

## C-Terminal Propeptide of BKA has a Protease Sensitive Structure Without any Inhibitory Effect on BKA

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### ABSTRACT

In our previous study, we compared the two  $\alpha$ -amylase enzymes from *Bacillus* sp.KR8104, BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193) which is the secreted form of it. The results indicated that the presence of 193 amino acids propeptide in the C-terminal of BKA $\Delta$ (N44) changed its enzymatic parameters like an uncompetitive inhibitor in comparison to BKA $\Delta$ (N44C193). In the present study, we cloned the DNA sequence of BKA $\Delta$ (N44) which codes the 193 amino acids propeptide in its C-terminal and the effect of this fragment as an inhibitor on BKA $\Delta$ (N44C193) was investigated. We also studied the possible foldase activity of the propeptide in BKA $\Delta$ (N44C193). Protease sensitivity of C-terminal 193 amino acid propeptide, BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193) was compared in order to explain why BKA $\Delta$ (N44C193) is the only secreted form of  $\alpha$ -amylase in the culture medium of *Bacillus* sp.KR8104. Circular dichroism indicated that the secondary structure of the C-terminal is mostly beta sheeted. At the end we proposed a possible regulatory role for the C-terminal propeptide of BKA.

**Keywords:**  $\alpha$ -Amylase, *Bacillus*, C-Terminal propeptide, Foldase activity, Uncompetitive inhibitor

### INTRODUCTION

In our previous studies we purified a 46 kDa protein with alpha amylolytic activity from *Bacillus* sp. KR8104 culture medium and crystallized it (PDBID: 3DC0). The purified  $\alpha$ -amylase is consisted of three domains (domains A, B, and C) like many other  $\alpha$ -amylases [11,14,18]. Catalytic domain A is made of a  $(\beta/\alpha)_8$  barrel structure, followed by a domain consisting antiparallel  $\beta$ -strands (domain C) with structural motif known as the Greek key [7]. A smaller domain (domain B) is present as a loop between the third  $\beta$ -strand and the third  $\alpha$ -helix of the  $(\beta/\alpha)_8$  barrel. In addition to these three domains, some  $\alpha$ -amylases such as  $\alpha$ -amylase from psychrophilic organism, *Alteromonas haloplanctis* [6], *Pseudomonas stutzeri* [16] and barley [21] have an extra truncatable portion at their carboxyl-terminal region which have known or unknown roles. Experimental data of the mentioned  $\alpha$ -amylases

showed that most of the carboxyl-terminal truncated enzymes retained the same level of their original activity after truncation [13,16,21]. The amino acid sequence obtained from X-ray crystallography of our purified enzyme (named BKA $\Delta$ (N44C193)) indicated that the crystallized enzyme has 422 amino acids [18]. Polymerase chain reaction by using *Bacillus* sp. KR8104 genomic DNA as template and primers based on *Bacillus subtilis amy* gene with GenBank accession No. EF051632.1 amplified an approximate 2 kb DNA fragment [18]. The nucleotide sequence of this DNA fragment that encodes an open reading frame (ORF) named *bka* (GenBank accession No. AF116581.1). Deduced amino acid sequence of *bka* showed that BKA is a polypeptide with 659 residues in its open reading frame. Amino acid sequence comparison between BKA and *Bacillus subtilis*  $\alpha$ -amylase (the most similar amino acid sequence to BKA) indicated that the first 33 amino acids in N-terminal of BKA probably comprises a signal peptide for secretion [18,19,20] and this signal is cleaved after secretion of the protein [22]. Remaining 11 residues from the cleavable N-terminal peptide of BKA will

