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Purification and Characterization of γ-Conglycinin of Soybean (*Glycine max*)

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大豆(*Glycine max*)의 γ-Conglycinin 의 精製와 特性에 關하여

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ABSTRACT

The physical and chemical properties of γ-conglycinin of soybean (*Glycine max*) were investigated. The soybean protein extracted from soybean meal using 0.2M NaCl solution at pH 4.5 was passed through a Sephadex G-150 column to isolate 7S globulin. γ-Conglycinin was isolated and purified from the 7S globulin with a DEAE Sephadex A-50 column chromatography. The protein preparation was pure on immunoelectrophoresis, polyacrylamide gel electrophoresis and gel isoelectric fousing. It had an isoelectric point at pH 5.4 and contained 16.12% nitrogen, 4.18% mannose and 1.21% glucosamine. Amino acid composition, in general, showed that γ-conglycinin contained higher contents of lysine, dicarboxylic acids and ammonia nitrogen, and lower contents of sulfur-containing amino acids and tryptophan. The subunits of γ-conglycinin were distributed in the range of pH 4.6-5.5. The subunits located in the pH region of 4.6-5.0 and 5.0-5.5 were glycopeptides (molecular weight of 38,000) and simple peptides (MW of 32,000), respectively.

INTRODUCTION

It is well established that most most of the protein of legume seeds is salt soluble globulin which is domposed of two major fractions, legumin and vicilin,[1] with sedimentation coefficients of approximately 11S and 7S, respectively,[2] and that these globulins are the major storage proteins of legumes including soybean (*Glycine max*).[3] Besides these major storage proteins, it is known[4,5] that other globulins having sedimentation values of 2S and 15S are also present in soybean. Legumin or 11S globulin from soybean has molecular weight in the region of310,000~380,000.[6,7] On the basis of biological, structural, and chemical composition data,[7] it seems that 11S globulin is found in many plants and this protein has similarity among different legumes. Smaller globulin, 2S, was isolated from soybean meal by Vaintraub and Shutov.[8] They reported that 2S globulin was composed of two fractions, 2.8S and 2.3S

whose molecular weights are 32600 and 18200, respectively. The 15S protein, which is often-accompanied with purified legumin from legumes as a minor component in the ultracentrifuge,[7] has not been well established.

Unlike legumin, the functional properties of vicilin are not well understood and the methods of isolation, purification and characterization of vicilin component(s) are just being developed, partly because of the complex ity of the protein. It is generally agreed[9~11] that 7S globulin is composed of at least two components. Catsimpoolas and Ekenstam[12] reported that 7S globulin had two distinct immunoelectrophoretic components, β-and γ-conglycinin, in which the former is the major 7S protein. The major 7S protein or β-conglycinin isolated from *Glycine max* has been characterized by Koshiyama.[13~20] The pretein was cold soluble and had a molecular weight of 180,000~210,000 at high ionic strength (I=0.5). The protein associated into a larger molecular species at low ionic strength (I=0.1).[13,14] However, β-conglycin in dissociated into 5S and 2S forms in 0.01M HCl, to 3S in sodium dodecyl sulfate and to 1~2S in urea.[15,16] It had an isoelectric point at pH 4.9 and contained 15.9% Kjeldahl nitrogen and 5% carbohydrate.[17~20]

Although much attention has been given to the characterization of β-conglycinin from various legumes,[7] little information is available concerning the properties of γ-conglycinin whose concentration in the crude 7S globulin fraction is approximately 10%, Recently, Koshiyama and Fukushima[21] isolated γ-conglycinin from soybean and reported some properties. It had a molecular weight of 104,000, a sedimentation value of 6.6S, an isoelectric point at pH 5.8 and a carbohydrate content of 5%(w/w). In contrast to the major 7S protein, γ-conglycinin did not aggregate at low ionic strength, at pH 7.6. The protein was reported to be differnt from an agglutinin.[21] The present study was carried out to develop a purification method and to investigate the physical and chemical properties of γ-conglycinin from *Glycine max*.

METERIAL AND METHODS

Materials

Soybean (*Glycine max*, cultivar Kwang-gyo) harvested in 1976 was obtained from Office of Rural Development, Suweon. Soybean meal was prepared by grinding and sieving through a 60-mesh sieve after removal of seed coat and defatting with n-hexane.

