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Characterization of α-Amylase-Inhibitor, a Lectin-Like Protein in the Seeds of *Phaseolus vulgaris*[1]

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ABSTRACT

The common bean, *Phaseolus vulgaris*, contains a glycoprotein that inhibits the activity of mammalian and insect α-amylases, but not of plant α-amylases. It is therefore classified as an antifeedant or seed defense protein. In *P. vulgaris* cv Greensleeves, α-amylase inhibitor (αAI) is present in embryonic axes and cotyledons, but not in other organs of the plant. The protein is synthesized during the same time period that phaseolin and phytohemagglutinin are made and also accumulates in the protein storage vacuoles (protein bodies). Purified αAI can be resolved by SDS-PAGE into five bands (M_r 15,000–19,000), four of which have covalently attached glycans. These bands represent glycoforms of two different polypeptides. All the glycoforms have complex glycans that are resistant to removal by endoglycosidase H, indicating transport of the protein through the Golgi apparatus. The two different polypeptides correspond to the N-terminal and C-terminal halves of a lectin-like protein encoded by an already identified gene or a gene closely related to it (LM Hoffman [1984] J Mol Appl Genet 2: 447–453; J Moreno, MJ Chrispeels [1989] Proc Natl Acad Sci USA 86:7885–7889). The primary translation product of αAI is a polypeptide of M_r 28,000. Immunologically cross-reacting glycopolypeptides of M_r 30,000 to 35,000 are present in the endoplasmic reticulum, while the smaller polypeptides (M_r 15,000–19,000) accumulate in protein storage vacuoles (protein bodies). Together these data indicate that αAI is a typical bean lectin-type protein that is synthesized on the rough endoplasmic reticulum, modified in the Golgi, and transported to the protein storage vacuoles.

The seeds of the common bean synthesize a number of proteins that are either true lectins (PHA-E and PHA-L)[3] or lectin-like proteins such as arcelin (23) and LLP (15, 32, 33). The genes for these proteins all show considerable sequence identity. Both PHA-E and PHA-L as well as arcelin are thought to be plant defense proteins that protect seeds against insect predation (13, 17, 23). Bean seeds also contain an αAI that inhibits insect but not plant α-amylases and is therefore also thought to be a plant defense protein.

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[3] Abbreviations: PHA, phytohemagglutinin; LLP, lectin-like protein; αAI, α-amylase inbibitor; SS buffer, succinate salt buffer: endo H, endoglycosidase H; TFMS, trifluoromethanesulfonic acid.

The αAI has been characterized in several varieties of beans (19, 21, 25). It is a glycoprotein (about 15% carbohydrate) and its native mol wt has been estimated to be 43 to 50 kD by gel filtration experiments (24, 25). The inhibitor is composed of subunits of M_r 15,000 to 18,000, and it has been proposed that it is either a trimer or a tetramer of identical polypeptides (24) or different polypeptides (19, 25). The inhibitor binds to animal α-amylases at a pH optimum of 5.6, forming a stable 1:1 (molar ratio) complex (26, 34). We recently reported (22) that the bean αAI is encoded by a gene that encodes a LLP described some years ago by Hoffman *et al.* (15) or by a closely related gene. The protein product (called LLP) of this gene remained unidentified until Vitale *et al.* (32) showed that it is a glycoprotein with four Asn-linked glycans and that it is synthesized on the rough ER. The protein has an apparent mol wt of 45,000 (gel filtration) or 40,000 (SDS-PAGE). Pulse-chase experiments indicated that the LLP protein left the ER and was either proteolytically processed or degraded (A Vitale, personal communication).

We report here the molecular characterization, biosynthesis, processing, and accumulation of αAI in developing bean cotyledons and show that all these processes resemble those observed for the polypeptides of phytohemagglutinin (6, 31). Our data are consistent with the interpretation that αAI is synthesized as a larger precursor (M_r 35,000) which is proteolytically processed to the polypeptides characteristic of αAI (M_r 15,000–18,000) prior to its accumulation in protein bodies. In a recent study, Ceriotti *et al.* (5) show that LLP is proteolytically processed to polypeptides of M_r 15,000 to 18,000 and accumulates in this form in protein bodies.

