新規食品保存剤としての応用を目指したモノメトキシポリエチレングリコールコンジュゲート化リゾチームの酵素化学的安定性

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Stability of the enzyme activity of a lysozyme by conjugating with monomethoxypolyethylene glycol for application as a new type of food additive for preservation

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我々の食生活に対する食品添加物の貢献は多大であり、抗菌、防腐、酸化防止等の目的で多くの食品添加物が利用されている。食品添加物による食品の品質保持をより卓越したものにするためには、摂取に対する安全性が十分に保証された上で、より長期にわたって機能を発揮できる安定な食品添加物を使用することが望まれる。

そこで我々は、有用な食品保存剤の一つであるリゾチームをより機能的な新規食品添加物にするために、両親媒性合成高分子化合物モノメトキシポリエチレングリコール (mPEG) を導入し、コンジュゲート (mPEG-リゾチーム) を創製した。mPEG-リゾチームにおいては、結合した mPEG が酵素作用の妨げになる可能性を有していること、および食品添加物としての使用を計画していることから、酵素活性面の性質を把握することは極めて重要である。これまでに、熱力学的解析により mPEG の導入がリゾチームを高度に安定化させることを報告していたが、mPEG-リゾチームの酵素活性上の安定性については未検討のままであった。

本研究では、mPEG-リゾチームが (i) 37℃ の熱条件下では20時間後も酵素活性を完全に保持し、80時間後も40%以上の活性を示したこと、および (ii) 90℃・30分の熱処理後も100%近い活性を保持し、98℃においても約70%の活性を示したこと、を見出した。また、mPEG-リゾチームが一部の有機溶媒中でも酵素活性を示すことも明らかになった。以上のことから、mPEG-リゾチームについて、我々の食生活を支える新しいタイプの食品添加物としての利用や食品製造への応用の可能性が高まったと示唆された。

INTRODUCTION

Food additives greatly contribute to the maintenance of food quality and are used for several purposes, such as

antimicrobial activity, preservation, and anti-oxidation. In addition, many proteins and enzymes are frequently used as food additives due to their attractive bioactivities. For more effective maintenance of food quality, it is appropri-

ate to use food additives with improved functions. Therefore, food additives that express their bioactivities for a longer period and are safe for consumption are desired.

The conjugation of protein/peptide food additives with polymer materials; i.e., protein conjugation, has received considerable attention as a technique for obtaining new types of food additives with improved functions. We have synthesized a lysozyme-monomethoxypolyethylene glycol conjugate (mPEG-lysozyme) as a food additive-polymer conjugate. Hen egg-white lysozyme is a relatively small protein of 129 amino acid residues with a molecular weight of 14.3 kDa and a muramidase that hydrolyzes the β -1,4-links between *N*-acetyl-muramic acid and *N*-acetyl-D-glucosamine in the peptidoglycans of bacterial cell walls.¹⁾ Therefore, lysozyme is a useful natural food additive for preserving the quality of foods because of its potent antimicrobial activity. mPEG is an amphipathic polymer with the general structure $\text{CH}_3(\text{OCH}_2\text{CH}_2)_n\text{OH}$, and its terminal hydroxyl group has been used to prepare derivatives that are useful for modifying proteins under mild conditions.²⁾ Most of the derivatives of mPEG used so far for preparing protein-polymer conjugates were designed to modify the amino groups in proteins through an amide linkage, and such modifications have been used extensively to resolve several disadvantageous features of proteins.^{2,3-5)} In our previous study, we determined the unique characteristics of mPEG-lysozyme: antimicrobial activity for Gram-negative bacteria and low immunogenicity.^{6,7)} In addition, we demonstrated that the thermodynamic stability of the lysozyme molecule was greatly improved by its conjugation with mPEG by calculating several of its thermodynamic parameters from a spectroscopic analysis, from which we also deduced the stabilizing mechanism.⁸⁾ However, the stability of the enzyme activity of mPEG-lysozyme remains unknown. Especially, we must demonstrate that mPEG-lysozyme has its enzyme activity for long period and under the severer conditions as a food additive. In this study, to obtain better understanding of the stability of the enzyme activity of mPEG-lysozyme, we clarified that mPEG-lysozyme indicated its enzyme activity for a longer period than native lysozyme and had the resistance against the heat-treatments. In addition, we studied the stability of mPEG-lysozyme in organic solvents since almost all natural enzymes are easily denatured and inactivated in the presence of organic solvents.

MATERIALS AND METHODS

Materials

Hen egg-white lysozyme (EC 3.2.1.17) was obtained

from Seikagaku Kogyo Co. (Tokyo, Japan). mPEG with a mean molecular weight of 5,000 was purchased from Polyscience Inc. (Warrington, USA). *Micrococcus luteus* cells were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dimethyl sulfoxide (DMSO) and acetonitrile were products of Nacalai Tesque (Kyoto, Japan). All other reagents used in this study were of high quality analytical grade.

