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TWO ISOFORMS OF NP24: A THAUMATIN-LIKE PROTEIN IN TOMATO FRUIT

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Abstract—Two isoforms (I and II) of the thaumatin-like protein NP24 have been discovered in extracts of tomato fruit. NP24 I increased *ca* 8-fold during ripening of tomato fruit, whereas NP24 II increased slightly as the fruit began to change colour and then remained constant. Both proteins were purified to homogeneity and one of them, NP24 I, was obtained in crystalline form. The proteins are similar except for differences in binding to cation exchangers and in two amino acids of the *N*-terminal amino acid sequences. The NP24s were tested as growth inhibitors of six different fungi and found to differ in effectiveness, but both proteins were most effective on *Phomo betae* and *Verticillium dahliae*. These proteins may be involved in disease resistance in tomatoes, but the increase in NP24 I during ripening suggests a possible role in fruit development and ripening. Copyright © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Suspension-culture cells of tomato and tobacco exposed to gradually increasing concentrations of NaCl or other water-potential-lowering agents accumulate a high level of a 24 kDa protein that has been named NP24 [1, 2]. It is a basic protein with unusually high proportions of the amino acids cysteine and proline and a high degree of homology with the sweet protein thaumatin [2]. The accumulation of NP24 during water stress suggests a possible role of the protein in cellular tolerance of dehydration, but its actual function is unknown. It is difficult to distinguish between product formation caused by water stress and that due to injury or damage accompanying the stress [3]. Similarity of NP24 with the tobacco protein osmotin [4] which is known to permeabilize fungal membranes [5], suggest an alternative function for NP24 in protection of plant cells against pathogens.

Control tomato and tobacco suspension cells contain some NP24, but the level of this protein is much higher in cells grown in medium with NaCl [1]. NP24 is also present in whole tomato plant and, as in tomato suspension cells, it increases especially in roots when the plants are stressed with NaCl [1]. NP24 was not previously studied in the fruit of tomato, but we have discovered that normal tomato fruit is a rich source of the protein. A protein that appears to be identical to the NP24 found in tomato cells and plants was obtained as crystals from highly concentrated extracts of ripe tomato fruit. Another major protein in extracts of tomatoes was determined to be an isoform of NP24. This paper describes the changes in the two NP24s during ripening of tomatoes, and the isolation and properties of the tomato fruit proteins.

RESULTS AND DISCUSSION

Occurrence of NP24 in tomato fruit

NP24 was discovered fortuitously in a tomato extract being prepared for the purification of the enzyme polygalacturonase (PG). The procedure consisted of homogenizing 1 kg of whole ripe tomatoes in 1 M NaCl at pH 6, concentrating the supernatant solution obtained by centrifugation to 25 ml by ultrafiltration, dialysing against 0.2 M NaCl, and chromatography on a column of DEAE-Sephadex A-50 [6]. The fractions containing PG were pooled, concentrated to 8 ml and stored at −20° for several weeks. A shimmering cloudiness in the solution when it was thawed was found to be due to fine crystals by microscopic examination (Fig. 1).

The crystals were collected by filtration and washed with cold water. They appeared to be insoluble in water in 0.15 M NaCl at neutral pH, but the crystals dissolved readily when the suspensions were acidified to pH 2.5. The solution could then be neutralized without recrystallization of the protein as long as the

