J. Microbiol. Biotechnol. (2000). **10**(2), 238–243

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Cloning and Characterization of *Pseudomonas mucidolens* Exoinulinase

KWON, YOUNG-MAN, HWA-YOUNG KIM, AND YONG-JIN CHOI*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Received: December 27, 1999

Abstract An exoinulinase (β-D-fructofuranosidase) gene was cloned by chromosome walking along the upstream region of the endoinulinase gene of *Pseudomonas mucidolens* isolated from soil. The exoinulinase gene consisted of an ORF of 1,506 bp encoding a polypeptide of 501 amino acids with a deduced molecular weight of 55,000. The exoinulinase produced by the recombinant *Escherichia coli* DH5α strain was also purified to homogeneity as determined by SDS-PAGE and a zymogram. The molecular weight of the purified exoinulinase according to both SDS-PAGE and gel filtration matched the deduced molecular weight of the protein described above, thereby indicating that the native form of the exoinulinase was a monomer. The purified enzyme hydrolyzed sucrose, raffinose, levan, in addition to inulin, with an S/I activity value of 2.0. Furthermore, no inulo-oligomers were liberated from the inulin substrate in the enzymatic reaction mixtures incubated for 90 min at 55°C. Taken together, these results indicate that the purified β-D-fructofuranosidase was an exoinulinase. The pH and temperature optima of the exoinulinase were pH 6.0 and 55°C, respectively. The enzyme had no apparent requirement for a cofactor, and its activity was completely inactivated by Ag^{+}, Hg^{2+}, and Zn^{2+}. Kinetic experiments gave K_m, V_{max}, and K_{cat} values for inulin of 11.5 mM, 18 nM/s, and 72 s^{-1}, respectively. The exoinulinase was fairly stable in broad pH conditions (pH 5–9), and at pH 6.0 it showed a residual activity of about 70% after 4 h incubation at 55°C.

Key words: Exoinulinase, inulin, *Pseudomonas mucidolens*, β-D-fructofuranosidase

Inulin is a nonstructural storage carbohydrate synthesized in various higher plants such as chicory, dahlia, and Jerusalem artichoke. It consists of linear chains of β-2,1-linked D-fructofuranose terminating in a glucose residue. Recently, there has been much interest in the production of natural and functional sweeteners through the enzymatic hydrolysis of inulin [15]. Enzymes that degrade inulin were first isolated from plants, however, most inulinases are now obtained from microorganisms such as *Kluyveromyces marxianus* [8], *Aspergillus ficuum* [4], and *Bacillus subtilis* [11]. Inulinase can be divided into two types according to the mode of action on inulin: the first, endoinulinase (EC 3.2.1.7; β-D-fructan fructanohydrolase), produces inulo-oligosaccharides from inulin and the second, exoinulinase (EC 3.2.1.26; β-D-fructofuranosidase), liberates fructose from the fructosyl terminal of inulin.

In previous work, we isolated a soil bacterium, *Pseudomonas mucidolens*, which had the ability to produce an endoinulinase [9]. The Pseudomonas strain was observed to grow well on a medium supplemented with inulin as the sole carbon source, thus implying that the bacterium should include an exoinulinase which is capable of hydrolyzing the inulo-oligomers produced by the action of endoinulinase and subsequently metabolizing the resulting monosaccharide as a carbon and energy source. Nevertheless, all previous efforts have failed to detect any exoinulinase activity in *P. mucidolens*.

In this work, an exoinulinase gene (*inu2*) from the *P. mucidolens* genome was identified and cloned by chromosome walking along the 5' upstream region of the endoinulinase gene (*inu1*) [3]. In addition to this, the exoinulinase was purified and characterized from a cell extract of *Escherichia coli* carrying the *inu2* gene. Biochemical studies on characteristics of the purified *inu2* gene product identified the enzyme as an exoinulinase.

MATERIALS AND METHODS

Strains and Growth Conditions

P. mucidolens, isolated from soil, was used as the source of the *inu2* gene, and grown at 45°C on a Luria-Bertani medium containing 1% inulin with agitation. *E. coli* DH5 [*sup*E44, △*lac*U169 (ϕ80 *lac*Z△M15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1] was used as the host strain for the plasmid preparation and for the exoinulinase production from the cloned *inu2* gene. Plasmid Bluescript

*Corresponding author
Phone: 82-2-3290-3417; Fax: 82-2-923-9923;
E-mail: choiyj@kuccnx.korea.ac.kr

KS (Stragene, U.S.A.) was used for the construction of the *P. mucidolens* genomic library.

