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ARTICLE

Hydrolytic and chromatographic studies on the PEGylation of dextranase from *Penicillium* sp.

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KEYWORDS

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Abstract Dextranases catalyze the hydrolysis of the α-l,6-glucosidic bond of the polysaccharide dextran. Dextranases have been isolated from bacteria, yeast and fungi. Purified dextranase enzyme from *Penicillium* sp. was PEGylated (polyethylene glycol modification) with mPEG (5000 Da) and showed an increase in the dextranase protein molecular weight as estimated by Superose 12 (23 ml) column and this increment in the molecular weight is directly proportional to mPEG (5000 Da) concentration until a complete dextranase enzyme PEGylation (disappearance of dextranase peak). The residual activity of partially PEGylated dextranase (mPEG 5000 of 5.8 mg/ml) was 33.8% and for the completely PEGylated dextranase (mPEG 5000 of 29 mg/ml) it was 25.75%. Dextranase PEGylated with mPEG (30,000 Da) showed a little PEGylation at mPEG concentration of 5.8 mg/ml but at a concentration of 29 mg/ml several PEGylated peaks were produced with a difference in dextranase activity toward dextran T500, retardation in the activity with the increasing in the molecular weight was clearly appeared with Sephadex G75 but for Sephadex G200 a little retardation than Sephadex G75 has been appeared.

1. Introduction

Dextranase (EC 3.2.1.11 α-D-glucan-6-glucanohydrolase) is an enzyme capable of hydrolyzing the α-1,6-glucosidic bond of dextran producing either glucose or isomaltose (endo-dextranase), or isomalto-oligosaccharides (exo-dextranase) depending on the enzyme source [1]. Dextranase plays an important role in sugar industry by reducing dextran formation, dental plaque treatment and production of low molecular weight clinical dextran [2–4].

PEGylation is a procedure of increasing interest for enhancing the therapeutic and biotechnological potential of peptides and proteins. When polyethylene glycol (PEG) is properly

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linked to a polypeptide, it modifies many of its features while the main biological functions, such as enzymatic activity or receptor recognition, may be maintained. PEG conjugation masks the protein's surface and enlarges the molecular size of the polypeptide, thus reducing its renal ultrafiltration, preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes [5]. PEG has also approved to be used as a vehicle or base in foods, cosmetics and pharmaceuticals, including injectable, rectal topical and nasal formulations. PEG shows little toxicity, and is eliminated from the body intact by either the kidneys (for PEGs < 30 kDa) or in the feces (for PEGs > 20 kDa) [6]. Mumtaz and Bachhawat [7] modified dextranase enzyme with PEG and used liposomes as carrier for delivering both free and PEG-modified dextranase to the liver of mice at similar rate and they found that the PEG-dextranase showed a greater intracellular stability as compared with the native enzyme. The PEG-dextranase could not only degrade the accumulated FITC-dextran but could also prevent further accumulation over a period. The low molecular mass endoglucanase from *Fusarium oxysporium* was chemically modified by two distinct types of amino acid specific modifiers, cyanuric chloride activated polyethylene glycol (CC-PEG) and polyethylene glycol succinimidyl succinate active ester (SS-PEG) specific for lysine attachment and maleimide polyethylene glycol (Mal-PEG) specific for cysteine attachment. Almost total activity loss occurred in the case of enzyme reaction with CC-PEG. In contrast there was no inactivation after enzyme reaction with SS-PEG and Mal-PEG. The modified endoglucanase showed remarkably enhanced stability against alkaline pH. It was also found that the modified enzyme cleaved preferably the internal glucosidic bonds of cellotetraose, cellopentaose and CMC. Thus the modified enzyme retains the endocharacter of the native enzyme [8]. L-asparaginase, an important enzyme used in leukemia treatment, was modified through polyethylene glycol (PEG) conjugation in order to reduce the hypersensitivity reactions caused by the enzyme. The PEGylated enzyme exhibited less immunogenicity with much longer half-time of plasmatic life [9]. This research is undertaken with the objective of studying the dextranase PEGylation with specific reference to its chromatographic and the hydrolytic properties.