MATERIALS AND METHODS

αAI Extraction

Midmature (20–30 d after pollination) cotyledons of seeds of the common bean (*Phaseolus vulgaris* cv Greensleeves) were homogenized in an ice-cold mortar with 3 mL of 10 mM β-mercaptoethanol per g of fresh weight. The homogenate was centhftiged at 12,000*g* for 10 min and the supematant was buffered by adding 0.2 M succinate, 0.1 M $CaCl_2$ (pH 3.8) (110 μL/mL) and was heated in a water bath at 70°C for 10 min. The protein precipitate was removed by centritugation (12,000*g* for 10 min), and the clear supernatant was brought to pH 5.6 with NaOH. This solution was used for further purification by affinity batch adsorption or for the αAI assay.

αAI Purification by Affinity Batch Adsorption

Porcine pancreas α-amylase (Boehringer Mannheim) was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The beads with immobilized enzyme were equilibrated with 15 mM succinate, 20 mM $CaCl_2$, 0.5 M NaCl (pH 5.6) (SS) buffer, and the inhibitor extract was added (0.2 mL extract per mL of beads). The mixture was tumbled at 37°C for 1 h, and then the beads were washed six times with SS buffer (5 mL/mL of beads). The inhibitor was released by tumbling 20 min with 0.12 M sodium citrate (pH 3.0) (1 mL/mL of beads). Then the suspension was centrifuged (1500*g* for 5 min) and the supernatant saved. The citrate washing was repeated, and both supernatants were pooled and filtered through a sintered glass funnel. αAI was concentrated by precipitation with trichloroacetic acid (25% final concentration).

α-Amylase Inhibitor Assay

The inhibitory activity assay was performed by adding different volumes of inhibitor extract to 80 ng of porcine pancreas α-amylase solution in a total volume of 1 mL of SS buffer with 1 mg of bovine serum albumin. The mixture was incubated at 37°C for 30 min and then the α-amylase reaction was initiated by adding 0.5 mL of potato starch in 50 mM sodium maleate, 10 mM $CaCl_2$ (pH 6.9). The concentration of starch was adjusted to give a zero-time A_{620} of 1.2 after reaction with the iodine reagent. The reaction was stopped after 5 min with 0.5 mL of iodine reagent (30). The blue color was diluted by adding 2.5 mL of water, and the A_{620} was determined. Inhibitory units (i.u.) were defined as the amount of αAI that completely inhibits 1 ng of porcine pancreas α-amylase in the assay conditions described above.

Purification of the Microsomal Fraction and Sucrose Gradient Fractionation

Bean cotyledons were homogenized in 0.1 M Tris-HCl, 1 mM EDTA with 12% (w/w) sucrose (pH 7.8) (1:10, fresh weight to volume ratio). The microsomal fraction was obtained by chromatography on Sepharose 4B as previously described (28), loaded on top of a 16 to 54% (w/w) linear sucrose gradient in 0.1 M Tris-HCl, 1 mM EDTA (6), and centrifuged at 150,000*g* for 2 h. The gradient was fractionated and an aliquot of each fraction subjected to SDS-PAGE and immunoblotting. For separating the ER membranes from the Golgi, the same procedure was followed except that EDTA was replaced by 2 mM $MgCl_2$ in all steps. Under these conditions, the ER of bean cotyledon bands at 1.13 $g \cdot cm^{-3}$ (medium with EDTA) or at 1.18 $g \cdot cm^{-3}$ (medium with 2 mM $MgCl_2$), while the Golgi membranes (marker enzyme inosine diphosphatase) remain at 1.14 $g \cdot cm^{-3}$ in both media (6).

Electrophoresis, Immunoblotting, and Endo H Treatment

SDS-PAGE (15% acrylamide) was performed according to Laemmli (18). Transfer to a nitrocellulose membrane and immunostaining was carried out as detailed in the Bio-Rad technical bulletin. Anti-αAI serum was obtained by injecting a rabbit with purified and chemically deglycosylated αAI from red kidney beans. Chemical deglycosylation of αAI was carried out by the method of Edge *et al.* (9). Goat anti-rabbit immunoglobulins coupled to horseradish peroxidase (Bio-Rad) was used as a secondary antibody.

