Highly Stabilized Papain Conjugated with Water-Soluble Phospholipid Polymer Chain Having a Reacting Terminal Group

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ABSTRACT: A novel water-soluble and biocompatible phospholipid polymer, poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC), was synthesized using a photoinduced living radical polymerization technique for modification of the enzyme. The PMPC had a reactive carboxylic group on a terminal, and its molecular weight and molecular weight distribution were regulated. The PMPC was reacted with the amino groups of papain via amide bonds to form a conjugate (P-PMPC). The modification degrees with PMPC chains on the conjugate were 22% (P-PMPC22) and 42% (P-PMPC42) versus the total number of amino groups of papain. The stability of the P-PMPC was evaluated in a buffered solution (pH 6.1) at 25 and 40°C. The helix content of the P-PMPC was slightly below that of native papain. However, the secondary structure of the P-PMPC was maintained at its initial level for 28 days at both temperatures. The enzymatic activity after the conjugation was about 40% of the native enzyme, but it was maintained about over 75% of the initial enzymatic activity even when it was stored at 40°C for 28 days. This result is due to inhibition of self-digestion and a change in the ternary structure of papain by the PMPC chains. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 827–832, 2004

Key words: photoinduced living radical polymerization; bioconjugation; phospholipid polymer; enzymatic activity

INTRODUCTION

Recently, accompanying the progress in biotechnology, we have been able to obtain useful biomolecules such as enzymes, antibodies, and bioactive proteins. To use these biomolecules, much attention has been paid to the development of biomolecules modified by conjugation with synthetic polymers.[1] Favorable characteristics of the conjugation may improve the stability of the native biomolecules and add new functions. In this step, the synthetic polymers do not show any adverse effects on the biomolecules after conjugation. One of the well-known polymers is poly(ethylene glycol) (PEG), and its conjugation has been widely studied.[2] The PEG-conjugated biomolecules generally have improved stability and solubility in both aqueous medium and various organic solvents.[3] On the other hand, from the viewpoint of polymer chemistry, the chemical structure of PEG is simple, so that further chemical modification is difficult. The oxyethylene repeating units are chemically inert, and only the terminal hydroxyl group can be changed to another functional group. That is, PEG has the following good properties as a conjugation polymer; reactivity of a terminal functional group and narrow molecular weight distribution. However, molecular design to give various functions by controlling the monomer structure and arrangement is quite difficult. We have newly focused on the usage of a vinyl polymer as a conjugate polymer. The vinyl polymers, which have reactive end groups, can be synthesized by conventional free radical polymerization using an initiator and a chain transfer agent.[4–6] These polymers have the potential to increase the variety of bioconjugation, but fewest these are water-soluble polymers, which can suppress the conformational change in biomolecules by conjugation.

We have been developing new bioconjugations with bioinspired water-soluble phospholipid polymers as a "nanoscaled molecular device." The phospholipid polymers, composed of a 2-methacryloyloxyethyl phosphorylcholine (MPC) moiety, show excellent biocompatibility as biomaterials.[7] In this study, a novel poly(MPC)(PMPC) with a reactive end group on one terminal was synthesized using living radical polymerization induced by photoirradiation. The molecular weight (MW) of PMPC could be regulated by the

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feeding ratio of monomer and a photoiniferter as a polymerization initiator.[8] Papain was used as a model enzyme, and modifications with the PMPC were carried out and its performance was evaluated with attention to the stability and enzymatic activity.

MATERIALS AND METHODS

Materials

MPC was synthesized by the method reported previously.[9] Sodium diethyldithiocarbamic acid, *N*-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were purchased from Kanto Chemicals (Tokyo, Japan). 4-Chloromethylbenzoic acid was purchased from Tokyo Kasei (Tokyo, Japan). Papain was purchased from Nacalai Tesque (Kyoto, Japan). Other organic solvents were purified by the usual methods.

