tissue used for preparation of mitochondria. The invaluable services of Mr. ALBERT HEINDEL in supervising the large-scale preparative work are gratefully acknowledged.

SUMMARY

DPNH oxidase has been fragmented with mixtures of cholate, ammonium sulfate and trypsin into a red particle with DPNH cytochrome *c* reductase activity (antimycin sensitive) and a green particle with cytochrome oxidase activity. This fragmentation probably accounts for the opening phenomenon in which the DPNH-oxidase activity of DPNH oxidase is abolished by exposure to deoxycholate, and two new activities emerge, *viz.* DPNH-cytochrome *c* reductase activity and ferrocytochrome *c* oxidase activity.

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A CRYSTALLINE TRYPSIN INHIBITOR FROM SWINE COLOSTRUM*

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A recent study of the resistance to pepsin of six naturally occurring trypsin inhibitors[1] revealed striking differences. The two most resistant were a pancreatic inhibitor of KUNITZ AND NORTHROP[2] and bovine colostrum inhibitor[3,4]. Since it was previously postulated[3] that the physiological role of the inhibitor in colostrum is to protect the antibodies of colostrum from digestion by trypsin, it appeared desirable to extend our studies to the trypsin inhibitor of swine colostrum. Newborn pigs are known to be very sensitive to infection when deprived of normal colostrum. One might therefore expect swine colostrum to have a high level of inhibitor, highly resistant to pepsin, and this has proved to be the case.

The present paper describes the occurrence, purification and some of the properties of swine colostrum trypsin inhibitor.

EXPERIMENTAL

Material

The swine colostrum was collected in the Department of Animal Husbandry, University of Wisconsin, Madison, Wisconsin. We are greatly indebted to Prof. R. H. GRUMMER and Mr. H. C.

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MARQUARDT for this material. Small samples of 3 to 5 ml of colostrum were collected from the same sow on 5 consecutive days after delivery. In addition the first day colostrum was collected from several sows (approximately 2 quarts) and pooled. It was kept frozen until used.

Analytical methods

Free trypsin inhibitor was determined either according to KUNITZ[5], or by our modification[1] of the PARKS AND PLAUT[6] method.

Purification

A combination of the standard chemical method[3] and the chromatographic method were used. Chromatographic purification was performed in a cold room at 1–2° on substituted celluloses: diethylaminoethylcellulose (DEAE) and carboxymethylcellulose (CM). Both were prepared according to PETERSON AND SOBER[7] and were gifts of Dr. E. A. PETERSON, to whom we express our sincere gratitude.

Step 1. Swine colostrum, 1,600 ml containing free inhibitor equivalent to 3.69 g of trypsin, was mixed with 3,200 ml of water, and 1,600 ml of 10% trichloroacetic acid. After 2 hours at room temperature, the mixture was filtered on a large Buchner filter. The filtrate was saved. The precipitate was suspended in water, adjusted to pH 6.0, and mixed with 1/3 volume of 10% trichloroacetic acid. The precipitate was separated by filtration and discarded. The filtrates were combined (total volume 7,660 ml), and 603 g of solid ammonium sulfate per liter were added, to attain 80% saturation. The precipitate was collected on a Buchner funnel using Whatman No. 50 paper.

Step 2. The precipitate was dissolved in water, diluted to 100 ml, and 100 ml of 5% trichloroacetic acid added. The mixture was heated to 80° and held 5 min at that temperature. A small precipitate was centrifuged off in a Sorvall high speed centrifuge (SS-1), and discarded. The liquid (186 ml) contained inhibitor equivalent to 2.9 g of trypsin. Potency was 2,900 units/E^*.

This solution was shaken thoroughly with an equal volume of ether to remove any contaminating lipid. The aqueous layer was separated and treated with solid ammonium sulfate to 80% saturation. The precipitate was filtered and redissolved in a small volume of 0.005 M phosphate buffer, pH 7.0; the solution was dialyzed against the same buffer for 48 hours. Total activity was equivalent to 2.5 g of trypsin, potency 3,000 units/E^*.

Step 3. This solution was placed on a DEAE column, 2.5 × 22 cm, previously perfused for 48 hours by the same buffer. The results of chromatography are shown in Fig. 1. The active component was slightly retarded at pH 7.0, and emerged at pH 6.5. The fraction from 60 to 387 ml was combined, lyophilized, dialyzed against water, and lyophilized once more. Total activity was equivalent to 1.5 g of trypsin, potency 4,500 units/E^*.

