ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ, 2011, том 47, № 4, с. 455–461

UDC 577.154.3:582.28

AN EXTRACELLULAR GLUCOAMYLASE PRODUCED BY ENDOPHYTIC FUNGUS EF6

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Received Aprel 12, 2010

A strain of endophytic fungus EF6 isolated from Thai medicinal plants was found to produce higher levels of extracellular glucoamylase. This strain produced glucoamylase of culture filtrate when grown on 1% soluble starch. The enzyme was purified and characterized. Purification steps involved $(NH_4)_2SO_4$ precipitation, anion exchange, and gel filtration chromatography. Final purification fold was 14.49 and the yield obtained was 9.15%. The enzyme is monomeric with a molecular mass of 62.2 kDa as estimated by SDS-PAGE, and with a molecular mass of 62.031 kDa estimated by MALDI-TOF spectrometry. The temperature for maximum activity was 60°C. After 30 min for incubation, glucoamylase was found to be stable lower than 50°C. The activity decrease rapidly when residual activity was retained about 45% at 55°C. The pH optimum of the enzyme activity was 6.0, and it was stable over a pH range of 4.0–7.0 at 50°C. The activity of glucoamylase was stimulated by Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, glycerol, DMSO, DTT and EDTA, and strongly inhibited by Hg^{2+}. Various types of starch were test, soluble starch proved to be the best substrate for digestion process. The enzyme catalyzes the hydrolysis of soluble starch and maltose as the substrate, the enzyme had K_m values of 2.63, and 1.88 mg/ml and V_{max}, values of 1.25, and 2.54 U/min/mg protein, and V_{max}/K_m values of 0.48 and 1.35, respectively. The internal amino acid sequences of endophytic fungus EF6 glucoamylase; RALAN HKQVV DSFRS have similarity to the sequence of the glucoamylase purified form *Thermomyces lanuginosus*. From all results indicated that this enzyme is a glucoamylase (1,4-α-D-glucan glucanohydrolase).

Glucoamylases (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.3), also referred to as amyloglucosidases or α-amylases, is a biocatalyst capable of hydrolyzing α-1,4 glycosidic linkages in raw (sparsely soluble) or soluble starches and related oligosaccharides with the inversion of the anomeric configuration to produce α-glucose. In addition to acting on α-1,4-linkages, the enzyme slowly hydrolyzes α-1,6-glycosidic linkages of starch [1–4]. These enzymes have many applications in industry, being used for dextrose production, in the baking industry, in the brewing of low calorie beer and in whole grain hydrolysis for the alcohol industry, but the most important application of glucoamylase is the production of high-glucose syrups. Others dextrose products formed as a result of the glucoamylase action are high fructose corn syrup and high conversion syrups. The conversion of starch to sugars is one of the most important biotechnological processes [5].

Many fungal species are capable of producing glucoamylase under different fermentation conditions and techniques. Most attempts involved seeking fungal species capable of hydrolyzing raw starch at elevated temperatures. The various fungi synthesizing glucoamylase that is active at higher temperatures include *Aspergillus awamori*, *A. foetidus*, *A. niger*, *A. oryzae*, *A. terreus*, *Mucor rouxians*, *M. javanicus*, *Neurospora crassa*, *Rhizopus delmar*, *R. oryzae* [6]; and *Arthrobotrys amerospora* [7, 8]. However, the industrial focus has been on glucoamylase from *A. niger* and *R. oryzae*. The employment of glucoamylases from these sources in the starch processing industries is due to their good thermostability and high activity at near neutral pH values [9, 10].

Endophytes are obviously a rich and reliable source of bioactive metabolites with huge medical, agricultural and industrial potentials [11]. Although the enzymes vary from isolate to isolate, the endophytic fungi tested all synthesize in vitro the enzymes necessary for penetrating and colonizing their plant hosts [12]. Such enzymes including pectinases, xylanase, cellulases and lipases, proteinase and phenol oxidase have been documented with some endophytes [11]. However, only little has been reported about glucoamylase production by endophytic fungi up to date.

The objective of this work is to isolate endophytic fungi capable to producing glucoamylase and describe the purification and partial characterization of a glucoamylase from endophytic fungi.

