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Isolation and Component of Rare Acidic Protein in Mammal Organ

Part 1 Isolation and Some Chemical Properties of Acidic Protein in Bovine Liver

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Introduction

The study on the existence and the isolation of acidic protein was carried on by some investigators[1~6] in biochemical and chemical fields. Recently, one kind of acidic protein was interested by a few of medical or physiological investigators especially. Moreover, this study becomes one important subject in medical and biochemical field, because this pretein may be related to transmission of information in nervous system. At first, a bovine liver was selected as one representative example of mammal organ. So, this rare acidic protein was tried to be isolated from the bovine liver which was possible to be obtained freshly at slaughterhouse. And some chemical properties of acidic protein were determined. Also, the existence of γ-carboxyglutamate was recognized as the chemical component of rare purified fractionated acidic protein by means of amino acid analyser and other analytical treatment. And other part related to this point may be published on the next paper.

This study was one part of investigatation that was carried out at Abteilung Enzymchemie, Institut für Biochemie, Georg-August Universität, Göttingen.

Experimental and Results

This experiment was repeated 5 times. So, the representative experimental results were published on this paper. The shown charts were representative among other many charts obtained. Also, some parts of this experimental procedure were described detailed. However, all detailed procedure could not be described depending upon the pagination. Then, other rest parts of this experimental procedure and the related parts may be published on other paper.

The fresh bovine liver was transported on ice from the slaughterhouse to the laboratory at the university eary morning. The only pure liver tissue of organ was separated exactly from epidermis, vein, artery, and other impure unexpected tissues. After the pure liver tissue 2.55 kg was separated from the whole fresh liver 4 kg on ice, the separated cut part of the pure tissue was homogenized with 0.1 M potassium phosphate buffer solution (KH_2PO_4, K_2HPO_4, adjusted to pH 7.1) 10 ℓ which contained 1 mM EDTA and 55% $(NH_4)_2SO_4$ at a refrigerated room (2°C ± 0.5°C). Each 2 ℓ was homogenized under the condition that rotation (6000 rpm) for 60 sec and successively stop for 2 min, rotation (10000 rpm) for 30 sec and successively stop for 2 min, and rotation (10000 rpm) for 30 sec were performed to keep the biochemical specificity. After the homogenized solution was allowed to stand at the refrigerated room overnight. And each 3 ℓ was centrifuged at 4.2°C and 4200 rpm for 35 min. After the collected centrifuged solution was centrifuged again, the supernatant part obtained was 4.27 ℓ. So, after $(NH_4)_2SO_4$ 1330 g was added in the 4.27 ℓ, the solution was allowed to stand at 4°C ± 0.5°C overnight. And the solution of 85% saturated $(NH_4)_2SO_4$ was centrifuged at 4.2°C and 4200 rpm for 2 hrs. The precipitate was dissolved in about 500 mℓ distilled deionized water. And it was dialyzed with distilled deionized water 5 ℓ at 4.0°C ± 0.5°C for 1 hr.

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Successively, each new distilled deionized 1 ℓ was changed four times during 4 hrs. Moreover, it was continued to dialyze with 0.01 M phosphate buffer, pH 6.8, 5 ℓ for 1 hr. and 0.1 M phosphate buffer, pH 7.1, 5 ℓ for 1 hr. And it was allowed to stand overnight at 4.0°C ± 0.5°C. Next, it was dialyzed with distilled deionized water 5 ℓ for 1 hr., 0.1 M phosphate buffer, pH 6.8, 5 ℓ for 1.3 hrs, and 0.1 M phosphate buffer containing 0.1 M NaCl 5 ℓ for 3 hrs at 4°C ± 0.5°C. The dialyzed solution was 1.06 ℓ. The protein content was 18.4 mg/mℓ (Biuret reaction, 546 nm).

The protein sample was fractionated with DEAE-Sephadex A-50 column 50 x 170 mm which was prepared from 22 g dry DEAE-Sephadex A-50, 0.1 M phosphate buffer, pH 7.1, containing 0.1 M NaCl, 500 mℓ, in a incubator at 100°C ± 1°C for 3 hrs. The spontaneous flow rate was about 250 mℓ/hr. Then, it was adjusted to 40 mℓ/hr for overnight running. This column chromatography was performed in circulation system of regurated temperature, 2°C ± 0.5°C. Of course, the collection or fractionation was performed at the same temperature. And the concentration of NaCl was changed step by step, 0.1 M, 0.17 M, 0.22 M (Peak A), and 0.60 M (Peak B). Then, the DEAE-Sephadex A-50 chromatographic result is shown in Fig. 1.

