Structural Relationship among the Rice Glutelin Polypeptides[1]

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ABSTRACT

When the glutelin protein fraction of rice (*Oryza sativa* L.) seeds was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, three size classes of proteins, 51 kilodaltons (kD), 34 to 37 kD, and 21 to 22 kD, as well as a contaminating prolamine polypeptide of 14 kD were detected. Antibodies were raised against these proteins and employed in studies to determine whether a precursor-product relationship existed among the glutelin components. Antibodies of the 34 to 37 kD and 21 to 22 kD polypeptides strongly reacted with the 51 kD protein, and conversely, anti-51 kD protein cross reacted with both of the putative subunits. Immunoprecipitaiton of *in vitro* translated products resulted in the synthesis of only the precursor form, indicating that the α and β subunits are proteolytic products of the 51 kD precursor protein. The poly(A)⁺ RNA directed *in vitro* translated product was about 2000 daltons larger than both the authentic glutelin precursor and the *in vitro* translated product from polysome run-off synthesis. Western blot analysis of the 34 to 37 kD and 21 to 22 kD polypeptides partially digested with *Staphylococcus aureus* V8 protease revealed distinct patterns indicating that these proteins are structurally unrelated. As observed for the glutelins, the rice prolamines are also synthesized as a precursor of 16 kD, 2000 daltons larger than the mature polypeptide. Addition of dog pancreatic microsomal membranes to a wheat germ protein translation system resulted in the processing of the prolamine preprotein but not the preproglutelin to the mature form.

The major storage proteins of rice endosperm are the glutelins which may constitute up to 75% of the grain protein (18, 19). In contrast, the alcohol-water soluble prolamines, which are the predominant storage proteins in most of the other major cereals, compose less than 5 to 10% of the total rice grain protein. Glutelins are synthesized at about 4 to 6 DAF,[2] whereas prolamine accumulation is first detected several days later (10, 22). These proteins are deposited exclusively into two different protein bodies which can be distinguished by morphological characters and size (17).

Rice glutelins have heterogeneous mol wt, the predominant species exhibiting sizes of 34 to 39 kD (α subunits) and 21 to 23 kD (β subunits). Wen and Luthe (21) have demonstrated the marked microheterogeneity of these subunits by isoelectric focusing. Results from *in vivo* pulse-chase labeling studies suggest that the α- and β-glutelin subunits may be proteolytic products of a larger precursor, 57 kD in size (10, 22). Direct evidence, however, for this precursor-product relationship is lacking. Here,

we have raised antibodies against the putative glutelin precursor and its two subunits and present immunological evidence that a precursor-product relationship exists among these glutelin components. Furthermore, the glutelin precursor as well as the prolamine polypeptides are synthesized *in vitro* as larger preproteins suggesting the presence of signal peptides and synthesis on RER.

MATERIALS AND METHODS

Chemicals. [1-¹⁴C]Iodoacetamide (23.6 mCi/mmol), ¹²⁵I-protein A (8.9 μCi/μg), and L-[³⁵S]methionine (1120 Ci/mmol) were obtained from New England Nuclear. ¹⁴C-Labeled protein mol wt standards were purchased from Bethesda Research Laboratories, Gaithersburg, MD. Canine pancreatic microsomal membranes for *in vitro* protein processing was from Amersham, England. All other chemicals and reagents used in this study were obtained from Sigma Chemical Co.

Plant Material. Rice (*Oryza sativa* L. cv. Biggs M-201) was grown in an environment controlled growth chamber in 25.4 cm pots containing 55% peat, 35% pumice, 7.5% pumice sand, and 2.5% sand under flooded conditions. The photoperiod was 14 h day (30°C) and 10 h night (25°C). The plants were fertilized twice with Peter's soluble fertilizers (10:30:20) supplemented with ferrous-chelate and micronutrients. Panicles were tagged on the day of anthesis and harvested at different stages of seed development. The seeds were frozen immediately in liquid N₂ and stored at −80°C.

