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ORIGINAL PAPER

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Construction and one-step purification of *Bacillus kaustophilus* leucine aminopeptidase fused to the starch-binding domain of *Bacillus* sp. strain TS-23 α-amylase

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Abstract The starch-binding domain of *Bacillus* sp. strain TS-23 α-amylase was introduced into the C-terminal end of *Bacillus kaustophilus* leucine aminopeptidase (BkLAP) to generate a chimeric enzyme (BkLAPsbd) with raw-starch-binding activity. BkLAPsbd, with an apparent molecular mass of approximately 65 kDa, was overexpressed in *Escherichia coli* M15 cells and purified to homogeneity by nickel–chelate chromatography. Native PAGE and chromatographic analyses revealed that the purified fusion protein has a hexameric structure. The half-life for BkLAPsbd was 12 min at 70°C, while less than 20% of wild-type enzyme activity retained at the same heating condition. Compared with the wild-type enzyme, the 60% decrease in the catalytic efficiency of BkLAPsbd was due to a 91% increase in K_m value. Starch-binding assays showed that the K_d and B_{max} values for the fusion enzyme were 2.3 μM and 0.35 μmol/g, respectively. The adsorption of the crude BkLAPsbd onto raw starch was affected by starch concentration, pH, and temperature. The adsorbed enzyme could be eluted from the adsorbent by 2% soluble starch in 20 mM Tris–HCl buffer (pH 8.0). About 49% of BkLAPsbd in the crude extract was recovered through one adsorption–elution cycle with a purification of 11.4-fold.

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Introduction

Leucine aminopeptidase (LAP; EC 3.4.11.1) catalyzes the hydrolysis of amino acid residues from the amino terminus of protein and peptides. LAPs are widely distributed in nature and play the key role in protein degradation and in the metabolism of biologically active peptides [1, 2]. To date, the biochemical and biophysical properties of bovine lens LAP have been extensively studied. The enzyme consists of six identical subunits with two zinc-binding sites in each subunit [3]. Based on X-ray structure and kinetic data of bovine lens enzyme, specific residues have been implicated in zinc-coordination and in catalysis [4–6]. The critical residues of bovine lens LAP are invariant in *Escherichia coli* PepA [7], tomato LAP-A [8], and *Bacillus kaustophilus* LAP (BkLAP) [9], suggesting that these enzymes might use a similar mechanism during the catalytic reaction.

Proteins are often associated into multidomains in order to perform tasks that require more than one function [10, 11]. Researchers have adopted the nature's strategy to develop many applications for chimeric proteins [12–15]. The use of genetic engineering techniques to construct chimeric proteins containing a functional domain together with a binding domain has already proven to be very useful, especially in protein purification. For example, the glutathione S-transferase gene fusion system is designed to express a gene of interest fused to the protein glutathione S-transferase and the recombinant protein is purified by affinity chromatography using a glutathione-sepharose column [16]. Similar systems have been developed on the basis of different fusion proteins; however, all these methods

use expensive materials such as sepharose, acrylic beads or glass beads, which require costly chemical modification, and in many cases the use of highly toxic compounds [17, 18]. Cellulose is an attractive matrix for enzyme immobilization mainly because of its highly suitable physical properties and its very low price. Therefore, many applications using cellulose-binding domain (CBD)-fused proteins have been developed, including CBD-alkaline phosphatase [19], CBD-protein A for IgG purification [20, 21], and CBD-streptavidin for biotinylated molecule applications [22]. Starch is another matrix suitable for enzyme immobilization. The starch-degrading enzymes are composed of a number of domains and the composition and arrangement of domains varies greatly between enzymes [23]. Besides a catalytic domain (domains A and B) and domains of unknown function (domains C and D), some amylolytic enzymes such as cyclodextrin glycosyltransferase [24] and *Aspergillus niger* glucoamylase [25] contain a starch-binding domain (SBD). SBDs are approximately 100 amino acids long and their sequences seem very well conserved among different enzymes as well as among different species [26]. The SBDs of *A. niger* glucoamylase, *Thermoanaerobacterium thermosulfurigenes* cyclodextrin glycosyltransferase, and *Bacillus* sp. strain TS-23 α-amylase were functionally expressed in recombinant *E. coli* [27–29]. Several studies have also shown that SBDs retain their binding activities towards raw starch when they were produced as fusion proteins [30–34]. These investigations indicate that the SBD of amylolytic enzymes should be an independent domain and maintain its original conformation even when it is separated from the catalytic center.

In this study, we report the construction and one-step purification of a chimeric enzyme (BkLAPsbd) that comprises the entire sequence of BkLAP and the SBD of *Bacillus* sp. strain TS-23 α-amylase. The chimeric enzyme retained LAP activity and had an enhanced thermostability. Moreover, the fusion protein could be recovered by adsorption–elution on the raw starch. The adsorption–elution procedure may be useful in various biotechnological applications when mild-conditions and nontoxic chemicals must be employed in order to avoid impairment of accompanying active ingredients.

