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Identification and Characterization of a Novel β-Galactosidase from *Victivallis vadensis* ATCC BAA-548, an Anaerobic Fecal Bacterium

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Victivallis vadensis **ATCC BAA-548 is a Gram-negative, anaerobic bacterium that was isolated from a human fecal sample. From the genomic sequence of *V. vadensis*, one gene was found to encode agarase; however, its enzymatic properties have never been characterized. The gene encoding the putative agarase (NCBI reference number ZP_01923925) was cloned by PCR and expressed in *E. coli* Rosetta-gami by using the inducible T_7 promoter of pET28a(+). The expressed protein with a 6×His tag at the N-terminus was named His_6-VadG925 and purified as a soluble protein by Ni^{2+}-NTA agarose affinity column chromatography. The purification of the enzyme was 26.8-fold, with a yield of 73.2% and a specific activity of 1.02 U/mg of protein. The purified His_6-VadG925 produced a single band with an approximate MW of 155 kDa, which is consistent with the calculated value (154,660 Da) including the 6×His tag. Although VadG925 and many of its homologs were annotated as agarases, it did not hydrolyze agarose. Instead, purified His_6-VadG925 hydrolyzed an artificial chromogenic substrate, *p*-nitrophenyl-β-D-galactopyranoside, but not *p*-nitrophenyl-α-D-galactopyranoside. The optimum pH and temperature for this β-galactosidase activity were pH 7.0 and 40°C, respectively. The K_m and V_{max} of His_6-VadG925 towards *p*-nitrophenyl-β-D-galactopyranoside were 1.69 mg/ml (0.0056 M) and 30.3 U/mg, respectively. His_6-VadG925 efficiently hydrolyzed lactose into glucose and galactose, which was demonstrated by TLC and mass spectroscopy. These results clearly demonstrated that VadG925 is a novel β-galactosidase that can hydrolyze lactose, which is unusual because of its low homology to validated β-galactosidases.**

Keywords: *Victivallis vadensis*, ZP_01923925, VadG925, β-galactosidase, lactose hydrolysis

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Introduction

Oligo- and polysaccharides containing D-galactose linked by a β-glycosidic bond are present in most organisms. Therefore, the corresponding glycosidases including β-galactosidase are as widely distributed as their substrates. β-Galactosidases (EC 3.2.1.23), a large family of proteins, have been widely studied and are known to catalyze both hydrolytic and transgalactosylation reactions. Their hydrolytic activity has been utilized in the food industry for decades to lessen the lactose content in milk. It is estimated that approximately 70% of the adult world population is not able to digest lactose because of the lack of β-galactosidase in the small intestine. Therefore, the absorption of milk and milk products causes symptoms such as diarrhea and flatulence (Stellmach, 1988). The role of intestinal β-galactosidase in the hydrolysis and consequently in the absorption of dietary lactose is well known (Wallenfels and Weil, 1972). In this sense, the pharmaceutical application of β-galactosidase to convert lactose into glucose and galactose to avoid these symptoms is indispensible.

The transgalactosylation activity of β-galactosidase has also been spot-lighted in recent years to synthesize pharmaceutically important galactooligosaccharides (GOSs) (Albayrak and Yang, 2002; Pocedičová *et al*., 2010), such as lactulose (Adamczak *et al*., 2009) and lactosucrose (Li *et al*., 2009). In these aspects, the classical β-galactosidases have become more important in bioindustries and are extracted most often from microorganisms, particularly from the fungi *Aspergillus* and *Penicillium* (Nagy *et al*., 2001; Haider and Husain, 2007), the yeast *Kluyveromyces* (Kim *et al*., 2003), the bacteria *Bacillus* and *Bifidobacterium* (Trân *et al*., 1998; Møller *et al*., 2001), and the archaea *Sulfolobus* and *Pyrococcus* (Petzelbauer *et al*., 2000).

