Analysis of Colostral Proteins in Calf Serum by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

Enzyme-linked immunosorbent assay method was improved to monitor the concentrations of colostral proteins in the range of 10 to 10³ ng/ml in calf serum. Colostral proteins were purified from fat-free colostrum, and antibodies against them were prepared from the rabbit anticostral protein sera. Concentration of each protein was determined by enzyme-linked immunosorbent assay without interference by calf serum proteins in a mixture of colostrum and precolostral calf serum. Changes in the colostral protein concentrations in the sera of five postcolostral calves were monitored by enzyme-linked immunosorbent assay. After feeding colostrum to the neonatal calf, serum IgG concentration increased rapidly within 16 h to 8.1 to 36.8 mg/ml and gradually declined until 3 d to the steady levels, 4.7 to 23.6 mg/ml. The concentrations of casein and P2 (colostral small proteins, which were eluted at the second peak in Sephadex G-100 gel filtration) also increased more rapidly within 16 h to 9.6 to 264.0 µg/ml and 31.5 to 1600 µg/ml, respectively, and steeply decreased to near the detection limit on 3 d after feeding. These results indicate that enzyme-linked immunosorbent assay is useful to measure and monitor the absorbed colostral proteins and also to survey calves receiving and not receiving colostrum.

INTRODUCTION

The relationship between the serum Ig concentrations and rates of morbidity and mortality in the neonatal calves have been argued (4, 14). The neonatal calf has very low concentrations of Ig in its serum. This hypogammaglobulinemia is improved by absorption of colostral immunoglobulins. Only then is resistance established against septicemia, enteritis, diarrhea, and other diseases (2, 4, 5, 9, 12, 14). Therefore, it is very important to determine and monitor colostral protein concentrations in the serum of calf to monitor its health.

A number of immunological and electrophoretical methods (2, 3, 7, 8, 10, 11, 12, 14, 15, 16, 18, 20, 21, 23) have been used to determine the gammaglobulin concentration of calf serum. However, these methods are not suitable for the quantitative determination at low concentration of IgG, and are time-consuming. For these reasons, development of a rapid, accurate method to measure the colostral proteins is necessary.

Enzyme-linked immunosorbent assay (ELISA) is the simple, quantitative and economical method. We have attempted to improve ELISA for the measurement of colostral proteins in the neonatal calf serum. This paper describes ELISA for bovine colostral IgG, casein, and small proteins and its availability in the field.

MATERIALS AND METHODS

Colostrum

Bovine colostrum from Holstein cows was kindly supplied by local dairy farms in the Hayakita town (colostrum A) in Hokkaido and the Agricultural Experiment Farm (colostrum B) in Hokkaido University. Colostrum was centrifuged at 6500 x g at 4°C for 30 min. The upper fat layer and sedimented pellet were discarded. The clear supernatant obtained was stored at -20°C until it was used.
Buffers

Two phosphate buffers were used for the fractionation of colostrum proteins: .01 M phosphate buffer for gel filtration and .005 M phosphate buffer for diethylaminoethyl cellulose chromatography. The following buffers were used for ELISA: .01 M phosphate buffer containing .15 M NaCl (PBS), .05% Tween 20 in PBS (.05% Tween 20-PBS), 1% egg albumin in PBS (E-alb-PBS), and .1 M citric acid-phosphate buffer containing 5 mM H₂O₂ and 2 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) reaction mixture.

The first peak contained Ig and other large molecules. The second peak contained low molecular weight proteins (we called it P2 proteins), and the third peak contained casein. The first peak was further purified on the DEAE-cellulose column (2.7 x 27 cm) by linear gradient elution method elevating NaCl concentration from 0 to .3 M at a flow rate of 40 ml/h. The IgG was eluted at the second peak. Purified colostral IgG, casein, and P2 each showed a single band on a 6% acrylamide gel in disc electrophoresis, and a single precipitin line in gel double diffusion test (13) (Figure 1).

Protein Determination

Protein concentration of eluate was monitored at 280 nm with a DU-7 Beckman spectrophotometer (Beckman Instruments, Fullerton, CA). Protein concentration of other samples was determined by the method of Kalckar (6).

Separation of Colostral Proteins

Colostral proteins, IgG, casein, and P2 (which was eluted at the second peak in Sephadex G-100 filtration, Pharmacia, Uppsala, Sweden), were separated as follows: 5 ml of the supernatant, which was dialyzed against .01 M phosphate buffer, was gel-filtrated on a column of Sephadex G-100, 2.7 x 60 cm, at a flow rate of 5 ml/h. Eluate was collected 3 ml/tube with a LKB Ultronac fraction collector (LKB, Bromma, Sweden). Three peaks of proteins appeared.

