Plant Physiol. (1987) 85, 529–536
0032-0889/87/85/0529/08/$01.00/0

Characterization of Osmotin[1]

A THAUMATIN-LIKE PROTEIN ASSOCIATED WITH OSMOTIC ADAPTATION IN PLANT CELLS

Received for publication May 1, 1987 and in revised form July 14, 1987

NARENDRA K. SINGH, CHARLES A. BRACKER, PAUL M. HASEGAWA, AVTAR K. HANDA, SCOTT BUCKEL, MARK A. HERMODSON, ED PFANKOCH, FRED E. REGNIER, AND RAY A. BRESSAN*
Center for Plant Environmental Stress Physiology/Department of Horticulture (N.K.S., P.M.H., A.K.H., R.A.B.), *Department of Biochemistry* (S.B., M.A.H., E.P., F.E.R.), *Department of Botany and Plant Pathology* (C.A.B.), *Purdue University, West Lafayette, Indiana 47907*

ABSTRACT

Cultured tobacco (*Nicotiana tabacum* var Wisconsin 38) cells adapted to grow under osmotic stress synthesize and accumulate a 26 kilodalton protein (osmotin) which can constitute as much as 12% of total cellular protein. In cells adapted to NaCl, osmotin occurs in two forms: an aqueous soluble form (osmotin-I) and a detergent soluble form (osmotin II) in the approximate ratio of 2:3. Osmotin-I has been purified to electrophoretic homogeneity, and osmotin-II has been purified to 90% electrophoretic homogeneity. The N-terminal amino acid sequences of osmotins I and II are identical through position 22. Osmotin-II appears to be much more resistant to proteolysis than osmotin-I. However, it cross-reacts with polyclonal antibodies raised in rabbits against osmotin-I. Osmotin strongly resembles the sweet protein thaumatin in its molecular weight, amino acid composition, N-terminal sequence, and the presence of a signal peptide on the precursor protein. Thaumatin does not cross-react with antiosmotin. An osmotin solution could not be detected as sweet at a concentration at least 100 times that of thaumatin which could be detected as sweet. Immunocytochemical detection of osmotin revealed that osmotin is concentrated in dense inclusion bodies within the vacuole. Although antiosmotin did not label organelles, cell walls, or membranes, osmotin appeared sparsely distributed in the cytoplasm.

When exposed to gradually increasing concentrations of NaCl, cultured tobacco cells undergo phenotypic adaptation and exhibit dramatically increased tolerance to NaCl characteristic of halophytic plant species (13). Salt adapted tobacco cells exhibit a number of physiological changes including considerable osmotic adjustment (1) which involves accumulation of ions and organic solutes (2) and compartmentalization of ions (ML Binzel, unpublished data). We have indicated that the process of cellular adaptation to osmotic stress must involve the differential expression of genetic information (13, 23). We have shown that specific alterations in gene expression of salt adapted cells of tobacco occur since several novel proteins are made by adapted cells including a predominant 26 kD protein (23). We recently named this protein osmotin (4) because it is synthesized and accumulated by cells undergoing gradual osmotic adjustment to either salt or desiccation stress but is not induced by osmotic shock (24).

[1] Supported by Purdue University Agricultural Experiment Station Program Improvement funds and by the United States Department of Energy Grant DE 13109. Journal paper No. 11,154, Purdue University Agricultural Experiment Station.

Osmotin is a cationic protein with a pI[2] value >8.2 and accounts for 10 to 12% of total cellular protein (as determined by spectrophotometric measurement of Coomassie stained gels) in NaCl and polyethylene glycol adapted tobacco cells (23). In unstressed unadapted cells, an immunologically related 26 kD protein with a lower pI is also synthesized but is not accumulated under normal growth conditions (23). Osmotin synthesis is regulated by abscisic acid, but its accumulation depends on adjustment of the cells to NaCl or to water stress (24). Others using cells obtained from our laboratory, have confirmed our observation of the accumulation of this protein in salt adapted cells (10, 16). We have isolated osmotin from NaCl adapted tobacco cells in sufficient quantities for further characterization. In this report we describe a simple and rapid procedure for purification of two forms of osmotin, some of their properties and localization within the cell, and the occurrence of significant amino acid sequence homology between osmotin, the sweet protein, thaumatin, and two other stress-related proteins.

MATERIALS AND METHODS

Cell Culture. Cell suspension cultures of tobacco (*Nicotiana tabacum* L. var Wisconsin-38) adapted to 25 g/L NaCl (S-25 cells) were maintained in our laboratory as described (12). These cells were harvested and stored as dry powders after washing in cold acetone (23).

Extraction of Osmotin. Acetone washed S-25 cells were suspended (100 mg/ml) in extraction buffer I (20 mM potassium phosphate buffer [pH 6.5], 5 mM EDTA, 1 mM PMSF) homogenized with a Teckmar Tissuemizer at 4°C three times for 1 min each. Suspended cell debris was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant containing soluble proteins was collected and designated fraction I. The cell pellet was resuspended in extraction buffer I, recentrifuged, and the supernatants were pooled. The remaining cell pellet was resuspended in extraction buffer II (20 mM potassium phosphate buffer [pH 6.5], 2 mM EDTA, 1 mM PMSF, 4 M urea, and 0.2% NP-40) and homogenized three times in the Tissuemizer as before and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant collected as detergent soluble protein was designated fraction II. Both fractions were processed further separately.

Ammonium Sulfate Fractionation. Fraction I was collected as an 80% precipitate by centrifugation at 10,000*g* at 4°C after discarding the 40% precipitate. This was resuspended in extraction buffer I and dialyzed for 20 h against five changes of 4 L distilled water at 4°C.

[2] Abbreviation: pI, isoelectric point; PMSF, phenylmethylsulfonylfluoride; Pth, *o*-phthaldehyde derivative.

Fraction II supernatant was precipitated with 80% ammonium sulfate, and a 10,000*g* floating pad (because of the presence of detergent) was collected. This pad was suspended in extraction buffer I and dialyzed separately as was fraction I.

After dialysis, fraction I was centrifuged separately at 10,000*g* for 10 min, and the clear supernatant was collected. Fraction II, containing a turbid precipitate, was dissolved by adding an equal volume of 2× fraction II resuspension buffer (40 mM potassium phosphate buffer [pH 6.0], 8 M urea, and 0.2% NP-40).

