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ISOLATION AND PURIFICATION OF COLOSTROKININ FROM BOVINE COLOSTRUM*

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Abstract—It was confirmed that a biologically active substance was released when pretreated bovine colostrum (colostrokininogen) was incubated with human salivary kallikrein. It was also demonstrated that colostrokininogen belongs to the immunolactoglobulin of colostric proteins. Crude colostrokinin was purified by means of adsorption on carboxymethylcellulose, Sephadex gel filtration (G-25, G-10 and G-15) and CM-Sephadex column chromatography. The active fraction obtained after CM-Sephadex column chromatography is homogeneous in thin-layer chromatography (TLC) and paper electrophoresis. The peptide spot and biological activity migrated similarly in paper electrophoresis. The vasodilator activity of 1 mg colostrokinin in dogs corresponds to that of 258 μg bradykinin. The pharmacological activities of colostrokinin qualitatively agreed with those of kinins, such as bradykinin. Vasodilatation in dogs, stimulation of smooth muscle of rats, and increase in capillary permeability in guinea pigs were caused by colostrokinin. Finally, it was indicated that colostrokinin might aid in the transport of high molecular weight substances across the neonatal gut of ungulates.

IT IS well known that kallikreins come from various sources or species and are capable of releasing from plasma proteins biologically active peptides, namely "kinins", which cause vasodilatation, smooth muscle stimulation, increase in capillary permeability, production of pain, and accumulation and migration of leukocytes.

Colostric proteins appear to be derived from the proteins of plasma,[1–3] but both colostric and plasma proteins are not identical electrophoretically.[4] In 1959, Werle[5] and Guth[6] observed that smooth muscle stimulating activity, resembling bradykinin activity, originated from bovine colostrum. This substance was called "colostrokinin" by Werle.[5]

We have been interested in the correlation between colostrum and the absorption of antibodies through the neonatal gut after ingestion of colostrum, which is a physiological phenomenon characteristic of the newborn. This paper describes the release of biologically active peptide when bovine colostrum was incubated with human salivary kallikrein, and the purification of crude colostrokinin. The physiological function of colostrokinin in the body is discussed.

MATERIALS AND METHODS

Bovine colostrum was generously supplied by the National Institute of Animal Industry (Chiba, Japan). Yellowish colostrum was pressed out within 24 hr after parturition and stored at −20°. Normal milk of cows was obtained commercially.

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Colostrum of women was supplied by the Morinaga Industry (Tokyo). The preparations of human salivary kallikrein used in these experiments were both the crude acetone powder and the fraction partially purified by diethylaminoethylcellulose treatment, according to a previously described method.[7] Synthetic bradykinin, kindly supplied by Sandoz (Switzerland), was used as a standard. Human mixed saliva was employed after filtration through Toyo Roshi No. 2 filter paper; it was necessary for this preparation to be as fresh as possible. Gastric juice and intestinal juice were obtained from the Keio Hospital (Tokyo, Japan).

Crude colostrokinin was prepared as follows: Colostrum was centrifuged at 11,000 *g* for 30 min. The middle layer was brought to pH 3·0 with 5 N HCl and warmed at 37° for 5 min. After the solution was readjusted to pH 5·5, low molecular weight impurities were removed by dialysis in cellulose tubing (Visking Company, Chicago) against tap water. Human salivary kallikrein was added (0·3 Frey unit/1 ml equivalent of original colostrum) to the dialyzed solution (the fraction inside the membrane) and the mixture was incubated at 37°, pH 7·6 to 7·8, for 1–2 hr. The pH was adjusted again to 4·5 with 5 N CH_3COOH, 2 vol. of ethanol was added to the solution, and the mixture was refluxed for 10 min. After cooling, the precipitate that formed was separated by centrifugation and was washed once with 66·7% (v/v) ethanol. The combined supernatants were evaporated under reduced pressure at a temperature not exceeding 40°. The residue was called "crude colostrokinin." In control experiments, the conditions were similar to those mentioned above, except for the absence of kallikrein or colostrum, or normal milk was employed in place of colostrum.

