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Isolation and Component of Rare Acidic Protein in Mammal Organ

Part 2 Isolation and Some Chemical Properties of Acidic Protein in Bovine Liver

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Introduction

The object and interest of this experiment were published by the author and the medical biochemists.[1-6] As one of the interest, such a rare acidic protein may be related to transmission of information in nervous system that is objective finally.

These experimental method and precedure were improved and changed partially for the previous publication.

At the first step, a liver organ was selected as one objective organ. So, the component, the chemical composition, and the physico-chemical properties of amino acids of the acidic protein that became the experimental target were determined with higher voltage paper electrophoresis, high voltage thin layer electrophoresis, polyacryl amide electrophoresis, amino acid analyser, precise amino acid analyser, high performance liquid chromatograph, and other instuments and treatments. Especially, γ-carboxy glutamate was determined with aspartic and glutamic acids. Also, as the chemical property of γ-carboxy glutamate, it was recognized that there was one specific result between alkaline hydrolysis and acidic hydrolysis. Namely, its acidic amino acid was possible to be detected under alkaline hydrolysis condition, but it was impossible to be detected under acidic hydrolysis condition. Its transformation from γ-carboxy glutamate to glutamate was interesting in biochemical reaction relating to physiological reaction in body.

These experiments were repeated several times to recognize the experimental results. This study was one part of investigation that was performed at Abteilung Enzymchemie, Institut für Biochemie, Georg-August Universität, Göttingen.

Some experimental results obtained at the Biochemie Institut were published on Archives Internationale de Physiologie et de Biochimie.

Laboratory of Biology

Experimental and Results

The preparation of material and the procedure of isolation were improved by the author partially.

The fresh warm bovine liver on ice was transported from the slaughet house to the laboratory of the university in the early morning. The pure liver tissue was separted exactly from epidermis, vein, artery and other unexpected tissue like the previous procedure.[8] During the experiment the procedure of isolation was performed on ice. Namely the author paid his attention about the purity and the denaturation by enzymic reaction enough.

The pure tissue 2.2 Kg was separated from the whole fresh liver held on ice. And the cut tissue was homogenized with 10 l 0.1 M potassium phosphate which contained 1 mM EDTA and 55% $(NH_4)_2SO_4$ solution (adjusted to pH 7.1) at a refrigerated room (2°C ± 0.5°C). The homogenization procedure was as follows: each 2 l was rotated at 8000 rpm for 60 sec and was stopped for 2 min to cool the contents in vessel, successively was rotated at 10000 rpm for 30 sec. And it was stopped for 2 min and rotated at 10000 rpm for 30 sec again. The homogenized solution was

allowed to stand the refrigerated room overnight.

The operation of the successive procedure to obtain the acidic protein fraction was performed according as the operation that was improved more and less. And, after each 2 l was centrifuged, $(NH_4)_2SO_4$ was added in the supernatant. It was allowed to stand at 4°C ± 0.5°C overnight. And, after the solution of 85% saturated $(NH_4)_2SO_4$ was centrifuged (4200 rpm) for 2 hrs at 4.2°C, the precipitate was dialyzed with distilled deionized water 5 l for 1 hr and four times each new one 1 l for 1 hr successively. Moreover, it was dialyzed with phosphate buffers of pH 6.8 and pH 7.1 successively until the next morning. And it was dialyzed with distilled deionized water, 0.1 M phosphate buffer (pH 6.8), and 0.1 M phosphate buffer (pH 6.8) containing 0.1 M NaCl at 4°C ± 0.5°C successively. The obtained protein sample was fractionated at 2°C ± 0.5°C with DEAE-Sephadex A-50 column that was prepared with published procedure.[8)]

The chromatograms that were eluted at two typical different (usual and slow) elution speeds were shown in Fig. 1 and Fig. 2 respectively. The latter chromatography was operated at unusual slow elution speed and unusual many fractionations. The latter procedure was better than the former one to obtain the pure fraction. However, it was very difficult that each important fraction had to be analyzed with several analyses, poly acrylamide gel, paper and thin layer electrophoreses, and others.