CM-Sephadex Purification. Fraction I protein, in 20 mM potassium phosphate (pH 6.0), and solubilized fraction II protein were applied directly to separate columns of CM-Sephadex A-25 equilibrated in 20 mM potassium phosphate (pH 6.0). The columns were washed with five bed volumes of washing buffer I (20 mM potassium phosphate [pH 7.0]) and five volumes of washing buffer II (20 mM potassium phosphate [pH 7.5]). Fraction I was eluted from the column with 20 mM potassium phosphate (pH 8.0) containing 0.5 M NaCl. Fraction II was eluted with 20 mM potassium phosphate (pH 8.0) containing 4 M urea, 0.5 M NaCl, and 0.1% NP-40. Both eluates were dialyzed separately overnight against three changes of 4 L distilled water. Fraction I was adjusted to pH 6.2 with 40 mM potassium phosphate buffer. Fraction II was brought to pH 5.5 with an equal volume of 100 mM sodium acetate (pH 5.5) and dissolved in 4 M urea and 0.1% NP-40.

Strong Cation HPLC Purification. SCX-polyamide on 17 μ Vydac silica was synthesized, and a preparative strong cation exchange HPLC column (25 × 1 cm i.d.) was packed. The column was equilibrated with 20 mM potassium phosphate (pH 6.2), and the flow was adjusted to 3 ml/min with a Spectraphysics high pressure pump. The proteins in fraction I were loaded in 10 to 20 ml volumes and eluted with a 40 min gradient of 0 to 0.5 M NaCl at a 3 ml/min flow rate. Proteins in fraction II were injected and eluted similarly except that the elution buffer contained 50 mM sodium acetate (pH 5.5), 4 M urea, and 0.1% NP-40. Ab-

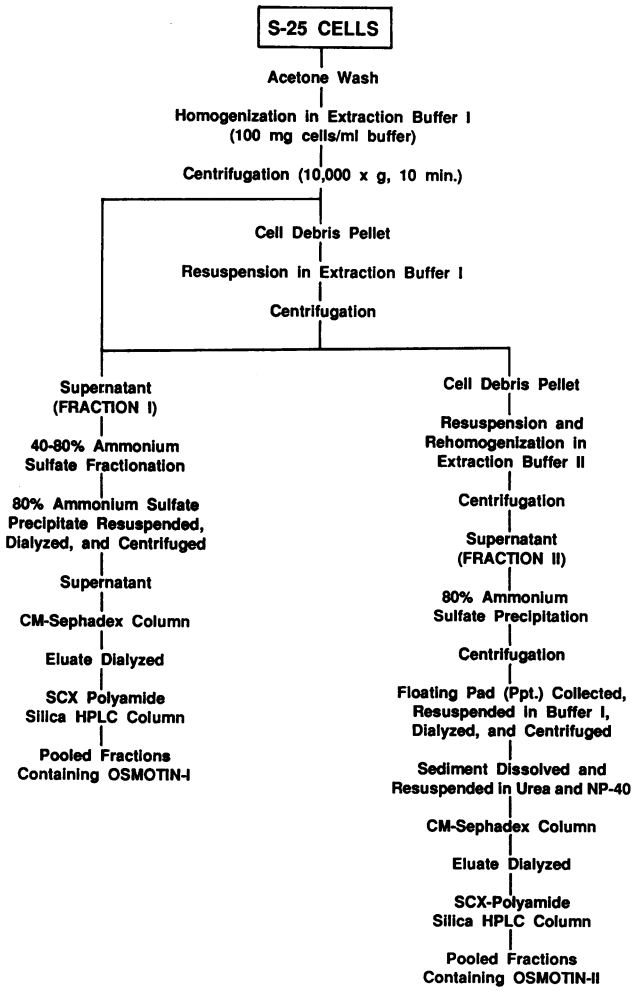


FIG. 1. Flow chart of steps in the isolation of two forms of osmotin.

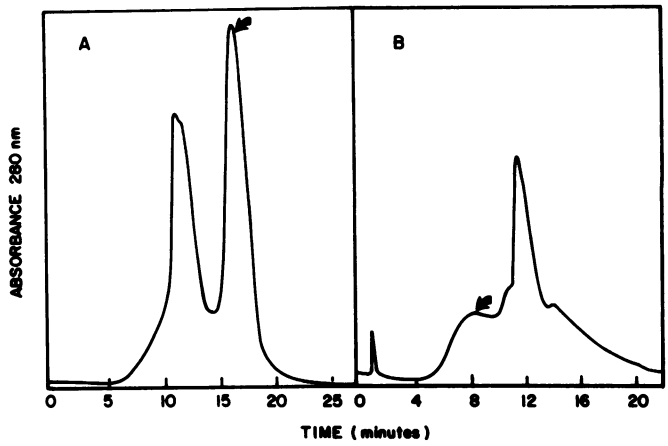


FIG. 2. Elution profile of protein monitored at 280 nm from a preparative SCX-polyamide HPLC column as described in "Materials and Methods." Osmotin-I appeared as a single predominant peak at 18 min (A) and osmotin-II appeared as a broad peak (mid point at 8 min) (B). Osmotin peaks are marked with an arrow.