Peak A (fraction number, 95 ~ 103) and Peak B (fraction number, 121 ~ 130) were lyophilized to keep the sample in frozen after dialysis respectively.

The one part of the typical fraction, Fr. 98 100μℓ or Fr. 125 100 μℓ, was determined with Biuret reaction

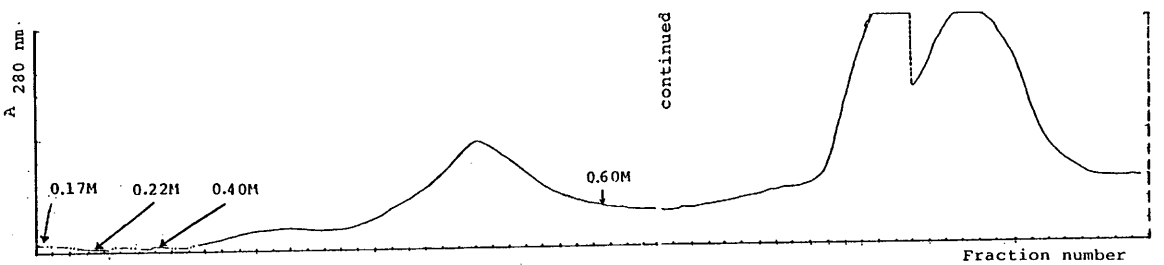

Fig. 1. DEAE-Sephadex A-50 chromatography

(546 nm) respectively. The protein content of Fr. 98 or Fr. 125 was 0.68 mg/mℓ or 0.44 mg/mℓ respectively.

And, alkaline hydrolysis with KOH was carried out against Fr. 98 or Fr. 125 respectively. For example, after 4N KOH 1 mℓ and Fr. 98 1 mℓ or Fr. 125 1mℓ were incubated at 110°C ± 1°C for 20 hrs in Teflon tube, each sample was neutralized with $HClO_4$ in ice and was centrifuged. And it was analyzed with higher voltage electrophoresis { paper, 3000V, 45 min, spotting → dried with blower → wetting with capillary phenomenon (pydrine → acetate buffer, pH 4.3) → electrophoresis (attend! mobile direction) → dried in oven (Umluft → Frischluft) 100°C, 5 min → dip (Ninhydrin 0.2% in acetone) → dried in oven (until the spot appeared, 100°C) } or high voltage thin layer electrophoresis (DC → Fertig Platten CEL 300-25, 500 V, 45 min, plate cooling system, spotting → dried with blower → spray (pyridine → acetic acid → water) → dried in oven, 15 min, 100°C → electrophoresis → spray (Ninhydrin Sprühreagenz 0.1%) → dried in oven, 5 or 10 min, 100°C.

The guide samples used usually were Aspartate (Asp), glutamate (Glu), γ-carboxy glutamate (Gla) in several concentrations and spotting amounts respectively or in mixing. Also, the relative interesting guide samples were serine, serine derivative (deriv.), threonine, threonine deriv., Asp, Glu, cysteine, cysteine deriv., and proline deriv. in mixing or individually.

Also, this high voltage thin layer electrophoresis could be applied on semiquantitative analysis. However, the volume of spray reagent had to be constant especially!

Moreover, Sephadex G-10 column (16 x 910 mm, prepared from Sephadex G-10 80 g and 0.05 M phosphate buffer, pH 7.13 at 110°C) was used to chromatography Gla, hydrolyzed A, and hydrolyzed B respectively. This procedure was useful to purify

the sample from contamination of salt or others. Of course, the fractionation of each sample with this column was performed to obtain high purity. This chromatography was carried out at the same temperature as the DEAE-Sephadex A-50 column. Then, the Sephadex G-10 chromatographic results are shown in Fig. 2.

