

Identification of a 28 kDa lychee allergen as a triose-phosphate isomerase

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Abstract

The aim of this study was to investigate the allergenic potency of the exotic fruit lychee (*Litchi chinensis* SONN.). For this purpose the lychee protein extract was separated by ion exchange chromatography and a purified allergen with a molecular weight of 28 kDa was identified by N-terminal sequencing and peptide mass fingerprinting. Both methods determined the lychee allergen as a triose-phosphate isomerase. To this protein 67% of patients showed allergic reactions respectively IgE binding of their sera. Similar enzymes from other plants were also recognized as allergens.

Keywords: *Lychee, 28 kDa allergen, triose-phosphate isomerase, Edman degradation, MALDI-TOF*

Introduction

Due to the fact that about 1% of adults and 2–8% of children suffer from food allergies, the investigation and identification of food allergens is becoming increasingly important. Owing to improved transport, the consumption of exotic fruits, such as kiwi, mango, and lychee has gained in significance. The lychee belongs to the family of the Sapindaceae, originating in South China, and today the lychee is found throughout the tropics.

Reports of adverse reactions to lychee refer to symptoms such as urticaria, erythema (Montag et al. 1996), pruritus, swelling of lips and fingers, dyspnoea and diarrhoea (Leep et al. 1992). Giannattasio et al. (1995) give a case report on a 34-year-old woman suffering from generalized urticaria, Quincke oedema and bronchospasm after eating lychee fruits. Furthermore, Fähr et al. (1995) report on a patient with anaphylactic shock reactions after eating lychee.

Little is known about the allergenic potency of lychee. Fähr et al. (1995) report on lychee cross-reactivities to various kinds of food and pollen. Wellhausen et al. (1996) describe the cross-reactivity of a minor allergen of birch pollen with a lychee allergen, both having a similar molecular weight of 35 kDa. The presence of allergen structures in the lychee fruit showing a homology to the pear allergen Pyr c 5 is suggested by Karamloo et al. (2001). Moreover Fähr et al. (1995) and Vieths et al. (1993)

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demonstrate the presence of profilin in the fruit, which is the only identified lychee allergen with nomenclature Lit c 1 (Fäh et al. 1995, ALLALLERGY 2004).

In general, any single food contains more than one allergen. In this study one particular allergen is purified and analysed by N-terminal sequencing and peptide mass fingerprinting with the goal of obtaining structural information to identify the allergen in a database.

Material and methods

Patients and human sera

Immunoblot examinations were performed by using sera from 39 patients suffering from adverse reactions to lychee (Table I). All sera were collected at the University Hospital Eppendorf, Department of Dermatology and Allergy, Hamburg, Germany. Clinical symptoms such as breathlessness, dermatitis, eczema, itching in mouth, rhinitis, sickness, stomach ache, swelling of lips and swelling of throat appear. Serum from a nonatopic patient was used as negative control. The amount of specific immunoglobulin E (IgE) in the sera was determined by an enzyme allergosorbent test (EAST) with lychee protein extract coupled to cyanogen bromide activated paper disks according to the manufacturer's instructions (Allergopharma, Reinbek, Germany). After sera characterization, a serum pool consisting of equal amounts of sera 1–21 was used for all immunoblot investigations. Sera 22–39 were no longer available for further investigation.

Lychee extract, electrophoresis, blotting and staining

Extracts were prepared from fresh lychees without peel and stone by a previously described low-temperature method (Vieths et al. 1992, Möller et al. 1997). Proteins were extracted from the obtained acetone powder with phosphate-buffered saline (PBS) for EAST and immunoblot investigations and with bidistilled water for ion exchange chromatography (IEC). For lychee the composition of both extracts is identical comparing protein and allergen pattern by SDS-PAGE and immunoblotting but PBS could disturb the IEC purification step.