DNA Manipulations

The genomic DNA was prepared using the method of Doi [2]. The plasmid preparation, DNA ligation, transformation, and agarose gel electrophoresis were performed according to the standard methods [14]. The Southern hybridization was performed essentially as described previously [3]. Dig-labeled DNA probes were prepared based on the Dig random primer method using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Germany), and then used as the hybridization probes for the Southern blot analysis and colony hybridization.

Nucleotide Sequencing

The nucleotide sequence of the *inu2* gene subcloned in the plasmid pBluescriptKS(+) was determined using the dideoxy chain termination method with an Applied Biosystems model 301 automated DNA sequencer (Perkin Elmer Co., U.S.A.). The nucleotide and deduced amino acid sequences were analyzed with the DNASIS and PC/GENE software programs.

The determined nucleotide sequence of the *P. mucidolens inu2* gene was assigned GenBank assession no. AF129819.

Protein and Enzyme Assays

The protein concentration was determined either by the method of Lowry [10] using bovine serum albumin as the standard or by measuring the absorbance at 280 nm. Unless otherwise noted, all enzymatic assays were carried out in a 50 mM citrate-phosphate buffer (pH 6.0) with 2% dissolved dahlia inulin (Sigma; average molecular weight, 5,400), 1% dissolved levan (Sigma; average molecular weight, 1×10^7), or 2% sucrose as the substrate. After the addition of the enzyme solution, the reaction mixture was incubated at 55°C for 20 min. The liberation of free fructose (and/or glucose) was determined using the DNS method [12]. One unit of exoinulinase or levanase activity was defined as the amount of enzyme required to liberate 1 μmol of fructose per min, and one unit of invertase was defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmol of sucrose per min. The S/I value of exoinulinase was calculated as the ratio of the total units of invertase activity to the total units of exoinulinase activity. The products of the enzymic reaction were also determined by HPLC, as described previously [7].

Enzymatic Properties

For determining the optimum pH, the exoinulinase activity was measured by incubating the enzyme with 2% inulin in either 50 mM citrate-phosphate buffers (pH 3.0 to 6.0) or 50 mM sodium phosphate buffers (pH 6.0 to 8.0) at 55°C for 20 min. The effect of the temperature on the activity of the exoinulinase was examined at temperatures ranging from 40 to 70°C. To measure the thermal stability of the exoinulinase, the enzyme solution was incubated at various temperatures, as indicated above, and samples were taken at every hour. The residual activity after the incubation was then assayed.

Purification of the Exoinulinase

Ammonium sulfate precipitation. The crude enzyme extract obtained by sonication of the *E. coli* DH5α/pEXI cells was fractionated with 30–55% ammonium sulfate. The pellet obtained by centrifugation (6,000 ×g, 30 min) was then dissolved in a small volume of 50 mM citrate-phosphate buffer (pH 6.0) and the resulting protein solution was desalted by dialyzing overnight at 4°C against 25 mM concentration of the same buffer.

DEAE-Sepharose CL-6B chromatography. i) NaCl gradient; The dialyzed protein solution (63 ml) was loaded on a Sepharose CL-6B anion-exchange column preequilibrated with 50 mM sodium phosphate buffer (pH 7.0). After washing the column with 500 ml of the same buffer supplemented with 0.1 M NaCl, proteins were eluted with a linear gradient of 0.1 to 0.3 M NaCl in 50 mM sodium phosphate buffer (pH 7.0).

ii) pH step gradient; The fractions exhibiting exoinulinase activity were pooled, dialyzed overnight at 4°C against 25 mM sodium phosphate buffer (pH 7.0), and loaded on a Sepharose CL-6B anion-exchange column preequilibrated with 50 mM sodium phosphate buffer (pH 7.0). Proteins were eluted step-wise with 50 mM sodium phosphate buffer from pH 7.0 to 6.0.

