

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ANALYSIS OF LISTERIOLYSIN O PRODUCED BY *LISTERIA MONOCYTOGENES*¹

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Accepted for Publication April 21, 1993

ABSTRACT

Listeriolysin O (LLO) is a heat-labile hemolysin produced by Listeria monocytogenes. Its hemolytic activity has been evaluated qualitatively by sodium dodecyl sulfate (SDS) electrophoresis and immunoblotting. In this experiment, an enzyme-linked immunosorbent assay (ELISA) was developed for quantitative analysis of LLO by using Streptolysin O (SLO) and antistreptolysin O (ASO) as the reagents. The selected coating and blocking buffers were 0.05 M Tris buffer (pH 8.5) and 0.25% casein solution with phosphate-buffered saline solution + 0.05% Tween 20 (PBS-T), respectively. A relationship between ASO and antibody was achieved with 5 mg/ml ASO and a 1:1,000 dilution of conjugate. The heat stability of LLO at 48, 62, 72, and 80C was examined by using this method and compared with a traditional hemolysis assay. Although the LLO is inactivated easily at those temperatures, the protein structure was not affected at temperatures lower than 80C for 3 min, pointing to a need for both hemolysis and ELISA to be conducted in determining both the activity and presence of LLO in foods.

INTRODUCTION

Listeriolysin O [LLO, molecular weight, 60,000 (Geoffroy *et al.* 1987)] is a water-soluble, heat-labile protein produced by *Listeria monocytogenes* that

¹Journal Paper No. J-15276 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project No. 3074.

possesses cardiotoxic activity. This protein is activated by sulfhydryl groups such as sodium thiosulfate. The primary sequence of the protein is almost identical to that of streptolysin O (SLO) (Mengaud *et al.* 1990), which means that LLO can be bound by antibodies specific for SLO (Njoku-Obi 1963). Many researchers have reported that the gene coding for the hemolysin produced by *Listeria monocytogenes* is at least partly responsible for the organism's virulence (Leimeister-Wachter *et al.* 1990; Sokolovic *et al.* 1990). When the bacteria escape from a host vacuole, this event is mediated partly by the action of LLO, which forms pores that enable the organism to escape (Mounier *et al.* 1990). According to Sokolovic *et al.* (1990), this protein seems to be produced even under heat-shock conditions and is the only major extracellular protein produced at this point. To evaluate LLO activity qualitatively, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting using anti-LLO antibodies conjugated with an enzyme such as horseradish peroxidase have been used (Parisius *et al.* 1986; Sokolovic *et al.* 1990). However, with these methods, it has been difficult to determine whether LLO is inactivated or actually denatured during a heat treatment such as heat shock. Enzyme-linked immunosorbent assay (ELISA) is a method that can be used for the quantitative analysis of protein because it is specific as well as accurate (Hornbeck 1991; Voller and Bidwell 1986). It is a procedure that can be expensive, however, inasmuch as it often requires preparation of antibodies specific for the antigen being studied. SLO reagent and anti-SLO (ASO) are standardized commercial products that have been used in hemolytic titrations in the clinic. As such, they offer an inexpensive and convenient alternative to the purification of antibody and antigen reagents for the measurement of LLO.

The objectives of this study were to develop an ELISA method for the quantitative measure of LLO by using commercial ASO and SLO reagents, to determine the heat stability of LLO by using this method, and to compare it with a traditional hemolysis assay for accuracy in predicting activity and presence of LLO after heating.

MATERIALS AND METHODS

Bacterial Culture Conditions for Production of Listeriolysin O

Listeria monocytogenes serotype 1 (ATCC 19111) was obtained from the American Type Culture Collection in Rockville, MD. One milliliter of the strain was incubated in 500 ml Brain Heart Infusion broth (BHI, Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose at 37C for 12 h. At the stationary

phase of growth, 0.5% glucose was added, and the cells were incubated for another 2 h. During incubation, the pH of the BHI culture with 0.5% glucose was adjusted to 7.0 with pH meter (Accumet 910, Fisher Scientific, Pittsburgh, PA) and readjusted whenever the pH of the culture dropped to 5.5 by adding 1 N NaOH.