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probably digested by proteases in culture medium just like digestion of the remaining amino acids after the signal peptide of *Bacillus subtilis*  $\alpha$ -amylase [22]. In our previous studies *bka* with deleted 44 residues in its N-terminal named *bka* $\Delta$ (N44) and *bka* with deleted 44 residues in N-terminal and 193 residues in its C-terminal named *bka* $\Delta$ (N44C193) were cloned and expressed in *E.coli*. Comparison of BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193) indicated that the presence of 193 residues in the C-terminal of BKA $\Delta$ (N44) has no effect on thermostability, product specificity, catalytic efficiency, EDTA sensitivity and its optimum pH relative to BKA $\Delta$ (N44C193) [18]. Nevertheless,  $K_m$  and  $k_{cat}$  of BKA $\Delta$ (N44) is less than BKA $\Delta$ (N44C193). It seems that presence of 193 amino acids in the carboxyl terminal of BKA $\Delta$ (N44) inhibits the enzymatic activity like an uncompetitive inhibitor. Here we cloned the DNA sequence that encodes carboxyl terminal 193 amino acid residues of BKA $\Delta$ (N44) alone and the effect of C-terminal as an inhibitor on BKA $\Delta$ (N44C193) activity was investigated. It has been approved that there are some truncatable carboxyl terminal portions in some proteins with proven chaperon like activity (foldase activity) [3] and their function is known as intramolecular chaperon. In order to prove whether the 193 amino acids carboxyl terminal of BKA is an intramolecular chaperon or not, refolding of BKA $\Delta$ (N44C193) in the presence and absence of C-terminal fragment was investigated. We determined the secondary structure of C-terminal fragment by circular dichroism spectroscopy. Protease sensitivity of C-terminal fragment relative to BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193) was also investigated in this study.

## MATERIALS AND METHODS

### Primers Design

Primers were designed according to the published sequence for *bka*. The forward primer, C-terminal F: 5'GGAATTCCATATGGATATTGCAAATGCGCCTCATG TC3' contained *NdeI* recognition site.

Reverse primer, C-terminal R: 5'CGCAAGCTTATGGGGAAGAGAACCGCTTAATC3' contained recognition site for *Hind III*.

The restriction enzyme sites (underlined) were added to the primers for subsequent cloning procedure.

### Gene Amplification and Cloning

PCR was performed in a 50  $\mu$ l total volume containing 1  $\mu$ g of template DNA (pET28a(+)) containing BKA $\Delta$ (44) encoding DNA, 1.5  $\mu$ M of each primer, 1.5 mM MgCl<sub>2</sub>, 150  $\mu$ M dNTP, 1X PCR buffer and 1.5 unit of Taq DNA polymerase (Sigma). The following conditions were used for amplification: initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min. The program followed by a final extension at 72 °C for 6 min. The PCR product was analyzed by electrophoresis in 1% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified by High pure PCR product purification kit (Bioneer, Korea) according to the manufacturer recommendation. The PCR products were digested with *NdeI* and *HindIII* and ligated to pET28a(+) (digested by the same restriction enzymes) using T4 DNA ligase (Invitrogen) at 14 °C overnight. *E.coli* BL21(DE3) competent cells were prepared by calcium chloride method and were used for transformation of recombinant pET28a(+) vector that had C-terminal coding DNA as insert. The transformed bacteria were selected by screening the colonies on LB media containing 50  $\mu$ g ml<sup>-1</sup> Kanamycin. The grown colonies were further analyzed by plasmid extraction, restriction enzyme digestion and PCR. Sequence analysis confirmed the correctness of the cloning procedure.

### Gene Expression and Protein Purification

The overnight culture in Luria-Bertani (LB) medium was diluted 1:100 with fresh media containing 0.05 mg ml<sup>-1</sup> kanamycin and incubated at 37 °C with shaking (180 rpm). After cells grew to an OD<sub>600</sub> of 0.5-0.6, the cells were induced with 1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG) and incubated at 37 °C. Cells were harvested before induction and also at times 4, 8 and 20 hours after induction by IPTG with centrifugation (8,000  $\times$  g, 15 min and 4 °C). Proteins in supernatant of the bacterial culture medium were precipitated by addition of ammonium sulfate salt to the crude culture supernatant to 85% saturation level at 4 °C for 2 h and analyzed by SDS-PAGE after concentration and desalination by dialysing in 20 mM Tris-HCl buffer, pH 7.4 in the presence of 1 mM protease inhibitor, phenylmethylsulphonyl fluoride (PMSF). The pellets were

