

Vincenzo Cunsolo<sup>1</sup>  
 Salvatore Foti<sup>1</sup>  
 Rosaria Saletti<sup>1</sup>  
 Leopoldo Ceraulo<sup>2</sup>  
 Vita Di Stefano<sup>2</sup>

<sup>1</sup>Dipartimento di Scienze  
 Chimiche, Università di Catania,  
 Catania, Italy

<sup>2</sup>Dipartimento di Chimica e  
 Tecnologie Farmaceutiche,  
 Università di Palermo  
 Palermo, Italy

## Detection and localisation of disulphide bonds in a synthetic peptide reproducing the sequence 1–30 of *Par j* 1.0101 by electrospray ionisation mass spectrometry

The structural characterisation of a synthetic peptide reproducing the sequence 1–30 of *Par j* 1.0101, a major allergenic protein present in the pollen of *Parietaria judaica*, by combined use of chemical and enzymatic cleavage, reversed-phase high-performance liquid chromatography and electrospray ionisation mass spectrometry (ESI-MS), is described. Direct ESI-MS of the synthetic peptide after reaction with methyl iodide showed that the product is a mixture of two peptides: one form in which two out of the four cysteine residues present in the sequence are oxidised and a minor amount of another form in which all the cysteines are fully reduced. It was ascertained, using the combined procedure indicated above and without prior separation of the two species, that the disulphide bond in the partially oxidised form is located between cysteines 29 and 30. These results show the usefulness of this approach for characterising synthetic peptides containing multiple cysteine residues in the sequence.

**Keywords:** Synthetic peptide / *Par j* 1.0101 / *Parietaria judaica* / Disulphide bridges / Structural characterisation / Electrospray ionisation mass spectrometry  
 PRO 0102

### 1 Introduction

The pollen of the weed *Parietaria judaica* (Pj) is the main cause of allergic reactions in the Mediterranean countries [1, 2], representing about 40% of the cases in pollen-allergic sensitive patients [3]. Its distribution is not restricted to the Mediterranean area since *P. judaica* and *P. officinalis* have also been reported as a cause of allergic reactions in Eastern Australia and in the United States, respectively [4, 5]. Investigation of the allergenic composition of the *Parietaria* pollen has shown that its aqueous extract is composed of a complex mixture of at least 26 antigens, nine of which bind specifically to IgE of allergic patients [6–8]. Among the last ones, a major allergen, named *Par j* 1.0101, has been cloned and its sequence has been determined [9]. With the aim of identifying the epitopes responsible for the specific binding to human IgE, some peptides with a different number of amino acid residues and sequence have been synthesised and tested. Among the synthesised peptides, one composed of 30 amino acid residues and corresponding to the N-terminal domain of *Par j* 1.0101, was capable of binding

specific IgE from a pool of sera of patients allergic to Pj pollen [10]. The sequence of the 1–30 peptide, which is now under investigation in the frame of a systematic study of the allergenic activity of *Parietaria* pollen, is shown in Fig. 1. Since the 1–30 peptide contains four cysteine residues, which are in the oxidised form in the native protein, we felt that an investigation of the presence of disulphide bonds in the synthetic peptide could be of interest.

In recent years, mass spectrometric techniques have emerged as a major tool for disulphide bond determination and localisation [11–14]. This paper is concerned with the detection and assignment of disulphide bonds in the 1–30 synthetic peptide by means of ESI-MS in combination with chemical and enzymatic cleavage and reversed-phase high-performance liquid chromatography (RP-HPLC).

