SPECIES SPECIFIC SIGNAL PEPTIDE CLEAVAGE OF PLANT STORAGE PROTEIN PRECURSORS IN THE ENDOPLASMIC RETICULUM

by

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Keywords: *Vicia faba*, *Phaseolus vulgaris*, *Hordeum vulgare*, vicilin, hordein, *lys* 3a, vectorial membrane transport, reconstituted microsomes

Smooth microsomal membranes were isolated from immature cotyledons of *Vicia faba* and *Phaseolus vulgaris* as well as from immature endosperms of Bomi barley and its mutant *lys* 3a. Isolated Bomi barley endosperm polysomes direct the synthesis of hordein precursor polypeptides. Functional reconstitution of Bomi barley rough microsomes employing such polysomes and smooth microsomal membranes resulted in the ability of the membranes to perform signal peptide cleavage and co-translational transport of hordein precursor polypeptides into the lumen of the microsomes. Only a small portion of the hordein precursor polypeptides was processed and transported when the reconstitution was performed with smooth microsomal membranes isolated from cotyledons of *Vicia faba* and *Phaseolus vulgaris* or from the endosperms of *lys* 3a. A precursor polypeptide to the 50 kD vicilin was translated in vitro from mRNA of immature *Vicia faba* cotyledons. In the presence of *Vicia faba* smooth microsomal membranes the precursor polypeptide was co-translationally processed to the size of the polypeptide synthesized by rough microsomal membranes. Smooth microsomal membranes from cotyledons of *Phaseolus vulgaris* and from the endosperms of Bomi or *lys* 3a barley failed to process detectable amounts of the vicilin precursor polypeptide. Dog pancreatic microsomal membranes cleaved significant amounts of the hordein precursor polypeptides whereas only a small portion of the vicilin precursor polypeptides was processed.

1. INTRODUCTION

The major storage proteins of legume cotyledons are the 7 S (vicilin) and 11 S (legumin) globulins, whereas hordein comprises the main storage polypeptides in the endosperm of barley caryopses. Cell fractionation and cytological studies have shown that these storage protein

polypeptides are deposited in vacuolar protein bodies (14, 17, 19, 47). The intracellular location of storage proteins has also been demonstrated by immunocytochemical studies in the electron microscope (21, 39).

Individual storage protein polypeptides of hordein and globulins are synthesized on the

Abbreviations: ER = endoplasmic reticulum; HEPES = N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid; IgG = immunoglobulin G; mb = membrane bound; M.W. = apparent molecular weight; SDS = sodium dodecyl sulphate; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis.

endoplasmic reticulum (7, 9, 10, 15, 16, 28, 42). Similar to the pathway for secretory proteins of animal cells, the storage protein polypeptides of plant seeds are synthesized as higher molecular weight precursors which are vectorially discharged into the lumen of the endoplasmic reticulum concomitantly with the cleavage of the signal peptide (10, 15, 16, 25, 48). Thereafter the storage protein polypeptides are transiently associated with the ER prior to sequestration into the protein bodies (8, 19).

Co-translational processing of plant storage protein polypeptides has been studied in vivo in *Xenopus* oocytes (2, 31) or in vitro by using ribosome denuded dog pancreas microsomes (3, 25). For elucidation of precursor processing of storage proteins in homologous systems, initially membrane bound polysomes or mRNA were supplemented with stripped microsome membranes which processed the newly synthesized precursor polypeptides to their mature form (12, 15). Processing has also been observed when rough microsomes were used to direct cell-free protein biosynthesis (10, 28, 48).

Ribosome-denuded ER membranes from storage protein synthesizing tissues of *Hordeum vulgare*, *Vicia faba*, *Phaseolus vulgaris* as well as from canine pancreas were used in this study for functional reconstitution of rough microsome membranes in vitro. Surprisingly large species specific quantitative differences were found in the ability of the membranes to co-translationally process the hordein and vicilin precursor polypeptides.

2. MATERIALS AND METHODS

2.1. Chemicals

ATP (disodium salt), GTP (disodium salt), HEPES, creatine phosphate (disodium salt), creatine kinase (E.C. No. 2.7.3.2.), spermidine phosphate, α -chymotrypsin (E.C. No. 3.4.21.1.), DTT and L-amino acids were obtained from Sigma Chemicals, USA. Poly (U) Sepharose 4 B and Protein A Sepharose CL 4 B were purchased from Pharmacia, Sweden. Rabbit reticulocyte lysate, canine pancreatic microsomal membranes, ^{35}S -methionine (spec. activity 1200-1400 Ci · mmol⁻¹), ^3H -leucine (specific activity 150-160 Ci · mmol⁻¹) and ^{14}C -methylated pro-

tein mixture (5 mCi · mmol⁻¹) were obtained from Radiochemical Center, Amersham, U.K. Anti-hordein IgG was a gift from Dr. CHRISTA DIERKS-VENTLING, Friederich Miescher Institute, Basel, Switzerland.

