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CHANGES IN THE SWEET PROTEINS (THAUMATINS) IN *THAUMATOCOCCUS DANIELLI* FRUITS DURING DEVELOPMENT

ALAN MACKENZIE, JOHN B. PRIDHAM and NICHOLAS A. SAUNDERS*

Department of Biochemistry, Royal Holloway College, University of London, Egham Hill, Egham, Surrey, TW20 0EX, U.K.

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Key Word Index—*Thaumatococcus danielli*; Marantaceae; ripening; proteins; thaumatins; sweet protein.

Abstract—The thaumatin content (forms TO, TI and TII) of fruits from *Thaumatococcus danielli* at various stages of maturation were examined. The amounts of all three forms of sweet protein increased during maturation to reach a total of about 50 mg/g in mature aril tissue in fruits from both the Ashanti and Kadjebe regions of Ghana. TO was a minor form in fruits at all stages of development from both regions. The major regional difference was that TII was absent from the Kadjebe fruits; however, the total level of sweet proteins was maintained by increased levels of TO and TI. Structural and immunological comparisons of the three thaumatin forms showed that TO is closely related to the other two forms which are known to differ at only five positions in their primary structure.

INTRODUCTION

In recent years the search for a non-toxic, low calorie sweetener has produced a number of materials of commercial interest. Amongst these are the sweet proteins monellin, miraculin and thaumatin which are all products of tropical plants.

Thaumatin was first isolated from the fruits of *Thaumatococcus danielli* Benth by Van der Wel and Loeve in 1972 [1] and was shown to consist of three forms TO, TI and TII. All three thaumatin proteins are basic and have M_r values of *ca* 22000. The primary amino acid sequence of TI has been determined using classical sequencing methods [2] and cDNA cloning studies [3] suggest that TI and TII are closely homologous but there are sequence differences involving five amino acids.

In the present study the thaumatin content of fruits at different developmental stages and from different regions of Ghana were compared, and the structural and immunological relationships between the well characterized thaumatin forms (TI and TII) and the third major sweet tasting protein, TO, were examined.

RESULTS AND DISCUSSION

Thaumatin levels in maturing fruit

Initial experiments indicated that repeated extractions of aril tissue with an alkaline buffer (0.01 M NaPi, pH 7.7) at high salt concentration (0.3 M NaCl) were necessary to extract *ca* 90% of the total protein. Aril extracts prepared from fruits at various developmental stages (see Table 1) were analysed by CM-cellulose chromatography. Figure 1 shows a typical separation of TO, TI and TII. Each of the three sweet-tasting protein peaks gave a single protein band when examined by PAGE in the presence or absence of SDS and by isoelectric focusing (Fig. 2). Table 2 shows the TO, TI and TII content of *Thaumatococcus danielli* fruits from the Ashanti region of Ghana at different stages of development. It is clear that there is a significant increase in total sweet protein levels over the whole ripening period. The thaumatin content also increases as a percentage of the total protein present, showing that the net synthesis observed is not merely a function of the general rise in protein content. There was no correlation between the ratios of TI and TII and fruit development and the proportion of TO was always small. The differences in the TI/TII ratios for the different batches of fruits are probably due to environmental factors.

Table 1. Letter code for stages of ripeness of *Thaumatococcus danielli* fruits

Ripeness stage	Estimated time after fruit set (days)	Pericarp colour	Seed colour
A	12	Green or green/orange	White
B	23	Orange or orange/red	Purple
C	70	Red	Aubergine
D	110	Dark red	Black

When fruits from the Kadjebe region were examined it was found that the total sweet protein content increased during maturation to a level similar to that found in Ashanti fruits, and again TO was a minor component. However, TII was absent at all stages of development (Table 3) and its absence was apparently compensated by increased synthesis of TI. This raises the possibility that there are taxonomic differences between plants from the two Ghanaian regions. Morphological differences have also been observed [4] which could be due to cultivation

*Present address: Department of Biochemistry, University of Manchester Medical School, Oxford Road, Manchester, M13 9PT, U.K.