suspended in 20 mM Tris-HCl buffer, pH 7.4, which also contained 1 mM PMSF and then sonicated on ice. Disrupted cells were removed by centrifugation ( $18,000 \times g$ , 15 min and  $4^\circ\text{C}$ ) and analyzed by SDS-PAGE. Soluble fractions after centrifugation were also analyzed by SDS-PAGE. Soluble fractions after sonication as the source of protein were loaded on a Ni Sepharose column (QIAGEN) ( $1\text{ cm}^3$ ) with flow rate of  $0.5\text{ ml min}^{-1}$  that had been equilibrated with 1 mM PMSF, 20 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, pH 7.4 and washed with washing buffer that contained 1 mM PMSF, 20 mM Tris-HCl, 300 mM NaCl, 30 mM imidazole, pH 7.4 with flow rate of  $2.5\text{ ml min}^{-1}$  and eluted with 6 ml elution buffer that contained 1 mM PMSF, 20 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 7.4 with flow rate of  $5\text{ ml min}^{-1}$ . Fractions were collected in 1 ml volumes and analyzed by SDS-PAGE.

### SDS-PAGE Analysis and Determination of Protein Concentration

Production, purity, relative molecular mass and protease digestion of the proteins were determined by SDS-PAGE (12% acrylamide gel) as described by Laemmli [12]. Samples were prepared in the presence of 5%  $\beta$  mercaptoethanol and 3% SDS by boiling at  $100^\circ\text{C}$  for 5 min. The gel was stained by Coomassie Brilliant Blue R-250 [24]. Solutions were dialyzed against 20 mM Tris-HCl buffer, pH 7.6, in order to remove their salts. Protein concentration was determined by the Bradford method [2].

### Circular Dichroism Spectroscopy

Circular dichroism spectra were recorded in a 0.2 cm path length cell under constant nitrogen flush using a Jasco 700 CD<sub>6</sub> dichrograph.

### Determination of $\alpha$ -Amylase Activity

$\alpha$ -Amylase activity was determined at  $50^\circ\text{C}$  in a 1 ml reaction mixture that contained 0.5 ml of 1% (w/v) potato starch solution in 20 mM Tris-HCl, pH 7.4.

The concentration of reducing sugars obtained from the catalyzed reaction for 3 min was measured by dinitrosalicylic acid (DNS) method according to Bernfeld [1]. One unit of  $\alpha$ -amylase was defined as the amount of enzyme that liberates  $1\ \mu\text{M}$  of reducing sugar/min with maltose as a standard.

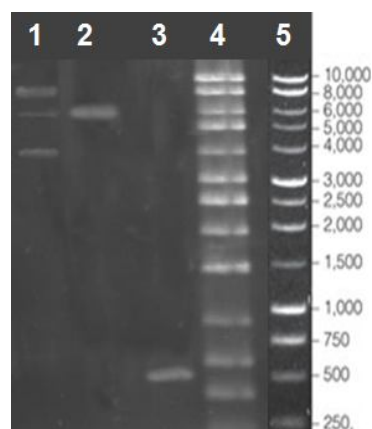
### Refolding of Unfolded Enzyme

Unfolding of BKA $\Delta$ (N44C193) was done by dissolving enzyme in 8 M urea. Refolding of unfolded enzyme was done by dilution of unfolded enzyme in Tris-HCl 20 mM pH 7.4 buffer until a negligible concentration of urea. After different times of incubation (1, 5 and 30 min), activity of the refolded enzyme was determined and compared with activity of intact BKA $\Delta$ (N44C193) with respect to its dilution factor.

## RESULTS

### Expression of C-Terminal Fragment and Investigation of its Secretion

Amplified DNA segment which encodes C-terminal was cloned into pET28a(+) vector (Fig. 1) downstream of T7 promoter and there was a six histidine residues tag at its carboxyl terminal which allowed its purification by affinity chromatography. Expression of C-terminal was examined at  $37^\circ\text{C}$  by adding IPTG to the final concentration of 1 mM.