Preparation of Crude Listeriolysin O

Crude listeriolysin O was prepared by a modified method of Bhakdi *et al.* (1984). Bacteria were sedimented in a Beckman J2-21 centrifuge (Beckman Spinco division, Palo Alto, CA) (rotor JA-10, 11,000 × g for 30 min), and the supernatant was filtered through a 0.45- μ m cellulose acetate membrane filter (COSTAR, Cambridge, MA). For inhibition of protease, phenylmethylsulfonyl fluoride (SIGMA Chemical Co., St. Louis, MO) was added to culture as a 1 mM solution. Fifty-three grams of ammonium sulfate (Fisher Scientific, Fair Lawn, NJ) were then added per 100 ml of solution to precipitate the proteins, and the sample was stirred in an ice bath for 60 min. The precipitate was collected by centrifuging at 13,000 × g for 30 min, resuspended in distilled water (final volume, 70–80 ml), and dialyzed with Spectra/Por membrane tubing (Spectrum Medical Industries, Inc., Houston, TX, MWCO : 25,000) overnight against 5 L of 50 mM NaCl-4 mM EDTA in the cold room. Twenty-five grams of polyethylene glycol (PEG) 4000 (Fisher Scientific, Fair Lawn, NJ) was added to 100 ml of sample solution and stirred at 4C for 30 min. After centrifugation at 30,000 × g for 60 min, the supernatant was discarded, and the precipitate was resuspended in 50 ml distilled water containing 3 mM NaN₃ and stored in the refrigerator until used. The final solution was able to hemolyze 50% of red blood cells (RBC) at 1:1,600 dilution.

Determination of Heat-Treated Listeriolysin O

A standard curve of optical density (OD) vs. SLO concentration was prepared by determining the OD of various concentration of SLO in 0.05 M Tris buffer (pH 8.5) (0, 2.5, 5, or 10 mg/ml) by ELISA. From this standard curve, the concentration of LLO was determined during heating. One milliliter of crude and concentrated listeriolysin O solution was heat-treated at 48, 62, 72, or 80C in a water bath for 3 min. The come-up time for each temperature was about 30 s. The temperature was detected with a J-type thermocouple (Omega Engineering Inc., Stamford, CT) and datalogger (Model LI-1000, LI-COR, Lincoln, NE). After heat treatment, each sample was immediately cooled in an ice bath and diluted 20 times with 0.05 M Tris buffer (pH 8.5). One-hundred microliters of the mixture was tested by ELISA, with OD readings used to determine the concentration of LLO from the standard curve.

Indirect ELISA

Antibody and Antigen. Streptolysin O (SLO, Difco Laboratories, Detroit, MI) and antistreptolysin O made from human blood (ASO, Difco Laboratories, Detroit, MI) were used as antigen and antibody, respectively. These reagents are shipped in lyophilized desiccated powder form.

Selection of Coating Buffer. Phosphate-buffered saline (pH 7.6) (PBS, Sigma, SIGMA Chemical Co., St. Louis, MO), 0.05 M Tris buffer (pH 8.5) (Sigma, SIGMA Chemical Co., St. Louis, MO), and 0.05 M bicarbonate buffer (pH 9.5) (Sigma, SIGMA Chemical Co., St. Louis, MO) were tested for their effect on binding of LLO onto wells of an activated polystyrene microtiter plate (96 wells, Corning Laboratory Sciences Company, Corning, NY). One-hundred microliters of 5 mg/ml ASO solution was used, and the dilution ratio of antibody conjugated with enzyme used for this experiment was 1:1,000.

Selection of Blocking Buffer. Casein (0.25% w/v) (Sigma Chemical Co., St. Louis, MO) and bovine serum albumin (0.25% w/v) (BSA, Sigma Chemical Co., St. Louis, MO) solution in PBS buffer containing 0.05% Tween 20 (Sigma, SIGMA Chemical Co., St. Louis, MO) (PBS-T) were examined to determine which provided more effective blocking of unbound sites on microplate.

