Purification and Characterization of Neomycin Phosphotransferase II from Genetically Modified Cottonseed (*Gossypum hirsutum*)

David C. Wood,*,† Linh V. Vu,† Nancee M. Kimack,† Glennon J. Rogan,‡ Joel E. Ream,‡ and Thomas E. Nickson‡

Monsanto Corporate Research and Monsanto Agricultural Group, Monsanto Company, St. Louis, Missouri 63198

Expression of the *nptII* gene [encoding the neomycin phosphotransferase II (NPTII) enzyme] is frequently employed as a selectable marker for plant transformation in a variety of species, including cotton. Therefore, characterization of this enzyme was considered an important part of the regulatory approval process designed to assess the safety of products derived from genetically modifed plants. Because of the role of cottonseed in animal feeds and other commercial uses, a purification scheme for NPTII from cottonseed was developed employing solvent extraction, a series of precipitations, DEAE-Sepharose chromatography, and affinity chromatography which yielded a highly enriched preparation that retained enzymatic activity. Further purification was obtained by SDS–PAGE, electrotransfer to PVDF membrane, and direct sequencing of the appropriate band. NPTII derived from cottonseed was characterized by purification behavior, NH_2-terminal sequence, SDS–PAGE, immunoblot, ELISA, and enzymatic activity and was found by these criteria to be chemically equivalent to the enzyme derived from an *Escherichia coli* expression system.

Keywords: *Neomycin phosphotransferase II (NPTII); genetically modified cotton; protein purification; affinity chromatography*

INTRODUCTION

Neomycin phosphotransferase II (NPTII) is a bacterial enzyme that confers resistance to some aminoglycoside antibiotics by phosphorylation of the 3′-hydroxyl group of the aminoglycoside (Sarwar and Akhtar, 1990). The gene encoding this enzyme is a commonly used selectable marker for cell transformation in bacterial and eukaryotic molecular biology (Balbas and Bolivar, 1990; Southern and Berg, 1982; Fraley et al., 1985). Previous studies have shown that expression in cotton of certain proteins derived from *Bacillus thuringiensis* (*B.t.*) led to effective levels of control of agronomically important lepidopteran pests (Dulmage, 1981; Hofte and Whitely, 1989; Perlak et al., 1990). A critical step in the methodology used to produce these genetically modified cotton plants employed the NPTII gene as a selectable marker, resulting in the low-level expression in cotton of the bacterial NPTII enzyme.

Sufficient quantities of heterologously expressed proteins such as NPTII or the *B.t.* proteins in a purified form are necessary to conduct appropriate safety assessments for the regulatory approval process for insect-resistant cotton and other genetically modified crop plants. Because of the extremely low expression level of NPTII in the genetically modified cotton tissue, an *Escherichia coli* based expression system was employed to produce NPTII in large quantities for the safety assessment studies (Fuchs et al., 1993). To demonstrate the equivalence of NPTII derived from microbial and cotton expression, the purification of sufficient NPTII from the cotton plant to carry out a comparison of the enzyme from the two sources was undertaken.

* Author to whom correspondence should be addressed at AA4I, Monsanto Company, 700 Chesterfield Parkway N., St. Louis, MO 63198 [telephone (314) 537-6242; fax (314) 537-7223].

† Monsanto Corporate Research.

‡ Monsanto Agricultural Group.

This paper describes the purification of neomycin phosphotransferase II from genetically modified cottonseed. All of the experiments were carried out using line 81 cottonseed (Perlak et al., 1990). This line expresses a truncated form (HD-1) of one of the insecticidal proteins produced by *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Fischoff et al., 1987) and had the highest expression levels of NPTII based on ELISA assay compared to other available lines. Although the NPTII enzyme has been studied for several years and has been purified from several sources (Sarwar and Akhtar, 1990; Umezawa et al., 1973; Matsuhashi et al., 1975, 1977; Goldman and Northrup, 1976; Ganelin et al., 1977; Perlin and Lerner, 1986), the enzymology of this important enzyme has proven difficult due to the extreme instability of enzyme activity (Goldman and Northrup, 1976). Due to instability and to the low expression level of NPTII in genetically modified cotton, the purification strategy employed was based on a substrate affinity separation step compatible with cottonseed extracts which allowed high-fold purification with acceptable yield, in a manner analogous to the previously cited isolations of this enzyme from *E. coli* and from other bacteria.