Purification of Glutelin and Prolamine Proteins. Preparation of a crude glutelin fraction and subsequent alkylation were performed according to Sawai and Morita (15, 16). The reduced glutelin preparation was then fractionated by preparative SDS-PAGE and briefly stained with Coomassie brilliant blue. Gel slices containing the 51 kD, 34 to 37 kD, and 21 to 22 kD polypeptides were then rinsed extensively with 100 mM Tris-HCl, pH 8.0. The gel slices were mashed and then soaked overnight in 1% SDS, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 150 mM NaCl. Eluted proteins were collected by precipitation with 4 volumes of acetone followed by centrifugation at 28,000g for 15 min. The protein pellets were dried *in vacuo* and repurified a second time by SDS-PAGE. Prolamines were obtained from rice flour by extracting with 70% ethanol. This protein fraction when resolved by SDS-PAGE revealed a major polypeptide of 14 kD and few other contaminating high mol wt proteins. The 14 kD polypeptide was further purified on preparative SDS-PAGE as described above.

Antibody Production to Rice Glutelin and Prolamine Polypeptides. Rabbits were injected with about 400 μg of purified glutelin and prolamine proteins for 4 consecutive times at 2 week intervals. Antigen for the first injection was emulsified in Freund's complete adjuvant, whereas in subsequent injections, incomplete adjuvant was used. The IgG fraction of the sera was partially purified by (NH₄)₂SO₄ precipitation and DEAE-cellulose chromatography.

Western Blotting. Total rice seed proteins fractionated by SDS-

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[2] Abbreviation: DAF, days after flowering.

PAGE were electrophoretically transferred to nitrocellulose according to Burnett (6). Glutelin proteins were then identified by incubating the nitrocellulose sheet with purified glutelin antibodies (36 μg/ml) followed by incubation with 1 μCi of ^{125}I-protein A. The immunoreactive proteins were detected by autoradiography using a Cronex Lightening Plus (Du Pont) intensifying screen.

Proteolytic Digestions. Purified glutelin polypeptides (50 μg) were dissolved in 60 μl of SDS sample buffer (125 mM Tris-HCl (pH 6.8), 0.5% SDS, 10% glycerol, and 0.001% bromophenol blue) and boiled for 3 min at 100°C. The samples were cooled to room temperature before the addition of *Staphylococcus aureus* V8 protease to a final concentration of 15 μg/ml. After incubation at 37°C for 45 min the reactions were terminated by placing the tubes at 100°C for 2 min. About 15 μg of protein per sample were then fractionated on 15% SDS polyacrylamide gels and the peptide pattern visualized by silver staining (11) or Western blotting as described above.

Other Methods. The isolation of polysomes, total RNA and poly(A)$^+$ RNA from developing rice seeds and their *in vitro* translation in a wheat germ lysate were carried out as described previously (12, 13). *In vitro* protein processing using canine pancreatic microsomal membranes were carried out according to the manufacturer's (Amersham) guidelines. Translations employing rabbit reticulocyte lysate were performed according to the manufacturer's (Promega Biotec) specifications. The 51 kD glutelin precursor was labeled by reduction with [^{14}C]iodoacetamide (3). SDS-PAGE (9) was carried out on 12% resolving polyacrylamide gels (1.5 mm thick) at a constant power of 5 W. Immunoprecipitation of the total *in vitro* translation products were carried out according to Jonassen *et al.* (8).

RESULTS

The α- and β-glutelin subunits have been purified using ion exchange chromatography and more recently by chromatofocusing (21) but because of their insolubility in nondenaturing media, the separation of individual glutelin subunits was extremely difficult in our hands. However, purification of glutelins by SDS-PAGE was a relatively easy way of obtaining large quantities of purified proteins. The reduced glutelin fraction when resolved by SDS-PAGE revealed 4 major groups of bands having mol wt of about 51 kD, 34 to 37 kD, 21 to 22 kD, and 14 kD as well as a few faint higher mol wt polypeptides (Fig. 1b). The 14 kD polypeptide was found to be a prolamine based on the protein blotting studies (results not shown) and was the major contaminant of the glutelin fraction. In view of the simple mixture of proteins in this fraction the putative 51 kD precursor, α subunit (34–57 kD) and β subunit (20–22 kD) bands were easily purified to near homogeneity on preparative SDS polyacrylamide gels (Fig. 1, c–e). Antibodies were raised to the purified α and β subunits as well as the putative precursor as described in "Materials and Methods."