We have been studying the agar-degrading enzymes termed agarases from many bacterial sources (Temuujin *et al*., 2011). Agar is a polysaccharide found in the cell walls of some red algae and consists of 2 different components, agarose and agaropectin. Agarose consists of a linear chain of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactose (Duckworth and Yaphe, 1972). Therefore, agarases are classified into 2 groups based on their mode of action, namely, α-agarase (EC 3.2.1.158) and β-agarase (EC 3.2.1.81), which hydrolyze α-1,3 linkages and β-1,4 linkages in agarose, respectively (Araki, 1959). Contrasting to β-agarase found in several taxonomically diverse genera (Allouch *et al*., 2003), α-agarase has been reported in only 2 microorganisms: *Alteromonas agarilytica* (Potin *et al*., 1993) and *Thalassomonas* sp. (Ohta *et al*., 2005). Because of the rarity of α-agarase, we surveyed the GenBank database

and found one candidate annotated as a putative α-agarase (ZP_01923925) reported from the genomic sequencing of *Victivallis vadensis* ATCC BAA-548, an anaerobic bacterium (van Passel *et al*., 2011). The cloning, expression, and enzymatic characterization of ZP_01923925, which we named VadG925, from *V. vadensis* ATCC BAA-548, is described in this paper.

Materials and Methods

Bacterial strains and plasmids

Victivallis vadensis ATCC BAA-548 was purchased from the American Type Culture Collection, USA. The bacterium is a strictly anaerobic, cellobiose-degrading, Gram-negative bacterium which was isolated from a human fecal sample (Zoetendal *et al*., 2003). *Escherichia coli* strains DH5α and Rosetta-gami and the pT&A and pET28a(+) cloning vectors were purchased commercially.

Materials

Chemicals including *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-α-Gal) and *p*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-Gal) were purchased from Sigma Chemical Co. (USA). Restriction endonucleases, T4-DNA ligase, and Taq DNA polymerase were purchased from TaKaRa Shuzo, Japan. The primers for PCR were obtained from DyneBio Inc., Korea. The T&A cloning vector was purchased from RBC (Taiwan). Silica gel 60 on glass plates was purchased from E. Merck AG (Darmstadt, Germany).

Media and cultivation

For *V. vadensis* ATCC BAA-548, a bicarbonate-buffered medium with the following composition was used (per L): 0.53 g $Na_2HPO_4{\cdot}2H_2O$, 0.41 g KH_2PO_4, 0.3 g NH_4Cl, 0.11 g $CaCl_2{\cdot}2H_2O$, 0.1 g $MgCl_2{\cdot}6H_2O$, 0.3 g NaCl, 1 ml selenite-tungstate solution, 1 ml trace element solution, 1 ml vitamin solution, 0.5 mg resazurin, 4 g $NaHCO_3$, 0.5 g $Na_2S{\cdot}9H_2O$, 0.2 g yeast extract, and 3.2 g cellobiose. The trace elements, selenite-tungstate solution, and vitamins were described in ATCC media #2362. All compounds were heat sterilized, excluding the vitamins and the $Na_2S{\cdot}9H_2O$, $NaHCO_3$, and cellobiose solutions, which were sterilized in the different bottles. The basal and separate media components were mixed anaerobically under 80% N_2 and 20% CO_2. Culture incubations were performed in the serum bottles sealed with butyl rubber stoppers at 37°C with agitation. *E. coli* was usually maintained at 16°C on Luria-Bertani (LB) medium (Sambrook and Russell, 2001) and also cultured at 16°C after induction with 0.03 mM IPTG under slow agitation.

DNA manipulations

The genomic DNA was isolated from the *V. vadensis* ATCC BAA-548 cells by the method of Sambrook and Russell (2001), with modifications as follows: proteinase K treatment and cell lysis, RNAse A digestion, phenol-chloroform extraction, and then ethanol precipitation. DNA preparation and manipulations in *E. coli* were also performed by the methods of Sambrook and Russell (2001). DNA samples were digested with restriction endonucleases and ligated using T4 DNA ligase according to the supplier's recommendations. DNA digests were analyzed by horizontal agarose gel electrophoresis in Tris-acetate-EDTA buffer (Sambrook and Russell, 2001).