MATERIALS AND METHODS

Seed Extraction. Delinted cottonseed (500 g) from line 81 (Perlak et al., 1990) was ground into a coarse powder in 250 g batches using a 1 qt Waring blender at high speed for 30 s. The ground seed was extracted at room temperature using 3 × 1 L volumes of reagent grade acetone (Fisher Scientific A18-1) by stirring in a beaker, followed by filtration and air-drying on a Büchner funnel. All subsequent steps were carried out at 4 °C unless otherwise stated. The dried acetone powder was extracted with 3750 mL of lysis buffer composed of 100 mM Tris, 100 mM boric acid, pH 7.8, 2.5 mM EDTA, 2 mg/mL ascorbic acid, 5 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin, and 1 μg/mL aprotinin using an Ultra-turrax homogenizer. Cell debris was removed by centrifugation for 20 min at 12000*g*, and the supernatant was filtered through

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cheesecloth. A 10% solution of polyethylenimine (PEI) was added to a final concentration of 0.15% v/v and stirred for 20 min. This PEI precipitate was removed by centrifugation and saved for later extraction. From the PEI supernatant fraction, protein was precipitated by the addition of 70% ammonium sulfate with stirring for 2 h. The PEI precipitate was extracted with lysis buffer containing 1 M NaCl and the solubilized protein was precipitated by addition of 70% ammonium sulfate as for the PEI supernatant. The ammonium sulfate pellets derived from both PEI fractions were combined, solubilized in approximately 500 mL of 50 mM Tris, pH 8.0, and 1 mM DTT (DEAE-Sepharose running buffer), and dialyzed vs two 4 L changes of the same buffer overnight.

DEAE-Sepharose Chromatography. After dialysis, the turbid solution was adjusted to pH 8.0, and any precipitate was removed by centrifugation. The conductivity was adjusted to 2.5–3.0 mS/cm by addition of cold 1 mM DTT, and the protein solution was loaded onto a DEAE-Sepharose Fast Flow column (9.0 cm diameter × 11 cm, bed volume 710 mL) at a flow rate of 160 mL/h. After loading, the column was eluted with 1–2 column volumes of DEAE running buffer followed by a 3500 mL linear gradient from 0 to 0.5 M NaCl. Fractions were assayed by immunoblot and a pool was made and stored at 4 °C. The same fractions were later assayed by ELISA for NPTII and HD-1 proteins.

Amikacin Affinity Chromatography. Amikacin Affi-Gel was prepared as described in Bio-Rad Product Bulletin 1085. All operations were carried out at 4 °C or on ice. In a typical preparation, Affi-Gel 10 was thoroughly washed on a Büchner funnel with cold deionized water to remove the storage solvent. The gel slurry was then transferred to a vial containing a solution of 100 mg/mL amikacin (Sigma A3650) in 100 mM sodium bicarbonate buffer, pH 8.6. These steps were completed in 5–10 min. The mixture of the gel and antibiotic solution was rocked overnight at 4 °C to ensure complete reaction. The coupled gel was stored at 4 °C prior to use. The ratio of gel to antibiotic was 1 mL of slurry to 100 mg of antibiotic in all preparations; thus, 20 mL of resin using 2 g of amikacin was prepared.

Chromatography of NPTII samples was performed at room temperature. A 2 mL column of amikacin affinity resin was poured and equilibrated with 25 mM Tris-HCl, pH 7.6, 50 mM KCl, and 2.5 mM DTT (affinity running buffer). The column was calibrated for NPTII elution by bind/release of a sample of NPTII generated from an *E. coli* overexpression system (Fuchs et al., 1993). Control experiments to determine run-to-run carryover of NPTII on this amikacin affinity column showed negligible carryover, indicating that the same column could be used for isolation of cottonseed NPTII without risk of contamination from *E. coli* derived NPTII. A 130 mL aliquot of the DEAE-Sepharose pool was dialyzed vs affinity running buffer and concentrated to 60 mL using an Amicon YM10 membrane. This sample was applied to the column at 0.4 mL/min. When the A_{280} had returned to near baseline, the column was eluted with 8 mM kanamycin B sulfate (Sigma B5264) in affinity running buffer. Fractions were collected (2.4 mL) and assayed by immunoblot and ELISA for NPTII. Fraction 38 was the fraction most enriched for NPTII of the eluent and was used for further analyses.