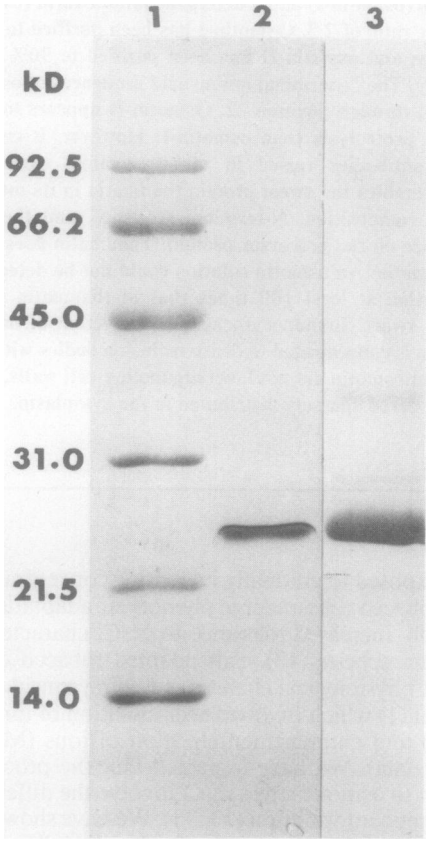


FIG. 3. SDS-PAGE on 12% polyacrylamide showing purified osmotin-I (lane 2) and osmotin-II (lane 3) from the HPLC elution shown in Figure 2. Mol wt standards are shown in lane 1. The gel was stained with Coomasie brilliant blue.

sorbance of the eluates was monitored at 280 nm and recorded on a Hewlett-Packard model 2600 integrator. Since we have no functional assay for osmotin, SDS-PAGE was used to identify chromatographic fractions containing osmotin. Pooled fractions containing osmotin were dialyzed and rechromatographed under the same conditions to obtain the finally purified osmotin.

Amino Acid Composition. After final purification on HPLC osmotin from fraction I was extensively dialyzed against distilled water and lyophilized. The protein (0.5 mg) was hydrolyzed in constant boiling 6 N HCl in evacuated sealed tubes at 110°C for 24 and 48 h. The amino acid composition was determined on Durrum D-500 amino acid analyzer according to the manufacturer's instructions. The cysteine content of the protein was determined as cysteic acid by the method of Hirs (14). Tryptophan was determined by the method of Edelhoch (8).

N-Terminal Amino Acid Sequence. Purified osmotins from both fractions I and II were carboxymethylated with [^{14}C]iodoacetamide by a modified procedure of Gurd (11). The protein was reduced in 1% DTT in 4 M guanidine hydrochloride in 0.1 M Tris-HCl (pH 8.5). [^{14}C]Iodoacetamide was added to the protein after removal of the DTT on a G-25 spin column, and the reaction was allowed to continue in the dark. Labeled protein was dialyzed against 8% formic acid and lyophilized. The protein and the fragments were degraded in a Beckman model 890C sequencer according to the method of Mahoney *et al.* (19). Pth-amino acids were identified and quantitated by HPLC. The number of cysteine residues was determined by measurement of ^{14}C in a scintillation spectrometer.

Electrophoresis, *in Vitro* and *in Vivo* Labeling, Immunoprecipitation and Proteolytic Digestion. Purified osmotin from both fractions I and II was labelled with ^{125}I using iodobeads by the method of Markwell (20). *In vitro* translation of mRNA from S-25 cells was performed with rabbit reticulocyte lysate according to the method of Pelham and Jackson (21). *In vivo* labeling was performed with $^{35}So_4$ as described (23). Radioiodinated osmotin protein, the *in vitro* translation products and, *in vivo* ^{35}S labeled products were immunoprecipitated with anti-osmotin-I, and SDS-PAGE was performed as described previously (24).

Radioiodinated osmotin-I, osmotin-II, and a larger protein which coimmunoprecipitated in the osmotin-II fraction were separately removed from an SDS-PAGE gel, and the proteins were eluted in 2% SDS and 5% β-mercaptoethanol overnight at 37°C. The ^{125}I-labeled proteins were dialyzed extensively against distilled water and equal cpm were used for proteolytic digestion of protein by trypsin and *Staphalococcus aureus* V-8 protease at 37°C for 6 h as described by Cleveland *et al.* (5).

Immunocytochemistry. S-25 cells from log phase cultures were collected by low speed centrifugation and immediately fixed in 3% paraformaldehyde-1% glutaraldehyde in K phosphate buffer (pH 7.2) at ambient temperature. After 5 min, the cells in fixative were placed on ice for the duration of the 1 h fixation. Cells were postfixed with 2% OsO_4 in buffer at 0°C, washed in water, then suspended and pelleted in 2% agar for further processing. After dehydration in a graded ethanol series, the samples were embedded in LR-White acrylic resin (Polysciences) and polymerized overnight at 55 to 60°C. Thin sections were treated briefly with 0.1% BSA in PBS (pH 7.2), then 1:100 or 1:500 rabbit antiosmotin-I for 2 h at 20 to 22°C. After being washed with buffer and mild detergent to remove nonspecific antibody binding, the sections were treated with goat anti-rabbit antibody conjugated

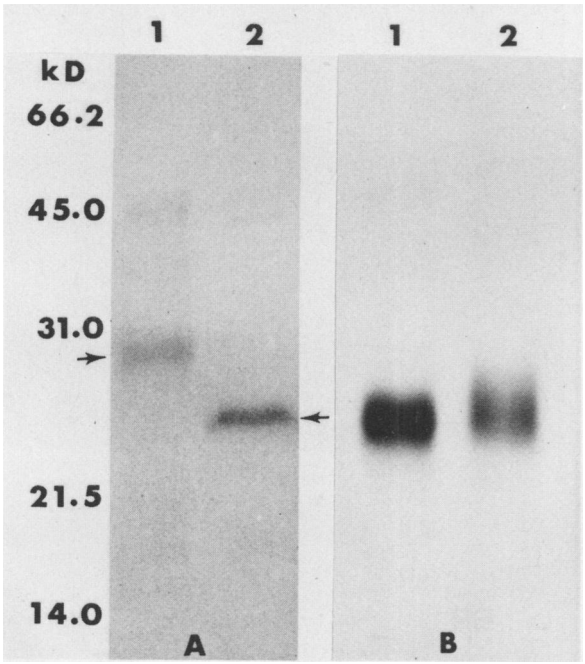


FIG. 4. Fluorograms of immunoprecipitated (antiosmotin-I) osmotin-I and osmotin-II. A, Fluorogram showing the immunoprecipitated ^{35}S-labeled precursor of osmotin synthesized *in vitro* in rabbit lysate (lane 1), and the immunoprecipitated osmotin labeled *in vivo* with ^{35}S (lane 2); B, fluorogram showing immunoprecipitation of ^{125}I-labeled purified osmotin-I (lane 1) and osmotin-II (lane 2). Immunoprecipitated osmotin-II could be separated into a 26 kD band and a slightly larger mol wt minor band (less than 10% of total) by a longer electrophoresis time. This higher mol wt protein was used also in proteolysis shown in Figure 5.