2. Materials and methods

2.1. Materials and equipments

Purified dextranase enzyme from *Penicillium* sp. was used as the PEGylated substrate. mPEG-SPA (Mwt. of 5000 and 30,000 Da) was purchased from Nektar™. Superose 12 HR10/30 column (Pharmacia Biotech.); Superose 6 HR10/30 column; Superose 12 prep. grade column (100 ml); Dextran 5 of average Mwt., 5150; Dextran 10 of average Mwt.,10450, Dextran 20 of average Mwt., 21450; Dextran 70 of average Mwt., 70300; Dextran 150 average Mwt.,147550; Dextran T250 of average Mwt., 238200 and dextran T500 were Pharmacia biotechnology products. Amicon Ultrafiltration cell using Amicon Millipore membrane of 10 pk was also used to concentrate enzyme samples. All chromatographic processes were carried on ÄKTA FPLC (Amershampharmacia Biotech.).

2.2. Dextranase assay and protein measurement

Dextranase enzyme activity was measured by incubating the enzyme with buffered dextran solution (2% dextran T250 dissolved in 0.05 M acetate buffer pH5) for 10 min at 55 °C [10,11]. The activity was measured by detecting the produced reducing sugar using DNSA (3,5- dinitrosalicylic acid) according to Miller [12]. One enzyme unit is defined as the amount of enzyme which liberates 1 μ mole of reducing sugar (glucose equivalent) per min at standard conditions. Protein had been measured according to Bradford [13].

2.3. PEGylation technique

The PEGylation procedure was done by mixing 0.5 ml of dextranase (2 mg/ml) in 20 mM Tris buffer (pH 8) with 0.5 ml of mPEG in an Eppendorff tube at room temperature for 10, 20, 30, 60 and 180 min. Two hundred microliter of the reaction mixture were added to 50 μl of 0.2 M Bis–Tris buffer (pH 5), to lower the pH to stop the reaction, and then 200 μl of this mixture was applied to Superose 12 HR10/30 column and the column was equilibrated with 20 mM Bis–Tris buffer (pH 5) containing 0.1 M NaCl. This experiment was done for mPEG 5000 and 30,000 Da with different concentrations (from 5.8 up to 116 mg/ml).

The pH adjustment of dextranase enzyme to 8 was done using 20 mM Tris buffer pH 8 by exchanging the buffer solution using concentration with Amicon Ultrafiltration cell and dilution using Tris buffer several times to a final product of 2 mg/ml dextranase in Tris buffer (pH 8). mPEG was dissolved in the same buffer.

2.4. Molecular weight estimation

Molecular weight determination was carried out using SDS/PAGE Phastgel gradient 8-25 electrophoresis using low molecular weight protein kits to detect the change in the molecular weight of dextranase after PEGylation.

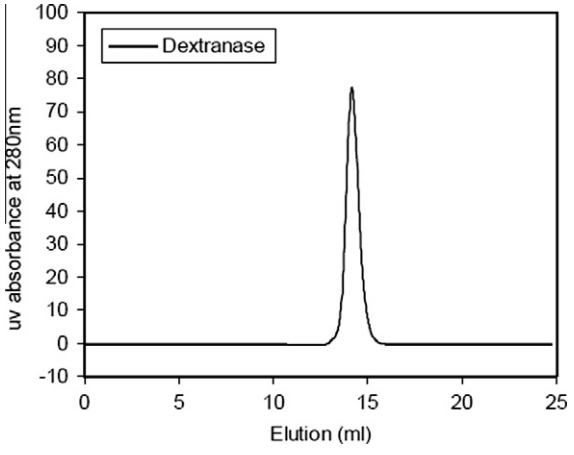

Figure 1 Size exclusion chromatography on Superose 12. A sample of 200 μL of a previously purified dextranase (0.45 mg/ml protein) was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm.

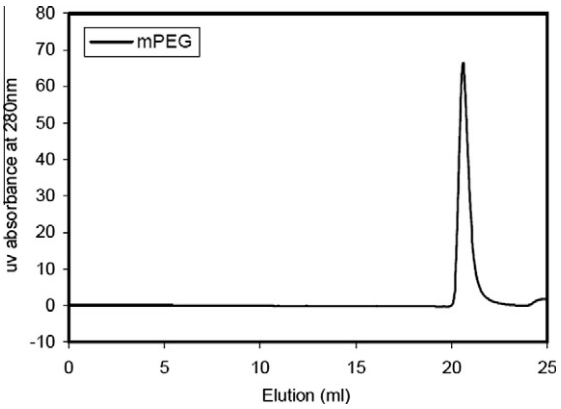

Figure 2 Size exclusion chromatography on Superose 12. A sample of 200 µL of mPEG 5000 (11.6 mg/ml) was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm.

