Dehydration of Atractylon Autoxidation Product B with Phosphorus Oxychloride——$POCl_3$ (0.8 g.) was added dropwise with stirring to a solution of atractylon autoxidation product B (0.10 g.) in dry pyridine (2 ml.) at 0°. The mixture was set aside for 15 hr. at 0° and for a further 2 hr. at room temperature, poured on crushed ice, and extracted with Et_2O. The product was crystallized from EtOH to give the dehydrated product (Ⅸ) as colorless needles, m.p. 106~108°, *Anal.* Calcd. for $C_{15}H_{18}O_2$: C, 78.23; H, 7.88. Found: C, 78.44; H, 7.87, UV: λ_{max}^{EtOH} 275 mμ (log ε 4.32), IR (KBr) cm^{-1}: 1765, 1665, 1649 (butadienolide), 895 (vinylidene).

The dehydrated product (0.08 g.) was dissolved in *N* NaOH (5 ml.) on the steam-bath. Acidification with HCl deposited a precipitate which was crystallized from AcOEt giving the autoxidation product B (Ⅴ) as colorless needles, m.p. 193~195°. The identity was established by the usual criteria.

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Summary

Atractylon, the sesquiterpenoid oxide isolated from the rhizomes of *Atractylodes japonica* and its related plants (Compositae), has been proven to have constitution Ⅰ on the basis of spectral properties and its conversion into 8,12-oxido-eudesmane. Autoxidation of atractylon has given two crystalline compounds, the autoxidation product A and B, which have been established as shown in formulae (Ⅳ and Ⅴ), respectively, from the degradative and spectral evidence.

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106. Motosuke Kikutani and Kazuko Hirose: Studies on the Harderian Gland. III.*1 Purification and Properties of the Principle in Bovine Harderian Gland that increases the Serum Alkaline Phosphatase Activity.

(*Faculty of Pharmacy, University of Nagasaki**2)

It was previously reported that six protein fractions affecting serum alkaline phosphatase activity of rabbit were extracted and isolated from bovine Harderian gland.*1 Of these, fractions designated as A-40, CS-3, and CS-5*3 increased serum alkaline phosphatase activity and, threefore, each of them is considered to be the effective principle, because this enzyme activity level decreased markedly in a rabbit from which the Harderian gland was removed.[1]

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*1 Part Ⅱ: Yakugaku Zasshi, **81**, 1154 (1961).

*2 Showa-machi, Nagasaki (菊谷元資, 広瀬和子).

*3 A-40=40% EtOH precipitated fraction; CS-3=30% $(NH_4)_2SO_4$ pptd. fraction; CS-5=50% $(NH_4)_2SO_4$ pptd. fraction.

1) M. Kikutani, Y. Takeuchi, Y. Nakamura: Yakugaku Zasshi, **80**, 1115 (1960).

Among these three fractions, fraction CS-5 was investigated, because it was isolated in a comparatively good yield without contaminating impurities. CS-5 was not homogeneous but a mixture of proteins and was separated at least into four components by paper electrophoresis. The chromatographic purification of CS-5 was carried out with DEAE-cellulose column and a greater part of the activity was eluted into CS-5d fraction.

This paper presents the results of chromatographic purification of CS-5, and of the electrophoretic and sedimentation analyses of fraction CS-5d.

Experimental

Materials——Lyophilized fraction CS-5 was used, which was obtained by means of fractional precipitation with $(NH_4)_2SO_4$ as described in the preceding paper.*1

Protein concentration was determined by spectrophotometric measurement at 276 mμ, using an extinction coefficient for each fraction with a Shimadzu QR-50 spectrophotometer.

Preparation of the Adsorbent and the Adsorption Procedure——DEAE- and CM-cellulose were prepared with Toyo Roshi Powder (100~200 mesh) by Peterson's method.[2]

In the adsorption procedure, 1 g. of DEAE- or 1 g. of CM-cellulose suspended in 0.05*M* acetate buffer (pH 5.8) was added to the same buffer solution in which 10 mg. of CS-5 was dissolved to make a total volume of 20 ml. Then the mixture was shaken for 10 min. and centrifuged. In order to ascertain adsorption, protein concentration of the supernatant was measured. A good adsorbent was examined to measure the eluting degree in various buffer solutions of different pH.