Anticoestrostral Sera

One milligram of each antigen (the colostral supernatant, casein, or P2) with Freund's complete adjuvant (Difco, Detroit, MI) was subcutaneously injected into a rabbit. The injection was repeated four times with 1-wk interval. A booster injection with .3 mg antigen without adjuvant was carried out 35 d after the first injection. Rabbits were bled 4 d after the booster injection. Blood was allowed to stand overnight at 4°C. Then, the resultant sera were separated by centrifugation at 1000 x g at 4°C for 15 min. Anticoestrostral IgG, anticasein, or antiP2 was purified by affinity chromatography with Sepharose 4B column, which coupled 1 mg of antigen. Antibodies were mixed and labeled (conjugate mixture) with the method of Avrameas and Ternynck (1).

![Figure 1. Confirmation of purified bovine colostral proteins using gel diffusion precipitin analysis. 1: IgG, 2: Casein, 3: P2 (eluted at the second peak in Sephadex G-100 gel filtration). C = Bovine colostrum and Ab = antibovine colostrum.](image)
Enzyme-Linked Immunosorbent Assay

A flat-bottomed microwell plate with 96 wells was incubated with 20 ng (in 100 μl) of anticostral IgG, 100 ng (in 100 μl) of anti-casein, or 50 ng (in 100 μl) of anti-P2 at 4°C overnight. The well was washed three times with 200 μl of 0.05% Tween 20-PBS. A 100 μl of E-alb-PBS was added to a well and incubated at 37°C for 30 min. After washing the plate three times with buffer, colostrum, calf serum, or standard solution diluted with E-alb-PBS was added to the wells. Plates were incubated at 37°C for 2 h, then the wells were washed three times with 200 μl of 0.05% Tween 20-PBS. Then 100 μl of conjugate mixture, which was diluted to 10³ fold with E-alb-PBS, was added to each well. After 2 h of incubation at 37°C, the plates were washed and 100 μl of the reaction mixture was added. After 1 h of incubation at 37°C, the optical density in each well was measured at 405 nm with a Titertek multiscanplate reading spectrophotometer (Efalb Oy., Helsinki, Finland).

Experimental Designs

**Determination of Colostral Proteins in Model System.** Interference of serum proteins in ELISA of colostral proteins was tested by using the following mixtures: 1) precolostral calf serum containing 10% of PBS; 2) precolostral calf serum containing 10% of colostrum A or B; 3) PBS containing 10% of colostrum A or B. On 4 to 8 replications, colostral protein concentrations (mean ± SE), percent difference (PD), and intraassay coefficient of variation (CV%) on ELISA for IgG, casein and P2. Percent difference was calculated: PD = mixture 3/ (mixture1 + mixture 2) × 100 – 100.

**Determination of Colostral Proteins in Calf Serum.** Five Holstein calves (animal number 8 to 12) were fed colostrum twice daily (2 L/40 kg body weight/time). Blood samples were obtained at 0, 4, 8, 12, and 16 h and 1, 2, 3, 5, and 7 d after the first feeding. Then sera were separated and analyzed on the colostral protein concentrations.

**RESULTS**

**Standard Curve**

Figure 2 shows standard curves against IgG, casein, and P2. They were highly reproducible. The magnitude of the standard errors was very small at each point. The measurable range of these proteins were 10 to 10³ ng/ml.

**Determination of Colostral Proteins in Model System**

Results of ELISA of IgG using the mixtures from 3 × 10⁵ to 5 × 10⁵-fold are shown in Table 1. Percents of difference were −2.6 to 13.1 and −8.6 to .7 in the colostrum A and B, respectively. Range of CV% was 1.3 to 24.3, and values from mixture 3 and systems were not different. Variations of IgG concentration as repeated 16 times for the precolostral calf serum, colostrum A, and colostrum B were 5.0 ± .2 (CV% 15.7), 59.8 ± 1.4 (CV% 9.3), and 31.9 ± 1.3 (CV% 16.3) mg/ml, respectively.

Table 2 shows results of ELISA of casein in the mixtures diluted 8 × 10³, 10⁴, and 2 × 10⁴-fold. Percents of difference were −.8 to 7.9 and −1.8 to 10.7 in colostrum A and B, respectively. Range of CV% was 3.2 to 16.0, and no difference was observed between mixtures 2 and 3. Variations of casein concentrations, as repeated 16 times for the colostrum A and B, were 4.5 ± .1 (CV% 9.0) and 3.3 ± .09 (CV% 10.4) mg/ml, respectively. Casein was not detected in the precolostral serum.