The specificity of α and β subunit antibodies was examined with the Western blot technique. Both antibodies exhibited high specificity and showed only a slight amount of cross-reactivity (Fig. 2). The cross-reactivity may be due to the presence of some common antigenic epitopes in both subunits or slight cross contamination of the glutelin subunits in our purified preparations. Both antibodies recognized the 51 kD polypeptide implying a direct immunological relationship among these components. Antibodies raised against the 51 kD protein also strongly reacted with all components in question (results not shown). In contrast, all antibody preparations failed to recognize the 14 kD prolamine present in the reduced glutelin fraction (Fig. 1b). The
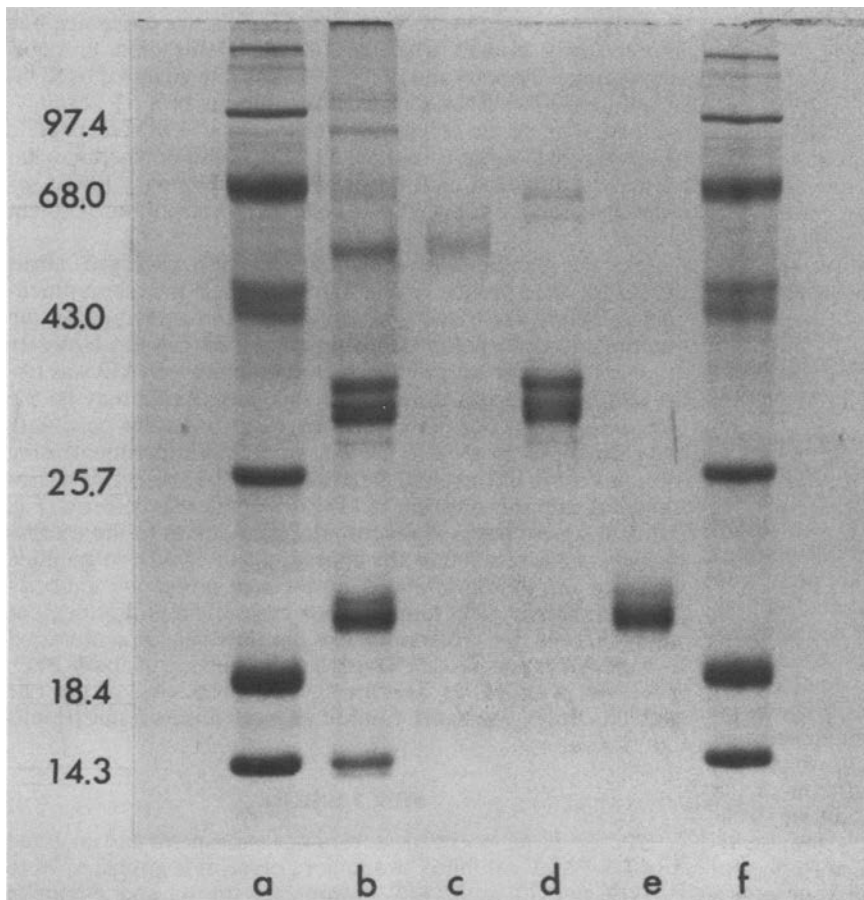

FIG. 1. SDS-PAGE analysis of rice glutelins. A crude glutelin fraction obtained by sequential solvent extraction was subjected to alkylation and resolved by SDS-PAGE (lane b). Polypeptides corresponding to the 51 kD (lane c), 34 to 37 kD (lane d), and 21 to 22 kD (lane e) glutelin polypeptides were twice purified by preparative SDS-PAGE. The proteins were resolved on 12% SDS polyacrylamide gels and stained with Coomassie brilliant blue. Lanes a and f are mol wt markers (lysosyme, 14,300; β-lactoglobulin, 18,400; α-chymotrypsinogen, 25,700; ovalbumin, 43,000; BSA, 68,000; and phosphorylase b, 97,400).