Synthesis of 4-(*N,N*-diethyl)dithiocarbamoylmethylbenzoic acid (BDC)

Sodium *N,N*-diethyldithiocarbate trihydrate (268 mmol) was coupled with 4-chloromethylbenzoic acid (229 mmol) in dry acetone (420 mL) in the dark and stirred at room temperature for 24 h. The white precipitates were filtered and dissolved in chloroform. The chloroform solution was repeatedly washed with 3 % (w/w) aqueous citric acid solution. The chloroform phase was dehydrated with anhydrous sodium sulfate and the solvent was finally evaporated and dried under reduced pressure. The chemical structure of BDC was confirmed by ^{1}H NMR (400 MHz, chloroform-d): δ (ppm) 1.29 (d, 6H, —N—CH_2—CH_3), 3.75–4.01 (d, 4H, —N—CH_2—), 4.63 (d, 2H, -benzyl—CH_2—), 7.51–8.08 (d, 4H, -*benzyl*).

Preparation of PMPC with a terminal carboxyl group (PMPC-COOH)

The BDC (6.00 mmol) was dissolved in tetrahydrofuran (THF) (30 mL), and MPC (90.5 mmol) was dissolved in ethanol (150 mL). After purging with Ar gas to remove oxygen in the solution, the solutions were put into glass tubing to be mixed together, and then the tubing was sealed. The photoirradiation was carried out using a high-pressure mercury lamp (Riko, Chiba, Japan) at room temperature for 3 h. The mixture was poured into chloroform to precipitate PMPC-COOH. The obtained PMPC-COOH was dried under reduced pressure for 2 days. The chemical structure of PMPC-COOH was confirmed by ^{1}H NMR as shown in Figure 2. (400 MHz, ethanol-d_6): δ 1.29 (d, 6H, —N—CH_2—CH_3), 1.87–1.93 (d, 3H, α-CH_3), 3.21–3.30 (d, 9H, —N(CH_3)—), 3.60–3.73 (d, 4H, —CH_2—N—), 4.07–4.32 (d, 4H, —O—CH_2— CH_2—P), 7.89 (d, 6H, *benzyl*).

Conjugation of PMPC-COOH with papain

The carboxyl group at the terminal of PMPC-COOH was reacted with an amino group of papain after activating the carboxyl group of PMPC-COOH. The PMPC-COOH (4.16 mmol) and NHS (42.5 mmol) were dissolved in methanol (150 mL). After cooling at 4°C, DCC (41.8 mmol)/methanol (3.0 mL) solution was added as a condensing agent. This reaction was carried out at 4°C for 1 h and continued at room temperature for 23 h. After cooling at −10°C, dicyclohexylurea was removed by filtration. The activated PMPC-COOH was recovered by precipitation in dioxane. The obtained polymer was dried under reduced pressure for 2 days. Papain (120 mg) was dissolved in 0.1 *M*, pH 7.0 phosphate buffered solution (120 mL) and activated PMPC-COOH (600 mg and 300 mg, respectively) was added to the solution. The reaction was carried out at room temperature for 2 h. To remove the byproduct NHS and unreacted native papain and PMPC-COOH, the solution was dialyzed using a membrane with a molecular weight (MW) cut off of 5.0 $\times$ 10^4 at 4°C for 3 days. Finally, the conjugated papain, P-PMPC, was obtained by a freeze-drying method.

Determination of amino groups in papain

The remaining amino groups in the P-PMPC were quantitatively determined by sulfo-succinimidyl-4-*O*-(4,4′-dimethoxytrityl)-butyrate) (sulfo-SDTB) method.[10] Sulfo-SDTB (4.4 mg) was dissolved in *N,N*-dimethylformamide (DMF) (1.0 mL) and diluted with 50 m*M* of pH 8.2 bicarbonate buffer (50 mL). One milliliters of the solution was added into the conjugated papain (8.6 mg)/50 m*M* of pH 8.2 bicarbonate buffer (1.0 mL) solution. After 2 h of reaction, this solution was dialyzed using a semipermeable cellulose membrane with a molecular weight cutoff of 1.0 $\times$ 10^4 at 4°C for 2 days. To leave the 4,4′-dimethoxytrityl cation, 37% perchloric acid aqueous solution (2.5 mL) was added. The remaining amino groups in the P-PMPC were determined by UV measurement at 498 nm. The modification degree of the P-PMPC was calculated to be the amount of amino groups in native papain. A schematic representation of the synthesis of PMPC-COOH and its conjugation of it with papain is shown in Figure 1.

