In Vitro Synthesis of Zein-like Protein by Maize Polyribosomes

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SUMMARY

Free and membrane-bound polyribosomes were isolated in an undegraded form from developing maize kernels. Translation of the membrane-bound polyribosomes in vitro produced one main radioactive protein. This protein was soluble in 70% ethanol and had the same mobility in electrophoresis on sodium dodecyl sulfate-gels as a zein standard. The ratio of [^{14}C] leucine to [^{14}C] lysine incorporated into the 70% ethanol extractable protein was similar to the mole fraction ratio of these amino acids in zein. The zein-like protein may represent as much as 50% of the total protein synthesized by the membrane-bound polyribosomes.

INTRODUCTION

Two major classes of storage protein are synthesized in the developing endosperm of maize (1). One of these is a 70% ethanol-soluble protein known as zein, much of which is compartmentalized in distinct protein bodies (2). Electron micrographs of developing maize endosperm reveal a close association between these protein bodies and the rough endoplasmic reticulum (3), however, the role of membrane-bound polyribosomes in the synthesis of zein or any other plant storage protein has not been demonstrated.

The discovery in recent years of "opaque" mutants which accumulate small amounts of zein but increase the content of non-zein protein has greatly improved the nutritional quality of maize seed protein (4). However, virtually nothing is known about how these mutations alter storage protein synthesis, nor how zein synthesis is controlled.

Protein synthesized by a protein body fraction derived from developing wheat endosperm has been reported, but no attempt was made to analyze the types of protein produced (5). An attempt to repeat these experiments using a protein

body fraction from corn endosperm was unsuccessful (6).

This communication is the first report of the successful *in vitro* synthesis of a plant storage protein, and demonstrates the role of membrane-bound polyribosomes in zein synthesis.

MATERIALS AND METHODS

Free and membrane-bound polyribosomes were isolated in an undegraded form from frozen 23-day post-pollination kernels of maize. Kernels were ground in 3-5 volumes of buffer A (0.2 M tris-HCl, pH 8.5; 0.2 M sucrose; 0.06 M KCl; 0.05 M $MgCl_2$; 0.005 M dithiothreitol), strained through 4 layers of cheesecloth, and centrifuged at 500 x g for 5 min. in a Sorvall SS-34 rotor. The supernatant was centrifuged at 37,000 x g to separate the free and membrane-bound polyribosomes (7, 8). The supernatant containing free polyribosomes was decanted and layered over 4 ml of 1.75 M sucrose in buffer B (0.04 M tris-HCl, pH 8.5; 0.02 M KCl; and 0.01 M $MgCl_2$). The 37,000 g pellet was resuspended in buffer A containing 1% Triton X-100. After 15 min. the insoluble material was removed by centrifugation at 37,000 x g for 10 min. The supernatant containing detergent-solubilized membrane-bound polyribosomes was also layered over 4 ml of 1.75 M sucrose in buffer B. Polyribosomes were pelleted by centrifugation for 75 min. at 229,000 x g avg. in a 65 rotor of a Beckman L2-65 ultracentrifuge. Polyribosome pellets were resuspended in buffer B and layered on linear 15-60% sucrose gradients and centrifuged at 189,000 x g avg. for 45 min. in a Beckman SW 50.1 rotor (8, 9). The optical density at 254 nm was recorded with an ISCO Model UA-5 absorbance monitor.

Protein synthesis was conducted by adding maize polyribosomes to an *in vitro* system derived from wheat germ (10). The reaction mixture contained: 1-2 A_{260} units of polyribosomes, 20 ug tRNA, 0.12 ml of S23[1], 0.048 M KCl, 0.004 M Mg-acetate, 0.001 M ATP, 35 uM GTP, 0.011 M creatine phosphate, 0.016 mg. creatine kinase, 0.035 M tris-acetate at pH 8.0, 0.003 M dithiothreitol, 45 uM each of 19 amino acids, and either 0.125 uCi of [^{14}C]leucine (175 mCi/mM) or [^{14}C]-lysine (50 mCi/mM) (Schwartz Bioresearch Inc.) in a final volume of 0.28 ml. Assays were incubated for 60 min. at 30° C.

