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Sequential immobilization of urease to glycidyl methacrylate grafted sodium alginate

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ABSTRACT

Objective of this study is to realize appropriate enzyme immobilization onto a suitable support material and to develop a model which enables reactions catalyzed with different enzymes arranged in order. Thence, this model was potential for developing a multi-enzyme system. The reactions need more than one enzyme can be realized using immobilized form of them and the enzymes will be in one support at wanted activities. In this study, sodium alginate was used as immobilization material and glycidyl methacrylate was grafted onto sodium alginate. Thus reactive epoxy groups were added to sodium alginate which also has carboxyl groups. Average molecular weight of sodium alginate was determined using Ubbelohde viscosimetri. The molecular mass of sodium alginate was calculated as 15,900 Da. Graft polymerization was made in two steps. Firstly, sodium alginate was activated with benzophenone using UV-light at 254 nm. Secondly, glycidyl methacrylate was grafted under UV-light at 365 nm onto activated sodium alginate. Grafted glycidyl methacrylate was determined gravimetric and titrimetric. Additional groups after grafting were showed with FT-IR spectrum. 1-Ethyl-3-(3-dimetylaminopropyl)-carbodiimide was used for immobilization urease from carboxyl groups at pH 5.0. Suitable 1-ethyl-3-(3-dimetylaminopropyl)-carbodiimide/–COOH ratio was found 1/10 and immobilized product activity was 197 U/g support. Reaction medium pH was 8.0 for immobilization from epoxy group. Optimum immobilization reaction time was found as 2 h and immobilized product activity was 285 U/g support. Sequential immobilization of urease to glycidyl methacrylate grafted sodium alginate was made from –COOH and epoxy groups, respectively.

1. Introduction

Enzymes are well-known green catalysts that possess a high degree of specificity. The specificity involves discrimination between substrates (substrate specificity), similar parts of molecules (regiospecificity), and optical isomers (stereospecificity) [1,2]. The mildness and specificity of enzymes endow them with a high efficiency for applications in fine-chemical and pharmaceutical synthesis, food processing, biosensors fabrication, bioremediation, and protein digestion in proteomic analysis [3,4]. However, the price and applications of enzymes are limited by their stability and non-reusability. Enzyme immobilization is an effective way to overcome these limitations to some extent. First, the multiple-point attachment to the support can restrict the undesirable conformational change of enzyme proteins in unfriendly environments. Second, insoluble supports can be recycled much more easily than soluble enzymes. Immobilization is advantageous because it extends the stability of the bioactive species by protecting the active material from deactivation, enables repeated use, it provides significant reduction in the operation costs, facilitates easy separation, and speeds up recovery of the bioactive agent [5–7]. There are many methods, such as adsorption, entrapment, covalent binding, for enzyme immobilization [8,9]. When the support contains the relevant functional groups, covalent immobilization of enzymes becomes feasible. A range of functional groups which can be used in the covalent immobilization of enzymes include epoxy, amino, hydroxyl, carboxyl and phenolic groups [10]. So far, commonly one enzyme and different materials have been used for immobilization. Multi-enzyme system can be modeled with enzyme immobilization. Different functional groups can be used for selective immobilization of different enzymes which form multi-enzyme system. Modification of polymers is suitable way of making multi-enzyme systems.

Modification of polymers has received much attention recently. Among the methods of chemical polymer modification grafting represents one of the most promising approaches since graft copolymerization will impart a variety of new functional groups to a polymer [11]. In order to modify the properties of a polymer in a desired way so called photografting is performed by irradiating it in a solvent containing selected monomers with appropriate radi-

