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Construction and characterization of chimeric enzymes of the *Agrobacterium tumefaciens* and *Thermotoga maritima* β-glucosidases

K. Goyal, Y.-K. Kim, M. Kitaoka, K. Hayashi*

Enzyme Laboratory, National Food Research Institute, Tsukuba, Ibaraki 305-8642, Japan

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Abstract

The β-glucosidases of *Agrobacterium tumefaciens* and *Thermotoga maritima* display quite distinct characteristics, both in terms of their pH optima, temperature optima and substrate specificities. Despite such differences, the encoded enzymes show homologies of approximately 45 and 37% in the amino acid sequences of the N- and C-terminal regions, respectively. Based on the amino acid alignment of these two enzymes, three chimeric β-glucosidase genes were constructed by shuffling selected DNA regions. The chimeric genes were expressed in *Escherichia coli* BL 21(DE3) and two catalytically active enzymes were obtained. Unlike other chimeras which display profiles intermediate to those of their respective parents, these chimeric enzymes displayed quite different profiles. The observed thermal stability of the more catalytically active chimeric enzyme was lower than that observed for either parental enzyme, however, the kinetic parameters were more similar to those of the *T. maritima* β-glucosidase.

Keywords: Chimeric; Gene shuffling; *Thermotoga maritima*; β-Glucosidase; Thermostable; *Agrobacterium tumefaciens*

1. Introduction

Enzymes catalyze a rich variety of metabolic transformations, and as such, these catalysts also play a significant role in industry. However, the industrial application of these biocatalysts is dependent on the development of novel enzymes with desirable activities and properties [1,2]. In recent years, protein engineering has become an increasingly important tool in the development of novel hybrid enzymes with useful catalytic functions [3–8]. The construction of chimeric enzymes has proven to be one of the most sensitive methods employed in the study of structure and function relationships in the parent proteins [9–15]. In addition, the construction of chimeric enzymes has facilitated progress towards the production of enzymes with improved catalytic activities and thermal stabilities. Several enzymes with improved properties and thermal stabilities have already been produced by gene shuffling experiments and these enzymes have also proved useful in mechanistic studies [16–22].

Using this strategy, we focused on the construction of chimeric β-glucosidases of *Agrobacterium tumefaciens* and *Thermotoga maritima*. β-Glucosidases of *A. tumefaciens* and *T. maritima*, which belong to the family three hydrolases, have already been characterized in our laboratory [23,24]. The β-glucosidases of *A. tumefaciens* (cbg1) and *T. maritima* (bglb) have 45% homology in the N-terminal region and 37% homology in the C-terminal region, in terms of their

* Corresponding author. Tel.: +81-298-38-8071; fax: +81-298-38-7321.
E-mail address: khayashi@nfri.affrc.go.jp (K. Hayashi).

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amino acid sequences. Despite these similarities, the β-glucosidases of *A. tumefaciens* and *T. maritima* display quite distinct characteristics.

Based on the amino acid alignment of the *A. tumefaciens* and *T. maritima* β-glucosidases, three shuffling sites were selected for the construction of chimeric enzymes. In order to investigate the respective contributions of the N- and C-terminal domains to the catalytic activities and thermal stabilities of both β-glucosidases, structure comparisons were made with other known family three glycosyl hydrolases [25].

2. Materials and methods

2.1. Bacterial strains and plasmids

The genomic DNA of *T. maritima* strain MSB8 was obtained from Prof. Dr. Karl O. Stetter of Regensburg University, Germany. The plasmid coding β-glucosidase of *A. tumefaciens* strain B3/73 was kindly supplied by Dr. L.A. Castle of Missouri University, USA. *Escherichia coli* DH5α F^{-}ϕ80d *lac*ZΔM15 *rec* A1 *end* A1 *gyr* A96 *thi*-1 *hsd* R17 ($r_k^- m_k^+$) *sup* E44 *rel* A1 *deo*R Δ(*lac* ZYA-*arg*F) U169 and *E. coli* BL21(DE3) F^{-} *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) were employed as the hosts for the β-glucosidase genes from *A. tumefaciens* and *T. maritima*, respectively. *Pcbg1* and *pbglb* were the recombinant plasmids used to carry the *A. tumefaciens* (*cbg1*) and *T. maritima* (*bglB*) genes, respectively.