Molecular cloning and expression of VadG925

VadG925 (ZP_01923925) is a protein with 1,427 amino acids, and thus a long DNA sequence (3,300 bp) had to be cloned. First, the 1,248-bp N-terminal region of VadG925 was amplified by PCR with the following two synthetic oligonucleotides: sense primer, 5′-GCGGCA*CATATG*CCGGCGAA TCCGCCCG-3′, with an *Nde*I site (italicized) and antisense primer, 5′-GCAGTAC*GGATCC*CGGATCGCCGCGTC-3′, with a *Bam*HI site (italicized), under previously described conditions (Oh *et al*., 2007). Second, the 1,148-bp middle region of VadG925 was amplified by PCR with the following oligonucleotides: sense primer, 5′-GACGCGGCGATCCG *GGATCC*GTACTGC-3′, with a *Bam*HI site (italicized) and antisense primer, 5′-CGCGGCA*CCCGGG*TTGCCGCCGG GCTC-3′, with a *Sma*I site (italicized). Lastly, the 2,042-bp C-terminal region of VadG925 was amplified by PCR with the following oligonucleotides: sense primer, 5′-GAGCCC GGCGGCAA*CCCGGG*TGCCGCG-3′, with a *Sma*I site (italicized) and antisense primer, 5′-CTGCGG*AAGCTT*GCG TCGGTGAAGGCGG-3′, with a *Hin*dIII site (italicized). The PCR products were double digested with appropriate restriction enzymes and then ligated into the pET28a(+) expression vector digested with *Nde*I-*Hin*dIII by 4-fragment ligation. The resulting plasmid, pET28a-VadG925, in which VadG925 could be expressed with 6 His residues in its N-terminus, was transformed into *E. coli* Rosetta-gami.

Purification of His_6-tagged VadG925 (His_6-VadG925)

The *E. coli* transformants were cultured in 100 ml of LB medium supplemented with kanamycin (50 μg/ml) in a 500-ml Erlenmeyer flask at 16°C and 150 rpm to an A_{600} (absorbance at 600 nm) of 0.6. IPTG (0.03 mM) was then added, and the culture was allowed to grow for an additional 24 h at 16°C under slow agitation. Cells were harvested by centrifugation (5,000×*g*, 10 min), resuspended in 5 ml of buffer A (0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 5 mM imidazole, and then disrupted by sonication (3 times for 1 min and 60 W). Crude cellular extract was obtained after centrifugation at 20,000×*g* for 20 min. The cell extract was then loaded onto a Ni^{2+}-NTA agarose affinity column for purification, as described by the supplier (Novagen, purification kit). The column was washed 3 times with 10 ml of buffer A containing 5 mM imidazole. The protein was eluted with 1.0 ml of buffer A containing 250 mM imidazole, and the eluents were then collected in 500 μl fractions. The purified protein was concentrated with an Amicon concentrator (10-kDa cutoff) and resuspended in buffer A to a final concentration of 10 μg/μl.

Protein analysis and electrophoresis

Protein concentration was measured using the Bradford protein microassay kit (Bio-Rad) with bovine serum albumin as

the standard protein (Bradford, 1976). The purified protein was applied to 0.1% sodium dodecyl sulfate (SDS)-6% polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (1970). After electrophoresis, protein bands were detected by staining with Coomassie Brilliant Blue R-250. The molecular masses of standard markers were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (20.1 kDa).

Agarase assay by the dinitrosalicylic acid (DNS) method

The agarase activity was measured under standard conditions as follows: In a glass test tube, 100 µl of enzyme solution was mixed with 3.9 ml of 50 mM sodium phosphate buffer (pH, 7.0) containing 0.2% agarose. After incubation at 40°C for 15 min, the sample was mixed with 4 ml of DNS reagent solution (6.5 g of DNS, 325 ml of 2 M NaOH, and 45 ml of glycerol in 1 L of distilled water) and heated in boiling water for 10 min until the color developed. The reaction was stopped by cooling the test tube in an ice-cold water bath. The absorbance at 540 nm (A_{540}) was then recorded (Zhang and Sun, 2007).

Determination of α- and β-galactosidase activity by chromogenic substrates

The α- and β-galactosidase hydrolytic activity was determined by measuring the release of *p*-NP from *p*NP-α-Gal and *p*NP-β-Gal at 420 nm, respectively. The reaction mixture consisting of 500 µl of reaction buffer (100 mM potassium phosphate; pH 7.0, 10 mM KCl, 1 mM $MgSO_4$) and 200 µl of *p*NP-β-Gal or *p*NP-α-Gal (4 mg/ml) as the artificial chromogenic substrate was prewarmed at 40°C for 5 min, rapidly mixed with 500 µl of enzyme solution, and incubated at 40°C for 15 min. The reaction was stopped by adding 500 µl of stop solution (1 M Na_2CO_3), and A_{420} was recorded. One unit of enzyme was defined as the amount that produced an A_{420} of 0.001 after a 15-min incubation.