Gel permeation chromatography (GPC) measurement

The MW of PMPC-COOH was measured by GPC measurement (flow rate, 0.5 mL/mim; detector, RI; eluent, 0.1 *M* LiBr aq. Column, Shodex OHpak SB-803

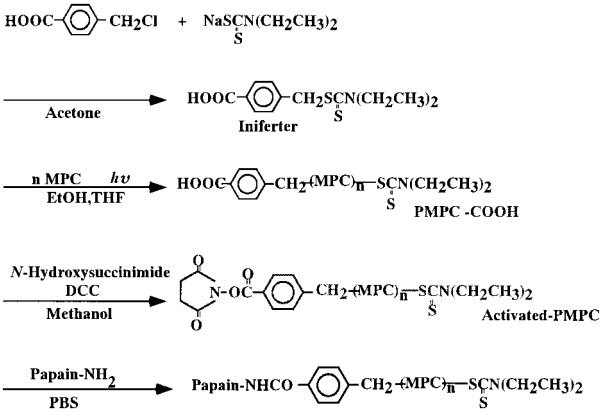

Figure 1 Schematic representation of synthesis of papain conjugated with PMPC-COOH (P-PMPC).

HQ). The state of the P-PMPC in an aqueous medium was evaluated by GPC measurement (flow rate, 0.5 mL/min; detector, UV; eluent, 0.1 *M* PBS + 0.1 *M* Na_2SO_4; column, TOSOH G2000SW).

Evaluation of secondary structure of the papain

The secondary structure of native papain and P-PMPC was evaluated by measurement of the helix content with a circular dichroism (CD) spectropolarimeter (J-720W, JASCO, Tokyo, Japan). The measurement was carried out at 25 and 40°C, and the concentrations of papain without and with modification with PMPC were 3.10×10^{-8} mol/L.

Measurement of enzymatic activity

The enzymatic activity of native papain and P-PMPC was determined using benzoyl-L-arginine-ethylester (BAEE) as a substrate. The BAEE was dissolved in 0.1 *M* of pH 6.1 PBS and the native papain and the P-PMPC/0.1 *M* of pH 6.1 PBS, which contains 1 m*M* EDTA, and 5 m*M* L-cysteine were added to the BAEE solution. The rate of enzymatic reaction was followed by the change in absorbance at 258 nm. The reaction was carried out at 25 and 40°C. Native papain and the P-PMPC solutions were stored at 25 and 40°C. We adjusted the initial activity of native papain and P-PMPC to be 18 unit/mL. Therefore, because the net activity of the papain before and after conjugation was different, the their concentrations were different.

RESULTS AND DISCUSSION

Generally, polymers with a terminal carboxyl group were synthesized from vinyl compounds using 2,2′-azobis-isobutyronitile as a radical polymerization initiator and 3-mercaptopropionic acid (MPAc) as a chain transfer agent.[11,12] During polymerization, the propagation of growth is initiated by MPAc and the polymer chain has a carboxyl group. The carboxyl group can be used for conjugation with proteins via a carbodiimide coupling process. However, it is difficult to control the molecular weight and the molecular weight distribution of the polymer because of the early termination of the polymer radical. In this study, we selected photoinduced living radical polymerization using BDC as a photoiniferter. The photoiniferter is an initiator that induces radical polymerization that proceeds via initiation, propagation, primary radical termination, and transfer to the initiator.[8] By changing the ratio of monomer and photoiniferter, the molecular weight of the polymer can be regulated. Moreover, because this photoiniferter, BDC, has one carboxyl group, a reactive end group was easily introduced in the polymer chain.

