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Research Article

IDENTIFICATION OF ACTIVE SITE RESIDUES IN DEXTRANSUCRASE FROM *Weissella cibaria* JAG8

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Abstract: Dextransucrase isolated from *Weissella cibaria* JAG8 was subjected to chemical modification by bifunctional inhibitor *o*-phthalaldehyde to know the involvement of lysine and cysteine residues in its activity. The enzyme lost 97% of its activity in presence of 10 mM *o*-phthalaldehyde. The enzyme inhibitor complex gave absorbance maxima at 334 nm and fluorescence emission maxima at 418 nm, which confirmed the isoindole derivative formation. Sucrose protected the enzyme against *o*-phthalaldehyde inactivation. Denaturation with urea decreased the fluorescence emission showing that the native form of enzyme is essential for isoindole formation. Dextransucrase pre-treated with pyridoxal-5′-phosphate followed by *o*-phthalaldehyde treatment showed an increase in fluorescence intensity at 418 nm after dialysis, when compared with fluorescence intensity before dialysis, indicated that both the inhibitors bind to same lysine residues present at (or) near the active site of enzyme. The results showed that both lysine and cysteine are the key amino acid residues which are present at the active site and are essential for dextransucrase activity.

Keywords: *o*-phthalaldehyde; dextransucrase; *Weissella cibaria* JAG8; isoindole; fluorescence.

Introduction

Dextransucrase (EC 2.4.1.5) is an extracellular enzyme which catalyses the formation of dextran from sucrose (Robyt and Walseth, 1979; Kobayashi and Matsuda, 1980). The enzyme exists in either single or multiple forms (Monsan *et al.*, 1987; Fu and Robyt, 1990; Goyal and Katiyar, 1994). Dextransucrase has been included in the glycoside hydrolase family 70 (Goyal *et al.*, 2007). Dextransucrase from *Weissella* species has recently gained importance for its high yielding dextran with unique properties which can be exploited in food and bakery industry (Schwab *et al.*, 2008). It was reported that dextran produced from *Weissella cibaria* species act as a perfect hydrocolloid with potential application in generation of gluten free cereal food products which can be used as food source for celiac disease patients (Galle *et al.*, 2010; Goggins and Kelleher, 1994).

Only very recently the three-dimensional structures of glycoside hydrolase GH (70) glucansucrases (GSs) have become available. These structures were of the glucosyl transferase (GTF)180-ΔN from *Lactobacillus reuteri* 180 (PDB:3KLK) (Vujicic-Zagar *et al.*, 2010), GTF-SI from *Streptococcus mutans* (PDB: 3AIE; (Ito *et al.*, 2011), and the glucan binding domain ΔN123-GBD-CD2 of the α(1→2) branching GS DSR-E from *Leuconostoc mesenteroides* NRRL B-1299 (PDB: 3TTQ) (Brison *et al.*, 2012). Complexes of GTF180-ΔN with the substrate sucrose (bound in sub sites Δ 1 and +1) and the acceptor maltose (bound in sub sites +1 and +2) confirmed the proposed double-displacement mechanism and revealed the acceptor-binding sub sites and revealed the presence of three domains (A, B and C)

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resembling those of GH13- amylases and two additional domains (IV and V) (Svensson, 1994). Four of the five domains appeared to be built up from discontinuous N- and C-terminal segments of the peptide chain; only domain C is formed from a single stretch of polypeptide. In the catalytic domain (A) of GTF180-ΔN, three acidic amino-acid residues (the nucleophile Asp1025, the general acid/base Glu1063 and the transition-state stabilizer Asp1136) constitute the catalytic machinery; they are present in the GH70 conserved sequence motifs II, III and IV, respectively (Mac-Gregor *et al.*, 1996; Pijning *et al.*, 2008).