Purification with Column Chromatography——Three grams of bufferized DEAE-cellulose in 0.05*M* acetate buffer (pH 5.8) was placed in a column (1.5×10.5 cm.) and the same buffer solution containing 30 mg. of the sample was passed through it. Elution of the column was made with stepwise increase in the concentration of acetate. Acetate buffers of 0.15*M* (pH 6.15), 0.25*M* (pH 6.5), and 0.5*M* (pH 6.9) were used as eluent. Each eluate was filtered and then lyophilized.

Paper Electrophoresis——Each of CS-5 and other fractions from the foregoing column chromatography was dissolved to make 4% solution of protein, and electrophoresis was carried out for 19 hr. in a Veronal buffer of pH 8.5, ionic strength of 0.045 at 400 v., 5.5 mA., with Toyo Roshi No. 2 of 5×40 cm. in size. After the electrophoresis, the paper was dried and stained with bromophenol blue solution.

Electrophoretic Analyses——Solutions of 1.5% were prepared by dissolving the sample in a Veronal buffer of pH 8.7, $\mu=0.2$, and acetate buffer of pH 4.9, 5.1, and 5.5, $\mu=0.2$. These solutions were dialyzed against several changes of buffer over a period of 24 hr. at 0° just before each experiment. Electrophoretic patterns of these dialyzed solutions were observed by the use of a Hitachi Tiselius Electrophoresis Apparatus, Type HT-B. For measuring the specific conductivity, Shimadzu Kohlrausch Bridge was used under the cell capacity of 3.0 ml. and the cell constant of 1.0404 in 0.1*N* KCl solution as a standard.

Ultracentrifugation——The experiments were performed in a Spinco Model E Ultracentrifuge and Hitachi Analysing Ultracentrifuge. For testing the homogeneity, these fractions were dissolved in a Veronal buffer (pH 8.09, $\mu=0.2$) so as to make approximately 1% concentration. For measuring the sedimentation constant, the samples were dissolved in a phosphate buffer (pH 6.8, $\mu=0.1$) to make approximate concentration of 0.5 and 0.2%. The apparent sedimentation constant was measured based on the rate of sedimentation with Hitachi Analysing Ultracentrifuge.

The molecular weight was calculated by Archibald's method[3,4] with Hitachi Analysing Ultracentrifuge.

Biological Assays——The samples dissolved in desalted water were injected subcutaneously in a rabbit. The serum alkaline phosphatase activity was measured by the method mentioned in the previous paper.[1]

Results and Discussions

Chromatographic Purification of Fraction CS-5 using a Column of Ion-exchange Cellulose

As Fig. 1 (a) shown, fraction CS-5 contains 2 or 3 components besides the main component, as indicated by the paper electrophoresis. Therefore, an attempt was made

2) E. A. Peterson, H. A. Sober : J. Am. Chem. Soc., **78**, 751 (1955).
3) W. J. Archibald : J. Phys. Colloid Chem., **51**, 1204 (1947).
4) S. M. Klainer, G. Kegeles : Arch. Biochem. Biophys., **63**, 247 (1956).

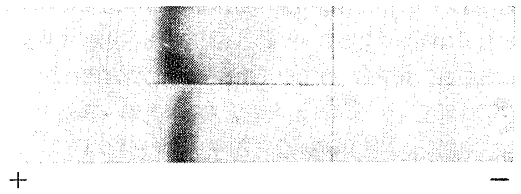

Fig. 1. Paper Electrophoretic Pattern

(a) Fract. CS-5
(b) Fract. CS-5d
Veronal buffer, pH 8.5, μ=0.045, 400 v., 5.5 mA, 19 hr.

purify CS-5 with ion-exchange cellulose. As a result of adsorption procedure, CS-5 was completely adsorbed on DEAE-cellulose in 0.05*M* acetate buffer and was eluted remarkably with 0.5*M* acetate buffer, but it was not adsorbed on CM-cellulose at all.

Therefore, CS-5 was purified on DEAE-cellulose by stepwise elution and CS-5 was divided into four components as shown in Fig. 2.

