A Soluble Auxin-Binding Protein from \textit{Hyoscyamus muticus} Is a Glutathione S-Transferase

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We have used the photoaffinity label azido-[\(^{3}\H]\text{IAA} \text{(5-N}_{3}\text{-[7-}\text{H}]\text{indole-3-acetic acid)}, a biologically active analog of indole-3-acetic acid, to identify auxin-binding proteins (ABPs) in the soluble fraction of \textit{Hyoscyamus muticus}. A \(25\text{-kD}\) polypeptide previously described \cite{Macdonald1991} has now been purified to homogeneity by conventional methods. Binding of azido-[\(^{3}\H]\text{IAA} \text{to the purified protein was reduced by active auxins but not by inactive indoles. Partial amino acid sequences of the purified protein showed high homology to glutathione S-transferase (GST) from tobacco (\textit{Pari}) and from maize (GT32). The conclusion that the \(25\text{-kD}\) ABP is a GST is further supported by high GST activity in fractions highly enriched in the \(25\text{-kD}\) polypeptide and recognition of the ABP by antibodies against GST from wheat and maize. Furthermore, purification of a protein from a soluble protein extract from \textit{H. muticus} by affinity chromatography on glutathione-agarose also yielded a \(25\text{-kD}\) polypeptide that was indistinguishable in its N-terminal amino acid sequence and biochemical characteristics from the protein purified by conventional methods. Possible functions of GST in auxin action are discussed.

Auxins are a class of plant hormones that influence a wide range of growth and developmental processes in plants \cite{Davies1987}. The chemical structure of the major natural auxin IAA has been known for more than 50 years, but the primary events leading to auxin action are still poorly understood. The models for auxin action have been strongly influenced by the mode of action of peptide and steroid hormones in animal cells. Hence, the first event leading to auxin action is thought to be the binding of IAA to a receptor protein. Several such ABPs have been identified in the membrane and soluble fraction of different plant species \cite{Jones1990}; Campos et al., 1992; Feldwisch et al., 1992), but whether one of these ABPs is an auxin receptor is still not clear.

The search for the auxin receptor has mainly focused on membrane proteins. IAA, however, does not necessarily need a receptor at the outer surface of the plasma membrane because the protonated (uncharged) form of this hydrophobic molecule penetrates the plasma membrane. In addition, many plant cells contain a specific uptake system for IAA \cite{Rubery1987}. Thus, the receptor for IAA may well be a soluble protein. Prasad and Jones \cite{Prasad1991} have described a soluble ABP localized in the nucleus that may be directly involved in the regulation of specific gene transcription.

Aside from possible receptor proteins, auxin is likely to interact with proteins involved in auxin transport and metabolism \cite{Cohen1982}. Recently, a soluble ABP from maize was characterized as a \(\beta\)-glucosidase with a possible role in the cleavage of auxin-sugar conjugates \cite{Campos1992}.

The photolabile, biologically active analog of IAA, azido-[\(^{3}\H]\text{IAA}, has been used for efficient screening for both membrane-bound and soluble ABPs \cite{Hicks1989; Jones1989; Macdonald1991; Feldwisch1992}. Using this labeling reagent, we identified several ABPs in the soluble fraction of \textit{Hyoscyamus muticus} \cite{Macdonald1991}. The present communication describes the purification of one of these proteins and its identification as a GST.

**MATERIALS AND METHODS**

**Cell Cultures**

The cell-suspension culture of \textit{Hyoscyamus muticus} and the culture conditions were as described by Gebhardt et al. \cite{Gebhardt1983}, except that no auxin was added to the medium.

**Extraction of Soluble Protein**

Cells were collected and homogenized 14 d after subculture as described by Macdonald et al. \cite{Macdonald1991} in 20 mM Tris/HCl, pH 8.0. Polyclay AT (Serva, 2.5\%, w/v) was added at least 1 h before use, and diethyldithiocarbamate (1.8 mg/mL), \(\beta\)-mercaptoethanol (0.3\%, v/v), and PMSF (0.5 mM) were added immediately before use. The homogenate was squeezed through Miracloth (Calbiochem, La Jolla, CA) and the filtrate was centrifuged at 23,500g for 1 h at 4°C. The supernatant was poured through filter paper prior to ammonium sulfate precipitation.

