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Antibacterial activity of lysozyme-binding proteins from chicken egg white

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ABSTRACT

The purpose of this study was to establish a method for determining the bacteriolytic activity after separation of lysozyme-binding proteins from egg white. Lysozyme-binding proteins such as ovotransferrin and ovalbumin were separated by non-denaturing two-dimensional electrophoresis (2DE) and transferred to a membrane. The lysozyme activity of the separated and immobilized egg white proteins was assessed directly to produce a non-denaturing 3D map of the egg white proteins by incorporating an axis that combined each spot's lysozyme-activity with the non-denaturing 2DE pattern. Lysozyme-ovotransferrin and lysozyme-ovalbumin complexes could be reconstructed *in vitro* after the cathode end fraction containing lysozyme was added to purified ovotransferrin and ovalbumin, respectively. These complexes retained lysozyme activity even after separation by non-denaturing 2DE. Furthermore, when the lysozyme-ovotransferrin complex from egg white was extracted after separation by isoelectric focusing by replacing the cathodic sodium hydroxide solution with phosphoric acid solution, the complex possessed bacteriolytic activity against both *Bacillus subtilis* and *Escherichia coli.* These methods can be applied to investigate protein complexes possessing bacteriolytic activity against a wide range of both Gram-positive and Gram-negative bacteria.

1. Introduction

Lysozyme exerts a lytic activity (lysozyme activity) against the peptidoglycans forming within the cell walls of Gram-positive bacteria (Vocadlo et al., 2001). Since lysozyme is a basic protein, it possesses a positive charge in near-neutral pH buffer solutions. Thus, the lytic activity of native lysozymes can be examined after separation by acid-polyacrylamide gel electrophoresis (one-dimensional electrophoresis, 1DE) (Hultmark et al., 1980; Sidhan and Gurnani, 1981; Lockey and Ourth, 1996). However, this method cannot estimate the isoelectric point (pI) differences among proteins. Chicken egg white contains not only lysozyme but also ovotransferrin, ovalbumin, and ovomucoid, which form complexes with lysozyme (Alleoni, 2006; Damodaran et al., 1998; Matsuda et al., 1982). These complexes retain the lysozyme activity when its catalytic site is exposed on their surface. Hence, these complexes must be separated under physiological (*e.g.* non-denaturing) conditions to examine the lysozyme activity. Furthermore, since ovotransferrin, ovalbumin, and ovomucoid are acidic proteins, the pI of the resulting complexes can shift toward the acidic side compared with that of lysozyme alone. Thus, two-dimensional electrophoresis (2DE) combining isoelectric focusing (IEF) and size-based separation techniques could be an optimal solution for separating lysozyme complexes. We have previously reported that protein and protein complexes from biological samples can be separated using non-denaturing 2DE, and transferred to a membrane (Shimazaki and Sakikawa, 2010; Shimazaki and Michhiro, 2013). Because proteins are immobilized on the surface of the membrane after separation and transfer, lysozyme complexes in egg white can interact with peptidoglycan molecules of Gram-positive bacteria. Thus, the lysozyme activity of protein complexes from egg white can be examined after the egg white proteins are separated by non-denaturing 2DE, and transferred to the membrane. Furthermore, a non-denaturing 3D map of egg white proteins can be constructed by combining an axis defining lysozyme activity with the non-denaturing 2DE pattern. Ovalbumin-lysozyme and ovotarnsferrin-lysozyme complexes have been previously formed *in vitro* (Ehrenpreis and Warner, 1956; Santos et al., 2018). To verify isoelectric point (pI) shifts and lysozyme activity of the complexed proteins, it is necessary that complexes are constructed after the purified lysozyme obtained from egg white is mixed with pure ovotransferrin and ovalbumin. Then, the complexes are separated by non-denaturing 2DE and their lysozyme activity is examined. In addition, it has been reported that lysozyme possesses antibacterial activity against Gram-positive species such as

Abbreviations: PVDF, polyvinylidene difluoride; 2DE, two-dimensional electrophoresis; TEMED, N,N,N′; *N′*, tetramethylenediamine; Tris, 2-amino-2-hydroxymethyl −1,3-propanediol

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Bacillus subtilis, but does not inhibit the growth of Gram-negative species such as *Escherichia coli* (Vocadlo et al., 2001; Wellman-Labadie et al., 2008). By contrast, ovotransferrin possesses antibacterial activity against a wide spectrum of bacteria including *E. coli* (Schade and Caroline, 1944; Ko et al., 2008), and, lysozyme has been shown to improve this activity (Ko et al., 2009). Thus, the complex of lysozyme and ovotransferrin is thought to have enhanced antibacterial activity against a wide range of bacteria including Germ-positive and Germ-negative species. We have previously reported that after separation by isoelectric focusing (IEF), the complex of lysozyme and ovotransferrin can be extracted by replacing the cathodic sodium hydroxide solution by phosphoric acid solution (Shimazaki et al., 2018). Moreover, the antibacterial activity of this complex against various microorganisms can be examined after extraction. The purpose of the present study was to establish a method for determining the bacteriolytic activity after proteins and/or protein complexes from egg white are separated by non-denaturing electrophoresis. After the native proteins in egg white are separated by non-denaturing 2DE and are transferred to a membrane, the lysozyme activity of the separated and immobilized proteins and/or protein complexes were examined. We found that the complex of lysozyme and ovotarnsferrin possessed bacteriolytic activity against both *B. subtilis* and *E. coli* when it was applied to bacteria after separation and extraction by non-denaturing IEF.