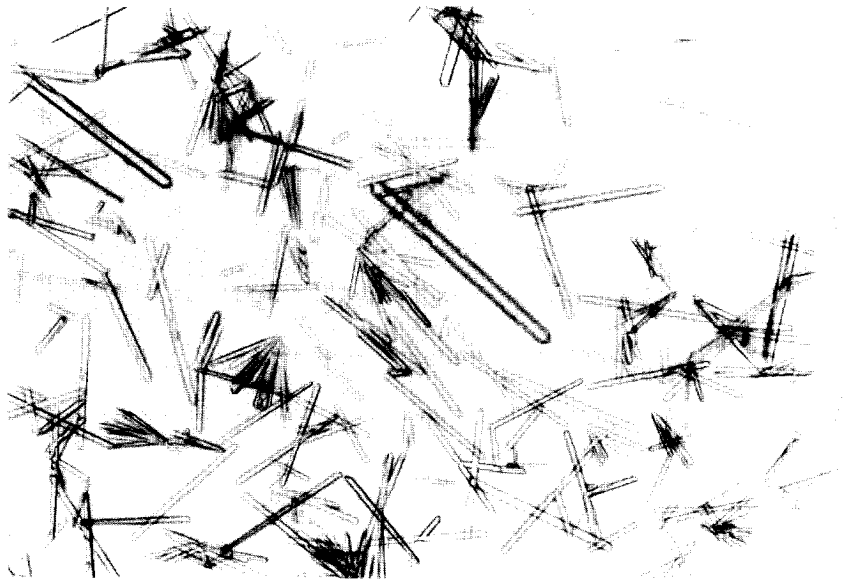

Fig. 1. Crystals of tomato NP24 I (×40 magnification).

protein concentration was less than 2 mg ml^{-1}. At higher levels of protein, crystallization and/or precipitation occurred even at room temperature as the pH was raised over 5.

Chromatography of an aliquot of the solubilized protein on a column of Sephadex G-75 revealed a M_r of *ca* 21 k. The protein eluted immediately before PG during ion-exchange chromatography on a Mono S column, suggesting that it is highly basic, like PG [7]. Attempts to identify enzymic activity in the protein were unsuccessful. The *N*-terminal amino acid sequence of the protein matched the published sequence for tomato cell NP24 [2]. Based on the *N*-terminal sequence and the preliminary physical evidence, the crystalline protein from tomato fruit was identified as NP24.

Another prominent basic protein was routinely observed in Mono S profiles of tomato extracts. Evaluation of this protein with a limited number of substrates failed to detect enzyme activity. The M_r of the protein was *ca* 21 k according to gel filtration on Sephadex G-75, and the *N*-terminal amino acid sequence resembled that for NP24. The similarities in properties and amino acid sequences suggested that this protein is an isoform of NP24. It was therefore designated NP24 II and the crystalline isoform first discovered was designated NP24 I.

In order to determine the location of the NP24 isoforms within tomato fruit, whole ripe tomatoes were separated into outer wall pericarp, inner wall pericarp including the radical wall, and the locular gel [8]. Extracts were prepared from each of these components as described in Experimental and analysed for NP24 I and II. The highest levels of both isoforms were found in the outer pericarp, with smaller amounts in the inner pericarp, but these proteins were not detected in the locular gel (Table 1). This is in contrast to the related protein thaumatin which occurs, not in the pericarp,but in a jelly-like aril at the base of a large seed in the fruit of *Thaumatococcus danielli* [9].

Table 1. Distribution of NP24 I and NP24 II in ripe tomato fruit

Fruit component	NP24 I ($\mu g\ g^{-1}$ fr. wt)	NP24 II ($\mu g\ g^{-1}$ fr. wt)
Outer pericarp	7.1	11.2
Inner pericarp	2.6	3.2
Locular gel	0	0

Changes in the levels of NP24 isoforms in tomatoes during ripening

Tomatoes (cv. Better Boy) were analysed for NP24 I and II at four stages of ripeness ranging from mature green to ripe red. NP24 II was relatively high in green tomatoes and it increased somewhat as the fruit turned pink, but not during further ripening (Fig. 2). In contrast, NP24 I was low in green tomatoes and it increased substantially during ripening, with the largest increase occurring between the pink and red stages (Fig. 2). The overall increase in NP24 I was *ca* 8-fold, but it was still lower than NP24 II at the red stage of ripeness.