2.2. Construction of the chimeric enzymes

Three gene shuffling sites were selected for the construction of chimeric β-glucosidases. The construction of chimeric enzymes was carried out using a three-step polymerase chain reaction (PCR) targeted at the homologous regions of the respective genes as previously reported [21]. KOD-plus DNA polymerase of high fidelity (TOYOBO Biochemicals, Osaka, Japan) was used in the PCR reactions with a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA).

The first PCR steps amplified the selected N- and C-terminal domains of *A. tumefaciens* and *T. maritima*. The denaturation and annealing steps were performed at 98°C for 1 min and 55°C for 1 min, respectively and primer extension was carried out at 68°C, repeating the sequence for 20 cycles. The amplified PCR products were separated by agarose gel electrophoresis and purified using a QIAquick gel extraction Kit (QIAGEN, Germany). The DNA sequences of primers for constructing three chimeric genes are listed in Table 1.

The purified product was used as template DNA for the second stage-overlapping PCR, without any primers present. In this stage, strands having similar base pairs overlapped and acted as primers for each other.

In the third PCR, the combined fragment was amplified using forward and reverse primers. The same set of nos. 1 and 8 primers listed in Table 1 is used to amplify each three chimeric genes. The conditions used for denaturation, annealing and primer extension were identical to the first PCR.

2.3. Cloning and DNA sequencing

The amplified PCR products were cloned into a *pCR-XL-TOPO* vector using the TOPO XL PCR Cloning Kit and TOP10 cells [F^{-} *mcr*A Δ(*mrr-hsd* RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*AI *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (str^R)

Table 1
DNA sequences of used primers[a]

Primer no.	DNA sequence
1	GCT AGC ATG ATC GAC GAT ATT
2	TCA TCA CGA AGC CGT CGA AGC CCC ATT C
3	CTT CGA CGG CTT CGT GAT GAG CGA CTG GTA
4	TCT TTC CCA CGA GAA CGT CCG CAA GCG C
5	GGA CGT TCT CGT GGG AAA GAT TAA TCC CTC C
6	AGA CTT CCT TTC CCG CCC TGT CGC CTA T
7	CAG GGC GGG AAA GGA AGT CTC ACA GGT CTA C
8	AAG CTT TCA TGG TTT GAA TCT C

[a] DNA sequences coding for the β-glucosidase of *T. maritima* are underlined. Annealing positions of the primers are illustrated in Fig. 2.

*end*A1 *nup*G] (Invitrogen, Carlsbad, CA, USA) [21,24]. The recombinant plasmids were purified using a QIAminiprep Kit (QIAGEN, Germany). The inserts were then sequenced using a Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems, CA, USA) with an automated DNA sequencer (ABI PRISM^{TM} 310 Genetic Analyser, Perkin-Elmer, Applied Biosystems, Foster City, CA).

2.4. Recombinant gene expression

In order to express the recombinant chimeric genes as shown in Fig. 2, the N- and C-terminal regions of *pAt217/238Tm*, *pAt625/534Tm* and *pAt727/632Tm* were digested by the *Nhe* I and *Hin*d III restriction enzymes, respectively. The digested gene was ligated with a previously hydrolyzed plasmid, *pET28a*(+), using the same restriction enzymes. These constructed plasmids were transformed separately into *E. coli* BL21(DE3) to obtain the active enzyme. Transformants BL21(DE3)*pAt217/238Tm*, BL21(DE3)*pAt625/534Tm* and BL21(DE3)*pAt727/632Tm* were grown overnight at 37°C in 100 ml of Luria–Bertani (LB) medium containing 30 μg ml^{-1} kanamycin. A 10 ml of this culture was used to inoculate 1 l of LB medium containing 30 μg ml^{-1} kanamycin. The resulting culture was grown at 30°C until the OD_{600} reached 0.6. The target protein was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to give a final concentration of 1 mM, and incubation was continued for a further 10–12 h. The *E. coli* cells were harvested by centrifugation (11,000 × *g* for 10 min at 4°C) in the late log phase of the growth. The cells were resuspended in 50 mM of MOPS buffer (pH 6.5) and sonicated (Branson sonifier 250/450 D). The cell debris were removed by centrifugation (15,000 × *g* for 10 min at 4°C) and the supernatant was used as the crude enzyme in the subsequent purification steps.

