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Novel α-Glucosidase from Extreme Thermophile *Thermus caldophilus* GK24

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α-Glucosidase of an extreme thermophile, *Thermus caldophilus* GK24 (TcaAG), was purified 80-fold from cells to a homogeneous state and characterized. The enzyme exhibited optimum activity at pH 6.5 and 90°C, and was stable from pH 6.0 to 8.5 and up to 90°C. The enzyme had a half-life of 85 minutes at 90°C. An analysis of the substrate specificity showed that the enzyme hydrolyzed the non-reducing terminal unit of α-1,6-glucosidic linkages of isomaltosaccharides and panose, α-1,3-glycosidic bond of nigerose and turanose, and α-1,2-glycosidic bond of sucrose. The gene encoding the TcaAG was cloned, sequenced, and expressed in *E. coli*. The nucleotide sequence of the gene encoded a 530 amino acid polypeptide and had a G+C content of 68.4% with a strong bias for G or C in the third position of the codons (93.6%). A sequence analysis revealed that TcaAG belonged to the α-amylase family. We suggest that this monomeric, thermostable, and broad-acting α-glucosidase is a departure from previously exhibited specificities. It is, therefore, a novel α-glucosidase.

Keywords: α-Glucosidase, Thermostable enzyme, *Thermus* sp., Gene cloning, Transglucosylation

Introduction

α-Glucosidase is a typical exo-type amylolytic hydrolase that releases α-glucose from the non-reducing end unit of substrates (oligosaccharides or polysaccharides). Many have been reported from microorganisms, plants, and mammals (Vihinen *et al.*, 1989), and they are diverse in substrate specificity and transglucosylation activities. The majority of α-glucosidases (α-D-glucoside glucohydrolase, EC 3.2.1.20) have been reported to show preference for maltose hydrolysis (Kelly *et al.*, 1983). Yet another group, for example dextrin 6-α-D-glucanohydrolase (oligo-1,6-glucosidases, EC 3.2.1.10), acts exclusively on the α-1,6-glucosidic linkage of isomaltosaccharides (Suzuki *et al.*, 1982). α-Glucosidase, which has the broadest substrate specificity reported, is from *Bacillus* sp. SAM1606. It could even hydrolyze the α-1,α-1-glucosidic linkage of α,α-trehalose, as well as the α-1,3, α-1,4 and α-1,6 linkages of nigerose, maltose and isomaltose, respectively, and 1-*O*-aryl α-glucosides (Nakao *et al.*, 1994a,b). Differences in substrate specificities and transglucosylation activities could be due to the corresponding differences in the structures of the substrate-binding and catalytic sites of the enzymes.

During our studies on carbohydrate-metabolizing enzymes from an extreme thermophile (Chang *et al.*, 1988), *Thermus caldophilus* GK24, we found an enzyme exhibiting α-glucosidase activity towards isomaltose and *p*-nitrophenol-α-D-glucopyranoside as substrates from the cellular fraction. We purified this novel thermostable α-glucosidase, whose efficient hydrolysis of sucrose, nigerose, and turanose distinguishes it from other oligo-1,6-glucosidases, with which it shares a similar catalytic property on isomalto-oligosaccharides. In addition to being one of the most thermostable α-glucosidase reported, its catalytic pH optima ranked it high for synergism in α-amylolysis for enhanced liquefaction process in the starch industry. A comparison of the primary structure of this novel α-glucosidase with others could provide useful information on structure-function relationships of α-glucosidases.

We report here the biochemical characteristics of TcaAG, and its gene cloning, sequencing, and expression in *E. coli*.

Materials and Methods

Materials and bacterial growth *p*-Nitrophenyl α-D-glucopyranoside (*p*-NPG), 4-Methylumbelliferyl- α-D-glucoside, and isopropyl β-thiogalactoside (IPTG) were purchased from Sigma. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was purchased from Promega. The PGO-assay kit was from Sigma. All enzymes for DNA manipulations were obtained from either POSCOCHEM (Korea) or BM (Germany). DEAE-Sephacel, Phenyl-Sepharose CL-4B, MonoQ, Sephacryl S300 H/R, Superose

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12 were purchased from Pharmacia LKB Biotechnology. Ingredients of *E. coli* media were purchased from Difco. All other chemicals were of analytical grade. *T. caldophilus* GK24 cells (kindly supplied by Professor T. Ohta, Faculty of Agriculture, The Tokyo University, Tokyo, Japan) were grown at 75°C in a medium (pH 7.2), containing 0.8% polypeptone, 0.4% yeast extracts and basal salts (Taguchi *et al.*, 1982; Park *et al.*, 1993), using a 10-liter stirred-tank fermentor (Korea Fermentor Co. Ltd., Inchon, Korea).