Vasodilatation in anesthetized dogs was measured by the method previously described.[7] The magnus method was performed in essentially the usual manner. Smooth muscle stimulating activity of samples was determined on the isolated uterus from rats weighing 120–180 g with standard synthetic bradykinin. The uterus was suspended in a 50-ml bath of oxygenated modified Locke–Ringer's solution* at 24°. Contractions of tissue were transmitted to a transducer (Nihon Koden & Company, Tokyo), amplified, and recorded electromanometrically on an ink writing polygraph. The activity on the rat ileum was estimated in a manner similar to that employed with the uterus, except that Tyrode's solution was used. Guinea pigs weighing about 300 g were used to assay of increases in capillary permeability. A 5% solution of pontamine sky blue 6B in physiological saline was injected i.v. in a dose of 0·6 ml/kg. After 30 min, solutions containing graded concentrations of bradykinin (standard) or colostrokinin (sample) in 0·1 ml of physiological saline or 0·1 ml of physiological saline (control) were injected intradermally in the depilated skin of the back. The animals were killed 25–30 min later and the blue-stained skin was cut into pieces with scissors. Dye was extracted according to Nitta *et al.*[8] The skin pieces were treated with 10 vol. of a mixture of absolute ethanol and dioxane (1:1), 10 vol. of dioxane, 10 vol. of a mixture of chloroform and methanol (2:1), and finally with a $\frac{1}{4}$ vol. of absolute ethyl ether. To the residual skin, 30% aqueous pyridine was added and the mixture was heated in a water bath at 80° for 30–60 min. After centrifugation, the concentration of dye in the clear but colored pyridine supernatant was estimated at 620 mμ using a Hitachi model EPU-2A spectrophotometer (Tokyo). Colostrokinin activity was

* NaCl, 8·8 g; KCl, 0·4 g; $CaCl_2$, 0·4 g; $MgCl_2$, 0·01 g; $NaHCO_3$, 0·4 g; KH_2PO_4, 0·02 g; Na_2HPO_4, 0·08 g; and glucose, 0·5 g; made to 1000 ml with deionized water.

measured with a calibration curve obtained with bradykinin (0·5 –2·5 μg) and expressed in microgram equivalents of bradykinin.

Colostrum after centrifugation (11,000 *g* for 30 min) was acidified to pH 4·6 with 5 N HCl. The supernatant (colostric whey) was separated from a small amount of the precipitate (casein, etc.) by recentrifugation. After the pH of colostric whey was adjusted to 7·0, solid $(NH_4)_2SO_4$ was gradually added to the solution at 5° with stirring, to 30, 50 and 100 per cent saturation. After acid treatment (pH 3·0, 37°, 5 min), the release of biologically active substance from each fraction was performed in the manner described above. Electrophoresis of colostric whey proteins was carried out on polyacrylamide gel according to the method previously described.[7]

Crude colostrokinin was purified as follows: Crude colostrokinin solution was diluted to 1:5 (X*/V) with deionized water, and carboxymethylcellulose (Serva, 0·52 m-equiv./g, H-form) was added to the solution at the ratio of 50 mg/1X. The mixture was adjusted with HCl to pH 5·0 and stirred for 2 hr at room temperature. After adsorption, the mixture was poured into a column and the resultant column was washed with 0·05 M ammonium formate (pH 5·0) to remove impurities. The buffer was added until the absorbance of the effluent at 280 mμ was less than 0·05. Then the fraction containing biological activity was eluted with 0·3 M ammonium formate (pH 5·0). This fraction was concentrated at a temperature not exceeding 40°, using a vacuum pump connected to columns of dried calcium chloride and sodium hydroxide. Sephadex G-25, G-15 and G-10 were employed in gel filtrations. Sephadex G-25 was used for removal of macromolecules (proteins) from solutions containing relatively small molecules (peptides, salts, etc.). The fractionation of these low molecular weight substances was performed by passage through Sephadex G-15. Gel filtration on Sephadex G-10 resulted in the separation of peptide fractions from a large amount of salts. As the eluant, 0·02 M ammonium formate (pH 5·0) was used. The elution rate was kept at 25 ml/hr in the Sephadex G-25 column and at 12·5 ml/hr in others with a micropump (LKB, Sweden). The void volume (Vo) of Sephadex columns was determined with dextran (mol. wt., 2×10^6, Pharmacia, Sweden). With CM-Sephadex C-25 (4·5 $\pm$ 0·5 m-equiv./g; Pharmacia, Sweden) column chromatography, the column was pre-equilibrated with 0·3 M ammonium formate (pH 5·0). The procedure was similar to general ion-exchange column chromatography. The eluants were 0·3 M ammonium formate (pH 5·0), 0·1 M NaCl in 0·3 M ammonium formate (pH 5·0) and 0·1 M NaCl in 0·5 M ammonium formate (pH 5·0) respectively.

