ISOLATION AND IMMUNOCHEMICAL
DETERMINATION OF SOW COLOSTRUM
TRYPSIN INHIBITOR

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Trypsin inhibitor from sow colostrum was isolated by ion exchange chromatography on DEAE-Sephadex A-50 followed by gel filtration chromatography on Sephadex G-100 and affinity chromatography. Antiserum against sow colostrum trypsin inhibitor was produced by immunization with the purified inhibitor, and made specific by absorption with normal porcine serum. The specific antiserum was used for immunoquantitation by single radial immunodiffusion (SR1). In sow colostrum whey, good agreement was found between the results obtained by SR1 and the total trypsin-inhibiting activity as determined by radial diffusion in a casein-containing agarose gel ($r = 0.97, n = 10$). In sow’s milk there was only a very low inhibiting activity, and no colostral inhibitor was demonstrable by SR1. Also in baby-pig urine agreement was found between the two methods ($r = 0.97, n = 14$). In baby-pig serum such an agreement was not seen, undoubtedly because of the presence of genuine serum trypsin inhibitors. By the SR1 technique it is possible specifically to determine the colostral inhibitor even in the presence of other trypsin inhibitors.

Key words: Sow colostrum trypsin inhibitor; trypsin inhibitor immunoquantitation; affinity chromatography.

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A trypsin inhibitor specific for porcine colostrum and with a high degree of resistance to pepsin and acid was first isolated and described by Laskowski et al. (1957). The inhibitor concentration in colostrum was highest on the first day after parturition and fell gradually to about zero by the fifth day. The colostral inhibitor is assumed to protect maternal antibodies during absorption from the gut (Baintner 1973), and was claimed (Baintner 1970) to be excreted in the urine of the newborn piglets shortly after colostrum ingestion. This latter supposition agrees with the findings of Carlsson & Karlsson (1972, 1973) and Carlsson et al. (1974). Gel filtration studies by Carlsson et al. revealed a molecular weight of about 18,000 for the colostral inhibitor and about 70,000 for serum inhibitors. By preparative electrophoresis of sow colostrum Carlsson & Karlsson (1973) found the trypsin-inhibiting activity to be located in the γ-region. After concentration of the electrophoretic fractions a weak in-
hibiting activity was found also in those fractions which had a migration rate corresponding to the serum inhibitors (α-region).

All these investigations were made by measuring the trypsin-inhibiting capacity of the samples by means of different substrates. By such methods it is not possible to differentiate the colostral inhibitor from other trypsin inhibitors. The aim of the present investigation was to isolate sow colostrum trypsin inhibitor and to work out a specific immunochemical method for measuring it in biological fluids.

**Materials and Methods**

Porcine colostrum for isolation of trypsin inhibitor was collected from 10 Danish landrace sows immediately after parturition. Sow's milk was collected 20-40 days after parturition. Blood and urine samples from neonatal piglets were collected at intervals during the period of colostrum feeding. Blood was obtained by puncture of the anterior vena cava, and urine by vesicocentesis. All samples were frozen as soon as possible and stored at -20°C until used.

Preparation of colostral and milk whey partly freed from β-lipoproteins. Sow colostrum was thawed, pooled, and centrifuged at 18,000 g for one hour at 4°C. The fat layer at the top and the pellet at the bottom were discarded. Colostrum whey was produced from the de-fatted colostrum according to the method of Aalund (1968) and used for the preparation of trypsin inhibitor after precipitation of β-lipoproteins by the method of Burstein (1960). This treatment, which served to reduce the viscosity of the whey before chromatography, had no effect on the trypsin-inhibiting capacity.

Chromatographic Methods

Ion exchange chromatography with step-wise elution was performed at 4°C on DEAE-Sephadex A-50 (Pharmacia, Sweden). A 0.1 M tris-HCl 0.2 mM CaCl$_2$ buffer pH 8.2, was used for swelling the gel and for initial elution. The same buffer with addition of NaCl (0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 0.6 M) was used for the step-wise elution. The protein content in the effluent from the chromatography column (5 x 30 cm) was monitored continuously at 280 nm on an LKB 8300 Uvicord (LKB-product AB, Sweden).