MATERIALS AND METHODS

Fungal strain and culture condition. Isolated strains of endophytic fungi were obtained from stock cultures of

Research Centre of bioorganic Chemistry (RCBC), Chulalongkorn University. The fungal cultures were grown on a screening starch agar plate. After that, the cultures were incubated at room temperature for 8–10 days. The media were flooded with iodine solution (0.1% I_2 Iand 1.0% KI) for 15 s and the iodine solution was removed. Clear zone were observed around the growing colony and the colonies which have a largest clear zone were isolated for further studied. The selected isolate of endophytic fungi was prepared on PDA at 25°C. Unless otherwise stated, actively growing fungal mycelium from a 7 day old culture was transferred to a 250-ml Erlenmeyer flask containing 100 ml of Mandels' medium [13]; composed of (g/l): urea – 0.3, $(NH_4)_2SO_4$ – 1.4, KH_2PO_4 – 2.0, $CaCl_2 \cdot 2H_2O$ – 0.4, $MgSO_4 \cdot 4H_2O$ – 0.3, peptone – 1.0; (mg/l): $FeSO_4 \cdot 7H_2O$ – 5.0, $MnSO_4 \cdot 4H_2O$ – 1.6, $ZnSO_4 \cdot 7H_2O$ – 1.4, $CoCl_2 \cdot 6H_2O$ – 2.0, and Tween 80 – 2.0 ml/l, pH 5.5 to which 1% soluble starch per l of distilled water. The incubation was carried out at room temperature on a rotary shaker at 200 rpm for 14 days. After the cultivation was completed, the culture was filtrated through a Whatman filter paper No.1 and the filtrate was used as the crude enzyme.

Molecular Identification. Genomic DNA was prepared from the fresh mycelial culture of selected isolate of endophytic fungi and extracted with cetyltrimethylammonium bromide (CTAB) as described in [14]. PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 50 μl which comprised approximately 100 ng genomic DNA, 1x PCR Master Mix (Fermentas, Califonia, USA), and the primer ITS1F [15]; and ITS4 [16]. The amplification was performed in a thermocycler TGradient (Biometra, Germany) with 94°C for 5 min, followed by 38 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, with final extension of 72°C for 5 min. PCR product was purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and sequenced externally by Macrogen (Seoul, Korea) using the same primer as for amplification.

Assay of glucoamylase. Glucoamylase activity was determined by measuring the production of reducing sugar using 3,5-dinitrosalicylic acid (DNS) as described by Miller [17]. The assay was carried out at 50°C, using a 1.0% starch solution in 0.1 M sodium acetate buffer, pH 5.0. One unit of glucoamylase activity was defined as the amount of enzyme that releases 1 μmol of glucose per minute.

Purification of glucoamylase. All of the procedures were performed at the 4°C, unless otherwise stated.

$(NH_4)_2SO_4$ precipitation. To 5.0 l of culture supernatant, $(NH_4)_2SO_4$ was added to give 80% saturation. After standing overnight the precipitate formed was collected by centrifugation at 10000 g for 20 min (Beckman Coulter, USA), and dissolved in 50 mM sodium acetate buffer, pH 5.0. The dissolved sample was dialyzed against the same buffer and concentrated by lyophilization (Labconco, USA).

Anion exchange chromatography. The sample solution was applied to a column (1.6 × 20 cm) of a Q Sepharose Fast Flow (Sigma, USA) equilibrated with 50 mM sodium acetate buffer, pH 5.0. Elution was undertaken with the same buffer at a flow rate of 1.0 ml/min. A linear gradient of 0–1.0 M NaCl in the same buffer was then applied. Fractions of 10.0 ml each were collected and assayed for glucoamylase activity. The fractions containing glucoamylase activities from the column were pooled and dialyzed against the same buffer for further purification.

Gel filtration chromatography. The active fraction from Q Sepharose Fast Flow was applied to a column (1.6 × 60 cm) of Superdex 75 high resolution (Amersham Pharmacia Biotech, Sweden) equilibrated with 50 mM sodium acetate buffer, pH 5.0 containing 100 mM NaCl at a flow rate of 1.0 ml/min. Fractions of 5.0 ml were collected and assayed for glucoamylase activity. The active fractions containing glucoamylase activities from the column were pooled and dialyzed against the same buffer for further analysis.