Sephacryl S-200 chromatography. The fractions exhibiting exoinulinase activity eluted from the above DEAE-Sepharose CL-6B chromatography were pooled, concentrated with Centricon (3,000 cutoff, Amersham, U.K.), and loaded on a Sephacryl S-200 HR column preequilibrated with 50 mM citrate-phosphate buffer (pH 6.0). Three fractions showing exoinulinase activity were then pooled, concentrated, and loaded onto a second Sephacryl S-200 HR column.

N-Terminal Amino Acid Sequencing

The N-terminal amino acid sequence of the purified exoinulinase was determined using a 476A gas-phase protein sequencer (Applied Biosystems Inc., U.S.A.).

RESULTS

Cloning and Sequencing of Exoinulinase Gene

In our previous work, we isolated a gene encoding an endoinulinase (*inu1*) from the *P. mucidolens* genomic DNA and cloned this *inu1* gene into *E. coli* [3]. Accordingly, this seemed to indicate that the Pseudomonas strain might also contain a gene encoding an exoinulinase. Nevertheless, no

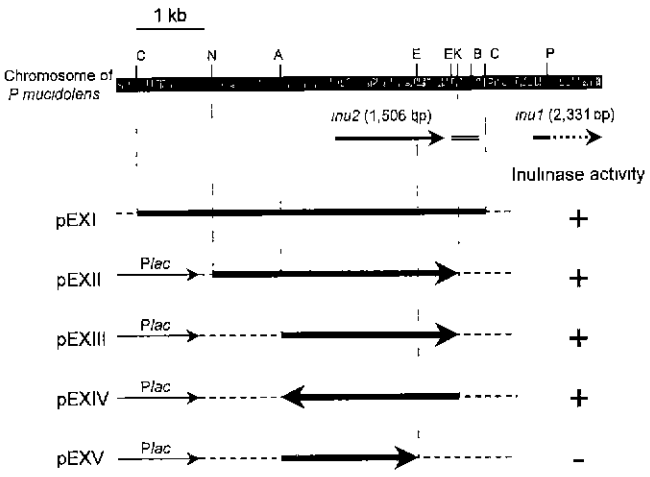

Fig. 1. Linear restriction map of plasmid pEXI containing the 4.3 kb *Cla*I DNA fragment and its derivatives.

The inulinase activity of the *E. coli* recombinants carrying the various constructs was assayed as described in Materials and Methods. The *Eco*RI-*Bam*HI fragment probe for the Southern blot is lined as (=). The thin lines represent the vector sequences. A, *Apa*LI; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; P, *Pvu*II.

Fig. 2. Nucleotide and deduced amino acid sequences of the *P. mucidolens inu2* gene.

The adenine-rich region and CATC sequence are boxed. The putative Shine-Dalgarno (SD) and N-terminal amino acid sequence of the purified exoinulinase are underlined. The sequences of the *Eco*RI-*Bam*HI fragment for the Southern blot probe are represented in italics

exoinulinase activity has been detected from the bacterial cells so far. However, in the course of sequence analysis of the *inu1* gene, a region containing a conserved sequence (Box D in Fig. 3) for β-fructofuranosidases was discovered at ~1.3 kb upstream of the *inu1* gene (Fig. 1).

To identify the whole gene for the potential β-D-fructofuranosidase, chromosome walking along the upstream region of the *inu1* gene was performed using a 300 bp Dig-labeled *Eco*RI-*Bam*HI fragment containing the discovered conserved sequence as the probe. As shown in Fig. 1, a 4.3 kb *Cla*I fragment was hybridized by the probe, isolated, and then cloned into pBluescriptKS(+), resulting in the recombinant plasmid pEXI.

The *E. coli* DH5α strain transformed with pEXI produced an enzyme capable of hydrolyzing sucrose, levan, and inulin. Surprisingly, however, it was found that the inulin hydrolyzing activity of the cloned gene (named *inu2*) was in the 2.5 kb *Apa*LI-*Kpn*I subcloned fragment located upstream of the probe sequence. There was no further investigation of the function(s) of the ~1.3 kb DNA between the *inu1* and *inu2* genes. As illustrated in Fig. 1, plasmids pEXIII and pEXIV carry the same 2.5 kb *Apa*LI-*Kpn*I fragment, yet in the opposite direction relative to the pBluescript *lac* promoter. Nevertheless, both plasmids exhibited the same level of inulin hydrolyzing activity on the respective *E. coli* transformants, thereby indicating that the *inu2* gene on the *Apa*LI-*Kpn*I fragment was expressed by its own promoter.