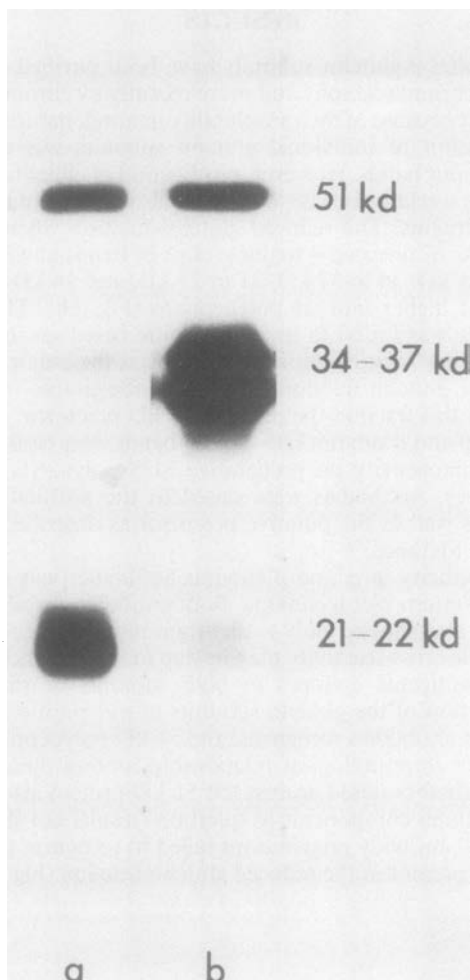FIG. 2. Western blot showing the specificity of the α- and β-glutelin subunit antibodies. A total of 50 μg rice seed proteins per lane were fractionated on a 12% SDS polyacrylamide gel and the resolved proteins electrophoretically transferred to nitrocellulose filter paper. The nitrocellulose paper was incubated with antisera raised against either the purified α subunits (lane b) or β subunits polypeptides (lane a). The immunoreactive bands were visualized by incubating the nitrocellulose paper with ^{125}I-protein A followed by autoradiography.

immunological relatedness shared by these components suggests that the 51 kD protein is indeed the precursor form of the mature glutelin subunits.

Proteolytic Digestion of the Rice Glutelin. The relatedness of these glutelin components was also analyzed by peptide mapping. The peptide patterns as generated by treatment with *S. aureus* V8 protease of the putative precursor and the two subunit polypeptides are shown in Figure 3A. Complex peptide patterns were obtained for the putative precursor and α subunit and in most instances common peptide bands were detected among these two polypeptides (Fig. 3A, lanes f and g). In contrast, the proteolytic peptide pattern obtained for the β subunit was relatively simple, reflecting the fact that this subunit contains fewer acidic amino acids (21) and hence less sites for cleavage by *S. aureus* V8 protease. Nevertheless the few peptide bands generated from the β subunit appear common to the precursor peptide map. Figure 3B shows the proteolytic peptide patterns of the precursor protein when visualized by immunoblotting techniques using purified α subunit and β subunit polyclonal antibodies. The peptide patterns of the putative precursor polypeptide are different when visualized with the anti-α or anti-β

antibodies. However, a composite of the digestion patterns obtained with both antibody preprations is consistent with patterns obtained from the whole putative precursor. Therefore, the α- and β-glutelin subunits appear to be structurally unrelated.