Protein determination. Protein concentrations in the enzyme preparations were determined by the method of Bradford [18]; with reference to a standard calibration curve for bovine serum albumin (Fluka, USA). During the column chromatographic separations the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

Molecular mass determination. *SDS polyacrylamide gel electrophoresis.* The gel was prepared with 0.1% SDS in 12.5% separating gels and 5.0% stacking gels. Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli [19]. Samples to be analyzed were treated with sample buffer and boiled for 5 min before application to the gel. Electrophoresis was run from the cathode to anode at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Amersham Pharmacia Biotech, Sweden). High and low molecular weight standards were used to determine the subunit molecular weight of the enzyme. After electrophoresis, proteins in the gel were visualized by staining with coomassie blue R-250.

Mattrix-assised laser desorption ionization time of flight. The purified glucoamylase from Superdex 75 HR column was analyzed. Desalting of enzyme was performed using reusable reverse-phase cartridge. The desalted enzyme was dried using a freeze dryer. The dried enzyme was dissolved in 50% acetonitrile in trifluoroacetic acid. Mass spectra of glucoamylase were acquired using a MALDI-TOF mass spectrometer operating in linear and reflectron modes (Biflex, Bruker, Germany).

Effect of temperature on glucoamylase activity and stability. The optimum temperature for enzyme activity was determined by monitoring each activity at 0.1 M acetate sodium acetate buffer pH 5.0 at various temperatures from 30 to 80°C. Stability was measured by incubating the enzyme in 0.1 M acetate buffer pH 5.0 for 30 min at tem-

peratures from 30 to 80°C. Following incubation, the enzyme solution was cooled, and the remaining activity was determined under standard enzyme assay conditions.

Effect of pH on glucoamylase activity and stability. The optimum pH of activity was determined by monitoring each activity at 50°C at various pH values ranging between 3.0 to 9.0. The following buffers were used: 0.1 M sodium acetate buffer (pH 3.0–6.0); 0.1 M phosphate buffer (pH 6.0–7.0) and 0.1 M Tris-HCl buffer (pH 7.0 9.0). The glucoamylase stability was examined at the pH values 3.0–9.0. Enzyme samples were pre-incubated in the above-cited buffers at 30°C for 1 h before adding the substrate. After adjustment of the pH the residual activity was determined under standard enzyme assay conditions.

Effect of metals and reagents. The effects of various metal ions and reagents at 1 mM on glucoamylase activity were determined by preincubating the enzyme with the individual reagents in 0.1 M sodium acetate buffer pH 5.0 at 50°C for 30 min. Activities were then measured at 50°C in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was recorded as 100%.

Substrate specificity of enzyme activity. The effects of different raw starches as substrate were determined by the reaction of enzyme preparation in the presence of various raw starches (soluble starch, corn, tapioca, wheat, rice, sticky rice starch). 1ml of enzyme was added to 1 ml of 1% (w/v) raw starches in 0.1 M sodium acetate buffer pH 5.0 and incubated at 50ºC for 10 min. The reaction was stopped by placing them in boiling water for 5 min. The amount of reducing sugar was assayed by standard procedure as described earlier. The control was reaction mixture with soluble starch.

Kinetic properties of gluamylase. Kinetics constants as K_m, V_{max} and V_{max}/K_m were measured by estimating hydrolysis with soluble starch and maltose as substrate. Initial velocity studies were carried out under the standard condition. The concentrations of substrate were varied from 0.2–20 mg. The Lineweaver-Burk of initial velocity against substrate concentration was ploted.

Internal amino acid sequence of glucoamylase by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). *In situ* (*in gel*) *trypsinization*. The sample preparation process followed the published method of Mortz [20]. Each band in the electrophoretic gel was excised, cut into small pieces (ca. 1 mm^3) and washed with 100 μl deionized water. The gel pieces were destained by adding 200 μl of a 2 : 1 (v/v) ratio of acetonitrile: 25 mM NH_4HCO_3 for 15 min, and this step was performed several times until the gel pieces were completely destained. The supernatant was removed and gels were then dehydrated by adding 200 μl acetonitrile for 15 min prior to drying in a vacuum centrifuge. Then 50 μl of a 10 mM DTT solution in 100 mM NH_4HCO_3 was added, and the proteins were reduced for 1 h at 56°C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile and the solvent evaporated off before adding 10 μl of a trypsin solution (proteomics grade, Sigma) (10 ng/μl in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4°C, 30 μl of 50 mM NH_4HCO_3 was added and the digestion allowed to proceed at 37°C overnight. The supernatant was then harvested following centrifugation at 10000 g for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetronitrile containing 5% (v/v) formic acid for 10 min with shaking, and subsequently pooled with the supernatant and taken to dryness.