2.2. Plant Materials

Immature seeds from *Vicia faba* L. var. minor, cv. Fribo and *Phaseolus vulgaris* L. and endosperms from *Hordeum vulgare* cv. Bomi and its mutant *lys 3a* were obtained as previously described (48).

2.3. Preparation of polysomes and mRNA

RNA was prepared as described in (26, 42) and membrane bound polysomes according to (9).

2.4. Isolation of rough and smooth microsomes

Rough microsomes were prepared from 20-36 g fresh weight cotyledons and endosperms and smooth microsomes were obtained by stripping the ribosome studded membranes with EDTA according to (15, 16).

2.5. Cell-free protein synthesis

In vitro translation of free mRNA, and mRNA associated with membrane bound polysomes or rough microsomes was performed with the wheat germ system (9, 34). A commercially available rabbit reticulocyte lysate translation system was alternatively used for the cell-free protein synthesis programmed by polyadenylated mRNA (0.04-0.06 A₂₆₀/20 µl assay). Reconstitution of rough microsomes was performed by the addition of 0.7 OD₂₆₀ smooth membranes in 25 µl assays. Canine pancreatic microsomal membranes were used according to the protocol supplied by Amersham, U.K. Post-translational treatments with α -chymotrypsin were performed at 0 °C for 2 h.

2.6. Isolation of storage protein polypeptides

Hordein polypeptides were isolated from the cell-free translation assays by extraction at 60 °C

for 30 min with isopropanol: 2-mercaptoethanol: H₂O (55:2:43, v/v/v). Immunoaffinity isolation of hordein and vicilin polypeptides using anti-hordein-IgG or anti-vicilin-IgG, respectively, were done according to (30, 48).

2.7. SDS-PAGE and fluorography of in vitro synthesized polypeptides

Samples were denatured by boiling in 25 µl 0.2 M-TRIS-HCl, pH 8.7, 5 mM-EDTA, 0.5 M-sucrose, 0.05% bromophenol blue, 1% methionine (w/v), 2% SDS (w/v) for 2 min. After addition of 10 µl 0.5 M-iodoacetamide electrophoresis was performed on 12.5% acrylamide gels according to CHUA and BENNOUN (20). The gels were

prepared for fluorography by incubation for 20 min in 1 M-sodium salicylate as described by CHAMBERLAIN (18) and exposed to RP Royal X-Omat film (Kodak) at -70 °C for 1-20 days, and then developed.

3. RESULTS

3.1. Reconstitution of rough microsomes in vitro

Messenger RNA or initially membrane bound polysomes from *Vicia faba* cotyledons and barley endosperms were used as templates to direct cell-free protein synthesis in a wheat germ translation system (Table I). Comparison of the efficiencies of the templates in the presence and

Table I.

Translation efficiencies of templates used for the functional reconstitution of rough microsomes in vitro

Template	OD 260 · assay ⁻¹	Co-translational addition	cpm · 10 ⁻⁶ · OD 260 ⁻¹
<u>Bomi barley</u> mRNA	0,06	- <u>smooth microsomes</u> Vicia faba Phaseolus vulgaris Bomi barley mutant Risø 1508	18 10 7 7 6
mb polysomes	0,50	- <u>smooth microsomes</u> Vicia faba Phaseolus vulgaris Bomi barley mutant Risø 1508	2 2 2 2 2
<u>Vicia faba</u> mRNA	0,06	- <u>smooth microsomes</u> Vicia faba Phaseolus vulgaris Bomi barley mutant Risø 1508	14 8 10 6 5
mb polysomes	1,25	- <u>smooth microsomes</u> Vicia faba Phaseolus vulgaris Bomi barley mutant Risø 1508	3 3 3 3 2

