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Characterization of the thermophilic isoamylase from the thermophilic archaeon *Sulfolobus solfataricus* ATCC 35092

Tsuei-Yun Fang [a,*], Wen-Chi Tseng [b], Ching-Ju Yu [a], Tong-Yuan Shih [a]

[a] *Department of Food Science, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 202, Taiwan*
[b] *Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan*

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Abstract

Isoamylase catalyzes the hydrolysis of α-1,6-glucosidic linkages of starch and related polysaccharides. In this study, the *treX* gene (GenBank accession no. AE006815 REGION: 9279 ... 11435) encoding the thermophilic isoamylase was PCR-cloned from the genomic DNA of *Sulfolobus solfataricus* ATCC 35092 to an expression vector with a T7*lac* promoter. Both wild-type and His-tagged isoamylases were expressed in *Escherichia coli*. The wild-type isoamylase was purified sequentially using heat treatment, nucleic acid precipitation, ion-exchange chromatography, and gel filtration chromatography while the His-tagged isoamylase was purified from the cell-free extract directly by metal chelating chromatography. Both enzymes were active only under their homo-trimer forms. In the absence of NaCl, both enzymes became inactive monomers. In addition, both enzymes were more stable when being stored at room temperature than at 4 °C. They had an apparent optimal pH of 5 and an optimal temperature at 75 °C. The enzyme activities remained unchanged after a 2 h incubation at 80 and 75 °C for the wild-type and His-tagged enzymes, respectively. These thermophilic isoamylases showed a potential to be used in industry to degrade the branching points of starch at a high temperature.
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Keywords: Thermophilic; Isoamylase; Trehalose; Starch; *Sulfolobus solfataricus*; *E. coli*

1. Introduction

Isoamylase (EC 3.2.1.68) catalyzes the hydrolysis of α-1,6-glucosidic linkages of amylopectin and related polysaccharides. The enzyme is able to hydrolyze both inner and outer branching points of amylopectin, and is commonly used in combination with other enzymes, such as α-amylase, β-amylase, and glucoamylase to produce amylose, maltose, and glucose from starch. Several bacteria are known to be capable of producing isoamylases, such as *Bacillius amyloliquefaciens* [1], *Escherichia coli* [2–4], *Flavobacterium odoratum* [5,6], *Pectobacterium chrysanthemi* [7], and *Pseudomonas amylodermosa* [8–10]. In addition, the *treX* gene on the genomes of *Sulfolobus acidocaldarius* ATCC 33909 [11] and *Arthrobacter* sp. Q36 [12] was also suggested to be an isoamylase gene. The *treX* gene along with the *treZ* and *treY* genes, which encode trehalosyl dextrin forming enzyme (TDFE) and trehalose forming enzyme (TFE), respectively, constitute an operonic structure and were involved in the trehalose biosynthesis [11,12]. TDFE mainly catalyzes an intramolecular transglycosylation reaction to form trehalosyl dextrins from dextrins by converting the α-1,4-glucosidic linkage at the reducing end to an α-1,1-glucosidic linkage. On the other hand, TFE mainly cleaves the α-1,4-glucosidic linkage next to the α-1,1-glucosidic linkage of trehalosyl dextrins to produce trehalose and the dextrins with lower molecular weight. The *treX* gene also exists on the genomes of thermophilic archaeon *S. solfataricus* ATCC 35092, also known as P2, and *S. tokodaii* strain 7, whose whole genomes have been sequenced recently [13,14]. Although isoamylases from many psychrophiles and mesophiles have been purified and characterized [6,7,15–18], the thermophilic isoamylases

* Corresponding author. Tel.: +886 2 2462 2192x5141;
fax: +886 2 2463 4203.
E-mail address: tyfang@mail.ntou.edu.tw (T.-Y. Fang).

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from thermophilic archaeon have been rarely studied. Only very few properties of recombinant thermophilic isoamylases from *S. acidocaldarius* and *Rhodothomus marinus* were documented [19]. Enzymes used in starch processing usually have different temperature and pH requirements based on their thermostabilities and physicochemical properties [20–23]. In addition, more than two enzymes are usually required to process starch to low-molecular products, such as glucose, fructose, maltose, trehalose or cyclodextrins [21,24,25]. Recently, many thermophilic archaeal amylolytic enzymes have been characterized, and most of them have shown a similar optimal pH of activity (pH 5–6.5) and a good thermostability [20–23,26]. Using the thermophilic enzymes from thermophilic archaeon on starch processing has several advantages. First, the reaction can be carried out at high temperatures to lower the viscosity of reaction mixture and to decrease the risks of microbial contaminations in the reaction mixtures [25,27]. Secondly, the same pH could be used throughout the whole biotransformation, and the acidification or neutralization step in the current starch processes could be avoided [20,28]. In this study, we PCR-cloned the *treX* gene from the genomic DNA of *S. solfataricus* ATCC 35092 and expressed the cloned gene in *Escherichia coli*. In addition to characterizing the general properties of the thermophilic isoamylase in both wild-type and His-tagged forms, we also found that the thermophilic isoamylase was not stable at 4 °C and only active under its homo-trimer form.

2. Experimental

2.1. Materials

The genomic DNA of *S. solfataricus* ATCC 35092 was obtained from the American Type Culture Collection (Manassas, VA). *E. coli* BL21-CodonPlus (DE3)-RIL was obtained from Stratagene (La Jolla, CA). Plasmid pET-15b and His·Bind resin were from Novagen (Madison, WI). Vent DNA polymerase and mung bean nuclease were purchased from New England BioLabs (Beverly, MA). T4 DNA ligase and restriction enzymes were supplied by Promega (Madison, WI). Bovine serum albumin (BSA), benzamidine, phenylmethylsulfonyl fluoride (PMSF), amylose, and pullulan were from Sigma (St. Louis, MO). Oyster glycogen was from USB (Cleveland, OH). Amylopectin was from Fluka (Buchs, Switzerland). Ultrafree-15 and Microcon Centrifugal Filter Units were obtained from Millipore (Bedford, MA). Q Sepharose Fast Flow, Sephacryl S-200 HR, disposable PD-10 desalting columns, and protein low-molecular-weight standards were from Amersham Pharmacia Biotech (Piscataway, NJ). BioSep-SEC-S4000 column (300 mm × 7.8 mm) was obtained from Phenomenex (Torrance, CA).

