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Asparagine 42 of the conserved endo-inulinase INU2 motif WMNDPN from *Aspergillus ficuum* plays a role in activity specificity

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ABSTRACT

Endo-inulinase INU2 from *Aspergillus ficuum* belongs to glycosidase hydrolase family 32 (GH32) that degrades inulin into fructo oligosaccharides consisting mainly of inulotriose and inulotetraose. The 3D structure of INU2 was recently obtained (Pouyez et al., 2012, Biochimie, 94, 2423–2430). An enlarged cavity compared to exo-inulinase formed by the conserved motif W-M(I)-N-D(E)-P-N-G, the so-called loop 1 and the loop 4, was identified. In the present study we have characterized the importance of 12 residues situated around the enlarged cavity. These residues were mutated by site-directed mutagenesis. Comparative activity analysis was done by plate, spectrophotometric and thin-layer chromatography assay. Most of the mutants were less active than the wild-type enzyme. Most interestingly, mutant N42G differed in the size distribution of the FOS synthesized.

1. Introduction

Inulin, a storage carbohydrate in plants such as chicory, Jerusalem artichoke and dahlia, is composed of linear chains of ß-(2-1) linked fructose residues terminated by a glucose residue. It can be hydrolysed by endo-inulinases in short-chain fructoinulooligosaccaharides (FOS). Over recent years, different applications of inulin have sparked great interest for the production of ethanol, fructose syrup or inulooligosaccharides (IOS) used as prebiotics in food industries such as those producing baked goods, milk desserts and chocolate [1,2]. The positive effects of IOS on human health have been widely described [3]. Major IOS obtained after inulin hydrolysis with endo-inulinase show a degree of polymerization of 3 or 4.

Inulinases belong to glycoside hydrolase family 32 (GH32) and can be divided into exo- and endo-inulinases. Exo-inulinases (EC 3.2.1.80) cleave the terminal fructose whereas endo-inulinases (EC 3.2.1.7) hydrolyse inulin by endocleavage, producing fructo oligosaccharides consisting mainly of inulotriose and inulotetraose [4,5]. This means that these enzymes hydrolyse inulin between the third and fourth residues. Classically, random attack of inulin by endo-inulinase yielded principally inulotriose (F3) and inulotetraose (F4) in equal amounts.

In the GH32 family, three acidic residues, an aspartate or a glutamate in the WMN(D/E)PN motif, an aspartate in the RDP motif and E in the ECP motif have been identified as the essential residues for enzyme activity based on mutational and/or crystallographic data [6–8]. They are respectively a catalytic nucleophile, a transition state stabilizer and a general acid/base catalyst [7].

The difference in the mechanism of action between exo- and endo-inulinases is thought to be due to a larger pocket in the endo-enzymes [9]. Recently, we described the 3D structure of endo-inulinase INU2 from *Aspergillus ficuum* and identified an enlarged cavity compared to exo-inulinase formed by the conserved motif W-M(I)-N-D(E)-P-N-G, the so-called loop 1 and loop 4. These two loops among the four identified are conserved among all the endo-inulinases with known amino acid sequence. Docking studies of the substrate-like kestopentaose revealed five subsites and their constitutive residues [10]. Based on these recent results, we investigated the importance of 12 residues, located around the catalytic pocket, on the activity and specificity of INU2 from *A. ficuum*. The effect of each mutation on the enzyme activity was characterized. Most affected the enzyme activity, while one in particular changed the size distribution of the FOS synthesized.

2. Materials and methods

2.1. Strains, plasmids, and culture conditions

Escherichia coli DH10B (Gibco BRL) was used as the host strain for plasmid amplification. *Pichia pastoris* X-33 (Invitrogen, Leek, Netherlands) was used for the expression of recombinant endo-inulinases.

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Table 1
Oligonucleotides employed for mutagenesis. Forward (F) and reverse (R) sequences are shown with the mutations in bold letters.