The authentic γ-carboxy glutamate was prepared by the complete chemical synthetic procedure in München Universität. The purity and some chemical properties could be understood with the shown chart in Fig. 3 (nano mole order). But the purity was

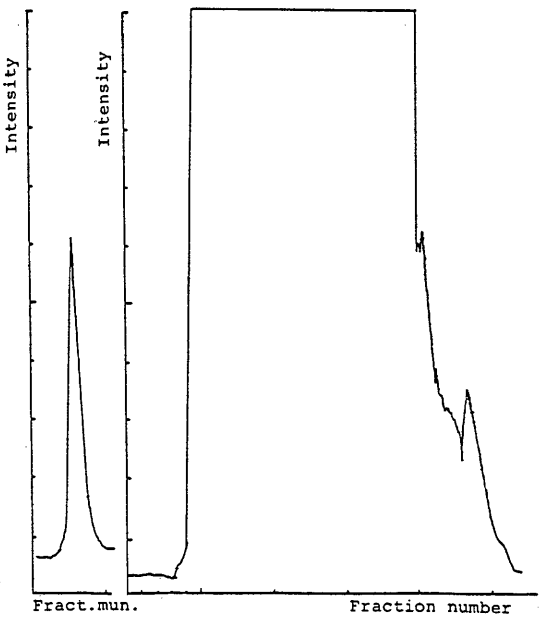

Fig. 2. Sephadex G-10 chromatography

guaranteed in picko mole order using fluorescence analyser. So, this Gla was used as the standard sample in various analytial procedures in this experiment.

The hydrolysis procedure with alkaline solution was performed in special polytetrafluoroethylene tube which was set in hard glass test tube under high reduced pressure. Namely, after the sample and alkaline solution poured into the reaction tube was frozen in liquid nitrogen, the reaction tube had to be sealed under high reduced pressure before the contents melted again. Then, after sample solution 1 mℓ and 4N KOH 1 mℓ were sealed in the tube, the reaction tube was heated in oven at 110°C ± 1°C for 20 hrs.

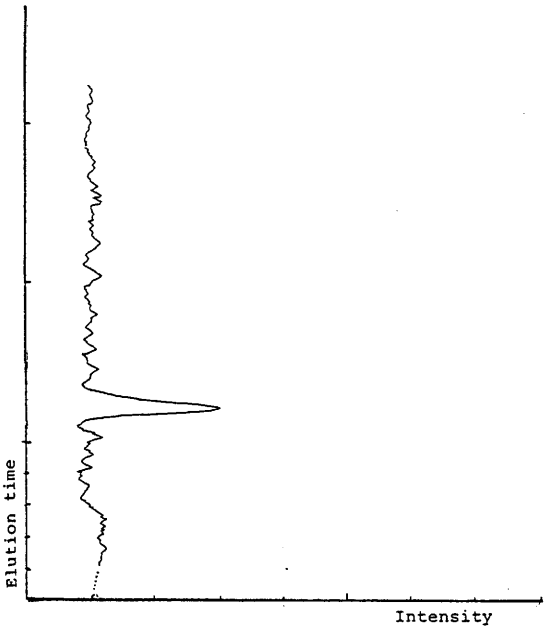

Fig. 3. Chromatogram of γ- carboxyglutamte

After the hydrolyzed solution was neutralized with 70% $HClO_4$ in crushed ice using pH meter, moreover, fine neutralization was performed with 2N KOH and 10% $HClO_4$. The neutralized solution was centrifuged at 0°C ~ 3°C for 5 min at 4000 rpm. The chemical components of the supernatant 100 μℓ were determined with precise amino acid analyser which designed by Merck Co. The elution time of alkaline hydrolyzed components of purified acidic protein was shown in Fig. 4.

