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Stabilization of cellobiase by covalent coupling to soluble polysaccharide

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Abstract

Cellobiase from *Aspergillus niger* was glycosylated by covalent coupling to cyanogen bromide activated dextran. The conjugated enzyme retained 62% of the original specific activity exhibited by the native cellobiase. The optimum pH as well as the pH stability of the conjugated form remain almost the same as for the native enzyme. Compared to the native enzyme, the conjugated form exhibited a higher optimal reaction temperature and energy of activation, a higher K_m (Michaelis constant) and lower V_{max} (maximal reaction rate), and improved thermal stability. The thermal deactivation of the native and conjugated cellobiase obeyed the first-order kinetics. The calculated half-life values of heat inactivation at 60, 70 and 80 °C was 10.7, 6.25, and 4.05 h, respectively, whereas at these temperatures the native enzyme was less stable (half-life of 3.5, 1.69, and 0.83 h, respectively). The deactivation rate constant at 80 °C for the conjugated cellobiase is about 7.9 $\times 10^{-2}$ h^{-1}, which is lower than that of the native enzyme (36.0×10^{-2} h^{-1}). The activation energy for denaturation of the native enzyme is about 10.58 kcal/mol, which is 7.25 kcal/mol lower than that of the conjugated enzyme. The effect of different surfactants and some metal ions on the activity of the conjugated cellobiase has been investigated.

Key words: Cellobiase – glycosylation – thermal stability-properties

Introduction

The stabilization of enzymes has received much attention in recent years (Arnold 1996). Stabilization against thermal inactivation can be performed in several ways such as cross-linking to a water-insoluble carrier with a bifunctional reagent, and covalent coupling to natural and synthetic polymers and entrapment in gels (Chibata 1986). Thermal stabilization by chemical modification with low molecular weight monofunctional reagents; cross-linking with bifunctional reagents and polymer attachment has been reported (Wasserman 1984).

Many proteins containing carbohydrate residues exhibit increased thermal stability towards heat and storage, which in many cases seems to be due to the carbohydrate part of the molecule (Klibanov 1983). Most glycoproteins exhibit high water solubility, and thus it was considered promising to obtain stabilized water-soluble enzymes through covalent attachment to carbohydrates (Kawamura *et al.* 1981; Woodward and Zachry 1982; Lendwers and Crichton 1984; Lenders *et al.* 1985; Srivastava 1991).

Klibanov (1983) has reported on the mechanism involved in the carbohydrate-induced stability of glycosylated proteins by rigidification of the conformation. On the other hand, Srivastava (1991) argued that the hydration effect of the attached carbohydrate may be responsible for improving the stability of conjugated enzymes. Hydrogen bonding between the polysaccharide and the protein surface (Blomhoff and Christensen 1983) and intra as well as intermolecular crosslinks between protein and polysaccharides has been suggested as causes of thermal stabilization of the synthetic glycoproteins (Lendewrs and Crichton 1984).

Cellobiase (β-glucosidase, β-D-glucosidase glucohydrolase, EC 3.2.1.21), catalyses the final step of cellulose hydrolysis (i.e., the breakdown of cellobiose to glucose). The supplementation of this enzyme to cellulase preparations, in order to obtain higher rates and extent of saccharification of cellulose, has been recommended (Wood and Wiseman 1982).

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With a view to enhancing the stability of enzymes, the stabilization of cellobiase by immobilization onto water-insoluble supports has been published several times (Desai *et al.* 1986, Simos and Georgatsos 1990; Abdel-Fattah *et al.* 1997). However, there are few reports about the stabilization of cellobiase by covalent coupling to soluble polysaccharides (immobilization to water-soluble carrier) (Lenders *et al.* 1986).

The present study deals with the stabilization of *Aspergillus niger* cellobiase by covalent coupling to soluble polysaccharide. The catalytic properties and the stability of the conjugated enzyme have been compared to those of the native enzyme.

Materials and methods

Cellobiase from *Aspergillus niger* was obtained from NOVO (Denmark). This crude enzyme was fractionated with ammonium sulphate as follows: A certain amount of the enzyme solution (10 mg protein/ml) was kept in a cooling water bath at 5.0 °C, then ammonium sulphate was added to a final saturation of 80% with stirring for 2 h. The precipitated fraction was collected by centrifugation in a refrigerated centrifuge (5000 rpm for 15 min), dissolved in 0.05 M acetate buffer (pH 5.0), dialysed against distilled water and lyophilized. This partially purified enzyme (specific activity 37 U/mg protein) was used for preparation of the conjugated cellobiase.

