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Enhanced activity and stability of cellobiase (β-glucosidase: EC 3.2.1.21) produced in the presence of 2-deoxy-D-glucose from the fungus *Termitomyces clypeatus*

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ABSTRACT

Generally less glycosylation or deglycosylation has a detrimental effect on enzyme activity and stability. Increased production and secretion of cellobiase was earlier obtained in the presence of the glycosylation inhibitor 2-deoxy-D-glucose in filamentous fungus *Termitomyces clypeatus* [Mukherjee, S.; Chowdhury, S.; Ghorai, S.; Pal, S.; Khowala, S. *Biotechnol. Lett.* **2006**, *28*, 1773–1778]. In this study the enzyme was purified from the culture medium by ultrafiltration and gel-permeation, ion-exchange and high-performance liquid chromatography, and its catalytic activity was six times higher compared to the control enzyme. K_m and V_{max} of the purified enzyme were measured as 0.187 mM and 0.018 U mg^{-1}, respectively, using *p*NPG as the substrate. The enzyme had temperature and pH optima at 45 °C and pH 5.4, respectively, and retained full activity in a pH range of 5–8 and temperatures of 30–60 °C. Interestingly less glycosylated cellobiase was resistant towards proteolytic as well as endoglycosidase-H digestion and showed higher stability than native enzyme due to increased aggregation of the protein. The enzyme also showed higher specific activity in the presence of cellobiose and *p*NPG and less susceptibility towards salts and different chemical agents. The β-glucosidase can be considered as a potentially useful enzyme in various food-processing, pharmaceutical and fermentation industries.

1. Introduction

The enzyme β-glucosidase catalyzes the hydrolysis of glycosidic linkages formed between the hemiacetal-OH group of a cyclic aldose or glucose and the –OH group of another compound viz., sugar, amino-alcohol, aryl-alcohol or primary, secondary or tertiary alcohols. Both the hydrolytic and synthetic activities of cellobiase are crucial for various biotechnological applications.[1] The role of cellobiase in cellulose hydrolysis is significant because cellobiose is an inhibitor of both *endo*- and *exo*-glucanases. It must be removed to allow efficient and complete saccharification of cellulose[2] by increasing the rate of cellulose hydrolysis[3] and thereby making the reaction more economical for production of alcohol. Cellobiase is also responsible for removing the aglycone moiety from flavanoids and isoflavanoid glucosides, which are phenolic and phytoestrogen glucosides that occur naturally in fruits, vegetables, tea, red wine and soybeans.[1] Detoxification of cassava, aroma enhancement and removing bitter compounds from citrus fruit juices or unripe olives are some other applications of cellobiase in the food-processing industry.[4] The non-toxic enzymic preparation 'Barlican' from *Trichoderma reesei*, containing cellulose hydrolyzing enzymes, has been reported to be safe for use as a feed additive.[5] Synthetic activity of cellobiase has potential applications in preparations of agrochemicals and drugs.[6] Alkyl glucosides are used as nonionic and biodegradable surfactants, as well as serving as precursors for the synthesis of other pharmaceutical compounds.[7]

It is well known that DG inhibited O-linked glycosylation in *T. reesei*[8] and mammals.[9] Inhibition of the O-glycosylation pathway generally affects protein production and secretion. The extent of

Abbreviations: DG, 2-deoxy-D-glucose (2-deoxy-D-*arabino*-hexose); C, cellobiase produced in the absence of DG; Cg, cellobiase produced in the presence of DG; BG, β-glucosidase; *p*NPG, *p*-nitrophenyl β-D-glucopyranoside; *o*NPG, *o*-nitrophenyl α-D-glucopyranoside; DEAE, diethylaminoethyl; GPC, gel-permeation chromatography; IEC, ion-exchange chromatography; HPGPLC, high-performance gel-permeation liquid chromatography; NBT, *p*-nitrobluetetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indoyl phosphate; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; Gdn·HCl, guanidium·HCl; GOD–POD, glucose oxidase–peroxidase enzyme; DHB, 2,5-dihydroxybenzoic acid; TFA, trifluoroacetic acid.

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glycosylation has a profound effect on the activity and stability of the secreted proteins.[10] Cellobiase production and secretion from *Termitomyces clypeatus* was highly stimulated after the addition of the glycosylation inhibitor DG in the growth medium.[11] Here we report the purification and characterization of cellobiase produced in the presence of DG, which was catalytically more efficient and stable than the control enzyme reported earlier.[12]

2. Results and discussion

In *T. clypeatus*, in the presence of the glycosylation inhibitors 2-deoxy-D-glucose, tunicamycin, 1-deoxynojirimycin and D-glucono-δ-lactone, total cellobiase activity was increased respectively by 50, 1.8, 2.4, 1.3-fold with respect to the control medium.[11] Almost 95–97% of the total protein and enzyme was released in the growth medium containing DG as compared to 71–76% in the absence of the inhibitor, although growth was restricted. Total protein content (in extracellular, intracellular and cell-bound preparations) in the fungus was also increased by 17% in the presence of DG. It was concluded that cellobiase was underglycosylated significantly in the presence of DG.

2.1. Purification of extra-cellular cellobiase (Cg) produced in the presence of DG

Cellobiase was found to be co-aggregated with sucrase in the fungus,[12,13] so a defined medium was chosen for growth and purification that avoided steps favouring aggregation. Purification of the cellobiase is given in Table 1, Figure 1. The enzyme was concentrated through ultrafiltration using a PM-10 membrane, and the specific activity (57.14 U mg^{-1}) of the cellobiase in the culture medium increased in the retentate to 79.2 U mg^{-1} (step 2, Table 1) with 98% cellobiase present in the fraction. In Sephacryl S-200 chromatography (Fig. 1A), the retentate showed a single protein peak (pool E1) with cellobiase and protein recovery of 14.27% and 26.7%, respectively. After IEC (Fig. 1B), cellobiase (pool E2) was eluted with a specific activity of 18.1 U mg^{-1} with 6.9% protein recovery. In HPGPLC (Fig. 1C) ~55% of the loaded protein was pooled (pool E3) with a specific activity of 17.9 U mg^{-1}. Separation of the cellobiase from sucrase in subsequent steps of purification is reported to show significant decrease in specific activity;[12] similarly the specific activity of Cg decreased from step 3 onwards during purification, unlike any regular protein purification profile. At the same time, the ratio of cellobiase to sucrase (C S^{-1}) gradually increased in every step as reported earlier. (C S^{-1}) was 78.6 in the culture filtrate, increased to 138.9 in the retentate (step 2) and was found to be at 1221 in step 3. In step 4 when Cg was completely separated from sucrase by IEC, the specific activity of the enzyme in pool E2 decreased to 18.1 U mg^{-1} with a cellobiase recovery of 2.18%. In HPGPLC (pool E3), the specific activity was measured as 17.9 U mg^{-1}. This observation was similar to that of earlier studies of the co-aggregation of intra- and extracellular cellobiase with sucrase produced in the absence of DG[12] and intracellular β-glucosidase produced in the presence of DG.[14] In *T. clypeatus*, where the kinetic activity and stability of the enzyme co-aggregates with different C S^{-1} ratios obtained at different stages of purification, were significantly altered due to separation

