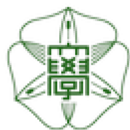
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Title	Rapid and Simple Purification of Lysozyme from the Egg Shell Membrane
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1
2 **Food Science**
3
4 # Rapid and simple purification of lysozyme from the egg shell
5 # membrane
6
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22
23 **Running title:** Rapid Purification of ESM Lysozyme
24

Summary

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. In the food industry, lysozyme is used as an additive mainly in the production of wine and beer. Lysozyme was found to be localized in the egg shell membrane. In this study, we found that lysozyme was easily purified from the egg shell membrane and that the enzyme also had an antibacterial activity. Furthermore, we found that the antibacterial activity of purified lysozyme from the egg shell membrane was lower than that of purified lysozyme from the egg white at alkaline pH. The method for rapid purification of lysozyme developed in this study should contribute to food industry.

Keywords: lysozyme, eggshell membrane, rapid and simple purification

49

50

51

52 ***Introduction***

53 Hen eggshell membrane (ESM) is a light pink membrane that contains a dozen kind
54 of proteins such as some chemical defense proteins, lysozyme and ovotransferrin [1].
55 Lysozyme (EC 3.2.1.17) is a hydrolytic enzyme that cleaves the β-(1, 4)-glycosidic
56 bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, a
57 major bacterial cell wall polymer. Lysozyme is comprised of three major types of
58 lysozyme, including the c-type (chicken or conventional type), g-type (goose-type) and
59 i-type (invertebrate type) lysozymes [2]. Lysozyme also has an antifungal activity like
60 RNase-7, chitotriosidase, lactoferrin, antileukoprotease, calprotectin, histatins, defensins,
61 cathelicidins, dermcidin and hGAPDH (2-32) [3].
62 In the food industry, lysozyme is used as an additive mainly in the production of wine
63 and beer [4] and as an inhibitor for Clostridia growth during cheese maturation [5].
64 Lysozyme was found to be localized in the ESM [6] and was partially purified from the
65 ESM [7]. Lysozyme has also been purified from the hen egg white (EW) using anion
66 exchange chromatography [8], ultrafilteration and affinity chromatography [9] and
67 direct crystallization [10].
68 In this study, we easily purified lysozyme from the ESM and found that the
69 antibacterial activity of purified lysozyme from the ESM was lower than that of purified
70 lysozyme from the egg white at alkaline pH. The method for rapid and simple
71 purification of lysozyme would be useful in studies of ESM lysozyme in the food
72 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

97 amino acid sequencing by the Edman degradation method using an automated protein
98 sequencer (Applied Biosystems Model 473). Phenylthiohydantoin amino acids were
99 identified using high-performance liquid chromatography (Applied Biosystem 120A
100 analyzer).
101
102 *Detection of antibacterial activity*
103 Antibacterial activity was measured by adaptation of the assay described by Shugar [12],
104 in which the change in optical density at 450 nm was measured using a microplate
105 reader after lysozyme from the ESM had been incubated with a cell suspension of
106 *Micrococcus luteus* at 37°C for 30 min in 50 mM phosphate buffer at pH 7.0 [13-15].
107
108 *Statistical analyses*
109 Data are expressed as means ± S.E. Statistical analyses were performed using analysis
110 of variance (one-way ANOVA) followed by unpaired Student's *t*-test.
111
112 ***Results and discussion***
113 To establish a rapid purification of lysozyme from the ESM, incubation time, pH and
114 concentration of sodium chloride were examined. The ESM was incubated in 50 mM
115 phosphate buffer at pH 7.5 containing 150 mM sodium chloride for 1, 2, 4, 8, 16 and 24
116 hours at 37°C. As shown in Fig.1A, lysozyme was extracted from the ESM, and the
117 amount of extracted lysozyme was increased in a time-dependent manner at a peak of 8
118 hours. The ESM was then incubated in the same phosphate buffer in the presence of 0,
119 25, 50, 100, 150 and 200 mM sodium chloride for 24 hours at 37°C. As shown in Fig.1B,
120 the amount of extracted lysozyme was increased in a dose-dependent manner with a

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

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

148

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

150

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154

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201

202 **Figure legends**

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

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

206 B. ESMs were incubated in 50 mM sodium phosphate buffer at pH 7.5 containing 0–
207 200 mM sodium chloride at 37°C for 24 hours and subjected to SDS-PAGE.

