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Role of N- and C-terminal domains and non-homologous region in co-refolding of *Thermotoga maritima* β-glucosidase

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Abstract

Thermotoga maritima β-glucosidase consists of three structural regions with 721 amino acids: the N-terminal domain, middle non-homologous region and a C-terminal domain. To investigate the role of these domains in the co-refolding of two fragments into catalytically active form, five sites coding the amino acid residue at 244, 331 in the N-terminal domain, 403 in the non-homologous region, 476 and 521 in the C-terminal domain were selected to split the gene. All the 10 resultant individual fragments were obtained as insoluble inclusion bodies and found to be catalytically inactive. However, the catalytic activity was recovered when the two fragments derived from N-terminal and C-terminal peptides were co-refolded together. It is quite interesting to find that not only the complement polypeptides such as N476/477C but also the truncated combination (N476/522C, amino acid residues from 477 to 521 is truncated) and overlapped combination (N476/245C and N476/404C, amino acid residues from 245 to 476 and from 404 to 476 are overlapped) also gave catalytically active enzymes. Our results showed that folding motifs consisted of the complete N-terminal domain play an important role in the co-refolding of the polypeptides into the catalytically active form.
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Keywords: β-Glucosidase; *Thermotoga maritima*; N- and C-terminal domains; Non-homologous region; Co-refolding; Gene splitting

1. Introduction

One of the key problems in protein biochemistry is the correct folding of the recombinant/engineered protein, which is functionally active after over-expression of gene of interest. Under ideal conditions, this process occurs spontaneously and the final conformation is driven solely by the amino acid sequence [1]. The process by which protein molecules attain their native conformations with minimum conformational entropy, i.e. protein folding, is a subject of fundamental and practical importance, yet it remains one of the key unresolved issues in protein biochemistry [2]. Mechanisms by which proteins acquire their specific, biologically active, three-dimensional structure are currently being studied by a variety of biophysical and computational approaches [3,4]. All the folding studies are very much limited to small single polypeptide chain molecules and not much is understood for large multidomain proteins. Of particular interest is the fragment complementation effects obtained when fragments are mixed of two types of mutants, one of which cuts out the N-terminal region of the polypeptide, while the other contains a point-mutant or a deletion within the C-terminal region, especially for larger proteins [5,6]. Previous studies showed that co-refolding in vitro may be initiated by collapse of hydrophobic regions into the interior of the molecule followed by the formation of stable secondary structures that provide a framework for subsequent folding and formation of covalent interactions such as disulfide bonds, that stabilize the polypeptide in particular conformations [7]. One way of checking this model is to isolate the polypeptide fragment corresponding to such a region and

Abbreviations: CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; CD, circular dichroism; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; HEPES, 2-[*N*-hydoroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; IPTG, isopropyl thiogalactoside; MES, 2-[*N*-morpholino] ethanesulfonic acid; MOPS, 3-[*N*-morpholino] propanesulfonic acid; *p*NP, *p*-nitrophenyl

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to test whether this fragment by itself is able to fold into the conformation it adopts in the native protein. There are very few studies using this experimental approach to investigate the structure and folding except for the case of β_2 subunit of *Escherichia coli* tryptophan synthetase [8].

The enzyme β-glucosidase (EC 3.2.1.21) is one of the components of the cellulase enzyme complex required for the hydrolysis of cellulose to glucose by catalyzing the final step, which converts cellobiose to glucose [9,10]. β-Glucosidases of *Thermotoga maritima* and *Agrobacterium tumefaciens*, which belong to the family 3 glycosyl hydrolases, have already been characterized [11,12]. Several chimeric β-glucosidases were constructed between β-glucosidases of *A. tumefaciens* and *T. maritima* by substituting different segments from one enzyme with that of the other and the enzyme characteristics of parental and chimeric enzymes were characterized [11,13]. Many attempts to get active chimeric β-glucosidases, shuffled in the N-terminal domain, were unsuccessful in our laboratory even after solubilization of proteins in the presence of molecular chaperones. Fragments complementation seems to be an important phenomenon in the folding of larger multi-domain proteins. The current study was undertaken to investigate the role of the two terminal domains and non-homologous region in proper co-refolding for the recovery of an active enzyme. In this study, we have split the gene at five sites of non-homologous region, N- and C-terminal domains followed by construction of N- and C-terminal peptides and studied the function of these regions with respect to co-refolding of the β-glucosidase with catalytic activity.

2. Material and methods

2.1. Bacterial strain and plasmids

The genomic DNA of *T. maritima* strain MSB8 (GenBank Accession No. CQ893499) was kindly supplied by Prof. Dr. Karl O. Stetter of Regensburg University, Germany. Plasmid DNA was prepared using a QIAprep Spin Plasmid Kit (Qiagen, Hilden, Germany). *pDrive* cloning vector was obtained from Qiagen (Hilden, Germany) and expression vector *pET28a* (+) was from Novagen (Madison, WI, USA). *E. coli* BL21 (DE3) and EZ competent cells (Stratagene, Lajolla, CA, USA) were used as the host for cloning and expression, respectively.

