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Note

Purification and Characterization of α-Mannosidase from Onion, *Allium cepa*

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Abstract: α-Mannosidase (ALMAN) extracted from onion (*Allium cepa*) was purified by column chromatography such as hydrophobic and gel filtration. ALMAN is an acidic α-mannosidase that exhibits maximum activity against *p*NP-α-Man at pH 4.0–5.0 at 50°C. Amino acid sequence analysis of ALMAN was consistent with α-mannosidase deduced from *Allium cepa* transcriptome analysis. The gene *alman* was amplified by PCR using mRNA extracted from onions, and a full-length gene of 3,054 bp encoding a protein of 1,018 amino acid residues was revealed. ALMAN is classified as Glycoside Hydrolase Family (GH) 38 and showed homology with other plant-derived α-mannosidases such as tomato and hot pepper.

Key words: α-Mannosidase, onion, *Allium cepa*

Plant α-mannosidases (EC 3.2.1.24) have been well characterized to date in several vegetables, such as bean, ginkgo, and tomato [1−3]. Genetic suppression of α-mannosidase in tomatoes and hot peppers is known to cause delayed ripening, suggesting an important role for this enzyme in plant physiology [4,5]. In our preliminary survey of vegetable glycosidases, we observed that α-mannosidase activity was particularly high in bulb vegetables such as onion and garlic. Vegetables are classified into climacteric types, which undergo additional ripening using ethylene after harvest, and non-climacteric types, which do not. Tomatoes are climacteric while the onions are non-climacteric. It has also been reported that onions have unique physiology such as ethylene suppressing sprout growth [6]. Regarding the source of enzyme isolation, plant GH38 α-mannosidases have been reported from seeds or fruits [1−5], while the nature and genes of the enzyme in bulbs are unknown. Onion (*Allium cepa*) is a popular bulb vegetable, but the enzymatic properties of α-mannosidase were unknown, and the gene had not been identified. In this report *Allium cepa* α-mannosidase (ALMAN) was characterized and the gene cloned.

Japanese yellow onion of the cultivar "Sapporo-ki" was peeled and homogenized by a cooking mixer in an equal volume (g/mL) of chilled water containing protease inhibitors (1 mM PMSF, 1.5 μM pepstatin and 14 μM E-64). The homogenate was filtered through nonwoven paper and the filtrate was centrifuged. Proteins in the supernatants were precipitated by adding ammonium sulfate of 70 % saturation followed by centrifugation for 20 min at 8,000 rpm. The pellets were dissolved in 10 mM acetate buffer (pH 6.0) containing 1.5 M ammonium sulfate and the protease inhibitors, then applied to a column (2.5 × 18 cm) of Butyl Toyopearl 650M that was equilibrated in the same buffer. Proteins were eluted in a decreasing gradient of ammonium sulfate (1.5 to 0 M) in the buffer. Effluents containing the enzyme were pooled, concentrated by a centrifugal ultrafiltration tube (cut by 30 kDa, Macrosep®, Pall Co. USA, Port Washington, NY USA), then applied to a column (1.8 × 95 cm) of Toyopearl HW55F that was ran in the buffer containing 0.1 M NaCl. Eluate containing ALMAN was concentrated and used for further experiments.

Protein concentration was determined by a method developed by Bradford using bovine serum albumin as a standard [7]. PAGE in non-denaturing and denaturing conditions were performed according to the methods described by Davis and Laemmli, respectively [8, 9].

The activity of ALMAN was measured by incubating the enzyme and 0.8 mM *p*NP-α-Man (Fujifilm Wako Pure Chem. Co., Japan) in total 100 μl of 20 mM sodium acetate buffer (pH 4.0) at 40℃ for certain period. The reaction was stopped by adding 100 μ L of 1 M Na_2CO_3 then absorption at 405 nm was measured. Hydrolysis of mannobiose was determined according to the methods described in our previous paper [10].