Covalent coupling of cellobiase to activated dextran. Dextrans of average molecular weights (MW) of 4.0, 7.0, and 23.0×10^4 were used in the present studies, all were obtained from Sigma. *A. niger* cellobiase was coupled to soluble dextran by the method reported by Srivastava (1991) as follows: To 100 ml of 1% (w/v) dextran solution, 0.125 g solid cyanogen bromide was added and the mixture was allowed to stand for 45 min with gentle shaking, the pH was maintained at 10.5 with 0.5 M NaOH. Then 0.125 g more of solid cyanogen bromide was added maintaining similar conditions as above. The activation process was allowed to occur at room temperature for one h. The excess CNBr was removed by dialysing the solution against distilled water. The enzyme sample (50 mg) was added and the coupling reaction was allowed to proceed at 4 °C for 16 h.

Purification of dextran-cellobiase conjugates. The dextran-cellobiase conjugates were concentrated by lyophilization. The concentrated conjugates (5 ml) were separated from the unconjugated enzyme by size exclusion chromatography on a column (2.0 cm × 40 cm) of Sephadex G-100, eluted with 0.05 M citrate-phosphate buffer (pH 5.0). The distribution of the enzyme activity as well as carbohydrate in the column fractions was determined. A mixture containing 5 mg cellobiase in 10 ml (1%, w/v) dextran solution was chromatographed on the same column for comparison of the distribution of the native enzyme in the column.

Assay for cellobiase activity. The cellobiase activity was determined according to the method reported by Berghem and Pettersson (1974) as follows: To one ml of 0.4% cellobiose dissolved in 0.05 M citrate-phosphate buffer (pH 5.0), 0.5 ml of diluted enzyme solution was added. The mixture was incubated in a water bath for 30 min at 50 °C. The reaction was stopped by heating the reaction mixture in a boiling water bath for 5 min. The glucose released was determined by glucose oxidase/peroxidase reagent. One unit of the enzyme activity (U) was defined as µmoles of glucose released per min under the assay conditions.

Thermal stability. Thermal stability of both native and conjugated cellobiase were tested by incubating the enzymes in 0.05 M citrate-phosphate buffer (pH 5.5) at a designated temperature for 1 to 8 h before activity assay.

Protein estimation. Protein was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard protein. All the results reported are the mean of at least three separate experiments.

Results

The variation of activity retained of the glycosylated cellobiase was affected by the MW of the carbohydrate residue covalently linked to the enzyme molecule. The results (Table 1) indicated that the retained specific activity with dextran of average MW of 230000 (22.94 U/mg protein) was about 73% and 89.5% of those recorded for other dextrans of average MW of 40000 and 70000 respectively. However, the retained activity, after heat treatment at 80 °C for 60 min, with the enzyme coupled to the higher molecular weight dextran (MW 230000) was 123% and 111% of those recorded for the conjugates with dextran of average MW 40000 and 70000 respectively. Since the present study deals with the stabilization of *Aspergillus niger* cellobiase, cellobiase conjugated to dextran of average MW of 230000 (which showed the highest stability) was used through this study.

Properties of the native and conjugated cellobiase

The glycosylated enzyme retained about 62% of the original specific activity exhibited by the free enzyme (37 U/mg protein).

The optimum pH of the native and conjugated cellobiase was studied using citrate buffer (0.05 M,

Table 1. Comparison of cellobiase activity and stability of *Aspergillus niger* cellobiase covalently coupled to cyanogen bromide-activated dextran of different molecular weights.

Molecular weight of Activated dextran	Specific activity (U/ mg protein)	Recovered specific activity* (%)	Thermal stability** at 80°C
MW, 40000	31.80	84.94	71.60
MW, 70000	28.49	77.00	79.30
MW, 230000	22.94	62.00	88.00

* This refers to the specific activity of the native enzyme. ** The recovered activity after treatment for one h at 80°C.

