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Title	Rapid and Simple Purification of Lysozyme from the Egg Shell Membrane
Author(s)	Kozuka, Miyuki; Murao, Sato; Yamane, Takuya et al.
Citation	Journal of Nutritional Science and Vitaminology, 61(1), 101-103 https://doi.org/10.3177/jnsv.61.101
Issue Date	2015-05
Doc URL	https://hdl.handle.net/2115/59544
Type	journal article
File Information	manuscript.pdf



Rapid and simple purification of lysozyme from the egg shell membrane

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Running title: Rapid Purification of ESM Lysozyme

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27 ***Summary***

28 Lysozyme (EC 3.2.1.17) is a hydrolytic enzyme that cleaves the β -(1, 4)-glycosidic
29 bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, a
30 major bacterial cell wall polymer. In the food industry, lysozyme is used as an additive
31 mainly in the production of wine and beer. Lysozyme was found to be localized in the
32 egg shell membrane. In this study, we found that lysozyme was easily purified from the
33 egg shell membrane and that the enzyme also had an antibacterial activity. Furthermore,
34 we found that the antibacterial activity of purified lysozyme from the egg shell
35 membrane was lower than that of purified lysozyme from the egg white at alkaline pH.
36 The method for rapid purification of lysozyme developed in this study should contribute
37 to food industry.

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39 ***Keywords:*** lysozyme, eggshell membrane, rapid and simple purification

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Introduction

Hen eggshell membrane (ESM) is a light pink membrane that contains a dozen kind of proteins such as some chemical defense proteins, lysozyme and ovotransferrin [1]. Lysozyme (EC 3.2.1.17) is a hydrolytic enzyme that cleaves the β -(1, 4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, a major bacterial cell wall polymer. Lysozyme is comprised of three major types of lysozyme, including the c-type (chicken or conventional type), g-type (goose-type) and i-type (invertebrate type) lysozymes [2]. Lysozyme also has an antifungal activity like RNase-7, chitotriosidase, lactoferrin, antileukoprotease, calprotectin, histatins, defensins, cathelicidins, dermcidin and hGAPDH (2-32) [3].

In the food industry, lysozyme is used as an additive mainly in the production of wine and beer [4] and as an inhibitor for Clostridia growth during cheese maturation [5]. Lysozyme was found to be localized in the ESM [6] and was partially purified from the ESM [7]. Lysozyme has also been purified from the hen egg white (EW) using anion exchange chromatography [8], ultrafiltration and affinity chromatography [9] and direct crystallization [10].

In this study, we easily purified lysozyme from the ESM and found that the antibacterial activity of purified lysozyme from the ESM was lower than that of purified lysozyme from the egg white at alkaline pH. The method for rapid and simple purification of lysozyme would be useful in studies of ESM lysozyme in the food industry and for antifungal therapies.

Materials and Methods

Materials

Egg white lysozyme and *Micrococcus luteus* were purchased from Wako Pure Chemicals (Osaka, Japan). A Seq-Blot PVDF membrane was obtained from Bio-Rad (Hercules, CA, USA). All other chemicals were of analytical grade and purchased from Wako Pure Chemicals.

Polyacrylamide gel electrophoresis

Slab gel electrophoresis was carried out using 15% polyacrylamide gels in a buffer containing 25 mM Tris, 192 mM glycine at pH 8.3 and 0.1% SDS by the method of Laemmli [11]. Proteins in gels were stained with Coomassie Brilliant Blue G-250.

Purification of lysozyme from the ESM

The isolated ESM from egg shells was washed by water and dried up at room temperature. The ESM (0.25 g) in 5 ml of 50 mM phosphate buffer at pH 7.0 containing 0 to 200 mM sodium chloride was incubated for 1-24 hours at 37°C. After centrifugation of the mixture at 1,000×g for 5 minutes at 4°C, supernatants were obtained and dialyzed against 50 mM phosphate buffer at pH 7.0.

Determination of amino acid sequence of lysozyme C from the ESM

Purified lysozyme was blotted onto a PVDF membrane (Bio-Rad) and subjected to

amino acid sequencing by the Edman degradation method using an automated protein sequencer (Applied Biosystems Model 473). Phenylthiohydantoin amino acids were identified using high-performance liquid chromatography (Applied Biosystem 120A analyzer).

Detection of antibacterial activity

Antibacterial activity was measured by adaptation of the assay described by Shugar [12], in which the change in optical density at 450 nm was measured using a microplate reader after lysozyme from the ESM had been incubated with a cell suspension of *Micrococcus luteus* at 37°C for 30 min in 50 mM phosphate buffer at pH 7.0 [13-15].

Statistical analyses

Data are expressed as means $\pm$ S.E. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test.

