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Immobilization of urease on cation-exchange membranes prepared by radiation-initiated graft copolymerization of acrylic acid on polyethene thin films

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Summary

A covalent immobilization of urease was conducted on carboxylic cation-exchange membranes (CEM) prepared by radiation-initiated graft copolymerization of acrylic acid (AA) on polyethene (PE) thin films. Six types of CEM with different grafting degree (from 26.5 to 95.2%) were used as carry. The carboxyl groups were activated by the carbodiimide method in order to carry out a covalently immobilization. The amount of bound protein and the enzyme activity were determined in each immobilized system. It was established that the urease, immobilized on CEM with 64.2% grafting degree, featured the highest relative activity – 80.32%. The amount of bound protein on this membrane type was 6.01 mg /cm^2. The basic characteristics of the immobilized and the free enzymes were determined (pH_{opt}, T_{opt} and pH_{stab}). It was found out that the immobilized urease had greater thermal and storage stability in comparison with the free enzyme. It was proven that CEM with a grafting degree of 64.2% would be a suitable carrier for urease immobilization.

Introduction

Enzymes are being used increasingly as analytical reagents. Although most biocatalysts are employed, many successful attempts have been made, in recent years, to use immobilized enzymes in clinical and industrial analysis [1,2]. One of the most important requirements to be achieved with immobilization is an increased stability of the enzyme. The immobilized enzyme will then be preferred for routine analysis due to the increased possible number of reutilizations and the lower cost per analysis.
Selection of the carrier to which the enzyme is ultimately attached has an important bearing on the activity and propriety of enzyme. A great number of synthetic and natural polymers are utilized as matrices for enzyme immobilization. The presence of appropriate reactive groups in the radiation-initiated grafted copolymers has made them an interesting object of research with respect to enzyme immobilization, etc

[3-8]. The preparation of different types of ionic polymers via radiation-initiated graft copolymerization of functional monomers is still a subject of intense investigations. [9-13]. The multiple potential applications of radiation grafted copolymers and their industrial utilization as carriers for preparation of biomaterials could be found in some references. [10, 14, 15]. The immobilization of biologically active substances onto radiation-initiated grafted copolymers, featuring high purity, good hydrophilicity, biocompatibility and mechanical strength, enables the preparation of new materials with biotechnological and medical significance [16-18].
The urease immobilization is of a particular interest regarding the preparation of enzyme membranes with different practical applications [19]. Membrane-immobilized urease opened the way for constructing urea sensors [20] , heavy metal sensors [21] and membrane bioreactors [16] applied for urea determinations and urea removal, respectively. A lot of research efforts were aimed to found out the optimal conditions for urease immobilization on polymer carry, including matrices made by grafting of different monomers [10, 23-25].
The main objective of the present work was to investigate the suitability of membranes prepared from radiation-grafted copolymers based on polyacrylic acid – polyethene low density for being used as carriers for urease immobilization.

Experimental Part

Materials

Carboxylated cation-exchange membranes, having different degree of grafted acrylic acid (AA) on thin (40μm) polyethene low density (PE) films, were used as supports for urease immobilization. The PE (Ropoten T, Brand FV-03-223) is commercial product of LukOil Neftochim AD Burgas).
The ion-exchange membranes were prepared via radiation-grafted copolymerization of acrylic acid (BASF, Germany) on the polymer, mentioned above, using the direct method of multiple radiation treatment with γ-rays from ^{60}Co radioactive source (the dose rate was 3.5κGy/h, a dose from 1 to 35 κGy/h) in an inert (N_2) atmosphere at T=25^oC. A 40 mass% water solution of AA was used. The homopolymerization of AA was suppressed by adding an inhibitor - $NH_4(FeSO_4).6H_2O$ with 1,5 mass% concentration regarding the water-acid solution.
The carboxylated copolymers were activated with N,N$^/$-dicyclohexylcarbodiimide (Merck, Germany). The enzyme to be immobilized was urease, prepared by Merck (Germany). 3% solution of carbamide in 0.06M phosphate buffer (pH5.8), 1N solution of HCl, Nessler's reagent, 2% solution of Na_2CO_3 in a 0.1N solution of NaOH, 1% solution of potassium-sodium tartratum, 0.5% solution of $CuSO_4$ in potassium-sodium tartratum and 1N solution of Folin reagent were used to determine the activities of the immobilized and the free enzyme and the amount of protein bound to the membranes. All reagents were chemical grade (Merck, Germany).