**Fig. 1.** Lane 1: pET28a(+) with inserted C-terminal DNA coding sequence between *NdeI* and *HindIII* restriction sites of pET28a(+) in its multiple cloning site; lane 2: pET28a(+) with inserted C-terminal DNA coding sequence between *NdeI* and *HindIII* restriction sites of pET28a(+) in its multiple cloning site which is digested with *HindIII*; lane 3: Amplified DNA segment which encodes C-terminal; lane 4: 1Kb DNA ladder; lane 5: DNA size (bp) for each band of DNA ladder.

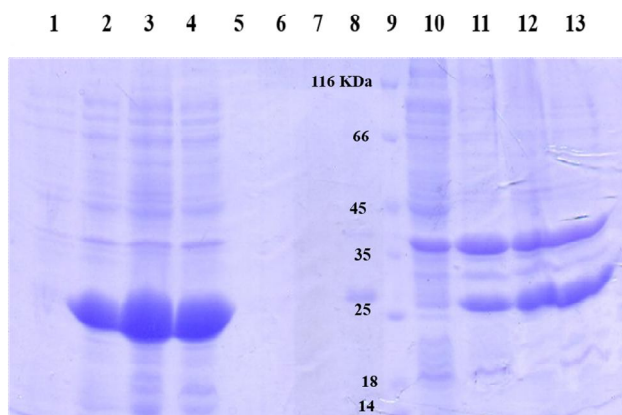
The presence of expressed C-terminal in different fractions of *E. coli* BL21 culture (culture medium, insoluble fraction after sonication, soluble fraction after sonication) was investigated at different times (before induction, 4, 8 and 20 h after induction). Different fractions of *E. coli* BL21 culture which didn't have the amplified DNA as insert in pET28a(+) were investigated under the same conditions as the controls (Fig. 2). The results indicated that C-terminal was just present in soluble fraction of *E. coli* BL21 after sonication [18]. Calculating molecular mass of the recombinant C-terminal from its amino acid sequence indicated that C-terminal is a 21 kDa protein. Purification of C-terminal protein was accomplished by means of the immobilized metal affinity chromatography (IMAC) and results are demonstrated in (Fig. 3). Secondary structure prediction by means of Wisconsin package (GCG 9.0) suggested a high content of  $\beta$ -sheet-forming residues in C-terminal and far-UV circular dichroism spectra of the protein approved this prediction too (Fig. 4). These data indicated the C-terminal as a typical  $\beta$ -pleated protein (50-70%) with a low  $\alpha$ -helical content (10%).

### C-Terminal Protein Inhibitory Function

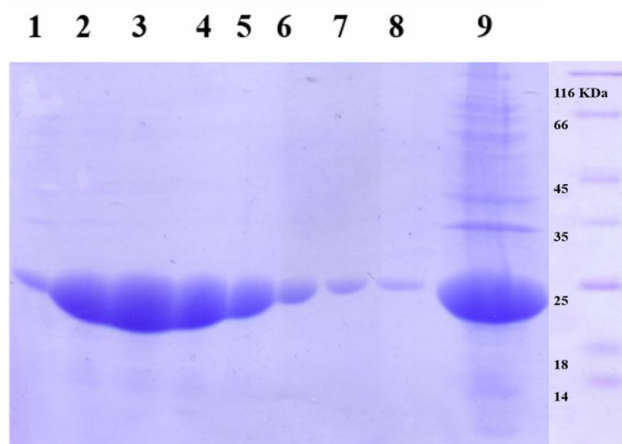
In order to study the probable inhibitory effect of the C-terminal on amylase activity as an uncompetitive inhibitor which was proposed in our previous study [18], we determined the catalytic activity of BKA $\Delta$ (N44C193) in presence of different concentrations of C-terminal fragment as inhibitor. These results indicated that C-terminal in different concentrations (0, 0.25, 2.5 and 25  $\mu\text{g ml}^{-1}$ ) and different times of incubation (1, 30, 60, 120 and 240 min) had no inhibitory effect on BKA $\Delta$ (N44C193) activity.

