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Isolation of *Anti-Br. abortus* strain 19 Factor in Bovine Serum with Ammonium Sulfate

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Summary

In order to isolate and purify Anti-st. 19 factor in bovine serum which is bactericidal for *Br. abortus* avirulent st. 19 but not for *Br. abortus* virulent st. 544, fractionation of bovine serum with ammonium sulfate was performed.

From the results, it became apparent that Anti-st. 19 agent was not in a single fraction but in two fractions which were salted out by different saturations of ammonium sulfate.

One is the globulin factor precipitating between 25 and 33 per cent saturation and the other is albumin factor precipitating between 50 and 60 per cent saturation. It was also proven that Anti-st. 19 action occured in co-operation with both the gloublin and the albumin factors under the existence of Mg^{++}. So, it may be thought that Anti-st. 19 action is a type of complement fixation reaction which depends on the globulin factor as antibody and on the albumin factor as a complement.

In our previous papers (1～4), it was shown that avirulent *Brucella abortus* st. 19 was very sensitive to the bactericidal action of normal bovine serum but virulent st. 544 was not and that Mg^{++} and a non-permeable factor participated in this anti-st. 19 action. It was also proven that the anti-st. 19 factor was heatlabile, relatively acid-labile and removable by an absorption with heat-killed brucella organisms (2).

On the other hand, most of well known bactericidal factors in normal serum belong to proteins such as lysozyme, antibody and complement (5, 6, 7). So the non-permeable factor participating in the anti-st. 19 action may be a protein or proteins. In this paper, isolation and purification of the factor in bovine serum were performed by salting-out techniques with ammonium sulfate.

Materials and Methods

Suspension of Br. abortus st. 19

The cell suspension was prepared with Tris-HCl buffer (pH 7.2) containing

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0.005 M $MgCl_2$ (Mg-Tris buffer) according to the methods described in the previous paper (4).

Serum fractionation

Fresh bovine serum was chilled to 0°C and saturated ammonium sulfate solution was added slowly up to the 33 per cent saturation. This mixture was allowed to stand overnight at 4°C and centrifuged at 4°C for 10 min. at 16,000 g. The precipitate obtained was dissolved in a small amount of cold Mg-Tris buffer and dialyzed against the buffer for 2 days at 4°C. This is a 33 per cent saturation fraction. The supernatant was saturated up to the 50 per cent with saturated ammonium sulfate solution and the precipitate formed was separated and dialyzed as above mentioned. This is a 50 per cent saturation fraction.

The supernatant from the 50 per cent saturation was also saturated entirely with solid ammonium sulfate. The precipitate formed was collected with filter paper (Toyo filter paper, No. 5c) and dialyzed. This is a 100 per cent saturation fraction. The filtrate was dialyzed to obtain a non-protein fraction.

Protein concentration

The protein concentration of the three fractions was spectrophotometrically estimated and adjusted to 10 mg per ml with Mg-Tris buffer. The non-protein fraction was concentrated to the initial serum volume with polyethylen glycol G–6000. These materials were filtrated with Sartorius membranefilter (pore size 0.45 μm) to be sterilyzed and stored at –20°C until the experiments were performed.

Estimation of anti-st. 19 activity

The amount of 0.1 ml of the suspension containing 10^2 viable st. 19 was added to 0.4 ml of preparations consisting of the single four fractions or of combinations of equal volumes of each fraction. These mixtures were set at 37°C for 3 hrs for sensitization. The number of viable cells was counted before and after sensitization, and the anti-st. 19 activity was calculated as follows:

$$\text{Anti-st. 19 activity} = \left(1 - \frac{\text{No. of viable cells after sensitization}}{\text{No. of viable cells before sensitization}}\right) \times 100$$

Relative activity of the action was also expressed by the ratio of values of the tested specimens to that of the control.

Results

Anti-st. 19 activity of serum fractions

As shown in Table 1, no anti-st. 19 activity was detected when single fractions were tested but some combinations of the fractions showed various activities in

TABLE 1. *Anti-st. 19 Activities of Bovine Serum Fractoins Salted out with Ammonium Sulfate*

Fraction and Combination	Anti-st. 19 activity
33% saturation fraction	0
50% saturation fraction	0
100% saturation fraction	0
Non-fractionable fraction	0
33% and 50% saturation fractions	12
33% and 100% saturation fractions	97
33% saturation and non-fractionable fractions	0
50% and 100% saturation fractions	31
50% saturation and non-fractionable fractions	12
100% saturation and non-fractionable fractions	0
33%, 50% and 100% saturation fractions	92
33% and 50% saturation and non-fractionable fractions	0
50% and 100% saturation and non-fractionable fractions	0
33%, 50% and 100% saturation and non-fractionable fractions	90

which only three combinations containing both 33 per cent and 100 per cent saturation fractions showed high activity. So, it became clear that these two fractions participated in the anti-st. 19 action of the serum.

Further studies on isolation of Anti-st. 19 factor

In order to furtherpurification, the concentration of ammonium sulfate in salting-out was changed. The 33 per cent saturation fraction was divided into two fractions precipitating between 0 and 25 per cent saturation and between 25 and 33 per cent saturation. These fractions were added respectively to the 100 per cent saturation fraction to detect their anti-st. 19 activities.

As shown in Table 2, the fraction precipitating between 25 and 33 per cent saturation showed high activity but the fraction precipitating between 0 and 25 per cent saturation showed less activity. That is, the anti-st. 19 factor in 33 per cent saturation fraction was mainly included in the fraction precipitating between 25 and 33 per cent saturation, which shall be called globulin factor.