The 3.2 kb *Nco*I-*Kpn*I insert in the plasmid pEXII was used to determine nucleotide sequence of the *inu2* gene (Fig. 2). The *inu2* gene consisted of an ORF of 1,506 bp encoding a 501 amino acid polypeptide. The deduced molecular weight and pI value of the polypeptide were calculated to be 55,797 and 5.04, respectively. The calculated molecular weight matched the size of the enzyme as determined by SDS-PAGE (Fig. 4). The translation start site (ATG) was located at position 1,451 in the sequence, as shown in Fig. 2. This was also confirmed by the N-terminal amino acid sequencing of the purified *inu2* gene product (MDKVY). A SD-like sequence (GAAGG) was identified at positions 1,435–1,440, 10 bp upstream of the translation initiation site. Furthermore, a CATC sequence was also found at positions 1,377–1,381 which is known to be a common promoter for some chromosomally-encoded Pseudomonas genes [13]. A multiple sequence alignment with *B. subtilis* levanase and *K. marxianus* inulinase revealed a considerable homology between them, showing a 56.3% identity with *B. subtilis* levanase [11], 30.7% with *K. marxianus* inulinase [8], and 27.4% with the endoinulinase of *P. mucidolens* (unpublished data). Six highly conserved blocks of inulin, levan, and sucrose hydrolyzing enzymes were also observed in the *inu2* gene product (Fig. 3). Among the conserved sequences, Box A (WMNDPNG) containing the proposed active site residue of Asp and Box E (ECP) containing the Cys residue have previously been reported as the critical components of the enzyme's catalytic domain. This was supported by chemical modification experiments with the purified *inu2* gene product. Among the chemicals examined, only the Cys-specific modifiers (*p*-hydroxymercury-benzoate and *N*-bromosuccinimide) and an Asp-specific chemical

Fig. 3. Comparison of amino acid sequence of exoinulinase with other β-D-fructofuranosidases.

The conserved regions of the β-fructosidase motif identified by Gunasekaran *et al.* [6] are boxed. The identical amino acids are indicated by asterisks. As for the sequence of *B. subtilis* levanase, only the first 528 amino acids are shown. PMINU2, *P. mucidolens* exoinulinase, BSLEV, *B. subtilis* levanase (accession no. A27286): KMEXI, *K. marxianus* inulinase (accession no CAA40488).

(Woodward's reagent K) inactivated the enzyme activity, resulting in residual activities of less than 15% of the activity shown in the absence of the modifiers (data not shown).

Purification of Exoinulinase

The cloned *inu2* gene was constitutively expressed and the synthesized enzyme was mostly accumulated in the cytoplasm of the *E. coli* DH5α/pEXI cells. The highest activity (35–40 U/mg of protein) was attained when the cells were grown at 37°C for 18 h in a M9 medium supplemented with 1.5% inulin, 1.25% peptone, and 100 μg/ml ampicillin.

The purification of the exoinulinase started from the cell extract obtained from the culture as described above and

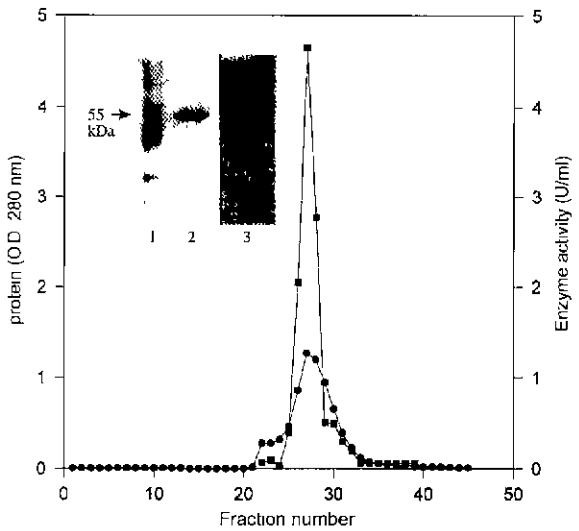

Fig. 4. Second gel filtration of exoinulinase on Sephacryl S-200HR.