The hydrolysis procedure with acidic solution was performed in special reaction tube under high reduced pressure. This case had to be paid much attention than the case of alkaline solution. After the sample, acidic solution, was poured into the reaction tube, the contents in the tube had to be frozen completely with liquid nitrogen. So, the frozen acidic solution had to be sealed during the solution was frozen. The HCl releases at melted state. After the sample solution many fractionated fractions was performed in orderly as follows; the peak fraction and the several fractions of the both outsides of the peak fraction were electrophorezed at the first step, the several fractions of the both outsides of the determined fractions of the first step were electrophorezed at the second step, the several fractions of the both outsides of the deter-

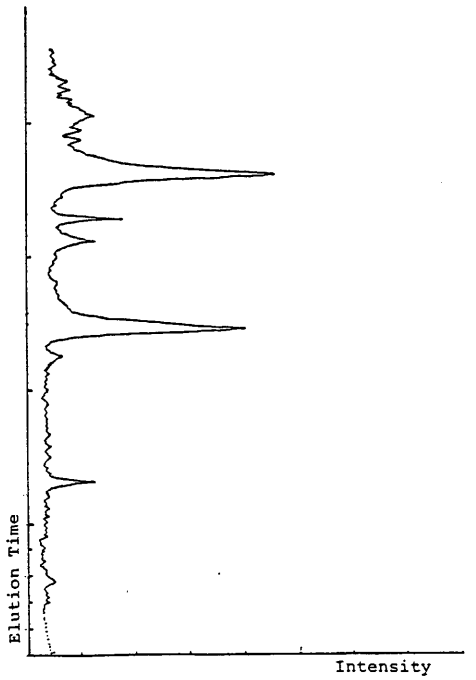

Fig. 4. Chromatogram of alkaline hydrolyzed protein

1 mℓ and 4N HCl 1 mℓ were poured in the reaction tube and it was recognized the complete freezing, the tube sealed under high reduced pressure. The sealed tube was allowed to stand in oven at 110°C ± 1°C for 20 hrs. After the hydrolyzed solution was neutralized with 6N KOH in crushed ice using pH meter, moreover, fine neutralization was performed with 10% $HClO_4$ and 2N KOH. The solution was centrifugued according to the procedure of neutralization of alkaline hydrolyzed solution. After the precipitate was separated, $HClO_4$ 1 mℓ was added into the supernatant in crushed ice. Then, the solution was centifuged again like the above described centrifugation procedure. After the precipitate was separated, the supernatant was neutralized with 2N KOH using pH meter. Its solution was centrifuged again like the above described procedure. It should be noted that the production of precipitate was affected with the temperature. Also, the volumes of solution and supernatant should be determined to decide the concentration of component. The elution time of acidic hydrolyzed fraction of purified acidic protein was shown in Fig. 5.

The alkaline hydrolyzed protein was determined in

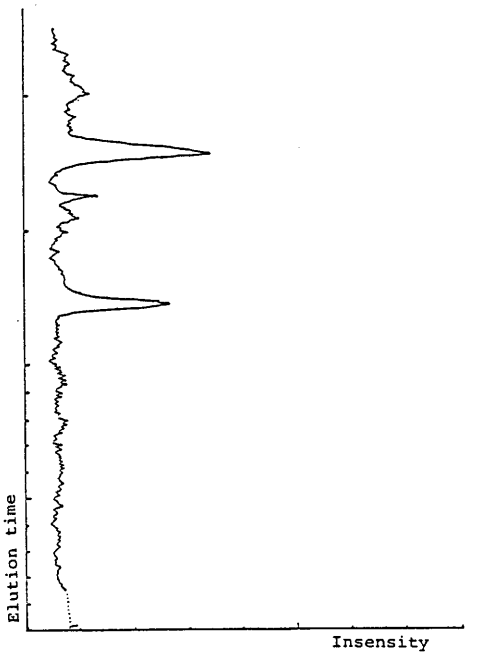

Fig. 5. Chromatogram of acidic hydrolyzed protein

comparison with Gla-containing amino acid mixture by high performance liquid chromatography (HPLC). Asp, Thr, Ser, Glu and Gla were recognized. HPLC of Gla-containing amino acid mixture which was standard is shown in Fig. 6. The detailed description may be reported on other paper closely.

