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음이온 계면활성제에서 파파인 효소의 안정도에 관한 연구

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A Study on the Stabilization of the Papain Enzyme in the Moderately Concentrated Anionic Surfactant System

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요 약: 일반적으로 음이온 계면활성제는 효소의 disulfide bond를 분해시켜 효소의 활성이 없어진다. 따라서 특정한 캡슐에 효소를 포집하여 안정도를 증대시킨다. 본 연구에서는 polyethylene glycol (PEG), polypropylene glycol (PPG), 그리고 PEG-PPG-PEG block copolymer 등의 폴리올을 이용하여 papain 효소의 안정도를 증대시켰다. Energy dispersive spectroscopy (EDS)와 confocal laser scanning microscope (CLSM) 분석을 통하여 폴리올은 고분자층과 효소의 중간에 위치하며, 이들은 완충액으로 작용하여 효소의 안정도를 증대시키는 것으로 확인하였다. 또한, 이온 복합체를 이용하여 다층 캡슐을 제조하여 wash-off 형태의 세정제에 응용하였다. 세정제 내에서 계면활성제와 물은 효소캡슐의 표면에 분산되었으며, 캡슐의 중앙부분으로 서서히 침투되었다. 반면에 본 연구에서 사용된 sodium lauroyl sarcosinate와 polyguaternium-6는 물이 효소부분으로 침투하지 않는 것을 *in vivo* 시험을 통하여 확인하였다.

Abstract: Even in the moderately concentrated anionic surfactant system, some special encapsulation method can shield the papain enzyme from proteolytic attacks. The stabilization of enzyme has been a major issue for successful therapies. In this study, we first stabilized an enzyme, papain in the microcapsules by using polyols, polyethyleneglycol (PEG), polypropyleneglycol (PPG), and PEG-PPG-PEG block copolymer. In the analysis of EDS and CLSM, it was demonstrated that polyols are effectively located in the interface of papain and polymer. Polyols located in the interface had an ability to buffer the external triggers by hydrophobic partitioning, preventing consequently the catalytic activity of papain in the microcapsules. Second, we introduced multi-layer capsulation methods containing ion complex. Such a moderately concentrated anionic surfactant system as wash-off cleansers, surfactants and waters can cause instability of entrapped enzymes. Surfactants and water in our final products swell the surface of enzyme capsules and penetrate into the core so easily that we can not achieve the effect of enzyme, papain. In this case, the ion complex multi-layer capsule composed of sodium lauroyl sarcosinate and polyquaternium-6 could effectively prevent water from penetration into the core enzyme, followed by *in vivo* test, and evaluate the stratum corneum (SC) turn-over speed.

Keywords: *papain enzyme, encapsulation ion complex, sodium lauroyl sarcosinate, polyquaternium-6, anionic surfactant*

1. Introductions

Wash-off type facial cleansing products such as cleansing foams are widely used. They are sometimes considered as a part of skin care and asked for some specific efficacy over their original cleansing power. Customers expect such effects as moisturization, whitening, brightening while wash-off cleansing.

In this article, we focused on the papain enzyme as an skin brightening active ingredient in wash-off

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cleansing foams. As widely known, papain enzymes are efficiently acted on the skin surface and help the SC turn-over. They can make skin bright.

But enzyme-based products easily become useless because of their denaturation.

Proteins are apt to adhere to surfaces, which results in many applications, for example in chromatography, immunoassays, biocatalysis, and biosensors[1-4]. When it comes to enzyme, however, this property may lead to another serious problem, that is, easy molecular deformation. This is closely related to the absorption of enzyme to a specific substrate and collapse of its three dimensional (3D) conformational structutre, that is denaturation. Therefore, both the storage and operational stabilities seriously affect the usefulness of enzyme based products. Enzymes should retain their catalytic abilities in the period between manufacture and eventual use. At the same time, enzymes should persist in their activities during an operation.

Previously, we first stabilized an enzyme, papain in the microcapsules by using polyols, polyethylene glycol (PEG), polypropyleneglycol (PPG), and PEG-PPG-PEG block copolymer[5]. In the analysis of confocal laser scanning microscopy, it was demonstrated that polyols are effectively located in the interface of papain and polymer. Polyols located in the interface had an ability to buffer the external triggers by hydrophobic partitioning, preventing consequently the denaturation of papain in the microcapsules.

With this stabilization method, we are very successful in some skin care products such as cleansing powders and emulsions[6]. Cleansing powders are non water-based. Emulsions usually contain surfactants in somewhat low concentration (less than 5 %).

Common skin cleansing products contain 30 ~ 70 % of water and more than 15 % of surfactants. Such a moderately concentrated anionic surfactant system as wash-off cleansers, surfactants and waters can cause instability of entrapped enzymes. As shown in Table 1, 30 days later, the residual activity of papain drops to 0 ℃ at both room temperature and 40 ℃ in pH 9.3 soap type wash-off cleansers. Surfactants and water in our final products swell the surface of enzyme capsules and penetrate into the core so easily that we can not achieve the effect of enzyme, papain.