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ation sources. The energy sources commonly used are high-energy electrons, X-rays, ultraviolet (UV) and visible light. Many different routes can be employed to introduce graft chains onto the surface of polymers depending on the system on the goal of later application [12,13]. Energy of UV has been extensively applied for surface graft polymerization of polymers with the aid of a photoinitiator, such as benzophenone (BP). UV-induced graft polymerization is a powerful technology, with low cost of operation and potentially reducing or even avoiding negative effects onto the bulk polymers. BP abstracts hydrogens from material and radicals occur. Graft polymerization starts from the radicalic centers. After proper modification, polymeric material can become a good carrier for binding of enzyme [14]. Glycidyl methacrylate (GMA) is an attractive monomer and BP is an efficient initiator for the photochemical grafting [15]. GMA, the ester of methacrylic acid and 2,3-epoxy-propanol, bears a reactive epoxide group which reacts with sulfhydryl- [16], amino- [17,18], carboxyl- [19,20] or hydroxylgroups [21] to form stable covalent bonds with biomolecules without any linker. Such epoxy-modified polymers are stable during long storage periods and relatively resistant against hydrolysis. Biomolecules like proteins are covalently coupled by opening the epoxide bridge in alkaline media.

Alginate is a water soluble linear polysaccharide extracted from brown seaweed and is composed of alternating blocks of $1 \rightarrow 4$-linked α-L-guluronic and β-D-mannuronic acid residues. The gelation of alginate can be carried out under an extremely mild environment and uses non-toxic reactants. The most important property of alginates is their ability to form gels by reaction with divalent cations such as Ca^{2+} [22–25].

Urease is an enzyme frequent in biological systems where it catalyses the hydrolysis of urea. It is related to protein intake, nitrogen metabolism and excretion as follows:

$$(NH_2)_2CO + 3H_2O \overset{\text{UREASE}}{\longrightarrow} HCO_3^- + 2NH_4^+ + OH^-$$

Immobilized form of urease has found broad applications, such as blood detoxification in artificial kidneys, the removal of urea from beverages and foods in food industry, and the reduction of urea content in effluent treatment in agriculture. The immobilization of enzyme on natural or synthetic membranes has found interesting applications in biosensors or membrane bioreactors. One technique employed for the immobilization is grafting, by which inert supports become available for enzyme attachment [26].

In this study, GMA was grafted to sodium alginate and then urease was selectively immobilized from epoxy- and carboxyl-groups on GMA grafted alginate. Using this model, multi-enzyme systems will be developed on one support and this type support could be used in medical applications. This system is important because the enzymes do not immobilize randomly. The desired activity and/or amount of two or more enzymes can be immobilized to support, respectively. This study is a model for enzyme immobilization.

2. Materials and methods

2.1. Materials, reagents and UV bulb

Low viscosity sodium alginate from *Laminaria hyperborea* was purchased from Manutex RSX, Kelco International, Ltd., Portugal. Sodium alginate was dissolved in distilled water, extracted with methanol, lyophilized and milled. NaOCl, NaOH, phenol, GMA, 1-ethyl-3-(3-dimetylaminopropyl)-carbodiimide (EDC), BP, albumin from bovine serum fraction V (BSA) were purchased from Merck (Darmstadt, Germany). Urease (E.C. 3.5.1.5) from Jack beans (Sigma-type III), acetone and methanol were obtained from Sigma Chemical Company (St. Louis, USA). Other chemicals used were of analytical grade.

Phenol reagent: 100 g/l phenol and 0.5 g/l sodium nitroprusside solutions were mixed equal volumes.
Hypochlorite reagent: 26 g/l sodium hypochlorite and 50 g/l sodium hydroxide solutions were mixed equal volumes.

Phenol and hypochlorite reagents are light sensitive and were stored in dark.

Bulb is 4 W and complete spectrum emission from 365 nm to visible light.

2.2. Viscosity measurement for determination of molecular weight of sodium alginate

Aqueous solutions of sodium alginate containing sodium chloride (0.1 M) were analyzed in an Ubbelohde viscometer, Schott-Gerate. Sodium alginate solution was transferred into the viscometer which was immersed in a precision water bath to maintain the temperature at 25 ± 0.1 °C and after equilibration for 10 min, the flow time was determined between the two etched marks. Serial dilution was performed in situ and five readings were taken for each dilution and averaged. The viscosity average molecular mass, M_W was evaluated using the Mark–Houwink–Sakurada equation (Eq. (1)) [27]:

$$\log[\eta] = \log K + a \times \log M_W \quad (1)$$

η: intrinsic viscosity; a: 1.13 for *Lamineria*; K: 6.9×10^{-6} for *Lamineria*; M_W: molecular weight.