Removal of high-mannose glycans was done with endoglycosidase H as described by Trimble and Maley (29). To 50 μg of protein in 50 μL of solution, we added 60 μg of SDS in 50 μL of water (1.2-fold weight excess of SDS) and heated for 2 min in a boiling water bath. The clear solution was brought to a final concentration of 0.1% Triton X-100 and 0.25% β-mercaptoethanol and heated in a boiling water bath for another 2 min. Twenty μL of endo H buffer (1 M Na-acetate, pH 5.8) and 10 munits of enzyme were added. Digestion was for 48 h at 37°C under a toluene atmosphere. After 24 h, another 10 munits of enzyme were added.

For preparative purification of the polypeptides of αAI after electrophoresis, the bands were visualized with KCl (14) and cut out of the gel. The gel pieces were minced and extracted by diffusion in 20 mM Tris-HCl, 0.5 M NaCl (pH 7.5), overnight at 4°C.

RNA Extraction and in Vitro Translation

RNA was extracted from bean cotyledons according to Prescott and Martin (27). Poly A^+-RNA was purified using an affinity paper (Hybond-mAP, Amersham) according to the manufacturer's instructions and translated using the rabbit reticulocyte lysate system (16) in the presence of [^{35}S]methionine. The translation product was immunoprecipitated with the anti-αAI serum followed by the addition of immobilized Protein A (Protein A Sepharose CL-4, Pharmacia) as described previously (10). The total and immunoprecipitated products were analyzed by SDS-PAGE and fluorography (3).

In Vivo Incorporation of [^{3}H]Glucosamine

Cotyledons were labeled with 5 μCi each of [^{3}H]glucosamine (40 mCi/mol) for 20 h as described (6).

Isolation of Protein Storage Vacuoles (Protein Bodies)

Protein storage vacuoles were isolated from the meal of dry seeds by the nonaqueous glycerol method of Begbie (1).

Measurement of Dry Weight and Protein

Dry weight was determined by weighing after heating the tissues overnight to 80°C in a vacuum oven. Protein was determined colorimetrically according to Lowry *et al.* (20) using bovine serum albumin as a standard.

RESULTS

Purification and Subunit Analysis of the αAI

We purified αAI from bean cotyledons by extraction at low ionic strength followed by heat treatment after adding a pH 3.8 succinate buffer. This gave a partially purified extract that showed inhibition of porcine pancreas α-amylases, but not of barley α-amylase (Fig. 1) as expected for the bean αAI. The

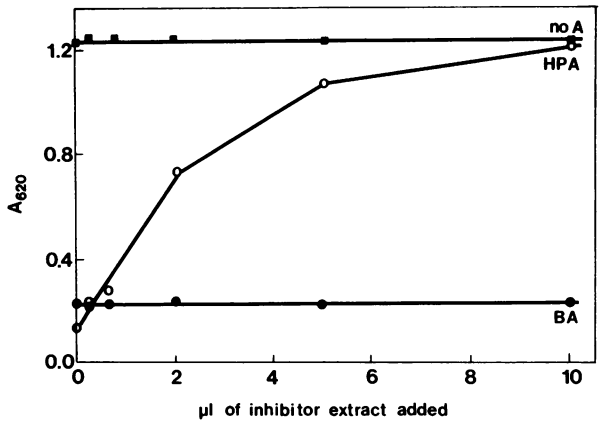

Figure 1. Assay of α-amylase inhibitory activity. The figure represents the amount of starch (measured as A_{620} after reaction with the iodine reagent) remaining after 5 min incubation with porcine pancreas α-amylase (HPA), barley amylase (BA), or no amylase (no A) *versus* the amount of inhibitor extract (diluted 1/10) added to the assay medium.

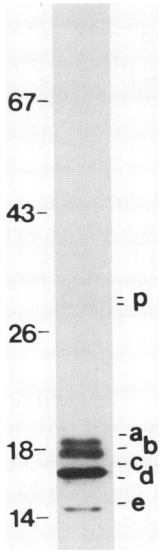

Figure 2. SDS-PAGE of αAI purified by affinity batch adsorption. Numbers represent approximate $M_r \cdot 10^{-3}$. p = aggregates.

