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ISOLATION OF WHEAT RIBOSOMES FREE OF HIGH MOLECULAR WEIGHT INHIBITORS OF THE NATURAL MESSENGER TRANSLATION

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The cell-free extract from wheat germ contains an inhibitor interfering with translation of a natural template (BMV RNA). The inhibitor affects neither the translation of poly(U) nor the aminocyclation of tRNA. It exhibits the activity of protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37). The inhibitor is found in lipoprotein aggregates which can be separated from ribosomes on Sepharose 2B column. Ribosomes purified on the Sepharose are several times more active in translation of BMV RNA than those isolated by conventional methods.

We have shown previously that the wheat germ cell-free system contains low-molecular-weight inhibitors of translation which diminish the protein synthesis directed both by natural and synthetic templates (Zagórska, 1978b; Rychlik & Zagórska, 1978). It seems that these inhibitors affect the aminoacyl-tRNA synthetases, leading to diminished formation of aminoacyl-tRNA. Beside these dialysable inhibitors, high-molecular-weight components, diminishing translation of natural mRNA but not that of a synthetic template, poly(U), were detected in the cell-free system (Rychlik & Zagórska, 1978). These inhibitors, found in the ribosomal fraction, strongly interfered with translation of natural mRNA in the cell-free system composed of isolated ribosomes and postribosomal supernatant.

The present communication describes the separation of wheat germ ribosomes from the fraction containing high-molecular-weight inhibitors of translation. Such purified ribosomes are several times more active in protein synthesis than those isolated by standard methods.

From the inhibitor-containing fraction, we purified a polypeptide revealing activity of protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37), which, when added to the incorporation system, inhibited translation of BMV RNA.  

* This work was supported by the Polish Academy of Sciences within the project no. 09.7.1, and in part by the U.S. Department of Agriculture, Grant no. FG-Po-334.

1 Abbreviations: BMV, Brome mosaic virus; DMSO, dimethyl sulphoxide; DTT, dithiothreitol (Cleland reagent); HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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It seems that mode of action of this protein kinase resembles that of cyclic 3',5'-AMP-independent protein kinase, implicated in the regulation of globin translation in the reticulocyte lysates (Datta et al., 1977).

MATERIALS AND METHODS

Chemicals. [γ-32P]ATP synthesized according to Post & Sen (1967) was kindly provided by Dr. T. Mańkowski of this Institute. Medical X-Ray films (super R blue base) were from Fotopan (Warsaw, Poland). DMSO was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) or from Reachim (U.S.S.R.). PPO was from POCh (Gliwice, Poland). Sepharose 2B was from Pharmacia (Uppsala, Sweden); other reagents were as described previously (Rychlik & Zagórski, 1978).

Cell-free extract, ribosomes and postribosomal supernatant. The wheat germ cell-free extract and postribosomal supernatant used in the translation experiments were isolated and activated by extensive dialysis as described previously (Zagórski, 1978b; Rychlik & Zagórski, 1978). All preparative steps were carried out at 0 - 4°C. The buffer used for isolation of subcellular fractions (buffer A) was composed of 3 mm-Mg/acetate, 50 mm-K/acetate, 10 mm-Tris/acetate, pH 7.5, and 1 mm-DTT. The ribosomes were isolated by two different methods.

(a) Direct ultracentrifugation of the cell-free extract. Undialysed cell-free extract prepared as previously described (Zagórski, 1978b; Rychlik & Zagórski, 1978), was submitted to ultracentrifugation in MSE 65 titanium rotor (10 × 10 ml) at 110 000 g for 3 h. The ribosomal pellet was suspended in a small volume of buffer A and centrifuged at 23 000 g for 10 min.

This preparation, called ribosomes I, was additionally purified by centrifugation in 10 × 10 ml MSE 65 titanium rotor (110 000 g, 4 h) through a 4 ml layer of 2 m-sucrose in buffer A. This step was repeated twice. The ribosomal pellet was suspended in buffer A and spun down as above. The final preparation was called ribosomes II.

(b) Gel filtration on the Sepharose 2B column. Twenty ml of non-dialysed cell-free extract prepared according to Rychlik & Zagórski (1978) by grinding 20 g of wheat germ with 60 ml of buffer A (final protein concentration in the 23 000 g extract was <0.5 mg/ml) was applied on the Sepharose 2B column (3.4 × 45 cm) equilibrated with buffer A. The column was developed with the same buffer at a flow rate of 40 ml/h. Fractions of 10 ml each were collected and their optical density at 260 nm was measured. The ribosomal peak (peak B on Fig. 1) was collected and centrifuged at 23 000 g for 20 min. Finally, the ribosomes were concentrated by ultracentrifugation as described in section (a). This preparation was called ribosomes III.