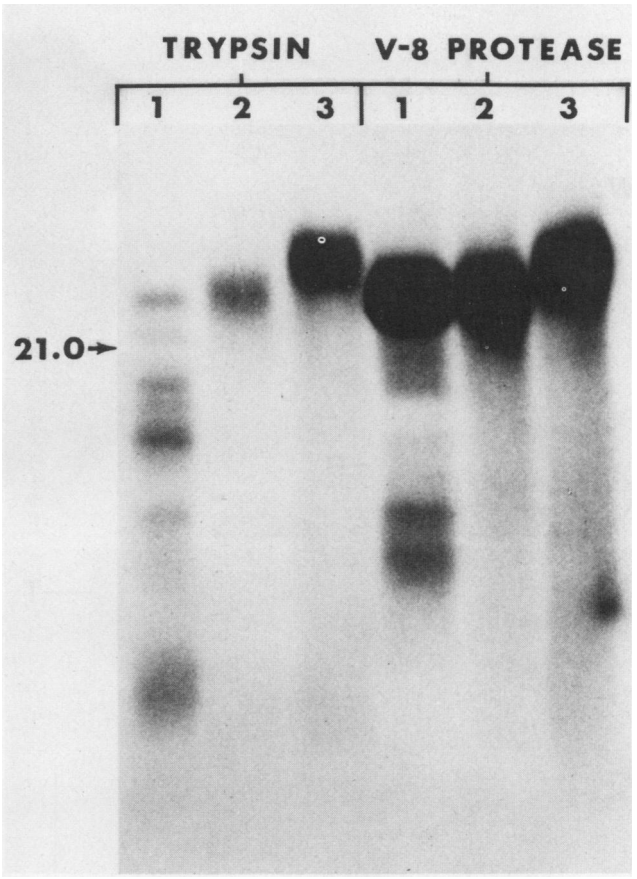
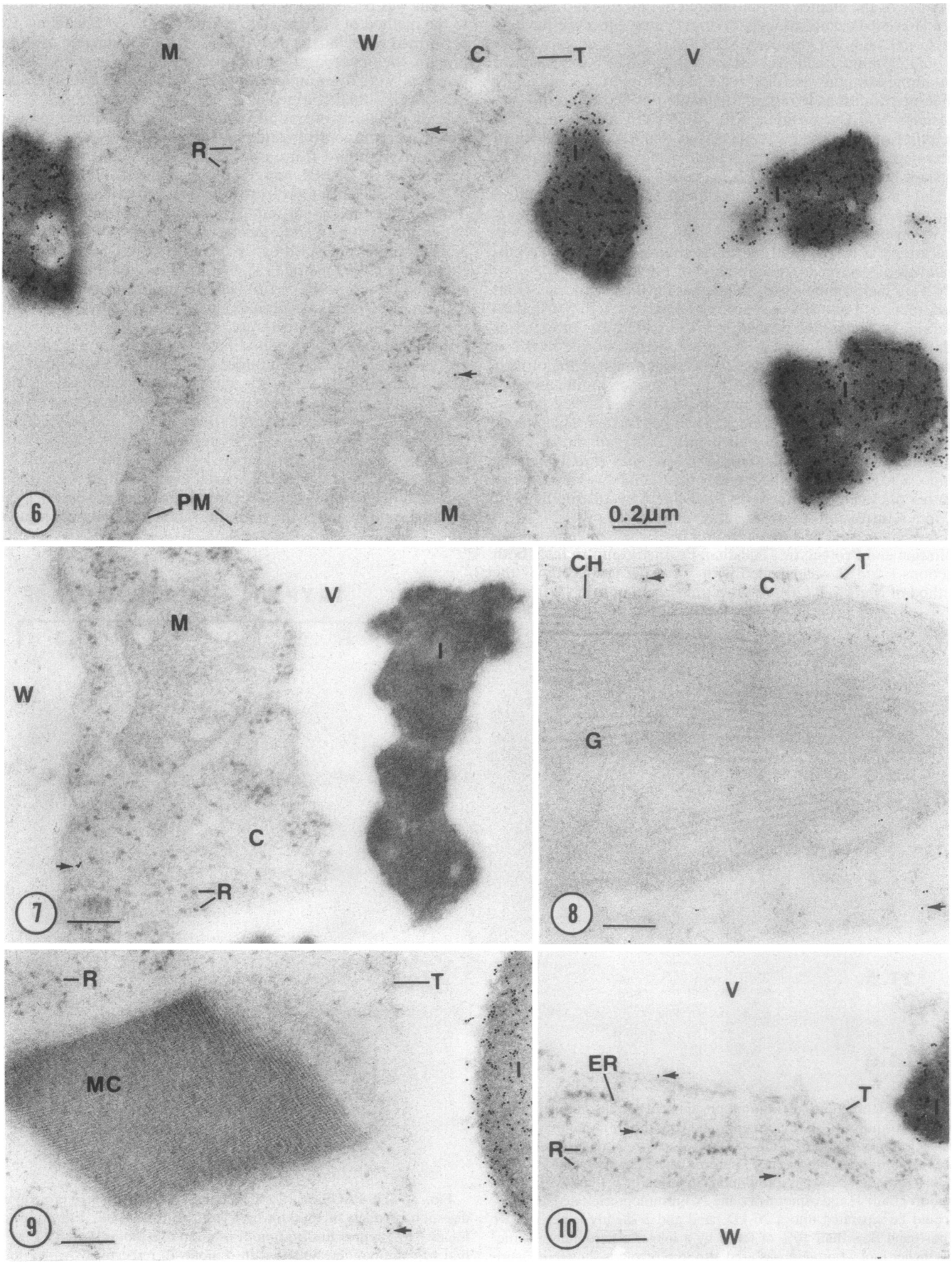
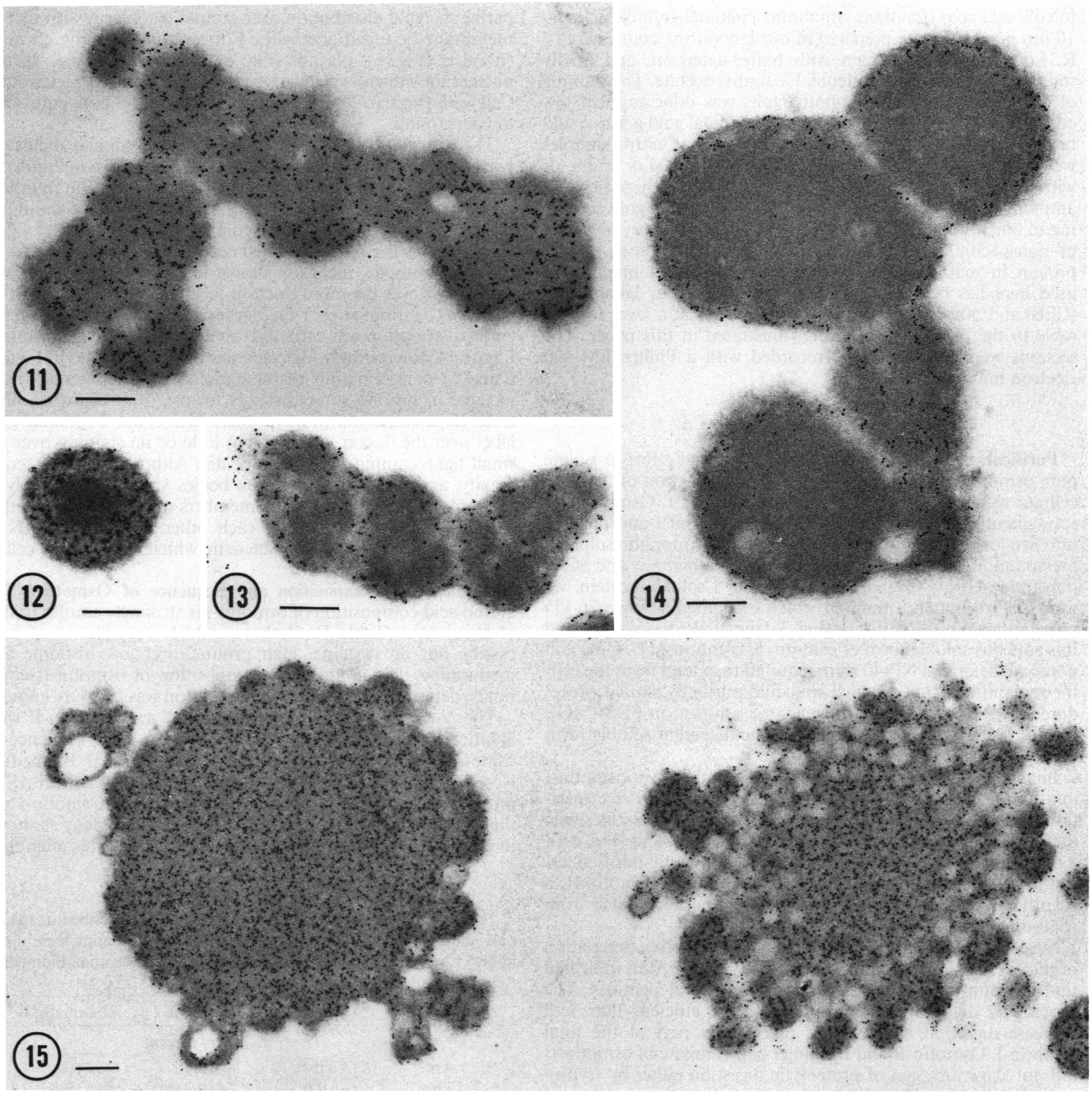


