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Partial Separation of Serum-soluble Antigen of *Leucocytozoon caulleryi*

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Abstract

The serum-soluble antigens of chickens infected with *Leucocytozoon caulleryi* were partially separated from chicken serum components by means of ammonium sulfate precipitation, molecular sieve chromatography (Sephacryl S-300) and diethylaminoethyl (DEAE)-cellulose chromatography, and the results of such separation were evaluated by specific activity of separated parts and immunoelectrophoresis. Precipitation with 50% saturated ammonium sulfate and sequential elution with 0.01 M Tris-HCl buffer containing 0.2 M sodium chloride from DEAE-cellulose column was proved to be the best method for the separation of the antigen.

Key words: *Leucocytozoon caulleryi*, chicken, protozoa, serum-soluble antigen, partial separation.

Introduction

Leucocytozoon caulleryi, a causative agent of chicken leucocytozoonosis, was first described by Mathis and Leger (1909), and in Japan by Akiba *et al.* (1958). The prevalence of this protozoan disease has been recognized in various Asian countries and it seriously affects poultry industry through a reduction of egg production, weight loss and sometimes death of chickens.

Serum-soluble antigens of *L. caulleryi* have been demonstrated in sera of infected chickens (Morii, 1972) and their characteristics were reported previously (Morii, 1974). However, the exact role of the antigen in the course of the infection is not cleared as yet. Before testing for protective activity of the antigen, we carried out the partial separation of serum-soluble antigen from the sera of chickes infected with *L. caulleryi* to reduce the contamination by host serum components.

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Materials and Methods

Parasites: The strain of *L. caulleryi* used in the present experiment was derived from a naturally infected chicken in Gifu, in July 1982. It has been maintained in our laboratory by cyclic passage in specific-pathogen-free (SPF) chickens and colonized *Culicoides arakawae*. The procedures of rearing, breeding, and feeding of *C. arakawae* and preparation technique of sporozoite suspension for inoculation to chickens were the same as described previously (Isobe *et al.*, 1984).

Chickens: Eighteen 20 to 93-day-old male SPF chickens of PDL-1 strain were used. They were derived from a flock of White Leghorn and maintained at our laboratory in the same manner described by Furuta *et al.* (1980). All chickens were reared in the chicken house isolated from biting midges and supplied feed and water *ad libitum*.

Preparation of serum containing serum-soluble antigen of L. caulleryi: Eight chickens were inoculated with 1.8×10^3 to 2.4×10^4 sporozoites intravenously. The sera, rich in objective antigens, were collected from them 13 to 14 days after sporozoite inoculation. They were pooled and stored at -35°C until use. The frozen sera were thawed and then

clarified by centrifugation at 8,000 x g for 30 min at 4°C. The resulting supernatant was used to examine the properties of the serum-soluble antigens.

Antiserum against serum-soluble antigen: Ten chickens were inoculated with 2 to 6 x 10^3 sporozoites intravenously. Sera were collected from them by cardiac puncture 20 to 23 days after sporozoite inoculation. They were pooled and stored at −35°C until use. The clarification method of sera was the same as above.

Fractionation of antigen with ammonium sulfate: Ten ml of sera containing serum-soluble antigen were precipitated with 33% saturated ammonium sulfate (SAS). The precipitate was dissolved in 15 ml of phosphate buffered saline (PBS, pH 7.2) and reprecipitated with 33% SAS three times. The final precipitate was dissolved in 5 ml of PBS, dialyzed for 48 hr against 4 liter of 0.01 M Tris-HCl buffer (pH 8.0) and concentrated to 4 ml by pervaporation with carboxymethyl cellulose. To the supernatant of the final 33% SAS precipitation step, SAS was added to yield 50% SAS precipitated and supernatant fractions. The supernatant fraction was dialyzed for 48 hr and concentrated to 5 ml as above. The resultant precipitate was dissolved in 10 ml of PBS and reprecipitated with 50% SAS three times. The final precipitate was dissolved in 5 ml of PBS and dialyzed, and concentrated to 4 ml as above.

Separation of antigen by molecular sieve chromatography: Three ml of the fraction precipitated with 50% SAS was subjected to Sephacryl S-300 (Pharmacia Fine Chemicals) chromatography using a 1.5 x 90 cm column and 0.01 M Tris-HCl buffer containing 0.3 M sodium chloride. A flow rate of 7.0 ml/hr and fraction volume of 3.0 ml/tube were maintained in the system. Each fraction was analyzed for protein content by absorbance at 280 nm in a Hitachi Spectrophotometer 101. The peak fractions were pooled, concentrated by pervaporation as above, and assayed for antigenic activity and protein concentration. Antigenic activity was determined by the agar gel precipitaion test (Isobe and Akiba, 1982) and protein concentration was measured by the method of Lowry *et al.* (1951).

