J Ind Microbiol Biotechnol (2004) 31: 273–277
DOI 10.1007/s10295-004-0146-5

ORIGINAL PAPER

Yu-Wen Hua · Meng-Chun Chi · Huei-Fen Lo
Wen-Hwei Hsu · Long-Liu Lin

Fusion of *Bacillus stearothermophilus* leucine aminopeptidase II with the raw-starch-binding domain of *Bacillus* sp. strain TS-23 α-amylase generates a chimeric enzyme with enhanced thermostability and catalytic activity

Received: 26 January 2004 / Accepted: 8 May 2004 / Published online: 10 July 2004

Abstract *Bacillus stearothermophilus* leucine aminopeptidase II (LAPII) was fused at its C-terminal end with the raw-starch-binding domain of *Bacillus* sp. strain TS-23 α-amylase. The chimeric enzyme (LAPsbd), with an apparent molecular mass of approximately 61 kDa, was overexpressed in IPTG-induced *Escherichia coli* cells and purified to homogeneity by nickel-chelate chromatography. The purified enzyme retained LAP activity and adsorbed raw starch. LAPsbd was stable at 70°C for 10 min, while the activity of wild-type enzyme was completely abolished under the same environmental condition. Compared with the wild-type enzyme, the twofold increase in the catalytic efficiency for LAPsbd was due to a 218% increase in the k_{cat} value.

Keywords Leucine aminopeptidase · *Bacillus stearothermophilus* · Amylase · Raw starch-binding domain · *Bacillus* sp. strain TS-23 · Thermostability

Introduction

The aminopeptidases, a group of enzymes that catalyze the removal of amino acids from the amino terminus of proteins, are critical components of the protein degradation machinery. Leucine aminopeptidase (LAP) is the most extensively studied of these enzymes due to its many potential commercial applications. LAP has been shown to improve flavor development [27], to improve the taste of debittered protein hydrolysates [20], and to convert L-homophenylalanyl amide into L-homophenylalanine, the versatile intermediate for a class of angiotension I-converting enzyme inhibitors [7]. LAPs are found in a wide variety of bacterial species and are classified into three groups on the basis of their molecular structures [4]. As a member of group III, the cobalt-coordinated *Bacillus stearothermophilus* LAP II (LAPII) is a dimeric enzyme with an apparent molecular mass of 46 kDa [24]. Recently, a gene encoding for LAPII was cloned [17] and expressed at high-levels in *Escherichia coli* [8].

Several chimeric proteins have been constructed for a variety of amylolytic enzymes. Studies of these proteins have focused on secretion of the enzyme [6] as well as changes in substrate [16, 26] or product specificities [18]. It was observed that the chimeric proteins of different enzyme species did not produce the functions expected from the original enzymes [5, 15, 23], since the native polypeptide-folding patterns were usually not maintained in the hybrid proteins. Earlier, an α-amylase gene from *Bacillus* sp. strain TS-23 was cloned and expressed in recombinant *E. coli* [10]. Deletion analysis revealed that the last 100 amino acids of the enzyme functioned as a starch-binding domain (SBD) [13]. The SBDs of *Bacillus macerans* cyclodextrin glucanotransferase (CGTase) and *Aspergillus niger* glucoamylase retained their starch-binding activity when they were produced as chimeric proteins with β-galactosidase [3]. Fusion of the SBD of *B. subtilis* CGTase with an α-amylase of the same species also resulted in a chimeric enzyme having both raw-starch binding and -digesting abilities [19]. Additionally, the SBD of *Bacillus* sp. strain TS-23 α-amylase was functionally expressed in *E. coli* [12]. The results of those studies strongly suggested that the SBDs of amylolytic enzymes could function independently from the catalytic domains. Potential applications for chimeric proteins containing the starch-binding tail

Y.-W. Hua · M.-C. Chi · L.-L. Lin (✉)
Department of Applied Chemistry, National Chiayi University,
300 University Road, 60083 Chiayi, Taiwan
E-mail: llin@mail.ncyu.edu.tw
Fax: +886-5-2717901

H.-F. Lo
Department of Food and Nutrition,
Hungkuang University, Taichung, Taiwan

