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# Possible Active Site of the Sweet-tasting Protein Thaumatin

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## Abstract

Epitopes on thaumatin and monellin were studied using the PEPSCAN-technology. The antibodies used were raised against thaumatin. Only antibodies that, in an ELISA, both recognized thaumatin and monellin were used in the PEPSCAN-analyses. On thaumatin two major overlapping epitopes were identified. On monellin no epitopes could be identified. The identified epitope region on thaumatin shares structural features with various peptide and protein sweeteners. It contains an aspartame-like site which is formed by Asp21 and Phe80, tips of the two extruding loops KGDAALDAGGR<sub>19-29</sub> and CKRFRGPP<sub>77-84</sub>, which are spatially positioned next to each other. Furthermore, sub-sequences of the KGDAALDAGGR<sub>19-29</sub> loop are similar to peptide-sweeteners such as L-Asp-D-Ala-L-Ala-methyl ester and L-Asp-D-Ala-Gly-methyl ester. Since the aspartame-like Asp21-Phe80 site and the peptide-sweetener-like sequences are also not present in non-sweet thaumatin-like proteins it is postulated that the KGDAALDAGGR<sub>19-29</sub> and CKRFRGPP<sub>77-84</sub> loop contain important sweet-taste determinants. This region has previously not been implicated as a sweet-taste determinant of thaumatin. *Chem. Senses* 20: 535-543, 1995.

## Introduction

The proteins thaumatin and monellin, isolated from the African fruits *Thaumatococcus danielli* Benth and *Dioscorea oppositifolia* Diels, respectively, are potently sweet-tasting proteins (Morris and Cahan, 1972; Van der Wel, 1972; Van der Wel and Loeve, 1972). The sweet-taste of thaumatin and monellin is already registered at a concentration of  $10^{-8}$  M which is, on a molar basis, approximately 100 000 times more potent than sucrose (Morris and Cahan, 1972; Van der Wel and Loeve, 1972; Brouwer *et al.*, 1973). Only humans and old world monkeys perceive these proteins as sweet.

Thaumatin is a single chain protein of 207 residues which contains eight disulphide bridges (Iyengar *et al.*, 1979).

Monellin consists of an A-chain of 44 residues and a B-chain of 50 residues which are non-covalently associated (Bohak and Li, 1976; Frank and Zuber, 1976; Hudson and Biemann, 1976; Kohmura *et al.*, 1990). Recently, high-resolution three-dimensional structures of monellin and thaumatin have been determined (De Vos *et al.*, 1985; Ogata *et al.*, 1987, 1992; Somoza *et al.*, 1993). The structural basis for the sweetness of thaumatin and monellin is still unknown (cf. Kurihara, 1992; Somoza *et al.*, 1993).

Thaumatin, monellin and the low molecular weight sweetener aspartame (L-Asp-L-Phe-methyl ester) have similar antigenic determinants. A number of antibodies raised against thaumatin have been shown to cross-react with monellin

(Hough and Edwardson, 1978; Van der Wel and Bel, 1978; Kim *et al.*, 1991; Mandal *et al.*, 1991). Furthermore, a number of antibodies raised against monellin have been shown to cross-react with thaumatin (Kim *et al.*, 1991; Mandal *et al.*, 1991). Some of the anti-thaumatin and anti-monellin antibodies also compete for aspartame (Hough and Edwardson, 1978; Kim *et al.*, 1991). It has been hypothesized that the epitope shared by thaumatin, monellin and aspartame is involved in receptor binding. However, although electrophysiological and competition studies suggest that thaumatin and monellin may bind to the same receptor (Brouwer *et al.*, 1973; Van der Wel and Arvidson, 1978), it is not yet possible to determine which receptor(s) are recognized by these sweeteners since the structure of the thaumatin, monellin and aspartame receptor is still unknown.

Different strategies have been used to study the structural basis for the sweetness of thaumatin and monellin. Initially, the structures of thaumatin and monellin have been compared. However, although the three-dimensional structure of both proteins has been resolved no obvious similarities between the two proteins have been found (cf. Kim *et al.*, 1988, 1991; Ogata *et al.*, 1992; Somoza *et al.*, 1993).

Thaumat-I has a very high isoelectric point ( $pI = 12$ ). Using chemical modification studies the role of the 10 lysines and 11 arginines in the sweetness of thaumatin has been studied (Van der Wel and Bel, 1976). Based on these studies and additional modification studies of the tyrosines it was postulated that only particular residues, notably Tyr95, Lys97 and Lys106, are important for sweetness (Van der Wel and Bel, 1976; Van der Wel, 1980).