Preparation of γ-congIycinin

Defatted soybean meal was extracted wi h 0.2M NaCl, pH 4.5, at 30°C for 1hr and the supernatant was dialyzed overnight against distilled water.[22] The precipitated globulin was eluted on a Sephadex G-150 column(3×100cm) with 0.035M phosphate buffer, pH7.6, containing 10mM β-mercaptoethanol[23] at a flow rate of 20m*l*/hr. Crude 7S globulin was rechromatographed on the Sephadex G-150 column, dialyzed against distilled water, and lyophilized.

The purified 7S globulin was dissolved in 0.05M Tris-HCl buffer, pH 7.6, containing 10mM β-mercaptoethanol and 0.2M NaCl and applied on a DEAE-Sephadex A-50 column (2.2×100 cm) which was equilibrated with the same buffer. The fractionation of globulin was performed with a stepwise elution with 0.2~0.3M NaCl at a flow rate of 30m*l*/hr. Each fraction was subjected to polyacrylamide gel electrophoresis. The γ-conglycinin fraction collected was dialyzed against distilled water and lyophilized.

Gel electrophoresis

Disc polyacrylamide gel eletrophoresis was performed by the method of Davis[24] using 5% acrylamide gel. Sodium dodecyl sulfate gel electrophoresis was carried out according to the procedure of Fairbanks et al.[25] Standard proteins used for the determination of molecular weight of γ-conglycinin were bovine serum albumin, ribonuclease A pepsin, trypsin and cytochrome c.

The gels were stained with either Amido black 10B[26] or with Coomassie blue solution[25] for protein. Periodic acid-Schiff base reagent[27]

was used for staining of glycoprotein. The scanning of the gels was carried out with a densitometer(Densitrol MDU-330, Toyo Inc', Japan) at 620nm using a 0.2×2mm slit.

Crossed immunoelectrophoresis

Soybean meal was extracted with 0.035M phosphate buffer, pH 7.6,[23] without β-mercaptoethanol. The extract was homogenized with Freund's complete adjuvant before injection to rabbit. The serum thus obtained was mixed with equal volume of phosphate buffered saline solution(pH 7.4, NaCl 8.0g, KCl 0.2g, anhydrous Na_2HPO_4 1.15g and KH_2PO_4 0.2g in 1l H_2O), and was partially purified for γ-globulin by saturated ammonium sulfate solution.[28]

Crossed immunoelectrophoresis was carried out according to the procedure of Bg-Hansen[29] using 1% agarose gel. The gels were stained with Coomassie blue solue solution.

Analytical gel isoelectric focusing

Gel isoelectric focusing of 7S globulin and γ-conglycinin in sucrose solution was performed with 7.5% polyacrylamide gel containing 1% ampholine (pH range of 3.5~10).[30]

Determination of carbohydrate

To determine the carbohydyrate moiety of γ-conglycinin, the rprotein was hydrolyzed with 4N HCl under nitrogen for 4hr at 108°C. The hydrolysate was eluted on a Dowex 50×4 column with 0.3N HCl.[31] Each fraction was analyzed for carbohydrate with phenol-sulfuric acid method[32] and for hexosamine with the method of Rondle and Morgan.[33]

The contents of hexose and hexosamine were determined using mannose and glucosamine as standard, respectively.

Amino acid analysis

Amino acid analysis for γ-conglycinin was performed with an amino acid autoanalyzer (Hitachi KLA-313, Japan). Methionine and 1/2 cystine were oxidized with performic acid[34] before analysis. Tryptophan was determined colorimetrically.[35]

N-terminal amino acids

N-terminal amino acid of γ-conglycinin were identified with[36] and dinitrophenyl[37] compounds using two dimensional thin layer chromatography technique.[38]

Two dimensional electrophoresis of γ-conglycinin subunits

Gel isoelectric focusing of γ-conglycinin in the presence of 9.7M urea was carried out according to the procedure of O'Farrel.[39] After the first run, the gel was subjected to the second run[39] on an SDS-polyacrylamide gel plate which was prepared according to Fairbanks et al.[25]

RESULTS AND DISCUSSION

Isolation of 7S glabulin

A gel filtration profile on Sephadex G-150 of soybean protein extracted with 0.2M NaCl at pH 4.5 is given in Fig. 1, which shows three major protein peaks. The first fraction eluted