inhibitor was isolated from this extract by affinity batch adsorption using porcine pancreas α-amylase coupled to agarose beads. The material adsorbed by the beads was analyzed by SDS-PAGE, which yielded a pattern of five polypeptide bands (labeled a to e in Fig. 2) in the range of M_r 14,000 to 19,000 and a weak doublet with a relative mobility corresponding to about M_r 32,000 (marked P in Fig. 2). It has been proposed that the 32 kD polypeptides are undissociated aggregates of the smaller polypeptides (19). To check this possibility, we obtained the putative aggregates and individual polypeptides separately by cutting out the gel pieces and eluting the polypeptides. The individual polypeptides were then subjected to another round of SDS-PAGE (Fig. 3). The putative aggregates (polypeptides with M_r 32,000) partially dissociated, giving rise to smaller polypeptides, while the

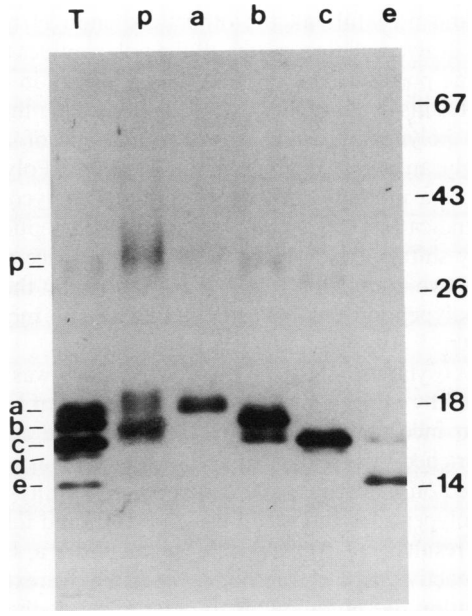

Figure 3. Analysis of the αAI polypeptide bands. Different fractions (obtained by excising bands from a preparative gel) enriched in the different polypeptides p, a, b, c, and e were run again on SDS-PAGE in parallel with the total αAI(T) and immunoblotted. Numbers indicate approximate $M_r \cdot 10^{-3}$.

smaller polypeptides (M_r 14,000–19,000) partially associated, giving rise to some larger polypeptides (Mr 32,000). We interpret this as evidence that there is always some aggregation of the α-Al polypeptides even after heating at 100°C in the presence of SDS and mercaptoethanol.

As previously reported (22), the N-terminal sequencing of the polypeptides a, b, c, and e gave only two amino acid sequences. Because of the similar mobilities of the polypeptides, we were unable to separate them clearly after SDS-PAGE followed by transfer to the polyvinylidene difluoride membrane. Nevertheless, the bands were excised and subjected to N-terminal amino acid sequencing. Polypeptide a gave a unique sequence corresponding to amino acids 101 to 115 of the derived amino acid sequence of the LLP gene (15). This was also the dominant sequence in polypeptides d and e, but a second contaminating sequence was also found. The second sequence corresponds to amino acids 24 to 34 of the derived sequence of LLP. Polypeptides b and c had as their dominant sequence amino acids 24 to 34 of the derived sequence of the LLP gene, and the other sequence as a contaminant. These results imply that the five polypeptides, a to e, may be glycoforms of two different polypeptides, since it is known that αAI is a glycoprotein (24, 25).

Glycosylation Status of αAI Polypeptides

We examined the glycosylation status of polypeptides by treating the polypeptide mixture and the individual polypeptides after electroelution from the appropriate gel slices with endo H to remove high-mannose glycans, and with TFMS to remove both the high-mannose and complex glycans. The resulting polypeptides were analyzed by SDS-PAGE. The results (Fig. 4) show that only polypeptide a is endo H sensitive

(lanes 4 and 5), while all the others are endo H resistant. Chemical deglycosylation causes all the polypeptides to move to the same position, just below d. Deglycosylation of polypeptide a results in a mobility that is slighlly greater than endo H-treated polypeptide indicating the presence of a single complex glycan as well as a high-mannose glycan. Polypeptide c undergoes a mobility shift upon chemical deglycosylation that also indicates a single complex glycan. Polypeptide b has a mobility shift upon deglycosylation that is twice as large, indicating two complex glycans. It is noteworthy that treatment of polypeptide e with TFMS caused a slight increase in its mobility.

The glycosylation status of the polypeptides was further investigated by purifying αAI from cotyledons that had been allowed to incorporate [^{3}H]GlcN for 20 h. The cotyledon extract enriched in αAI by acid and heat treatment was then fractionated on a porcine pancreas aamylase affinity column and the polypeptides analyzed by SDS-PAGE and fluorography. The results (Fig. 5) show that polypeptides a, b, and c were radioactive, and e was not (even after overexposure). The resolution did not allow us to determine whether d was also labeled.