Figure 1. SDS-PAGE of 55% isopropanol-soluble translation products from Bomi barley membrane bound (mb) polysomes in the presence of smooth microsomal membranes isolated from immature cotyledons of *Vicia faba* and *Phaseolus vulgaris* and from immature endosperms of Bomi barley and mutant *lys 3a* (Risø 1508). Track 1 – in vivo labelled hordein polypeptides, track 2 – translation products of Bomi polysomes, track 3 – translation products of rough microsomes from Bomi barley. Translation products programmed by Bomi mb polysomes in the presence of smooth microsomal membranes from *Vicia faba* (tracks 4, 8), Bomi barley (tracks 5, 9), mutant Risø 1508 (tracks 6, 10) and *Phaseolus vulgaris* (tracks 7, 11). Tracks 8-12 show the translation products after post-translational treatment of the assays with 1.5 mg · ml⁻¹ α-Chymotrypsin. Track 12 shows complete digestion of hordein polypeptides synthesized by membrane bound polysomes. B, C – b and c hordein polypeptides.

absence of smooth microsomal membranes showed that the polysome directed protein synthesis was not inhibited by the co-translational addition of smooth membranes whereas addition of microsomal membranes to the cell-free protein synthesizing system directed by mRNA resulted in an inhibition of the translations by 40-70% (Table I).

3.2. SDS-PAGE of hordein polypeptides synthesized in vitro on reconstituted rough microsomes

Initially membrane bound polysomes from Bomi barley endosperms were used as a template for the cell-free translation of hordein polypeptides. SDS-PAGE and fluorography shows that the hordein polypeptides synthesized by poly-

somes (Figure 1, track 2) have a lower mobility than the hordein polypeptides synthesized by the rough microsomes (Figure 1, track 3), indicating that signal peptide cleavage by the endoplasmic reticulum membrane had occurred. The processed polypeptides have the same apparent molecular weights as the hordein polypeptides synthesized in vivo (Figure 1, track 1).

Smooth microsomal membranes isolated from Bomi barley endosperms or from the endosperms of the barley mutant *lys 3a*, as well as from immature cotyledons of *Phaseolus vulgaris* and *Vicia faba* were added to the cell-free translation system. The newly synthesized hordein precursor polypeptides are co-translationally processed by the Bomi barley microsomal membranes as indicated by their comigration with the hordein polypeptides synthe-

Figure 2. SDS-PAGE of immunoaffinity isolated vicilin polypeptides translated in vitro from mRNA in the presence of smooth microsomal membranes isolated from immature cotyledons of *Vicia faba*, *Phaseolus vulgaris*, and from immature endosperms of Bomi barley and mutant *lys 3a* (Risø 1508). ^3H -leucine was used as the label. Track 1 – translation products of mRNA. Tracks 2, 7 – translation products directed by rough microsomes. Translation products programmed by mRNA in the presence of smooth microsomal membranes from *Vicia faba* (track 3), *Phaseolus vulgaris* (track 4), mutant *lys 3a* (track 5) and Bomi barley (Risø 1508) (track 6).

sized on the rough microsomes and with native hordein (Figure 1, tracks 3, 5). No detectable processing was seen when membranes isolated from *lys 3a* endosperms, *Vicia faba* or *Phaseolus vulgaris* cotyledons, respectively, were used (Figure 1, tracks 4, 6, 7).

To analyse those hordein polypeptides which are located inside the microsomal vesicles, the cell-free translation assays were post-translationally treated with $1.5 \text{ mg} \cdot \text{ml}^{-1}$ α -chymotrypsin. This protease concentration is more than sufficient to digest unprotected hordein polypeptides outside the reconstituted microsomes (Figure 1,

tracks 2, 12). Reconstitution of rough microsomes using polysomes and smooth microsomal membranes from Bomi barley significantly protected the newly synthesized hordein polypeptides from proteolytic attack (Figure 1, track 9), demonstrating that functional reconstitution comprising signal peptide cleavage and co-translational transport across the membrane was achieved. Only a small amount of α -chymotrypsin resistant polypeptides could be detected when Bomi barley polysomes were translated in the presence of smooth microsomal membranes isolated from the cotyledons of the two legumes

or from the barley mutant *lys 3a* (Figure 1, tracks 8, 10, 11). Thus, these results show that only the homologous wild type barley membranes are highly efficient to process and protect newly synthesized hordein polypeptides.