Resolution of antigens on diethylaminoethyl-cellulose (DEAE-cellulose): Three and half ml of the fraction precipitated with 50% SAS was placed on a DEAE-cellulose column (1.5 x 30 cm) equilibrated with 0.01 M Tris-HCl buffered solution of pH 8.0. Fractions were eluted stepwise with 0.01 M Tris-HCl buffer containing 0.1 M, 0.2 M and 0.3 M sodium chloride. Each fraction was analyzed for protein content and the peak fractions were pooled, and treated as above.

Immunoelectrophoresis: Immunoelectrophoresis was carried out in 1% special noble agar (Difco) adjusted the pH to 8.4 with a barbital buffer, μ=0.05, at 4 mA/cm for 2 hr to observe the separation of serum-soluble antigen from serum components. Antiserum (prepared in rabbits) against chicken whole serum and antiserum (prepared in chickens) against *L. caulleryi* were used as antibodies. Fractions used as antigens were the fraction precipitated with 50% SAS, the peak fractions eluted with 0.01 M Tris-HCl buffer containing 0.2 M sodium chloride from DEAE-cellulose column, the peak fractions eluted from Sephacryl S-300, and sera containing serum-soluble antigen.

Results

Fractionation of antigen with ammonium sulfate: Antigenic activities were detected in both precipitates and supernatant fluids treated with 33% or 50% SAS. However, the fraction precipitated with 50% SAS showed the highest specific activity (Table 1).

Molecular sieve chromatography of antigens: Figure 1 shows the elution pattern in molecular sieve chromatography. Only one peak of ultraviolet absorbing material was resolved. The antigenic activity of this peak was 1:32, but the specific activity was not so high as that of the fraction precipitated with 50% SAS (Table 1).

Table 1 Protein concentration, antigen activity and specific activity of each fraction of serum-soluble antigen from chickens infected with *Leucocytozoon caulleryi*

Procedure for separation	Fraction	Protein concentration (mg/ml)	Antigen activity*	Specific activity†
None (pre)	whole serum	34	1 : 64	1.9
Ammonium sulfate precipitation	50% supernatant	13	1 : 8	0.6
	50% precipitin	9	1 : 64	7.1
	33% precipitin	11.5	1 : 16	1.4
DEAE-cellulose chromatography	I	0.27	1 : 2	7.4
	II	2.25	1 : 16	7.1
	III	0.455	—	—
Molecular sieve chromatography	I	76	1 : 32	4.2

*: Agar gel precipitation titer.
†: Antigen activity per milligram of protein antigen.

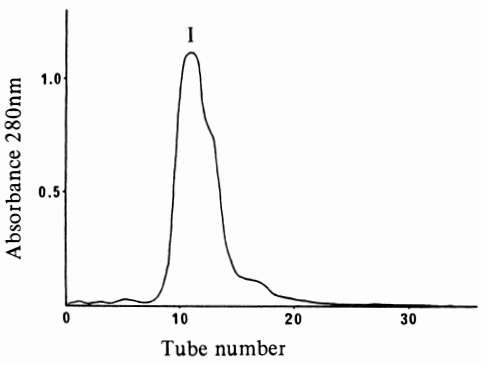

Fig. 1 Elution profile of gel filtration of the fraction precipitated with 50% saturated ammonium sulfate on Sephacryl S-300: Column, 1.5 x 90 cm; fraction volume, 3.0 ml/tube; flow rate, 7.0 ml/hr.

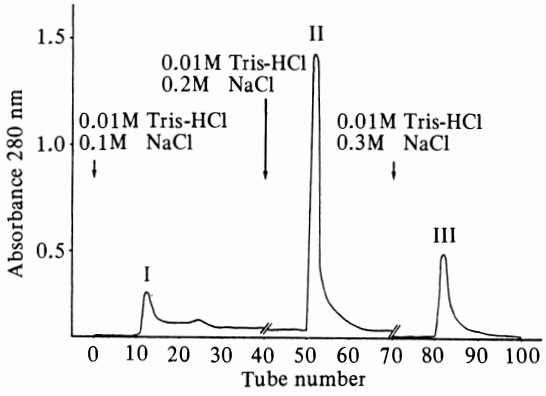

Fig. 2 Elution profile of ion exchange chromatography of the fraction precipitated with 50% saturated ammonium sulfate on DEAE-cellulose: Column, 1.5 x 30 cm; fraction volume, 3.0 ml/tube; flow rate, 7.0 ml/hr.