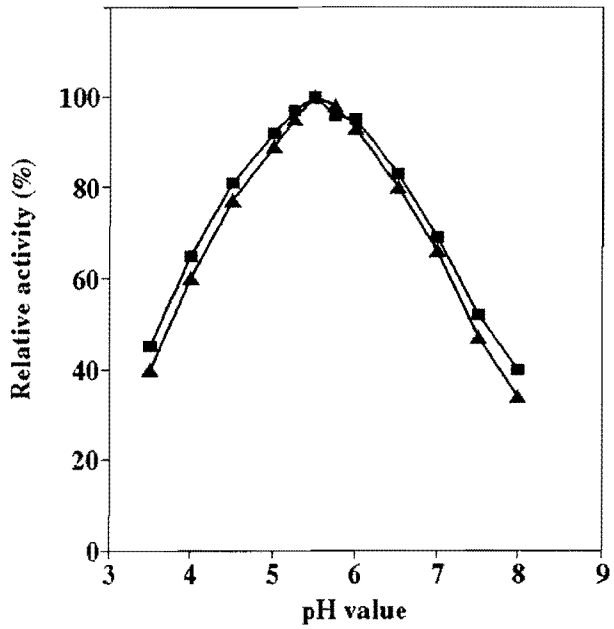

Fig. 1. Effect of pH on the activity of the native (■) and conjugated (▲) cellobiase.

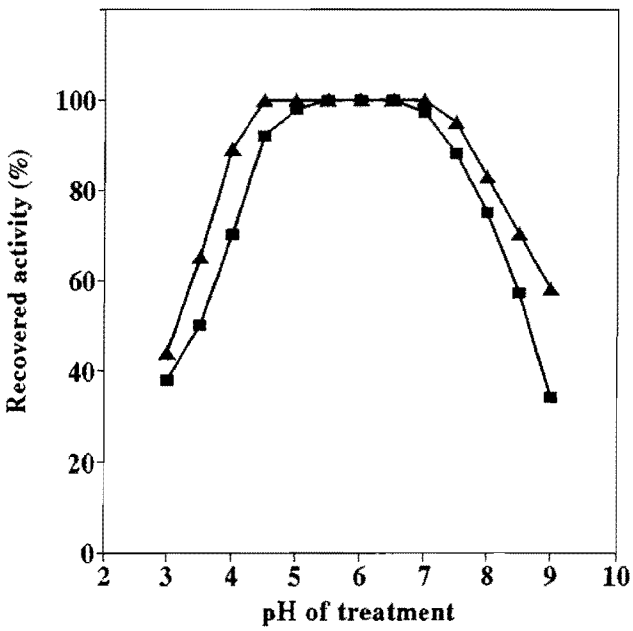

Fig. 2. pH stability of the native (■) and conjugated (▲) cellobiase.

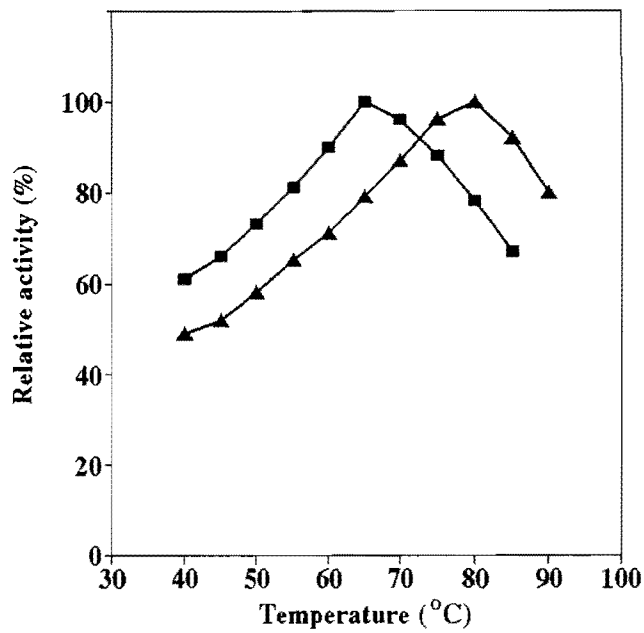

Fig. 3. Effect of temperature on the activity of the native (■) and conjugated (▲) cellobiase.

pH 3.5–5.5), citrate-phosphate buffer (0.05 M, pH 6.0–7.5), and 0.05 M phosphate buffer, pH 7.0–8.0). The results (Fig. 1) indicated that the two forms of cellobiase were optimally active at pH 5.5. The pH stability of the two forms of cellobiase was investigated by preincubating the enzyme samples at the designed pH for 60 min. The results (Fig. 2) showed no change of the pH stability of the enzyme after the glycosylation process.