Results and discussion

To establish a rapid purification of lysozyme from the ESM, incubation time, pH and concentration of sodium chloride were examined. The ESM was incubated in 50 mM phosphate buffer at pH 7.5 containing 150 mM sodium chloride for 1, 2, 4, 8, 16 and 24 hours at 37°C. As shown in Fig.1A, lysozyme was extracted from the ESM, and the amount of extracted lysozyme was increased in a time-dependent manner at a peak of 8 hours. The ESM was then incubated in the same phosphate buffer in the presence of 0, 25, 50, 100, 150 and 200 mM sodium chloride for 24 hours at 37°C. As shown in Fig.1B, the amount of extracted lysozyme was increased in a dose-dependent manner with a

peak at 150 mM sodium chloride and then decreased at 200 mM sodium chloride, indicating that the optimal concentration of sodium chloride is 150 mM. After determination of the N-terminal amino acid sequence of lysozyme, lysozyme from the ESM was identified as egg white lysozyme [16] (Fig.1C). Purity of isolated lysozyme was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue, and a single band on SDS-PAGE was obtained in the presence of 2-mercaptoethanol (2-ME), being more than 92% of purification (Fig. 2). These findings suggest that ESM lysozyme exists in hen ESM in a monomeric form. Using this method, the overall yield of ESM lysozyme from 1 g of the ESM was approximately 2 mg. Compared with purification methods of lysozyme from the EW using column chromatography [8, 9] and crystallization [10], our method is simple and rapid because purified lysozyme is obtained from ESM merely incubated with buffer. Antibacterial assays were carried out to compare the antibacterial activity of lysozyme purified from the ESM to that of lysozyme purified from the EW. The results presented in Fig. 3A showed that ESM and EW lysozymes contained the same or similar levels of antibacterial activities under the conditions of pH 4-7 but that the antibacterial activity of ESM lysozyme was lower than that of EW lysozyme at alkaline pH. ESM and EW lysozymes also contained the same or similar levels of antibacterial activities under the conditions of 25-50°C, but the antibacterial activity of ESM lysozyme was lower than that of EW lysozyme at low and high temperatures (Fig. 3B). These results suggest that ESM lysozyme is readily denatured in comparison with EW lysozyme. Furthermore, it is known that pH in the EW of old eggs changes to alkaline pH [17]. Since ESM lysozyme is localized in the eggshell matrix and acts as a first defense against bacteria [6], ESM lysozyme may not possess antibacterial activity in an old egg shell membrane, permitting bacteria to

invade into the egg white through the egg shell membrane. The method for rapid purification of lysozyme from the eggshell membrane would there be useful for antifungal therapies and for the food industry.

Abbreviations: ESM, eggshell membrane; EW, egg white; 2-ME, 2-mercaptoethanol.

Acknowledgements

This work was supported by the Scientific Research Fund from Hokkaido System Science and Hokkaido Wako Junyaku.

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Figure legends

Fig. 1. Determination of conditions for rapid purification of lysosome from the ESM

A. ESMs were incubated for 1–24 hours at 37°C in 50 mM sodium phosphate buffer at pH 7.5 containing 150 mM sodium chloride and subjected to SDS-PAGE.

B. ESMs were incubated in 50 mM sodium phosphate buffer at pH 7.5 containing 0–200 mM sodium chloride at 37°C for 24 hours and subjected to SDS-PAGE.

C. The N-terminal sequence of purified protein was determined by the Edman degradation method, and the sequence identified is shown by an underline. All of the sequence in Fig. 3C is from the sequence of egg white lysozyme.

Fig. 2 Rapid purification of lysozyme from the ESM

Two µg each of purified enzymes in buffers in the presence or absence of 2-ME was separated by electrophoresis on a 15% polyacrylamide gel containing SDS and stained with Coomassie Brilliant Blue G-250.

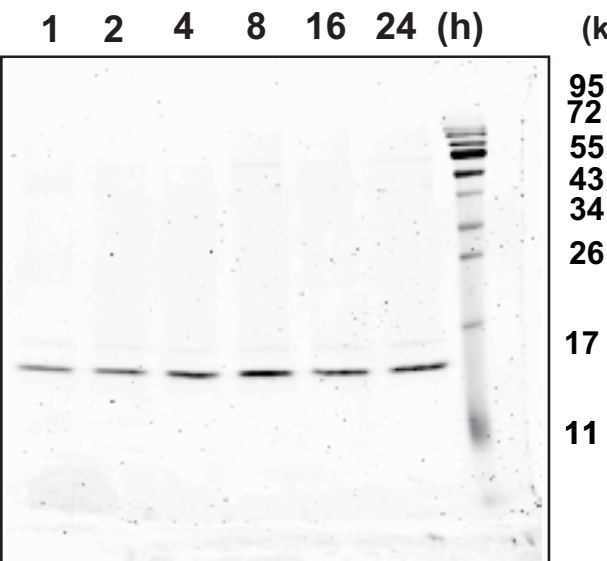
Fig. 3. Comparison of the antibacterial activities of lysozymes purified from the ESM and EW.

A. pH dependency of the antibacterial activity of purified lysozymes (0.5µg) was measured under conditions at pH 4–8 using 50mM sodium citrate buffer, pH4-5 and 50mM phosphate buffer, pH6-8. * $p<0.05$, $n=5$.