Gel filtration chromatography was performed at 4°C on a 2.5 x 60 cm column of Sephadex G-100 (Pharmacia, Sweden) in 0.1 M tris-HCl 0.2 mM CaCl$_2$ buffer pH 8.2, and on a 1.5 x 30 cm column of Sephadex G-75 with the same buffer. A constant outflow rate of 12 ml/h was maintained and the protein concentration in the effluent was recorded as described above. Concentration of the chromatographic fractions was made by vacuum dialysis.

Affinity chromatography was performed according to Chauvet & Acher (1972) with some modifications. Five grams of cyanogen bromide activated Sepharose 4 B (Pharmacia, Sweden) was washed and reswelled with 1:1 M HCl on a sintered-glass filter (G 3) and then rapidly washed with about 0.5 l of cold 0.1 M NaHCO$_3$, 0.3 M NaCl buffer pH 9.0. The activated Sepharose was suspended in 20 ml of the same buffer containing 200 mg porcine trypsin ("Trypure", Novo, Denmark) and gently stirred at room temperature overnight. The trypsin-Sepharose was washed with 1:1 0.1 M NaHCO$_3$, 0.3 M NaCl buffer pH 9.0, resuspended in 0.5 M ethanolamine pH 9.0, and gently stirred at 4°C overnight. The trypsin-coupled gel was washed five times with, alternately, a 0.1 M acetate 0.3 M NaCl 0.01 M CaCl$_2$ buffer pH 4.0, and a 1 M NaCl solution, and then transferred to a column of 1.5 x 15 cm. The column was washed with 200 ml 0.1 M HCl 0.5 M NaCl 0.01 M CaCl$_2$ solution at room temperature and subsequently equilibrated with the acetate buffer. The partially purified colostral inhibitor was dialysed against the acetate buffer and applied to the column, which was left overnight at room temperature. The column was then washed with the acetate buffer until no more protein could be measured in the effluent. After that it was washed with 0.5 M NaCl solution and again with the acetate buffer. Inhibitor was eluted by a 0.1 M HCl 0.5 M NaCl 0.01 M CaCl$_2$ solution. The effluent was collected in tubes containing 0.5 M tris-HCl buffer pH 8.5, for neutralizing the acid. The active fractions were pooled, concentrated, and dialysed against a 0.1 M tris-HCl 0.2 mM CaCl$_2$, 15 mM NaN$_3$ buffer pH 8.2, and stored at +4°C.

**Determination of Activity**

Radial diffusion assay for semiquantitative evaluation of trypsin-inhibiting activity in colostrum samples and chromatographic fractions was performed according to the method of Carlsson & Karlsson (1972) using casein as a substrate.

Quantitative determination of the trypsin-inhibiting activity was performed by the radial diffusion technique just referred to, though with the following modifications: After the casein agarose had solidified, wells with a diameter of 4 mm were punched in the gel (Fig. 1). Of each sample a series of two-fold dilutions in saline were prepared in tubes. Each dilution was mixed with an equal volume of a saline solution of trypsin (50 μg/ml)
whereafter 15 μl of each mixture was placed in a well. After incubation for 3 hours in a humid atmosphere at 37°C the plates were fixed and dried. Staining was performed for 5 min in a solution of Coomassie Brilliant Blue R. Trypsin activity would manifest itself as circular unstained zones around the wells, and, conversely, absence of such zones would indicate inhibition of the trypsin (Fig. 1). Inhibiting activity was expressed by the amount of trypsin inhibited per ml (μg trypsin inhibited/ml sample). Values between those pertaining to two consecutive sample dilutions were obtained by interpolation between the areas of the zones concerned. In serial dilutions with more than one dilution giving a partially blocked zone the inhibitor concentrations were expressed by the lowest amount of trypsin used per ml sample to give less than 5 per cent inhibition.