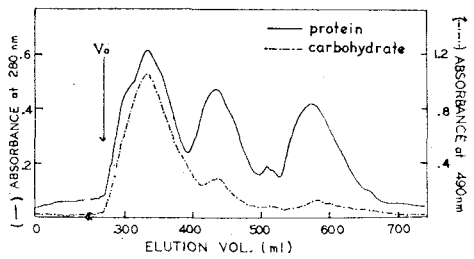

Fig. 1. Gel filtration profile on Sephadex G-150 of soybean protein extracted with 0.2M NaCl at pH 4.5. Vo represents the void volume of the gel.

near a void volume of the gel containing a considerable amount of carbohydrate was separated into two sub-fractions: one was turbid fraction and the other clear fraction. By comparing the mobility on gel electrophoresis with that of Hill and Breidenbach,[40,41] the turbid fraction was identified as 7S globulin. The turbidity occurring in 7S globulin might be a complex of protein and phytate[42] which is formed at the isoelectric point(*i.e.*, 4,5) of soybean protein during extraction. This complex could be removed either by centrifuge at 18,000 G or by adding Dowex 1×10.[42] Although the 7S globulin with a molecular weight less than 200 000

is expected to have about 400m*l* of the elution volume, the experiment showed the smaller value. It may imply that the Stokes' radius of 7S golbulin is larger than the actual dimension of the protein molecule.[43]

The clear fraction of the first protein peak (Fig. 1) and the second protein peak were unk nown and had a higher mobility on the gel electrophoresis than 7S globulin. The third peak was 2S globulin, judged by the gel electrophoresis mobility.[41,42]

Rechromatography of the turbid and clear fractions of the first protein peak(Fig. 1) on a Sephadex G-150 column revealed that the turbid fraction gave a single peak, whereas the clear fraction gave two peaks which had the same mobility on the gel electrophoresis. These results demonstrated that the unknown protein fraction eluted with 7S globulin could be removed by rechromatography on a Sephadex G-150 column.

Purification of γ-conglycinin

The result of DEAE-Sephadex A-50 chromatography of purified 7S globulin is shown in Fig. 2. A minor peak eluted at 0.23M NaCl

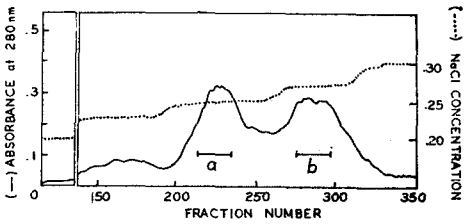

Fig. 2. DEAE-Sephadex A-50 chromatography of purified 7S globulin.

concentration could not be detected on the gel electrophoresis. The two protein fractions, "a" and "b" (Fig. 2), were eluted at 0.25M and 0.27M NaCl concentration, respectively. Both fractions were pure on the gel electrophoresis and the fraction "a" showed a higher mobility. Catsimpoolas and Exenstam[12] reported that γ-conglycinin had a higher mobility on the gel electrophoresis than B-conglycinin. Based on these results, the fraction "a" corresponded to γ-conglycinin and the fraction "b" to β-conglycinin.

Crossed immunoelectrophoresis showed that total soybean extracted with 0.2M NaCl had four major precipitins and five minor precipitins. It is known[12,14] that a precipitin with the smallest mobility on immunoelectrophoresis is γ-conglycinin. By comparing the mobility of the precipitins of total soybean protein, 7S globulin and the fractions "a" and "b" (Fig. 2), the fraction "a" was identified as γ-conglycinin and the fraction "b" as β-conglycinin. Both fractions "a" and "b" were immunoelectrophoretically pure.

Isoelectric point of γ-conglycinin

Analytical gel isoelectric focusing of 7S globulin and γ-conglycinin on polyacrylamide gel is shown in Fig. 3. 7S globulin showed two protein peaks which had isoelectric points at pH 4.8 and 5.4 (a in Fig.3). However, γ-conglycinin showed a single peak with an isoelectric point at pH 5.4 (b in Fig. 3).