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

211

212 **Fig. 2** Rapid purification of lysozyme from the ESM

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

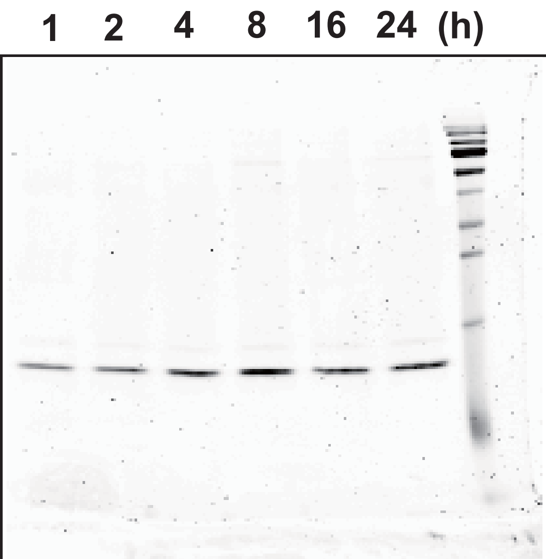
216

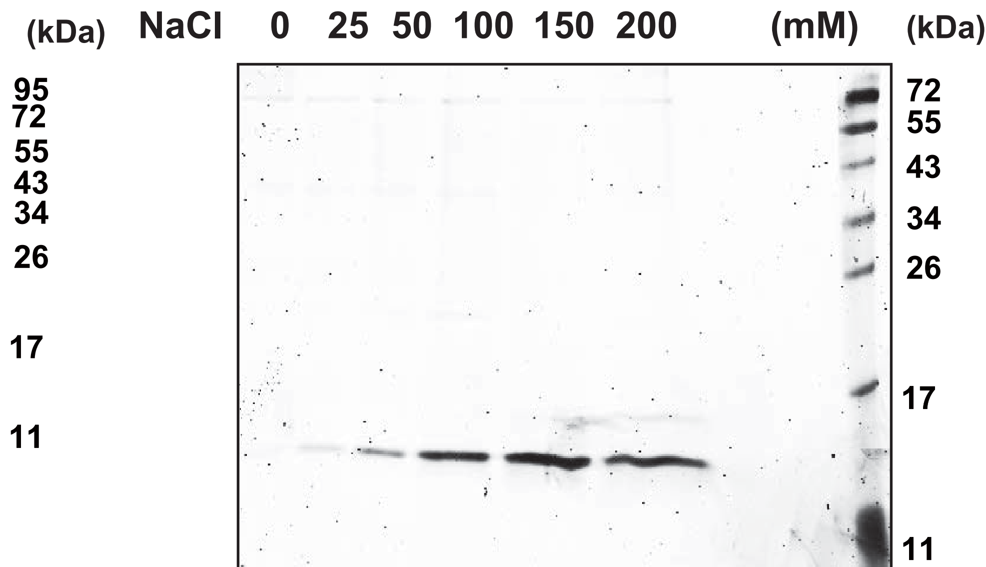
217 **Fig. 3.** Comparison of the antibacterial activities of lysozymes purified from the ESM
218 and EW.

219 A. pH dependency of the antibacterial activity of purified lysozymes (0.5μg) was
220 measured under conditions at pH 4–8 using 50mM sodium citrate buffer, pH4-5 and
221 50mM phosphate buffer, pH6-8. $^{*}p<0.05$, n=5.

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

Fig.1





C

MRSLLILVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRG 40

YSLGNWVCAAKFESNFNTQATNRNTDGSTDYGILQINSRW 80

WCNDGRTPGSRNLCNIPCSALLSSDITASVNCAKKIVSDG 120

NGMSAWVAWRNRCKGTDVQAWIRGCRL 147

Fig.2

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

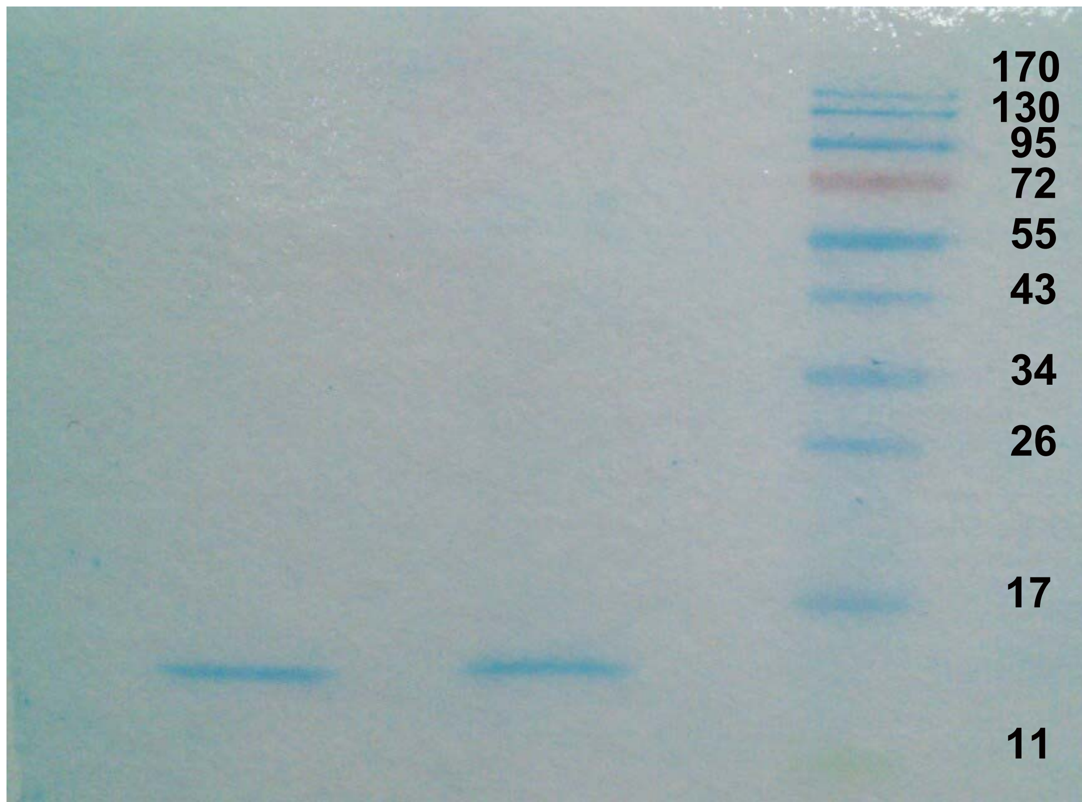


Fig.3

A

Antibacterial activity

0.25
0.2
0.15
0.1
0.05
0

pH4 pH5 pH6 pH7 pH8

*

ESM
EW

B

Antibacterial activity

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0

4℃ 25℃ 37℃ 50℃ 80℃

**
***

ESM
EW