**Immunoelectrophoretic Methods**

The equipment and reagents for immunoelectrophoresis were essentially as described by Weke (1973 a). The electrophoresis was carried out in 1 per cent agarose (w/v) (“Indubiose” A 37, L’industrie Biologique Française S.A.) in 0.016 M Barbital-Na 0.003 M Barbital 0.003 M sodium azide pH 8.6. The same buffer four times concentrated was used in the electrophoresis vessels. Rocket immunoelectrophoresis and line immunoelectrophoresis were performed as described by Weke (1973b) and Kroli (1973). The electrophoresis were performed in a 0.1 cm thick gel on 10 × 10 cm glass slides at 2 V/cm for 18 hours. After completion of the electrophoresis process the plates were pressed, washed, dried, and finally stained with Coomassie Brilliant Blue R.

*Single radial immunodiffusion* (SRI) for immunoquantitation (Mancini et al. 1965) was made in the same agarose gel plates as described above, supplemented with polyethylene glycol 6000 to a concentration of 2.5 per cent. Purified colostral trypsin inhibitor and a pool of sow colostrum (n = 10) were used for reference.

**Preparation of Antisera**

Antisera were prepared by immunization of rabbits according to the method of Harboe & Ingild (1973). To each of 8 rabbits, at least 3 intradermal injections with 50 μg purified trypsin inhibitor were given at intervals of 2 weeks. The antisera were absorbed with a pool of adult porcine normal serum and subsequently tested by rocket and line immunoelectrophoresis against sow colostrum.

*Protein determination.* Total protein was determined by the method ofLowry et al. (1951) using bovine serum albumin as standard.

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**Results**

**Purification of Colostral Trypsin Inhibitor**

By ion exchange chromatography on colostrum whey fractions with strong trypsin-inhibiting activity were obtained only in the descending part of the fall-through peak. These fractions were pooled, concentrated, and gel-filtered on Sephadex G-100. The elution pattern is shown in Fig. 2 A. After rechromatography on Sephadex G-100 the fractions with strong inhibiting activity were further purified by affinity chromatography and used for immunization. The elution zones with trypsin inhibiting activity from concentrated fractions obtained by Sephadex G-100 chromatography of whole colostrum whey are indicated in Fig. 2 B.

**Immunoechemical Determination of Colostral Trypsin Inhibitor**

From just one of eight immunized rabbits was a serum obtained which gave distinct precipitation lines when tested by immunoelectrophoresis against the immunization material, i.e., purified trypsin inhibitor, or against sow colostrum and porcine serum. With colostrum whey this antiserum gave one....
distinct and one faint line. With the purified colostral trypsin inhibitor used for immunization it gave one strong and one weak line and with porcine serum one weak line. Partial identity was found between the distinct colostrum line and the strong line developed with the purified inhibitor; furthermore the spur from the colostrum precipitate showed identity reaction with the weak line of the purified inhibitor (Fig. 3). A pattern of identity was also seen between the faint colostrum line and the serum line. Specificity for sow colostrum inhibitor was obtained after absorption of the antiserum with appropriate amounts of porcine normal serum (Fig. 4). The distinct precipitate was iden-
Fig. 3. Line immunoelectrophoretic comparison of:
A: 5 μl colostrum whey. B: 5 μl purified sow colostrum trypsin inhibitor (85 μg/ml). C: 5 μl purified sow colostrum trypsin inhibitor (50 μg/ml).
D: 5 μl porcine serum pool. E: 5 μl colostrum whey. The antibody gel contained antiserum against purified sow colostrum trypsin inhibitor, 10 μl/cm². Anode at the top.

Fig. 4. Line immunoelectrophoretic comparison of:
A: 15 μl colostrum whey. B: 15 μl purified sow colostrum trypsin inhibitor (85 μg/ml). C: 15 μl baby pig urine. D: 15 μl porcine serum pool. The antibody gel contained antiserum against purified sow colostrum trypsin inhibitor after absorption with 10 per cent (v/v) porcine serum pool, 13 μl/cm². Anode at the top.