Enzyme assay α-Glucosidase activity was measured as the amount of *p*-nitrophenol released from *p*-nitrophenyl-α-D-glucopyranoside. The reaction mixture (0.5 ml) that contained 40 mM phosphate buffer pH 6.8, 10 mM *p*-nitrophenyl-α-D-glucopyranoside, and the enzyme was incubated at 75°C for 10 min (Suzuki *et al.*, 1976). One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 μmole *p*-nitrophenolate per minute under the above condition. When determining the effects of various metal ions, the reaction mixtures containing cations (1 mM) were used. The apparent kinetic parameters were determined by measuring the release of *p*-nitrophenol or glucose from *p*-NPG, or oligosaccharides by the glucose oxidase/peroxidase procedure (Sigma Kit No. 510-DA) (Dahlqvist 1968), respectively. The hydrogen peroxide, formed during the dehydrogenation of glucose by glucose oxidase, is used by peroxidase to oxidize *o*-dianisidine to a brown compound. The color intensity was measured at 450 nm. K_m and V_{max} were calculated from the initial velocity. The molar activity, k_{cat}, was calculated from V_{max} and the molar concentration of TcaAG in the reaction mixture, E_o, using the equation $k_{cat} = V_{max}/E_o$. Protein concentrations were quantified by the method of Bradford with bovine serum albumin as a standard protein (Bradford, 1976).

Enzyme purification All purification steps were performed at room temperature. *T. caldophilus* GK24 cells (50 g) were suspended in 40 mM potassium phosphate, pH 7.0 (buffer A), and disrupted by sonication. Cell debris was removed by centrifugation at 12,500 × *g* for 1 h, and the supernatant (100 ml) was used as the cell-free extract for enzyme purification. Solid ammonium sulfate was slowly added on the cell-free extract to a 50% saturation. The precipitate formed was collected by centrifugation at 12,500 × *g* for 30 min, followed by dialysis against buffer A. The dialyzed solution was then applied to a DEAE-Sephacel column (2.5 × 30.0 cm) that had been equilibrated with buffer A. Active fractions were eluted with a linear salt gradient from 0.1 to 0.3 M NaCl in buffer A. These were pooled (15 ml), dialyzed against buffer A, and applied to a Phenyl-Sepharose column (Pharmacia), equilibrated with 1.0 M ammonium sulfate in buffer A. The enzyme was eluted with a decreasing linear gradient of ammonium sulfate from 1.0 M to 0 M in buffer A. The active fractions were collected, pooled (6 ml), dialyzed against buffer A, and applied to a Mono Q column (Pharmacia), equilibrated with buffer A. After washing the column with buffer A, the enzyme was eluted with a linear gradient of NaCl from 0.1 to 0.35 M in buffer A. The active fractions were pooled (2 ml), concentrated by ultrafiltration with Amicon membrane YM10, and dialyzed against buffer A. The enzyme solution was then applied to a Sephacryl S-300 column (Pharmacia), equilibrated with buffer A that contained 0.15 M KCl, and eluted with the same buffer. The chromatography columns were controlled by FPLC systems (Pharmacia Biotech). Active fractions were pooled (1 ml), dialyzed against buffer A, and stored at 4°C.

Electrophoretic analysis The molecular mass of the purified enzyme was estimated by SDS-PAGE on 10% (w/v) gel (Laemmli, 1970). After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. Molecular weight markers (Pharmacia LKB) were used as standard proteins. The α-glucosidase activity staining was performed in 10% polyacrylamide gels that contained SDS by a slightly modified method (Bibel, 1998). Following renaturation of the enzyme by washing the gel with Triton X-100 (2%), the gel was incubated with 40 mM potassium phosphate buffer pH 7.0 that contained 3 mM 4-methylumbelliferyl-α-D-glucoside at 65°C for 15-20 min. The α-Glucosidase activity was identified by fluorescence under UV (366 nm) illumination.

Substrate specificity and transglucosylation reaction Estimation of the substrate specificity was made using purified TcaAG at a concentration of 10 mM oligosaccharide substrates in 40 mM potassium phosphate buffer (pH 7.0), incubated for 1 h at 75°C. For the transfer reaction, the mixtures containing either isomaltose alone, or in combination with acceptor molecules in 40 mM potassium phosphate buffer, pH 7.0, were incubated at 75°C for 72 h. The mixtures were then boiled for 10 minutes to stop the reaction and centrifuged. Carbohydrate products from the substrates hydrolysis and transglucosylations were analyzed by Dionex high performance anion-exchange chromatography with pulsed amperometric detector (HPAE-PAD) on a column (0.4 × 25 cm) of CarboPac PA-1 (Dionex, Camberly) eluted at 1 ml/min with a gradient of sodium acetate (2-20 min increasing from 0 to 300 mM in 60 mM NaOH).