Purification of NP24 isoforms

Extraction of tomato pericarp tissue with 0.15 M NaCl at pH 1.6 was a convenient and efficient method for solubilizing the NP24s. The proteins were then adsorbed on S-Sepharose by addition of the gel to the pH 1.6 extract. The gel was washed with H_2O to

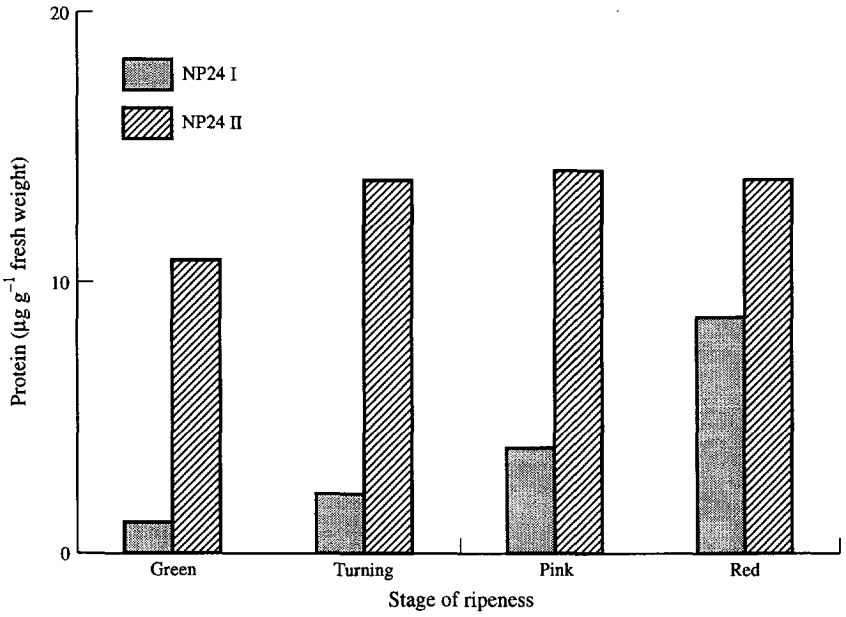

Fig. 2. Changes in NP24 I and NP24 II during ripening of tomatoes.

Protein	Sequence
NP24 I	ATIEVRNNXPYTVWAASTPIGGGRR
NP24 II	ATFEVRNNXPYTVWAAFTPIGGGRR

Fig. 3. *N*-Terminal amino acid sequences of NP24 I and II.

remove the NaCl and then with 0.02 M MES (pH 6) to raise the pH to 6. Subsequent elution with a linear gradient of NaCl separated the two NP24s (Fig. 3). The peak from the S-Sepharose column containing NP24 I was contaminate primarily by PG. The two components could be separated by gel filtration, but it was easier to concentrate the solution to a small volume and to allow crystallization of NP24 I to occur at low temperature. The crystals that formed after several weeks were collected by filtration and dried under vacuum. The yield of NP24 I was 14 mg from 2 kg of tomato tissue.

NP24 II in the protein peak from the S-Sepharose column (Fig. 3) was accompanied by a large amount of pectinesterase, another major basic protein in tomatoes. Chromatography of this mixture on a column of DEAE-Sephadex A-50 separated NP24 II from pectinesterase (data not shown). Final purification of NP24 II was accomplished by chromatography on a Mono S column, which removed a few minor proteins eluting immediately before and after NP24 II. The yield of NP24 II was 25.2 mg from 2 kg of tomato tissue.

Properties of the NP24 isoforms

The purified NP24 isoforms were homogeneous according to the following chromatographic and electrophoretic criteria. A single and sharp peak was obtained for each protein by ion-exchange chromatography on a Mono S column. The proteins also yielded sharp elution peaks on gel filtration using sephadex G-75. SDS–PAGE showed a single protein band for each isoform. The M_rs of the proteins appeared to be similar, but the values varied with the method of measurement. Values of a 21 k were obtained for both proteins from gel filtration data, but SDS–PAGE yielded values of 26 k. The predicted amino acid sequence derived from the cDNA clone for tomato root NP24 indicated a protein of 226 amino acid with a M_r of 24,226 [2].