W.-H. Hsu
Institute of Molecular Biology,
National Chung Hsing University, 402-27 Taichung, Taiwan

include enzyme immobilization to starch or recovery and purification of target proteins from cell-free extracts. In this investigation, we constructed a raw-starch-binding LAP (LAPsbd) by introduction of the SBD of *Bacillus* sp. strain TS-23 α-amylase into the C-terminus of *B. stearothermophilus* LAP II. The results showed that the chimeric enzyme retained LAP activity and had an enhanced thermostability.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Escherichia coli NovaBlue (Novagen, Wis., USA) was used as the host for routine plasmid propagation and DNA cloning. *E. coli* M15 (Qiagen, Calif., USA) was used for T5 RNA polymerase/promoter system protein expression studies. Plasmids used were pTS917 [10] and pQE-LAPII [8]. *E. coli* cells carrying plasmids were grown oxically at either 28 or 37°C in Luria–Bertani (LB) medium supplemented with 100 μg ampicillin/ml for Novablue strain or 100 μg ampicillin/ml and kanamycin (25 μg/ml) for M15 strain.

DNA methods

General DNA techniques were done essentially as described previously [21]. The PCR products were analyzed on a 1% agarose gel and purified by the Geneclean III kit (Bio 101, Calif., USA). Oligonucleotide primers were synthesized by Mission Biotechnology (Taipei, Taiwan). The construct was confirmed by dye-terminator cycle sequencing with an automated 373A sequencer (Perkin-Elmer, Foster City, Calif., USA).

Construction of expression plasmid

Two primers containing *Pst* I restriction site were synthesized in order to amplify a DNA fragment encoding the last 100 amino acids of *Bacillus* sp. strain TS-23 α-amylase (Fig. 1). PCR amplification was carrid out using a DNA thermal cycler (Model 2400; Perkin-Elmer, Conn., USA) with a reaction mixture (100 μl) containing 1.25 mM deoxynucleotides, 20 mM of each primer, 100 ng template, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 2 units *Pfu* DNA polymerase. The DNA was amplified as described by [12], digested with *Pst* I and inserted into the respective site of pQE-LAPII, yielding yield pQE-LAPsbd.

Expression and purification of LAPsbd

For high-level expression of LAPsbd, *E. coli* strain M15 (pQE-LAPsbd) was grown on LB medium containing the above-mentioned antibiotics to an optical density at 600 nm of approximately 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cultivation was continued at 28°C for 12 h. The cells were harvested by centrifugation at 12,000 *g* for 10 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. LAPII and LAPsbd were purified by a previously described procedure [8].

Electrophoresis and activity staining

SDS-PAGE was carried out using the Laemmli buffer system [9]. Prior to electrophoresis, the samples were heated at 100°C for 5 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue R-250 dissolved in 50% methanol–10% acetic acid, and destained in a solution of 30% methanol and 10% acetic acid. 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).

Leucine aminopeptidase activity in the gel was detected with the procedure described by Manchenko [14]. PAGE was performed in a vertical mini-gel system (Mini-Protean III system; Bio-Rad Laboratories, Richmond, Calif., USA) with a 10% non-denaturing polyacrylamide gel. Electrophoresis was run at 4°C and a constant voltage of 100 V for 3 h. To detect LAP activity, the gels were immediately immersed into 0.4%

Fig. 1a,b Construction of His_6-tagged leucine aminopeptidase (LAP)II/starch-binding domain (SBD) fusion protein. **a** The last 100 amino acids of *Bacillus* sp. TS-23 α-amylase. The first amino acid of the putative SBD is *underlined*. **b** Primers used for PCR amplification of a 324-bp DNA fragment encoding for the SBD. The synthetic oligonucleotides used are *bold* and the created restriction enzyme sites are in *italics*

(A)

515-TSNVTFTVNNATTTSGQNVYVVANIPELGNWNTANAIKMNPSSYPTWKATIALPQ

570-GKAIEFKFIKKDQAGNVIWESTSNRTYTVPFSSTGSYTASWNVP*

(B)

5′-***CTGCAG*ACGTCAAACGTCACATTT**-------GCGAGCTGGAATGTGCCACTGCAG-3′

3′-GACGTCTGCAGTTTGCAGTGTAAA-------**CGCTCGACCTTACACGGT*GACGTC***-5′

L-leucyl-2-naphthylamide and 0.6% fast black K in 100 mM phosphate buffer (pH 5.8), and were incubated at 55°C until dark blue bands appeared.

