Subtilisin-catalyzed religation of proteolyzed hen egg-white lysozyme: investigation of the role of disulfides

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Background: The use of proteases to form, instead of break, peptide bonds has expanded the repertoire of techniques available for protein semisynthesis. Several groups have previously reported the use of proteases in aqueous–organic solvents to form single amide bonds within proteins, but low yields and lengthy reaction times make this an impractical approach to protein synthesis. We recently found that proteolyzed triose phosphate isomerase can be re-ligated rapidly and efficiently by subtilisin, in mixed aqueous–organic solvent systems.

Results: We now report the use of subtilisin to resynthesize hen egg-white lysozyme from a mixture of its proteolyzed fragments in high yield and with rapid reaction times. This enzymatic religation can also be achieved after reduction of the four disulfide bonds present in lysozyme, with the same efficiency as that observed for the disulfide-containing proteolysis mixture.

Conclusions: For egg-white lysozyme, the subtilisin religation reaction can be used to re-synthesize a proteolyzed protein even after reduction of disulfide bonds. The utility of this reaction in more generalized protein semisynthesis reactions is currently being explored.

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Introduction

In recent years there has been continued effort to use enzymes as practical and efficient catalysts for organic chemical transformations [1]. In particular, the use of proteases for peptide bond formation has expanded the techniques available for use in protein semisynthesis [2–6]. Several groups have previously reported the use of proteases in aqueous–organic solvents to form single amide bonds within proteins, but low yields and lengthy reaction times made this seem to be an impractical approach to protein synthesis [7–11]. The use of a mutated subtilisin (subtiligase) as a catalyst for amide bond formation in aqueous solution overcame some of these disadvantages [12].

We recently reported the use of subtilisin to form three amide bonds between the proteolyzed fragments of triose phosphate isomerase (TIM) in mixed aqueous–organic solvents [13]. The observed protease-catalyzed amide bond reformation has been attributed to an increase in the pK_a of the carboxy-terminal carboxyl group upon addition of an organic cosolvent, which shifts the equilibrium toward synthesis [7], and the ability of the proteolyzed TIM fragments to form a stable, non-covalent assembly [13]. Our synthesis was shown to be both rapid and efficient, opening the door to a more serious consideration of this approach for the semi-synthesis of large proteins. We now report the use of subtilisin, in mixed aqueous–organic solvent systems, to resynthesize a protein with an entirely different folding motif from TIM, hen egg-white lysozyme, from a mixture of its proteolyzed fragments. In addition, we have found that this enzymatic religation can be achieved after reduction of the four disulfide bonds present in lysozyme, with the same efficiency as that observed for the disulfide-containing proteolysis mixture.