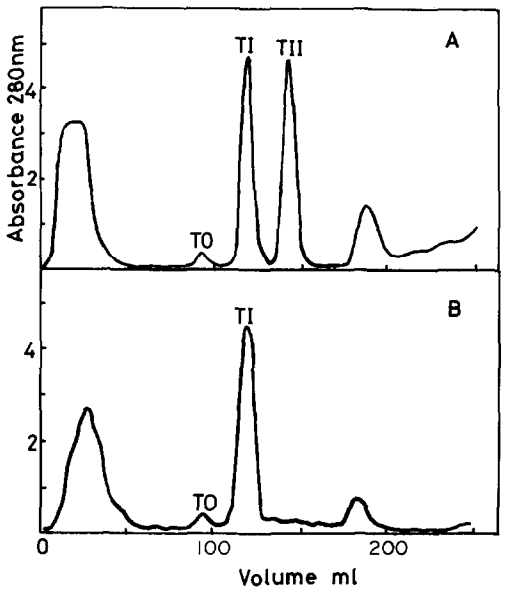

Fig. 1. Chromatography of aril extracts from *Thaumatococcus danielli* fruits on a CM 52 cellulose column. Elution was effected with 0.01 M NaPi buffer (pH 7.2, 56 ml) followed by a linear gradient of NaCl (0–0.2 M) in NaPi buffer (200 ml). Peaks TO, TI and TII were sweet tasting. A, Ashanti region fruits; B, Kadjebe region fruits.

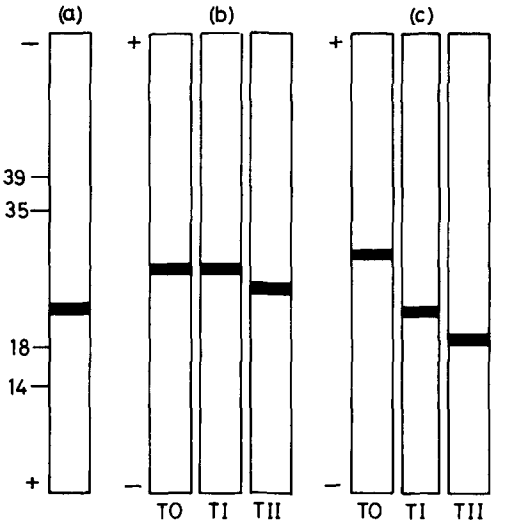

Fig. 2. Analysis of thaumatins, TO, TI and TII from Ashanti fruits (obtained from a CM 52 cellulose column; Fig. 1) by polyacrylamide gel electrophoretic techniques (a) with SDS; (b) without SDS and (c) isoelectric focusing. The positions and M_r ($\times 10^{-3}$) of protein markers are shown in gel (a). Protein bands were detected with Coomassie brilliant blue.

in different geographical environments. However, the observed different sweet protein profiles may indicate that the plants are different genotypes, the TII gene having been deleted from the Kadjebe plants.

Structural comparison of TO, TI and TII

Amino acid and *N*-terminal analyses of TI and TII confirmed the findings of Van der Wel and Loeve [1]. These studies were extended to TO which had the same *N*-terminal group (alanine) as TI and TII and a very similar amino acid composition (Table 4), suggesting a close structural relationship of all three forms. Limited proteolysis of the three sweet protein forms with *Staphylococcus aureus* V8 protease followed by SDS-PAGE analysis also yielded identical peptide patterns (unpublished) in each case.

SDS-PAGE showed that all three thaumatin forms had identical mobilities (Fig. 2a) and were of similar size (M_r 20 200 ± 250). However, in the absence of SDS (Fig. 2b)

Table 2. Total protein and thaumatin contents of *Thaumatococcus danielli* (Ashanti region) fruit arils at different stages of development (see Table 1)

	mg/g aril tissue						
Ripeness stage	Total protein	TO	TI	TII	Total sweet protein	% sweet protein	TI/TII
Sample 1							
A (16)	40.9	0.1	3.6	2.3	6.0	14.6	1.5
B (14)	66.0	0.4	10.6	5.3	16.3	24.8	2.0
C + D (17)	101.0	0.6	32.0	21.0	53.6	53.1	1.5
Sample 2							
A (20)	30.7	0.1	1.0	1.6	2.7	8.7	0.6
B (47)	46.9	nd	2.5	4.1	6.6	14.2	0.6
C + D (15)	79.2	1.3	16.6	24.1	42.0	53.4	0.7
Sample 3							
A (16)	12.0	0.1	0.4	0.3	0.8	6.3	1.3
C (14)	72.6	0.8	14.2	13.2	28.2	39.1	1.1
D (17)	66.7	0.5	9.0	22.9	32.4	48.7	0.4

Figures in parentheses represent numbers of arils extracted for each developmental stage.