Activation of the cation-exchange membranes

The carboxyl groups were activated with N, N$^/$- dicyclohexylcarbodiimide [26]. Three membranes with 1.5 cm^2 surface area each were placed in 10 ml 1% ethylacetate solution of N, N$^/$- dicyclohexylcarbodiimid. Thus the membranes were incubated for 80 min at pH 4.75 and at 20^oC. After that the membranes were washed in a beaker, containing 50 ml $CH_3COOC_2H_5$, and finally with bidistilled water.

Urease immobilization

The activated CEM were incubated in a 0.1% solution of urease in a phosphate buffer (pH 5.8) for 24 hours at 4°C. The immobilized system – enzyme plus membrane – was washed with bidistilled water and 0.06M phosphate buffer and then was stored at 4°C in 0.06M phosphate buffer (pH 5.8).
All solutions, which were used during the immobilization, had been prepared with bidistilled water.

Analyses

The degree of hydrophilicity of the membranes were determined on the basis of the methods, described in reference [27]. The concentration of carboxyl groups in the modified membranes was quantified through residual titration. This method is based on the neutralization of the free carboxyl groups with NaOH and then the excess of NaOH is titrated with a solution of HCl. The amount of bound protein was determined via the Lowry method. [28]. The activities of the immobilized and the free urease were established spectrophotometrically (Specol 11, Carl Zeiss Jena) using Nessler's reagent at 480 nm. The procedure is described in reference [29].

Results and discussion

All research efforts were aimed try to find the suitable polymer matrices, with determined grafting degree of AA for prepares an enzyme membrane. The laboratory made membranes were produced by radiation grafting of AA on thin PE films [27, 30]. By varying the reaction conditions of the radiatiation copolymerization grafted copolymers wiht degrees of grafting from 26.5 to 95.2% were obtained. The method of multiple grafting used, involving post-polymerization effect, provides a possibility for the monomer to penetrate into the polymer matrix together with the stepwise generation of free radicals. As a result, the grafting proceeds with the polymer matrix, thus reducing the thickness of the non-grafted layer. This is crucial for the properties of the cation-exchange membranes obtained. Six types of CEM (with different grafting degree of AA) were used (Table 1).The presence of carboxyl groups in the grafted layer premised the hydrophilisity of membranes. The water molecules are hydrated the “fixed” carboxyl groups. The water contents of these membranes were shown in Table 1.

Table 1. Main characteristics of the membranes, used as supports for urease immobilization

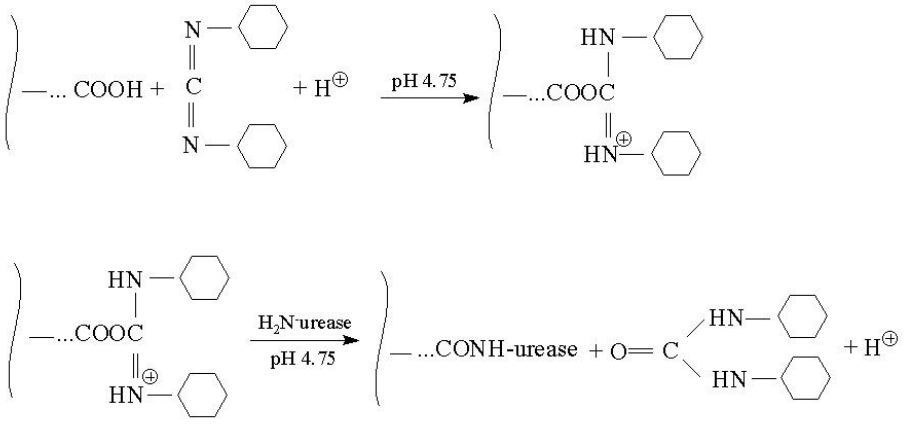
№	Dose (KGy)	Grafting Degree (%)	Water content (%)	Amount of carboxyl groups (mgeq/g)
1	1.1	26.5	6.9	6.71
2	2.2	44.4	10.7	7.12
3	5.0	50.5	17.2	7.67
4	10.0	64.2	22.7	8.95
5	15.0	73.2	24.1	9.15
6	20.0	95.2	27.2	9.49

It was shown that the increase of grafting degree (irradiation dose) of AA had increased the membrane hydrophilisity.

