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Further Purification and Properties of Kininogenase from the Guinea Pig's Coagulating Gland[1)]

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A purified kininogenase preparation which was homogeneous in disc-electrophoresis and ultracentrifugal analysis was obtained from a guinea pig's coagulating gland by the purification methods of DEAE-cellulose chromatography and Sephadex G-200 gel filtration. The apparent molecular weight of it was estimated to be 31000 by ultracentrifugation and about 40000 by gel filtration, and its isoelectric point was found to be pH 3.4 in electrofocusing analysis with carrier ampholyte. Its amino acid composition was similar to those of the other glandular kallikreins, and it was found to be a glycoprotein containing about 14% of carbohydrates. The kininogenase hydrolyzed N-α-tosyl-L-arginine methyl ester and N-α-benzoyl-L-arginine ethyl ester, having the *K*m values of 7.80×10^{-4} and 2.13×10^{-4} M, respectively. The pH optimum appeared to be 9.0 in the esterolytic response. This enzyme seemed to be rather stable in heat treatment because about 60% of the esterolytic activity was retained after a treatment at 75° for 60 min.

Following the demonstration of a new potent kininogenase in the accessory sex glands of the guinea pig by Schachter and his co-workers in 1962,[3)] Moriwaki and Schachter described the extraction of the kininogenase separately from the kininase which existed together in the coagulating gland.[1)] They also reported that, though this kininogenase (CGK) seemed to be one of the glandular kallikrein, it released bradykinin from dog kininogen and it was not susceptible to some kallikrein inhibitors.

In the course of the succeeding investigation on this enzyme, we were able to obtain it in homogeneous state. The present paper deals with the further purification of CGK and some of its physicochemical properties.

Material and Method

Crude Extract of Guinea Pig's Coagulating Gland——Male guinea pigs, weighing 350—400 g, were sacrificed by a blow on the head and exsanguinated by section of the jugular vein, and the coagulating glands were taken out immediately. After rinsing away the adherent blood in cold saline, the glands were stored at −20° until the extraction process began. The preparation method of crude extract of the gland was almost identical with that of Moriwaki and Schachter,[1)] but the wet glands were homogenized with a glass-homogenizer in cold distilled water (5 g wet gland/25 ml). The absorbance at 280 nm (E_{280}) of this extract corresponding to 1 g of the wet gland was about 100, and its vasodilator activity was 12.4 KU/E_{280}. After dialysis of the extract against distilled water at 4°, the clear supernatant was concentrated to 1/10—1/20 of the volume of the supernatant with the Diaflo system (membrane type UM-2, Diaflo Co., U.S.A.) under N_2 gas pressure. This concentrated crude extract was submitted to the further purification described below.

Purification Procedure——The concentrated crude extract was applied on a DEAE-cellulose column, 1.5×25 cm size, equilibrated with 0.02 M phosphate buffer (pH 7.0), and a gradient elution from 0 to 0.4 M NaCl in the above buffer was performed. Protein concentration was estimated from the absorbance at 280 nm measured with a Hitachi EPU spectrophotometer. The kinin forming and the N-α-tosyl-L-arginine methyl ester (TAME) hydrolyzing activities of each fraction were assayed, and the active fractions were

1) The previous paper on this subject: C. Moriwaki and M. Schachter, *J. Physiol.*, **219**, 341 (1971).
2) Location: *12 Funakawara-machi, Ichigaya, Shinjuku-ku, Tokyo.*
3) K.D. Bhoola, R. May May Yi, J. Morley, and M. Schachter, *J. Physiol.*, **163**, 269 (1962).

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pooled, dialysed overnight against distilled water at 4°, and concentrated. This preparation was again submitted to rechromatography with DEAE-cellulose using the same procedures described above, but slower NaCl gradient from 0.1 to 0.3 M.

After the rechromatography with DEAE-cellulose, the active fractions were concentrated and it was gel filtrated on Sephadex G-200 column, 1.5×70 cm. The elution was performed with 0.05 M ammonium formate, pH 6.0, at a flow rate of 8 ml per hr. The active fraction of this step was submitted to the following analysis as the purified CGK.