Chemical modification studies provide a great deal of information regarding the structural and functional aspects of enzyme. Modification of enzyme with chemical reagents is versatile and rather simple technique for the identification of functional groups in enzyme but also in stability of enzyme. Generally, the modification of enzyme involves binding of chemical moiety to the side chain of amino acid residues leading to change in its property besides binding to functional group belonging to a particular amino acid residue with out affecting other functional groups (or) the conformation of enzyme molecule. One such chemical inhibitor is *o*-phthalaldehyde which is a fluorogenic bifunctional inhibitor used for the identification of lysine and cysteine residues at the active site (Blaner and Churchich, 1979; Placzewski et al., 1983; Mooser *et al.*, 1991; Puri *et al.*, 1985). It was reported that *o*-phthalaldehyde specifically binds to the sulfhydryl group of cysteine and å-amino group of lysine and gives a fluorescent, isoindole derivative (Bhagwar and Krishna, 1986; Chen *et al.*, 1991; Sheikh and Katiyar, 1992; Sheikh and Katiyar, 1993). The formation of isoindole derivative is possible only if the two functional groups are in close proximity (2.6-3.4A^{o}) to each other (Goyal and Katiyar, 1995; Goyal and Katiyar, 1998) thus providing vital information pertaining to distance and orientation of these residues at the active site of enzyme. In the present study dextransucrase isolated from *Weissella cibaria* JAG8 (GenBank accession no KC110687) which exist in single form was subjected to active site mapping analysis by chemical modification. The enzyme was modified with a bifunctional reagent *o*-phthalaldehyde for identification of lysine and cysteine residues.

Materials and Methods

Enzyme preparation and activity assay – Dextransucrase was produced from *Weissella cibaria* JAG8 and purified by fractionation with Polyethylene glycol 400 as reported by Rao and Goyal (2013). The purified enzyme with specific activity of 20 U mg^{-1}, 0.44 mg mL^{-1} protein and molecular weight of 177 kDa, was used for the chemical modification studies unless otherwise stated. One unit (U) of dextransucrase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min under optimized condition (Goyal and Katiyar, 1995). The assay mixture (1.0 mL) contained 5.0% (w/v) substrate sucrose in 20 mM sodium acetate buffer pH 5.4 and the enzyme solution. The reaction mixture was incubated at 35°C for 15 min. The enzyme activity was determined by measuring the amount of reducing sugar released. The reaction mixture (100 µL) was analysed for reducing sugar by method as described by Nelson and Somogyi's method (Nelson, 1944; Somogyi, 1945). The protein concentration was determined by using Lowry's method (Lowry *et al.*, 1951). All the reagents such as *o*-phthalaldehyde, pyridoxal-5′-phosphate (PLP), urea, β-mercaptoethanol, EDTA, cysteine were purchased from Sigma Chem. Co., (USA). For all the inactivation reactions, were carried out in 20 mM sodium acetate buffer pH 5.4 at 35°C unless otherwise stated.

Modification of dextransucrase with o-phthalaldehyde : The enzyme was treated with *o*-phthalaldehyde ranging from 2-10 mM concentration at 35°C. The reaction of enzyme with *o*-phthalaldehyde was stopped at different time intervals by transferring four parts (40 µL) of enzyme incubated with *o*-phthalaldehyde to one part (10 µL) of 100 mM cysteine to stop the reaction. 20 µL of the above mixture was incubated with 5.0% (w/v) sucrose solution in 100 µL reaction mixture for 15 min and the enzyme activity was determined by measuring the amount of reducing sugar released.

UV-Visible absorbance of dextransucrase-o-phthalaldehyde complex : Dextransucrase with 20 U

mg^{-1} and 0.44 mg mL^{-1} was incubated with 10 mM *o*-phthalaldehyde for 1h at 35°C and the absorbance scan was carried out from 200-600 nm. *o*-Phthalaldehyde specifically binds to the sulphydryl group of cysteine and amino group of lysine and results in the formation of isoindole derivative (Goyal and Katiyar, 1995; Goyal and Katiyar, 1998), which can be detected by the absorbance maxima at 334 nm.

Fluorescence spectroscopy analysis of dextransucrase-o-phthalaldehyde complex : The enzyme was inactivated by incubating with 10 mM *o*-phthalaldehyde in 20 mM sodium acetate buffer (pH 5.4) for 1 h at 35°C. The absorbance scan showed absorbance maxima at 334 nm. The fluorescence emission spectrum of dextransucrase-*o*-phthalaldehyde complex was recorded at excitation wavelength 334 nm.