### 2 Materials and methods

N-Tosyl-L-phenylalanine chloromethyl ketone/treated trypsin, cyanogen bromide (CNBr), DTT, methyl iodide, ammonium acetate and calcium chloride were purchased

**Correspondence:** Dr. Leopoldo Ceraulo, Dipartimento di Chimica e Tecnologie Farmaceutiche, Università di Palermo, Via Archirafi, 32, I-90123, Italy  
**E-mail:** lceraulo@unipa.it  
**Fax:** +39-091-6236110

**Abbreviation:** CNBr, cyanogen bromide

10  
 GLN-GLU-THR-CYS-GLY-THR-MET-VAL-ARG-ALA-LEU-MET-PRO-CYS-LEU-  
 20 30  
 -PRO-PHE-VAL-GLN-GLY-LYS-GLU-LYS-GLU-PRO-SER-LYS-GLY-CYS-CYS

**Figure 1.** The sequence of the 1–30 peptide reproducing the N-terminal domain of *Par j* 1.0101.

from Sigma (Milan, Italy); TFA was provided by Fluka (Milan, Italy). High-performance chromatography grade H<sub>2</sub>O and CH<sub>3</sub>CN were obtained from Romil (Cambridge, UK). All other chemicals were of the highest purity commercially available and were used without further purification. The 1–30 peptide was prepared by solid phase synthesis performed in a Milligen 9050 synthesiser (Burlington, MA, USA) as previously described [15].

## 2.1 Methylation of peptide 1–30

The lyophilised peptide (500 µg) was dissolved in 0.1 M Tris-HCl at a concentration of 1 mg/mL and the pH adjusted to 8.5 by NaOH. 2 µL of methyl iodide (50-fold molar excess over the thiol-group concentration) were added and the sample was repeatedly shaken at room temperature for 15 min to disperse the reagent. The reaction was stopped by cooling in liquid nitrogen and the product was freeze-dried.

## 2.2 Tryptic digestion of the methylated peptide 1–30

The methylated peptide (250 µg) was dissolved in 20 mM ammonium acetate (pH 8.3) containing 1 mM calcium chloride at a concentration of 1 mg/mL. Trypsin was dissolved in the same buffer and added at an enzyme-to-substrate ratio of 1:50 (mol/mol). The solution was incubated at 37°C for 4 h. The digestion was stopped by cooling in liquid nitrogen and the mixture was immediately freeze-dried.

## 2.3 Cyanogen bromide cleavage of the methylated peptide 1–30

The methylated peptide (250 µg) was dissolved in 70% aqueous formic acid at a concentration of 1 mg/mL. CNBr (50-fold molar excess over the methionine concentration) dissolved in 150 µL of 70% formic acid was added and the mixture allowed to react for 24 h at room temperature in the dark under nitrogen atmosphere. The solution was then diluted 20-fold with water and freeze-dried. The latter procedure was repeated twice.

## 2.4 HPLC

HPLC runs were performed using a Varian 9010 (Sunnyvale, CA, USA) equipped with a Varian 9050 detector and a Varian 4400 integrator. Samples were dissolved in aqueous 0.05% TFA at a concentration of 1 mg/mL and filtered on Ultrafree-MC Millipore (Bedford, CA, USA). For the purification of the active 1–30 peptide, the sample was

loaded onto a RP-Vydac C18 (1.0 × 25 cm) column. The column was eluted at room temperature with a gradient of solvent B (95% CH<sub>3</sub>CN-5% H<sub>2</sub>O containing 0.05% TFA) in A (0.05% aqueous TFA) from 5% to 25% in 15 min and then to a final concentration of 40% of B in A in 50 min. The flow rate was 3 mL/min. Peaks were detected by their absorption at 224 nm. Fractions were collected manually and freeze-dried. The purification of the methylated peptide 1–30 was performed under the same conditions, eluting the column with a gradient of solvent B in A from 5% to 60% in 40 min.

For the separation of the tryptic and CNBr fragments of the methylated peptide 1–30, samples were loaded onto a RP-Vydac C18 (0.46 × 25 cm) column. The column was eluted at room temperature with 95% of solvent A and 5% of solvent B for 5 min, and then with a linear gradient of B in A to a final concentration of 47% of B in A in 55 min. The flow rate was 1 mL/min. Peaks were detected by their absorption at 224 nm. Fractions were collected manually and freeze-dried.

