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SYNTHESIS OF GLYCININ IN A WHEAT GERM CELL-FREE SYSTEM

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SUMMARY

Polyribosomes isolated from the cotyledons of developing soybean seeds were translated in a wheat germ cell-free system. When the radioactive translation products synthesized in the cell-free system were fractionated by centrifugation on sucrose density gradient, a radioactive peak which overlapped an authentic glycinin was detected. This radioactive co-sedimentable material was judged to be also a glycinin by its behavior toward polyacrylamide gel electrophoresis and immunoprecipitation.

INTRODUCTION

The molecular mechanism of the biosynthesis of a single polypeptide has been reasonably well understood, while much less is known concerning the process of subunit assembly in the biosynthesis of multisubunit proteins. Glycinin, a major component of soybean storage proteins (so-called 11S globulin), consists of at least six nonidentical polypeptides (1,2) and is localized in subcellular structure called protein bodies (3,4). Thus, glycinin probably is formed in the seed at least in three steps, i.e., synthesis of each subunit polypeptide (or precursor polypeptide), assembly of the subunits (including the formation of interchain disulfide bonds), and accumulation into protein bodies. A step of glycosylation, one of post-translational modification, can be precluded here, since glycinin has been found not to be glycosylated (5). In the present study, we attempted to provide a cell-free system for the synthesis of complete glycinin in order to investigate the molecular mechanism of the process of subunit

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assembly. The synthesis of a complete form of glycinin in a wheat germ cell-free system, including dialysis procedure, is demonstrated here with an analysis of the translation products by means of sucrose gradient centrifugation, polyacrylamide gel electrophoresis and immunoprecipitation.

MATERIALS AND METHODS

L-[U-^{14}C]Leucine (251 mCi/mmol) was purchased from the Radiochemical Centre, Amersham. Pancreatic RNase A was purchased from Worthington Biochemical Corp.

Free and membrane-bound polyribosomes were isolated from developing soybean seeds (cv. Tsuru-no-ko) according to the method of Blobel and Potter (6) as follows. The cotyledons were isolated from the seeds (150-300 mg each) and ground to a fine powder in a mortar containing liquid nitrogen. Three volumes of 0.2 M Tris-HCl buffer (pH 7.8) containing 60 mM KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol and 0.25 M sucrose were added, and the mixture was allowed to thaw and warm to 0-5°C. A discontinuous sucrose gradient consisted of 4 ml of 2 M sucrose overlayed with 3 ml of 1.38 M sucrose in 20 mM Tris-HCl buffer (pH 7.8) containing 20 mM KCl, 5 mM magnesium acetate and 6 mM 2-mercaptoethanol. Centrifugation was carried out at 2°C and 40,000 rpm for 8.5 h in the RP65T rotor (Hitachi). The membranous material at the lower interface and the pellet were termed membrane-bound and free polyribosomes, respectively. The membrane-bound polyribosomes were collected by centrifugation at 45,000 rpm for 4 h in the RP65T rotor. Each polyribosome pellet was suspended in a small volume of 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 M KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, 40% glycerol and 0.5% Triton X-100. A higher yield of free polyribosomes was obtained than that of membrane-bound polyribosomes.

A cell-free system for protein synthesis used for translation of polyribosomes was basically the same as that of Roberts and Paterson (7). Cell-free synthesis was performed in 0.5 ml reaction mixture as described previously (8). About 2 A_{260} units of free polyribosomes was used. After incubation, 9 volumes of high-salt buffer (0.035 M potassium phosphate (pH 7.6) and 0.4 M NaCl) were added to the reaction mixture, polyribosomes removed by centrifugation and EDTA (final 25 mM) and pancreatic RNase (final 25 μg/ml) were added to the supernatant, followed by incubation at 37°C for 15 min. After incubation, the sample was concentrated to approximately 1 ml by ultrafiltration, authentic glycinin (0.5 A_{280} unit) added to the sample, followed by dialysis against 100 ml of the high-salt buffer containing 20% glycerol at 20°C for 24 h and then 40 h with a change of buffer. After dialysis, the sample was concentrated to 0.4 ml and subjected to analysis on the sucrose gradient centrifugation.

Centrifugation was performed at 20°C and 36,000 rpm for 16 h in the RPS40T rotor (Hitachi) using linear sucrose density gradient (10-30%, w/v) in the high-salt buffer. After centrifugation, the gradient was fractionated into each 0.6 ml fraction and measured at 280 nm simultaneously using an ISCO density gradient fractionator.

Polyacrylamide gel electrophoresis was performed in 6.5% gels according to the method of Davis (9).