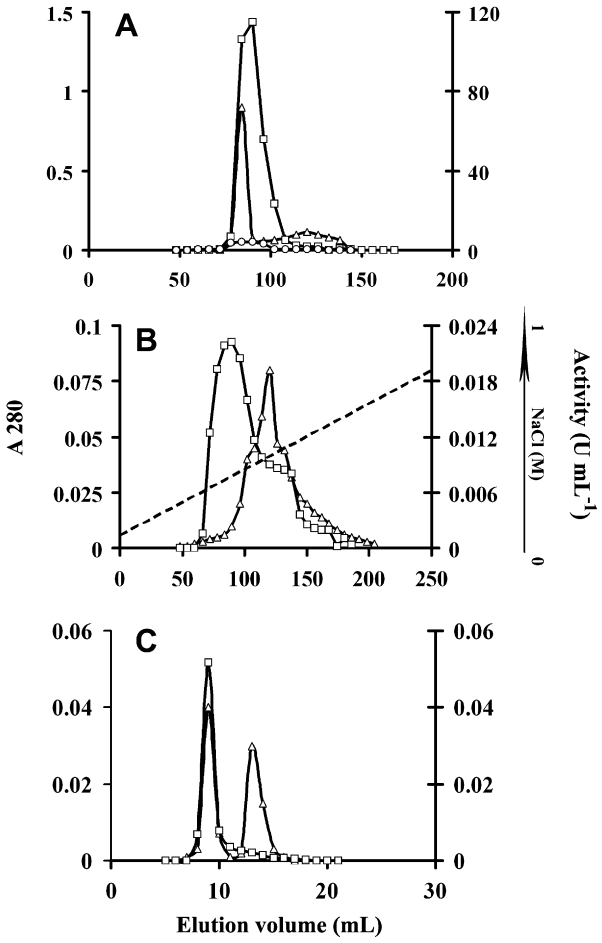

Figure 1. (A) Sephacryl S-200 chromatography of PM-10 retentate. Extracellular protein (3 mg mL^{-1}) was applied to the column in batches, and fractions (3 mL) were monitored for A280 (-Δ-), cellobiase (-□-) and sucrase (-○-). (B) Anion-exchange chromatography of pool E1. Cellobiase (4.3 mg protein) from pool E1 obtained from GEC was equilibrated with 0.01 M acetate buffer, pH 5.0 and applied to IEC. Enzyme fractions (3 mL) collected after elution by salt were monitored for A280 (-Δ-) and cellobiase (-□-). (C) HPGPLC of pool E2. Cellobiase (0.2 mg protein) from pool E2 obtained from DEAE A-50 chromatography was equilibrated with 0.1 M acetate buffer, pH 5.0 and applied to the column. Enzyme fractions (1 mL) were monitored for A280 (-Δ-) and cellobiase (-□-).

Table 1
Purification of extracellular cellobiase in the presence of 2-deoxy-D-glucose

Purification steps	Total protein (mg) [recovery%]	Cellobiase			C S^{-1}
		Total activity (units)	Specific activity (U mg^{-1})	Recovery (%)	
Step 1					
Culture filtrate (2400 mL)	1680 [100]	96,000	57.14	100	78.6
Step 2					
Ultrafiltration (PM-10) filtrate (1898 mL)	544.3 [32.4]	1920	3.53	2	126
Retentate (455 mL)	1187.8 [70.7]	94,080	79.2	98	138.9
Step 3					
Sephacryl-S-200 chromatography of retentate pool E1: (78L–108 mL)	448.6 [26.7]	13699.2	30.53	14.27	1221
Step 4					
DEAE sephadex A-50 chromatography of pool E1 pool E2 (CFS) (66–102 mL)	115.92 [6.9]	2098.15	18.1	2.18	—
Step 5					
HPGPLC Protein Pak 125, pool E3 (9′)	63.75 [3.79]	1141.12	17.9	1.19	—

from sucrase. In this study the change in activity due to disaggregation of Cg and S followed the same trend, but the values were different due to different degrees of aggregation of Cg as observed during purification.

2.2. Characterization of the purified extracellular cellobiase

Purified enzyme preparation from HPGPLC (step 5, pool E3) was used for all the studies unless otherwise specified.

2.2.1. Molecular size of cellobiase

Cg showed a molecular size of around 57.3 kDa in HPGPLC (Fig. 1C) and 116 kDa in western blotting (Fig. 2A). Earlier C was reported as an oligomeric enzyme with monomeric size unit of 14 kDa in HPGPLC and a single band of 56 kDa in SDS–PAGE, which showed ladder-like bands of 14 kDa, 28 kDa, 42 kDa and 56 kDa on denaturation by a mixture of 1 M Tris–HCl (pH 8.0), 0.1 M EDTA, 1 M DTT and 8 M urea.[15] In this study Cg produced protein bands of 118 kDa, 110 kDa, 70 kDa, 60 kDa and 28 kDa on denaturation by a Tris–EDTA–DTT–urea mixture (Fig. 2B). On enzymatic deglycosylation, Cg showed 8 bands corresponding to masses of 116 kDa, 75 kDa, 56 kDa, 50 kDa, 44 kDa, 30 kDa, 28 kDa and 18 kDa, whereas C displayed protein bands of 110 kDa, 75 kDa, 60 kDa, 50 kDa, 37 kDa, 30 kDa, 28 kDa, 18 kDa and 12 kDa (Fig. 2C). The results clearly indicate that Cg is aggregated to a substantially higher degree than C due to underglycosylation induced by DG. Higher aggregation was observed for glucoamylase from *Aspergillus niger*[16] upon deglycosylation, and a lower degree of aggregation for the glycosylated enzyme was due to the presence of hydrophilic sugar components providing repulsive interactions, loss of which caused aggregation. Similarly, glucose oxidase from *A. niger* and yeast invertase were more prone to aggregation after deglycosylation.[17] The presence of glycosylation improved the solubility of unfolded or partially folded invertase molecules from yeast, leading to suppression of aggregation.[18] Many recombinant versions of human proteins including α-antitrypsin (α-AT) underwent aggregation upon storage due to the lack of carbohydrates in the recombinant protein structures.[19,20]