## 2.5 ESI-MS

ESI mass spectra were recorded on a HP 59987A instrument (Hewlett Packard, Palo Alto, CA, USA) coupled with a HP 5989A quadrupole analyser. Peptide samples were dissolved in H<sub>2</sub>O-CH<sub>3</sub>OH-CH<sub>3</sub>COOH (50:50:1) at a concentration of 50 ng/µL. The solution was infused into the ESI ion source at a flow rate of 5 µL/min. Scanning was performed from *m/z* 100 to 1500. Calibration was performed using the multiply-charged ions from a separate introduction of a solution of horse heart cytochrome c, gramicidin S and tetraethyl ammonium hydroxide for relatively high-molecular weight peptides, and a mixture of valine, tri-tyrosine and hexa-tyrosine for the small ones.

## 3 Results and discussion

Final purification of the synthetic 1–30 peptide was obtained by RP-HPLC. The chromatogram showed two peaks, with the most abundant fraction corresponding to the active peptide. The ESI mass spectrum of the active fraction (Fig. 2) exhibits two series of ions, carrying 3, 4 and 5 charges. The maximum charge state correlates well with the five basic sites present in the sequence (3 Lys, 1 Arg and the *N*-terminal amino group). The deconvoluted spectrum shows a molecular ion at 3271.6 Da, which is close to the *M<sub>r</sub>* of the peptide (3271.9 Da, calculated for the fully reduced peptide), accompanied by the Na-cationised peak at 3293.3 Da. The ESI mass spectrum of the inactive component indicated a mass of 3322.6 Da,

