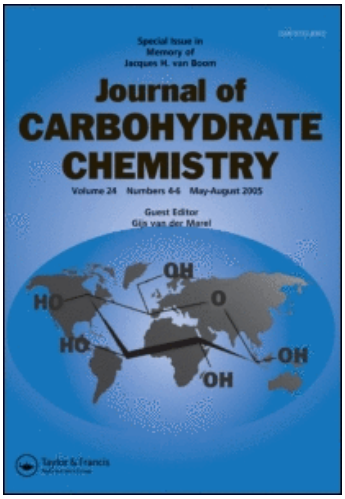

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

Functional Stabilization of Cellulase from *Aspergillus niger* by Conjugation with Dextran-aldehyde

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Online publication date: 08 September 2010

To cite this Article Arslan, Asli , Kuzu, Huriye and Altikatoglu, Melda(2010) 'Functional Stabilization of Cellulase from *Aspergillus niger* by Conjugation with Dextran-aldehyde', Journal of Carbohydrate Chemistry, 29: 5, 222 — 235
To link to this Article: DOI: 10.1080/07328303.2010.508140
URL: http://dx.doi.org/10.1080/07328303.2010.508140

Journal of Carbohydrate Chemistry, 29:222–235, 2010

ISSN: 0732-8303 print / 1532-2327 online
DOI: 10.1080/07328303.2010.508140

Functional Stabilization of Cellulase from *Aspergillus niger* by Conjugation with Dextran-aldehyde

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The covalent conjugates of cellulase from *Aspergillus niger* were prepared with various molar ratios by using dextran. The conjugate (n_E/n_D: 1/5) showed higher activity than purified enzyme at all temperatures after 1 h of incubation and its activity could also be measured at higher temperature. Also, this conjugate lost only 60% activity in 2 h at 70°C in comparison to the purified enzyme, which lost all its activity. In addition, conjugation protected cellulase against denaturation in the presence of sodium dodecylsulfate (residual activity of about 80%) and inactivation by air bubbles (residual activity of about 50% for 4 h).

Keywords Cellulase; Dextran-aldehyde; Conjugate; Enzyme stability; Incubation

INTRODUCTION

The chemical modification of enzymes with carbohydrates was shown as a useful strategy for improving the stability of biocatalysts due to formation of additional inter- and intramolecular bridges in the glycosylated enzyme molecule.[1] Generally, polysaccharides are more effective than mono- or disaccharide stabilizers because multipoint attachment of such polyhydroxyls increases the rigidity as well as hydration of the enzyme molecule. It was reported that neutral polysaccharides (dextran, levan) improved the enzyme stability. Dextran contains vicinal hydroxyl groups that can readily be modified

Received March 22, 2010; accepted July 10, 2010.
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with periodate to form aldehyde groups. The oxidized dextran-bearing aldehyde groups serve as a good macromolecular cross-linker for polysaccharides with free amino groups.[2] On the other hand, polyaldehydes can be further modified with different reagents.[3] Dextran aldehyde is a flexible polymer and it has been used to cross-link immobilized multimeric enzymes, generate hydrophilic environments, and even reduce the interaction of supports with macromolecules.[4–7] Dextran aldehyde was successfully adapted to suit the particular enzyme and prevent its inactivation by subunit dissociation, even when boiled in the presence of SDS and mercaptoethanol.[8]

The electrostatic and hydrogen interactions between polysaccharides and the protein may contribute to enzyme stabilization.[4,9,10] The operational stability of industrial enzymes remains a critical issue in biotechnology, though dramatic advances have taken place in the field of protein stabilization using protein engineering, immobilization techniques, stabilizing additives, and chemical modification.[5,11,12] There are multiple inactivation factors for enzymes during their industrial applications. Among them, interactions between soluble enzymes and interfaces of organic solvent drops in biphasic systems and gas bubbles in stirred systems may be very important.[4,5,13]