**Ammonium Sulfate Precipitation**

Ammonium sulfate precipitation was performed as described previously \cite{Macdonald1991}. The pellets were

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Abbreviations: ABP, auxin-binding protein; azido-[\(^{3}\H]\text{IAA}; 5-N_{3}\text{-[7-}\text{H}]\text{indole-3-acetic acid}; BCIP, 5-bromo 4-chloro 3-indolyl phosphate-toluidine salt; CDNB, 1-chloro 2,4-dinitro-benzen; GST, glutathione S-transferase; 1-NAA, 1-naphtylacetic acid; 2-NAA, 2-naphtylacetic acid; NBT, p-nitro blue tetrazolium chloride.
resuspended on ice in a minimum volume of 20 mM Tris/HCl, pH 7.0, and desalted by dialysis against the same buffer overnight.

**Anion-Exchange Chromatography I**

The dialysate was loaded onto a Sepharose Q column (2.5 cm × 8 cm, Pharmacia) previously equilibrated with 20 mM Tris/HCl, pH 7.0. The column was washed with the same buffer until the A₂₅₀ returned to the baseline. Bound proteins were eluted with a linear gradient of 0 to 350 mM NaCl in 20 mM Tris/HCl, pH 7.0. The gradient volume was 450 mL and the fraction size 8 mL. Aliquots (25 μL) of individual fractions were labeled with azido-[³H]IAA and analyzed by SDS-PAGE and fluorography. Fractions containing the 25-kD polypeptide were pooled, concentrated to 1 mL. All concentration steps were performed using an Amicon Ultrafiltration Cell with Amicon Diaflo YM 10 membranes (molecular mass cutoff 10 kD).

**Gel-Filtration Chromatography**

The concentrated fractions from anion-exchange chromatography were loaded onto a Sephacryl S-300 column (1.6 cm × 100 cm, Pharmacia) equilibrated with 200 mM Tris/HCl, pH 7.5. Elution was at a flow rate of 30 mL/h, the fraction size was 2.5 mL. Fractions containing the protein of interest as assayed by SDS-PAGE and fluorography were pooled.

**Anion-Exchange Chromatography II**

The combined fractions from the gel-filtration column were dialyzed against 20 mM Tris/HCl, pH 7.5, and loaded onto a Q-300 HPLC column (Synchropak, 250 × 10 mm) equilibrated with 50 mM NaCl in 20 mM Tris/HCl buffer, pH 7.5. The column was washed for 10 min. Bound proteins were eluted within 60 min with a linear gradient of 50 to 250 mM NaCl in 20 mM Tris/HCl, pH 7.5. The gradient volume was 300 mL and the flow rate 5 mL/min. Fractions were collected at 1-min intervals.

