

Youji Shimazaki^{1,2}
Yoshiko Ochi²
Kennosuke Fujimura²

¹Department of Chemistry and
biology, Graduate School of
Science and Engineering, Ehime
University, Matsuyama, Japan

²Faculty of Science, Ehime
University, Matsuyama, Japan

Received August 25, 2017

Revised January 29, 2018

Accepted January 30, 2018

Research Article

Microscale isolation of native forms of lysozyme from chicken egg white by gel isoelectric focusing

To separate and extract the native states of lysozyme from chicken egg white, a hybrid method for the mobilization of proteins after non-denaturing gel isoelectric focusing (IEF) combined with detection of lysozyme activity was developed. When the proteins in the chicken egg white were first separated using non-denaturing gel IEF, a lysozyme was obtained at the top of the gel column at the cathode end of the IEF. And, when the IEF-separated proteins of the egg white were mobilized by replacing the cathodic sodium hydroxide solution with phosphoric acid solution, an additional active state of the lysozyme that could be bound to proteins, such as ovotransferrin, was extracted from the solution. Furthermore, it was shown that the addition of lysozyme, obtained via IEF, to pure ovotransferrin generated a complex manifesting lysozyme activity, clearly indicating a successful reconstruction of the lysozyme-ovotransferrin complex *in vitro*. Therefore, the obtained results demonstrated that the native states of lysozymes, such as lysozyme and the lysozyme-ovotransferrin complex, can be effectively separated and extracted using non-denaturing gel IEF. Thus, this method can be applied to separate and extract different charge states of native proteins that retain their biological activities.

Keywords:

Cathodic mobilization / Complex form / Isoelectric focusing / Lysozyme activity / Ovotransferrin

DOI 10.1002/elps.201700445



Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Lysozyme from chicken egg white exists in several states, including the lysozyme and a complex of lysozyme and ovotransferrin (ovt) [1–4]. Since these states of lysozyme possess different charge states, a separation method that retains not only the lysozyme activity but also its different states is required. It has been reported that gel isoelectric focusing (IEF) can be applied to separate hemoglobin protein complexes, because of which the isoelectric point (pI) values of single hemoglobin and the hemoglobin-haptoglobin complex are changed [5]. In addition, we reported that the activities of enzymes such as esterase are retained after separation by non-denaturing gel IEF [6, 7]. Therefore, non-denaturing gel IEF is thought to be suitable for examining the different

charge states of lysozymes while retaining their bacteriolytic activity. Lysozyme from chicken egg white comprises 129 amino acids, has a molecular mass of 14.3 kDa and a pI of 10.7 [1, 2]. Accumulated evidence indicates that the lysozyme can be purified from egg white using affinity methods involving magnetic beads [8–11]. The lysozyme is thought to be separated from egg white in the basic area of the IEF gel and/or at the top of the IEF column at the cathode end because the pI of lysozyme alone is 10.7. In addition, because it has been reported that lysozyme activity is kept with 0.1 M trimethylamine (pH 10.9) [12]. Therefore, the lytic activity of lysozyme can be retained even after separation in the basic area of the IEF gel and/or at the cathode end of the IEF gel. In contrast, other different charge states of lysozymes, such as lysozyme-ovt complex, are thought to be separated within the IEF gel. Furthermore, it has been reported that after the separation by IEF, proteins are mobilized by replacing the cathodic sodium hydroxide solution by solutions containing another anion such as phosphoric acid [13]. Owing to this, proteins such as lysozymes that are separated within the IEF

Correspondence: Dr. Youji Shimazaki, Department of Chemistry and Biology, Graduate School of Science and Engineering (Science), Ehime University, Matsuyama City, 790-8577 Japan
Fax: +81-89-927-9590
E-mail: shimazaki.yoji.my@ehime-u.ac.jp

Abbreviations: EU, enzyme units; Ovt, ovotransferrin

Color Online: See the article online to view Figs. 1–6 in color.

gel can migrate to the cathode and can be extracted within the solution. The extracted lysozyme is thought to retain its lytic activity because of its separation and mobilization using non-denaturing IEF. However, due to the fact that the extracted lysozyme is diluted within the solution, it is necessary that the protein be concentrated. Polyethylene glycol (PEG) has been used for protein precipitation in fluidic samples [14]. Thus, lysozymes in solution can be concentrated by PEG precipitation. After the lysozymes are concentrated, their activity and structures can be examined. In addition, other native minor proteins that retain their biological activity, such as bacteriolytic activity, are concentrated after separation and mobilization using non-denaturing gel IEF.

The purpose of the present study is to develop a hybrid method combining the mobilization of proteins after non-denaturing gel IEF and the measurement of the lysozyme activity. To this end, different states of lysozyme, such as a single lysozyme and lysozyme-ovt complex, were extracted after separation and mobilization using non-denaturing gel IEF. The activity of the extracted lysozymes was measured by using specific methods. The outcomes of this research indicate that the developed method can be successfully applied to separate and extract other proteins that have different states with the retention of their biological activities, for instance, bacteriolytic activity.

2 Materials and methods

2.1 Chemicals and sample preparation

Acrylamide, carrier ampholyte (Pharmalyte, pH 3–10) and polyclonal anti-lysozyme (Hen egg white) antibody were purchased from Daiichi Pure Chemicals Co. Ltd. (Osaka, Japan), GE healthcare (Uppsala, Sweden) and Rockland Inc. (Gilbertsville, PA), respectively. Polyvinylidene difluoride (PVDF) and protein supports were purchased from Merck-Millipore (Bedford, MA) and Beckman coulter, Inc. (Brea, CA), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Osaka, Japan). A freshly-laid fertilized chicken egg was used for the sample preparation. For the preparation of 0.1 M Tris-HCl (pH 7.2 and 7.0) solutions, 0.1 M Tris was adjusted to pH 7.2 and 7.0, respectively, using HCl. To prepare the 50 mM Tris-HCl (pH 7.2) solution, 0.1 M Tris-HCl (pH 7.2) was diluted by factor of 2 using deionized water. After the yolk and egg white were separated, the egg white was two-fold diluted with 50 mM Tris-HCl (pH 7.2). The homogenate was centrifuged for 20 min at $6000 \times g$ to obtain the cytosol fraction.