2.2. Construction of gene fragments

The plasmid DNA preparation, DNA electrophoresis and other basic DNA manipulations described in this study have been described previously [14]. The five different sites at 244 and 331 (N-terminal domain), 403 (non-homologous region), and 476 and 521 (C-terminal domain) were selected for gene splitting. In order to amplify the gene fragments, six forward and six reverse primers were designed according to the nucleotide sequence (Table 1). Primer 1 with *Nhe* I restriction site was used as the forward primer for five N-terminal fragments and primer 2 with *Xho* I restriction site was used as the reverse primer for five C-terminal fragments. Primers 3, 5, 7, 9 and 11 with *Hin*d III restriction site were used as the reverse primers for N-terminal fragments. Similarly, primers 4, 6, 8, 10 and 12 were used as the forward primers for five C-terminal fragments with *Nco* I restriction site. All 10 fragments were amplified using PCR consisted of denaturation at 98 °C for 1 min, annealing at 58 °C for 1 min and primer extension at 68 °C for 1 kb/min with 20 cycles [13]. PCR was carried out with a GeneAmp PCR System 9700 (Applied Biosystems, Foster city, CA) using high fidelity KOD-Plus DNA Polymerase (Toyobo Biochemicals, Osaka, Japan). As a result of amplification, 10 fragments (N244, N331, N403, N476 and N521; 245C, 332C, 404C, 477C and 522C) were obtained (Fig. 1). After digestion and gel purification, the amplified fragments were ligated to the *pDrive* vector and plasmids were transformed into *E. coli* cells. The recombinant plasmids were isolated from positive clones and DNA sequence of all recombinant genes was confirmed.

Table 1
Sequence of oligoprimers used in the construction of various DNA fragments

Primer	Sequence (5′–3′)
1.N-terminal-FWD	GCT AGC ATG GAA AGG ATC GAT GAA
2. C-terminal-REV	CTC GAG TGG TTT GAA TCT CTT CTC TCC CT
3.N244-REV	AAG CTT CTA GTA CCA GTC GCT CAT CAC GA
4. 245C-FWD	CCA TGG CGG GAG ACA ACC CTG TAG AA
5. N331-REV	AAG CTT CTA TGC TTC GTA GGC GAC TTC C
6. 332C-FWD	CCA TGG GTG CGG AGG GTG TTG TCC TT
7. N403-REV	AAG CTT CTA CTC ATA AGT GGA AGC GAG TT
8. 404C-FWD	CCA TGG AGT ACA TAA AAA AGA TGA GAG AAA
9. N476-REV	AAG CTT CTA TAG CTC GTC ATC GGA GAG GT
10. 477C-FWD	CCA TGG AAC TCA TAA AAA CCG TCT CGA
11. N521-REV	AAG CTT CTA CGC CTG CCA GAC GAG AAG A
12. 522C-FWD	CCA TGG GAC AGG AGA TGG GAA GAA TA

Restriction enzyme sites are underlined. Numbers 244, 245, 331, 332, 403, 404, 476, 477, 521 and 522 represent different N- and C-terminal fragments obtained as a result of gene splitting.

2.3. Over-expression of plasmids

In order to over-produce each peptide fragment for the gene fragments of interest, all 10 fragments were excised from *pDrive* vector with four restriction enzymes (*Nhe* I, *Hin*d III, *Nco* I and *Xho* I) and the digested genes were ligated with a previously hydrolyzed plasmid *pET28a* (+) vector using the same restriction enzymes. For ligation, High T4 DNA ligase (Toyobo, Japan) was used and constructed plasmids were transformed separately into *E. coli* BL-21 (DE3) cells and expressed as described earlier [15].

2.4. Denaturation and purification of insoluble fragments

Recombinant N- and C-terminal peptide fragments, consisting of a tag of six histidine residues at the N-terminus of N-terminal and six at the C-terminus of C-terminal fragments, were produced as inclusion bodies, respectively. Inclusion body pellets were solubilized in an 8 M urea solution containing 50 mM Tris/HCl (pH 8.0), 1 mM EDTA and reduced for 1 h at room

