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Halophilic characterization of starch-binding domain from *Kocuria varians* α-amylase

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ABSTRACT

The tandem starch-binding domains (KvSBD) located at carboxy-terminal region of halophilic α-amylase from moderate halophile, *Kocuria varians*, were expressed in *E. coli* with amino-terminal hexa-His-tag and purified to homogeneity. The recombinant KvSBD showed binding activity to raw starch granules at low to high salt concentrations. The binding activity of KvSBD to starch was fully reversible after heat-treatment at 85 °C. Circular dichroism and thermal scanning experiments indicated that KvSBD showed fully reversible refolding upon cooling after complete melting at 70 °C in the presence of 0.2–2.0 M NaCl. The refolding rate was enhanced with higher salt concentration.

1. Introduction

Halophilic and halo-tolerant microorganisms have been the resource of halophilic enzymes that can function in the presence of high salt concentrations or under low-water activity conditions often associated with inclusion of organic solvents, surfactants and detergents, under which most "normal" enzymes become non-functional [1–3]. We have been studying the distinct characteristics of halophilic proteins, including nucleoside diphosphate kinases [4–7], β-lactamase [8,9] and α-amylase secreted from *Kocuria varians* [10]. The most striking features of halophilic proteins, especially the secretory proteins present in the periplasmic space or the culture medium of moderate halophiles, are their high aqueous solubility in both the native and denatured structures and the high reversibility of refolding from the denatured states without forming aggregation [6,8]. These halophilic properties, i.e., the ability to function in the wide range of salt concentrations or tolerate to the conditions of low-water activity, and high reversibility of refolding, make halophilic proteins attractive for the industrial applications.

Several halophilic and halo-tolerant amylolytic and pullulytic enzymes were isolated from extremely and moderately halophilic microorganisms [10–14] and poly-extremophilic microorganisms, e.g., thermophilic halophile [15–17]. We have previously reported the cloning and expression of the gene coding for α-amylase (KVA) from moderate halophile, *K. varians*, isolated from soy sauce mash [10]. In addition to the α-amylase catalytic region, we found two tandem domains, each consisting of about 90 amino acid residues, at carboxy-terminal portion of this halophilic α-amylase. The amino acid sequence of the tandem domains showed high similarity with the carboxy-terminal starch-binding domain (SBD) of *Bacillus* sp. 195 α-amylase, which is classified carbohydrate binding module (CBM) family 25 [18,19]. CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity (CAZy, http://www.cazy.org/) [20]. KVA was found to be unable to digest raw starch granules, when this carboxy-terminal region was deleted [10]. The amino acid composition of this region revealed typical acidic characteristics of halophilic proteins [10]. These biochemical data and the sequence similarity of this region with SBDs from other bacteria [18,21] strongly suggested that these tandem domains function as a halophilic starch-binding module, which allows KVA to bind and digest raw starch granules in the salinity environments. Here, we carried out the recombinant expression of

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Table 1
Properties of KvSBDs, BaSBDs and BhCBM25.

SBD	Total residues	Asp + Glu	Lys + Arg	Acidic/basic ratio	Cal. pI	[a]Chance of sol./insol. prediction (%)	Identity to KvSBD1 (%)	Identity to KvSBD2 (%)
KvSBD1	90	9 + 6	0	Infinity	3.4	97.9 sol.	100	79.5
KvSBD2	88	7 + 8	0	Infinity	3.0	97.0 sol.	79.5	100
BaSBD1	89	3 + 0	2 + 1	1.0	7.1	97.3 insol.	43.4	46.7
BaSBD2	91	3 + 0	2 + 2	0.8	8.2	97.8 insol.	65.1	61.1
BhCBM25	95	6 + 5	2 + 2	1.6	5.3	63.2 insol.	36.3	41.8

[a] Chance of solubility was predicted according to Wilkinson and Harrison [23]. Sol, soluble; insol, insoluble.

this region in *E. coli* to examine the starch-binding activity and its halophilic characteristics.

2. Materials and methods

2.1. Bacterial strains and plasmid constructions

K. varians ATCC21971 and *Bacillus* sp. no. 195 strains were used to isolate their gene regions for α-amylases and starch binding-domains. *E. coli* BL21(DE3) was used for recombinant protein expression.

For the expression in *E. coli* of tandem starch binding-domain from *K. varians* α-amylase with N-terminal hexa-His-tag (His-KvSBD12), tandem KvSBD12 gene region was amplified by PCR from a plasmid DNA pBR322-kva encoding whole *kva* gene [10] with forward primer (5′-TATACATATGGGCACCGGCTCCGGCGACGA-3′) and reverse primer (5′-TTTCTCGAGTCAGCAGGGACTGCCTGCGGT-3′). The amplified fragment was digested with *Nde*I/*Xho*I and ligated to *Nde*I/*Xho*I-digested pET15b (Novagen) to construct pET15b-SBD12. To express KvSBD1 with hexa-His-tag (His-KvSBD1), KvSBD1 gene region was amplified by PCR with forward primer (5′-GGAATTCCATATGCTCGCGCTGCACTACGCCGCCGAC-3′) and reverse primer (5′-AAACTCGAGTCAGCAGGGATCGCCCTCGCT-3′) and ligated to pET15b as above to construct pET15b-SBD1.