2.2 Gel isoelectric focusing of egg white proteins

For separation of chicken egg white proteins by non-denaturing IEF using a previously reported method [13, 15]. Sucrose was added to egg white at a final concentration of

40% (m/v). For separation of lysozyme from egg white by non-denaturing IEF, IEF was done on gel column (30 mm length $\times$ 1.3 mm id) within a glass capillary (45 mm length $\times$ 1.3 mm id). A mixed solution of 4 or 8% (m/v) acrylamide (0.2 or 0.4% (m/v) *N,N'*-methylenebisacrylamide (Bis), respectively) containing 5% (v/v) pharmalyte (pH 3–10), 0.05% (m/v) ammonium persulfate and 0.029% (v/v) TEMED was used. The electrode solutions were 0.04 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). The protein samples were applied to cathode or anode end of the IEF gel. Constant current of 0.1 mA/capillary was applied to reach a voltage 300 V. And, then the constant voltage at 300 V was applied for 40 min. To determine *pI* within the non-denaturing IEF gel, the pH gradient was measured. For this purpose, the gel was cut into 3-mm segments and each segment was put into a 0.5 mL microtube. 100 μ L aliquot of water was added to the microtube and incubated for 2 h. Then, the pH of the solution was measured.

2.3 Cathodic mobilization of protein after gel isoelectric focusing

To mobilize egg white proteins by non-denaturing IEF using a previously reported method [13], the 0.04 M NaOH electrolyte was replaced with 0.01 M H_3PO_4 after separating egg white proteins by IEF. During mobilization, a current of 0.1 mA per non-denaturing IEF gel column was applied, and less than 150 mW of power was used. Proteins (μ g) were collected at the cathode end of the IEF gel after protein mobilization for 0 (0), 0–5 (5), 5–15 (15), 15–30 (30), 30–60 (60), and 60–90 (90) min. After proteins at the cathode end of the IEF gel were collected, an equivalent volume of 60% (m/v) PEG was added to each fraction. The mixture was then kept at 4°C for more than 2 h, and centrifuged for 30 min at $10\,000 \times g$ for protein precipitation. The precipitate was dissolved in 10 μ L of 0.1 M Tris-HCl (pH 7.2) (the cathode end of IEF fraction). The lysozyme activity and molecular mass of the obtained precipitate were determined. an equivalent volume of 60% (m/v) polyethylene glycol (PEG) 4000 was added to the protein samples. The mixture was then kept at 4°C for more than 2 h, and centrifuged for 30 min at $10\,000 \times g$ for protein precipitation. The precipitate was dissolved in 10 μ L of 0.1 M Tris-HCl (pH 7.2). The lysozyme activity and molecular mass of the obtained precipitate were determined.

2.4 Molecular mass determination of the proteins

To determine the molecular masses of the proteins, the proteins in the obtained samples were separated by one-dimensional SDS-PAGE containing 14% (m/v) acrylamide (0.7% (m/v) Bis). The separated proteins in the gel were stained with 0.1% (m/v) CBB. After 0–2 μ g of the purified lysozyme from chicken egg white was separated using one-dimensional SDS-PAGE and stained by CBB, the intensity of the lysozyme spots was analyzed using the ImageJ

program (Rasband, W.S., U.S. National Institute of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>) to construct the calibration curve. The amounts of lysozyme within the collected fractions at 0 (0) min and 0–5 (5), 15–30 (30), 30–60 (60) and 60–90 (90) min after protein mobilization were estimated using the calibration curve. Spots of ovt on 2-DE were determined after purified ovt and the mixture of egg white proteins and the purified ovt were separated using 2DE combined with non-denaturing IEF and a 2nd-dimensional SDS-PAGE. To identify the lysozyme, immunoblotting was performed as follows. The proteins separated by one-dimensional SDS-PAGE or 2DE were transferred from the gel to a PVDF membrane using a semi-dry transblotting apparatus for immobilization [6]. After blotting, the PVDF membrane was soaked in 5 mL of 1% (m/v) bovine serum albumin (BSA) in 0.1 M Tris-HCl (pH 7.0) for 1 h. After washing with the buffer, a buffer containing polyclonal anti-lysozyme antibody and BSA was added to the membrane. The membrane was then incubated for more than 2 h at room temperature. After washing with the buffer, the membrane was incubated with peroxidase-conjugated goat anti-rabbit antibody (1/100 dilution) for 1 h at room temperature. After washing in the buffer, immunoreactive spots were stained with 0.2 mM diaminobenzidine- H_2O_2 in the Tris-HCl (pH 7.2) at room temperature. To analyze the specific spots that reacted to the polyclonal anti-lysozyme antibody, the membrane was incubated with a peroxidase-conjugated goat anti-rabbit antibody without incubating with the polyclonal anti-lysozyme antibody. The stained spots were further analyzed using diaminobenzidine- H_2O_2 .

2.5 Reconstruction of the protein complex with lysozyme and ovt

For the reconstruction of the protein complex with the lysozyme and pure ovt, 20 μg of the purified ovt was added to 10 μL of the cathode end fraction containing 8–16 μg of protein and incubated with 50 mM Tris-HCl (pH 7.2) for longer than overnight at 4°C. Solutions containing 20 μg of the purified ovt and the fraction containing 8–16 μg of protein obtained from cathode end were used as controls. After the addition of sucrose to the protein mixture at a final concentration of 40% (m/v), the mixtures were allowed to be separated by non-denaturing IEF. The IEF gel was placed on the top of a 2-DE, which was then run with 5.7% (m/v) acrylamide (0.28% (m/v) Bis). The separated proteins in the 2DE gel were stained with 0.1% (m/v) CBB. To identify ovt and the protein complex containing ovt in 2DE gel using zinc acetate, 2DE gel was soaked into 10 mL of 1% (m/v) sodium carbonate for 15 min. After washing, the gel was soaked into 10 mL of 4.4% (m/v) zinc acetate solution for 5 min. White bands were observed on the gel after more than ten washings. To analyze the constituent proteins and to test the lysozyme activities of the protein complexes on the 2DE gel, the proteins were homogenized with 50 μL of 0.1 M Tris-HCl (pH 7.2) and extracted from the non-denaturing 2DE in the solutions.