The other chemical properties of acidic protein were studied with polyacrylamide gel electrophoresis. After each fraction eluted from Sephadex column was checked with absorption at 280 nm, they were determined by usage of the polyacryamide gel electrophoresis. Then, the electrophoretic mobilities of the fractionated sample fractions related to the objective protein were more faster than bovine serum albumin (BSA) that was used as one standard sample. The

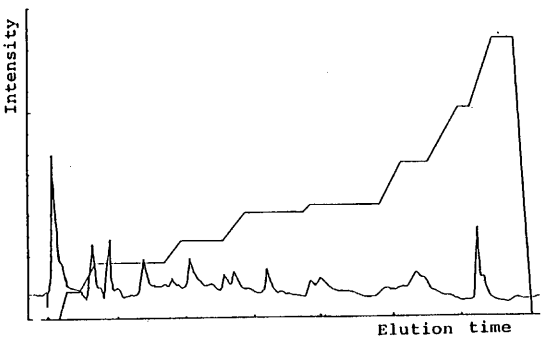

Fig. 6. HPLC of Gla-containing amino acid mixture

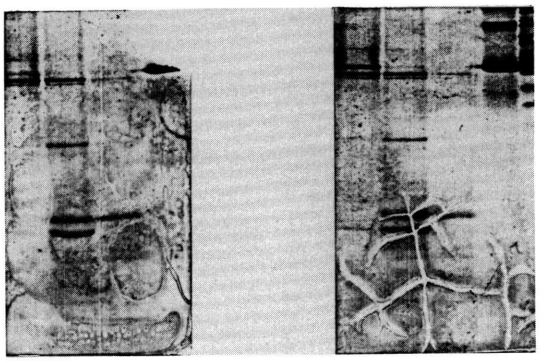

Fig. 7. 7.5% polyacrylamide gel of purified rare protein

The mobilities and the fragments of the related important fractions are compared with BSA.

representative results were shown in Fig. 7.

The determination procedure of each fraction of mined fractions of the second step were electrophorezed at the third step, and so on. All many important fractions were electrophorezed according to such a method.

The electrophoretic analyses of unusal acidic protein fractions were performed with 7.5% polyacrylamide gel as one analytical method. Also, 15% polyacrylamide gel method was applied for it. The former was better than the latter. The components of 7.5% polyacrylamide gel were shown in Table 1.

Table 1 Components of 7.5% Polyacrylamide Gel

TRENN GEL		SAMMEL GEL	
30% Acrylamid	7.5 mℓ	Destillierted Wasser	8.0 mℓ
Destilliertes Wasser	14.7 mℓ	30% Acrylamid	1.0 mℓ
1.4 M Tris-HCl Puffer (pH 8.9)	7.5 mℓ	1.4 M Tris-HCl Puffer (pH 8.9)	0.5 mℓ
0.06% N,N,N,N-Tetramethyl ethylendiamin	20 μℓ	0.06% N,N,N,N-Tetramethyl ethylendiamin	20 μℓ
5% ammoniumpersulfat	0.4 mℓ	5% Ammoniumpersulfat	0.2 mℓ

Bromophenol blue (BPB) was used as a marker dye. And bovine serum albumin (BSA) was used as a standard protein sample of mobility, molecular weight, and other chemical properties in electrophoresis. Also, 1.5 M saccharose 100 μℓ, 0.05% BPB 50 μℓ, and sample 300 μℓ, 100 μℓ, 50 μℓ were respectively used for electrophoresis at 30 mA, 60 V for 2.5 hrs ~ 3.5 hrs. The fast moving fraction was one objective substance in comparison with BSA.

Discussion and Conclusion

Some new points, some improved procedures, importances and characters in this experiment were discovered as regards the recognition of existence of rare acidic protein. So, the representative several points are shown in the following several sections. Also, the author paid attention to the following many points.

This liver that was selected as one representative sample of mammal organ had to be fresh and clean as possible to avoid biochemical emzymic reaction, microbial action, and other changes. The investigator must pay his attention for defense of occurrence of these negative phenomena in his experimental procedure, but many investigators neglect such an important points generally.

Next, it was general case that the contamination of epidermis, vein, artery and other impure tissue existed in the separated tissue. The very small amount of contaminator exerts very important influence on the analysis of amino acid sequence, component, and molecular structure of tissue. This remove of these contaminators was very difficult partially, the author gave attention to the change and the contamination.