Table 3. Total protein and thaumatin contents of *Thaumatococcus danielli* (Kadjebe region) fruit arils at different stages of development (see Table 1)

	mg/g aril tissue					
Ripeness stage	Total protein	TO	TI	TII	Total sweet protein	% sweet protein
A (17)	34.3	0.4	10.4	0	10.8	31.3
B (18)	47.1	0.3	19.2	0	19.5	41.5
C+D (18)	87.2	1.9	53.8	0	55.7	63.8

Figures in parentheses represent numbers of arils extracted for each developmental stage.

Table 4. Amino acid analyses of thaumatins TO, TI and TII

Amino acids (mol/mol protein)	TO	TI	TII
Lysine	11.0	11.0	11.0
Histidine	0.0	0.0	0.0
Arginine	11.9	11.7	11.2
Aspartic acid	23.5	22.9	21.0
Threonine	17.5	19.0	19.4
Serine	13.4	15.6	15.3
Glutamic acid	11.1	10.0	10.0
Proline	12.2	12.2	12.1
Glycine	24.0	22.9	21.5
Alanine	16.9	16.1	15.3
Cysteine	14.9	14.7	14.1
Valine	9.4	9.5	9.7
Methionine	2.0	2.0	2.0
Isoleucine	7.6	7.6	7.2
Leucine	10.0	9.3	8.7
Tyrosine	8.3	8.6	7.2
Phenylalanine	10.8	10.6	10.1
Tryptophan	2.3	2.4	2.5

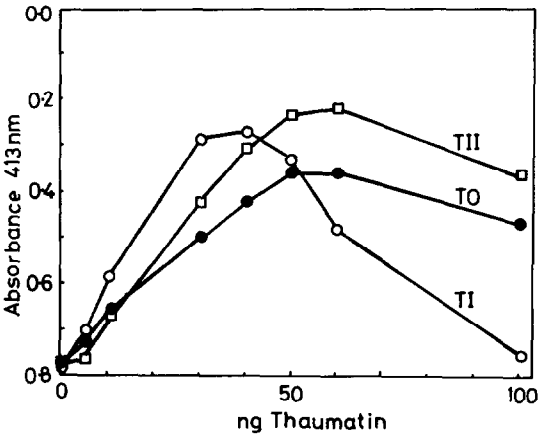

Fig. 3. Micro-complement fixation analysis of the thaumatin forms. Each assay mixture contained a fixed quantity of anti-TII antiserum (100 μl:400 ng/ml), diluent (300 μl), complement (100 μl:34.5C'H_{50}/ml), thaumatin (100 μl:1–200 ng), sensitized sheep erythrocytes (100 μl:5×10^7 cells/ml). After incubation cell lysis was estimated by measurement of $A_{413\,nm}$ of the cell free supernatant.

small differences in mobility were observed. The three thaumatin forms could also be separated by non-equilibrium PAGE isoelectric focusing (Fig. 2c) which showed that the order of basicity was TO < TI < TII and furthermore suggested greater charge differences between TO and TI than TI and TII.

The quantitative precipitin analysis gave a value of 100% for the cross-reactivity between the antiserum to TII and the other thaumatin forms. However, the more sensitive micro-complement fixation procedure showed a small difference between the three thaumatins (Fig. 3) with an index of dissimilarity of *ca* 1.25 between TO and TI and *ca* 1.09 between TI and TII. The index of dissimilarity is the factor by which the antiserum concentration must be raised in order for a heterologous antigen to produce a complement fixation curve with a peak height equal to that of the homologous antigen. From these values cross-reactivities of 96% and 98% respectively, were derived. By analogy with the lysozyme system studied by Prager and Wilson [5] this should result from *ca* six amino acid differences between TI and TII. This result is in good agreement with the sequence data reported by Iyengar *et al.* [2] and Edens *et al.* [3] which suggest five amino acid differences between the two forms. On the basis of the cross-reactivity values it can, therefore, be postulated that TO and TII differ by *ca* eight amino acids.