In this study, we introduced multi-layer capsulation methods containing ion complex. In this case, the ion complex multi-layer capsule composed of sodium lauroyl sarcosinate and polyquaternium-6 could effectively prevent water from penetration into the core enzyme, followed by *in vivo* test, and evaluate the SC turn-over speed.

Table 1. Residual Activities of Papain Microcapsules in pH 9.3 Soap Type Wash-off Cleansers

Storage time (days) \ Condition	Room tempreature	40 ℃
10	100 %	67 %
30	0 %	0 %

2. Experimentals

2.1. Preparation of Multi-layer Capsule

The multi-layer capsules were produced by the fluid bed process. The fluid bed coater, GLATT coater GPCG-120 used. Details are as follows: 600 g of papain microcapsules (which were produced by the method cited in the reference[5]) and 156 g of hydroxypropyl methyl cellulose (Amerchol Corporation, USA) are dispersed in 340 g of water and 340 g of ethyl alcohol. Papain/HPMC dispersion is loaded on 925 g of sugar (Sugar sphere25, ISP, Italy) by bottom spray coating process. The solvents are dried while the coating process. We obtained multi-layer capsules by repeating the fluid bed process mentioned above.

Below is the list of composition of each layer :

* 1st layer: 87.5 g of shellac in 1375 g of ethyl alcohol solution.
* 2nd layer: 87.5 g of stearic acid in 875 g of ethyl alcohol solution.
* 3rd layer: hydroxypropyl methyl cellulose in 340 g of water and 340 g of ethyl alcohol solution
* 4th layer: 175 g of polyquaternium-6 (Ciba Specialty Chemicals Corporation, Swtzerland) in 437.5 g of water solution.
* 5th layer: the same as the 3rd layer
* 6th layer: 70 g of sodium lauroyl sarcosinate (Croda Inc., UK) in 65.6 g of ethyl alcohol and 372 g of water solution.

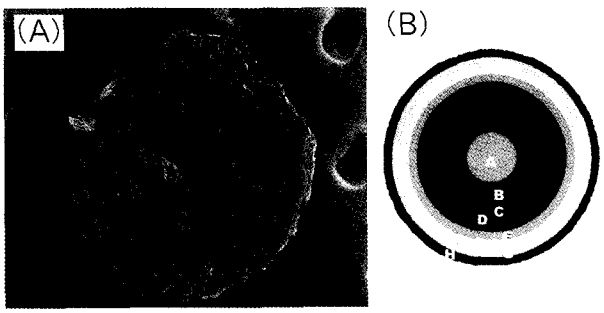

Figure 1. (A) A scanning electron microscope image of the cross section of an ion complex multi-layer capsule. (B) Schematic diagram of a multi-layer capsule A: sugar (as a seed) & papain microcapsule, HPMC, B: 1st layer : shellac, C: 2nd layer: stearyl acid, D: 3rd layer : HPMC, E: 4th layer : polyquaternium-6, F: 5th layer : HPMC, G: 6th layer : sodium lauroyl sarcosinate, H: 7th layer : HPMC.

* 7^{th} layer: the same as the 3rd layer

The yield was always above 90 wt% weight content.

2.2. Measurement of Enzyme Activities

The enzyme activity of papain or its capsule was measured by the Pierce method using bicinchoninic acid (BCA protein assay kit, Pierce, Prod no. 23225)[7]. First, 50 mg of the prepared papain multi-layer capsules were dispersed in 0.5 mL of dimethylsulfoxide (DMSO, Aldrich, USA) and stirred at 37 ℃ for 1 h. 1 mL of 0.5 wt% sodium lauryl sulfate-0.05 N NaOH solution was added to the mixture. After 1 h at room temperature, filtering followed by mixing with BCA solution After equlilibrating at 37 ℃ for 30 min, the activity of papain was determined by UV absorbance at 562 nm. The stability was defined by residual activity compared with initial activity.

2.3. Microscopic Observations

The cross section of multi-layer capsules was observed with a scanning electron microscope (SEM, JSM-6300, JEOL, Japan).

2.4. *In vivo* Tests

5-[dimethylamino]naphthalene-1-sulfonyl chloride (dancyl chloride, Sigma, USA) was patched for 24 h as big as 20 mm diameter circle in the forearms of 22 candidates. The cleansing products containing 0.5 wt% of the multi-layer papain capsules were applicated in the fluorescent dancyl chloride dyed forearms for 30 s and washed off for another 30 s. During 3 weeks, SC turn-over degree, from which the enzyme activity could be estimated, was analyzed by image analyzer (Image Pro. Plus Ver.4.5). *In vivo* tests were supported by IEC KOREA.