Protein of ALMAN was reduced and carbamidomethylated in the gel after PAGE, then digested with trypsin. Internal amino acid sequences of the tryptic peptides were identified by LC-MS/MS (Triple TOF 6600, AB Sciex, Japan) with ProteinPilot Software (AB Sciex) for data processing. Since onion α-mannosidase was not found in existing protein databases, amino acid sequence of ALMAN

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Abbreviations: CBB, Coomassie brilliant blue; DDBJ, DNA Data Bank of Japan; E-64, *N*-[*N*-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl]-agmatine; LC-MS/MS, liquid chromatography triple quadrupole mass spectrometry; Man, D-mannose; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; *p*NP, *p*-nitrophenyl; SDS, sodium dodecyl sulfate; SWATH：sequential window acquisition of all theoretical fragment ion spectra; TLC, thin-layer chromatography.

Table 1. Purification of ALMAN.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	664	338	0.5	100	1
$(NH_4)_2SO_4$ precipitation	85.8	88.8	1.0	26	2
Butyl Toyopearl	7.3	57.1	7.8	16	15
Toyopearl HW55F	0.4	21.0	47.7	6	94

One unit (U) was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min.

was determined by informatics works with the aid of *Allium cepa* transcriptome analysis (Bioproject Accession: PRJNA246669) [11]. By using the Prosite of ExPASy (http://prosite.expasy.org/) the protein function was predicted from the amino acid sequence.

Onion RNA was extracted from an onion bulb by using RNAzol RT (Molecular Research Center, USA) and cDNA prepared by a reverse transcriptase. Gene *alman* was amplified by PCR using a set of primers AC-01 (TCCATATGGCGACTACTACTTATCCTC) and AC-02 (AGCTCGAGAGGAACGTCTACTTCAAG) those contained *Nde* I and *Xho* I restriction sites respectively (underlined), with the onion cDNA as the template. The PCR product was inserted to T vector pMD20 (Takara Bio Inc., Shiga, Japan) after adding dA and introduced to *E. coli* DH5α. A plasmid DNA obtained from the transformants were used for sequencing.

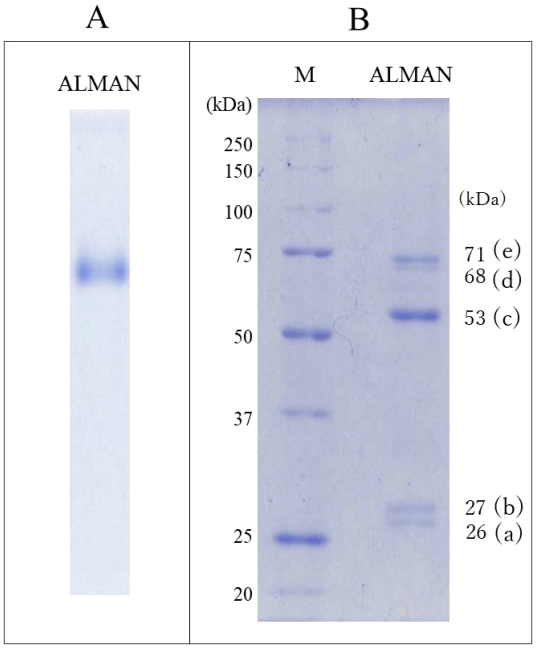

Fig. 1. PAGE of ALMAN in non-denatured (A) and SDS-denatured (B) conditions.
Gels were stained with CBB.

ALMAN was purified by two steps of column chromatography (Table 1, Fig. S1: see J. Appl. Glycosci. Web site). It showed a single band on PAGE in non-denaturing condition, however five protein bands (71, 68, 53, 27, and 26 kDa) were observed on SDS-PAGE (Fig. 1). This suggests that there were proteolytic cleavages of the original protein. In tomato and chili pepper α-mannosidase, two polypeptides of approximately 70 and 50 kDa were reported [5,12]. In purification of ALMAN the low-molecular-weight polypeptides did not pass through a 30 K molecular filtration filter in their native state and were recovered on the high-molecular side, indicating that they are associated with the totality of α-mannosidase.