This step was performed after the extraction of the purified ovt spots stained with zinc acetate that were used as controls. For the real samples, after proteins obtained from cathode end, separated by non-denaturing 2DE and stained with zinc acetate: similar separation area of the 2DE gel was excised, homogenized with 50 μL of 0.1 M Tris-HCl (pH 7.2) and extracted in the solution. Lysozyme activity of the obtained protein mixtures was established on the basis of the degradation of cell wall structure. To analyze the constituent proteins involved in the protein complexes on the 2DE gel, they were separated by SDS-PAGE and stained either with CBB or silver. For silver staining, gel was washed with water three times. The gel was soaked in 10 mL of 10% (v/v) glutaraldehyde solution and incubated for 30 min. After three washes with water, the gel was further drenched in 10 mL of ammoniacal silver nitrate solution containing 0.64% (m/v) silver nitrate and 1% (v/v) ammonium hydroxide and incubated for 5 min. Next, the gel was again washed with water three times and soaked in 10 mL of 0.05% (v/v) of formaldehyde solution. When the protein bands were stained, the gel was drenched in photographic fixing solution and washed with water.

2.6 Lysozyme activity detection on Petri dishes

The antibacterial activity against *Bacillus subtilis*, (natto) was analyzed using the following methods combined with non-denaturing IEF and culture. After 100 mL of culture medium containing 0.5 g NaCl, 1.0 g tryptone, 0.5 g dried yeast extract, 1.5 g agar powder, and 0.8 mM NaOH was autoclaved at 123°C for 30 min, it was poured into Petri dishes to a height of 10 mm. To make a groove of IEF gel, a glass of rod gel was placed on the surface of the culture medium, and the agar medium was solidified. To obtain *Bacillus subtilis*, (natto), a sample of the race surface was obtained with a cotton bud, and this was dissolved in sterile water. 200 μL of water containing *Bacillus subtilis*, (natto) was then homogeneously spread over the surface of the medium using a bacterial spreader. The proteins obtained at the cathode end of the IEF gel were applied to a piece of filter paper, and the paper was placed on agar medium containing *Bacillus subtilis*, (natto) (Fig. 1A). The IEF gel containing the separated proteins of egg white was placed on the agar medium containing *Bacillus subtilis*, (natto) (Fig. 1B). This was then incubated for longer than overnight.

2.7 Lysozyme activity analysis using degradation of cell wall component

To determine the activity of the lysozyme from egg white, 10 μL samples of the obtained precipitate were added to 200 μL of 0.33 mM potassium phosphate buffer (pH 6.2) containing 0.01% (m/v) *Micrococcus luteus* suspension, and changes in absorbance at 450 nm were monitored by UV-vis spectrometry (Biospec-1600, Shimadzu) at 0, 1, 2, 3, 20, and 40 min at 25°C. The absorbance changes in the

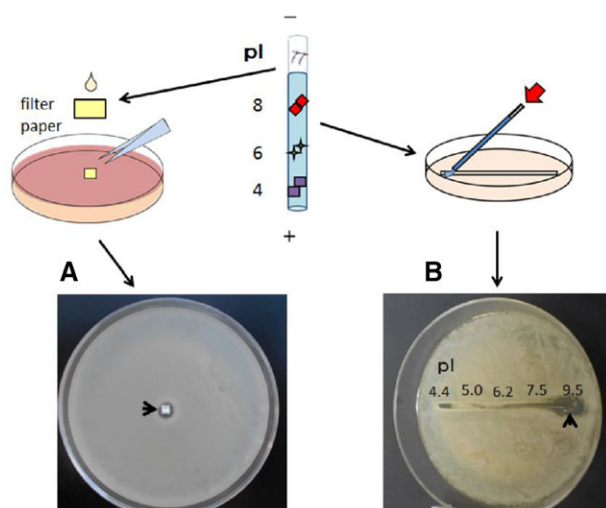


Figure 1. Bacteriolytic activity against *Bacillus subtilis* (natto) of the proteins obtained at the cathode end of the IEF gel (A), and of the proteins within the IEF gel (B) after separation of egg white proteins by non-denaturing IEF. Growth inhibition of *Bacillus subtilis* (natto) is shown by arrowheads.

Micrococcus luteus suspension in the absence of samples were used as controls. The slope ($A_{450\text{ nm}}/\text{min}$) of the linear portion of the curve for the absorbance at 450 nm versus time was used to calculate the biological activity of the lysozyme in enzyme units (EU). A decrease of $0.001 \times A_{450\text{ nm}}/\text{min}$ was defined as 1 EU. To calculate the relative biological activity of the lysozyme, the maximum biological activity of the lysozyme in the fractionated samples was assigned the value 1.0.