2.5. Purification of the β-glucosidases

The β-glucosidases of *A. tumefaciens* and *T. maritima* were purified as reported previously [23,24]. In the first-step, the expressed chimeric β-glucosidases were purified using metal chelate chromatography. A 6 × His-tag was added in the amino terminus to bind with a Ni-NTA agarose slurry. The protein was then eluted in a batch procedure using imidazole gradients, as described in the QIAexpressionist^{TM} instructions (QIAGEN, Germany). The active fractions were pooled, diluted five times with water and finally applied to a HiLoad Q Sepharose column (Pharmacia) equilibrated with 20 mM of MOPS buffer (pH 6.5). The enzymes were eluted with a linear gradient from 0 to 0.5 M NaCl using an FPLC system (Pharmacia LKB Biotechnology Inc.). The fractions with β-glucosidase activity eluted as single peaks and the enzyme purity was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a Phast-System (Pharmacia) by 1 μg on a PhastGel Gradient 10–15 (Pharmacia) [26].

2.6. Determination of enzyme activity

β-Glucosidase activity was measured at 30°C using *p*-nitrophenyl β-D-glucopyranoside as the substrate, monitoring the amount of *p*-nitrophenyl released at 405 nm. The assay mixture, consisting of 2 mM *p*-nitrophenyl β-D-glucopyranoside in 50 mM MOPS buffer (pH 6.5) and 0.02% BSA, was incubated with the enzyme for 10 min in a total volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml of 0.2 M glycine–NaOH (pH 10.5). One unit of β-glucosidase is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenyl per minute under the conditions described above.

2.7. Effects of pH and temperature

To determine the optimum pH, enzyme activity was determined at 30°C using various buffers at 50 mM concentrations, viz. sodium phosphate (pH 1.1–3.1), sodium citrate (pH 3.16–4.12), sodium acetate (pH 3.75–5.71), 2-[*N*-morpholino]ethanesulfonic acid (MES) (pH 5.14–7.17), 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 6.2–8.18) and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (pH 6.48–8.56). Similarly, the pH stability of the enzyme was determined by preincubating the enzyme at 30°C for 30 min with the buffers described above, and the remaining activity was determined using the standard procedure.

The temperature optimum of the enzyme was determined using the standard assay at pH 6.5 (50 mM MOPS) with temperatures ranging from 0 to 100°C. To determine the thermal stability, the enzyme was

incubated for 30 min at different temperatures (between 0 and 100°C). After cooling the sample on ice for 10 min, the remaining activity was determined using the standard procedure. All buffers were prepared by adjusting the pH at room temperature and were used in the standard assay at 30°C. All chemicals used were obtained from Nacalai Tesque (Japan).

2.8. Kinetic parameters

For determination of the kinetic parameters (K_m and k_{cat}), purified enzymes were assayed with various substrates at pH 6.5. The concentration of each substrate was between one-half and two times that of the observed K_m values. The rate of substrate hydrolysis was monitored at 405 nm using a Beckman spectrophotometer (model DU 640) with temperature-controlled cell holders maintained at 30°C. The rate observed before 10% of the substrate was hydrolyzed was used to calculate the apparent K_m and k_{cat} values. Values for K_m and k_{cat} and their standard errors were calculated using the nonlinear regression analysis program "Grafit" [27].