2.2. Amplification of the treX gene

The *treX* gene was amplified by the polymerase chain reaction (PCR). Two primers were designed on the basis of the *treX* sequence of *S. solfataricus* P2 (GenBank accession no. AE006815 REGION: 9279 . . . 11435). In order to clone *treX* gene into pET-15b vector, the *Xho*I and *Bam*H I restriction sites were included in the forward and reverse primers, respectively. The primer sequences are as follows: 5*XtreX* (forward primer): 5′-CCC GGG TCG ACT CGA GAT GGC ATT ATT CTT CAG AAC TAG AG-3′; 3*BtreX* (reverse primer): 5′-TTA GCA GCC GGA TCC TCA TAA TTC TAT CCT CCT ATA AAC TAA -3′, where the *Xho*I and *Bam*H I restriction sites were in boldface. The reaction was carried out in 100 μl of reaction mixture containing the genomic DNA of *S. solfataricus* ATCC 35092, two primers, dNTPs, Vent DNA polymerase, and Vent DNA polymerase buffer, and was performed using a GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA) according to the following conditions in sequence: 95 °C for 5 min, an amplification, and a final extension at 72 °C for 10 min. The amplification profile was 35 cycles of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C.

2.3. Construction of the expression vector for thermophilic isoamylase

The 2.2 kbp PCR-amplified fragment was purified and then digested with *Xho*I and *Bam*H I. The digested fragment was inserted into the pET-15b vector, resulting in a recombinant vector designated as pET-15b-*treX*. The amplified *treX* gene was fused in frame with the His-tag coding sequence on pET-15b; therefore, the expressed thermophilic isoamylase possessed a His-tag on its N-terminal region. The sequences of the entire *treX* gene and the fused His-tag coding region on pET-15b-*treX* were confirmed by DNA sequencing. In an effort to express a wild-type thermophilic isoamylase, which contains no extra His-tag coding sequence derived from the above cloning steps, the pET-15b-*treX* was digested with *Nco* I and *Xho*I to remove the His-tag coding sequence, made blunt by mung bean nuclease and ligated, resulting in a recombinant clone designated as pET-15b-ΔH-*treX*. DNA sequencing was also performed to ascertain the removal of the His-tag coding sequence.

2.4. Expression of the thermophilic isoamylase by E. coli

The pET-15b-*treX* or pET-15b-ΔH-*treX* vector was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL to express isoamylase. One single colony from a newly transformed culture plate was inoculated into 10 ml of terrific broth (TB) medium supplemented with 100 μg/ml ampicillin plus 34 μg/ml chloramphenicol, and grown at 37 °C until the OD_{600} reached 0.6. Cells were collected by centrifugation and resuspended in 4 ml of fresh TB medium. A volume of 3 ml resuspended culture was then added into 600 ml of fresh TB medium containing 100 μg/ml of ampicillin plus 34 μg/ml chloramphenicol. The culture was then induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) when the OD_{600} reached 0.6. After a further culture at 20 °C

for 16 h, the cells was then harvested by centrifugation and stored at −70 °C before further purification proceeded.

2.5. Preparation of cell-free extract

Frozen cells (8 g) expressing the wild-type and His-tagged thermophilic isoamylases were suspended in 24 ml of lysis buffers A and B, respectively. Lysis buffer A contained 20 mM Tris–HCl (pH 8.0), 0.2 M NaCl, 1 mM benzamidine, 0.05 mM PMSF, and 0.1% Triton X-100. Lysis buffer B contained 20 mM Tris–HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl, 1 mM benzamidine, 0.05 mM PMSF, and 0.1% Triton X-100. The suspended cells were disrupted using a French Press disruptor (Sim-Aminco, Rochester, NY) at 20,000 psi. The cell-free extract was then prepared by removing the insoluble fractions from the supernatant of the above mixture by centrifugation at 10,000 × *g* for 2 h.

2.6. Purification of the wild-type enzyme

Heat treatment was first used to precipitate most of the undesired proteins by incubating the cell-free extract at 80 °C water bath for 1 h followed by centrifugation to remove the heat-labile proteins. A 10% (w/v) streptomycin sulfate stock solution was then added to a final concentration of 1% (w/v) to precipitate the nucleic acids. After centrifugation at 10,000 × *g* for 1 h, the supernatant was dialyzed against 20 mM Tris–HCl buffer (pH 8.5), and subsequently loaded onto a Q Sepharose column (1.6 cm × 10 cm), which was equilibrated with a buffer of the same composition as the above dialysis buffer. The column was then washed thoroughly with the same buffer until the absorbance of 260 nm became almost undetectable. Finally, a linear gradient of 0–0.5 M NaCl in the above buffer was used to elute the bound proteins. The eluted fractions containing enzyme activity were collected and concentrated for further purification by gel filtration. The concentrated sample was loaded onto a Sephacryl S-200 HR column (1.6 cm × 60 cm) previously equilibrated with a buffer containing 20 mM Tris–HCl (pH 8.0) and 0.2 M NaCl. The eluted fractions containing enzyme activity were collected for the characterization of isoamylase.

2.7. Purification of the His-tagged enzyme

The cell-free extract was diluted with an equal volume of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) prior to being loaded onto a His·Bind column (1.6 cm × 5 cm), which was previously equilibrated with the same binding buffer. The column was first washed with the binding buffer to remove the unbound proteins, then washed with a wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9), and finally eluted with an elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) according to the protocols provided in the Novagen His·Bind Kits. The eluted fractions containing enzyme activity were collected and dialyzed against a buffer containing 20 mM Tris–HCl (pH 8.0) and 0.2N NaCl.

2.8. Enzyme activity assay

The assay of isoamylase activity was modified from the procedures described by Harada et al. [16]. The reaction mixture contained 350 μl of 0.5% amylopectin solution, 100 μl of 0.25 M citrate phosphate buffer (pH 5), and 50 μl of enzyme solution. The mixture was incubated at 75 °C for 30 min followed by an addition of 0.5 ml of 0.01N I_2–0.1N KI solution. Afterwards, the whole mixture was diluted to 10 ml with distilled water before the measurement of the absorbance at 610 nm. One unit (U) of isoamylase activity was defined as the amount of enzyme that caused an increase in the absorbance of 0.1/h.

In order to determine the substrate specificity, amylopectin, amylose, glycogen, or pullulan was used as substrate, and the reducing-end formation was determined by the DNS method [29] with glucose as standards. The enzyme reaction conditions were the same as described above.