Thin-layer chromatograms were developed by the ascending method by the following two techniques: (1) phenol–water (3:1) was used with glass plates (250μ thick layer) for 2 hr; (2) butyl alcohol–acetic acid–water (4:1:1) was used with Eastman chromatogram sheets (100 μ thick layer, Eastman Kodak Company) for 3·5 hr. The spots were stained by spraying with ninhydrin (0·2% in *n*-butyl alcohol) or trinitrobenzene sulfonate (0·1% in 0·2 M $Na_2B_4O_7$ 10 H_2O–0·8 M KH_2PO_4 buffer). Paper electrophoresis was run on Toyo Roshi No. 51 paper in two ways: (1) in 2 N acetic acid, pH 2, for 2 hr at constant voltage (400 V); (2) in 0·1 M barbital–0·1 N HCl buffer, pH 7·4, for 5 hr at constant current (1 mA/cm). The spots were stained with ninhydrin or trinitrobenzene sulfonate. Bradykinin (Sandoz, Switzerland) was the control sample.

* X refers to milliliter equivalents of original colostrum.

The vasodilator activities of two peptides eluted from the paper were measured by assay in dogs.

RESULTS AND DISCUSSION

Crude colostrokinin dilated the peripheral blood vessel of dogs (Table 1) and contracted the uterus of rats (Fig. 1). These activities were distinctly higher than controls. Thus the biologically active material was released by incubation of pretreated bovine colostrum with human salivary kallikrein. Vasodilator activity in dogs was

TABLE 1. VASODILATOR ACTIVITY RELEASED FROM BOVINE COLOSTRUM BY HUMAN SALIVARY KALLIKREIN

	Substrate		Kallikrein added	Released kinin activity
	Colostrum (X)	Normal milk (X)	(FU/1X)	(μg·Br/1X)
Colostrum + kallikrein	100		2·33	0·53
Control group				
Colostrum only	100	—	—	0·0538
Kallikrein only	—	—	2·33	0·0091
Milk + kallikrein	—	60	3·88	0·0363
Milk only	—	60	—	0·0054

* FU = Frey unit; X = ml equivalents of starting colostrum or milk; μg.Br = μg equivalents of bradykinin. Incubation: 37°, pH 7·6–7·8, 2 hr.

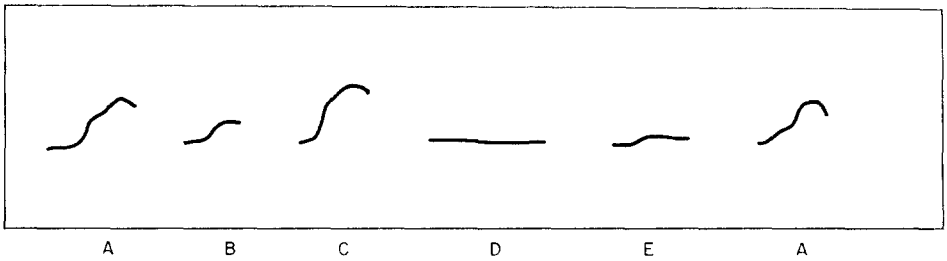

FIG. 1. Smooth muscle stimulating activity released from bovine colostrum by human salivary kallikrein. (A) 0·05 μg syn. bradykinin; (B) 0·025 μg syn. bradykinin; (C) incubation of pretreated colostrum (0·625 X) with kallikrein (1·45 FU); (D) incubation of kallikrein (7·05 FU) only; (E) incubation of pretreated colostrum (2·0 X) only. X refers to ml equivalents of original colostrum. Assays were performed with the rat uterus in a 50-ml bath of modified Locke–Ringer's solution at 24°.