Materials and methods

Materials, strains, and plasmids

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Restriction and modification enzymes were obtained from Promega Life sciences (Madison, WI, USA). Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resin was acquired from Qiagen Inc. (Valencia, CA, USA). All other chemicals were commercial products of analytical grade or molecular biological grade.

Escherichia coli Novablue (Novagen Inc., Madison, WI, USA) was used for the preparation and construction of plasmids. T5 RNA polymerase-mediated overexpression of wild-type BkLAP and its fusion protein was performed in *E. coli* M15 (Qiagen). *E. coli* XL1-Blue (Stratagene Inc., La Jolla, CA, USA) was used for site-directed mutagenesis. Plasmids used were pTS-917 [35] and pQE-LAP [36]. Host cells conferring the recombinant plasmids were grown aerobically in Luria-Bertani (LB) medium supplemented with 100 μg ampicillin/ml for strains Novablue and XL1-Blue or 100 μg ampicillin/ml and 25 μg kanamycin/ml for strain M15.

Construction of expression plasmid

Two primers with *Kpn* I and *Hin*d III restriction sites, respectively, were synthesized for amplification of a DNA fragment encoding the last 102 amino acids of *Bacillus* sp. strain TS-23 α-amylase (Fig. 1a, b). PCR amplification was carried out with a DNA thermal cycler (Model 2400; Perkin-Elmer, CO, USA) in a reaction mixture (100 μl) containing 100 ng pTS-917, 20 mM of each primer, 1.25 mM deoxynucleotides, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), and 1.5 units *Pfu* DNA polymerase. Up to 25 cycles of amplification were employed under the following conditions: 94°C for 2 min, 55°C for 1.5 min, and 72°C for 2 min. The amplified DNA was digested with *Kpn* I and *Hin*d III, and inserted into the respective sites of pQE-LAP to yield pQE-LAP-sbd. To construct the fusion between BkLAP and the SBD of *Bacillus* sp. strain TS-23 α-amylase (Fig. 1c), the stop codon (TAA) of *B. kaustophilus lap* gene on pQE-LAP-sbd was mutated to Ser codon (TCA) with the complementary primers, 5′-CGGTTTGAATCAGGTACCTGGGTG-3′ and 5′-CACCCAGGTACCTGATTCAAACCG-3′ (nucleotides underlined represent the mutated codon). Mutagenesis was done by the QuikChange method (Stratagene) according to the manufacture's protocol. Briefly, Plasmid pQE-LAP-sbd was PCR-amplified in the elongation process using *PfuTurbo* DNA polymerase. The product was treated with *Dpn* I endonuclease specific for methylated and hemimethylated DNA, hence the parental DNA template was digested. The nicked vector DNA carrying the desired mutation was proliferated in *E. coli* XL1-blue supercompetent cells. The selected clones were verified for the required mutation by the dideoxy-chain termination method with a Dye terminator cycle sequencing kit and an automated DNA sequencer. The mutated plasmid was designed pQE-LAPsbd.

Metal-chelate column chromatography of BkLAP and BkLAPsbd

E. coli M15 harboring pQE-LAP or pQE-LAPsbd was grown at 37°C in 100 ml of LB medium supplemented

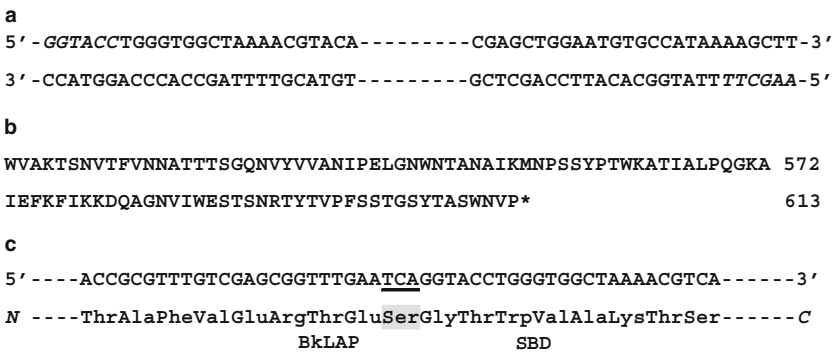

Fig. 1 Construction of *B. kaustophilus* LAP fused to starch-binding domain of *Bacillus* sp. strain TS-23 α-amylase. **a** Primers used for PCR amplification of 306-bp DNA fragment encoding for the SBD. The created restriction enzyme sites are in italics. **b** The last 102 amino acids of *Bacillus* sp. strain TS-23 α-amylase. **c** The sequence between BkLAP and *Bacillus* sp. strain TS-23 α-amylase

with the above-mentioned antibiotics to an attenuance at 600 nm of approximately 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and the cultivation continued at 28°C for 12 h. The cells were harvested by centrifugation at 12,000*g* for 20 min at 4°C, resuspended in 3 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl; pH 7.9), and disrupted by sonication. The resulting extracts were clarified by centrifugation at 12,000*g* for 20 min, and the supernatants were then mixed with Ni^{2+} -NTA resin pre-equilibrated with the binding buffer. After three volumes of washing buffer (50 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl; pH 7.9), the His_6-tagged enzymes were eluted from the resin by a buffer containing 0.5 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl (pH 7.9).