Protein extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% Bis-Tris gels (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. Afterwards, the proteins were visualized by silver staining (Heukeshoven & Dernick 1986) or transferred onto Protran nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by semidry blotting (Kyhse-Andersen 1984) with a discontinuous buffer system (Vieths et al. 1992). For immunoblotting, the membrane was incubated with pooled sera (1:15 diluted). Serum from a nonatopic patient was used as negative control. Immunostaining was performed according to Vieths et al. (1992) and Möller et al. (1997). Molecular weights were determined by a protein standard mixture (14.4–94.0 kDa) after silver staining or, on nitrocellulose membranes, after Ponceau S staining. The membrane was incubated for 30 min with a Ponceau S solution (2% Ponceau S in 3% trichloroacetic acid in bidistilled water) followed by a treatment with 3% acetic acid in bidistilled water to destain the membrane background.

The peptide mass fingerprint (PMF) was performed with electrophoretically separated proteins after coomassie brilliant blue (CBB) staining and in-gel digestion.

Table I. Characteristics of sera.

No.	EAST-class/spec. IgE (UmL ⁻¹)	Molecular weight of detected allergens in immunoblot (kDa)
1	2/ 1.06	25, 28, 32, 35, 40
2	3/ 3.93	14, 25, 35, 40, 55, 70
3	2/ 0.98	14, 35, 40, 55, 60, 65, 70
4	2/ 1.84	35, 55, 60, 65
5	2/ 0.93	25, 35, 55, 60, 65, >70
6	4/ >17.5	25, 28, 32, 40, 43, 55, 70, >70
7	2/ 1.03	35, 55, 60, 65
8	3/ 5.72	14, 28, 32, 35, 40, 55, 60, 70, >70
9	3/ 10.1	14, 25, 28, 32, 35, 40, 43, 55, 60, 70, >70
10	2/ 0.69	32, 43, 55, 70, >70
11	2/ 1.03	25, 28, 32, 35, 40, 43, 55, 70, >70
12	4/ >17.5	25, 28, 32, 35, 40, 43, 55, 70, >70
13	2/ 1.34	14, 25, 28, 32, 35, 43, 55, 65, 70, >70
14	3/ 3.78	14, 25, 28, 32, 35, 43, 55, 70, >70
15	3/ 4.27	35, 40, 55, 70, >70
16	2/ 1.04	32, 35, 43, 55, 70, >70
17	3/ 4.27	14, 35, 55, 65
18	2/ 1.72	14, 28, 32, 35, 40
19	3/ 3.89	14, 25, 28, 32, 35, 40, 43, 55, 70, >70
20	2/ 3.30	40, 55, 70, >70
21	3/ 9.50	25, 28, 32, 40, 43, 55, 70, >70
22	3/ 8.87	14, 40, 55, 70, >70
23	3/ 3.56	25, 32, 40, 43, 55, 70, >70
24	2/ 1.40	14, 28, 43, 55, 65
25	3/ 7.84	35, 40, 55, 70, >70
26	2/ 2.76	28, 40, 43, 55, 65
27	2/ 0.74	28, 40, 43, 70
28	2/ 1.63	14, 28, 35, 43, 55
29	3/ 3.71	28, 35, 40, 43, 55, 70, >70
30	2/ 0.91	25, 28, 35, 40, 43, 55, 70, >70
31	3/ 11.78	14, 28, 40, 43, 55, 70, >70
32	2/ 1.04	28, 40, 43, 55, 70, >70
33	2/ 1.09	28, 35, 40, 43, 55, 70, >70
34	4/ >17.5	28, 32, 35, 40, 43, 55, 70, >70
35	4/ >17.5	25, 28, 32, 35, 40, 43, 55, 70, >70
36	2/ 1.23	25, 28, 40, 43, 55, 70, >70
37	4/ >17.5	28, 40, 43, 55, 70, >70
38	3/ 3.84	25, 28, 40, 43, 55, 70, >70
39	2/ 0.82	14, 25, 28, 35, 40, 43, 55, 70, >70

For this purpose, a CBB solution according to Fling and Gregerson (1986) was used for staining and 30% methanol in bidistilled water was used to destain the gel background (Rosenfeld et al. 1992).

For sequencing, the proteins were transferred onto polyvinylidene difluoride (PVDF)-membrane (Amersham Biosciences, Uppsala, Sweden) after SDS-PAGE and stained with CBB as described by Ward et al. (1990).