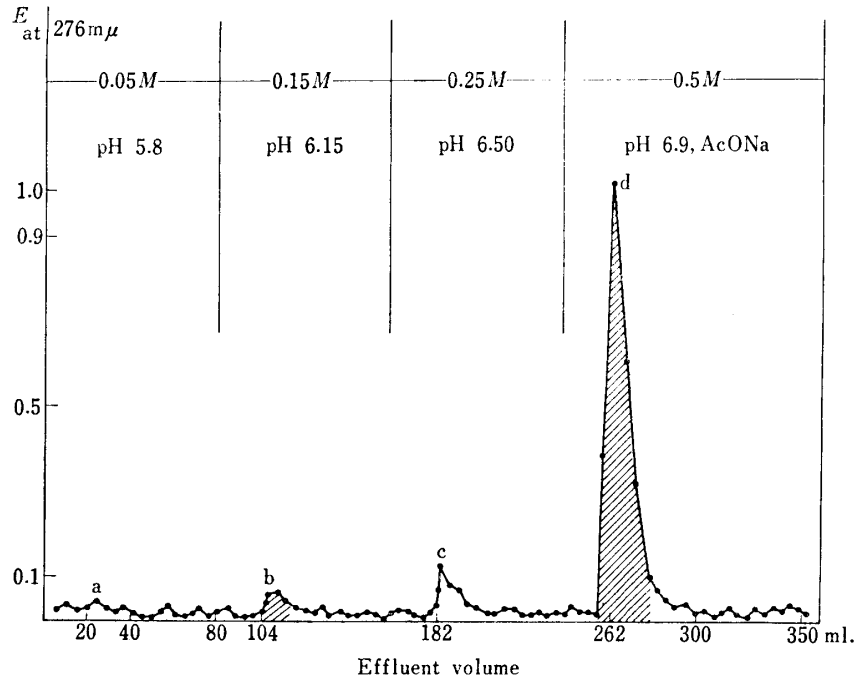

Fig. 2. Stepwise Elution Diagram of Harderian Gland Extract (Fract. CS-5) on a DEAE-Cellulose

30 mg. of CS-5 in 4 ml. 0.05*M* acetate buffer applied to 3 g. of adsorbent; effluent collected in 2 ml. fractions; flow rate, 14 ml./hr.; DEAE-cellulose column, 1.5×10.5 cm.; ▨, this fraction increased alkaline phosphatase activity.

It was found that CS-5b fraction, the eluate in 0.15*M* acetate buffer, and CS-5d fraction, the eluate in 0.5*M* acetate buffer, had the activity that increase the serum alkaline phosphatase activity, as shown in Table I.

Table I. Effect of Each Fraction obtained from CS-5 on Serum Alkaline Phosphatase Activity

Fraction No.	Protein concn.[a] (mg.)	Phosphatase act. dec. or inc. % (dose : 0.25 mg./kg.)
CS-5	29.364	+13.43
a	—	—
b	1.449	+18.09
c	2.066	−16.00
d	16.928	+19.17

a) Calculated from calibration curve of protein at 276 mμ.

As the rate of nitrogen recovery of CS-5d by this procedure was approximately 60%, it seems that fraction CS-5d had almost activity that increases the serum alkaline phosphatase activity contained initially in CS-5.

Ultracentrifugal and Electrophoretic Analyses

The sedimentation and electrophoretic patterns of CS-5d are illustrated in Figs. 1 (b), 3, and 4. They are shown to have a single symmetrical peak in each.

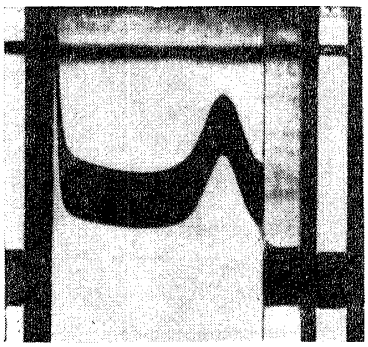

Fig. 3. Ultracentrifuge Sedimentation Diagram of Fract. CS-5d

Veronal buffer, pH 8.09, 64 min. after reaching full speed (56,100 r.p.m.).

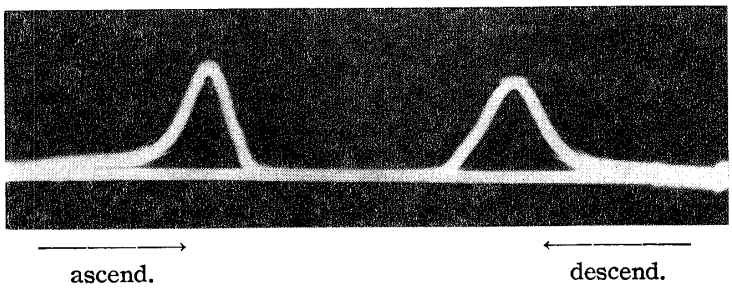

Fig. 4. Electrophoretic Pattern of Fract. CS-5d

Veronal buffer, pH 8.7, μ=0.2, 44 v., 10 mA, 60 min.