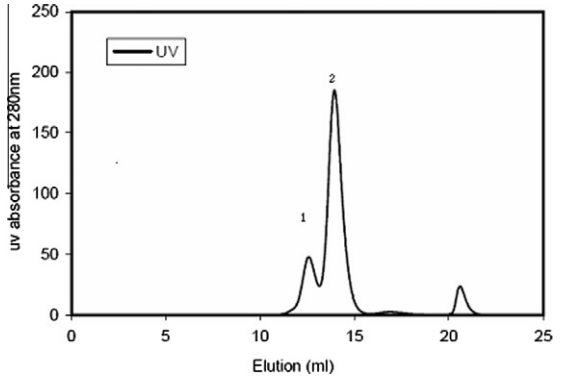

Figure 3 Size exclusion chromatography on Superose 12. A sample of 200 µL of a reaction mixture of dextranase (2 mg/ml) with mPEG 5000 (11.6 mg/ml) for 10 min was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm. The same chromatogram has been seen with 20, 30, 60 and 180 min.

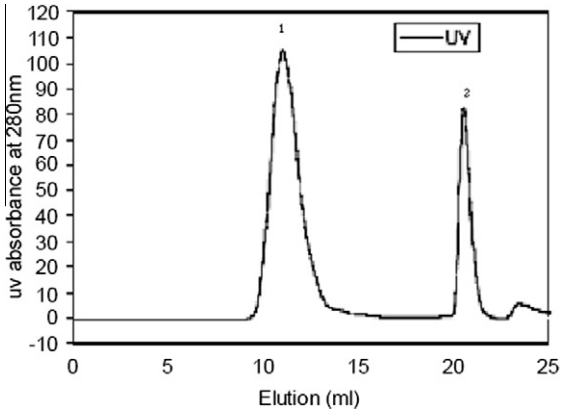

Figure 4 Size exclusion chromatography on Superose 12. A sample of 200 µL of a reaction mixture of dextranase (2 mg/ml) with mPEG 5000 (58 mg/ml) for 10 min was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm.The same results were found with incubation periods of 20, 30, 60 and 810 min.

3. Results

Pure dextranase enzyme in a concentration of 0.45 mg/ml (200 µl) was loaded to a Superose 12 HR 10/30 with a total volume of about 23 ml. The column was equilibrated with 20 mM Bis–Tris buffer (pH 5) containing 0.1 M NaCl. A single peak was produced (Fig. 1). Two hundred microliter of a solution of 11.6 mg/ml of mPEG (5000 Da) was also loaded to the same column under the same conditions and a single peak was produced with an elution volume (21 ml) more than the pure dextranase enzyme (Fig. 2). Moreover, dextranase enzyme in a concentration of 2 mg/ml (in 20 mM Tris buffer, pH8) was mixed with 11.6 mg/ml of mPEG (dissolved in Tris buffer, pH8) for different incubation periods, 10, 20, 30, 60 and

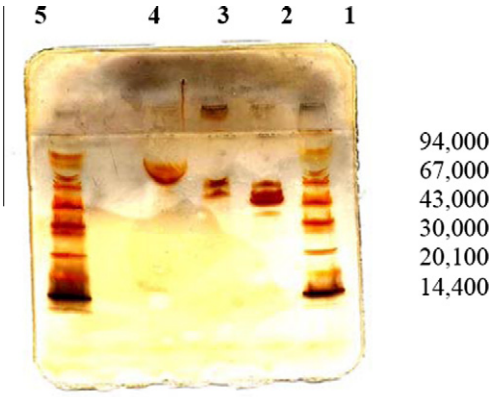

Figure 5 SDS/PAGE of samples from PEGylated of dextranase. Lane 1&5, LMW caliberation kit proteins (Amersham Pharmacia Biotech.); Lane 2, the residual dextranase after PEGylation with mPEG 5000 (5.8 mg/ml); Lane 3, the PEGylated dextranase by mPEG 5000 (5.8 mg/ml); lane 4, the PEGylated dextranase by mPEG 5000 (29 mg/ml). Protein stained with silver nitrate.