Optimization of Concentration of Enzyme-Conjugated Antibody. Even though polyclonal antibodies would have been equally acceptable for use in this assay, we chose monoclonal antibodies to reduce the possibility of cross-reaction and false positive results. Monoclonal antihuman immunoglobulin (γ -chain specific) conjugated with GG-5-alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was diluted to 1:500, 1:1,000, 1:2,000, 1:3,000, or 1:4,000 with PBS-T.

First, ASO solutions (3.9, 15.6, 62.5, or 250 μ g/ml of PBS-T) were bound on microplates for 2 h at 25C and rinsed three times with deionized distilled water. After blocking with casein solution for 10 min, the microplates were bound with 100 μ l of alkaline phosphatase-conjugated antibody solution. One milligram per milliliter of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) with 1 M diethanolamine buffer + 0.5 mM MgCl₂ reaction mixture (pH 9.8) was used as the substrate solution.

Method. A modified ELISA was used according to the method of Hornbeck (1991). One hundred microliters of sample or standard solution was added into the microtiter plate, incubated at 37C for 30 min, transferred to 4C, and incubated for 16 h. The antigen-bound microplate was rinsed three times with deionized distilled water, filled with blocking buffer, and incubated for 30 min at 25C. After

the blocking step, the plate was rinsed three times with deionized distilled water. ASO diluted in PBS-T was added into each well, and the plate was incubated at room temperature for 2 h. After binding of antibody, the plate was rinsed three times in deionized distilled water, and the wells were filled with blocking buffer and incubated for 10 min at 25C. After blocking, the plate was rinsed three times with deionized distilled water. After the final rinse, residual liquid was removed by wrapping the plate in tissue paper and gently tapping it face down onto several paper towels. As the next step, 100 μ l of alkaline phosphatase-linked antibody solution was added into each well, and the plate was incubated at 25C for 2 h. After incubation, the plate was rinsed three times with deionized distilled water, and 100 μ l of *p*-nitrophenyl phosphate as a substrate was added as the substrate. The enzymatic reaction was carried out at 30C for 30 min and stopped by addition of 50 μ l of 3 N NaOH. The optical density was detected at 405 nm by using a Kinetic Microplate Reader spectrophotometer (Molecular Devices, Palo Alto, CA).

Preparation of Red Blood Cells (RBC) for Hemolysis Assay

Defibrinated sheep blood (Adam Scientific, Warwick, RI) was centrifuged at $600 \times g$ for 5 min with a Beckman Model TJ-6 centrifuge. The red blood cells were resuspended in phosphate-buffered saline solution (PBS, pH 7.4) and washed with gentle stirring. The cells then were centrifuged at $600 \times g$, and the washing steps were repeated two times. The final cell concentration was adjusted to 2.0×10^8 cells per ml of red blood cell (RBC) solution in PBS containing 3 mM NaN_3 , and the resulting solution was stored at 4C.

Hemolysis Assay of Heat-Treated LLO

To detect hemolytic activity, samples were heat-treated 48, 55, or 62C for various periods in a water bath. After heat treatment, each sample was immediately cooled in an ice bath, and the hemolysin activity was assayed as a modified titration method of Bhakdi *et al.* (1984). Briefly, samples were diluted with distilled water and 0.5-ml aliquots of each diluted sample were added into 0.5 ml of 20 mM dithiothreitol in PBS solution and incubated for 10 min in a 37C water bath to activate the listeriolysin O. One milliliter of sheep red blood cell suspension was added, and the mixture was incubated for 60 min at 37C. After hemolysis, the mixture was diluted to 1:1 with PBS solution and resuspended with a vortex mixer for even turbidity of the solution. The turbidity was detected spectrophotometrically at 650 nm by using a Spectro 20 spectrophotometer (Bausch & Lomb Analytical Systems Div., Rochester, NY), and the hemolytic activities were calculated as:

$$\text{Hemolysis \%} = \left(1 - \frac{\text{OD}_s}{\text{OD}_t}\right) \times 100$$

OD_s : difference of optical density at 650 nm between sample and 100% hemolyzed RBC solution

OD_t : difference of optical density at 650 nm between nonhemolyzed and 100% hemolyzed RBC solution

When the RBC solution was not hemolyzed at all, the OD value was 0.62 ± 0.02 . Therefore, the dilution ratio of sample solution required for 50% hemolysis fo RBC resulted in an OD value of 0.31 ± 0.02 .