Activity Assay——Kinin forming activity was determined by measuring the amount of kinin yielded from 15 mg of the kininogen which was prepared from heated bovine plasma using the method of Moriwaki and Schachter.[1] It was preliminarily confirmed that this substrate was free from kallikrein and kininase, and 100 and 35 ng of bradykinin equivalent kinin were liberated from 1 mg of this substrate by excess amounts of trypsin and hog pancreatic kallikrein, respectively. The kinin amount liberated in 1 min at 30° was assayed on a loop of isolated guinea pig's ileum suspended in a 10 ml bath of Mg^{2+}-free Tyrode's solution. Contractile responses were recorded isometrically via a force-displacement transducer (Nihon-Koden, Tokyo), and the activity was given in μg bradykinin equivalent.

The esterolytic activity on TAME (Foundation for Protein Research, Osaka) was determined using the method of Moriwaki, *et al.*[4] and expressed in μmoles TAME digested in 1 min. The vasodilator activity was determined as described earlier.[5]

Electrophoresis——Disc-electrophoresis was carried out on 5% (w/v) polyacrylamide gel with 0.05 M Veronal buffer at pH 8.6 in the manner of Davis,[6] and vertical plate polyacrylamide-agarose electrophoresis was performed as described before.[7] Gels of polyacrylamide or polyacrylamide-agarose were stained with amido black 10B for protein detection and with the formazan system for the activity detection.[7]

Ultracentrifugal Analysis and Determination of Isoelectric Point——Ultracentrifugation of the purified CGK was performed with a Spinco model E apparatus, and isoelectric focusing with carrier ampholytes for pH 3—10 and 3—5 were performed with an apparatus of LKB (Sweden) under cooling for 40—44 hr.

Analysis of Amino Acid Composition and Sugar Content——The purified CGK was hydrolyzed in an evacuated test tube (below 2 mmHg for 5 min) for 24 and 36 hr at 110° with redistilled 6 N HCl. Amino acid analysis was carried out by the modified method of Spackman, *et al.*[8] with an amino acid analyzer (Nihon-Denshi, Type 5AH). The content of tryptophane was determined spectrophotometrically.[9]

Glucosamine content was determined by the Elson-Morgan reaction as modified by Svennerholm[10] after hydrolysis in 2 N HCl at 110° for 16 hr. Hexose as the neutral sugar component in the purified CGK was determined using the technique of Hodge and Hofreiter,[11] and the procedure of Aminoff[12] was followed for the estimation of sialic acid content. As the authentic substances, glucosamine, sialic acid (Seikagaku Kogyo, Tokyo) and glucose (Tokyo Kasei, Tokyo) were used.

Result

Purification of CGK

Fig. 1 shows the elution patterns of protein and kininogenase activity on the first DEAE-cellulose chromatography of the crude CGK, and it was discovered that the kininogenase appeared at around 0.25M NaCl concentration. The active fractions, No. 44—47, were rechromatographied over DEAE-cellulose and most of the protein was recovered as almost a single peak at 0.25M NaCl in 0.02M phosphate buffer, but the activity peak shifted slightly from the protein peak. The result of Sephadex G-200 gel filtration of this active fraction was given in Fig. 2. An active protein peak was separately obtained from a minor inactive protein fraction by this process. The protein elution pattern of the active fraction was almost symmetric and the activities appeared coincidently with the protein elution. This seems to suggest the homogeneity of this preparation. By these combined procedures, the kinino-

4) C. Moriwaki, N. Inoue, Y. Hojima, and H. Moriya, *Yakugaku Zasshi*, **91**, 413 (1971).
5) H. Moriya, K. Yamazaki, and H. Fukushima, *J. Biochem.* (Tokyo), **58**, 201 (1965).
6) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
7) Y. Fujimoto, H. Moriya, K. Yamaguchi and C. Moriwaki, *J. Biochem.* (Tokyo), **71**, 751 (1972).
8) D.H. Spackman, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
9) T.W. Goodwin and R.A. Morton, *Biochem. J.*, **40**, 628 (1964).
10) L. Svennerholm, *Acta Soc. Med. Upsal.*, **61**, 287 (1956).
11) J.E. Hodge and B.T. Hofreiter, "Methods in Carbohydrate Chemistry," Vol. 1, ed. by R.L. Whistler, and M.L. Walfolm, Academic Press, 1962, p. 388.
12) D.A. Aminoff, *Biochem. J.*, **81**, 384 (1961).