Immunological cross-reactivity studies suggest that Tyr57-Phe58-Asp59 of thaumatin and Tyr63-Ala64-Ser65-Asp66 of the A-chain of monellin are important for the sweet-taste of thaumatin (Kim *et al.*, 1991). Furthermore, a detailed study of the three-dimensional structure of the above mentioned monellin loop indicates that the Tyr63-Asp66 pair, whose side-chains are solvent exposed (Somoza *et al.*, 1993), is structurally similar to aspartame (Tancredi *et al.*, 1992). However, site-directed mutagenesis studies suggest that Asp66 of the A-chain is not essential for sweet-taste, but indicate that Asp7 of the B-chain is the only aspartate-residue that is essential for sweet taste (Kohmura and Ariyoshi, 1990; Kohmura *et al.*, 1991, 1992a,b). Thus, there exists no consensus on the sweet-taste receptor binding sites of thaumatin nor monellin (cf. Somoza *et al.*, 1993). The results presented in the present paper suggest a new location for the sweet-taste determinant of thaumatin.

## Experimental

### Materials

Thaumat-I was purified by the method of Van der Wel and Loeve (1972). Monellin used was obtained from Unilever Vlaardingen (cf. Van der Wel and Bel, 1978). Bovine serum albumin (BSA) was purchased from Sigma. Standard RPMI medium: RPMI 1640 (Flow) was made up with 10% fetal calf serum, 1% L-glutamine, 1% Na pyruvate and 1% penicillin-streptomycin (5000 units/ml); selective HAT medium: standard RPMI medium supplemented with 2% hypoxanthine-desoxy thymidine, 1% aminopterin, 5% horse serum, 10% human epithelial cell supernatant; fusion reagent: 40 g polyethyleneglycol MW 4000 (Merck), 5 ml dimethylsulphide (BHD) in 60 ml  $H_2O$ . The solution was autoclaved for 20 min at  $121^\circ C$  and stored at  $4^\circ C$ .

### Production of polyclonal antiserum

Rabbits and sheep were subcutaneously immunized with 100  $\mu g$  thaumat-I emulsified in complete Freund's adjuvant. With 2–3-week intervals the animals were boosted with 100  $\mu g$  thaumat-I emulsified in incomplete Freund's adjuvant.