LC/MS/MS and peptide blasting. The likely amino acid sequence of each internal fragment of the trypsinized was analyzed by LC/MS/MS mass spectrometry. The extracted tryptic peptides were then subjected to LC-nano ESI/MS/MS (Sigma-Aldrich, USA). All collected LC/MS/MS data were processed and submitted to a Mascot (http://www.matrixscience.com) search of the NCBI database (http://blast.ncbi.nlm.nih.gov). The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage sites, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ±0.2 Da peptide tolerance and MS/MS tolerance, and ESI-TRAP fragmentation scoring [20].

RESULTS AND DISCUSSION

Screening of endophytic fungi for glucoamylase activity. Isolated strained of endophytic fungi were investigated for their ability to hydrolyze starch after 8 days of growth on starch agar. When the medium was flooded which iodine solution, clear zones were observed around growing colonies of the fungi. Isolate EF6 produced clear zones, with sizes around 15 mm diameter. The clear zones suggested that isolate EF6 can produce amylolytic enzymes to digest soluble starch in starch agar. Differences in the ability of the endophytic fungi to hydrolyze soluble starch could be due to the amount of enzyme excreted into starch agar and the different growth rates of the strains [21]. Isolate EF6 showed the largest clear zones when compared with other positive strain and were chosen for further investigation.

Molecular identification of endophytic fungi EF6. The mycelium of endophytic fungus isolate EF6 was prepared by slide culture technique and observed under light microscope. Isolate EF6 is mycelia sterile because it showed septate hyphae with non spore formation were then identified by molecular technique. Endophytic fungi EF6 was sent for identification by molecular methods at the Macrogen, Seoul, Korea The rDNA ITS region of endophytic fungi EF6 was amplified with the conserved fungal primer ITS_{1F} and ITS_4. Endophytic fungi EF6 produced a single ITS band, containing a part of the 18S, ITS1, 5.8S and 28S rDNA. The nucleotide sequence data reported in this

Purification table of glucoamylase from endophytic fungi EF6

Purification step	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield, %	Purification, fold
Cuture filtrate	519.35	27.157.85	52.29	100.00	1.00
80% $(NH_4)_2SO_4$ Precipitation	302.51	22.593.04	74.69	83.19	1.43
Q Sepharose FF	23.47	7.808.44	332.70	28.75	6.36
Superdex 75 HR	3.28	2.486.19	757.98	9.15	14.49

paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB284189.

Purification of glucoamylase. Endophytic fungus EF6 was cultivated in the of Mandels medium [22] containing 2% soluble starch per liter, at pH 5.0, 200 rpm for 10 days which was optimal condition determined by glucoamylase activity measuring the production of reducing sugar using DNS method [17]. The myceliums were removed by filtration, and the crude enzyme in the supernatant fraction was collected. Through the step of $(NH_4)_2SO_4$ precipitation to give 80% saturation, and dialyzed against 50 mM sodium acetate buffer, pH 5.0. The purification of glucoamylase was increased by 1.43 fold and 83.19 % yield was obtained, the glucoamylase was concentrated by lyophilization to dryness. Then concentrated glucoamylase solution was applied onto Q Sepharose chromatography. The major active peak contained glucoamylase activity was adsorbed onto the Q Sepharose column and eluted from the column at 0.75–0.80 M NaCl, whereas the minor active peak was not adsorbed on the resin and eluted with most of the loading protein in the starting wash period. Then active fractions were pooled, and dialyzed against 50 mM sodium acetate buffer, pH 5.0, for this step the enzyme with 6.36 purification fold and 28.75% yield was obtained. Before the next Superdex 75 chromatography, the glucoamylase was concentrated by lyophilization to dryness. Superdex 75 chromatography column profiles of glucoamylase separation which was well isolated from other proteins. The fraction which contains glucoamylase was the major peak. The overall purification steps of glucoamylase from endophytic fungi EF6 are summarized in Table 1. Finally, the glucoamylase was purified to 14.49 fold with the yield of 9.15%. Specific glucoamylase activity was increased from 52.29 of the crude enzyme to 757.98 U/mg protein of the purified enzyme. Although the recovery rate was relatively low, the purification steps were far more rapid than some other methods [5, 23–28].