The Sephacryl S-200HR was equilibrated with a 50 mM sodium phosphate buffer (pH 6.0). The elution was performed with the same buffer at a flow rate of 7.5 ml/h. A 9% SDS-PAGE and zymogram of the purified exoinulinase are indicated in the figure. The zymography was performed as described previously [3]. Lane 1, Molecular weight markers (97.4, 66.2, 55.0, 42.7, 40.0, 31.0, 21.5 kDa): lane 2, purified exoinulinase, lane 3, zymogram band of purified exoinulinase. ●, protein concentration; ■, enzyme activity.

the result is summarized in Table 1. The final second Sephacryl S-200 chromatography resulted in an apparently pure enzyme preparation, giving a single protein band on SDS-PAGE gel visualized using Coomassie brilliant blue R-250 staining (Fig. 4).

The molecular mass of the enzyme was estimated to be 55 kDa by both SDS-PAGE and gel filtration using Sephacryl S-200HR, thereby indicating that the enzyme has a monomeric structure (data not shown).

Enzyme Properties

The substrate specificity of the purified enzyme, described in Table 2, suggested that the enzyme could be classified as a β-D-fructofuranosidase: the purified enzyme hydrolyzed sucrose and raffinose as well as inulin. Therefore, to further determine whether the enzyme acted on a fructan substrate endo- or exo-hydrolytically, inulin was hydrolyzed

Table 1. Summary of purification of exoinulinase.

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)	Purification fold
Cytoplasmic extract	65,430	1,768	37	100.0	1
Ammonium sulfate fractionation	48,158	499	97	73.6	3
DEAE-Sepharose CL-6B (pH 7.0, NaCl 0.1–0.3 M)	8,025	4.8	1,672	12.3	45
DEAE-Sepharose CL-6B (pH 7.0–6.0)	2,045	1.2	1,704	3.1	46
Sephacryl S-200 1st	583	0.10	5,830	0.9	157
Sephacryl S-200 2nd	376	0.04	9,400	0.6	254

Table 2. Substrate specificity of exoinulinase.

Substrate (2%, w/v)	Relative activity (%)
Dahlia inulin	100
Chicory inulin	80
Jerusalem artichoke inulin	57
Levan	15
Melizotise	0
Raffinose	93
Sucrose	188

under optimal conditions using the purified enzyme, and the hydrolyzed products were then analyzed by HPLC. As shown in Fig. 5, fructose appeared from the very beginning, and was the only sugar released during the entire period of the enzymic reaction. Liberation of any inulo-oligomers from the inulin substrate was not detected from the enzymatic reaction mixtures incubated for 90 min at 55°C. This demonstrated that the enzyme attacked the polymers of D-fructose exohydrolytically. Moreover, the S/I ratio of the enzyme was calculated to be about 2 (see Table 2). Thus, it appears that the enzyme of *P. mucidolens* is a true exoinulinase. Interestingly, the exoinulinase showed significantly different activities toward inulin substrates, exhibiting the highest activity with dahlia inulin and the lowest with Jerusalem artichoke inulin (Table 2).

It should also be noted that the purified exoinulinase did not have any fructosyltransferase activity, a common side action of many β-fructofuranosidases in high sucrose concentrations and at low reaction temperatures (data not shown) [5].

The exoinulinase showed a broad pH-activity profile with an optimum pH of 6.0. At the optimum pH, the enzyme was found to be most active at 55°C and fairly stable up to 50°C, yet after 4 h of incubation at 55°C the exoinulinase became slightly inactivated, showing a residual activity of 70% (data not shown). Metal ions such as Ag^{+}, Cu^{2+}, Fe^{3+}, and Hg^{2+} almost completely inhibited the enzyme activity at a concentration of 5 mM, and no metal

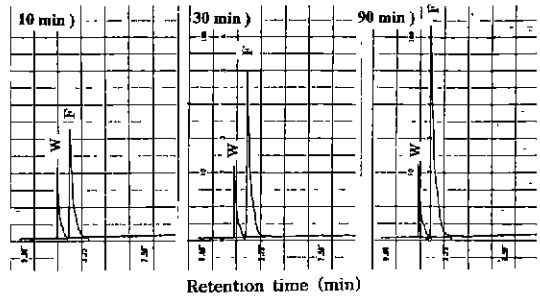

Fig. 5. HPLC analysis of exoinulinase action.
Inulin (2%, w/v) was used as the substrate. The enzyme reaction was carried out at 50°C for 10, 30, and 90 min, respectively. Only fructose was released during the whole course of the enzymic reaction. F, fructose; W, water.