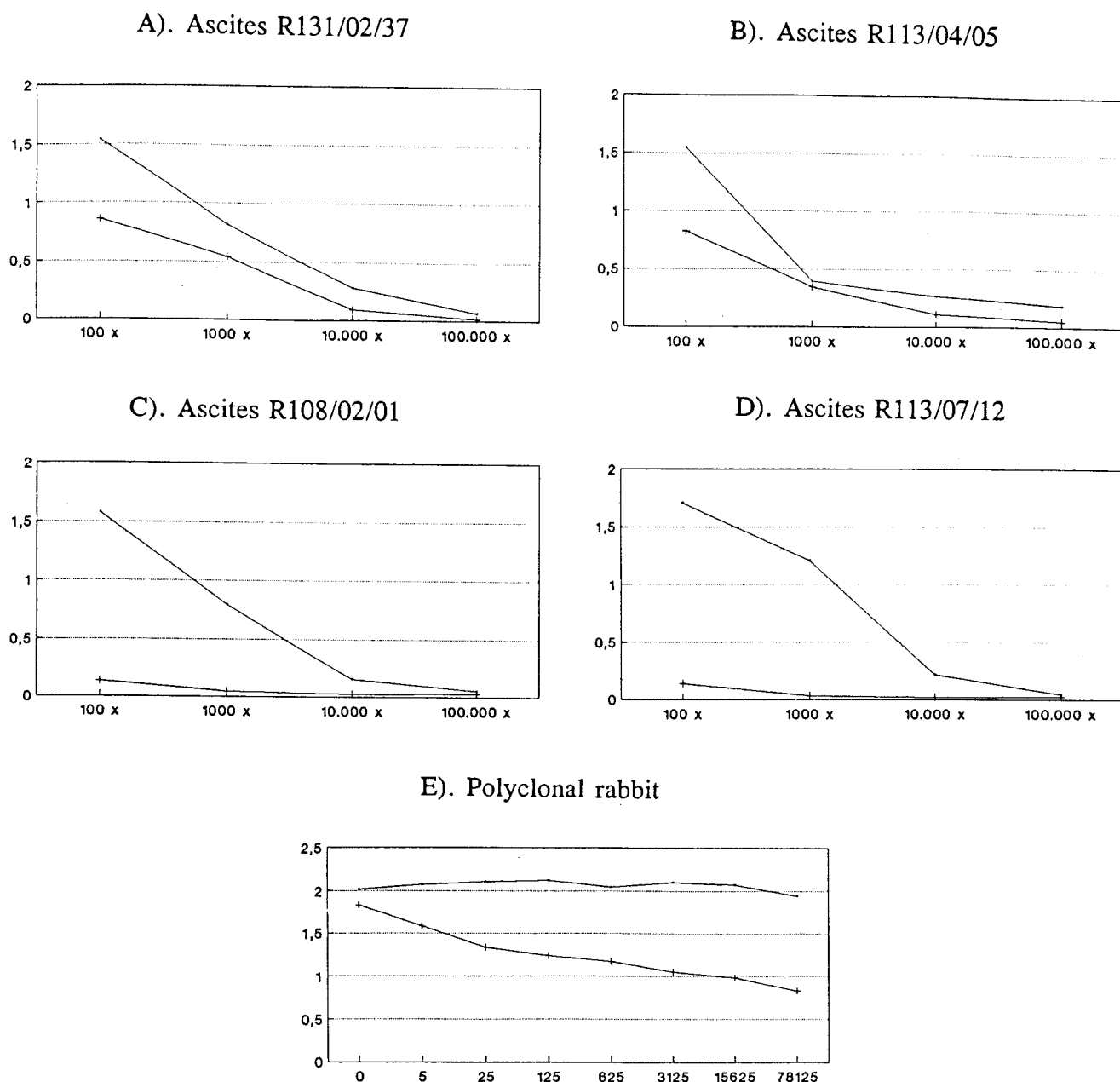
### Production of monoclonal antibodies

#### *Immunization in vivo*

Young adult female BALB/c mice, each 2–4 months old, were immunized by multiple injections with thaumat-I. The primary immunization was initiated by intraperitoneal injection of 100  $\mu g$  thaumat-I, precipitated with aluminum potassium sulphate. A booster with 50  $\mu g$  thaumat-I intraperitoneal was administered 2 weeks later. The last booster, 100  $\mu g$  thaumat-I in PBS, was given intravenously 3 weeks later, 3 days before the spleen cells were fused with SP 2/0 plasmacytoma cells according to Köhler and Milstein (1975).

#### *Immunization in vitro*

An *in vitro* immunization was carried out with spleen cells from an unimmunized Balb/c mouse. The spleen cells were aseptically isolated and suspended in a medium containing: 30 ml MEMS medium (Gibco) enriched with  $10^{-4}$  M 2-mercaptoethanol and 20% fetal calf serum, 10 ml RPMI 1640 medium pre-incubated with mouse thymus cells for 48 h and 10  $\mu l$  thaumat-I (1 mg/ml). The final density of spleen cells in this immunizing medium was  $60 \times 10^5/ml$ .



**Figure 1** ELISA on thaumatin and monellin: **(A)** ascites R131/02/37; **(B)** ascites R113/04/05; **(C)** ascites R108/02/01; **(D)** ascites R113/07/12; **(E)** polyclonal rabbit antiserum. The dilution of the antibodies is shown on the x-axis. The O.D. (405 nm) is shown on the y-axis; dotted marker is ELISA on thaumatin; crossed marker is ELISA on monellin.

and culturing was continued for 4 days. Next the cell suspension was centrifuged for 10 min at 250 g, washed with serum-free medium and next fused with SP 2/0 plasmacytoma cells according to Köhler and Milstein (1975).

### Screening of anti-thaumatococcus antibody producing hybridomas

The hybridomas were tested with  $^{125}\text{I}$ -labelled rabbit-anti-mouse or goat-anti-mouse immunoglobulins (Nordic). For

iodination of antibodies to  $^{125}\text{I}$  the iodogen method was used (Fraker and Speck, 1978). The Radio-Immuno-Assay (RIA) was carried out in 96-well polyvinyl plates (Falcon). Thaumatin was absorbed to the plastic by filling the wells with 25  $\mu\text{l}$  thaumatin 100  $\mu\text{g}/\text{ml}$  PBS for 60 min. The wells were filled completely with 1% BSA in PBS for 15 min to prevent non-specific adsorption. After washing with PBS, 20  $\mu\text{l}$  hybridoma supernatant (or diluted ascites fluid) were incubated for 60 min. After washing with PBS, 20  $\mu\text{l}$   $^{125}\text{I}$ -labelled

anti-mouse Ig (approximately 20 000 c.p.m.) was added and incubated for 60 min. Bound radioactivity in the wells was counted in a gamma-counter (Packard auto-gamma 5266).

