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# Development of High-Performance and Rapid Immunoassay for Model Food Allergen Lysozyme Using Antibody-Conjugated Bacterial Magnetic Particles and Fully Automated System

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## Abstract

A high-performance and rapid chemiluminescence immunoassay for model food allergen lysozyme, one of the major allergenic components in egg white, using antibody-conjugated bacterial magnetic particles and a fully automated system was developed. This system contains a reaction station, tip rack, and an eight-tip pipettor that is able to attach and detach a strong magnet to the tip surface. The immunoreaction time was shortened to 5 min, and the assay was completed within 20 min. The lower detection limit for lysozyme was 10 ng/mL. This system can be used to perform 24 samples in 60 min within 10% coefficient of variation.

**Index Entries:** Sandwich immunoassay; food allergen; lysozyme; bacterial magnetic particles; fully automated system.

## Introduction

A food allergy is defined as an adverse reaction to food triggered by specific allergenic components. Crossed immunoelectrophoresis, radioimmunoelectrophoresis, and IgE inhibition enzyme-linked immunosorbent assay (ELISA) have been used to estimate the allergenicity of food allergens *(1–3)*. However, their major disadvantage is the reliance on human serum from allergenic individuals as a reagent. Such human serum is

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difficult to obtain, often poorly characterized, and inherently variable *(1)*. Recently, Koppelman et al. *(3)* reported that a sandwich ELISA using IgG purified from immunized animals showed similar results to an IgE inhibition ELISA using human serum in terms of its sensitivity and specificity in detection of hazelnut proteins in food products.

On the other hand, Kushimoto and Aoki *(4)* reported that the allergens obtained from gluten, gliadin, and glutenin by pepsin digestion of wheat yielded the other allergenic fragments that elicit IgE antibodies, and these allergens were different from wheat and gluten allergens obtained by simple extraction. The allergen could arise on digestion of the food. Therefore, the preparation of various IgG antibodies is required for detection of food allergens. However, sandwich ELISA is not appropriate for this purpose because it is time-consuming. A rapid, simple, high-performance assay system needs to be developed.

Magnetic bacteria synthesize intracellular magnetite particles *(5–8)*. The amount of antibody coupling with BMPs is higher than that with artificial magnetite particles of the same size *(9)*, because the bacterial magnetic particles (BMPs) are small (50–100 nm) and are superior to artificial magnetite particles in their dispersion ability as covered with a lipid membrane *(10)*. BMPs permit the development of highly sensitive and rapid chemiluminescence enzyme immunoassays, because they have higher relative surface area and antibodies can be efficiently chemically coupled to their surfaces *(11)*. We have constructed a fully automated, reliable immunoassay system for human serum insulin using antibody and BMP complexes in which 1 ng/mL of insulin was detected in 60 min *(12)*. Furthermore, BMPs have been applied to genotyping of microbes and fish in which target-specific DNA probes were immobilized on BMPs and DNA-BMPs were used for magnetic capture hybridization *(13,14)*.

Two to 8% of children and 1 to 2% of adults are reported to have food allergies. The most common allergenic foods for children are eggs and milk. Lysozyme is one of the major allergenic components in egg white *(15)*. In the present study, lysozyme was targeted as a model allergen. We report on a high-performance and rapid immunoassay system for lysozyme using antibody-conjugated BMPs and a fully automated system.

## Materials and Methods

### *Materials*

Rabbit anti-lysozyme IgG fraction (polyclonal) was obtained from Rockland (Gilbertsville, PA). Lysozyme was purchased from Seikagaku (Tokyo, Japan). Alkaline phosphatase (ALP) was purchased from Toyobo (Osaka, Japan). Lumi-phos 530 was obtained from Wako (Osaka, Japan). Sulfo-succinimidyl 6-[3'-(pyridyldithio)-propionamido]hexanoate (Sulfo-LC-SPDP) and sulfo succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) were purchased from Pierce (Rockford, IL).

Other reagents were commercially available analytical reagents or laboratory-grade materials. Deionized distilled water was used in all procedures.