Fig. 5. Rocket immunoelectrophoresis of two different colostrum samples (A, B), purified sow colostrum trypsin inhibitor (C), and porcine serum pool (D). The samples were applied under a weak current. The antibody gel contained antiserum against purified sow colostrum trypsin inhibitor after absorption with 10 per cent (v/v) porcine serum pool. 13 μl/cm². Anode at the top. Note the “side diffusion” and the cathodic movement of the precipitate.

tified as an inhibitor-antiinhibitor complex on account of the agreement found between the results obtained on examination of colostrum and milk by, respectively, single radial immunodiffusion and radial diffusion assay (RDA) (Table 1). A correlation coefficient of 0.97 (n = 10) was found.

The results of comparative examinations on serum and urine from neonatal piglets at different intervals after their first colostrum meal are given in Table 2. There was good agreement between the results obtained by the two methods as far as the urine samples are concerned, (r = 0.97, n = 14), but discrepancy regarding the serum samples.

As seen from Fig. 5 there was a tendency to “side diffusion” of the inhibitor and cathodic movement of the precipitate during the electrophoretic runs. This was also observed after application of the samples under a weak current. Of the inhibitor fractions represented in Fig. 2, only those from the low mol. wt. zone showed reaction with the specific antiinhibitor. By gel filtration of the
TABLE 2. Trypsin Inhibitor Concentrations in Serum and Neonatal Piglets (I-V) at Different Intervals (Hours) after Their First Colostrum Meal. The Concentrations Were Measured by Radial Diffusion Assay in Casein-containing Agarose (RDA) and by Single Radial Immunodiffusion (SRI)

<table>
<thead>
<tr>
<th>Pig No. hours</th>
<th>Serum</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RDAa</td>
<td>SRI</td>
</tr>
<tr>
<td></td>
<td>mg trypsin inhibited</td>
<td>µg trypsin inhibited</td>
</tr>
<tr>
<td>I - 5</td>
<td>1.6</td>
<td>59</td>
</tr>
<tr>
<td>II - 5</td>
<td>3.2</td>
<td>51</td>
</tr>
<tr>
<td>III - 5</td>
<td>3.2</td>
<td>49</td>
</tr>
<tr>
<td>IV - 5</td>
<td>6.4</td>
<td>40</td>
</tr>
<tr>
<td>V - 5</td>
<td>3.2</td>
<td>1.6</td>
</tr>
<tr>
<td>I - 28</td>
<td>1.6</td>
<td>3</td>
</tr>
<tr>
<td>II - 28</td>
<td>3.2</td>
<td>NM</td>
</tr>
<tr>
<td>III - 28</td>
<td>6.4</td>
<td>NM</td>
</tr>
<tr>
<td>IV - 28</td>
<td>3.2</td>
<td>NM</td>
</tr>
<tr>
<td>V - 28</td>
<td>3.2</td>
<td>4</td>
</tr>
<tr>
<td>I - 51</td>
<td>3.2</td>
<td>NM</td>
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<td>IV - 51</td>
<td>3.2</td>
<td>NM</td>
</tr>
<tr>
<td>V - 51</td>
<td>3.2</td>
<td>NM</td>
</tr>
</tbody>
</table>

a) Lowest amount of trypsin used per ml sample to give less than 5 per cent inhibition. NM = not measurable, ND = not done.

purified colostral trypsin inhibitor on Sephadex G-75 no further separation according to mol. wt. was accomplished.