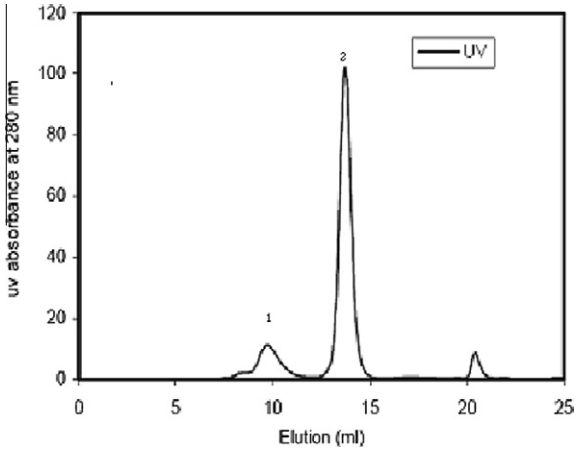

Figure 6 Size exclusion chromatography on Superose 12. A sample of 200 µL of a reaction mixture of dextranase (2 mg/ml) with mPEG 30,000 (11.6 mg/ml) for 10 min was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm. The same chromatogram has been produced with different incubation periods (20, 30, 60 and 180 min.

Table 1 Determination of dextranase activity and specific activity for different peggulated dextranase with different concentration of mPEG (5000).

Sample	Activity (U/ml)	Specific activity (U/mg protein)	Residual activity (%)
Original dextranase	4884.82	18787.77	100
PEGylated with 5.8 mg/ml mPEG 5000	1651.40	6351.53	33.81
PEGylated with 29 mg/ml mPEG 5000	1248.95	4803.96	25.57

Table 2 Effect of PEGylation (11.6 mg/ml mPEG 30,000 Da) on dextranase activity upon different substrates.

Substrate	Activity (U/ml)	Specific activity (U/mg prptein)	% to each control
Enzyme + T500 (control)	4884.82	18787.77	100
PEGylated enzyme + T500	1074.16	6767.19	36
Enzyme + Sephadex G200 (control)	2123.32	13376.89	100
PEGylated enzyme + SephadexG200	815.99	5197.39	38.85
Enzyme + Sephadex G75 (control)	1079.55	6801.17	100
PEGylated enzyme + Sephadex G75	355.81	2266.31	33.32

180 min, and it was found that the PEGylation has been occurred partially (three peaks with 12.5, 14.5 and 21 elution volume corresponding to mPEG-dextranase, free dextranase and free mPEG, respectively) without any change with different incubation periods (Fig. 3).

Results in Fig. 4 showed a complete PEGylation that occurred when a solution of 2 mg/ml of dextranase was mixed with equal volume of solution of 58 mg/ml of mPEG 5000 Da (both the enzyme and the mPEG were dissolved in Tris buffer, pH 8). The complete PEGylation was not affected by the time of incubation. The dextranase activity of unPEGylated, partially PEGylated and complete PEGylated peaks has been measured in terms of specific activity and residual activity (%) in relation to the original dextranase enzyme that also measured. It has been found that the unPEGylated dextranase peak showed a residual activity of 100% and the partially PEGylated dextranase peak (5.8 mg/ml mPEG 5000 Da) showed a residual activity of 33.81 but the completely PEGylated peak (29 mg/ml mPEG 5000 Da) showed a residual dextranase activity of 25.6% (Table 1). The molecular weight of these three peaks have been estimated by SDS/PAGE electrophoresis using Phast gel gradient 8-25 and compared to LMW proteins kit and it has been found that the unPEGylated dextranase peak had the same molecular weight of the original enzyme, but the partially PEGylated and the completely PEGylated had higher molecular weights in the order of partially and completely PEGylated dextranase (Fig. 5).

On the other hand, high molecular weight mPEG (30,000 Da) had also been used to PEGylate dextranase enzyme. mPEG in concentration of 11.6 mg/ml was mixed with dextranase enzyme of a concentration of 2 mg/ml for different incubation periods (10, 20, 30, 60 and 180 min). It was found that a very little portion of the enzyme has been PEGylated and with prolonged incubation time, no further effect on the degree of PEGylation had been observed (Fig. 6). Dextranase activity was measured for the PEGylated part with dextran T500, Sepadex G75 and Sephadex G200. It was found that the PEGylated dextranase retained a residual activity of 36%, 12% and 27% for dextran T500, Sephadex G75 and Sephadex G200, respectively (Table 2).