### *Preparation of BMPs and Conjugation of Antibody onto BMPs*

BMPs were isolated from the magnetic bacterium *Magnetospirillum magneticum* AMB-1 by a method previously described *(11)*. The BMP-antibody conjugation was performed according to Tanaka and Matsunaga *(12)*. Sulfo-SMCC (1 mg) was added to 500 µL of antibody (anti-lysozyme IgG antibody) solution (1 mg/mL of phosphate-buffered saline [PBS]) and incubated for 0.5 h at room temperature. The sample was purified on an NAP-5 column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Sulfo-LC-SPDP (10 m*M*) was added to 1 mL of BMP suspension (1 mg/mL) and incubated for 0.5 h at room temperature. After incubation, modified BMPs were isolated magnetically from the reaction mixture using a neodymium-boron (Nd-B) magnet (produced by TDK, Tokyo, Japan) and washed three times with 1 mL of PBS. The modified BMPs were dispersed in 1 mL of 20 m*M* dithiothreitol in acetate buffer (100 m*M* sodium acetate; 100 m*M* NaCl, pH 4.5) and incubated for 0.5 h at room temperature. After washing, BMPs having thiol groups were incubated with the sulfo-SMCC-modified antibody solution for 20 h at 4°C. Antibody-conjugated BMPs (antibody-BMPs) were washed three times with PBS to remove excess antibody.

### *Preparation of ALP-Conjugated Antibody*

A mixture containing 500 µL of ALP from calf intestine and aqueous anti-lysozyme antibody (500 µL; 1 mg/mL) was dialyzed for 12 h against PBS. ALP was crosslinked to antibodies by the addition of 25% (v/v) glutaraldehyde solution (8 µL) and subsequently incubated for 2 h at room temperature. The solution was dialyzed against PBS (1000 mL) for 12 h and then against Tris-HCl buffer, pH 8.0, for 24 h.

### *Sandwich Immunoassay Using Automated System*

The sandwich immunoassay was performed with an automated immunoassay system *(12)*. The automated immunoassay system contains a reaction station, tip rack, and an eight-tip pipettor that is able to attach and detach a strong magnet to the tip surface for use with 96-well microtiter plates mounted in the reaction station. Each reagent is dispensed in each eight-well row of the microtiter plate. Figure 1 illustrates the assay.

Lysozyme solution (50 µL) that was prepared in washing buffer (PBS containing 0.1% bovine serum albumin and 0.05% Tween-20) was added to 50 µL of antibody-conjugated BMPs. The mixture was dispersed by the pipettor and incubated at 30°C (the first immunoreaction). Immunocomplexes were separated magnetically using an Nd-B magnet and then washed by automated resuspension (20 cycles of pipet action) in 100 µL of washing buffer. To the washed complexes, 100 µL of ALP-antibody was added and dispersed with the automated pipet and incubated (the second

