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PROPERTIES OF COLOSTROKININ FROM BOVINE COLOSTRUM*

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Abstract—Various biological and chemical properties of colostrokinin were studied. Vasodilatation in dogs, stimulation of smooth muscles of rats and increasing capillary permeability in guinea pigs were observed as the pharmacological actions. Among these, the change in capillary permeability is the strongest when compared to the activity of bradykinin. Colostrokinin activity is destroyed by chymotrypsin, but only slightly by trypsin. It is more stable in acid than in alkaline solution. This substance is dialyzable, and is soluble in water, in 67% ethanol and in 75% acetone, but not in absolute acetone. The isoelectric point is close to pH 7·4. Phenylalanine and serine were detected as the *N*-terminal and the C-terminal amino acids respectively. The molecular weight, on the basis of amino acid analysis, was calculated to be about 2000. Colostrokinin absorbs light close to $\lambda = 260$ mμ.

THE BIOLOGICAL activity of colostrokinin resembles that of bradykinin. This peptide is released when pretreated bovine colostrum is incubated with human salivary kallikrein. Colostrokinin has been purified by ion-exchange chromatography and gel filtration.[1] In this paper, various pharmacological, chemical and physical properties of colostrokinin are described.

MATERIALS AND METHODS

The colostrokinin preparations used in this study were prepared and purified as previously described.[1] Vasodilatation, smooth muscle stimulation and increase in capillary permeability were assayed in the manner already described, and thin-layer chromatography and paper electrophoresis were carried out as previously mentioned.[1]

Trypsin (Merck, Germany and N.B.C., U.S.A., twice crystallized) and chymotrypsin (Eisai Laboratory, Japan) were used as the enzyme preparations. In the experiments with serum, fresh human blood was centrifuged at 1000 *g* for 30 min and the resultant supernatant (serum) was used.

For dialysis, the sample solution was placed into cellulose tubing (Visking Company, Chicago) and dialyzed against deionized water at 7°. After dialysis, biological activity distributed inside and outside of the membranes was estimated by vasodilatation in dogs, and the recovery of activity and the ratio of activity inside to outside were determined.

One-half ml water (pH 5), 66·7% ethanol, 99·5% ethanol, 75% acetone, or absolute acetone was placed in five test tubes with 5 mg (0·355 μg·Br) of the sample. Insoluble materials, if necessary, were separated by centrifugation and dissolved in water (pH 5).

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Biological activity distributed in both soluble and insoluble fractions was measured by vasodilatation in dogs.

The procedures using Sephadex G-10 and G-15 (Pharmacia, Sweden) were almost identical. The eluant was 0·05 M ammonium formate (pH 6·0). The elution rate was controlled at 12·5 ml per hr with a micropump (LKB, Sweden). The void volume (Vo) of the columns was examined with Blue Dextran (mol. wt. 2×10^6, Pharmacia, Sweden).

Determination of the isoelectric point was performed according to Kunkel and Tiselius.[2] The sample (295 μg·Br/A_{260}) and dextran were applied to a line at the middle of Toyo Roshi No. 51 paper (9·0 × 23·5 cm) and then subjected to paper electrophoresis for 5 hr at constant current (1 mA/cm) and constant ionic strength (μ = 0·1). The buffers were 0·1 M glycine–0·1 N NaOH (pH 11) and 0·1 M barbital–0·1 N HCl (pH 6·8 to 9·0). After electrophoresis, the dried papers were immersed in 1% bromphenol blue in ethanol saturated with mercuric chloride for 15 min and rinsed with absolute ethanol. Since the technique followed separation of the dye from a dye-dextran complex, the position of dextran at times had to be confirmed during rinsing with ethanol. One of two samples which were run simultaneously at pH 7·4 was stained with the dye and the other was used for the assay.