In Vitro **Translation.** In previous studies, SDS-PAGE of *in vitro* translation products of rice polysomes (10, 22) revealed the synthesis of a polypeptide of 56 to 57 kD, the putatve glutelin precursor. Since no direct evidence was obtained in these studies that this *in vitro* translated product was indeed the glutelin precursor, this question was addressed here. Poly(A)$^+$ RNA was isolated from 15 DAF rice seeds, translated in a wheat germ or rabbit reticulocyte protein synthesizing system and resulting products analyzed by SDS-PAGE (Fig. 4). A translation product of about 53 kD was detected, and as observed by others, no bands corresponding to the α and β subunits were evident. Interestingly in the wheat germ system the putative polypeptide did not represent the predominant translation product. Several low mol wt translation products of 12 kD or less appeared conspicuously (results not shown). *In vitro* translations employing a rabbit reticulocyte lysate greatly reduced the appearance of the lower mol wt translational products (Fig. 4c) and increased the amount of [^{35}S]methionine incorporation in the putative precursor polypeptide. Moreover, efficient synthesis of polypeptides with mol wt greater than 50,000 were obtained using the rabbit reticulocyte lysate system rather than the wheat germ extract system. Samples of the reticulocyte driven assays were incubated with either α or β subunit antibodies and the immunoprecipitated polypeptides analyzed by SDS-PAGE. Both the α and β subunits antibodies immunoprecipitated the same 53 kD polypeptide (Fig. 4, d and e) confirming the precursor nature of this polypeptide. In contrast, no bands were detected when the translation products were treated with the same amount of preimmune IgG. When compared to the authentic ^{14}C-labeled precursor polypepide (Fig. 4f), the *in vitro* translated precursor (Fig. 4g) was larger by 1000 to 2000 D. This size difference was more clearly evident when the *in vitro* translation products employing polysomes and poly(A)$^+$ RNA were analyzed by SDS-PAGE. Poly(A)$^+$ RNA directed the synthesis of a 53 kD polypeptide, whereas the polysomes synthesized a 51 kD polypeptide (Fig. 5A). This suggests that the 51 kD glutelin polypeptides are initially synthesized as a larger species possessing a leader sequence of about 2 kD which facilitate their transport into protein bodies.

Like the glutelins, the prolamines are also packaged, albeit separately, into protein bodies. Interestingly in the rabbit reticulocyte system no translational products corresponding to the mature prolamine polypeptides were observed (Fig. 4c). However a major translational product corresponding to 16 kD was observed, leading us to assume that this polypeptide may be the precursor for the rice prolamines. In order to test this possibility we carried out an *in vitro* protein processing experiment using dog pancreatic microsomal membranes. In the absence of microsomal membranes only the 16 kD polypeptide was detected (Fig. 5B, lane a). Addition of microsomal membranes to the translation reactions resulted in the appearance of 14 kD polypeptide that was immunoprecipitated by the rice prolamine antibody (Fig. 5B, lane b). The results of our experiment clearly demonstrate that the rice prolamines, like the glutelins, are synthesized as a precursor polypeptide having a signal peptide of about 2 kD. However, attempts to demonstrate the processing of the rice glutelins under the same conditions were unsuccessful (results not shown).