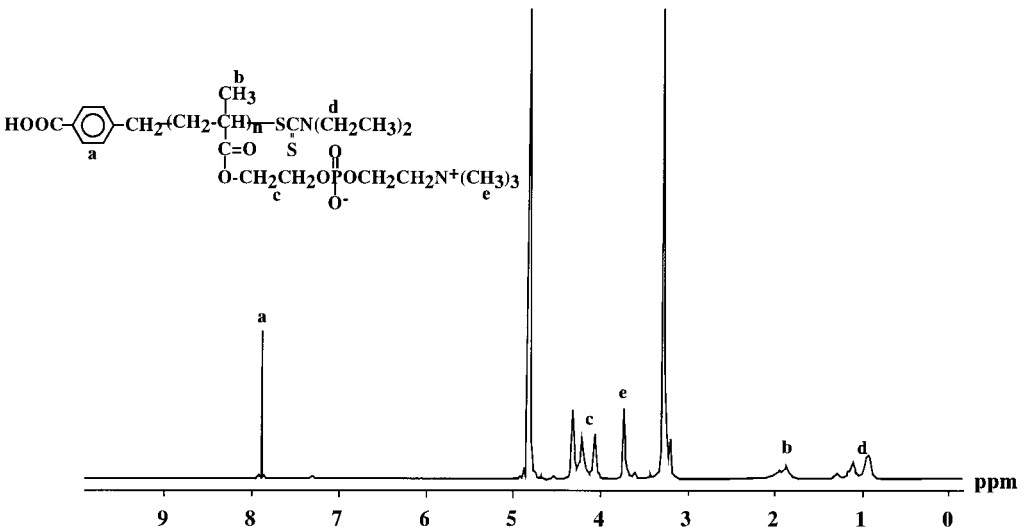

Figure 2 ^{1}H NMR spectrum of PMPC-COOH.

TABLE I
Characterization of PMPC-COOH

[MPC]/ [Iniferter]	Polymerization time (h)	M_w[a]	M_w/M_n[a]	Yield (%)
15.1	3	5.0×10^3	1.23	85

[a] Poly(ethylene oxide) standard.

The characterization of PMPC-COOH is shown in Table I. The molecular weight distribution was very narrow, which indicates that this polymer was better adapted to conjugate with proteins.

Table II shows the conditions for conjugation of PMPC-COOH with papain. The PMPC-COOH was reacted with an amino group of papain via an amide bond and the biological data of P-PMPC are summarized. The modification degree was 22% (P-PMPC22) and 42% (P-PMPC42) vs the total number of amino groups of papain. These values were almost the same as that in the feed. The enzymatic activity remaining after conjugation were 41.5% (P-PMPC22) and 34.1% (P-PMPC42), respectively. The decrease in the enzymatic activity after conjugation is due to a change in the ternary structure of papain, reduction of substrate diffusion to the active site and a decrease in the diffusion of the P-PMPC. The helix content of conjugated papain was slightly below that of native papain. This indicates that even though PMPC-COOH was conjugated, the secondary structure was maintained and PMPC-COOH did not have adverse effects on papain.

We have studied the interaction between plasma proteins and MPC polymers.[13–15] These proteins were hardly adsorbed on the MPC polymer surface. Even when even the protein was adsorbed on the MPC polymer surface, the secondary structure of adsorbed protein did not change. Moreover, water-soluble MPC polymer could stabilize the antibody and the enzyme-linked antibody for the enzyme-linked immunosorbent assay method.[16,17] These mild interactions between MPC polymers and proteins are attributed to the fact that the water state does not change even when the MPC polymer is present in the aqueous system.[15,18]

Figures 3 and 4 show the change in the helix content of native papain and the P-PMPC stored at 25 and 40°C, respectively. The helix content represents stability of enzyme. The helix content of native papain was almost 25%, but it decreased gradually and could not be detected after 21 and 14 days storage at 25 and 40°C, respectively. This phenomenon indicates that the secondary structure of native papain completely collapsed during these periods. On the other hand, although the helix content of the P-PMPC was 21%, that is, slightly decreased by the conjugation, it was maintained for 28 days when stored both at 25 and 40°C. This result indicates that the change in the secondary structure did not occur for 28 days, even when it was stored at high temperature, 40°C. The PMPC chains did not show any adverse effects on the stability of papain. Figures 5 and 6 show the enzymatic activity profile of native and conjugated papain stored at 25 and 40°C, respectively. The enzymatic activity of native papain decreased with the storage period at both temperatures. These results corresponded to the time dependence of the helix content. The enzymatic activities of P-PMPC22 and P-PMPC42 were maintained at over 75 and 85% of the initial enzymatic activity, respectively, even when stored at 40°C for 28 days. This is due to inhibition of self-digestion and structure change in papain by conjugation with the PMPC chains. When PMPC-COOH was conjugated with papain, 42% of the modification degree was good