2.9. Protein concentration measurement

Protein concentration was quantitated by Bradford's method [30] with BSA as standards.

2.10. Mass spectrometric analysis

The mass spectrometric analysis was performed on a MicroMass (Altrincham, UK) Quattro-BIO-Q mass spectrometer as previously described [31].

2.11. HPLC analysis of the molecular weight of thermophilic isoamylase

The molecular weights of both wild-type and His-tagged isoamylases were estimated using a BioSep-SEC-S4000 column (300 mm × 7.8 mm), which is a silica-based gel filtration column. The mobile phase contained 20 mM Tris–HCl (pH 7.5) plus 0.2 M NaCl, and the flow rate was controlled at 0.5 ml/min. The analysis was carried out under a Hitachi HPLC L-7000 (Tokyo, Japan) equipped with an UV detector.

3. Results and discussion

3.1. Comparisons of the amino acid sequences of isoamylases from different sources

The thermophilic isoamylase from *S. solfataricus* ATCC 35092 possesses 718 amino acids and a calculated molecular weight of 83091 Da, which was deduced from the nucleotide sequence of *treX* gene and analyzed by Compute pI/Mw tool (http://tw.expasy.ch/tools/pi_tool.html) [32], respectively. Isoamylase from *S. solfataricus* ATCC 35092 shared at least 74% identity with the other three isoamylases from *Archaea*, and it also shared more than 32% identity with the other six isoamylases from *Bacteria* (Table 1). According to the

Table 1
A pairwise comparison between the amino acid sequences of the archaeal and bacterial isoamylases

Domain	Organism	Amino acid identity (%)[c]									
		S so	*S sh*	*S t*	*S a*	*A sp.*	*E c*	*P c*	*P a*	*F o*	*R m*
Archaea	*S. solfataricus*[a]	100	95	80	74	50	42	40	32	33	53
	S. shibatae[a]		100	80	74	50	43	40	32	34	53
	S. tokodaii[a]			100	79	52	42	39	32	33	54
	S. acidocaldaricus[a]				100	50	42	38	31	31	53
Bacteria	*Arthrobacter* sp.[a]					100	45	45	29	31	54
	E. coli[a]						100	61	31	32	46
	P. chrysanthemi[a]							100	30	31	42
	P. amylodermosa[a]								100	62	31
	F. odoratum[a]									100	32
	R. marinus[b]										100

[a] The deduced amino acid sequences were obtained from the PIR-NREF protein database on the website http://pir.georgetown.edu/pirwww/search/pirnref.shtml under the following NREF ID: NF00192949 for *S. solfataricus*, NF01037729 for *S. shibatae*, NF00783228 for *S. tokodaii*, NF00190766 for *S. acidocaldarius*, NF00422023 for *Arthrobacter* sp., NF00694882 for *E. coli*, NF01056861 for *P. chrysanthemi*, NF00059064 for *P. amylodermosa*, and NF00577098 for *F. odoratum*.

[b] The deduced amino acid sequence of isoamylase from *R. marinus* was from Tsutsumi et al. [19].

[c] Identity values were calculated using the CLUSTAL W program on the website http://services.bioasp.nl/blast/cgi-bin/clustal.cgi, and the identity matrix was chosen to run each pairwise alignment.

information from CAZy, a WWW resource on glycosyl hydrolases at http://afmb.cnrs-mrs.fr/CAZY/, these isoamylases have been previously classified in family 13 of glycosyl hydrolases based on their amino acid sequence similarities according to the classification developed by Henrissat [33–35]. Families 13, 70 and 77 in this classification constitute the α-amylase family, which contains functionally and structurally related enzymes [36]. Fig. 1 shows the four highly conserved amino acid sequences in the α-amylase family enzymes. Seventeen out of thirty amino acid residues on the four conserved regions are identical among archaeal and bacterial isoamylases (Fig. 1). The four conserved regions also contain three essential amino acids, Asp375, Glu435, and Asp510 (numberings according to the enzyme from *P. amyloderamosa*) (Fig. 1), which have been shown to play a catalytic role in the active site of α-amylase family [36–38].

		Region 1		Region 2		Region 3		Region 4
a. Generalized sequence		XDXXXNH		GXRXDXXZZ		XXXOEZZZ		XXBBHD
b. Isoamylases		* * **		* *****		* ****		**
S. solfataricus	285	IDVVYNH	359	GFRFDLAAA	395	KLIAEPWD	466	YVTSHD
S. shibatae	285	IDVVYNH	359	GFRFDLAAA	395	KLIAEPWD	466	YVTSHD
S. tokodaii	283	IDVVYNH	357	GFRFDLAAA	393	KLIAEPWD	464	YITSHD
S. acidocaldarius	279	IDVVYNH	353	GFRFDLASA	389	KLIAEPWD	460	YITSHD
Arthrobacter sp.	265	LDVVYNH	339	GFRFDLAAA	375	KLIAEPWD	446	FVTAHD
E. coli	260	LDIVLNH	332	GFRFDLAAV	367	KLIAEPWD	438	LVTAHD
P. chrysanthemi	258	LDVVFNH	330	GFRFDLATI	365	KLIAEPWD	436	MLTSHD
P. amylodermosa	291	MDVVYNH	371	GFRFDLASV	431	DLFAEPWA	505	FIDVHD
F. odoratum	311	VDVVYNH	391	GYRFDLASV	439	DLIAEPWA	513	FITAHD
R. marinus	278	VDVVYNH	353	GFRFDLAAA	387	KLIAEPWD	458	FVTAHD

Fig. 1. A comparison of the conserved amino acid sequences in the active sites of α-amylase family enzymes. (a) A generalized sequence of four conserved regions [36]. X represents a hydrophobic residue, B represents a hydrophilic residue, O represents a Gly or Ala residue, and Z represents a residue important for specificity. (b) Isoamylases form different strains. The identical residues among these isoamylases were marked with asterisks (*). The three proposed catalytic residues were shown in bold face, which were Asp, Glu, and Asp in regions 2, 3, and 4, respectively. The sources of the deduced amino acid sequences of isoamylases were identical to those shown in Table 1. These isoamylases were all classified in family 13 of glycosyl hydrolases.