Protein methods

Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical min-gel system (Mini-Protean III system; Bio-Rad Laboratories, Richmond, CA, USA) with 8% nondenaturing polyacrylamide gel. Electrophoresis was done at 4°C and a constant voltage of 100 V for 6 h.

Sodium dodecylsulfate-PAGE (SDS-PAGE) with 4% polyacrylamide stacking and 10% polyacrylamide separating gels was performed using Laemmli buffer system [37]. Before electrophoresis, the enzymes were mixed with 2×SDS-sample buffer, heated at 100°C for 5 min, and centrifuged at 12,000*g* for 10 min. Protein bands were stained with 0.25% Coomassie Brilliant Blue dissolved in 50% methanol-10% acetic acid, and destained in a 30% methanol-10% acetic acid solution. Protein markers were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa).

Protein concentrations were measured by the Bradford method [38] with Bio-Rad protein assay reagent, and bovine serum albumin was used as the standard. Chromatographic analyses of BkLAP and BkLAPsbd were carried out on a TSK gel SW guard column (4 cm×8 mm) and a TSK G-3000 SW (30 cm×8 mm) (TosoHaas, Japan) loaded on a Hitachi D-7000 high-performance liquid chromatography system (Hitachi Ltd., Tokyo, Japan). The peaks were detected at wavelength of 280 nm, and acquisition and processing of data were completed by Hitachi B-7000 software with an AID interface. The mobile phase used was 0.05% (w/v) sodium azide in 20 mM Tris–HCl buffer (pH 8.0). The buffer solution was degassed with a Branson 2510 ultrasonic system (Branson Ultrasonic Corp., Danbury, CT, USA) and filtered with 0.45 μm sterile units (Millipore Co., Bedford, MA, USA) right before it was employed. A typical analysis was completed in 40 min with a flow rate of 0.6 ml/min. Both high and low molecular mass calibration curves were constructed by measuring the elution volumes of standard compounds, calculating the $*K_{av}$ value for each, and plotting the K_{av} value versus the logarithm of each molecular mass. The standard compounds included bovine serum albumin (67.0 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextrin 2000 (2,000 kDa).

$$*K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where K_{av}, V_e, V_o, and V_t represent the partition coefficient of protein, the elution volume of protein, column void volume, and total bed volume, respectively.

Enzyme assay, kinetic characterization, and thermostability

LAP activity was assayed following the procedure of Lin et al. [36]. One unit of LAP activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitroanilide (*p*-NA) per min at 55°C.

To determine thermostabilities of BkLAP and BkLAPsbd, protein concentrations were adjusted to 20 μg/ml with 20 mM Tris–HCl buffer (pH 8.0). The enzyme solutions were incubated at 70°C for designed

time periods. Aliquots (50 μl) of the enzyme solution were withdrawn to determine the residual activity under the standard assay conditions.

The K_m and k_{cat} values were estimated by measuring *p*-NA production in 0.5 ml reaction mixtures with various concentration of the substrate (0.1–2.0 Km) in 20 mM Tris–HCl buffer (pH 8.0) and a suitable amount of enzyme. The K_m and k_{cat} values were determined by fitting the initial rates as a function of substrate concentration to the Michaelis-Menten equation.

Starch-binding assays

BkLAPsbd concentrations ranging from 1 μM to 30 μM were mixed with 20 mg of prewashed granular corn starch and incubated at 4°C for 2 h. The binding was terminated by sedimentation of the starch. After centrifugation at 12,000*g* for 10 min, the nonbound protein concentration was determined by the Bio-Rad protein assay kit, and the amount of adsorbed protein was calculated from the difference between the initial and final protein concentrations in the supernatant.

