STUDY OF IMMOBILIZATION OF PROTEASE AND SORPTION OF BSA ON CELLULOSE, CELLULOSE DERIVATIVES, AND GRAFT COPOLYMERS

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In order to develop novel polymeric supports based on cellulosics, cellulose extracted from pine needles, a perennial resource material available in huge quantities as waste, was graft copolymerized with vinyl monomers. Cellulose, cellulose derivatives, and their graft copolymers with highest percent grafting (P_g) were used as supports for immobilization of an industrially important protease enzyme and the protein bovine serum albumin (BSA) by a specific sorption method. The *Manachini method* was used to determine activity of the immobilized enzyme. Sorption of protein was characterized by activity of protein concentration by the *Lowry method*. Cellulose itself was found to be effective as a polymeric support to retain a good amount of protease and BSA, whereas cellulose derivatives were effective to adsorb BSA only. Among cellulose graft copolymers, those based on methyl methacrylate proved to be better sorbents.

Keywords: Graft copolymers; Sorption; Immobilized enzyme; Sorbents

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INTRODUCTION

Polymer supports are finding increased use in protein sorption, separation, and enrichment technologies. Despite their high potential for successful use, the high cost, restricted availability, difficulty in recovery from reaction mixtures, and above all, the fragile nature of the enzymes restricts their use (Kennedy et al. 1985; Kierstan et al. 1985; Trevan 1980). Immobilization is a means by which enzymes and cells are transferred into heterogeneous catalysts where they are confined to a restricted region and can be repeatedly used without contamination (Rosevear et al. 1988; Hayashi et al. 1990). In addition, the reaction product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries). Furthermore, the immobilized enzyme has a longer half-life and predictable decay rate. They are more stable at higher temperature (Tanksale et al. 2001) and are active over a wide pH range (Reshmi et al. 1991). Therefore, immobilized enzymes are getting special attention in several areas of modern biotechnology, including in the chemical industry, pharmaceuticals, cheese making, food industry, clinical chemistry, alcohol production, waste water treatment, and in agriculture (Bu'lock et al. 1987). Immobilized proteases are especially employed in specific applications of cheese making, milk clotting, wine making, peptide synthesis, many biochemical and clinical procedures, and in biochemical processes, including esterifica-

tion and transesterification (Taylor et al. 1977; Kise et al. 1990, 1991; Garg et al. 1993). Sorption of an enzyme on a carrier molecule is the simplest approach and involves interaction between the outer surface of carrier and the biocatalyst. However, the enzyme is prone to physical abrasion, exposed to microbial attack, and has a relatively low surface area available for sorption. Cellulose acetate, cellulose butyrate, ethyl cellulose (Gil et al. 1996) and poly(hydroxyethyl-acrylate)-co-cellulose (Beddows et al. 1984) can be given as examples of cellulose derivatives that were used for the immobilization of urease and trypsin, respectively. Trypsin was the model proteolytic enzyme chosen for the immobilization, because it is highly efficient and has been described as specific for catalyzing the breakdown of peptide linkages (Kunitz et al. 1936). Besides, this enzyme has been successfully used, when immobilized on a synthetic polymer, to cure inflammation in medical treatment (Watanabe et al. 1988).

This paper considers immobilization of protease enzyme and protein bovine serum albumin (BSA) on some candidate polymers based on cellulosics. Cellulose and its derivatives can be expected to be very suitable supports for proteins and enzyme due to their hydrophilicity and the fact that they can offer a friendly environment for enzymes, as well as offering a support upon which interactions can take place between biomolecules, including enzymes and proteins. Enzyme and protein sorption has been characterized by measuring activity of the enzyme and protein concentration.

EXPERIMENTAL

Materials

Cellulose was extracted from pine needles by ammonia digestion by an earlier reported method (Chauhan et al. 2002). It was graft copolymerized with vinyl monomers, methyl methacrylate (GMA) (Chauhan et al. 2005a), glycidyl methacrylate (MMA) (Chauhan et al. 2005b), and 2-hydroxyethyl methacrylate (HEMA) (Sharma et al. 2009) by initiation with benzoyl peroxide (BPO). Under the optimum reaction conditions evaluated for the grafting of each vinyl monomer alone, comonomers (CM) as acrylamide (AAm), acrylic acid (AAc), and acrylonitrile (AN) at five different concentrations were also co-grafted along with vinyl monomer onto cellulose, and results have been discussed elsewhere (Chauhan et al. 2005a, 2005b and Sharma et al. 2009). The graft copolymers yielding the highest percent grafting (P_g) in the cited studies were used as supports for immobilization of an industrially important enzyme, protease, as well as for sorption of BSA.

