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SYNTHESIS OF SP II ALBUMIN, β-AMYLASE AND CHYMOTRYPSIN INHIBITOR CI-1 ON POLYSOMES FROM THE ENDOPLASMIC RETICULUM OF BARLEY ENDOSPERM

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Free and initially membrane bound polysomes were isolated from 20 day old endosperms of Bomi and Hiproly barley and used as templates in an in vitro protein synthesizing system based on wheat germ extract. Three ^{35}S-labelled translation products were identified as SP II albumin, β-amylase and chymotrypsin inhibitor CI-1 among the polypeptides synthesized by the polysomes of the endoplasmic reticulum. Identification employed immunoaffinity isolation. Protein Z was not detectable among the in vitro translation products of either initially membrane bound or free endosperm polysomes.

Compared to Bomi barley the Hiproly endosperm is enriched for translatable mRNA coding for SP II albumin, β-amylase and chymotrypsin inhibitor CI-1. In vitro and in vivo synthesized β-amylases have identical molecular weights. Only one form of SP II albumin is produced in vitro, confirming that the lower molecular weight SP II B albumin is a proteolytic cleavage product of the SP II A albumin.

Abbreviations: DTT = dithiothreitol; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; HKM buffer A and B = HEPES-K-Mg acetate buffer (see 2.2.); IgG = immunoglobulin G; PRB-buffer = Tris-HCl buffer pH 7.7 containing 2% Triton X-100 and 0.15 M-NaCl (see 2.2.); SDS-PAGE = Sodium dodecylsulfate polyacrylamide gel electrophoresis; TCA = trichloroacetic acid.

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1. INTRODUCTION

Of the major storage proteins in the barley grain, the hordein polypeptides are synthesized on the endoplasmic reticulum, co-translationally transported into its lumen and from there transferred into the vacuoles of the cells (4, 6, 7, 8). It has recently been shown that the protein bodies deposited in the vacuoles of the barley endosperm contain additional polypeptides besides hordein (6). For a further understanding of storage protein deposition and of the possibilities for their nutritional improvement it is desirable to learn which other proteins are synthesized on the rough endoplasmic reticulum and from there transferred into the protein bodies of the vacuoles. We have therefore investigated whether the lysine-rich SP II albumin, the chymotrypsin inhibitor CI-1, β-amylase and protein Z are synthesized on polysomes of the endoplasmic reticulum.

The SP II albumin has been isolated from mature Hiproly barley caryopses (16) and its increased presence in the mutant line was found to account for 37% of the difference in crude protein lysine between Hiproly and varieties with a normal lysine content (17). SP II albumin acts as a serine protease inhibitor with strong effects on subtilisin and weak effects on chymotrypsin (22). It shows homology in amino acid sequence with a chymotrypsin inhibitor I from potato tubers (21, 22). The latter exhibits immunochemical identity with a chymotrypsin inhibitor from tomato leaves (20) which is synthesized as a precusor protein and deposited in the vacuoles of the leaf cells and considered as a storage protein (19, 20, 23). Apart from SP II albumin Hiproly barley endosperms are enriched in chymotrypsin inhibitor CI-1 – also with a high lysine content –, in β-amylase and in protein Z (1, 13, 17).

2. MATERIALS AND METHODS

2.1. Plant material

Hiproly and Bomi barley (Hordeum vulgare L.) plants were grown in the field. Spikes were harvested 20 days after anthesis, frozen in liquid nitrogen and stored in plastic bags at −70 °C for up to 3 months.

2.2. Chemicals

HEPES, ATP (disodium salt), GTP (sodium salt), creatine phosphate (disodium salt), creatine kinase (EC 2.7.3.2.), DTT, spermidine phosphate and L-amino acids were purchased from Sigma Chemicals. Protein-A coupled Sepharose and Sephadex G-25 were obtained from Pharmacia Fine Chemicals. ^{35}S-methionine and [N-methyl-^{14}C] dansyl chloride (specific activity 900 and 43.9 mCi/mmole, respectively, (^{14}C)methylated protein mixture for SDS-PAGE molecular weight standards (CFA 626) with a specific activity of 10–50 μCi/mg, were obtained from Radiochemical Center, Amersham, U.K.

