J. Appl. Glycosci., **62**, 15−19 (2015)
doi: 10.5458/jag.jag. JAG-2014_008

Regular Paper

Characterization of Lysozyme from Brown Eared Pheasant Egg White

(Received September 12, 2014; Accepted December 1, 2014)
(J-STAGE Advance Published Date: December 9, 2014)

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Abstract: Lysozyme was purified from brown eared pheasant (*Crossoptilon mantchuricum*) egg white using pH treatment and cation exchange chromatography resulting in an 80-fold enhancement of the specific activity. The enzyme exhibited hydrolytic activity toward glycol chitin and chitooligosaccharides [$(GlcNAc)_n$ (n=5 and 6)]. The enzyme catalyzed degradation of bacterial cells of not only *Micrococcus luteus* but also *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 upon treatment with chloroform/tris(hydroxymethyl)aminomethane-HCl. The pH optimum of the glycol chitin hydrolytic reaction was 5.5 at 37°C. The optimal temperature for activity was 53°C in 50 mM sodium acetate buffer (pH 5.5). The enzyme mainly hydrolyzed the fourth glycosidic linkage from the nonreducing end of $(GlcNAc)_6$. The anomeric form of the products indicated it was a retaining enzyme.

Key words: brown eared pheasant, chitin, *Crossoptilon mantchuricum*, lysozyme, subsite

INTRODUCTION

Lysozyme (EC 3.2.1.17) hydrolyzes the β-1,4 glycosidic linkage between *N*-acetyl muramic acid and *N*-acetyl glucosamine (GlcNAc) in bacterial cell wall peptidoglycan. The enzyme is widespread in animals, plants, invertebrates, bacteria, and bacteriophages.[1)] In the animal kingdom, many lysozymes have been purified from various avian egg whites, and their reaction mechanism and protein structures have been investigated in detail. Avian egg white lysozymes are divided into C-type (chicken type) and G-type (goose type) based on their amino acid sequences and catalytic mechanism. C-type lysozyme consists of about 130 amino acid residues (molecular weight of 14 kDa) and catalyzes the hydrolytic reaction with retention of anomeric configuration via two key acidic amino acid residues in the active site. C-Type lysozymes have been purified from various avian egg whites with hen egg white lysozyme (HEWL) being a representative example. [2,3)]

G-type lysozyme has been purified from the egg white of the Embden goose, black swan, ostrich, cassowary, and rhea.[1)] G-type lysozyme is about 20 kDa, and hydrolyzes the substrate with inversion of the anomeric configuration.[4)] C-type lysozyme possesses lytic activity not only toward the bacterial cell wall but also toward chitin, a linear GlcNAc polysaccharide linked by β-1,4 glycosidic bonds. On the other hand, G-type lysozyme slightly expressed hydrolytic activity toward chitin oligosaccharides, but the enzyme displayed weak chitin hydrolytic activity.[5−7)]

We have a long standing interest in the diversity of substrate specificity and reaction mechanism of avian egg white lysozymes. Recently, Ishikawa Zoo kindly donated various avian eggs which did not use extrication. We successfully purified lysozyme from brown eared pheasant (*Crossoptilon mantchuricum*) using these eggs. In this study, we characterized the properties of the enzyme toward various polymeric and oligomeric substrates.

MATERIALS AND METHODS

Materials. Brown eared pheasant eggs were donated from Ishikawa Zoo. $(GlcNAc)_n$ (n = 5 and 6), HEWL and *Micrococcus luteus* were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Unless otherwise stated, reagents were of analytical grade and were obtained commercially.