In the mass determination by MALDI-TOF, no clear peak was visible. Non-availability of a well-standardized matrix composition for aggregated glycoproteins may be the reason for failure in mass determination by MALDI analysis. It may be mentioned here that the size of the monomeric unit of intracellular β-glucosidase purified in the presence of DG in the same medium was characterized as a 6688 dalton protein.[14] It, therefore, seemed quite logical that the monomeric unit of Cg should be a small protein, but due to substantial high aggregation its unit size could not be ascertained.

Table 2
Kinetic parameters of purified cellobiase

	Cellobiase	K_m (mM)	V_{max} (U mg^{-1})	$V_{max}\,K_m^{-1}$ (U mg^{-1} mM^{-1})
Cg	Step 5: pool E3	0.187	0.018	0.096
C	Pool EFC	4.762	0.076	0.016

Table 3
Substrate specificity of the purified enzyme

Substrate(s) (1%)	Substrate specificity (U mg^{-1})	
	Cg	C
o-Nitrophenyl α-D-galactopyranoside	–	–
Methyl β-D-glucopyranoside	–	–
Carboxymethyl cellulose	–	–
Methyl α-D-galactopyranoside	–	–
Maltose	–	–
Trehalose	–	–
Cellobiose	21.43	2.35
*p*NPG	17.9	2.41
Sucrose	–	–

2.2.2. Kinetic parameters

K_m and V_{max} of purified Cg are presented in Table 2. The substrate affinity of purified Cg increased by 25 times to 0.187 mM, leading to an overall increase in catalytic efficiency of Cg by six times as compared to C. Such a high decrease in K_m value due to underglycosylation is not known for any glycosidase, though the K_m of partially deglycosylated TP (turnip peroxidase isozyme) was reduced to 25 mM, which is half of that obtained for the native TP (55 mM).[21] The partial removal of carbohydrate chains from isoperoxidase (EC 1.11.1.7) purified from avocado leaves (*Persea americana* Mill. cv. Topa Topa) led to a marginal decrease in the K_m .[22] It may be possible that after removal of the carbohydrate moiety, the substrates had better accessibility to their binding sites, resulting in increased affinity and activity for the deglycosylated enzyme.

2.2.3. Substrate specificity

Purified cellobiase had a narrow substrate specificity and could hydrolyze only cellobiose and *p*NPG and showed no activity on *o*NPG, methyl α-D-galactopyranoside, maltose, sucrose, trehalose

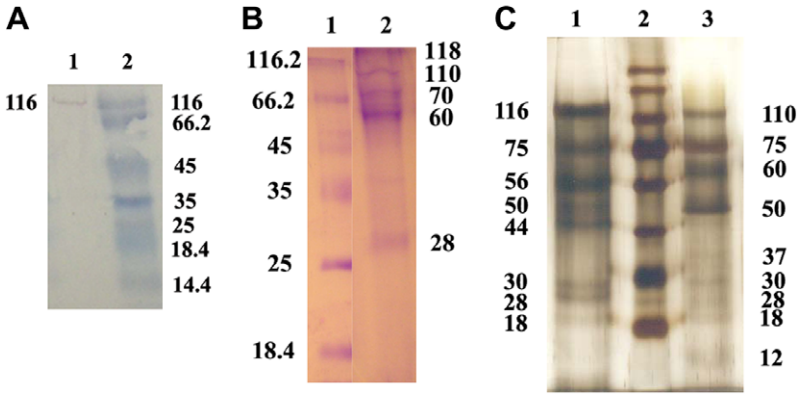

Figure 2. (A) Western blotting of Cg. Lane 1: purified Cg (60 μg protein); Lane 2: 5 μg marker proteins comprising mixture of denatured β-galactosidase 116 kDa; serum albumin 66.2 kDa; ovalbumin 45 kDa; lactate dehydrogenase 35 kDa; Restriction endonucleases (Bsp 981) 25 kDa; β-lactoglobulin 18.4 kDa; lysozyme 14.4 kDa. (B) SDS–PAGE (10%) of denatured Cg**:** Lane 1: 5 μg marker proteins comprising mixture of denatured β-galactosidase 116.25 kDa; serum albumin 66.2 kDa; ovalbumin 45 kDa; lactate dehydrogenase 35 kDa; restriction endonucleases (Bsp 981) 25 kDa; β-lactoglobulin 18.4 kDa; Lane 2: purified Cg (200 μg protein); enzyme was denatured as described in Section 4. (C) Silver staining of deglycosylated cellobiase: Lane 1: deglycosylated Cg (50 μg protein), Lane 2: molecular weight marker SM0671 (170 kDa; 130 kDa; 100 kDa; 70 kDa; 55 kDa; 40 kDa; 35 kDa; 25 kDa; 15 kDa), Lane 3: deglycosylated C (50 μg protein).

and carboxymethyl cellulose, etc. (Table 3). Specific activities of Cg for cellobiose and *p*NPG were 9.1 and 7.4 times higher compared to C and exhibited better activity towards cellobiose (21.43 U mg^{-1}) than with *p*NPG (17.9 U mg^{-1}), whereas C showed a similar specific activity for *p*NPG and cellobiose.[23]