2. Materials and methods

2.1. Chemicals and sample preparation

Acrylamide, carrier ampholyte (Pharmalyte, pH 3–10) and polyvinylidene difluoride (PVDF) were purchased from Daiichi Pure Chemicals Co. Ltd. (Osaka, Japan), GE healthcare (Uppsala, Sweden) and Merck-Millipore (Bedford, MA, USA), respectively. Polyclonal anti-lysozyme (Hen egg white), polyclonal anti- transferrin (human) and polyclonal anti-ovalbumin (Hen egg white) antibodies were purchased from Rockland Inc. (Gilbertsville, PA, USA), Dako (Glostrup, Denmark) and Novus Biologicals (CO, USA), respectively. *Escherichia coli* (Bacterial strain LE392) was purchased from Promega Co. (Madison, WI, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Osaka, Japan). A freshly –laid fertilized chicken egg was used for the sample preparation. After the yolk and the egg white were separated, the egg white was two-fold diluted with 0.05 M Tris-HCl solution, pH 7.2. The homogenate was centrifuged for 20 min at 6000 ×*g* to obtain the cytosol fraction.

2.2. Separation and immobilization of egg white proteins

Chicken egg white proteins were separated by non-denaturing 2DE using a previously reported method (Manabe et al., 1982; Shimazaki et al., 2003). Sucrose was added to egg white at a final concentration of 40% (m/v). For separation of egg white proteins by non-denaturing isoelectric focusing (IEF), IEF was done on gel column (30 mm length × 1.3 mm id) within a glass capillary (45 mm length × 1.3 mm id). A mixed solution of 4% (w/v) acrylamide (0.2% (w/v) Bis, respectively) containing 5% (v/v) pharmalyte (pH 3–10), 0.05% (w/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 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. The IEF gel was placed on the top of a 2DE, which was then run with 6.6% (w/v) acrylamide (0.33% (w/v) Bis). The proteins separated by 2DE were transferred from the gels to a PVDF membrane using a semi-dry transblotting apparatus for immobilization [5,6]. For the detection of proteins on the membrane, the membrane was soaked in 0.5% Ponceau S in 10 mL 0.1 M acetate buffer, pH 5.2. And, then the membrane was washed with H_2O. For identification proteins on the membrane, the membrane was soaked in 5 mL of 1% (w/v) bovine serum albumin (BSA) in 0.1 M Tris-HCl buffer, pH 7.0, for 1 h after transblotting. After washing with the buffer, a buffer containing 1% BSA and polyclonal anti-lysozyme, transferrin or ovalbumin antibody was added to the membrane. The membrane was then incubated for > 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 buffer at room temperature.

2.3. Lysozyme activity analysis using degradation of cell wall component

To determine the lysozyme activity of the separated proteins on the membrane, spots (5 × 5 mm) of egg white proteins were excised from the membrane. The excised spots were washed with 200 µL of 0.1 M Tris-HCl buffer. The spots were then soaked in 1% BSA and incubated for 1 h for blocking. After washing with the buffer, 200 µL of 0.33 mM potassium phosphate buffer, pH 6.2, containing 0.01% (m/v) *Micrococcus luteus* suspension was added to the excised spots. Changes in absorbance at 450 nm were monitored by UV–vis spectrometey (Biospec-1600, Shimazu) at 0, 1 and 3 min at 25 °C. The absorbance changes in the *Micrococcus luteus* suspension in the absence of the spot were used as a control. The slope ($A_{450\ nm}$/min) of the linear portion of the curve for the absorbance at 450 nm *vs* time was used to calculate the biological activity of the lysozyme in enzyme units (EU). A decrease of 0.001 × $A_{450\ nm}$/min was defined as 1 EU.

2.4. Reconstruction of the protein complex with the single lysozyme and other egg white proteins

To obtain lysozyme from egg white, proteins at the cathode end of the IEF gel were collected after egg white proteins were separated by non-denaturing IEF. After an equivalent volume of 60% (w/v) polyethylene glycol (PEG) 4000 was added to the collected protein fraction, the mixture was kept at 4 °C for > 2 h, and centrifuged for 30 min at 10,000 ×*g* for protein precipitation. For the reconstruction of the protein complex with the single lysozyme and pure ovotransferrin or pure ovalbumin, 50 µg of the purified ovotransferrin or purified ovalbumin was added to 10 µL of the cathode end fraction containing 8–16 µg of lysozyme and incubated with 50 mM Tris-HCl buffer, pH 7.0, for longer than overnight at 4 °C. Solutions containing 50 µg of the purified ovotransferrin or the purified ovalbumin 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 2DE, which was then run with 6.6% (w/v) acrylamide (0.33% (w/v) Bis). After the proteins separated by 2DE were transferred from the gels to the membrane, proteins on the membrane was stained with Ponceau S. The lysozyme activities on the spots were analyzed using degradation of cell wall component.

2.5. Cathodic mobilization and extraction of protein complex

To mobilize egg white proteins by non-denaturing IEF using a previously reported method (Shimazaki et al., 2018), 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 < 150 mW of power was used. Proteins were collected at the cathode end of the IEF gel after protein mobilization for 0, 0–10, 10–20, 20–30, and 30–60. After proteins at the cathode end of the IEF gel were collected, an equivalent volume of 60% (w/v) PEG was added to each fraction. The mixture was then kept at 4 °C for > 2 h, and centrifuged for 30 min at 10,000 ×g for protein precipitation. The precipitate was dissolved in10 µL of 0.1 M