N-Terminal amino-acid analysis The purified enzyme was electrophoretically transferred to a sheet of polyvinylidene difluoride (PVDF) membrane (BioRad Lab) from SDS-PAGE gel (10%). The N-terminal amino-acid sequence of the excised CAG band was analyzed by the Edman degradation method with an Applied Biosystems model 476A Protein/Peptide sequencer (Applied Biosystems Inc., CA).

Molecular cloning and sequencing of DNA *T. caldophilus* GK24 chromosomal DNA was used as the source for isolation of the α-glucosidase gene. *Escherichia coli* strain DH5α was used as the host for gene cloning, subcloning, and sequencing, and *E. coli* BL21(DE3) was used as a host for expression. The vector plasmids that were used are pUC18, pUC19 and pUC119. Southern hybridization was performed with Hybond N^+ (Amersham), and DNA probes labeled and detected with a DIG-Labeling and Detection kit (Boehringer Mannheim). DNA was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with a Prism Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems), and One-Shot Sequencing cum Silver staining System (Bioneer Corporation, Korea). DNA digestion with restriction endonucleases, and ligation of DNA fragments with T4 DNA ligase were performed according to the suppliers specifications. Other procedures (Rodriguez *et al.*, 1983; Sambrook *et al.*, 1989) were carried out for DNA recombinant experiments and chromosomal

DNA preparation. Cells were suspended in a TE buffer that contained lysozyme and RNase. After incubation at 37°C for 30 min, 1.5 ml of 25% SDS and 100 μl of proteinase K (10 mg/ml) was added and further incubated for 12 h. The solution was extracted with phenol/chloroform, and DNA was precipitated with 2 vol. of ethanol. Washed DNA was dissolved in TE and stored at 4°C.

An α-glucosidase-Specific DNA fragment was initially amplified from *T. caldophilus* GK24 genomic DNA by a polymerase chain reaction, PCR (Turbo Thermal Cycler, Bioneer Inc.), using two degenerate oligonucleotides, 5'-CAGCGSGCSGT NATHTACCAGGTN3' and 5'-NTCCCARTTNAGRTCNGGY-TG-3', where N,S,H Y, R and D denotes all four bases, g/c, a/t/c, c/t, a/g, and g/a/t, respectively. These primers correspond to the determined N-terminal amino-acid sequence of TcaAG (Table 4) and a conserved amino-acid sequence shown in Fig 3. The amplified product was cloned into pUC18 and sequenced on both strands. Genomic DNA from *T. caldophilus* GK24 was digested with restriction enzymes. The resulting fragments were separated on a 1% agarose gel, transferred to a Hybond N^+ membrane, and hybridized with a DIG-labeled PCR fragment as probe at 68°C in order to obtain 3.1 kb genomic clones. A mini gene pool of *T. caldophilus* was constructed by ligating purified DNA fragments that hybridized with the probe into pUC18. *E. coli* DH5α cells, transformed with the mini gene pool, were screened by colony hybridization. Among the positive signals, a clone was sequenced completely on both strands.

Expression of the TcaAG gene in *E. coli* and its purification The DNA that corresponds to the mature protein open reading frame, ORF, was amplified by PCR. The primer, 5'-GAACATATGAGCTGGTGGCAAAGGGCG-3' was synthesized, based on the sequence at the N-terminus. It contained an *NdeI* site in front of the ATG start codon. The antisense primer, 5'-GCAAAG CTTCTAGTCTAGCCGCACCGC-3', was derived from the sequence in the vicinity of the designated stop codon. It contained a new *HindIII* site just after the termination codon. The amplified product was double digested with *HindIII* and *NdeI*. It was then cloned into the expression vector, pET22b(+) (Novagen), that had been previously digested with the same enzymes. *E. coli* BL21(DE3) cells, harboring a recombinant plasmid carrying the TcaAG gene, were grown for 3 h at 37°C in 200 ml Luria broth containing 100 μg/ml ampicillin. The T7*lac* promoter was induced by the addition of 1 mM isopropyl-thio-β-D-galactopyranoside. The cells were allowed to grow for 12 h with vigorous shaking, collected by centrifugation (10,000 × *g* for 10 min), and suspended in 20 ml 40 mM potassium phosphate, pH 7.0. The suspension was sonicated and centrifuged (12,500 × *g* for 30 min). The supernatant was incubated for 30 min at 85°C and centrifuged to remove denatured *E. coli* debris. The TcaAG produced was analyzed by SDS-PAGE, as well as by *p*-NPG hydrolysis.