Purification of lysozyme. Brown eared pheasant egg white lysozyme (BEPEWL) was purified with cation exchange chromatography according to the method developed by Thammasirirak *et al.*[7)] First, the yolk and egg white were carefully separated from two eggs, and an equal volume of 50 mM sodium phosphate buffer (pH 7.0) was added into the egg white. The egg white solution was stirred for 30 min at 4°C, and then the solution was centrifuged at 9,800 × G. Next, the pH of the supernatant was adjusted to 4.0 with 1 M HCl. After treating the solution as described Thammasirirak *et al.*, the pH of the sample was adjusted to 6.0 with 1 M NaOH.[7)] Finally, a pH 7.0 solution was obtained after adjustment with 1 M NaOH. The supernatant was loaded onto a CM-TOYOPEARL® 650M column (2.8 × 24 cm, Tosoh Co., Tokyo, Japan) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) at 25°C. After washing with the same buffer, chromatography was performed using a liner gradient of NaCl solution from 0 to 0.3 M in 50 mM sodium phosphate buffer (pH 7.0). Finally, the lysozyme was eluted with 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 M

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NaCl. Active enzyme fractions were collected, then the solution was desalted using an Amicon Ultra-4 3k (Millipore Co., Carrigtwohill, Ireland). The purity of the proteins was determined by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE).[8] XL-Ladder Marker (SP-2110 Broad Range; APRO Life Science Institute, Tokushima, Japan) was used as the standard molecular markers for SDS-PAGE. Protein concentrations were determined by the absorbance at 280 nm by comparison with an authentic bovine serum albumin solution.

Substrates for lytic activity. *Salmonella enterica* serovar Typhimurium JCM6977 was incubated in Trypticase Soy Broth (100 mL, Becton, Dickinson and CO., Spark, MD) at 37°C for 20 h. *Escherichia coli* O157:H7 (nonpathogenic mutants) was grown in Luria broth (100 mL) at 37°C for 2 h. After autoclaving the stationary phase cultures at 121°C for 15 min, the cells were collected by centrifugation at 3,500 × G. To disrupt the cellular outer membrane, the cell pellets were treated with chloroform/tris(hydroxymethyl)aminomethane-HCl as described by Nakimbugwe *et al.*[9]

Assay of enzymatic activity. Bacterial cells were suspended in 50 mM sodium phosphate buffer (pH 7.0). The absorbance of the cell suspension first adjusted to 0.75 prior to measurement of the lytic activity at 540 nm. One unit of lytic activity was defined as a decrease of 0.1 absorbance units at 540 nm for 1 min at 30°C.

The enzyme activities toward 0.1% glycol chitin were measured as the increase in reducing sugar by using GlcNAc as the standard.[10] The reaction was carried out in 50 mM sodium acetate buffer (pH 5.5) at 37°C.

Effects of pH and temperature on enzymatic activity. Enzymatic activity was measured under standard conditions of 0.1% glycol chitin hydrolysis while the pH of the reaction mixture was changed varied. The buffer systems used were sodium acetate (pH 3.5−5.5), sodium phosphate (pH 6.0−8.0), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (pH 9.0) and 3-cyclohexylaminopropanesulfonic acid (pH 10.0−11.0). The optimum temperature of the activity was measured at various temperatures in 50 mM sodium acetate buffer (pH 5.5).

(GlcNAc)$_n$ degradation determined by high performance liquid chromatography (HPLC). Lysozyme (0.024 mg) catalyzed degradation of 0.65 mM (GlcNAc)$_n$ (n = 4, 5 and 6) were carried out in 25 mM sodium acetate buffer (pH 5.5) for 30 min at 37°C. The reaction mixture was diluted with distilled water (1:2) and boiled for 5 min to inactivate the enzyme. The enzymatic products from the (GlcNAc)$_n$ starting material were determined by HPLC using a Shodex Asahipak NH2-50 4E column (4.6 × 250 mm, Showa Denko K.K., Tokyo, Japan), eluted with acetonitrile-water (70:30 v/v) at a flow rate of 1.0 mL/min at 30°C. The products were detected at 215 nm with a UV-VIS monitor (L-4250, Hitachi High-Tech Fielding Co., Tokyo, Japan).

Analysis of anomeric products. The anomeric forms of the hydrolytic products from (GlcNAc)$_6$ (2.6 mM) were determined using an isocratic HPLC method.[11] The enzymatic reaction was performed in 50 mM sodium acetate buffer (pH 5.5) at 27°C. A total of 1 min and 38 min after the addition of BEPEWL (0.4 mg), an aliquot of the reaction solution was immediately injected into a TSK-GEL AMIDE-80 column (4.6 × 250 mm, Tosoh Co.), and eluted with acetonitrile−water (7:3 v/v) at a flow rate of 1.0 mL/min at 25°C to separate the (GlcNAc)$_n$ anomers. The substrate and products were detected as described above.