The maximum amount of bound protein B_{max} and K_d values were determined by nonlinear regression of the binding isotherms using a modified model for saturation binding, comprising one binding site as developed by Swillens [39]. The following equation describes the well-known binding model derived from the law of mass action:

$$B = \frac{B_{max}F}{K_d + F} \quad (1)$$

where B (μmol), B_{max}, and K_d (μmol) represent the bound protein, the maximum amount of bound protein, and the equilibrium dissociation constant, respectively. F is calculated by subtracting B from the total protein added (T) as follows:

$$F = T - B \quad (2)$$

Combing Eqs. 1 and 2 yields

$$B = \frac{B_{max}(T - B)}{K_d + (T - B)} \quad (3)$$

Equation 3 was used to calculate the nonlinear regression curve. The units of the B_{max} and K_d values calculated were converted into μmol/g and μM, respectively.

Adsorption–elution purification of BkLAPsbd

To prepare the crude BkLAP and BkLAPsbd solutions for adsorption–elution purification, the IPTG-induced *E. coli* M15 cells harboring pQE-LAP or pQE-LAPsbd (harvested from 100 ml of fermentation broth) was suspended in 10 ml of 20 mM Tris–HCl buffer (pH 8.0), disrupted by sonication, and the extracts were clarified by centrifugation. Then, raw corn starch was added to the crude solutions to a final concentrations of 1, 2, 3, 4, 5, 6, and 8% (w/v). The reaction mixtures (1 ml) were incubated at 4°C with agitation for 2 h. After centrifugation at 12,000*g* for 5 min, the LAP activity and protein concentration of the supernatants were measured and the adsorption efficiency was calculated. The adsorption efficiency (%) is defined as [(original LAP activity − LAP activity in the supernatant)/original LAP activity]×100%.

To recover the adsorbed enzyme, the starchy pellet was washed once with 1 ml of cold Tris–HCl buffer (20 mM, pH 8.0). It was then resuspended in 1 ml of elution buffers at 4°C, stirred for 1 h, and centrifuged. LAP activity in the supernatant was assayed and compared with the adsorbed activity to calculate the elution efficiency, which is defined as (the recovered LAP activity/the adsorbed LAP activity)×100%.

Results

Production and purification of BkLAP and BkLAPsbd

In a recent study, we have constructed an expression plasmid, pQE-LAP, for the heterologous production of BkLAP in recombinant *E. coli* [36]. The mRNA of this construct encodes ten additional amino acids, MRGSHHHHHH, at the N-terminus of BkLAP facilitating one-step purification of the enzyme by Ni^{2+} -NTA resin. The insertion of PCR-amplified DNA fragment encoding the SBD of *Bacillus* sp. strain TS-23 α-amylase into pQE-LAP coupled with site-directed mutagenesis of the stop codon of *B. kaustophiluslap* gene generated a fusion protein having both His tag and SBD. Analysis of the total proteins from IPTG-induced *E. coli* M15 (pQE-LAPsbd) revealed a predominant protein band with an apparent molecular mass of approximately 65 kDa (data not shown), which compared well with the calculated mass of affinity-tagged translational product of the fusion gene. A 54-kDa protein band was also found in the extract of *E. coli* M15 (pQE-LAP). BkLAP and BkLAPsbd in the crude extract were further purified by metal chelate chromatography. As shown in Fig. 2, the expected proteins were observed in the respective lanes. The purification procedure resulted in yields of 41% and 27% of BkLAP and BkLAPsbd, respectively, from the crude extracts. The specific activities for purified BkLAP and BkLAPsbd were 108.6 and 37.6 U/mg protein, respectively.

The molecular masses of BkLAP and BkLAPsbd were determined by chromatographic analysis. To make the molecular mass determination more accurate, calibration curves with correlative coefficients of 0.97 and 0.99 for high molecular mass and low molecular mass, respectively, are established using protein standard compounds. BkLAPsbd was eluted at 12.3 min, whose molecular mass was determined to be around 400 kDa. As a control, BkLAP had a retention time of 13.1 min

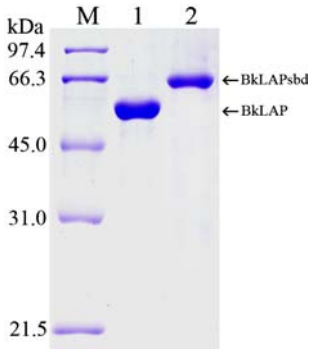

Fig. 2 SDS-PAGE analysis of purified BkLAP and BkLAPsbd. Lanes: M, standard marker proteins; 1, purified BkLAP; 2, purified BkLAPsbd

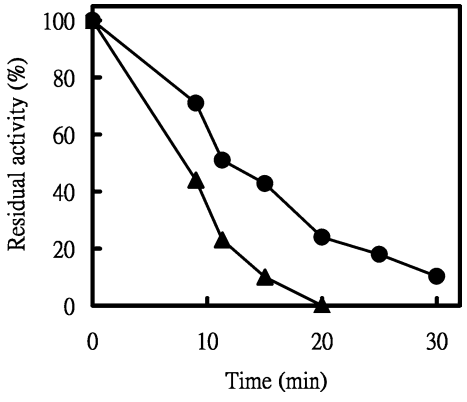

Fig. 3 Thermostability of BkLAP (*filled triangle*) and BkLAPsbd (*filled circle*). The performed temperature was 70°C and the residual activity was assayed as described in Materials and methods. The data represent the average values of three measurements