Step 4. The material from step 3 was dialyzed against 0.005 M phosphate buffer pH 5.5, and chromatographed on a CM column, 2 × 20 cm, previously equilibrated with the same buffer (Fig. 2). Only part of peak 2, the fraction from 417 to 477 ml, was pooled and lyophilized. Total activity was equal to 0.8 g of trypsin, potency 5,500 units/E^*, **.

* One unit/E means that 1 μg of trypsin is inhibited by 1 ml of solution of optical density 1 at 280 mμ. The figures for potency given in the text refer to the casein method.

** It is interesting to note that only a small increase in potency from 5,500 to 5,600 units/E was achieved between step 4 and crystallization. Several attempts to crystallize the inhibitor after step 4 were unsuccessful. It may be that the interfering contaminant contained no optically absorbing amino acids and therefore escaped detection.

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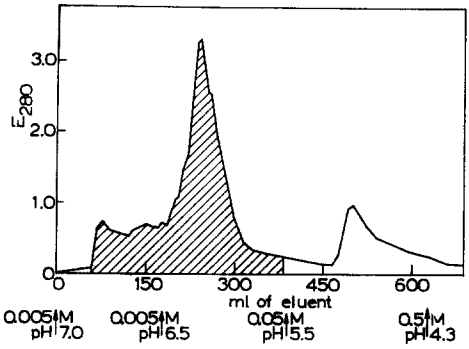

Fig. 1. Step 3. Chromatographic pattern of the solution of inhibitor. Column 2.5 × 22 cm of DEAE-Solka-Floc. Eluent was a mixture of NaH_2PO_4 and Na_2HPO_4 of indicated molarity and pH. Flow rate was about 5 ml per hour.

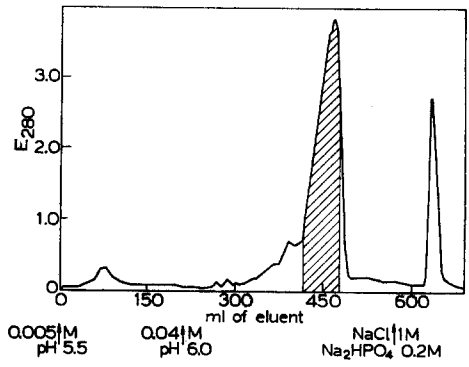

Fig. 2. Step 4. Chromatographic pattern. Column 2 × 20 cm of CM-cellulose. Eluent sodium phosphate of indicated pH and molarity. Other notations as in Fig. 1.

Abscissa: milliliters of eluent; ordinate: optical density at 280 mμ. The shaded area represents the fraction used for further purification.

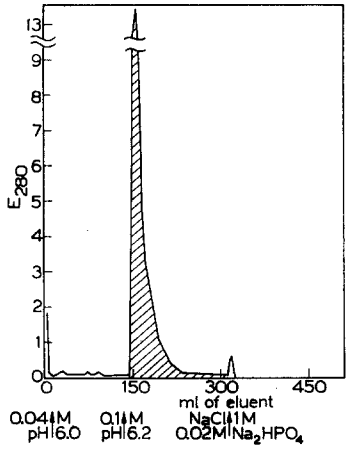

Fig. 3.

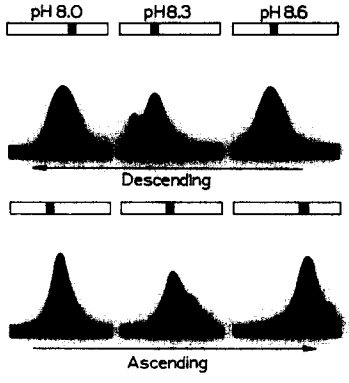

Fig. 4.

Fig. 3. Step 5. Chromatographic pattern of the solution of the trypsin inhibitor-trypsin complex. Column 1.2 × 18 cm of CM-cellulose. Other conditions and notations as in Figs. 1 and 2.

Fig. 4. Electrophoretic patterns of peak 2 of Fig. 3 (1.5% solution with respect to protein) in 0.1 *M* barbiturate buffer of indicated pH, periods of 130 to 150 minutes, at 6.3 volts/cm.

Fig. 5. Step 5. Second chromatography of the trypsin inhibitor-trypsin complex. Column 2.5 × 25 cm of CM-cellulose. All other conditions and notations as in Fig. 1.

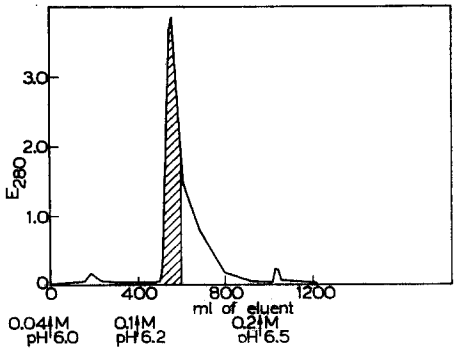

Fig. 5.