Cellulose phosphate

Cellulose phosphate was prepared (Guthrie 1971) as an ammonium salt by the reaction of cellulose with phosphoric acid and urea at elevated temperature (130° to 150°C). Cellulose in a mixture of 50% urea, 18% H_3PO_4, and 32% water was heated at 150° to 175°C for 1.0h. Filtrate content was washed with distilled water, and the resultant component was labeled as cellulose phosphate.

Oxycellulose

Permanganate-oxidized cellulose was prepared by an earlier reported method (Swenson 1967). Cellulose (10.0 g) was stirred with 300.0 mL water. An acidified solution of $KMnO_4$ was prepared by dissolving $KMnO_4$ (4.0 g) in 8% H_2SO_4 (50.0 mL). This was added to cellulose slurry drop-wise from a separating funnel over a period of 2h. The mixture was allowed to stand over night until the permanganate colour disappeared. The product was filtered and suspended in 2% H_2SO_4. Colour of the permanganate was removed from cellulose by addition of small amount of H_2O_2, and product was then filtered and washed with distilled water until free from acid and manganese sulphate.

Methods

Cellulose, cellulose derivatives, and their graft copolymers with highest percent grafting (P_g) were used as supports for protease immobilization.

Protease assay materials

The protease assay was conducted by using the Manachini method (Manachini et al. 1988). The following reagents were used

i) Casein solution: 0.5% (W/V) of Casein (Hammartein) dissolved in 50 mM
ii) Tris-HCl buffer (*p*H = 8.0)
iii) 5% (W/V) of Trischloroacetic Acid (TCA)
iv) Standard: Tyrosine 10-100 μgm/mL

Procedure

Known weights of cellulose, cellulose graft copolymers, and cellulose derivatives (50.0 mg) were immersed in 1.0 mL of protease enzyme (50μL) for 24 h. After 24 h, matrices were filtered and washed with Tris-HCl. These matrices were dipped in 4.0 mL of Casein solution. The reaction mixture was incubated at 55°C for 10 min. The reaction was stopped by addition of 5.0 mL of 5% TCA. It was again vortexed and allowed to stand for 20 min. The contents were filtered through Whatman No. 1 filter paper, and the absorbance of the supernatant was measured at 275*nm* (taken on UV-Vis Spectro-photometer model Thermo Nicolet Evolution 300). Percent enzyme activity was calculated from a standard curve prepared by the same procedure using Tyrosine 10-100 μgm/mL (Fig. 1).

Protein assay

A protein assay was carried out by the Lowry method (Lowry et al. 1951). The following reagents were used:

i) 1% $CuSO_4$
ii) 2% Sodium Potassium Tartarate
iii) 2% Sodium Carbonate in 0.1N NaOH.
iv) Lowry Alkaline Reagent [mixture of 1mL of (i) and 1mL of (ii) + 98mL of (iii)].
v) Folin-Ciocalteau's Phenol reagent (1:1 with distilled water).
vi) Standard: Bovine Serum Albumin (BSA) 10-100 μgm/mL.

Procedure

Samples of cellulose, cellulose graft copolymer, and cellulose derivatives (50.0 mg) were taken and dipped in 1.0 mL of BSA (100 μgm/mL) for 24 hrs. The samples were filtered after 24 hrs. and washed with 5.0 mL of distilled water. In filtered matrix, 3.0 mL of Lowry alkaline reagent was added. After mixing thoroughly on a vortex mixer it was allowed to stand for 15 min. at room temperature. To this was added 0.3 mL of Folin-Ciocalteau's phenol reagent, and the contents were vortexed and allowed to stand for 30 min. for maximum colour development. Optical density was measured at 670*nm* (taken on UV-Vis Spectrophotometer model Thermo Nicolet Evolution 300) against a reagent blank. The concentration of protein in the samples was calculated from a standard curve prepared by the same procedure using BSA from 10-100 μgm/mL (Fig. 2).