The nature of the glycans was also examined by immunostaining the polypeptides with an antiserum that recognizes the xylose-containing complex glycans of plants (11). We found that polypeptides a, b, and d stained positive, but c and e did not (data not shown).

Together these results are consistent with the following interpretation. Polypeptides a, d, and e are glycoforms of a peptide that is the C-terminal portion of LLP or a closely related protein: e has no glycans, d has one complex glycan, and a has a complex and a high-mannose glycan. Polypeptides b and c are glycoforms of the N-terminal portion of LLP or a closely related polypeptide: b has two complex glycans, and c has a single complex glycan that lacks xylose (as indicated by its lack of reactivity with the antiserum against complex glycans).

Level of Expression of αAI in Different Organs

Partially purified extracts of αAI were made from cotyledons, embryonic axes, seed coats, and pods 23 d after fertilization, and from leaves, stems, and roots of the same bean plants. The extracts were analyzed by immunoblot and α-amylase inhibitory assay. The inhibitor was detected only in cotyledons and embryonic axis (Fig. 6), its concentration (on a total protein basis) being threefold higher in the former. The immunoblot shows that the intensities of bands a, b, and c relative to d and e are much higher in the embryonic axis. Thus, there is a higher proportion of doubly glycosylated polypeptides in the axis, indicating that in the axis glycosylation is more efficient than in the cotyledons. This may be caused by the much lower level of synthesis of the glycoproteins phaseolin and PHA in the axis compared to the cotyledons, resulting in a relatively more abundant supply of glycans in the ER of the axis cells.

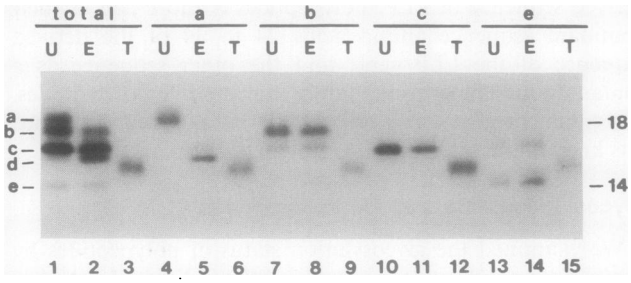

Figure 4. Glycan analysis of the αAI polypeptides. Total αAI and fractions enriched in different polypeptides (as in Fig. 3) were treated with endo H (E), deglycosylated with TFMS (T) or left untreated (U), analyzed by SDS-PAGE, and immunoblotted. Numbers in the right margin indicate approximate $M_r \cdot 10^{-3}$1.

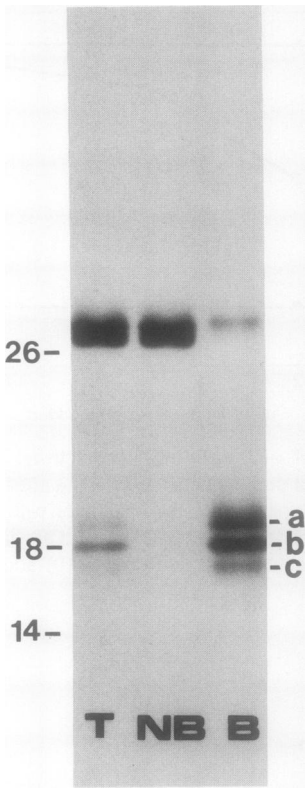

Figure 5. Incorporation of [^{3}H]GlcN into αAI. Cotyledons were labeled for 20 h and an αAI-rich extract prepared and fractionated on immobilized porcine pancreas α-amylase into a bound (B) fraction and a nonbound (NB) fraction. Total (T) is shown for comparison. Overexposure of the fluorograph did not result in a radioactive band for polypeptide e.

Accumulation of αAI during Seed Development

Extracts made from embryonic organs (cotyledons and axis) during different stages of development of the seed (ranging from 13–40 d after pollination) were analyzed by SDS-PAGE and immunoblot and for inhibitory activity. The αAI was detected in the seeds 17 d after pollination, and the amount increased (on a dry-weight basis) until 27 d after pollination, when it reached a plateau (Fig. 7). Afterward, the inhibitor content remained the same or decreased slightly (on a dry-weight basis) during the drying of the seed.