2.3. Photografting procedures

2.3.1. One step photografting

Sodium alginate (100 mg) was dissolved in 5 ml distilled water. BP (46.6 mg) and GMA (337 mg) were also dissolved in 5 ml methanol. The two solutions were mixed in a quartz vessel and sparged with nitrogen gas for 5 min at 25 °C. Then it was exposed at room temperature to UV radiation at 254 and 365 nm for 10 min under nitrogen atmosphere at 25 °C. The reaction mixture was added into 20 ml methanol and stirred at 4 °C for 24 h. Precipitate was filtered and washed three times with 10 ml methanol to remove GMA homopolymer, unreacted BP and unreacted monomer. The precipitate was dried until a constant weight. Grafting procedure was repeated at 0 and 50 °C. The grafting percentage of GMA was calculated gravimetric according to the Eq. (2) and volumetric methods:

$$G(\%) = \frac{W_a - W_b}{W_a} \times 100 \quad (2)$$

where W_b and W_a are the sodium alginate mass before and after the grafting process, respectively. The BP concentration in the washing solution was spectrophotometrically determined at 256 nm from BP standard curve and reacted BP concentration was calculated.

2.3.2. Two-step photografting

Sodium alginate (100) mg was dissolved in 5 ml distilled water. BP (46.6 mg) was also dissolved in 5 ml methanol. The two solutions were mixed in a quartz vessel and sparged with nitrogen gas for 5 min at 25 °C. Then it was exposed at room temperature to UV radiation at 254 nm for 90 s under nitrogen atmosphere at 25 °C. The reaction mixture was added into 20 ml methanol and stirred at 4 °C for 24 h. Precipitate was filtered and washed three times with 10 ml methanol to remove unreacted BP. The BP concentration in the washing solution was spectrophotometrically determined at 256 nm from BP standard curve and amount of bounded BP was calculated. BP activated sodium alginate was dissolved in 5 ml distilled water. GMA (337 mg) was also dissolved in 5 ml methanol. The two

solutions were mixed in a quartz vessel and sparged with nitrogen gas for 5 min at 25 °C. Then it was exposed at room temperature to UV radiation at 365 nm under nitrogen atmosphere for 10 min at 25 °C. The reaction mixture was added into 20 ml methanol and stirred at 4 °C for 24 h. Precipitate was filtered and washed three times with 10 ml methanol to remove GMA homopolymer and unreacted monomer. The precipitate was dried until a constant weight. The grafting percentage was calculated according to Eq. (2) and volumetric GMA determination method.

2.4. FT-IR spectroscopic measurements

A Fourier transform infrared spectrophotometer (Perkin Elmer Spectrum 100 Series) was employed. One milligram of GMA grafted sodium alginate was ground into powder with high purity infrared grade KBr powder (100 mg) and pressed into a pellet for measurement. Spectrum was recorded in the range of 400–4000 cm^{-1} with a resolution of 2 cm^{-1}.

2.5. Determination amount of grafted GMA

Dried GMA grafted sodium alginate (50 mg) was dissolved in 10 ml distilled water containing $Na_2S_2O_3{\cdot}5H_2O$ (2 mmol) and acetic acid (1 mmol) and 10 ml acetone was added. The solution was incubated at 40 °C for 20 min and cooled. It was titrated with 0.1N NaOH. Grafted GMA was determined with standard curve against GMA standard solution [28].