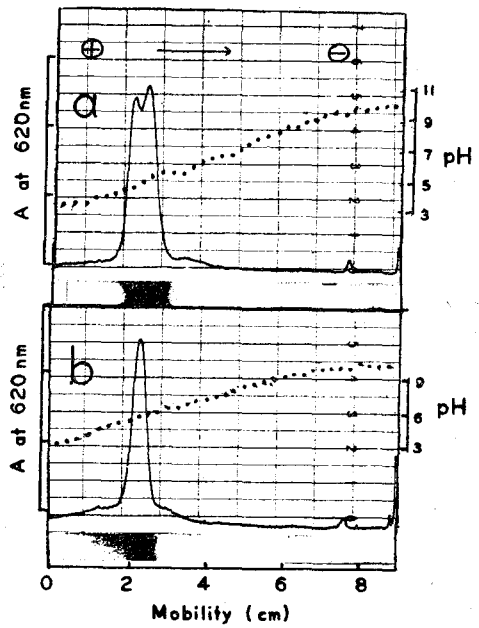

Fig. 3. Analytical gel isoelectric focusing on polyacrylamide gel of total 7S globulin (a) and γ-conglycinin. (b) The dotted line represent the pH gradient along the gel column after electrofocusing.

Although the isoelectric point of β-conglycinin (*i.e.*, 4.8) was similar to that(pH 4.9) reported by Koshiyama,[17] the isoelectric point of γ-conglycinin (pH 5.4) was somewhat lower than the value of 5.8 in literature.[21]

Carbohydrate moiety of γ-conglycinin

Column chromatography of the hydrolyzate of γ-conglycinin on a Dowex 50×4 column is given in Fig. 4. Paper chromatogram of hexose and hexosamine fractions showed that hexose was mannose and hexosamine was glucosamiine. The contents of mannose and glucosamine were 4.18% and 1.21%, respectively. These values were similiar to those of β-conglycinin, that is, 3.75% mannose and 1.19% glucosamine.[18~20]

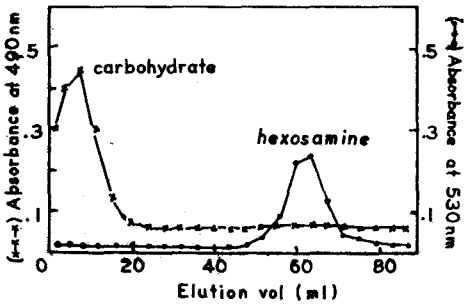

Fig. 4. Column chromatography of the hydrolyzate of soybean γ-conglycinin on Dowex 50×4.

Amino acid composition of γ-conglycinin

Amino acid composition of γ-conglycinin with that of β-conglycinin is presented in Table 1, which shows that both β-and γ-conglycinin contained substantial quantities of the dicarboxylic acids and/or their amides and small amounts of methionine, cysteine and tryptophan. These characteristics of amino acid compositions seem to be common in variety of legume vicilin.[7] The higher contents of dicarborylic acids and amonia nitrogen of γ-conglycinin reflect its role

Table 1. Amino acid compositions of γ-and β-conglycinin (gr./100gr. protein)

Amino acid	γ-conglycinin Author	β-conglycinin Koshiyama(36)
LYS	8.14	7.01
HIS	1.88	1.67
ARG	8.66	8.82
ASN	12.60	14.13
THR	2.20	2.81
SER	4.17	6.77
GLE	18.76	20.50
PRO	3.62	4.33
GLY	2.72	2.85
ALA	3.03	3.70
VAL	4.84	5.08
MET	0.74	0.25
IIE	5.36	6.40
LEU	9.20	10.25
TYR	2.82	3.61
PHE	6.83	7.39
1/2CYS	0.36	0.26
TRY	0.83	0.32
NH_3	2.62	1.71
Total	99.38	107.86

* Kjeldahl nitrogen contents ot β-and γ-conglycinin were 15.91% and 16.12% respectively.

Table 2. Color intensity of N-terminal amino acid derivatives of γ-conglycinin

N-Terminal amino acid	Dansyl derivatives	Dinitrophenyl derivatives
Aspartic acid	+	+
Serine	++	+
Glutamic acid	+	+
Glycine	++	++
Tyrosine	+	+
Valine	+	+

as a stroage protein.

The Kjeldahl nitrogen content for γ-conglycinin was 16.12%, which was higher than the reported value of 15.91% for β-conglycinin.[17] This appears to be due to the higher content of ammonia nitrogen in γ-conglycinin than β-conglycinin(Table. 1).

