Investigations on Possible Roles of C-Terminal Propeptide of a Ca-Independent α-Amylase from Bacillus

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Previously, an extracellular α-amylase (BKA) had been purified from the culture of Bacillus sp. KR8104. Subsequently, the crystal structure of the active enzyme revealed a 422 amino acids polypeptide. In this study, the bka was cloned into E. coli, which encoded a polypeptide of 659 amino acids including two additional fragments: one 44 residues N-terminal fragment and another 193 residues C-terminal fragment. In order to investigate the role of the C-terminal fragment, two constructs with and without this region [BKA∆(N44) and BKA∆(N44C193)] were designed and expressed in E. coli BL21. The optimum pH, thermal stability, and the end-products of starch hydrolysis were found to be similar in both constructs. The K_m and V_max values for BKA∆(N44) were lower than BKA∆(N44C193), using either starch or ethylidene-blocked 4-nitrophenyl-maltoheptaoside as a substrate.

Keywords: α-Amylase, Bacillus sp. KR-8104, biochemical characterization, secretion, truncation

α-Amylase (α-1,4-glucan-4-glucanohydrolases, E.C. 3.2.1.1) is an endo-acting enzyme that catalyzes the hydrolysis of α-1,4-glycosidic linkages in starch and related oligosaccharides [4, 19]. Although amylases can be isolated from different sources, including plants, animals, and microorganisms, microbial enzymes, especially those obtained from the genus Bacillus, have been widely used for the commercial production of the enzyme [9, 15]. X-Ray crystallographic studies have shown that α-amylases have three domains, named A, B, and C. The central (α/β)_8-barrel, domain A, forms the core of the molecule and contains the active site of the enzyme. Domain B, a protrusion between the third strand and the third helix of the (α/β)_8-barrel, is probably responsible for the differences in stability and substrate specificity. Domain C is arranged at a very common structural motif known as the Greek key [7].

Bacillus sp. KR-8104, an aerobic bacterium growing optimally at 37°C, has been previously isolated and purified, and its crystal structure has been deposited into a protein data bank (PDB ID code 3DC0). With regard to the crystal structure of the secreted protein in the medium, we identified a truncated C-terminal region. Cloning, expression, and purification of different forms of BKA in E. coli were conducted in order to identify the role of the BKA C-terminal region. The effects of the C-terminal region on the enzyme activity, thermostability, and substrate specificity were investigated by comparing two truncated forms of the enzyme. In addition, the possible function of the C-terminal in enzyme secretion is discussed.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from the corresponding companies: yeast extract, tryptone, and agar (Liophilchem, Italy); Tris (Applichem GmbH, Germany); starch, 3,5-dinitrosalicylic acid (DNS), ethylidene-blocked 4-nitrophenyl-maltoheptaoside (EPS), different oligosaccharides G1–G7, ethylenediaminetetraacetic acid (EDTA), and all chemicals for gel electrophoresis (Sigma, USA); phenylmethyl-sulfonyl fluoride (PMSF) (Merek, Germany); Ni-NTA column (Qiagen, Germany); agarose, IPTG, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), kanamycin, and PCR reagents (Fermentas, EU); and restriction enzymes and T4-DNA ligase were purchased from Roche (Germany). All other chemicals used were of analytical grade.

Media, Bacterial Strains and Plasmids

The growth media for microorganisms were YNA (Yeast extract, 5 g/l; Nutrient agar, 28 g/l, and Agar, 8 g/l) and Luria Broth (LB)
Cloning of Complete Bacillus sp. KR-8104 α-Amylase Gene

Genomic DNA from Bacillus sp. KR-8104 was isolated by a high pure PCR template preparation kit (Roche, Germany). Two oligonucleotides were designed and synthesized based on the sequence of α-amylase from Bacillus subtilis (GenBank Accession No. EF051632.1 and AF116581.1). An approximately 2 kb DNA fragment was obtained using the following two primers: BSUA-F (5'-GGTGCAAGAATGTTGCAAAC-3') and BSUA-R (5'-CCTCAATGGGGAAGAGAACGCTTAATC-3'). Bacillus sp. KR-8104 chromosomal DNA was used as a template. Then, the desired fragment was extracted from the gel with a PCR purification kit (Bioneer, Korea). The extracted fragment was subsequently ligated to pTZ57R/T and transformed into E. coli XL-1 blue by electroporation method [23]. White colonies on LB agar medium supplemented with ampicillin (100 mg/ml), IPTG (100 mM), and X-gal (20 mg/ml) were selected. The resulting recombinant plasmid was used for sequencing. The complete nucleotide sequence has been submitted to the GenBank database and assigned under accession number EU717848.3.