RESULTS AND DISCUSSION

Selection of Coating Buffer

In general, aqueous diluents at neutral or alkaline pH of various buffer solutions have been used successfully for ELISA of most proteins, but the coating effect of each buffer is different, according to the protein. For example, Barlough *et al.* (1983) showed that carbonate buffer (which is the most popular coating buffer for ELISA) used to bind coronavirus antigens resulted in diffuse and nonspecific binding but that sodium phosphate buffer, sodium chloride, or distilled water gave excellent results with that antigen. In our study, PBS buffer (pH 7.5)

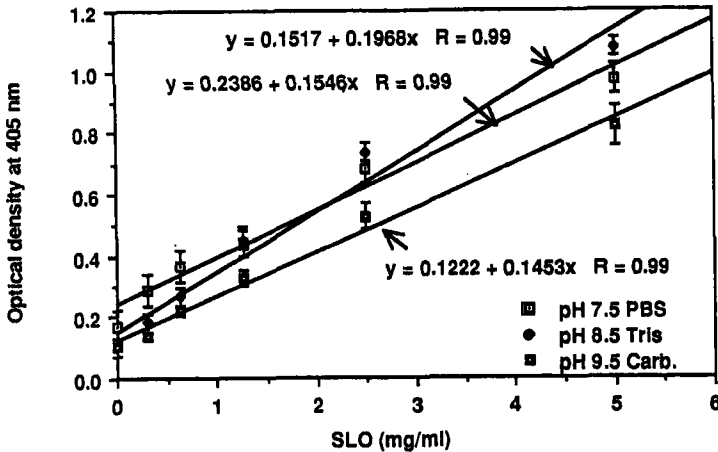


FIG. 1. EFFECT OF TYPE OF COATING BUFFER ON SLO BINDING
For conditions used, see 'Materials and Methods' section.

50 mM Tris buffer (pH 8.5), and 50 mM carbonate buffer (pH 9.5) were tested as coating buffers. Figure 1 indicates that Tris buffer was the most effective because the slope of the line obtained with this buffer was greater (indicating greater SLO binding) and that the optical density without SLO was less than with of any other buffers (indicating minimal background). Therefore, we selected the 50 mM Tris buffer (pH 8.5) as the coating buffer.

Selection of Blocking Buffer

A 0.25% (w/v) BSA solution and a 0.25% (w/v) casein solution were tested for their ability to block effectively any residual binding capacity and to prevent nonspecific adsorption while enhancing specific interaction of antigen and antibody. In this ELISA method, the blocking step was used twice: the first was after binding of SLO and, the second, after binding of ASO. Table 1 shows that

TABLE 1.
COMPARISON OF BSA AND CASEIN SOLUTIONS FOR THEIR BLOCKING EFFECT
IN ELISA¹

ASO (mg/ml)	Optical density at 405 nm			
	0.25 % BSA		0.25 % Casein	
	1st, 2nd ²	2nd ³	1st, 2nd	2nd
5.00	0.698	>3.000	0.192	2.720
2.50	0.164	1.224	0.040	0.641
1.25	0.031	0.120	0.008	0.022
0.61	0.008	0.030	0.002	0.012

¹ The binding step of SLO was omitted in this experiment.

² The blocking steps were done twice; before addition each concentration of ASO (1st step) and after binding of ASO for 2 h (2nd step).

³ The blocking step was done just after binding of ASO for 2 h (2nd step).

the casein solution was more effective in blocking than the BSA solution inasmuch as addition of ASO resulted in lower absorbance readings when casein was used as the blocking buffer when compared with BSA.

Determination of Optimum Concentration of Anti-ASO Antibody Conjugated with Alkaline Phosphatase by a Criss-Cross Matrix Analysis

Serial dilution titration analyses were performed to determine the optimal concentration of enzyme-conjugated antibody that would result in detection of SLO. According to Table 2, an ASO dilution of 1:1,000 resulted in the greatest optical density reading at 250 $\mu\text{g/ml}$ ASO, and a dilution of 1:500 resulted in greatest optical density at 62.5 $\mu\text{g/ml}$ ASO. Because the concentration of ASO used in our experiments was greater than 125 $\mu\text{g/ml}$, an enzyme dilution rate of 1:1,000 was established. At this dilution, the enzymatic activity was 630 units/ml (one unit refers to the amount of enzyme necessary to hydrolyze 1.0 μmole of ρ -nitrophenylphosphate to form ρ -nitrophenol and inorganic phosphate per min).