FIG. 5. SDS-PAGE on 18% acrylamide of radioiodinated proteolytic digestion products of osmotin by trypsin and *S. aureus* V-8 protease. Lanes 1 to 3 represent digestion of osmotin-I, osmotin-II, and a higher mol wt coimmunoprecipitate with osmotin-II, respectively, with either trypsin or V-8 protease as indicated.



FIGS. 6–10.



FIGS. 11–15. Osmotin localization in vacuolar inclusions from S-25 tobacco cells. Inclusions in different cells differ in size, complexity, and OsO_4-UAc staining pattern, and reveal various distribution patterns of the colloidal gold marker. The complex inclusion at the right in Figure 15 represents an oblique section through the peripheral region of an inclusion similar to the one at the left.

Figs. 11–14. ×50,000; Fig. 15. × 36,000. Scale bars = 0.2 μm.

FIGS. 6–10. Electron micrographs of S-25 cells stained with antibodies and colloidal gold conjugate. All magnified ×50,000. Scale bar = 0.2 μm. Labels on all figures have been positioned so that they do not cover or interfere with any gold grains.

FIG. 6. Portions of two adjacent cells treated with antiosmotin and goat anti-rabbit-gold. Osmotin is indicated by the small black grains concentrated over the vacuolar inclusions (I), and to a lesser extent (arrows) in the ground cytoplasm (C). W, cell wall; M, mitochondrion; R, ribosome; PM, plasma membrane; T, tonoplast; V, vacuole.

FIG. 7. Control cell stained with rabbit anti-α-zein, showing essentially no staining. Background label (arrow).

FIG. 8. Stained with antiosmotin. Colloidal gold grains (arrows) occur at a low level over ground cytoplasm but not over the chloroplast (CH). G, grana thylakoids.

FIG. 9. Antiosmotin staining over vacuolar inclusion and in ground cytoplasm (arrow) but not over crystalloid in the microbody (MC).

FIG. 10. Low level osmotin staining (arrows) over the ground cytoplasm, tonoplast, and near endoplasmic reticulum (ER).

to colloidal gold (fractions containing colloidal gold particles 6–10 nm diameter were prepared in our laboratory, courtesy of C. R. Lending), washed again with buffer-detergent, and finally stained for 10 min with aqueous 1% uranyl acetate. The staining of cells and processing of photographs was done to yield low contrast images so that the very small colloidal gold grains could be seen against the background of cell structure. Control samples were treated with preimmune serum diluted 1:100 or 1:500, or with anti-α-zein diluted 1:100 or 1:500. Both of these primary antibody controls were treated with goat anti-rabbit gold according to our normal procedure and demonstrated a very low level of nonspecific background labeling. α-Zein is a seed storage protein in maize endosperm, and the anti-α-zein preparation used here has been used routinely in one of our laboratories (CEB) at 1:500 to stain maize protein bodies at a level comparable to the osmotin localization illustrated in this paper. The sections were examined and recorded with a Philips EM-400 electron microscope.

