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The immobilization of trypsin on soap-free P(MMA-EA-AA) latex particles

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Abstract

Poly(methyl methacrylate-ethyl acrylate-acrylic acid) latex particles with narrow size distribution and with surface carboxyl groups were produced by soap-free emulsion polymerization, and covalent immobilization of trypsin onto these particles was carried out by using the water-soluble carbodiimide (EDC) as an activating agent under various conditions. Different immobilization methods were employed and the factors affecting the efficiency and activity of the immobilized enzyme, such as the amount of trypsin and EDC, pH and temperature of the immobilization reaction were investigated. Results showed that both relatively high immobilization efficiency and high enzyme activity were achieved when pre-adsorption method was employed. The immobilization efficiency decreased as the trypsin amount increased, and increased as pH and temperature increased. When the EDC amount varied, the immobilization efficiency first increased significantly and then decreased slowly. A maximum of enzyme activity can be obtained at the optimum value of 958.0 mg trypsin/g dried particles and 372.5 mg EDC/g dried particles at 25 °C and pH 5.0. The immobilized trypsin exhibited much higher relative activity than its free counterpart.

Keywords: Soap-free latex particles; Trypsin; Enzyme immobilization; Covalent coupling; Methyl methacrylate based copolymer

1. Introduction

Trypsin is a highly efficient proteolytic enzyme that has been described as specific for catalyzing the breakdown of peptide linkages [1]. This enzyme has been used as an adjuvant in clinics to cure inflammation and dyspepsia, and also had found applications in tanned leather and raw silk treatment. In recent years, interest has increased in the potential utilization of this enzyme in food processing [2], enzyme reactors [3], and peptide synthesis [4]. However, trypsin is one of the least stable neutral proteases. Its rapid autolysis in solution makes it difficult to control the reaction conditions and as a consequence, the catalytic efficiency of this enzyme decreased and the cost of its use was increased [5].

Immobilized enzymes are currently a topic of active research in enzyme technology for their enhanced stability over their soluble counterparts. Puvanakrishnan et al. [6] immobilized trypsin on sand by a bifunctional agent with five different modes and established methods to estimate the proteolytic activity and protein content of immobilized enzyme. Tyagi and his coworkers [7] modified trypsin by pyromellitic dianhydride (PMDA) to increase its surface negative charges and then bind it to ion-exchangers, and this reversible immobilizing approach makes it possible to control the strength of binding by varying the extent of chemical modification. Ge et al. [8] developed a new method to immobilize trypsin on sliced shrimp-shell chitin and investigated its potential application by the continuous hydrolysis of casein in a pilot-scale column reactor. Silylate magnetic iron beads and silylated magnetic chitosan had also been used as carriers to immobilize trypsin by using formaldehyde or glutaraldehyde as coupling agent to facilitate the separation of the enzyme [9]. However, only a few of practical applications of the immobilized enzymes have been reported, and the key problem that should be solved urgently was still the choice of appropriate support material and simple immobilization method [8,10].

Submicron sized latex particles produced by emulsion polymerization can provide a large specific surface area. Recent advances in polymerization technology have enabled the preparation of a variety of latex particles, and among them,

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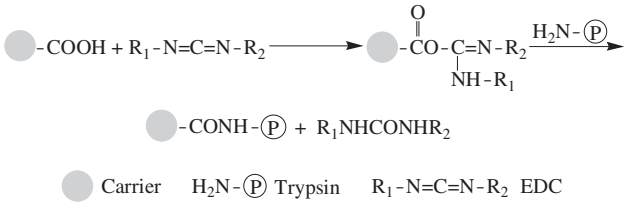

Fig. 1. Reaction of enzyme immobilization.

monodispersed and functionalized particles have attracted much attention. At present, most of functional polymer latices are prepared in the presence of an emulsifier [11–14], which is adverse to their applications. Moreover, most of the investigations were mainly focused on the styrene polymers. Compared with styrene, methyl methacrylate (MMA) is more hydrophilic, and it is supposed that PMMA particles with surface carboxyl groups may have many promising applications in biomedical and biochemical fields [15,16].

In our previous work [15], P(MMA-EA-AA) latex particles with narrow size distribution and with surface carboxyl groups were synthesized by a batch soap-free emulsion polymerization, and the diameter of particles can be well controlled in the range from 300 to 600 nm. In this study, trypsin was covalently immobilized onto these particles by using different methods, and the influences of trypsin and carbodiimide amount, pH and temperature of the reaction on immobilization efficiency and enzyme activity were investigated.