RESULTS AND DISCUSSION

Sorption of Protease

Results of protease immobilization are presented in Table 1. Figure 1 shows a calibration curve for tyrosine.

Table 1. Immobilization of Protease by Cellulose, Cellulose Graft Copolymers, and Cellulose Derivatives

Sr. No.	Graft Copolymer	Change in OD	% Relative Enzyme Activity
1.	**Cellulose**	**0.814**	**65.4**
2.	Cell-*g*-poly(GMA)	0.600	48.2
3.	Cell-*g*-poly(GMA-*co*-AAc)	0.130	10.4
4.	Cell-*g*-poly(GMA-*co*-AAm)	0.213	17.1
5.	Cell-*g*-poly(GMA-*co*-AN)	0.109	8.8
6.	**Cell-*g*-poly(MMA)**	**0.632**	**50.8**
7.	Cell-*g*-poly(MMA-*co*-AAc)	--	--
8.	Cell-*g*-poly(MMA-*co*-AAm)	0.472	37.9
9.	**Cell-*g*-poly(MMA-*co*-AN)**	**0.732**	**58.8**
10.	Cell-*g*-poly(HEMA)	0.242	19.4
11.	Cell-*g*-poly(HEMA-*co*-AAc)	0.366	29.4
12.	Cell-*g*-poly(HEMA-*co*-AAm)	0.201	16.1
13.	Cell-*g*-poly(HEMA-*co*-AN)	0.169	13.6
14.	Cellulose phosphate	0.076	6.1
15.	Oxycellulose	0.054	4.3
16.	Test	1.245	100

As previously mentioned, cellulose offers a large hydrophilic surface area despite its insolubility in common solvents. The presence of hydroxyl groups on the anhydroglucose unit assists in complexation both with small and large molecular weight molecules. Further, the low cost, renewable, and non-toxic nature makes it a suitable candidate as the structural foundation for supports for protein enrichment and separation technologies. Grafting improves some of its molecular interaction parameters such as surfactant sorption and also improves stability, hence, has potential to improve cellulose-based materials in their role as supports.

However, in the present study it has been observed that cellulose itself is effective as a polymeric support to retain a good amount of protease (65.4%). Use of cellulose derivatives such as cellulose phosphate and oxycellulose has been reported in protein separation, but in the present study a very high selectivity towards protease was observed, as very low amounts were adsorbed on such substrates.

It is understandable that the activity of enzyme was high in cellulose graft copolymer of all three methacrylates and followed the order: cell-*g*-poly(MMA) > cell-*g*-poly(GMA) > cell-*g*-poly(HEMA). This was observed despite the fact that HEMA and GMA have additional groups like -OH and epoxy that are known *anchors* for molecular sorption due their activity. Cell-*g*-poly(GMA) showed activity very near to that of Cell-*g*-poly(MMA), yet it was less than that of cellulose itself. On the other hand, the effect of comonomer grafting from a binary system affording supports like cell-*g*-poly(MMA-*co*-CM), it is not of much use in the immobilization of enzyme, as indicated by the lowered activity of immobilized enzyme in almost all cases. Only immobilization onto binary graft copolymer cell-*g*-poly(MMA-*co*-AN) was somewhat encouraging. This is perhaps due to the hydrophobicity introduced by the incorporation of poly(AN).