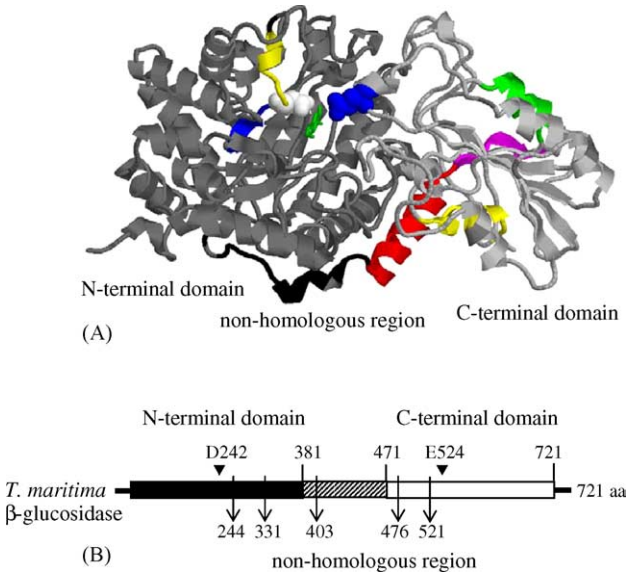

Fig. 1. (A) Three-dimensional structure of barley β-glucosidase representing the key structural regions used in this fragment complementation study. The two catalytic residues, Glu491 and Asp285, are shown in space fill model in blue and white, respectively, and the bound glucose (green) shown in ball & stick model. The structural regions, helices I (magenta), H (red), G (yellow) and M (green) and strand g (blue) are shown to represent the gene splitting sites. The non-homologous region (black) consists only 16 residues in barley enzyme but contains 90 residues in the *T. maritima* enzyme. (B) Schematic diagram for producing various peptide fragments of the two terminal domains and non-homologous region of *T. maritima* β-glucosidase. Numbers 244, 331, 403, 476 and 521 represent the positions of amino acid residues at the splitting sites, respectively. D242 and E524 represent two catalytic residues in N- and C-terminal domains, respectively. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of the article.)

temperature by adding 0.05% (v/v) 2-mercaptoethanol and then oxidized by the addition of glutathione (oxidized form) at a concentration of 0.08% (w/v) for 1 h. The insoluble portion was removed by centrifugation at 15,000 × *g* for 20 min. The denatured fragments were purified using metal chelate chromatography followed by ion-exchange chromatography. The purification steps were carried out by using the buffer containing 8 M urea [11]. The purity of the eluted fractions was assessed with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Refolding and co-refolding by slow dialysis

Each of the purified N-(0.2 mg/ml) and C-terminal (0.2 mg/ml) fragments was subjected to refolding by slow dialysis at 4 °C against 50 mM of different buffers acetate (pH 5.0), MOPS (pH 7.0) or CHES (pH 9.0) containing 1 mM EDTA, 0.05% 2-mercaptoethanol, 0.16% oxidized glutathione and urea where the urea concentration was decreased from 8 to 0 M over 4 days [15,16]. The catalytic activity was checked at the end of the refolding. After mixing the equimolar of N- and C-terminal peptides, co-refolding was also carried out by the same procedure. The native [11] and four co-refolded enzymes, N476/477C, N476/245C, N476/404C and N476/522C, were purified by ion-exchange chromatography and subjected to SDS-PAGE.

2.6. Enzyme assay

Using *p*NP-β-D-glucopyranoside as the substrate, β-glucosidase activity was measured at 30 °C by monitoring the amount of *p*-nitrophenyl released at 405 nm [11,12].

2.7. Circular dichroism

Secondary and tertiary structural elements of native and co-refolded enzymes were monitored by Circular Dichroism Spectrometer (Jasco J-820; Tokyo, Japan) at 25 °C. The protein concentration of each enzyme was 0.1–0.2 mg/ml. All data were averaged from five acquisitions between 200 and 250 nm at a scan rate of 20 nm/min with a 0.1 cm path cell and the data is presented as mean residue elipticity.

2.8. Effects of pH, temperature and alcohol on the enzyme activity

To determine the optimum pH of native and co-refolded enzymes, activity was determined at 30 °C using various buffers at 50 mM concentrations, viz. sodium citrate (pH 2.2–4.2), sodium acetate (pH 3.7–5.8), MES (pH 5.1–7.2), MOPS (pH 6.2–8.2), HEPES (pH 6.6–8.5) and CAPS (pH 9.4–11.4). Similarly, the pH stability was determined by preincubating the enzyme at 30 °C for 30 min with the buffers described above, and the residual activity was determined using standard assay procedure. The temperature optimum of each enzyme was determined using the standard assay conditions by incubating the reaction mixture containing 50 mM acetate buffer (pH 3.7) at different temperatures ranging from 30 to 95 °C for 30 min. For determination of thermal stability, the enzyme was incubated for 30 min at different temperatures. After cooling the sample on ice for 10 min, remaining activity was determined using the standard assay procedure. To investigate the effect of straight chain alcohols on the catalytic activity of co-refolded enzymes, transglycosylation activity was measured and compared with the native enzyme as described earlier [12,13].