### Purification of monoclonal antibodies

Monoclonal specific antibody secreting cell-lines were obtained through sub-cloning, ascites production and protein-A affinity purification. In short, positive hybridomas were cultured in 15-ml bottles. A culture was diluted to 50 viable cells/10 ml standard medium and 0.1 ml was transferred to each well of a 96-well plate. Positive hybridomas were stored in liquid nitrogen. For ascites production  $10^6$  monoclonal specific antibody secreting hybridoma cells in serum-free medium were injected intraperitoneally in about 9-week-old Balb/c mice. The ascites fluid was 'tapped' and the monoclonal antibodies were affinity purified on a protein A column (Pharmacia).

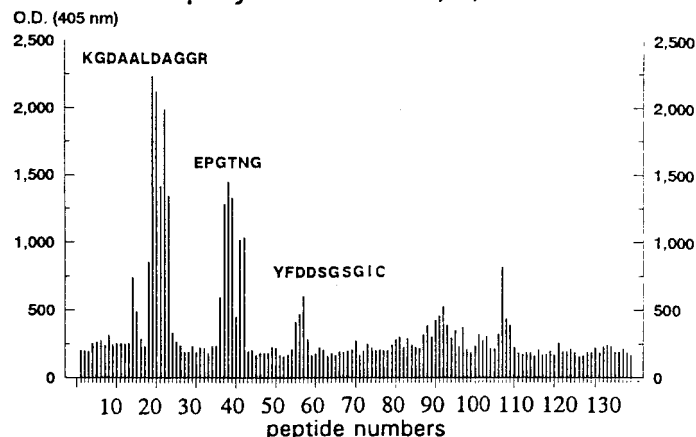
### ELISA of anti-thaumatins antibodies on thaumatin and monellin

Thaumatins and monellins were dissolved in coating-buffer (10 µg protein/ml 0.25 M phosphate-buffer, pH 7.5). The 96 wells were coated with 100 µl thaumatins or monellins solution (overnight, 4°C). The antibodies (serum or ascites) were diluted in phosphate buffer (0.04 M phosphate, 0.15 M NaCl, 0.1% BSA, pH 7.4). After rinsing (rinsing-buffer: 0.2 M Tris/citric acid, 0.1 M NaCl, 0.05% Tween-20, pH 7.4), antibody (100 µl/well) was incubated with the coated protein (1 h, 20°C). After rinsing peroxidase-conjugate, goat-anti-mouse (GAMPO, 1/10 000), goat-anti-rabbit (GARPO, 1/2000) or rabbit-anti-sheep (RASHPO, 1/10 000), dissolved in 0.2 M Tris/HCl, 0.2 M NaCl, 1% PEG-6000, 5% BSA, pH 7.4, was added (100 µl/well, 1 h, 20°C). After rinsing enzyme-substrate (10 ml 0.1 M Na-acetate/citric acid, pH 5.5 to which 100 µl 0.025 M TMB.2 HCl and 100 µl 0.3% H<sub>2</sub>O<sub>2</sub>) were added (100 µl, 30 min). The reaction was stopped with 50 µl 2 N H<sub>2</sub>SO<sub>4</sub> and measured in an ELISA-reader (450 nm).

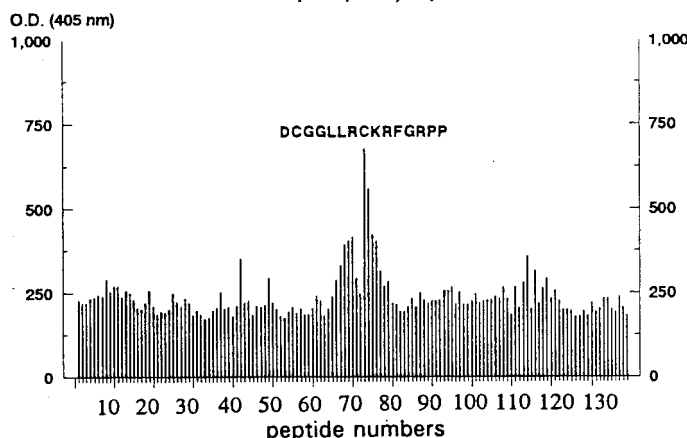
### Epitope mapping by PEPSCAN-method

Using the PEPSCAN technique hundreds of peptides can be synthesized simultaneously. These peptides are covalently linked to solid supports which are placed into holders that fit into 96-well plates. In an ELISA-assay these peptides are incubated with antibodies of interest. In this way linear epitopes of proteins can be systematically analysed (Geysen