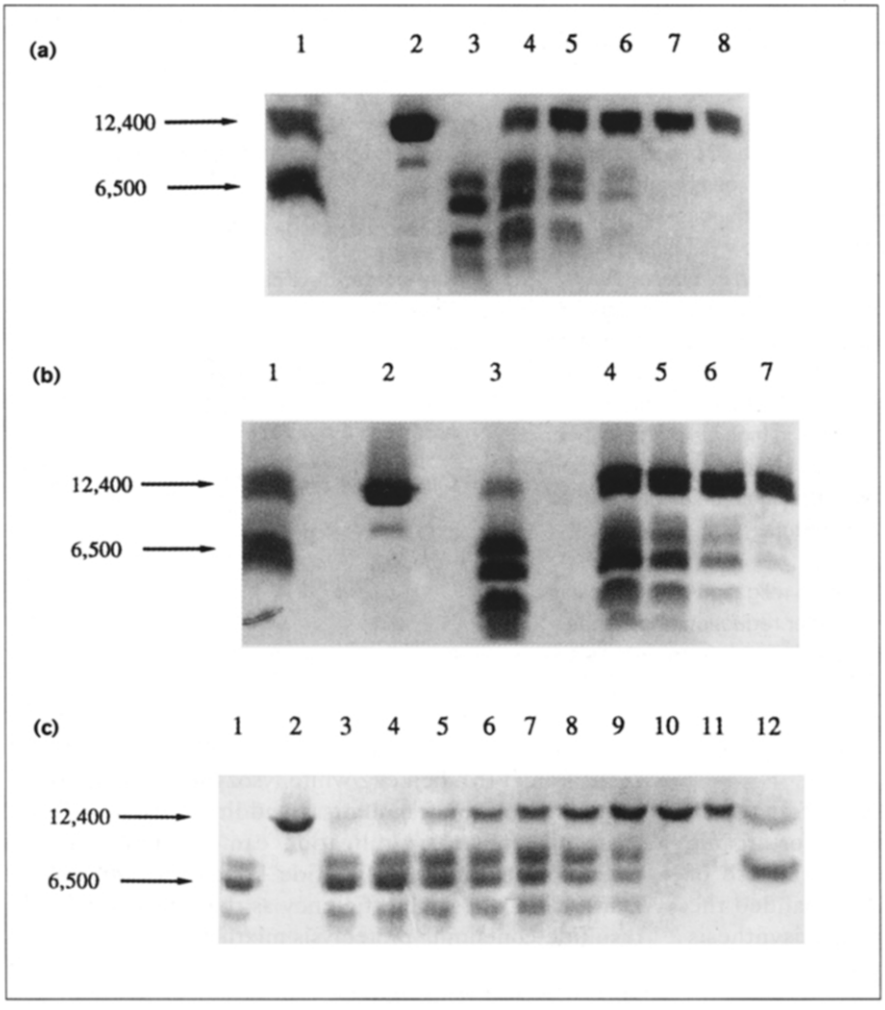
Results and discussion

Limited proteolysis and religation

Hen egg-white lysozyme is a monomeric protein of 129 amino acid residues, which is cross-linked by four disulfide bonds [14–19]. When this enzyme was submitted to limited proteolysis with 0.01 equivalents of subtilisin Carlsberg for 24 h at 37 °C, peptide fragments (Fig. 1a, lane 3) were generated corresponding to cleavage at two sites as determined through amino-terminal sequencing of the gel-purified peptides; the cleavage sites were at Gln41–Ala42 and Leu56–Gln57. In our previous work with TIM, the amide bonds available for proteolytic cleavage were those located in solvent exposed loops [13]; similarly, the cleavage sites within lysozyme were at the solvent-exposed ends of β-sheets (Fig. 2).

To determine if lysozyme could be resynthesized in a manner similar to TIM, the proteolysis mixture of five fragments, (see Materials and methods for details), which still contained subtilisin, was treated with increasing concentrations of organic solvents (Fig. 1). Complete resynthesis of native lysozyme was observed when the concentration of organic solvent was ~80–90 %

Figure 1



Organic cosolvents promote the religation of lysozyme by subtilisin. **(a)** Lane 1, molecular weight markers; lane 2, native lysozyme; lane 3, subtilisin-cleaved lysozyme; lanes 4–8, subtilisin-cleaved lysozyme with 50–90 % dimethylsulfoxide (DMSO; the concentration of DMSO was 50 % for lane 4 and increased in steps of 10 % to 90 % in lane 8). **(b)** Lane 1, molecular weight markers; lane 2, native lysozyme; lane 3, subtilisin-cleaved lysozyme; lanes 4–7, subtilisin-cleaved lysozyme with 60–90 % acetonitrile (the concentration of acetonitrile was 60 % for lane 4 and increased in steps of 10 % to 90 % in lane 7). **(c)** Lane 1, subtilisin-cleaved lysozyme; lane 2, native lysozyme; lanes 3–11, subtilisin-cleaved lysozyme with 10–90 % glycerol (the concentration of glycerol was 10 % for lane 3 and increased in steps of 10 % to 90 % in lane 11), lane 12, molecular weight markers.