Cellulose, one of the most abundant polymers in nature, consists of flat chains of repeating monomers (poly-1,4-β-glucose). In nature, cellulose never occurs as a single chain; from the moment of synthesis, it exists as a composite of many chains, termed microfibrils.[14] In a typical cellulose-degrading ecosystem, a variety of cellulolytic bacteria and fungi work in concert with related microorganisms to convert insoluble cellulosic substrates to soluble sugars, which are then assimilated by the cell. In order to catalyze this process, the cellulolytic microbes produce a variety of different enzymes, known as cellulases.[15] Cellulases are well-established agents in the textile industry for fiber and fabric modifications.[16] In the pulp and paper industry, cellulases also play an important role for improvement of properties of virgin and recycled fibers. In the food industry, these enzymes have been used as processing aids in seed oil extraction.[17–19] Cellulases, which are members of the glycoside hydrolase family, are responsible for the hydrolysis of the β-1,4-glycosidic bonds in cellulose.[20,21] The cellulolytic complex from fungi is well defined and is composed of three major components: endoglucanase (1,4-β-D-glucan glucanohydrolase EC 3.2.1.4), cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucohydrolase EC 3.2.1.21).[21,22] These fungal extracellular enzymes are widely used in the textile processing industry. However, if these enzymes are to become successfully commercialized in the future, an acceptable level of stability must be ensured.[23,24] In our earlier studies, horseradish peroxidase was successfully modified by covalent bonding with dextran aldehyde, and it was found that conjugation of purified enzyme with dextran makes it more stable over a wide range of temperature and pH

and for a longer incubation period.[4] This present paper deals with the covalent attachment of a dextran-aldehyde polymer to cellulase from *Aspergillus niger* (EC 3.2.1.4) and the effect of this transformation on enzyme stability properties.

MATERIAL AND METHODS

Materials

Cellulase from *A. niger* (M_w = 31 kDa) (FL.22178), Sephadex G-50 column material (FL.84946), and carboxymethyl cellulose (CMC) (FL.21902) were purchased from Fluka. Dextran from *Leuconostoc mesenteroides* (M_w = 75 kDa, SI.D3759) were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals used were all analytical grade.

Enzyme Purification

The protein content of the purchased cellulase enzyme was determined as 28% (A_{280}/1.34). HPLC chromatograms also confirmed that there were impurities in the commercial enzyme. For conjugate synthesis it was decided that using purified enzyme would be better. Purchased cellulase was purified by gel permeation chromatography using Sephadex G-50 column. For elution, the column was washed with 0.01 M phosphate buffer, pH 7.0, and fractions were collected in tubes. Absorbances at 280 nm were measured with UV spectrophotometer and appropriate fractions were pooled for the conjugate synthesis.[4]

Preparation of Dextran-aldehyde Derivative

Freshly prepared $NaIO_4$ solution (8 g dissolved in 70 mL distilled water) was added slowly over dextran solution (3.33 g dissolved in 30 mL distilled water) and kept stirred in darkness for 24 h at rt. At the end of this period, the solution was dialyzed for 24 h against distilled water and aldehyde derivative of dextran was recovered by freeze drying.[16,25]

Preparation of Cellulase-dextran-aldehyde Conjugates

Appropriate amounts of dextran-aldehyde derivative were calculated according to the following formula with constant enzyme concentration (2 mg/mL):

$$n_E/n_D = c_E M_D/c_D M_E = 1/1, 1/5, 1/10, 5/1, 10/1$$

c : concentration(mg/mL); M : molarweight

Calculated amounts of dextran-aldehyde for the ratios 1/1, 1/5, 1/10, 5/1, and 10/1 were freshly prepared in 0.01 M phosphate buffer, pH 7.0, as well

as purified enzymes. Afterwards the reaction was initiated by mixing enzyme (2 mL) and dextran-aldehyde (2 mL) solutions together and kept in a water-bath at 25°C for 16 h. As a result, a Schiff base was formed between aldehyde groups of dextran derivative and amine groups of enzyme. Afterwards 5.6 mL of cold (4°C) 100 mM sodium bicarbonate, pH 8.5, was added to increase the pH of the reaction media. To reduce the schiff base that occurred and the unreacted aldehyde groups of dextran derivative, 9.6 mg of sodium borohydride was added. This solution was stirred for 15 min and then 9.6 mg of sodium borohydride was again added. Reduction was continued at 4°C for 15 min and finally the pH of the solution was adjusted to 7.0.[16,26]

Characterization of the Conjugates

The molecular masses of dextran aldehyde, purified enzyme, and conjugates were estimated by HPLC using column Shim-Pack Diol-300 (7.9 mm ID × 50 cm) with Shim-Pack Precolumn Diol (4.0 mm ID × 5 cm) at rt. The fractions were eluated at 1 mL/min^{-1} with 0.1 M PBS buffer (pH 7) containing 0.15 M NaCl and 7.5 mM NaN_3. The eluate was monitored at 280 nm with Shimadzu SPD-10AV VP Model UV–vis Detector. The particle size of the enzyme and conjugates were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 4.0-mV He-Ne laser at a wavelength 633 nm and a temperature of 25°C. All the solutions were filtered through 0.2 μm RC-membrane filters (Sartorius) before DLS measurement.