HKM buffer A: 20 mM-HEPES, pH 7.6, 100 mM-K-acetate, 50 mM-Mg-acetate, 0.2 M-sucrose and 2 mM-DTT.

HKM buffer B: 20 mM-HEPES, pH 7.6, 100 mM-K-acetate, 5 mM-Mg-acetate and 2 mM-DTT.

PRB buffer: 50 mM-Tris-HCl, pH 7.7, 0.15 M-NaCl, 2% Triton X-100, 10 mM-methionine.

2.3. Purification of monospecific IgG

Antisera towards SP II albumin were obtained as described previously (16). Specific antisera towards chymotrypsin inhibitor CI-1, protein Z and β-amylase were a generous gift from Dr. J. HEJGAARD, Department of Biochemistry and Nutrition, Technical University of Denmark, DK-2800 Lyngby.

SP II albumin antisera were tested for monospecificity by crossed immunoelectrophoresis (16) and purified by Protein-A Sepharose affinity chromatography (10). The purified antibodies were lyophilized and redissolved in PRB buffer.

2.4. Preparation of ^{14}C-dansylated SP II albumin

^{14}C-dansylated SP II albumin was prepared by reacting 2.5 mg SP II albumin dissolved in 500 μl 0.1 M-$NaHCO_3$ with 2.5 μCi [N-methyl-^{14}C]-dansyl chloride in 50 μl acetone for 1.5 hours at 0 °C. Uncoupled dansyl chloride was removed by Sephadex G-25 gel filtration with 0.1 M-NH_4HCO_3 as eluant.

2.5. Polysome isolation

Free and initially membrane bound polysomes were isolated by a procedure slightly modified from (4, 18). All operations were carried out at 2–4 °C. Endosperms were squeezed out from the spikes and ground in a precooled mortar containing 1 ml HKM buffer A per spike. After the initial grinding further homogenization was done by three strokes in a Potter-Elvehjem homogenizer, the shaft being rotated at 500 rpm. The homogenates were centrifuged at $500 \times g$ for 5 min in a Sorvall SS-34 rotor to remove most of the starch, cell walls and unbroken cells. The supernatant was then centrifuged at $37{,}000 \times g$ for 10 min to separate the free and membrane bound polysomes.

The $37{,}000 \times g$ supernatant containing the free polysomes was layered over 2 ml 1.75 M-sucrose in HKM buffer B and centrifuged at $96{,}000 \times g$ for 18 hours in a Spinco 50 rotor to pellet the polysomes. The $37{,}000 \times g$ pellet was resuspended in HKM buffer A containing 1% Triton X-100 to solubilize the initially membrane bound polysomes and these were then pelleted likewise at $96{,}000 \times g$. The polysome pellets were resuspended in HKM buffer B.

2.6. Cell-free protein synthesizing system

In vitro protein synthesis was performed as described earlier (5), except that the (Mg^{++}) concentration was raised from 1.8 to 2.0 mM.

2.7. Isolation of in vitro synthesized proteins by monospecific antibodies and protein-A Sepharose

Immunoaffinity isolation of specific proteins was carried out as recommended by Dr. P. STRØMAN, Chr. Hansen Laboratorium A/S, Copenhagen. The procedure was as follows: Aliquots representing 8.5×10^5 cpm (Bomi and Hiproly membrane bound polysomes, Hiproly free polysomes) and 5.9×10^5 cpm (Bomi free polysomes) were adjusted to identical concentration of wheat germ extract. The aliquots were diluted with PRB buffer to a total volume of 600 µl and incubated for 30 min at room temperature in order to dissolve the membranes. Insoluble material was removed by centrifugation ($13{,}000 \times g$, 10 min) and the supernatant was incubated for 1 hour at room temperature with 5 µl PRB buffer containing 50 µg (0.3 nano moles) of monospecific anti SP II albumin. The antigen-IgG complex was coupled to protein-A by addition of 50 µl hydrated Protein-A Sepharose corresponding to 5 nanoequivalents protein-A. The sample was shaken gently for 30 min. The antigen-IgG-protein-A Sepharose complex was sedimented by centrifugation ($13{,}000 \times g$, 1 min). The supernatant was used for subsequent immunoaffinity isolation of β-amylase, protein Z and chymotrypsin inhibitor CI-1 employing anti-β-amylase, anti-protein Z and anti-chymotrypsin inhibitor CI-1 sequentially. The Sepharose pellets were washed with PRB buffer. Washings were repeated 3 times with PRB buffer and 2 times with 10 mM-Tris/HCl pH 7.4.