3.2. Expressions, purifications, and storages of the wild-type and His-tagged thermophilic isoamylases

The expression levels of both wild-type and His-tagged isoamylases were higher in the absence of IPTG than in the presence of IPTG induction. Although the amount of the expressed His-tagged isoamylase in the cell-free extract was about three times more than that of the wild-type isoamylase (Fig. 2, lanes 1 and 5), the yield of the active wild-type and His-tagged isoamylases expressed in *E. coli* were 5000 and 4200 U/g of wet cells, respectively. The wild-type isoamylase was purified from the cell-free extract of *E. coli* sequentially by heat treatment, nucleic acid precipitation, ion-exchange chromatography, and gel filtration chromatography while the His-tagged isoamylase was purified from the cell-free extract directly by metal chelating chromatography (Table 2). Both purified isoamylases showed a single band on SDS-PAGE, indicating that a high purity of protein was obtained (Fig. 2, lanes 4 and 6). The apparent molecular weights of purified wild-type and His-tagged enzymes estimated under denatured conditions by SDS-PAGE were both around 77 kDa (Fig. 2, lanes 4 and 6). The purified wild-type isoamylase, however, has a molecular mass of 82,955 Da under an electrospray ionization mass spectrometric analysis. This result is close to the theoretical molecular weight (82,960 Da) with the removal of the N-terminal methionine. The molecular weights of the purified isoamylases under non-denatured conditions were then analyzed by HPLC using the BioSep-SEC-S4000 gel filtration column. The wild-type isoamylase showed only one peak at 17.66 min (Fig. 3a), while the His-tagged isoamylase appeared in two peaks at 17.69 and 19.33 min, respectively (Fig. 3b). In a

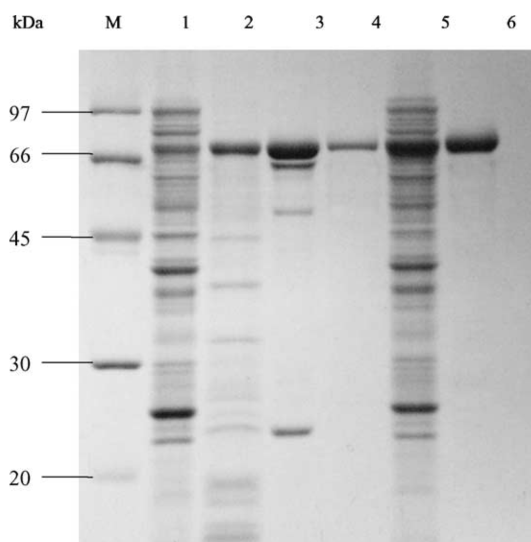

Fig. 2. SDS-PAGE analysis of expressions and purifications of the wild-type and His-tagged isoamylases expressed in *E. coli* BL21-CodonPlus (DE3)-RIL carrying the pET-15b-ΔH-*treX* and pET-15b–*treX*, respectively. Lane M: the molecular weight standards; lanes 1 and 5: the crude cell-free extracts of the wild-type and His-tagged isoamylases, respectively; lane 2: the partially purified fraction of the wild-type isoamylase after a heat treatment; lane 3: the partially purified fraction of the wild-type isoamylase after ion-exchange chromatography; lane 4: the purified fraction of the wild-type isoamylase after gel-filtration chromatography; lane 6: the purified fraction of the His-tagged isoamylase after metal-affinity chromatography.

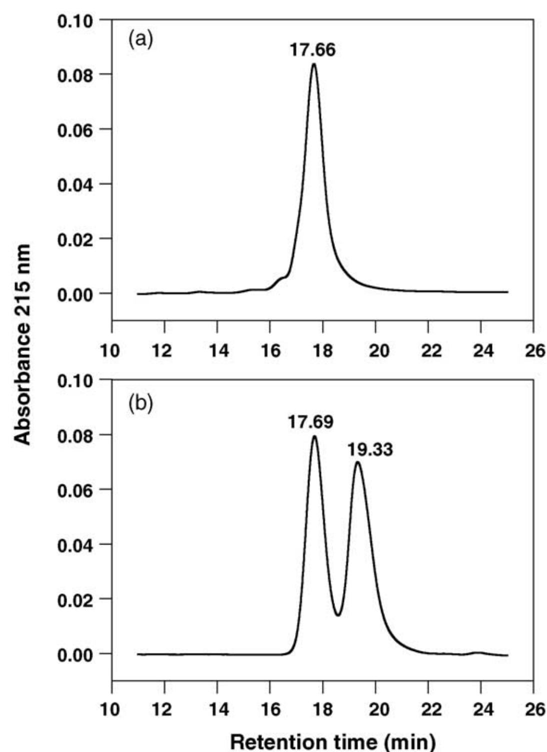

Fig. 3. HPLC analysis of the purified wild-type and His-tagged isoamylases using the BioSep-SEC-S4000 size-exclusion column. The HPLC profiles of the purified wild-type (panel a) and His-tagged (panel b) enzymes were obtained when the mobile phase contained 20 mM Tris–HCl buffer (pH 7.5) plus 0.2 M NaCl, and the flow rate was 0.5 ml/min.

comparison to the calibration curve derived from the molecular weight standards, the molecular weight of the wild-type isoamylase under non-denatured conditions was around 230 kDa, and those of the His-tagged isoamylase were 230 and 76 kDa (Fig. 4). Since the theoretical molecular weight of one subunit is around 83 kDa, the estimated 230 and 76 kDa were corresponding to 2.8 and 0.9 subunits. These results suggested that the purified wild-type isoamylase was a homo-trimer, while the purified His-tagged isoamylase contained both a homo-trimer form and a monomer form. We also found that the monomer contained no isoamylase activity. In fact, the partially purified wild-type isoamylase eluted from the ion exchange chromatography also contained both trimer and monomer forms (data not shown). The monomer form of wild-type isoamylase was also inactive. Because the Sephacryl S-200 HR gel filtration column that we used can separate the trimer from the monomer, the purified wild-type isoamylase contain only its trimer form. The isoamylase activity was found in the trimer form but not in the monomer form of both wild-type and His-tagged enzymes, suggesting that the trimer formation may produce new binding sites or reveal active sites as previously described by Marianayagam et al. [39].