RESULTS AND DISCUSSION

Purification and basic properties of BEPEWL.

Table 1 shows the total protein and activity of BEPEWL at each step of the purification. After the pH treatment during the lysozyme purification, the viscosity of the crude sample was decreased as reported previously.[7] Using CM-TOYOPEARL® 650M chromatography, the fractions eluted in 0.5 M NaCl had high bacterial cell wall lytic activity as shown in Fig. 1. After collecting the active fractions (63rd to 70th fractions), the samples were desalted using an Amicon Ultra-4 3k. The samples from all purification steps were analyzed by SDS-PAGE as shown in Fig. 2. After purification using CM-TOYOPEARL® 650M chromatography, BEPEWL was observed as a 14 kDa protein on SDS-PAGE. Overall, the lysozyme was purified 80 fold as shown in Table 1. In purification step, total activity was gradually increased with increasing pH value of the solution. Previously, it was reported that HEWL was inactivated by forming complex between HEWL and ovomucin (180−210 kDa) which was

Table 1. Purification of lysozyme from brown eared pheasant egg white.

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
Crude	14874	11800	0.8
pH 4 Treatment	9995	14475	1.4
pH 6 Treatment	7616	14322	1.9
pH 7 Treatment	3676	14799	4.0
CM-Toyopearl® 650M	283	18018	63.6

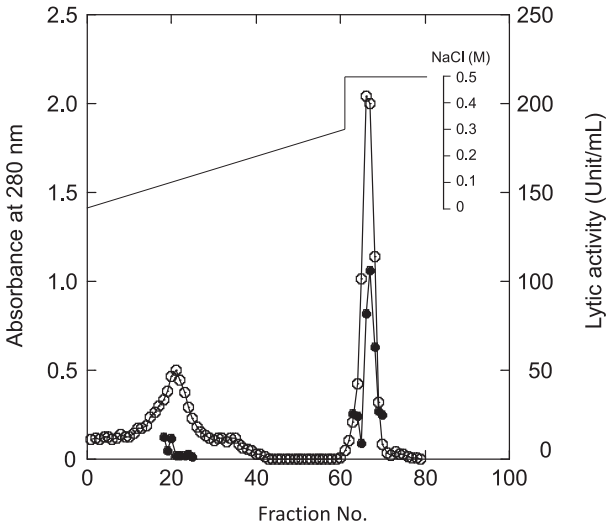

Fig. 1. Purification of lysozyme from brown eared pheasant egg white using cation exchange chromatography.

Symbols indicate absorbance at 280 nm (open circles) and lytic activity (closed circles). Chromatography was performed using CM-TOYOPEARL 650M® with a liner gradient of NaCl solution from 0 to 0.3 M in 50 mM sodium phosphate buffer (pH 7.0). The lysozyme was eluted with 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl solution from fraction 60. One fraction volume was 6 ml and the flow rate was 30 mL/h.

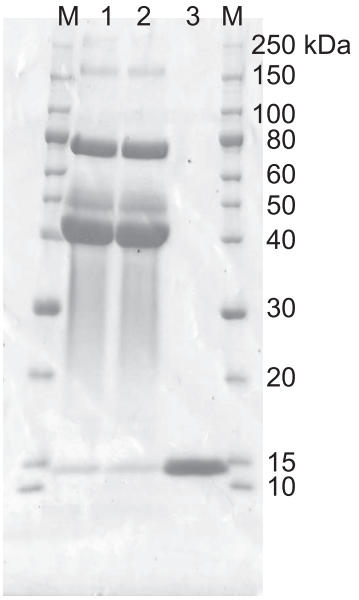

Fig. 2. SDS-PAGE of proteins at various stages of purification.

Lane M, XL-Ladder Marker (SP-2110 Broad Range; APRO Life Science Institute); 1, crude extract from brown eared pheasant egg white; 2, pH 7 treatment; 3, purified BEPEWL.