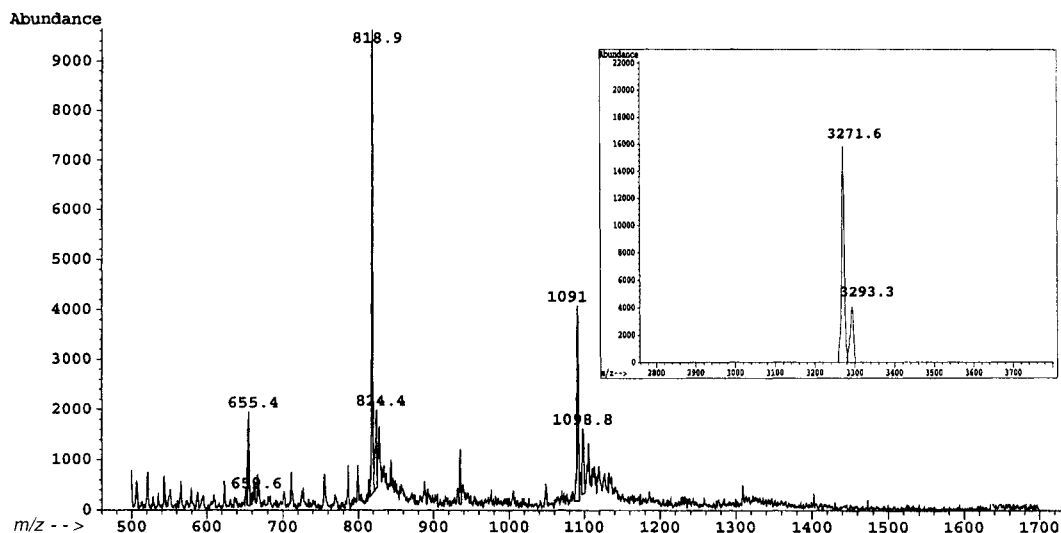


**Figure 2.** ESI mass spectrum and (insert) deconvoluted spectrum of the synthetic peptide 1–30.

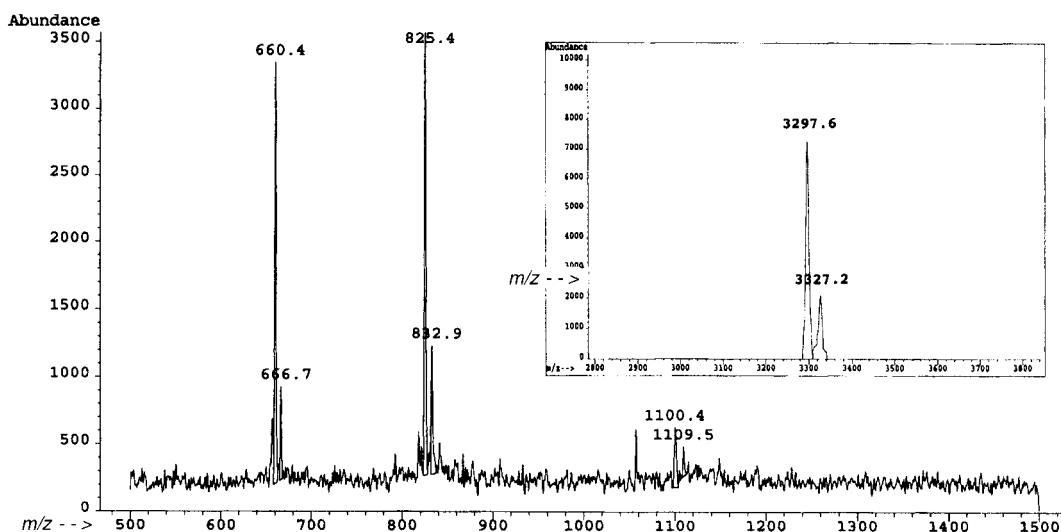


**Figure 3.** ESI mass spectrum and (insert) deconvoluted spectrum of the synthetic peptide 1–30 after reaction with methyl iodide.

probably corresponding to a peptide in which a blocking group was not removed or to a peptide having an error in the sequence.

Since the resolution of the ESI quadrupole instrument is not sufficiently high to discriminate between fully reduced and partially oxidised forms when they are present as a mixture, methylation by methyl iodide of the free thiol groups in the active peptide was performed in order to verify the oxidation state of the four cysteines. Purification of the reaction product was performed by HPLC, to give a single chromatographic peak. The ESI spectrum of the purified product presents two series of signals (Fig. 3). Deconvolution of the first set of peaks at  $m/z$  660.4,

825.4 and 1100.4 yields a mass value of 3297.6 Da, differing by 25.7 Da from the calculated molecular weight of the fully reduced peptide. This  $M_r$  corresponds to a peptide in which two cysteine residues are methylated and the other two are in the oxidised form (calculated average  $M_r$  3298). Deconvolution of the signals at  $m/z$  666.7, 832.9 and 1109.5 gives rise to a peak at 3327.2 Da, corresponding to the  $M_r$  of a peptide in which all the cysteines are methylated (calculated average  $M_r$  3328).

These results clearly show that the synthetic product comprises a peptide in which two out of the four cysteine residues present in the sequence are oxidised together with a minor amount of a form in which all the four

cysteines are fully reduced. Assuming that the intensity of the signals in the deconvoluted spectrum is proportional to the concentration of the species in solution, the spectrum in Fig. 3 indicates that the relative ratio in which the partially oxidised and fully reduced forms are present in the mixture is about 4:1. ESI-MS spectra of samples obtained from different preparations, however, showed that the relative intensity of the signals corresponding to the partially oxidised and fully reduced forms was variable from the 4:1 ratio observed in this case to the 1:1 ratio found for sample obtained in other preparations, indicating that the relative amount of the two species is dependent on the particular preparation. To determine the location of the disulphide bond in the partially oxidised form, the mixture of fully methylated and partially methylated peptides was digested using trypsin. The peptide contains 3 Lys and 1 Arg residues and therefore five tryptic peptides should be generated (Table 1). Depending on the position of the disulphide bond, these peptides may be independent or two of them bridged together by a disulphide linkage. HPLC separation of the tryptic digest is shown in Fig. 4. The ESI mass spectrum of fraction 1 showed a signal at  $m/z$  717.5 corresponding to the protonated molecule of sequence 22–27 (fragment T3+4). Lack of cleavage at Lys23, which generally occurs when an acidic amino acid is adjacent to Lys or Arg, accounts for the presence of peptide T3+4 and also explains the absence in the chromatogram of peaks corresponding to peptides T3 and T4. The ESI spectrum of fraction 2 exhibits two signals at  $m/z$  1038.4 and 519.9 corresponding to the ions of the Cys4-methylated fragment 1–9 (T1), carrying 1 and 2 charges, respectively.