### C-terminal Protein Foldase Activity

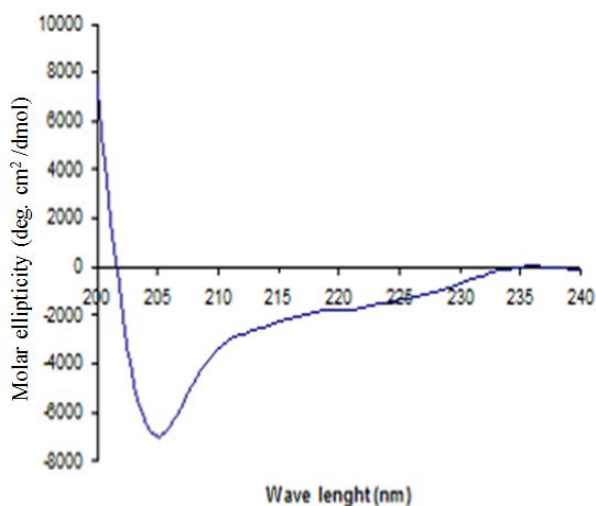
In order to investigate possible foldase activity of the C-terminal, BKA $\Delta$ (N44C193) (10  $\text{mg ml}^{-1}$ ) was first unfolded in 8 M urea. Unfolding of BKA $\Delta$ (N44C193) in the presence of 8 M urea was accepted by complete loss of enzyme activity in this concentration of urea and also confirmed by CD and fluorescence spectra (data not shown). Activity of BKA $\Delta$ (N44C193) without unfolding was also determined under the same conditions as an original activity with respect to its dilution factor (protein concentration after dilution is 0.2  $\text{mg ml}^{-1}$ ). Comparison of the enzyme activity



**Fig. 2.** Lane 1: soluble fraction (before induction); lane 2: soluble fraction at 4 h after induction; lane 3: soluble fraction at 8 h after induction; lane 4: soluble fraction at 20 h after induction; lane 5: culture supernatant (before induction); lane 6: culture supernatant at 4 h after induction; lane 7: culture supernatant at 8 h after induction; lane 8: culture supernatant at 20 h after induction; lane 9: protein molecular-weight marker ( $\beta$  galactosidase 116 kDa, Bovine serum albumin 66 kDa, Ovalbumin 45 kDa, Lactate dehydrogenase 35 kDa, REase Bsp981 25 kDa,  $\beta$  lactoglobulin, 18 kDa, Lysozyme 14 kDa); lane 10: pellet after sonication (before induction); lane 11: pellet after sonication at 4 h (without induction); lane 12: pellet at 8 h (without induction); lane 13: pellet at 20 h (without induction).