2.8 Identification of lysozyme by MALDI-TOF MS

The peptide mass fingerprinting essentially followed a previously published protocol [16–18]. The protein spot was excised from the gel and digested in the gel by bovine trypsin (12.5 ng/ μL) at 37°C for 8 h. The digested polypeptides were recovered by 10 μL solution containing 0.1% (v/v) trifluoroacetic acid (TFA) and 50% (v/v) acetonitrile. The extracts were concentrated in a vacuum evaporator centrifuge and suspended in 20 μL of 0.1% (v/v) TFA. The polypeptides obtained were concentrated and desalted using C18 Zip Tip from Millipore (Bedford) and mixed with α -cyano 4-hydroxycinnamic acid. Mass analysis was done using MALDI-TOF MS operating in a positive ion reflector mode. The spectra were analyzed using the mass values for monoisotopic peaks that were used for searches (MASCOT, <http://www.matrixscience.com/>) against the Swiss Prot database. The database was searched using the following terms: taxonomy (bony vertebrates), trypsin digestion (one missed cleavage allowed), and mass tolerance of 150 ppm using internal calibration, oxidation of methionines and carbamidomethylation of Cysteine. The criteria used to accept

identification included the extent of sequence coverage, the number of peptides matched and the probabilistic score (the required probability for a random match was <0.05). For identification of lysozyme after separation by one-dimensional SDS-PAGE, the combined methods of peptide mass fingerprinting and immunoblotting were used in the present study.

3 Results and discussion

3.1 Lysozyme activity of egg white at the cathode end of non-denaturing IEF after separation by IEF

Bacteriolytic activity against *Bacillus subtilis* (natto) is shown in Fig. 1 after egg white proteins at the cathode end of the IEF gel (a) and the proteins within the IEF gel (b) were separated and obtained using non-denaturing IEF gel. Growth inhibition of *Bacillus subtilis* (natto) was clearly achieved with the proteins at the cathode end (a) and at a pI of more than 8 with the IEF gel (b). The growth inhibition is thought to originate from the antibacterial activity of egg white lysozyme, because the basic proteins of egg white retain their activity after separation by non-denaturing IEF. Figure 2 shows lysosome activity using *Micrococcus luteus* (a) and protein separation using SDS-PAGE (b) after egg white proteins were separated by IEF and precipitated using PEG. The lysosome activity in the presence of the proteins obtained at the cathode end (3.6) was six-fold higher than that in the absence of the proteins (0.6, control in Fig. 2A). A protein band at 14.4 kDa was specifically obtained at the cathode end of the IEF gel after egg white proteins were separated by non-denaturing IEF (lane 2 in Fig. 2B). Furthermore, monoisotopic peaks at m/z 993 251, 1045 426, 1325 720, 1333 760, 1428 789, 1675 628, and 1 753 710 were obtained after the protein band at 14.4 kDa

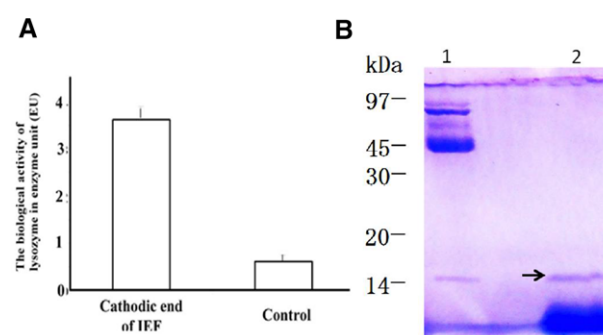


Figure 2. Lysosome activity using *Micrococcus luteus* (A) and protein separation using SDS-PAGE (B) when proteins are obtained at the cathode end of the IEF gel and precipitated using PEG after the separation of egg white proteins by IEF. The lysosome activity in the presence of the proteins obtained at the cathode end (cathodic end of IEF) is higher than that in the absence of the proteins (Control) (A). A protein band at 14.4 kDa is specifically obtained when proteins are obtained at the cathode end of the IEF gel and precipitated using PEG after the separation of egg white proteins by IEF (arrow, lane 2 in B). SDS-PAGE of egg white proteins without IEF separation indicates lane 1 in B.

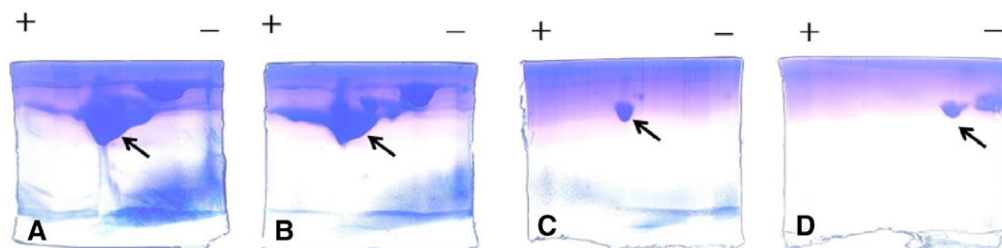


Figure 3. 2DE patterns combined with non-denaturing IEF and SDS-PAGE for the movement of egg white proteins in IEF and the mobilization steps. When NaOH as catholyte was replaced by H_3PO_4 after gel IEF separation of egg white proteins, the proteins were mobilized towards the cathode as time proceeded at 0 min (A), 30 min (B), 60 min (C) and 90 min (D). Arrow indicates ovalbumin in each pattern.

was digested with trypsin (Supporting Information Fig. 1). The peaks corresponded to polypeptides of lysozyme C from *Gallus gallus* (LYSC-CHICK, Swiss Prot ID). Thus, lysozyme that retains its activity can be isolated at the cathode end of the IEF gel using non-denaturing IEF.

3.2 Mobilization and extraction of egg white proteins from non-denaturing IEF after separation by the IEF

Figure 3 shows the movement of egg white proteins in IEF and the mobilization steps using 2DE combined with non-denaturing IEF and SDS-PAGE. When NaOH as catholyte was replaced by H_3PO_4 after gel IEF separation of egg white proteins (Fig. 3A), the proteins were mobilized toward the cathode as time proceeded (Fig. 3B, C, and D). It has been reported that human plasma proteins move towards the cathode, retaining their relative positions attained during the focusing step [13]. Thus, the proteins separated by

non-denaturing IEF gel are thought to be extracted. The extracted proteins can retain their physiological functions such as lysosome activity because the proteins are separated and extracted under non-denaturing conditions.

