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Study of cellulolytic enzyme immobilization on copolymers of N-vinylformamide

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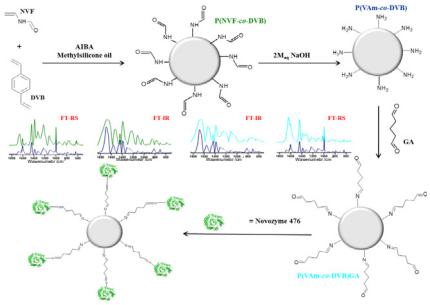
HIGHLIGHTS

- Copolymers of NVF with DVB showed high degree of crosslinking.
- Polymeric carriers were subjected to chemical modification.
- During hydrolysis amide groups were converted to amine groups.
- GA binded to P(VAm-*co*-DVB) by amide bond.
- Cellulase onto polymeric carriers was more efficient than native enzyme.

GRAPHICAL ABSTRACT



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ABSTRACT

This study was focused on finding of effective carriers suitable for the immobilization of cellulase. Copolymers of N-vinylformamide (NFV) and divinylbenzene (DVB) were synthesized by free radical crosslinking polymerization in inverse suspension. Methyl silicone oil was used as the continuous phase. Three polymeric carriers based on P(NVF-*co*-DVB) with varying degrees of crosslinking and spherical particles with different grain sizes were obtained. The formamide groups in these carriers were hydrolyzed to amino groups, yielding three P(VAm-*co*-DVB) polymers with vinylamine units. Enzyme, cellulase (Novozym® 476), was immobilized onto carriers with vinylamine (through glutaraldehyde) and vinylformamide groups (without glutaraldehyde). The efficiency of the enzyme immobilization was determined based on the enzymatic activity of the enzyme during the catalytic reaction relative to that of the native enzyme. All tested carriers were found to be effective carriers for the immobilization of cellulase. However, the catalytic activity of cellulase immobilized on the P(VAM-*co*-$DVB_{0.27}$)/2000/350 carrier was higher than that for the native enzyme. In addition, two molecular spectroscopy methods, Fourier-transform absorption infrared spectroscopy (FT-IR) and Fourier-transform Raman spectroscopy (FT-Raman), were used to analyze the carriers. These studies provided complete information regarding the structure of the studied copolymers.

Introduction

Currently, technological processes involving biocatalysts are used for the preparation of many products, e.g., chiral molecules, specialty and commodity chemicals, and for the conversion of

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unstable or complex molecules [1,2]. The reactions catalyzed by enzymes are carried out in environmentally friendly solvents under mild temperature and pressure conditions [3]. Thus, the reactions are typically simple to run in conventional manufacturing equipment and are cost-efficient.

In the food, textile, paper, or agriculture industry, commercially available oxidoreductase enzymes, particularly glycoside hydrolyses such as cellulases, that degrade cellulose to shorter carbohydrates (glucose) are widely used [4]. Cellulases include at least three basic fractions of enzymes: endo-(1,4) β-D-glucanase (EC 3.2.1.4), exo-(1,4) β-D-glucanase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) [5]. These enzymes are usually immobilized on insoluble carriers [6]. However, the immobilization of cellulase onto reversibly soluble methacrylate copolymers [7], nanofibrous PVA membranes [8], and silica gels [9,10] has also been studied.

There are no distinct criteria for the selection of a support for enzyme immobilization. Selections are made experimentally because the immobilization of an enzyme on a carrier can significantly alter the enzyme structure, leading to its deactivation. On the other hand, the proper size and shape of carriers allows for biocatalysts with improved activity and stability relative to those of the native enzyme to be obtained [11–13]. Biocatalytic processes are preferentially carried out using flow columns. Thus, spherically shaped carriers are favored [14]. Suspension polymerization of vinyl monomers, functional internal crosslinking monomers, allows for a spherical polymer network with specified physico-chemical properties to be obtained [15]. In addition, enzyme immobilization on carriers possessing active functional groups, i.e., primary amino, primary amide, and hydroxyl groups, involves the formation of stable biocatalytic systems possessing carrier–enzyme or carrier–spacer–enzyme covalent bonds [1,13]. The incorporation of so-called spacer molecules is readily applied because these molecules prevent the close approach of enzyme molecules to the carrier surface, thereby preventing diffusion and steric constraints from arising due to the multi-binding character of the enzyme on the carrier [16,17].