Preparative SDS–PAGE and N-Terminal Sequence Analysis. The peak fraction (no. 38) from the amikacin Affi-Gel column was applied to a 15 × 22 cm, 0.75 mm thick, 15% SDS acrylamide gel and electrophoresed using the standard Tris–glycine–SDS system. Replicate lanes were run for immunoblot and Coomassie staining analyses. Three gel lanes containing 50 μL of fraction 38 were electroblotted onto PVDF membrane, using the method of Matsudaira (1987), and stained with Ponceau S, and the band corresponding to NPTII was excised for subsequent N-terminal sequence analysis using an Applied Biosystems 470A gas phase sequencer with on-line HPLC detection of PTH amino acids employing an Applied Biosystems 120A PTH analyzer fitted with a Brownlee 2.1 mm PTH C_{18} column.

Immunoblot Analysis. Samples for analysis were electrophoresed on a 10–20% precast minigel (ISS/Enprotech) and electrotransferred to nitrocellulose for 1 h using a Hoeffer Transblot apparatus. The blot was blocked with 0.25% gelatin (Sigma G-6269) in Tris-buffered saline, pH 7.5, with 0.05% Tween 20 (TBST) for 20 min. The blot was then probed with anti-NPTII rabbit serum at 1:5000 dilution for 15 min, followed by a 5 min wash with TBST. NPTII bands were visualized using a 1:5000 dilution of anti-rabbit IgG alkaline phosphatase conjugate (Promega S373B) for 15 min followed by 5 min washes with TBST and alkaline phosphatase buffer (0.1 M Tris, 0.1 M NaCl, 0.005 M $MgCl_2$, pH 9.5) and addition of NBT and BCIP substrate (Promega S380C/S381C).

Table 1. NPTII Purification Table[a]

step	vol, mL	[NPTII], μg/mL	NPTII, μg	yield, %
lysate	2560	0.117	300	100
PEI supernatant	2380	0.115	274	91
DEAE load	1000	0.156	156	52
DEAE pool	340	0.764	260	87

[a] Aliquots of samples at various stages of purification were stored at −20 °C prior to ELISA assay for quantitation of NPTII mass.

Quantitation of NPTII by ELISA. The double-antibody sandwich ELISA described by Rogan et al. (1992) was employed. The method employs a protein A affinity-purified rabbit polyclonal IgG preparation and a horseradish peroxidase conjugate of the same antibody for visualization using 3,3′,5,5′-tetramethylbenzidene (TMB) as substrate.

NPTII Enzymatic Activity Assay. NPTII enzymatic activity was measured using the coupled spectrophotometric assay described by Goldman and Northrup (1976).

RESULTS AND DISCUSSION

A summary of the NPTII recovery data through the DEAE-Sepharose step is shown in Table 1. The ELISA assay of Rogan et al. (1992) was used to monitor purification yield rather than direct enzymatic activity assay due to the low concentration of NPTII present prior to the affinity chromatography step. From 500 g of delinted cottonseeds, recovery of NPTII through the DEAE pool stage was 260 μg by ELISA with a yield of 87%. The seed extraction with acetone was quite effective in removing lipid material, as judged by the decreased turbidity of the aqueous extract relative to seeds extracted without acetone treatment. Polyethylenimine and ammonium sulfate precipitations also resulted in decreased turbidity while giving good recovery of NPTII. Because purification of active enzymes from plant extracts often requires specialized conditions to minimize inactivation of the enzyme by organic compounds extracted from the plant tissue, the extraction buffer contained borate anion to minimize interference by gossypol, the most abundant phenolic compound in cotton, and ascorbic acid to act as an antioxidant (Loomis, 1974).