Determination of ASO and SLO Concentration for the Standard Curve

The standard curves of ASO concentration vs. SLO concentration were plotted by using the optimal enzyme conjugate concentration and appropriate blocking and coating buffer. Table 3 and Fig. 2 show the predicted equations of OD vs. concentrations of ASO and the standard curves, respectively. From these results, at 0.3 mg/ml of ASO, the linear relationship was detected at less than 0.63 mg/ml of SLO, and, at 1.25 mg/ml of ASO, it was detected at less than 1.25 mg/ml of SLO. In addition, the use of SLO (0.63–5.0 mg/ml) and ASO (5.0 mg/ml) resulted in a linear relationship, but the OD value obtained when

TABLE 2.
OPTIMIZATION OF ANTI-ASO ANTIBODY CONCENTRATION
(CONJUGATED WITH ALKALINE PHOSPHATASE) IN ELISA

ASO $\mu\text{g/ml}$	Optical density at 405 nm				
	1:500 ¹	1:1,000	1:2,000	1:3,000	1:4,000
250	2.528	1.248	0.458	0.367	0.225
62.5	1.078	0.514	0.180	0.144	0.089
15.6	0.055	0.021	0.004	0.012	0.005
3.9	0.007	0.005	0.003	0.003	0.000

¹ Dilution ratio of anti-ASO antibody conjugated with alkaline phosphatase.

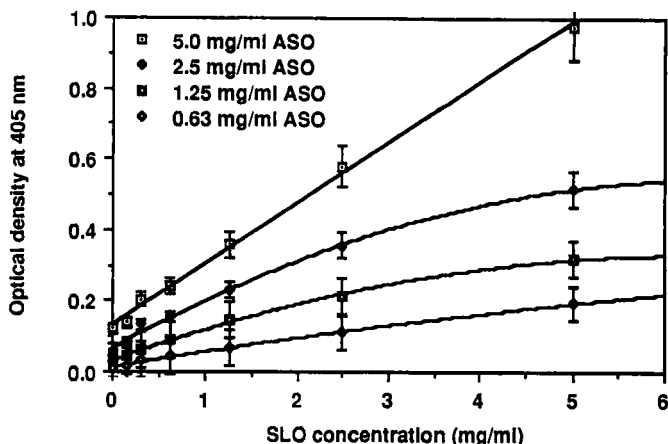


FIG. 2. STANDARD CURVES OF SLO ON ASO CONCENTRATION
The dilution ratio of anti-ASO antibody conjugated with enzyme was 1:1,000.

less than 1.25 mg/ml of ASO was used was below detection. Therefore, a concentration of ASO of 5.0 mg/ml was selected to be used in determining concentrations of SLO of up to 5.0 mg/ml of SLO.

Heat Stability of LLO

The stability of LLO was determined after heating by both ELISA and hemolysis analysis. From Fig. 3, the hemolytic activity was inactivated very rapidly at temperatures above 55°C. The concentration of LLO decreased with increasing temperature (Table 4).

In addition, the concentrations of LLO of the heat-treated samples were less than that of the standard curve of 5 mg/ml of ASO, which indicates that there was an interference effect in the sample solutions against detection of LLO. This interference could be attributable to possible binding of some components onto the wells, essentially competing with antibodies. Also, protein-protein interactions in the absorption process could occur (known as 'protein-stacking'). Because these are not stable, detachment of the protein from the surface could result (Kurstak 1986). In this experiment, the interference was defined as:

The interference (%) = 100 - (slope of sample/slope of SLO standard curve)

The interference percentages of the samples heated at 48, 62, and 72°C were calculated to be in the range of 19.8–24.8%, whereas the sample heated at 80°C resulted in the least interference (14.5%). From these data, some inhibitors can be denatured or inactivated by heating to decrease the interference effect.