Fractionation of antigens by DEAE-cellulose chromatography: The elution profile is shown in Figure 2. The second peak eluted with 0.01 M Tris-HCl buffer containing 0.2 M sodium chloride showed the highest ultraviolet absorbance. Antigenic activity was detected in peaks eluted with 0.01 M Tris-HCl buffer containing 0.1 M and 0.2 M sodium chloride respectively. The specific activities of both peaks were nearly the same (Table 1).

Immunoelectrophoresis: Figure 3 shows the behavior of each fraction compared with the pattern of whole chicken serum. The fraction eluted with 0.01 M Tris-HCl buffer containing 0.2 M sodium chloride from DEAE-cellulose column was highly separated from serum components. The serum-soluble antigen was found to give precipitated lines in the α and β region.

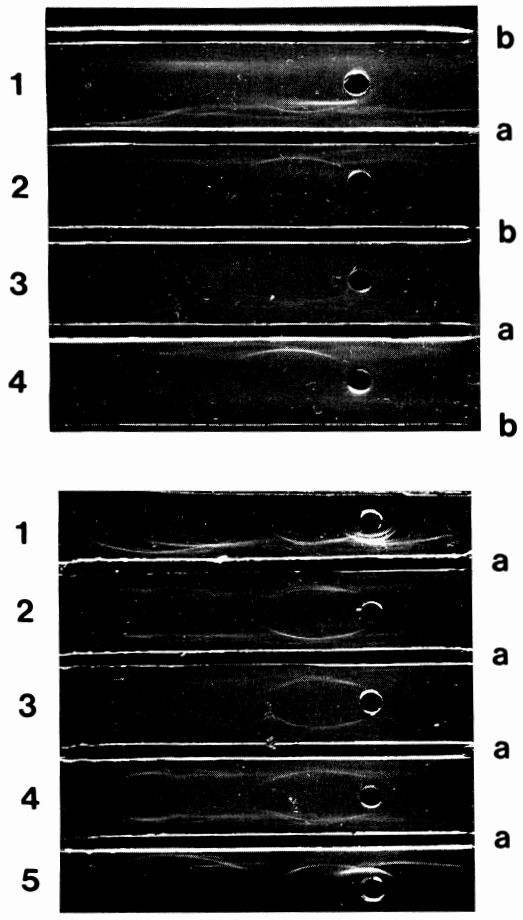

Fig. 3 Immunoelectrophoresis analysis of antigen fractions: Well 1 and 5, whole chicken serum containing serum-soluble antigen; well 2, the fraction precipitated with 50% saturated ammonium sulfate; well 3, the peak fractions eluted with 0.01 M Tris-HCl buffer containing 0.2 M sodium chloride from DEAE-cellulose column; well 4, the peak fractions eluted from Sephacryl S-300; trough a, antisera against chicken whole serum; trough b, antisera against *Leucocytozoon caulleryi*.

Discussion

Morii (1972) demonstrated the presence of serum-soluble antigens in the sera of chickens infected with *L. caulleryi*. Results of the present study demonstrate the partial separation of this antigen from chicken serum components. In the fractionation with SAS, antigenic activities were detected in both supernatant fluid and precipitate (Table 1), suggesting that serum-soluble antigen may be associated with both of globulin and alubmin fractions of chicken serum as mentioned by Morii (1974). However, the specific activity was the highest in the fraction precipitated with 50% SAS (Table 1). Therefore, precipitation with 50% SAS seems to be available for the first step in separation of the antigen.

In molecular sieve chromatography which followed the precipitation with 50% SAS, only one protein peak was obtained (Fig. 2) and the specific activity of this peak was lower than that of the fraction precipitated with 50% SAS (Table 1). Consequently, this method seems to be not so advantageous for separation of the antigen.

In a DEAE-cellulose chromatography which followed the precipitation with 50% SAS, three protein peaks were eluted and the second protein peak, eluted with 0.01 M Tris-HCl buffer containg 0.2 M sodium chloride, showed the highest ultraviolet absorbance (Fig. 1). The antigenic activity was detected in the first and second peaks and was higher in the second peak. The specific activity was not so different between these two peaks (Table 1). Therefore, the precipitation of the antigen with 50% SAS followed by the elution with 0.01 M Tris-HCl buffer containing 0.2 M sodium chloride from DEAE-cellulose column is recommended for separation of the antigen. Moreover, in electrophoresis, the contamination by serum components was the least in this part (Fig. 3). Then this part is presumed to be suitable for immunization to chickens as serum-soluble antigen of *L. caulleryi*.

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