DEAE-Sepharose chromatography resulted in significant purification of NPTII, as evidenced by the UV absorbance profile shown in Figure 1 and by the SDS–PAGE analysis shown in Figure 2. During column loading, a bright yellow band was adsorbed at the top of the column. Yellow and purple pigments were eluted with the protein in the salt gradient, but a significant quantity of yellow material was retained on the column. The column fractions were analyzed by immunoblotting, as shown in Figure 3, and fractions 83–97 were pooled. The same fractions were assayed by ELISA for both NPTII and *B.t.k.* HD-1 proteins, and those elution profiles are shown in Figure 1. Both proteins eluted in a single peak from the DEAE column, showing no obvious heterogeneity in this separation step. The apparent yield of NPTII from the DEAE column of

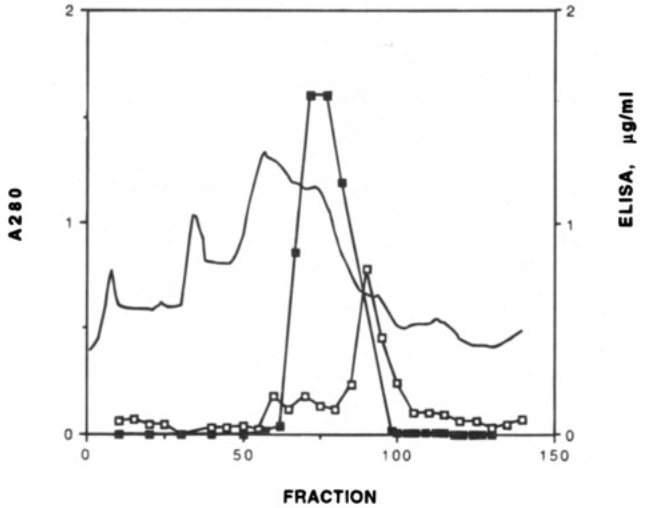

Figure 1. DEAE-Sepharose chromatography. The chromatography conditions are described under Materials and Methods. A pool of NPTII from fractions 83–97 was made based on immunoblot analysis. Fractions containing the *B.t.k.* HD-1 protein were also pooled. (—) A_{280}; (□) NPTII, µg/mL; (■) HD-1, µg/mL.

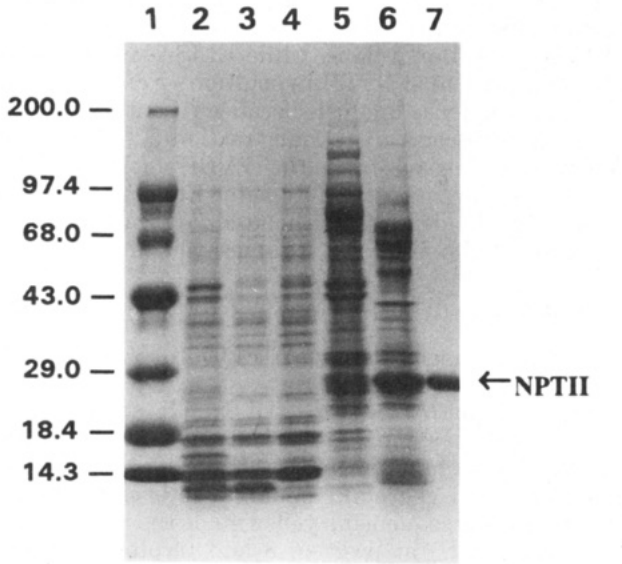

Figure 2. SDS–PAGE analysis of NPTII purification steps. Aliquots of the various pools were electrophoresed on a 4–20% precast minigel (Novex) and then stained with Coomassie. Lanes are as follows: 1, MW markers, BRL high range; 2, seed lysate; 3, PEI supernatant; 4, DEAE load; 5, DEAE pool; 6, amikacin column fraction 38; 7, *E. coli* NPTII marker, 5 µg.