2.2.4. pH and temperature optima and stability

Due to underglycosylation the pH optimum of Cg changed to pH 5.4, which was earlier observed at pH 5.0 in the absence of DG for C.[12] The optimum pH of recombinant phytase expressed in *Pichia pastoris* was shifted from pH 5.0 to 2.5 by deglycosylation.[24] Purified Cg was most stable at pH 5.4 and retained ~68–71% of its residual activity between pH 4.0 and 9.0 after 24 h of incubation at 30 °C (Fig. 3A). Only 8% activity was lost at pH 6.0, but residual activity was 5% at pH 10.4. Earlier purified C was found to be inactive at pH 3.5 and 8.0.[12] At pH 8.0, nearly a threefold decrease in enzymatic activity was observed for deglycosylated forms of stem bromelain from *Ananas comosus* as compared to that for the glycosylated enzyme.[25] When most of the carbohydrate moiety of β-fructofuranosidase $P\bar{1}$ from *Aureobasidium* sp. ATCC 20524 was removed, its stability at pH 4–5 and 50 °C was decreased.[26] A similar effect was seen in case of the glucosyltransferase from *Aureobasidium* as the pH and thermal stabilities were decreased after altered glycosylation.[27] The temperature optimum of Cg was determined as 45 °C. The enzyme showed more than 80% of its residual activity during 1 h incubation in the temperature range from 30 to 53 °C and 42% activity at 60 °C (Fig. 3B). The enzyme became inactive at 70 °C in 30 min, whereas C had temperature optimum at 47 °C and showed no activity at 60 °C.[12] The deglycosylated stem bromelain from *A. comosus* was found to show less activity at all temperatures,[25] whereas deglycosylated TP (TP2) was inactivated at a higher rate than that of the native TP when heated at 65 °C.[21] This was attributed to the crucial role played by the carbohydrate portion on the thermal stability of the protein.[28,29] Han and Lei reported that deglycosylation of the *A. niger* phytase expressed by *P. pastoris* and the commercial phytase (by *A. niger*) showed approximately 50% reduced thermostabilities.[30] A decrease in stability towards temperature was also observed after reduced glycosylation of isoperoxidase from avocado leaves.[22] It was suggested that the carbohydrate moiety located on the surface of the peroxidase molecule acted as a molecular shield.

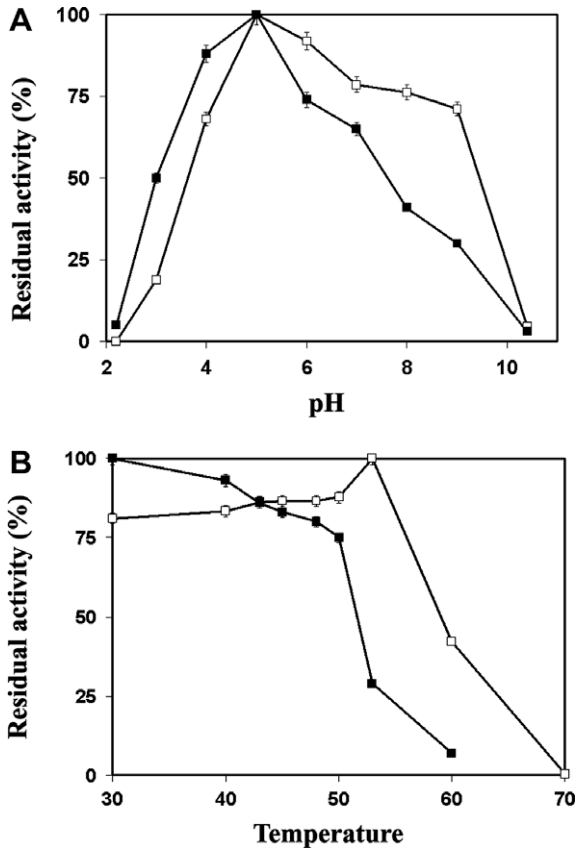

Figure 3. pH (A) and temperature (B) stability of cellobiase. Residual enzyme activities were measured at different temperatures and pH for purified Cg (-□-) and C (-■-). An average of three sets of experimental results is presented.

2.2.5. Susceptibility towards salts, chaotropic, reducing agents and products

Effects of some salts were observed on Cg, and results were compared with those of C (Table 4). In the presence of 20 mM Mg^{2+}, sodium azide Cg showed 97.8% and 77.8% residual activity), whereas C retained 86.09% and 73.34% activity. Cg was inhibited by 37% in the presence of PCMB (0.5 mM) in comparison with 62% inhibition of the control enzyme. In the presence of Mn^{2+} (20 mM), Zn^{2+} (2 mM) and K^{+} (20 mM) both the enzymes showed equal susceptibility, showing around 60–80% activity. C showed greater susceptibility towards EDTA (20 mM), Hg^{2+} and Cu^{2+} compared to Cg. Inactivation by the salts may be explained by destabilization of the enzyme structure by the ions.

Cg showed much better stability in the presence of chaotropic and reducing agents than C[12] (Table 4). Residual activity in the presence of 1–2 M and 4 M urea was higher by 20% and 30% compared to C, whereas in the presence of 1–2 M Gdn·HCl, residual activities of Cg and C were 83.3–45.5% and 70.0–35.0%, respectively. In the presence of 2-mercaptoethanol (20–50 mM) and DTT (1–2 mM), an increase in cellobiase activity was observed. Residual activities of Cg were 13% and 6% higher in the presence of 1–2 mM and 10–20 mM 2-mercaptoethanol respectively compared to C. Cg showed around 9% higher residual activity in comparison with C at 20–50 mM DTT. The deglycosylated glucosyltransferase from *Aureobasidium* became sensitive to metal ions and sulfydryl reagents than the native enzyme.[27] The glycosylated recombinant human interferon gamma receptors were reported be less resistant to chaotropes.[31] The stability or less susceptibility observed in cellobiase from *T. clypeatus* may be attributed to the higher aggregating property of Cg. In the presence of reducing agents the activities of Cg and C were increased. It might be possible that at lower concentration these agents are

Table 4
Effect of salts, chaotropic, reducing agents and chemicals on cellobiase activity

Salts/chaotropic/reducing agent(s)/product(s)	Concentration	Residual cellobiase activity (%)	
		Cg	C
$MgSO_4$	20 mM	97.8	86.09
Sodium azide	20 mM	77.8	73.34
PCMB	0.5 mM	63.1	37.68
KCl	20 mM	80.3	81.2
EDTA disodium salt	20 mM	69.1	86.56
$CuSO_4{\cdot}5H_2O$	2.0 mM	78.4	89.66
$ZnSO_4{\cdot}7H_2O$	2.0 mM	67.5	70.4
$HgCl_2$	2.0 mM	40.9	64.21
$MnCl_2$	20 mM	54.7	60.0
Urea	1–2 M	80.6–71.6	60.8–50.8
	4 M	69.0	37.6
Gdn·HCl	1–2 M	83.3–45.5	70.0–35.0
2-Mercaptoethanol	1–2 mM	129.9	116.7
	10–20 mM	79–22	72.8–16
DTT	20–50 mM	109.2–108.6	100.0
Glucose	20 mg mL^{-1}	14.6	42
DG	1 mg mL^{-1}	82.7	45

affecting the catalytic activity due to topological effects resulting from the breakage of disulfide linkages.