Determination of the *N*-terminal amino acid was performed by using the technique of Sanger.[3] Ten ml of 5% fluoro-2, 4-dinitrobenzene was added to the sample (22·5 μg·Br/mg, homogeneous on thin-layer chromatograms developed with the phenol system) in 4·7 ml of 0·13% trimethylamine. The solvents used for development of paper chromatograms were as follows; (1) *n*-butyl alcohol saturated with 0·1% ammonium hydroxide as the first solvent (descending method) and phosphate buffer (31·2 g $NaH_2PO_4 \cdot 2\ H_2O$ and 35·8 g $Na_2HPO_4 \cdot 12\ H_2O$ were dissolved and the solution was diluted to 200 ml with water) as the second (ascending technique); and (2) *n*-butyl alcohol–*n*-butyl acetate–0·1% ammonium hydroxide (1:2:3) by the descending method and cyclohexane–isopropyl alcohol–0·05 M potassium benzoate (120:72:8; v/v/v) by ascending chromatography. After extraction of the hydrolysate with ether, the residual aqueous phase was evaporated and the residue was subjected to one-dimensional descending paper chromatography in butyl alcohol–acetic acid–water (4:1:2). The quantitative analysis of the *N*-terminal amino acid with 1 μmole colostrokinin (1·99 mg/ml, 236 μg·Br/mg in the vasodilatation assay in dogs) was accomplished in a manner similar to the experiment described above. The spot on the paper corresponding to the end group was cut out beyond its visible periphery and a blank of similar size was also cut from the adjacent paper. Both pieces were dropped into 1·5 × 9·0 cm test tubes, 4 ml water was added and the tubes were placed in a water bath at 55–60° for 15 min to elute the color. After an additional 15 min at room temperature, the solutions were decanted into cuvettes, and the absorbance at 360 mμ was read in a Hitachi model EFU-2A spectrophotometer against the extract of blank paper. The recovery in this procedure was calculated from a value obtained with phenylalanine (1 μmole, 0·165 mg/ml) carried through the procedure. The sample (purity was the same as for the determination of the *N*-terminal group) was treated with 0·6 ml of anhydrous hydrazine for determination of the C-terminal amino acid.[1] Paper chromatograms were developed with the *n*-butyl alcohol and the phosphate buffer systems that were used in the determination of the *N*-terminal group. The quantitative analysis of the C-terminal amino acid with 1 μmole colostrokinin (purity

was the same as for the determination of the *N*-terminal group) was carried out in the manner described above. The spot corresponding to the end group was then treated as described for the quantitative measurement of the *N*-terminal group. The recovery in this instance was calculated by using a value obtained with serine (1 μmole, 0·105 mg/ml) as a standard.

For amino acid analysis, the sample (2130 $\mu g \cdot Br/A_{260}$) was dissolved in 6 N HCl. One portion was heated in a sealed tube at 100° for 25 hr and another for 36 hr under identical conditions. The molar ratio of amino acid residues was examined with an amino acid analyzer (LC-2 model, Yanagimoto, Japan); calculations were based upon phenylalanine for the 25-hr hydrolysis and on glycine for the 36-hr hydrolysis.

Determination of tryptophan was performed as follows.[5] One ml of the sample solution (2·32 mg, 2015 $\mu g \cdot Br/A_{260}$) was added to 9·0 ml of 21·4 N H_2SO_4 containing 30 mg diethylaminobenzaldehyde in a test tube. The mixture was allowed to stand at 25° for 12 hr in the dark. After addition of 0·1 ml of 0·04% $NaNO_2$, the mixture was kept at room temperature for an additional 30 min, the absorbance at 590 mμ was read, and the tryptophan content was calculated from a standard curve. The protein fraction (4·39 mg) obtained after Sephadex G-25 gel filtration during purification of colostrokinin[1] served as a control.