Kinetic Studies

To measure kinetic parameters, substrate (CMC) concentrations from 0.5% to 5% (W7V) were used at constant enzyme concentrations under the optimum conditions (at pH 5.0, 30°C). Rates were measured for eight different substrate concentrations in duplicate. The apparent kinetic Michaelise-Menten constants (K_m), V_{max}, and k_{cat} of purified cellulase and cellulase-dextran aldehyde conjugate (n_E/n_D: 1/5) were estimated by using linear regression plots of Lineweaver and Burk.

Activity Determination

A reaction mixture contained 0.5 mL of 2% CMC in 0.05 M sodium citrate buffer (pH 5), 0.5 mL of enzyme solution (2 mg/mL dissolved in citrate buffer, pH 5), and 9 mL citrate buffer. The reaction mixture was incubated in a water bath at 50°C for 30 min. The amount of reducing sugar that was liberated in the reaction mixture was measured by using the 3,5-dinitrosalicylate acid (DNS) method. The absorbance of the reference sample (substrate solution incubated without enzyme and enzyme solution in buffer) was subtracted

from those of the test samples. One unit of activity was defined as the amount of enzyme that released 1 μmol of glucose equivalents from substrate per minute.[27–29] For repeatability of activity measurements, five consecutive applications were carried out for each data point in same condition and recorded absorbance values were averaged.

Stability Studies

Thermal stability

Thermal stabilities of the synthesized conjugates and purified cellulase were evaluated with the activities determined according to the procedure described earlier. But in this procedure enzyme/conjugate solutions were kept at different working temperatures (30, 40, 50, 60, 70, 80°C) for 1 h and activity determination was performed at pH 5.0. Meanwhile, thermal stabilities of the enzyme and conjugate were monitored at 70°C. The enzyme and conjugate solutions (2 mg/mL in 0.05 M citrate buffer pH 5.0) were incubated at 70°C for different periods of time. Residual cellulase activity was assayed with periodically withdrawn aliquots (1 mL). The activity in the aliquot withdrawn initially at 0 h was used as control and considered 100%.

pH stability

The stabilities of the conjugate (n_E/n_D: 1/5) and purified cellulase against pH factor were determined according to the same procedure for activity determination described earlier. The enzyme and conjugate solutions were kept at different pH values for 1 h and the activity was determined at 50°C. All enzyme experiments were performed in citrate buffer (0.05 M) at pH ranging from 2.0 to 5.0, 0.01 M phosphate buffer of pH ranging from 6.0 to 8.0, and 0.01 M glycine buffer of pH 9.0.

Enzyme inactivation in stirred aqueous systems

Fifty milliliters of 0.05 M sodium citrate buffer at pH 5.0 containing purified cellulase and conjugate was placed in a graduated cylinder (diameter 5 cm) and then mechanically stirred at 1200 rpm to generate air-liquid interfaces. The experiment was carried out at pH 5.0 and 50°C. Samples were withdrawn at different time intervals and the activity was measured as previously described. All results reported represent averages of at least three experiments. Moreover, in all cases, the experimental error was not higher than 5%.

Stability in the presence of sodium dodecylsulphate (SDS)

Purified and conjugated enzyme preparations were incubated at 50°C in 0.3% (w/v) SDS in 0.05 M sodium citrate buffer, pH 5, for different periods of time. Aliquots were removed at scheduled times and assayed for activity.

