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"솔보포빅"한 고분자 마이크로 캡슐을 이용한 효소 안정화에 관한 연구

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Stabilization of Enzyme in "Solvophobically" Controlled Polymer Microcapsules

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요 약: 본 연구는 효소의 활성을 저해하는 주위 환경, 특히 열로부터 효소의 활성을 장기간 유지할 수 있는 효소 안정화 시스템에 대한 것으로, 이 시스템은 poly(ε-caprolactone) (PCL) 마이크로 캡슐로, 파파인 효소를 모델 효소로 하여, poly(propylene glycol) (PPG) 층이 코어 효소층을 둘러싸고 있는 형태로 설계되어 있다. 공촛점 현미경 및 장기 열 안정도 결과를 분석해본 결과, 파파인 효소가 소수성 PPG로 둘러쌓여 있고, 배타적 볼륨 효과(exclusive volume effect)에 의해 안정화 되어 있음을 밝힐 수 있었다. 이와 같이 향상된 효소의 열 안정도는 소수성 사슬이 긴 PPG를 사용할수록 증가됨을 알 수 있었으며, 이것은 효소와 PPG 계면 사이에서 PPG 층이 파파인 효소를 효과적인 형태 고정(conformational anchoring)을 통해 안정화한 것임을 알 수 있었다.

Abstract: This article describes an enzyme stabilization method that allows the use of enzymes irrespective of environmental factors, especially heat, while maintaining their activity for a long time. We have designed enzyme microcapsules that consist of papain enzyme cores, poly(propylene glycol) interlayers, and poly(ε-caprolactone) walls. By confocal laser scanning microscopy measurements and the thermal stability of papain-loaded microcapsules, it is demonstrated that the papain is surrounded by a hydrophobic polyol layer and stabilized by the exclusive volume effect. In our study, improved thermal stability can be obtained by using more hydrophobic long-chained polyols, which is understood to be attributed to the effective formation of a hydrophobic polyol layer between the papain and the polymer wall by means of conformational anchoring in the interface.

Keywords: *conformational anchoring, enzymes, exclusive volume effect, stabilization, thermal stability*

1. Introduction

It has been well known that proteins favorably adhere to surfaces, which makes many applications, such as, chromatography, immunoassays, biocatalysis, and biosensors[1-3]. When we think of enzymes, however, this property may lead to another serious problem, that is, an easy molecular deformation on the surface, especially at high temperature. This is closely related to the adsorption of enzymes to a specific substrate and the collapse of their 3-D conformational structure, denaturation. Although considerable progress has been made in the stabilization of enzymes, the problem of easy denaturation at high temperature still remains. Until now, unique approaches, including DNA shuffling, mutagenesis, nano- or micro-particulation, and colloidal surface immobilization have provided an essential progress in enzyme stabilization[4-8]. However, a lack of high enough stability, processability, and productivity in actual applications needs further efforts.

Therefore, in this article, we describe a useful method for the stabilization of enzymes, which allows us to use enzymes irrelative to the environmental factors, especially to heat, all while maintaining their

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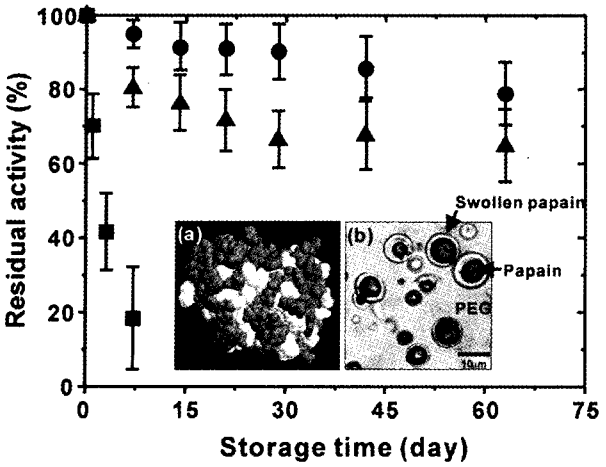

Figure 1. Thermal stability of papain enzyme in different dispersion media at 40℃: (square) in water; (triangle) in PEG of 400 g/moL; (circle) in PPG of 1103 g/mol. In this study, papain was selected as a model enzyme. The activity of papain dispersion was adjusted to *ca.* 100 tu/mg throughout the test, which corresponds to 1 wt% papain in the microcapsules. The optical micrographs in the figure box show the schematic molecular structure of papain (a) and its dispersion state in PEG (b).

activity for a long time. In the aspect of the stabilization of enzymes, our interest has been focused on the effect of polyols. At the end of 1990s, several papers have demonstrated fundamentally that the presence of polyols, such as glycerol, erythritol, sorbitol, and xylitol in aqueous formulations increases the thermal stability of enzymes and the extent is dependent on their stoichiometry[9-13]. As suggested by Lozano *et al.*, it has been understood that the polyols having preferential hydration are readily excluded from the immediate vicinity of enzymes in aqueous system[10,11]. Similarly, Kaushick et al. have reported that the polyols lower the surface tension of water and act via the so-called, solvophobic effect. Their understandings give us a hypothesis that if the solvophobic effect is maximized, the enzymes would be much more stable. Therefore, in this study, we try to reveal the importance of the hydrophobic polyols in stabilizing enzymes and build up a microcapsule system that can enhance the thermal stability of the enzymes therein. This study is expected to provide another useful approach to enzyme stabilization.