In conclusion, TI and TII are different gene products and TO, a structurally similar protein, is possibly the product of a third gene. The gene for TII in Kadjebe fruits appears to be either absent or non-functional. The thaumatins, therefore, represent a group of fruit proteins that are regulated during development.

EXPERIMENTAL

Materials. Fruits of *Thaumatococcus danielli* Benth at different developmental stages (Table 1) from the Ashanti and Kadjebe regions of Ghana were provided by Tate and Lyle Ltd. After collection fruits were transported to the UK by air and stored at −70° on arrival: 2–3 days elapsed between collection and storage. Analar grade reagents were obtained from BDH. *Staphylo-*

coccus aureus V8 protease was purchased from Miles. The following immunological reagents were supplied: Freund's adjuvant (Calbiochem); Sheep erythrocytes and guinea pig complement (Flow Laboratories, Irvine, U.K.); rabbit haemolytic serum (Wellcome Foundation Ltd., Dartford, U.K.).

Tissue preparation and protein extraction. Arils were separated from the fruits, diced at 4° and placed in 0.01 M NaPi (pH 6; 20 ml/g fr. wt tissue). The tissue fragments were homogenized by crushing in a pestle and mortar and sonicated for 30 sec at maximum amplitude, with a MSE sonicator. After centrifugation at 100 000 *g* for 90 min the pellets were re-extracted once with the original buffer and then twice with 0.01 M NaPi buffer, pH 7.7, containing 0.3 M NaCl. The protein contents of the combined supernatants were measured by the method of ref. [6].

Separation of thaumatins. The above supernatants were dialysed against H_2O and applied to a column (30 cm × 1 cm) of CM 52 cellulose (Na^+ form) which had been equilibrated with 0.01 M NaPi buffer, pH 7.2. The column was eluted with equilibration buffer (50 ml) followed by a linear gradient of 0–0.2 M NaCl in the same buffer (200 ml). The protein contents of the column fractions were measured at 280 nm and the sweet protein fractions identified by tasting.

Electrophoresis. PAGE was carried out according to ref. [7], SDS-PAGE by the method of ref. [8] and isoelectric focusing as described in ref. [9].

Amino acid analysis. The procedure of ref. [10], using a Jeol analyser (JLC-6AH), was followed. Tryptophan was estimated by the method of ref. [11].

N-terminal analysis. Samples were analysed by the method of ref. [12].

Peptide mapping. The method of ref. [13] for partial hydrolysis (*S. aureus* V8 protease) and peptide analysis was used.

Immunological methods. Rabbits were injected subcutaneously with 50 μg/kg of purified TI in complete Freund's adjuvant. Booster injections (50 μg/kg) in incomplete adjuvant were given weekly until a satisfactory titre was obtained. The resulting antiserum containing 1% NaN_3 was stored at 4°. Quantitative precipitin analysis was carried out according to ref. [4]. Purified TO, TI and TII, dissolved in 0.9% NaCl (200 μl) were mixed with anti-TII antiserum (titre 35 μg/ml; 200 μl). After incubation at 20° for 16 hr the ppts were pelleted by centrifugation, washed twice with cold 0.9% NaCl and the pellets finally dissolved in 0.5 M NaOH (400 μl). $A_{280\,nm}$ of these solns were measured. Quantitative micro-complement fixation analysis was performed according to ref. [14] using the diluent of ref. [15]. Reaction mixtures (final vol. 700 μl) contained diluent (0.14 M NaCl, 0.01 M Tris–HCl, pH 7.45, 0.5 mM $MgSO_4$, 0.15 mM $CaCl_2$; 300 μl), antiserum (titre 400 ng/ml; 100 μl), guinea pig complement (34.5 $C'H_{50}$/ml; 100 μl) and antigen soln (1–200 ng thaumatin; 100 μl). This mixture was preincubated for 18 hr at 4° and sensitized sheep erythrocytes (5×10^7 cells/ml; 100 μl) then added. The complete mixture was incubated for 1 hr at 37°. Reactions were stopped by cooling (0°) and the intact erythrocytes and cellular debris removed by centrifugation. *A* of the supernatants was measured at 413 nm.

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