analyzed as described previously by HPLC [13]. When the enzyme activity was very low, 5 ml of cell-free medium in which each strain was cultured for 3 days was concentrated to 130 μl using a Centricon™ (Millipore Co., USA), followed by measurement of the enzyme activity.

Primers for site-directed mutagenesis by inverse PCR*

Primer	Sequence	Position (5′-3′)**	Target
UPINUWX	AAC GAG CCA AAC GGC CTG ATT AAA	627-650	WMNEPNG
LWINUWX	CAT CNN GTA CTG GTC CGG TGT GAA	626-603	(W to X***)
UPINUED	AAC GAC CCA AAC GGC CTG ATT AAA	627-650	WMNEPNG
LWINUEX	CAT CCA GTA CTG GTC CGG TGT GAA	626-603	(E to D)
UPINUEX	AAC NNG CCA AAC GGC CTG ATT AAA	627-650	WMNEPNG
LWINUEX	CAT CCA GTA CTG GTC CGG TGT GAA	626-603	(E to X)

* The target amino acid residues and the altered nucleotides in the primers are shown by shadow with underline.
** Position refers to that in the *inuB* sequence deposited in GenBank (accession number AF006951).
*** X means any amino acid whose codon has a nucleotide G at the third position.

RESULTS

Effect of mutation of W17 to another amino acid in the WMN(D/E)PN motif. Since at least one W residue may be involved in the active center of Afi-Endo as analyzed by chemical modification with N-bromosuccinimide [11], the W17 residue in the WMN(D/E)PN motif was thought to be the first candidate responsible for loss of the activity by chemical modification. To investigate its role, Trp17 was replaced with Leu, Ser, Pro, Gln, Thr, or Met (giving variants W17L, W17S, W17P, W17Q, W17T, or W17M, respectively), by site-directed mutagenesis. Cell-free medium of each mutant exhibited no detectable activity, as analyzed by HPLC. When the cell free medium of the W17Q mutant was concentrated 38-fold, the enzyme activity was 6.9 U/ml, corresponding to 0.2% of the wild type activity.

Effect of E20D substitution on the enzyme activity. Many β-fructofuranosidases grouped in the glycosylhydrolase family 32, including invertases, inulinases, levanases, and sucrases, have an Asp residue in the WMN(D/E)PN motif as a catalytic site [3]. In contrast, Afi-Endo has Glu in place of Asp [4]. Therefore, it was interesting to analyze the effect of Glu20 mutation to Asp in the WMN(D/E)PN motif on the enzyme activity. The mutant enzyme (E20D) had an activity of 0.9 U/ml in the cell-free medium, which corresponds to 1.1% of the wild-type level. Furthermore, when the cell-free medium was concentrated by 130-fold, the activity of the E20D enzyme in the concentrate was 37 U/ml. Such a low level was unexpected, since many β-fructofuranosidases have Asp instead of Glu in the conserved WMN(D/E)PN motif [3]. This result indicated that Glu20 is essential for the endoinulinase activity.

Effect of Glu20 mutations to other amino acids. Since the Asp residue which corresponds to the Glu20 residue in Afi-Endo is directly involved in catalysis in the reaction mechanism of yeast invertase, as proposed previously [1], the Glu20 residue in Afi-Endo may also be involved in catalysis. When E20 was replaced with Ser, Leu, Thr, Gln, Ala, or Val (giving E20S, E20L, E20T, E20Q, E20A, or E20V variants, respectively), the corresponding variant enzymes showed nearly zero activity in cell-free medium, even after a 38-fold concentration.

DISCUSSION

In the present study, we have carried out site-directed mutagenesis to investigate the role of two target amino acids, Trp17 and Glu20, in the well conserved motif WMN(D/E)PN. All mutant enzymes with substituted Glu20 (except for E20D) showed no detectable activity, which is consistent with a previous result that Asp20, or Glu20, in the motif is a catalytic residue, as suggested by Reddy and Maley [1]. The common amino acid sequence in the motif of all endoinulinases that have been reported so far is WMNEPN, while that of other fructofuranosidases is WMNDPN. It is interesting that the Glu20 to Asp substitution caused a 90-fold reduction in the enzyme activity. Since the catalytic mechanism of glycosylhydrolases in most cases involves two acidic residues, one acting as a nucleophile and the other as a proton donor [1, 7], E20 may serve as a nucleophile in the catalytic process of inulin hydrolysis, as in the case of *S. cerevisiae* invertase. Glu20 replacement with Asp affects the position of the side-chain carboxyl group required for catalysis, because of the difference in the size of the side chains between Glu and Asp. Furthermore, when Glu20 was changed to other amino acids examined in this study, no activity was detectable, which is consistent with the previously proposed reaction mechanism.

The replacements of Trp17 by other amino acids results in an almost complete loss of activity. This result is consistent with the previous result of chemical modification of *Penicillium* endoinulinase with N-bromosuccinimide [11]. The exact role of Trp17 in the catalytic process of endoinulinase is not clear at present. Previous experiments on substrate specificity showed that endoinulinase hydrolyzes only inulin and inulooligosaccharides, whereas larger fructooligosaccharides apparently bound predominantly in nonproductive modes [4]. Relative activities toward inulin, inulopentose, inulotetraose, inulotriose, and inulobiose are 370 : 16 : 8 : 3 : 1, respectively. Hydrolysis patterns of inulooligosaccharides also showed that the enzyme hydrolyzes inulohexaose (DP6) to DP3, inuloheptaose (DP7) to DP3 and DP4, and inulooctaose (DP8) to DP3 and DP5. This means that the enzyme nearly quantitatively hydrolyzes an oligosaccharide between the third and fourth residues from its reducing terminus, just as expected if the enzyme has at least eight saccharide-binding subsites and cleaves its bound substrate between the third and fourth subsites. This proposed cleavage mode is very similar to that of lysozyme, whose catalytic mechanism is defined well. In the proposed catalytic mechanism of lysozyme, the imino group of Trp62 indole ring binds to the C-6 hydroxyl group of the third N-acetylglucosamine by a hydrogen bond for the proper positioning of substrate at the active center [15]. Just as in the case of lysozyme, a plausible role of Trp17 in Afi-Endo is that the residue binds the hydroxyl group of C-6 of the fructose ring of inulin chain by a hydrogen bond to give the proper positioning of inulin at the active center. Further kinetic studies and the determination of the crystal structure of the Afi-Endo will contribute to better understanding of the roles of Trp17 and Glu20 in the active center.

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