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Preparation of a pH-sensitive affinity precipitation polymer and its application in purification of trypsin☆

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ABSTRACT

Affinity precipitation is a potential protein purification technique, and preparation of affinity macroligands is a key problem. Until now, only a few polymers could be used for proteins purification in laboratory scale. In this paper, a reversibly water-soluble and pH-sensitive copolymer was synthesized by radical polymerization using methyl acrylic acid (MAA), methyl methacrylate (MMA), methacrylic acid 2-(dimethylamino) ethyl ester (DMAEMA) and *N*-hydroxymethyl acrylamide (NHMA). Its recovery was over 95% by adjusting pH to its isoelectric point (p*I* = 6.5). The classical epichlorohydrine activation method was used to activate the hydroxyl groups on the copolymer, and then *p*-amino benzamidine was coupled to the activated copolymer as an affinity ligand for trypsin adsorption. Various affinity macroligands with different ligand densities were prepared. The polymer with ligand density of 39.5 μmol/g, was used for purification of trpsin. The adsorption capacity of trypsin was found to be 132.5 mg/g polymer. Trypin was purified from 267.2 to 1637.1 IU/mg protein with 82.6% of activity yield and 6.13 folds of purification factor. The SDS-PAGE analysis demonstrated that trypsin with electrophoresis purity was obtained.

1. Introduction

Developing efficient protein purification techniques in large scale is still a challenge. For instance, a typical problem of the downstream process of bio-products is how to capture the highly diluted product from the complex process stream in the early stage [1]. Various chromatography techniques could not be used at large scale in early purification due to problems of column fouling and flow-rate limitation [2]. Since 1960s, bio-specific affinity separation methods in large scale have been investigated. Affinity precipitation is one of protein purification techniques.

Affinity precipitation is a technique combined specific affinity with precipitation with easy large scale operation. The polymer coupled with specific ligand is called "macroligand". The macroligand forms a complex with the target protein. Phase separation of the macroligand is induced by changes of the environment such as pH, temperature or ion strength etc.

Affinity macroligand polymers have to be recycled due to economic and environmental reasons. Until now, only a few of recyclable polymers have been used in laboratory scale to result in hindrance of affinity precipitation application. It is necessary to design and synthesize the recyclable polymers by simply changing the environmental conditions at a low cost. In this study, we developed a new pH-sensitive copolymer P_{MMDN} consisting of methyl acrylic acid (MAA), methyl methacrylate (MMA), methacrylic acid 2-(dimethylamino) ethyl ester (DMAEMA) and *N*-hydroxymethyl acrylamide (NHMA) as monomers. Recycle ability of the polymer was investigated and its application was examined by the immobilized *p*-aminobenzamidine as ligand to purify trypsin from its crude material.

2. Experiments

2.1. Reagents

p-Amino benzamidine (PABA) and *N*-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma. Pancreatic trypsin was obtained from DongWu SuZhou Biochemical Corporation. Azo-bis-isobutyronitrile (AIBN), methyl acrylic acid (MAA), methyl methacrylate (MMA), methacrylic acid 2-(dimethylamino) ethyl ester (DMAEMA) and epichlorohydrine (ECH) were reagent grade.

2.2. Methods

2.2.1. Synthesis of N-hydroxymethyl acrylamide (NHMA)

The synthesis procedure followed Shen Lili's method [3].