2.9. Kinetic parameters

To determine the kinetic parameters (K_m, k_{cat} and K_m/k_{cat}), the purified enzymes were assayed at 50 mM MOPS buffer (pH 6.5) with various substrates. 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 less than 10% of the substrate hydrolyzed was used to calculate the apparent K_m and k_{cat} values [17].

3. Results and discussion

3.1. Construction and over-expression of plasmids

In the family 3 glycosyl hydrolase, barley-β-D-glucan exohydrolase is the only enzyme whose three-dimensional structure is known. The enzyme consists of N-terminal $(\alpha/\beta)_8$ TIM barrel

and a C-terminal six-stranded β sandwich, connected by a helix-like strand of 16 amino acid residues. The catalytic center is located in the pocket at the interface of the two domains. Asp285 in the N-terminal domain acts as a catalytic nucleophile, while Glu491 in the C-terminal domain acts as a proton donor. Three-dimensional structural analysis indicates that the N-terminal domain forms an $(\alpha/\beta)_8$ barrel structure and this represents one of the typical folds observed in the glycoside hydrolases [18] (Fig. 1A). *T. maritima* β-glucosidase is a highly thermostable enzyme and has been previously cloned and characterized in our laboratory. Alignments of the amino acid sequences of *T. maritima* β-glucosidase with several families 3 β-glucosidases has shown that this enzyme consists of two functional domains, an N-terminal domain and a C-terminal domain, which are linked together by a non-homologous region. Hence, the N-terminal and C-terminal homologous regions of this enzyme are comparable to the N-terminal and C-terminal domains of barley β-D-glucan exohydrolase, catalytic residues were aligned to be Asp242 (D242, nucleophile/base in N-terminal domain) and Glu524 (E524, catalytic proton donor in C-terminal domain).

Practical interest in the protein folding problem stems from the fact that large proteins overproduced by genetically engineered cells are often obtained in non-native forms (e.g. inclusion bodies). In case of chimeric β-glucosidases shuffled in the N-terminal domain, solubilization of the inclusion bodies of chimeric enzymes in a buffer containing 8 M urea and subsequent refolding by slow dialysis was not successful in refolding into active forms [19]. Therefore, this study was undertaken to investigate the role of each domains and homologous region in proper refolding for the recovery of an active enzyme. The first splitting site coding amino acid residue of 244 was selected in the region between the strand g and the helix G of the $(\alpha/\beta)_8$-barrel domain, based on the X-ray crystal structure of barley β-glucosidase [18]. This splitting site is just two amino acids downstream of the catalytic residue of the nucleophile/base D242. The second splitting site coding amino acid residue of 331 was selected in the region between helix H3 and the strand i of the $(\alpha/\beta)_8$-barrel domain. This splitting site is 89 amino acids downstream of the catalytic residue D242. The non-homologous region is situated between the N- and C-terminal domains. This region consists of 90 amino acid residues compared to that of only 16 residues of the barley enzyme. Gene splitting regions correspond to *T. maritima* and catalytic residues are shown in Fig. 1B. To study the importance of this region, splitting site coding amino acid residue of 403 was selected. Analysis of domain homology data available in the Prodom protein domain database (Protein.toulouse.inra.fr/prodom/prodom.html) revealed that C-terminal domain of the *T. maritima* β-glucosidase has 116 more amino acid residues than the barley enzyme. Barley β-D-glucosidase consisted of 605 amino acid residues while that of *T. maritima* β-glucosidase is 721. The fourth splitting site coding the amino acid residue of 476 was selected in the region between strand m and the helix M of $(\alpha/\beta)_6$-sheet domain. It is 48 amino acid residues upstream of the catalytic residue E524. Fifth splitting site coding the amino acid residue of 521 was located after the N helix of the $(\alpha/\beta)_6$-sheet domain. It is just three amino acid residues upstream of the catalytic residue E524 (starting point of the C-terminal loop).

As a result of gene splitting, 10 individual fragments were obtained. N244 and N331 had a partial N-terminal domain. Similarly, 522C and 477C had a partial C-terminal domain. N403 had N-terminal domain with a partial non-homologous region and 404C had a partial non-homologous region with C-terminal domain. N476 and N521 had N-terminal domain and non-homologous region with a partial C-terminal region. In contrast, 245C and 332C had C-terminal and non-homologous region along with a partial N-terminal domain (Fig. 1B). The coding genes for all 10 peptide fragments were individually cloned into *E. coli pET28a* (+) and all expressed fragments were obtained as inclusion bodies that are catalytically inactive.