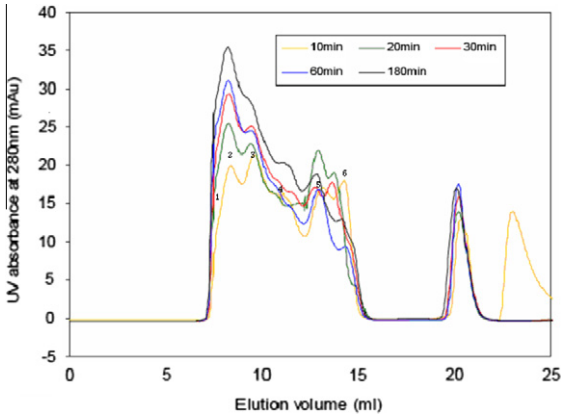

Figure 7 Size exclusion chromatography on Superose 12. A sample of 200 μL of a reaction mixture of dextranase (2 mg/ml) with mPEG 30,000 (29 mg/ml) for 10, 20, 30, 60 and 180 min separately was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm.

Table 3 Effect of different dextrans of different molecular weights on the activity of PEGylated dextranase (29 mg/ml mPEG 30,000) for 10 min using the same amount of protein in relation to the UV absorbance at 280 nm.

Dextran (MWt.)	Peak activity (U/ml)				
	2	3	4	5	6
5000	42.74	59.81	57.59	46.77	60.64
10,000	60.37	80.35	90.34	83.26	115.74
20,000	93.87	122.12	106.16	108.65	142.38
70,000	88.26	108.79	137.80	111.71	150.71
150,000	82.43	111.71	125.86	117.26	150.29
250,000	88.95	122.12	141.27	124.34	162.36

With the use of mPEG (30,000 Da) in a concentration of 29 mg/ml to PEGylate dextranase enzyme having a concentration of (2 mg/ml), the enzyme showed a PEGylation with dif-