2.8. SDS-PAGE and fluorography of proteins synthesized in vitro

In vitro synthesized polypeptides were prepared for SDS-PAGE by mixing an aliquot of the in vitro translation assay corresponding to 8×10^4 TCA precipitable cpm with 30 µl 0.1 M-Na_2CO_3, 0.1 M-DTT, 20 µl 30% sucrose, 5% SDS, 1 µl 1 M-methionine. The samples were boiled for 3 min and 15 µl 2 M-iodoacetamide were added 2 hours prior to electrophoresis. Samples corresponding to 4×10^4 cpm were applied to the gel.

The IgG-protein-A Sepharose complexes were treated with 15 µl 0.1 M-DTT, 0.1 M-Na_2CO_3 and 10 µl 30% sucrose, 5% SDS and boiled for 3 min. Then the samples were mixed with 8 µl 2 M-iodoacetamide and left for 2 hours before electrophoresis.

Electrophoresis was performed according to (9). ^{14}C-labelled SP II A- and SP II B albumin were co-electrophoresed as markers.

The gels were prepared for fluorography (3), and exposed to RP Royal X-omat film (Kodak) at −70 °C for up to 20 days, and then developed.

3. RESULTS AND DISCUSSION

3.1. Isolation and in vitro translation of free and membrane bound polysomes

Isolation of polysomes from the endosperms

Table I

Polysome yield and in vitro template activity of free and initially membrane bound polysomes from barley endosperms.
Template activities are expressed as hot 10 % TCA precipitable cpm per A_{260} unit of added polysomes in a standard reaction mixture (5) for cell-free protein synthesis employing ^{35}S-methionine.

Barley	Polysome yield A_{260} units spike^{-1}		^{35}S-methionine incorporated cpm A_{260} unit^{-1} 10^{-5}	
	membrane bound	free	membrane bound	free
Bomi	4.7	1.5	7.3	0.9
Hiproly	3.2	1.4	6.1	0.7

of one barley spike yielded 3 to 4 times more initially membrane bound polysomes than free polysomes (Table I) in agreement with previous results (4). The activity of the initially membrane bound polysomes in performing in vitro protein synthesis in the wheat germ reaction mixture was about 8 times higher than the activity of the free polysomes. The template activity of the polysomes isolated from the two genotypes was comparable.

3.2. SDS-polyacrylamide gel electrophoresis and fluorography of in vitro translation products

The preparations of free and membrane bound polysomes from Bomi and Hiproly endosperms contain messenger RNA coding for polypeptides distributed over the molecular weight range covered by the polyacrylamide gel (Figure 1, tracks 1 and 2).

3.2.1. Translation products of membrane bound polysomes

The in vitro synthesized polypeptide chains selected by anti-SP II albumin, anti-β-amylase and anti-chymotrypsin inhibitor CI-1 are displayed in Figure 1, tracks 5 to 12. Membrane bound polysomes from Hiproly endosperms include sufficient template activity for the three polypeptides to give a strong fluorographic image after 20 days exposure of the gel (Figure 1, tracks 5, 7 and 11). In the reaction mixtures employing membrane bound polysomes derived from Bomi barley endosperms, only β-amylase was synthesized in detectable amounts (track 8). After exposure of the gel for 3 months a polypeptide and band representing chymotrypsin inhibitor CI-1 emerged in track 12. The high amounts of translatable messenger RNA for SP II albumin, β-amylase and chymotrypsin inhibitor CI-1 in Hiproly endosperms correlate with the high content of these polypeptides measured in Hiproly kernels (13, 17). A single polypeptide with an apparent molecular weight of 12,000 is immunoaffinity isolated with anti-SP II albumin (Figure 1, track 5).