**Isolation of Tryptic Peptides and Amino Acid Sequencing**

Fractions from the HPLC anion-exchange chromatography containing the 25-kD polypeptide were pooled, concentrated in an Amicon Ultrafiltration Cell, and separated on a preparative SDS gel. The gel was briefly stained with Coomassie blue, and the prominent stained band was cut out and electroeluted with a Biotrap Electroelution System (Schleicher & Schuell) according to the protocol from the manufacturer. The electroeluted polypeptide (250 μg) was precipitated with methanol and glacial acetic acid (1.5 mL of methanol and 15 μL of acetic acid per mL of sample) for at least 2 h at −20°C, washed with methanol, and dried. The precipitate was redissolved in 500 μL of 50 mM ammonium hydrogencarbonate, pH 8.0, heated in a boiling water bath for 3 min, cooled and digested with 2 × 20 μg of trypsin (Boehringer Mannheim) for 2 × 2 h at 37°C, and lyophilized. The lyophilized material was dissolved in 100 mM sodium phosphate buffer, pH 2.2, loaded onto an Ultrasphere C₁₈ HPLC column (Beckman, 4.6 × 25 mm) equilibrated with the same buffer, and eluted with a gradient of 0 to 50% (v/v) acetonitrile at a flow rate of 1 mL/min. Peptides were monitored at 214 nm. Peptide-containing fractions were neutralized with 5 mM NaOH, lyophilized, redissolved in 0.1% (v/v) TFA in water, and loaded onto an Ultrasphere C₄ column (Beckman, 4.6 × 25 mm) for further purification. Elution was carried out with a gradient of 0 to 35% (v/v) acetonitrile containing 0.1% (v/v) TFA. The fractions containing peptides were lyophilized and redissolved in 100 μL of 0.1% TFA in water. One-third of each sample was subjected to amino acid sequence analysis by automated Edman degradation. For N-terminal sequencing, the electroeluted polypeptide was electrodialyzed against 20 mM ammonium acetate, pH 7.8 (Schleicher & Schuell Biotrap Electroelution System), and lyophilized. The samples redissolved in 20 mM ammonium acetate, pH 7.8, were directly used for sequencing.

**Photoaffinity Labeling**

Photoaffinity labeling was performed as described by Macdonald et al. (1991) with some minor changes. Briefly, the protein sample was mixed in a UV-transparent acrylic cuvette (Semadeni, Ostermundigen, Switzerland) with an equal volume of labeling buffer (100 mM sodium citrate buffer, 250 mM Suc and 0.5 mM MgSO₄, pH 4.5) containing 0.7 μM azido-[³H]IAA (16 Ci/mmol). Typically, 75 to 100 μg of a complex protein mixture in about 50 μL of the respective chromatography buffer were used. If purified GST was labeled, the protein was mixed with an equal amount of BSA (Sigma) and horse heart Cyt c (Sigma). Where competing agents were used, they were added to the protein mixture in 10% DMSO at a final concentration of 0.5 mM prior to the addition of azido-[³H]IAA. Samples were then mixed and kept on ice for 20 min. The samples were frozen in liquid nitrogen for 30 s before irradiation on a short-wave transilluminator (UVTM 25, Hoefer Scientific Instruments) for 1 min.

**SDS-PAGE, Fluorography, and Analytical IEF**

The methods used for SDS-PAGE and fluorography were as described by Macdonald et al. (1991). Analytical IEF was performed on Servalyt Precotes pH 3 to 10 (Serva) according to the manufacturer's instructions. The gel was stained with Coomassie blue as described by the manufacturer.

**Western Blotting**

Proteins from unstained gels were blotted onto nitrocellulose membranes (Bio-Rad) at 50 V for 1 h using a mini-blot apparatus (Bio-Rad). The transfer buffer was 20 mM Tris, 150 mM Gly, and 20% (v/v) methanol. The membranes were stained for proteins with a Ponceau S solution (Sigma, 0.5% [w/v] in water). After destaining in water, the blot was blocked in 3% (w/v) gelatin (Bio-Rad) in TBS (20 mM Tris/HCl, pH 7.5, containing 150 mM NaCl) for at least 30 min. The blot was incubated for 2 h at room temperature with GST antibodies, which were diluted 500-fold with 1% (w/v) gelatin in TBS. The blot was then washed three times for 15 min.
min in TTBS (TBS with 0.1% [v/v] Tween 20) and 15 min in TBS. Goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Bio-Rad) was diluted 1000-fold with 1% (w/v) gelatin in TBS and incubated with the blot at room temperature for an additional 2 h. The blot was washed as described above and finally developed with the NBT/BCIP color reagents as described by Ausubel et al. (1987).