Step 5. Crystallization of the swine colostrum trypsin inhibitor-trypsin complex. The lyophilized powder was dissolved in water and mixed with an equivalent amount of crystalline trypsin dissolved in 0.0025*N* HCl. The mixture was adjusted to pH 5.5, and dialyzed against 0.005*M* phosphate buffer, pH 5.5. It was chromatographed on a CM column 1.2 × 18 cm, previously adjusted to the same buffer (Fig. 3). Peak 2 was pooled, dialyzed and lyophilized. It was analyzed for tryptic and inhibitory activity, and was inactive. Electrophoretic analysis revealed the presence of one contaminant (Fig. 4), and showed that the complex had an isoelectric point close to 8.3. In view of the heterogeneity of this preparation, one more attempt was made to remove the contaminant by chromatography on CM, using a larger column (Fig. 5). Only the fraction 186 to 225 ml was pooled, dialyzed against distilled water and lyophilized. The complex was redissolved in 1 ml of 0.1*N* tris(hydroxymethyl)aminomethane buffer, pH 8.3, cooled to 0°, and treated with 0.7 ml 95% ethanol. The heavy precipitate was centrifuged off at —10° C, and washed with 2 ml of 33% alcohol. The precipitate was redissolved in 1 ml of water, a small amount of insoluble material was removed by centrifugation, and the liquid was treated with 1 ml of 40% alcohol at 0°, to bring the final alcohol concentration to 20%. The mixture was exposed to —10° for 24 hours. The precipitate was crystalline, but could be photographed only after centrifugation and suspension in 70% alcohol (Fig. 6), because in lower alcohol concentration, the precipitate dissolved when warmed to room temperature.

Step 6. Crystallization of free swine colostrum trypsin inhibitor. The crystalline complex was dissolved in 2 ml of water and treated with 2 ml of 5% trichloroacetic acid for 30 min at 25° and centrifuged. The precipitate contained active trypsin. The supernatant was heated 5 min at 80°, centrifuged, and the small precipitate discarded. The liquid was adjusted to pH 3.0, and 80% saturated with ammonium sulfate. The mixture was centrifuged and the liquid was discarded. The precipitate was dissolved in 0.2 ml of acetate buffer, pH 6.0, and treated with 0.6 ml of saturated ammonium sulfate. The precipitate which formed, after standing for several days at room temperature, changed into not so well defined plates, Fig. 7.

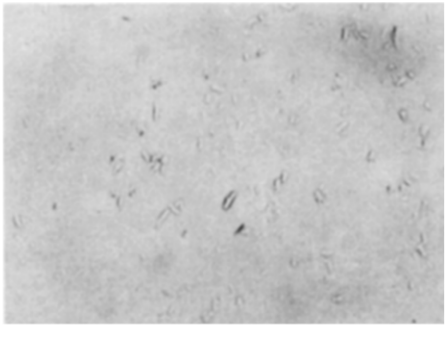

Fig. 6. Crystalline trypsin inhibitor-trypsin complex (× 200).

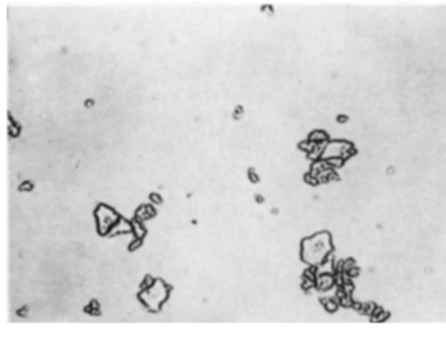

Fig. 7. Crystalline free trypsin inhibitor from swine colostrum (× 200).

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RESULTS AND DISCUSSION

Occurrence

The level of trypsin inhibitor in swine colostrum during the first 5 days after parturition was determined by both methods[1,5] and the average values are given in Fig. 8. As previously observed[3] with human and bovine colostrum, the swine inhibitor almost disappears by the fifth day. Maximum activity is present in the colostrum of the first day, as in bovine colostrum. The amount of inhibitor is, however, considerably higher in the sow than in the species previously studied; in relative numbers: human 1, cow 10, sow 67. In actual figures, 1 ml of the first day swine colostrum inhibits, on an average, 2 mg of crystalline trypsin.