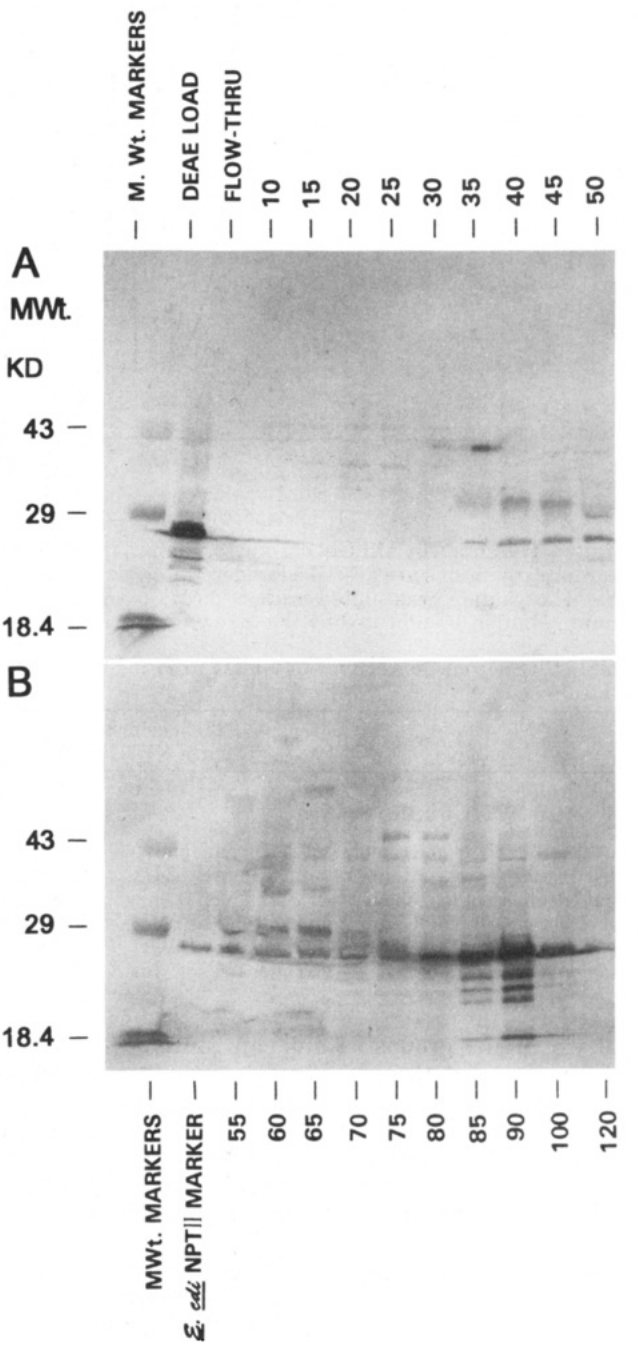

Figure 3. NPTII immunoblot analysis. Panels A and B represent two different gels run in parallel and electrotransferred at the same time for immunoblot analysis according to the procedure described under Materials and Methods. Fraction numbers are indicated for each lane.

greater than 100% in Table 1 may be due to suppression of the ELISA signal by residual polyethylenimine in the DEAE load solution which is not present in the DEAE column pool.

NPTII from the DEAE column was further purified using amikacin affinity chromatography, with the resulting chromatogram shown in Figure 4. A large flow-through absorbance was observed, and a sharply eluting peak of absorbance was obtained upon elution with kanamycin B. The peak fraction in the eluent (no. 38) was greatly enriched in NPTII relative to the DEAE pool, as shown in Figure 2 by SDS–PAGE using Coomassie staining. The effective elution of NPTII by the antibiotic kanamycin B suggests that a biospecific bind/release mechanism was operating, as opposed to a strictly ion exchange interaction between NPTII and the immobilized, charged substrate. Neither the conductivity nor the pH of the buffered kanamycin B solution used for elution was significantly different from the column running buffer, which further supports the hypothesis that NPTII was bound to immobilized amikacin through the enzyme active site and was released by competitive binding of the substrate kanamycin. However, in agreement with Perlin and Lerner (1979), elution of NPTII from the amikacin column was also obtained by 250 mM NaCl in the absence of kanamycin, indicating that an increase in conductivity alone was sufficient, perhaps operating through a mechanism distinct from kanamycin elution.