Preparation of mPEG-lysozyme

To conjugate the lysozyme with mPEG, an active succinyl ester derivative of mPEG was prepared as previously reported.^{2,6)} The preparation of mPEG-lysozyme, in which mPEG was covalently attached to Lys 33, was performed under the mild conditions and the resultant mPEG-lysozyme was finally purified with use of columns of CM-Toyopearl 650M and Sephadex G-75 as described previously.⁶⁾ The concentration of lysozyme was measured spectrophotometrically using an absorption coefficient of $A_{1\text{cm}} = 26.9$ at 280 nm. The same value was used for mPEG-lysozyme because it has an identical UV-absorption spectrum to native lysozyme.⁶⁾

Assay of lysozyme activity

The enzyme activity of mPEG-lysozyme towards *M. luteus* cells was measured by a turbidometric method.⁹⁾ Two milliliters of a *M. luteus* cells suspension ($A_{450} = 1.0$) in 50 mM acetate buffer at pH 5.0 were incubated at 37°C. Then, 50 μl of enzyme solution (1.8×10^{-6} M) in the same buffer pre-incubated at 37°C for the given time were added to this substrate solution. The change in the absorbance of the reaction mixture was measured at 450 nm for 10 min using a UV-2200 SHIMADZU UV-VIS recording spectrophotometer. In the enzyme activity assay of native lysozyme, the enzyme reaction was performed in 50 mM acetate buffer at pH 6.0.

To measure the enzyme activity of mPEG-lysozyme in organic solvents, buffers containing the given concentrations of DMSO (0–50%) or acetonitrile (0–50%) were used. After adding the enzyme solution to the substrate solution, the reaction mixture was incubated at 25°C for 10 min, and the absorbance was measured at 450 nm. The assay of lysozyme was performed in triplicate.

Thermostability assay

Samples of enzyme solution (1.8×10^{-6} M) in 50 mM acetate buffer (pH 5.0) were kept at the given temperatures (25, 80, 85, 90, and 98°C) for 30 min. Each solution was cooled rapidly with tap water for 5 min, and the enzyme activity towards *M. luteus* cells was measured at pH 5.0. In the thermostability assay of native lysozyme, the enzyme reaction was performed at pH 6.0. The thermostability assay was performed in triplicate.

Statistical analysis

Enzyme assay data were expressed as means $\pm$ S.E.M. Statistical significant test was performed by Student's t-test. Differences with p values less than 0.05 were considered statistically significant.

RESULTS

We prepared a lysozyme derivative conjugated with mPEG, mPEG-lysozyme, using the "protein tailoring" method to obtain a food additive suitable for preservation. As previously reported, the optimal pH for the enzyme activities of mPEG-lysozyme and native lysozyme towards *M. luteus* cells are 5.0 and 6.0, respectively.⁶⁾ Therefore, the enzyme activity assays were performed under the optimal pH conditions for each lysozyme in this study. Figure 1 shows the effects of heat-treatment at 37°C on the enzyme activities of mPEG-lysozyme and native lysozyme. mPEG-lysozyme was stable and retained 100% of its enzyme activity towards *M. luteus* cells after being heated at 37°C for 20 hours, while native lysozyme lost half of its enzyme activity. In addition, mPEG-lysozyme also maintained higher enzyme activity than native lysozyme after heating at 37°C for 80 hours.

Figure 2 compares the thermostability of mPEG-lysozyme with that of native lysozyme. When the samples were heated at 90°C for 30 min, mPEG-lysozyme maintained most of its enzyme activity towards *M. luteus* cells. Moreover, mPEG-lysozyme retained 70% of its enzyme activity after heat-treatment at 98°C for 30 min, while only 10% of the activity of native lysozyme was retained.

The enzyme activity of mPEG-lysozyme in organic solvents is compared with that of native lysozyme in Figure 3. Native lysozyme showed a markedly reduced enzyme activity towards *M. luteus* cells in organic solvents such as DMSO and acetonitrile. On the other hand, mPEG-lysozyme retained high activity in both DMSO and acetonitrile. In particular, in DMSO, mPEG-lysozyme retained nearly 80% of its enzyme activity towards *M. luteus* cells at a concentration of 30% DMSO, while native lysozyme only retained 20% of its activity, as shown in Figure 3 (a).