Effect of sucrose and denaturants on o-phthalaldehyde binding with dextransucrase : For conducting protection experiment the enzyme (0.44 mg mL^{-1}, 20 U mg^{-1}) was pre-incubated with 5.0 mM EDTA in 20 mM sodium acetate buffer (pH 5.4) at 35°C for 30 min, followed by treatment with 150 mM sucrose solution for 15 min at 35°C prior to the addition of 10 mM *o*-phthalaldehyde for 60 min and the residual enzyme activity was determined. The assay mixture contained 5.0 mM of Ca^{2+} ions for reactivation of the enzyme. The enzyme pre- incubated with EDTA followed by treatment with Ca^{2+} ions without *o*-phthalaldehyde was used as control. In case of denaturant, the enzyme was treated with 4 M urea at 35°C for 30 min, followed by treatment with 10 mM *o*-phthalaldehyde. A control was used which contained only dextransucrase. All the reactions were carried out using 20 mM sodium acetate buffer (pH 5.4) at 35°C. The fluorescence emission spectra of the above samples were carried out at excitation wavelength 334 nm.

Spectral analysis of o-phthalaladehyde reaction with dextransucrase in presence of β-mercaptoethanol - Dextransucrase was treated with 10 mM *β*-mercaptoethanol for 30 min followed by incubating with 10 mM *o*-phthalaldehyde at 35°C for 30 min. A control was run in the absence of *β*-mercaptoethanol. The fluorescence emission spectra of the above samples were recorded at excitation wavelength of 334 nm.

Effect of pyridoxal-5′-phosphate (PLP) pre-treated dextransucrase on fluorescence emission spectra of dextransucrase reaction with o-phthalaldehyde : The enzyme was incubated with 25 mM PLP for 30 min prior to incubation with 10 mM *o*-phthalaldehyde at 35°C for 1 h. The incubation of enzyme with two inhibitors was monitored by fluorescence emission spectra on excitation at 334 nm before and after dialysis along with control containing only the enzyme. Fluorescence emission spectra were recorded on spectrofluorimeter (Jobin Yvon Horiba; Model Fluoro Max-3). Absorption spectra were recorded on spectrophotometer (Cary-100, Varian). Both the fluorescence and absorbance were recorded in Quartz cuvettes of 1 cm path length.

Results

Modification of Dextransucrase with *o*-Phthalaldehyde

Inactivation of dextransucrase was augmented with increase in concentration of *o*-phthalaldehyde displaying 97.0% loss in enzyme activity at 10 mM in 60 min (Figure 1a). The absorbance maximum of the enzyme-*o*-phthalaldehyde (isoindole derivative) complex (Goyal and Katiyar, 1995) was observed at 334 nm (Figure 1b). This showed that the inactivation of enzyme was due to the reaction of *o*-phthalaldehyde with dextransucrase leading to the formation of isoindole derivative. This was further confirmed by increase in fluorescence emission maxima at 418 nm, when excited at 334 nm (Figure 1c). The fluorescence intensity did not change when recorded even after 24 h. The isoindole ring formation was confirmed in which the sulfhydryl group of cysteine and ε-amino group of lysine are involved. This also showed that two functional groups are 2.6-3.4 °A apart (Goyal and Katiyar, 1998). The presence of cysteine residues was confirmed by using 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) and iodoacetic acid with 98.7% and 98.9% inhibition at 10 mM and 25 mM concentration respectively (data not shown). The thio-nitrobenzoate complex thus formed showed absorbance maxima at 406 nm, where as iodoacetic acid formed thioacetate complex with dextransucrase with absorbance maxima at 323 nm respectively (data not shown).