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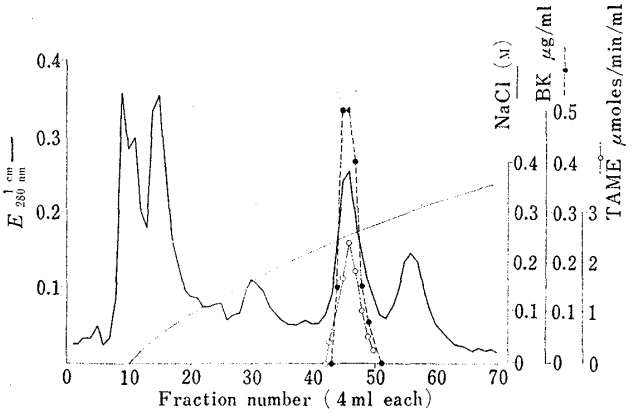

Fig. 1. DEAE-Cellulose Column Chromatography of the Concentrated Crude Extract from the Coagulating Gland

column: 1.5×25 cm
eluant: 200 ml of 0.02M phosphate buffer, pH 7.0, gradient from 0 to 0.4M NaCl
volume of mixing chamber: 300 ml

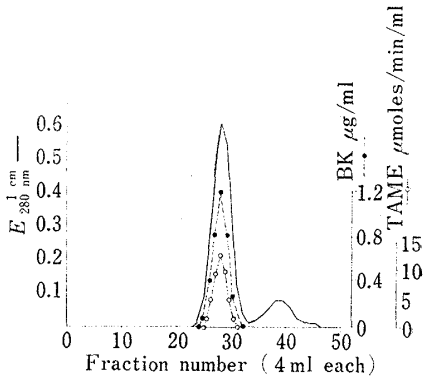

Fig. 2. Sephadex G-200 Gel Filtration of the Active Fraction in DEAE-Cellulose Rechromatography

column: 1.5×70 cm
eluant: 0.05M ammonium formate, pH 6.0
flow rate: 8 ml/hr

TABLE I. Purification of Coagulating Gland Kininogenase

Step	Recovery (%)		Activities		
	Protein	Activity[a]	Vasodilator KU/E_{280}	Kinin releasing[a] BK μg/E_{280}	Esterolytic TAME μmoles/min/E_{280}
Water extract	100	100	12.44(1)[b]	0.11(1)[b]	1.14(1)[b]
Dialysis	55.5	105.1	29.04(2.3)	0.27(2.4)	2.0 (1.8)
DEAE-C (1st)	6.54	73.85	186.80(15.0)	2.07(18.5)	18.0 (15.9)
DEAE-C (2nd)	2.38	50.19	250 (20.1)	2.50(22.3)	25.0 (21.9)
Gel fil.	1.75	38.15	420 (33.8)	5.38(48.0)	38.0 (33.3)

a) Kinin released from bovine pseudo-globulin (30°, 1 min) was assayed on isolated quinea pig ileum.
b) Purification factor is shown in parenthesis.

genase was purified about 40—50 times with an activity recovery of about 40% (Table I). The purified CGK contained neither caseinolytic activity determined by the Kunitz's method,[13] nor kininase activity which was assayed by using bradykinin as the substrate.

Homogeneity of Purified CGK

The purified CGK obtained by Sephadex gel filtration gave a single protein band in both disc-electrophoresis (Fig. 3) and vertical polyacrylamide-agarose gel electrophoresis. Furthermore, the enzymatically active band detected by the formazan system was identical with the protein band.