Enzyme assay and kinetic characterization

Leucine aminopeptidase activity was assayed following the procedure of Kuo et al. [8]. One unit of LAP activity is defined as the amount of enzyme that produces 1 μmol *p*-nitroanilide (*p*-NA) per min at 60°C. Protein concentration was measured with the Bio-Rad protein assay reagent and bovine serum albumin was used as the standard.

To determine the thermostability of the purified LAPII and LAPsbd, the enzymes were adjusted to 100 μg protein/ml with 50 mM Tris-HCl buffer (pH 8.0). The enzyme solutions were incubated at 70°C for the designated time periods. After incubation, 100 μl of the enzyme solution was withdrawn and residual activity was measured under the standard assay conditions.

K_m and k_{cat} values were estimated by measuring *p*-NA production in 0.5-ml reaction mixtures containing various concentration of the substrate (0.3∼2.0 K_m) in 50 mM Tris–HCl buffer, pH 8.0, and a suitable amount of enzyme. Samples were incubated for 10 min at 60°C. The K_m and k_{cat} values were calculated from the rate *p*-NA production using the Michaelis-Menten equation.

Adsorption of raw starch

The Starch-binding assay was done essentially according to the procedures described by Lo et al. [13]. The adsorption rate is defined as [(protein concentration in the original enzyme solution − residual protein after adsorption)/protein concentration in the original enzyme solution] × 100. Briefly, approximately 234 μg LAPsbd/ml in 50 mM Tris-HCl (pH 8.0) was separately mixed with 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 mg corn starch to a final volume of 1 ml. The mixtures were shaken at 4°C for 3 h and then centrifuged to sediment the adsorbent. Aliquots (150 μl) of the resulting supernatants were taken for protein determinations. The protein concentrations were determined colorimetrically from the cleared supernatants at 595 nm in a Hitachi U-1800 spectrophotometer. Assay tubes in the absence of LAPsbd were used as the blanks.

Results and discussion

Expression and purification of LAPsbd

In a previous study, we constructed a bacterial expression plasmid, pQE-LAPII, for the heterologous production of *B. stearothermophilus* LAP II [8]. The mRNA of this construct encodes ten additional amino acids (MRGSHHHHHH) at the N-terminus of LAPII, which facilitates one-step purification of recombinant proteins with nickel nitrilotriacetate (Ni^{2+}-NTA) resin. In order to overproduce LAPsbd, the PCR-amplified DNA fragment encoding the SBD of *Bacillus* sp. strain TS-23 α-amylase was restricted with *Pst* I and inserted into the respective site of pQE-LAPII to obtain pQE-LAPsbd. Analysis of the total proteins from IPTG-induced *E. coli* M15 (pQE-LAPsbd) revealed a predominant protein band with apparent M_r of approximately 61 kDa (Fig. 2), which compared well with the calculated mass of the affinity-tagged translational product of the fusion gene. As a control, a 44.5-kDa protein band was also observed in *E. coli* M15 (pQE-LAPII). LAPII and LAPsbd in the crude extracts were further purified by a Ni^{2+}-NTA resin. As shown in Fig. 2, only one protein band was observed in the respective lanes. Activity staining showed that the chimeric protein retained LAP activity (Fig. 3), indicating that the chimeric environment does not affect functional expression of the enzyme in recombinant *E. coli*. Purified LAPII and LAPsbd had a specific activity of 413 and 528 U mg^{-1} protein (Table 1), respectively. The significant increase in the specific activity of LAPsbd indicated that the starch-binding region did not interfere with enzyme function; thus, in some applications it may not be necessary to remove the tail from the chimeric protein.

Characterization of LAPsbd

Small differences in domain structure may affect the fine structure of the active cleft. In the case of chimeric α-glucan phosphorylase, the presence of 78 residue at a position near the active site of the type L isozyme causes steric hindrance of large, branched glucan molecules approaching the active site [16]. However, LAPII and LAPsbd displayed similar substrate specificity (data not shown), implying that a steric effect did not occur in the