The *N*-terminal amino acid sequences for the NP24 isoforms are shown in Fig. 4. The sequence for NP24 I is identical to that reported for tomato cell NP24 (2) and osmotin [4]. The sequence for NP24 II differs from that for NP24 I in amino acids 3 and 17 (Fig. 4). NP24 I and NP24 II were easily separated by ion-exchange chromatography, indicating marked differences in the charge properties of the proteins. The pIs as determined by chromatofocusing on a Mono P column were found to be 9.7 and 10.4 for NP24 I and NP24 II, respectively.

Inhibition of fungal growth by tomato NP24s

The NP24s were evaluated for growth inhibition of six different fungi by Dr Willem Broekaert of the Katholieke Universitert Leuven, Heverlee, Belgium.

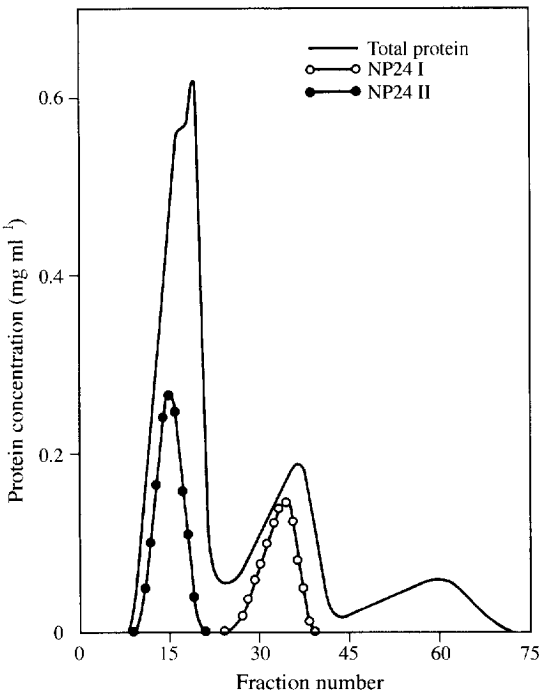

Fig. 4. Elution of tomato proteins from a column of S-Sepharose. The fraction volume was 14 ml.

The tests were conducted in SMF media [10] with and without the addition of 1 mM $CaCl_2$ and 50 mM KCl. The proteins were quite active in the absence of the cations on some of the fungi, particularly *Phoma betae* and *Verticillium dahliae* (Table 2). In the presence of $CaCl_2$ and KCl, however, the IC_{50} values were greater than 400 μg ml^{-1} for both proteins and all six fungi (data not shown). The effectiveness of other antifungal proteins has been reported to be very sensitive to the presence of cations [11].

There is evidence that other thaumatin-like proteins possess antifungal properties. Thaumatin itself has weak acitivty against *Candida albicans* [12]. Zeamatin, a protein from corn seeds known to have antifungal activity, was found to have a *N*-terminal sequence very similar to osmotin [12]. A protein isolated from tobacco plants inoculated with TMV causes lysis of sporangi and inhibition of growth of *Phytophthora infestans* and was identified as osmotin II [5]. Subsequently, Vigers *et al.* [13] found that osmotin was particularly effective in inhibiting the growth of *C. albicans*, *Neurospora crassa* and *Trichoderma reesi*. Overexpression of osmotin in transgenic potato plants leads to delayed development of disease symptoms after incubation with spore suspension of *P. infestans* [14], providing further evidence that osmotin may be involved in disease resistance.

Table 2. Inhibition of fungal growth by tomato NP24s

Fungus	IC_{50} (μg ml)	
	NP24 I	NP24 II
Fusarium colmorum	>400	120
Alternaria brassicola	15	75
Ascochyta pisi	>499	22
Phomo betae	1.5	6
Verticillium dahliae	0.8	3
Botrytis cinerea	>400	150

The protein concentrations (μg ml^{-1}) for 50% growth inhibition (IC_{50}) were determined as described by Terras *et al.* [10]. The values were determined after 72 hr of growth in the presence of two-fold serial dilutions of the proteins.