Properties of the inhibitor

Ultraviolet absorption curves of the crystalline swine colostrum inhibitor and its complex with trypsin (Fig. 9) are typical of protein absorption curves, and show considerable resemblance to the curves for soybean inhibitor and complex reported by KUNITZ[8]. The maximum absorption for the free inhibitor is at 277 mμ, probably indicating the absence of tryptophan.

Trypsin-inhibiting activity of the crystalline preparation was 5,600 units/E* by the method of KUNITZ[5] and 4,500 units/E by the manometric[1] method**. For comparison, bovine colostrum inhibitor[3] contains 4,600 units/E, pancreatic inhibitor[2] 2,780, and soybean inhibitor[5] 1,100. The factor for converting the E_{280} to milligrams of swine colostrum inhibitor is approximately 1.9. Insufficient material was available for a more accurate determination.

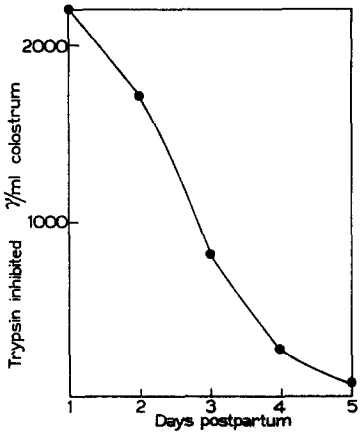

Fig. 8. Content of trypsin inhibitor in swine colostrum during the first five days after parturition.

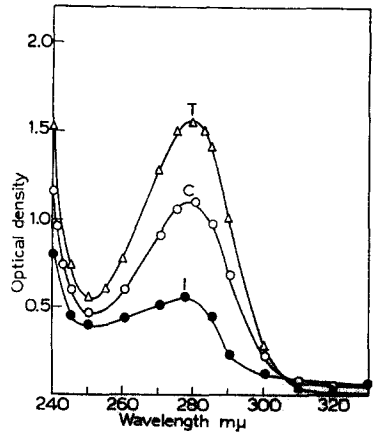

Fig. 9. Ultraviolet absorption spectra of the complex, of the free inhibitor, and of trypsin. Concentrations approximately 1 mg/ml, pH 7.0. ○—○ complex (C); ●—● inhibitor (I); △—△ trypsin (T).

* One unit/E means that 1/μg of trypsin is inhibited by 1 ml of solution of optical density 1 at 280 mμ. The figures for potency given in the text refer to the casein method.

** With all the other inhibitors tested, the two methods give closely similar activities. However the difference with the sow colostrum inhibitor between the two methods is definite and consistent. At present, this is not readily explicable.

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Purified swine colostrum (from step 4) was digested at pH 1.5 and 37° C with 0.01 and 0.05% pepsin, by the procedure previously described[1]. Its relatively slow digestion in comparison with bovine colostrum inhibitor is shown in Fig. 10. One hundred hours were required for 50% digestion with 0.01% pepsin. Exposure for the same period of time to acid at pH 1.5 without pepsin had no effect on the activity of this inhibitor.

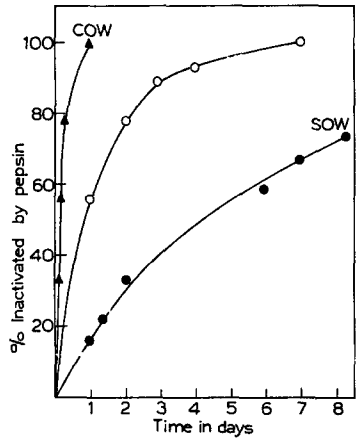

Fig. 10. Inactivation of colostrum inhibitor with pepsin at pH 1.5 and 37°. ●—● swine colostrum inhibitor, 0.01% pepsin; ○—○ swine colostrum inhibitor, 0.05% pepsin ▲—▲ bovine colostrum inhibitor, 0.01% pepsin.

This high degree of resistance to pepsin, as well as the high concentration of inhibitor in the swine colostrum, confirms our previous conclusion[1] that the failure of BARRACK *et al.*[9] to increase assimilation of γ-globulins in artificially fed newborn pigs by feeding the γ-globulins with soybean inhibitor, could have been caused by their choice of an inhibitor easily inactivated by acid and by pepsin[1,10].

ACKNOWLEDGEMENTS

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SUMMARY

Swine colostrum contains a trypsin inhibitor in much higher concentration than that previously reported in human or bovine colostrum. The concentration was highest (1 ml inhibited 2 mg of trypsin) on the first day after birth and fell gradually almost to zero by the fifth day. Like the majority of naturally occurring trypsin inhibitors of animal origin, the swine colostrum inhibitor is a small protein. Both the free inhibitor and the inhibitor-trypsin complex were obtained in crystalline form; the method of purification is described.

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