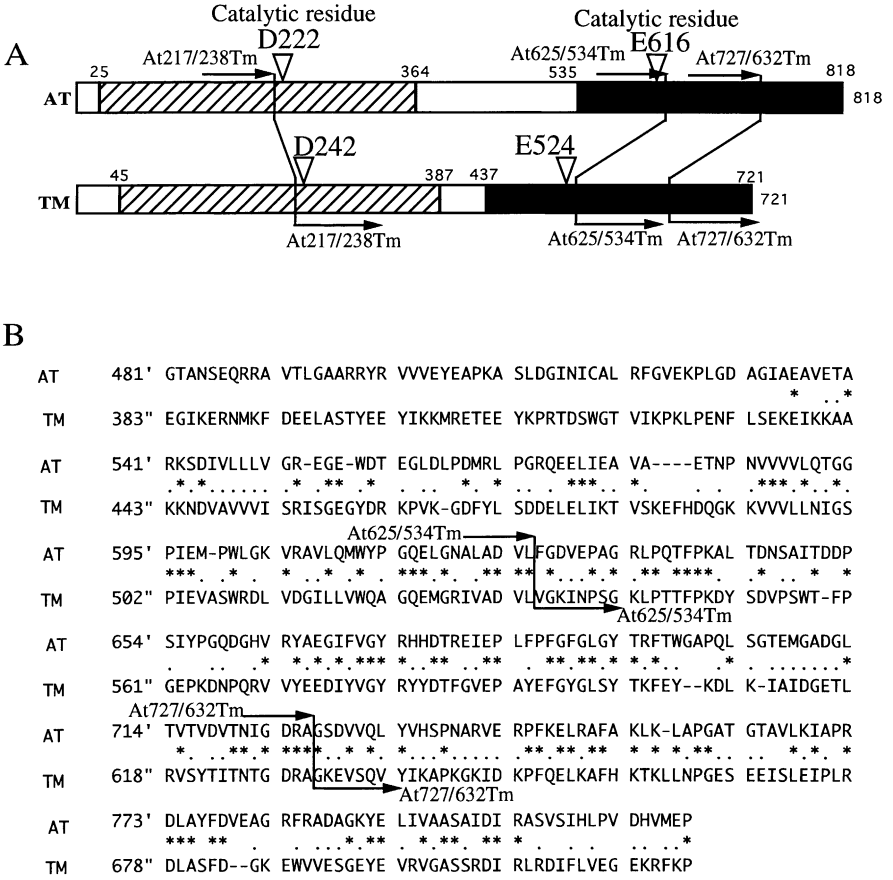

Fig. 1. Homology in the amino acid sequences of the β-glucosidases from *A. tumefaciens* and *T. maritima*. AT and TM denote *A. tumefaciens* and *T. maritima*, respectively. (A) Schematic representation of the sequence homology in the N-terminal (hatched) and C-terminal (solid) regions of the β-glucosidase genes. Position of catalytic nucleophile/bases of D222 and D242, and catalytic proton donors of E606 and E524 were indicated in the figure. (B) Amino acid alignment of *A. tumefaciens* and *T. maritima* β-glucosidases in the C-terminal region. Identical and similar amino acid residues are designated by asterisk (∗) and dot (·), respectively.

3. Results and discussion

3.1. Construction and expression of the chimeric β-glucosidases

The β-glucosidases of *A. tumefaciens* and *T. maritima* were previously cloned and characterized in our laboratory [23,24]. It was found that both enzymes have quite different characteristics. The β-glucosidase of *A. tumefaciens* from amino acid T25 to R364 and from E535 to P818 displays homologies of 45 and 37% with that of the β-glucosidase of *T. maritima* from T45 to K387 and from E437 to P721, respectively (Fig. 1). Based on the sequence alignment, three different sites of the β-glucosidase gene of *A. tumefaciens* were selected for gene shuffling with that of the *T. maritima* gene (Fig. 2). In comparison with the only one available X-ray crystal structure of the family three glycosyl hydrolases from barley, the first shuffling site (At217-238Tm) was located in the region between helix F and the strand G of the $(\alpha/\beta)_8$-barrel domain [25]. This shuffling site is just five amino acids upstream of the catalytic site of D222 in the *A. tumefaciens* β-glucosidase. The second shuffling site (At625-534Tm) was located after the N helix of the $(\alpha/\beta)_6$ sheet domain. This site is two amino acids upstream of the starting point of the C-terminal loop.