Isolation of SP II albumin yields two polypeptides A and B which are identical in amino acid sequence except that A has an extension of 8 to 11 amino acid residues at the N-terminal end (16, 21). SP II B albumin is thus considered a proteolytic break-down product of SP II A. As the latter is blocked at the N-terminal end only the SP II B fragment is accessible to effective in vitro dansylation with ^{14}C-dansylchloride (Figure 1, track 4). A faint band of slow migration and a band corresponding to the ^{14}C-dansylated SP II B albumin is obtained in the SP II A albumin preparation which had been dansylated (Figure 1, track 3). The migration of the in vitro synthesized SP II albumin (Figure 1, track 5) corresponds to this faint band. It can be concluded that only SP II A albumin is synthesized on the polysomes in vitro. If the SP II B fragment occurs as a native

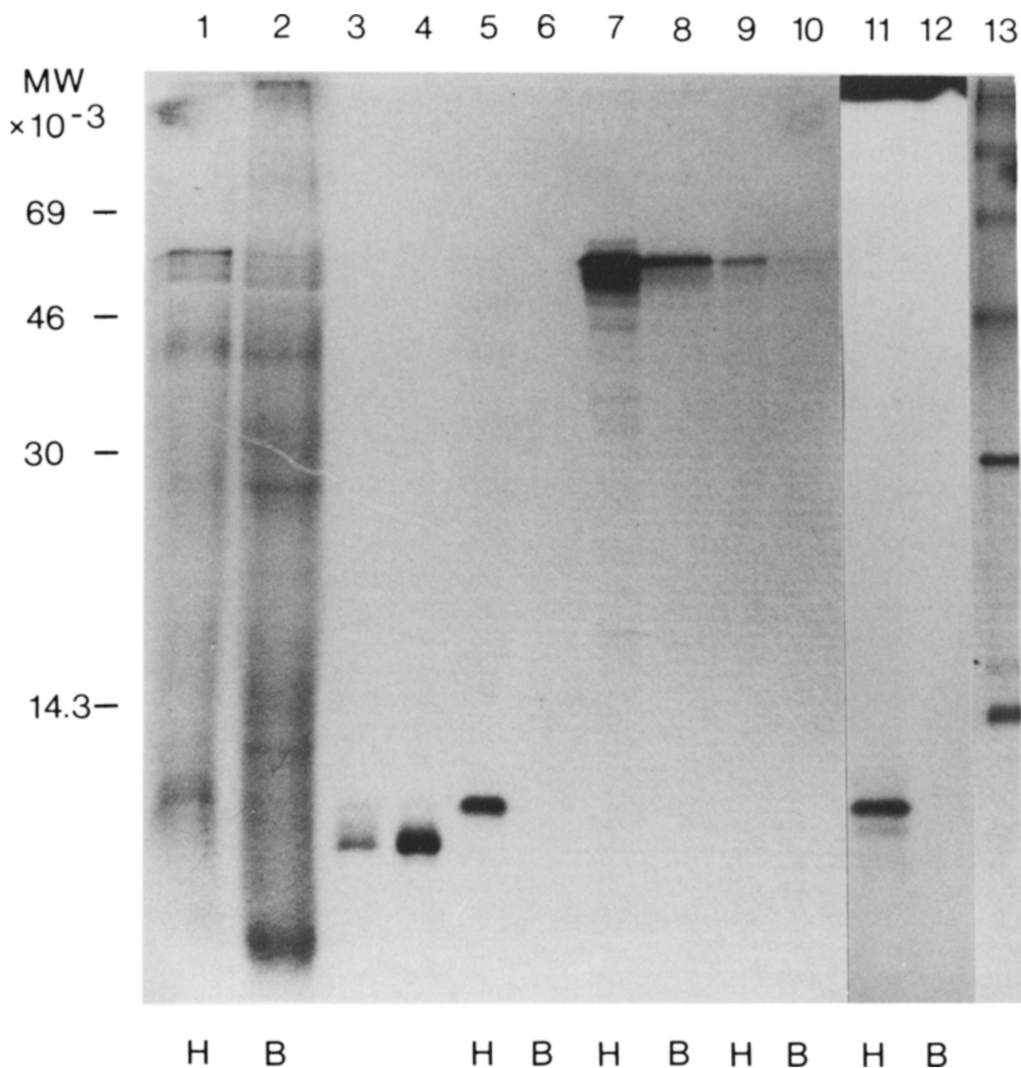

Figure 1. SDS-polyacrylamide gel electrophoresis of polypeptides synthesized in vitro using initially membrane bound polysomes as templates.