DISCUSSION

Glutelin proteins extracted from rice endosperm and analyzed by SDS-PAGE exhibited two major polypeptide groups of 34 to 37 kD and 20 to 22 kD, presumably the α- and β-glutelin
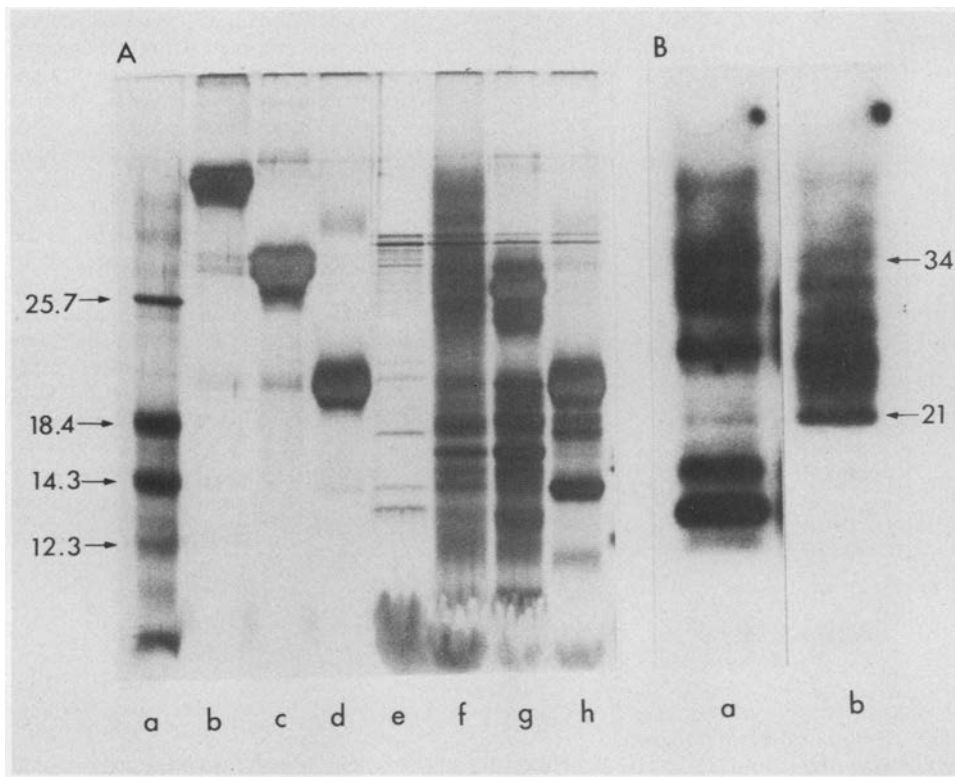
FIG. 3. SDS-polyacrylamide gel patterns of glutelin polypeptides obtained after partial digestion with *S. aureus* V8 protease. Panel A, peptide patterns generated by silver staining. Lane a, protein standards in kD; lanes b, c, and d, undigested 51 kD polypeptide, α subunit and β subunit, respectively; lanes f, g, and h, peptide patterns of 51 kD polypeptide, α subunit, and β subunit, respectively; and lane e, *S. aureus* V8 protease alone. Panel B, immunological detection of the peptide pattern generated by limited proteolysis of the 51 kD polypeptide. Lane a was probed with the α subunit antibody while lane b was incubated with the β subunit antibody.
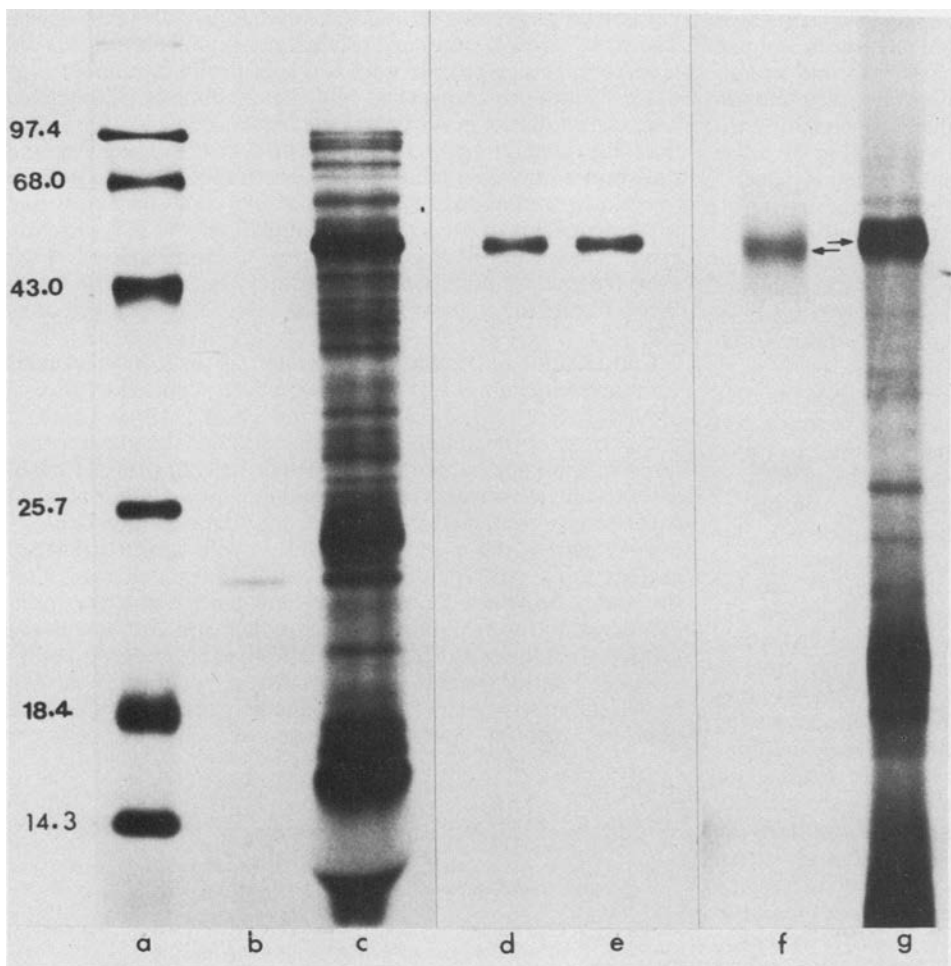