The activities of native and conjugated cellobiase were assayed at various temperatures (50–90°C). The native enzyme had an optimum temperature of about 65°C, whereas that of the conjugated enzyme was shifted to 80°C (Fig. 3). The temperature data were replotted in the form of Arrhenius plots (Fig. 4). The slope of a logarithmic Arrhenius plot is related to the activation energy for the molecule by the relationship: slope = activation energy/2.303 R, where R is the gas constant. The plots for both the native and conjugated enzymes were linear and the values of the energy of activation were calculated as 3.3 Kcal/mol for the

Table 2. Comparison of thermal stabilities of both native and dextran-conjugated cellobiase.

Property	Native enzyme	Conjugated enzyme
Optimum temperature (°C)	65	80
Energy of activation (Kcal/mol)	3.30	4.22
Half-life (h):		
60°C	3.50	10.71
70°C	1.69	6.25
80°C	0.83	4.04
Deactivation constant rate (h^{-1}):		
60°C	8.6×10^{-2}	2.8×10^{-2}
70°C	17.7×10^{-2}	4.8×10^{-2}
80°C	36.0×10^{-2}	7.9×10^{-2}
Activation energy of denaturation (kcal/mol)	10.58	17.83

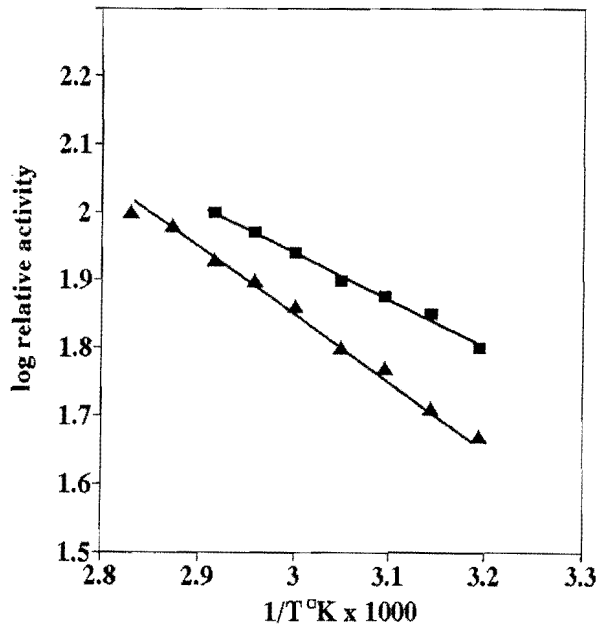

Fig. 4. Arrhenius plots, from data in Fig. 3.

native and 4.22 Kcal/mol for the conjugated cellobiase (Table 2).

Lineweaver-Burk plots of the native and glycosylated cellobiase gave K_m (Michaelis constant) of 1.5 mM and 2.20 mM, respectively with cellobiose. The V_{max} (the maximum reaction rate) of the native and conjugated cellobiase were 63 and 41 U/mg protein respectively.

The rates of heat inactivation of the native and conjugated cellobiase were investigated in the temperature range between 60 and 80°C (Fig. 5). When the log of

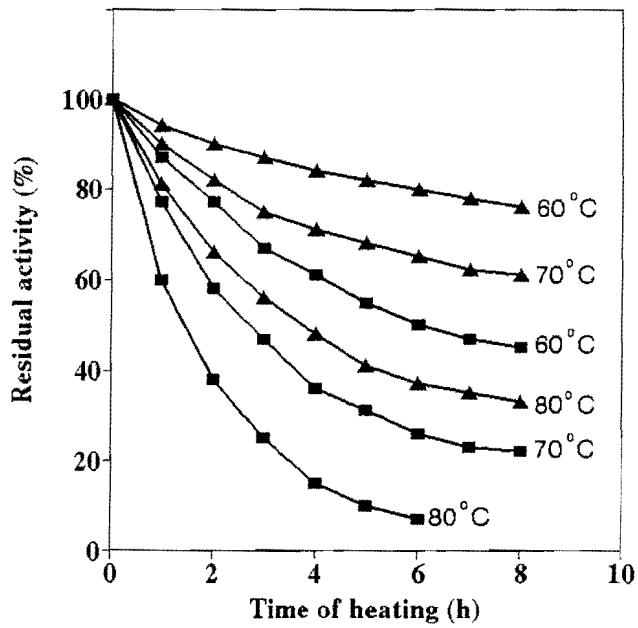

Fig. 5. Heat inactivation of the native (■) and conjugated (▲) cellobiase.