Next, the acidic protein fraction was hydrolyzed at 115°C ± 1°C for 24 hrs with 4 N KOH in Teflon tube under reduced pressure of glass closed system. The 4 N KOH 1 ml was added into the Teflon tube contained the dialyzed dried acidic protein fraction sample 1 mg. After hydrolysis, the hydrolyzed solution was neutralized with 70% $HClO_4$ on ice at the first step of neutralization. The fine neutralization was performed with 10% $HClO_4$ using pH meter. Also, if 10% $HClO_4$ was added excessively against neutral point, the excess acid was neutralized with 2 N KOH with pH meter exactly on ice. It was better that the centrifugation for precipitate had to be operated at low temperature. Because, the solubility of precipitate was influenced by temperature. So, the

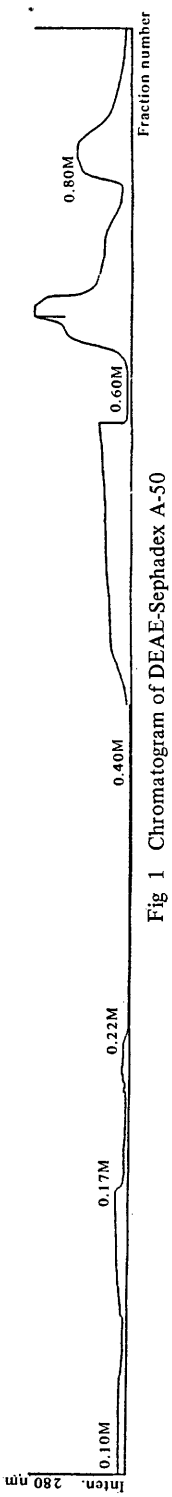


Fig 1 Chromatogram of DEAE-Sephadex A-50

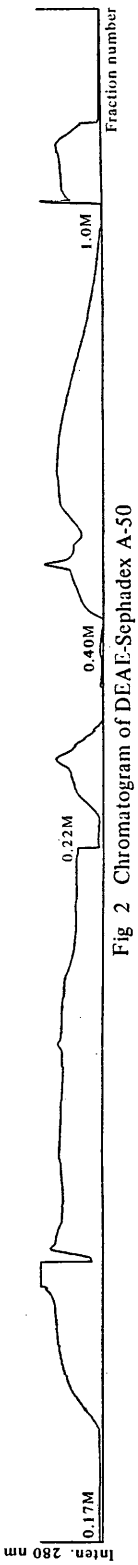
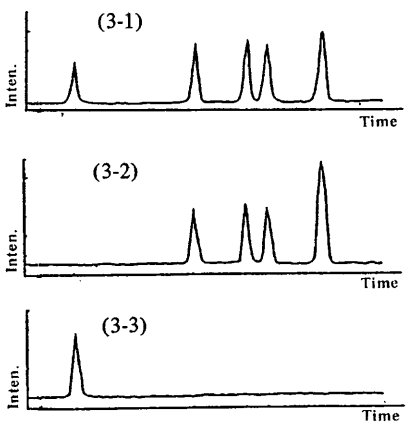


Fig 2 Chromatogram of DEAE-Sephadex A-50

neutralization was affected unexpectedly. The chromatography did not show the existence of γ-carboxyglutamate as below described. On the other hand, the hydrolysis with 4 N HCl in stead of 4 N KOH detected the existence of γ-carboxyglutamate under reduced pressure in glass closed system at 115°C ± 1°C for 24 hrs. And, the neutralization against HCl hydrolysis was performed with 6 N KOH firstly and with 2 N KOH (10% $HClO_4$, if alkali was excess.) exactly using pH meter. Before HCl hydrolysis, the glass tube had to be frozen with liquid nitrogen completely. And, after the freeze of the sample was recognized surely, the hydrolysis should be performed. Especially attention should be paid to such a volatile reagent.

The chromatograms of alkaline and acidic hydrolyzed acidic protein using amino acid analyser were shown in Fig. 3-1 and Fig. 3-2 respectively. At the same time, the chromatogram of the authentic γ-carboxyglutamate was shown in Fig. 3-3 to detect the precision of the analysis and the purity in relation to these samples.



Figs. 3-1, 3-2, 3-3 Chromatograms of alkaline, acidic hydrolyzed proteins, and τ-carboxyglutamate

Also, the chromatogram of alkaline hydrolyzed acidic protein using precise amino acid analyser was shown in Fig. 4. Moreover, the purity of the authentic γ-carboxyglutamate that was a standard sample was shown in Fig. 5 with the recognization of the precision and accuracy of the precise amino acid analyser.