Fig. 4 is the schlieren pattern of 0.2% (w/v) of purified CGK in 0.1M phosphate buffer. The pattern also indicates the homogeneity of the preparation. The *s* value at this protein concentration was found to be about 2.7 at 20° ($s_{20,w}$=2.7).

Isoelectric Point of CGK

As a preliminary experiment, the crude extract of CGK was fractionated by isoelectrofocusing with carrier ampholyte for pH 3—10. Several protein peaks were obtained, but the kinin forming activity was found only in a peak at pH 3. Refering to this result, the purified CGK was submitted to the same fractionation with carrier ampholyte for pH 3—5.

13) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).

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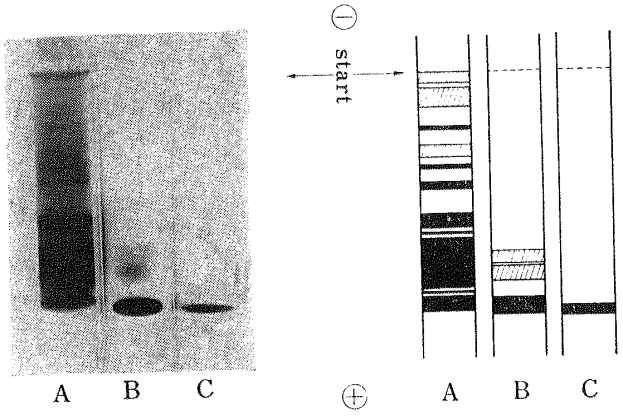

Fig. 3. Disc-Electrophoresis of CGK at Each Purification Step

(A): crude extract of CGK
(B): active fraction in DEAE-cellulose chromatography
(C): final preparation of CGK

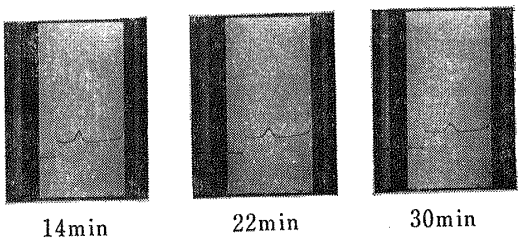

Fig. 4. Schlieren Patterns of CGK

Purified CGK (0.2% in 0.1M phosphate buffer, pH 6.8) was sedimented from left to right in a Spinco model E ultracentrifuge (55430 rpm, 18.4°, bar angle 65°).

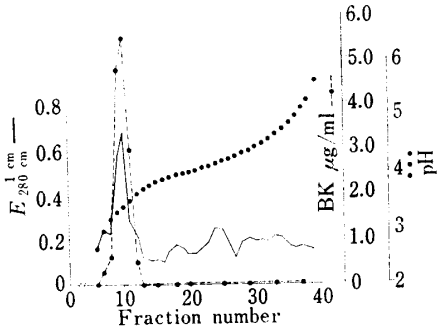

Fig. 5. Isoelectric Fractionation of CGK with Carrier Ampholyte (pH 3—5)

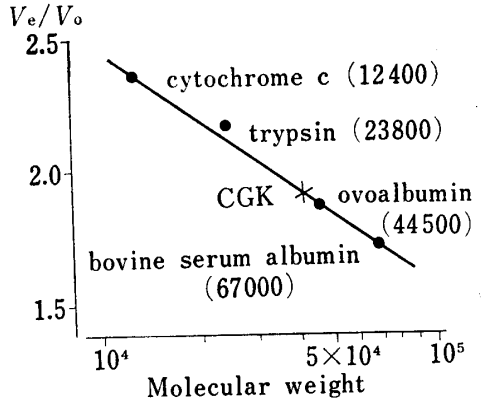

Fig. 6. Estimation of the Molecular Weight of CGK by Gel Filtration on Sephadex G-150

column: 1.5×80 cm
V_0: 48 ml
eluant: 0.45M NaCl containing 0.05M phosphate buffer, pH 7.0
flow rate: 10 ml/hr

A single active peak was found and the isoelectric point of CGK was determined as pH 3.4 (Fig. 5).