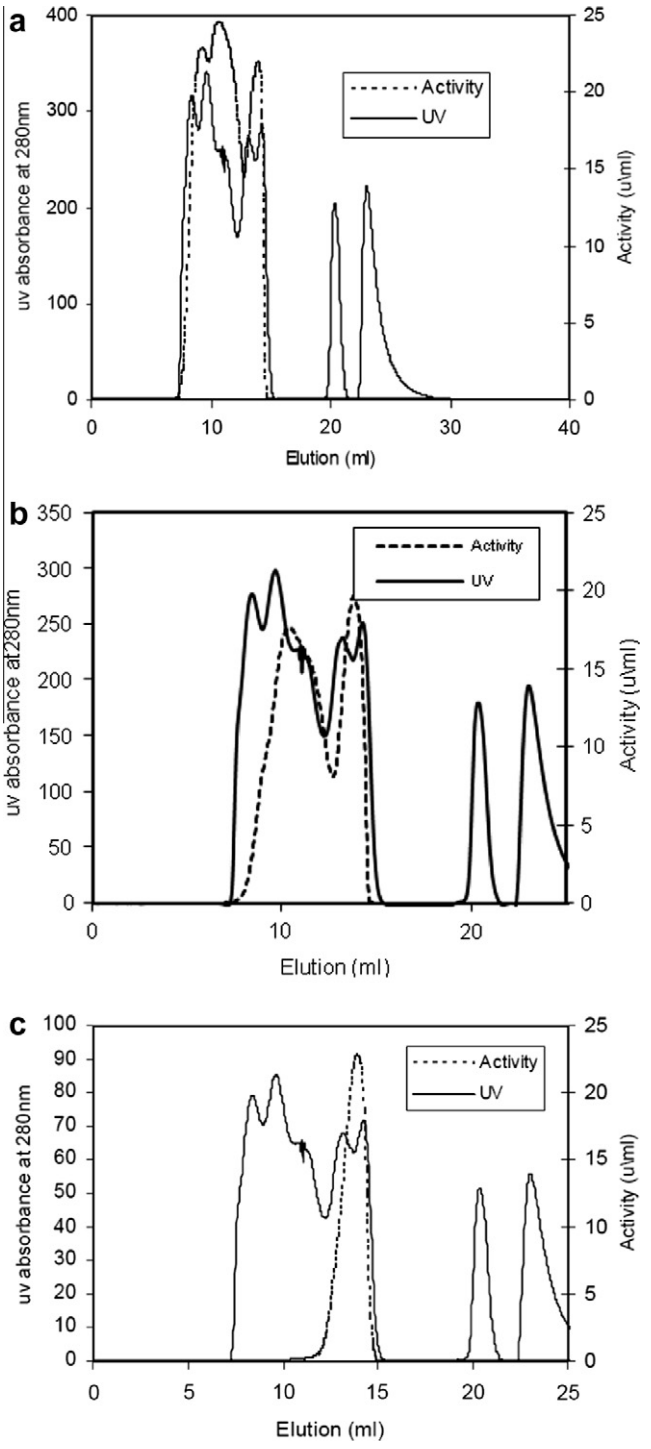

Figure 8 (a, b and c): Size exclusion chromatography on Superose 12. A sample of 200 µL of a reaction mixture of dextranase (2 mg/ml) with mPEG 30,000 (58 mg/ml) for 10 min was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm. The dotted line showed the dextranase activity when dextan T500 (a), Sephadex G200 (b) and Sephadex G75 (c) were used as a substrate, respectively.

ferent degrees. Fig. 7 showed the different PEGylated peaks (from Superose 12 column) produced from dextranase with mPEG 30,000 (29 mg/ml). The dextranase activity with the

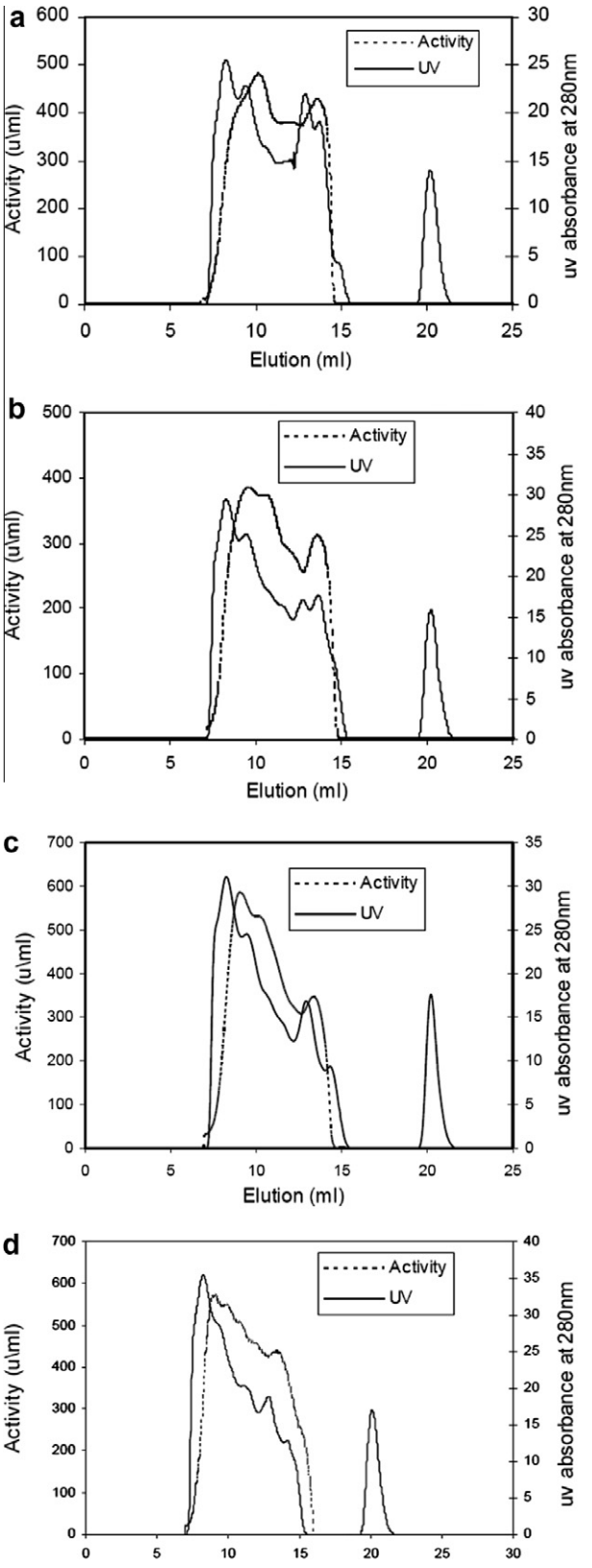

Figure 9 (a, b, c and d): Size exclusion chromatography on Superose 12. A sample of 200 µL of a reaction mixture of dextranase (2 mg/ml) with mPEG 30,000 (58 mg/ml) for 20, 30, 60 & 180 min, respectively, Linear flow rate 1 ml/min. UV absorbance at 280 nm. The dotted line showed the dextranase activity when Dextran T500 was used as a substrate.