Extracted proteins from the IEF gel were collected at cathode end of the IEF gel. Figure 4A shows relative lysozyme activity within the collected fractions at 0 (0) min and for 0–5 (5), 5–15 (15), 15–30 (30), 30–60 (60) and 60–90 (90) min. The lysozyme activities decreased over time proceeds; however, those in each fraction were clearly detected. The protein bands at 14.4 kDa, which were recognized by anti-lysozyme antibody, were clearly obtained when the proteins in the fractions at 0, 5, 30 and 60 min were separated by SDS-PAGE (Fig. 4B and C). When 260 μg of proteins obtained from chicken egg white were applied to non-denaturing gel IEF with 8.8 mm³ of the collected capacity, the estimated amounts of lysozyme were 13, 2, 0.4 and 0.5 μg within the collected fractions at 0, 5, 30 and 60 min, respectively. Thus, this method involves the mobilization and extraction of the proteins within IEF at the cathode end by replacing the cathodic sodium

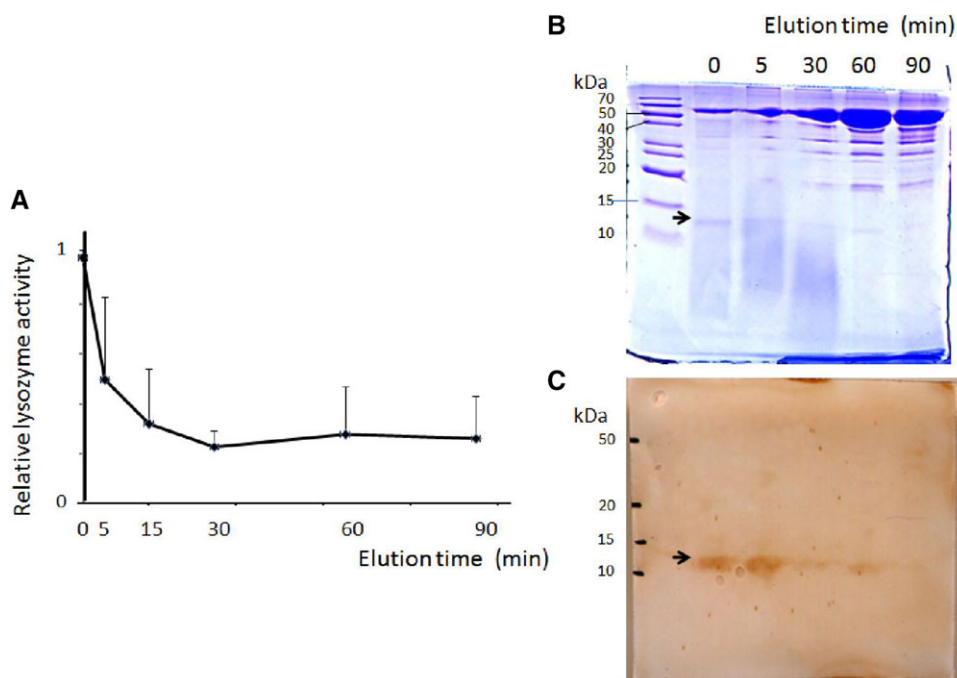


Figure 4. Relative lysozyme activity in the collected fractions with elution times of 0, 5, 15, 30, 60, and 90 min (A). SDS-PAGE analysis of proteins in the collected fractions with elution times of 0, 5, 15, 30, 60, and 90 min (B). Immunoblotting using anti-lysozyme antibody after separation by SDS-PAGE of proteins in the collected fractions with elution times of 0, 5, 15, 30, 60, and 90 min (C). Arrows indicate a 14.4 kDa protein spot.

hydroxide solution by phosphoric acid solution. Using this method, micrograms of lysozyme were obtained. It has been reported that 100 μ g of protein sample can be isolated by preparative capillary isotachopheresis in column coupling system, and hundreds of milligrams of biopolymers can be separated using a continuous free-flow electrophoresis [19]. Herein, using this gel IEF method, the collected capacities at the top of the gel column can be increased by increasing the diameter of the gel column and/or length of the collected space. In addition, it has been reported that protein-protein binding such as serum haptoglobin and hemoglobin induces changes in the *pI*, compared to that of hemoglobin [9]. Thus, complexes containing lysozyme in egg white can penetrate the non-denaturing IEF gel, because the *pI* of the complex is thought to be lower than that of lysozyme alone. Complexes separated by non-denaturing IEF can be eluted from the cathode end of the IEF gel. It has been reported that native proteins and protein complexes in solution can be separated and extracted by offgel IEF, because a maximum current of 50 μ A per IEF and 200 mW of power was used in this method [20–22]. In the present study, a current of 0.1 mA per non-denaturing IEF gel column was applied, and less than 150 mW of power was used. Thus, after IEF separation and extraction, proteins can retain their activities and protein-protein interactions. In addition, the present study firstly indicates that combination of micro-sized non-denaturing IEF and enrichment culture can be applied to examine the antibacterial activity of native proteins in the IEF gel. Furthermore, it takes from a few hours to 4 days to separate and extract proteins using offgel IEF [21], while proteins can be separated and extracted within 100 min when the target proteins are narrowed down.

3.3 Analysis of lysozyme-ovotransferrin complex by 2-DE combined with non-denaturing IEF and SDS-PAGE

Figure 5 depicts 2-DE pattern (A), immunoblotting using an anti-lysozyme antibody and peroxidase-conjugated goat anti-rabbit antibody (B) and immunoblotting using a

peroxidase-conjugated goat anti-rabbit antibody (C) after the egg white proteins were separated by 2DE combined with non-denaturing IEF and SDS-PAGE. Several immunoreacted spots (arrow, * and ** in Fig. 5) were obtained in the 2DE patterns in (Fig. 5B and C). Spots (* and ** in Fig. 5B and C) reacted to peroxidase-conjugated goat anti-rabbit antibody, and these reactions are considered to be non-specific interactions. However, the arrow spot in Fig. 5B specifically reacted to the anti-lysozyme antibody (Fig. 5B) when the membrane was incubated. The specifically reacted spot was located below the ovt spot (Fig. 5A). It has been reported that the interaction of ovt and lysozyme induces the formation of soluble complexes [3]. Thus, the complex of ovt and lysozyme exists in egg white. The complex can penetrate non-denaturing IEF gel, because the *pI* of the complex is thought to be lower than that of lysozyme. The complex can then be broken down into ovt and lysozyme during separation by SDS-PAGE. To examine the reconstruction of the ovt-lysozyme complex in vitro, lysozyme separated by non-denaturing IEF is mixed with the purified ovt.