Results and Discussion

Purification and properties of TcaAG We found an enzyme in the cellular fraction of *T. caldophilus* GK24 that catalyzes the hydrolysis of isomaltose and *p*-nitrophenol-α-D-glucopyranoside. The presence of the enzyme was confirmed by assaying the ability of the crude extract and chromatographic fractions to form glucose from isomaltose and *p*-nitrophenol from *p*-NPG. Identification of the single protein band, detected in the purified preparation with α-glucosidase, was confirmed by affinity staining with 4-methylumbelliferyl-α-D-glucoside (Fig. 1). TcaAG was extracted and purified as described in Materials and Methods. A summary of the purification steps is given in Table 1. TcaAG was purified 80-fold over the crude extract with a 0.8% yield. The molecular mass of the purified enzyme was estimated to be about 60 kDa by SDS-PAGE. The molecular masses of other reported monomeric members of the Family I α-glucosidases also range from 50-66 kDa (Oda *et al.*, 1993). The N-terminal peptide was determined as Ser-Trp-X-Gln-Arg-Ala-Val-Ile-Tyr-Gln-Val. This resembles the *Bacillus* sp., yeast, and insects enzyme appreciably. The optimum pH for activity was at 6.5 in 40 mM potassium phosphate buffer. The enzyme was fairly stable between pH 6 and 8.5. The effects of temperature on activity and stability are shown in Fig. 2. The optimum activity was recorded at 90°C, and the enzyme was stable at temperatures below 95°C. The enzyme had a half-life of 85 min at 90°C. Among metal ions tested, the enzyme activity was slightly activated by Ca^{2+} and Ni^{2+}, but strongly inhibited by Hg^{2+} (97%). The α-glucosidase from *Bacillus brevis* was also reported to be stimulated by the Ca^{2+} ion (McWethy *et al.*, 1979). Other metal ions did not significantly affect the activity of the bound enzyme.

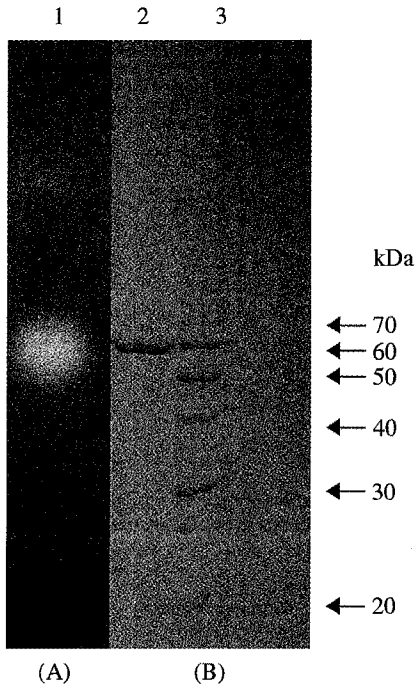

Fig. 1. Zymogram (A) and SDS-PAGE (B) of the purified GK24 α-glucosidase. Lanes: 1, Activity staining of SDS-PAGE; 2, *T. caldophilus* GK24 α-glucosidase; 3, Molecular weight marker.

Table 1. Summary of *T. caldophilus* GK24 α-Glucosidase Purification.

Purification steps	Total protein (mg)	Total activity (*U*)	Specific activity (*U*/mg)	Yield (%)	Purification fold
Crude protein	1875	23980	12.8	100	1
50% $(NH_4)_2SO_4$ ppt	750	21865	29.1	91.2	2.3
DEAE-Sephacel	510	20234	39.7	84.4	3.1
Phenyl Sepharose	35.5	7024	197.9	29.3	15.5
MonoQ	1.8	860	477.8	3.6	37.3
Sephacryl S-300	0.2	199.9	999.5	0.8	78.1

Data corresponds to a typical purification from 50g of wet cells. Enzyme assay was performed as described under Materials and Methods for *p*-NPG hydrolysis.