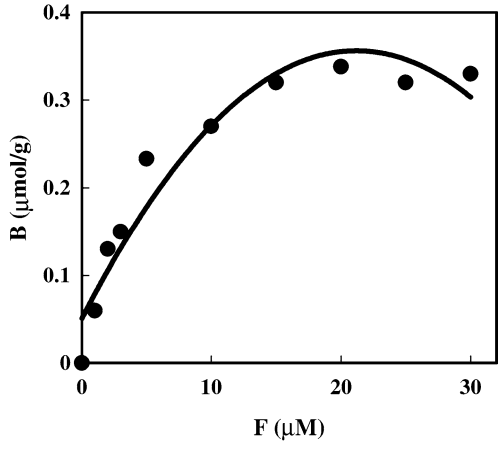

Fig. 4 Binding of purified BkLAPsbd to raw starch. BkLAPsbd at different concentrations was bound to raw corn starch until equilibrium as described in the Materials and methods. Protein concentrations were determined by direct measurement of bound BkLAPsbd on the starch granules

Table 1 Kinetic parameters of purified BkLAP and BkLAPsbd

Enzyme	K_m(μM)	k_{cat}(min^{-1})	k_{cat}/K_m(min^{-1} mM^{-1})
BkLAP	302 ± 15	0.12 ± 0.04	0.40
BkLAPsbd	578 ± 27	0.09 ± 0.01	0.16

referring to a molecular mass of 320 kDa. The data represents that both enzymes exist a hexameric structure. Nondenaturing PAGE analysis of BkLAP and BkLAPsbd confirmed the chromatographic data although protein band tailings were observed in both enzymes (data not shown). These results indicate that the presence of *Bacillus* sp. strain TS-23 α-amylase SBD at the C-terminus of *B. kaustophilus* LAP does not affect the oligomerization of the enzyme.

Characterization of BkLAPsbd

The optimum pH for BkLAP and BkLAPsbd was the same (pH 8.0), and the enzymes exhibited similar patterns in activity over a pH range of 7–10. The optimum activity for BkLAP was 55°C, while the maximum temperature for BkLAPsbd elevated to 65°C. Thermostability of each protein was also monitored in 20 mM Tris–HCl buffer (pH 8.0). Both proteins were found to be stable at 50°C with no change in activities even after incubation at this temperature for 90 min (data not shown). As shown in Fig. 3, BkLAPsbd exhibited a time-dependent decrease in LAP activity and had a half-life of 12 min at 70°C, while that of BkLAP was less than 9 min. As the wild-type enzyme, Mn^{2+} and Ni^{2+} ions had a stimulatory effect on the amidolytic activity of BkLAPsbd. Moreover, these two enzymes were most active against L-Leu- *p*-NA followed by L-Cys- *p*-NA.

To understand further the basis for variation in specific activity, steady-state kinetic parameters were determined for both BkLAP and BkLAPsbd using *p*-NA as the substrate. As shown Table 1, the catalytic efficiency of BkLAPsbd was reduced with more than 91% increase in K_m value, indicating that the presence of *Bacillus* sp. strain α-amylase SBD significantly affect the catalytic function of BkLAP.

Binding of purified BkLAPsbd to raw starch

Equilibrium adsorption isotherm was used to study the affinity of purified BkLAPsbd for raw corn starch. In general, at low protein concentrations, the amount of bound protein demonstrated a linear dependence on the free protein concentration. It almost approached the BkLAPsbd saturation, but not attained even at the highest level of protein concentration tested (Fig. 4).

The binding isotherm was used to calculate binding parameters. The K_d and B_{max} values were calculated from a nonlinear regression of the adsorption isotherm (Fig. 4). The affinity of BkLAPsbd to starch was 2.3 μM

and B_{max} value of the fusion protein was found to be 0.35 μmol/g.

Isolation of BkLAPsbd by adsorption–elution on raw starch

The effect of raw starch concentration on the adsorption of BkLAPsbd was performed at pH 8.0 and 4°C. With the increase of adsorbent concentrations form zero to 3%, there was a rapid increase in the adsorption efficiency (Fig. 5a). The efficiency was reduced and appeared to reach a plateau when the adsorbent concentration over 4%. As expected, no significant adsorption was observed in BkLAP even at high concentration of raw starch. SDS-PAGE analysis confirmed that the band density of BkLAPsbd in the crude enzyme solution decreased with the increase of raw starch concentrations (Fig. 5b).