Ion exchange chromatography

The lychee proteins were separated by anion exchange chromatography with a Resource QTM (6 ml) chromatography column from Amersham Biosciences (Uppsala,

Sweden) with a Tris-HCl-buffer system. Buffer I consists of 0.02 M Tris-HCl in bidistilled water (pH 8.0), whereas eluent II contains additionally 0.5 M NaCl. The detailed conditions of the continuously changing gradient elution are shown in Table III. The wavelength for protein detection was 280 nm. The obtained protein fractions were dialysed against bidistilled water applying Slide-A-Lyzer dialysis cassettes with a 3.5 kDa molecular weight cutoff (Pierce, Rockford, USA).

N-terminal sequencing and peptide mass fingerprint

The determination of N-terminal amino acid sequences by Edman degradation was carried out with protein bands, corresponding to 28 kDa allergens transferred onto PVDF-membrane and stained with CBB. This investigation was performed in cooperation with the University Hospital Eppendorf, Hamburg, Germany.

For the PMF the in-gel digestion was accomplished using trypsin according to Rosenfeld et al. (1992) with some modifications. Previous to the in-gel digestion the lychee extract was purified by ion exchange chromatography. The protein fraction containing the 28 kDa allergen was dialysed, separated by SDS-PAGE and stained with CBB. The spot corresponding to the 28 kDa allergen was excised, destained by washing three times for 20 min with 250 µl washing buffer (50% acetonitrile, 50 mM ammonium carbonate in bidistilled water pH 8.9) and dried in a vacuum drying oven. In the next step, the gel piece was rehydrated with 5 µl rehydration buffer (50 mM ammonium carbonate in bidistilled water pH 8.9) and incubated with a trypsin solution. For this purpose, 2 µl rehydration buffer containing trypsin were added. The trypsin activity was chosen with 0.0714 nkat (equivalent to 0.004285 U) relating to one protein band (1.3 µg protein, estimated). Subsequently the gel piece was immersed in rehydration buffer. After incubation overnight at 37°C the peptides were extracted with extraction buffer (100 µl, three times for 20 min; 60% acetonitrile, 0.1% TFA in bidistilled water), lyophilized, desalted with ZipTips_{C18} (Millipore, Billerica, MA, US) using the following solutions: wetting solution: 50% acetonitrile in bidistilled water; equilibration solution: 0.1% TFA in bidistilled water; wash solution: 0.1% TFA in bidistilled water; elution solution 1: 50% acetonitrile/0.1% TFA in bidistilled water; elution solution 2: acetonitrile. Prior to desalting, the sample was incubated with 4 µl 0.1% TFA in bidistilled water, and was subsequently lyophilized again.

Before the MALDI-TOF-measurement, 2 µl of a solution consisting of 60% acetonitrile/0.1% TFA in bidistilled water was added to the peptides. As matrix α -cyano-4-hydroxy-cinnamic acid was used. PMF was determined in cooperation with the Institute of Organic Chemistry, University of Hamburg, Germany.

Results

Allergen pattern

The lychee allergen pattern was investigated by means of immunoblot. IgE-binding proteins with molecular weight (MW) from 14–70 kDa were detected (see Figures 1 and 2). The allergen patterns differ depending on the patient's serum used for immunoblotting. IgE-binding proteins with molecular weights of 14, 25, 28, 32, 35, 40, 43, 55, 60, 65, 70 and > 70 kDa were observed. The number of sera staining one particular allergen is shown in Table II.