On the basis of domain homology data obtained from the ProDom protein domain database (protein.toulouse.inra.fr/prodom/prodom.html), it was apparent that C-terminal domain of the β-glucosidases of *A. tumefaciens* and *T. maritima* have more amino acid residues than that of the barley enzymes. The β-glucosidase from barley is consisted with 630 amino acid residues while those of *A. tumefaciens* and *T. maritima* are 818 and 721, respectively. Therefore, to investigate the structure and function relationships of these additional amino acid residues in the C-terminal domain, a third shuffling set (At727-632Tm) was designed in this region. In the latter two shuffling sets (At625-534Tm and At727-632Tm), the two catalytic sites, D222 and E616, are preserved.

The constructed chimeras were expressed in *E. coli* and purified to homogeneity. Enzyme purity was established by SDS-PAGE analysis, which gave a single band (Fig. 3). Of the three chimeric enzymes

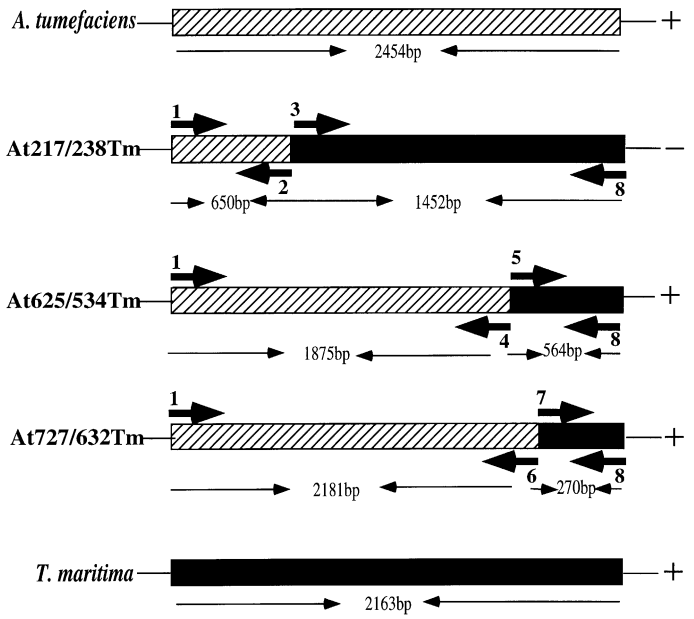

Fig. 2. Schematic representation of the parental and chimeric β-glucosidase genes. Hatched and black-colored regions are derived from *A. tumefaciens* and *T. maritima*, respectively. Eight primers used for constructing chimeric gene are illustrated. DNA sequences of the primers are listed in Table 1. (+) Activity and (−) no activity

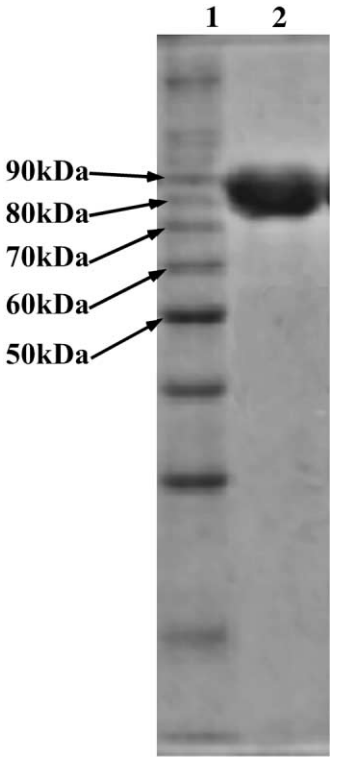

Fig. 3. SDS-PAGE analysis of the purified chimeric β-glucosidase (At625/534Tm). Lane 1: 10 kDa protein marker; lane 2: purified chimeric At625/534Tm β-glucosidase.

constructed, one chimera (At625/534Tm) was catalytically active, one chimera (At217/238Tm) was catalytically inactive, and a very low but unstable level of activity was observed for the third chimera (At727/632Tm).