Table 3. Kinetic parameters of exoinulinase.

Substrate	K_m (mM)	V_{max} (nM/s)	K_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Inulin	11.5	18	72	6.3
Sucrose	24.4	10	40	1.6

The molecular weight of inulin was assumed to be 5 400.

ion was found to increase the exoinulinase activity (data not shown).

Kinetic Characterization

The apparent K_m and V_{max} values for inulin and sucrose were calculated from double-reciprocal plots. As shown in Table 3, the higher affinity of the exoinulinase for inulin than for sucrose is further evidence to indicate that the enzyme is an exo-acting enzyme. From the data, the turnover number (K_{cat}) and specificity constants (K_{cat}/K_m) for each substrate were calculated, and the kinetic parameters obtained are summarized in Table 3.

Discussion

In the previous work, we isolated a bacterium from soil that produced high levels of endoinulinase [9], and identified the isolate as a strain of *P. mucidolens*. Subsequently, the endoinulinase gene (*inu1*) was isolated and cloned in *E. coli* [3].

However, no exo-acting inulinase activity was ever detected from the Pseudomonas strain. Currently, the reason for this failure to detect any exoinulinase activity can not be clearly explained. However, during the sequence analysis of the above *inu1* gene, a common conserved sequence for genes for β-D-fructofuranosidases was accidentally identified in the upstream region of the *inu1* gene (see Fig. 1). Accordingly, in the present work, an exoinulinase gene (*inu2*) was cloned by the chromosomal walking method and expressed in *E. coli* DH5α. The two inulinase genes, *inu1* and *inu2*, were oriented in the same direction on *P. mucidolens* genomic DNA. However, they both had their own promoters and were separated by a space of about 1.3 kb where another unidentified ORF was detected.

As mentioned above, the *inu2* gene was confirmed as being expressed in *E. coli*, using its own promoter. Nevertheless, in the 5' noncoding region of the *inu2* gene, there was no recognizable sequence homologous to the promoters of enteric bacteria [1]. However, at positions 1,377–1,381, a CATC sequence was found which is a common promoter to some chromosomally-encoded Pseudomonas genes [13], and may, therefore, provide the minimal requirement for participation of RNA polymerase. It should also be noted that a common adenine-rich run

was located upstream of the CATC sequence although its relationship to the promoter activity was not investigated in the context of the *inu2* sequence.

A standard method to discriminate between the specificity of β(2-1) hydrolyzing β-fructofuranosidases for high molecular weight substrates versus the low molecular weight substrates is to determine the ratio of invertase to inulinase activity (S/I values). S/I values for invertases are relatively high numbers ranging from 1,600 to 28,300. Conversely, S/I values of true inulinase are low numbers; typically, S/I values between 1.5 and 20 are reported for most inulinases from microorganisms [15]. The S/I value for the INU2 enzyme of *P. mucidolens* was determined to be 2, thereby indicating that this enzyme was more selective of inulin than sucrose. In addition, the enzyme's higher specificity for inulin was also reflected by the ratio of the specificity constants for inulin and sucrose (α ratio) of 3.9. The α values of invertases or sucrases are less than one [4]. The INU2 enzyme of *P. mucidolens* was, therefore, classified as an exoinulinase.

The complete inactivation of the exoinulinase caused by 5 mM Ag^{+} or Hg^{2+} was indicative that the SH group might be essential for its catalytic activity. In fact, one of the four Cys residues (C205) residing in the conserved E box, indicated in Fig. 3, might be the active site of this enzyme. This conclusion was further supported by the chemical modification experiment in which treatment with *p*-hydroxymercurybenzoate, a Cys-specific modifier, totally inactivated the exoinulinase activity (data not shown). Most of the kinetic parameters of the present exoinulinase were found to fall within the ranges previously reported [15].

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