The authentic γ-carboxy glutamate (Gla) was prepared by organic chemical synthesis as one very important standard sample in this experiment. The amount of standard sample is required to get in gram order at least, because many analytical reactions had to be performed. The standard sample has to be constant against various determinations. Generally, the usage of the standard sample that was prepared at the same time is better than the standard sample prepared at each time. Also, such an authentic synthetic standard sample is better than the standard sample isolated from the natural product. Because, the latter in large scale does not keep the extra high purity generally. And the separation of very small amount of

impure material is so difficult. However, in the case of simple structural compound except complicate structure, the chemical synthesis is more efficient than the isolation from natural product. Of course, its chemical synthesis was not so easy. On the other hand, the isolation and purification of Gla in gram order from the natural product were very difficult impossibly.

After the homogenization and the extraction of the acidic protein, the centrifugation and the precipitation of it were performed. The above described procedure is usual one that many investigators carried on. But, in this experiment, the first precipitate was dissolved again. Successively the second homogenization and the second centrifugation were performed. This procedure was important and improved points to obtain the high pure sample. Moreover, if this procedure was repeated again, the recovery may decrease. It was considered that the performance of other procedure might be better than performance of the same procedure three times and more. However, the repeat once was useful to obtain the high pure sample in comparison with the non-repeat.

Gla was recognized with alkaline hydrolysis from the isolated acidic protein which was rare in natural organ. This evidence of existence of Gla meaned that Gla was one component of the acidic protein. So, in the sequence of amino acid residure in acidic protein, Gla was situated on one decided position. Perhaps, Gla may be situated on terminal position than intermediate position. On the other hand, the mobility of the fragment was very fast in electrophoresis. It meant the acidity of the fragment. Also, the molecular weight of the fragment may be not large. This hypothsis was induced from this experimental results, but the direct proof was not yet performed. And it may be performed closely by high precise amino acid sequence analyser with computer directly.

On the other hand, amino acid anlyser proved that the acidic protein contained Asp, Thr, Ser, Glu, and Gla exactly. The peaks of Asp, Thr, Ser, Glu and Gla were recognized with those authentic standard samples respectively. The chromatographic determinations regarding the component of rare acidic protein showed the five identified peaks and other peaks which were not yet identified. As these identifications were not so easy, they may be identified with other high precise amino acid analyser.

The acidic hydrolysis did not show the existence of Gla against the isolated acidic protein. The result of acidic hydrolysis was different from the result of alkaline hydrolysis. This result induced one hypothesis. This acidic hydrolysis under reduced pressure at 110°C might make possible the conversion of Gla into the other compound. The other compound may be Glu, because the peak and the aera of Glu became high and large in comparison with the chart of alkaline hydrolysis. If the cautious conclusion is required, the direct conversion reaction of Gla must be proved prudently. Then, this physicochemical conversion reaction was tried to determine under several reaction conditions. So, the results may be published closely on the other paper. Also, the following hypothesis has to be detected closely and may be proposed in near future. As one reason of this conversion, the hypothesis induced the theory as follows; the acidic condition changed from dicarboxylic to monocarboxylic and might produce the release of carbon dioxide from one carboxylic radical among two carboxylic radicals of Gla. It is not so difficult that such a reaction of decarboxylation may be activated under the proton rich condition. The electron distribution is localized for the unbalance between two carboxylic radicals and one amino radical. So, carbon dioxide may be possible to release easily. On the other hand, Asp, Thr, Ser and Glu were stable under this proton rich condition like condition of alkaline hydrolysis. Then, it was hypothesized that Gla was possible to be transformed into Glu. Thus, the same sample produced the two differnt results under the two kinds of condition. The difference or change was depended upon the condition that free proton affected the electron of molecule. The change that the molecular structure of Gla was transformed into the molecular structure of Glu under decarboxylation was illustrated. This illustration may be shown closely on the other paper.

The thin layer electrophoresis was useful to identify the component of alkaline hydrolyzed protein. The 0.5 mm thickness of layer was better than the 1 mm thickness. Much sample against 1 mm thickness was

impossible to separate as the tailing appeared. Less sample against 0.5 mm thickness showed nice result that each spot separated completely.

The volume of spraying of spray reagent was studied on the basic viewpoint. From the determined results, the volume controlled whether the detection was successful or was unsuccessful. So, the volume had to be constant for success. Also, the measured spray bottle made by the author was useful for semiquantitative analysis.

Then, many successful results against the identification of component of the alkaline hydrolyzed protein were obtained with the two kinds of electrophoretic method, thin layer electrophoresis and paper electrophoresis.