In an earlier study, in which we studied the folding mechanism of a β-glucosidase, we showed that the folding information was distributed unevenly in the protein. In this study, the enzymes were composed of four regions; an N-terminal catalytic domain, a non-homologous region, a C-terminal domain of unknown function and C-terminal residues. The four different constructs truncated at the non-homologous region resulted in active enzymes with slight modifications in character. The chimeric enzymes shuffled at the C-terminal domain of unknown function formed active enzymes [18,19]. Removal of five amino acid residues at the C-terminal (viz. RGRAR) required co-expression with a GroEL/ES molecular chaperone to produce an active enzyme. However, the two chimeric enzymes shuffled at the N-terminal catalytic domain were only found in inclusion bodies even though they were co-expressed with the GroEL/ES molecular chaperone [22]. Solubilization of the inclusion body in the buffer containing 8 M urea and subsequent refolding of these two proteins by slow dialysis was not successful in producing active enzymes [28].

Similar results were also obtained when the chimeric gene shuffled at the N-terminal catalytic domain, At217/238Tm, was expressed.

3.2. pH and thermal profiles of the chimeric and parental enzymes

The β-glucosidase of *A. tumefaciens* and *T. maritima* show marked differences in their pH optima and pH stabilities. The pH optima for the *A. tumefaciens* and *T. maritima* enzymes are pH 7.2–7.4 and 3.2–3.5, respectively. The observed optimum pH for chimera At625/534Tm was around pH 6.2–6.5 (Fig. 4A), which is closer to that of the β-glucosidase of *A. tumefaciens*. Similarly, the pH stability of this chimeric enzyme was found to lie in the range from pH 5 to 9, whereas the *A. tumefaciens* and *T. maritima* enzymes displayed stability within the pH ranges from 4 to 11 and 3 to 12, respectively (Fig. 4B).

The correlations between temperature and activity for the parental and chimeric enzymes are shown in Fig. 5. The temperature optima for the *A. tumefaciens* and *T. maritima* enzymes are 65 and 85°C, respectively. Unlike other chimeras that show profiles intermediate to those of their parent enzymes [18–22], this chimera does not. The observed temperature optimum for the At625/534Tm chimeric enzyme is around 45°C, which is lower than that of the parental enzymes (Fig. 5A).

With regard to heat stability, our results demonstrate that the temperature optimum for the chimeric enzyme does not correlate with the thermal stabilities of the parental enzymes. The chimeric enzyme At625/534Tm is stable up to 37°C, retains about 90% of its maximum activity at 40°C, and is inactivated at 50°C (Fig. 5B). In contrast, the β-glucosidases of *A. tumefaciens* and *T. maritima* are stable up to temperatures of 55 and 75°C, respectively.