Molecular Weight of CGK

The approximate molecular weight of CGK was determined by gel filtration on a Sephadex G-150 column according to Andrews.[14] On the basis of the elution volume, the molecular weight of CGK was estimated to be about 40000 from the linear relationship between the elution volumes of the authentic proteins and logarithms of their molecular weights (Fig. 6).

The molecular weight of CGK was also estimated from the result of ultracentrifugal analysis using the formula of Svedberg and Pederson.[15] It was calculated to be 31000 assuming that the $\bar{v}$ was 0.749 as in the other kallikreins.[16]

Amino Acid Composition and Sugar Content of CGK

For the amino acid analysis, 23.8 μg of the purified CGK was hydrolyzed and the result of the 24 hr hydrolyzate was shown in Table II. The molar ratio of amino acids were estimated

14) P. Andrews, *Biochem. J*, **91**, 222. (1964).
15) T. Svedberg and K.O. Pederson, "The Ultracentrifuge," Oxford Univ. Press ,London, 1940.
16) H. Fritz, I. Eckert, and E. Werle, *Z. Physiol. Chem.*, **348**, 1120 (1967).

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based on the molecular weight of 31000 which was determined in the ultracentrifugal analysis. The amino acid content was 87%.

The content of amino sugar was 6—7%, and that of hexose and sialic acid were 6.0 and 2.4%, respectively.

Table II. Amino Acid Composition of the Purified CGK (24 hr hydrolyzate)

Amino acid	Mole ratio	No. of a.a.	Hog panc.[16] kallikrein
Lys.	1	4	13
His.	2.47	10	10
Arg.	1.49	7	4
Asp.	7.95	32	33
Thr.	3.21	13	18
Ser.	3.15	12—13	17
Glu.	6.32	25	28
Pro.	4.75	31	20
Gly.	5.15	20—21	27
Ala.	3.82	15	16
Cys.	2.11	8— 9	10
Val.	4.97	20	13
Met.	1.77	7	5
Ile.	2.32	9	14
Leu.	6.11	24—25	24
Thr.	1.53	6	9
Phe.	1.03	4	13
Trp.		7	9

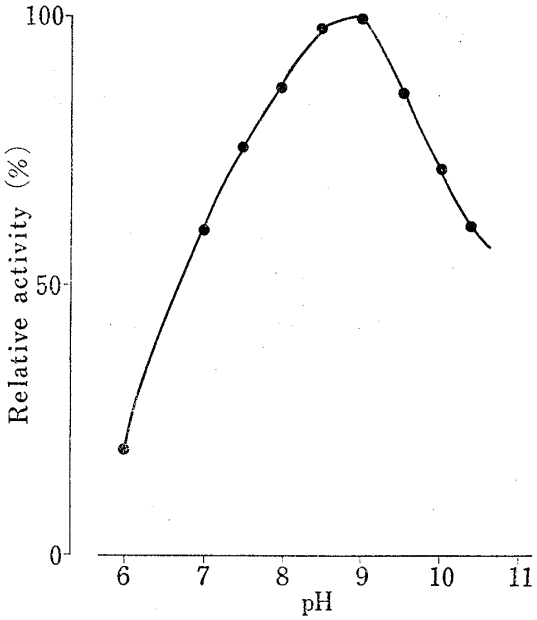

Fig. 7. Optimum pH to the Hydrolysis of TAME by CGK

10 μmoles of TAME was incubated with CGK at 30° for 30 min, and MeOH liberated was measured by chromotropic acid method.[9]

Optimum pH of CGK

The effect of pH on TAME hydrolyzing activity of CGK was studied using Britton-Robinson's wide range buffer and the maximum activity was found at about pH 9 (Fig. 7). This result agrees well with the values described by Webster and Pierce[17] for other glandular kallikreins.

Kinetics

The Michaelis constants (*K*m) of CGK towards BAEE and TAME were determined using the methods of Schwert and Takenaka[18] and Hummer.[19] From the Lineweaver-Burk plots, the *K*m values for BAEE and TAME were found to be 2.13×10^{-4} and 7.80×10^{-4}M, respectively.