Restricted glycosylation of Cg resulted in less susceptibility towards DG and in more susceptibility towards glucose compared to C (Table 4). In the presence of 20 mg mL^{-1} of glucose, Cg retained 14.6% activity, whereas C showed 42% activity under the same conditions. The activity of Cg and C decreased in a similar fashion up to 0.1 mg mL^{-1} of DG. At 1 mg mL^{-1} of DG, residual activities of Cg and C were 82.7% and 45%, respectively.

2.2.6. Susceptibility towards tryptic and endoglycosidase-H digestion

Contrary to the characteristics of any less/deglycosylated enzyme reported so far, Cg was not at all susceptible towards the hydrolytic action of trypsin and endoglycosidase-H until 36 h, whereas C gradually lost activity after 30 min and 5 h in the presence of trypsin (Fig. 4A) and endoglycosidase-H, respectively (Fig. 4B).[23] Interestingly during endoglycosidase-H digestion, the residual activities of Cg and C were gradually increased upto 2 h and 3 h, respectively. The carbohydrate moiety exerted a protective effect against proteolysis of native TP,[21] which was also observed for other glycoproteins.[32] It was suggested that the carbohydrate moiety may block the access of proteases to the potential proteolytic cleavage sites or may stabilize the conformation of the domain where the cleavage sites are exposed.[33] Moreover, carbohydrate removal may produce protein conformational changes exposing amino acid sequences to be recognized by proteases and eventually get destroyed.[34] Han and Lei reported that deglycosylation of the *A. niger* phytase expressed by *P. pastoris* and the commercial phytase (by *A. niger*) with *endo*-H resulted in less than 20% reduction in their original activities.[30] The enzymatic activity of human thyroid peroxidase (hTPO) was inhibited by *endo*-H deglycosylation, inducing a modification of the tertiary structure of hTPO, which affected the enzymatic site.[35] While studying *endo*-H treated enzymes, Chu et al. showed that the presence of carbohydrate did not influence the conformation of the polypeptide backbone but considerably enhanced protein stability towards heat and resistance to proteolysis.[36] Removal of oligosaccharides from a recombinant monoclonal antibody also caused significant structural changes as evidenced by the susceptibility of the deglycosylated antibody to trypsin.[37] However, exactly the reverse situation was observed in case of cellobiase as it became resistant towards proteolysis after less glycosylation, which appears unique. Stability to trypsin and endoglycosidase-H may be attributed to the increased aggregation property of Cg after restricted glycosylation.

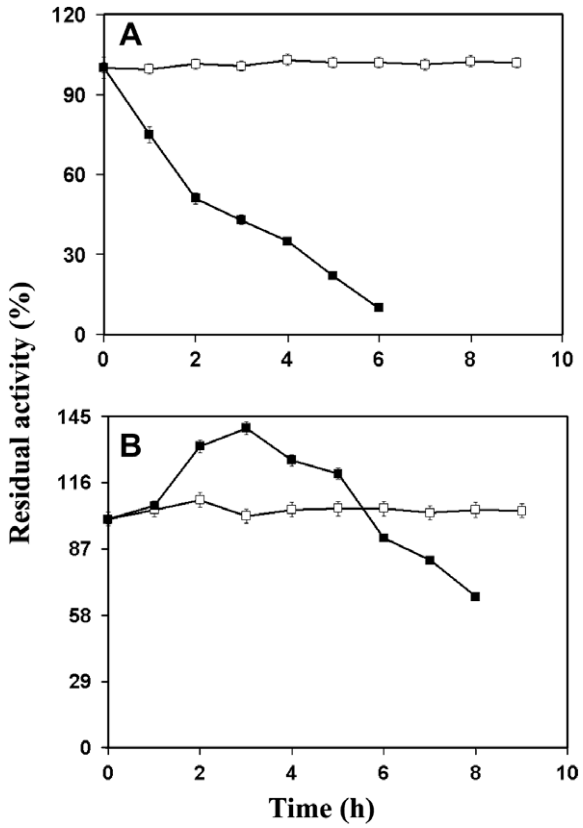

Figure 4. Susceptibility to trypsin (A) and *endo*-H (B) digestion. 50 mU of purified Cg (-□-) and C (-■-) were incubated with trypsin (5 μg) and *endo*-H (0.3 mU μg^{-1} enzyme) and residual activity was measured. An average of three sets of experimental results is presented.

Table 5
Amino acid analysis of cellobiase

Amino acid	nmol (%)
Lys	24
Leu	23
His	15
Phe	7
Tyr	6
Ile	5
Gly	5
Met	3
Ala	2
Asn/Asp	2
Gln/Glu	2
Val	1
Arg	1
Cys	1
Thr	1

2.2.7. Amino acid analysis of cellobiase

Amino acid analysis (Table 5) of Cg revealed that the enzyme contained 48% hydrophobic amino acids (Gly 5, Ala 2, Val 1, Met 3, Ile 5, Leu 23 and Phe 7), whereas the percent contribution of polar amino acids was 52 (Asn/Asp 2, Gln/Glu 2, His 15, Arg 1, Tyr 6, Cys 1, Lys 24 and Thr 1). The presence of a high percent of hydrophobic residues justified the strong aggregating nature of cellobiase.