RESULTS

Purification of Osmotin I and II. Our initial effort to isolate pure osmotin protein indicated that only a fraction of the total cellular osmotin is soluble in extraction buffer I. Osmotin was subsequently isolated from cells adapted to NaCl and purified into two forms as outlined in Figure 1. After ammonium sulphate precipitation, CM-Sephadex column chromatography and SCX-polyamide HPLC (Fig. 2A) of this buffer I soluble protein, we were able to obtain a fraction which contained a single 26 kD protein shown by SDS-PAGE (Fig. 3, lane 2). We have designated this aqueous soluble form of osmotin as osmotin-I (4). Urea and nonionic detergent NP-40 were required to extract from the cells the remaining osmotin which we could purify by similar procedures (Fig. 2B) to near homogeneity as shown on SDS-PAGE (Fig. 3, lane 3). We have designated the detergent soluble form of osmotin as osmotin-II.

Immunological Cross-Reactivity of Osmotins. Antiosmotin-I immunoprecipitates a 28.5 kD protein from the *in vitro* translation products of poly A^+ RNA from adapted cells (S-25), while the *in vivo* labeled osmotin appears as a 26 kD band (Fig. 4A). Antiosmotin-I antibodies cross-reacted with both purified osmotin-I and osmotin-II (Fig. 4B). In addition to osmotin-II, a slightly larger protein was always co-immunoprecipitated from osmotin-II preparations.

Susceptibility to Proteolytic Digestion of Osmotins. Separation of trypsin and V-8 proteolytic peptides by SDS-PAGE indicated that osmotin-I was digested to produce several peptides. The proteolytic digestion by trypsin was more efficient than V-8 protease digestion which could digest only part of the total osmotin-I. Osmotin-II and the larger coprecipitate of osmotin-II did not show any sign of proteolytic digestion either by trypsin or by V-8 protease (Fig. 5).

Intracellular Localization of Osmotin. Immunocytochemical localization showed that the osmotin is concentrated in electron-dense inclusions in the vacuoles (Figs. 6, 9–15). Control sections treated with preimmune rabbit serum (not illustrated) or with rabbit anti-α-zein (Fig. 7) showed no staining in the vacuolar inclusions and extremely low background overall. In addition to the concentrated staining in vacuolar inclusions, osmotin was also detected in the ground cytoplasm, but at a lower level (Figs. 6, 8–10) that was nevertheless above background. We could not detect any preferential localization within the cytoplasm. Occasional gold grains appeared over the tonoplast and in the cytoplasm, but rarely over ribosomes, although a few grains were close to endoplasmic reticulum. Rarely were any gold grains found over the plasma membrane (Figs. 6, 7, 10), indicating that this membrane did not attract any labeling above background. The cytoplasmic labeling was not to be confused with the significantly lower background labeling; this could be seen by comparing the gold distribution over ground cytoplasm with that on membrane-bounded organelles. For example, staining on mitochondria (Fig. 6), plastids (Fig. 8), microbodies (Fig. 9), and nuclei (not shown) was nearly void, comparable to background. Cell walls (Figs. 6, 7, 10) also failed to stain, and were equivalent to background.

The distribution of gold label within the vacuolar inclusions varied from cell to cell. In some, the colloidal gold was uniformly distributed throughout the inclusions (Figs. 6, 11). But in others, it was preferentially distributed in certain regions of the inclusions. In Figure 12, the dense core of the inclusion lacks gold label whereas the lighter mottled cortex was strongly labeled. In the more complex inclusion shown in Figure 13, the label was distributed over the more electron-lucent areas while little or no label was distributed over the darker core granules. This preferential distribution was carried to an extreme in the inclusions in Figure 14. Interestingly, the vacuolar inclusion bodies in Figures 6 and 11 consist mainly of the darker staining material which, in these inclusions, stains strongly for osmotin. Figure 15 illustrates large and complex inclusions which contain colloidal gold label over the darker portions, but little or no staining over the small light-staining peripheral locules. Although the size, complexity, and number of inclusion bodies varied considerably in the samples we examined, the members of a population within a single cell were similar to each other. So, inclusion body morphology was a unit characteristic which varied from cell to cell but not within a cell.

Amino Acid Composition and Sequence of Osmotins. The amino acid composition of osmotin-I is strikingly similar to that of thaumatin (Table I). Both have high proportions of half-cystine but no cysteine, high proline, and low histidine and methionine. The amino acid composition of osmotin-II could not be determined because the preparation was not pure enough.

The *N*-terminal sequences of osmotin-I and osmotin-II were identical through the first 22 amino acids. The sequence of osmotin I was used in a search of the National Biomedical Research Foundation protein sequence database with the FASTP program developed by Lipman and Pearson (18). Osmotin-I and the thaumatins have significant and striking homology as shown in the amino acid sequence alignment (Fig. 16). This alignment

Table I. *Amino Acid Composition*

The composition of osmotin was determined as described in "Materials and Methods." The compositions of the thaumatins were determined from the sequences as presented in the National Biomedical Research Foundation Protein Sequence Database.

Amino Acid	Osmotin I	Thaumatin I	Thaumatin II
		mol percent	
Asx	11.4	10.6	10.4
Thr	9.0	9.7	9.4
Ser	4.2	6.8	6.1
Glx	6.6	4.8	6.5
Pro	8.9	5.8	5.6
Gly	12.8	11.6	11.3
Ala	6.9	7.7	7.5
Cys[a]	8.4	7.7	7.5
Val	3.6	4.8	4.7
Met	1.0	0.5	0.5
Ile	3.9	3.9	3.8
Leu	3.6	4.3	5.2
Tyr	2.8	3.9	3.8
Phe	5.7	5.3	5.2
His	0.8		
Lys	2.4	5.3	5.2
Arg	5.4	5.8	6.1
Trp	3.2	1.4	1.4

[a] One-half cystine.