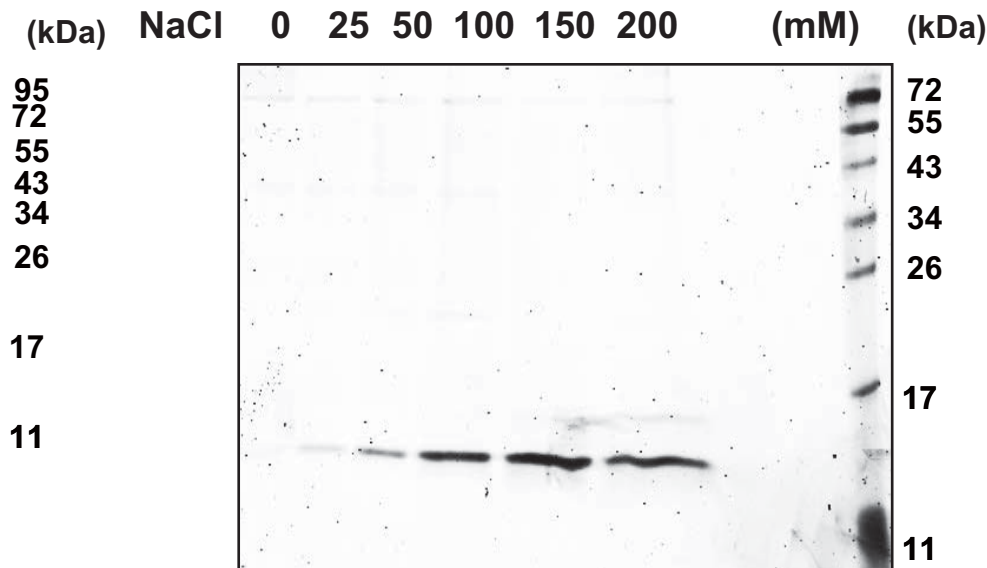
B. Temperature dependency of the antibacterial activity of purified lysozymes (0.5µg) was measured under conditions at 4–80° C using block incubator. ** $p<0.01$, *** $p<0.001$, $n=5$.

Fig.1

A



B



C

MRSLLILVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRG 40

YSLGNWVCAAKFESNFNTQATNRNTDGSTDYGILQINSRW 80

WCNDGRTPGSRNLCNIPCSALLSSDITASVNC AKKIVSDG 120

NGMSAWVAWRNRCKGTDVQAWIRGCRL 147

Fig.2

2-ME **-** **+** **M** **M.W. (kDa)**

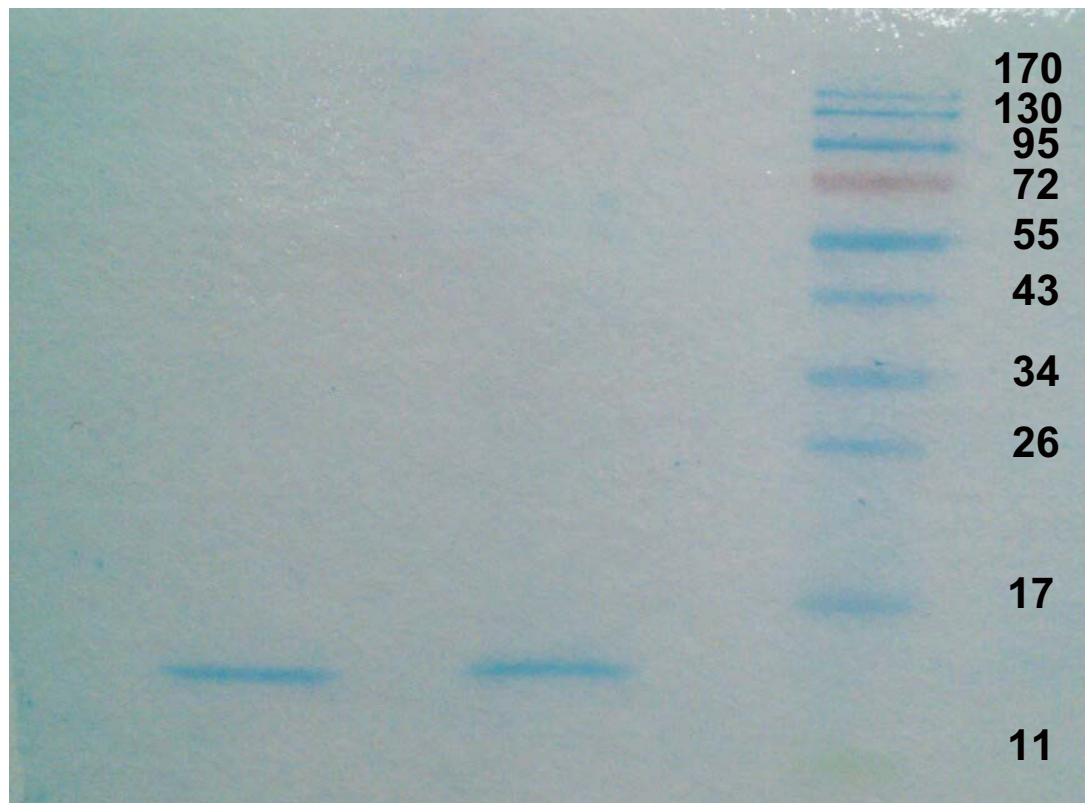


Fig.3