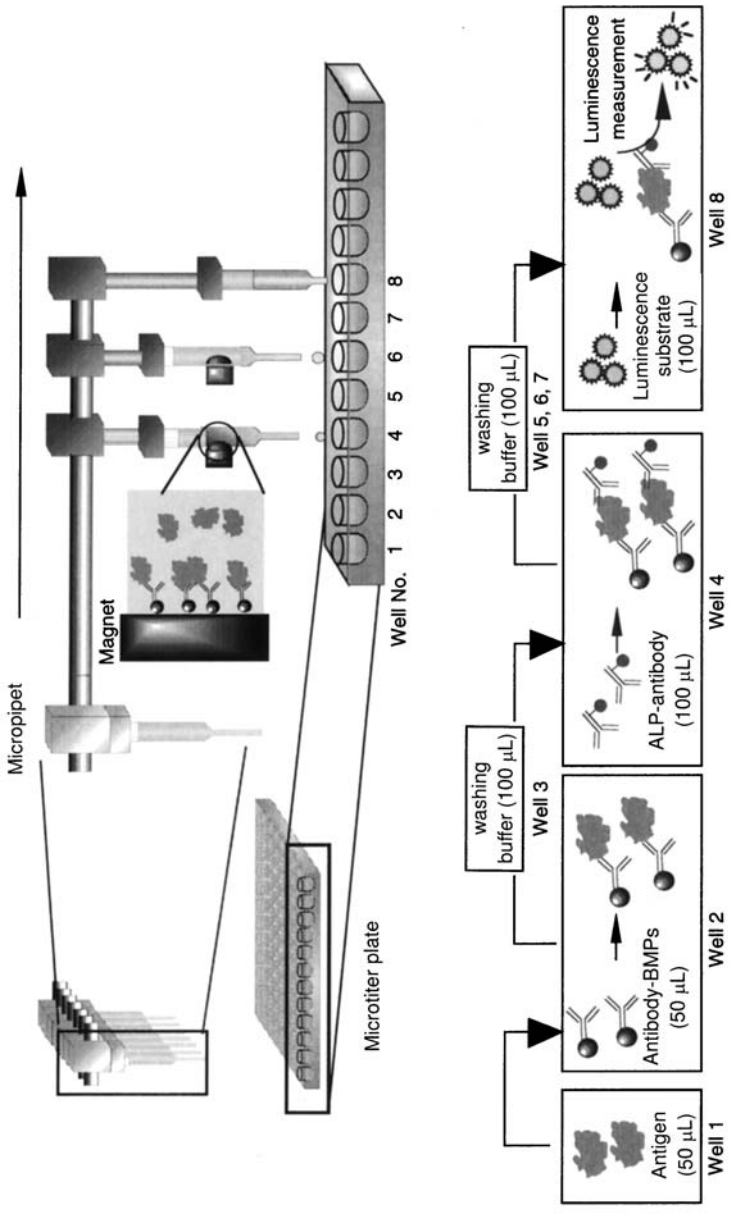


Fig. 1. Fully automated sandwich immunoassay system using antibody-BMPs and ALP-antibody.

immunoreaction). After incubation, antigen-antibody complexes were separated magnetically and washed three times by repeated pipetting in washing buffer. Antigen-antibody complexes were finally suspended in 100 µL of Lumi-phos 530, and the luminescence intensity was measured.

Optimization of the amount of antibody-BMPs added into lysozyme solution was first carried out with the immunoreaction for 20 min and the subsequent immunoreaction with 100 µL of ALP-conjugated antibody solution (10 µg/mL) for 20 min. The amount of ALP-antibody in the second immunoreaction was also optimized after the first immunoreaction between lysozyme and antibody-BMPs for 20 min. The time for the first immunoreaction was fixed under the optimized conditions for amounts of antibody-BMPs and ALP-antibody in which the second immunoreaction was performed for 20 min.

The precision (coefficient of variation [CV]) of this assay method was calculated as follows: standard deviation/mean value × 100.

## Results and Discussion

### *Optimum Assay Conditions for Rapid Fully Automated Sandwich Immunoassay*

Figure 2 shows the relationship between luminescence intensity and BMP concentration in the sandwich immunoassay using the automated system. The luminescence increased with increasing antibody-BMP concentration in the range between 100 and 600 µg of BMP/mL. However, the increase was not proportionate to antibody-BMP concentration. A large number of antibodies normally result in faster immunoassay completion. Excess antibody-BMPs in solution, however, physically block luminescence. This phenomenon in Fig. 2 may be a result of BMPs blocking the luminescence or particle aggregation. The most effective amount of antibody-BMPs was 600 µg/mL in this assay without the influence of BMP shadowing on luminescence at measurement. Therefore, in the immunoassay a concentration of 600 µg/mL of BMPs was used.

ALP-antibody concentration affects the luminescence based on the amount of ALP-antibody bound to antigen-antibody-BMP conjugates. Our previous reports *(11,12)* indicated that higher ALP-antibody concentrations induced high background in immunoassays using BMPs; therefore, sandwich immunoassays using 0–100 µg/mL of ALP-antibody were performed to determine the level of ALP-antibody to employ in the assay. Luminescence increased with increasing ALP-antibody concentrations to 80 µg/mL. Therefore, 80 µg/mL of ALP-antibody was used for further experiments (data not shown).

To optimize the immunoreaction time, the time course of luminescence intensity based on the reaction of lysozyme and antibody-BMPs was investigated. The luminescence intensity in the presence of 10 µg/mL of lysozyme increased up to the reaction time of 20 min and remained constant for times longer than 10 min (Fig. 3). The immunoreaction time was