Effects of temperature and pH on the β-galactosidase activity

The effects of temperature and pH on the activity of the protein were determined under the standard assay conditions. The effect of pH on β-galactosidase activity was assayed by replacing sodium phosphate buffer (pH 7.0) with 50 mM each of the following buffers: sodium citrate buffer (pH 4.0–5.0), standard assay buffer (pH 7.0), sodium phosphate buffer (pH 6.0–8.0), and glycine-NaOH buffer (pH 9.0–11.0) at 40°C. The effect of temperature on β-galactosidase activity was determined by performing the enzyme reaction at various temperatures (4°C, 20–70°C) at pH 7.0. The thermal stability of β-galactosidase was determined by preincubating the enzyme solution at the indicated temperature (4°C, 20–70°C) at pH 7.0 for 1 h and then measuring the residual enzyme activity. The relative activity was defined as the percentage of activity determined with respect to the maximum β-galactosidase activity.

Estimation of kinetic parameters

To estimate kinetic parameters, the enzymatic assay was performed at substrate (*p*NP-β-Gal) concentrations ranging from 1 to 8 mg/ml. The rest of the procedure was performed as described in the enzyme assay section. K_m and V_{max} values were calculated from the Lineweaver-Burk plot. The reaction time of the assay was sufficiently short (2 min) to limit substrate utilizations below 5% (Segel, 1976).

Enzymatic product analysis

For the complete hydrolysis of lactose, the reaction mixture contained 20 µl of enzyme, 40 µl of 20 mM Tris-Cl; pH, 7.0, and 40 µl of 1% (w/v) substrate and was incubated at 40°C for 24 h. The hydrolyzed product was analyzed by Silica Gel 60 TLC plate. The reaction products were developed with *n*-butanol-acetic acid-water (2:1:1). The sugars on the plate were detected by heating at 140°C for 5 min after spraying with 10% sulfuric acid. The molecular mass distribution of the products was determined using a direct matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer (Autoflex III; Bruker, USA).

Results and Discussion

In silico analysis of VadG925 (ZP_01923925)

V. vadensis ATCC BAA-548 is a Gram-negative, anaerobic bacterium that was isolated from a human fecal sample by combining enrichments in liquid and soft-agar basal media (Zoetendal *et al.*, 2003). From the entire genomic sequence of *V. vadensis*, one candidate annotated as an agarase gene was identified. The gene product ZP_01923925 was composed of 1,427 amino acids, and its molecular weight was calculated as 159.7 kDa from the deduced amino acid sequence. It was originally annotated as a putative α-agarase with a conserved pfam07488 motif between Arg-710 and Leu-791. The pfam07488 motif has been found at the N-terminus of glycosyl hydrolase family 67 α-glucuronidases, components of an ensemble of enzymes central to the recycling of photosynthetic biomass that act by removing the α-1,2-linked 4-*O*-methyl glucuronic acid from xylans. Recently, the whole-genome sequence record was removed because it had been superceded by a new assembly of the genome, and then ZP_01923925 was annotated as an endo-β-agarase with the NCBI reference sequence ZP_01923925.1. For convenience, we named the protein VadG925.

Table 1. Protein purification table for His_6-VadG925 from *E. coli*/pET28a-VadG925

Purification procedure	Volume (ml)	Total protein (mg)	Specific activity (unit/mg)	Total activity (unit)	Purification fold	Yield (%)
Whole cell	200	220	0.038	8.36	1	100
Cell-free extract	6	26.82	0.25	6.71	6.6	80.2
Ni^{2+}-agarose affinity chromatography	6	6	1.02	6.12	26.8	73.2

One unit of enzyme was defined as that amount that produced an A_{420} of 0.001 after a 15-min incubation.

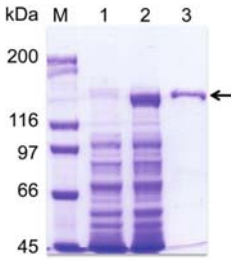


Fig. 1. Confirmation of the overexpression and purification of VadG925 by SDS-PAGE analysis. The soluble cell-free crude extract prepared from of *E. coli*/pET28a-VadG925 was used to purify His_6-VadG925 by Ni^{2+}-NTA agarose affinity column chromatography, and then the protein samples were separated by 6% SDS-PAGE. Lanes: M, molecular weight standards; 1 and 2, total protein of *E. coli*/pET28a-VadG925 before and after induction with IPTG, respectively; 3, purified His_6-VadG925. The His_6-VadG925 protein is indicated by a thick arrow.