FIG. 4. *In vitro* translation of rice endosperm poly(A)$^+$ RNA and immunoprecipitation of the glutelin precursor polypeptide. A sample of 1 μg of rice endosperm poly(A)$^+$ RNA at 15 DAF was translated in a rabbit reticulocyte lysate (lanes b, c, d, and e) or wheat germ lysate (lane g) and products analyzed by SDS PAGE. Lane a, protein standards in kD; lane b, control translations without any added poly(A)$^+$ RNA; lanes c and g; total translation products; lanes d and e, translation products immunoprecipitated by α subunit and β subunit antibodies, respectively; and lane f, ^{14}C-labeled authentic glutelin precursor polypeptide.
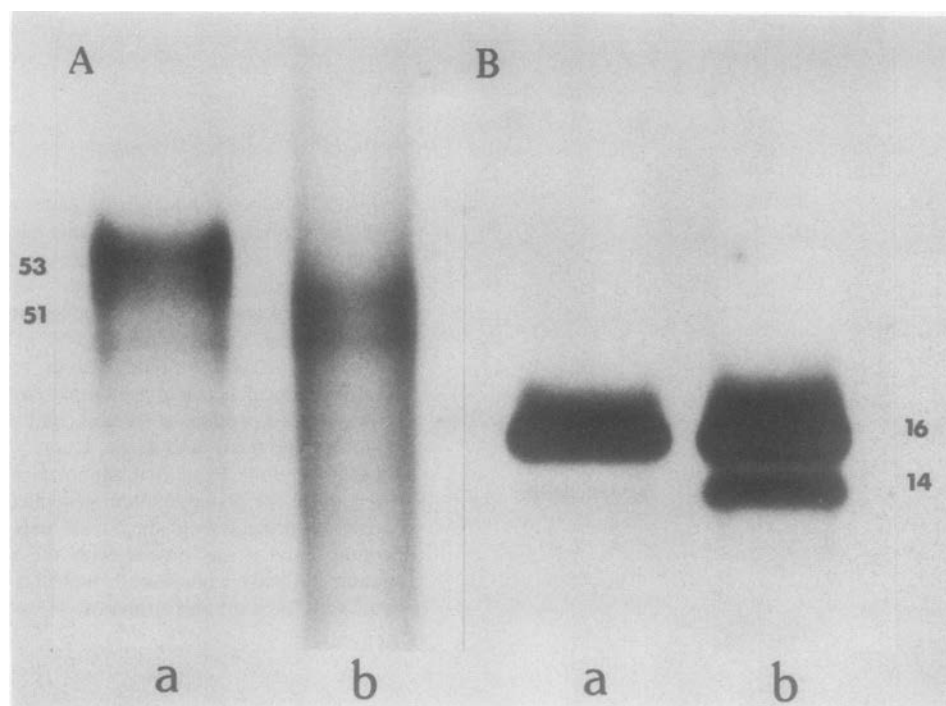
FIG. 5. SDS-Polyacrylamide gel patterns of immunoprecipitated rice glutelin and prolamine showing the presence of a putative signal peptide. Panel A, in vitro translational products synthesized with poly(A)$^+$ RNA (lane a) or polysomes (lane b) were immunoprecipitated with the glutelin α subunit antibody. Panel B, in vitro translations were carried out either in the absence (lane a) or in the presence (lane b) of dog microsomal membranes and the products were immunoprecipitated with anti-prolamine and analyzed by SDS-PAGE. The numbers on each side of panel A and B represents the mol wt in kD of the immunoprecipitated polypeptides.