2.2.8. N-Terminus sequencing of cellobiase and sequence analysis

The N-terminus of Cg was determined as [AAPPPPPPPPP-WAAAANTTLRL]. The first 22 amino acids did not reveal any conserved domain in BLAST. Sequence similarity was found with the hypothetical protein of *Coccidioides immitis* RS and *Oryza sativa* Indica Group through non-redundant PSI-BLAST, whereas iteration from SWISS–PROT showed similarity with one membrane protein 3 from *Zea mays,* indicating to an extent the novel characteristics of the glycoprotein.

3. Conclusions

The novelty of the work lies in the high activity and stability of underglycosylated cellobiase (Cg) produced in *T. clypeatus* in the presence of a glycosylation inhibitor due to high aggregation of the enzyme. Restricted glycosylation resulted in increased stability of the enzyme towards pH, temperature and other agents such as Mg^{2+}, sodium azide, PCMB, Gdn·HCl, urea, DTT and 2-mercaptoethanol. In addition, Cg was superior in terms of catalytic activity compared to the control enzyme. The observations altogether were quite an exception to the general trend where restricted

glycosylation led to loss in enzyme activity and stability. The amino acid sequences and N-terminal analysis revealed that the enzyme was novel, which appeared as an interesting model for the study of glycosylation and for aggregation of proteins from filamentous fungi. The intracellular β-glucosidase from the same medium[14] was identified as a novel small protein with transglycosylation activity. With increased substrate affinity, catalytic activity and stability of the extracellular enzyme can be exploited in the food-processing, pharmaceutical or fermentation industries where β-glucosidase activity plays a crucial role.

4. Experimental

4.1. Materials

Cellobiose, *p*NPG, SDS, DEAE-sephadex A-50, PROT-SIL1, IgG conjugated with alkaline phosphatase, NBT and BCIP were obtained from Sigma–Aldrich Chemical Co., USA. Sephacryl S-200 was purchased from Pharmacia (Sweden). The deglycosylation kit was purchased from Calbiochem (E. Merck KGaA, Germany). A dialysis bag (6.5 kDa cutoff), composed of regenerated cellulose, was purchased from Spectrapore, USA. Ultrafiltration membranes were obtained from Millipore (USA). Molecular weight markers were purchased from Fermentas, Canada. All other biochemicals used were of AR quality and were obtained from the local market.

4.2. Organism and growth condition for enzyme production

T. clypeatus was grown at 30 ± 2 °C on a rotatory shaker (at 350 rpm and throw of 3.5 cm) for 4 days in 5 × 500 mL (2-L flask) in a synthetic medium containing (%, w v^{-1}), cellobiose, 1; $NH_4H_2PO_4$, 2.5; sodium succinate, 0.5 and salts at pH 5.0 as described earlier.[11] Sterile solutions of DG were added into the culture media before inoculation to attain a concentration of 1 mg mL^{-1}. Experiments were performed in parallel in a control medium (without using the glycosylation inhibitor). Cell-free culture filtrate was collected as the extracellular enzyme.

4.3. Enzyme purification

The culture filtrate (2400 mL) was condensed to 455 mL by ultrafiltration using a PM-10 membrane. The retentate was further concentrated by lyophilization and was subjected to gel filtration in batches of 2.5 mL (7.5 mg protein) to a Sephacryl S-200 column (1.6 × 100 cm) equilibrated with 100 mM acetate buffer, pH 5.0 at 7 mL h^{-1}. Fractions (3 mL) were monitored for protein (A_{280}) and enzyme activities. Enzyme peaks were collected as pool E1 (78–108 mL) and desalted through dialysis against 10 mM acetate buffer, pH 5.0. It was then subjected to ion-exchange chromatography on a DEAE-sephadex A-50 column (3.2 × 18 cm) pre-equilibrated with 10 mM acetate buffer, pH 5 at 18 mL h^{-1}. The proteins were eluted out with a linear gradient of NaCl from 0 to 1.0 M in the 10 mM acetate buffer, pH 5. The fractions that eluted were assayed for cellobiase activity. Cellobiase was collected as the eluate (66–102 mL) from the column, dialyzed, and after lyophilization subjected to high-performance gel-permeation liquid chromatography (HPLC; WATERS, USA) through a gel-filtration column (Protein Pak 125, 7.8 × 300 mm, Waters). The mobile phase used was 100 mM acetate buffer and 100 mM NaCl at pH 5.0. Samples were collected at a flow rate of 1 mL min^{-1}. The molecular weight of the enzyme was approximated from HPGPLC by a plot of the log of molecular weight versus K_{av} values of standard proteins of 66 kDa, 44.6 kDa, 29 kDa and 16.9 kDa corresponding to 8.863 min, 9.256 min, 10.734 min and 11.507 min, respectively. C was purified in a parallel set according to the methods described by Mukherjee et al.[12]

4.4. Enzyme assays and protein estimation

Cellobiase activity was measured as β-glucosidase by the amount of *p*-nitrophenol liberated from *p*NPG and measured at 400 nm.[11] The assay was carried out in the reaction mixture containing 2 mM *p*NPG in 0.1 M NaOAc buffer, pH 5.0. Incubation was done at 45 °C for 10 min. The reaction was terminated by the addition of 0.5 mL of Na_2CO_3 (1 M). The intensity of the yellow colour developed by liberation of *p*NP was measured. A unit of enzyme activity was expressed as the amount of enzyme that produced 1 μmol of *p*NP per min under the assay conditions. Sucrase activity was assayed by measuring the liberation of glucose in the assay mixture (40 μL) of sucrose (4 mM) in 0.1 M NaOAc buffer, pH 5.0 by the GOD–POD reagent.[38] The mixture was incubated at 45 °C for 5 min, and the reaction was terminated by keeping the solution in a boiling water bath for 5 min. The solution was cooled to room temperature, and 1 mL of GOD–POD reagent was added to it. The intensity of the colour was measured at 505 nm after 30 min. The units of sucrase activity were expressed in terms of μmol of glucose liberated per min under assay conditions. Protein concentration was determined using the method of Bradford with bovine serum albumin as a standard.[39]

4.5. Western blotting of cellobiase

SDS–PAGE of extracellular cellobiase was performed in a 10% polyacrylamide gel slab according to Laemmli and Favre.[40] Electrotransfer of protein bands was done according to Towbin et al.[41] The protein bands were electrotransferred onto a nitrocellulose membrane (Amersham, UK) in a semidry transblot apparatus (Bio-Rad) at a constant current of 170 mA for 1 h. Blotted protein bands were detected by reaction with anti-cellobiase serum according to Blake et al.[42] Antirabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody. The colour was developed with NBT and BCIP in 100 mM NaCl and 5 mM $MgCl_2$.