**Table 1.** Position sequence and calculated monoisotopic mass of the tryptic fragments of the peptide 1–30

| Fragment | Position | Sequence                                        | Calculated monoisotopic $M_r$                |
|----------|----------|-------------------------------------------------|----------------------------------------------|
| T1       | 1–9      | Gln-Glu-Thr-Cys-Gly-Thr-Met-Val-Arg             | 1023.4 <sup>a)</sup><br>1037.4 <sup>b)</sup> |
| T2       | 10–21    | Ala-Leu-Met-Pro-Cys-Leu-Pro-Phe-Val-Gln-Gly-Lys | 1302.7 <sup>a)</sup><br>1316.7 <sup>b)</sup> |
| T3       | 22–23    | Glu-Lys                                         | 275.1                                        |
| T4       | 24–27    | Glu-Pro-Ser-Lys                                 | 459.2                                        |
| T3+T4    | 22–27    | Glu-Lys-Glu-Pro-Ser-Lys                         | 716.3                                        |
| T5       | 28–30    | Gly-Cys-Cys                                     | 281.1 <sup>a)</sup><br>309.1 <sup>b)</sup>   |
| TC2      | 10–17    | Ala-Leu-Met-Pro-Cys-Leu-Pro-Phe                 | 890.7 <sup>a)</sup><br>904.7 <sup>b)</sup>   |

a) Cysteine in the reduced form

b) Cysteine methylated

The ESI mass spectrum of fraction 3 shows two correlated charge states at  $m/z$  1317.9 and  $m/z$  659.5, corresponding to the Cys14-methylated sequence 10–21 (T2), carrying 1 and 2 charges, respectively. The spectrum of fraction 4 gave a signal at  $m/z$  905.5, due to the protonated form of the Cys14 methylated sequence 10–17 (TC2), originated by cleavage at the Phe17-Val18 linkage. Cleavage at the carbonyl site of Phe is frequently observed during tryptic digestion and is attributed to the activity of pseudo-trypsin which is generated slowly by autolysis of trypsin [16].

There was no evidence in the chromatogram of the presence of tryptic peptides corresponding to fragments T1 and T2 bridged together by a disulphide bond or to fragments T1 or T2 bridged by a disulphide bond to fragment 28–30 (T5), as would occur if Cys4 and Cys14 were bridged together or if one of these two cysteines was bridged to Cys29 or Cys30. Therefore these results show that Cys4 and Cys14 are totally methylated, indicating that, in the original peptide mixture, they should be in the reduced form, both the partially oxidised and in the fully reduced peptide. Consequently, location of the disulphide bond exclusively between cysteines 29 and 30 could be inferred from the above data. However, these data do not provide direct evidence for the position of the disulphide bond because fragment 28–30 (T5) was not detected either in the oxidised cysteine, or in the methylated form.

In order to unambiguously localise the disulphide bond, complementary sequence data were obtained by CNBr cleavage of the mixture of the fully methylated and partially methylated peptides. Because of the presence of two methionine residues in the sequence, three fragments should be generated by CNBr cleavage (Table 2). The HPLC separation of the CNBr peptides is shown in Fig. 5. The ESI mass spectrum of the first fraction in the chromatogram presents a signal at  $m/z$  541.4, which corresponds to the  $MH^+$  ion of the 8–12 (B2) peptide sequence with the homoserine lactone form, and another at  $m/z$  271.2 due to the doubly charged molecular ion. The ESI analysis of fraction 2 shows signals at  $m/z$  491.3, 654.8 and 981.5, which give a deconvoluted mass value of 1961.2 Da, corresponding to the  $M_r$  of fragment 13–30 (B3) with one cysteine methylated and two cysteines in the oxidised form. This fragment originates from CNBr cleavage of the partially oxidised peptide and indicates that the disulphide bond is localised within the 13–30 peptide sequence. By comparison with the results obtained by tryptic cleavage, the methylated Cys residue can be identified as Cys14, so that the disulphide bond in the partially oxidised peptide is unequivocally located in the Cys29-Cys30 position.