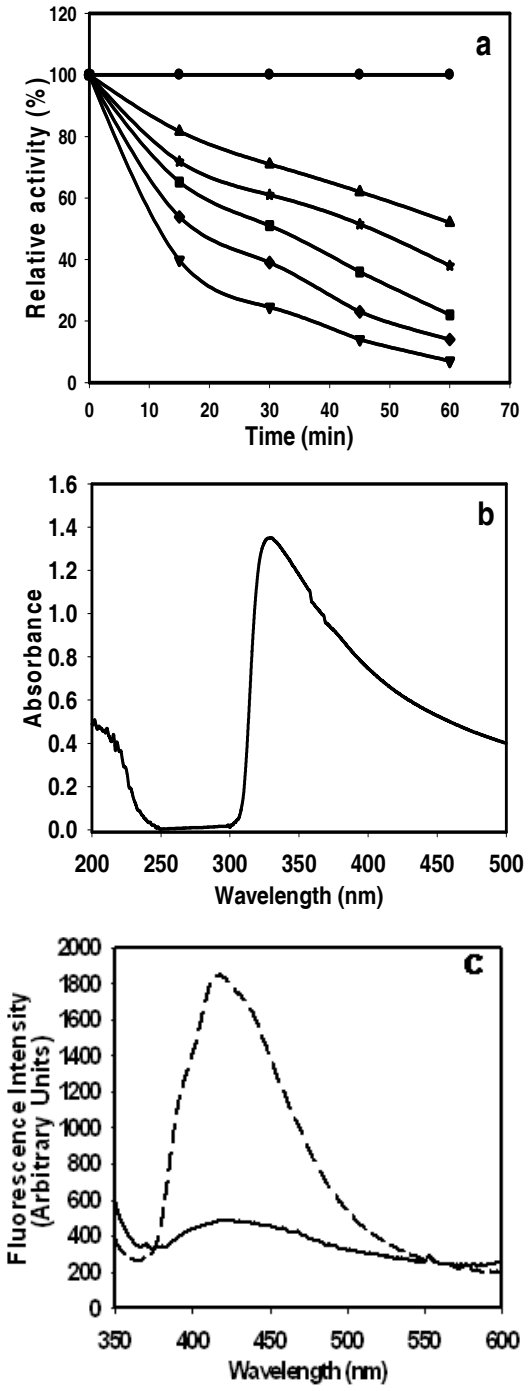

Figure 1: **Effect of *o*-phthalaldehyde on dextransucrase from *W. cibaria* JAG8. (a) Time dependent inactivation of dextransucrase by *o*-phthalaldehyde. The enzyme (0.44 mg mL^{-1}, 20 U mg^{-1}) was incubated with 0 (•), 2 (▲), 4 (*), 6 (■), 8 (♦), 10 mM (▼) *o*-phthalaldehyde at 35°C for 60 min. Aliquots were with drawn at the indicated time intervals and residual enzyme activity was determined, (b) Absorption spectrum of dextransucrase-*o*-phthalaldehyde complex using 10 mM *o*-phthalaldehyde. The resulting isoindole derivative was characterized by absorption spectrum at wavelength of 334 nm, (c) Fluorescence emission spectra of dextransucrase (——) and dextransucrase-o-phthalaldehyde complex (— — —) on excitation at 334 nm**

Effect of Sucrose and Denaturants on Dextransucrase binding with *o*-phthalaldehyde

Dextransucrase on treatment with *o*-phthalaldehyde led to the formation of isoindole derivative which was detected by increase in fluorescence intensity at 418 nm. The enzyme pre-treated with EDTA, followed by incubation with sucrose and then with *o*-phthalaldehyde led to decrease in fluorescence intensity as compared with the control (in absence of sucrose) (Figure 2). Dextransucrase is a metalloenzyme. Ca^{2+} ion has been reported to be associated with the catalytic sites of enzyme enhancing the activity of dextransucrase from *L. mesenteroides* B-512F (Miller and Robyt, 1986). Patel et al., (2011) reported that Ca^{2+} ion stabilize the three dimensional structure of enzyme and significantly enhance the dextransucrase activity. EDTA, being a chelating agent of metal ions, inhibits the activity of enzyme by binding to the Ca^{2+} ions. Addition of Ca^{2+} ions to EDTA inactivated enzyme causes regain of its enzyme activity (Goyal and Katiyar, 1998). Inactivation of dextransucrase with 5.0 mM EDTA at 35°C led to 75% inactivation in 30 min (Table 1). Further increase in EDTA significantly decreased the inactivation of enzyme. The addition of 5.0 mM $CaCl_2$ to the EDTA treated enzyme led to around 46.5% of enzyme reactivation and no further significant increase in the reactivation was observed with increase in the concentration of Ca^{2+} ions (Table 1). The effect of sucrose on dextransucrase inactivation by *o*-phthalaldehyde is shown in Table 1. A concentration of 300 mM sucrose solution has provided maximum protection to enzyme with 94.3% of reactivation and 76.5% in case of 150 mM sucrose concentration. In case of control, the enzyme pre-treated with EDTA caused inactivation, but it still allowed the binding of *o*-phthalaldehyde with free lysine and cysteine residues that led to the formation of isoindole derivative and thus giving fluorescence intensity (Figure 2). Whereas presence of sucrose blocked the inhibitor *o*-phthalaldehyde from binding to the enzyme which resulted in reduced fluorescence intensity (Figure 2). Enzyme treated with urea followed by treatment with *o*-phthalaldehyde led to decrease in fluorescence emission, when compared with