**Fig. 3.** Lane 1 to 8: purified C-terminal protein; Lane 9: Unpurified supernatant after sonication.

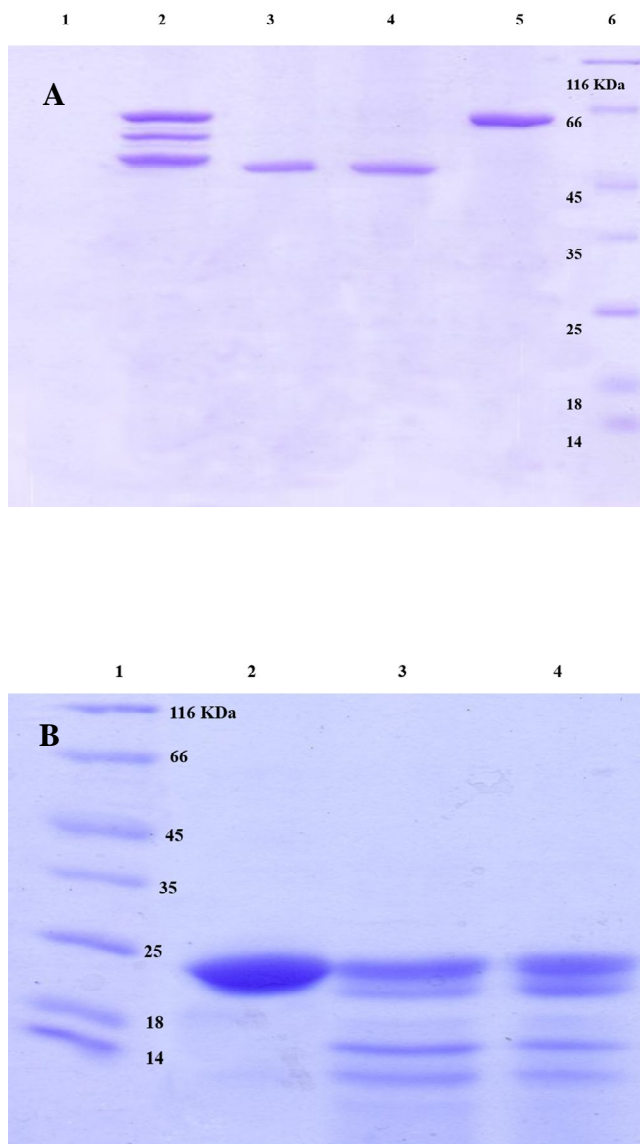


**Fig. 4.** Far-UV circular dichroism spectra of C-terminal protein in 20 mM Tris-HCl pH 7.4. Data are expressed in terms of the mean residue ellipticity,  $\theta$ .

after refolding and its original activity indicated that unfolded enzyme gains 100% of its original activity after refolding without C-terminal protein as a probable foldase. This data proposes that BKA $\Delta$ (N44C193) can fold by itself and its folding is not dependent on any foldase and the function of C-terminal as a foldase for  $\alpha$ -amylase does not seem probable.

#### C-Terminal has a Protease Sensitive Structure Relative to BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193)

C-Terminal, BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193) were partially digested with chymotrypsin and analyzed by SDS-PAGE (Fig. 5). These data indicate that C-terminal has a protease sensitive structure compared with BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193). Another interesting feature of the data is the high protease stability of BKA $\Delta$ (N44C193) relative to BKA $\Delta$ (N44). Due to high protease sensitivity of C-terminal protein and with respect to the fact that the only difference between BKA $\Delta$ (N44C193) and BKA $\Delta$ (N44) is the presence of C-terminal at carboxyl terminal of BKA $\Delta$ (N44), it seems that 193 amino acids portion in the carboxyl terminal of BKA $\Delta$ (N44) is the protease sensitive portion of it. This proposal is in good agreement with the



**Fig. 5.** A, SDS-PAGE analysis of partially digested BKA $\Delta$ (N44) and BKA $\Delta$ (N44C193). Lane 1: chymotrypsin; lane 2: Partially digested BKA $\Delta$ (N44) with chymotrypsin; lane 3: Partially digested BKA $\Delta$ (N44C193) with chymotrypsin; lane 4: Undigested BKA $\Delta$ (N44C193); lane 5: Undigested BKA $\Delta$ (N44); lane 6: protein molecular-weight marker. B, SDS-PAGE analysis of partially digested C-terminal. Lane 1: protein molecular-weight marker; lane 2: undigested C-terminal; lane 3 and 4: Partially digested C-terminal with chymotrypsin.