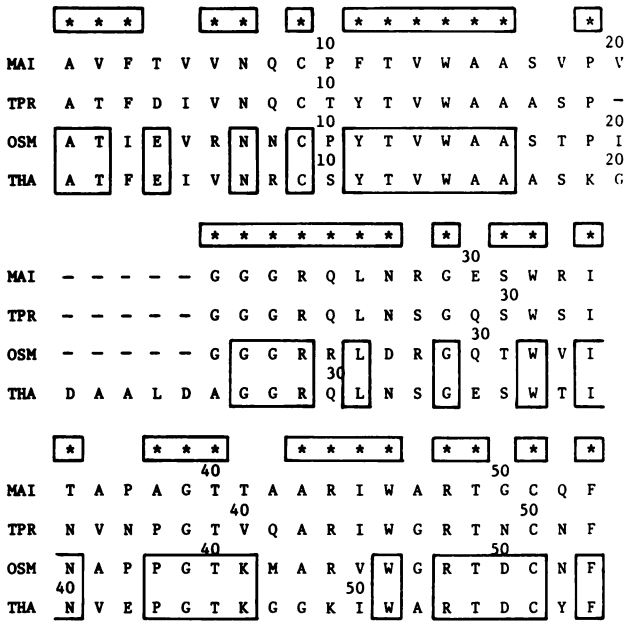


FIG. 16. Amino acid sequence alignment of maize trypsin/α-amylase inhibitor (MAI), tobacco pathogenesis-related protein (TPR), osmotin I (OSM) and thaumatin (THA). Homologous amino acids are enclosed in boxes. Asterisks in boxes indicate where at least three of the proteins have homologous sequences. Gaps (-) are indicated which produce optimal alignment.

revealed an identical sequence of 6 consecutive amino acids, a sequence with 11 out of 16 identical amino acids and 29 out of the total 53 (55%) amino acids in the known osmotin sequence identical with thaumatin. The amino acid sequence of osmotin was 62% homologous with TPR protein, and 59% homologous with MAI protein (Fig. 16), both of which are induced by stress (7, 22). Another well characterized proteinase inhibitor protein, inhibitor I from potato (6), did not have significant amino acid sequence homology with osmotin. Although this protein is also sequestered in the vacuole it has a considerably lower mol wt than osmotin.

DISCUSSION

Osmotin is a protein that accumulates during adaptation of cells to high osmotic stress including salt or polyethylene glycol (23). Thaumatin is a sweet protein present in the fruits of a tropical plant *Thaumatococcus daniellii* (25). There is no known obvious reason for thaumatin and osmotin to have similar primary structures, but the amino acid sequence homology is significant and the proteins share several common features, such as similar mol wt, the presence of several disulfides and the lack of sulfhydral residues, a high proportion of proline, and a basic pI. Although osmotin is low in lysine compared to thaumatin, it is possible that most of the Asx and Glx in osmotin are Asn and Gln as is the case in thaumatin (15). Furthermore, thaumatin has a 22 amino acid hydrophobic signal peptide sequence (9). We have demonstrated also that osmotin is produced as a precursor protein in an *in vitro* translation system which is 2.5 kD larger than the mature protein (Fig. 4A). Although thaumatin and osmotin exhibit these similarities, antiosmotin-I does not cross-react with thaumatin. In a blind, randomly ordered test of the taste of thaumatin and osmotin, 16 of 16, 14 of 16, and 6 of 16 individuals identified 100, 10, and 1 μM thaumatin, respectively, as sweet. None of 16 of the same individuals identified 100 μM osmotin or water as sweet. The sweetness of thaumatin is reported to be associated with both high lys content and certain lys residues (22). The lower lys content of osmotin could reflect a loss of these 'sweet' sequences. There are two other proteins which have reported sequences which are highly homologous to thaumatin and thus to osmotin; a tobacco mosaic virus-induced protein in tobacco (7) and a maize proteinase inhibitor (22) (Fig. 16). Although neither the function or the factors regulating the synthesis of thaumatin are known, its homology with three stress-induced proteins suggest that its synthesis may also be controlled by stress-related conditions.

Osmotin-I and -II have the same N-terminal amino acid sequence through the first 22 amino acids. Both forms, when extracted from S-25 cells, appear to have basic pIs of about 8.2. Osmotin-II is less susceptible to protease digestion. Since osmotin-II appears to be particulate it is conceivable that it might have undergone some posttranslation modifications which make the protein inaccessible to proteases. Even osmotin-I, which could be partially digested by *Staphalococcus* V-8 protease and trypsin, appeared to be resistant to cleavage by CNBr and endopeptidase lys-C (data not shown). Most of the peptides of osmotin-I which were produced by proteolysis contained the N-terminal sequence of intact osmotin suggesting that osmotin-I is also partially resistant to several proteases, at least on the N-terminal end (data not shown). It is possible that the different osmotin forms are coded by different genes or at least arise from messages which have been processed differently. Using an osmotin cDNA sequence we have found recently that at least two sizes of mRNA for osmotin are made (data not shown).

We know that unadapted cells synthesize a 26 kD protein which does not accumulate and has a lower pI value than either osmotin-I or II extracted from adapted cells (23, 24). We do not know the physiological function of osmotin yet, but its synthesis is coincident with osmotic adjustment in the tobacco cells (1). Osmotin is induced by ABA which is known to accelerate adaptation of cells to salt (17) and to induce osmotic adjustment (3). However, accumulation of osmotin is dependent upon simultaneous exposure to NaCl (24), except in cells which have been grown in the presence of NaCl for a long time period and then grown in medium without salt. These cells and cells cultured from plants regenerated from them accumulate osmotin-I without salt exposure (4).

The occurrence of osmotin in vacuolar inclusion bodies and the induction of its synthesis by ABA suggest that osmotin may be some sort of storage protein, the accumulation of which is linked to reduced growth and stress adaptation. The predominant storage proteins of plants occur in mature seeds. However, a 26 kD protein was not found in mature seeds of tobacco (data not shown). The immunological cross-reactivity of osmotin to any seed storage proteins is still undetermined. Since osmotin was found in several unrelated plant species (24) it is not a typical seed storage protein since these are usually species specific. Osmotin could be involved in osmotic adjustment by the cells either by facilitating the accumulation or compartmentation of solutes or by being involved in metabolic or structural alterations during osmotic adjustment. Its homology with MAI and TPR suggests that it may have a more general type of defense function.