Table III. Heat Stability of Purified CGK

	% to the activity determined at 30°	
	10 min	60 min
50°	90.2	77.5
60°	76.5	64.7
75°	69.6	59.8

17) M.E. Webster and J.V. Pierce, *Proc. Soc. Exptl. Biol. Med.*, **107**, 186 (1961).
18) G.W. Schwert and Y. Takenaka, *Biochem. Biophys. Acta*, **16**, 570 (1955).
19) B.C.W. Hummer, *Can. J. Biochem. Physiol.*, **37**, 1393 (1959).

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Heat Stability of CGK

This enzyme remained rather stable during heat treatment. As shown in Table III, about 60% of the esterolytic activity (TAME) was sustained even after treatment of the purified CGK dissolved in 0.1M phosphate buffer (pH 8.0) at 75° for 60 min.

Discussion

In the paper of Bhoola, *et al.*,[3] they noted that the kininogenase activity in the accessory sex gland of the guinea pig was extremely potent. The vasodilator activity in 1 g of the wet coagulating gland was determined to be about 1500 KU in our investigation without any special activation process. No other tissues from various animals contain such potent kininogenase. However, one of the difficulties on the purification of this enzyme was the presence of strong kininase in the same gland. Moriwaki and Schachter[1] found that the dialysis of a crude extract of the dry gland against distilled water was quite effective to eliminate the kininases. This fact was confirmed again on the fresh gland homogenate in the present study and this process could possibly be utilized for other sources of glandular kallikrein. An abundance of active kininogenase in the gland and the easy elimination of kininase made it rather facile to purify this kininogenase. A homogeneous fraction in disc-electrophoretic and ultracentrifugal analysis was obtained by chromatographies with DEAE-cellulose and Sephadex with a good yield.

The molecular weight of this enzyme was calculated to be 30000—40000 and this result indicates the similarity of CGK to other glandular kallikreins, but it differs from those in plasma.[20–22]

The same tendency was also observed in the pH optimum of CGK. However, it was rather stable to heat, unlike the other glandular kallikreins, except those from human urine (Fujimoto, *et al.*, unpublished observation). The kallikreins are known to be a glycoprotein. Fritz, *et al.*[16] mentioned that purified hog pancreatic kallikrein contained 3.4% of hexose, 2.9% of amino sugar and 0.8% of sialic acid, and amino acid content was 94%. Purified CGK seemed to contain more sugar than the hog pancreatic kallikrein, while the amino acid composition of CGK was similar to the kallikrein.[21]

The Michaelis constant of CGK towards TAME was about 10 times bigger than that of hog pancreatic kallikrein (0.6×10^{-4}M),[23] while *K*m values towards BAEE of these kallikreins were similar. The ratio of the esterolytic activities on BAEE and TAME of CGK was 0.75. The value of hog pancreatic kallikrein was about 30,[22] but those of other glandular kallikreins and trypsin were less than 1 as CGK. In this respect, CGK is quite different from pancreatic kallikrein. This kininogenase released bradykinin like trypsin or plasma kallikrein, but it has already been mentioned that CGK was not susceptible when treated by Trasylol and potato kallikrein inhibitor.[1] So far, it is clear that, though CGK is one of the glandular kallikreins, it seems to be quite a unique one.

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20) J.V. Pierce, "Handbook of Experimental Pharmacology, Vol. XXV. Bradykinin, Kallidin and Kallikrein," ed. by O. Eichler, A. Farah, H. Herken, and A.D. Welch, Springer-Verlag, Berlin, 1970, p. 27.
21) M.E. Webster, "Handbook of Experimental Pharmacology, Vol. XXV. Bradykinin, Kallidin, and Kallikrein," ed. by O. Eichler, A. Farah, H. Herken, and A.D. Welch, Springer-Verlag, Berlin, 1970, p. 138.
22) E.K. Frey, H. Kraut, and E. Werle, "Das Kallikrein-Kinin-System und Seine Inhibitoren," Ferdinand Enke Verlag, Stuttgart, 1968.
23) E. Habermann and W. Klett, *Biochem. Z.*, **346**, 133 (1966).

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