After the purification by metal chelating chromatography, the His-tagged enzyme showed only one protein band under SDS-PAGE analysis (Fig. 2, lane 6); however, the specific activity of this enzyme preparation was only 710 U/mg, about one seventh of that of the purified wild-type enzyme

Table 2
Summary of purification steps of the wild-type and His-tagged isoamylases expressed in *E. coli* BL21-CodonPlus (DE3)-RIL

Enzyme	Purification step	Total protein (mg)	Total activity (U)	Activity recovery (%)	Specific activity (U/mg)	Purification fold
Wild-type						
	Crude extract	920	39900	100	44	1
	Heat treatment[a]	48	33600	84	700	16
	Q Sepharose	4.4	11300	28	2550	57
	Sephacryl S-200	0.69	3180	8.0	4600	106
His-tagged						
	Crude extract	860	33600	100	39	1
	His·Bind	40	28200	84	710	18

[a] The crude extract of wild-type enzyme was heated at 80 °C for 1 h and then centrifuged to remove the precipitates.

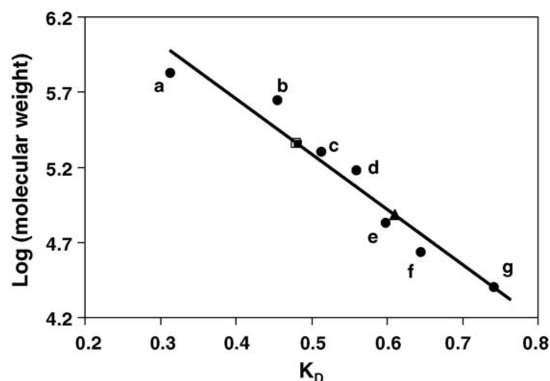

Fig. 4. Determination of the molecular weights of the wild-type and His-tagged isoamylases by HPLC analysis. The protein standards (●) included (a) thyroglobin (669 kDa), (b) apoferritin (443 kDa), (c) β-amylase (200 kDa), (d) alcohol dehydrogenase (150 kDa), (e) albumin (67 kDa), (f) ovalumin (43 kDa), and (g) chymotrypsinogen A (25 kDa). The trimer of the wild-type enzyme (□), the trimer of the His-tagged enzyme (■), and the monomer of the His-tagged enzyme (▲) were plotted with the protein standards. The K_D values were calculated using the equation $K_D = (V_e - V_o)/(V_T - V_o)$, where V_e is the elution volume and V_T and V_o represent the total liquid volume and void volume of the column, respectively. The mobile phase and the flow rate were the same as those used in Fig. 3.

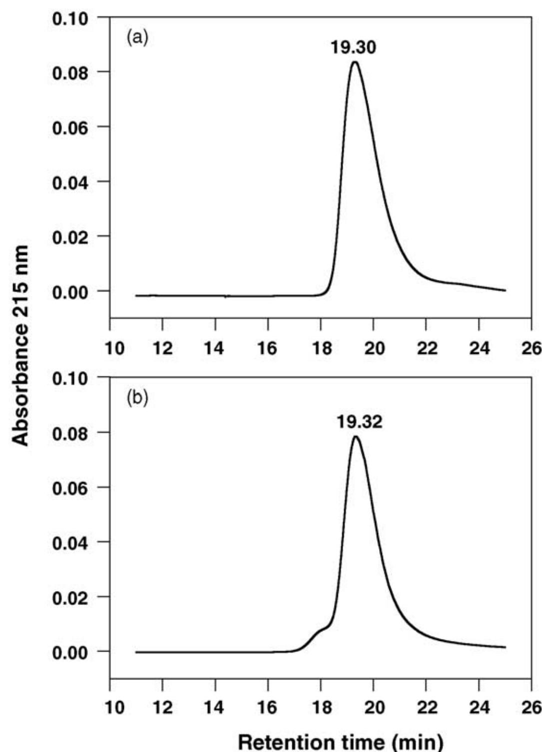

Fig. 5. The HPLC analysis of the desalted wild-type and His-tagged isoamylases using the BioSep-SEC-S4000 size-exclusion column. The desalted wild-type (panel a) and His-tagged (panel b) isoamylases were analyzed under the same conditions as those used in Fig. 3.

(Table 2). The evidence that about 50% of the purified His-tagged enzyme was in the inactive monomer form (Fig. 3b) cannot fully explain its low specific activity, suggesting that the existence of N-terminal His-tag might affect the activity of the thermophilic isoamylase. The lower specific activity of the purified His-tagged isoamylase could also explain why the His-tagged isoamylase expressed in *E. coli* had less activity yield (4300 U/g) than that of the wild-type enzyme (5000 U/g), despite that the protein expression level of the His-tagged isoamylase was three times higher than that of the wild-type enzyme as found in the cell-free extract (Fig. 2, lanes 1 and 5).

We had attempted to remove the 0.2 M NaCl from the purified wild-type and His-tagged isoamylases by the PD-10 desalting columns, which were equilibrated with 20 mM Tris–HCl buffer (pH 8.0). After desalting, both enzymes completely lost their activities. Under the HPLC analysis, these desalted enzymes appeared as a single peak at 19.30 and 19.32 min, respectively, which was corresponding to the inactive monomers (Fig. 5). In addition, we also found that both wild-type and His-tagged thermophilic isoamylases were more stable when being stored at room temperature than at 4 °C (Fig. 6). A 7-day storage at 4 °C caused about 80% of activity loss for both enzymes. Therefore, we stored both purified enzymes in 20 mM Tris–HCl buffer (pH 8.0) plus 0.2 M NaCl at room temperature.

3.3. Substrate specificity

Four different α-glucans were incubated with the wild-type or His-tagged isoamylase to determine the substrate specificity (Table 3). The wild-type and His-tagged enzymes had essentially the same substrate specificity. Amylopectin was preferentially hydrolyzed while glycogen was not hydrolyzed efficiently. Pullulan was not cleaved even at a high enzyme concentration of 600 U/ml. Surprisingly, amylose was also hydrolyzed, suggesting that the α-1,4 glycosidic linkage could be cleaved by the thermophilic isoamylase. In

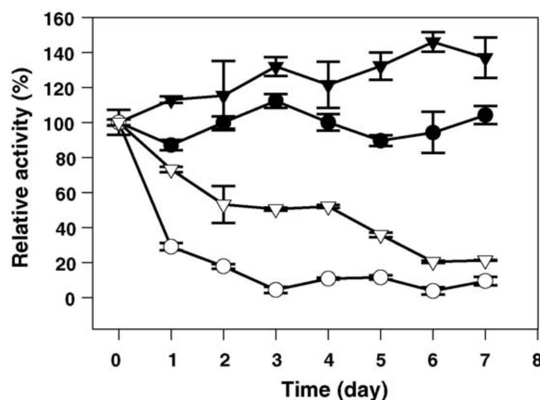

Fig. 6. Effect of temperature on the storage stabilities of the wild-type and His-tagged isoamylases. The enzymes were stored in 20 mM Tris–HCl buffer (pH 8.0) containing 0.2 M NaCl at 4 °C and room temperature, respectively. (●) The wild-type enzyme at room temperature; (▼) the His-tagged enzyme at room temperature; (◯) the wild-type enzyme at 4 °C; and (▽) the His-tagged enzyme at 4 °C. Data represent the mean ± S.D. from triplicate experiments.