3.2. Denaturation, purification and refolding of insoluble fragments

All insoluble N- and C-terminal fragments were denatured and purified using metal chelate affinity and ion exchange column chromatography. SDS gel electrophoresis results showed good agreement with the calculated MW of the fragments (data not shown). Purified insoluble fragments were mixed and then subjected for co-refolding by slow dialysis to obtain catalytically active proteins. Co-refolding was carried out at different pH conditions (pH 5.0, 7.0 and 9.0) and the catalytic activity of enzymes showed best at pH 7.0. No catalytic activity was observed when 10 fragments were apparently refolded, individually. The refolded fragments were monitored for intact secondary structure by far-UV CD measurements (data not shown). Attempts to monitor the equilibrium unfolding in presence of chaotropes were unsuccessful since these fragments were quite big to follow two-state transitions during unfolding–refolding reactions. However, the catalytic activity was observed when the two fragments were co-refolded together. Fifteen co-refolded enzymes were found to be catalytically active, 2 of them were less active and other 13 co-refolded fragments were catalytically inactive (Table 2).

All five fragments co-refolded with the N-terminal fragment (N244) obtained by splitting the gene coding the amino acid residue at 244 in $(\alpha/\beta)_8$-barrel (N-terminal domain) were catalytically inactive. These results were expected since the splitting site was very close to the catalytic residue D242 (nucleophile/base) and would dissect the active site. However, we were speculative to see whether any other nucleophilic residue would perform the function of D242 that would constitute the active site. Similarly, three truncated types, one complement type and one overlapped type of co-refolded enzymes were obtained by splitting the gene coding the amino acid residue at N331. All these five co-refolded enzymes showed no catalytic activity. These results suggest that splitting the gene in the $(\alpha/\beta)_8$-barrel strongly inhibit the co-refolding into catalytically active form of the enzyme. Previous study revealed that all four chimeric enzymes shuffled in the $(\alpha/\beta)_8$-barrel domain were expressed only as inclusion bodies and were catalytically inactive [13], although several other chimeric enzymes that

Table 2
Recovery of the catalytic activity when the two fragments were co-refolded

N-terminal fragments	C-terminal fragments				
	245C	322C	404C	477C	522C
N244	X	X	X	X	X
N331	X	X	X	X	X
N403	⊙	⊙	○	△	△
N476	⊙	⊙	⊙	○	○
N521	⊙	⊙	⊙	⊙	○

(**X**) No catalytic activity was detected (<0.1%); (△) faint catalytic activity was obtained (1.0–0.1%); (◯) catalytic activity was obtained (>1.0%), (▩) combination of truncated peptides; (□) combination of complement peptides; (⊡) combination of overlapping peptides; (▣) characterized co-refolded enzymes. The ratio of enzyme recovery after co-refolding was: 476N/245C (32%), 476N/404C (29%), 476N/477C (34%) and 476N/522C (8%). This data was calculated from the results of FPLC.

have been constructed in the C-terminal region was resulted in catalytically active enzymes [20,21].

Splitting the gene in the non-homologous region (amino acid residues at 403) gave five co-refolded fragments with catalytic activity. Three of them (two of the overlapping type, N403/245C and N403/332C, and one of the complement types, N403/404C) were found to be catalytically active, whereas two truncated types, N403/477C and N403/522C, in which few amino acid residues in the C-terminal domain were deleted, showed very low catalytic activity. This result suggests that the complete set of amino acid residues of the $(\alpha/\beta)_8$-barrel domain is essential to obtain catalytically active form of the enzyme by co-refolding. All the 10 co-refolded enzymes (7 overlapped type, 2 complement type and 1 truncated type) obtained by splitting the gene at 476 and 521 of C-terminal domain showed catalytic activity. This result shows that splitting the gene in the C-terminal domain may not play an important role in the co-refolding of the polypeptides into the catalytically active form. In our earlier studies on shuffling of the family 3 glycosidases of *C. gilvus* and *A. tumefaciens*, it was found that catalytically active chimeras could be obtained only when shuffled at the C-terminal domain [22,23]. And on gene splitting at the non-homologous region of *A. tumefaciens* β-glucosidase resulted in the formation of only two active enzymes out of 16 mixed and co-refolded fragments [15]. In another study, it has been reported that the deletion of about 100 amino acid residues near the C-terminal region of the α-amylase gene did not affect enzyme activity [24]. Cyclomaltodextrin glucanotransferases lacking 30 amino acids [25] and an endoglucanase lacking 75 amino acids [26] from the C-terminal end showed no enzyme activity.