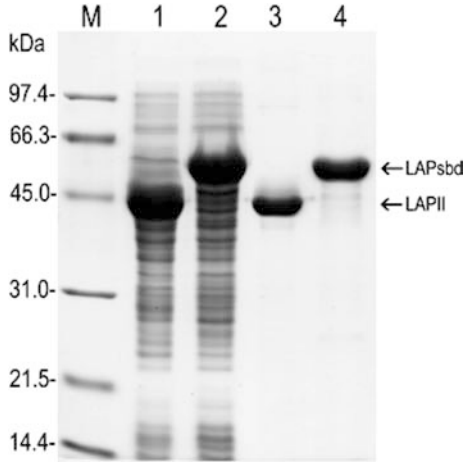

Fig. 2 Analysis of LAPII and LAPsbd by SDS-PAGE. Lanes: *M* standard marker proteins, *1* total proteins from *E. coli* M15 (pQE-LAPII) 2 total proteins from *E. coli* M15 (pQE-LAPsbd), *3* purified LAPII, *4* purified LAPsbd

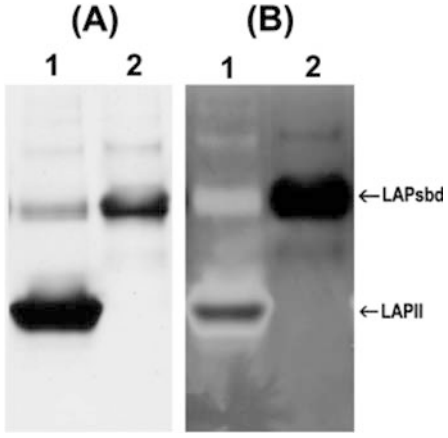

Fig. 3a,b Analysis of LAPII and LAPsbd by non-denaturing PAGE. The purified enzymes were separated on 10% polyacrylamide gels and visualized by Coomassie brilliant blue staining (**a**) and activity staining (**b**). Lanes: *1* purified LAPII, *2* purified LAPsbd. *Dark bands* Proteins exhibiting LAP activity

Table 1 Specific activity and kinetic parameters of purified LAP and LAPsbd

Enzyme	Specific activity (U mg^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
LAP	413 ± 25	0.94 ± 0.08	18.41 ± 5.61	19.6
LAPsbd	528 ± 27	1.02 ± 0.11	40.17 ± 9.87	39.4

chimeric enzyme. It is worth noting that the maximal activity for the wild-type enzyme was 60°C, while the optimal temperature of LAPsbd was elevated to 75°C. The thermostability of each protein was also monitored in 20 mM Tris-HCl buffer (pH 8.0). The proteins were found to be stable at 50°C with no decrease in activities even after incubation at this temperature for 90 min (data not shown). As shown in Fig. 4, LAPsbd displayed a half-life of 30 min, while that of LAPII was less than 5 min. Based on these measurements, we determined that LAPsbd displayed significantly higher amidolytic activity and thermostability than the parental enzyme. In some cases, the chimeric environment did not affect proper functioning of β-galactosidase [2] and glucoamylase I [22], while a remarkable decrease in catalytic activity was observed in a fusion protein of cyclodextrin glucanotransferase [19]. Clearly, the introduction of SBD of *Bacillus* sp. strain α-amylase into LAPII does not affect its catalytic function. It has been reported that the SBD of *B. macerans* CGTase is involved in the thermostability of the enzyme [1]. Although the SBD of *Bacillus* sp. strain TS-23 α-amylase is not important for thermostability [13], this domain contributes greatly to the thermostability of LAPsbd.

Steady-state kinetic parameters were determined using *p*-NA at the concentrations of 0.2–3.8 mM. LAPsbd exhibited a similar K_m value with an increased catalytic efficiency relative to the wild-type enzyme (Table 1). It has been demonstrated that the glucoamylase fusion protein exhibits similar Michaelis–Menten kinetic parameters to those of the parental enzyme [22]. The V_{max} values for maltose of the chimeric α-glucosidases were significantly changed whereas the K_m values were similar to that of the wild-type enzyme [25]. In our study, the catalytic efficiency of LAPsbd was higher than that of LAPII. This implies that fusion of SBD with LAPII causes a conformational change in the catalytic cleft of the enzyme.