Mutant	Orientation	Sequence
Inu M41A	F	ccggaccaatattgg**gcg**aacgagccaaacggcc
	R	ggccgtttggctcgtt**cgc**ccaatattggtccgg
Inu N42G	F	gaccagtactggatg**gga**gagccaaacggcctg
	R	caggccgtttggctc**tcc**catccagtactggtc
Inu E43D	F	ccggaccagtattggatgaac**gat**ccaaacggcctg
	R	caggccgtttgg**atc**gttcatccaatactggtccgg
Inu Q59A	F	cctggcacctgttctt**tgc**gcacaatccgacggcc
	R	ggccgtcggattgtgc**gca**aagaacaggtgccagg
Inu P62G	F	Ttctttcaacacaat**ggc**acggccaatgtatggggcaatatttgctgggg
	R	Ccccagcaaatattgccccatacattggccgt**gcc**attgtgttgaaagaa
Inu W67A	F	ccgacggccaatgta**gcg**ggcaatatatgctgggggc
	R	gcccccagcatatattgcc**cgc**tacattggccgtcgg
Inu I70A	F	ccaatgtatggggcaac**gca**tgctgggggcacgctacg
	R	cgtagcgtgccccccagca**tgc**gttgccccatacattgg
Inu F99A	F	ggatgagaacggagtcgaagcg**gct**accggtaccgcc
	R	ggcggtaccggt**agc**cgcttcgactccgttctcatcc
Inu R175A	F	cgggcggccttgagagt**gcg**gatccaaaggtattcttcc
	R	ggaagaatacctttggatc**cgc**actctcaaggccgcccg
Inu N265A	F	ggatcccctgccggtggt**gcc**ggggtgctagctatcaccgg
	R	ccggtgatagctagcacccc**ggc**accaccggcaggggatcc
Inu R295A	F	ggctggacaatgggg**gct**gatttcgatggagctctgagc
	R	gctcagagctccatcgaaatc**agc**cccattgtccagcc
Inu D298A	F	ggacaatgggcgtgatttc**gct**ggagctctgagctggg
	R	cccagctcagagctcc**agc**gaaatcacgcccattgtcc

The plasmid used in this study was pPICZαA [11] for expression in *P. pastoris*.

E. coli strains were grown at 37 °C in low-salt Luria–Bertani (LB) medium (DIFCO) containing 100 μg/ml ampicillin for selection of recombinant clones.

P. pastoris was grown in flasks shaken at 30 °C in buffered YEPS medium containing 1% yeast extract, 2% peptone, and 1% sorbitol. The transformants were selected on the appropriate medium containing 25 μg/ml zeocin. Recombinant cultures of *P. pastoris* were grown in flasks at 30 °C in BMGY and BMMY media containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate at pH 6.0, 1.34% YNB, 4×10^{-5} % biotin and 1% glycerol or 0.5% methanol.

2.2. Recombinant DNA techniques

Standard recombinant DNA techniques (preparation and transformation of competent *E. coli* cells, DNA cloning, restriction enzymes digestion, ligation) were performed according to published procedures [12].

2.3. Site-directed mutagenesis

All mutations were performed using the "quickchange® site-directed mutagenesis kit" (Stratagene). The mutagenic primers used to produce the desired gene alteration based on the induced amino acid alterations (mutated bases shown in bold) are shown in Table 1. Beckman Coulter Genomics performed the sequencing.

A molecular model of the N42G mutant was built from the X-ray structure of the wild-type enzyme (PDB: 3SC7). The EsyPred3D program was used [13].

2.4. Expression of recombinant and wild-type enzymes

P. pastoris X-33 cells were transformed by electroporation with 10 μg of *Sac*I-linearized plasmid DNA carrying wild-type or mutated endo-inulinase encoding genes. Freshly transformed cells were plated onto solid YEPS medium containing 25 μg/ml zeocin. Positive transformants were checked for the endo-inulinase gene by PCR analysis. Recombinant cultures were grown in flasks (shaking at 150 rpm) at 30 °C in 10 ml of BMGY medium for 1 day, reaching A600 = 2.0–6.0 (approximately 16–18 h). The cells were harvested and resuspended to an A600 of 1.0 in 50 ml of BMMY medium. Methanol (100% v/v) was added to the cultures to a final concentration of 0.5% (v/v) every 24 h to maintain induction. Proteins were recovered from the culture supernatants by centrifugation at 4000 rpm for 20 min and then filtered on 0.22 μMillipore filters (Millipore).

2.5. Inulinase purification

After filtration, supernatant was dialyzed at 4 °C in 10 mM phosphate buffer at pH 7.5. The resulting enzyme was applied to a DEAE sepharose CL-6B column pre-equilibrated with the same buffer. Endo-inulinase was eluted with a linear gradient of NaCl from 0 to 500 mM in the same buffer. The protein was then dialyzed against a 50 mM sodium acetate buffer (pH 5.0) in order to keep the protein in its optimal conditions. The purity of the protein was checked on SDS–PAGE stained with Coomassie Blue.

2.6. Enzymatic determination

2.6.1. Plate assay

An inulin-agar plate was prepared with 20 mM phosphate buffer (pH6) containing 4% inulin. Equal amounts of proteins from the *P. pastoris* supernatant were spotted onto the inulin-agar plate and incubated at 50 °C for 12 h.

2.6.2. Spectrophotometric assays

Inulinase activity was assayed by measuring the amount of reducing sugars released from inulin using Somogy–Nelson's method [21]. The reaction mixture was composed of 60 μl of suitably diluted protein, 440 μl of 4% inulin from a solution of dahlia tubers (Sigma Chemical Co.) in 50 mM phosphate buffer at pH 6. The reaction was carried out for 10 min at 50 °C. Inulinase activity was determined spectrophotometrically by recording the increase in optical density (OD) at 520 nm.