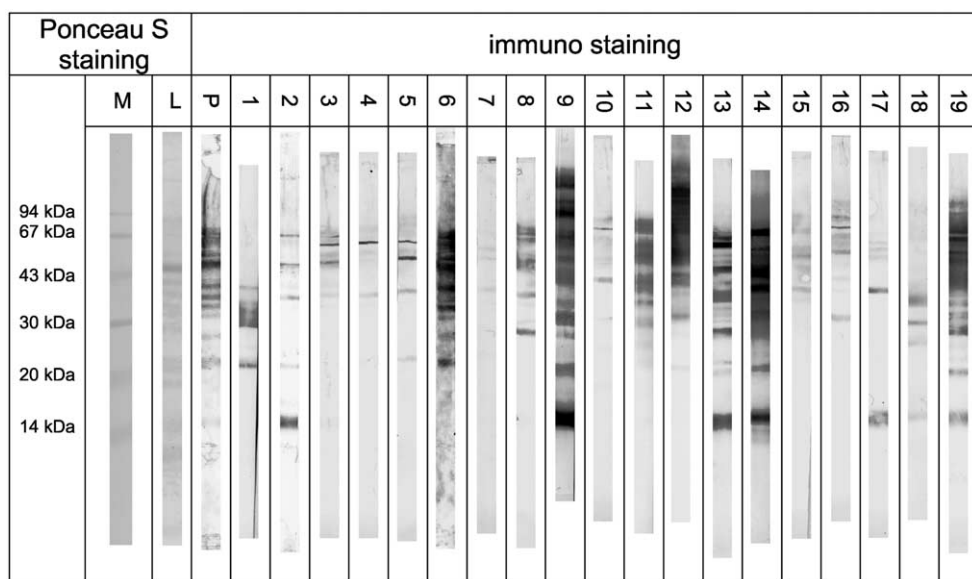


Figure 1. Immunoblot with sera 1–19. M, molecular weight marker; L, lychee; P, pool serum; 1–19: sera 1–19.

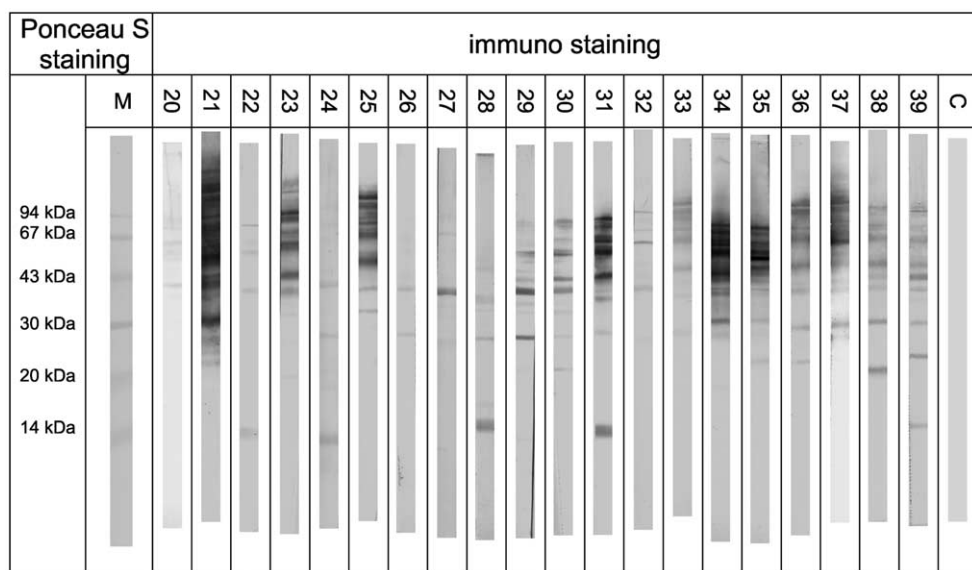


Figure 2. Immunoblot with sera 21–39. M, molecular weight marker; 21–39: sera 20–39; C, control.

Table II. Frequency of detection.

MW (kDa)	14	25	28	32	35	40	43	55	60	65	70	>70
No. of detecting sera	14	17	26	16	25	29	26	36	6	8	30	28
ratio (%)	36	44	67	41	64	74	67	92	15	21	77	72

MW: molecular weight; No: number.

Allergens with molecular masses of 28, 35, 40, 43, 55 and 70 kDa were detected by more than 50% of sera and therefore appear to be major lychee allergens. We focused further investigations on the 28 kDa allergen, to which 67% of the sera were reactive.

Purification by ion exchange chromatography

A chromatogram obtained from the anion exchange chromatography (conditions I, Table III) is depicted in Figure 3. From the separated lychee extract, seven fractions (A–G) were collected, dialysed and investigated by SDS-PAGE with subsequent silver staining and immunoblotting (Figure 4). By means of immunoblotting, the 28 kDa allergen could be detected in fraction C. The fraction contains other allergens and non-IgE-binding proteins with significantly different molecular weights; hence, the 28 kDa protein band could be excised without contamination. The piece of gel containing the 28 kDa allergen was cut out and used to determine the PMF.