2. Material and Methods

2.1. Materials

Papain(ca. 1 × 10^3 tu/mg, total mesophilic Count/g: <100, CPC Wolfgang Muhlbauer GmbH), poly(ε-caprolactone) (PCL, 8 × 10^4 g/moL, Aldrich) were all regent grades and used without any further purification. Polypropylene glycol was also purchased from Aldrich (PPG, 400, 1 × 10^3, 2 × 10^3, 3.5 × 10^3 g/moL). Methylene chloride (MC, J.T. Baker) and polyethylene glycol (PEG, 400 g/moL, Aldrich) were used for papain microcapsules preparation. Poly(vinyl alcohol) (PVA, Mw = 8.8 × 10^4 9.2 × 10^4 g/moL, degree of saponification 87~89%, Kuraray) was used as a microcapsule stabilizer. N,N-dimethylated Casein (Sigma), sodium phosphate dibasic anhydrous (Sigma), Trichloroacetic acid (TCA, ACS grade, Sigma-aldrich), Citiric acid (Monohydrate, Sigma), L-tyrosine (minimum 98% (TLC), Sigma), BCA protein assay kit were used for papain activity measurement. Fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RBITC) were purchased from Fluka.

2.2. Measurements of Enzymatic Activity

Enzymatic activity of papain or its capsules is measured with the time intervals by the method using casein as a substrate. First, a given amount of sample is dissolved in 0.2 mL of the 1% N,N-dimethylated casein in 50 mM potassium phosphate buffer (pH 6.0). After one hour, 0.3 mL of 30% TCA solution is added to the reaction mixture and statically equilibrated at 40℃ for 30 min. After centrifuging and filtering, the activity of papain is determined by UV absorbance at 562 nm. Then, thermal stability is defined by residual activity compared with initial activity.

2.3. Preparation of Microcapsules

The microcapsules are produced by the conventional solvent evaporation method[18-20]. Details are as follow. 8.9 g of PCL and 1 g of PPG (selectively, 400, 1 × 10^3, 2 × 10^3, 3.5 × 10^3 g/moL) are dissolved in 40 g of MC. 0.1 g of powder-typed papain pre-wetted in 2 g of PEG is added to PCL/PPG/MC solution. Then, this mixture is emulsified in 1 wt% PVA aqueous solution with a MX-5 homogenizer (Nihonseiki) for 5 min at 5 × 10^3 rpm. Then, the MC in the dispersion droplets is

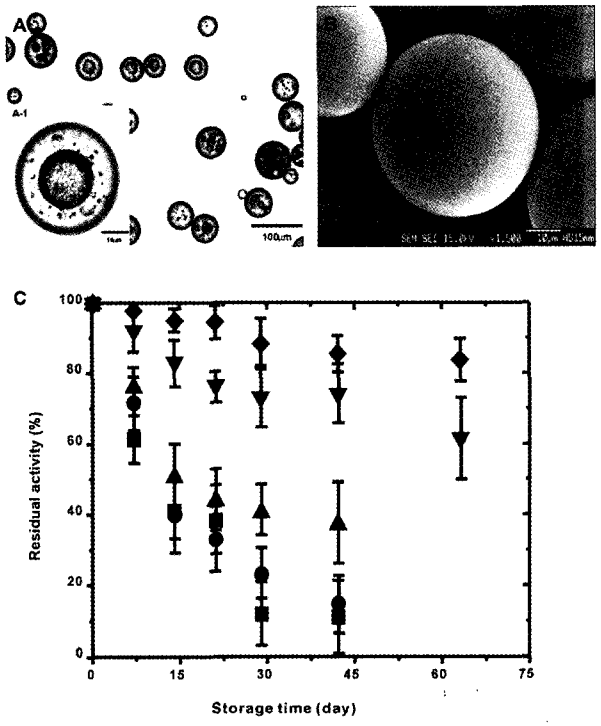

Figure 2. (A) An optical micrograph of papain-loaded PCL microcapsules dispersed in water. (B) A scanning electron microscope image of papain-loaded PCL microcapsules. (C) Effect of molecular weight of PPG on the thermal stability of papain loaded in PCL microcapsules in state of water suspension at 40℃: (square) in the absence of PPG; (circle) in the presence of 400 g/mol of PPG; (triangle) in 1103 g/moL of PPG; (reverse triangle) in 2103 g/moL of PPG; (diamond) in 3.5103 g/moL of PPG. The activity of papain in the suspension was also adjusted to *ca.* 100 tu/mg throughout the test.

evaporated completely at 30℃ for 1 h. The microcapsules produced are repeatedly washed by decantation in water and dried at ambient temperature. The yield is always above 10 wt% solid content.