subunits, and a putative precursor at 51 kD. Antibodies raised against the α and β subunits cross reacted with the 51 kD polypeptide, and conversely, anti-51 kD polypeptide reacted with both the α- and β-glutelin subunits (data not shown). These results indicate that there is a direct structural relationship among these components. The results of the in vitro protein synthesis experiments further substantiates this relationship. Immunoprecipitation of the in vitro translation products by glutelin antibodies resulted in the isolation of a 53 kD product (Fig. 4), a size similar to the putative glutelin precursor. No major in vitro translation products corresponding to the size of the α- and β-glutelin subunits were detected on these gels. The immunological relatedness of the putative precursor and glutelin subunits, in vitro synthesis of only the putative precursor, and the results of in vivo pulse-chase labeling studies of others (10, 22) establish that the α- and β-glutelin subunits are products of a posttranslational cleavage of the 51 kD polypeptide.

The ability to isolate the glutelin precursor indicates that this protein is fairly stable in vivo and that proteolytic cleavage into the glutelin subunits occurs posttranslationally and not co-translationally. Yamagata et al. (22) have proposed that this processing step occurs only after the presursor reaches the protein bodies and this may be true for the oat globulin precursor (2). Our observation that the in vitro translated glutelin precursor is slightly larger (1000–2000 D) than the authentic ^{14}C-labeled glutelin precursor and the polypeptide synthesized by polysomes suggests the presence of a signal sequence (Figs. 4 and 5A) which has been found for all protein body-localized storage proteins studied to date (4). The addition of dog microsomal membranes to the in vitro translation reactions did not result in the processing of the glutelin precursor. This might indicate that the conditions employed in this experiment may not have been optimal for efficient processing of the glutelin precursor. However, the fact that we were able to demonstrate under the same conditions the processing of the prolamine preprotein suggests that the signal peptides of these proteins may be sufficiently different thereby accounting for their reactivity to the signal peptidases present in the dog microsomal membrane preparations. DNA sequence analysis of full length glutelin and prolamine cDNA clones should establish the nature of the signal peptides of these prepro-

teins.

The protein profile of the in vitro translation products revealed many distinct polypeptide bands. In addition to a prominent 53 kD glutelin preprotein, several lower mol wt products are evident. The 16 kD in vitro translated product presumably represents the preprolamine polypeptide since it is specifically immunoprecipitated by anti-prolamine (Fig. 5B). The amount of [^{35}S]methionine incorporation in the prolamine band is considerably higher than the glutelin preprotein (Fig. 4). However, in vivo, the prolamines only constitute a small fraction of the total protein in endosperm tissue (18). This discrepancy could be simply due to the capacity of in vitro translations systems to efficiently synthesize smaller polypeptides relative to larger species. However, the glutelin preprotein mRNA may require specific translation factors for efficient synthesis as seen for the oat globulins (7).

Conservation of peptide sequences among the storage proteins from several legumes and cereals have been reported by several groups (1, 5, 14, 20, 21, 23). Wen and Luthe (21) have made a comparison of the amino acid composition of the glutelin subunits to homologous proteins from oat, coconut, broad bean, soybean and other plants and observed that the β-glutelin subunit was strongly conserved in these plants. Partial amino acid sequence data of the β-glutelin subunit have confirmed this homology to the pea legumin (23). These observations imply that the storage proteins are coded by ancient genes which have been conserved during evolution. Because of their similarities with the legume globulins with respect to their physical properties, processing and packaging into protein bodies, it would be interesting to determine what extent the rice glutelin genes share sequence homology with the legume globulin genes.

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