RESULTS AND DISCUSSION

After the occurrence of covalent bonding between aldehyde groups of dextran derivative and primary amino groups of enzyme, the peak of aldehyde, which was observed at about 2939 cm^{-1} at Fourier transform infrared (FTIR), disappeared. The percentage of dextran modification was approximately 83%; as reported in the literature, each glucose consumed two molecules of oxidizing reagent.[6]

One of the most fundamental parameters for characterizing macromolecules is their molecular weight and molecular weight distribution.[30] However, gel permeating chromatography has been a widely used technique for estimating these characteristics of proteins, polysaccharides, and protein-polymer conjugates in their native forms based on their elution positions.[1,31] HPLC method is used for the determination of soluble aggregates. Figure 1 shows the HPLC results of cellulase-dextran aldehyde conjugates at different ratios of molecular concentrations of components (n_E/n_D) at pH 7.0. The retention time (RT) of conjugates with n_E/n_D: 5 and n_E/n_D: 10 did not change when compared to purified cellulase (the RT of the conjugates n_E/n_D: 5, n_E/n_D: 10, and enzyme is equal to 17.2 min). Successive addition of dextran-aldehyde did lead to noticeable change in RT of conjugate (n_E/n_D: 1/1, 1/5, 1/10) peak areas, so the growth rates of conjugates with n_E/n_D: 1/1, 1/5, and 1/10 increased.

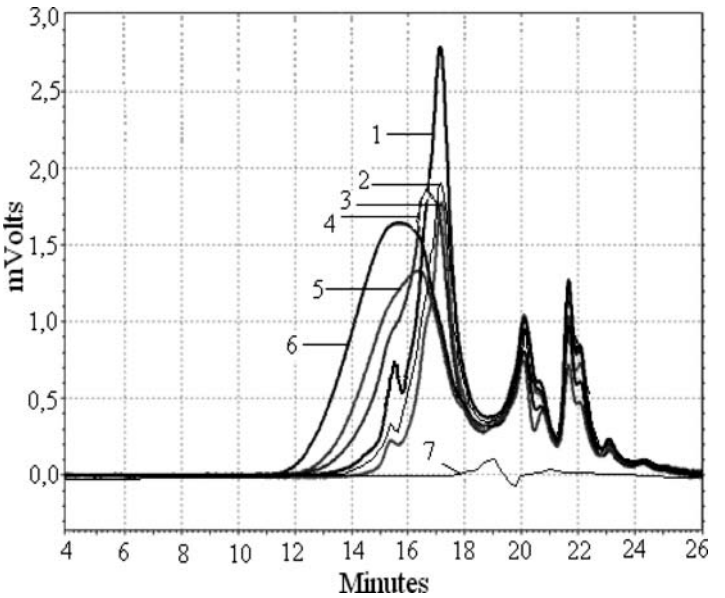

Figure 1: HPLC chromatograms of cellulase (1), dextran aldehyde (7), and conjugates prepared at the ratios of n_E/n_D: 10/1 (2), 5/1 (3), 1/1 (4), 1/5 (5), and 1/10 (6) at pH 7.

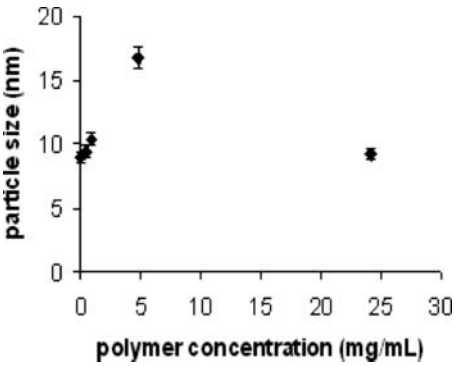

Figure 2: Dependence of the particle sizes of the cellulase-dextran aldehyde mixture on the amount of added polymer at constant concentration of the cellulase (2 mg/mL). Each data point represents the average value of three independent experiments with error bars indicated.

Elution positions of the conjugates, which were placed in front of cellulase, relate the formation of covalently bonded macromolecules with higher molecular weights than the constituents. HPLC analysis results indicate that enzyme bonded with dextran aldehyde forms stable covalent conjugates. It has been reported that the current cellulase was a monomeric protein in numerous investigations.[27,32] It is active as a monomer but it tends to form complexes with dextran aldehyde molecules, as suggested in the HPLC.