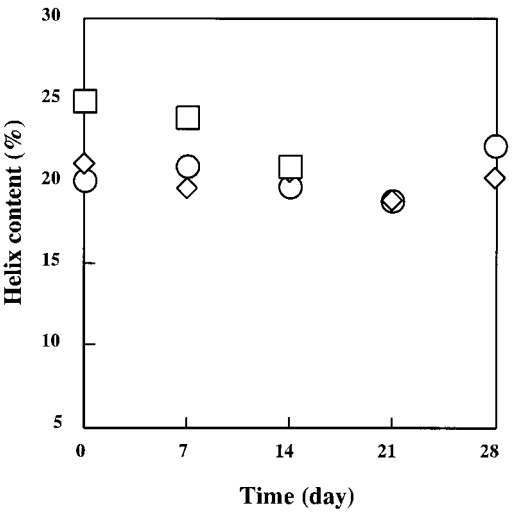

Figure 3 Storage time dependence of the helix content of papain at 25°C. (□): native, (◇): P-PMPC22, (○): P-PMPC42.

TABLE II
Experimental Conditions of the Conjugation and Characterization of the Conjugated Papain

Abb.	Papain (mg)	Activated PMPC-COOH (mmol)	Modification ratio[a] (%)	Residual of helix content (%)	Residual of enzymatic activity (%)
P-PMPC22	120	0.06	22.0	84.3	41.5
P-PMPC42	120	0.12	41.8	71.7	34.1

[a] Amino acid residues in papain are 212 and total number of amino groups is 35.

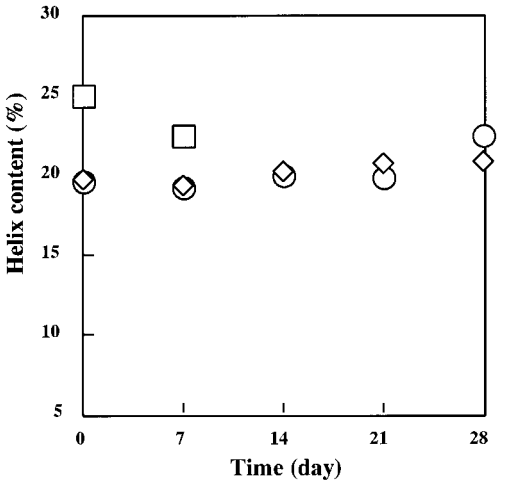

Figure 4 Storage time dependence of the helix content of papain at 40°C. (□): native, (◇): P-PMPC22, (○): P-PMPC42.

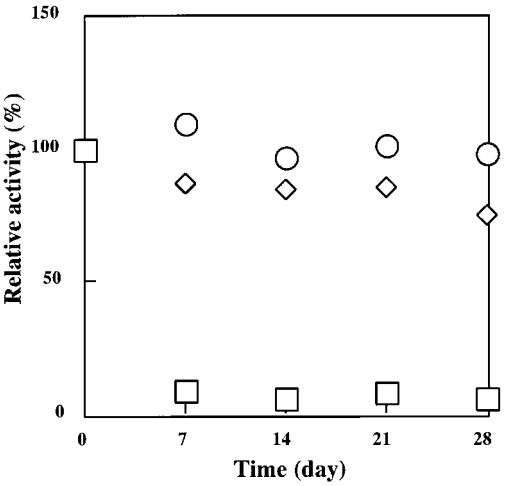

Figure 6 Relative remaining enzymatic activity of papain stored at 40°C. (□): native, (◇): P-PMPC22, (○): P-PMPC42.