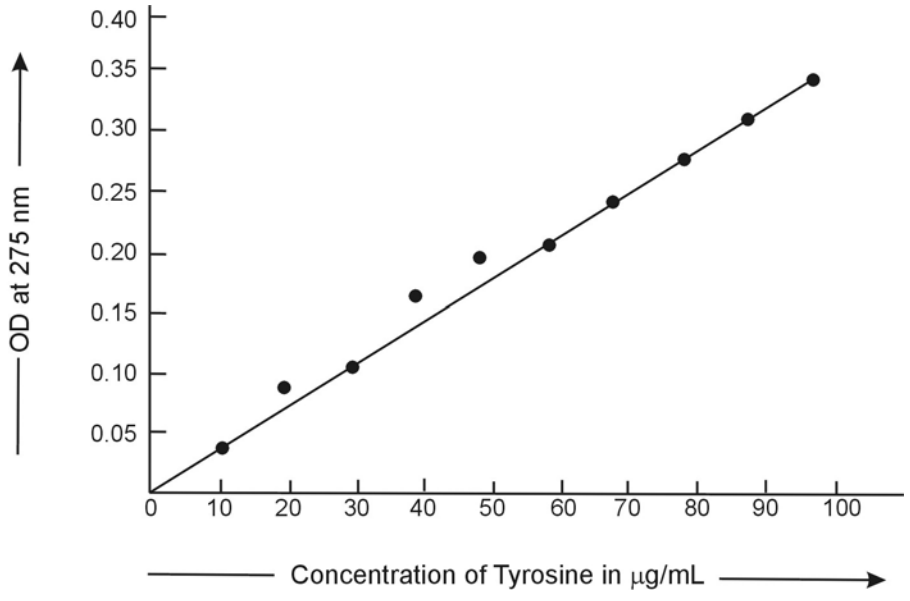

Fig. 1. Standard Curve of Tyrosine (10-100μg/mL), OD at 275nm

Further, it can be concluded that immobilization of protease is a surface phenomenon. Since grafting opens up the cellulose matrix, immobilization of enzyme should have been increased due to increase in the bulk surface area as a result of the grafting process. This concept is further supported by the fact that immobilization is facilitated by sorption processes more on symmetrical surfaces like cellulose in comparison with brush-shaped or comb-shaped graft copolymers and also in lesser steric polymers like graft copolymers of poly(MMA). However, grafting did not improve the immobilization of proteases.

Sorption of Bovine Serum Albumin

Sorption of bovine serum albumin (BSA) onto different polymeric carriers or supports again showed that native cellulose remains a very good matrix for its sorption. The percent relative activity was observed to be 80.4% (Table 2). Figure 2 shows the corresponding calibration curve.

Table 2. Immobilization of Protein (BSA) by Cellulose, Cellulose Graft Copolymers, and Cellulose Derivatives

Sr. No.	Graft Copolymer	Change in OD	% Relative Enzyme Activity
1.	**Cellulose**	**0.365**	**80.4**
2.	Cell-g-poly(GMA)	0.135	29.7
3.	**Cell-g-poly(GMA-co-AAc)**	**0.206**	**45.4**
4.	Cell-g-poly(GMA-co-AAm)	0.072	15.8
5.	Cell-g-poly(GMA-co-AN)	0.073	16.1
6.	**Cell-g-poly(MMA)**	**0.390**	**85.9**
7.	Cell-g-poly(MMA-co-AAc)	0.367	80.8
8.	Cell-g-poly(MMA-co-AAm)	0.145	81.9
9.	Cell-g-poly(MMA-co-AN)	0.381	83.9
10.	**Cell-g-poly(HEMA)**	**0.210**	**46.3**
11.	Cell-g-poly(HEMA-co-AAc)	0.176	38.8
12.	Cell-g-poly(HEMA-co-AAm)	0.137	30.2
13.	Cell-g-poly(HEMA-co-AN)	0.117	25.8
14.	**Cellulose-Phosphate**	**0.376**	**82.8**
15.	**Oxycellulose**	**0.385**	**84.8**
16.	**Test**	**0.454**	**100**

For the graft copolymers again the order of reactivity followed more or less the same pattern. So poly(MMA)-based graft copolymers absorbed more protein than poly(HEMA), which in turn were better absorbents than poly(GMA). Further, for the same series of polymer, comonomers usually helped in lower sorption but in this case cell-g-poly(MMA-co-AN)-based copolymers they showed high activity, as compare to

copolymers based on cell-g-poly(MMA-co-AAm) and cell-g-poly(MMA-co-AAc). Very encouraging results were observed for the protein sorption on cellulose-derivatized sorbents (cellulose phosphate = 82.8 and oxycellulose = 84.8). It can be concluded that grafting onto this particular backbone is not a very technologically and economically viable exercise, since in both the cases cellulose and in the later case cellulose and its derivatives act as potential polymeric supports.