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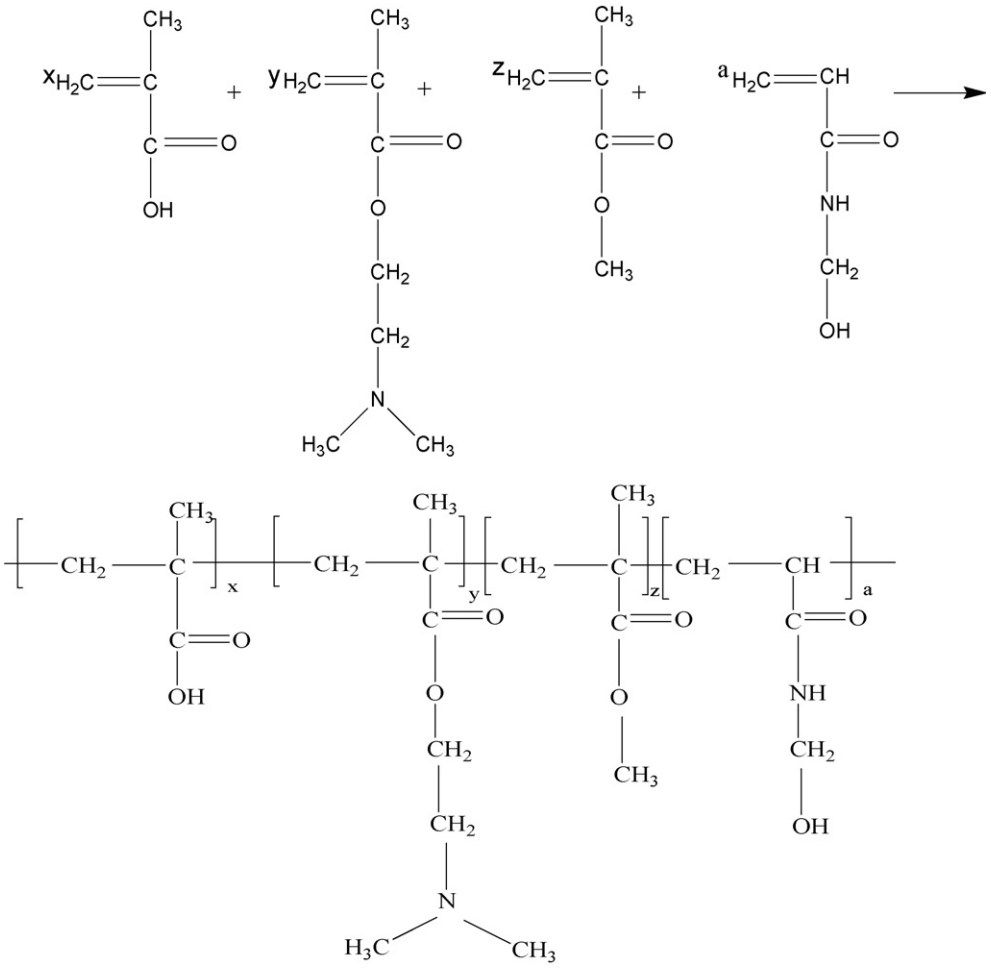

Fig. 1. Reaction formula.

2.2.2. P_{MMDN} copolymer polymerization

The mixture of specified amount of four monomers (MAA, MMA, DMAEMA and NHMA), AIBN as polymerization initiator and absolute ethanol as solvent were poured into a flask with a nitrogen maintained for 15 min. The reaction was carried out for 24 h at 60 °C and the products was precipitated from reaction solution and washed three times with acetone and absolute ethanol to remove residual monomers. The solid product was dried under vacuum condition. The possible reaction formula is as Fig. 1.

2.2.3. Characterization of P_{MMDN} copolymer

2.2.3.1. Testing the isoelectric point of P_{MMDN} copolymer. To study the precipitation behavior of the reversibly soluble polymers, the absorbance of the polymer solution was measured by a spectrophotometer at 420 nm [4]. Polymer P_{MMDN} was dissolved in 0.2 M sodium phosphate buffer containing 0.28 M NaCl in 2% (w/v) concentration (pH 7.2). The isoelecric point of the copolymer was defined as the pH at which the absorbance of the supernatant at 420 nm becomes to minimum. Each sample was tested in triplicates.

2.2.3.2. Testing the recovery of P_{MMDN} copolymer. The recovery of the polymer was determined by weight of the polymer after pI precipitation and dry. The recovery is defined as the ratio of the amount of recovered polymer to the initial amount of polymers.

2.2.3.3. FT-IR and ^{1}H NMR spectra. FT-IR spectroscopy was carried out using a Magna-IR 550 (Thermo Nicolet Corp., USA). The dry power of polymer was mixed with KBr and pressed into a tablet form. The FT-IR spectrum was then recorded. The ^{1}H NMR spectrum was carried out using a BRUKER AVANCE 500 (Germany).

2.2.4. Hydroxyl group activation and determination of epoxy group density of P_{MMDN}

The hydroxyl groups on the copolymer were activated by epichlorohydrine. 1 g polymer was dissolved in 100 ml NaOH (1.0 M), and 0.5–1.2 ml epichlorohydrine and 0.1 g $NaBH_4$ were added into it and then reaction carried out for 2 h at 40 °C. The reaction formula is as Fig. 2.

The determination of epoxy group density was carried out according to Chinese Standard GB1677-81 with HCl titration in acetone.

2.2.5. Immobilization of ligand on P_{MMDN} polymer

1 g activated polymer was dissolved in 100 ml water, and pH of the solution was adjusted to 11 with 1 M NaOH. 10 mg *p*-aminobenzamidine was added into it. Then the reaction carried out in a shaker at 100 rpm for 24 h (80 °C). After reaction finished, pH of the solution was adjusted to 6.5 to precipitate macroligands. The macroligands was washed with an excess of distilled water and store at 4 °C. The immobilization reaction process is as Fig. 3.

The ligand density on polymer P_{MMDN} was determined by mass balance of *p*-aminobenzamidine before and after reaction. Concentration of ligand in solution was measured spectrophotometrically at 292 nm, compared with calibrate curve.