2.6. Urease immobilization from –COOH groups

Dried GMA grafted sodium alginate (100 mg) was dissolved in 5 ml (pH 5, 0.02 M) phosphate buffer solution and EDC (10 mg) was added. pH of the solution was adjusted to 5.0 with 0.1 M HCl. The solution was shaken gently in a water bath for 15 min at 30 °C. The activated GMA grafted sodium alginate was precipitated with 10 ml acetone and washed with acetone. The activated GMA grafted sodium alginate was separated and dissolved in 5 ml (pH 5, 0.02 M) phosphate buffer solution and 2 ml (contain 1.02 mg/ml protein according to the Bradford method [29]) of urease solution was added. pH of the solution was again adjusted to 5.0 with 0.1 M HCl. The solution was shaken in a water bath for 2 h at 30 °C. Then it was dropped into cold 100 ml (0.1 M) $CaCl_2$ solution and alginate beads were formed. The beads were stirred for 1 h at 4 °C. The beads were washed three times with cold 50 ml (0.1 M) $CaCl_2$ solution for 30 min to remove unbound urease. The amount of protein uncoupled to GMA grafted sodium alginate was determined according to the Bradford method [29]. Beads activities were determined by the Berhelot method [30].

The same procedure was made without EDC to show urease was not immobilized from –COOH and epoxy groups and/or immobilized noncovalently at pH 5.

2.7. Urease immobilization from epoxy group

Dried GMA grafted sodium alginate (100 mg) was dissolved in 5 ml (pH 8, 0.02 M) phosphate buffer solution and 2 ml (contain 1.02 mg/ml protein according to the Bradford method [29]) of urease solution was added. The solution was shaken at 30 °C for 2 and 18 h. The solution was dropped into cold 100 ml (0.1 M) $CaCl_2$ solution and alginate beads were formed. The beads were stirred for 1 h at 4 °C and washed three times with cold 50 ml (0.1 M) $CaCl_2$ solution for 30 min to remove unbound urease. The amount of protein uncoupled to GMA grafted sodium alginate was determined according to the Bradford method [29]. Beads activities were determined by the Berhelot method [30].

Table 1
Flow time averages of each sodium alginate dilutions, relative viscosity and specific viscosity values.

Concentration of sodium alginate (%)	Average of flow times (s)	η_r	η_{sp}
0.6	85.9	3.06	3.43
0.7	105	3.73	3.90
0.8	128	4.55	4.44
0.9	153	5.44	4.93
1.0	181	6.44	5.44

η_r: (flow time of sodium alginate)/(flow time of 0.1 M sodium chloride).
η_{sp}: $(\eta_r - 1)$/(% concentration of sodium alginate).

2.8. Sequential immobilization of urease to GMA grafted sodium alginate

Firstly, immobilization of urease was realized from –COOH groups according to Section 2.6. Occurred urease immobilized GMA grafted sodium alginate beads were dissolved in 0.1 M sodium citrate at 4 °C. The solution was dialyzed against (pH 8, 0.02 M) phosphate buffer solution at 4 °C to remove citrate molecules. Secondly, 2 ml (contain 1.02 mg/ml protein according to the Bradford method [29]) of urease solution was added to dialysate and immobilization from epoxy groups was carried out according to Section 2.7.

2.9. Urease activity

Urease activity was determined by the Berhelot method [30]. Ten microliters of urea (1.1 mM), 1.94 ml (pH 7, 50 mM) phosphate buffer and 50 μl of urease solution were added to test tube. The mixture was shaken for 10 min at 37 °C. Phenol reagent (0.5 ml) and hypochloride reagent (0.5 ml) were added to mixture. The mixture was shaken for 10 min at 55 °C and cooled. Urease activity was determined spectrophotometric at 630 nm. The standard curve for urease activity was prepared with different concentration ammonium chloride solution.

Urease immobilized alginate beads activities were measured with the same procedure. Phosphate buffer (pH 7, 50 mM, 50 μl) was added instead of urease solution (50 μl) and urease immobilized alginate beads were used as urease preparate.

One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol urea per minute at 37 °C under standard assay conditions.

2.10. Determination of protein content

The protein content was determined by the method of Bradford [29]. Solution (0.1 ml) which was contained urease was added 2 ml of Coomassie Brilliant Blue solution (40 mg of Coomassie Brilliant Blue G-250 dissolved in 50 ml 95% ethanol and, finally, 55 ml 88% H_3PO_4 added. The resulting solution was diluted to a final volume of 1 l.). The Coomassie Brilliant Blue forms a blue complex with the proteins. After 10 min of incubation at room temperature, the absorbance was measured at 595 nm, against an albumin standard solution of BSA (0.01–0.15 mg/ml).