GST Enzyme Assay

GST activity was measured spectrophotometrically at 340 nm according to Edwards and Owen (1986) with horse liver GST (Sigma) as a positive control. Depending on the purification state, 1 to 100 µg of protein were diluted to 1 mL using potassium phosphate buffer, pH 7.4. The protein solution was then mixed in a UV-transparent acrylic cuvette (Fisher) with 1 mL each of 3 mM CDNB and 10 mM reduced GSH in potassium phosphate buffer, pH 7.4. The formation of the color reaction product was measured at 340 nm. The value of the nonprotein blank was subtracted and the values were expressed as difference in A min⁻¹ mg⁻¹ of protein.

GSH-Agarose Affinity Chromatography

Soluble proteins from a H. muticus cell-suspension culture were prepared as described above. The proteins were precipitated with 80% ammonium sulfate, redissolved in PBS, and dialyzed against PBS, Triton X-100 was added to 1% (v/v) and the extract was loaded onto a GSH-agarose column (2 mL, Sigma) previously equilibrated with PBS containing 1% (v/v) Triton X-100. The column was washed with 20 mL of PBS. Bound proteins were eluted with 10 mL each of 0.1% (w/v) Triton X-100. The column was washed with 20 mL of potassium phosphate buffer, pH 7.4. The protein solution was then mixed in a UV-transparent acrylic cuvette (Fisher) with 1 mL each of 3 mM CDNB and 10 mM reduced GSH in potassium phosphate buffer, pH 7.4. The formation of the color reaction product was measured at 340 nm. The value of the nonprotein blank was subtracted and the values were expressed as difference in A min⁻¹ mg⁻¹ of protein.

RESULTS

Purification of the 25-kD Polypeptide

The 25-kD ABP was purified from suspension cultures of H. muticus by the consecutive use of ammonium sulfate precipitation, conventional anion-exchange chromatography, gel-filtration chromatography, and anion-exchange chromatography on HPLC. Figure 1 shows the purification, starting with the first anion-exchange chromatography step. Aliquots of individual fractions from the different columns were labeled with azido-[³H]IAA and analyzed by SDS-PAGE and fluorography to detect the ABPs. The 25-kD ABP eluted as a broad peak from both anion-exchange columns (Fig. 1, A and C), suggesting the presence of isoforms with slightly different isoelectric points. This suggestion was supported by analytical IEF of the pooled fraction after gel-filtration chromatography, which showed a number of bands over a pH range of 4.5 to 5.5 (data not shown). Labeling of the 25-kD polypeptide from different fractions in all cases showed a correlation between azido-[³H]IAA labeling and staining with Coomassie blue, suggesting that the different isoforms had similar affinity for the auxin (data not shown).

The native molecular mass of the 25-kD polypeptide as estimated by gel-filtration chromatography was 45 kD, indicating that the native protein probably is a dimer.

Analysis of Partial Amino Acid Sequences

The 25-kD polypeptide electroeluted from a preparative SDS gel was subjected to N-terminal sequencing. A sequence of 36 amino acids was obtained and compared with the protein sequences in the SwissProt data bank. Significant homologies were found to the N-terminal sequences of GST from tobacco (ParB; Takahashi and Nagata, 1992; 55% identity and 75% similarity) and GST from maize (termed GST-III by Grove et al., 1988, and GT32 in the data bank; 47% identity and 70% similarity) (Fig. 2).

Tryptic peptides were generated and partially sequenced after purification by reverse-phase HPLC. All sequences had high homology with corresponding sequences of both ParB and GT32 (Fig. 2, only the comparison with the ParB sequence is shown).
ammonium sulfate precipitate to the purest fraction (Fig. 3). The specific activity of our purest fractions was about 50% of the activity of the reference GST from equine liver. GST activity was not altered by IAA up to 0.5 mM (data not shown), but was abolished by boiling the samples for 30 s. Fractions not containing the 25-kD polypeptide showed no GST activity above background levels.