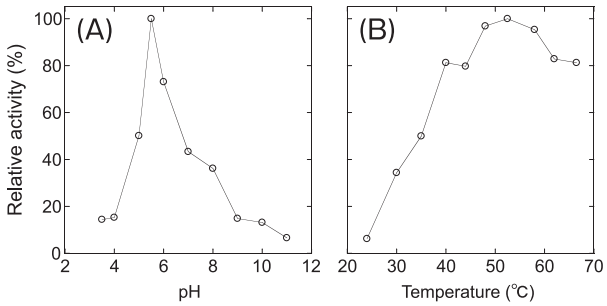

Fig. 3. pH and temperature dependence of the chitinolytic activity of BEPEWL.

(A) The effect of pH on activity, (B) The effect of temperature on activity. The substrate is 0.1% glycol chitin.

Table 2. Lytic activity (U/mg) of BEPEWL and HEWL toward *Micrococcus luteus* cells and *Salmonella enterica* serovar Typhimurium JCM6977 and *Escherichia coli* O157:H7 treated with chloroform/Tris-HCl.

	Micrococcus luteus	*Salmonella enterica* serovar Typhimurium	*Escherichia coli* O157:H7
BEPEWL	4.7	0.1	0.5
HEWL	6.4	2.4	0.5

One unit of lytic activity is defined as a decrease of 0.1 absorbance units at 540 nm for 1 min at 30°C.

the structural glycoprotein of hen egg white.[12] In the SDS-PAGE, ovomucin-like protein was found at 180 kDa in crude and pH7 solutions, but the protein was disappeared after CM- TOYOPEARL® 650M chromatography. Thus, we considered the ovomucin-like protein and lysozyme were formed an inactive complex in crude sample, and ovomucin-like protein was gradually uncoupled from the complex with proceeding purification steps.

Glycol chitin degradation activity of BEPEWL.

BEPEWL hydrolyzed 0.1% glycol chitin in 50 mM sodium acetate buffer (pH 5.5), with a specific activity of 0.036 mM/min/mg. Figure 3 shows the pH and temperature dependence of BEPEWL. The optimum pH was pH 5.5 and the maximum activity was observed at 53°C in 50 mM sodium acetate buffer (pH 5.5) for the BEPEWL catalyzed hydrolysis of 0.1% glycol chitin.

Lytic activity toward bacterial cells.

Generally, lysozymes act on the cell wall of Gram-positive bacteria but not on those of Gram-negative bacteria. Lysozyme was not able to attack the peptidoglycan of Gram-negative bacteria because of the shielding provided by the various lipopolysaccharides present in the outer membrane.[1] However, mutants of *S. typhimurium* and *E. coli*, which were designed with truncated lipopolysaccharides forming activities, were sensitive to the lytic activity of lysozyme.[13] These Gram-negative bacteria are major sources of food pollutants in avian egg whites. Recently, the substrate specificity of various lysozymes was investigated by using the cell wall from a Gram-negative bacteria treated with chloroform/tris(hydroxymethyl)aminomethane-HCl to remove the outer and inner membranes.[9] This approach prompted us to investigate the substrate specificity of avian lysozymes toward bacterial cell wall from the food pollutant sources, *S. typhimurium* and *E. coli* O157:H7. Nakimbugwe et al. also reported that lysozyme inhibitors which produced by Gram-negative bacteria were extracted with chloroform/tris(hydroxymethyl)aminomethane-HCl buffer, suggesting that removal of the inhibitors was important for estimation of the bacterial cell wall lytic activity by lysozymes.[9] Thus, we carefully prepared the bacterial cell walls with washing chloroform/tris(hydroxymethyl)aminomethane-HCl buffer.

Table 2 shows the lytic activities of BEPEWL and HEWL toward bacterial cells. Both enzymes displayed the highest lytic activity toward *M. luteus* cells, and had the lowest lytic activity toward *E. coli* O157:H7. The lytic activities of HEWL and BEPEWL toward *S. typhimurium* JCM6977 varied suggesting that there were differences in their cell wall recognition mechanism.

(GlcNAc)$_n$ hydrolysis by BEPEWL.