Figures 6A and 6B show CBB and zinc acetate staining, respectively, after a single lysozyme obtained at the top of the IEF gel column at the cathode end was mixed with the purified ovt and separated by non-denaturing 2-DE. Spots at *pI* 5.5–7.0 were obtained when the mixture was separated by non-denaturing 2DE (Fig. 6A, 6B and Supporting information Fig. 2A). White colored spots of ovt were obtained on the non-denaturing 2DE as a result of the bonding formed between zinc and ovt (Fig. 6B). The constituent proteins were analyzed by SDS-PAGE, and bands at 76 and 14.4 kDa were obtained (Fig. 6C) after the excision of the spots at *pI* 5.5–7.0. The bands at 76 and 14.4 kDa could be attributed to the mixed ovt and lysozyme, respectively. In contrast, spots at *pI* 5.5–7.0 were also obtained when the purified ovt was separated by non-denaturing 2DE (Supporting Information Fig. 2B). However, only 76 kDa band was obtained after the spots at *pI* 5.5–7.0 were excised and the constituent proteins were analyzed by SDS-PAGE (data not shown). When a lysozyme obtained at the top of the IEF gel column at the cathode end was separated by non-denaturing 2-DE, no spots were

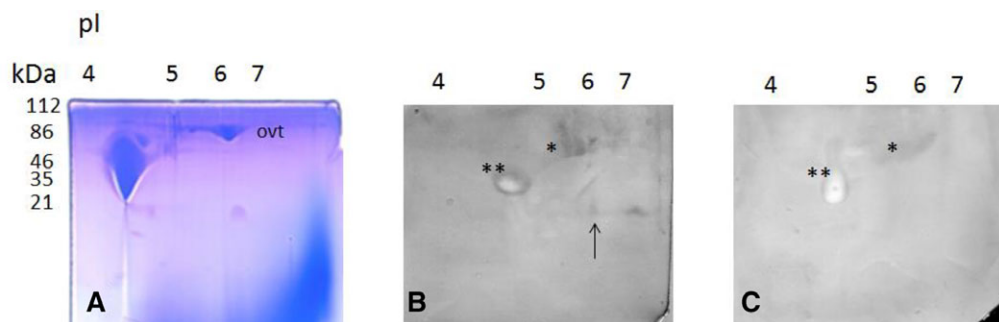


Figure 5. 2-DE pattern (A), immunoblotting using anti-lysozyme and peroxidase-conjugated goat anti-rabbit antibodies (B) and immunoblotting using peroxidase-conjugated goat anti-rabbit antibody (C) after the egg white proteins were separated by 2DE combined with non-denaturing IEF and SDS-PAGE. Spots (* and **) indicate nonspecific reactivity to the peroxidase-conjugated goat anti-rabbit antibody. Arrow and Ovt indicates lysozyme and ovotransferrin spot, respectively.