One part of physicochemical properties of acidic protein was determined by the polyacryamide electrophoresis. This determination was applied to each fraction of acidic protein eluted from Sephadex column. The determination procedure for many fractions was improved by the author like the above descriptions. So, the fractions that contained much sample were determined early. And the fractions that contained less sample were determined late. Such an analytical procedure was one of the method improved by the author. This determination method performed according to the necessity of the objective compound is useful to reasonable anslysis.

The chromatographic procedures were improved by the author. For example, when DEAE Sephadex A-50 column was prepared, dry DEAE Sephadex A-50 was treated with two kinds of phosphate buffer solution in oven. Namely, one kind was the phosphate buffer solution contained 0.10 M NaCl, another kind was the phosphate buffer solution which did not contain 0.10 M NaCl. The latter was used in the general case. However, the former was better than the latter.

Summary

The bovine liver was selected as the mammal organ, because it could be obtained under most fresh condition. One kind of acidic protein, rare acidic protein, was fractionated and isolated by means of partition column chromatography (main column: DEAE-Sephadex A-50, others) from the fresh bovine liver that the epidermis, vein, artory and other impure tissue were cut off carefully. These acidic fractions were more faster than bovine serum albumin in screening of polyacrylamide gel electrophoresis. And the electrophretical fast fractions were lyophilized and were purified. Then, each purified fraction that the electrophretical property was equal was combined. So, its fraction was hydrolyzed to analyze the component with 2 N KOH.

In the typical chemical components of acidic protein of the only liver tissue isolated, γ-carboxy glutamate, asparatic acid, and glutamic acid were detected by amino acid analyses, high voltage paper or thin layer electrophoreses, precise amino acid analysis, and high performance liquid chromatography. Especially, the isolation of γ-carboxy glutamate that several chemical properties were determined is one important projective point.

References

1) A. Gardemann, D. F. Domagk; *Arch. Biochem. Biophys.* **220** 347 1983.
2) H. Guetekin, D. F. Domagk; *Z. Physiol. Chem.* **364** 1134 1983.
3) P. V. Hauschka, J. P. Lian, P. M. Gallop; *Proc. Nat. Acad. Sci.* USA 72 3925 1975.
4) P. V. Hauschka, P. A. Friedman, H. P. Traverso, P. M. Gallop; *Biochem. Biophys. Res. Comm.* **71** 1207 1976.
5) T. Kato, Y. Kato, H. Kasai, T. Okuyama; *J. Biochem.* **82** 1297 1977.
6) J. Stenflo, P. Fernlund, W. Egan, P. Roeps Troff; *Proc. Nat. Acad. Sci.* USA **71** 2730 1974.
7) K. Horitsu, H. Guetekin, G. F. Domagk; *Archives Internationales de Physiologie et de Biochimie*, 1984, 92(3) B88.

Foot note – As the pagination for one report is limited by the budget, this report had to be shortened.

Keisuke HORITSU

哺乳動物器官の希有酸性蛋白質の単離と組成
第1報　牛肝臓の酸性蛋白質の単離と化学的性質

堀　津　圭　佑

（昭和60年9月28日受理）

牡牛肝臓は哺乳動物器官として撰択された．それは大変新鮮な状態で得ることができたためであった．ある種の酸性蛋白質，希有酸性蛋白質，は分配カラムクロマトグラフ法（主カラム：DEAE—Sephadex A—50，その他）で表皮，静脈，動脈，その他不純組織を注意深く切除した新鮮牡牛肝臓から分画，単離された．それらの酸性画分はポリアクリルアミドゲル，電気泳動の選別では牡牛血清アルブミンより速く移動した．電気泳動的速動画分は凍結乾燥され，精製された．電気泳動的性質の等しい各精製画分は集められた．そしてその画分は組成分析のため，2N KOH で加水分解された．

単離された肝臓組織そのものの酸性蛋白質の典型的化学成分には，γ—カルボキシグルタミン酸，アスパラギン酸，グルタミン酸がアミノ酸分析機器，高電圧沪紙および薄層電気泳動，精密アミノ酸分析機器，高性能液体クロマトグラフにより検出された．特にいくつかの化学的性質が測定されたγ—カルボキシグルタミン酸の単離は1つの重要計画点である．