The quantity of the carboxyl groups was determined by residual titration for each membrane type (Table 1). It was found out that the increase of the grafted component had increased the amount of carboxyl groups. The lowest grafting degree (27.6%) refers to the lowest amount of carboxyl groups 6.71 mgeq/g, whereas the highest degree (95.2%) – to 9.49 mgeq/g.

One of the most widely used methods for covalent immobilization of enzymes on carboxylated supports is the carbodiimide method [26]. In the present work N,N/-dicyclohexylcarbodiimide was used as an activator of the carboxyl groups on the supports. The enzymatic basic amino groups can form amide bonds with the carboxyl groups of the activated membranes. The reaction could be conducted either with water-soluble or water-insoluble carbodiimides. Unlike most of the reactions, where amides are formed, this reaction requires mild conditions and subacidic pH (4.7). The activation of the carboxyl groups, as well as their subsequent bonding with the enzyme NH_2 groups is shown below in scheme 1.

Two experiments were conducted (using different concentration of N,N/-dicyclohexylcarbodiimide for each experiment - 1 и 5 mass%) in order to find out which concentration would be expedient. Urease was immobilized on two membranes with 64.2% grafted AA. The activities of the immobilized enzymes were found to be similar for each concentration of the activator. That was why all other experiments were conducted using 1 mass% solution of N,N/-dicyclohexylcarbodiimide.



Scheme 1. Reaction of membrane activation and reaction of enzyme immobilization

Seta Kűpcű et al. [26] have also found out that 1 mass% is the optimum concentration for the activation of the carboxyl groups in the support.

One very important aspect of an enzyme immobilization is to define the most suitable carrier. A series of experiments were conducted where urease was immobilized on membranes with different grafting degree of AA. The amount of bound protein and the enzyme activity were determined in each immobilized system. The results are presented in Figure 1 and 2.

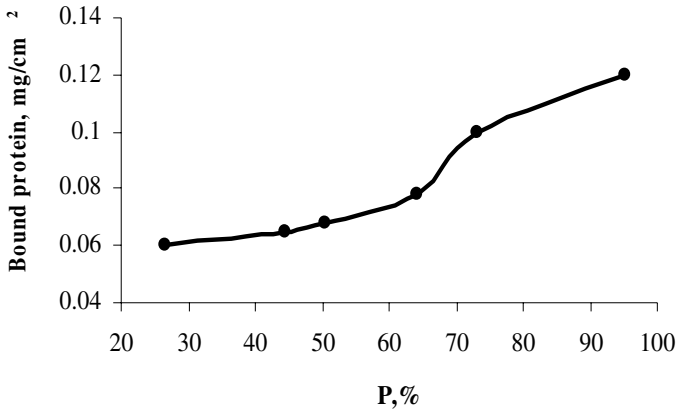


Figure 1. Dependence of the bound protein on the degree of grafting of acrylic acid

As can be seen from Figure 2 the relative enzyme activity had been increasing to the point of 64.2% grafting degree, after which the enzyme activity decreased. This could be explained with the local accumulation of protein (the highest amount of bound

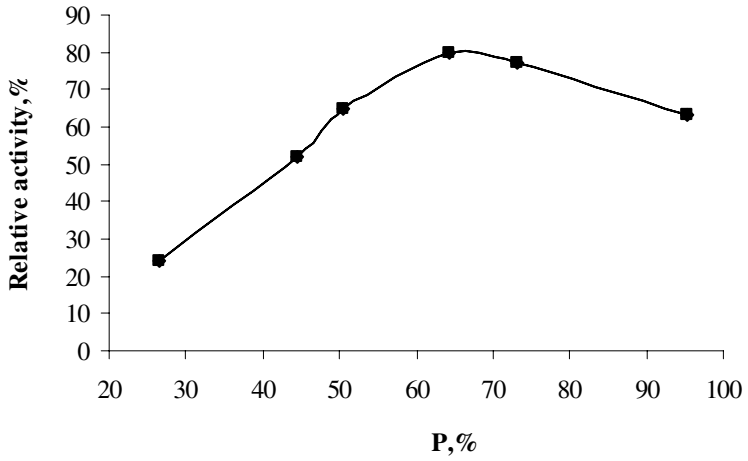


Figure 2. Dependence of the activity of immobilized urease on the grafting degree (P, %) of acrylic acid onto polyethene films