Molecular mass determination. Molecular weight of extracellular glucoamylases of endophytic fungi EF6 was determined by SDS-PAGE and it was about 62.2 kDa (Fig.1a). These values are in agreement with the literature

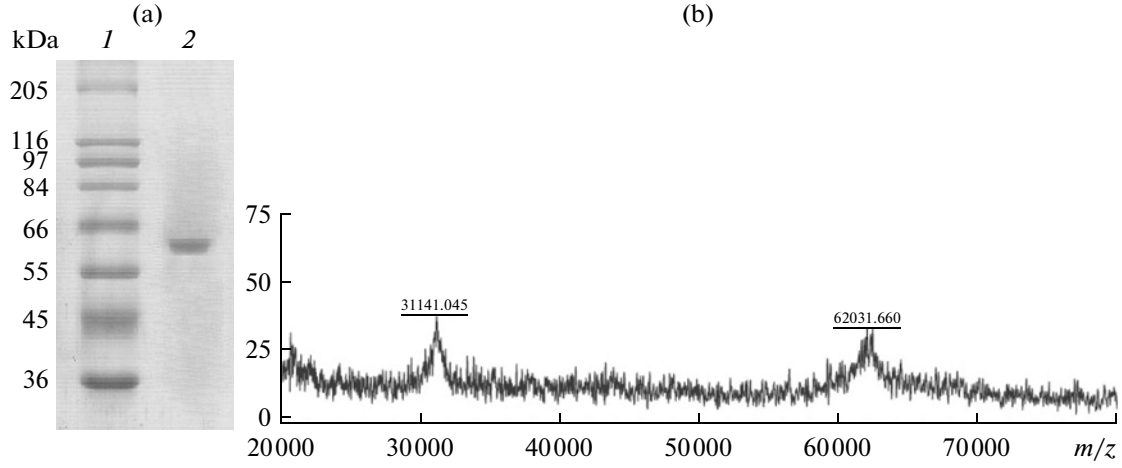

Fig. 1. SDS-PAGE of purified glucoamylase from endophytic fungi EF6: (a): *1* – molecular weight marker of protein standard, i.e., myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), and glyceraldehydes-3-phosphate dehydrogenase (36 kDa), *2* – purified glucoamylase from endophytic fungi EF6. (b) – MALDI-TOF mass spectrum of purified glucoamylase from Superdex-75 high resolution column.