In addition, the proteins of fraction C were pooled, dialyzed and purified again with the same column but different conditions of elution (conditions II, Table III). In this manner the proteins could be separated more specifically. The proteins are separated into seven fractions and immunoblot analysis was done of all fractions. Thereby a 28 kDa allergen could be visualized in fraction 1 and 3, and in very small amounts in 2. The proteins were blotted onto PVDF membrane, stained with CBB and the protein bands corresponding to the 28 kDa allergen were excised and applied for Edman degradation.

Identification by N-terminal sequencing and PMF

By N-terminal sequencing of the two spots in fraction 1 and 3 representing the 28 kDa allergen 13 amino acids were determined. The obtained N-terminal sequences of the

Table III. Conditions of chromatography.

Time (min)	Buffer I	Buffer II	Flow rate (mLmin ⁻¹)
Conditions I			
0	100	0	0.3
0.5	100	0	1
5	100	0	1
55	50	50	1
65	0	100	1
75	0	100	1
80	100	0	1
100	100	0	1
Conditions II			
0	100	0	0.3
0.5	100	0	1
5	100	0	1
160	65	35	1
170	0	100	1
180	0	100	1
185	100	0	1
205	100	0	1

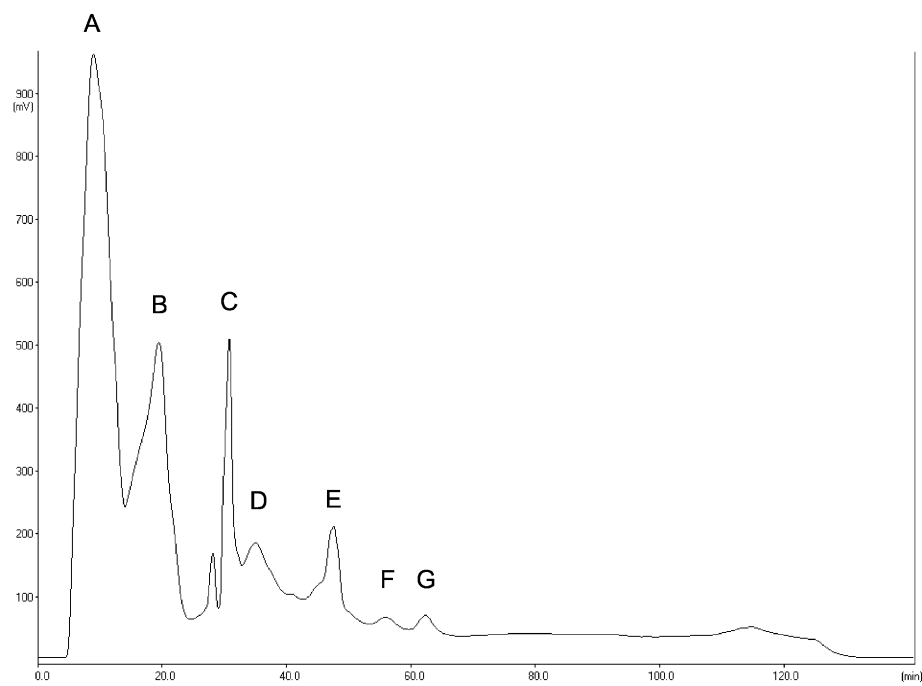


Figure 3. Ion exchange chromatography 1, conditions I see Table III.