To investigate the effect of overlapping and truncation of amino acid residues on the co-refolded enzymes, four co-refolded enzymes N476/245C and N476/404C (231 and 72 residues were overlapped, respectively), N476/477C (complement) and N476/522C (46 residues truncated) were selected to

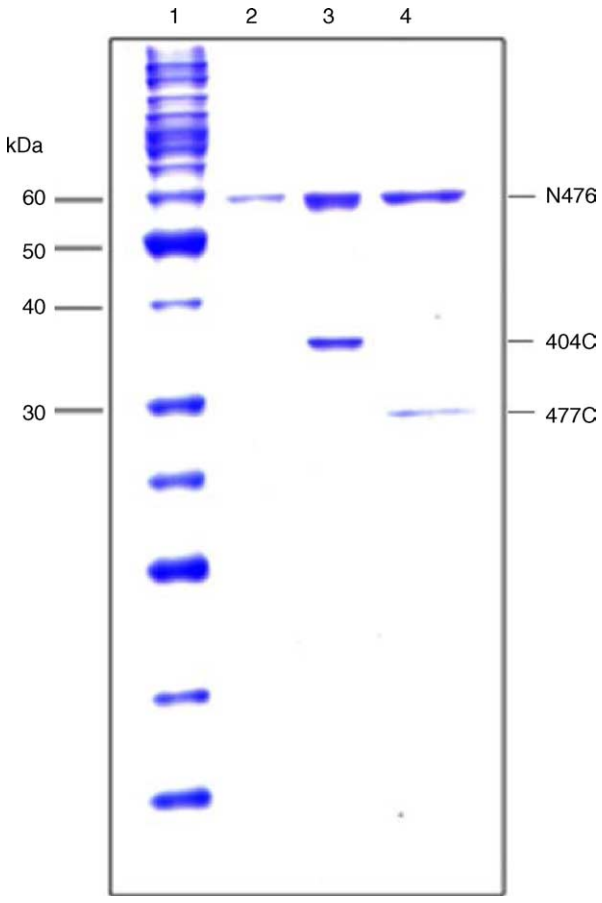

Fig. 2. SDS-PAGE analysis of the co-refolded enzymes. Lane 1: 10 kDa protein markers, lane 2: N476/245C, lane 3: N476/404C and lane 4: N476/477C.

investigate the enzyme character (Table 2). The native and four co-refolded enzymes were purified using an anion exchange column chromatography to homogeneity and enzyme purity was confirmed by SDS-PAGE analysis, which showed two bands corresponding to N- and C-terminal fragments of each co-refolded enzymes. Separation of two bands of N476 and 245C peptides turned out difficult since the MW of the two fragments is close to each other (Fig. 2). The MW of enzyme fragments was consistent with the values calculated using the ExPASy ProtoParam tool. It is of interest to note that N476/245C with 232 overlapping amino acid residues in the $(\alpha/\beta)_8$-barrel domain and non-homologous region and N476/404C with 73 overlapping residues in the non-homologous region was found to be catalytically active. Thus, these results indicate that these extra residues did not inhibit the folding ability. Another co-refolded enzyme N476/522C was also found to be catalytically active although 45 amino acid residues in the C-terminal domain were deleted. Therefore, this portion of C-terminal domain is not important for co-folding property of the enzyme.

The secondary structural details of the native and co-refolded enzymes were characterized by circular dichroism spectroscopy (Fig. 3). The results revealed that all four enzymes including N476/477C (complement fragments) co-refolded in a similar way as that of the native enzyme showing no essential structural changes. Previous study on two subunits of tryptophan

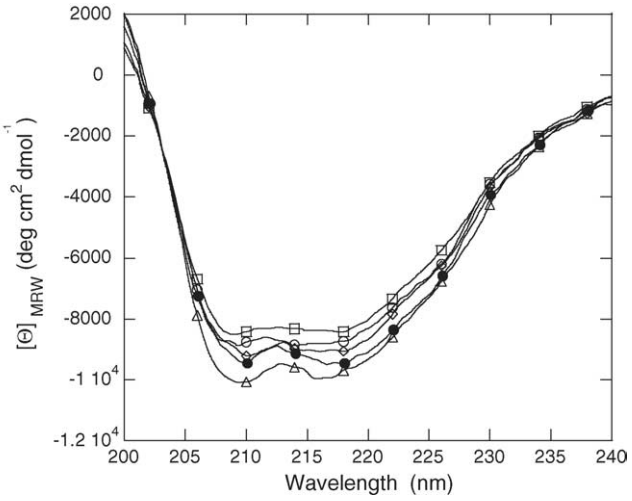

Fig. 3. Far-UV circular dichroism spectra of the native and co-refolded enzymes. Protein concentrations were 0.1–0.2 mg/ml in 20 mM MOPS buffer (pH 6.5) CD spectra were determined in a cell with 0.1 cm path length. Native (●), N476/245C (◯), N476/404C (□), N476/477C (△) and N476/522C (◊).

synthase showed that the fragments produced by a single site of cleavage were able to refold after urea treatment to produce an active enzyme with the same amount of α-helix as the native enzyme, but the enzyme cleaved at more than one site was not able to refold after urea treatment [27–29]. They also observed that there was only a small difference between the CD spectra of two mixed fragments, suggesting that only a small change in secondary structure or conformation took place after co-refolding of the two fragments. Thus, the two fragments appear to fold independently and may be considered to be independent domains by this criterion [30,31]. Less information is available on the folding and assembly of larger multidomain proteins and more detailed study on refolding kinetics or the structural analysis is needed to clarify this point.

