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Mechanistic investigation of capability of enzymatically synthesized polycysteine to cross-link proteins

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ABSTRACT

Background: Previously, we had reported that α-chymotrypsin–catalyzed polymerization of l-cysteine ethyl ester in a frozen buffer provided poly-l-cysteine (PLCys) in good yield, of which degree of polymerization had been determined to be 6–11. Almost all of SH groups in PLCys were in free forms. Such a multi-thiol peptide may cross-link proteins through thiol/disulfide (SH/SS) exchange reactions, considering the knowledge that other synthetic multi-thiol additives changes properties of protein materials.
Methods: This study explored the capability of PLCys to cross-link proteins using lysozyme as a model protein which has four disulfide bonds but no free SH group. The protein was incubated with PLCys at neutral pH and at below 70 °C to avoid PLCys-independent, β-elimination-mediated cross-linkings. Protein polymerization was analyzed by SDS-PAGE and SEC. PLCys peptides involved in the protein polymer, which were released by reduction with dithiothreitol, were analyzed by RP-HPLC.
Conclusions: Addition of urea and thermal treatment at 60 °C caused PLCys-induced lysozyme polymerization. Compared with free cysteine, a higher level of PLCys was required for the polymerization probably due to its low water solubility. RP-HPLC analyses suggested that PLCys played a role in the protein polymerization as a cross-linker.
General significance: Enzymatically synthesized PLCys shows promise as a peptidic cross-linker for the production of protein polymers with novel physiochemical properties and functionalities.

1. Introduction

Protein cross-linking plays an important role in the functionality of food and non-food proteins. Cross-linking of proteins leads to the creation of macromolecular assemblies with new or modified physicochemical properties and functionalities [1]. Recently, research into enzymatic cross-linking has become prominent in many fields including food technology as well as biochemical and biomedical research [2–5]. For example, the transglutaminase-catalyzed formation of 3-dimensional protein networks is based on the condensation of Gln and Lys residues by transamidation [6,7]. Indeed, the application of transglutaminase in food processing has been reported to improve the technical characteristics of proteins, such as gel formation as well as emulsifying and rheological properties [2,3].

Conventionally pre-existing disulfide (SS) bonds also contribute to protein cross-linking. In contrast to the irreversible covalent bonds found in other types of protein linkages, the SS bonds can be reversibly cleaved by reducing agents. In addition, the reshuffling reaction between thiol (SH) groups and intra- and intermolecular SS bonds occurs with thermal treatment of protein solutions [8]. Not only this reaction but also disulfide formation are pH-dependent [8,9]. Many researchers have characterized the relationship between the SS bonds in gluten and the extensibility and elasticity of wheat dough [10–13]. Thermal treatment of gluten consisting of monomeric gliadin and polymeric glutenin induces intra- and intermolecular SH/SS exchange reactions, resulting in the incorporation of gliadin into the glutenin network. The structure, density, and strength of the gluten network affect the property of dough, which determines the bread quality. Thus, the effects of redox agents on gluten have been studied [14–16]. It has also been reported that the addition of free Cys into a gliadin solution followed by gentle stirring causes polymerization of the protein even at 40 °C for 30 min [17]. Protein films derived from Cys-mediated gliadin polymerization show water vapor permeability comparable to glutenin films. Such films made from cross-linked food proteins have received considerable attention as a promising

Abbreviations used: Cys-OEt, l-cysteine ethyl ester; DMSO, dimethyl sulfoxide; DP, degree of polymerization; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; IAM, iodoacetamide; MALDI-TOF MS, matrix assisted laser deso′rption/ionization time of flight mass spectrometry; PLCys, poly-l-cysteine; *S*-CM, S-carbamoylmethyl; SEC, size exclusion chromatography

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material for use in food packaging and agricultural applications.

We recently reported that α-chymotrypsin-catalyzed polymerization of L-cysteine ethyl ester (Cys-OEt) in a frozen buffer solution produces water-insoluble poly-L-cysteine (PLCys), reaching 85% yield on a substrate basis [18]. One advantage of this synthetic method is its simplicity, not requiring blocking/deblocking or any harmful reagents. The degree of polymerization (DP) of the synthesized PLCys was from 6 to 11 as determined using MALDI-TOF MS. Most of the SH groups in PLCys were found to be in the free form. Recently, other researchers also reported that papain- and proteinase K-catalyzed synthesis of PLCys from Cys-OEt can be successfully accomplished at 40 °C [19,20]. Furthermore, Ma *et al.* demonstrated that PLCys in the solid state is stable at broad temperatures up to 200 °C [20]. If PLCys can join the intermolecular SH/SS exchange together with proteins, it would work as a cross-linker providing protein polymers with novel physicochemical properties and functionalities.

In the present study, we investigated whether PLCys could help protein cross-linking through SH/SS exchange and function as a cross-linker. To focus on the effects of SH groups in PLCys, lysozyme was chosen as a model protein, which has four SS bonds but no free SH group [21,22]. In addition, its molecular size of 14.3 kDa makes it facile to determine polymerization of the protein by polyacrylamide gel electrophoresis.