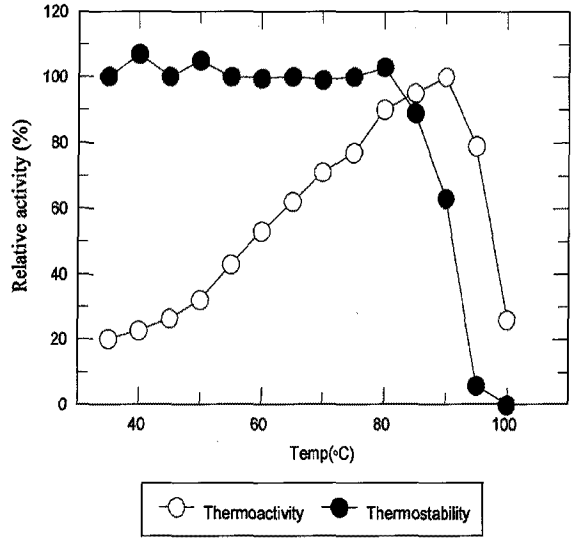

Fig. 2. Effect of temperature on *p*-NPG-hydrolyzing activity and on the stability of *T. caldophilus* GK24 α-glucosidase. The relative activity of the purified enzyme was determined at various temperatures. For a stability test, the reaction mixture was incubated without *p*-NPG at different temperatures (35-100°C) for 1 h. The residual activity of the enzyme was then measured by reaction with *p*-NPG at 75°C, as described in Materials and Methods.

Substrate specificity and kinetic studies Substrate specificity, expressed as the conversion rate to glucose from various substrates, is shown in Table 2. The TcaAG released glucose from isomaltose, panose, isomaltotriose and isomaltotetraose. No glucose was produced with either treated starch or pullulan. Also, TcaAG liberated glucose from the following saccharides: sucrose, nigerose and turanose. Glucose production from isomaltose was the highest.

Exo-type action The hydrolytic trend of isomaltooligosaccharides and panose at time intervals were monitored by thin layer chromatography (data not shown). With the exception of isomaltose, only two products could be observed in the digest of each substrate at the end of the reaction. One was glucose, the sole product from isomaltose. The other is maltose from panose, or an isomaltosaccharide, one glucose unit less than the initial substrate. These products increased with time, maltose formation being parallel with glucose from panose. Hydrolysis of panose did not generate isomaltose, indicating that each isomaltosaccharide is hydrolyzed at the non-reducing terminal bond. This is typical of *Bacillus* sp. oligo-1,6-glucosidases (Suzuki *et al.*, 1989).

The apparent kinetic constant for the thermostable α-glucosidase activity was determined by measuring the initial rate of *p*-nitrophenol, or glucose release from *p*-NPG, or preferred oligosaccharides. The rate of enzymatic hydrolysis on substrate concentration followed Michaelis-Menten kinetics. A linear relationship of 1/V versus 1/[S] were obtained. The K_m value for *p*-NPG was 14.2 mM. The K_m and molecular activities of the recombinant TcaAG for oligosaccharides are given in Table 3. It is evident from the affinity and specific constant that isomaltose and sucrose are the best substrates, while turanose is a poor substrate. As the turnover number for various substrates are similar (max:72.66/56.94, 28%), the catalytic efficiency of the enzyme appears to depend mainly on the affinity for substrates (max: 1.57/0.26, a 6-fold difference in affinity). With the exception of *Bacillus amyloliquefaciens* (Urlaub *et al.*, 1978) and *Bacillus* sp. SAM1606 α-glucosidases (Nakao *et al.*, 1994), the majority of the bacterial α-glucosidases shows little or no activity towards sucrose. As in yeast, and the previously mentioned bacterial enzymes, sucrose is a very good substrate for TcaAG, unlike many of the oligo-1,6-glucosidases that share a similar catalytic property on isomaltooligosaccharides and panose. We suggest that TcaAG is a sucrase-isomaltase.

Cloning and sequencing of the TcaAG gene A 501-base TcaAG specific oligonucleotide probe for gene cloning was prepared by PCR on the basis of a determined N-terminal protein sequence of the *T. caldophilus* enzyme, and an internal conserved amino acid sequence (Lee *et al.*, 2000). Analysis of the deduced amino acid sequence of the PCR product showed a homology greater than 70% with reported bacterial, yeast, and insects α-glucosidase sequences. The probe hybridized