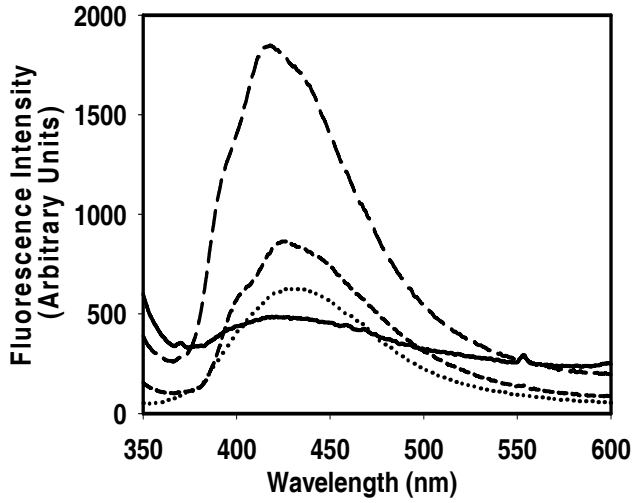

Figure 2: **Effects of sucrose and denaturants on dextransucrase reaction with *o*-phthalaldehyde. The enzyme (0.44 mg mL^{-1}; 20 U mg^{-1}) was pre-incubated with 5.0 mM EDTA for 30 min followed by incubation with 300 mM sucrose for 15 min and finally with 10 mM *o*-phthalaldehyde in 20 mM sodium acetate buffer (pH 5.4) for 30 min at 35°C. The fluorescence spectra of control was run with out sucrose (— — —) and with 300 mM sucrose (· · ·) were recorded with excitation wavelength 334 nm. The fluorescence emission spectra of pure enzyme (——) and enzyme denatured by 4M urea (- - -) followed by *o*-phthalaldehyde treatment were recorded**

Table 1
Effect of Substrate Sucrose on Dextransucrase Inactivation by *o*-phthalaldehyde

*Reagent**	*Relative Activity (%)*
Control	100
Enzyme + EDTA 5 mM (30 min) at 35°C	24.9
EDTA treated Enzyme + $CaCl_2$ 5 mM (60 min) at 35°C	44.5
Enzyme + EDTA 5 mM (30 min) + Sucrose 150 mM (15 min) + $CaCl_2$ 5mM (60 min) + o-phth (10 mM) 60 min at 35°C	76.5
Enzyme + EDTA 5 mM (30 min) + Sucrose 300 mM (15 min) + $CaCl_2$ 5 mM (60 min) + o-phth (10 mM) 60 min at 35°C	94.3

* *Reagents were incubated with dextransucrase (0.44 mg mL^{-1}) for indicated time period followed by incubation with 10 mM o-phthalaldehyde for 60 min at 35°C. Appropriate controls in each case without o-phthalaldehyde were run in parallel.*

control indicating that proximal integrity of lysine and cysteine residues at the active site of native enzyme is essential for isoindole complex formation (Goyal and Katiyar, 1998).

Spectral Analysis of Dextransucrase Modified by *o*-phthalaladehyde in Presence of β-mercaptoethanol

The fluorescence emission maximum of *o*-phthalaladehyde-modified dextransucrase was obtained at 418 nm (λ_{em}) upon excitation at 334 nm. This showed the formation of isoindole derivative, which involves the participation of proximal thiol and ε-amino groups of cysteine and lysine, respectively. The molar transition energy (E_T) was calculated by the following equation (Goyal and Katiyar, 1998).