After ALMAN was subjected to SDS-PAGE, each of the five protein samples was subjected to amino acid sequencing by LC-MS/MS. As a result, all five samples contained a partial amino acid sequence of the same gene product, α-mannosidase (*Allium cepa* Transcriptome ID: Locus_2733.4) [11] (Table S1). Comprehensive detection and quantification of all compounds present in a sample (MS/MSALL) by SWATH Acquisition of proteins excised from undenatured PAGE gels revealed that the relative value of α-mannosidase (Transcriptome ID: Locus_2733.4) was the highest (as 100 %) among the onion transcriptome, and another sequence (Locus_50048.1) with the next highest value (27 %). The latter locus was thought to be the C-terminal sequence lacking in Locus_2733.4 because the two loci partially overlapped. There were no other candidates with the relative value of 10 % or more.

The optimum pH of ALMAN was on the acidic side between pH 4.0 and 5.0, and even at pH 2.0 it showed 70 % of its activity. The enzyme activity was highest at 50˚C, while preheating at 60 ˚C for 30 min reduced the activity to less than 50 %, and above 70 ˚C there was no activity. Regarding

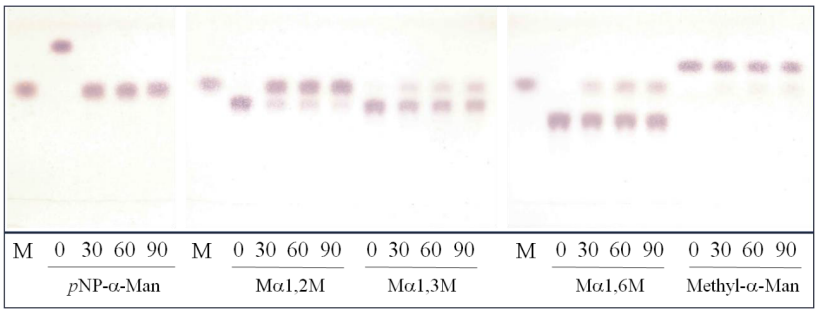

Fig. 2. TLC analysis of ALMAN reaction products.
The enzymes were reacted with 5.8 mM of each substrate in 20 mM acetate buffer (pH 4.0) at 40 ˚C for the times indicated. Sugars were developed in a solvent containing water, 1-butanol, and 2-propanol (4:3:12). M; α-D-Man as a standard. Mannobiose is shown as MM with its linkage.