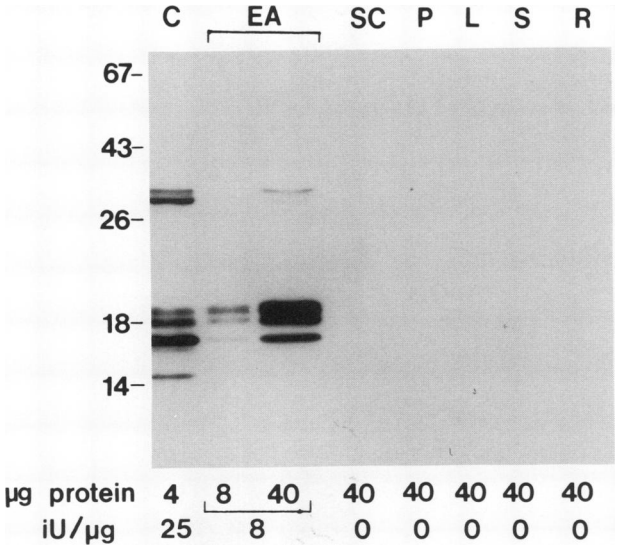

Figure 6. Organ and tissue specificity of αAI expression. Extracts from cotyledons (CC), embryonic axis (EA), seed coat (SC), pods (P), leaves (L), stems (S), roots (R) were either developed with SDS-PAGE and immunoblotted or assayed for inhibitory activity. Numbers in the left margin indicate approximate $M_r \cdot 10^{-3}$

Evidence for a Precursor of αAI

To find out if αAI is synthesized as a preprotein, we isolated poly A^+-RNA from cotyledons in three stages of development corresponding to numbers 3, 4, and 5 of Figure 7. The *in vitro* translation of this RNA and immunoprecipitation of the *in vitro* translation product gave rise to only one band of M_r 28,000, which we suggested to be a precursor form of αAI since its size matches the expected LLP gene product (22).

The putative precursor has been detected with the anti-αAI serum in the microsomal fraction of bean cotyledons as glycosylated products of M_r 35,000–40,000 (22). This fraction also contained polypeptides of lower mol wt matching the size of the mature αAI subunits. When the microsomal fraction was analyzed on isopycnic sucrose gradients in the presence of 1 mM EDTA, both the putative precursor and the subunits banded at a similar density of about 1.13 to 1.14 g·cm^{-3} (Fig. 8A). When the experiment was repeated in the presence of 2 mM Mg^2+, the high M_r polypeptides shifted to a higher density (1.18 g·dcm^{-3}), while the low mol wt polypeptides stayed at the same density (Fig. 8B). Analysis of marker enzymes on such gradients shows the ER marker enzyme NADH-Cyt *c* reductase undergoes a similar density shift (from 1.13–1.18 g·cm^{-3}) when $MgCl_2$ is substituted for EDTA, while the Golgi-marker enzyme inosine diphosphatase remains at the same density (1.14 g·cm^{-3}) (6). The results indicate, therefore, that the M_r 30,000 to 35,000 polypeptides are associated mainly with the ER, while the 14 to 19 kD polypeptides that do not shift in density may be associated with Golgi-derived membrane vesicles. These data are consistent with the interpretation that the M_r 30,000 to 35,000 polypeptides are processed to low mol wt polypeptides in the Golgi apparatus.