RESULTS AND DISCUSSION

We have reported that colostrokinin released from the protein fraction (the colostrokininogen fraction) in bovine colostrum by human salivary kallikrein is a low molecular weight active substance.[1] The responses to colostrokinin were different from those of kallikrein. The pharmacological activities of colostrokinin qualitatively agreed with those of kinins, such as bradykinin (i.e. vasodilatation in dogs, contractions of the rat uterus and the rat ileum, and increase in capillary permeability in guinea pigs). Table 1 summarizes the results of assays of colostrokinin activity. The

TABLE 1. PARALLEL ASSAY OF COLOSTROKININ ACTIVITY

Expt. No.	Biological activity (μg·Br/1X)*				Ratio (A = 1)		
	Vasodilatation in dogs	Contraction of the rat uterus	Contraction of the rat ileum	Increasing capillary permeability in guinea pigs			
	A	B	C	D	B/A	C/A	D/A
1	0·228	0·0265	0·184	2·32	0·116	0·807	10·2
2	0·389	0·0318	—	—	0·0818	—	—
3	0·196	0·0416	—	—	0·212	—	—
4	0·831	—	—	6·84	—	—	8·23
5	0·107	—	—	0·776	—	—	7·25

*1X = 1 ml equivalent of original colostrum.

actions of colostrokinin are expressed in microgram equivalents of bradykinin. The ratios were obtained by dividing the value for each assay by that from vasodilatation in dogs. It seems that those indexes far from unity were indicative of the difference between colostrokinin and bradykinin. In terms of bradykinin equivalents, the increase in capillary permeability was the strongest action. The vasodilator activity of 1 μg colostrokinin was found to be equivalent to that of 0·258 μg bradykinin. Colostrokinin activities (i.e. vasodilatation in dogs, contractions of the rat uterus and the rat

ileum, and increased capillary permeability in guinea pigs) relative to bradykinin activities (bradykinin activity = 1) were found to be 0·258, 0·0297 to 0·0573, 0·208, and 1·87 to 2·63 respectively. Thus the increase in capillary permeability produced by colostrokinin was stronger than that of bradykinin.

Colostrokinin activity was destroyed by chymotrypsin (Eisai Laboratory, Japan); however, a part of the colostrokinin activity remained unaffected by trypsin after incubation for 48 hr. This result did not depend on the purity of the preparations of colostrokinin and trypsin. The presence of colostrokinin activity after incubation with trypsin suggested either that basic amino acids in the colostrokinin molecule were situated so that they resisted attack by trypsin, or that the degradated fragments of colostrokinin possessed biological activity. These possibilities were investigated with the aid of paper electrophoresis; a stained spot and a section containing activity moved the same distance on the filter paper, and neither positions changed after digestion by trypsin. Consequently, it is assumed that the structure of the colostrokinin molecule made it partly resist to trypsin. Bradykinin activity decreased only slightly after incubation with trypsin (Merck.) This finding was probably caused by the impurities in the preparation of trypsin, which has also been reported by other investigators.[6, 7] Figure 1 shows the influence of serum on colostrokinin (partially purified)

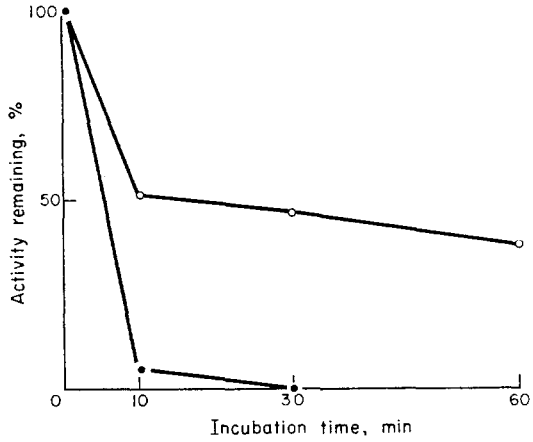

FIG. 1. Effect of human serum on pharmacological activities of colostrokinin and bradykinin. Incubation: 37°, pH 7·6–8·0 (0·1 M phosphate buffer); assay, contraction of the isolated rat uterus. ○—○, Colostrokinin (1 μg·Br) + fresh serum (0·2 ml); ●—●, syn. bradykinin (1 μg) + fresh serum (0·2 ml).

and bradykinin activities. Colostrokinin activity was partly destroyed by serum, although the degradation rate was not so rapid as that of bradykinin. The activities of colostrokinin and bradykinin were more stable at acid pH than at alkaline pH values. Colostrokinin activity was fairly stable at acid pH values, a finding that was independent of the purity of the preparation.