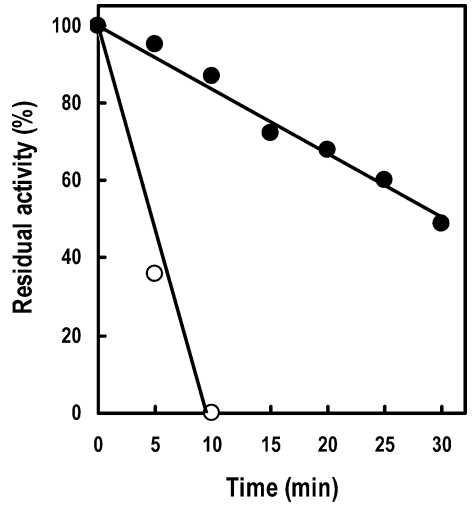

Fig. 4 Effect of temperature on the stability of LAPII (*open circles*) and LAPsbd (*filled circles*). The reaction temperature was 70°C and the residual enzyme activities were assayed as described in Materials and methods. The data represent the average values of three measurements

Adsorption of LAPsbd to raw starch

The raw-starch-binding activity of the chimeric protein was determined with an adsorption isotherm over a range of starch concentrations. As expected, no detect-

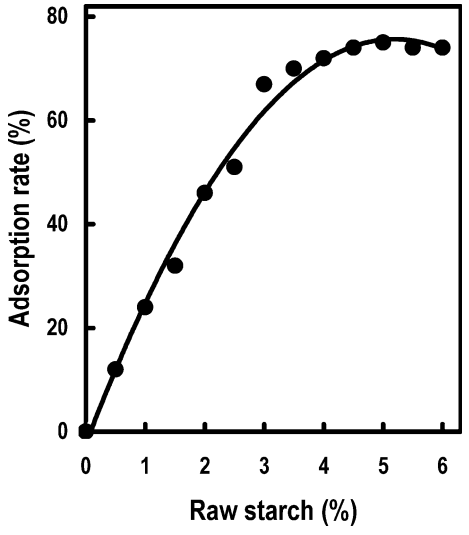

Fig. 5 Binding of purified LAPsbd to raw starch. The data represent the average values of three measurements

able adsorption was observed in LAPII (data not shown). There was a linear increase in the adsorption rate at these raw starch concentrations of 0–3% (Fig. 5). The rate of increase was reduced and appeared to reach a plateau when the raw starch concentration was over 4%.

In conclusion, we have shown that a LAPII fusion protein containing 100 amino acids from the C-terminus of *Bacillus* sp. strain TS-23 α-amylase has the ability to adsorb raw starch. The strong folding ability of *B. stearothermophilus* LAP II and the functionally independent characteristic of the SBD of *Bacillus* sp. strain TS-23 α-amylase may have played an important roles in starch adsorption. We previously demonstrated that recombinant α-amylase of *Bacillus* sp. strain TS-23 can be recovered from crude *E. coli* extract by adsorption to starch granules followed by gentle elution with a Tris-HCl buffer [11]. The conditions for recovery of chimeric proteins from starch, however, remain to be optimized.

Acknowledgements This work was supported by a research grant (NSC 92-2313-B-415-005) from the National Science Council of the Republic of China.