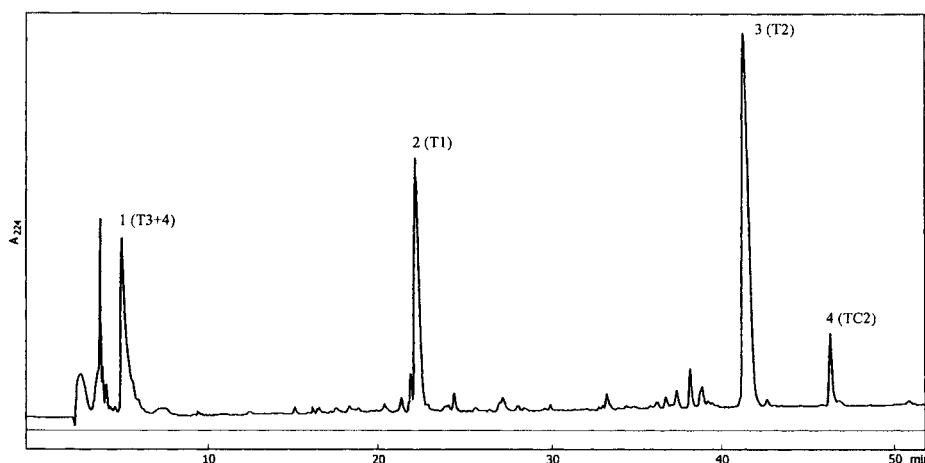


**Figure 4.** HPLC separation of the tryptic digest of methylated peptide 1–30.

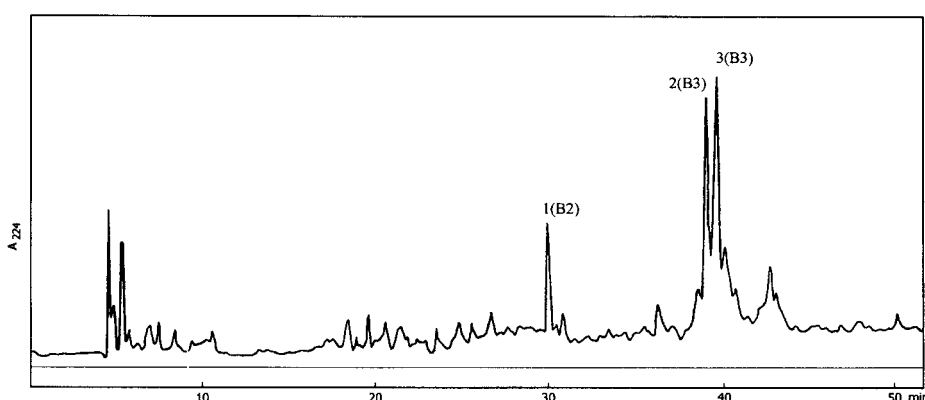


**Figure 5.** HPLC separation of the CNBr fragments from methylated peptide 1–30.

**Table 2.** Position, sequence and calculated monoisotopic mass of the CNBr fragments of the peptide 1–30

| Fragment | Position | Sequence                                                                | Calculated monoisotopic $M_r$ <sup>a)</sup>  |
|----------|----------|-------------------------------------------------------------------------|----------------------------------------------|
| B1       | 1–7      | Gln-Glu-Thr-Cys-Gly-Thr-Met                                             | 720.3 <sup>b)</sup><br>743.3 <sup>c)</sup>   |
| B2       | 8–12     | Val-Arg-Ala-Leu-Met                                                     | 540.3 <sup>c)</sup>                          |
| B3       | 13–30    | Pro-Cys-Leu-Pro-Phe-Val-Gln-Gly-Lys-Glu-Lys-Glu-Pro-Ser-Lys-Gly-Cys-Cys | 1961.0 <sup>d)</sup><br>1991.0 <sup>e)</sup> |

a) Homoserine lactone form

b) Cysteine in the reduced form

c) Cysteine methylated

d) One cysteine methylated and two in the oxidised form

e) Three cysteines methylated

The ESI spectrum of fraction 3 shows a signal at  $m/z$  498.8 and 664.7, whose deconvolution gives a mass value of 1991.1 Da, attributable to the  $M_r$  of 13–30 peptide fragment (B3) with all the three cysteines methylated. This

fragment originates from CNBr cleavage of the fully reduced form. The chromatogram shows no evidence for the presence of peptide B1 bridged by a disulphide bond to peptide B3, further substantiating the conclusion that the disulphide bond in the partially oxidised peptide is specifically located in the Cys29-Cys30 position.