Table 2. Substrate Specificity of α-Glucosidase from *T. caldophilus* GK24

Substrate	Glycosidic linkage	Relative glucose yield(%)
Isomaltose	Glu-α-1,6-Glu	100.0
Isomaltoriose	Glu-[α-1,6]-Glu-α-1,6-Glu	77.0
Isomaltotetraose	Glu-[α-1,6]$_3$-Glu	50.5
Panose	Glu-[α-1,6]-Glu-α-1,4-Glu	55.0
Gentibiose	Glu-β-1,6-Glu	0.0
Leucrose	Glu-α-1,5-Fru	0.0
Maltose	Glu-α-1,4-Glu	0.0
Lactose	Gal-β-1,4-Glu	0.0
Cellobiose	Glu-β-1,4-Glu	0.0
Nigerose	Glu-α-1,3-Glu	86.0
Turanose	Glu-α-1,3-Fru	54.8
Laminaribiose	Glu-β-1,3-Glu	0.0
Sucrose	Glu-α-1,2-Fru	65.0
Trehalose	Glu-α-1,1-Glu	0.0
Pullulan		0.0
Starch	Poly[Glu-α-1,4-Glu]	0.0

Enzyme (0.3 μg) was incubated at 75°C for 1 h in the presence of 10 mM of each substrate. The reaction was stopped by boiling the mixture. The liberated glucose yield were estimated by the HPAEC-PAD system, as described in Materials and Methods. Substrate specificity was expressed as glucose yield relative to that from isomaltose.

with about a 3.1 kb *HindIII* DNA fragment of the *T. caldophilus* chromosomal DNA. The chromosomal DNA, digested with *HindIII,* was size-fractionated on a 1% agarose gel, and an insert size of approximately 3.0-3.5 kb was constructed at the *HindIII* site of pUC18 and used to transform *E. coli* cells. After screening the recombinant plasmids by Southern hybridizations, positive clones were selected, and from one clone (pAGON41) a 3.1 kb *HindIII* fragment was obtained. The complete nucleotide sequence and deduced amino acid sequence of pAGON41 are shown in Fig. 3.

The putative coding region of the TcaAG gene was 1590 bp, starting from an ATG codon at nucleotide 1221 and ending in a TAG codon at 2810 (Fig. 3). The open reading frame was neither immediately flanked by consensus Shine-Dalgarno or prokaryotic promoter sequences. The frame corresponded to a protein of 530 amino acid residues with an identical N-terminal amino acid sequence as in the purified enzyme. The calculated molecular mass of the enzyme was 61,430 Da. This agrees with the one that was determined from the purified TcaAG. The G + C content of the cloned gene was 68.4%, which is typical of *Thermus sp.* proteins (Kwon *et al.*, 1997). The codon composition favors guanosine, or cytosine in the wobble position (93.6%).

Amino acid sequence similarity An extensive data base search for proteins with the TcaAG sequence similarity revealed high sequence similarities (55%) with *Bacillus* sp. SAM1606 α-glucosidase, oligo-1,6-glucosidases of *B. cereus* ATCC7064 and *B. thermoglucosidasius* KP1006 (Watanabe *et al.*, 1990; Watanabe *et al.*, 1991; Nako *et al.*, 1994b), and maltase from *Saccharomyces carlsbergensis* [(Hong *et al.*, 1986) (Fig. 4). The sequence similarity (42%) was also found with maltase from insects (Ohash *et al.*, 1996). As expected of a Family I α-glucosidase, TcaAG had no sequence similarity to the mammalian and fungal α-glucosidases (Hunzuiker *et al.*, 1986; Hoefsloot *et al.*, 1989; Kimura *et al.*, 1992; Sugimoto *et al.*, 1996), while Family II α-glucosidases share a similarity to each other. A strong evolutionary relationship in the origin of these enzymes with α-amylase was suggested by a secondary structural analysis, which showed that they exhibit an α-amylase-type (β/α) 8-barrel (or TIM barrel) structure (MacGregor *et al.*, 1989; Jesperson *et al.*, 1991). Hence, the enzymes were grouped together in one family, the α-amylase family. TcaAG also had the six conserved regions (R1-R6, Fig. 4) and contained a TIM barrel structure in its N-terminal domain (not shown), as predicted by the method (Kyte *et al.*, 1982). TcaAG could be assigned as a member of the α-amylase family. Based on the comparison with the catalytic domain among similar enzymes, we suggest that Asp-198, Glu-265, and Asp-327 in the TcaAG gene are involved in catalysis, and His-100 and His-326 in substrate binding.