for stabilization of papain at better than 22% of what. At 25°C, the enzymatic activity of P-PMPC was decreased at first, but it increased again. During this process, the helix content of P-PMPC did not decrease, as shown in Figure 3. We considered that the soluble state of P-PMPC in aqueous medium influenced this phenomenon. Figure 7 shows the GPC chart of P-PMPC42. The shoulder peaks at early elution time were observed. These peaks, which corresponded to the molecular aggregate of P-PMPC, gradually decreased and finally disappeared. By combination of these results on enzymatic activity and aggregation behavior, the mechanism of the storage period dependence of enzymatic activity could be explained as follows. At first, the P-PMPC formed an aggregate. The aggregate of the P-PMPC dissociated gradually with time. The initial enzymatic activity of the P-PMPC was adjusted to be the same as that of native papain. Because the enzymatic activity of P-PMPC was lowered by conjugation by about 34–42% of that of native papain, the concentration of the P-PMPC in the storage solution was higher than that of native papain. At first, a decrease in the enzymatic activity of P-PMPC is due to formation of aggregates, and the ternary structure of the P-PMPC gradually altered with the storage period. However, dissociation of the aggregation was induced by the PMPC chains, which became highly hydrated.Accompanying this phenomenon, both the mobility of P-PMPC and the diffusivity of the substrate increased. Therefore, the apparent enzymatic activity increased again. This explanation will be clarified by evaluation of the ternary structure of P-PMPC and will be reported elsewhere. At 40°C, the mobility of the P-PMPC was high compared with that at 25°C. A decrease in the enzymatic activity was not observed, and the initial level was maintained during measurement period.

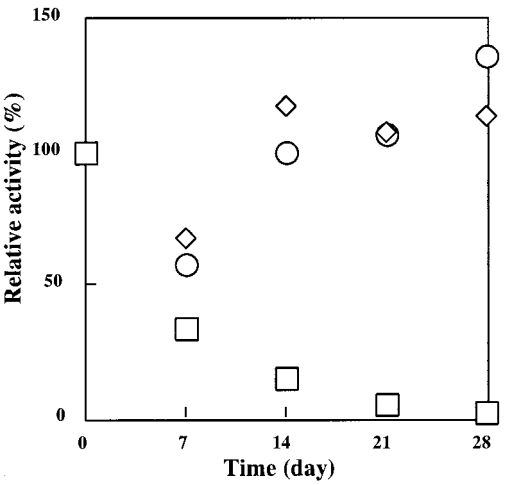

Figure 5 Relative remaining enzymatic activity of papain stored at 25°C. (□): native, (◇): P-PMPC22, (○): P-PMPC42.

CONCLUSIONS

A water-soluble and biocompatible phospholipid polymer with a carboxyl group (PMPC-COOH) could

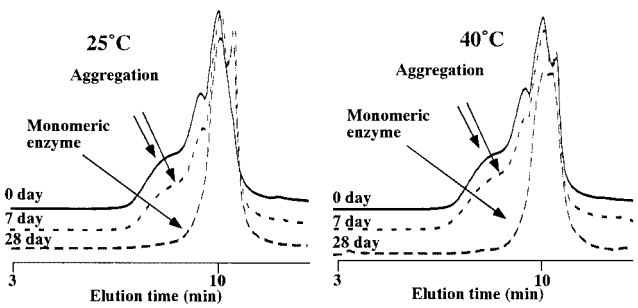

Figure 7 Change in GPC elution profile of P-PMPC stored at 25 and 40°C.

be synthesized by photoinduced living radical polymerization. The PMPC-COOH could react with the enzyme, papain, to prepare the bioconjugate (P-PMPC) under mild conditions. The enzymatic activity of P-PMPC was reduced by about 60% compared with that of native papain, but it maintained even when stored at 40°C for 28 days. We conclude that PMPC-COOH is a useful polymer for conjugation with the enzyme and for improvement of the stability of the conjugated enzyme.

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