Hot 5% CCl_3COOH-insoluble and hot 70% ethanol-soluble protein from the *in vitro* assays were dialyzed in an SDS buffer (0.05 M tris-HCl, pH 6.9; 0.5% SDS; 1% 2-mercaptoethanol) and analyzed by electrophoresis in 15% SDS-gels (15% acrylamide; 0.3% bisacrylamide; 0.1% SDS; 0.375 M tris-HCl, pH 8.5; 0.025% TEMED) at 4 ma/gel for 3 hr. (11). The hot acid-insoluble protein from reaction mixtures without polysomes was used as a control. A purified zein preparation (Nutritional Biochemicals Corporation) was solubilized in SDS buffer and used as a standard. The zein standard was stained with Coomassie blue and scanned at 600 nm.

The incorporation of [^{14}C]leucine and [^{14}C]lysine into protein was determined by filter paper disk techniques. One half of a reaction mixture was spotted on a cellulose disk and the hot acid-insoluble radioactivity determined (12). The other half of the reaction mixture was adjusted to 70% ethanol and extracted at 60° C for 90 min. The hot ethanol-soluble protein was spotted on cellulose disks and processed through a zein purification procedure (13). The assays were conducted in triplicate and radioactive samples were counted to 7% relative standard error. Lysine counts were multiplied by 3.5 to correct for the difference in specific activity of the two amino acids in the reaction mixtures.

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[1] *Abbreviations*: S23 is a post 23,000 x g supernatant obtained from wheat germ; Sodium dodecyl sulfate, SDS: N,N,N',N'-tetramethylenediamine, TEMED.

RESULTS AND DISCUSSION

Typical density-gradient profiles of free and initially membrane-bound polyribosomes are depicted in Fig. 1. Both free and membrane-bound polyribosomes gave A_{260}/A_{280} ratios of 1.7, indicating a high degree of purification (14). The free polyribosomes showed a normal size distribution with the 10 or 11-mer as the size-class of maximum absorbance (Fig. 1-A). The membrane-bound polyribosomes contained several distinct polyribosome size-classes. Fig. 1-B shows a major absorbance peak at the 8-mer. Several other large absorbance peaks were separated which sedimented deeper in the gradient, but their size-classes were not resolved. The membrane-bound polyribosomes were susceptible to ribonuclease (Fig. 1-C), as were the free polyribosomes (data not shown). Further analysis of the membrane-bound polyribosomes will be presented separately (manuscript in preparation).

Both classes of polyribosomes were found to actively synthesize protein

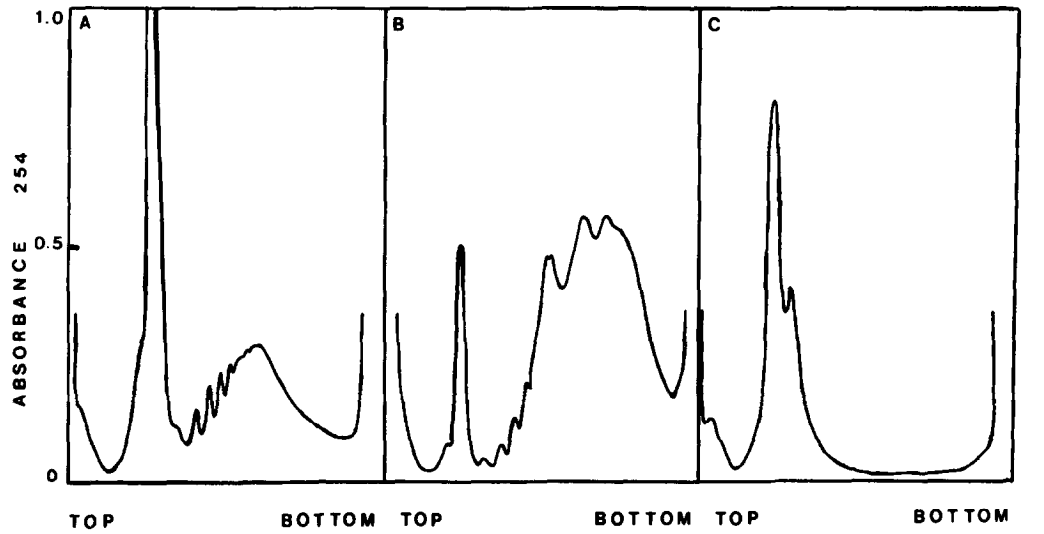


Figure 1

Free and initially membrane-bound polyribosomes from 23-day post-pollinated maize kernels. Polyribosome pellets were resuspended in buffer B (A, 1.92 A_{260} units of free polyribosomes; B, 2.1 A_{260} units of membrane-bound polyribosomes) or buffer B plus 1 ug/ml pancreatic ribonuclease (C, 0.55 A_{260} units of membrane-bound polyribosomes) and separated on 15-60% sucrose gradients. The optical density at 254 nm was scanned with an ISCO UA-5 absorbance monitor.