## 4 Concluding remarks

A synthetic peptide reproducing the sequence 1–30 of *Par j* 1.0101, a major allergenic protein present in the pollen of *P. judaica*, has been synthesised and structurally characterised by ESI mass spectrometry. Direct ESI-MS of the methylated peptide allowed us to demonstrate that two of the four cysteines present in the sequence were partially oxidised. The assignment of the disulphide bond was achieved by combined use of chemical and enzymatic cleavage, RP-HPLC and ESI-MS. The results demonstrated that, among the four cysteines contained in the sequence of the peptide, the disulphide linkage is located between cysteines 29 and 30. The results show the usefulness of this approach for characterising synthetic peptides containing multiple cysteine residues in the sequence.

Mass spectral data were obtained on instruments of the "Italian Mass Spectrometry Network-CNR, Nodo 7 LSMO-CT". Financial support of MURST is gratefully acknowledged.

Received April 19, 2001

## 5 References

- [1] Serafini, U., Gola, G., in: *La Pollinosi*, Vallecchi, Firenze, Italy 1952.
- [2] Raffaelli, M., *Webbia* 1977, 31, 49–68.
- [3] Accorsi, C. A., Baudini Mazzanti, M., *Webbia* 1980, 34, 643–661.
- [4] Bass, D., Baldo, B. A., *Med. J. Aust.* 1984, 140,, 511–520.
- [5] Charpin, J., Charpin, H., *J. Allergy Clin. Immunol.* 1980, 65, 80–81.
- [6] Menegozzo, M., Geraci, D., Ruffilli, A., *Immunochemistry* 1976, 13, 475–476.
- [7] Geraci, D., Oreste, U., Ruffilli, A., *Immunochemistry* 1978, 15, 491–498.
- [8] Feo, S., Cocchiara, R., Geraci, D., *Mol. Immunol.*, 1984, 21, 25–36.
- [9] Costa, M. A., Colombo, P., Izzo, V., Kennedy, H., et al., *FEBS Lett.* 1994, 341, 182–186.
- [10] Colombo, P., Kennedy, D., Ramsdale, T., Costa, M. A., et al., *J. Immunol.* 1998, 160, 2780–2785.
- [11] Takao, T., Yoshida, M., Hong, Y. M., Aimoto S., Shimonishi, Y., *Biomed. Mass Spectrom.* 1984, 11, 549–556.
- [12] Akashi, S., Hirayama, K., Seino, T., Ozawa, S., et al., *Biomed. Environ. Mass Spectrom.* 1988, 15, 541–546.
- [13] Smith, D. L., Zhou, Z., in: McCloskey, J. A., (Ed.) *Methods in Enzymology*, Vol. 193: *Mass Spectrometry*, Academic Press, San Diego 1990, p. 374.
- [14] Nguyen, D. N., Becker, G. W., Rigglin, R. M., *J. Chromatogr.* 1995, 705, 21–45.
- [15] Tancredi, T., Salvadori, S., Amodeo, P., Picone, D., et al., *Eur. J. Biochem.* 1994, 242, 241–247.
- [16] Keil-Dlouha, V., Zylber, N., Imhoff, J.-M., Tong, N. T., Keil, B., *FEBS Lett.* 1971, 16, 291–295.

This article is published jointly with the *European Journal of Mass Spectrometry (EJMS)*, ISSN: 1356–1049). Copyright is entered for *Proteomics*. Therefore, when citing this article please refer to the *Proteomics* citation.