Authentic glycinin was prepared from dry soybean seeds (cv. Tsuru-no-ko) using two cycles of sucrose gradient centrifugation described by Hill and Breidenbach (10) and followed by DEAE-Sephadex column chromatography described by Catsimpoolas et al. (11). Rabbits were immunized with the authentic glycinin solution and an equal volume of Freund's complete adjuvant (Difco). A single precipitation band was observed between the antiserum and the whole soybean extract in a double gel immunodiffusion (data not shown).

RESULTS AND DISCUSSION

Figure 1 shows the sedimentation profiles of the translation products directed by free polyribosomes in the wheat germ cell-free system. A radioactive peak which overlapped an authentic glycinin (11S peak) was detected in the sucrose gradient. The amount of radioactivity sedimented at 11S peak was about 12.9% of the total radioactivity applied on the gradient. There were no significant differences in the appearance of radioactivity at 11S peak and in the amino acid incorporating activity between free and membrane-bound polyribosomes. The absence of carrier authentic glycinin did not affect the appearance of radioactivity at 11S peak (Table I, 2nd line). In the mixing test (Table I, 3rd line), the radioactive top fraction was collected from the sucrose gradient after centrifugation, as shown in Fig. 1, except authentic glycinin was not added prior to the dialysis procedure. To this top fraction authentic glycinin was added and then centrifuged on a similar sucrose gradient. There was no signifi= cant radioactivity at 11S peak. These results indicate that the radioactivity sedimented at 11S peak was not due to some non-specific association of the radioactive translation products with the carrier of authentic glycinin. The radioactive materials at 11S peak were then analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 2, the mobility of the radioactive material

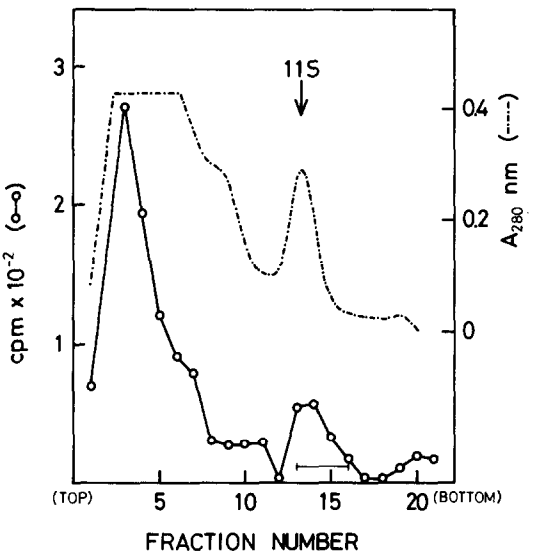

Figure 1. Sucrose density gradient centrifugation of ^{14}C-labeled translation products. After centrifugation, carrier bovine serum albumin (0.2 mg protein) and then 0.6 ml of cold 10% trichloro= acetic acid (TCA) were added to each fraction. The precipitate formed was collected, solubilized with an aliquot of 0.1 N NaOH and applied to a filter paper disk. The disk was washed with cold 5% TCA and counted in 10 ml of a toluene-based scintillation mixture. Arrow indicates the sedimentation position of an authentic glycinin (11S).

Table I

Formation of presumed glycinin under various conditions.

Condition	Radioactivity at 11S peak (%)*
Control	100
Minus authentic glycinin	98
Mixing test	31
Plus anti-glycinin rabbit serum	53
Blank**	23

* Control was regarded as 100%.
** When the in vitro translation product was treated with 7 M urea and 0.2 M 2-mercaptoethanol prior to dialysis, the radioactivity and UV absorbance at 11S peak decreased significantly. Both the formation and reconstruction of glycinin seem to be prevented in such a condition, since various kinds of wheat germ proteins besides the subunit polypeptides of glycinin coexist in the system. Therefore, this small amount of radioactivity at 11S peak was regarded as a back ground level.

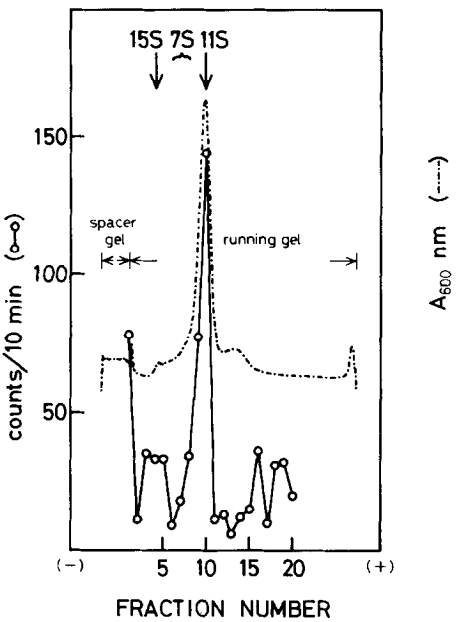

Figure 2. Polyacrylamide gel electrophoresis of ^{14}C-labeled translation products. The radioactive material sedimented at 11S peak (underlined part in Fig. 1) was electrophoresed as described in the text. After electrophoresis, the gel was cut into 3 mm slices and dissolved with 0.5 ml of 30% H_2O_2 at 60°C overnight, then counted in 15 ml of a Triton X-100-toluene-based scintillation mixture. The gel in which the authentic glycinin preparation was electrophoresed was stained with Amidoblack. Arrows indicate the migration position of the major storage proteins of soybean, so-called 7S, 11S (glycinin) and 15S components.

coincided with that of authentic glycinin. No significant radioactivity was detected apart from the bands of glycinin.