3. Results and discussion

3.1. Determination of molecular weight of sodium alginate

The flow time averages of each sodium alginate dilutions, relative viscosity (η_r) and specific viscosity (η_{sp}) values were given in Table 1. Average of flow times of 0.1 M sodium chloride was 28.1 s.

A graph was prepared between different concentrations of sodium alginate values and their specific viscosity values for calculation of intrinsic viscosity ([η]) (Fig. 1).

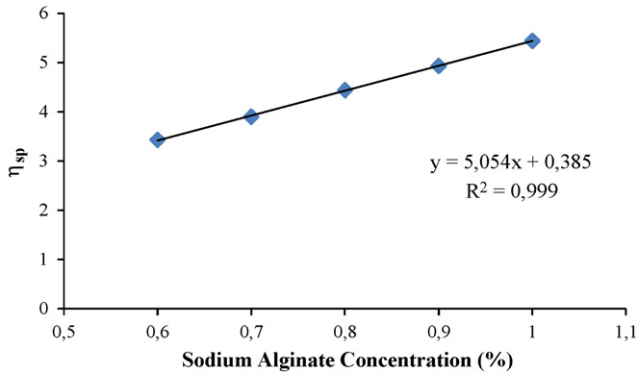

Fig. 1. Graph of different concentrations of sodium alginate and their specific viscosity values for calculation of intrinsic viscosity.

The straight line was extended to y-axis, $x = 0$, and $[\eta]$ was calculated as 0.385 dl/g. $[\eta]$ was used in Mark–Houwink–Sakurada equation and M_w of one chain of sodium alginate was found 15,900 Da. Sodium alginate is formed guluronic and mannuronic acids. Their M_w is 198 g/mol. 15,900 was divided into 198 and 80.3 was found. This was average monomer number in one chain of sodium alginate. BP and GMA concentrations at graft polymerization studies were calculated according to ~80 monomers unit.

3.2. Photoinduced graft polymerization

Graft polymerization was made one- and two-step at 254 and 365 nm. 7.73 and 8.50 mg of GMA was grafted to 100 mg sodium alginate at one step grafting for 254 and 365 nm, respectively. In the first step of two-step grafting, BP (13.4 mg) was reacted with sodium alginate. The BP concentration was calculated with standard curve of BP. BP was prepared in the grafting solvent system and spectral analysis of BP solution was done. The solution was given a peak at 256 nm. Then standard curve was prepared with different concentration of BP at 256 nm. In the second step of two-step grafting, 11.1 mg of GMA was grafted to 100 mg sodium alginate. Two-step grafting was given the best result for GMA grafting to sodium alginate and used for following grafting studies.

Grafting studies were made at 0 and 50 °C. The grafting percentages were lower than grafting at 25 °C. At lower temperature, the grafting reaction was confirmed slower. At higher temperature, the radicals were died away so quickly. 25 °C was determined the optimum temperature for grafting.

3.2.1. Effect of irradiation time on BP activation (first step)

Sodium alginate and BP solution were irradiated for 30, 60, 90, and 120 s. Amount of reacted BP was maximum at 90 s (Fig. 2).

BP was bounded with sodium alginate at UV irradiation. Then one radical was occurred on BP and other radical was occurred on sodium alginate. The maximum bounded BP was at 90 s. Most of BP radicals were reacted each other and semipinacols were occurred at 120 s. Semipinacol are not UV sensitive and they cannot made radicals like BP at UV irradiation [31]. 87.8% of bounded BP was removed between 90 and 120 s. 28.8% of added BP was bounded to sodium alginate at 90 s.

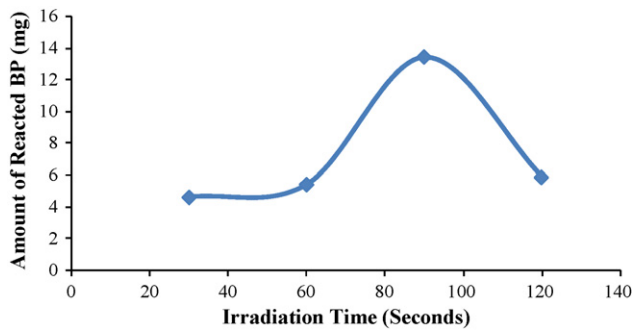

Fig. 2. Effect of irradiation time on BP activation.