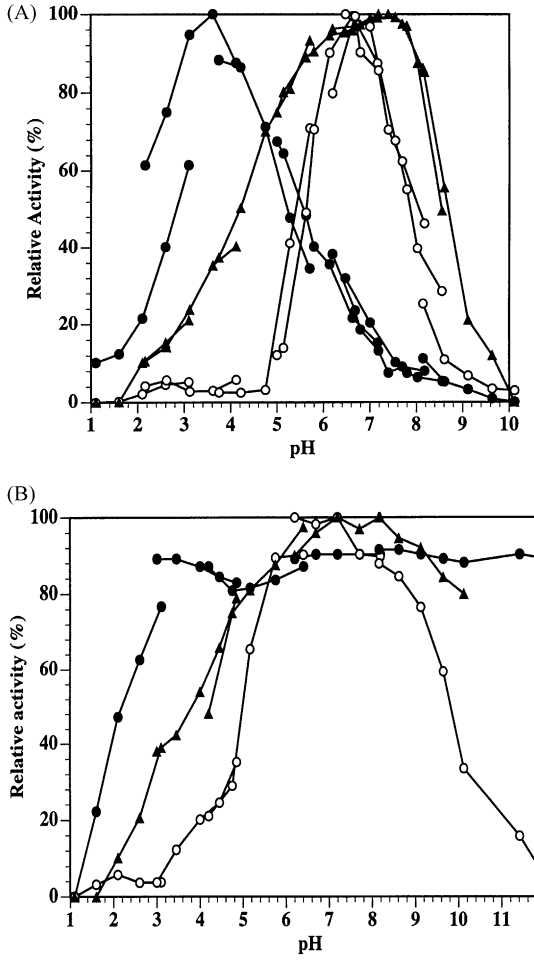

Fig. 4. pH activity (A) and pH stability (B) profiles of the chimeric and parental β-glucosidases. The pH was adjusted with the following buffers: phosphate (pH 1.1–3.1), citrate (pH 2.16–4.12), acetate (pH 3.75–5.71), MES (pH 5.0–7.7), MOPS (pH 6.2–8.18), HEPES (6.48–8.56) and CHES (pH 8.15–10.12). For pH stability experiments, the enzymes were incubated at 30°C at different pH for 30 min. then the residual activities were determined using the standard assay conditions. *A. tumefaciens* (▲); At625/534Tm (○); *T. maritima* (●).

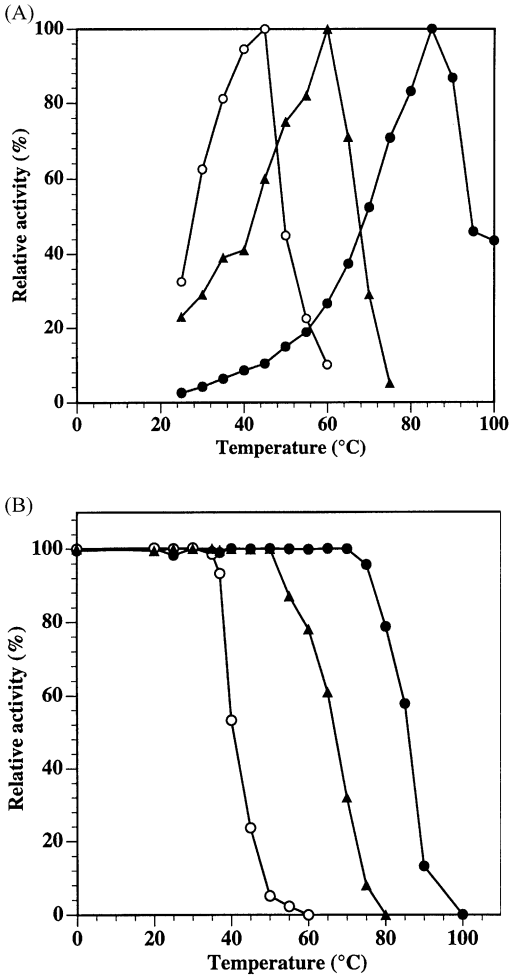

Fig. 5. Temperature optimum (A) and heat stability (B) profiles of the chimeric and parental β-glucosidases. For the estimation of thermal stabilities of the β-glucosidases, each enzyme was preincubated for 30 min at various temperatures. The residual activities were then determined using the standard assay conditions. *A. tumefaciens* (▲); At625/534Tm (○); *T. maritima* (●).