DISCUSSION

The present paper describes an immunochemical method for determination of sow colostrum trypsin inhibitor independently of all other inhibitors. This is not possible with methods based on trypsin-inhibiting capacity. The acid stability of the trypsin inhibitor (Laskowski et al. 1957) permitted the use of the affinity chromatography method of Chauvet & Acher (1972) for its final purification, including an elution procedure with hydrochloric acid as the elution agent. By this procedure it was easy to break the bindings between the trypsin inhibitor and porcine trypsin covalently bound to the Sepharose matrix, a problem which had been found difficult in some preliminary investigations. Gel filtration on Sephadex G-100 before affinity chromatography helps separating the specific colostrum inhibitor (mol. wt. 18,000) from small amounts of other inhibitors, e.g., serum inhibitors (mol. wt. about 70,000), which can be found also in colostrum (Carlsson et al. 1974, Carlsson & Karlsson 1973). Nevertheless a faint immunoprecipitate was found when rabbit natsera raised against purified colostral inhibitor were tested against adult normal porcine serum. This precipitating antigen was found to be immunologically identical with an antigen in colostrum. No attempt was made to identify this precipitate; it may be due to antibodies induced by small amounts of an unidentified trypsin inhibitor contaminating the purified colostral inhibitor preparation used as antigen, and occurring in adult pig serum too, or to some other protein unspecifically bound to Sepharose and eluted together with the inhibitor.

Low molecular weight and slow electrophoretic velocity was described by Weeke (1973 b) as a cause of "side diffusion" of proteins during rocket immunoelectrophore-
sis. Carlsson & Karlsson (1973), using gel filtration studies, demonstrated that the colostral trypsin inhibitor had a mol. wt. of about 18,000, and by agarose gel electrophoresis Carlsson et al. (1974) found it to be placed in the \( \gamma \)-globulin region. These circumstances will probably explain the “side diffusion” of the inhibitor and the tendency to cathodic movement of the precipitate.

The double precipitate (Fig. 3 and 4) formed by the purified colostral inhibitor is assumed to be an artefact caused by partial proteolytic degradation during purification. In immunoelectrophoresis of erythrocyte membrane proteins artefacts were described by Bjerrum & Bog-Hansen (1975) to be caused by leucocyte proteases contaminating the erythrocyte preparations. Also in colostrum-protein preparations there is a risk of contamination with leucocytes and leucocyte proteases, which may be able to degrade the inhibitor during the separation procedure. The little peak of trypsin-inhibiting activity found, after concentration, in some early fractions from the Sephadex G-100 filtration of colostrum whey (Fig. 2) was probably caused by the same inhibitor as found by Carlsson & Karlsson (1973) in the \( \alpha \)-globulin region after pevicon electrophoresis of sow colostrum. This conclusion is supported by the fact that these fractions did not react immunochemically with antisera against colostral inhibitor. The discrepancy between the results obtained on examination of sera from suckled neonatal piglets by, respectively, radial diffusion assay and SRI is presumably due to the strong inhibiting activity shown by Carlsson & Karlsson (1972, 1973) and Carlsson et al. (1974) for an inherent feature of foetal and neonatal pig serum. The occurrence, in RDA, of more than one partially blocked zone in a series of dilutions was associated with serum samples only and should probably be ascribed to differences between the binding constants for serum and colostral trypsin inhibitors. There was a fine agreement between the results of the two methods when applied to urine from the same piglets, which confirms that only the colostral inhibitor is eliminated in the urine. The quantitative immunochemical determinations of colostral inhibitor in serum and urine from neonatal piglets verify the conclusions by Baintner (1970) and Carlsson & Karlsson (1972, 1973) that in neonatal piglets the colostral inhibitor is very efficiently eliminated by the kidneys, in that the low concentration of the colostral inhibitor in serum from suckled neonatal piglets as compared to the high concentration in the urine implies a very quick elimination. This agrees with the finding by Carlsson et al. (1974) that on intravenous injection of colostrum into 10-day-old piglets most of the trypsin-inhibiting activity appeared in the urine within a few hours of the injection.

By the immunochemical method described, a specific, quantitative determination of sow colostrum trypsin inhibitor in biological fluids is easily performed.

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REFERENCES


Carlsson, L. C. T. & Karlsson, B. W.: Trypsin in-
hibiting capacity in serum, digestive contents, urine and organs of the developing pig. Enzymologia 43: 8–103, 1972.