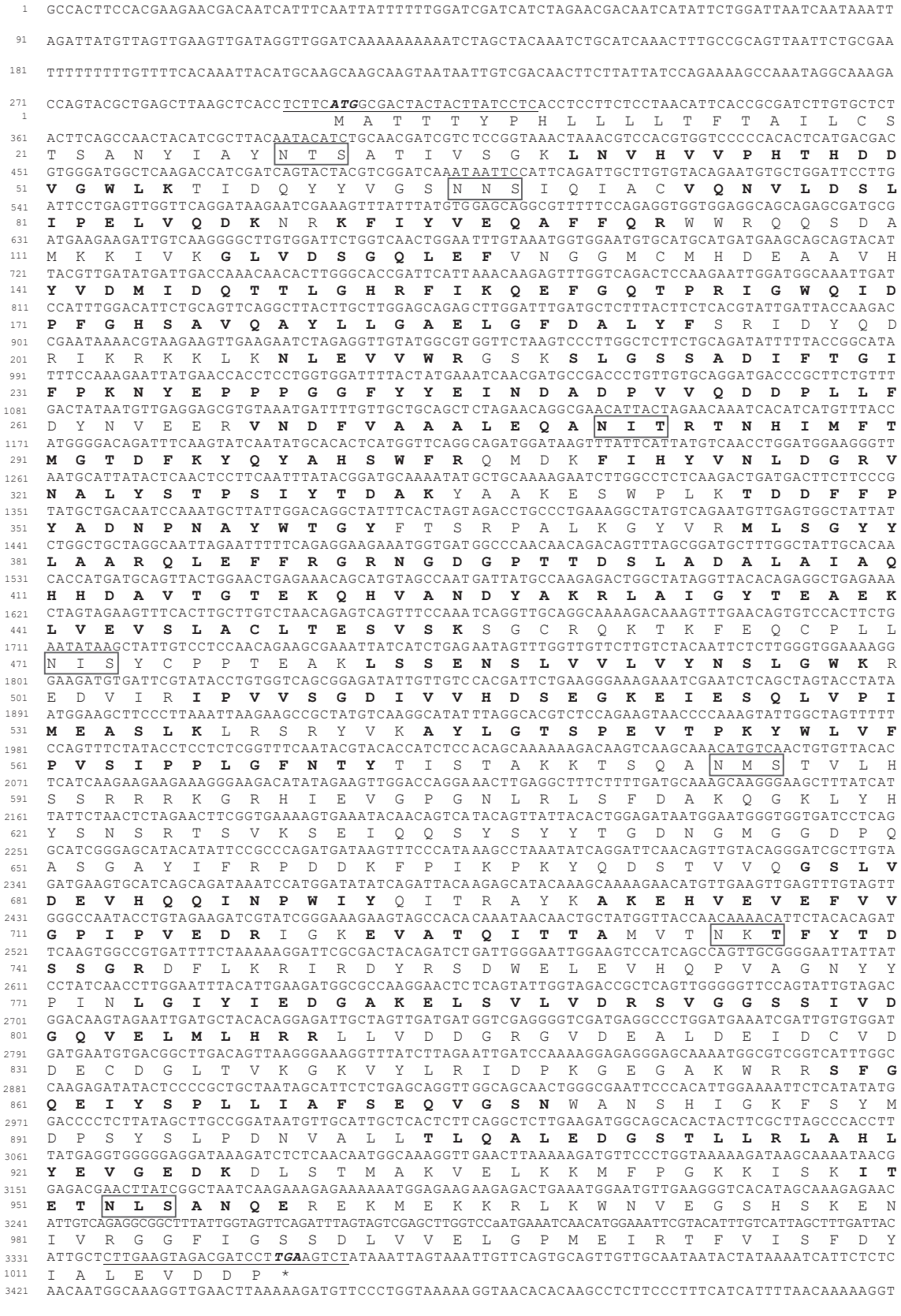

Fig. 3. Nucleotide sequence of *Allium cepa* α-mannosidase (*alman*).

Amino acids residues identified by LC-MS/MS are shown in bold. The putative N-glycosylation sites are boxed. Horizontal bars indicate the positions of PCR primers. Initiation and termination of the ORF are shown in italics.

substrate specificity, ALMAN showed the highest activity towards *p*NP-α-Man with K_m 1.69 mM and V_{max} 8.2×10^{-2} μM/min, which was comparable to that of *Capsicum* α-Man [5]. When mannobioses (Sigma-Aldrich Co., USA) were the substrate, the activity was shown in the order of α1-2 (3.3 %), α1-3 (0.8 %), and α1-6 (0.2 %) (numbers indicate relative activity to *p*NP-α-Man) (Fig. 2). Transglycosylation was not observed with mannobiose substrates. Activity towards methyl α-mannoside was negligible. ALMAN was inhibited by swainsonine with IC_{50} of 0.2 μM, while 1-deoxymannojirimycin, an inhibitor of 1,2-α-mannosidase [13], did not show effective inhibition.

PCR primers were designed based on the sequences of these two loci, and PCR was performed using mRNA extracted from onions as a template, resulting in a gene containing a 3,054 bp ORF (Fig. 3). All the amino acid sequences in Table S1 were identified in the ORF. The gene *alman* (DDBJ accession number LC779624) encoded a protein consisting of 1,018 amino acid residues with an estimated molecular mass of approximately 116 kDa. ALMAN is classified as Glycoside Hydrolase Family (GH) 38 and showed 64, 56, 58, and 65% identity with α-mannosidase from tomato [4], bean [14], rice [15], and chili pepper [5], respectively (Fig. S2: see J. Appl. Glycosci. Web site). The presence of mannose-related glycans or naturally occurring α-mannosides in onion is unknown, and the results obtained in this study will be used to investigate the relationship between ALMAN and onion growth in future studies.

CONFLICTS OF INTERESTS

The authors declare no conflict of interests.

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