Table 3
Hydrolysis of various substrates by the wild-type and His-tagged isoamylases[a]

Enzyme	Substrate	Relative rate of hydrolysis (%)
Wild-type		
	Amylopectin	100
	Glycogen	0.96
	Amylose	12.0
	Pullulan	ND[b]
His-tagged		
	Amylopectin	100
	Glycogen	0.93
	Amylose	12.2
	Pullulan	ND[b]

[a] A final concentration of 0.35% amylopectin, glycogen, amylose, or pullulan was incubated with 12, 600, 120, and 600 U/ml enzyme, respectively, at 75 °C and pH 5.
[b] Not detected.

addition, there was no glucose detected by glucose oxidase method [40] in each reacted mixture. These results indicated that the thermophilic enzyme in this study was an isoamylase.

3.4. *Effects of metal ions on the activities of the wild-type and His-tagged isoamylases*

In order to determine the effects of metal ions on the activities of the wild-type and His-tagged isoamylases, both purified enzymes were dialyzed first against a buffer containing 20 mM Tris–HCl (pH 8.0), 0.2 M NaCl, and 5 mM EDTA, and then were dialyzed against the above buffer without EDTA. These enzymes were also kept at a high concentration of 5000 U/ml. Both enzymes were diluted 50-fold with 20 mM Tris–HCl buffer (pH 8.0) just before the experiments were carried out. Because only one-tenth volume of the enzyme was added to the activity assay mixture, the final concentration of NaCl in the control sample was only 0.4 mM. The addition of K^+, Na^+, Ca^{2+}, Ni^{2+} or EDTA had neither activation nor inhibition effect on both wild-type and His-tagged isoamylase activities (Fig. 7). The addition of Hg^{2+} had a strong inhibition effect at 1 and 5 mM while the addition of Cu^{2+} showed strong inhibition only at 5 mM on both wild-type and His-tagged isoamylase activities. The addition of 5 mM Mg^{2+} slightly inhibited the wild-type enzyme activity, but had neither activation nor inhibition effect on the His-tagged enzyme activity. These results suggested that these metal ions were not required for the thermophilic isoamylase activity.

3.5. *Effects of pH and temperature on the activities and stabilities of the wild-type and His-tagged isoamylases*

Both wild-type and His-tagged enzymes showed an optimal activity at pH 5 (Fig. 8a). The wild-type enzyme remained stable in the pH range from 5 to 10, while the His-tagged enzyme remained stable in the pH range only from 7 to 10 (Fig. 8b). This result indicated that the addition of His-tag at the N-terminal of the thermophilic isoamylase affects its pH stability in the low pH range. In the low pH range, we found that the loss of isoamylase activity was due to the formation of the inactive monomer. Therefore, the addition of His-tag at the N-terminal of the thermophilic isoamylase might alter its trimer stability.

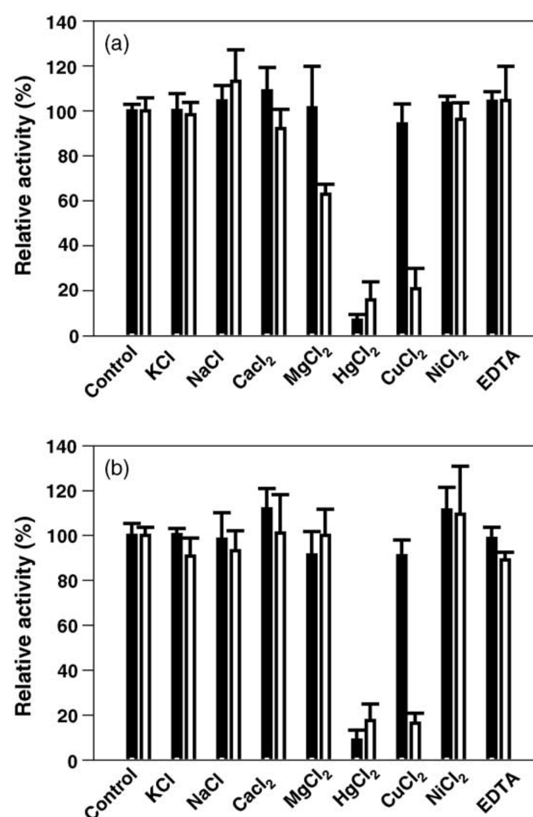

Fig. 7. Effects of metal ions on the activities of the wild-type and His-tagged isoamylases. The enzyme activities of the wild-type (panel a) and His-tagged (panel b) isoamylases were assayed at 75 °C, pH 5 in the presence of additional 1 (solid bars) and 5 (hollow bars) mM EDTA, KCl, NaCl, $CaCl_2$, $MgCl_2$, $HgCl_2$, $CuCl_2$, or $NiCl_2$. The relative activity was expressed as the percentage of the activity in the control experiment that contained 0.4 mM NaCl inherited from the stock enzyme. Data represent the mean ± S.D. from triplicate experiments.

Both enzymes had an optimal activity at temperature of 75 °C (Fig. 9a). The enzyme activities remained unchanged after a 2-h incubation at 80 and 75 °C for the wild-type and His-tagged enzyme, respectively (Fig. 9b). The good thermostabilities of both enzymes indicated that the active recombinant isoamylases were well folded, and that the wild-type isoamylase should have the same structure as that produced directly from *S. solfataricus* ATCC 35092. The relative activity of the His-tagged isoamylase was slightly lower than that of the wild-type enzyme under the incubation at temperatures ranging from 80 to 95 °C, indicating that the addition of His-tag at the N-terminal of the thermophilic isoamylase slightly lowers its thermostability.