Physiological functions of thaumatin-like proteins

Osmotin and NP24 were discovered in osmotically stressed plants and suspension cells [1, 4] and interest in these proteins has centred on their roles in cellular tolerance of dehydration. The findings that they possess antifungal properties [4, 12, 13] suggest an additional or alternative function in the control of pathogens. Gene expression during stress can be due not only to the stress, but also to injury or damage that occurs during stress such as dehydration [3]. Furthermore, the expression of water-deficit-associated genes may occur independently of the stress conditions [3] and the functions of the products are not obvious. It is becoming evident that thaumatin-like proteins are present in fruit tissues. Thaumatin, which is highly homologous with osmotin and NP24, is a major protein in normal ripe fruit of a West African shrub [9]. The function of thaumatin in this fruit is unknown, but its intense sweetness [15] suggests a possible role in attracting animals for seed dispersal. The cloning of a gene from avocado that encodes a thaumatin-like protein [16] suggests the presence of a related protein in this fruit. Expression of the mRNA homologous to this protein increased during normal ripening of avocado [16]. The results of the present study show that tomato fruit contains high levels of the NP24 isoforms and that one of them, NP24 I, increases significantly during tomato ripening. The roles of thaumatin-like proteins in fruits may be the same as in other plant tissues, but it appears that their formation may be ripening related and thus could be involved in the ripening process.

EXPERIMENTAL

Assays for NP24s. NP24s were assayed by FPLC (Pharmacia) on a Mono S HR 5/5 column using 30 ml of a linear gradient of 0–0.5 M NaCl in 0.02 M MES, pH 6. NP24 I and II eluted at 0.07 and 0.22 M NaCl, respectively.

Purification of NP24s. Pericarp tissue (2 kg) from ripe Better Boy tomatoes was homogenized with a polytron in 2 l of cold H_2O. The homogenate was adjusted to pH 3 by addition of M HCl. Solid NaCl was added to yield a concn of 0.15 M and the pH was further lowered to 1.6 with HCl. After stirring for 30 min, the homogenate was centrifuged at 8000 *g* and

the supernatant soln was saved. A suspension of 80 ml S-Sepharose Fast Flow (Pharmacia) in 200 ml H_2O was adjusted to pH 1.6 and added to the extract. The slurry was stirred for 30 min and the gel allowed to settle. The supernatant soln was decanted and the gel poured into a 2.5 × 40 cm glass column. The gel was washed with 200 ml H_2O and then with 400 ml 0.02 M MES, pH 6. Proteins were eluted from the column with 1 l of a linear gradient of 0–0.5 M NaCl in 0.02 M MES, pH 6. The frs (14 ml) were assayed for NP24 I and II by the FPLC method.

The frs from the S-Sepharose column containing NP24 I (28–37) were pooled and concd to 7 ml. The soln was stored at −12° for 3 weeks. The sample was then allowed to thaw at room temp. and the crystals were collected by filtration. The protein was recrystallized by dissolving in 5 ml 0.15 M NaCl at pH 2 followed by adjusting the pH to 6 and storage at −20° for several days.

The frs containing NP24 II (11–18) were pooled and the soln was concd to 10 ml by ultrafiltration using a PM-10 membrane (Amicon). The soln was dialysed against 0.2 M NaCl and applied to a 5 × 70 cm column of DEAE-Sephadex A-50 equilibrated with 0.2 M NaCl containing 0.02 M NaOAc, pH 6. Elution of protein was conducted with 0.2 M NaCl containing 0.02 M NaOAc, pH 6. The frs (25 ml) were analysed for NP24 II and those containing the protein were pooled and ultrafiltered to 5 ml. Aliquotes (1 ml) were then subjected to FPLC on Mono S. Elution was conducted with 30 ml of a linear gradient of 0–0.25 M NaCl in 0.02 M MES, pH 6. The frs (1 ml) containing NP24 II were combined from the 5 chromatographic runs and concd to 2 ml by ultrafiltration.

Other methods. Protein concns were measured by *A* at 280 nm and according to ref. [17]. SDS–PAGE was conducted on Phast Gel gradient 10–15 gel in a Phast System (Pharmacia) and stained with Coomassie blue. *N*-Terminal protein sequences were determined on a model 470 A protein sequencer (Applied Biosystems) by Dr John Wunderlich, University of Georgia.

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