3.3. Substrate specificities of the chimeric and parental enzymes

The kinetic parameters of the β-glucosidases of *A. tumefaciens*, *T. maritima* and the chimeric enzyme, At625/534Tm, were investigated using various aryl glycosidases as substrates. The observed K_m and k_{cat} values for pNP-β-D-glucopyranoside, pNP-β-D-xylopyranoside, pNP-β-D-fucopyranoside and pNP-α-L-arabinofuranoside are given in Table 2. From Table 2 it can be seen that the specificity of chimeric enzyme is different from that of the parent enzymes. The K_m values for pNP-β-D-glucopyranoside for the parent enzymes, *A. tumefaciens* and *T. maritima*, are 0.012 and 0.0039 mM, respectively, which is the lowest K_m previously reported for any glycosidase. However, the observed K_m value for the chimeric enzyme At625/534Tm for pNP-β-D-glucopyranoside

Table 2
Kinetic parameters of the parental and chimeric enzymes[a]

Substrate	*A. tumefaciens*	*T. maritima*	At625/534Tm
pNP-β-D-glucopyranoside			
K_m (mM)	0.012	0.0039	0.081
k_{cat} (s^{-1})	95.4	6.4	3.3
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	7950	1640	41
pNP-β-D-xylopyranoside			
K_m (mM)	0.005	2.64	0.95
k_{cat} (s^{-1})	28.9	18.4	0.16
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	5780	6.96	0.17
pNP-β-D-fucopyranoside			
K_m (mM)	0.08	42.6	0.24
k_{cat} (s^{-1})	22.1	27.6	0.013
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	280	0.648	0.054
pNP-α-L-arabinofuranoside			
K_m (mM)	0.24	18.9	0.66
k_{cat} (s^{-1})	119	9.0	0.025
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	495	0.47	0.038

[a] Standard errors are within 10% of the given values.

was 0.081 mM, which is slightly higher than that observed for the parent enzymes. In comparison, the k_{cat} value of the chimeric enzyme for pNP-β-D-glucopyranoside was 3.38 s^{-1}, which is lower than that observed for the β-glucosidases of *A. tumefaciens* (95.4 s^{-1}) and *T. maritima* (6.4 s^{-1}). The k_{cat}/K_m ($mM^{-1}\,s^{-1}$) values for the *A. tumefaciens* enzyme towards pNP-β-D-glucopyranoside, pNP-β-D-xylopyranoside, pNP-β-D-fucopyranoside and pNP-α-L-arabinofuranoside are 7950, 5780, 280, and 440, respectively, while for the *T. maritima* enzyme they are 1640, 6.96, 0.649 and 0.047, respectively. The k_{cat}/K_m ($mM^{-1}\,s^{-1}$) values for the chimeric enzyme At625/534Tm towards the same substrates were 40, 1.72, 0.053 and 0.038, respectively. The above data indicates that the specificity of the chimeric enzyme is slightly closer to that of the *T. maritima* β-glucosidase since they showed more or less the same specificity toward the investigated substrates though the chimeric enzyme contains only 10.3% amino acid residues of *T. maritima* enzyme. The shuffled region of C-terminal domain may play a significant role on the enzyme substrate specificity. The chimeric enzyme At625/534Tm does differ in specificity from that of the β-glucosidase of *A. tumefaciens* in that the chimeric enzyme is poor at hydrolyzing pNP-β-D-xylopyranoside.

4. Conclusions

A catalytically active protein was obtained by exchanging the F626 to P818 amino acid residues of the β-glucosidase of *A. tumefaciens* with those of *T. maritima*. The resulting chimeric enzyme showed different characteristics in terms of its pH and thermal profiles relative to the parental enzymes. The observed K_m value of the chimeric enzyme for pNP-β-D-glucopyranoside was 0.081 mM, which is slightly higher than that of the parental enzymes. The k_{cat} value for the chimeric enzyme toward this substrate was 3.28 s^{-1}, which is lower than that of the β-glucosidases of *A. tumefaciens* (95.4 s^{-1}) and *T. maritima* (6.4 s^{-1}). The chimeric β-glucosidase also hydrolyses pNP-β-D-xylopyranoside, pNP-β-D-fucopyranoside and pNP-α-L-arabinofuranoside, but the specificities are different to those of the parental enzymes.

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