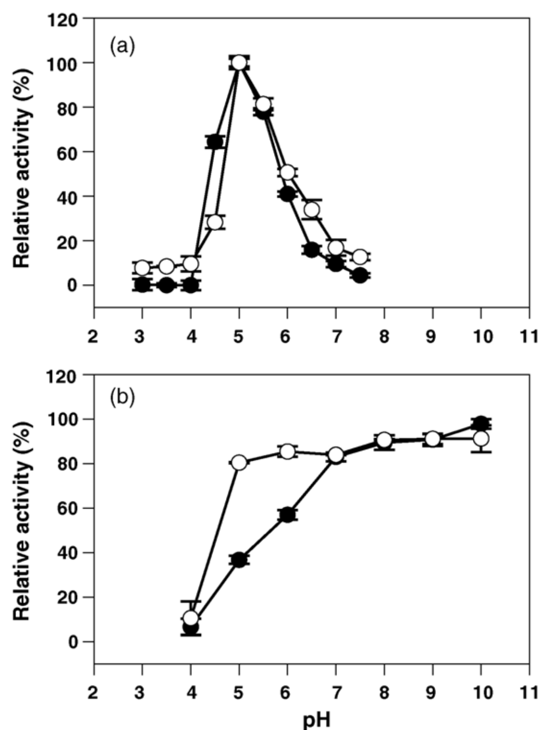

Fig. 8. Effect of pH on the activities and stabilities of the wild-type and His-tagged isoamylases. The following buffers with a concentration of 50 mM were used in different pH ranges: citrate–phosphate buffer (pH 3.5–7), Tris–HCl buffer (pH 7.5–8.5), and $NaHCO_3$–Na_2CO_3 buffer (pH 9.5–11). Panel (a) shows the effect of pH on the activities of the wild-type (◯) and His-tagged (●) isoamylases. For determination of the optimal pH, each enzyme (20 U/ml) was assayed at different pHs under the standard conditions. Panel (b) shows the effect of pH on the stabilities of the wild-type (◯) and His-tagged (●) isoamylases. For determination of pH stability, each enzyme (200 U/ml) was incubated at various pHs at room temperature for 24 h, and the remaining activities were assayed under the standard conditions. Data represent the mean ± S.D. from triplicate experiments.

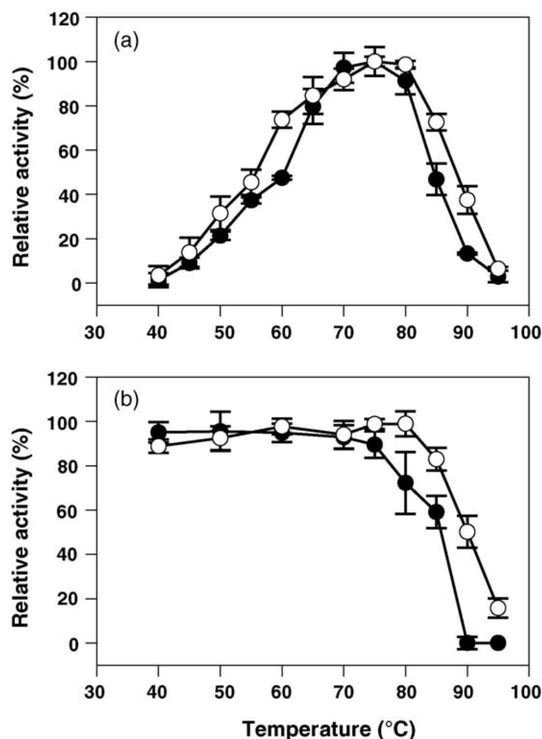

Fig. 9. Effect of temperature on the activities and thermostabilities of the wild-type and His-tagged isoamylases. Panel (a) shows the effect of temperature on the activities of the wild-type (◯) and His-tagged (●) isoamylases. For determination of the optimal temperature, each enzyme (20 U/ml) was assayed at different temperatures under the standard conditions. Panel (b) shows the effect of temperature on the thermostabilities of the wild-type (◯) and His-tagged (●) isoamylases. For determination of the thermostability, each enzyme (200 U/ml) was incubated at various temperatures at pH 5 for 2 h, and the remaining activities were assayed under the standard conditions. Data represent the mean ± S.D. from triplicate experiments.

A comparison of the enzymatic properties of the thermophilic isoamylases from different sources was listed in Table 4. In general, these thermophilic isoamylases from different sources possess a similar optimum pH. The optimum

Table 4
Comparison of the enzymatic properties of the thermophilic isoamylases from different sources

Properties	Recombinant			Natural
	S. solfataricus[a]	*S. acidocaldarius*[b]	*R. marinus*[b]	*R. marinus*[b]
Optimum temperature[c]	75 °C (pH 5)	70 °C (pH 5.5)	85 °C (pH 5)	NR
Optimum pH[d]	5 (75 °C)	5.5 (70 °C)	5 (50 °C)	5 (50 °C)
Thermostability[e]	80 °C, 2 h (99%)	NR[f]	NR	NR
pH stability	5–10	NR	NR	NR
Metal ion inhibition	Hg^{2+}, Cu^{2+}	NR	NR	NR
Ca^{2+} activation	No	NR	NR	NR
Molecular weight (SDS-PAGE)	77 kDa	NR	80 kDa	80 kDa
Molecular weight (mass spectrometry)	83 kDa	NR	NR	NR
Molecular weight (native)	249 kDa	NR	NR	NR

[a] This study.
[b] Tsutsumi et al. [19].
[c] The pH values used to measure the optimum temperature are shown in parentheses.
[d] The temperatures used to measure the optimum pH are shown in parentheses.
[e] The residual activitiy is shown in the parenthesis.
[f] Not recorded in the published literatures.

temperatures, however, vary with the sources and were 85, 75, and 70 °C for the enzymes from *R. marinus, S. solfataricus,* and *S. acidocaldarius*, respectively.

4. Conclusions

The thermophilic isoamylase from *S. solfataricus* ATCC 35092 was cloned and expressed in *E. coli*. We characterized the enzymatic properties of this recombinant enzyme in both wild-type and His-tagged forms. We found that the thermophilic isoamylases were active only in their trimer forms. The recombinant isoamylases that we cloned and expressed had a similar optimum pH to other natural or recombinant thermophilic isoamylases. The results from this study also suggested that these recombinant isoamylases that we cloned and expressed could be used in industry to degrade the branching points of starch at a high temperature.