To investigate the effect of pH on the adsorption of BkLAPsbd, 20 mg raw starch was suspended in 1 ml crude enzyme solution at specific pH values and at 4°C with shaking for 2 h. The reaction mixtures were centrifuged and the LAP activity remaining in the supernatant was then measured. The results showed that the optimal adsorption to the adsorbent occurred at pH range of 7–9 (Fig. 6a). The adsorption decreased at lower pH with 21% retention at pH 4.0.

The effect of temperature on the adsorption of BkLAPsbd was evaluated at pH 8.0. As shown in Fig. 6b, more than 70% adsorption was observed at the temperature up to 40°C but declined to only 20% at 70°C.

Four buffers were used to elute the adsorbed enzyme from the raw starch. The elution of BkLAPsbd from the adsorbent by 20 mM Tris–HCl buffer was 19.2%, indi-

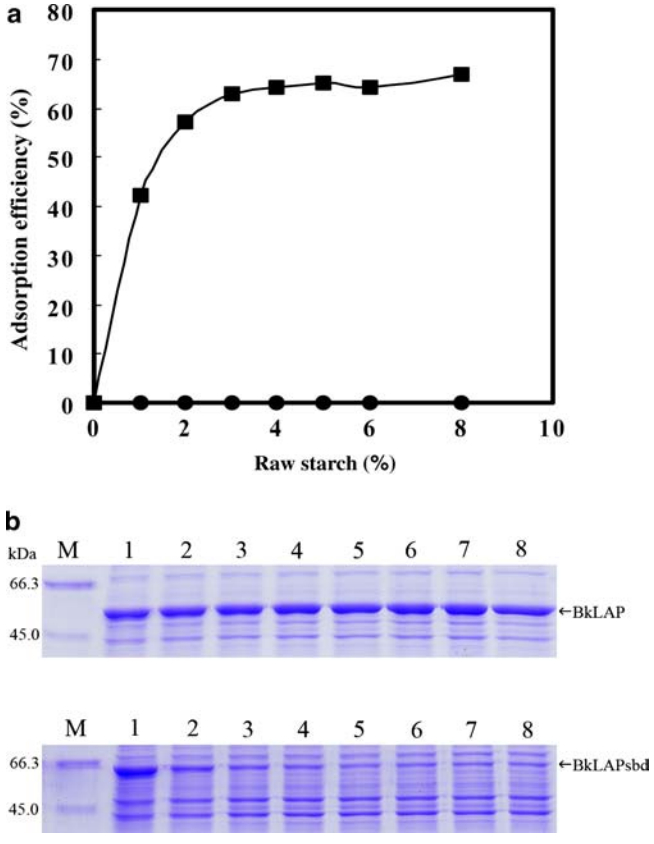

Fig. 5 Adsorption of BkLAPsbd to raw starch. **a** Effect of starch concentrations on the adsorption. The crude BkLAPsbd solution was mixed with raw corn starch up to 8% and the adsorption efficiency was calculated as described in Materials and methods. **b** Analysis of the resultant supernatants by SDS-PAGE. Lanes: M, standard marker proteins; 1, 5 μl of the crude BkLAPsbd solution; 2, 5 μl of the crude BkLAPsbd solution with 1% raw starch; 3, 5 μl of the crude BkLAPsbd solution with 2% raw starch; 4, 5 μl of the crude BkLAPsbd solution with 3% raw starch; 5, 5 μl of the crude BkLAPsbd solution with 4% raw starch; 6, 5 μl of the crude BkLAPsbd solution with 5% raw starch; 7, 5 μl of the crude BkLAPsbd solution with 6% raw starch; 8, 5 μl of the crude BkLAPsbd solution with 8% raw starch. (*filled circle*), BkLAP; (*filled square*), BkLAPsbd