Immunological Characterization

Further evidence for the GST nature of the 25-kD ABP came from its immunological characterization. Western blot analysis of the fractions used for GST activity determination was performed with an antibody against a GST from wheat (kindly provided by Felix Mauch, University of Zurich). The antibody specifically recognized the 25-kD protein (Fig. 4C). Similar results were obtained with two different antibodies against GST from maize (kindly provided by Klaus Kreuz, Ciba Basel) (data not shown).

Purification of GST by Affinity Chromatography on GSH-Agarose

GST was purified from extracts of *H. muticus* by affinity chromatography on GSH-agarose. The procedure is shown in Figure 3. The specific activity of our purest fractions was about 50% of the activity of the reference GST from equine liver. GST activity was not altered by IAA up to 0.5 mM (data not shown), but was abolished by boiling the samples for 30 s. Fractions not containing the 25-kD polypeptide showed no GST activity above background levels.

Figure 3. Analysis of GST activity in fractions obtained during purification of the 25-kD ABP. Protein solutions were mixed with GSH and CDNB and A340 was measured for 2 min. The blank (no protein) was subtracted and the results were expressed as difference in A mg⁻¹ protein min⁻¹. Column 1, 45 to 65% ammonium sulfate cut; column 2, pooled fractions after first anion-exchange chromatography; column 3, pooled fractions after gel-filtration chromatography; column 4, pooled fractions after second anion-exchange chromatography.

Figure 2. Comparison of partial amino acid sequences of the 25-kD ABP (lower sequences) with the protein sequences of GT32 (GST-III) and ParB (upper sequences). The N-terminal sequences are compared in the upper part of the figure. In the lower part of the figure, partial sequences of the 25-kD ABP are compared with the protein sequence of ParB. Vertical bars indicate identical amino acids; colons indicate conservative amino acid replacements.

Figure 4. Analysis of fractions obtained during purification of the 25-kD ABP by SDS-PAGE, fluorography, and immunoblotting. A, SDS-polyacrylamide gel stained with Coomassie blue; B, fluorography of the gel shown in A; C, corresponding immunoblot decorated with an antibody against a GST from wheat. Molecular masses (kD) of two marker proteins are indicated at the left side of panel A. Lanes 1 to 3 contain 75 µg of protein, lane 4 contains 5 µg. Lane 1, proteins after ammonium sulfate precipitation (45-65% saturation); lane 2, proteins after first anion-exchange chromatography; lane 3, proteins after gel-filtration chromatography; lane 4, proteins after second anion-exchange chromatography.
eluate were analyzed by SDS-PAGE (Fig. 5A), with the antibody against GST from wheat (Fig. 5B), and by labeling with azido-[3H]IAA (Fig. 5C). On a western blot, the antibody against wheat GST strongly reacted with the prominent protein of 25 kD in the eluate fraction. In addition, the immunoreactive polypeptide was labeled with azido-[3H]IAA. N-terminal sequencing of the 25-kD polypeptide purified on GSH-agarose (24 amino acid residues) revealed identity with the 25-kD polypeptide purified by conventional methods (data not shown).

**Competition for Azido-[3H]IAA Labeling**

Previously, we have shown that labeling of the 25-kD polypeptide in crude extracts with azido-[3H]IAA was inhibited by auxin analogs (Macdonald et al., 1991). IAA, 1-NAA, 2-NAA, and, to a lesser extent, 2,4-D competed with azido-[3H]IAA for binding, whereas D- and L-Trp did not. We repeated the competition experiments with the purified 25-kD polypeptide and with GST purified on GSH-agarose and confirmed the data of Macdonald et al. (1991). Figure 6 shows the competition experiment with GSH-agarose-purified GST. IAA competed better than 1- and 2-NAA, 2,4-D competed weakly, and Trp did not compete. To reduce nonspecific labeling of the purified GST, we added the same amount of BSA and Cyt to the labeling mixture. BSA is known to bind IAA (Venis, 1984) under some conditions. However, Figure 6 shows that under our assay conditions, azido-[3H]IAA labeled BSA only weakly and did not label Cyt c at all.