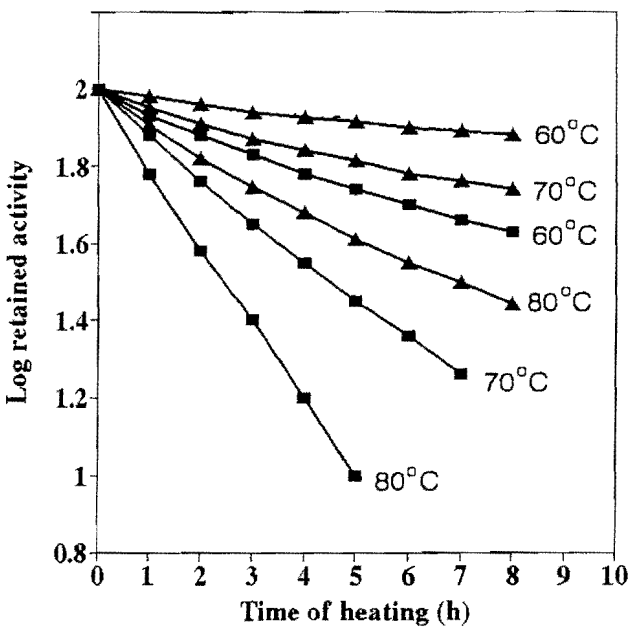

Fig. 6. Log of activity retained as a function of time for the native (■) and conjugated (▲) cellobiase.

the activity retained was plotted against time at the temperature used for inactivation, the two forms of cellobiase gave straight-line plots (Fig. 6). This indicated that the thermal inactivation process of the conjugated enzyme corresponded well to the theoretical curves of a simple first-order reaction, i.e., a monomolecular denaturation mechanism is involved (Kawamura *et al.* 1981).

Table 3. Effect of surfactants and various metal ions on the activity of native and conjugated cellobiase

Substance	Concentration	Relative Activity (%)	
		Native	Conjugated
None	–	100	100
Tween 80	0.05%	77	100
Triton X-100	0.05%	83	95
Sodium dodecyl sulphate	0.01%	56	90
Urea	2 M	40	88
	4 M	27	53
	6 M	00	33
$MgSO_4$	25 mM	70	94
	100 mM	24	60
KCl	25 mM	96	98
	100 mM	93	94
$CuSO_4$	10 mM	38	75
$CaCl_2$	10 mM	96	100

Treatment at 55 °C for 60 min

In general, covalent attachment of cellobiase to dextran protected the enzyme against heat inactivation. For example, the calculated half-life value shows that the heat inactivation of the conjugated enzyme at 60, 70 and 80 °C was 10.71, 6.25, and 4.05 h respectively (Table 2), whereas at these temperatures the native enzyme was less stable (half-life of 3.5, 1.69, and 0.83 h, respectively). The deactivation rate constant at 80 °C for the conjugated cellobiase is about 7.9 $\times 10^{-2}$ h^{-1}, which is lower than that of the native cellobiase (36.0×10^{-2} h^{-1}). Using Arrhenius equation, the activation energy for denaturation of the native enzyme is about 10.58 kcal/mol, which is 7.25 kcal/mol lower than that of the conjugated enzyme (Table 2). Cellobiase has thus been stabilized by glycosylation (immobilization on a water-soluble polysaccharide).

The conjugated cellobiase activity was not reduced in the presence of various surfactants, while the native enzyme showed a remarkable loss of activity in the presence of sodium dodecyl sulphate (Table 3). The effect of urea denaturation of the native and conjugated cellobiase was investigated at different concentrations (2 to 6 M). The native enzyme lost 73 and 100% of its original activity when treated with 4 and 6 M urea, respectively, however, under identical conditions the conjugated enzyme retained 53 and 33% of the original activity. Treatment of the native enzyme with 25 and 100 mM $MgSO_4$ (high water-binding salt) showed about 30% and 76% losses of its original activity, respectively. Under the same conditions, however, the conjugated cellobiase was more stable (the retained activity was 94% and 60%, respectively).