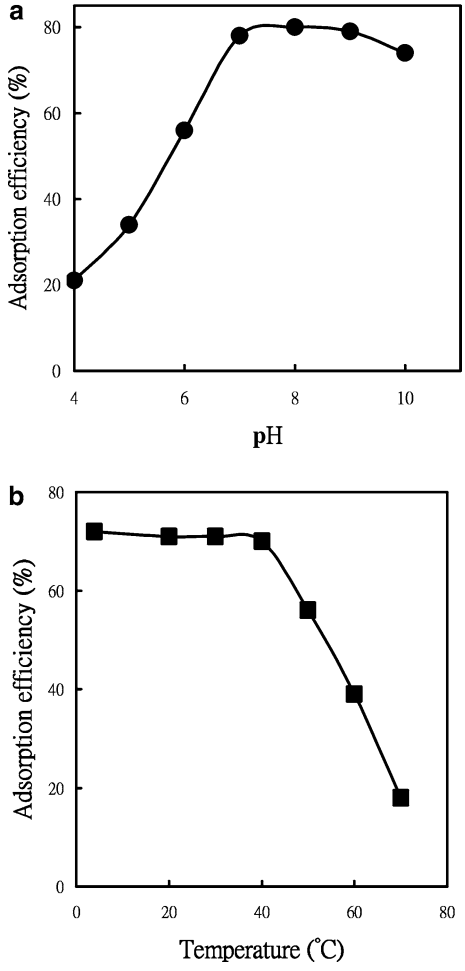

Fig. 6 Effect of pH and temperature on the adsorption of BkLAPsbd to raw starch. The experiments were performed in 20 mM phosphate buffer (pH 4–6) and 20 mM Tris–HCl buffer (pH 7–10) at 4°C (**a**) and in 20 mM Tris–HCl buffer (pH 8.0) at different temperatures (**b**). The data is the representative of three measurements

Table 2 Purification scheme of BkLAPsbd by adsorption–elution on raw starch

Step	Activity (U/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme solution	6.1 ± 1.9	613.6	175.3 ± 7.1	3.5	1	–
Elution	14.9 ± 2.3	297.8	7.5 ± 0.8	39.7	11.4	49

[a] Raw corn starch (5 g) was used to adsorb BkLAPsbd in 100 ml crude enzyme solution. The eluant was 20 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 2% soluble starch

[b] Yield is defined as (the recovered activity/total activity)×100%

cating that there is a loose interaction between the enzyme and raw starch. The addition of 2% sucrose into the buffer had no obvious effect on the elution of BkLAPsbd. The presence of 2% maltose in 20 mM Tris–HCl buffer only increased the elution efficiency to 25.3%. Among the elution buffers used, 2% soluble starch in 20 mM Tris–HCl buffer gave the highest elution (63.9%). SDS-PAGE analysis of the eluants confirmed the above findings and the purified fusion protein appeared to have an apparent molecular mass of 65 kDa (Fig. 7). It is worth to note that there was no significant difference in elution efficiency when the elution temperature was below 40°C.

Under optimum conditions, the scale-up purification cycle resulted in a substantial increase in the specific activity (from 3.5 to 39.7 U/mg protein) with respect to the crude enzyme (Table 2). Also, approximately 49% of BkLAPsbd in the crude extract was recovered through the adsorption–elution procedure.

Discussion

We successfully introduced the raw-starch-binding ability into *B. kaustophilus* LAP. Acquisition of the ability to BkLAP is very interesting from the standpoint of industrial application. The presence of *Bacillus* sp. strain TS-23 α-amylase at the C-terminus of BkLAP seems not interfere its oligomerization, suggesting that the folding of BkLAP is strong enough to retain its function even with the extra domain. Unfortunately, BkLAPsbd displayed lower LAP activity compared to the parental enzyme. To date, several fusion proteins have been constructed in a variety of starch-degrading enzymes. However, the chimeric proteins usually do not produce the functions expected from the original enzymes since the native polypeptide-folding patterns do not maintain in the hybrid proteins [30, 33, 40, 41]. In contrast, fusion of *Bacillus stearothermophilus* LAPII with the SBD of *Bacillus* sp. strain TS-23 α-amylase results in a chimeric enzyme having raw-starch-binding ability and proper catalytic function comparable to the wild-type enzyme [34]. In this case, however, a defective fusion enzyme was produced after introducing the same SBD into BkLAP. It is difficult to figure out the reason for the opposite result without the support of three-dimensional structures of these two fusion proteins. Further structural determinations should help to clarify the conflicting result.

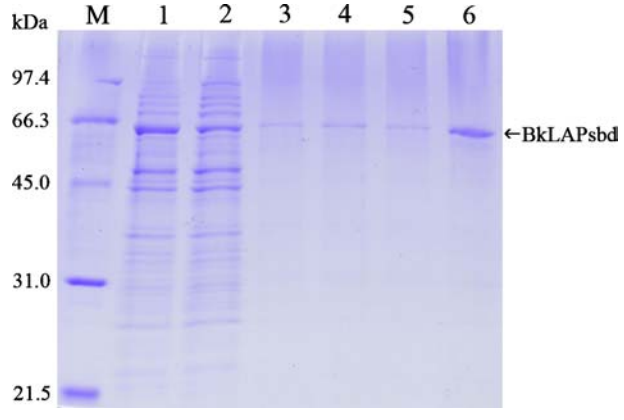

Fig. 7 SDS-PAGE analysis of crude enzyme solution, the supernatant after raw starch adsorption, and the purified BkLAPsbd. Lanes: 1, standard marker proteins; 2, 5 μl of crude enzyme solution; 2, 5 μl of the supernatant after raw starch adsorption; 3, 5 μl of 20 mM Tris–HCl buffer (pH 8.0)-eluted enzyme solution; 4, 5 μl of 20 mM Tris–HCl buffer (pH 8.0) containing 2% sucrose-eluted enzyme solution; 5, 5 μl of 20 mM Tris–HCl buffer (pH 8.0) containing 2% maltose-eluted enzyme solution; 6, 5 μl of 20 mM Tris–HCl buffer (pH 8.0) containing 2% soluble starch-eluted enzyme solution