Despite high sequence similarities that are exhibited by the α-glucosidases from various sources, the enzymes show great differences in substrate specificities. TcaAG is patently an oligo-1,6-glucosidase by virtue of its characteristic action on isomaltosaccharides and panose (Suzuki *et al.*, 1982; Suzuki *et al.*, 1983; Nakao *et al.*, 1994b). However, it differs from them by its action on sucrose, in addition to a relatively higher specificity for α-1,3 glucosidic linkage. The *Bacillus* sp.SAM1606 enzyme additionally hydrolyses maltose and

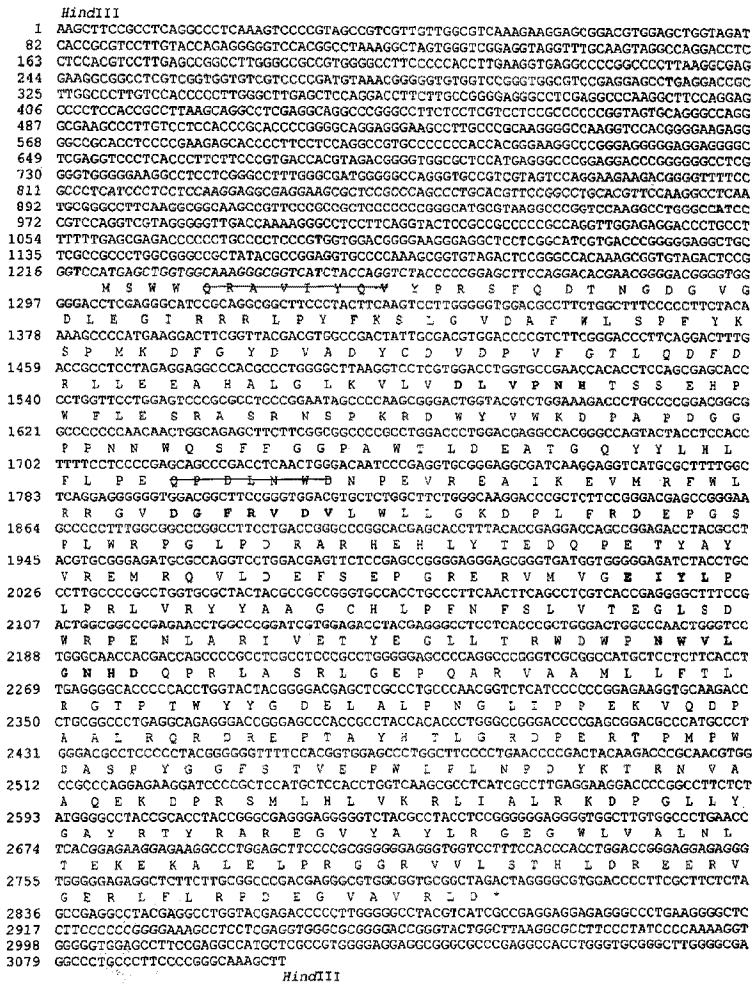

Fig. 3. Nucleotide sequence of the *T. caldophilus* GK24 α-glucosidase gene, and its deduced amino acid sequence. The amino acid used for the design of PCR primers are underlined. The α-glucosidase conserved residues are highlighted in black.

trehalose (Nakao *et al.*, 1994). During the course of this study, an unusual thermostable oligo-1,6-glucosidase gene was reported from *Bacillus flavocaldarius* KP1228 (Kashwabara *et al.*, 1998), which strangely shares a 98.3% identity with the TcaAG sequence, while showing only a 40-42% identity with other *Bacillus* sp. oligo-1,6-glucosidases that normally exhibits a 62-72% identity among themselves (Nakao *et al.*, 1994b). Unlike other bacillary counterparts, the *B. flavocaldarius* enzyme specificity resembles those of TcaAG, but with contrasting affinities. *B. flavocaldarius* enzyme transglucosylation activity was also not reported. A comparative X-ray crystallographic and site-directed mutagenesis studies should be able to elucidate the differences in substrate specificities among the enzymes from various sources. As for the increased thermostability of TcaAG over and above the *Bacillus sp.* enzymes, the proline theory (Suzuki *et al.*, 1982) should, in part, hold for the TcaAG that contains 47 proline residues (8.9 mol/100 mol) in contrast to the 35 proline residues (6.0 mol/100 mol) in the SAM1606 α-glucosidase sequence.

Expression of the TcaAG gene in *Escherichia coli* and purification of the recombinant enzyme The complete TcaAG gene was subcloned into the pET22b (+), and the pETAGLU1 obtained was used for the production of the enzyme in *E. coli* BL21(DE3) cells under the control of the *T7* promoter (Lee and Kim, 1999). Full IPTG-dependent induction was achieved after 3 h and TcaAG was produced to a level as high as 10% of the total cellular protein. As expected, TcaAG could be purified easily from induced *E. coli* cells, harboring the plasmid, by heat treatment of the cell free extract (85°C, 30 min). The purified recombinant enzyme has identical characteristics as its progenitor. It showed the same mobility as the original enzyme on SDS-PAGE. The recombinant enzyme showed a ten-fold increase in activity compared to native enzyme, and exhibited the same specificity towards isomaltosaccharides, panose, sucrose, nigerose, and turanose. Its pH and temperature optimum were also the same as for the native enzyme. Recombinant TcaAG exhibited transglucosylation activities with either sucrose or isomaltose, singly or in combination with other substrates.