when incubated in a heterologous *in vitro* protein synthesizing system derived from wheat germ (Table 1). SDS-gel electrophoresis of [^{14}C]leucine-labeled protein was done to determine if zein synthesis had occurred *in vitro*. Since zein is isolated on the basis of its solubility in hot 70% ethanol (13), both

Table 1

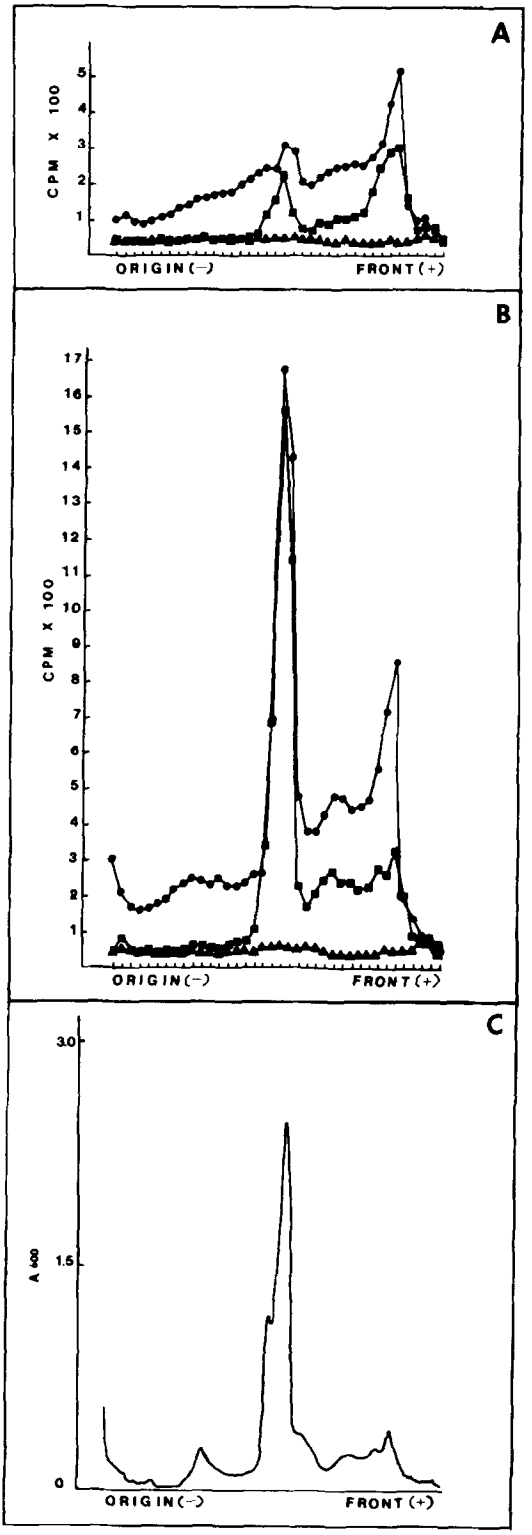
Comparison of [^{14}C] Leucine and [^{14}C] Lysine Incorporation into Protein Synthesized by Free and Membrane-bound Polyribosome Fractions.

Polyribosome Type	Hot Acid-Insoluble Radioactivity (CPM)		Hot 70% Ethanol-Soluble Radioactivity (CPM)		
	Leucine	Lysine	Leucine	Lysine	Lysine/Leucine
Free	18,602	12,764	1,615	193	0.012
Membrane-Bound	35,358	7,469	18,810	332	0.0175

Distribution of radioactivity in hot acid-insoluble and hot 70% ethanol-soluble protein synthesized by free and membrane-bound polyribosome fractions. Assays and extraction procedures are described in Methods and Materials. The radioactive counts were the averages of triplicate assays, and were counted to a relative standard error of 7%. Lysine counts were multiplied by 3.5 to correct for the difference in specific activity between lysine and leucine in reaction mixtures. The counts per minute are expressed per total reaction mixture.

the acid-insoluble and hot ethanol-soluble protein were electrophoresed. A purified zein preparation was electrophoresed as a standard (Fig. 2-C) and revealed one intensely stained region consisting of two bands. Other minor bands migrating faster and slower than the two main bands also appeared in the gel, but they represented only a small fraction of the total protein.