The particulate nature of osmotin-II suggests that it should be associated with a cell organelle or other discrete structure. However, our immunocytochemical evidence indicates no association of the antiosmotin antibody with organelles. Since the antibody to osmotin-I cross-reacts with osmotin-II we would expect to find some labeling in organelles if they contained either form of osmotin, but we do not. If there is a delicate association between osmotin and membrane structures, we are unable to detect it. We cannot be certain which osmotin is being detected by the present methods. Since osmotin-I is easily solubilized in aqueous solutions, it is possible that a considerable proportion of it is lost by leaching during the fixation and washing procedures we used to prepare the cells for electron microscopy. So, the images could represent the staining of only the less soluble osmotin-II, or of both osmotins. It is less likely that it represents the staining of

osmotin-I alone; after all, osmotin-II is 'particulate,' and the vacuolar inclusions are particles. But, whether the vacuolar and cytoplasmic sites of osmotin revealed by the colloidal gold staining contain one and the same form of osmotin remains uncertain. We hope these questions and others will be more accessible with the freeze-substitution technique which immobilizes cellular structures instantaneously by rapid freezing, processes them in a nonaqueous environment, and provides superior preservation of delicate proteins.

Acknowledgments—We would like to thank M. Goodwin for assistance in amino acid sequencing. We thank R. A. Bentlage for processing and staining the cells for electron microscopy and immunocytochemistry. Portions of this work were conducted with the facilities of the Electron Microscopy Center in Agriculture at Purdue University.

LITERATURE CITED

1. BINZEL ML, PM HASEGAWA, AK HANDA, RA BRESSAN 1985 Adaptation of tobacco cells to NaCl. Plant Physiol 79: 118–125
2. BINZEL ML, PM HASEGAWA, D RHODES, S HANDA, AK HANDA, RA BRESSAN 1987 Solute accumulation in tobacco cells adapted to NaCl. Plant Physiol. In press
3. BRESSAN RA, NK SINGH, AK HANDA, A KONONOWICZ, PM HASEGAWA 1985 Stable and unstable tolerance to NaCl in cultured tobacco cells. *In* M Freeling, ed, Plant Genetics. Alan R Liss, Inc, New York, pp 755–769
4. BRESSAN RA, NK SINGH, AK HANDA, R MOUNT, J CLITHERO, PM HASEGAWA 1987 Stability of altered genetic expression in cultured plant cells adapted to salt. *In* L Monti, E Porceddu, eds, EEC Symposium on "Drought Resistance in Plants: Genetic and Physiological Aspects," pp 41–57
5. CLEVELAND DW, SG FISHER, MW KIRSCHNER, UK LAEMMLI 1977 Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 252: 1102–1106
6. CLEVELAND TE, RW THORNBURG, CA RYAN 1987 Molecular characterization of wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. Plant Mol Biol 8: 199–207
7. CORNELISSEN BJC, RAM HOOFT VAN HIEVJSDUIJNEN, JF BOL 1986 A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. Nature 321: 531–532
8. EDELHOCH H 1967 Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry 6: 1948–1954
9. EDENS L, L HESLINGA, R KLOK, AM LEDEBOER, J MAAT, MY TOONEN, C VISSER, CT VERRIPS 1982 Cloning of cDNA encoding the sweet-tasting plant protein thaumatin and its expression in *Escherichia coli*. Gene 18: 1–12
10. ERICSON ME, SH ALFINITO 1984 Protein produced during salt stress in tobacco cell culture. Plant Physiol 74: 506–509
11. GURD FRN 1967 Carboxymethylation. Methods Enzymol 9: 532–541
12. HASEGAWA PM, RA BRESSAN, AK HANDA 1980 Growth characteristics of NaCl-selected and non-selected cells of *Nicotiana tobacum* L. Plant Cell Physiol 21: 1347–1355
13. HASEGAWA PM, RA BRESSAN, AK HANDA 1987 Cellular mechanism of salinity tolerance. HortScience. In press
14. HIRS CHW 1967 Determination of cystine as cysteic acid. Methods Enzymol 11: 59–62
15. IYENGER RB, P SMITS, H VAN DER WEL, FJC VANDER OUDERAA, JH VAN BROUWERSHAVEN, P RAVENSTIEN, G RICHTERS, PD VAN WASSENAAR 1979 The complete amino acid sequence of the sweet protein thaumatin I. Eur J Biochem 96: 193–204
16. KING GJ, CE HAUSEY JR, VA TURNER 1986 A protein induced by NaCl in suspension cultures of *Nicotiana tabacum* accumulates in whole plant roots. Plant Mol Biol 7: 441–449
17. LAROSA PC, AK HANDA, PM HASEGAWA, RA BRESSAN 1985 Abscisic acid accelerates adaptation of cultured cells to salt. Plant Physiol 79: 138–142
18. LIPMAN DJ, WR PEARSON 1985 Rapid and sensitive protein similarity searches. Science 227: 1435–1441
19. MAHONEY WC, RW HOGG, MA HERMODSON 1981 The amino acid sequence of the D-galactase-binding protein from *Escherichia coli* B/r. J Biol Chem 256: 4350–4356
20. MARKWELL MAK 1982 A new solid-state reagent to iodinate proteins. 1. Conditions for the efficient labelling of antiserum. Anal Biochem 125: 427–432
21. PELHAM HRB, RJ JACKSON 1976 An efficient mRNA dependent translation from reticulocyte lysate. Eur J Biochem 67: 247–256
22. RICHARDSON M, S VALDES-RODRIGUEZ, A BLANCO-LABIA 1987 A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. Nature 327: 432–434
23. SINGH NK, AK HANDA, PM HASEGAWA, RA BRESSAN 1985 Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiol 79: 126–137
24. SINGH NK, PC LAROSA, AK HANDA, PM HASEGAWA, RA BRESSAN 1987 Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. Proc Natl Acad Sci USA 84: 739–743
25. VAN DER WEL H, K LOEVE 1972 Isolation and characterization of thaumatin I and II. The sweet-tasting protein from *Thaumatococcus daniellii*. Eur J Biochem 31: 221–225