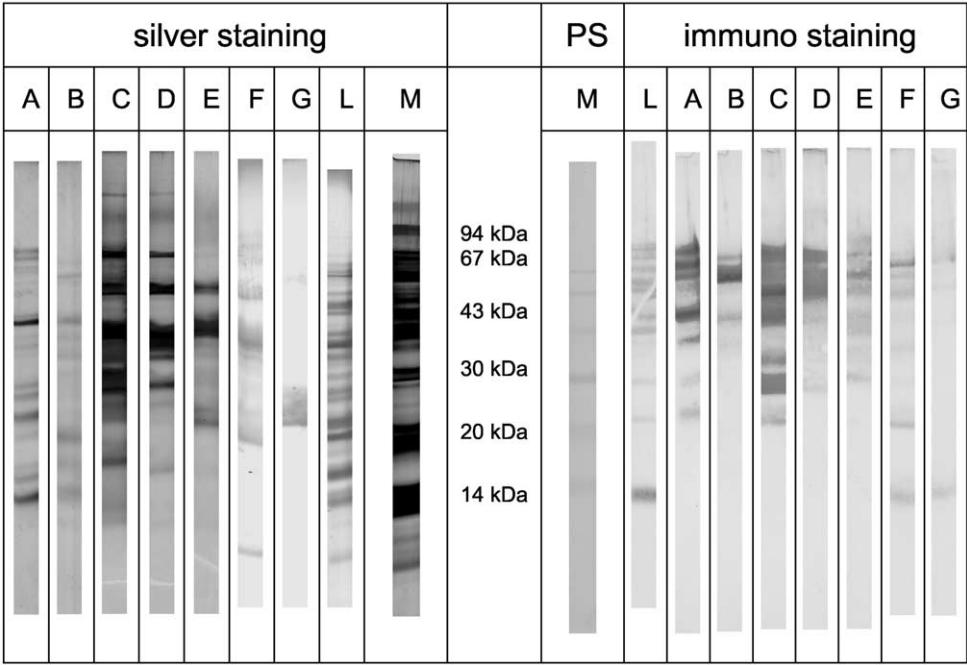


Figure 4. SDS-PAGE and immunoblot of fractions A–G. M, molecular weight marker; L, lychee; A–G, fractions; PS, Ponceau S staining.

Table IV. N-terminal amino acid sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13
28 kDa lychee allergens	A ¹	R	K	F	F/I	V	G	G	N	W	K	X	N
triose-phosphate isomerase	A ²	R	K	F	F	V	G	G	N	W	K	C	N

¹First amino acid from the N-terminus; ²Second amino acid from the N-terminus.

two IgE-binding proteins are identical, whereby the amino acids in position five and twelve could not be analysed precisely (Table IV). The amino acid in position five might be phenylalanine or isoleucine. In position twelve no amino acid could be identified and an "X" is used as substitute symbol.

The identical sequences of the IgE-binding proteins exhibit a high homology to a triose-phosphate isomerase (EC 5.3.1.1). In Table IV, the triose-phosphate isomerase sequence from mouse-ear cress (*Arabidopsis thaliana*) is shown, and triose-phosphate isomerases from other organisms show comparable amino acid sequences. The sequences are identical except for the two unidentified amino acids in position five and twelve. The fact that both IgE-binding proteins exhibit the same N-terminal sequence indicates that two isoallergens are presented in the fruit. The isoelectric point (pI) of the two IgE-binding proteins in fractions 1 and 3 are 7.4 and 7.0 respectively.

This apparent homology of the lychee allergens to triose-phosphate isomerases was confirmed by the determination of the characteristic PMF. For this purpose the lychee extract was fractionated by means of IEC, without separation of the isoallergens because the separation is not necessary for this investigation. After in-gel digestion with trypsin the resulting peptides were investigated by MALDI-TOF mass spectrometry (Figure 5). The tryptic peptides matched those of a triose-phosphate isomerase confirming the results obtained by Edman degradation.

Discussion

In general, food allergens exhibit molecular masses of 10–70 kDa (Taylor et al. 1987) and individual foods contain several allergens irrespective of whether the origin is a fruit, a vegetable or an animal. The aim of this study was to identify an important lychee allergen, and for the study, the 28 kDa allergen that was detected by 67% of patients' sera in immunoblot was chosen.

Prior to the allergen purification and characterization, the proteins must be isolated from the fruits in a native form. The low-temperature method (Vieths et al. 1992, Möller et al. 1997) allows the separation of interfering phenolic compounds from the proteins and the reduction of enzymatic activities. Allergens with molecular weights of 28, 35, 40, 43, 55, 70 kDa are important lychee allergens because they could be stained in immunoblot with more than 50% of patients' sera. The allergen with a molecular weight of about 14 kDa was identified by Fähr et al. (1995) and Vieths et al. (1993) as a profilin, and regarding this pool of patients, this allergen could be stained by 36% of patients' sera. Other lychee allergens have not yet been identified. In the next step the 28 kDa allergen was isolated by ion exchange chromatography because this method is characterized by mild conditions, which makes it possible to isolate an allergen in its native form.