Figure 2 presents the dependence of the particle size of the cellulase-dextran aldehyde mixtures on the amount of added polymer to constant amount of cellulase. It can be seen that the particle sizes of conjugate depended on the n_E/n_D ratio. Particle size increased as dextran concentration increased and reached a maximum value at n_E/n_D: 1/1. Particle size of the conjugate with molar ratio n_E/n_D: 1/1 was higher (*ca*. twofold) than pure enzyme. Particle size decreased after the excessive addition of dextran (n_E/n_D: 1/5). This may be explained as aggregation can be minimized by increasing concentration of polysaccharide. The reduction in the size of the conjugate had effectively improved the stability and efficiency of bioconjugates.[33]

To investigate the mechanism of enzymatic conversion, a kinetic model has been used to fit the experimental data. The kinetic parameters K_m and V_{max} of purified cellulase and cellulase-dextran aldehyde conjugate (n_E/n_D: 1/5) were determined from Lineweaver-Burk double reciprocal plots of cellulase activity at 30°C using various concentrations of CMC as substrate (Fig. 3). The apparent kinetic parameters are reported in Table 1. As shown in the table, the chemical conjugation increased the catalytic constant (k_{cat}) and decreased the values of the apparent Michaelis-Menten constant (K_{m}) for CMC. Thus, the catalytic efficiencies (k_{cat}/K_{m}) of cellulase-dextran aldehyde conjugate were higher than that of purified cellulase. The K_m was found to decrease by 2.07

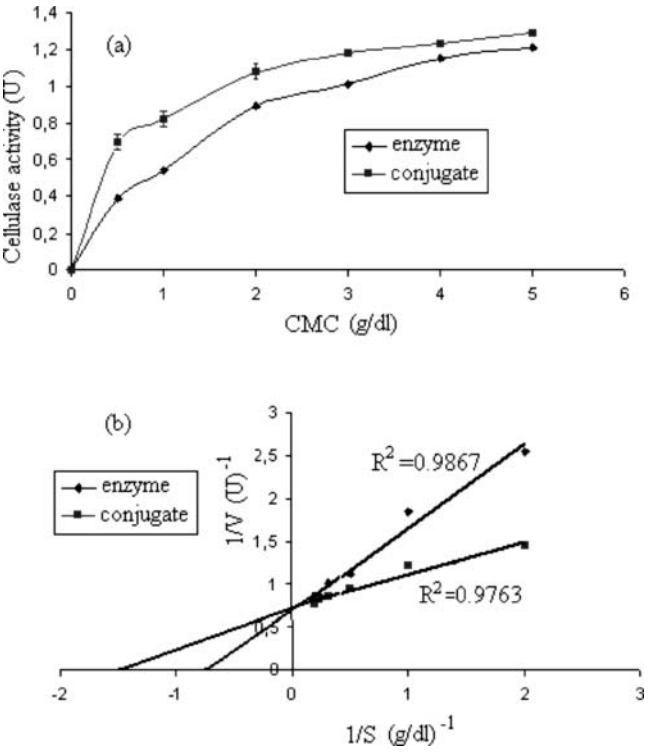

Figure 3: Kinetic assays of the cellulase and conjugate (n_E/n_D: 1/5). (a) Effect of substrate concentration (CMC) on the activities of the cellulase and conjugate (n_E/n_D: 1/5). (b) Double reciprocal plot for determining the V_{max} and K_m values of cellulase and conjugate (n_E/n_D: 1/5).

times at optimal pH and temperature. Error values given in the table are the calculated standard deviations.

To reveal thermal stabilities of conjugates, their activities were determined after 1 h of incubation at different temperatures for pH 5 (Fig. 4). Thermal stabilities of conjugates were influenced by n_E/n_D ratios used in the synthesis reactions. It was observed that conjugates had higher resistance against the

Table 1: Kinetic constants of purified cellulase and conjugate (n_E/n_D: 1/5)

Parameter	Purified cellulase	Cellulase-dextran conjugate
K_m(g dl^{-1})	1.45 ± 0.01	0.70 ± 0.0
V_{max} (μmol min^{-1})	0.674 ± 0.01	0.683 ± 0.01
k_{cat} (s^{-1})	62.4 ± 0.5	63 ± 0.7
K_{cat}/K_m (dl g^{-1} s^{-1})	43.03 ± 0.2	90 ± 1.02

± SD, n = 3.