N-terminal amino acids of γ-conglycinin

N-terminal amino acids of γ-conglycinin which were determined by dansyl and dinitrophenyl derivatives were aspartic acid, serine, glutamic acid, glycine, tyrosine and valine(Table. 2). Serine and glycine showed a stronger colorintensity in dansyl derivatives and glycine in dinitrophenyl derivatives than other N-terminal amino acids.

The N-terminal amino acids for β-conglycinin were reported to be aspartic acid, serine, glycine, tyrosine, valine, leucine and alanine.[17,23] These results together with those for γ-conglycinin may imply that the subunits of β-and γ-congly cinin could have similarity.

Subunits of γ-conglycinin

An SDS-polyacrylamide gel electrophoretic scan of γ-conglycinin showed that γ-conglycinin was composed of six subunits(Fig. 5).

The molecular weights for subunits A and B (Fig. 5) were 38,000 and 32,000, respectively (Fig. 6). A small peak with the lowest mobility (C in Fig. 5) may be a thiol-disulfide polymer [45] and its molecular weight was estimated to be over 180,000. The molecular weights of other small peaks with high mobility(D,E,F in Fig. 5) were too small to be determined from Fig. 6 (probably less than 11,000).

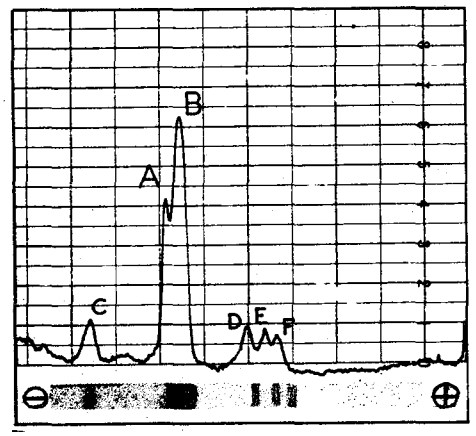

Fig. 5. A electrophoretic scan of sodium dodecyl sulfate gel with 5.6% acrylamide of γ-conglycinin.

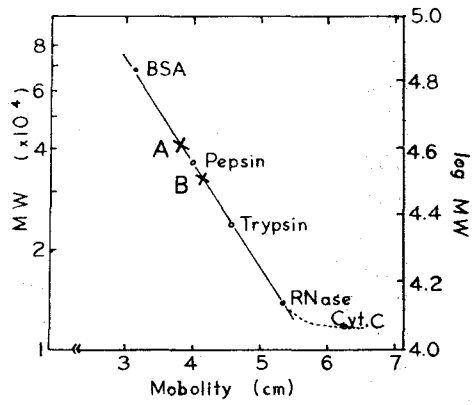

Fig. 6. Determination of the molecular weight of the subunits peak A and B liberated from γ-conglycinin on sodium dodecyl sulfate gel with 5.6% acrylamide.

Polyacrylamide gelisoelectric focusing, in the presence of 9.2M urea, of γ-conglycinin resulted

in eight main bands and five small bands at isoelectric points in the region of pH 4.6~5.5 (Fig. 7), indicating that γ-conglycinin consists of acidc subunits. Of these, the bands in the region of pH 4.6~5.0 were stained with periodic acid-Schiff base reagent, indicating that subunits with isoelectric points at pH 4.6~5.0 were glycoproteins. The subunits with isoelectric points at 5.0~5.5 were simple peptides.

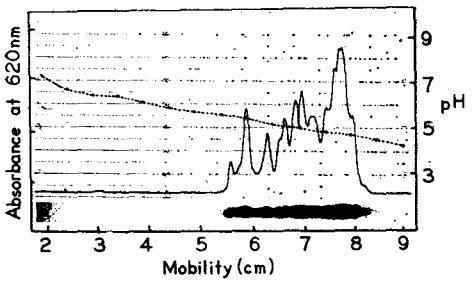

Fig. 7. A scan of polyacrylamide gel isoelectric focusing containing 9.2M urea of the subunits of γ-conglycinin. The dotted line represents the pH gradient.