Gene cloning and purification of VadG925

From the amino acid sequence, VadG925 was supposed to be cleaved between Ala-21 and Ala-22; thus, the gene was cloned to be translated from the 22nd Ala into pET28a(+) under the T_7 promoter, and then the recombinant plasmid was introduced into *E. coli* strain Rosetta-gami. The VadG925 was purified as a soluble protein from cell-free crude extract with a 6×His tag at its N-terminus (His_6-VadG925) by Ni^{2+}-NTA agarose affinity column chromatography. The purification of the enzyme was 26.8-fold, with a yield of 73.2% and a specific activity of 1.02 U/mg (Table 1). The purified enzyme exhibited a single band with a molecular mass of approximately 155 kDa (Fig. 1), which is consistent with the calculated value of 154,660 Da based on its 1,406 amino acid residues plus 6 His residues.

Enzymatic property of VadG925

The purified His_6-VadG925 was used for its ability to hydrolyze the artificial chromogenic substrates *p*NP-α-Gal and *p*NP-β-Gal. The protein eluted by imidazole from the Ni^{2+}-NTA agarose column could actively hydrolyze *p*NP-β-Gal, but the cell-free extract prepared from the *E. coli*/pET28a control did not exhibit any detectable activity (Fig. 2A). Additionally, His_6-VadG925 did not hydrolyze *p*NP-α-Gal (data not shown), indicating that it recognizes and acts specifically on the β-glycosidic bond of galactose.

Because VadG925 was annotated as an agarase, the agarase activity of His_6-VadG925was measured using agarose as the substrate. Although VadG925 and many homologs were annotated as agarases, no agarase activity was detected from