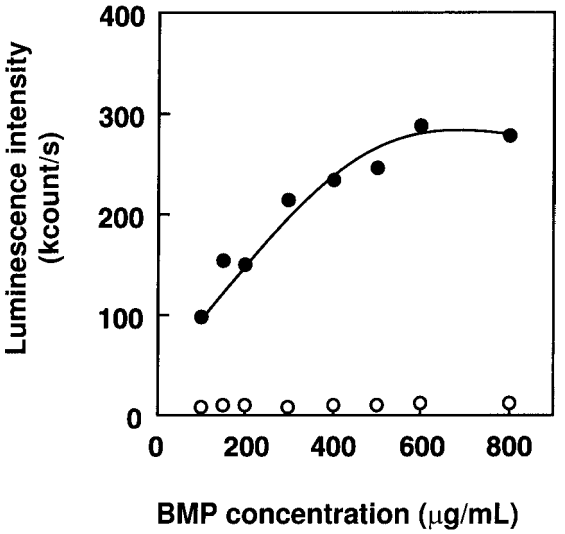


Fig. 2. Correlation between antibody-BMP concentrations and luminescence intensity. Lysozyme solution (50 μL: ○, 0 μg/mL; ●, 10 μg/mL) was mixed with 50 μL of antibody-BMP solution for 20 min. Antigen-antibody complex was collected with an Nd-B magnet and washed with PBS. BMPs were collected and dispersed by pipetting in 100 μL of ALP-conjugated antibody solution (10 μg/mL). Antigen- antibody complexes were dispersed in 100 μL of Lumi-phos 530 and luminescence intensity was measured.

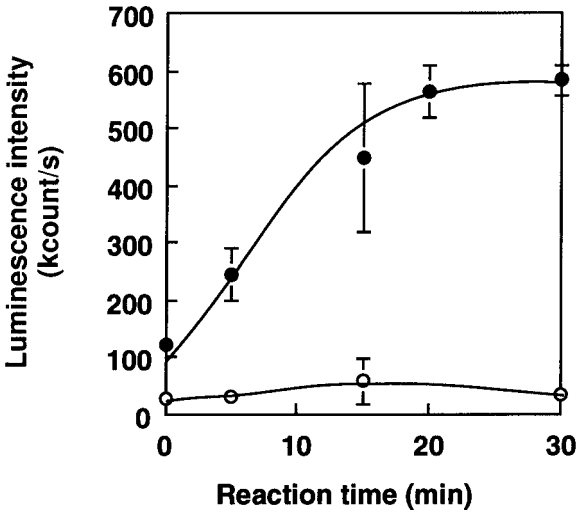


Fig. 3. Time course of the luminescence intensity in immunoreaction based on the lysozyme and antibody-BMP. Lysozyme solution (50 μL: ○, 0 μg/mL; ●, 10 μg/mL) was mixed with antibody-BMPs (50 μL, 600 μg/mL). BMPs were dispersed by pipetting in 100 μL of ALP-conjugated antibody solution (80 μg/mL) for 20 min. Antigen-antibody complexes were dispersed in 100 μL of Lumi-phos 530 and luminescence intensity was measured.

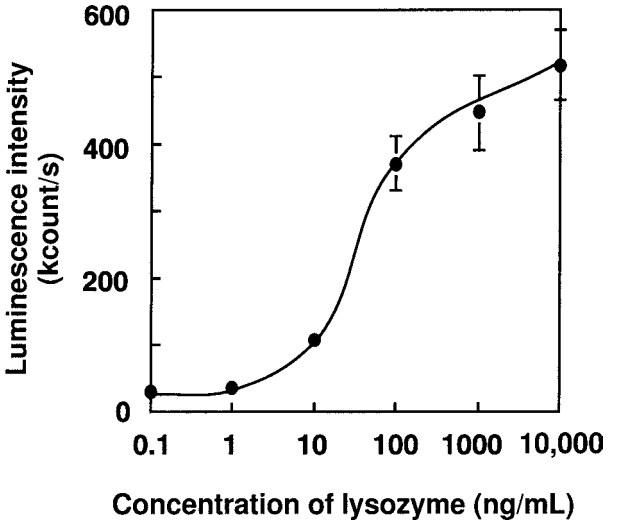


Fig. 4. Relationship between lysozyme concentration and luminescence intensity. The first and second immunoreactions were carried out for 5 min. Lysozyme solution (50 µL) was mixed with antibody-BMPs (50 µL, 600 µg/mL). BMPs were dispersed by pipetting in 100 µL of ALP-conjugated antibody solution (80 µg/mL). Antigen-antibody complexes were dispersed in 100 µL of Lumi-phos 530 and luminescence intensity was measured.

fixed at 5 min in the assay, because the minimum detectable limit obtained with the immunoreaction times at 5 min was not significantly different from that at 20 min (data not shown).