higher than rat uterus stimulating activity. Doses used in each assay of vasodilatation in dogs and contraction of the isolated rat uterus were 0·003 to 0·04 and 0·2 to 1·6 ml equivalents of original colostrum, respectively, although the activity found depended on the starting material. The pharmacological activities of colostrokinin qualitatively agreed with those of kinins, such as bradykinin, since colostrokinin produced vasodilatation in dogs contraction of the isolated rat uterus, and increase in capillary permeability in guinea pigs; the activities per 1 ml of starting colostrum were found to be equiactive with those of 0·19 to 1·65,0·0125 to 0·105 and 0·748 to 6·18 μg bradykinin respectively.

Studies on the reaction of colostrokininogen with kallikrein indicated the following.

The colostrokininogen fraction became less active when its viscosity was high. This problem was solved by previous centrifugation at 11,000 *g* for 30 min. Acid treatment of the colostrokininogen fraction was carried out to inactivate kallikreinase (enzymes which destroy kallikrein) and kininase.[9,10] Incubation with kallikrein required 1 hr for complete liberation of kinin activity. The amount of released activity was not affected by the purity of the kallikrein preparations used in our experiments.

As shown in Table 1, little or no biological response could be observed when normal milk was used as a substrate instead of colostrum, even in the presence of a large amount of kallikrein. The results suggest that colostrokininogen is one of the proteins characteristic of colostrum.

Colostric proteins derived from serum proteins have been reported to agree with neither serum proteins nor milk whey proteins electrophoretically.[4] Colostric whey proteins are composed largely of euglobulin and β-lactoglobulin, and the marked difference in the composition of both colostric and milk whey proteins is that colostrum is rich in euglobulin, which is called "immuno-lactoglobulin" because it contains antibody activity.[4,11] It is concluded from the following experimental results that colostrokininogen belongs to the immuno-lactoglobulin fraction of colostrum. The pH of colostrum was adjusted to 4·6 and the resulting precipitate was removed by centrifugation. The supernatant (colostric whey) was fractionated with ammonium sulfate; each fraction, precipitated at 30, 50 and 100 per cent saturation with ammonium sulfate, was incubated with human salivary kallikrein and the released activity was measured. The activity released from the fraction obtained at 30 per cent saturation with ammonium sulfate was almost equal to that from the whole colostric whey (Table 2). Little response was observed with the other fractions. These results suggest that colostrokininogen is the immuno-lactoglobulin, not β-lactoglobulin, which usually precipitates at 30 to 50 per cent saturation with ammonium sulfate.

TABLE 2. VASODILATOR ACTIVITY RELEASED FROM COLOSTRIC WHEY FRACTIONATED WITH AMMONIUM SULFATE*

Fraction	Released kinin activity (μg·Br/1X)
Colostric whey	0·207
0–30% $(NH_4)_2SO_4$	0·20
30–50% $(NH_4)_2SO_4$	0·0053
50–1000% $(NH_4)_2SO_4$	0·001

* μg·Br = μg equivalents of bradykinin; X = ml equivalents of starting colostrum. Incubation: 37°, pH 7·6–7·8, 2 hr.