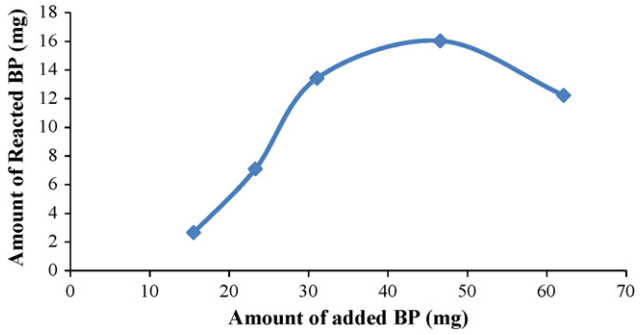

Fig. 3. Effect of amount of BP on grafting.

3.2.2. Effect of amount of BP on grafting

Different amounts of BP (15.5, 23.3, 31.1, 46.6, and 62.1 mg) were used for activation. Amount of bounded BP was increased with increasing BP concentration up to 46.6 mg of BP. Then it was decreased (Fig. 3).

The direct effect of higher amount of BP is to increase reaction rate, but the indirect effect to increase light absorption, lowering the UV intensity at the interface of the BP solution and sodium alginate [32].

3.2.3. Effect of irradiation time on GMA grafting to sodium alginate

GMA was grafted to sodium alginate at different irradiation times to determine optimum reaction time for grafting. The amount of grafted GMA was reached a saturation value at 30 min (Fig. 4).

The optimum UV irradiation time was 30 min. After 30 min, amount of GMA might be decreased and radical end groups of grafting GMA were bounded each other. The grafting reaction was stopped.

3.2.4. Effect of amount of GMA on grafting

The grafting percentage of GMA was increased with increasing amount of GMA. The grafted GMA was reached to maximum value with added 337 mg of GMA and amount of grafted GMA was not changed dramatically at higher GMA concentrations (Table 2). Amount of added GMA was selected as 337 mg of GMA.

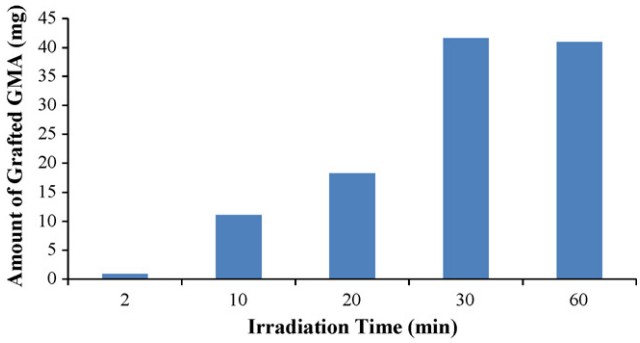

Fig. 4. Effect of irradiation time on GMA grafting.

Table 2
Effect of amount of initial GMA according gravimetric and titrimetric methods.

Amount of initial GMA (mg)	Amount of GMA grafted to 100 mg sodium alginate (mg) (gravimetric)	Amount of GMA grafted to 100 mg sodium alginate (mg) (titrimetric)
126	0	1.56
169	5.1	9.89
253	22.8	18.7
337	41.7	52.0
506	43.2	51.2

3.3. Determination of amount of GMA in GMA grafted sodium alginate

Grafting percentage of GMA was determined gravimetric and titrimetric. Titrimetric method was made according to reaction between epoxy group and sodium thiosulfate. NaOH was produced and reacted with CH_3COOH. Remained CH_3COOH was titrated with 0.1N NaOH and GMA content were calculated from mole ratios. [28]. GMA content of GMA grafted sodium alginate (Section 3.2.3 samples) was calculated according to titrimetric method and results were also given in Table 2. The results of titration and gravimetric methods were compared and the results were closed to each other. These methods were corrected each other. According to these results, sodium alginate polymer was formed 80 units of mannuronic and guluronic acid residues. It was activated with BP at each 6 units and 4 GMA was grafted to each activated unit.