### *Fully Automated Sandwich Immunoassay for Lysozyme*

The relationship between luminescence intensity and concentration of lysozyme was investigated using anti-lysozyme antibody-BMPs and ALP-labeled anti-lysozyme antibody (Fig. 4). Luminescence intensity increased with increasing concentration of lysozyme solution. Luminescence intensity was dependent on lysozyme concentration in the range between 10 ng/mL and 100 µg/mL. The minimum detectable concentration of lysozyme was 10 ng/mL. Luminescence intensity at <1 ng/mL did not show a significant difference from that of a lysozyme concentration of 0 ng/mL. This limit is acceptable for the detection of food allergens *(16)*. In this assay, the second immunoreaction time was fixed for 5 min, because the minimum detectable concentration of lysozyme when using an immunoreaction time of 5 min was equal to that for 20 min.

One assay is completed within 20 min as follows: magnetic separation steps and pipetting steps, 8.5 min; first immunoreaction time, 5 min; second immunoreaction time, 5 min; and luminescence measurement steps, 1.5 min. The fully automated immunoassay system was equipped with an eight-tip pipettor, and, therefore, this system can assay 24 samples within 60 min. The precision (CV) at each lysozyme concentration was within 10%.

Lysozyme, ovalbumin, ovotransferrin, and ovomucoid are major allergens in egg white. In the present study, we constructed an assay system for lysozyme because lysozyme is found in the lowest concentration of the four in egg white and the assay for lysozyme required higher sensitivity than that for the other three allergens. Detection methods for allergens in food must be sufficiently sensitive to detect trace amounts (<10 ppm) of the offered food *(16)*. In this system, the minimum detectable concentration of lysozyme was 10 ng/mL, and it is sufficiently sensitive for the detection of lysozyme in food.

In conclusion, a fully automated chemiluminescence sandwich enzyme immunoassay for a food allergen lysozyme using antibody-BMP complexes was developed. This fully automated system allows rapid detection of lysozyme, within 20 min for eight samples. This system could be applicable to the multiple detection of antigens. Future studies must be directed toward detecting lysozyme in foods using this assay system.

## References


1. Ebbehøj, K., Dahl, A. M., Frøkiær, H., Nørggard, A., Poulsen, L. K., and Barkholt, V. (1995), *Allergy* **50,** 133–141.
2. Lehrer, S. B., McCants, M. L., and Salvaggio, J. E. (1985), *Int. Arch. Allergy Appl. Immunol.* **77,** 192–194.
3. Koppelman, S. J., Knulst, A. C., Koers, W. J., Penninks, A. H., Peppelman, H., Vlooswijk, R., Pigmans, I., Duijn, G., and Hessing, M. (1999), *J. Immunol. Methods* **299,** 107–120.
4. Kushimoto, H. and Aoki, T. (1985), *Arch. Dermatol.* **121,** 355–360.
5. Blakemore, R. P. (1975), *Science* **190,** 377–379.
6. Matsunaga, T., Tadokoro, F., and Nakamura, N. (1990), *IEEE Trans. Magnet.* **26,** 1557–1559.
7. Matsunaga, T., Sakaguchi, T., and Tadokoro, F. (1991), *Appl. Microbiol. Biotechnol.* **35,** 651–655.
8. Sakaguchi, T., Burgess, J. G., and Matsunaga, T. (1993), *Nature (Lond.)* **365,** 47–49.
9. Nakamura, N., Hashimoto, K., and Matsunaga, T. (1991), *Anal. Chem.* **63,** 268–272.
10. Balkwill, D. L., Maratea, D., and Blekemore, R. P. (1980), *J. Bacteriol.* **141,** 1399–1408.
11. Matsunaga, T., Kawasaki, M., Yu, X., Tsujimura, N., and Nakamura, N. (1996), *Anal. Chem.* **68,** 3551–35454.
12. Tanaka, T. and Matsunaga, T. (2000), *Anal. Chem.* **72,** 3518–3522.
13. Takeyama, H., Tuzuki, H., Chow, S., Nakayama, H., and Matsunaga, T. (2000), *Marine Biotechnol.* **2,** 309–313.
14. Matsunaga, T., Nakayama, H., Okochi, M., and Takeyama, H. *Biotechnol. Bioeng.*, in press.
15. Holen, E. and Elsayed, S. (1990), *Int. Arch. Allergy Appl. Immunol.* **91,** 136–141.
16. Taylor, S. L. and Nordlee, J. A. (1996), *Food Technol.* **61,** 231–238.