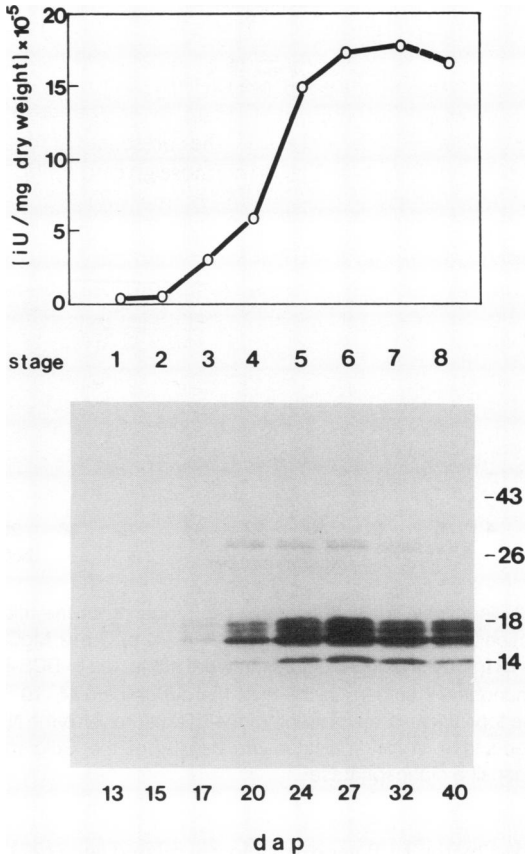

Figure 7. Developmental sequence of αAI accumulation during seed maturation. Samples of bean seeds were taken at indicated stages (dap = days after pollination) and αAI was determined either by inhibitory activity assay or SDS-PAGE (same amount of dry weight of seed loaded) and immunoblot. Numbers in the right margin indicate approximate $M_r \cdot 10^{-3}$.

α-Amylase Inhibitor is a Vacuolar Protein

Protein storage vacuoles (protein bodies) were isolated by the method of Begbie (1) from seed meal, and the polypeptides fractionated by SDS-PAGE. The Coomassie blue stained gel shows the characteristic presence of the storage protein phaseolin and of PHA (Fig. 9, lane 1). The immunoblot shows the presence of the five polypeptides of αAI (Fig. 9, lane 2) in the protein body fraction. Proteins that accumulate in vacuoles are synthesized as preproteins on the rough ER, and their transport to the vacuoles is mediated by the Golgi apparatus (7).

DISCUSSION

Several reports have been published about the structural characteristics and subunit composition of the αAI (19, 24, 25), postulating a trimer or tetramer structure on the basis of the mol wt of the subunits and of the native inhibitor as determined by gel filtration. However, some authors have pointed out that the mol wt of αAI could be lower than calculated due to the abnormal behavior of glycoproteins in gel filtration columns (25). The finding that the αAI of bean

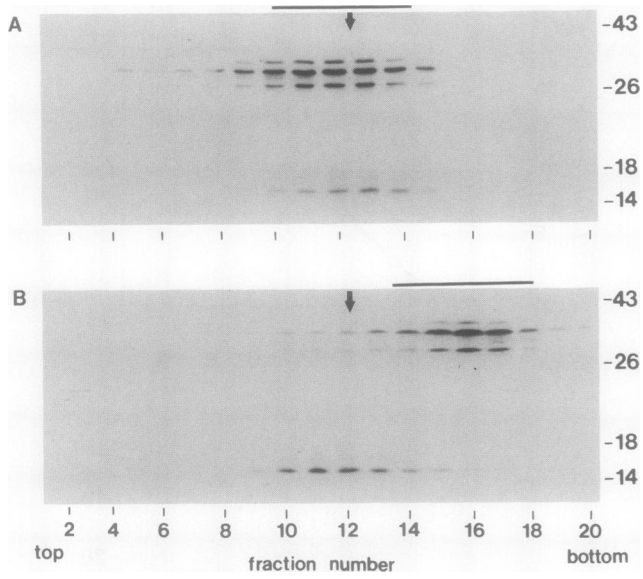

Figure 8. Isopycnic sucrose gradient centrifugation of the microsomal fraction in the presence of 1 mM EDTA (A) or 2 mM $MgCl_2$ (B). Aliquots of fractions along the gradient were subjected to SDS-PAGE and immunoblot. Numbers in the right margin indicate $M_r \cdot 10^{-3}$. Bar along the top indicates the position of the ER-marker enzyme NADH-Cyt *c* reductase. Arrow indicates the density of the Golgi-marker enzyme inosine diphosphatase.

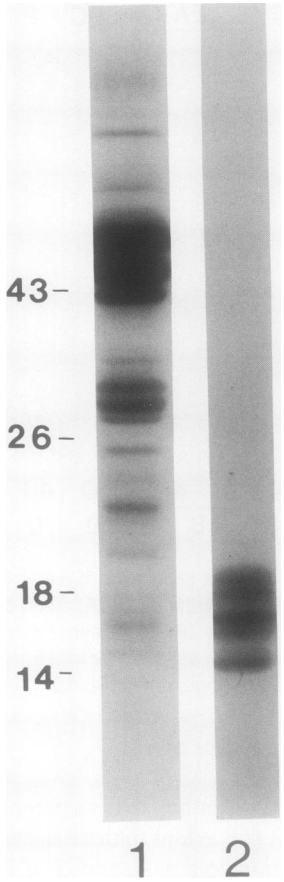

Figure 9. SDS-PAGE and immunoblot of a protein body fraction. Lane 1: Coomassie brilliant blue stain; lane 2: immunoblot developed with antibodies to αAl.