Average yield of ribosomes I, II, III from 10 g of wheat germ was equal to 60, 20, 60 mg of protein, respectively.

The peak preceding the ribosomal fraction, eluted in the void volume (peak A on Fig. 1), was collected separately, submitted to centrifugation at 23 000 g for 20 min and the aggregates obtained were tested for the activity of translation inhibitor. Extraction of the aggregates with 2 m-LiCl and chromatography on hydroxyl-
apatite and CM-Sepharose allowed us to demonstrate the presence of an electrophoretically homogeneous polypeptide (mol. wt. about 20,000) showing activity of protein kinase and inhibiting translation of BMV RNA (to be published).

**Translation assay and aminoacyl-tRNA synthesis.** Overall conditions for the incorporation of [³⁵C]leucine into BMV RNA-directed products, poly(U)-directed polyphenylalanine synthesis, and formation of [³⁵C]phenylalanine tRNA were described previously (Rychlik & Zagórska, 1978). Details concerning incorporation mixtures are included in the legends to Figures. Radioactivity was measured as described previously (Rychlik & Zagórska, 1978).

**Protein kinase assay.** The assay mixture, 50 μl, contained 33 μM-[γ-³²P]ATP (spec. act. 1.0 Ci/mmol); 20 mm-HEPES/KOH buffer, pH 7.5; 60 mm-Mg/acetate; 100 μg of casein as a substrate, and 200 ng of purified translation inhibitor; it was incubated at 31°C. At indicated time intervals, 4 μl samples were withdrawn, spotted onto Whatman 3MM filter paper discs, washed for 10 min in 10% trichloroacetic acid - 3% phosphoric acid mixture, and then washed according to Mans & Novelli (1961).

**Isolation of BMV, BMV RNA and wheat germ tRNA.** These preparations were obtained as described previously (Zagórska, 1978b).

**Identification of polypeptides synthesized under direction of BMV RNA.** This was performed according to Laemmli (1970). Autoradiography was according to Bonner & Laskey (1974). Appropriate portions of the incorporation mixture (cf Rychlik & Zagórska, 1978) were made up to 15 μl with H₂O, and supplemented with 5 μl of an 8% solution of SDS in 250 mm-Tris/HCl, pH 6.8, containing 30% glycerol, 3 μl of β-mercaptoethanol and 1 μl of 0.1% bromophenol blue. Samples were boiled for 2 min and submitted to electrophoresis. The electrophoresis was run at 30 mA for about 4 h, till the front migrated for 11 cm into the separating gel (12.5% acrylamide). Gel thickness was 1.1 mm.

After electrophoresis, gels were impregnated with PPO and submitted to fluorography at −60°C for one to two weeks.

**Protein estimation.** Protein content was estimated by the method of Lowry et al. (1951), as described previously (Zagórska, 1978b).

**RESULTS AND DISCUSSION**

The system composed of ribosomes isolated by direct ultracentrifugation and postribosomal supernatant activated by dialysis (Rychlik & Zagórska, 1978), when tested for amino acid incorporation into proteins, was less active than the system containing the unfractionated cell-free extract. Moreover, the ribosomes isolated by direct ultracentrifugation stimulated incorporation only in a limited range of ribosome concentration, not exceeding 60 μg per 25 μl of incorporation mixture. At higher ribosome concentration, inhibition of the BMV RNA translation was quite often observed.
It was assumed that the ribosomal preparation contains some inhibitory material cosedimenting with ribosomes during ultracentrifugation. To check this, the cell-free extract was filtered through the Sepharose 2B column (Fig. 1) before sedimentation of ribosomes. Of the three peaks separated on the column, peak B contained ribosomes. Peak C represented the soluble portion of the cell-free extract. Peak A, which was excluded in the void volume, contained heavy aggregates composed of lipids, proteins and RNA. When the proteins extracted from these aggregates by 4 M-urea and 2 M-LiCl were submitted to polyacrylamide-gel electrophoresis under conditions described by Giseland & Staehelin (1967), their pattern was entirely different from that of 80S ribosomal proteins (not shown). It was, therefore, concluded that peak A did not represent the polysomal fraction or aggregates of 80S ribosomes.