Cg was denatured by a mixture of 1 M Tris–HCl (pH 8.0), 0.1 M EDTA, 1 M DTT and 8 M urea according to the method described earlier.[15]

4.6. Deglycosylation of cellobiase

Deglycosylation of the HPLC-purified cellobiase was done according to denaturing standard protocol given with the Glycoprotein Deglycosylation Kit. Around 50 μg of glycoprotein was treated with 1 μL of each of N-glycosidase F, α 2–3, 6, 8, 9 neuraminidase, *endo*-α-*N*-acetylgalactosaminidase, β1, 4-galactosidase and β-*N*-acetylglucosaminidase and was incubated for 3 h at 37 °C. The deglycosylated enzyme was then silver stained after separation in 10% SDS–PAGE.

4.7. Silver staining

Fixing solution, sensitizer solution, silver solution and developer solution were prepared according to the technical bulletin supplied with silver stain kit PROT-SIL1 (ProteoSilver Silver Stain Kit) and staining was done according to the manual.

4.8. Mass analysis of cellobiase

HPLC-purified 1 μL cellobiase enzyme (step 5, 20 pmol μL^{-1}) was mixed with 3.0 μL 'super-DHB' matrix solution. The 'super-DHB' matrix consisted of a 9:1 (w/w) mixture of DHB and 2-hydroxy-5-methoxybenzoic acid, since this ratio was found to be the most effective. Stock solutions of DHB (10 mg mL^{-1}) were prepared in 50% v v^{-1} aqueous acetonitrile containing 0.1% (v v^{-1}) TFA, and that of 2-hydroxy-5-methoxybenzoic acid (10 mg mL^{-1}) was made

in high-purity abs EtOH. The mixture was vortexed, and approximately 1.5 μL of the matrix–enzyme mixture was spotted onto the MALDI target plate. The plate was allowed to dry at the room temperature, and mass analysis was performed on a MALDI-TOF/TOFMS (4700 Proteomics Analyzer, Applied Biosystems Inc.) in the positive-ion mode, accumulating 2000 laser shots.

4.9. Amino acid analysis and N-terminal sequencing of the cellobiase enzyme

Amino acid analysis was performed in a PICO·TAG system according to the PICO·TAG operation manual (Waters, USA). The N-terminal amino acid sequence of the HPLC-purified sample was determined with a Procise cLC 491 Protein Sequencing System (Applied Biosystems Inc). The NH_2-terminal amino acid sequence of cellobiase was identified by Edman degradation on a pulsed liquid-phase protein sequencer with on-line detection of phenylthiohydantoin-amino acids by a 120A PTH Analyzer (Applied Biosystems). Sequence was analyzed for any conserved domain through CDD search in BLAST. PSI-BLAST was done with the NCBI algorithm keeping the filter on low complexity regions. All other parameters were kept at default values.

4.10. Kinetic parameters

All enzyme assays were performed in NaOAc buffer (0.1 M) at pH 5.0 unless otherwise specified. Various concentrations of the substrate *p*NPG (between 0.01 and 2 mM) were used for enzyme assay to determine K_m and V_{max} of the cellobiase enzyme, and the results were calculated via a Lineweaver–Burk plot. An average of three sets of experimental results is presented.

4.11. Effect of pH and temperature

The pH and temperature optima of the enzyme were determined by measuring the enzyme activity at various pH levels (pH 2–11) and at temperatures of 20–70 °C in 0.1 M NaOAc buffer at pH 5.0. The following buffers were used: (pH 2–4) HCl-sodium–citrate buffer 100 mM, (pH 5) NaOAc buffer 100 mM, (pH 6–7) phosphate (KH_2PO_4/K_2HPO_4) buffer 100 mM, (pH 8) Tris–HCl buffer 100 mM and (pH 9–10) glycine–NaOH buffer 100 mM. pH stability was determined by incubating the purified enzyme in buffers of pH ranging from 2.0 to 11.0 for 24 h at 30 °C, and for temperature stability for 60 min at the desired temperature, followed by measuring the residual activity. An average of three sets of experimental results is presented.

4.12. Effect of salts, chaotropic, reducing agents and products on the enzyme activity

Residual cellobiase activity was measured after incubating the enzyme in the presence of salts, chaotropic and reducing agents for 30 min. The ionic solutions used in this study were prepared in distilled water. Purified cellobiase was incubated in the presence of glucose (0–25 mg mL^{-1}) and DG (0–1.5 mg mL^{-1}) for 30 min, and the residual activity was determined. The activity of the samples incubated without agents was taken as 100%. An average of three sets of experimental results is presented.

4.13. Susceptibility towards tryptic digestion and endoglycosidase-H

Purified Cg (50 mU) was incubated with 5 μg of trypsin in Tris buffer (pH 8.0) at 37 °C. The purified enzyme (0.6 μg protein) in 50 mM NaOAc buffer at pH 5.0 was treated with 0.3 mU of endoglycosidase-H μg^{-1} protein at 37 °C. Aliquots were taken at different time intervals and were assayed for cellobiase activity as described earlier. Activity of the samples incubated without trypsin and endoglycosidase-H was taken as 100%. An average of three sets of experimental results is presented.

4.14. Substrate specificity

Cellobiase activity was measured in the presence of substrates (*p*NPG, *o*NPG, methyl β-D-glucopyranoside, methyl α-D-galactopyranoside, maltose, cellobiose, sucrose, trehalose, carboxymethyl cellulose) by the method of Nelson[43] and Somogyi[44] using a substrate concentration of 1% (w v^{-1}) at pH 5.0 and 0.1 M NaOAc buffer, except for *p*NPG and *o*NPG, for which the assay procedure is given above.[11] An average of three sets of experimental results is presented.