3.4. IR-spectroscopy of GMA grafted sodium alginate

IR-spectroscopy of the GMA grafted sodium alginate was analyzed by IR-spectroscopy (Fig. 5).

The epoxide group is characterized by a peak at 945 cm^{-1}. 1722 cm^{-1} due to stretching vibrations of C=O of ester group [33]. Among the characteristic vibration of the GMA is the methyl vibration at 2924 cm^{-1} [34]. These peaks showed that GMA was grafted to sodium alginate.

3.5. Enzyme immobilization to GMA grafted sodium alginate

Urease was immobilized to GMA grafted sodium alginate from epoxy group of GMA and –COOH group of alginate. First, urease was immobilized from –COOH with EDC activation at low pH and then immobilized from epoxy group at high pH. This ordered immobilization system allows to made multi-enzyme system. More than one enzyme could be immobilized to supports at wanted activities sequentially. This is a model system for enzymology and can be developed more than two enzymes.

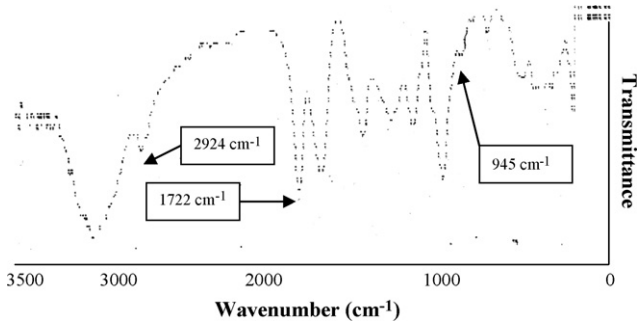

Fig. 5. FT-IR spectrum of GMA grafted sodium alginate.

Table 3
Effect of different EDC/–COOH ratios to immobilization.

EDC/–COOH	Activity of immobilized urease (U/g support)	Remained activity (%)	Bounded protein (mg)	Bounded protein (%)
1/1	36.9	2.93	1.53	75.1
1/5	63.9	5.03	1.55	76.2
1/10	197	18.3	1.31	64.3
1/20	127	14.2	1.08	53.2

3.5.1. Urease immobilization from –COOH groups

Different EDC/–COOH ratios were investigated. Urease immobilization results were given at Table 3. Optimum EDC/COOH ratio was 1/10. 64.3% of added urease was immobilized to sodium alginate. At 1/20 (EDC/COOH) ratio, amount of EDC was low according to other EDC/COOH ratios and urease was bounded to sodium alginate at lower grade. But at 1/5 and 1/1 (EDC/COOH) ratios, more urease was immobilized according to bounded protein results. In this case, urease was bounded to sodium alginate from multi-point and 3D structure of urease was changed. So the activity of immobilized urease was decreased [35].

Activation of supports which contain –COOH groups with EDC and enzyme immobilization can be made at pH 4.5–5.0 [36,37]. Some authors defend that the reaction should be made at weak acidic or neutral conditions [38]. Immobilization with EDC was provided high selectivity. It reacts with amino and substituent amino containing nucleophilic groups. It also reacts with –SH group of cystein and –OH group of tyrosine but the reaction rate is very low [39]. There are several ways of enzyme immobilization using EDC in literature. The first way, EDC, support and enzyme were put to reaction vessel at the same time. The second way, support was activated with EDC and unreacted EDC is washed. Then enzyme was added to reaction vessel. This method is two steps. In the first way, enzyme also bounds to support but enzymes bound to each other from –COOH group. Because EDC activates –COOH groups of enzymes, too. So 3D of enzyme changes and activity of enzyme decreases. So we immobilized urease according to second method.