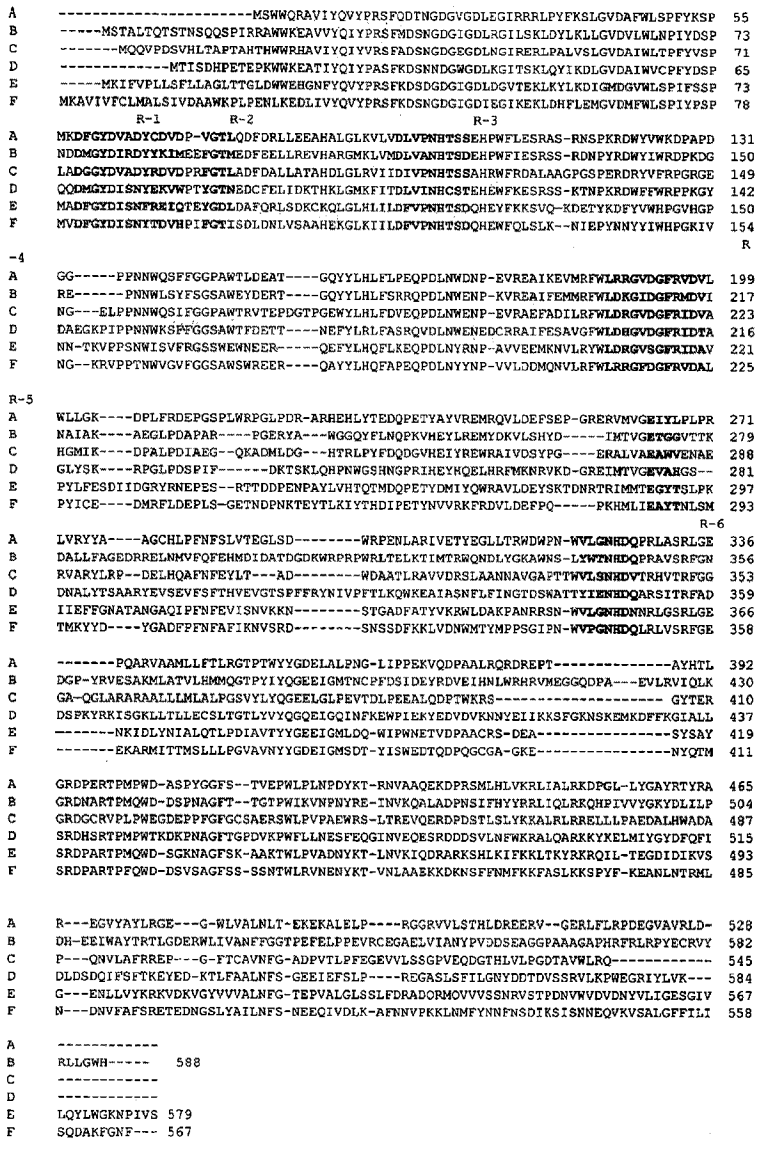

Fig. 4. Comparison of the deduced amino acid from GK24 α-glucosidase with those from various microbial sources: A,TcaAG (THIS STUDY); B, *Bacillus sp.* SAM1606 (Bac.SAM1606); C, *Thermonospora curvata*; D, Yeast; E, Mosquito and F, Honey-Bee. The six conserved regions among the enzymes that belong to the α-amylase family (R1-R6) are highlighted in bold letters.

This is true of *Bacillus* sp. SAM1606 α-glucosidase (Nakao *et al.*, 1994a). TcaAG is the most thermostable α-glucosidase with transglucosylation activity that has been reported (further work and identification of the products will soon be reported).

In conclusion, we identified and characterized a novel thermostable α-glucosidase from *T. caldophilus* GK24, and over-expressed the corresponding cloned gene in *E. coli*. This is the first report on an α-glucosidase sequence from the *Thermus* sp. The characteristics of TcaAG should make it a good candidate for enzyme engineering studies and industrial applications. It should be especially helpful in the starch hydrolysis process, along with amylases for enhanced glucose production, and enzymatic synthesis of novel oligosaccharides.

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