Figure 4 shows the HPLC chromatograms of $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ hydrolysis by BEPEWL. Table 3 lists the $(GlcNAc)_n$ concentrations in the reaction mixtures at 30 min based on the HPLC chromatograms. In the $(GlcNAc)_6$ hydrolytic reaction, $(GlcNAc)_4$ and $(GlcNAc)_2$ were the main products in the reaction while smaller amounts of GlcNAc, $(GlcNAc)_3$, and $(GlcNAc)_5$ were observed. GlcNAc and $(GlcNAc)_4$ were abundantly produced from the hydrolysis of $(GlcNAc)_5$. In the case of the $(GlcNAc)_4$ reaction, no lower molecular weight products were detected under the same condition.

Figure 5 shows the HPLC chromatogram for the analysis of the anomeric products from $(GlcNAc)_6$ hydrolysis by BEPEWL. As shown in this figure, the ratio of α-$(GlcNAc)_6$ and β-$(GlcNAc)_6$ was 1.0:0.7 before addition of the enzyme. After 1 min of reaction, β-$(GlcNAc)_4$ and α and β-$(GlcNAc)_2$ were produced. The α and β forms of $(GlcNAc)_2$ were derived from the reducing end of the $(GlcNAc)_6$. Also, the β-$(GlcNAc)_6$ slightly decreased in the enzymatic reaction

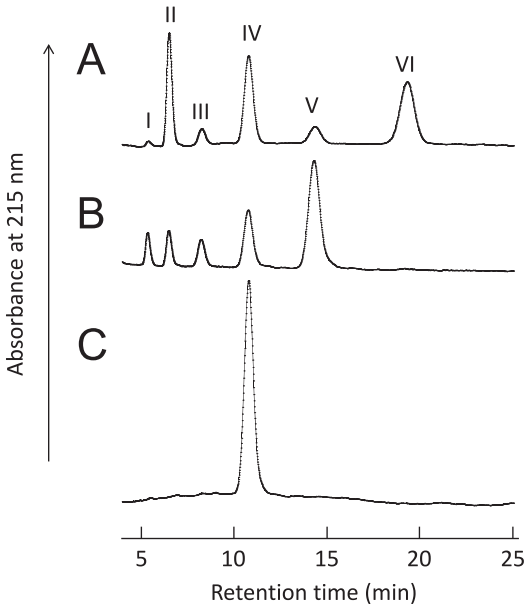

Fig. 4. HPLC chromatograms of the products from $(GlcNAc)_n$ (n = 4−6) hydrolysis catalyzed by BEPEWL

$(GlcNAc)_6$ (A), $(GlcNAc)_5$ (B), and $(GlcNAc)_4$ (C) hydrolytic reaction catalyzed by BEPEWL. The enzymatic reaction was performed in 50 mM sodium acetate buffer (pH5.5) for 30 min at 37°C. Substrate and enzyme concentrations were 0.650 mM and 0.024 mg.

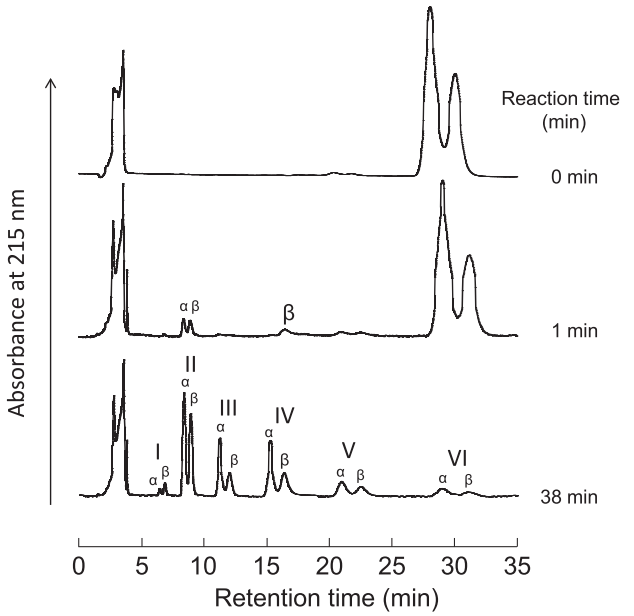

Fig. 5. Anomeric analysis of the $(GlcNAc)_6$ hydrolysis catalyzed by BEPEWL.