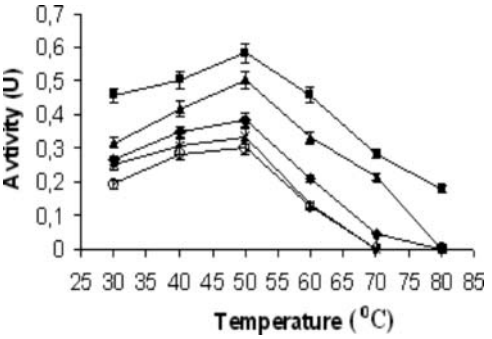

Figure 4: Thermal stability of cellulase and cellulase-dextran conjugates with different molar ratios (n_E/n_D: 1/5, 1/1, 5/1, 10/1) at pH 5; activity determination with 1 h incubation at working temperature. (♦) free enzyme; (■) n_E/n_D: 1/5; (▲) n_E/n_D: 1/1; (*) n_E/n_D: 5/1; (o) n_E/n_D: 10/1. Each data point represents the average value of three independent experiments with error bars indicated.

temperature. Conjugates showed a higher and broader activity/temperature profile than those of the purified enzyme. High resistance against the temperature may be due to formation of thick polymer cover around enzyme molecules. The conjugate n_E/n_D: 1/5 showed the highest thermal resistance and displayed activity also at high temperatures (70°C, 80°C) after 1 h of incubation. From Figure 4, we can also conclude that cellulase-dextran aldehyde conjugate (n_E/n_D: 1/5) showed higher thermal stability than purified cellulase. The results are also consistent with the results of kinetic studies (Table 1).

Figure 5 shows the significant increase in stability of the conjugate; n_E/n_D: 1/5 lost 60% of its activity after 2 h of incubation at 70°C when compared to the purified enzyme, which lost all its activity under the same conditions, whereas conjugate was still stable and showed good activity values for 4 h at 70°C.

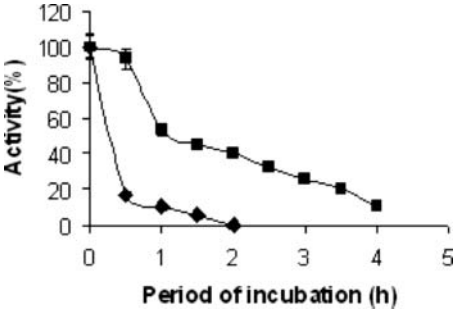

Figure 5: Thermal stability of *A. niger* cellulase at 70°C. The conjugate n_E/n_D : 1/5 and free cellulase were incubated at 70°C for varying times. The residual activity was determined by estimating liberated glucose at 540 nm as described in the materials and methods section. (♦) free enzyme; (■) conjugate n_E/n_D: 1/5. Each data point represents the average value of three independent experiments with error bars indicated.

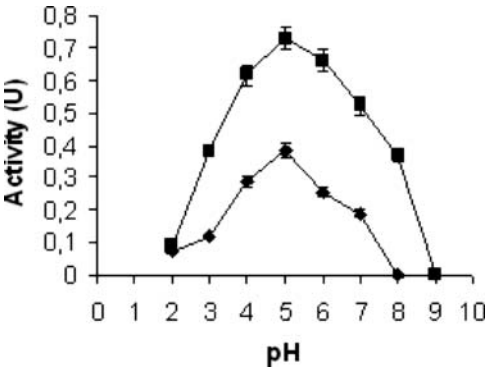

Figure 6: pH stability of purified enzyme and its conjugate with dextran at 50°C; activity determination with 1 h incubation at working temperature. (♦) purified cellulase; (■) cellulase-dextran aldehyde conjugate (n_E/n_D: 1/5). Each data point represents the average value of three independent experiments with error bars indicated.

The advantage of using cellulase at higher temperature has been shown in the case of the Kallar grass (*Laptochloa fusca*) cellulase hydrolysis by *Chaetomium thermophilum*.[34] Thermal stability by conjugated *A. niger* cellulase in our case seems to have reasonably good values for 4 h at 70°C. Similar findings were reported for cross-linking by glutaraldehyde.[29] The thermal stabilization shown by the cellulase-dextran conjugate results from the contribution of several factors on the maintenance of the active conformation of the enzyme. Among these, the most important factor is the conformational stabilization of cellulase molecules due to intramolecular cross-linking caused by the multipoint attachment of one molecule of polymer to several activated sugar residues from the same polypeptide chain.[35,36]