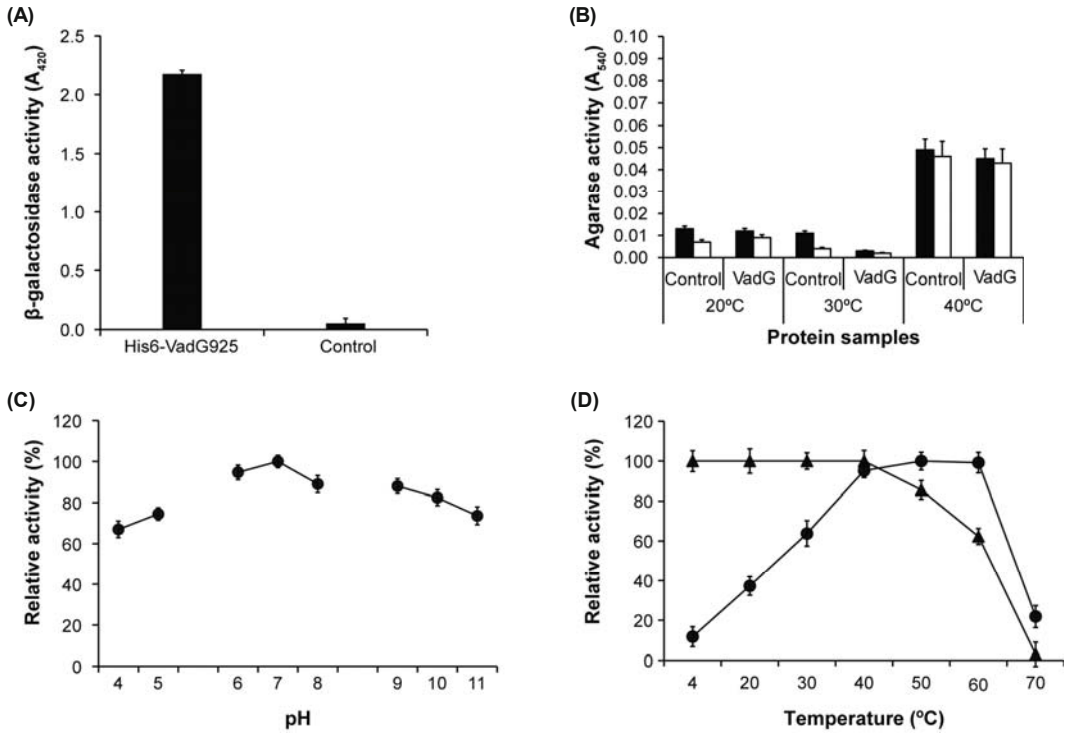


Fig. 2. Determination of enzymatic property of the His_6-VadG925. (A) The α- and β-galactosidase hydrolytic activities of His_6-VadG925 were determined by measuring the release of *p*-NP from *p*NP-α-Gal and *p*NP-β-Gal as the substrates at 420 nm, respectively. Because the activity toward *p*NP-α-Gal was not detectable, only the activity toward *p*NP-β-Gal is presented. The soluble cell-free crude extract of *E. coli*/pET28a was used as the control. (B) The agarase activity of the His_6-VadG925 at various temperatures was measured under standard conditions as described previously. A_{540} was recorded, and no difference was observed between the reaction with the protein sample of *E.coli*/pET28a and the purified His_6-VadG925. Control; the soluble cell-free crude extract of *E. coli*/pET28a, VadG: the purified His_6-VadG925 protein. (C) The β-galactosidase activity of the purified His_6-VadG925 was measured with the artificial chromogenic substrate *p*NP-β-Gal (4 mg/ml) at various pH values ranging from 4.0 to 11.0 at 40°C. (D) The β-galactosidase activity (●) of the purified His_6-VadG925 was also measured at various temperatures ranging from 4°C to 70°C in 50 mM artificial substrate buffer at pH 7.0. The thermal stability (▲) of the β-galactosidase was determined by preincubating the enzyme solution at the indicated temperature for 1 h and then measuring the residual enzyme activity. The highest agarase activity was considered as 100% when calculating the relative activities. All data shown are mean values from at least three replicate experiments.