Attempts to quantify the binding of [3H]IAA to the purified polypeptide by equilibrium dialysis (Reinard and Jacobsen, 1989) at pH 4.5 and 7.0 failed, which indicated low affinity between the polypeptide and the auxin under the conditions of the in vitro binding assay. The apparent displacement constant (K) for IAA in a nonequilibrium assay has been reported to be around 100 μM (Macdonald et al., 1991), which may suggest a substrate role for IAA binding to GST.

**DISCUSSION**

In an earlier paper (Macdonald et al., 1991), the use of azido-[3H]IAA to search for ABPs in the soluble fraction of *H. muticus* was described. We report now the purification of one of the proteins described therein, a 25-kD polypeptide. Its N-terminal sequence and sequences of trypsic peptides share significant homology with GST from maize and tobacco. This finding, based on sequence homology, was supported by the GST activity of the purified 25-kD ABP and its recognition by antibodies against GST from wheat and maize. Furthermore, the same polypeptide (based on sequence data and biochemical characterization) was isolated by affinity chromatography on GSH-agarose.

The biological relevance of the finding that GST from *H. muticus* binds auxins in vitro is, at this time, difficult to assess. Nevertheless, there are a number of interesting considerations that we will test in the future. GST catalyzes the conjugation of various electrophilic molecules with GSH (tripeptide: γ-Glu-Cys-Gly). One known function of GST in plants is the detoxification of herbicides by conjugation to the tripeptide (for a review, see Timmerman, 1989). By analogy, auxins may become conjugated to GST, either for direct modulation of hormone activity or for temporary storage. Although conjugates of IAA with GSH or with Cys, a possible degradation product of GSH-IAA, have not yet been identified in plants, it is possible that metabolization of the conjugate is a very rapid process and that the intermediates are only transient. The putative GSH-IAA conjugate may rapidly be transacylated to CoA-IAA, which, as an activated form of IAA, in turn could be esterified with myo-inositol or Glc (Kopcewicz et al., 1974). Kowalczyk and Bandurski (1990) described an alternative, CoA-independent pathway for the synthesis of hormone activity or for temporary storage. Although conjugates of IAA with GSH or with Cys, a possible degradation product of GSH-IAA, have not yet been identified in plants, it is possible that metabolization of the conjugate is a very rapid process and that the intermediates are only transient. The putative GSH-IAA conjugate may rapidly be transacylated to CoA-IAA, which, as an activated form of IAA, in turn could be esterified with myo-inositol or Glc (Kopcewicz et al., 1974). Kowalczyk and Bandurski (1990) described an alternative, CoA-independent pathway for the synthesis of auxin.
IAA esters. Which of these two pathways is used in vivo remains to be elucidated.

Another explanation for the binding of IAA to GST is that the auxin binds as a nonsubstrate ligand. GST is known to have a high affinity for different hydrophobic and amphipathic compounds without using them as a substrate (Ketley et al., 1975; Reinemer et al., 1991). Auxin binding to GST as a nonsubstrate ligand may also represent temporary storage and modulate hormone activity. Binding of nonsubstrate ligands is thought to occur at a different site than that occupied by GSH, but seems to reduce GST activity (Reinem er et al., 1991). We did not observe an inhibition of GST activity by IAA.

Another interesting but speculative function of GST is its possible involvement in plant development by regulating the level of cellular GSH. It has been suggested that the redox potential of suspension-cultured carrot cells is effective in switching between somatic embryogenesis and cell proliferation (Earnshaw and Johnson, 1985). In differentiating cells, the ratio GSH to GSSG, which is a marker for the redox state, was found to be low, whereas in proliferating cells the ratio was markedly increased. These marked changes in the ratio were due to removal of GSH by a change in GST activity. It remains to be determined whether auxin is directly involved in modulating GST activity by binding to the transferase or, as shown by Takahashi and Nagata (1992), by increasing the transcriptional activity of GST genes.

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