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References

1. Bhatia, Y.; Mishra, S.; Bisaria, V. S. *Crit. Rev. Biotechnol.* **2002**, *22*, 375–407.
2. Bhat, M. K.; Bhat, S. *Biotechnol. Adv.* **1997**, *15*, 583–620.
3. Barnett, C.; Berka, R.; Fowler, T. *Biotechnology (N Y)* **1991**, *9*, 562–567.
4. Bajaj, B. K.; Pangotra, H.; Wani, M. A.; Sharma, A.; Sharma, P. *J. Sci. Ind. Res.* **2009**, *68*, 242–247.
5. Coenen, T. M.; Schoenmakers, A. E.; Verhagen, H. *Food Chem. Toxicol.* **1995**, *33*, 859–866.
6. Fischer, L.; Bronmann, R.; Kengen, S. W. M.; DeVos, W. M.; Wagner, F. *Biotechnology (N Y)* **1996**, *14*, 88–91.
7. Otto, R. T.; Bornsheuer, U. T.; Syldatk, C.; Schmic, R. D. *Biotechnol. Lett.* **1998**, *20*, 437–440.
8. Kubicek, C. P. *J. Gen. Microbiol.* **1987**, *133*, 1481–1487.
9. Ishii, S.; Volpe, J. J. *J. Neurosci. Res.* **1990**, *26*, 419–427.
10. Kruszewska, J. S.; Perlińska-Lenart, U.; Górka-Nieć, W.; Orłowski, J.; Zembek, P.; Palamarczyk, G. *Acta Biochim. Pol.* **2008**, *55*, 447–456.
11. Mukherjee, S.; Chowdhury, S.; Ghorai, S.; Pal, S.; Khowala, S. *Biotechnol. Lett.* **2006**, *28*, 1773–1778.
12. Mukherjee, S.; Basak, S.; Khowala, S. *Enzyme Microb. Technol.* **2001**, *29*, 213–224.
13. Mukherjee, S.; Basak, S.; Khowala, S. *Biotechnol. Prog.* **2002**, *18*, 404–408.
14. Pal, S.; Banik, S. P.; Ghorai, S.; Chowdhury, S.; Khowala, S. *Bioresour. Technol.* **2010**, *101*, 2412–2420.
15. Saha, R.; Roy, S.; Sengupta, S. *Biotechnol. Prog.* **2002**, *18*, 1240–1248.
16. Jafari-Aghdam, J.; Khajeh, K.; Ranjbar, B.; Nemat-Gorgani, M. *Biochim. Biophys. Acta* **2005**, *1750*, 61–68.
17. Takegawa, K.; Fujiwara, K.; Iwahara, S.; Yamamoto, K.; Tochikura, T. *Biochem. Cell Biol.* **1989**, *67*, 460–464.
18. Schulke, N.; Schmid, F. X. *J. Biol. Chem.* **1988**, *263*, 8832–8837.
19. Kwon, K. S.; Yu, M. H. *Biochim. Biophys. Acta* **1997**, *1335*, 265–272.
20. Vemuri, S.; Yu, C. T.; Roosdorp, N. *Pharm. Biotechnol.* **1993**, *5*, 263–286.
21. Duarte-Vá zquez, M. A.; García-Almendá rez, B. E.; Rojo-Domínguez, A.; Whitaker, J. R.; Arroyave-Herná ndez, C.; Regalado, C. *Phytochemistry* **2003**, *62*, 5–11.
22. Sanchez-Romero, C.; Garcia-Gomez, M. L.; Pliego-Alfaro, F.; Heredia, A. *Physiol. Plant.* **1994**, *92*, 97–101.
23. Mukherjee, S. Ph. D. Thesis, Jadavpur University, 2002.
24. Guo, M.; Hang, H.; Zhu, T.; Zhuang, Y.; Chu, J.; Zhang, S. *Enzyme Microb. Technol.* **2008**, *42*, 340–345.
25. Khan, R. H.; Rasheedi, S.; Haq, S. K. *J. Biosci.* **2003**, *28*, 709–714.
26. Hayashi, S.; Nonoguchi, M.; Shimokawa, Y.; Takasaki, Y.; Imada, K. *J. Ind. Microbiol. Biotechnol.* **1992**, *9*, 251–255.
27. Hayashi, S.; Ueda, Y.; Yokoi, H.; Takasaki, Y.; Imada, K. *Mem. Fac. Eng. Miyazaki. Univ.* **2000**, *29*, 65–70.
28. Nie, G.; Reading, N. S.; Aust, S. D. *Arch. Biochem. Biophys.* **1999**, *365*, 328–334.
29. Tigier, H. A.; Quesada, M. A.; Heredia, A.; Valpuesta, V. *Physiol. Plant.* **1991**, *83*, 144–148.
30. Han, Y. W.; Lei, X. G. *Arch. Biochem. Biophys.* **1999**, *364*, 83–90.
31. Fountoulakis, M.; Gentz, R. *Nat. Biotechnol.* **1992**, *10*, 1143–1147.
32. Varki, A. *Glycobiology* **1993**, *3*, 97–130.
33. Schwarz, R. T.; Datema, R. *Methods Enzymol., Part D* **1982**, *83*, 432–443.

34. Barriocanal, J. G.; Bonifacino, J. S.; Yuan, L.; Sandoval, I. V. *J. Biol. Chem.* **1986**, *261*, 16755–16763.
35. Giraud, A.; Franc, J.-L.; Long, Y.; Ruf, J. *J. Endocrinol.* **1992**, *132*, 317–323.
36. Chu, F. K.; Trimble, R. B.; Maley, F. *J. Biol. Chem.* **1978**, *253*, 8691–8693.
37. Liu, H.; Gaza-Bulseco, G.; Xiang, T.; Chumsae, C. *Mol. Immunol.* **2008**, *45*, 701–708.
38. Chowdhury, S.; Ghorai, S.; Banik, S. P.; Pal, S.; Khowala, S. *Process Biochem.* **2009**, *44*, 1075–1082.
39. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
40. Laemmli, U. K.; Favre, M. *J. Mol. Biol.* **1973**, *80*, 575–599.
41. Towbin, E.; Stachelin, T.; Gordon, J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350–4354.
42. Blake, M. S.; Johnston, K. H.; Russell-Jones, G. J.; Gotschlich, E. C. *Anal. Biochem.* **1984**, *136*, 175–179.
43. Nelson, N. *J. Biol. Chem.* **1944**, *153*, 375–380.
44. Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19–23.