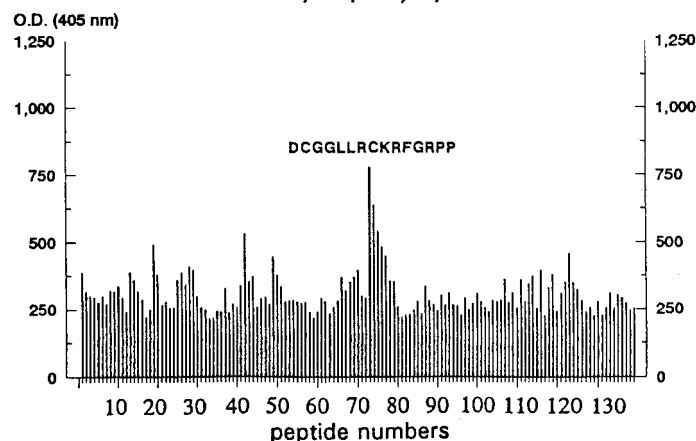
### A) PEPSCAN Thaumatin polyclonal rabbit, 1/25



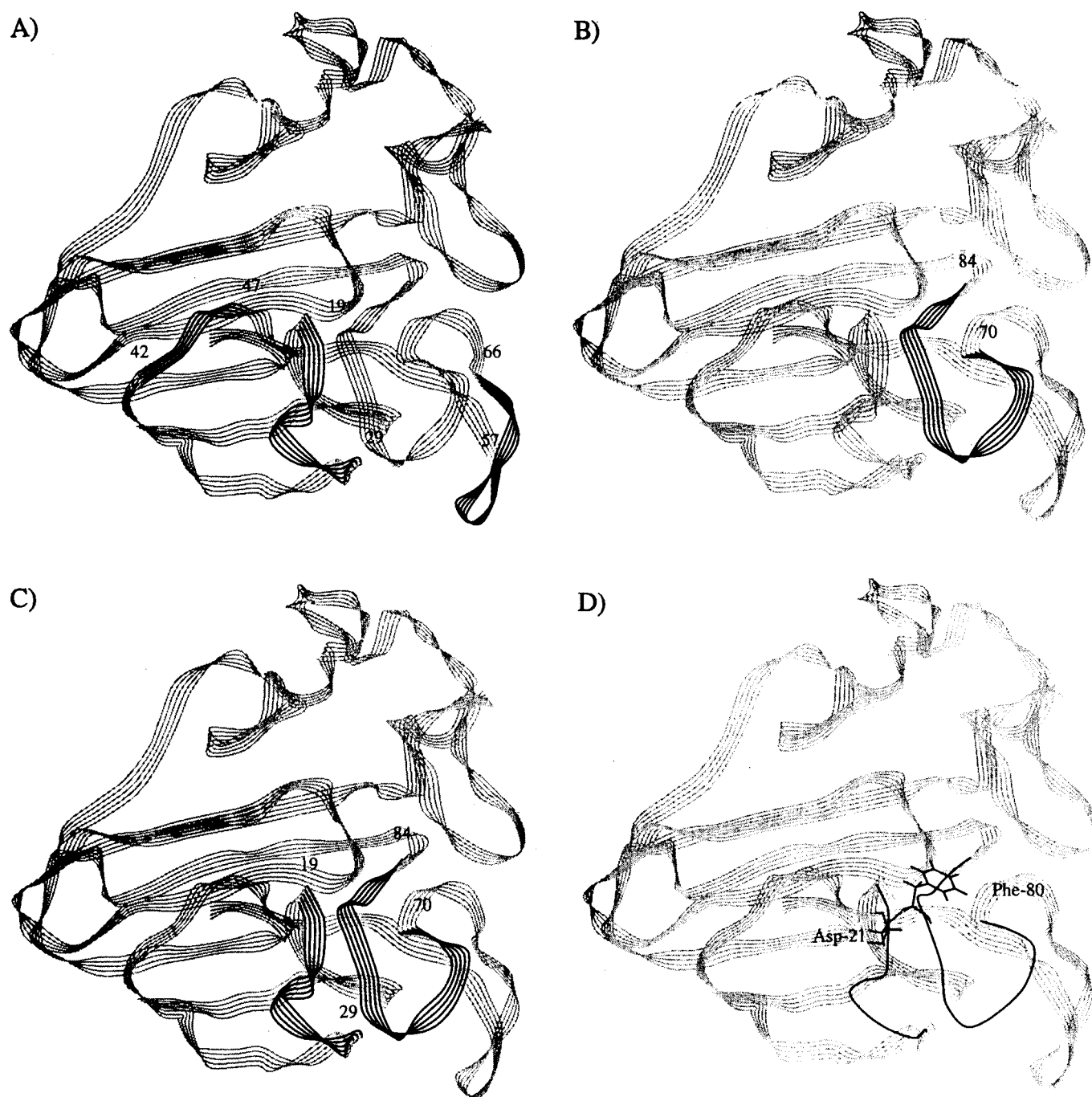
### B) PEPSCAN Thaumatin R131/02/37, 1/35



### C) PEPSCAN Thaumatin R113/04/05, 1/25



**Figure 2** PEPSCANS on all overlapping 12-mers of thaumatins: (A) polyclonal rabbit antiserum; (B) ascites R131/02/37; (C) ascites R113/04/05. The secondary antibody was RAMPO (1/1000) or SWARPO (1/1000).