TABLE 2. *Anti-st. 19 Active Fraction in 33% Saturation Fraction of Bovine Serum*

Fractions added to 100% saturation fraction	Anti-st. 19 activity	Relative activity
0–25% saturation fraction	10	13
25–33% saturation fraction	95	119
(Control) 0–33% saturation fraction	80	100

In the next experiments, the 100 per cent saturation fraction was divided into three fractions precipitating between 50 and 60 per cent saturation, between 50 and 67 per cent saturation and between 67 and 100 per cent saturation. These fractions were added to the 33 per cent saturation fraction and their anti-st. 19 activities were detected.

Table 3 indicates that the first fraction showed the highest activity and the next showed a relatively high activity but the other fraction showed much less activity. Thus, the 50 to 60 per cent saturation fraction is thought to take part in the anti-st. 19 action of the 100 per cent saturation fraction. We shall call this fraction albumin factor.

TABLE 3. *Anti-st. 19 Activities of Bovine Seurm 100% Saturation Fraction*

Fraction added to 33% saturation fraction	Anti-st. 19 activity	Relative activity
50–67% saturation fraction	74	112
67–100% saturation fraction	19	28
50–60% saturation fraction	95	139
(Control) 50–100% saturation fraction	68	100

Anti-st. 19 activity of Globulin and Albumin factors.

Protein concentrations of 2-fold serially diluted globulin and albumin factors were measured at 280 nm on a spectrophotometer and the anti-st. 19 activities were detected on all of the combinations of the diluted factors.

As shown in Table 4, the O.D. value of minimum protein concentration enough to give the anti-st. 19 activity of over 90 was 0.63 in both factors, while the minimum protein concentration of fresh bovine serum was 1.76 at O.D. value as shown in Table 5. Comparing these two O.D. values, it is thought that the anti-st. 19 activity of the globulin and albumin factors was three times as high as that of normal bovine serum.

TABLE 4. *Anti-st. 19 Activities of Combined Mixtures of Various Concentration of Globulin and Albumin Factors*

Protein Concentration (mg/ml)		Albumin factor				
		5	2.5	1.25	0.63	0.31
Globulin factor	5	100*	98	91	95	82
	2.5	92	91	99	84	88
	1.25	95	86	94	83	73
	0.63	91	89	92	91	74
	0.31	82	78	85	80	72

* Anti-st. 19 activity

TABLE 5. *Anti-st. 19 Activities of Fresh Bovine Serum*

Serum dilution	Anti-st. 19 activity	Protein concentration
1:2	99	
1:4	100	
1:8	97	
1:16	98	3.52 mg/ml
1:32	93	1.76
1:64	82	0.88
1:128	64	0.44

Discussion

In order to isolate and purify Anti-st. 19 factor, fractionation of bovine serum was done with ammonium sulfate. From the results, it became clear that the anti-st. 19 agents were included in two fractions which were salted out at different saturations of ammonium sulfate respectively. One is Globulin factor, precipitated between 25 and 33 per cent saturation and the other is Albumin factor, precipitated between 50 and 60 per cent saturation. Each of them alone did not act on *Br. abortus* st. 19 but a mxiture of them effected the organisms remarkably.

Braun (8) showed the striking bactericidal effects of human and equine γ-globulin on *Brucella abortus* which did not require CO_2. Yotis and Ekstedt (9) found that both rabbit and horse serum globulins were bactericidal for staphylococcus organisms. Evans *et al* (10) showed that macroglobulins were bactericidal for *Veillonela alcalescens*. These facts are different from the authers' findings in which the bovine globulin fraction by itself did not have a bactericidal action for *Br. abortus*. It has been also known that globulins of porcine, ovine and bovine sera did not affect *Br. abortus* (8) or *staphylococcus* (9). Thus, it is thought that the role of globulins in the serum bactericidal action varies between the species of animals.

On the other hand, complement, spermine and hemoglobin have been known as bactericidal factors included in albumin fractions of serum. Rozansky *et al* (11) reported that spermine itself inhibited the growth of some gram-negative bacteria and two neisseria organisms. Hobson (12) showed that a low concentration of hemoglobin had a lethal action on some gram-negative bacteria. However, Albumin factor which participated in the anti-st. 19 action of bovine serum did not possess the bactericidal action by itself. From these facts, Albumin fractor is distinctly different from spermine and hemoglobin.

Hirsch (5) and Rowly (13) reported that a mixture of immunoglobulin and complement killed *Salmonella, Escherichia coli* and so on. Nelson *et al* (14) showed that the fraction salted out between 50 and 60 per cent saturation of ammonium sulfate contained a lot of C2 and C9 constitutents of complement. Judging from these facts and our finding that the anti-st. 19 action occured in co-operation with

both Globulin and Albumin factors, it is thought that the albumin factor is a part of a complement and that the globulin factor is a natural antibody against st. 19 of *Br. abortus*. In previous papers, we reported that Mg^{++} was necessary for this anti-st. 19 action (3, 4). Considering that Mg^{++} is essential for complement fixation reaction (15), it may be thought that the anti-st. 19 action is a type of complement fixation reaction. This assumption is supported by results in our previous paper (2) in which Anti-st. 19 action in bovine serum was heatlabile and removable by an absorption with heat-killed brucella organisms.

The rate of purification of Globulin and Albumin factors was three times as high as in normal serum. It is thought that this low rate depends on the fact that the distribution of the anti-st. 19 agents was in a wide range of serum protein, because fractions precipitated between 0 and 25 per cent saturation and 67 and 100 per cent saturation of ammonium sulfate also showed the anti-st. 19 activities, of which values were 10 and 19 respectively.

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