$$E_T = 2.985\ \lambda_{em} - 1087.28$$

The molar transition energy of dextransucrase and *o*-phthalaladehyde adduct (Figure 3) was found to be 160.4 kJ mol^{-1} which is close to synthetic isoindole in dioxane (157.5 kJ mole^{-1}) indicating that the microenvironment around lysine and cysteine residues is relatively in hydrophobic environment (Goyal and Katiyar, 1998). Fluorescence emission spectra of enzyme modified with *o*-phthalaladehyde in presence of β-mercaptoethanol showed a shift in peak with maximum at 457 nm (λ_{em}) upon excitation at 334 nm (Figure 3). The E_T was found 276.8 kJ mole^{-1}

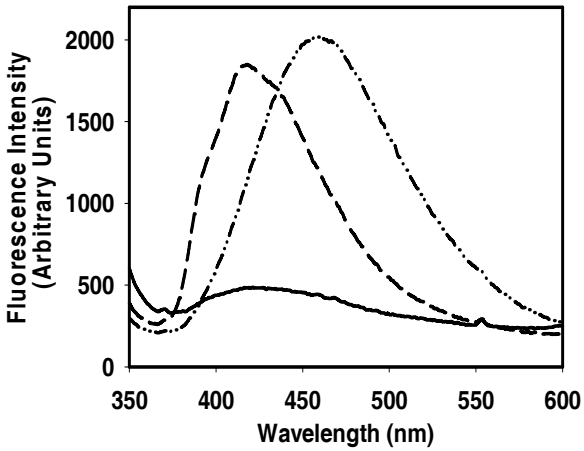

Figure 3: **Fluorescence emission spectra of dextransucrase-*o*-phthaladehyde adduct in the presence and absence of β-mercaptoethanol. The enzyme (0.44 mg mL^{-1}; 20 U mg^{-1}) in 20 mM sodium acetate buffer pH (5.4) was incubated with 10 mM *o*-phthalaldehyde for 30 min at 35°C. In another set of experiment the enzyme was preincubated with 10 mM β-mercaptoethanol followed by treatment with 10 mM *o*-phthaladehyde. The resulting isoindole derivatives were characterized by fluorescence emission spectrum in the presence (— ·· — ·· —) and absence of β-mercaptoethanol (— — —) at excitation wavelength 334 nm. The emission spectrum of pure enzyme (——) was recorded at excitation wave length, 334 nm**

to which is close to isoindole in water (Blaner and Churchich, 1979; Placzewski *et al.*, 1983), indicating that the isoindole derivatives are relatively in hydrophilic environment.

Effect of PLP Pre-treated Dextransucrase on Fluorescence Emission Spectra of Dextransucrase-*o*-phthalaldehyde Adduct

Dextransucrase pre-incubated with 25 mM PLP followed by treatment with *o*-phthalaldehyde before dialysis did not show any fluorescence but after the dialysis there was increase in the fluorescence intensity at 418 nm. PLP inactivates the enzyme reversibly and is removed after dialysis (Goyal and Katiyar, 1995). The control containing the enzyme treated only with *o*-phthalaldehyde, exhibited fluorescence emission maximum at 418 nm (Goyal and Katiyar, 1995), when excited at 334 nm (Figure 4). This showed that PLP and *o*-phthalaldehyde are binding to the same lysine residue.

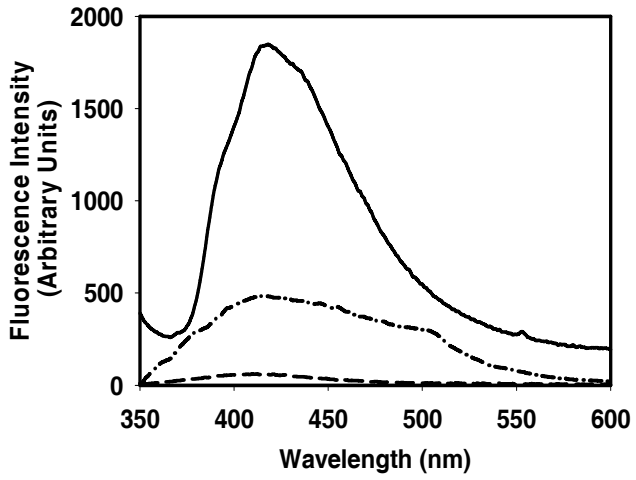

Figure 4: **Effect of PLP pretreatment of dextransucrase on fluorescence emission spectra of dextransucrase-*o*-phthalaldehyde adduct. The enzyme was incubated with 25 mM PLP for 1 h, followed by treatment with 10 mM *o*-phthalaldehyde for 30 min at 35°C. The fluorescence emission spectra were recorded, before dialysis (— — —) and after dialysis (— · — · —). The enzyme treated with 10 mM *o*-phthalaldehyde for 30 min without PLP treatment was used as control (————)**