DISCUSSION

It was revealed that mPEG-lysozyme retained its ability to catalyze the hydrolysis of beta-1, 4-glycoside linkages in *M. luteus* cells for longer period (Figure 1) and under higher temperatures (Figure 2), compared to the native lysozyme that is currently used as a food additive for preservation. Considering that disruption of the conformational structure of a protein causes the disappearance of its

function in many cases, we assumed that the stability of the three-dimensional structure of the lysozyme molecule in mPEG-lysozyme would be markedly increased by conjugating it with mPEG. In fact, we previously demonstrated that the temperature of the midpoint of the denaturation-transition of lysozyme was greatly improved by conjugating it with mPEG, and the stable nature of mPEG-lysozyme was mainly ascribed to the large energy difference between the folded and the unfolded state, which was induced by lowering the energy level of the folded state.⁴⁾ Moreover, it was suggested that the bound mPEG did not physically inhibit the binding action of lysozyme with its substrate and the interactions formed between lysozyme and mPEG did not affect the catalytic action of lysozyme from the evidence that mPEG-lysozyme indicated its enzyme activity under the severe conditions (Figures 1 and 2). Since our present dietary habits are supported by many food additives, the use of food additives with improved functions is important for improving our dietary habits. When the lysozyme derivative which has the excellent enzyme stability by conjugating with mPEG is used as a food additive for preservation, it seems that mPEG-lysozyme contributes to the maintenance of food quality for longer period. Considering that food additives that adjust the pH of acidifying foods are frequently used for maintaining food quality, mPEG-lysozyme, which can express its enzyme activity in more acidic media, is also suggested to be useful for such functions.

In the food industry, there are numerous advantages of employing enzymes as catalysts in organic solvents or aqueous solutions containing organic solvents instead of water.¹⁰⁾ If enzymes can retain their activity in organic sol-

Figure 1 Stability of the enzyme activity of mPEG-lysozyme.

The enzyme activities of mPEG-lysozyme (open circles) and native lysozyme (closed circles) towards *M. luteus* cells were spectrophotometrically measured at 37°C after pre-incubating at 37°C. The enzyme activities of the two enzymes before incubation were taken as 100%. Results show mean $\pm$ S.E.M. of three independent measurements (*p<0.05, **p<0.01).

Figure 2 Thermostability of the enzyme activity of mPEG-lysozyme.

The thermostabilities of mPEG-lysozyme and native lysozyme were measured as described in the text. The stabilities of both enzymes are shown in terms of the remaining activity towards *M. luteus* cells after heat-treatment. The enzyme activities of the untreated samples were taken as 100%. Results show mean $\pm$ S.E.M. of three independent measurements (** $p < 0.01$).

Figure 3 Enzyme activity of mPEG-lysozyme in organic solvents.

In the enzyme activity in organic solvents assay, buffers containing the given concentrations of (a) DMSO or (b) acetonitrile were used. The enzyme activities of mPEG-lysozyme (open circles) and native lysozyme (closed circles) towards *M. luteus* cells were spectrophotometrically measured at 25°C. The enzyme activities of the two enzymes before incubation were taken as 100%. Results show mean $\pm$ S.E.M. of three independent measurements (* $p < 0.05$, ** $p < 0.01$).

vents, improvements in reaction conditions and product yields will be realized because it makes it easy to purify and collect the reaction products. Thus, when an enzyme reaction involves water-insoluble substrates, it is desirable to carry out the reaction in the presence of an organic solvent, and microbial contamination is also greatly decreased in organic solvents.¹⁰⁾ However, in general, enzymes lose their ability to maintain their three-dimensional structures, and so their functions decrease immediately in organic solvents. When enzymes are chemically modified with amphipathic compounds, they often become soluble in both aqueous solutions and organic solvents.^{11–14)} To shed further light on the possible applications of lys-

ozyme conjugated with mPEG, we measured the enzyme activity of mPEG-lysozyme in organic solvents. mPEG-lysozyme was stable and retained its enzyme activity in the presence of organic solvents (Figure 3). In mPEG-lysozyme, the lysozyme molecule was protected from physical contact with denaturing factors because the strongly hydrated mPEG chains created an aqueous shell around the lysozyme molecule.¹⁰⁾ Moreover, the stability of the lysozyme molecule was enhanced by the formation of new hydrogen bonds and hydrophobic interactions between the surface of the lysozyme molecule and the introduced mPEG, which was supposed to prevent protein denaturation.⁸⁾ Therefore, mPEG-lysozyme is also expected to be

useful in various other fields such as the food industry, food processing, and bioreactor technology.

Although mPEG-lysozyme must be examined its general and special toxicity before it is used, mPEG-lysozyme with improved stability, less immunogenicity, and stronger antimicrobial activity against Gram-negative microorganisms has potential to expand the applications of lysozyme as a new type of food additive.^{6,7)} Since the conjugation effects seen in mPEG-lysozyme can be applied to other food additives, a new type of food additive with high stability and novel functions may take the place of the present food additives in the near future.

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