The hot acid-insoluble and hot 70% ethanol-soluble protein synthesized by the membrane-bound polyribosomes included a major radioactive peak (Fig. 2-B) that coincided with the two main bands of the zein standard (Fig. 2-C). This peak represented 34% of the acid-insoluble radioactive protein and 57% of the total hot ethanol-soluble radioactive protein. Both the hot acid-insoluble and


A
CPM X 100
ORIGIN(−)
FRONT(+)
B
CPM X 100
ORIGIN(−)
FRONT(+)
C
A 400
ORIGIN(−)
FRONT(+)

hot ethanol-soluble protein fractions contained radioactive material of smaller molecular weight migrating nearer the front. This low molecular weight protein may represent incomplete polypeptide chains which were not removed during dialysis.

SDS-gels of the ethanol-soluble products of the free polyribosomes showed a radioactive peak corresponding to the two zein bands (Fig. 2-A), although it represented a smaller fraction of the total radioactivity (Fig. 2-A cf. Fig. 2-B). These results demonstrate that a zein-like protein was synthesized *in vitro*, and it was the major product of the membrane-bound polyribosomes.

Since zein has no known enzymatic activity, other methods of identification were necessary. Attempts were made to characterize the radioactive products based on the unusual amino acid composition of zein. Zein contains 20.2 mole % leucine, but only 0.25 mole % lysine (15). If zein were the major product of the membrane-bound polyribosomes, the protein synthesized *in vitro* should be labeled more extensively by [^{14}C] leucine than by [^{14}C] lysine. In addition, the ratio of lysine/leucine incorporated into the 70% hot-ethanol fraction should approximate the mole fraction ratio reported for these two amino acids in zein.

Table 1 shows typical results of [^{14}C] leucine and [^{14}C] lysine incorporation into hot acid-insoluble and hot ethanol-soluble protein produced by equal amounts of either free or membrane-bound polyribosomes. When [^{14}C] leucine in the hot acid-insoluble protein was used as a measure of total incorporation, then the membrane-bound polyribosomes incorporated more than 50% of the [^{14}C]-leucine into ethanol soluble protein (18,810/35,358 x 100). The free polyribosomes, however, incorporated only 9% (1615/18,062 x 100) of the [^{14}C] leucine into ethanol soluble protein

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Figure 2

SDS-gel electrophoresis of [^{14}C] leucine labelled products of free and membrane-bound polyribosomes. Figure 2-A depicts the hot acid-insoluble (●) and the hot 70% ethanol-soluble (■) protein synthesized by the free polyribosomes. Figure 2-B depicts the hot acid-insoluble (●) and the hot 70% ethanol-soluble (■) protein synthesized by the membrane-bound polyribosomes. The hot acid-insoluble protein from control mixtures (▲) did not contain polyribosomes (Fig. 2-A, Fig. 2-B). Figure 2-C, the zein standard, was stained with Coomassie blue and scanned at 600 nm.

These results suggest that the ethanol-soluble protein was synthesized predominantly by the membrane-bound polyribosomes.

The ratio of [^{14}C] lysine to [^{14}C] leucine in the ethanol soluble protein synthesized by the free and membrane-bound polyribosomes gave values of 0.012 and 0.0175 respectively. These values approximate the lysine/leucine mole fraction ratio of 0.0125 present in zein (15). If it is assumed that complete polypeptide chains were synthesized in vitro, as is suggested by Fig. 2, then these ratios indicate that the protein produced in vitro had the lysine and leucine composition expected of zein (15).

The solubility in 70% ethanol, identical mobility in SDS-gels, and lysine/leucine ratio, strongly suggest that the protein synthesized in vitro is zein. Experiments to further characterize the products based on immunoprecipitation are in progress.

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