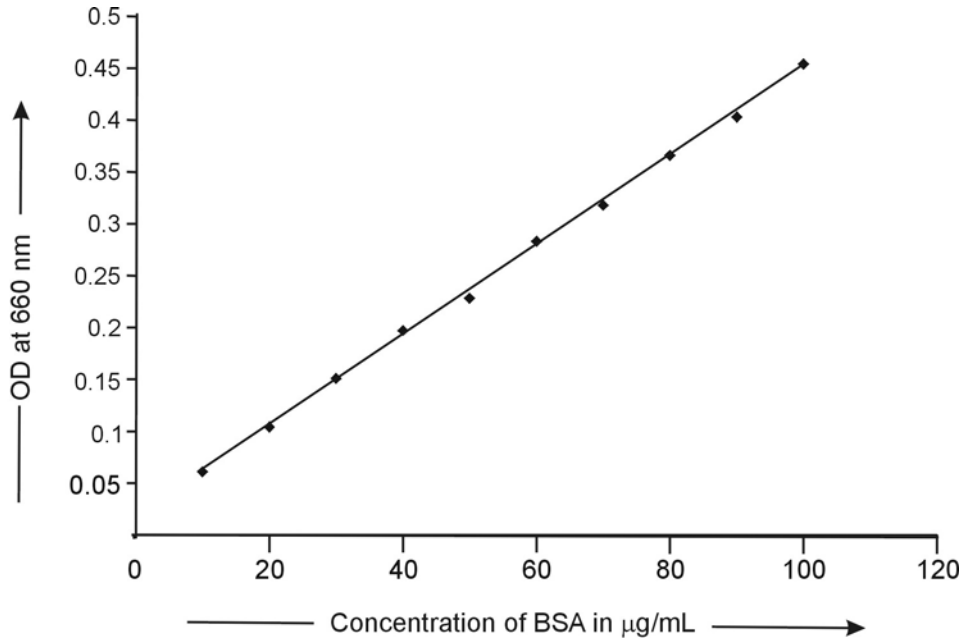

Fig. 2. Standard curve for Bovine Serum Albumin (BSA) (10-100µg/mL), optical density (OD) at 660nm

CONCLUSIONS

1. Cellulose itself is an effective polymeric support to retain a satisfactory amount of protease (65.4%) and bovine serum albumin (BSA); the % relative activity of BSA was observed to be 80.4%.
2. The activity of enzyme in cellulose graft copolymer followed the order: cell-*g*-poly(MMA) > cell-*g*-poly(GMA) > cell-*g*-poly(HEMA). Cell-*g*-poly(MMA) activity was less than that of cellulose itself. On the other hand, effects of comonomer (CM) grafting from binary system affording supports such as cell-*g*-poly(MMA-*co*-CM) were not of much use to immobilize sufficient amounts of enzyme, as reflected in the lowered activity of immobilized enzyme in almost all cases, except that the binary graft copolymer cell-*g*-poly(MMA-*co*-AN] was somewhat encouraging.
3. Cell-*g*-poly(MMA) absorbed more protein than cell-*g*-poly(HEMA), which in turn were better absorbents than cell-*g*-poly(GMA). Further, for the same series of polymer, comonomers usually helped in lower sorption, but in this case Cell-g-poly(MMA-co-AN) based copolymers showed higher activity, as compared to copolymers based on cell-g-poly(MMA-co-AAm) and cell-g-poly(MMA-co-AAc). Cell-*g*-poly(MMA), and cell-*g*-poly(MMA-*co*-CM) showed higher activity than that

of cellulose itself. The order for protein sorption can be formulated as cell-g-poly(MMA) > cell-g-poly(MMA-co-AN) > cell-g-poly(MMA-co-AAm) > cell-g-poly(MMA-co-AAc) > cellulose.

4. Use of cellulose derivatives such as cellulose phosphate and oxycellulose have been reported in protein separation, but in the present study very high selectivity towards protease was observed, as a very low amount on the same was adsorbed. But very encouraging results were observed for the protein sorption on cellulose-derivatized sorbents (cellulose phosphate = 82.8 and oxycellulose = 84.8).

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