These results demonstrate that glycinin-like material was formed in *in vitro* system directed by the polyribosomes isolated from the developing soybean seeds. This was further supported by the immunological analysis of the radioactive materials at 11S peak. As shown in Table I (4th line), when the *in vitro* translation product was treated with the anti-glycinin serum prior to centrifugation on sucrose gradient, the radioactivity at 11S peak decreased significantly.

In the present experiment, dialysis was carried out to allow formation of disulfide bridges between the subunit polypeptides

synthesized in the cell-free system. Such disulfide bridge formation during dialysis has recently been reported by Bester et al. (12) in thyroglobulin synthesis in a reticulocyte cell-free system. They suggested that limited hybridization between carrier and newly synthesized half-molecules of thyroglobulin occurred during dialysis. However, this may not be the case for glycinin synthesis, since it is known that glycinin can dissociate into its subunit polypeptides in the presence of both urea and 2-mercaptoethanol (13). This suggests that radioactive translation products sedimented at 11S peak consist of all newly synthesized polypeptides. Further detailed analysis of the composition of the radioactive polypeptides of 11S translation products may shed some light on this point.

The synthesis of subunit polypeptides in a cell-free system has been demonstrated in studies investigating the biosynthesis of various seed storage proteins having subunit structure (8,14-21). However, the synthesis of complete multimeric proteins has not been demonstrated in those studies. This is the first report of the detection of a complete form of multisubunit protein synthesized in a cell-free system including dialysis procedure. This system is expected to be useful in subsequent studies in elucidating the mechanisms of subunit assembly into complete multisubunit protein in addition to the biosynthesis of its subunit polypeptides.

REFERENCES

1. Catsimpoolas, N. (1969) FEBS Lett. 4, 259-261.
2. Kitamura, K., Takagi, T., and Shibasaki, K. (1976) Agric. Biol. Chem. 40, 1837-1844.
3. Catsimpoolas, N., Campbell, T. G., and Meyer, E. W. (1968) Plant Physiol. 43, 799-805.
4. Koshiyama, I. (1972) Agric. Biol. Chem. 36, 62-67.
5. Koshiyama, I., and Fukushima, D. (1976) Cereal Chem. 53, 768-769.

6. Blobel, G., and Potter, V. R. (1967) J. Mol. Biol. 26, 279-292.
7. Roberts, B. E., and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. USA. 70, 2330-2334.
8. Mori, T., Wakabayashi, Y., and Takagi, S. (1978) J. Biochem. 84, 1103-1111.
9. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427.
10. Hill, J. E., and Breidenbach, R. W. (1974) Plant Physiol. 53, 742-746.
11. Catsimpoolas, N., Rogers, D. A., Circle, S. J., and Meyer, E. W. (1967) Cereal. Chem. 44, 631-637.
12. Bester, A. J., Pammenter, M., Gevers, W., and Van Jaarsveld, P. (1977) Biochem. Biophys. Res. Commun. 78, 230-236.
13. Kitamura, K., Takagi, T., and Shibasaki, K. (1977) Agric. Biol. Chem. 41, 833-840.
14. Sun, S. M., Buchbinder, B. U., and Hall, T. C. (1975) Plant Physiol. 56, 780-785.
15. Burr, B., and Burr, F. A. (1976) Proc. Natl. Acad. Sci. USA. 73, 515-519.
16. Larkins, B. A., jones, R. A., and Tsai, C. Y. (1976) Biochemistry 15, 5506-5511.
17. Luthe, D. S., and Peterson, D. M. (1977) Plant Physiol. 59, 836-841.
18. Burr, B., Burr, F. A., Rubenstein, I., and Simon, M. N. (1978) Proc. Natl. Acad. Sci. USA. 75, 696-700.
19. Sun, S. M., Mutschler, M. A., Bliss, F. A., and Hall, T. C. (1978) Plant Physiol. 61, 918-923.
20. Hall, T. C., Ma, Y., Buchbinder, B. U., Pyne, J. W., Sun, S. M., and Bliss, F. A. (1978) Proc. Natl. Acad. Sci. USA. 75, 3196-3200.
21. Beachy, R. N., Thompson, J. F., and Madison, J. T. (1978) Plant Physiol. 61, 139-144.