3. Results and Discussion

In order to confirm a importance of the hydrophobic polyols in stabilizing enzymes preliminarily, we selected the papain[14-16] as a model enzyme (Figure 1a) that is one of the most instable enzymes and observed its stability at different dispersion media (Figure 1b). As shown in Figure 1, the thermal stability of the papain maintained for more than 2 months with swelling part-

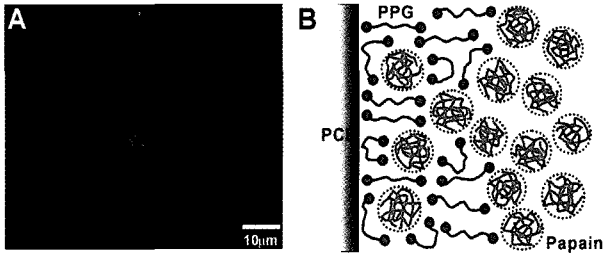

Figure 3. (A) A CLSM image of a PCL microcapsule containing FITC-labeled papain and RBITC-labeled PPG (3.5103 g/mol) at the same time. (B) a schematic understanding for solvophobic effect in the microcapsule system composed of enzyme, polyol, and polymer.

ially its surface by polyol molecules and without disturbing seriously its initial enzymatic activity. On the basis of this result, we can get a methodology that if the solvophobic effect is maximized in a confined system, the enzyme located therein can have a high stability, which enlarges its limited applications. To meet all the requirements, a capsule system is the best candidate. So, we designed a capsule structured with a core of enzymes and polyols and with a shell of biodegradable polymer. Here, it is expected that the enzymes should be completely surrounded by polyol molecules in the microcapsule. In this condition, a strong solvophobic effect can be imparted to the enzymes. Figure 2A and 2B represent typical optical microscope (Olympus BH-2) and scanning electron microscope (JSM-6330F, JEOL) images of the microcapsules that appear to contain the papain and PPG in the PCL shell in the size range of 20~50 μm (Figure A-1). Each microcapsule has several separated spherical domain(s), which indicates that the papain dispersions themselves are capsulated by PPG and PCL. However, with water-soluble polyols, such as PEG, glycerol, and butylene glycol, we cannot get such capsule structure, because they are likely to diffuse out during emulsifying process. In order to confirm the effect of hydrophobic polyols analytically, the papain microcapsules were prepared with changing the molecular weight of PPG. Then, they were dispersed evenly in neutralized polyacrylic acid (0.01 wt%, Carbopol 941, Noveon) aqueous gels (ca. 2.5 × 10^4 cps) and their enzymatic activity was measured with the time intervals at 40℃, and compared with initial activity. Figure 2C shows the thermal stability. It is noteworthy that

as the molecular weight increases, the thermal stability enhances dramatically. This result indicates that in the presence of water, the stabilization of the papain is attributed to the solvophobic effect induced in the interface of papain and PCL by PPG. Especially when the hydrophobicity is fortified by increasing the chain length of PPG, we can obtain much enhanced thermal stability.

Our next attention is naturally turned to the capsule morphology. We carried out experiments to visualize the exact location of each component in the microcapsules with confocal laser scanning microscope (CLSM, MRC-1024, Biorad). First, papain and PPG (3.5×10^3 g/moL) were labeled with fluorescein isothiocyanate (FITC, Fluka) and rhodamine B isothiocyanate (RBITC, Fluka), respectively, following the method reported by Lamprecht and Schreiber[20-22]. Then, the labeled components are encapsulated using the above-mentioned sampling process. Adjusting the laser in the green/red fluorescence mode, which yields two excitation wavelengths at 488 and 514 nm, FITC-labeled papain and RBTC-labeled PPG can be visualized with green channel and red channel, respectively, as shown in Figure 3A. From the CLSM image, we can observe exactly that the papain is located in the center and surrounded richly by PPG layer in the microcapsule. In such capsule morphology, the PPG molecules can immobilize effectively the papain in the interface, which is attributed possibly by the molecular buffering of hydrophobic, long chained PPG, as presented schematically in Figure 3B. In this case, we may also consider together the conformational anchoring of polyols in the hydrophobic PCL interface[23-26]. As a result, this study demonstrates nicely that the solvophobic effect induced by the hydrophobic polyol molecules in the microcapsules can be proposed to be an operative force for the stabilization of enzymes, which can be realized only in the capsulation or its extended system.

4. Conclusion

In conclusion, we described a new strategy to allow simple and effective stabilization of enzymes in microencapsulation. We have made the enzyme microcapsules that consist of enzyme cores, polyol inter-layers, and polymer walls. In this way, instable enzymes are likely to be surround by hydrophobic polyol layer and stabilized by imparting an effective solvophobic effect to them. Since enzymes can be used in a reaction system without losing their activity for a long time, such enzyme microcapsules are expected to find applications for controlled enzymatic reactions of pharmaceuticals, foods, and cosmetics.

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