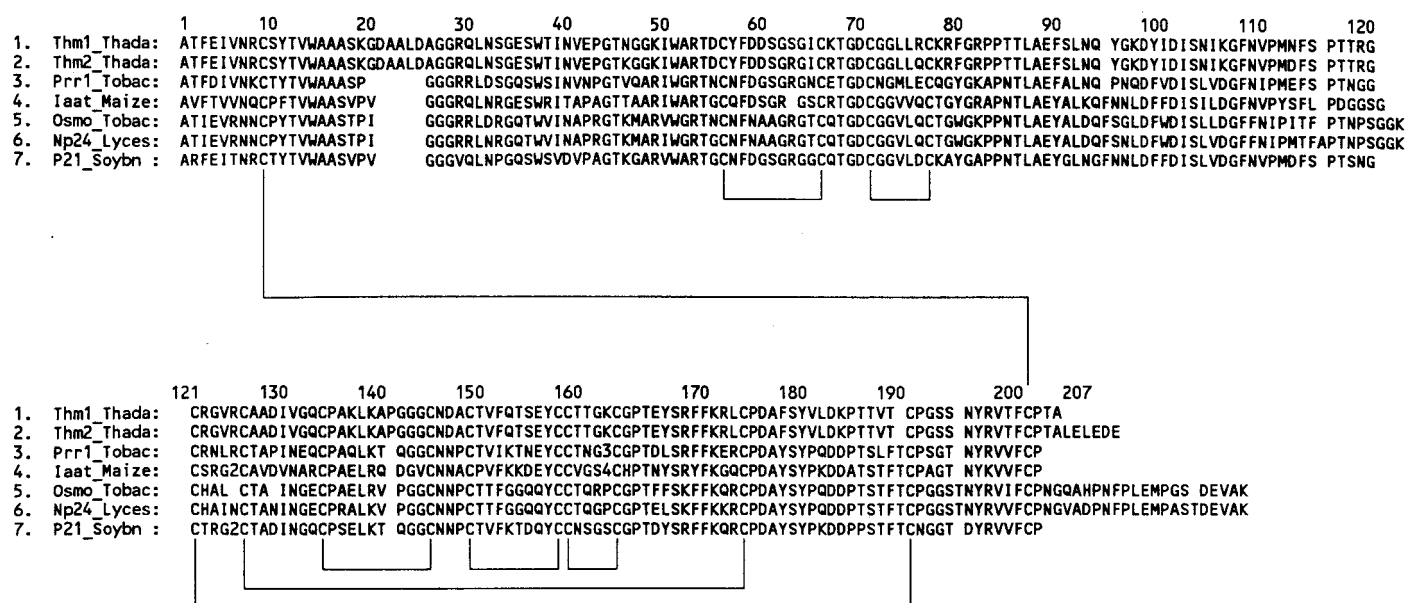


**Figure 3** An C-alpha backbone ribbon of thaumatin-I derived from the X-ray diffraction pattern of this protein as determined by De Vos *et al.* (1985). Thick lines correspond to **(A)** KGDAALDAGGR<sub>19-29</sub>, YEPGTNG<sub>42-47</sub> and YFDDSGSGIC<sub>57-66</sub>; **(B)** DCGGLLRCK<sub>70-78</sub> and CKRFRPP<sub>77-84</sub>; **(C)** representation of the parallel location of the KGDAALDAGGR<sub>19-29</sub> and DCGGLLRCKRFRPP<sub>70-84</sub> loop; **(D)** Model of the side chains of Asp21 and Phe80. Thin lines correspond to KGDAALDAGGR<sub>19-29</sub> and DCGGLLRCKRFRPP<sub>70-84</sub>.

*et al.*, 1984). All overlapping 12-mers (1-12, 2-13, etc.) of thaumatin and monellin were synthesized on polyethylene rods as described previously (Geysen *et al.*, 1984). In an ELISA-assay the peptides were incubated with the various anti-thaumatin antibodies.