Colostrokinin was dialyzed through cellulose membranes (Visking Company). The longer the period of dialysis, the more colostrokinin passed through the membranes. The recovery of activity after dialysis, however, diminished to nearly 50 per cent when the period of dialysis was prolonged for over 16 hr.

Colostrokinin was found to be soluble in 66·7% ethanol and in 75% acetone, and was insoluble in absolute acetone. With 99·5% ethanol, 24 per cent of the starting activity was present in the soluble fraction.

The elution volume (Ve) of colostrokinin in Sephadex G-10 gel filtration was equal to the void volume (Vo) of the column. In Sephadex G-15, the elution pattern of colostrokinin was situated at almost the same position as those of kallidin, bradykinin and oxytocin. These findings suggest that the molecular weight of colostrokinin is between 1000 and 2000.

In thin-layer chromatography, the R_f values of colostrokinin were found to be 0·56 in the phenol system and 0·57 in the butyl alcohol system.[1] The isoelectric point of colostrokinin lies between pH 7·2 and 7·4 (Fig. 2). The mobility distance of colostrokinin was determined relative to that of dextran. Both colostrokinin and dextran were detected with bromphenol blue.

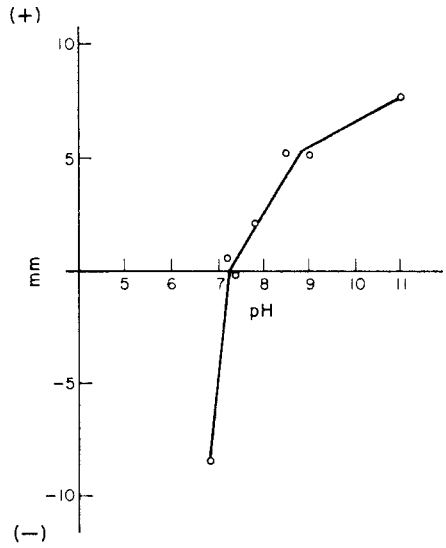

FIG. 2. Mobility values of colostrokinin on filter paper plotted against pH. The conditions employed were: 1 mA/cm, $\mu = 0{\cdot}1$. Buffer: 0·1 M barbital–0·1 N HCl (pH 6·8 to 9·0); 0·1 M glycine–0·1 N NaOH (pH 11). ○—○, Mobility distance of colostrokinin relative to the mobility distance of dextran.

The *N*-terminal amino acid of colostrokinin is phenylalanine (Fig. 3 and Table 2). In Fig. 3, spot C corresponds to phenylalanine; spots A and D correspond to *p*-nitroaniline and *p*-nitrophenol respectively. Spots B and E are suggested to be the derivatives or impurities of *p*-nitroaniline and *p*-nitrophenol, respectively, because spots B and E were observed when *p*-nitroaniline and *p*-nitrophenol were chromatographed independently. After extraction of the hydrolysate with ether, no DNP-amino acid was formed from the aqueous layer. The experiment employing hydrazine degradation revealed that the C-terminal amino acid of colostrokinin is serine. In Fig. 4, spot E is serine; spots A and C correspond to *p*-nitroaniline and *p*-nitrophenol respectively. Spot B is suggested to be the derivative or impurity of *p*-nitroaniline, as in the case of the *N*-terminal group. Spot D is judged to be the di-DNP form of acidic amino acid

hydrazide, because the spot did not agree with any of the mono-DNP forms of standard amino acids, and the spot was brown in color. This result was unexpected because of the trypsin-like specificity of kallikrein. Therefore, it is assumed either that colostrokinin is situated in the C-terminal position of colostrokininogen or that it