3.3. SDS-PAGE of vicilin synthesized in vitro on reconstituted rough microsomes

Translation of mRNA from *Vicia faba* cotyledons in the wheat-germ system, resulted in the synthesis of the larger precursor polypeptides to the 50 kD vicilin (Figure 2, track 1). Translation on rough microsomes resulted in the formation of Mr 50 kD polypeptide, indicating co-translational signal peptide cleavage on the endoplasmic reticulum membrane (Figure 2, track 2). When smooth microsomal membranes from *Vicia faba* were added co-translationally to the mRNA directed translation system, only the processed form of the vicilin polypeptide was identified (Figure 2, track 3). When the *Vicia faba* mRNA is translated in the presence of *Phaseolus vulgaris*, mutant *lys 3a* and Bomi barley membranes, respectively, the major translation product is the precursor to the 50 kD vicilin polypeptide, indicating that significant signal peptide cleavage did not occur (Figure 2, tracks 4, 5, 6). The small amount of unprocessed polypeptide to be seen in the rough microsome directed translation (Figure 2, track 7) is probably due to release of initially membrane bound polysomes from the endoplasmic membrane preparation prior to translation. The three prominent primary translation products of about 20,000 M.W. in tracks 1 and 4-7 which are recognized by the monospecific anti-vicilin IgG are considered to be premature terminated polypeptides. They are not observed in significant amounts after mRNA translation in the reticulocyte lysate system (Figure 3, tracks 1 and 2).

3.4. SDS-PAGE of vicilin and hordein polypeptides synthesized in vitro in the presence of dog pancreatic microsomal membranes

Messenger RNA from *Vicia faba* cotyledons was translated in the rabbit reticulocyte lysate system and the newly synthesized vicilin poly-

Figure 3. SDS-PAGE of immunoaffinity isolated vicilin and hordein polypeptides synthesized in the rabbit reticulocyte lysate system from *Vicia faba* and barley mRNA, respectively. Tracks 1, 2, 3: Vicilin polypeptides. Tracks 4, 5: hordein polypeptides. Dog pancreatic microsomal membranes were added co-translationally in the reaction mixtures for tracks 1 and 4, while the products of tracks 2 and 5 were made without the addition of membranes. Track 3: translation product of rough microsomal membranes from *Vicia faba*.

peptides were immunoaffinity isolated (Figure 3, tracks 1, 2, 3). In the presence of dog pancreatic microsomal membranes, only a small portion of the newly synthesized precursor for the 50 kD vicilin polypeptide was processed (tracks 1, 2) to the size of the polypeptides synthesized by *Vicia faba* rough microsomes (track 3). When the translation of mRNA from Bomi barley

endosperms was performed in the presence of dog pancreatic microsomal membranes many of the newly synthesized hordein polypeptides comigrate with native hordein (Figure 3, tracks 4, 5). More processed polypeptides are present in the B-hordein region than among the C-hordein polypeptides. The latter appear weak on the fluorogram. We conclude that there is a significantly greater ability of the dog pancreatic microsomal membrane to process newly synthesized hordein than vicilin polypeptides.

4. DISCUSSION

Rough endoplasmic reticulum membranes from immature cotyledons of *Vicia faba* and *Phaseolus vulgaris* as well as from the endosperms of Bomi barley and the hordein deficient mutant *lys 3a* were isolated by a procedure, which has been shown by electron microscopy to yield highly purified membrane preparations (15, 16, 40). By stripping the rough membranes with EDTA, smooth membranes are obtained and then added to the cell-free translation systems containing mRNA or initially membrane bound polysomes from Bomi barley endosperms or *Vicia faba* cotyledons. The translation of mRNA by membrane bound polysomes was not affected, whereas a significant inhibition of the translation of mRNA in the presence of smooth membranes was observed in the wheat germ translation system (Table I). Since the polysome preparation used in this study predominantly re-initiates protein synthesis as evidenced by the production of the precursor polypeptides (Figure 1), it is unlikely that the membranes inhibit the initiation of translation.

The smooth microsomal membranes from Bomi barley endosperms transport and process the hordein precursor polypeptides, whereas the membranes from the hordein deficient mutant *lys 3a* as well as those from immature cotyledons of *Vicia faba* and *Phaseolus vulgaris* did not effectuate vectorial transport and cleavage of the precursor polypeptides in significant amounts. CAMERON-MILLS and INGVERSEN (15) demonstrated functional reconstitution of Bomi rough microsomes from polysomes and stripped microsomes. A proportion of all the synthesized hordein polypeptides was discharged vectorially

into the microsomal lumen, since these are protected from degradation by post-translationally added chymotrypsin. In their experiments the polysome and microsome translation products were similar in size and larger than native hordein. It will be interesting to find out under which conditions signal peptide cleavage does not immediately follow the vectorial discharge. In the present experiment these two separable processes were closely linked.