The ELISA of P2 was carried out in the mixtures diluted with 4 × 10⁴, 8 × 10⁴, and 10⁵-fold (Table 3). Percents of difference ranged...
TABLE 1. Enzyme-linked immunosorbent assay of IgG in the mixture of colostrum and precolostral calf serum.

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>1&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Colostrum A (× 10^-5 mg/ml)</th>
<th>Colostrum B (× 10^-5 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2a&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3a&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>(× 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>X</td>
<td>SE</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>1.55</td>
<td>.13</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td>(21.3)&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td>(10.9)</td>
</tr>
<tr>
<td>4</td>
<td>1.24</td>
<td>.06</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>(12.1)</td>
<td></td>
<td>(7.8)</td>
</tr>
<tr>
<td>5&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.13</td>
<td>.03</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>(4.4)</td>
<td></td>
<td>(2.5)</td>
</tr>
</tbody>
</table>

1Mixture 1 = 10% phosphate-buffered saline (PBS) (NaCl 8.0 g, KCl .2 g, NaHPO<sub>4</sub> • 12 H<sub>2</sub>O 2.9 g and KH<sub>2</sub>PO<sub>4</sub> .2 g were diluted in 1 L, pH 7.0 to 7.2) in precordial calf serum.

2Mixtures 2a and 2b = 10% colostrum A and B in PBS, respectively.

3Mixtures 3a and 3b = 10% colostrum A and B in precordial calf serum, respectively.

4Percent difference was computed by mixture 3 (a or b)/(mixture 1 + mixture 2 (a or b))× 100 - 100.

5Mean ± SE and CV% (in parentheses) in six repeated assays.

6Repeated counts were 4 with this diluted rate.
TABLE 2. Enzyme-linked immunosorbent assay of casein in the mixture of colostrum and precolostrum calf serum.

<table>
<thead>
<tr>
<th>Dilution rate (X 10^-3)</th>
<th>Colostrum A (X 10^-4 mg/ml)</th>
<th>% Difference</th>
<th>Colostrum B (X 10^-5 mg/ml)</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2a³</td>
<td>3a³</td>
<td></td>
<td>2b³</td>
</tr>
<tr>
<td>8³</td>
<td>5.24 .09</td>
<td>5.20 .24</td>
<td>-0.8</td>
<td>3.96 .23</td>
</tr>
<tr>
<td>10²</td>
<td>4.41 .17³</td>
<td>4.76 .31</td>
<td>7.9</td>
<td>3.27 .16</td>
</tr>
<tr>
<td>20³</td>
<td>2.35 .08</td>
<td>2.38 .09</td>
<td>1.3</td>
<td>1.68 .06</td>
</tr>
</tbody>
</table>

1 Mixture 1 = 10% phosphate-buffered saline (PBS) (NaCl 8.0 g, KCl .2 g, NaHPO₄ · 12H₂O 2.9 g and KH₂PO₄ .2 g were diluted in 1 L, pH 7.0 to 7.2) in precolostral calf serum.
2 Mixtures 2a and 2b = 10% colostrum A and B in PBS, respectively.
3 Mixtures 3a and 3b = 10% colostrum A and B in precolostrum calf serum, respectively.
4 Percent difference was computed by mixture 3 (a or b)/{mixture 1 + mixture 2 (a or b)} × 100 – 100.
5 Repeated counts were 4 with this diluted rate.
6 Mean ± SE and CV% (in parentheses) in six repeated assays.
<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>1</th>
<th>Colostrum A ($10^{-5}$ mg/ml)</th>
<th>Colostrum B ($10^{-5}$ mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2a</td>
<td>3a</td>
</tr>
<tr>
<td>$(X \times 10^3)$</td>
<td></td>
<td>X</td>
<td>SE</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>4.19</td>
<td>.31</td>
</tr>
<tr>
<td>(21.0)</td>
<td></td>
<td>(12.3)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>1.73</td>
<td>.15</td>
</tr>
<tr>
<td>(24.9)</td>
<td></td>
<td>(20.3)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>1.26</td>
<td>.11</td>
</tr>
<tr>
<td>(22.9)</td>
<td></td>
<td>(38.9)</td>
<td></td>
</tr>
</tbody>
</table>