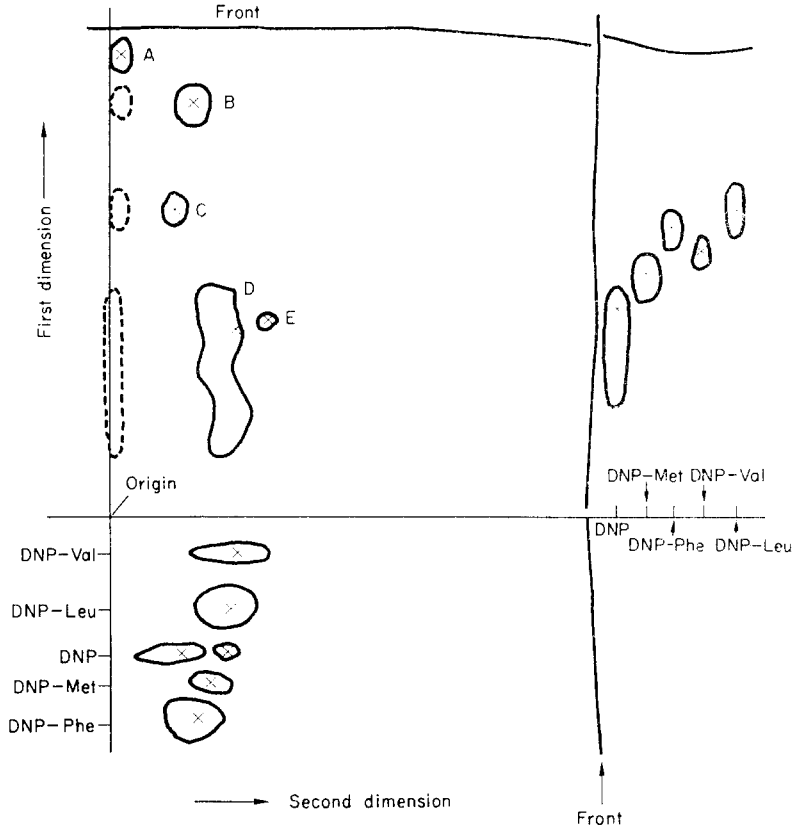

FIG. 3. Two-dimensional paper chromatography of DNP-amino acids in the detection of the *N*-terminal amino acid of colostrokinin. First dimension, *n*-butyl alcohol saturated with 0·1% ammonium hydroxide; second dimension, phosphate buffer. Standard DNP-amino acids were applied and chromatographed only in the first or second dimension. The broken line indicates the positions of materials after chromatography in the first dimension.

Rf values:

Spot	First dimension	Second dimension
A	0·82	0
B	0·732	0·162
C	0·581	0·125
D	0·371	0·245
DNP-valine	0·564	0·262
DNP-leucine	0·643	0·250
DNP-methionine	0·462	0·206
DNP-phenylalanine	0·605	0·176
Dinitrophenol	0·432	0·128,* 0·235*

*Standard dinitrophenol moved so unpredictably that the *Rf* values cannot be considered constant. Spot C is phenylalanine. Spots A and D correspond to *p*-nitroaniline and *p*-nitrophenol respectively. Spots B and E are suggested to be the derivatives or impurities of *p*-nitroaniline and *p*-nitrophenol respectively.

TABLE 2. DETECTION OF DNP-AMINO ACID OF *N*-TERMINAL POSITION IN BUTANOL AND CYCLOHEXANE SYSTEMS*

Spot	First dimension (butanol system)	Second dimension (cyclohexane system)
Spot C (in Fig. 3)	1·45	1·0
DNP-phenylalanine	1·59	1·2
DNP-methionine	1·29	1·7
DNP-valine	1·37	4·2
DNP-leucine	1·68	4·9

*Moving distance (dinitrophenol = 1).