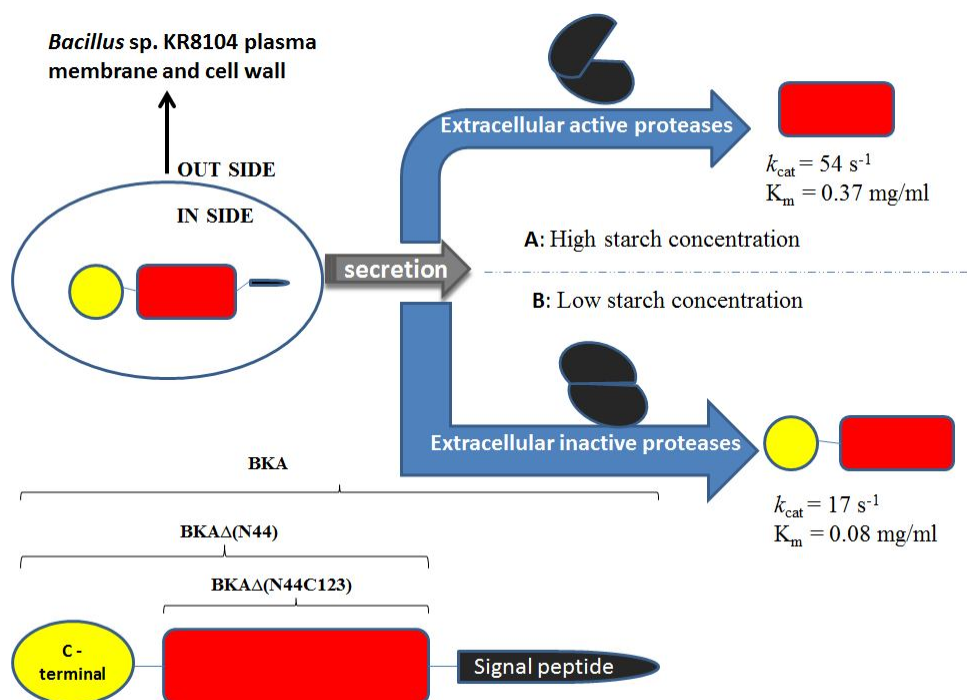


presence of a proteolysis-stable polypeptide with an equal molecular mass to BKAΔ(N44C193) according to SDS-PAGE analysis of partially digested BKAΔ(N44).

## DISCUSSION

Foldase activity or intramolecular chaperone function has been attributed to carboxyl terminal propeptides of many enzymes [5,15]. Although BKAΔ(N44C193) can fold properly in *E. coli* without any need to C-terminal but it is probable that its folding is mediated by a general chaperon in the bacteria. Our study showed that BKAΔ(N44C193) can fold properly by itself *in vitro* without any need to a foldase. So, C-terminal portion function as a foldase is not probable. We also showed that C-terminal fragment has not any inhibitory effect on BKAΔ(N44C193) activity and the reduction in  $K_m$  and  $k_{cat}$  of BKAΔ(N44) is not because of the inhibitory effect of 193 amino acids in the carboxyl terminal portion of the enzyme. It seems that the presence of 193 amino acids propeptide in the carboxyl terminal of BKAΔ(N44) will reduce  $K_m$  and  $k_{cat}$  of the enzyme with a noninhibitory mechanism. High content of  $\beta$ -sheet in secondary structure of C-terminal (50-70%) is resemble to the  $\beta$ -sheet content of secondary structure of truncatable 192 amino acids carboxyl terminal propeptide of  $\alpha$ -amylase from *Alteromonas haloplanctis* [6] that its function as a secretion helper has been proven. It has been verified that the presence of a phenylalanine or tyrosine is critical for the function of carboxyl terminal propeptides that have proven function as autotransporter domain [9] for secretion of proteins from outer membrane of gram negative bacteria [4,8,10]. Amino acid sequence comparison of C-terminal propeptide of *A. haloplanctis* and C-terminal propeptide of *Bacillus* sp. KR8104  $\alpha$ -amylase indicates that the latter has not the conserved tyrosine or phenylalanine at its carboxyl terminal. Although the C-terminal portion of BKA has not the conserved tyrosine or phenylalanine amino acids at its carboxyl terminal, our recent studies showed that it has a helper role in secretion of BKA from *E. coli* with unknown mechanism (Khajeh *et al.* unpublished data). Extracellular media of *Bacillus* sp. KR8104 is highly proteolytic like *Bacillus subtilis* [23] and our present studies indicated that C-terminal has a sensitive structure to protease digestion relative to BKAΔ(N44) and BKAΔ(N44C193). It may