Two dimensional polyacrylamide gel electrophoresis of γ-conglycinin subunits(Fig. 8) showed 6~8 bands, which in the region of pH 4.6~5.0 (B in Fig. 8) were stained with periodic acid-Schiffbase reagent. These results were in agreement with those found in Fig. 7. The bands having the fastest mobility (C in Fig. 8) were simple peptides and A in Fig. 8 might be a thiol-disulfide complex.[45]

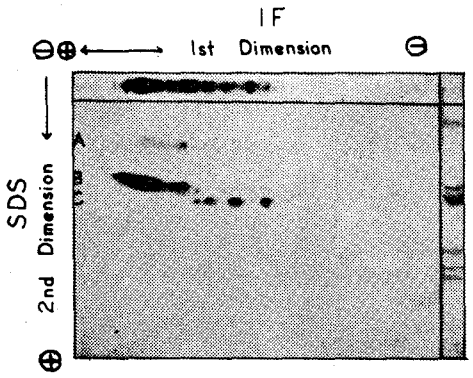

Fig. 8. Two dimensional polyacrylamide gel electrophoresis of γ-conglycinin subunits. Eirst run was subjected to gel isoelectric focusing containing 9.2M urea and second run to sodium dodecyl sulfate gel electrophoresis. The gel was stained with Coomassie blue.

Based on the N-terminal amino acids of γ-codglycinin (Table 2) and the results in Figs. 7 and 8, it seems that γ-conglycinin has 6~13 subunits. However, the thirteen bands of γ-conglycinin subunits on gel isoelectric focusing (Fig. 7) appears to be due to the heterogeneity of carbohydrate in glycoprotein[45] and/or of amide group of dicarboxylic acids.[47] It remains, therefore, to be elucidated how many bands appeared in gel isoelectric focusing are true subunits of γ-conglycinin.

The chemical composition and N-terminal amino acids of γ-conglycinin suggest that the subunits of γ-conglycinin could have with those of γ-conglycinin as suggested by Hill and Breidenbach[40,41] and Lee *el al.*[48]

要　　約

大豆의 γ-conglycinin을 精製하고 그의 物理一化學的 性質을 硏究하여 다음의 結果를 얻었다.

Gamma-conglycinin은 大豆의 pH 4.5, 0.2M Nacl 추출액을 Sephadex G-150으로 분리하여 얻은 7S globulin을 DEAE-Sephadex A-50으로 column chromatography 하여 精製하였다.

정제된 γ-conglycinin은 免疫電氣泳動, polyacrylamide gel電氣泳動과 gel isoelectric focusing 상으로 純粹하였다.

等電點은 pH 5.4이었으며 窒素 16.12%, mannose 4.18, glucosamine 1.21%의 含量이었다. 아미노산 組成은 一般的으로 lysine, dicarboxylic acid와 암모니아態 窒素의 含量이 높았고 含黃아미노산과 tryptophane의 含量은 낮은 편이었다.

Subunit의 等電點은 pH 4.5~5.5에 分布하였는데 그중 pH 4.6~5.0에 位置한 것은 分子量 38,000의 糖 peptide이었으며 pH 5.0~5.5의 것은 分子量 32,000의 單純 peptide이었다.

REFERENCES

1) Osborne, T.B. and Campbell, G.F.: J. Am. Chem Soc., **20**, 419(1898).