3.3. Effects of pH, temperature and salt concentration on the activity of co-refolded fragments

The influence of pH on β-glucosidase activity was determined using a series of buffers (data not shown). The optimum pH for all four co-refolded enzymes was around 3.7, which is similar to that of the native enzyme. The pH stability of three co-refolded enzymes was found to be in the range from pH 3.2–11.4 like the native enzyme, whereas N476/404C displayed stability between pH 2.7 and 11.4. The correlations between temperature and activity for the native and co-refolded enzymes are shown in Fig. 4A and B. N476/245C and N476/404C were optimally active at 75 °C, whereas the other two co-refolded enzymes were active at 80 °C compared to that of 85 °C of native enzyme. Ther-

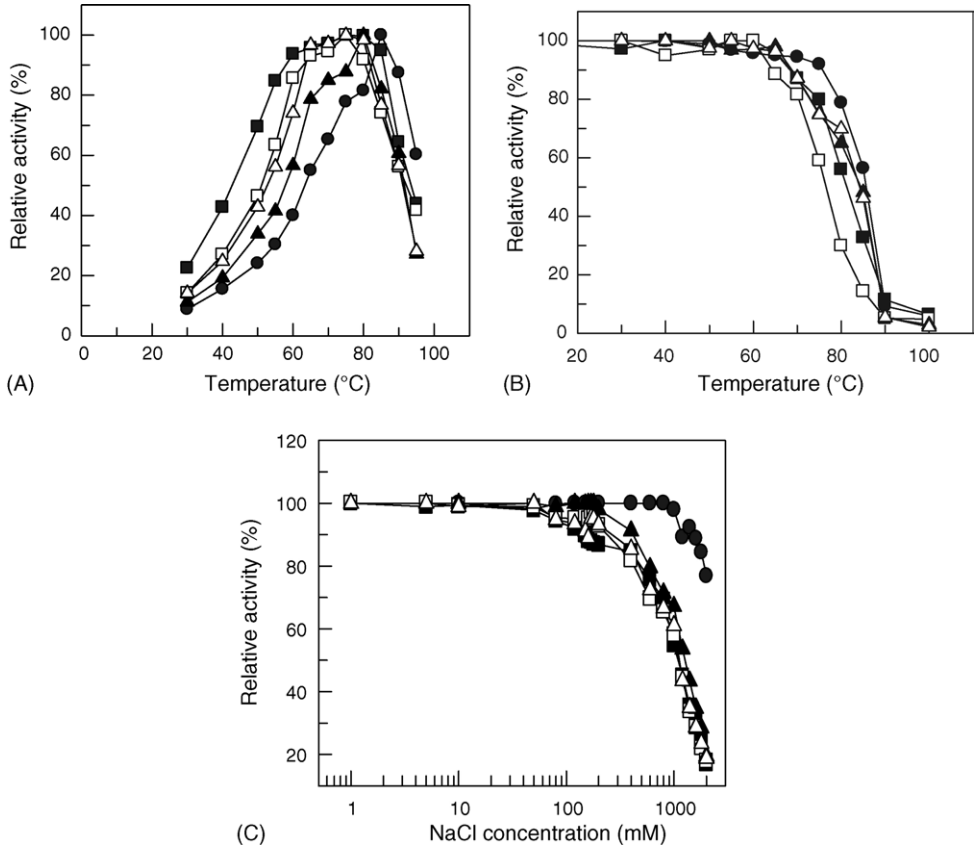

Fig. 4. Effects of temperature and NaCl on catalytic activity of native and co-refolded enzymes (A) optimum temperature profile (B) thermal stability and (C) NaCl. For the estimation of thermal stabilities of the β-glucosidases, each enzyme was preincubated for 30 min at various temperatures. In each case 5 μg/ml proteins were used in this experiment. The residual activities were then determined using the standard assay conditions. The effect of NaCl was determined by measuring the enzyme activity in the presence of different concentration of NaCl. Native (●), N476/245C (■), N476/404C (□), N476/477C (▲) and N476/522C (△).