Co-translational processing of precursor polypeptides to maize zein showed that smooth microsomal membranes isolated from three mutants defective in zein synthesis were able to cleave the zein precursor polypeptides (13). Previous (15) and our present results give an example of a plant mutant (*lys 3a*) with an impaired ability of the endoplasmic reticulum to co-translationally transport storage polypeptides and cleave the signal peptide. Functional reconstitution of the mutant membranes by adding components from the wild type membrane could provide an assay aiding the isolation and characterization of membrane components important for signal peptide cleavage and/or co-translational transport. The barley and *Phaseolus vulgaris* membranes did not process the newly synthesized precursor to the 50 kD vicilin polypeptide in amounts comparable to the *Vicia faba* membranes (Figure 2). Dog pancreatic microsomal membranes cleaved the precursor polypeptides to hordein and vicilin although the hordein precursor polypeptides were much more efficiently processed (Figure 3). The amount of co-translational transport of the newly synthesized polypeptides closely paralleled the amount of signal peptide cleavage obtained by the different membranes. Further studies are required to determine if the species specific effects observed are due to differences in the components participating in the vectorial membrane transport or to differences in the signal peptidases. Such analyses may further elucidate the causal relationships between the two processes.

Dog pancreatic microsomal membranes process a wide variety of precursor polypeptides (3, 25, 29, 45). Signal peptide cleavage of animal pre-proteins by dog pancreas membranes occurs at the correct site as shown by amino acid

Figure 4. Comparison of signal peptide sequences of vicilin from *Pisum sativum* (33) and of B-hordein from *Hordeum vulgare* (11). Cleavage of the hordein signal peptide is most likely between the alanine and glutamine residue.

sequencing of radioactively labelled and processed pre-proinsulin and other pre-secretory proteins (5, 32, 44). Pre-prolactin and precursors of bovine lactoproteins are processed by deoxycholate solubilized signal peptidase from dog pancreas (29) and from bovine mammary microsomal membranes (23). However, less than 50% of the precursor molecules reacted with the enzyme, and pre-proteins other than pre-prolactin were less effective substrates for the same enzyme preparation (29). Nuclease treated dog pancreas membranes or salt washed dog pancreatic microsomes supplemented with signal recognition particles processed co-translationally precursor polypeptides to storage globulins of *Pisum* (25) and *Vicia faba* (3). Apparently, the signal peptides of different animal and plant pre-secretory proteins are recognized by the dog pancreas signal peptidases. Equally promiscuous are *Xenopus* oocytes which process precursor polypeptides to the storage proteins of *Zea mays*, *Phaseolus vulgaris*, *Pisum sativum* and *Vicia faba* (2, 31, 35). Comparison of the signal peptide sequences of one vicilin from *Pisum sativum* and one B-hordein (Figure 4) reveals amino acid identity in 5 consecutive positions. Both signal peptides are hydrophobic but only the hordein signal peptide contains a positively charged lysine residue. If the species specific signal peptide cleavage is dependent on the structure of the signal peptide, the homologous regions of the two peptides cannot play an important role in this process. Among the different known eukaryotic signal sequences for transport across the endoplasmic reticulum no unique consensus sequence is apparent, although common features exist in their primary structure (1, 6, 24, 41). As in prokaryotes, different signal peptidases are likely to act on

different pre-sequences. The signal peptidase for the *E. coli* prolipoprotein is different from that cleaving the phage M13 procoat protein signal peptide (46). Certain amino acid deletions and insertions in the signal peptide of the outer membrane protein in *E. coli* prevent the export of this polypeptide (22). Also exchange of a single amino acid within the signal peptide of the maltose binding protein of *E. coli* is sufficient to block the secretion process due to inaccessibility of the altered peptide to the signal peptidase (4).

BURR and BURR (12) obtained different processing efficiencies for signal peptide cleavage of precursor polypeptides to maize endosperm zein polypeptides by using microsomal membranes from different tissues of maize. Membranes from the endosperm processed the precursor polypeptides more efficiently than microsomal membranes isolated from either shoot or nucellar tissue.

Expression of a β -phaseolin gene from French beans in tobacco seeds produced glycosylated product (43), while only transcripts were detected when a zein gene was introduced into sunflower tissue (36). Judging from the species specific membrane transport and precursor polypeptide cleavage found in this study it is conceivable that the sunflower endoplasmic reticulum is unable to process the precursor for the zein polypeptide whereby the transport pathway of the zein to the vacuolar protein body is obstructed and the translation suppressed.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. RENATE MANTEUFFEL and Dr. CHRISTA DIERKS-VENTLING for providing the immunoglobulin preparations. We are very grateful to Professor D. VON WETTSTEIN and Professor K. MÜNTZ for continuous encouragement and critical comments.

This work was supported by the Biomolecular Engineering Programme of the Commission of the European Communities with contract No. 6B 1-4-024-DK to Professor D. VON WETTSTEIN.

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Accepted by H. KLENOW