Discussion

The reason for investigating the stabilization of cellobiase is to assess its potential usefulness in the saccharification of cellulose with the cellulase enzymes obtained from *Trichoderma*. Covalent coupling of soluble polysaccharide to proteins has been reported as a common technique for improving their thermal stability (Blomhoff and Christensen 1983). This method has been applied to cellobiase using cyanogen bromide activated dextran of different molecular weights. Among all preparations the enzyme coupled to activated dextran of molecular weight of 230000 showed the highest thermal stability. The conjugated enzyme retained 62% of the original specific activity. The reason of the drop of the specific activity after glycosylation may be attributed to the rigidification of the enzyme protein conformation (Lendewrs and Crichton 1984). Consequently, the usual binding of the enzyme to the substrate was impeded or prevented. On the other hand, Gottschalk and Jaenicke (1991) reported that the covalent attachment of the enzyme to the immobilization matrix would also lead to a decrease in catalytic activity owing to the decrease in the flexibility of the enzyme molecule. The drop of the specific activity after the glycosylation of other enzymes were previously reported (Lendewrs and Crichton 1984; Srivastava 1991).

It was found that there was no change of the pH optimum for activity and pH stability of the native and glycosylated enzyme. This means that the ionization of the amino acid residues at the active site remains unaffected by the glycosylation process. Similar results were reported for other glycosylated amylase (Srivastava 1991). In contrast, pH optimum of *Bacillus subtilis* protease was shifted to acidic range after immobilization to a water-soluble carrier (Troitskii *et al.* 1987).

The increase of the optimum temperature is probably a consequence of enhanced thermal stability. The higher value of the activation energy obtained for the conjugated cellobiase indicates that the applied glycosylation procedure introduced changes in the structure of the enzyme molecule which impeded the enzyme catalyzed reaction. A similar explanation has been given by other investigators (Krajewska *et al.* 1990, Birnbaum 1994).

Covalent coupling of cellobiase to activated dextran appeared to enhance its thermal stability as reflected from the increased half-life for thermal deactivation, decreased rate constant of deactivation, and higher value of activation energy of denaturation.

The mechanism involved in the carbohydrate-induced stability of glycosylated proteins by rigidification of the conformation has already been discussed (Klibanov 1983). On the other hand, Srivastava (1991) argued that the hydration effect of the attached carbohydrate may be responsible for improving the stability of conjugated

enzymes. Hydrogen bonding between the polysaccharide and the protein surface (Blomhoff and Christensen 1983) and intra as well as intermolecular crosslinks between protein and polysaccharides have been suggested as causes of thermal stabilization of the synthetic glycoproteins (Lendewrs and Crichton 1984).

The apparent increase of the K_m value after covalent coupling to the activated dextran is most likely a consequence of conformational changes in the enzyme introduced by the glycosylation procedure which renders its active site less accessible to the substrate. Consequently, the maximum reaction rate of the enzyme-catalyzed reaction was lower than the native enzyme (Gottschalk and Jaenicke 1991). The increase of K_m value and decrease of the V_{max} after glycosylation of other enzymes were similarly reported (Vegarud and Christensen 1975).

The higher recovered activity of the conjugated form, after treatment with $MgSO_4$, compared to the native enzyme could be attributed to the hydrophilic nature of the polysaccharide attached to it. Perutz (1978) reported that protein hydration may have a positive effect on stability. Therefore, treatment of proteins with high water-binding salts (like $MgSO_4$) reduces the water shield surrounding them and consequently, the stability was negatively affected. In case of the conjugated cellobiase, and due to the hydrophilic nature of the polysaccharide attached to it, the water shield was probably preserved. A similar explanation was given by others (Blomhoff and Christensen, 1983, Srivastava 1991).

The results revealed that the glycosylation of cellobiase (immobilization onto a water-soluble carrier) formed stable covalent bonds that led to achievement of resistance against chemical and thermal denaturation. The conjugated enzyme acquired a higher temperature optimum (80 °C). This provides an additional advantage for practical application by reducing the probability of microbial attack for the reaction mixtures. In addition, the conjugated enzyme acquired a pH optimum (pH 5.5) very close to that of the most widely used *Trichoderma* cellulase (optimum pH 5.0). All these criteria could, therefore, be successfully utilized in the practical application.

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