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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.

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