is encoded by the LLP gene or a closely related gene (22) may help to clarify this point. The LLP mature polypeptide has a molecular mass of 24.7 kD, and even if glycosylated in its five potential sites, its molecular mass is not expected to exceed 35 kD, which is the M_r value found for the putative precursor (by SDS-PAGE) in our experiments. The proteolytic processing of this precursor would produce a heterodimer (corresponding to the N-terminal and C-terminal pieces of LLP). This type of processing is identical to that observed for several other vacuolar proteins including jack bean α-mannosidase (12) and pea legumin and vicilin (8).

Immunoblots of membrane fractions indicate the presence of three polypeptides in the M_r 30,000 to 35,000 range (see Fig. 8). Treatment with endo H results in a major polypeptide of M_r 25,000 (22) indicating that the three polypeptides differ by the number of glycans present (*i.e.* they are glycoforms). Evidence of glycoforms was also obtained by an analysis of the carbohydrate status of the αAI polypeptides. It appears, therefore, that all the glycosylation sites are not always filled. The amino acid sequencing data (22) and the glycan analysis presented here are consistent with the interpretation that polypeptides b and c represent glycoforms of the N-terminal portion of LLP (with two and one glycans, respectively), while a, d, and e are glycoforms of the C-terminal portion of LLP with two, one, and no glycans, respectively.

According to the postulated processing of the LLP (22), the subunits should be 77 and 146 amino acids long. However, we observed that after deglycosylation the different αAI subunits have an M_r of 15,000 when analyzed by SDS-PAGE. This could be caused by the abnormal behavior of the polypep-tides in SDS-PAGE. We cannot rule out unusual processing events in which a piece of one polypeptide is transferred and re-ligated to the other, as has been shown to occur for concanavalin A (4). The excellent similarity between the amino acid composition of αAI and LLP protein suggests that there is no extensive loss of polypeptide fragments during the proteolytic processing of αAl.

The conclusion that the M_r 35,000 polypeptide in the membrane fraction is a precursor of the αAl polypeptides would be strengthened by pulse-chase experiments showing that radioactivity disappears from the precursor and appears in the smaller polypeptides. Such experiments were attempted several times, but were not successful. Although the antiserum recognizes polypeptides on immunoblots and can be used to immunoselect *in vitro*-synthesized protein, we were unable to immunoselect *in vivo*-synthesized protein, even after denaturation with SDS. This made it impossible to carry out *in vivo* pulse-chase experiments as we have done for other proteins (8, 28).

The results reported here complement results of Ceriotti *et al.* (5). These workers made antibodies against a fusion protein of the LLP gene in an expression vector. With these antibodies, they were able to show that the initial translation product of LLP in developing bean seeds is a glycoprotein of M_r 40,000 that is proteolytically processed after leaving the ER to polypeptides of M_r 15,000 to 19,000. These polypeptides

accumulate in the protein storage vacuoles. The two approaches, independently pursued, provide complementary data and lead to the same conclusion: αAI has LLP as its precursor (22) and LLP is processed to polypeptides that have the same size as αAI (5).

Our finding that αAI is a vacuolar protein, therefore, led us to look for the precursor of αAI in the ER, using the conventional method of shifting the density of the ER by using two different media, one with 1 mM EDTA and one with 2 mM $MgCl_2$. Vacuolar proteins have been shown to be synthesized on the rough ER and pass through the Golgi before arrival in the vacuoles (7). The results show that the larger polypeptides shift in density in the same way as the marker enzyme NADH-Cyt *c* reductase, while the smaller polypeptides that are associated with the membrane fraction remain at the same density. This finding indicates that cross-reacting polypeptides of the right size (putative precursors) are indeed associated with the ER, and that processing of the precursor may take place when these precursors reach the Golgi apparatus. We have previously shown that processing of other vacuolar proteins generally occurs in the vacuoles themselves (8). It appears that processing of αAI may occur sooner along the transport pathway than processing of other proteins.

ACKNOWLEDGMENTS

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