For the expression of tandem SBD region (BaSBD12) from non-halophilic *Bacillus* sp. no. 195 α-amylase [18], which shows the highest sequence similarity to KvSBD region by database examination, halophilic fusion protein expression vector pBF [22] was used to express His-β-lactamase-BaSBD12 fusion protein (His-BLA-BaSBD12), since BaSBD12 region was predicted to lead to insoluble expression in *E. coli* cells (Table 1) [23]. Gene region for BaSBD12 was amplified by PCR from a plasmid DNA encoding whole *Bacillus* α-amylase gene with forward primer (5′-AAACCCGGGGTGTACTACTCGACGTCGAAGGGCTGGAGC-3′) and reverse primer (5′-AAAACTAGTTCAGGCGCAGGGGTTTCCCGTGCT-GACCAC-3′). The amplified fragment was digested with *Sma*I/*Spe*I and ligated to *Sma*I/*Spe*I-digested pBF to construct pBF-BaSBD12. KvSBD12 was also expressed as a BLA-fusion protein as follows: KvSBD12 region was amplified by PCR with forward primer (5′-AAAGGTACCGGCACCGGCTCCGGCGACGAC-3′) and reverse primer (5′-AAAACTAGTTCAGCAGGGACTGCCTGCGGT-3′) and ligated to *Kpn*I/*Spe*I-digested pBF to construct pBF-KvSBD12.

2.2. Expression and purification of His-SBD and His-BLA-SBD fusion proteins

Expression vector, pET15b-SBD12, was transformed to *E. coli* BL21(DE3) and His-KvSBD12 protein was expressed in LB-ampicillin (100 μg/ml) with 0.1 mM isopropyl-1-thio-β-galactopyranoside at 18 °C. Harvested cells from 250 ml culture were sonicated in 25 ml of 50 mM Na-phosphate buffer, pH 7.2, containing 0.15 M NaCl. After centrifugation at 12,000 × *g* for 15 min, the supernatant was diluted twice with the same buffer solution and filtrated by 0.45 μm membrane filter. Imidazole (final concentration 20 mM) was added to filtrate and the sample was applied to His Trap FF column (1 ml resin, GE Healthcare) equilibrated with 50 mM Na-phosphate buffer, pH 7.2, containing 20 mM imidazole and 0.15 M NaCl. After extensive washes, the bound proteins were eluted with a step wise of imidazole (each 3 ml of 50, 100, 200 and 300 mM) in the same buffer. The major fractions of His-KvSBD12 protein was diluted twice with distilled water and applied to Resource Q column (GE Healthcare). His-KvSBD12 was eluted with a linear NaCl gradient of 0–0.8 M. His-KvSBD1 was also purified by the same protocol as described above using *E. coli* BL21(DE3) harboring plasmid pET15b-SBD1.

His-BLA-KvSBD12 and His-BLA-BaSBD12 fusion proteins were purified as follows. Homogenate preparations and Ni-column chromatography were performed in the same way as described above. The major fractions of fusion protein were adjusted to NaCl concentration at 1.0 M and applied to Dextrin Sepharose High Performance column (GE Healthcare) equilibrated with 50 mM Na-phosphate buffer, pH 7.2, containing 1.0 M NaCl. The column was extensively washed with same buffer and the bound fusion protein was eluted from resin with 0.1 M maltose in the same buffer. The purified fusion protein thus obtained was extensively dialyzed against 50 mM Tris–HCl buffer, pH 7.0.

2.3. Binding assay

The starch-binding isotherms were used to calculate binding parameters, K_d and B_{max} (maximum amount of bound protein) of His-KvSBD12 according to the methods of Paldl et al. [24] and Swillens [25]. Wheat raw starch was washed with distilled water several times and dried at room temperature. His-KvSBD12 (1–10 μM) was incubated at 25 °C for 1 h in the binding assay mixture (100 μl) containing 2 mg of wheat raw starch, 50 mM Tris–HCl, pH 7.0, and 0.2 or 2.0 M NaCl. After centrifugation to precipitate raw starch, protein amount in supernatant was measured by BCA protein assay [26]. GraphPad Prism version 5.04 (GraphPad Software, Inc.) was used for data analyses.

The binding assay of His-BLA-KvSBD12 and His-BLA-BaSBD12 in the presence of 0–2.0 M NaCl was carried out as follows: fusion proteins (10 μM of SBD regions/100 μl assay mixture) was incubated at 25 °C for 1 h in 50 mM Tris–HCl buffer, pH 7.0, 2 mg of wheat raw starch and 0–2.0 M NaCl. Protein amount in supernatant after centrifugation was measured by Pierce 660 nm protein assay (Thermo Scientific).

2.4. Thrombin-digestion of BLA-SBD fusion proteins and heat-treatment

His-BLA-SBD fusion protein (1.6 mg) was digested with 32 unit of bovine thrombin (GE Healthcare) at 22 °C for 16 h in the reaction mixture (400 μl) containing 50 mM Tris–HCl buffer, pH 8.0, 0.1 M NaCl and 1 mM $CaCl_2$.

The digested samples (0.5 mg protein/ml) were heat-treated at 85 °C for 5 min, cooled on ice for 10 min, and centrifuged at 16,000 × *g* for 10 min to remove aggregated proteins. The