By means of Edman degradation and PMF determination, the two 28 kDa lychee isoallergens showed a high homology to a triose-phosphate isomerase. This enzyme

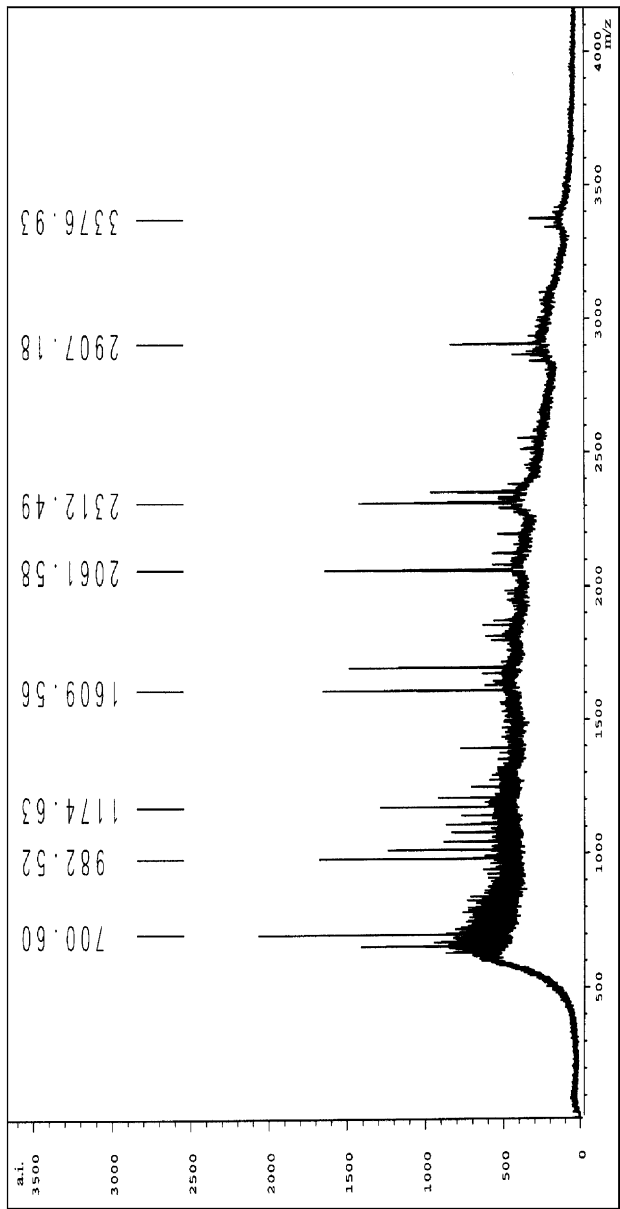


Figure 5. MALDI-TOF spectrum.

was also described as an allergen in wheat and latex. Sander et al. (2001) investigated wheat flour allergens in the content of workplace-related respiratory symptoms of bakers. With sera of patients with baker's asthma, several allergens could be detected. For identification mass spectrometric fingerprinting was used and the obtained masses matched triose-phosphate isomerase from *H. vulgare*. Posch et al. (1997) identified an IgE-binding latex protein as triose-phosphate isomerase.

Besides the identification of allergens, a further interesting point is the occurrence of cross-reactions among allergens of different origin. These reactions are based on the presence of the same allergic structures in different foods, pollen etc.

In this connection, Niggemann et al. (2002) report on a 12-year-old girl with symptoms such as swelling of lips, pruritus, generalized urticaria, and dyspnea after eating raw lychee. Despite a positive skin prick test to raw lychee, no IgE to lychee could be detected but IgE to latex could be. Inhibition experiments showed a cross-reactivity of lychee to latex. If this cross-reaction between lychee and latex depends on the occurrence of triose-phosphate isomerase in both allergen sources, this interesting question has to be investigated.

These investigations point out the occurrence of a triose-phosphate isomerase in lychee fruit which is an important lychee allergen and may be involved in cross-reactions to other food.

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