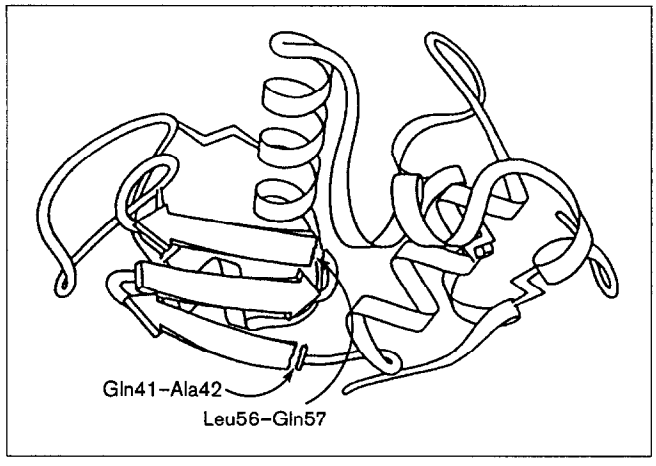
acetonitrile, glycerol or dimethyl sulfoxide; the fragment bands of lysozyme were converted to one band corresponding to native lysozyme within ~5 min (Fig. 1). High-performance liquid chromatography (HPLC) of the 90 % acetonitrile treated reaction mixture resulted in a 60 % yield of resynthesized lysozyme.

Several methods were used to confirm that covalent bonds had been formed in the course of lysozyme religation. Both native and reformed lysozyme (crude and purified material) had identical electrophoretic mobility when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Native and reformed lysozyme (purified material) were also analyzed by electrospray mass spectrometry, and both gave an identical mass of 14 304 Da, whereas the proteolysis mixture produced a series of fragments of lower molecular weight. Amino-terminal sequencing also detected only the amino terminus of the intact protein and none of the fragment amino-termini that were observed in the cleavage mixture.

Circular dichroism (CD) spectroscopy and a lysozyme activity assay were also used to show that the reformed lysozyme had not been denatured under the conditions for resynthesis. The CD spectra of both native and reformed lysozyme (purified material) were quite similar, suggesting that the addition of organic solvents did not denature the protein, and that fragment self-assembly correctly guided the religation process. To investigate this possibility further, reformed lysozyme was assayed for its ability to cleave the bacterial cell wall polymer N-acetyl-D-glucosamine–N-acetyl muramic acid (NAG–NAM) at the $\beta(1\rightarrow4)$ linkage. Reformed lysozyme was found to have 88 % of the activity of the native enzyme (Table 1) [20,21].

Factors that aid in shifting the protease reaction equilibrium toward synthesis include both the introduction of organic cosolvent to increase the pK_a of the carboxy-terminal carboxyl group [7], and the tight self-assembly of proteolyzed fragments [13]. Size-exclusion chromatography was used to determine that fragment

Figure 2



Structure of hen egg-white lysozyme showing the two sites that subtilisin cleaves and then religates: Gln41–Ala42 and Leu56–Gln57.

association was occurring between the proteolyzed fragments of lysozyme. The apparent molecular weight of the fragment mixture as determined by this technique was found to be identical to that of the native enzyme. In addition, the proteolysis mixture was assayed for its ability to cleave the β(1→4) NAG–NAM linkage [20,21]. The enzymatic activity of the proteolysis mixture was found to be 95 % of the native enzyme activity for this substrate (Table 1). These results confirm that specific self-assembly occurs, correctly forming the active-site cleft of lysozyme in the proteolyzed sample.

Role of disulfides in religation

The disulfide linkages of lysozyme are essential for its stability and biological activity [22,23]. We postulated that the presence of disulfide bonds within the proteolyzed fragments of lysozyme would aid in the assembly of the fragment mixture. To determine whether the stabilization due to the disulfide bonds had an amplifying effect on the resynthesis of proteolyzed lysozyme, protease-catalyzed resynthesis was explored with a proteolysis mixture in which the disulfide bonds of the fragments had been reduced after proteolysis. The proteolysis mixture was first reduced with β-mercaptoethanol [24–28], and then added to a 90 % solution of acetonitrile, glycerol or dimethyl sulfoxide. Interestingly, under these conditions lysozyme religation was found to proceed with an efficiency comparable to that of the disulfide-containing mixture (Fig. 3). The reduced, religated lysozyme obtained from this reaction was analyzed for free thiol content and for enzymatic activity (Table 1). Both before and after ligation, more than 99 % of the theoretical thiol content of the lysozyme sample was detected, and each of these samples had very low enzymatic activity as was observed for reduced native lysozyme. Upon reoxidation of the reduced native, reduced proteolyzed and reduced religated lysozyme, ~50 % of the enzymatic activity was restored.