Roman numerals indicate the degree of polymerization of GlcNAc. The reaction products were analyzed using a TSK-GEL AMIDE-80 column (4.6 × 250 mm, Tosoh Co.), and eluted with acetonitrile−water (7:3 v/v) at a flow rate of 1.0 mL/min at 25°C. Substrate and enzyme concentrations were 2.6 mM and 0.4 mg.

Table 3. Products of the reaction of $(GlcNAc)_{4-6}$ with BEPEWL after 30 min.

	GlcNAc	$(GlcNAc)_2$	$(GlcNAc)_3$	$(GlcNAc)_4$	$(GlcNAc)_5$	$(GlcNAc)_6$
$(GlcNAc)_4$	nd	nd	nd	0.65		
$(GlcNAc)_5$	0.21	0.14	0.08	0.19	0.36	
$(GlcNAc)_6$	0.03	0.46	0.05	0.3	0.06	0.22

The concentrations (mM) were determined from HPLC of the reaction solution (Fig. 4). nd, not detectable.

(α:β = 1.0:0.6). These results suggested that the enzyme slightly recognized β-anomeric form of $(GlcNAc)_6$ in the hydrolytic reaction. Previously, we reported that REX (Reducing end xylose-releasing exo-oligoxylanase) strongly recognized β-anomeric form of the xylooligosaccharides and an inverting chitinase from *Vibrio proteolyticus* preferred to hydrolyze α-$(GlcNAc)_6$ rather than β-$(GlcNAc)_6$.[14,15] On the other hand, the β-anomeric form of $(GlcNAc)_4$ was produced by the hydrolytic reaction at the 4th glycosidic linkage from the nonreducing end of the substrate. We also observed a similar HPLC profile of $(GlcNAc)_6$ hydrolytic reaction by HEWL (data not shown).[11] Therefore, these results suggested that the hydrolytic reaction catalyzed by BEPEWL proceeded with retention of the β-anomeric form of the substrate. The results of Table 3 and Fig. 5 indicated that the enzyme had at least 4 subsites at the nonreducing end of the active site.

BEPEWL is a C-type lysozyme.

In 1967, the three dimensional structure of the complex between HEWL and $(GlcNAc)_3$ was elucidated by X-ray crystallography.[16] HEWL possesses at least 6 subsites based on the complex structures and hydrolytic patterns of $(GlcNAc)_n$.[3] The cleavage point is located between the 4th and 5th subsites from the nonreducing end.[11] A three dimensional X-ray crystal structure of a G-type lysozyme was also determined, and the active site was elucidated based on the complex structure between the goose egg white lysozyme and $(GlcNAc)_3$.[17] However, G-type lysozyme hydrolyzes the 3rd and 4th glycosidic linkage of $(GlcNAc)_6$ with anomeric inversion.[6] We considered these differences important in discriminating between C- and G-type lysozymes. In BEPEWL, the cleavage patterns of $(GlcNAc)_5$ and $(GlcNAc)_6$ were in accordance with the reaction catalyzed by HEWL. Anomeric analysis of the products from $(GlcNAc)_6$ indicated the cleavage mainly occurred at the 4th glycosidic linkage from the nonreducing end of the substrate in the same reaction manner as that catalyzed by HEWL.[11] In this study, transglycosylation was not observed in $(GlcNAc)_{4-6}$ reaction by the enzyme. However, our results indicated that the enzyme had similar activity of HEWL, suggesting that the enzyme would express transglycosylation activity depending on the experimental condition.

In conclusion, our studies on BEPEWL suggest that the enzyme should be categorized as a C-type lysozyme, judging from its molecular weight and reaction mechanism. To elucidate the reaction mechanism of BEPEWL in detail, determination of the amino acid sequence and subsite binding free energies of the enzyme are now in progress.

ACKNOWLEDGEMENTS

We are grateful to Mr. Hiroshi Domae and Mr. Hideo Mima (Ishikawa Zoo) for donating various avian eggs. This work was supported in part by Japan−Mexico Training program for the Strategic Global partnership 2012.

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