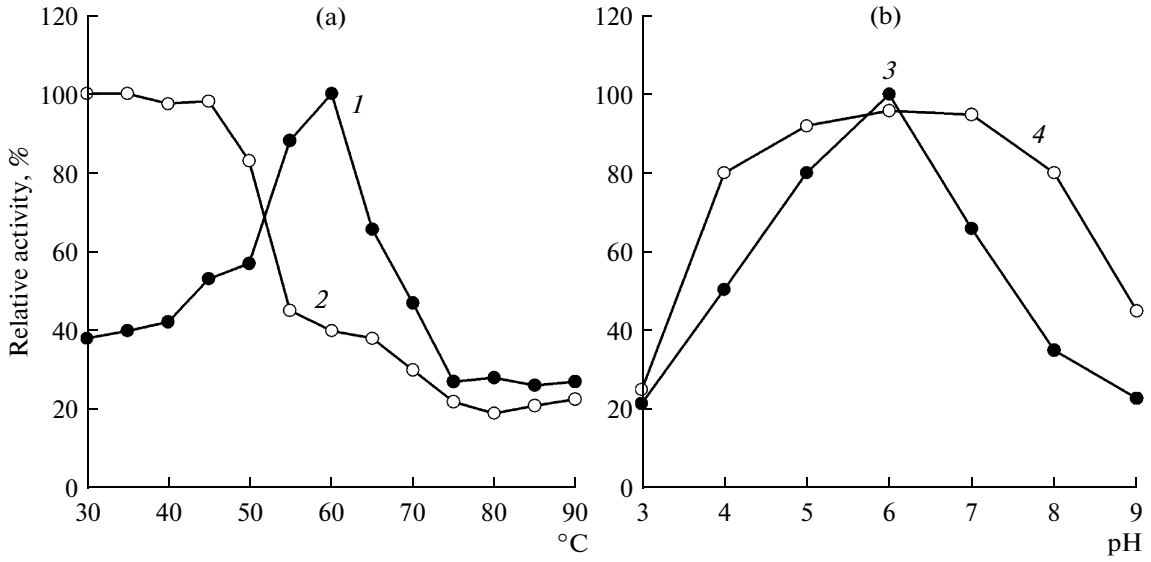

Fig. 2. Effect of temperature (a) and pH (b) on glucoamylase activity and stability; (a): *1* – temperature activity, *2* – temperature stability. The following buffer systems were used 0.1 M acetate buffer pH 5.0 for 30 min; (b): *3* – pH activity, *4* – pH stability. The following buffer systems were used: 0.1 M sodium acetate buffer (pH 3.0–6.0), 0.1 M phosphate buffer (pH 6.0–7.0) and Tris-HCl buffer (pH 7.0–9.0). The values shown represent averages from triplicate experiments.

that reports that the molecular masses of fungal glucoamylases are in the range 45–90 kDa [29]; as those of *Termomyces lanuginosus* [30]; *Chaetomium thermophilum* [31]; and *Sclerotinia sclerotiorum* [26]. However, a higher value of MW of 125 kDa was reported for *A. niger* glucoamylase [32]. To confirm the mass of analyse, mass spectrometry was used (Fig. 1b). The *m/z* of 62.031 and 31.141 Da were recorded and were related to singlet and doublet charge species of purified glucoamylase, respectively. This result is supported the MW which performed by SDS-PAGE.

Effect of temperature on glucoamylase activity and stability. To determine the effect of temperature on purified glucoamylase from endophytic fungi EF6, enzyme activities were estimated over the temperature range of 30–90°C. This enzyme exhibited optimum activity in the range from 55–60°C (Fig. 2a). Similar finding were also reported for glucoamylase from *A. oryzae* [33]; *Monascus* sp. [34]; and *M. javanicus* [35]. After 30 minutes for incubation, glucoamylase was found to be stable at temperature lower than 50°C. The activity is rapidly decreased and the remaining residual activity was about 45% of original at 55°C (Fig. 2a). On the other hand, the enzymes which was isolated from several mesophilic fungi, was shown to be highly susceptible to thermal denaturation at temperature above 50°C [28, 36].

Effect of pH on glucoamylase activity and stability. The optimum pH of the purified glucoamylase from endophytic fungi EF6 was determined by measuring its activity at different pH values. The result suggested that purified glucoamylase was active with pH optimal of 6.0 (Fig. 2b). Similar results were obtained for extracellular glucoamylase from *Humicola grisea* [23]; and *A. awamori* [37]. Most fungal starch-degrading enzymes have optimum pH values of 5.0–7.0 [38]. Generally, glucoamylases obtained from fungi are active at acidic pH values. However, several forms have variable values of pH optimum. The pH stabilities of the enzyme at 50°C were determined by incubating the enzyme in the respective buffers of different pH values for 1 h. The enzyme was stable over an acidic pH range of 4.0–7.0 at 50°C (Fig. 2b).

Effect of metals and reagents. The effect of various metal ions on purified glucoamylases activity was determined using soluble starch as substrate. The assays were carries out in the presence of 1 mM concentrations of each of the metal ions and reagents. The activity assayed in the absence of cations or reagents was taken as 100%. The inhibitory effect has been shown in the present of Hg^{2+} (8.4%). Moreover, Cu^{2+} (65.3%) and Fe^{2+} (75.5%) have inhibited the glucoamylases activity. The chelating agent; EDTA (100.3%) did not affect glucoamylases activity which indicating that glucoamylases is not a metalloprotein. Furthermore, DTT (106.9%) was not an inhibitor suggested that disulfide bonds were not involved for enzyme activity. Activation by Ca^{2+} (107.5%), Co^{2+} (102.4%), Mn^{2+} (121.5%), Mg^{2+} (121.5%), glycerol (104.5%) and DMSO (104.5%) may be explained by stabilization of the enzyme structure. Glucoamylase of *Chaetomium thermophilum* was more active in the presence of Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}, and inhibited by Fe^{2+}, Ag^{+}, and Hg^{2+} [39]. Mn^{2+} and Zn^{2+} enhanced glucoamylase activity from *Himicola grisea* var. *thermoidea* [27]; and lower concentrations of Mn^{2+} and Ca^{2+} enhanced the activity of glucoamylase of *Sclerotinia sclerotiorum* [26].