protein was detected for 73.2% и 95.2%), which led to some diffusion limitations for the substrate molecules to penetrate to the enzyme active centers. Relative activity characterizes the efficiency of immobilized enzyme system. It was calculated as ratio of specific activity of immobilized enzyme to specific activity of free enzyme. The high relative activity of the immobilized urease (80.32%) testifies to the suitability of

the 64.2% grafted CEM because there are only minor interior and exterior diffusion resistances regarding the substrate. Furthermore, there is a considerable amount of bound protein (6.01 mg/cm^2).

All other experiments concerning the evaluation of the main parameters of the immobilized enzyme – pH and thermal optimum, pH and thermal stability, as well as storage stability, of immobilized urease on CEM with grafted degree of AA - 64.2%.

The pH optimum of the immobilized urease was determined (Figure 3) by measuring the enzyme activity in buffer solutions with different pH (from 4 to 8). The highest enzyme activity of immobilized urease was observed at pH 6.0. This result was compared to the pH optimum of free urease (pH 5.8). The comparison led to the conclusion that the pH optimum of the immobilized urease had shifted to the alkali part of the pH scale toward to the pH optimum of the free urease. This is due to the residual electric charge on the membrane surface, which affects the distribution of H+ and OH- ions in the vicinity of the immobilized enzyme molecules.

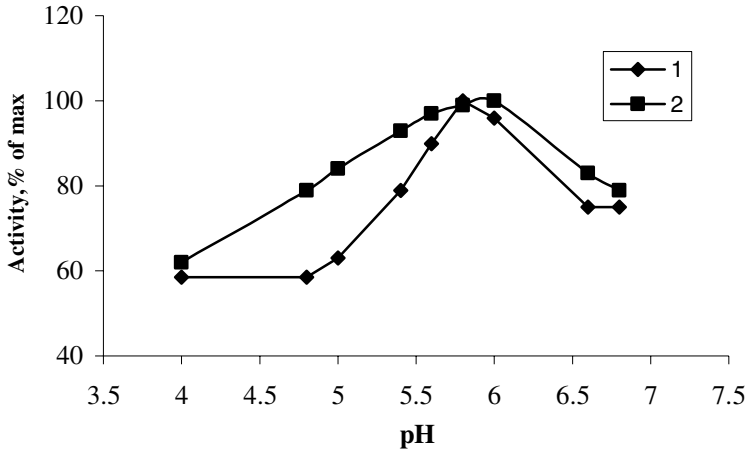


Figure 3. Dependence of the activity of free (1) and immobilized (2) urease (CEM with P=64.2%) on pH of the buffer solution

The thermal optimum of the immobilized enzyme was also determined by measiring the enzyme activity at different temperatures - from 20 to 40°C at pH 6.0 (Figure 4). The thermal optimum was found to be 30°C (only two degrees more than the thermal optimum of free urease - 28°C).

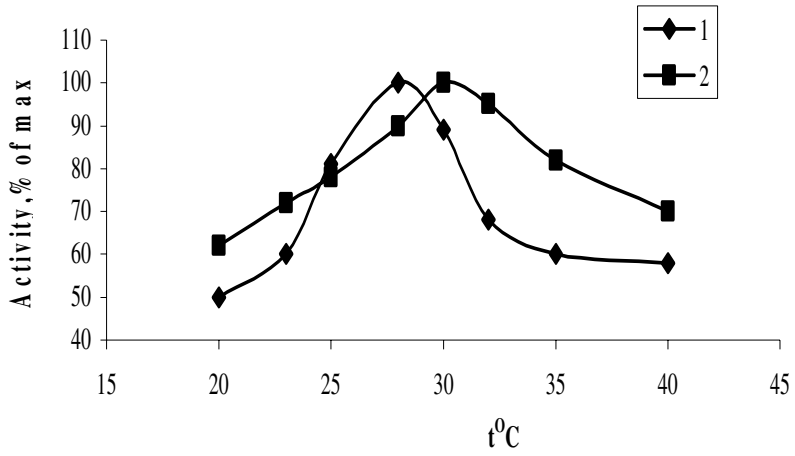


Figure 4. Dependence of the activity of free (1) and immobilized (2) urease (CEM with P=64.2%) on temperature