Many polysaccharide-degrading enzymes display a modular structure in which a catalytic module is attached to one or more noncatalytic modules. Deletion of noncatalytic modules often leads to a reduction in the structural integrity of the protein at elevated temperatures; hence, they have been termed "thermostabilizing domains' to reflect this destabilization [42, 43]. Mutational studies indicate that the SBD of *Bacillus macerans* cyclodextrin glycosyltransferase is involved in the stability and integrity of the enzyme [44]. Although the SBD of *Bacillus* sp. strain TS-23 α-amylase has been demonstrated not important for its thermostability [45], this domain does contribute greatly to the thermostability of BkLAPsbd. Consistently, the thermostability of *B. stearothermophilus* LAPII is improved by introducing the same domain into the C-terminus of the enzyme [34]. Thus, in some applications, it may not be necessary to remove the tail from the fusion enzyme.

As shown in Fig. 6, the optimal adsorption of BkLAPsbd for the adsorbent occurred at pH range of 7–9. To our knowledge, no report has been dealt with the pH effect on the adsorption of fusion protein onto raw starch, while the starch adsorption of *Aspergillus* glucoamylase is pH dependent and reaches a maximum around 3.5 [46]. *Pseudomonas amyloderamosa* isoamy-

lase also shows a maximal adsorption at pH values between 3 and 5 [47]. These findings suggest that electrostatic interactions might play an important role in the adsorption process of raw-starch-binding enzymes. The adsorption temperature is critical for BkLAPsbd (Fig. 6b). The adsorption efficiency reached 72% when the performed temperature was below 40°C. Other investigations also demonstrate that lower temperature is favor for the adsorption of amylolytic enzymes to the adsorbent [47–49]. It has been reported that albumin undergoes reversible conformational and functional changes in the two thermodynamically independent domains at the temperature of above 50°C [50]. These changes will affect its adsorption-desorption behavior toward silica particles [51]. Accordingly, it is possible that BkLAPsbd undergoes a conformational change at the temperature greater than 40°C and this change has a deleterious effect on the adsorption of the fusion enzyme onto raw starch.

On the basis of primary and tertiary structure similarities, carbohydrate-binding modules can be grouped into approximately 30 families and the three-dimensional structure of representatives of approximately half of the modules have now been resolved [52]. These modules are predominately β-stranded and their ligand-binding sites reflect the structure of the target polysaccharide [52]. Ligand-binding is dominated by hydrophobic interactions between the sugar rings and the aromatic residues on the surface of the protein-binding site, and the orientation and the nature of the amino acids in the binding site can play an important role in ligand specificity [24, 53]. Based on the characteristics of carbohydrate-binding modules, starch and its derivatives have been used for the elution of α-amylase [54], β-amylase [55], glucoamylase [46], isoamylase [47], and cyclodextrin glucanotransferase [33] from the adsorbents. Like the above-mentioned enzymes, BkLAPsbd was eluted from the adsorbent by soluble starch in 50 mM Tris–HCl buffer. It is worth to note that maltose in Tris–HCl buffer did not increase the elution efficiency of BkLAPsbd. This may be that the fusion situation changes the conformation of *Bacillus* sp. strain TS-23 α-amylase SBD to which disrupt the normal interactions between raw starch and BkLAPsbd.

In conclusion, the excellent chemical and physical properties of starch together with its abundant in nature make this polysaccharide a useful component for industrial applications. The expression of *B. kaustophilus* LAP in *E. coli* as fusion with the family 13 carbohydrate-binding module of *Bacillus* sp. strain TS-23 α-amylase enables us to purify the recombinant enzyme by adsorption–elution on raw starch. One problem for operating the adsorption–elution purification of amylolytic enzymes is the biodegradation of the adsorbent [56, 57], while this would not happen in this application since the purification process is performed at 4°C and the fusion enzyme has no amylolytic activity. However, in this case, it is necessary to remove the tag after protein preparation to possibly regain the full LAP activity. Removal of the tag could be accomplished by insertion of a unique amino acid sequence that is recognized and cleaved by a site-specific protease. An expression vector set combining N- or C-terminal fusion to the SBD of *Bacillus* sp. strain TS-23 α-amylase with the possibility of tag removal by factor Xa is currently under construction. The vector set will offer the option to produce active proteins of interest by cloning the corresponding genes into the multiple cloning sites of expression plasmid.

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