Substrate specificity. The ability of hydrolysis on various substrates by glucoamylase was determined using var-

Endophytic fungi EF6		5						10						15				Acession Number
Thermomyces lanuginosus	305	R	A	L	A	N	H	K	Q	V	V	D	S	F	R	S	319	Q58HN1
Aspergillus shirousami	296	R	A	L	A	N	H	K	E	V	V	D	S	F	R	S	310	P22832
Aspergillus niger	297	R	A	L	A	N	H	K	E	V	V	D	S	F	R	S	311	P69328
Aspergillus awamori	296	R	A	L	A	N	H	K	E	V	V	D	S	F	R	S	310	Q76L97
Fusarium verticillioides	297	R	A	L	A	N	H	K	E	V	V	D	S	F	R	S	311	B0Z3Y3
Aspergillus oryzae	300	R	A	L	A	N	H	K	E	V	V	D	S	F	R	S	314	O59846
Talaromycets emersonii	302	R	A	L	A	N	H	K	V	V	T	D	S	F	R	S	316	Q9C1V4
Aspergillus flavus	299	R	A	L	A	N	H	K	V	V	T	D	S	F	R	S	313	B8NX52
Penicillium notatum	295	R	A	L	S	N	H	K	E	Y	V	D	S	F	R	S	308	Q76KF7
Neurospora crassa	309	R	A	L	A	N	H	K	V	V	V	D	S	M	R	–	322	P14804

Fig. 3. Amino acid sequences from the fragment obtained by tryptic digestion of the purified glucoamylase from endophytic fungi EF6. Comparisons are made with other glucoamylase family. Shaded regions represent regions of identity.

ious substrates. The results suggested that glucoamylase has the highest hydrolyzing activity on soluble starch (100.0%). Tapioca (95.8%), corn (88.3%), rice (67.3%), wheat (64.7%), and sticky rice (55.4%) flour also showed similar activity but slightly lower. This differential rate of digestion was due to the existence of starch granules, which in turn depends upon its botanical source [40]. These results suggest that hydrolysis of raw starches is not only a property of the enzyme but that it also depend on the type of starch [21].

Kinetics properties. Reaction kinetics of the purified glucoamylase was determined from Lineweaver-Burk plots with soluble starch and maltose as substrate under defined assay conditions. The enzyme had K_m values of 2.63, and 1.88 mg/ml and V_{max}, values of 1.25, and 2.54 U/min/mg protein, and K_m/V_{max} values of 0.48 and 1.35, respectively. The enzyme possesses greater affinity to maltose which was confirmed by the values of catalytic efficiency. Therefore these data are consistent with values expected for complete hydrolysis, the small difference observed in the yields of the product in the case of starch could be due to the inability of the enzyme to attack α-D-1,6-glucosidic linkages [41].

Identification of glucoamylase. The internal sequence analysis of the purified glucoamylase was obtained by digestion with trypsin and sequence analysis with LC-MS/MS and the identified tryptic fragment was found to be RALAN HKQVV DSFRS. Then this peptide sequence was used to perform the database searching using SwissProt database. The search result is shown in Fig. 3. From the results of database searching, partial amino acid sequences of purified protein is similar to glucoamylase from *T. lanuginosus*, *A. shirousami*, *A. niger*, *A. awamori*, *F. verticillioides*, *A. oryzae*, *T. emersonii*, *A. flavus*, *P. notatum*, and, *Neurospora crassa*. From all result suggested that the purified enzyme from endophytic fungi EF6 is glucoamylase (1,4-α-D-glucan glucanohydrolase).

ACKNOWLEDGMENTS

The authors wish to thank the Chulalongkorn University graduate school thesis grant, the National Research University Project of CHE, the Ratchadaphiseksomphot Endowment Fund (AG001B, AM1019A), and the Thai Government Stimulus Package 2 (TKK2555), for financial support of this research, as well as the Research Institute of Biotechnology and Genetic Engineering and Biotechnology program, the Faculty of Science, Chulalongkorn University for support and facilities. We also wish to thank Dr. Robert Butcher (Publication Counselling Unit, Chulalongkorn University) for his constructive comments in preparing this manuscript.

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