Table 1

The enzymatic activity of oxidized and reduced forms of native, proteolyzed and religated hen egg-white lysozyme.

Lysozyme species	Percent lysozyme activity[a]
Native	100
Proteolyzed	95
Religated	88
Native reduced	11
Proteolyzed reduced	4
Religated reduced	2
Native reoxidized	42
Proteolyzed reoxidized	52
Religated reoxidized	44

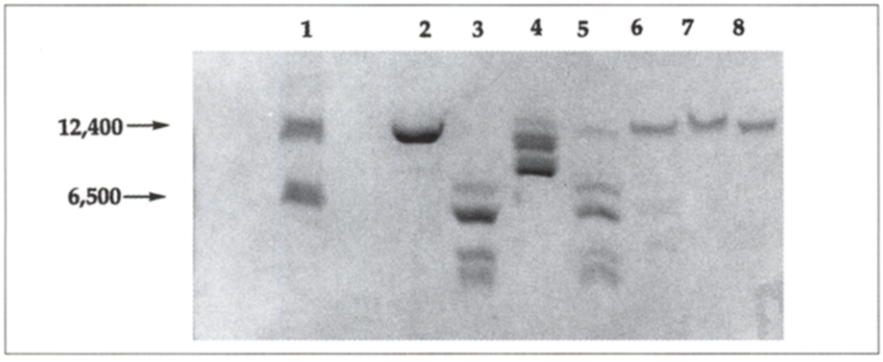
[a]All values are based on the activity of the native lysozyme sample.

It has been observed that the disulfide bonds of lysozyme are essential for enzymatic activity, but not for the correct folding of the protein [22,23]. Our results demonstrate that the disulfide bonds are also not a necessary prerequisite for the accurate re-association of proteolyzed lysozyme fragments into a structure that allows efficient religation of the correct peptide bonds to take place. Although the protein may fold without the disulfides, the long substrate-binding cleft and active site of lysozyme are formed by regions of secondary structure in the protein that are held together by disulfide bonds. It is possible, therefore, that reduction of the disulfide bonds in this region could induce changes in protein architecture that might have profound effects on the ability of lysozyme to bind and catalyze the cleavage of the β(1→4) linkage of NAG–NAM, but not affect folding or religation reactions.

Significance

Chemical synthesis of large proteins would be useful for many purposes, including the construction of novel protein molecules that contain non-natural amino acids. One approach to the problem of a general method for assembly of synthetic proteins is to ligate large fragments of proteins together using enzymes. This can be achieved either by modifying the enzyme to increase the efficiency of peptide ligation compared to protease activity [12] or by performing the reaction in the presence of carefully chosen organic cosolvents, which shift the equilibrium towards synthesis [13]. We have demonstrated that fragments resulting from cleavage at two sites within lysozyme can be religated in the presence of subtilisin and either 90 % acetonitrile, glycerol, or dimethyl sulfoxide (Fig. 4), and that the four disulfide bonds present within the lysozyme structure are not required for the resynthesis of biologically active lysozyme. Thus,

Figure 3



Organic cosolvents promote the religation of subtilisin-cleaved lysozyme following reduction of its fragments. Lane 1, molecular weight markers; lane 2, native lysozyme; lane 3, subtilisin-cleaved lysozyme; lane 4, subtilisin-cleaved lysozyme (non-reducing sample buffer); lane 5, subtilisin-cleaved lysozyme, reduced with dithiothreitol (DTT) in non-reducing sample buffer; lanes 6–8 contain subtilisin-cleaved and DTT-reduced lysozyme with 90 % acetonitrile (lane 6), 90 % glycerol (lane 7) or 90 % dimethylsulfoxide (lane 8).

it may be possible to use this religation approach to easily include non-natural amino acids even in peptide fragments that are normally disulfide-linked to other fragments of the enzyme. The utility of this religation reaction in more generalized protein semisynthesis applications is currently being explored.