TABLE 3.
PREDICTED EQUATIONS OF STANDARD CURVES AT VARIOUS
CONCENTRATIONS OF ASO

ASO (mg/ml)	Predicted equations ¹
0.63	$Y = 0.015 + 0.043X - 0.002X^2$
1.25	$Y = 0.068 + 0.144X - 0.011X^2$
2.50	$Y = 0.034 + 0.093X - 0.007X^2$
5.00	$Y = 0.134 + 0.172X$

¹ X: Concentration of SLO (mg/ml)

Y: Optical density at 405 nm

The optimal conditions for quantitative determination of LLO by an ELISA method were established. The selected coating buffer and blocking buffer were 0.05 M Tris buffer (pH 8.5) and 0.25% casein solution in PBS-T. The optimal dilution ratio of anti ASO antibody conjugated with alkaline phosphatase was 1:1,000, and the optimal concentration of ASO for constructing a standard curve was 5 mg/ml. According to the results, the structure of LLO was stable at temperatures less than 80C, since LLO was still detectable by ELISA even though its activity had been eliminated according to the hemolysis assay. Thus, it is important to note that measurement of activity solely by this ELISA method may lead to false negative results as far as activity is increased. Similarly, simply relying on the hemolysis assay could be deceptive if evidence for the presence of hemolysis strains of *Listeria monocytogenes* is being sought.

In general, evaluation of the hemolytic activity of listeriolysin of *Listeria monocytogenes* is usually determined in vitro by the CAMP test in BHI agar by using sheep blood cells for β -hemolysis (Groves and Welshimer 1977; Skalka *et al.* 1982) and by microtitration assays (Bhakdi *et al.* 1984). These methods do not offer an accurate measure of LLO produced because conditions such as pH and presence of oxidant often affect them (Geoffroy *et al.* 1987; Jenkins *et al.* 1964). Assays such as ELISA provide a quantitative measure of the production of LLO while not being affected by these factors. In this ELISA, the effective concentration range of SLO was 500 $\mu\text{g}/\text{well}$ through 15.6 $\mu\text{g}/\text{well}$. Since the protein composition in SLO was 48%, the minimum concentration of SLO that can be detected by this test can be estimated to be less than 0.075 $\mu\text{g}/\mu\text{l}$.

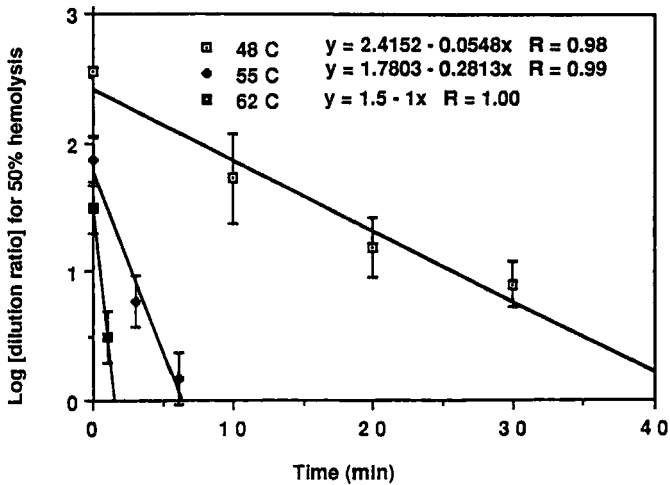


FIG. 3. EFFECT OF HEATING ON STABILITY OF LLO AT 48, 55, and 62C

TABLE 4.
EFFECT OF HEAT TREATMENT ON STABILITY OF LLO

	25 C	48 C	62 C	72 C	80 C
LLO (mg/ml) ¹	0.18 ± 0.02 ²	0.17 ± 0.03	0.17 ± 0.05	0.15 ± 0.03	0.05 ± 0.02

¹ Corresponding concentration of LLO was calculated from the standard curve by using SLO (Figure 2).

² Average values of three replications ± standard deviation.

Although this ELISA does not measure hemolytic activity by itself, this method coupled with hemolytic assays can provide an indication of activity where the sensitivity or accuracy of hemolytic assays may be questionable.

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