As the analytical method for detection of the component, higher voltage paper electrophoresis was applied according to the following procedure: spotting

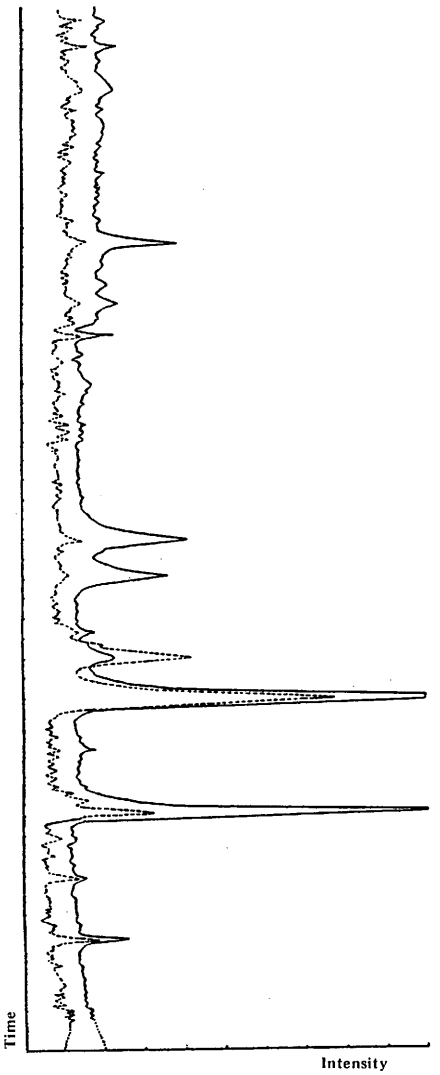


Fig. 4. Chromatogram of alkaline hydrolyzed protein

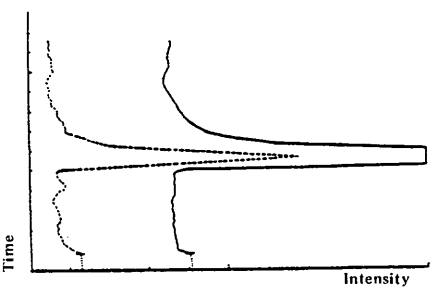


Fig. 5. Chromatogram of τ-carboxyglutamate

of sample on the approximate middle line of large high quality filter paper (ca. 50 x 100 cm, attend! sizing direction and anode-cathode) → dried with blower → repeating several times → dipped the front of paper → wetting with the solvent (pyridine – acetate buffer, pH 4.3) that capillary phenomenon is responsible for the rise → electrophoresis (3000 V, 45 min, attend! mobile direction) → dry in oven (Umluft → Frischluft) at 100°C for 5 min → wet with the solvent (ninhydrin 0.2% in acetone) → dried in oven (until the spot appeared) at 100°C.

Also, as other detection method high voltage thin layer electrophoresis was performed as follows: spotting of sample on thin layer (Fertig Platten CEL 300, ca. 20 x 20 cm, spotting line on 7 ~ 7.5 cm from cathode side was better than middle line.) → dried with blower → repeating several times → spray the constant volume of the solvent (pyridine – acetic acid – water) → dried in oven at 100°C for 15 min → electrophoresis (500 V, 45 min, plate cooling system with circulation of ice – water) → spray the constant volume of Ninhydrin Spruhregenz (0.1%) → dried in oven at 100°C for 5 min or 10 min. One representative obtained result among many results was shown in Fig. 6.

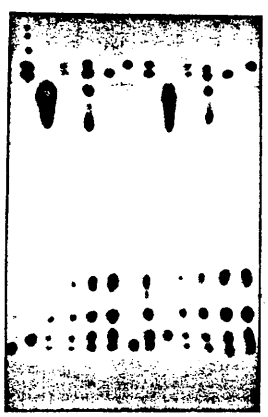


Fig. 6. High voltage thin layer electrophoresis

In these paper and thin layer electrophoreses, the guide samples used usually were aspartate (Asp), glutamate (Glu), γ-carboxyglutamate (Gla), in several concentrations and spotting amounts respectively or in mixture. Also, the interesting guide samples used were threonine, threonine derivative (deriv.), serine, serine deriv., proline, proline deriv., glycine, alanine, cysteine, valine, and methionine in mixture or individually.

These experiments were very important and useful to compare with the results of chromatographies for amino acid analyses. Also, these electrophoretic results were useful to detect the rare acidic protein and one acidic amino acid, Gla, in the case of amount of mikro order or above. Moreover, these results of precise amino acid analyser and high performance liquid chromatograph were useful to detect them in the case of amount of nano order or above. Then, this experiment was checked at the both orders at least.

And, as the previous publication,[8] the properties of acidic protein were determined with polyacrylamide gel electrophoresis. And, the components of polyacrylamide gel were changed partially as Table 1.