We hypothesize that the semisynthetic antibiotic amikacin (Haas and Dowding, 1975) may have advantages over gentamicin C_{1a} employed by Goldman and Northrup (1976) as a reagent for coupling to Affi-Gel

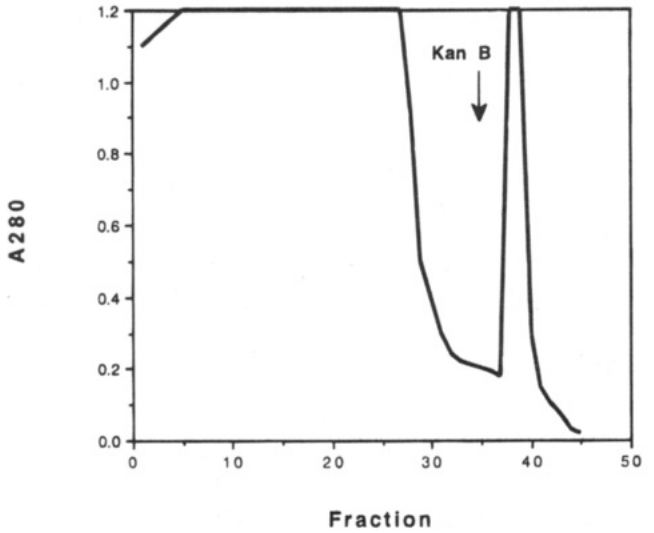

Figure 4. Amikacin Affi-Gel chromatography. The chromatography conditions are described under Materials and Methods. The column was eluted with 8 mM kanamycin B in running buffer as indicated by the arrow.

Table 2. Enzymatic Activity of NPTII Purified from Cottonseed

sample	NPTII specific activity[a] (U/mg of NPTII protein)
E. coli NPTII	8.0
cotton NPTII, fraction 38	8.1

[a] A unit (U) = micromoles of neomycin and ATP-dependent NADH oxidized per minute at approximately 24 °C. Data represent the mean of four measurements of NPTII enzymatic activity for fraction 38. NPTII protein content of cotton NPTII was estimated to be 10% of total protein by SDS–PAGE. *E. coli* NPTII was obtained from 5 Prime - 3 Prime, Inc., of West Chester, PA.

10 via amino groups. Amikacin contains a primary amino group at the end of a five-atom spacer, unlike other primary amino groups on aminoglycosides, including gentamycin C_{1a}. This side chain primary amino group should be acylated by the resin faster than any other amino group on the molecule. Thus, the coupling efficiency to the activated ester of Affi-Gel 10 as well as the conformational accessibility of the 3′-OH of amikacin to the active site of NPTII during chromatography may be improved relative to other choices of aminoglycosides for affinity chromatography.

After amikacin affinity chromatography, the eluted fraction was evaluated for NPTII enzymatic activity (Goldman and Northrup, 1976) and compared with NPTII derived from recombinant *E. coli*. NPTII purified from cotton was found similar in specific activity to the *E. coli* NPTII reference standard when compared by an activity per milligram of NPTII protein basis, as shown in Table 2.

The pool from amikacin affinity chromatography was further purified prior to NH_2-terminal sequence analysis by preparative SDS–PAGE and electrotransfer to PVDF membrane. A Coomassie stained lane identical to the electroblotted sample is shown in Figure 5A, and immunoblot analysis of fractions 37 and 38 is shown in Figure 5B. These data clearly show a major band with the same gel mobility as NPTII standard from *E. coli*. In the immunoblot shown, the NPTII band was the first to show a signal during the alkaline phosphatase reaction, while additional bands appeared later. The relative intensity of the contaminant bands is overestimated in Figure 5B due to the limited dynamic