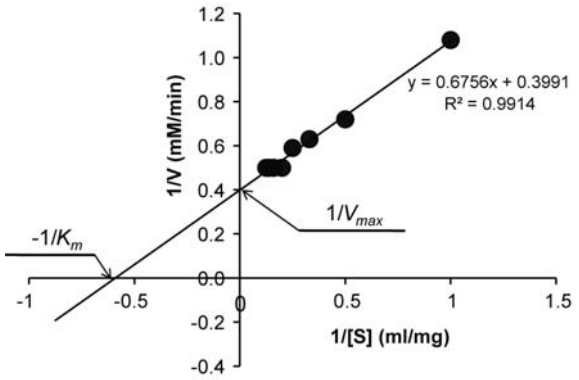


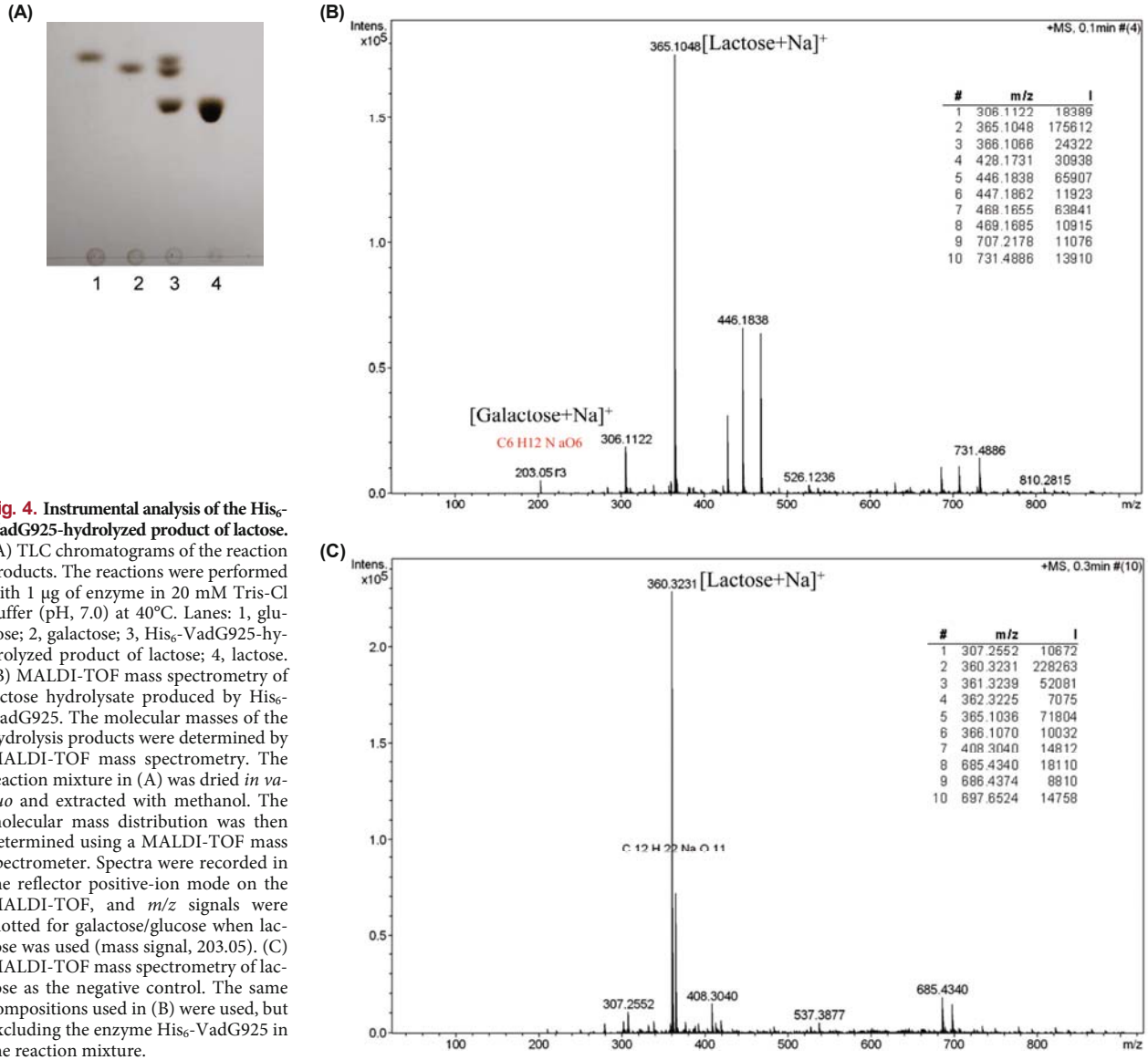
Fig. 3. **Lineweaver–Burk plots to determine the kinetic parameters of His_6-VadG925 β-galactosidase acting on the artificial substrate *p*NP-β-Gal.**

His_6-VadG925, clearly indicating that VadG925 is not an agarase, but probably a β-galactosidase (Fig. 2B).

Effects of temperature and pH on β-galactosidase activity of VadG925

The β-galactosidase activity of His_6-VadG925 towards *p*NP-β-Gal was measured at various pH values at 40°C for 15 min. As shown by the pH profile of β-galactosidase in Fig. 2C, the enzyme exhibited maximal activity at pH 7.0, and the enzyme was active over the wide pH range of 6.0–10.0.

The optimum temperature for β-galactosidase activity was investigated (Fig. 2D). Within the range of the tested temperatures, the enzyme exhibited maximal activity at 50°C and retained 95% and 99% of its maximum activity at 40°C and 60°C, respectively. The stability of His_6-VadG925 against heat treatment is presented in Fig. 2D. The enzyme was



#	m/z	I
1	306.1122	18389
2	365.1048	175612
3	366.1066	24322
4	428.1731	30938
5	446.1838	65907
6	447.1862	11923
7	468.1655	63841
8	469.1685	10915
9	707.2178	11076
10	731.4886	13910

#	m/z	I
1	307.2552	10672
2	360.3231	228263
3	361.3239	52081
4	362.3225	7075
5	365.1036	71804
6	366.1070	10032
7	408.3040	14812
8	685.4340	18110
9	686.4374	8810
10	697.6524	14758

Fig. 4. **Instrumental analysis of the His_6-VadG925-hydrolyzed product of lactose.** (A) TLC chromatograms of the reaction products. The reactions were performed with 1 μg of enzyme in 20 mM Tris-Cl buffer (pH, 7.0) at 40°C. Lanes: 1, glucose; 2, galactose; 3, His_6-VadG925-hydrolyzed product of lactose; 4, lactose. (B) MALDI-TOF mass spectrometry of lactose hydrolysate produced by His_6-VadG925. The molecular masses of the hydrolysis products were determined by MALDI-TOF mass spectrometry. The reaction mixture in (A) was dried *in vacuo* and extracted with methanol. The molecular mass distribution was then determined using a MALDI-TOF mass spectrometer. Spectra were recorded in the reflector positive-ion mode on the MALDI-TOF, and *m/z* signals were plotted for galactose/glucose when lactose was used (mass signal, 203.05). (C) MALDI-TOF mass spectrometry of lactose as the negative control. The same compositions used in (B) were used, but excluding the enzyme His_6-VadG925 in the reaction mixture.

stable against heat treatment for 60 min until 40°C; however, it retained 85% and 62% of its maximum activity at 50°C and 60°C, respectively. Combining those results, we performed all enzyme reactions for VadG925 at 40°C and pH 7.0.