Table 1 Components of Polyacrylamide Gel

TRENN GEL		SAMMEL GEL	
30% Acrylamid/ 0.8% Bis	15 ml	Destilliertes Wasser	8.0 ml
Destilliertes Wasser	7.5 ml	30% Acrylamid/ 0.8% Bis	1.0 ml
1.4 M Tris-HCl Puffer (pH 8.9)	7.5 ml	1.2 M Tris-HCl Puffer (pH 6.7)	0.5 ml
N,N,N N-Tetra-methyl ethylendiamin	50 μl	N,N,N,N-Terta-methyl ethylendiamin	20 μl
5% Ammonium-persulfat	0.4 ml	5% Ammonium-persulfat	0.2 ml

The fast moving fraction was one objective substance in comparison with bovine serium albumin that was used as reference sample.

Discussion and Conclusion

The fresh warm bovine liver obtained at the slaughter house was quickly transported on ice to the laboratory of the university in the early morning. The fresh the better. And, the contaminations of epidermis, vein, artery, and unnecessary organ tissue had to be excluded to keep the high purity of material. It is very important that a very small amount of unexpected material decides the degree of purity of the objective material. Also, the material had to be kept at low temperature, because the denaturation or the activation of enzymic reaction that might change the composition had to be avoided. In spite of usual negligence the author paid enough his attention to exclude the contamination, enzymic reaction, and denaturation. The isolation procedure was carried on ice.

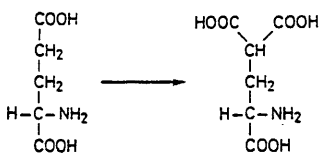
The fractionation was repeated several times to collect the similar objective fraction. Moreover, the repeating means to recognize the reproducibility of the experiment.

The important fractions which contained the objective acidic protein were the 0.22 M and 0.40 M NaCl fractions mainly. The small amount of acidic protein was contained in 0.17 M NaCl fraction partially. The 1.0 M NaCl fraction contained the acidic protein little. Of course, the pure acidic fractions were collected only with monitor.

On the other hand, the profiles of chromatograms of DEAE-Sephadexes in Germany often were different from those in Japan. It will be depended upon some fine conditions. However, the problem did not produce fundamentally, because the important fractions were all analyzed with several methods. So, the experimental results themselves were shown on this paper like the previous paper.[8)]

The two kinds of hydrolyses were considered as typical hydrolysis conditions, alkaline hydrolysis, OH^- rich, and acidic hydrolysis, H^+ rich. The hydrolysis conditions were changed into 115°C for 24 hrs that more and less high temperature long time than the condition published on the previous paper.[8)] This try was to check the reaction condition. The obtained results were equal to the published results.

In comparison between the chromatogram of the alkaline hydrolyzed fraction and it of the acidic hydrolyzed fraction, the former did not show the peak of γ-carboxyglutamate (Gla), but the latter showed the peak of Gla. This difference in the both fractions showed the specific characteristics of the stability of radical and skeleton in H^+ or OH^- rich environment. This difference is interest point, and it indicates that the amino acid posses acidic property. It means the existence of acidic radical in the compound. So, it is thought that its radical is carboxy at first, for example. And, if it posses carboxy radical, its molecular structure is Gla. Then, it is deduced that the acidic protein contains Gla. Namely, the tissue contains Gla similarly as one hypothesis. Then, it is illustrated in some figures. Now, the hypothesis is developed once more. Alkaline hydrolysis did not release CO_2 from Gla. However, acidic hydrolysis released CO_2 from it. These reactions could be recognized by amino acid analyser. The chemical synthetic reaction that glutamate was transformed to Gla as illustration shown in Fig. 7 was considered at first. And it was possible. But, when the reaction occurred enzymically in organ tissue, the reaction might be changed. Namely, the



γ-CARBOXYGLUTAMATE

Fig. 7. Carboxylation of Glu

biological synthesized Gla might not be free in biosystem. Perhaps, it might be bound in one low potential, stable, biological form. So, it was considered that CO_2 became incorporated into Glu reversely in biosystem. Perhaps, it might be as follows: L-glutamate might change to γ-carboxy L-glutamate with Vitamin K dependent carboxylase biochemically. So the hypothetical illustration was shown in Fig. 8.