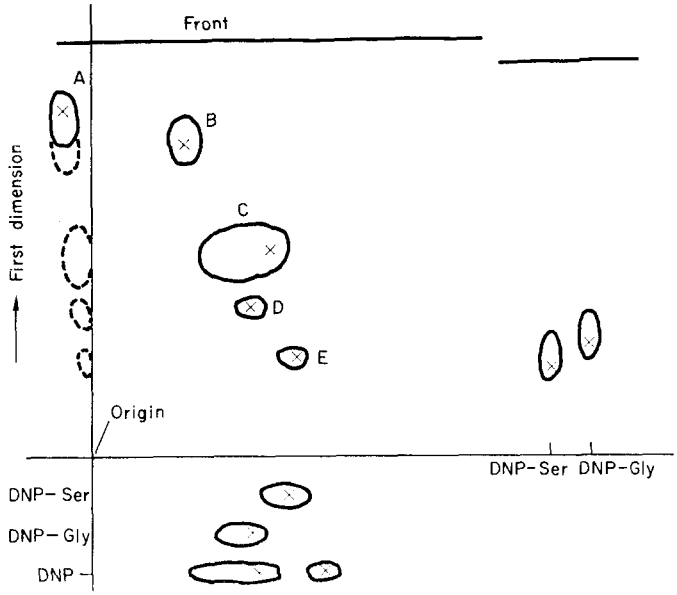

FIG 4. Two-dimensional paper chromatography of DNP-amino acids in the detection of the C-terminal amino acid of colostrokinin. First dimension, *n*-butyl alcohol saturated with 0·1% ammonium hydroxide; second dimension, phosphate buffer. Standard DNP-amino acids were applied and chromatographed only in the first or second dimension. The broken line indicates the positions of materials after chromatography in the first dimension.

Rf values:

Spot	First dimension	Second dimension
A	0·841 (1·76)	(0)
B	0·763 (1·60)	(0·650)
C	0·477 (1·00)	(1·00)
D	0·350 (0·734)	(0·874)
E	0·234 (0·491)	(1·07)
DNP-serine	0·236 (0·477)	(1·18)
DNP-glycine	0·281 (0·532)	(0·977)
Dinitrophenol	0·528 (1·00)	(1·00)

The numbers in parentheses refer to the mobility distance of each spot relative to that of dinitrophenol. Spot E is serine. Spots A and C correspond to *p*-nitroaniline and *p*-nitrophenol respectively. Spot B is suggested to be the derivative or impurity of *p*-nitroaniline.

might be released by a typical degradation mechanism.[8, 9] The relationship between the pharmacological and physiological activities and the terminal amino acids of the kinins has not yet been elucidated. Some investigators have reported that a basic amino acid at the *N*-terminal position of kinins was necessary for the maintenance of activity,[10] but it is reasonable to assume that this is not the case here.

Table 3 shows the results of amino acid analysis of colostrokinin. The amino acids detected were lysine, histidine, arginine, aspartic acid, serine, glutamic acid, proline, glycine, valine, isoleucine and phenylalanine. From this result, the molecular weight of colostrokinin was calculated to be 1992. The experiment with dimethylamino-benzaldehyde indicated that colostrokinin did not contain tryptophan. This agreed with the finding that the ultraviolet absorption spectrum of colostrokinin possessed a maximum absorbance at 260 mμ (Fig. 5).

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TABLE 3. MOLAR RATIO OF AMINO ACID RESIDUES OF COLOSTROKININ

Amino acid	Molar ratio		
	Found		Supposed
	25-hr Hydrolysis	36-hr Hydrolysis	
Lysine	1·90	1·85	2
Histidine	1·30	1·23	1
Arginine	1·03	1·05	1
Aspartic acid	1·15	1·10	1
Serine	0·88	0·83	1
Glutamic acid	2·90	2·83	3
Proline	2·10	2·22	2
Glycine	0·95	1·00	1
Valine	1·01	1·01	1
Isoleucine	0·98	1·00	1
Leucine	2·20	1·91	2
Phenylalanine	1·00	1·12	1

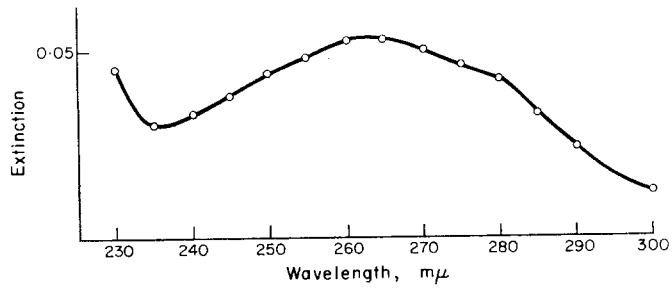

FIG. 5. Ultraviolet absorption spectrum of colostrokinin.

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