explain why C-terminal was not present in secreted form of BKA from *Bacillus* sp. KR8104 that we have shown in our previous study [18]. In this stage of our studies, we showed that the truncatable, highly  $\beta$ -sheeted 193 amino acids propeptide in carboxyl terminal of BKA had not any inhibitory or foldase function on the enzyme. We showed that the reduced  $k_{cat}$  and  $K_m$  of BKAΔ(N44) ( $k_{cat} = 17 \text{ s}^{-1}$  and  $K_m = 0.08 \text{ mg ml}^{-1}$  for starch substrate) in relation to BKAΔ(N44C193) ( $k_{cat} = 54 \text{ s}^{-1}$  and  $K_m = 0.37 \text{ mg ml}^{-1}$  for starch substrate) [18] could just be observed when the C-terminal is covalently bonded to the enzyme. Furthermore, it is the first time that truncatable carboxyl terminal portion of an  $\alpha$ -amylase function as an inhibitor on activity of the enzyme has been investigated. With respect to the fact that  $k_{cat}/K_m$  of BKAΔ(N44) and BKAΔ(N44C193) are similar (142 for BKAΔ(N44) and 146 for BKAΔ(N44C193)) [18] and also the presence of C-terminal in BKAΔ(N44) decreases  $K_m$  of the enzyme for starch substrate in comparison to BKAΔ(N44C193) (about three times) with the expense of reduction in the enzyme  $k_{cat}$  (about three times), we are proposing that C-terminal might have a regulatory role for the amylase activity. It seems *Bacillus* sp. KR8104 can convert its secreted  $\alpha$ -amylase from BKAΔ(N44) form with lower  $k_{cat}$  and  $K_m$  to BKAΔ(N44C193) with higher  $k_{cat}$  and  $K_m$  by means of some extracellular proteases and using higher sensitivity of C-terminal portion of BKAΔ(N44) to proteolysis in relation to BKAΔ(N44C193) (the portion of BKAΔ(N44) which has  $\alpha$ -amylolytic activity). In this proposed model, activity of some proteases which are involved in BKAΔ(N44) process to BKAΔ(N44C193) may be regulated with the amount of starch in the extracellular medium of *Bacillus* sp. KR8104. When the concentration of starch is enough in the medium, the proteases activity degrades C-terminal and changes secreted BKAΔ(N44) to BKAΔ(N44C193) (Fig. 6A). This form of enzyme with higher  $k_{cat}$  converts starch to permeable products with ideal pace. Under these conditions the amount of starch is not rate limiting and higher  $K_m$  of BKAΔ(N44C193) has no negative effect on degrading starch. Therefore, higher  $k_{cat}$  of the enzyme makes this form of enzyme suitable for best activity under these conditions. On the other hand when starch concentration in the medium is decreased, activity of these proteases might be stopped. So the dominant form of enzyme under these conditions



**Fig. 6.** A, On condition that concentration of starch is low, extracellular proteases are inactive and the amylase enzyme is secreted in the medium in its BKA $\Delta$ (N44) form after degradation of the signal peptide (44 amino acids). B, C-terminal portion of secreted amylase enzyme is digested by extra cellular active proteases when starch concentration is high in extracellular medium of the bacteria (because of its high sensitivity to proteolysis). BKA $\Delta$ (N44C193) is the only present form of  $\alpha$ -amylase in the extracellular medium under these conditions.

would be BKA $\Delta$ (N44) (Fig. 6B). This form of enzyme is suitable for converting limited amounts of starch to permeable products in such a way that critical amounts of starch can be used for aliveness of the organism (because of the lower  $K_m$  of BKA $\Delta$ (N44)). In starch limiting conditions this organism can maintain its aliveness by changing its amylase enzyme from BKA $\Delta$ (N44C193) mode with higher  $k_{cat}$  and  $K_m$  to BKA $\Delta$ (N44) with lower  $k_{cat}$  and  $K_m$  which can convert little amount of starch to products with lower speed. In these conditions the ability of using small amounts of starch as energy source is more important than the rate of conversion. Now different studies are in progress in our lab to prove this proposal including; cultivating *Bacillus* sp. KR8104 in media with different starch concentrations. Protease enzymes purification from extracellular medium at

different starch concentrations and investigation of changes in protease concentrations under these conditions by proteomics techniques are also suggested. At the end, *in vitro* digestive effects of these purified proteases on BKA $\Delta$ (N44), BKA $\Delta$ (N44C193) and carboxyl terminal propeptide of BKA are very beneficial to investigate the credibility of our proposed model. Molecular dynamics studies are also useful in this part of study.

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