Effect of immobilization time was investigated. Immobilization times were selected as 0.5, 1, 2 and 4 h. Optimum immobilization time was 2 h. Effect of amount of urease was also investigated. 1.5, 3, 5 and 10 mg urease were used. 3 mg urease was found as optimum. Immobilization of urease was made at room temperature in most studies [40,41]. So 30 °C was selected as reaction temperature.

At low pH values, urease was immobilized to GMA grafted sodium alginate according to bounded protein values but activity was lower than other pH values assays (Table 4). The same profile can be seen in literature [42–44]. The aim of study was made pH selectivity for immobilization. Activation with EDC and immobilization were made at pH 5 because of making pH selectivity. Urease activity for pH 5 was lower than other pH values assays but the activity was enough for immobilization.

Table 4
Effect of pH at activation with EDC and immobilization.

pH of activation with EDC	Immobilization pH	Urease immobilized GMA grafted sodium alginate (U/g support)	Bounded protein (mg)
5.0	5.0	197	1.31
5.0	6.5	202	1.06
5.0	8.0	72.9	0.51
6.5	6.5	232	0.92
8.0	8.0	53	0.21

Table 5
Effect of reaction time at immobilization from epoxy group.

Immobilization time (hours)	Urease immobilized GMA grafted sodium alginate (U/g support)	Remained activity (%)	Bounded protein (mg)	Bounded protein (%)
2	285	37.9	0.892	43.9
18	116	6.78	2.03	100

A blank assay was made. Urease was tried to immobilize from epoxy groups at pH 5. No urease activity and protein were determined. So we showed that urease was immobilized from –COOH groups with EDC at pH 5. Urease was not immobilized from epoxy groups at pH 5.

3.5.2. Urease immobilization from epoxy groups

Epoxy groups hydrolyze at extreme pH. So they are very stabile groups. Immobilization from epoxy group occurs at two steps. At the first step, enzyme adsorbs to support. At the second step, epoxy group of GMA grafted sodium alginate reacts with amino group of enzyme and covalent bound was occurred. Occurred C–N bound is very stabile [38]. Reaction temperature was selected 25 or 30 °C by authors [43,45,46]. 30 °C for reaction temperature was used for immobilization.

The most important parameter is reaction time for enzyme immobilization from epoxy group. Optimum reaction time was 2 h. Most of immobilization reactions from epoxy group were more than 15 h [41,43,44,46]. But long immobilization time was caused decreasing enzyme activity. The reaction was occurred spontaneously and no chemical agent was used for immobilization. Activity and remained activity (%) of urease immobilized GMA grafted sodium alginate was fairly well and suitable for practical applications.

3.5.3. Sequential immobilization of urease

First, urease was immobilized from –COOH groups of GMA grafted sodium alginate at pH 5 and then the enzyme was immobilized from epoxy groups of GMA grafted sodium alginate at pH 8, selectively. After the first immobilization process, urease, immobilized from –COOH groups, activity was 199 U/g support. After the second immobilization process, total immobilized urease activity was 476 U/g support. Urease, immobilized from epoxy groups, activity was 285 U/g support according to Table 5. Remained activity was 191 U/g support for the first immobilization process. Urease, immobilized from –COOH groups, activity was lost 4.02% of activity during second immobilization process.

4. Conclusion

–COOH and epoxy groups of GMA grafted sodium alginate were selected for urease immobilization. Immobilization of urease was made from –COOH group at low pH. Immobilization from epoxy group was made at high pH. By this way, enzyme immobilization could be done according to pH selectivity during immobilization. We used urease for immobilization. Urease was immobilized from –COOH and epoxy groups, respectively. This immobilization processes were made sequentially. Urease, immobilized from –COOH, activity was 199 U/g support. After second immobilization process, urease, immobilized from –COOH, activity was decreased to 191 U/g support according to calculations. Only 4.02% of urease activity was lost. Additional second immobilization process was caused to decrease urease activity which was immobilized from –COOH groups. This decreased value of activity was acceptable and wanted enzyme activity can be adjusted.

Two or more different enzyme can be immobilized using this method. The importance of this study is that two or more enzyme can be immobilized at intended activity to a support using graft polymerization. Multi-enzyme systems can be developed.

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