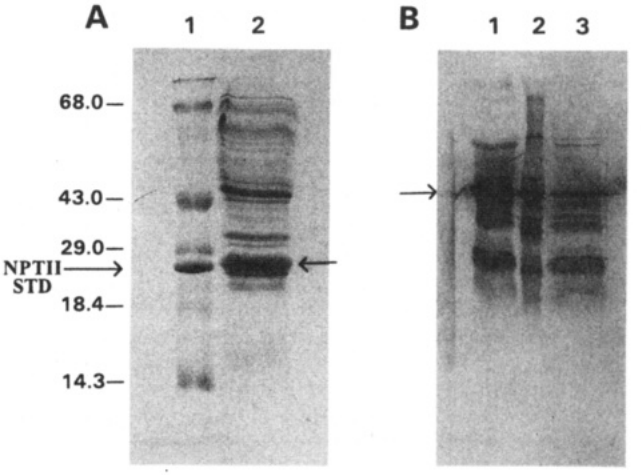

Figure 5. Preparative SDS–PAGE of NPTII. The migration position of NPTII is indicated by an arrow. (A) Coomassie staining; lane 1, MW markers + *E. coli* NPTII; lane 2, amikacin column fraction 38 (50 μL). (B) Immunoblot analysis; lane 1, amikacin column fraction 38; lane 2, *E. coli* NPTII marker; lane 3, amikacin column fraction 37.

range of the immunoblot method. For this reason, immunoblotting was used only as a rapid procedure for monitoring purification progess and for analysis of NPTII molecular weight, while ELISA was employed for quantitation of NPTII in solution. Fraction 38 was used for sequence analysis because it contained NPTII at higher concentration than fraction 37. The NPTII band was excised from the PVDF membrane and sequence analyzed using automated Edman degradation chemistry to determine the amino-terminal protein sequence. The following sequence was identified:

obtained sequence MIEQDGL(H)AGSPAA(W)

theoretical sequence MIEQDGLHAGSPAAW

In addition to the expected NPTII sequence, the electroblotted sample also contained minor protein contaminants to the primary NPTII signal, which was observed as background signal. The background and relatively low sequencing yields made the identification of histidine (H) at position 8 and tryptophan (W) at position 15 in the sample tenuous. A second purification of cottonseed NPTII was subsequently carried out using a fresh seed extract and a new lot of amikacin Affi-Gel. When this amikacin eluent was electrophoresed, electroblotted, and sequence analyzed, the purity and mass of NPTII were significantly greater, and all of the amino acid residues through 15 places were clearly identified. In addition, NPTII derived from a recombinant *E. coli* expression system (Fuchs et al., 1993) contained an N-terminal sequence identical to the theoretical sequence derived from the gene DNA sequence (data not shown).

The initial yield of PTH amino acids obtained from gas phase sequence analysis was used to make an estimate of NPTII present at the DEAE pool step (Table 1). From a sequencer initial yield of 21 pmol, with the assumption of 10% recovery during electrotransfer/sequencing and 100% recovery at all other post-DEAE steps, the mass of NPTII present at the DEAE pool step was back-calculated to be 250 μg, which was in good agreement with the value of 260 μg obtained by ELISA (Table 1). Even though quantitation based upon sequence analysis is highly protein dependent, and necessarily contains several assumptions, the data support

the accuracy of the ELISA assay for quantitation of NPTII during this purfication.

SUMMARY

Neomycin phosphotransferase II was purified from cottonseed derived from genetically modified cotton plants which express this commonly used selectable marker. Purification of NPTII with retention of enzymatic activity required initial removal of seed lipids and phenolics for the subsequent chromatographic separations to perform efficiently. In the chromatography steps, NPTII from genetically modified cottonseed behaved similarly to the enzyme as isolated from *E. coli*, indicating the physical properties of NPTII from the two sources to be equivalent. Analysis by SDS–PAGE, immunoblot, ELISA, enzymatic activity, and N-terminal sequencing also was consistent with the equivalence of NPTII whether expressed in the prokaryote, *E. coli*, or in the eukaryote, *Gossypum hirsutum* (cotton). While these analytical methods provide a reliable data set for comparison of two proteins, they do not have sufficient resolution to identify protein microheterogeneity which might exist. However, the low molar concentration of any single chemical isoform of NPTII would seem to make removal of such species of minimal value to the assessment of protein safety in feeding studies.

It should be noted that the NPTII protein is also expressed in the Flavr Savr tomato from Calgene, Inc., which has recently been approved for sale by the Food and Drug Admininstration, which has given Food Additive Status to NPTII as expressed in cotton, canola, and tomato (Food and Drug Administration, 1994). Due to the general utility of the *nptII* gene as a selectable marker in plant transformation, we anticipate the submission for regulatory approval of additional genetically modified plants that have been produced using the NPTII gene.

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