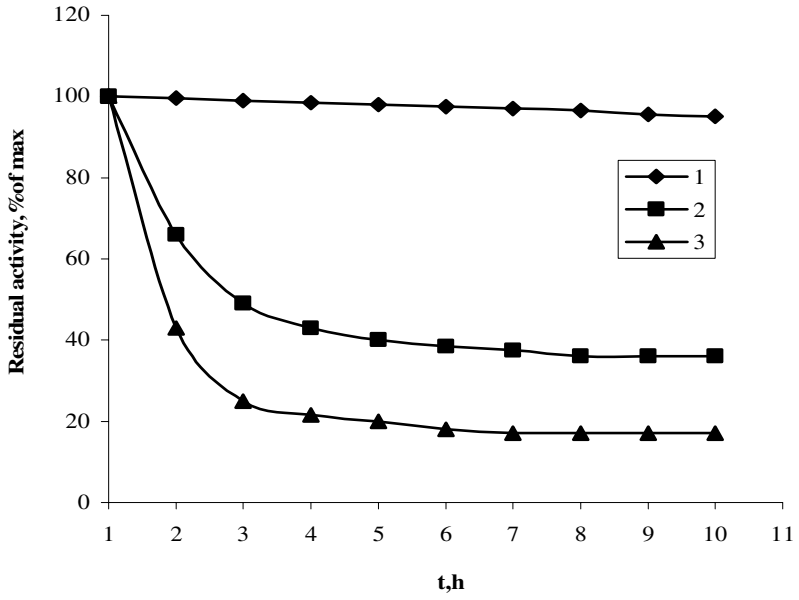


Figure 5. Dependence of the residual activity of the immobilized urease (CEM with P=64.2%) on the incubation time at 30 (1), 40(2) and 60(3)°C

The thermal stability of the immobilized urease was compared to the stability of the free enzyme at 28°C (t_{opt}). The results clearly showed the advantage of the immobilized system since the free urease lost 20% of its initial activity after 10 hours of incubation at 28°C. At 40°C free urease was inactivated for 5h.

Figure 6 displays the residual activity of the immobilized enzyme on membrane with 64.2% grafted AA for a storage period of 56 days at 4°C in a buffer solution (pH 6.0).

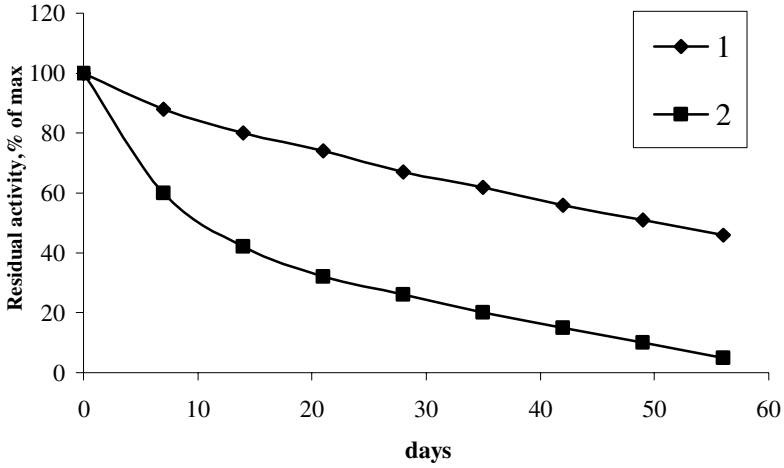


Figure 6. Residual activity of the immobilized urease (1) (CEM with P=64.2%) and free urease (2) during storage in a buffer solution (6.0) at 4°C

As can be seen from the trend line the enzyme activity has decreased twofold during that period. Since the free enzyme has lost 95% of its initial activity for the same storage period, this confirms the advantages of the immobilized system over the free enzyme form once more.

Conclusions

An enzyme polymer membrane was prepared by a covalent immobilization of urease on membrane obtained from radiation grafting of acrylic acid on thin polyethene films. The experimental results showed that membranes with 64.2% grafted degree of AA would be the most suitable support for an enzyme immobilization. The immobilized urease featured high relative activity – 80.32% and a considerable amount of bound protein – 6.01 mg/cm^2.

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