Enzyme kinetics

Based on the results obtained above, an enzyme reaction was performed in artificial substrate buffer (pH, 7.0) at 40°C. The K_m and V_{max} values for the artificial chromogenic substrate were 0.0056 M (1.69 mg/ml) and 30.3 U/mg, respectively (Fig. 3).

Lactose hydrolysis by the purified VadG925

Because we confirmed that VadG925 has β-galactosidase but not agarase activity, we tested its enzymatic activity towards lactose as the natural substrate. When the reaction mixture was separated on TLC, 3 spots with identical R_f values to those of lactose, galactose, and glucose (Fig. 4A) were identified. MALDI-TOF mass spectra revealed that the major product had molecular ions at an m/z of 203.05, corresponding to the sodium adduct of galactose/glucose $[M+Na]^+$ (Fig. 4B). The negative control without enzyme was also analyzed by MALDI-TOF (Fig. 4C), and only molecular ions at an m/z of 360.32, corresponding to the sodium adduct of lactose $[M+Na]^+$, were found. These results clearly demonstrate that VadG925 is a β-galactosidase that can hydrolyze lactose into glucose and galactose. Therefore, we conclude that VadG925 is a novel β-galactosidase that recognizes D-galactose linked by a β-glycosidic bond and thus hydrolyzes the β-bond of lactose.

When we surveyed the homologs of VadG925 in the GenBank database by the BlastP program, the distribution of 101 Blast hits on the 1st page of the search result revealed all of the sequences had significant homology to VadG925 with E-values lower than $6e^{-41}$, and most were annotated as agarases. However, only the AgaB protein among the 101 hits was functionally validated as an agarase from *Vibrio* sp. strain JT0107 (Sugano *et al.*, 1994). Actually, VadG925 exhibited 32% amino acid identity with AgaB, with an E-value of $7e^{-70}$. It has also 32% of identity with the NP_627690 which is annotated as a putative β-galactosidase belonging to glycosidic hydrolase family 42 in *Streptomyces coelicolor* (Bentley *et al.*, 2002). Therefore, the possibility that the homologs revealed in the BlastP search are types of agarase appears to be very low even though most are annotated as agarases.

In recent years, the isolation and characterization of novel β-galactosidases have become more frequent. Thus, a number of agarases and β-galactosidases have been reported, and the 3D structures of some of these enzymes have been studied. Bga is the β-galactosidase from *Arthrobacter* sp. C2-2, and its 3D structure was reported (Skálová *et al.*, 2005). However, VadG925 has only 10.52% and 18.29% amino acid identity and similarity with Bga, respectively. In addition, the catalytic residue of Bga, Glu-448, was not conserved (data not shown). LacA is another β-galactosidase from *Caldicellulosiruptor saccharolyticus* that has a known 3D structure (Park and Oh, 2010). VadG925 has 8.55% and 11.4% amino acid identity and similarity with LacA, respectively, and the catalytic residues (Glu-562 and Glu-929) were not conserved (data not shown). *In silico* analyses also revealed that VadG925 exhibited approximately 10% identity with the other functionally validated β-galactosidases. From these observations and results, we conclude that VadG925 is a novel β-galactosidase that has a distinct amino acid sequence.

According to recent reports, the K_m values of LacA β-galactosidase from *Bacillus licheniformis* DSM 13 (Juajun *et al.*, 2011) and a novel heterodimeric β-galactosidase from *Lactobacillus pentosus* KUB-ST10-1 (Maischberger *et al.*, 2010) toward *o*-nitrophenyl-β-D-galactopyranoside were 13.7 mM and 38 mM, respectively. Although the experimental conditions are not exactly the same, His_6-VadG925 showed a significantly lower K_m value (5.6 mM) then those by the above-mentioned enzymes, implying it is a good candidate for industrial application. We expect that VadG925 will be able to expand the range of potential applications of β-galactosidases in industrial processes.

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