The colostrokinin formed by kallikrein action on colostrum was purified by using crude colostrokinin as the starting material. Carboxymethylcellulose adsorption was performed at pH 5·0 for 2 hr at room temperature with stirring; under these conditions, most of the biologically active substance was adsorbed on CM-cellulose. The recovery of biological activity was, therefore, almost quantitative in all of our experiments. Five liter equivalents of starting colostrum was the largest quantity used in this procedure. The residue (4620 X) after treatment with CM-cellulose was filtered through a Sephadex G-25 column (1·5 × 30 cm, Vo = 30·0 ml). The elution pattern revealed two peaks consisting of proteins and peptides. Biological activity was

observed in a peptide fraction. Generally Sephadex G-25 gel filtration was utilized to eliminate lower molecular weight impurities from protein solution. In the purification of colostrokinin, passage through Sephadex G-25 served to remove high molecular weight proteins. This procedure was effective with materials containing large amounts of macromolecular impurities. The biologically active fractions were collected and concentrated under reduced pressure at temperatures not exceeding 40°. The active fraction from Sephadex G-25 gel filtration contained a large quantity of ammonium formate due to the high concentration of the buffer used with the CM-cellulose, the large volume of the effluent from the CM-cellulose column, and the insufficient evaporation of ammonium formate during concentrating of the effluent. Therefore, this fraction (7950 X, absorbance at 260 mμ was 63·5) was subjected to Sephadex G-10 gel filtration (column, 1·5 × 24·5 cm; Vo = 12·0 ml) to remove the relatively low molecular weight impurities, which consisted mainly of ammonium formate. In this step, the portion containing biological activity was separated from the others. The tail end of the elution pattern gave a positive response to Nessler's reagent, perhaps owing to ammonium formate. The active fractions were collected and lyophilized. The residue (15900 X) from Sephadex G-10 gel filtration was fractionated on a Sephadex G-15 column (1·0 × 55 cm, Vo = 17·0 ml). This procedure afforded different purification factors and different elution patterns among the various lots. The fractions with biological activity were collected and lyophilized. The fraction (31800 X) obtained after Sephadex G-15 gel filtration was further purified on a CM-Sephadex C-25 column. The sample was applied to the column (1·5 × 20 cm) equilibrated with 0·3 M ammonium formate (pH 5·0). Development was accomplished by using three eluants as described in the Methods section. The fraction with biological activity was eluted with 0·1 M sodium chloride in 0·5 M ammonium formate (pH 5·0). From this observation, it seems that the active substance is strongly adsorbed on CM-Sephadex C-25. Table 3 summarizes the results of the purification of crude colostrokinin. Crude colostrokinin was purified 5010-fold. The active fraction after CM-Sephadex column chromatography gave a single spot in both silica gel-thin layer chromatography using

TABLE 3. PURIFICATION OF COLOSTROKININ

Fraction	Scale of experiment (ml equivalents of colostrum)	Absorbance at 260 mμ (%)	Activity (%)	Purity (μg·Br/A_{260})*	Purification factor
Crude colostrokinin	2000	100	100	0·458	1
CM-cellulose adsorption Fractions 5–7	5000	9·37	96·8	4·72	10·3
Sephadex gel filtrations					
Sephadex G-25 Fractions 13–24	4620	2·75	71·5	11·8	25·8
Sephadex G-10 Fractions 8–12	7950	0·693	53·1	34·8	76·0
Sephadex G-15 Fractions 8–17	15,900	0·295	38·6	59·3	130
CM-Sephadex (C-25) column chromatography Fraction 50	31,800	0·00211	10·6	2292	5010

* μg·Br = μg equivalents of bradykinin.

phenol–water (3:1) or butyl alcohol–acetic acid–water (4:1:1) and paper electrophoresis (2 N acetic acid, pH 2). This single spot agreed with the section where biological activity was observed (Fig. 2). The absorbance of fraction No. 50 (2292 μg·Br/A_{260}) at 260 mμ corresponded to 8·88 mg. (Desalting was performed on a Sephadex G-10 column with deionized water.) The vasodilator activity of 1 mg of this fraction in dogs was found to be equivalent to that of 258 μg bradykinin.

The stability of colostrokinin activity in saliva, gastric juice and intestinal juice was examined. When crude colostrokinin was incubated with human saliva, its activity, was essentially stable for 30 min and then decreased to half of the activity after 60 min. as assayed in dogs. When incubated with gastric juice, colostrokinin was stable for 120 min. In this assay (vasodilator activity), no response to gastric juice itself was observed. The result of the incubation of colostrokinin with intestinal juice was nearly similar to that observed with saliva. The response to intestinal juice itself in this assay did not change in 120 min. Colostrokinin seems to be more stable *in vivo* than *in vitro*, owing to various inhibitors in colostrum[12, 13] or the digestive system.[14, 15] Therefore, it is considered possible that colostrokinin has an important role in some physiological conditions in the alimentary system.