Table 3
Kinetic parameters of the native and four co-refolded β-glucosidases[a]

Substrates	Native	N476/245C	N476/404C	N476/477C	N476/522C
*p*NP-β-D-glucopyranoside					
K_m (mM)	0.0039 ± 0.0012	0.0073 ± 0.0006	0.0096 ± 0.0006	0.0137 ± 0.0010	0.0088 ± 0.0007
k_{cat} (s^{-1})	6.4 ± 0.1	1.51 ± 0.04	1.46 ± 0.03	5.44 ± 0.14	1.38 ± 0.02
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	1640	207	152	397	157
*p*NP-β-D-xylopyranoside					
K_m (mM)	2.64 ± 0.12	3.87 ± 0.29	3.82 ± 0.34	6.74 ± 0.50	4.12 ± 0.40
k_{cat} (s^{-1})	18.4 ± 0.4	8.5 ± 0.3	5.90 ± 0.25	15.0 ± 0.7	6.21 ± 0.31
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	6.96	2.20	1.54	2.23	1.51

Standard errors are within 10% of the given values.
[a] No significant catalytic activity by the co-refolded enzymes were observed to the following substrates: *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-β-D-galactopyranoside.

mal stability experiments revealed that four co-refolded enzymes maintained its activity (up to 80%) even at 70 °C and completely inactivated at 90 °C, similar to the native enzyme. Little difference in temperature optimum of the co-refolded enzymes could be due to the presence of weak ionic interactions between the two polypeptide fragments compared to the native enzyme.

The effect of NaCl on the activity of co-refolded enzymes was investigated under the standard assay condition at different salt concentrations. No significant difference in the enzyme activity was observed below 100 mM NaCl but strong inhibition was observed at higher concentration such as 1 M (Fig. 4C) suggesting that the co-refolded enzymes have weak ionic and hydrogen bonding interaction than the native enzyme [32,33]. Previous studies showed that chimera shuffled in the C-terminal domain tend to fold and produce catalytically active enzymes while those shuffled in the N-terminal domain tend to loose their folding ability and form inclusion bodies which are catalytically inactive.

3.4. Co-refolded fragments retain substrate specificity and transglycosylation activity

The substrate specificity of the co-refolded and native enzymes was studied using various aryl glycosides as substrates (Table 3). The observed K_m values of all co-refolded enzymes were only two- to four-fold higher than that of the native enzyme towards two substrates, *p*NP-β-D-glucopyranoside and *p*NP-β-D-xylopyranoside. It is of interest that the k_{cat} value of N476/477C (complement peptide) was very close to the native enzyme toward both substrates compared to the overlapped and truncated enzymes. These results, suggest that splitting the gene at the C-terminal domain does not significantly affect the catalytic efficiency and that these regions may not play a significant role in defining the enzyme's substrate specificity. However, the catalytic efficiency of the enzyme is inhibited in the co-refolded enzymes of overlapping or truncation type, N476/245C, N476/404C and N476/522C. Co-refolded enzymes did not show any activity towards *p*NP-β-D-fucopyranoside, *p*NP-β-D-arabinofuranoside and *p*NP-β-D-xylopyranoside.

One unique characteristic feature of *T. maritima* β-glucosidase is its transglycosylation activity in the presence of alcohols. In fact, many enzymes in the glucosidase family 3 do not possess any transglycosylation activity. To investigate the effect of co-refolding on the transglycosylation activity, enzyme activity was determined in the presence of a series of straight-chain alcohols. It was demonstrated that as the chain length of the alcohol was increased, the rate of release of *p*-nitrophenol was also elevated. Maximal enzyme activation was achieved in the presence of 20 mM heptanol for both the native and co-refolded enzymes at 30 °C, suggesting that the transglycosylation activity was not affected in the co-refolded enzymes (Fig. 5).

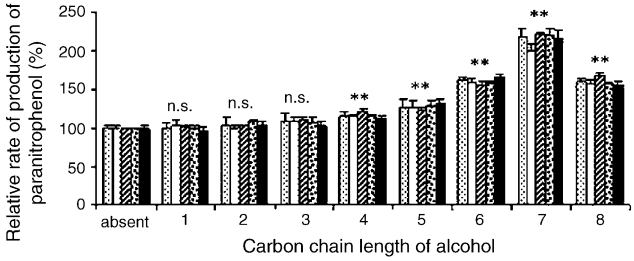

Fig. 5. Relative rates of the production of *p*-nitrophenyl from *p*NP-β-D-glucopyranoside with native and four co-refolded enzymes in the presence of 20 mM of the straight chain alcohols at 30 °C. n.s., non-significant; ** significantly different ($p < 0.01$) from the value of native and co-refolded enzymes. In each case 10 μg/ml proteins were used in this experiment. Native (⁘), N476/245C (▭), N476/404C (▨), N476/477C (▩) and N476/522C (■).

4. Conclusions

This fragment complementation study confirms that complete set of the amino acid residues in the N-terminal domain are important in the co-refolding of the two peptide fragments into catalytically active form. C-terminal domain is less significant in terms of enzyme folding. The non-homologous region plays an important role in bringing the other catalytic residue located on the C-terminal domain to the close proximity of substrate binding pocket.

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