Polysomes were isolated from the immature endosperms of Bomi (B) and Hiproly (H) barley and translated in vitro in a system based on wheat germ extract. Total in vitro translates (tracks 1 and 2) or immunoaffinity isolates (tracks 5 to 12) were fractionated on an SDS-polyacrylamide gel and the polypeptide bands visualized by fluorography. Specific antibody probes were as follows: anti-SP II albumin (tracks 5 and 6); anti-β-amylase (tracks 7 and 8); anti-z-protein (tracks 9 and 10); anti-chymotrypsin inhibitor CI-1 (tracks 11 and 12). Controls: ^{14}C-dansylated SP II A albumin (track 3); ^{14}C-dansylated SP II B albumin (track 4); molecular weight markers comprising: Lysozyme, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase b and myosin with molecular weights 14,300, 30,000, 46,000, 69,000, 92,500 and 200,000, respectively.

constituent of endosperm cells, it is a posttranslational derivative.

Track 7 in Figure 1 reveals a strong β-amylase band with a molecular weight of 58,000 compared to a molecular weight of 57,000 determined for in vivo synthesized β-amylase (24). Anti-β-amylase also selects polypeptides with molecular weights slightly lower than 58,000 (Figure 1, track 7). These polypeptide bands are most likely prematurely terminated β-

amylase chains, but large enough to contain the antibody binding sites.

Immunoaffinity isolation with anti-chymotrypsin inhibitor CI-1 results in two distinct polypeptide bands (Figure 1, track 11). The prominent band has an apparent molecular weight of 12,000. The second less prominent polypeptide band has a molecular weight which is lower than 12,000. Whether it results from in vitro translation of a different messenger RNA molecule or from posttranslational modification of the 12,000 molecular weight polypeptide remains to be investigated.

Protein Z with a molecular weight of 40,000 was originally identified as a barley endosperm protein which survives the brewing process and thus is present in beer made from barley malt (12). When isolated from barley kernels it is often found as a complex with β-amylase (11). No detectable amounts of protein Z was immunoisolated from the supernatant remaining after treatment with anti-SP II albumin and anti-β-amylase (Figure 1, track 9). Either the amounts of protein Z produced were insufficient to be detectable in the present experiment or the antigenic determinants of the in vitro product differ from those of the isolated polypeptide. Alternatively, protein Z may be synthesized at another stage of development or by the free polysomes. Track 9 does, however, include one labelled polypeptide band which migrates to the same position as β-amylase. This band is not present if the immunoaffinity isolation is performed in reverse order, beginning with anti-chymotrypsin inhibitor followed by anti-protein Z, anti-β-amylase and anti-SP II albumin. The presence of the band is thus most likely due to residual anti-β-amylase-β-amylase complexes remaining after treatment with protein-A Sepharose and therefore carried over to the next immuno isolation step.

The tracks of Figure 1 represent the translation products of initially membrane bound polysomes from 0.2–0.4 barley spikes. In vitro translation products representing free polysomes from 0.4 barley spikes were subjected to SDS-PAGE. The resulting fluorogram was blank after 20 days of exposure. Weak fluorographic images of the polypeptide bands present in Figure 1 appeared after 3 months exposure of the polyacrylamide gel containing the in vitro translation products of the free polysomes. This is readily explained by a small contamination of the free polysome preparation with initially membrane bound polysomes. The free polysomes did not contain detectable amounts of messenger RNA coding for protein Z.

4. CONCLUDING REMARKS

We conclude that SP II albumin, β-amylase and chymotrypsin inhibitor CI-1 are synthesized on the rough endoplasmic reticulum of the barley endosperm, and that the endosperm from Hiproly barley is enriched in translatable messenger RNA coding for the three polypeptides.

In analogy with hordein – a major group of storage proteins of the barley endosperm (15) – the following characteristics are expected to be associated with a storage protein: 1) it is synthesized on the rough endoplasmic reticulum (4), 2) its deposition is enhanced by an increase in nitrogen fertilizer (2, 13), 3) it is deposited as protein bodies in vacuoles (6, 7, 8, 14). The first two expectations are fulfilled by β-amylase (13) while SP II albumin and chymotrypsin inhibitor CI-1 comply with the first. It will be of interest to determine if SP II albumin, β-amylase and chymotrypsin inhibitor CI-1 are deposited in the vacuoles of the barley endosperm.

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