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References

[1] H. Urlaub, G. Wober, FEBS Lett. 57 (1975) 1.
[2] T. Romeo, A. Kumar, J. Preiss, Gene 70 (1988) 363.
[3] H. Yang, M.Y. Liu, T. Romeo, J. Bacteriol. 178 (1996) 1012.
[4] R. Jeanningros, N. Creuzet-Sigal, C. Frixon, J. Cattaneo, Biochim. Biophys. Acta 438 (1976) 186.
[5] K. Takahashi, J.I. Abe, T. Kozuma, M. Yoshida, N. Nakamura, S. Hizukuri, Enzyme Microb. Technol. 19 (1996) 456.
[6] J. Abe, C. Ushijima, S. Hizukuri, Appl. Environ. Microbiol. 65 (1999) 4163.
[7] W.J. Lim, S.R. Park, S.J. Cho, M.K. Kim, S.K. Ryu, S.Y. Hong, W.T. Seo, H. Kim, H.D. Yun, Biochem. Biophys. Res. Commun. 287 (2001) 348.
[8] T. Harada, K. Yokobayashi, A. Misaki, Appl. Microbiol. 16 (1968) 1439.
[9] A. Amemura, R. Chakraborty, M. Fujita, T. Noumi, M. Futai, J. Biol. Chem. 263 (1988) 9271.
[10] J.H. Chen, Z.Y. Chen, T.Y. Chow, J.C. Chen, S.T. Tan, W.H. Hsu, Biochim. Biophys. Acta 1087 (1990) 309.
[11] K. Maruta, H. Mitsuzumi, T. Nakada, M. Kubota, H. Chaen, S. Fukuda, T. Sugimoto, M. Kurimoto, Biochim. Biophys. Acta 1291 (1996) 177.
[12] K. Maruta, M. Kubota, S. Fukuda, M. Kurimoto, Biochim. Biophys. Acta 1476 (2000) 377.
[13] Q. She, R.K. Singh, F. Confalonieri, Y. Zivanovic, G. Allard, M.J. Awayez, C.C. Chan-Weiher, I.G. Clausen, B.A. Curtis, A. De Moors, G. Erauso, C. Fletcher, P.M. Gordon, I. Heikamp-de Jong, A.C. Jeffries, C.J. Kozera, N. Medina, X. Peng, H.P. Thi-Ngoc, P. Redder, M.E. Schenk, C. Theriault, N. Tolstrup, R.L. Charlebois, W.F. Doolittle, M. Duguet, T. Gaasterland, R.A. Garrett, M.A. Ragan, C.W. Sensen, J. Van der Oost, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 7835.
[14] Y. Kawarabayasi, Y. Hino, H. Horikawa, K. Jin-no, M. Takahashi, M. Sekine, S. Baba, A. Ankai, H. Kosugi, A. Hosoyama, S. Fukui, Y. Nagai, K. Nishijima, R. Otsuka, H. Nakazawa, M. Takamiya, Y. Kato, T. Yoshizawa, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, K. Aoki, S. Masuda, M. Yanagii, M. Nishimura, A. Yamagishi, T. Oshima, H. Kikuchi, DNA Res. 8 (2001) 123.
[15] B.M. Krohn, G.F. Barry, G.M. Kishore, Mol. Gen. Genet. 254 (1997) 469.
[16] T. Harada, A. Misaki, H. Akai, K. Yokobayashi, K. Sugimoto, Biochim. Biophys. Acta 268 (1972) 497.
[17] H.H. Sato, Y.K. Park, Starch 32 (1980) 132.
[18] S. Hizukuri, T. Kozuma, H. Yoshida, J. Abe, i.K. Takahash, M. Yamamoto, N. Nakamura, STARCH-STARKE 48 (1996) 295.
[19] N. Tsutsumi, H. Bisgard-Frantzen, A. Svendsen, US Patent 6,087,149 (2000).
[20] E. Leveque, S. Janecek, B. Haye, A. Belarbi, Enzyme Microb. Technol. 26 (2000) 3.
[21] M.J. van der Maarel, B. van der Veen, J.C. Uitdehaag, H. Leemhuis, L. Dijkhuizen, J. Biotechnol. 94 (2002) 137.
[22] C. Bertoldo, G. Antranikian, Curr. Opin. Chem. Biol. 6 (2002) 151.
[23] G.D. Haki, S.K. Rakshit, Bioresour. Technol. 89 (2003) 17.
[24] A. Biwer, G. Antranikian, E. Heinzle, Appl. Microbiol. Biotechnol. 59 (2002) 609.
[25] M. Kato, J. Mol. Catal. B: Enzym. 6 (1999) 223.
[26] T.Y. Fang, X.G. Hung, T.Y. Shih, W.C. Tseng, Extremophiles 8 (2004) 335.
[27] I. Di Lernia, A. Morana, A. Ottombrino, S. Fusco, M. Rossi, M. De Rosa, Extremophiles 2 (1998) 409.
[28] C. Schiraldi, M. De Rosa, Trends Biotechnol. 20 (2002) 515.
[29] G.L. Miller, Anal. Chem. 31 (1959) 426.
[30] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[31] L.F. Liu, Y.C. Liu, C.H. Hsieh, T.C. Tam, C.H. Chuang, C.D. Hsiao, M.F. Tam, Anal. Biochem. 307 (2002) 173.
[32] M.R. Wilkins, E. Gasteiger, A. Bairoch, J.-C. Sanchez, K.L. Williams, R.D. Appel, D.F. Hochstrasser, in: A.J. Link (Ed.), 2D Proteome Analysis Protocols, Humana Press, New Jersey, 1998, p. 531 (Chapter 54).
[33] B. Henrissat, Biochem. J. 280 (1991) 309.
[34] B. Henrissat, A. Bairoch, Biochem. J. 293 (1993) 781.
[35] B. Henrissat, A. Bairoch, Biochem. J. 316 (1996) 695.
[36] E.A. MacGregor, S. Janecek, B. Svensson, Biochim. Biophys. Acta 1546 (2001) 1.
[37] Y. Katsuya, Y. Mezaki, M. Kubota, Y. Matsuura, J. Mol. Biol. 281 (1998) 885.
[38] B. Svensson, Plant Mol. Biol. 25 (1994) 141.
[39] N.J. Marianayagam, M. Sunde, J.M. Matthews, Trends Biochem. Sci. 29 (2004) 618.
[40] E. Rabbo, T.C. Terkildsen, Scand. J. Clin. Lab. Invest. 12 (1960) 402.