On the other hand, the chemical modification reaction of CO_2 in aspartate might become as shown in

Fig. 9. And it might be possible to synthesize it. However, the reaction did not be tried to synthesize. Also, similarly, the biological modification of aspartate in biosystem could not be hypothesized, because, β-carboxyaspartate could not be recognized with amino acid analyser. It is reasonable that the occasion of chemical reaction is not always similar to the occasion of biological reaction. These problems

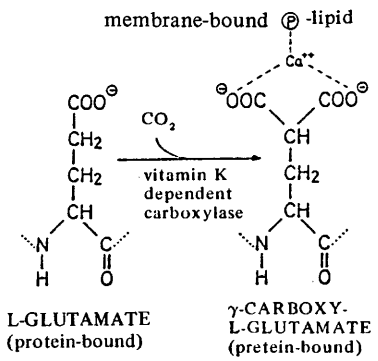


Fig. 8. Glu → Gla modification

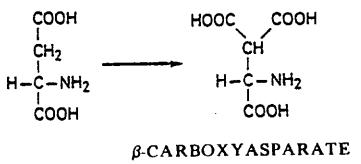


Fig. 9. Carboxylation of Asp

might be soluted in future.

And, it has been emphasized that the experiment had to been tried to determine according to the following conditions at least. A) the experiment has to be repeated at least twice, but twice and more is better. B) the used instruments are two and more kinds. C) the experiment is carried out with two and more suitable methods. D) the experiment has to be high precise and accuracy.

Summary

These experimental procedure and method were improved and changed partially for the previous paper.

The acidic protein fraction was isolated and purified from fresh bovine liver with the improved procedure at first. And, at second, the determinations of the component and the composition of the rare acidic protein were one of the object of this experiment. Then, the existences and some chemical properties of the main three amino acids, γ-carboxy glutamate, aspartic acid, and glutamic acid, were recognized and determined. And the experimental results regard to other amino acids may be reported on other publication.

The some chemical properties of isolated purified acidic protein that was fractionated respectively with the partion column were determined by polyacrylamide gel electrophoresis in comparison with bovine serium albumin when necessary. The objective fraction was more faster than bovine serium albumin.

The some chemical properties of amino acids existed in the acidic protein were determined by high voltage paper or thin layer electrophoresis, usual or precise amino acid analysis, and high performance liquid chromatography.

On the other hand, when the isolated and purified acidic protein was hydrolyzed with 2 N KOH in Teflon-glass reduced pressure sealed tube at 115°C ± 1°C for 24 hours, γ-carboxyglutamate, aspartic acid, and glutamic acid were determined as predominate important amino acid. But it was one changed method. Also, when the acidic protein was hydrolyzed with 2 N HCl under the above described condition, aspartic and glutamic acids were determined. However, γ-carboxy glutamate could not be determined.

References

1) A. Gardemann, G. F. Domagk; *Arch. Biochem. Biophys.* **220** 347 1983.
2) H. Guetekin, G. 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; *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.
8) K. Horitsu; *Bull. Tokyo Kasei Daigaku*, **26** 133 1986.

Foot note – As the pagination for one report was limited by the budget, this report had to be shortened. Moreover, the rigorous restriction is one person one contribution one year.

哺乳動物器官の希有酸性蛋白質の単離と組成
第2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61年9月25日受理）

これらの実験操作や方法は前報に対し部分的に改良や変更を行った．

酸性蛋白質区分はまず改良された操作で新鮮牛肝臓から単離精製された．次に希有酸性蛋白質の成分や組成の測定はこの実験の目的の一つであった．そこで主は3つのアミノ酸，γ-カルボキシグルタミン酸，アスパラギン酸，グルタミン酸の存在といくつかの化学的性質を確認し測定した．また他のアミノ酸に関する実験結果は他の刊行物で報告するであろう．分配カラムで個個に分画された単離精製酸性蛋白質の化学的性質は必要時牛血清アルブミンと比較しポリアクリルアミドゲル電気泳動で測定した．目的区分は牛血清アルブミンより早かった．

酸性蛋白質に存在するアミノ酸の化学的性質は高圧濾紙や薄層電気泳動，通常や精密アミノ酸分析，高性能液体クロマトグラフにより測定された．

他方，単離精製酸性蛋白質がテフロンガラス減圧封管中115°±1℃，24時間，2N KOHで加水分解された時，γ-カルボキシグルタミン酸，アスパラギン酸，グルタミン酸を優先的重要アミノ酸として測定した．しかし，それは一変法であった．また酸性蛋白質が上記の条件下2N HClで加水分解された時，アスパラギン酸とグルタミン酸を測定したがγ-カルボキシグルタミン酸は測定できなかった．