### Location of antibody binding peptides on the three-dimensional structure of thaumatin

The PEPSCAN-peptides that bound to the various antibodies are shown in the three-dimensional structure of thaumatin



**Figure 4** Alignment of the amino acid sequence of thaumatin and thaumatin-like proteins: thaumatin-I (Thm1\_Thada), thaumatin-II (Thm2\_Thada), pathogenesis-related protein R major form (Prr1\_Tobac), antifungal alpha-amylase/trypsin inhibitor (Iaat\_Maize), salt-stress-induced osmotin (Osmo\_Tobac), salt-stress induced Np24 pathogenesis-related protein (Np24\_Lyces), and P21 pathogenesis-related protein (P21\_Soybn). The lines represent the eight disulphide bridges in thaumatin-I. Insertions are indicated by numbers. Empty spaces are gaps.

using Sybyl<sup>®</sup> (version 6.0, Molecular Modeling Software, Tripos Associates, a subsidiary of Evans and Sutherlands, Inc., St Louis, USA). The PDB-file 1thi.pdb was obtained from the Protein Data Bank of the Brookhaven National Laboratory (Bernstein *et al.*, 1977). Sybyl<sup>®</sup> runs on a Silicon Graphics Computer System (IRIS Indigo Elan<sup>™</sup>, 3D graphics system).

## Results

From four *in vivo* fusion experiments and one *in vitro* fusion experiment four positive hybridoma cell-lines were subcloned, injected in mouse for ascites production and affinity purified: 131/02/37 (*in vitro* fusion) and hybridomas 113/04/05, 108/02/01 and 113/07/12 (*in vivo* fusion). Together with two polyclonal antibodies these monoclonal antibodies were tested in an anti-thaumatins and anti-monellin ELISA. Two of the four monoclonal antibodies and one of the two polyclonal antisera recognized both thaumatin and monellin: the monoclonal antibodies R113/04/05 and R131/02/37 and the polyclonal rabbit antiserum (Figure 1). The affinity of these antibodies for thaumatin and monellin was not determined.

The polyclonal rabbit antiserum and the monoclonal antibodies R131/02/37 and R113/04/05 were positive in the PEPSCAN-analysis, i.e. on thaumatin various epitopes were

identified (Figure 2), whereas on monellin no clear epitopes could be identified (not shown).

### PEPSCAN of thaumatin with polyclonal rabbit anti-thaumatins serum

The rabbit polyclonal antiserum especially binds to PEPSCAN-peptides 14–23 and PEPSCAN-peptides 36–42 and to a lesser extent to PEPSCAN-peptides 55–57, 90–92 and 106–109 (Figure 2A). In the three-dimensional structure the PEPSCAN-peptides 14–23, 36–42 and 55–57 are part of one region. The core sequences of these peaks are KGD-AALDAGGR<sub>19–29</sub>, EPGTNG<sub>42–47</sub> and YFDDSGSGIC<sub>57–66</sub> (Figure 3A).

### PEPSCAN of thaumatin with monoclonal antibodies R113/04/05 and R131/02/37

The monoclonal antibodies R113/04/05 and R131/02/37 especially bind to PEPSCAN-peptides 67–70 and 73–77 and to a lesser extent to PEPSCAN-peptides 19 and 42 (Figures 2B and 2C). The core sequence of the 67–70 and 73–77 peaks are DCGLLRCK<sub>70–78</sub> and CKRFRGPP<sub>77–84</sub>, respectively. In the three-dimensional structure these sequences are located in the same region as KGDALDAGGR<sub>19–29</sub>, EPGTNG<sub>42–47</sub> and YFDDSGSGIC<sub>57–66</sub>, which are recognized by the polyclonal rabbit antiserum (cf. Figures 3A and 3B).

A. putative sweet-taste receptor binding site		.KGDAALDAGGR.	
		↓ ↓ ↓ ↓ ↓ ↓ ↓	
		↓ ↓ ↓ ↓ ↓ ↓ ↓	
		↓ ↓ ↓ ↓ ↓ ↓ ↓	
		↓ ↓ ↓ ↓ ↓ ↓ ↓	
B. peptide sweeteners which carry similarity with the KGDAALDAGGR loop		DA	-OMe 25 x 0.6% sucrose
		DA	-OMe bitter
		DA	-OPr 170 x 0.6% sucrose
		DAA	-OMe 50 x 0.6% sucrose
		DAA	-OMe 5 x 0.6% sucrose
		DAA	-OMe bitter
		ADA	-OMe <1 x 0.6% sucrose
		ADA	-OMe 12 x 0.6% sucrose
		ADA	-OPr 30 x 0.6% sucrose
		ADAA	-OMe <1 x 0.6% sucrose
		DAAL	-OMe bitter
		DAAA	-OMe 0.5 x 0.6% sucrose
		DLA	-OMe bitter
		DAG	-OMe 3 x 0.6% sucrose
		DG	-OMe 8 x 0.6% sucrose
		DGA	-OMe 1 x 0.6% sucrose
C. aspartame		DF	-OMe 180 x 0.6% sucrose
		ADF	-OMe 1 x 0.6% sucrose
		ADF	-OMe 170 x 0.6% sucrose
		AADF	-OMe bitter