2. Experimental

2.1. Materials

Methyl methacrylate (MMA), ethyl acrylate (EA) and acrylic acid (AA) (all A. R. grade, First Chemical Reagent Factory, Tianjin, China) were purified by distillation under reduced pressure and kept in the refrigerator. Ammonium persulphate (APS) (A. R. grade, Aijian Modern Reagent Factory, Shanghai, China) was purified by recrystallization twice in water before use.

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Ultra Pure, BBI), Trypsin (Tissue Culture Grade, Amresco), *N*-Benzoyl-L-arginine ethyl hydrochloride (BAEE) (Biochemical Reagent, Shanghai Chemical Reagent Corporation, Shanghai, China), and Casein and 6-Aminocaproic acid (6-ACA) (Biochemical Reagent, Beijing Yili Fine Chemicals Co., Ltd., Beijing, China) were used without further purification. Phosphate buffer saline (PBS) solutions (66 mM) of different pH were prepared in our lab. All other reagents used were of analytical grade. Deionized and distilled water was employed.

2.2. Preparation and characterization of P(MMA-EA-AA) latex particles

P(MMA-EA-AA) latex was synthesized based on the reported method [15]. At first, 100 ml water and the mixture of 19 g of MMA, 1 g of EA, and 1.2 g of AA were added into the system in sequence. Then the reactor was immersed in a water bath in which the temperature was previously adjusted to 80 °C, and stirring speed was controlled at around 300 rpm. 0.285 g of APS in 24 ml water was introduced in three steps at different polymerization time: 16 ml at the beginning, 4 ml at 4 h and the last 4 ml at 6 h. After 7.5 h of polymerization, the polymerization was continued for an additional 0.5 h at 90 °C.

The size and morphology of latex particles were observed on Hitachi H-800 transmission electron microscope (TEM; Hitachi, Japan) using aqueous phosphotungstic acid as a staining agent. Monomer conversion (*Conv.*), particle diameter (D_p), the density of surface −COOH (S_d), and Zeta potential (ζ) were characterized as described elsewhere [15].

2.3. Immobilization of trypsin onto P(MMA-EA-AA) particles

Latex particles were purified three times by ultra-centrifugal washing with water at 4000 rpm, and the solid content of the latex was adjusted to about 2%. In the process of the immobilization, 1 g of the diluted latex was centrifuged at 12,000 rpm for 30 min and resuspended in 1 ml of PBS buffer at pH 5.0. Trypsin was covalently bonded onto the surface of the particles by three methods as follows: (the reaction of covalent coupling and three immobilization methods were showed in Figs. 1 and 2, respectively)

A. Pre-activation method: The activating reaction was allowed to proceed for 8 min after 5 mg of coupling agent (EDC) was

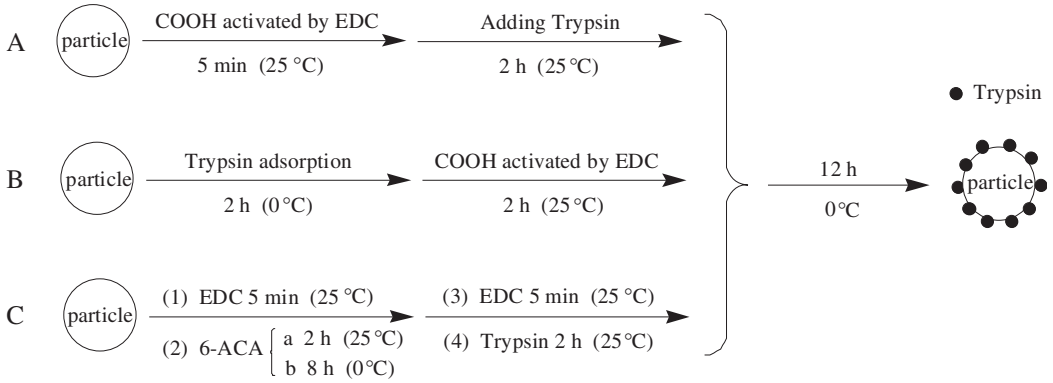

Fig. 2. Three methods of enzyme immobilization.