Discussion

Dextransucrase was strongly inhibited by the bifunctional inhibitor *o*-phthalaldehyde. The inactivation followed pseudo first order kinetics. Inactivation of enzyme involves the isoindole ring formation in which the sulfhydryl group of cysteine and ε-amino group of lysine are involved, which was confirmed by increase in fluorescence emission at 418 nm. Moreover, isoindole formation can occur only when the two functional groups are between 2.6-3.4 A° apart from each other (Chen *et al.*, 1991; Sheikh and Katiyar, 1993; Goyal and Katiyar, 1995). The formation of isoindole derivatives does not depend on the closeness of lysine and cysteine residues in the primary structure, but rather on the specific proximity of these residues in the tertiary structure of enzyme as reported (Blaner and Churchich, 1979; Goyal and Katiyar, 1995). The fluorescence intensity of isoindole complex, formed in presence of *o*-phthalaldehyde with denatured dextransucrase, was low when compared to native enzyme. This indicated that the enzyme should be in its native conformation for the integrity of lysine and cysteine residues at the active site which are involved in isoindole formation. EDTA pre-treated enzyme followed by incubation with sucrose reduced the fluorescence intensity, indicating that active site residues of the enzyme were being protected by sucrose and are not available for binding to *o*-phthalaldehyde (Goyal and Katiyar, 1998), which further confirmed that lysine and cysteine residues are present at the active site.

The molar transition energy of 160.4 kJ mol^{-1} which is close to synthetic isoindole in dioxane (Blaner and Churchich, 1979; Placzewski *et al.*, 1983), indicating that the microenvironment around lysine and cysteine residues is relatively in non polar hydrophobic environment. The hydrophobicity at the active site of dextransucrase indicates that the ε-amino group of lysine remains in deprotonated form to permit reaction with *o*-phthalaldehyde. However, the kinetics of inactivation by *o*-phthalaldehyde in presence of β-mercaptoethanol did not show any significant difference in the fluorescence intensity. This observation confirmed that other lysine residues modified in presence of β-mercaptoethanol do not contribute to loss of activity and thus are not critical for enzyme activity. It is known that inhibition by PLP can be reversed by dialysis. The enzyme on treatment with PLP followed by incubation with *o*-phthalaldehyde did not show any

fluorescence, but after dialysis of the same sample a fluorescence emission maxima at 418 nm was observed with reduced intensity as compared with control. This indicated that both the inhibitors bind to the same lysine residues involved in catalytic activity of dextransucrase. Similar results were reported earlier for dextransucrase from *L. mesenteroides* B-512F by Goyal and Katiyar (1998).

Conclusion

Dextransucrase was strongly inactivated by bifunctional inhibitor *o*-phthalaldehyde. The isoindole derivative formation confirmed that both lysine and cysteine are in close proximity (2.6-3.4 A°) at the active site. Sucrose protected the enzyme against *o*-pthalaldehyde inactivation confirming that both lysine and cysteine residues are present at the active site. Denaturation with urea before treating with *o*-phthalaldehyde resulted in decrease fluorescence indicated that enzyme should be in native conformation for isoindole complex formation. The molar transition energy of isoindole derivative was found to be 160.4 kJ $mole^{-1}$, indicated that microenvironment around the active site containing cysteine and lysine residues are hydrophobic in nature. PLP pre-treated enzyme on incubation with *o*-phthalaldehyde followed by dialysis showed fluorescence maxima at 418 nm confirmed that both PLP and *o*-phthalaldehyde binds to same lysine residues at the active site. The above results clearly indicated that both lysine and cysteine are the key amino acid residues which are present at the active site and are essential for dextransucrase activity.

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Abbreviations

PLP, pyridoxal-5´-phosphate; EDTA, Ethylene-diamine-tetra-aceticacid; *o*-phth, *o*-phthalaldehyde.

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