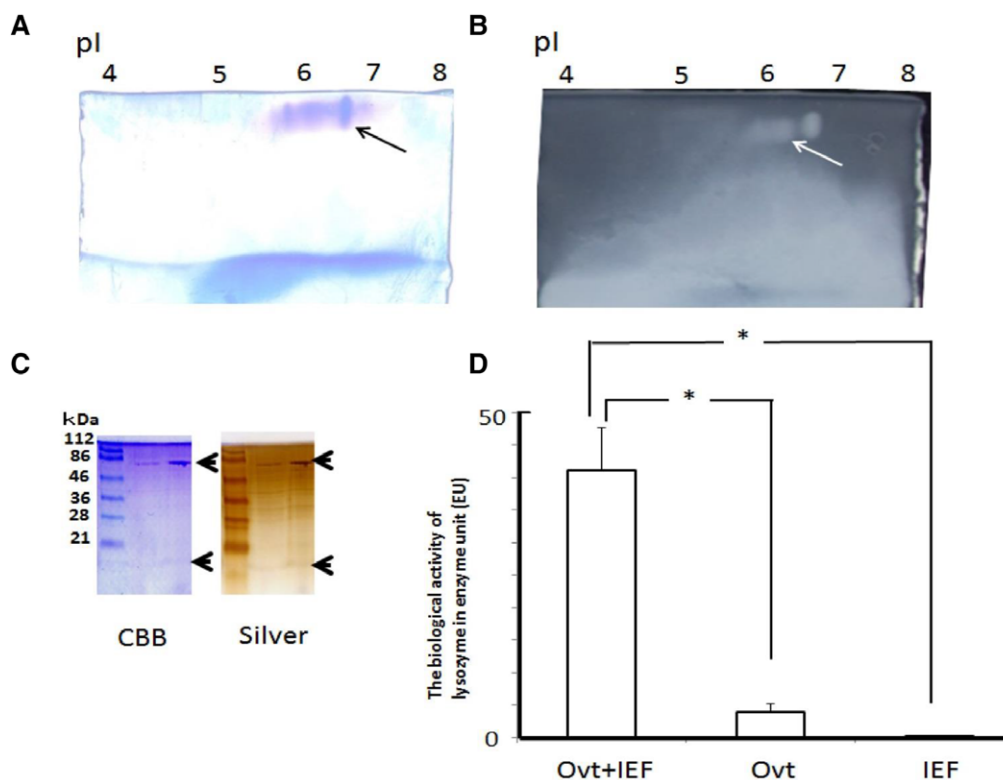


Figure 6. CBB (A) and zinc acetate staining (B), respectively, after a single lysozyme obtained at the top of the IEF gel column at the cathode end, was mixed with the purified ovt and separated by non-denaturing 2-DE. CBB or silver staining (C) of constituent proteins after the excision of spots on non-denaturing 2DE at pI 5.5–7.0 (arrow in b). Lysozyme activities (EU) of the extracted proteins (D) after the mixture of the purified ovt and the lysozyme obtained at the top of the IEF gel column at the cathode end (ovt + IEF), the purified ovt (ovt) or lysozyme obtained at the top of the IEF gel column at the cathode end (IEF) were separated by non-denaturing 2DE and zinc acetate staining, and then, extracted within the solution. Arrow indicates the mixture spot; Arrowhead indicates constituent proteins of spots at pI 5.5–7.0. * $p < 0.05$ (Student's *t*-test).

obtained (Supporting Information Fig. 2C). It can therefore be stated that the spots at pI 5.5–7.0 are caused by either the purified ovt or the complex between the ovt and lysozyme. Lysozyme activities (EU) of the extracted protein mixture in the solution are shown in Fig. 6D. They were assessed after the mixture of the purified ovt and the lysozyme obtained at the top of the IEF gel column at the cathode end (ovt + IEF), pure ovt (ovt), or lysozyme obtained at the top of the IEF gel column at the cathode end (IEF) were separated by non-denaturing 2DE and zinc acetate staining. It was noticed that the activity of the mixture (ovt + IEF) was 10.5 and 136 times higher than those of the pure ovt, and proteins in cathode end of IEF (IEF), respectively (Fig. 6D). When the lysozyme obtained at the top of the gel column at the cathode end was added to the purified ovt, ovt manifesting lysozyme activity was obtained. These results indicate that a complex between lysozyme and ovt can be reconstructed in vitro, and this complex can retain lysozyme activity even after separation by non-denaturing 2DE, staining with zinc acetate and extraction from the gel. It has been previously reported that lysozyme forms an electrostatically soluble complex with ovt [3, 4]. Indeed, the present study reinforces the idea of the reconstruction of ovt–lysozyme complex in vitro. Therefore, it

can be concluded that lysozyme–ovt complex from egg white was mobilized and extracted from the non-denaturing gel IEF and its lysozyme activity was retained after the extraction of the complex.

4 Concluding remarks

In summary, this study emphasized that the native states of lysozyme, such as a lysozyme and the lysozyme–ovt complex, can be separated and extracted using non-denaturing gel IEF. Lysozyme that retains its activity was obtained at the top of the gel column at the cathode end after the proteins from chicken egg white were separated using non-denaturing gel IEF. Lysozyme–ovt complex with lysozyme activity within the non-denaturing gel IEF was mobilized and extracted at the cathode end by replacing the cathodic sodium hydroxide solution with phosphoric acid solution; then, the complex was extracted within the solutions from the gel IEF. On the basis of the obtained results, it can be admitted that a hybrid method for the mobilization of proteins after non-denaturing gel IEF combined with detection of lysozyme activity was developed. Likewise, this method can be also applied to separate

and extract other proteins that have different states with their biological activities, for instance, bacteriolytic activity.

This work was partly supported by Japan Society for Promotion of Science (JSPS), Grants-in-Aid for Scientific Research (KAKENHI) Grant Number 25410145. The mass analysis of lysozyme was carried out on a MALDI-TOF MS (Voyager-DE PRO) at the Division of Applied Protein Research, Advance Research Support Center, Ehime University.

The authors have declared no conflict of interest.

5 References

- [1] Mine, Y., *Trends Food Sci. Technol.* 1995, **6**, 225–232.
- [2] Mine, Y., Ma, F., Lauriau, S., *J. Agric. Food Chem.* 2004, **52**, 1088–1094.
- [3] Ehrenpreis, S., Warner, R. C., *Arch. Biochem. Biophys.* 1956, **61**, 38–50.
- [4] Matsudomi, N., Takahashi, M., Kobayashi, K., *Agric. Biol. Chem.* 1991, **55**, 1651–1653.
- [5] Righetti, P. G., Conti, M., Gelfi, C., *J Chromatogr. A* 1997, **767**, 255–262.
- [6] Sakikawa, T., Shimazaki, Y., *Appl. Biochem. Biotechnol.* 2011, **165**, 69–74.
- [7] Shimazaki, Y., Miki, S., *J. Enzyme Inhib. Med. Chem.* 2013, **28**, 894–899.
- [8] Arica, M. Y., Yimaz, M., Yacin, E., Bayramoglu, G., *J. Chromatogr. B* 2004, **805**, 315–323.
- [9] Altintas, E. B., Denizli, A., *Int. J. Biol. Macromol.* 2006, **38**, 99–106.
- [10] Basar, N., Uzun, L., Güner, A., Denizli, A., *Int. J. Biol. Macromol.* 2007, **41**, 234–242.
- [11] Altintas, E. B., Tüzmen, N., Candan, N., Denizli, A., *J. Chromatogr. B* 2007, **853**, 105–113.
- [12] Habeeb, A. F., Bennett, J.C., *Biochim Biophys Acta* 1971, **251**, 181–184.
- [13] Manabe, T., Miyamoto, H., Iwasaki, A., *Electrophoresis* 1997, **18**, 92–97.
- [14] Ingham, K. C., *Methods Enzymol.* 1984, **104**, 351–356.
- [15] Shimazaki, Y., Sugawara, Y., Ohtuka, Y., Manabe, T. *Proteomics* 2003, **3**, 2002–2007.
- [16] James, P., Quadroni, M., Carafoli, E., Gonnet, G., *Biochem. Biophys. Res. Commun.* 1993, **195**, 58–64.
- [17] Pappin, C. J. C., Hojrup, P., Leaby, A. J., *Curr. Biol.* 1993, **3**, 327–332.
- [18] Corthals, G. L., Gygi, S. P., Aebersold, R., Patterson, S. D., In: Rabilloud, T, (Ed.), *Proteome Research*, Springer, Berlin Heidelberg 2000, pp.197–231.
- [19] Kašička, V., *Electrophoresis* 2009, **30**, 0S40–0S52.
- [20] Meert, C. D., Brady, L. J., Guo, A., Balland, A., *Anal. Chem.* 2010, **82**, 3510–3518.
- [21] Moreda-Pineiro, A., Garcia-Otero, N., Bermejo-Barrera, P., *Anal. Chim. Acta* 2014, **836**, 1–17.
- [22] Ferreira, R.A., Martins-Dias, S., *Anal. Biochem.* 2016, **509**, 100–103.