2) Danielsson, C.E.: Biochem. J., **44**, 387(1949).
3) Briatry, L.G., Coult, D.A. and Boulter, D.: J. Exp. Botany, **20**, 358(1969).
4) Wolf, W.J., Babcock, G.E. and Smith, A. K.: Arch. Biochem. Biophys., **99**, 265(1962).
5) Wolf, W.J.: Bakers Dig., **43**, 30(1969).
6) Koshiyama, I.: Intern. J. Peptide Protein Res., **4**, 167(1972).
7) Derbyshire, E., Wright, D.J. and Boulter. D.: Phytochemistry, **15**, 3(1976).
8) Vaintraub, I.A. and Shutov, A.D.: Biokhimiya, **34**, 984(1968).
9) Hasegawa, K., Kusano, T. and Mitsuda, H.: Agr. Biol. Chem., **27**, 878(1963).
10) Wolf, W.J. and Sly, D.A.: Arch. Biochem. Biophys., **127**, 338(1968).
11) Catsimpoolas, N.: Cereal Chem., **47**, 70(1970).
12) Catsimpoolas, N. and Ekenstam, C.: Arch. Biochem. Biophys., **129**, 490(1969).
13) Koshiyama, I.: Agr. Biol. Chem., **32**, 879 (1968).
14) Koshiyama, I.: Agr. Biol. Chem., **33**, 281 (1969).
15) Koshiyama, I.: Agr. Biol. Chem., **33**, 281 (1969).
16) Koshiyama, I.: Agr. Biol. Chem., **35**, 385 (1971).
17) Koshiyama, I.: Cereal Chem., **45**, 394(1968).
18) Keshiyama, I.: Agr. Biol. Chem., **30**, 646 (1966).
19) Koshiyama, I., Agr, Biol. Chem., **31**, 874 (1967).
20) Koshiyama. I., Arch. Biochem. Biophys., **130**, 370(1969).
21) Koshiyama, I. and Fukushima, D.: Phytochemistry, **15a** 157(1976).
22) Lee, C.Y., Kim, I.S. and Jo, D.H.: Korean Biochem. J., **2**, 129(1976).
23) Roberts, R.C. and Briggs. C.R.: Cereal Chem., **42**, 71(1976).
24) Davis, B.J.: Ann N.Y. Acad. Sci., **121**, 104(1964).
25) Fairbanks, G., Steck, T.L. and Wallach, D F..H.: Biochemistry, **10**, 2606(1971).
26) Woeller, E.H.: Anal. Biochem., **2**, 508(1961).
27) Segrest. A.K. and Jackson, R,L,: in Methods in Enzymology, Vol. 27, p.54(V. Ginburg, Ed , Acacemic Press, New York, 1973)
28) Kawamura, A., Jr.: Fluorescent Antibody Techniques and their Applications, p.11. University of Tokyo Press, Tokyo, 1969.
29) Bog-Hansen, T.C.: Anal. Biochem., **56**, 480 (1973).
30) Wrigley, C.W.: in New Techniques in Amino Acid Peptide and Protein Analysis, p.291 (A. Niederwieser and G. Pataki, Ed. Ann Arbor Science Publishers Inc., Michigan, 1973).
31) Gardell, S.: Acta Ccem. Scan., 19 207(1953).
32) Dubois, M., Gilles, K.A., Hamilton, J.K., Roberts, P.A. and Smith, F.: Anal. Chem., **28**, 350(1956).
33) Rondle, C.J.M. and Morgan, W.T.J.: Biochem. J., **61**, 586(1955).
34) Moore, S.: J. Biol. Chem., **238**, 235(1663).
35) Graham, C.E., Smith, E.P., Hier, S.W. and Klein, D.: J. Biol. Chem., **168**, 711(1947).
36) Gras, C. and Labouesse, B.: Eur. J. Biochem., **7**, 463(1969).
37) Niederwieser, A.: in Methods in Enzymoloyg, Vol. 25, p.89 (V. Ginburg, Ed., Academic Press, New York, 1971).
38) Deyl, Z. and Resmus, T.: J. Chromatog., **20**, 514(1965).
39) O'Farrell, P.H.: J. Biol. Chem., **250**, 4007 (1975).
40) Hill, J.E. and Breidenbach, R.W.: Plant Physiol., **53**, 742(1974).
41) Hill, J.E. adn Breidenbach, R.W.: Plant Physiol., **53**, 747(1974).
42) Smith, A.K. and Rackis, J.J.: J.Am. Chem. Soc., **79**, 633(1957).
43) Rackis, J.J., Anderson, R.L., Sasame, H.A. and Smith, A. K.: J. Agr. Food Chem., **9**, 409(1961).

44) Catsimpoolas, N., Leuthner, E. and Meyer, E.W.: Arch. Biochem., Biophys., **127**, 338 (1968).

45) Catsimpeelas, N., Ekenstam, C., Rogers, D A. and Meyer, E.W.: Biochem. Biophys. Acta, **168**, 122(1968).

46) Yamauchi F., Thanh, V.H., Kawase, M. and Shibasaki, K.: Agr. Biol. Chem., **40**, 691(1976).

47) Catsimpoolas, N. and Wang, J.: Anal. Bio chem., **44**. 436(1971).

48) Lee, C.Y., Kim, I.S. and Kim, S.U.: J Korean Agr. Chem. Soc., **20**, 26(1977).