Materials and methods

Materials

Hen egg-white lysozyme and subtilisin Carlsberg were purchased from Sigma (St. Louis, MO). All electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Sephadex G-50 and Sephadex G-25 was purchased from Sigma (St. Louis, MO), and Pharmacia (Upsala, Sweden), respectively. All other reagents and chemicals were purchased from Sigma (St. Louis, MO), Fisher (Pittsburgh, PA), or Aldrich (Milwaukee, WI), unless otherwise specified.

Limited proteolysis

Hen egg-white lysozyme (70 μmol) and subtilisin Carlsberg (0.7 μmol) were dissolved in 1 ml of 50 mM triethanolamine-HCl, pH 7.5 buffer, and the reaction was allowed to proceed at 37 °C for 24 h. SDS-PAGE electrophoresis was performed on the proteolysis mixture using a Bio-Rad vertical electrophoresis system with 0.8-mm thick, 17 % isocratic separating gels in a discontinuous buffer system. The acrylamide gels were stained for one hour with Coomassie brilliant blue R-25 dye to identify the presence of protein bands. Sites of proteolysis were determined by amino-terminal sequencing of the electroblotted bands from SDS-PAGE. From top to bottom of the gel, the bands were determined to consist of fragments from amino acids 42–129 (band 1), 57–129 (band 2), 1–56 (band 3), 1–41 (band 4) and 42–56 (which runs at the very bottom of the gel, and does not stain with Coomassie blue).

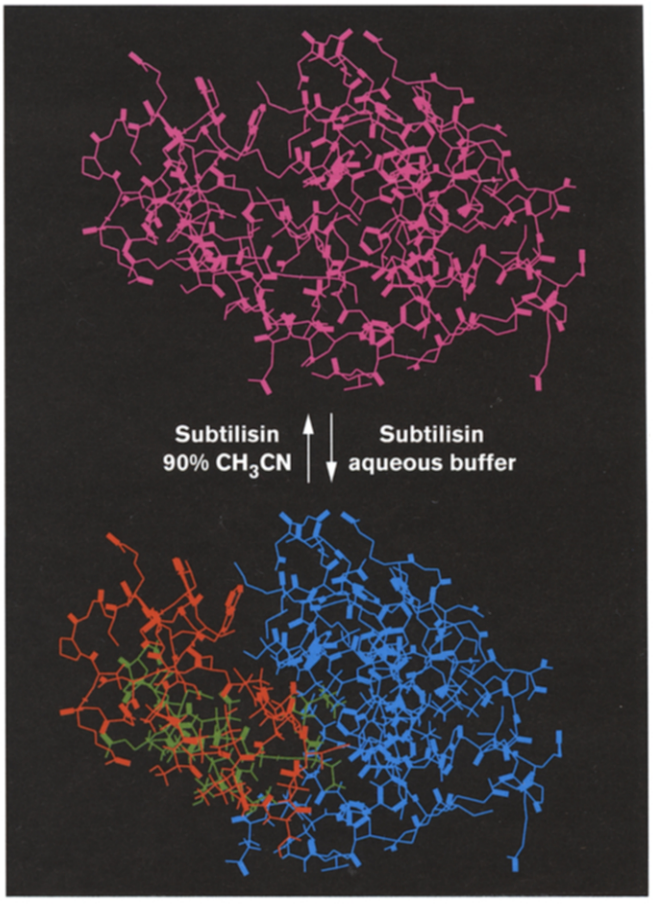
Size exclusion chromatography

Size exclusion studies were performed at 4 °C using Sephadex G-50 in a 1.6 cm by 90 cm column. The eluent, 50 mM triethanolamine-HCl, pH 7.5, was controlled at 0.3 ml min^{-1}, and was monitored for peptide or protein elution at 214 nm. A standard molecular weight curve was generated using bovine serum albumin, carbonic anhydrase, cytochrome *c*, and aprotinin. The column was loaded with 0.5 ml samples of 1 mg ml^{-1} native, proteolyzed, or religated lysozyme. The apparent molecular weights of each sample were obtained by comparison to the calibration curve.