Colostrokinin increased capillary permeability more than did bradykinin. This seems to suggest that its physiological significance is different from that of bradykinin.

We observed that groups of rats receiving colostrokinin (0·00413 μg·Br/animal) absorbed more ^{131}I-labeled human albumin from the gut than control groups.[16] Although more detailed experiments must be carried out in the future, these results are taken as support for our hypothesis of a physiological role for colostrokinin in altering permeability.

The activity of colostrokinin was studied when released from human colostrum by human salivary kallikrein. The activity was less and the degradation rate was faster than that found with bovine colostrum. Antibodies can be conveyed to the newborn of ungulates (e.g. calves) by the maternal colostrum; however, there is little such possibility in humans.[17] Thus the results obtained with human colostrum would appear to be reasonable. Some investigators have reported the absorption of proteins from the suckling gut;[18, 22] however, the mechanism has not yet been resolved. The mechanism of transport of proteins across the intestinal mucosa has been explained in two ways: (1) the cells of the mucosa of the neonatal gut are histologically of a particular type;[23, 24] and (2) a specific physiologically active substance exists in the alimentary system.[6, 25] Some studies support the first hypothesis.[23, 24] On the other hand, little experimental evidence is available to support the second possibility.

It is assumed that there may be some correlation between the absorption of high molecular weight substances and the physiological activity of colostrokinin in the alimentary tract. Colostrokinin, which is released as a result of the action of salivary kallikrein on colostrum ingested in suckling animals, might aid in the absorption of high molecular weight substances from the neonatal gut of ungulates. This problem is being pursued further.

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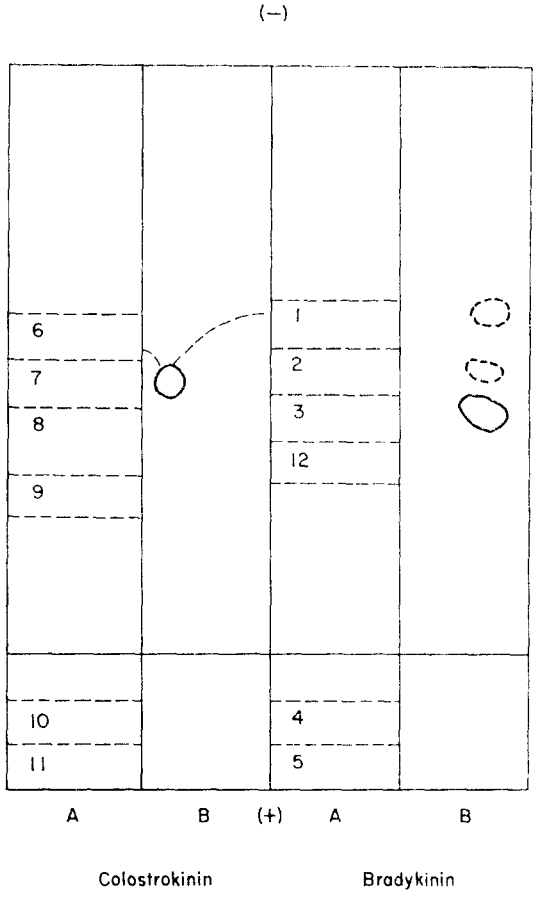

FIG. 2. Comparison between paper electrophoretic patterns of colostrokinin and bradykinin. The conditions employed were: 2 N acetic acid (pH 2·0), 400 V, 2 hr. (A) Assay by vasodilation in dogs; (B) detection with ninhydrin.

Distribution of activity:

Colostrokinin (μg·Br)	Bradykinin (μg)
6, 0·0484	1, 0·00971
7, 0·0641	2, 0·32
8, 0·0190	3, 1·22
9–11, 0	12, 0·00971
	4–5, 0

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