The pH stability profiles of cellulase and conjugate (n_E/n_D: 1/5) are shown in Figure 6. The dextran had a positive stabilization effect on the enzyme

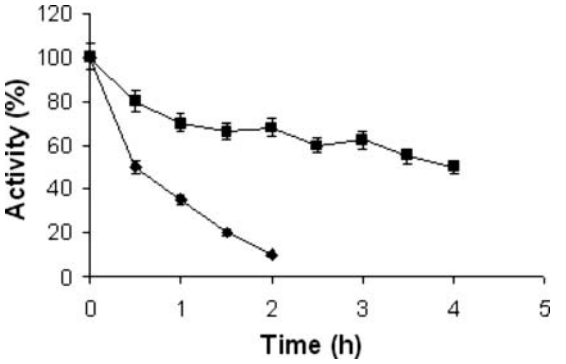

Figure 7: Inactivation course of purified enzyme and conjugate under strong stirring. Experimental conditions were 50°C and pH 5. (♦) purified cellulase; (■) cellulase-dextran aldehyde conjugate (n_E/n_D : 1/5). Each data point represents the average value of three independent experiments with error bars indicated.

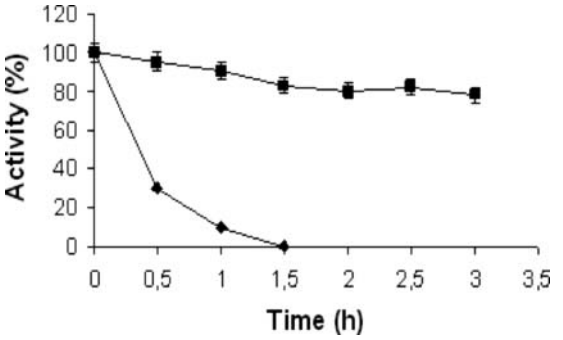

Figure 8: Kinetics of denaturation of purified enzyme and conjugate in the presence of 0.3% (w/v) SDS. Experimental conditions were 50°C and pH 5. (♦) purified cellulase; (■) cellulase-dextran aldehyde conjugate (n_E/n_D: 1/5). Each data point represents the average value of three independent experiments with error bars indicated.

activity at all used pH and especially at pH 8. The activity pH range was shifted through the basic pH. As shown in Figure 6, purified enzyme has quite low activity after 1 h incubation, whereas conjugate had higher resistance against the pH.

Conjugation of cellulase with dextran-aldehyde exerted a well-stabilizing effect against mechanical stirring in an aqueous system (air bubbles). As shown in Figure 7, enzyme-dextran conjugate (n_E/n_D : 1/5) was stable, showing good activity values (residual activity of about 50%) for 4 h, while the purified cellulase was almost fully inactive within the first 2 h. These values are in agreement with the values reported in cases of other enzymes with dextran aldehyde.[5,13]

Figure 8 reports the time course of denaturation of purified and conjugated enzyme in the presence of 0.3% (w/v) SDS at 50°C. As could be seen, purified enzyme was deactivated after 1 h of incubation in the surfactant media, but a residual activity of about 80% was determined for the cellulase-dextran aldehyde conjugate (n_E/n_D: 1/5) after 3 h of incubation under these conditions. Dextran-aldehyde conjugation conferred high stability to the enzyme structure in surfactant media. Whereas SDS disrupts the stabilizing hydrogen bonds in the enzyme,[1] the attached dextran-aldehyde chains maintain the active conformation of the conjugated cellulase, as is reflected by the high residual activity retained by this adduct after 3 h of incubation under these denaturing conditions.

CONCLUSION

In this work, cellulase from *A. niger* was conjugated with dextran aldehyde and obtained conjugates were compared for enzyme stabilization. The results

presented suggest that the covalent attachment of the polysaccharide to glycoenzymes,[35] targeting carbohydrate chains as modification points, might be a useful method for improving cellulase stabilization under various denaturing conditions. In addition, kinetic studies proved that dextran-conjugated cellulase showed greater affinity and catalytic efficiency than purified cellulase. These results are promising and warrant applications of cellulase in the textile industry as well as in detergents.

ACKNOWLEDGMENTS

The authors wish to commemorate this study to beloved professor Huriye Kuzu.

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