Reduction and reoxidation

Prior to or following limited proteolysis, the four disulfide bridges within the hen egg-white lysozyme were reduced using classical conditions [24–28]. The protein or proteolysis mixture (70 μmol), in 1 ml of 50 mM triethanolamine-HCl, pH 7.5 buffer, was added to 1.5 ml of 8 M urea, pH 8, and 2-mercaptoethanol (1 μl) (βME) was then added. The reaction was degassed with N_2 and allowed to stir at room temperature for 5 h. The reaction was adjusted to pH 3 with concentrated acetic acid, and the acidified reaction was desalted on a

Figure 4



Semisynthesis of proteins by subtilisin produces fully-folded, catalytically active proteins from small fragments. The three-dimensional structure of lysozyme is shown (top) and the three fragments of lysozyme that result from cleavage at Gln41–Ala42 and Leu56–Gln57 are color-coded (bottom).

Sephadex G-25 column equilibrated in 0.1 M acetic acid. The 0.5 ml G-25 column fractions were assayed for free thiol using Ellman's test [29]. Caution was used when running the G-25 column to ensure that βME did not elute with the reduced protein sample.

To reoxidize previously reduced lysozyme samples, the desalted sample, in 0.1 M acetic acid, was adjusted to pH 8.5 with 1.5 M Tris-HCl. The sample was stirred, and exposed to the air at room temperature for 15 h. After that time, the samples were again assayed to determine the concentration of free thiol.

Religation of proteolyzed hen egg-white lysozyme

After limited proteolysis, the mixture of lysozyme peptide fragments, in either the oxidized or reduced form, was added to the desired percentage (v/v) of organic cosolvent: acetonitrile, glycerol, or dimethyl sulfoxide. The pH of the reactions was measured to be 7.2 for the native religation reaction, and 5.7 for the reactions containing reduced proteolyzed lysozyme. Each reaction, which still contained subtilisin, was allowed to remain at room temperature, and, within ~5 min, the religation reaction was complete as judged by analytical SDS-PAGE.

High performance liquid chromatography

Purification of religated lysozyme was accomplished on a Vydac C8 reverse phase semi-preparative column, 2.2 x 25 cm, 8.0 ml min^{-1}, with a linear gradient (15–95 % over 60 min) of mobile phase A (100 % CH_3CN/0.1 % trifluoroacetic acid (TFA)) in the presence of mobile phase B (100 % H_2O/0.1 % TFA). The products were then analyzed by electrospray mass spectrometry (ES-MS). ES-MS was performed in a matrix of 50:50 CH_3CN and H_2O with 3 % acetic acid, (VG Platform, University of Nebraska Center for Mass Spectrometry).

Circular dichroism spectroscopy

Circular dichroism spectra were recorded on a Jasco J-600 spectropolarimeter. All spectra were recorded in 10 mM phosphate, pH 7.0 buffer, at 25 °C. The spectra were recorded using a 2 mm path length cell scanned from 200 to 260 nm. The spectra were an average of three scans with a resolution of 0.2 nm and a scan speed of 10 nm min^{-1}. All stock solutions were quantitated using amino acid analysis (Beckman 7300 amino acid analyzer).

Assay of hen egg-white lysozyme activity

The activity of native, religated, and proteolyzed hen egg-white lysozyme, in oxidized and reduced forms, was determined as a function of its ability to reduce the turbidity of a suspension of *Micrococcus lysodeicticus* cells (~80 mg) in 67 mM phosphate, pH 7.1 buffer [20,21]. 20 μg of each lysozyme sample was added to the cell suspension and the decrease in absorbance at 450 nm was monitored over time at 25 °C on a Carey 1E spectrophotometer using a cell path length of 1 cm.

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