Subcloning of Different Fragments of “bka” Gene

The pTZ57R/T containing the complete α-amylase gene was used as a template in a PCR reaction using the following synthetic oligonucleotides: forward primer A (5'-CATGCCATGGCACCTGCAAAAC-3') and reverse primer B (5'-CCAGAAGCTTGGGGAAAGAGAACCGCTTAATC-3'), C (5'-GGAGCGTTGTCCGTTGAGCCGCAC-3'), and E (5'-CCAGAAGCTTGGGGAAAGAGAACCGCTTAATC-3') were used to clone BKA (complete gene), BKA (C20), and BKA (MC193), respectively. The restriction enzyme cutting sites are underlined. The PCR profiles were as follows: 95°C for 5 min, 95°C for 45 s, 65.5°C for 1 min, and 72°C for 90 s for 35 cycles, and 72°C for 5 min. Amplified fragments were cloned into HindIII and Ncol sites of pET28-a (+) using T4-DNA ligase. The ligation mixture was transformed into E. coli XL-1 blue by a chemical method [3]. A master plate was prepared from all the cells possessing plasmid. Colonies containing the insert were chosen by the cracking method [kuchem.kyoto-u.ac.jp/seika/shiraishi/protocols/cracking.html]. Colonies were cultured in LB medium and purified by a Plasmid Mini Extraction kit (Bioneer, Korea). Plasmids including the gene were checked by restriction enzymes (Ncol and HindIII) digestion. The sequences were verified by sequencing and then transformed into E. coli BL21.

Subcloning of the BKAαN44C193 Gene

The pTZ57R/T vector containing the complete bka gene was used as a template for PCR. The BKAαN44C193 fragment was amplified using the upstream primer F (5'-CATGCCATGGGGCCATCAATGCA-3') and downstream primer E to generate a fragment of approximately 1.3 kbp. The resulting PCR product was digested with HindIII and Ncol and subcloned into pET28-a (+). The ligation reaction was carried out in the presence of T4-DNA ligase. The ligation mixture was then transformed into E. coli XL-1 blue. The product obtained by plasmid extraction from one of the recombinant colonies was transformed into E. coli BL21 (DE3). Subsequently, a bacterial colony containing the recombinant plasmid was chosen to express the protein of interest.

Expression and Purification of Different Forms of BKA in E. coli BL21 (DE3)

Cells containing the recombinant plasmids were grown at 37°C in 250 ml of LB medium supplemented with 50 mg/ml kanamycin to an OD600 of 0.5–0.7. IPTG was added to a final concentration of 1 mM to induce expression of α-amylase. After 6 h incubation at 25°C and 225 rpm, cultures were centrifuged at 7,000 rpm for 20 min at 4°C. The cells were then resuspended and sonicated in the lysis buffer (50 mM Tris-HCl, 300 mM NaCl, and 20 mM imidazole, pH 8.0) with a High Intensity Ultrasonic (800 W). The extract was centrifuged at 7,000 rpm and 4°C for 20 min to remove cell debris. In order to purify the his-tagged fusion protein, the culture broth obtained from the first centrifugation step was applied to a Ni-NTA column equilibrated with lysis buffer. The crude extract was loaded onto a Ni-NTA column and washed with 20 ml of washing buffer (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, pH 8.0). Proteins were then eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fractions containing α-amylase activity were pooled and dialyzed against 20 mM Tris-HCl, pH 7.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [11]. A high-molecular-weight marker (Fermentas, EU) was used and protein bands were stained with Coomassie brilliant blue R-250.

Biochemical Characterization and Determination of Protein Concentration

α-Amylase activity was determined in a reaction containing 25 µl of enzyme and 475 µl of starch 1% (w/v) as a substrate in Tris-HCl buffer, pH 7.2. The mixture was incubated at 50°C for 5 min using the DNS method [17]. One unit of α-amylase is defined as the amount of enzyme that liberates 1 µmol reducing sugar per minute under the assay conditions with maltose as a standard. α-Amylase activity was also assayed at 37°C, using ethylidene-blocked 4-nitrophenylmaltoheptaoside (EPS) with α-glucosidase as substrate at pH 7.2 in 20 mM Tris buffer. The initial rate (during the first 3 min after a 5 min delay time) was taken to measure enzymatic activity. The amount of p-nitrophenol was monitored at 405 nm and calculated using the molar extinction coefficient of 10.13 mM−1 cm−1 [10]. Protein concentration was determined using the Bradford method [1]. In all experiments, the enzyme was used at a concentration of 5 µg/ml. The effect of pH on α-amylase activity (BKAαN144C193 and BKAαN44) was examined in the pH range of 3–10 using a mix buffer system (50 mM glycine-HCl, pH 2–4; 50 mM sodium acetate, pH 4.0–6.0; 50 mM sodium phosphate, pH 5.8–8; glycine-NaOH buffer, pH 8.0–10.6) at 50°C. The enzyme activity was assayed by the DNS method. Kinetic parameters (Vmax, kcat, kcat/Km) for purified BKAαN144C193 and BKAαN44 were determined by using the Lineweaver–Burk plot. The results are mean value of three independent experiments and were repeated.