Carriers with reactive primary amino groups require the use of glutaraldehyde (GA) as a spacer molecule. In an aqueous medium, bifunctional glutaraldehyde reacts rapidly with the amino groups of the carrier and the α-amino groups of the enzyme [18,19] and creates stable imide bonds. The introduction of primary amino groups to the polymeric carrier is not easy and requires the use of carcinogen ethyleneimine. An alternative route is the use of N-vinylformamide (NVF) as the poly(vinylamine) PVAm precursor, which is readily hydrolyzed to PVAm [20]. Insoluble VAm carrier units can be obtained from NVF crosslinked with divinylbenzene (DVB) in a single-step hydrolysis process [21]. To date, on such supports, only α-amylase has been successfully immobilized [22].

Due to the interesting findings and applications previously described, polymer carriers based on DVB crosslinked with NVF via free radical crosslinking polymerization in the inverse suspension were synthesized in this work. The conditions of the aforementioned polymerization reaction (viscosity of the condensed phase and speed of stirring) were optimized to obtain spherically shaped particles of the P(NVF-*co*-DVB) carrier that, as a carrier–spacer–enzyme system, do not degrade (do not change structure) when applied in the alkali hydrolysis of microcrystalline cellulose. The crosslinking DVB monomer was used to secure the stability of the carrier grains formed during the aggressive alkaline hydrolysis reaction of the formamide groups [23]. Glutaraldehyde was used as the spacer molecule, whereas the commercially available cellulase Novozym® 476 was immobilized on the carrier–spacer system. Finally, the most efficient carrier for enzymatic catalysis (P(NVF-*co*-DVB$_{0.27}$)/2000/350) was spectroscopically characterized via Fourier-transform absorption infrared (FT-IR) and Fourier-transform Raman scattering (FT-Raman) spectroscopies, two complementary methods that are widely used in polymer chemistry not only for the identification of functional groups of organic compounds and for the investigation of conformation and backbone structures that are present in polymer systems [24] but also in monitoring polymer formation [25].

The combination of these two methods together with the development of imaging techniques, light-fiber optics and probes, and chemometric procedures yields valuable information in determining the distribution of copolymerized units, sequence length, chain branching, end-group composition, configurational and conformational isomerisms, hydrogen bonding, chain order and crystallinity, chain folding, and molecular orientations [26–28]. Such information can be obtained because absorption infrared spectroscopy mainly yields data about chemical species with a significant dipole moment (i.e., C=O, C–O, C–N, and N–H) that are commonly found in polymer side-chains [29]; in Raman spectra, on the other hand, bands due to the vibrations of the highly polarizable groups (i.e., C–C and C=C) present in polymer backbones are observed [30]. In the literature, an immense number of studies describe the application of these vibrational spectroscopic methods as routine analytical tools for analyzing polymers [31,32].

Experimental part

Reagents

N-vinylformamide (NVF) (Sigma–Aldrich), immediately before use, was distilled in vacuum over a Vigreoux column. 2,2′-Azobis(2-methylpropionamidine)dihydrochloride (AIBA), divinylbenzene (DVB), glutaraldehyde (GA 50%), and D-(+)-glucose monohydrate (Sigma–Aldrich) were used without further treatment. Oil POLSIL OM® 1000, POLSIL OM® 3000 (Silikony Polskie Sp. Z o. o.) and 3,5-dinitrosalicylic acid (Sigma–Aldrich) were also used as obtained. Microcrystalline cellulose (Sigma–Aldrich) was enzymatically hydrolyzed by Novozym 476 (Novozymes®).

Syntheses

Inverse suspension crosslinking copolymerization of NVF and DVB in silicone oil

Into a 250 mL thermostated four-neck round-bottom flask equipped with a reflux condenser, thermometer, stirrer and inert gas inlet, 50 mL of silicone oil (viscosity 1000 or 2000 cSt) was placed. Oil was deaerated at 80 °C under a flush of argon. A freshly prepared mixture of 5 g of NVF, 1.5 g of DVB (molar ratio NVF:DVB = 7:1), and 0.1197 g of AIBA (molar ratio AIBA:(DVB + NVF) = 1:100) was added under constant stirring (250, 350, or 500 rpm). All ingredients were mixed and heated to a temperature of 80 °C, at which the suspended particles were polymerized for 6 h under a gentle stream of argon. Thereafter, the product was filtered off, rinsed with toluene and hot water until the silicon oil was removed from the spheres. Finally, the particles were vacuum dried.

Depending on the experimental conditions, three polymeric carriers (P(NVF-*co*-DVBα)/η/rpm*) were synthesized (see Table 2 for parameters determining the crosslinking polymerization of NVF and DVB). Methyl silicone oil with a viscosity of 2000 cSt was prepared by mixing POLSIL OM® 1000 and POLSIL OM® 3000 in suitable proportions.

Swelling degree

The swelling degree (S_w) of the P(NVF-*co*-DVBα)/η/rpm* carriers (Table 2) was determined by the gravimetric method. The sample of the copolymer carrier was weighed (W_o) and immersed in deionized water at room temperature for 72 h. Then, the sample