![Graph](image)

Fig. 1. Fractionation of wheat germ cell-free extract on the Sepharose 2B column. Fractions from 0.35 to 0.42 of total volume of the column (peak A) were pooled and aggregates were spun down at 23,000 g for 20 min. The pellet (37 mg of protein) was suspended in buffer A and its activity was tested in incorporation experiments. Fractions from the shadowed area of peak B (0.51 - 0.71 of total volume of the column) were pooled, centrifuged at 23,000 g for 10 min, and the ribosomes were pelleted by ultracentrifugation. Then the ribosomes (66 mg of protein) were dissolved in a small volume of buffer A and used for the incorporation experiments.

The material found in peak A, when added to the incorporation system, diminished translation of BMV RNA but not that of a synthetic template. The formation of aminoacyl-tRNA was also unaffected by the presence of the material from peak A (Fig. 2). Translation of natural mRNA results both from specific initiation and elongation in polypeptide synthesis. In contrast to that, poly(U) translation occurring at high Mg²⁺ concentration may be taken as a measure of elongation alone. Therefore, the results presented in Fig. 2 seem to indicate that the translation inhibitors present in peak A prevented specifically the initiation of polypeptide synthesis and did not interfere with the elongation processes.
Fig. 2. The effect of material found in peak A on the wheat germ protein synthesizing system.

A. Translation of total BMV RNA. The incorporation mixture (50 μl) contained 17 μl of cell-free extract (680 μg of protein), 25 μg of wheat germ tRNA, 3.1 mM-Mg/acetate, 100 mM-K/acetate, 1 mM-ATP, 0.375 mM-GTP, 20 mM-creatine phosphate, 2.5 μg of creatine kinase, 20 mM-HEPES/KOH buffer, pH 7.6, 7 mM-Tris/acetate buffer, pH 7.6, 0.7 mM-DTT, 6.6 nmoles of [¹⁴C]leucine (spec. act. 12 mCi/m mole), 0.15 mM each of 19 unlabelled amino acids, and 7 μg of total BMV RNA. The incubation was performed at 31°C for 2 h with the indicated amounts of the material from peak A. Hot-trichloroacetic-acid-precipitable radioactivity was measured in 40 μl portions of the incubation mixture.

B. Translation of poly(U) and aminocacylation of tRNA. The incorporation mixtures (50 μl) contained 17 μl of cell-free extract (680 μg of protein), 8.9 mM-Mg/acetate, 0.32 n mole of [¹⁴C]phenylalanine (spec. act. 60 mCi/m mole) instead of [¹⁴C]leucine, 24 μg of poly(U) instead of BMV RNA, and other components as in Fig. 2A. The incubation conditions and radioactivity measurements were the same as in Fig. 2A (●).

In the aminocacylation assay, 39 μg of total wheat germ tRNA was incubated with 5 μg of post-ribosomal supernatant (125 μg of protein) and with 0.25 n mole of [¹⁴C]phenylalanine (spec. act. 60 mCi/m mole) and other components as in Fig. 2A in a final volume of 50 μl. After 30 min at 31°C, 40 μl portions were spotted on 3MM Whatman paper discs and radioactivity in [¹⁴C]phenylalanyl-tRNA was estimated as described previously (Rychlik & Zagórska, 1978) (△).

The inhibitory material cosedimented with the ribosomal fraction when the ribosomes were pelleted directly from the cell-free extract. At least a part of this material was found in peak A from the Sepharose column. As can be seen in Fig. 3A, the translational capacity of the cell-free extract was fully restored in the fractionated system containing ribosomes purified on the Sepharose column. The incorporation in this system was sensitive to the addition of translation inhibitors present in peak A (Fig. 3B).
Fig. 3. Comparison of translational capacity of ribosomes isolated by direct ultracentrifugation and by gel filtration.

A. The incorporation mixtures (25 µl) containing 8 µl of postribosomal supernatant (200 µg of protein) were supplemented either with ribosomes I (■) or II (○) isolated by direct ultracentrifugation, or with ribosomes isolated after gel filtration of the cell-free extract (▲). Total BMV RNA (3 µg) was used as a template. Other components as in Fig. 2A.

B. The incorporation mixtures (50 µl) containing 10 µl of postribosomal supernatant (250 µg of protein) and ribosomes isolated by gel filtration (150 µg of protein), were supplemented with the indicated amounts of the material from peak A. Total BMV RNA (7 µg) was used as a template. Other components as in Fig. 2A.