Electronic Supporting Information

For

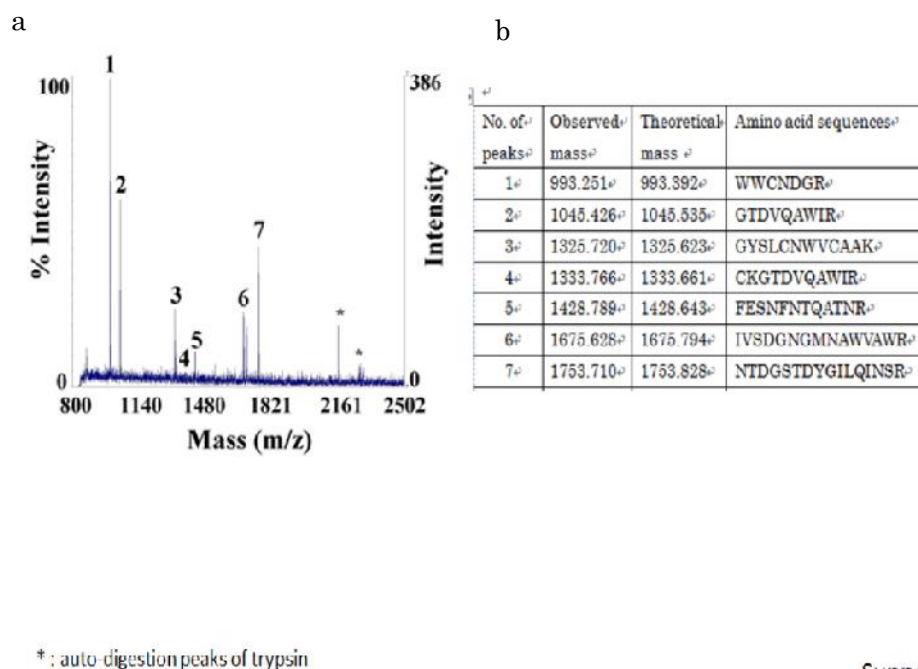
Microscale isolation of native forms of lysozyme from chicken egg white

by gel isoelectric focusing

Youji Shimazaki ^{a, b}, Yoshiko Ochi ^b and Kennosuke Fujimura ^b,

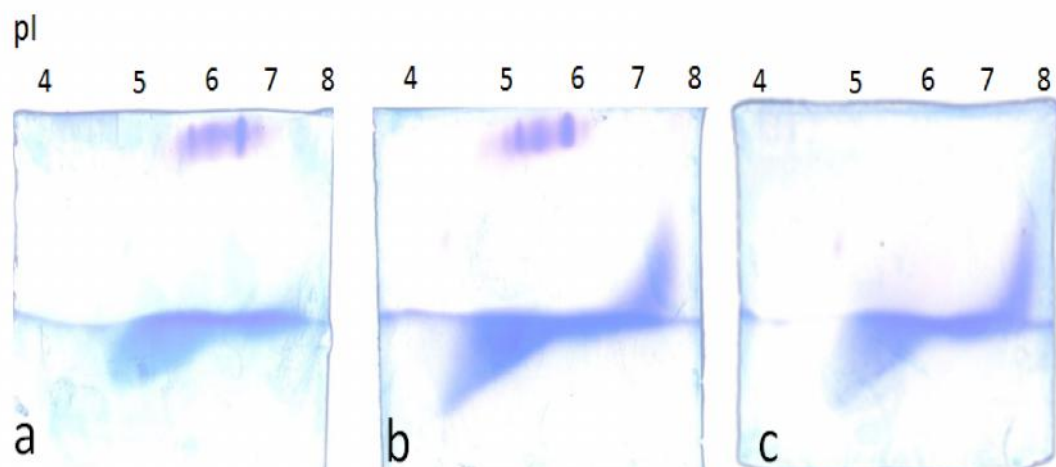
- a) Department of Chemistry and biology, Graduate School of Science and Engineering, Ehime University, Matsuyama, Japan
- b) Faculty of Science, Ehime University, Matsuyama, Japan

Correspondence: Dr. Youji Shimazaki: Department of Chemistry and Biology, Graduate School of Science and Engineering (Science), Ehime University, Matsuyama City, 790-8577 Japan; Tel: +81-89-927-9617, Fax: +81-89-927-9590, e-mail: shimazaki.yoji.my@ehime-u.ac.jp



Supporting Information Fig. S1

Figure S1. MALDI-TOF MS spectrum of the tryptic peptide mass from spot (arrow) in Fig. 2b (a). Comparison between the observed mass of the spot and theoretical tryptic peptide mass of lysozyme from chicken egg white (b).



Supporting Information Fig.2

Figure S2. CBB staining after lysozyme obtained at the top of the IEF gel column at the cathode end, was mixed with the purified ovt and separated by non-denaturing 2-DE (a), the purified ovt was separated by non-denaturing 2-DE (b), and lysozyme obtained at the top of the IEF gel column at the cathode end was separated by non-denaturing 2-DE (c).