References

1. Chang HY, Irwin PM, Nikolov ZL (1998) Effects of mutations in the starch-binding domain of *Bacillus macerans* cyclodextrin glycosyltransferase. J Biotechnol 65:191–202
2. Chen LJ, Ford C, Nikolov Z (1991) Adsorption to starch of a β-galactosidase fusion protein containing the starch-binding region of *Aspergillus* glucoamylase. Gene 99:121–126
3. Dalmia B, Schütte K, Nikolv Z (1995) Domain E of *Bacillus macerans* cyclodextrin glucanotransferase: an independent starch-binding domain. Biotechnol Bioeng 47:575–584
4. Gonzales T, Robert-Baudouy J (1996) Bacterial aminopeptidases: properties and functions. FEMS Microbiol Rev 18:319–344
5. Hellman J, Mäntsälä P (1992) Construction of an *Escherichia coli* export-affinity vector for expression and purification of foreign proteins by fusion to cyclomaltodextrin glucanotransferase. J Biotechnol 23:19–34
6. Juge N, Sógaard M, Chaix JC, Martin-Eauclaire MF, Svensson B, Marchis-Mouren G, Guo XJ (1993) Comparison of barley malt α-amylase isozymes 1 and 2: construction of cDNA hybrids by in vivo recombination and their expression in yeast. Gene 130:159–166
7. Kamphuis J, Meijer EM, Boesten WHJ, Broxterman QB, KapteinB, Hermes HFM, Schoemaker HE (1992) Production of natural and synthetic L- and D-amino acids by aminopeptidases and amino amidases. In: Rozzell JD, Wagner F (eds) Biocatalytic production of amino acids and derivatives. Wiley, New York, pp 178–206
8. Kuo LY, Hwang GY, Lai YJ, Yang SL, Lin LL (2003) Overexpression, purification, and characterization of the recombinant leucine aminopeptidase II of *Bacillus stearothermophilus*. Curr Microbiol 47:40–45
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685
10. Lin LL, Hsu WH, Chu WS (1997) A gene encoding for an α-amylase from thermophilic *Bacillus* sp. strain TS-23 and its expression in *Escherichia coli*. J Appl Microbiol 82:325–334
11. Lin LL, Lo HF, Chen JN, Ku KL, Hsu WH (2002) Isolation of a recombinant *Bacillus* sp. TS-23 α-amylase by adsorption-elution on raw starch. Starch/Stärke 54:338–342
12. Lin LL, Lo HF, Chi MC, Ku KL (2003) Functional expression of the raw starch-binding domain of *Bacillus* sp. strain TS-23 α-amylase in recombinant *Escherichia coli*. Starch/Stärke 55:197–202
13. Lo HF, Lin LL, Chiang WY, Chi MC, Hsu WH, Chang CT (2002) Deletion analysis of the C-terminal region of the α-amylase of *Bacillus* sp. strain TS-23. Arch Microbiol 178:115–123
14. Manchenko GP (1994) Handbook of detection of enzymes on electrophoretic gels. CRC Press, London, pp 3.4.11.1
15. Moraes LMP, Astolfi-filho S, Oliver SG (1995) Development of yeast strains for the efficient utilization of starch: evaluation of constructs that express α-amylase and glucoamylase separately or as bifunctional fusion proteins. Appl Microbiol Biotechnol 43:1067–1076
16. Mori H, Tanizawa K, Fukui T (1993) A chimeric α-glucan phosphorylase of plant type L and H isozymes: functional role of 78-residue insertion in type L isozyme. J Biol Chem 268:5574–5581
17. Motoshima H, Minagawa E, Tsukasaki F, Kaminogawa S (1997) Cloning of genes of the aminopeptidase T family from *Thermus thermophilus* HB8 and *Bacillus stearothermophilus* NCIB8924: apparent similarity to the leucyl aminopeptidase family. Biosci Biotechnol Biochem 61:1710–1717
18. Nakano YJ, Kuramitsu HK (1992) Mechanism of *Streptococcus mutans* glucosyltransferase: hybrid-enzyme analysis. J Bacteriol 174:5639–5646
19. Ohdan K, Kuriki T, Takata H, Okada H (2000) Cloning of cyclodextrin glucanotransferase gene from alkalophilic *Bacillus* sp. A2-5a and analysis of the raw starch-binding domain. Appl Microbiol Biotechnol 53:430–434
20. Rao MB, Tanksale AM, Ghatge MS, Desphade VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635
21. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 17.2–17.44
22. Sauer J, Christensen T, Frandsen TP, Mirgorodskaya E, McGuire KA, Driguez H, Roepstorff P, Sigurskjold BW, Svensson B (2001) Stability and function of interdomain linker variants of glucoamylase I from *Aspergillus niger*. Biochemistry 40:9336–9346
23. Shibuya I, Tamura G, Shima H, Ishikawa T, Hara S (1992) Construction of an α-amylase-glucoamylase fusion gene and its expression in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 56:884–889
24. Stoll E, Weder HG, Zuber H (1976) Aminopeptidase II from *Bacillus stearothermophilus*. Biochim Biophys Acta 438:212–220
25. Sugimoto M, Ohta T, Kawai F (2003) Change in maltose- and soluble starch-hydrolyzing activities of chimeric α-glucosidases of *Mucor javanicus* and *Aspergillus oryzae*. Biochim Biophys Acta 1645:1–5
26. Terashima M, Hosono M, Katoh S (1997) Functional roles of protein domains on rice α-amylase activity. Appl Microbiol Biotechnol 47:364–367
27. Toldrá F, Aristoy AC, Flores M (2000) Contribution of muscle aminopeptidases to flavor development in dry-cured ham. Food Res Int 33:181–185