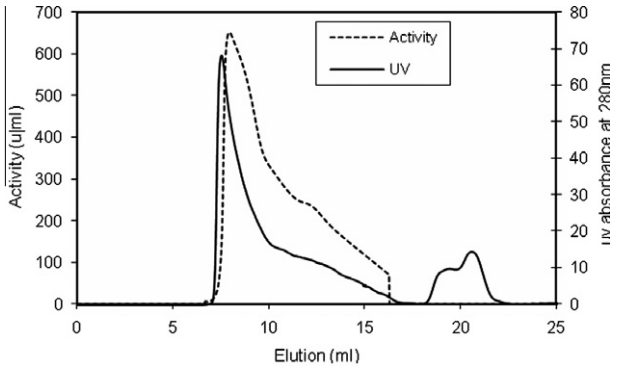

Figure 10 Size exclusion chromatography on Superose 12. A sample of 200 μL of a reaction mixture of dextranase (2 mg/ml) with mPEG 30,000 (116 mg/ml) for 30 min was loaded to Superose 12 FPLC column (~23 ml). Linear flow rate 1 ml/min. UV absorbance at 280 nm. The dotted line showed the dextranase activity when Dextran T500 was used as a substrate.

use of different dextrans with different molecular weights (dextran 5, 10, 20, 70, 150 and 250 kDa) has been investigated for the different produced peaks and it was found that the activity decreased with the increase of the molecular weight and peak 1 (fraction 1, 0.5 ml) showed no activity with all the dextrans used (Table 3).

A comparison of the activity of dextranase between dextran 500, Sephdex G75 and Sephadex G200 was done and it was found that with the exception of fraction no. 1 (first 0.5 ml) a dextranase activity was found with dextran 500 and in case of Sephadex G200 the first two fractions were found to have no activity and there was an activity with the others but in case of Sephadex G75 no activity has been found up to fraction no 8 (Fig. 8a, b and c). Fig. 9a, b, c and d represent the PEGylation of dextranase (2 mg/ml) with mPEG 30,000 Da for different incubation periods (20, 30, 60 and 180 min), respectively. Also, the dextranase activity was plotted for each PEGylation curve. On the other hand, Fig. 10 showed mPEG 30,000 Da of a concentration of 116 mg/ml incubated with dextranase for 30 min.

4. Discussion

Polyethylene glycol (PEG) is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnological applications [14]. Compared to native dextranase, an average increase in its molecular weight has been observed using gel filtration columns (Sephadex) and SDS/PAGE studies. These results are approximately equivalent to that investigated by Christakopolous et al. [8] when they studied the PEGylation of endoglucanase from *Fusarium oxysporium*.

The PEGylation progress for dextranase was also estimated using SDS/PAGE technique and it was found that the PEGylation increased with increasing the mPEG concentration and the period of incubation. Diwan and Park [15] studied the SDS/PAGE of lys-mPEG conjugate and the results gave a mixture of different lysosome-mPEG conjugates and their results with SE-HPLC showed the mono-, di-, and tri-PEGylated derivatives of lyzosome. Weider et al. [16] studied the electrophoresis of partially purified preparation of O-phenylealanine ammonia lyase using SDS/PAGE. They investigated that the PEGylated enzyme results in great decrease in electrophoretic mobility and formes a single band at the top of the gel. Fee [17] found a shift in the peak areas with time, showing a reduction in the native protein peak of α-lactalbumin and β-lactoglobulin when both PEGylated and examined by gel-filtration.

By increasing the degree of dextranase PEGylation the activity showed a considerable decrease and the stability increased. The cellulase complex from *Trichoderma reesei* was polyethylene glycol (PEG)-modified with considerable retention of endo-beta-1,4-glucanase activity, as evaluated by the carboxymethylcellulase (CMCase) assay. While resistance towards heat denaturation was the same for either form, susceptibility towards proteolysis was slightly greater for the PEG-conjugate, in contrast to most reports using other enzymes [18]. The loss of considerable activity following modification may be due to reaction of the coupling agent with essential sulfhydryl groups [19]. Moreover, Freitas et al. [20] investigated that uricase enzyme retained 87% and 75% of its activity when PEGylated with mPEG-pNP and mPEG-CN, respectively.

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