1. P2 = Colostral small proteins which were eluted at the second peak in Sephadex G-100 gel filtration.
2. Mixture 1 = 10% phosphate-buffered saline (PBS) (NaCl 8.0 g, KCl .2 g, NaHPO$_4$·12H$_2$O 2.9 g and KH$_2$PO$_4$.2 g were diluted in 1 L, pH 7.0 to 7.2) in precolostral calf serum.
3. Mixtures 2a and 2b = 10% colostrum A and B in PBS, respectively.
4. Mixtures 3a and 3b = 10% colostrum A and B in precolostral calf serum, respectively.
5. Percent difference was computed by mixture 3 (a or b)/(mixture 1 + mixture 2 (a or b)) × 100 − 100.
6. Mean ± SE and CV% (in parentheses) in eight repeated assays; other samples were tested six times.
from \(-1.0\) to \(14.3\) and \(-.7\) to \(3.9\) in colostrums A and B, respectively. Range of CV% was \(12.3\) to \(38.9\), which was a little higher than those values in Tables 1 and 2. Variations of P2 concentration, as repeated 22 times, were \(14.6 \pm .8\) (CV% 24.4) and \(13.2 \pm .7\) (CV% 24.7) mg/ml in the colostrum A and B, respectively.

**Determination of Colostral Proteins in Calf Serum**

Colostral IgG concentrations of precolostral calves individually varied from .13 to 7.6 mg/ml (Figure 3), but the other colostral proteins were not detected in all animals (Figure 4 and 5). After feeding, IgG concentration rapidly increased to the maximum (8.1 to 36.8 mg/ml) within 16 h. Higher initial IgG concentrations of neonatal Holstein calves 9 and 12 gradually decreased until 2 to 3 d to a steady level of 16.2 and 23.6 mg/ml. Lower initial IgG concentrations of calves 8, 10, and 11 slightly decreased until 2 to 3 d to range of 6.6 to 12.3 mg/ml afterwards; this level was maintained.

Concentrations of casein and P2 increased more rapidly than concentrations IgG after feeding. The maximum concentrations of casein and P2 in calves 8, 9, and 12 were 94.0 to 137.5 and 730 to 1600 µg/ml, respectively, while casein and P2 concentrations in calves 10 and 11 were lower. The concentrations of these proteins steeply decreased nearly to the detection limit 3 d after the first feeding.

Figure 3. Changes in concentrations of serum IgG in postcolostral calves. Each mark shows calf number 8 (△), 9 (○), 10 (●), 11 (●), and 12 (●). Arrows show colostrum ingestion (2 L/40 kg body weight/one time) within 24 h after birth. Until 7 d, ingestion was twice per day.

Figure 4. Changes in concentrations of serum casein in postcolostral calves. Each mark shows calf number 8 (△), 9 (○), 10 (●), 11 (●) and 12 (○). Arrows show colostrum ingestion (2 L/40 kg body weight/one time) within 24 h after birth. Until 7 d, ingestion was twice per day.

Figure 5. Changes in concentrations of serum P2 (eluted at the second peak in Sephadex G-100 gel filtration) in postcolostral calves. Each mark shows calf number 8 (△), 9 (○), 10 (●), 11 (●), and 12 (●). Arrows show colostrum ingestion (2 L/40 kg body weight/one time) within 24 h after birth. Until 7 d, ingestion was twice per day.
DISCUSSION

The single radial immunodiffusion (sRID) method has been extensively used to determine the IgG concentration (3, 7, 8, 12, 14, 20, 21). This method allows large number of samples to be processed at one time, but it is not suitable to determine the low concentration of IgG. Usually, detection limit of sRID is microgram order. In the present experiments, however, sensitivity of ELISA for the colostral proteins (IgG, casein, and P2) was 10 ng order, indicating higher sensitivity.

Although Fleenor and Stott (3) reported percent errors (PD in our text) of total IgG in fat-free colostrum was -34.33 to 13.94 and CV% was 66.4 in sRID. The variations of PD and CV% in ELISA of IgG were very small (-8.6 to 13.1 and 1.3 to 24.3, respectively) in our model system, indicating high reproducibility. Similar results were observed using ELISA to determine casein and P2.

In postcolostral calves, changing pattern of serum IgG as shown in Figure 3 was similar to those observed in previous studies (10, 15, 20, 21, 22, 23). These observations indicate that ELISA for colostral proteins is reliable.

In this study, the fate of individual colostral proteins was different. These differences would be explained by the difference in the absorption mechanism (16), half-life (8, 19), and clearance (15, 17). Change in each protein after absorption can be analyzed by ELISA. The ELISA for colostrum proteins may be applicable to survey the precolostral or postcolostral calf and also to check on its health, especially its immune status in the field.

Previous workers reported that IgG concentrations above 2 mg/ml in the neonatal calf remarkably decreased mortality from septicaemia; IgG concentration higher than that necessary to protect from the other diseases (4, 14). Moreover, the presence of the high concentration of protective antibodies against the pathogenic organisms is more effective. Because the specific antibody can be measured by ELISA, further improvement and application of ELISA for a specific pathogen will make it possible to predict the power of resistance to various diseases of the neonatal calves.

REFERENCES