Table 2. Residual Activities vs. Papain Concentrations (wt%) of Multi-layer Capsules in pH 9.3 Soap Type Wash-off Cleansers

Papain concentrations (wt%)	0.3	0.6	0.9
Residual activities (%)	0.3	0.5	0.6

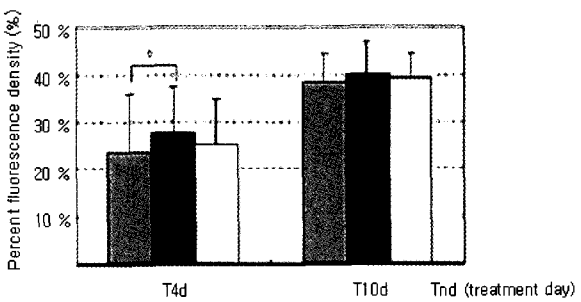

Figure 2. % Variance of fluorescence density (N) non-treated. (A) a wash-off cleanser treated. (B) a cleansing powder treated. A wash-off cleanser contains 0.3 % ion complex multi-layer papain capsules and a cleansing powder contains 0.5 % papain microcapsules.

3. Results and Discussion

In this study, we aim to demonstrate that enzymes can be stabilized using ion complex multi-layer capsulation method even in the moderately concentrated anionic surfactant system. Our scheme is to form an immediate barrier as soon as waters and surfactants penetrate into to the enzyme capsules. Water and surfactants in our final products may swell the 7^{th} layer of our enzyme capsules and sink into the 6^{th} layer, then immediately form the insoluble ion-complex with 4^{th} layer, which can block the continuous penetration into the core enzyme. Between the layers, shellac or HPMC was located as buffer.

Figure 1(A) represents a scanning electron microscope image of the cross section of an ion complex multi-layer capsule that appear to contain the papain microcapsule-sugar seed. Each layer was successfully loaded as in the schematic diagram of a multi-layer

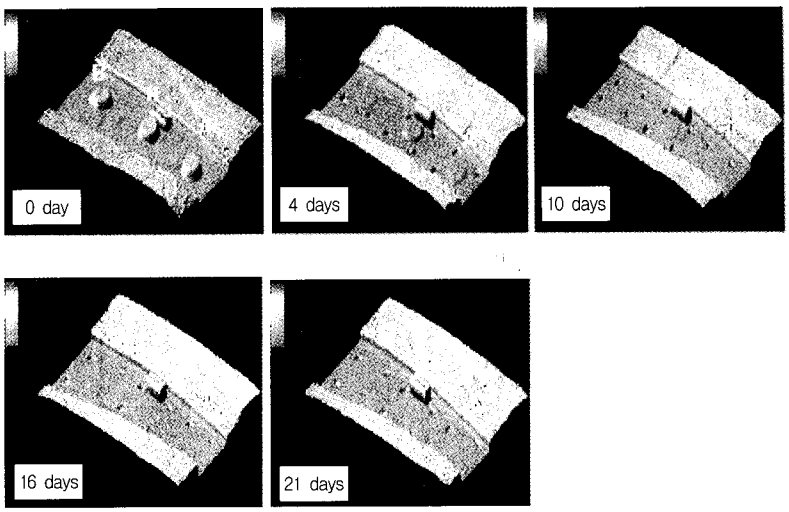

Figure 3. Results of image analyzation, conditions are the same as the Figure 2. The numbers labeled in each pictures are the times (day) during the treatment.

capsule (Figure 1(B)).

In our ion complex multi-layer capsulation system, the enzymatic activities could be detected directly from papain multi-layer capsules.

As shown in Table 2, we can see the positive relationship between the residual activities and papain concentrations. But the residual activities are somewhat low because of the difficulties of papain recovering procedure from our multi-layer system. So we need to evaluate the SC turn-over speed by *in vivo* tests.

The results of *in vivo* tests demonstrates that ion complex multi-layer capsules successfully stimulate the SC turn-over. As we can see in Figure 2 and Figure 3, a wash-off cleanser containing 0.5 % ion complex multi-layer papain capsules efficiently remove the aggregation of the SC. A cleansing powder containing 0.5 % papain microcapsules was chosen as control. The % variances of fluorescence density were meaningful in both cases. Figure 3 represents that the aggregation of the SC was almost disappeared after 16 days of treatment with papain multi-layer capsules as well as papain microcapsules. This means that papain enzymes are successfully stabilized by an ion complex multi-layer encapsulation method even in the surfactants based wash-off cleansers.

4. Conclusion

We introduced a new strategy to allow effective stabilization of enzyme. We have made ion complex multi-layer capsules that consist of enzyme cores, a cation layer and an anion layer. Between the layers exists shellac or HPMC. This system effectively bars water from penetration into the core enzyme even in the moderately concentrated cleansing products. Consumers can expect the real SC-disaggregation effect while comfortable washing.

Appendix

We can define variance of fluorescence (%) as Eq. (1).

Variance of fluorescence density (%)

$$= (T_{nd} - T_{0d}) / T_{0d} \quad \text{Eq. (1)}$$

Fluorescence density (T) is the intensity mean of degrees of whiteness when the original pictures are converted to black and white.

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