Then fraction CS-5d is affirmed to be almost homogeneous protein through ultracentrifugal and electrophoretic analyses.

The electrophoretic mobility of CS-5d was measured and the isoelectric point was found to be between pH 4.7 and 4.8. The lyophilized fraction CS-5d was almost insoluble in strong acidity and the neutral or weakly acid solution of it became cloudy in acidity below pH 4.7 and CS-5d precipitated in strong acidity. Since the mobility was not measured in acidity below pH 4.7, the isoelectric point was determined by drawing a curve as in Fig. 5 and getting the intersection of the curve and the horizontal axis.

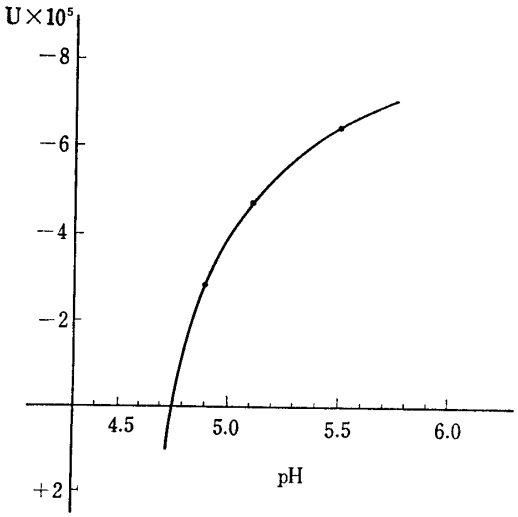

Fig. 5. Mobility-pH Curve of Fract. CS-5d

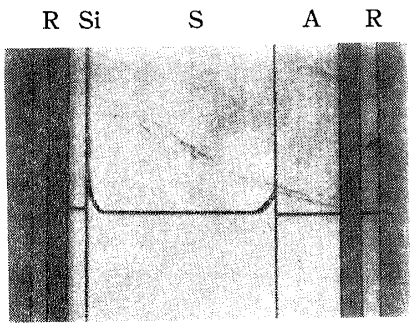

Fig. 6. Ultracentrifuge Diagram of CS-5d with Solution-air Meniscus and Solution-silicone Meniscus

Key: R, reference; A, air; S, solution; Si, silicone

The sedimentation constant of CS-5d was estimated and s_{20w} value was 2.26×10^{-13}. This value seems to indicate that its molecular weight is smaller than 30,000 and its molecular shape is assumed to be globular, because the concentration dependence of sedimentation rate is not relatively clear.

The molecular weight was estimated by the Archibald's method and an approximate value of 27,000 was obtained. A part of the sedimentation pattern and a part of the calculation are shown respectively in Fig. 6 and Table II.

TABLE II. Calculation of Molecular Weight of CS-5d by Archibald's Method

Time (min.)	Dimension of  (cm^2)	Value of dc/dx (cm.)	Molecular weight
16	1.32	1.95	2.97×10^4
26	1.79	1.94	2.88
36	2.02	1.74	2.74
46	2.19	1.64	2.67
56	2.39	1.53	2.61
66	2.63	1.43	2.70
			mean : 27,000

$$M=\frac{RT}{(1-V\rho)\omega^2}\cdot\frac{dc/dx}{xc}$$

M is the gram molecular weight, R the gas constant, T the absolute temperature, V the partial specific volume of solute, here it was taken as 0.720 tentatively, ρ the density of solution, ω the angular velocity, c the solute concentration, and x the distance measured from the center of rotation to the top of the liquid column.

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Summary

1. The fraction CS-5, extracted and isolated from bovine Harderian gland as the protein component increasing the serum alkaline phosphatase activity of rabbit was further purified by column chromatography using DEAE-cellulose. Resultant fraction CS-5d had almost activity that increases the serum alkaline phosphatase activity contained initially in CS-5, and was affirmed to be almost homogeneous protein through ultracentrifugal and electrophoretic analyses.

2. The sedimentation constant of CS-5d was 2.26×10^{-13}.

3. The isoelectric point of CS-5d was found to be between pH 4.7 and 4.8.

4. The molecular weight of CS-5d was estimated as be 27,000 by the Archibald's method.

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