It should be noted that the aggregates detected in peak A were not present in the freshly prepared cell-free extract. They were formed during extensive dialysis of the extract, required for activation of the incorporation system, as well as during prolonged ultrafiltration on the Sepharose column. Formation of these aggregates was a time-dependent process, occurring at low temperature and at relatively low ionic strength. At higher temperature and at elevated ionic strength, for example under the conditions appropriate for amino acid incorporation, the aggregates became partially solubilized, releasing a protein fraction which inhibited the translation of BMV RNA.

On the basis of the presented results, we assumed that the aggregates excluded in the void volume of Sepharose 2B column contained translation inhibitors specific for the initiation of polypeptide synthesis. On characterizing these inhibitors, we isolated and purified from the aggregates the polypeptide (mol. wt. 20 000) which inhibited the translation of BMV RNA, but did not affect poly(U) translation.

The purified polypeptide preferentially inhibited translation of BMV non-structural proteins, being less effective against translation of coat protein gene (Fig. 4).
Fig. 4. SDS-polyacrylamide-gel electrophoresis of polypeptides synthesized under direction of total BMV RNA.

A. The incorporation mixture (50 μl) containing 20 μl of cell-free extract, 12 μg of wheat germ tRNA, 3.1 mM-Mg acetate, 100 mM-K acetate, 80 mM-spermine, 1 mM-ATP, 0.375 mM-GTP, 20 mM-creatine phosphate, 2.5 μg of creatine kinase, 20 mM-HEPES/KOH buffer, pH 7.6, 7 mM-Tris/acetate buffer, pH 7.6, 0.7 mM-DTT, 4 nmole of [14C]leucine (spec. act. 211 mCi/mmole), was supplemented with 4 μg of total BMV RNA. The incubation was carried out at 31°C for 7 min, then K/acetate concentration was raised to 170 mM and the incubation was continued for another 2 h; then 10 μl portions of the mixture were submitted to electrophoresis, as described in Materials and Methods. Position of polypeptides la, 2a, 3a and 4a (coat protein) formed under direction of total BMV RNA (see Zagorski, 1978a) are indicated by arrows.

B. The incorporation mixture as in Fig. 4A was supplemented with 4 μg of protein kinase isolated from peak A. The incubation and electrophoresis were carried out as in Fig. 4A. The incorporation without inhibitor was equal to 212, and in the presence of inhibitor to 148 pmol of [14C]leucine per 50 μl.

It should be noted that this polypeptide exhibited the activity of protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37), (Fig. 5); its specific activity when measured in the presence of casein as a substrate was equal to 215 nmoles of 32P incorporated per mg of enzyme protein per minute.

High-molecular-weight translation inhibitors were detected in other cell-free systems studied, as reticulocyte lysates (Kramer et al., 1976; Farrell et al., 1977; Haro & Ochoa, 1978), Friend leukemia cells (Pinphanichakarn et al., 1977), Artemia
Fig. 5. Time-course of phosphorylation of casein by protein kinase isolated from peak A. Phosphorylation of casein by the translation inhibitor was measured as described in Materials and Methods.

Salina embryos and wheat germ (Sierra et al., 1977). It was observed that these inhibitors exhibited the activity of protein kinase, too. Our results suggest that involvement of protein kinase in regulation of protein biosynthesis is an important cellular mechanism functioning not only in animals but also in plants.

M.M.Z. is grateful to the Polish IAESTE Committee for the financial support within the framework of the international student exchange programme. Skillful technical assistance of Miss E. Borkowska is acknowledged. Authors are thankful to General Mills Inc. (U.S.A.) for the generous gift of wheat germ used in these studies.

REFERENCES


IZOLOWANIE Z FRAKCIJ ZARODKÖW PSZENICY RYBOSOMÓW WOLNYCH OD WYSOKOCZÄSTECZKOWYCH INHIBITORÓW TRANSLACJI NATURALNYCH INFORMACYJNYCH RNA

Streszczenie

W ekstrakcie bezkomórkowym z zarodków pszenicy występuje inhibitor translacji naturalnych mRNA (BMV RNA). Inhibitor ten nie hamuje translacji poli(U) ani syntez aminocyto-rrNA. Inhibitor wykazuje aktywność kinazy białkowej (fosfotransferazy ATP : białko, EC 2.7.1.37). Inhibitor występuje w agregatach białkowo-lipidowych, które można oddzielić od frakcji rybosomowej przez filtrację na Sepharose 2B. Rybosomy oczyszczone od inhibitory wykazują kilkakrotnie wyższą aktywność w translacji BMV RNA niż rybosomy izolowane metodami konwencjonalnymi.

Received 5 October, 1978.