2. Materials and methods

2.1. Materials

L-Cysteine ethyl ester hydrochloride was purchased from Tokyo Kasei Co. (Tokyo, Japan). α-Chymotrypsin (EC 3.4.21.1) type II from bovine pancreas, 3 × crystallized from 4 × crystallized chymotrypsinogen, dialyzed essentially salt-free and prepared as lyophilized powder, which was 54 U/mg protein determined at 25 °C and pH 7.8 with *N*-benzoyl-L-tyrosine ethyl ester as a substrate, lysozyme from chicken egg white, L-arginine ethyl ester dihydrochloride and ProteoMass™ peptide MALDI-MS calibration kit were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 2,5-Dihydroxybenzoic acid, DMSO, trifluoroacetic acid (TFA) and urea were purchased from Kanto Chemical Co. (Tokyo, Japan). Iodoacetamide (IAM) and 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). 2,4,6-Trinitrobenzenesulfonic acid sodium salt dihydrate was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Standards for size exclusion chromatography were from Bio-Rad (Hercules, CA, USA). All other regents used were of analytical grade.

2.2. Enzymatic polymerization of Cys-OEt and preparation of PLCys

Conditions for enzymatic polymerization of Cys-OEt were as follows: 100 mM Cys-OEt and 20 μM α-chymotrypsin were mixed in 100 mM Na-phosphate buffer (pH 8.0), and then the mixture was immediately frozen at −20 °C and stayed for 3 days. To stop the reaction, 1/5 vol of 5 N HCl was added into the frozen reaction mixture and then it was thawed, preventing its temperature from reaching 10 °C. The precipitated PLCys was collected, rinced and dried as described previously [18]. It was kept in the dark at 4–8 °C until before use for various analyses. Quantitative assays of the DTNB-reactive SH groups in PLCys were performed as described previously [18]. After PLCys was dissolved in DMSO, about 65% of Cys residues in PLCys could be determined using DTNB [18]. Based on this, theoretical Cys residues in PLCys were estimated.

2.3. Reaction of PLCys with lysozyme

Prepared PLCys was suspended in 200 mM Na-phosphate, pH 7.0, so as to contain DTNB-reactive SH groups of 21 mM (theoretical Cys residues: 33 mM). This suspension was mixed with 6 mg/mL (about 0.4 mM) of lysozyme in pure water with or without 8 M urea in a 1:1 vol ratio, each of their final concentrations becoming half. The mixture was incubated for 30 min at 30 or 60 °C. After the reaction mixture was cooled on ice for 5 min, it was mixed with 1/5 vol of 200 mM IAM in 100 mM Na-phosphate buffer (pH 8.0), and then the pH was adjusted to 8.0 by the addition of 1 N NaOH. It was incubated for 1 h at room temperature in the dark to modify remaining free SH groups. The sample was centrifuged at 15,000 × *g* for 30 min at 25 °C. The supernatant was analyzed by SDS-PAGE, SEC, and ultrafiltration followed by RP-HPLC.

2.4. Ultrafiltration of lysozyme-PLCys reaction mixture

The supernatant of the lysozyme-PLCys reaction mixture was loaded onto the membrane filter of an Amicon Ultra centrifugal filter unit of 30 kDa molecular weight cut off (Millipore Ireland Ltd. Cork, Ireland). To prevent aggregation of proteins, L-arginine ethyl ester dihydrochloride (at final concentration of approx. 1 M) was added into the sample [23]. After centrifugation at 5000 × *g* at 25 °C, the ultrafiltrate was transferred to an Amicon Ultra filter of 10 kDa for further ultrafiltration. Each of the higher molecular weight (> 30 kDa) and 10–30 kDa fractions was rinsed with 4 M urea/0.05 N HCl (pH 4.0) five times, and then, was concentrated by about 30-fold in the ultrafiltration device. Such a mild acidic conditions was used to inhibit β-elimination of SS bonds.

2.5. SDS-PAGE

The supernatant of lysozyme-PLCys (or Cys) reaction mixture was analyzed by SDS-PAGE [24] with a separating gel of 12.5% polyacrylamide. Prior to electrophoresis, the sample was mixed with an equal volume of 60 mM Tris-HCl (pH 7.4) containing 2% SDS, 20% glycerol, and 0.02% bromophenol blue with or without 1.5% DTT, and then incubated for 1 h at room temperature in the dark. Twenty microliters of each sample was loaded. Gels were stained by Coomassie Brilliant Blue (CBB) R-250.

2.6. Size exclusion chromatography (SEC)

The supernatant of the lysozyme-PLCys (or Cys) reaction mixture was filtered through a 0.45 μm filter (DISMIC-13cp, Advantec Toyo, Tokyo, Japan), and then, the filtrate was loaded (20 μL) on a Showdex KW 803 column (Showa Denko, Tokyo, Japan). The proteins were eluted at room temperature with 2% SDS at flow rate of 1 mL/min using a Hitachi L-2700 pump, and detected at 280 nm by an L-2400 UV detector. Chromatograms were analyzed using the data processing software Chromato-PRO (Run Time Corporation, Kanagawa, Japan). Proteins were classified into (1) monomers of about 14 kDa and (2) polymers of broad molecular sizes from 28 kDa to 110 kDa. The proportion of lysozyme in these two groups was estimated from peak areas, based on the peak area of intact (control) lysozyme as 100%.

2.7. RP-HPLC

For investigation of PLCys involved in the polymerized lysozyme complex, each sample was mixed with an equal volume of 0.1 M DTT in 50 mM Na-phosphate (pH 8.0) containing 4 M urea, and then incubated for 1 h at room temperature in