**Figure 5** Similarity of the KGDAALDAGGR<sub>19-29</sub> loop with various sweet/bitter-tasting peptides. D-residues are underlined. Ome = methyl ester-group; OPr = propyloxy-group (sweetness of peptides from Ariyoshi *et al.*, 1991).

## Discussion

All major epitopes that were identified in the PEPSCAN-analysis, i.e. KGDAALDAGGR<sub>19-29</sub>, EPGTNG<sub>42-47</sub> and YFDDSGSGIC<sub>57-66</sub>, DCGLLRCK<sub>70-78</sub> and CKRFG-RPP<sub>77-84</sub>, are located within one region. Particular sites within this region are structurally similar to aspartame and/or various other peptide sweeteners.

The sequence YFDDSGSGIC<sub>57-66</sub>, part of a large extruding loop which is one of the most flexible regions of thaumatin (Ogata *et al.*, 1992) and a small peak in the PEPSCAN-analysis (Figure 2A), has already been implicated in sweet-taste receptor binding. It is a part of a thaumatin fragment which is recognized by an antibody that also recognizes monellin (Kim *et al.*, 1991). The Tyr and Asp in the YFD<sub>57-59</sub> sequence, being similar to aspartame (Asp-Phe-OMe), have been postulated as the sweet-taste receptor binding sites (Kim *et al.*, 1991).

The sequence KGDAALDAGGR<sub>19-29</sub>, a large extruding loop, is not present in non-sweet thaumatin-related proteins which are in this region, except for this loop, very similar to thaumatin (Richardson *et al.*, 1987; Ogata *et al.*, 1992) (Figure 4). Furthermore, small sweet/bitter-tasting peptides

like DA-OMe, DA-OPr, DAA-OMe, DAAL-OMe and DAG-OMe (Ariyoshi *et al.*, 1991), which contain D-residues, are similar to KGDAALDAGGR<sub>19-29</sub> (Figure 5). This sequence has previously not been implicated in sweet-taste.

The CKRFGRRPP<sub>77-84</sub> sequence is an extruding loop which, in the three-dimensional structure, is located parallel to the KGDAALDAGGR<sub>19-29</sub> loop (Figure 3C). A tip of the KGDAALDAGGR<sub>19-29</sub> loop, Asp21, is facing Phe80 of the CKRFGRRPP<sub>77-84</sub> loop (Figure 3D). The backbone distance between Asp21 and Phe80, which is approximately 4 angström, is similar to the backbone distance between the Asp and Phe in the dipeptide sweetener aspartame (Asp-Phe-OMe) (cf. Kim *et al.*, 1991; Tancredi *et al.*, 1992). The similarity of the KGDAALDAGGR<sub>19-29</sub> loop with various peptide sweeteners and the similarity of the Asp21-Phe80 pair with aspartame may suggest that the KGDAALDAGGR<sub>19-29</sub> and CKRFGRRPP<sub>77-84</sub> loop are important sweet-taste determinants.

Recently, it has been suggested that aspartame directly binds G-proteins (Naim *et al.*, 1994). This implicates that the aspartame-like Asp21-Phe80 pair in thaumatin also binds these G-proteins. However, direct binding of large proteins such as thaumatin to intracellular G-proteins seems unlikely,

although antifungal thaumatin-homologous proteins seem to act by causing membrane permeabilization (Vigers *et al.*, 1991). Further studies will be required to conclude that the aspartame-like Asp21-Phe80 pair and/or the various other peptide-sweetener like sites part of the KGDAALDAGGR<sub>19-29</sub> loop are indeed important sweet-taste determinants.

## Concluding remarks

Different antibodies that both recognize thaumatin and monellin recognize a region on thaumatin that contains structural features that are similar to aspartame and structural features that are similar to other types of peptide sweeteners. It is postulated that the aspartame-like Asp21-Phe80 pair and/or the various other peptide-sweetener like sites in the KGDAALDAGGR<sub>19-29</sub> and CKRFGRPP<sub>77-84</sub> loops in thaumatin are important sweet-taste determinants which were so far not implicated as such.

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