Journal of Chromatography B, 872 (2008) 107–114

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantitative analysis of backbone-cyclised peptides in plants

Ivana Saska[a], Michelle L. Colgrave[a,1], Alun Jones[a], Marilyn A. Anderson[b], David J. Craik[a,*]

[a] Institute for Molecular Bioscience, University of Queensland, 306 Carmody Road, Brisbane, Queensland 4072, Australia
[b] Department of Biochemistry, La Trobe University, Melbourne, Victoria 3086, Australia

ARTICLE INFO

Article history:
Received 9 May 2008
Accepted 17 July 2008
Available online 29 July 2008

Keywords:
Cyclotides
MALDI-TOF MS
Plant peptides
Quantification

ABSTRACT

Progress in understanding the biosynthetic pathway of the cyclotides has been hampered as this unique family of cyclic plant peptides are notoriously difficult to analyse by standard proteomic approaches such as gel electrophoresis. We have developed a simple, rapid and robust strategy for the quantification of cyclotides in crude plant extracts using MALDI-TOF MS making use of generic peptides similar in mass to the analyte as internal standards for calibration. Linearity ($r^2 > 0.99$) over two orders of magnitude (down to femtomole levels) was achieved in plant extracts, allowing quantitative analysis of transgenic and endogenous peptide expression.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Cyclisation of a protein backbone has the capacity to increase a protein's thermodynamic stability and decrease its susceptibility to enzymatic degradation [1,2]. Understanding the biosynthesis of backbone-cyclised (circular) peptides which occur naturally in bacteria, plants and mammals [3,4] is therefore an area of significant interest but progress has been impeded by difficulties associated with quantitatively analysing circular peptides in complex mixtures. By far the largest family of circular peptides is the cyclotides-insecticidal peptides [5,6] produced by plants from the Rubiaceae and Violaceae families, with linear ancestral cyclotides also recently reported in the grasses (Poaceae; [7]). Typically ~30 residues in length, cyclotides are characterised by the conserved spacing of six cysteine residues, which form a knot of three disulfide bonds at their core, and a head-to-tail cyclised protein backbone. The structure of the prototypic cyclotide kalata B1 is shown in Fig. 1(a).

Owing to their compact nature and the fact that each plant produces a large suite of closely related cyclotides [8,9] it has not been possible to characterise cyclotides in plant extracts using methods such as gel electrophoresis and immunoblotting. This is highlighted by the immunoblot in Fig. 1(b) in which polyclonal antibodies raised against kalata B1 were tested against an extract from *Oldenlandia affinis* (Rubiaceae) leaves as well as the purified peptide. *O. affinis* endogenously produces large amounts of the prototypic cyclotide kalata B1 in addition to a suite of other related cyclotides [8]. The RP-HPLC traces in Fig. 1(c) confirm that kalata B1 is present in the *O. affinis* extract, where it is easily identified as a prominent peak that co-elutes with purified kalata B1. Despite its apparent abundance, kalata B1 antibodies do not detect the mature peptide either in the *O. affinis* extract or as purified peptide although they do identify the cyclotide precursor protein (~12 kDa) in the leaf extract [10]. Similarly cyclotides are not detected by protein dyes such as Coomassie blue or silver stain.

Many proteomic studies utilise electrophoresis-based fractionation followed by mass spectrometry to analyse changes in protein abundance under different conditions [11–14]. This approach is not amenable to the detection of backbone-cyclised peptides, such as cyclotides, and possibly overlooks many other peptides present in plants. Nevertheless, previous studies have shown that although cyclotides lack termini they are amenable to analysis by mass spectrometry using both electrospray [9,15,16] and MALDI [17] ionisation techniques. Recently we also showed that cyclotides can be detected in crude leaf extracts using matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) [10,17]. Here we describe and validate a procedure for the quantitative analysis of cyclotides in plants using MALDI-TOF MS calibrated relative to an internal standard. Both endogenous and transgenic kalata B1 production is reproducibly quantified from crude extracts in a rapid process that could

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; kB1, kalata B1.

* Corresponding author. Tel.: +61 7 3346 2019; fax: +61 7 3346 2029.
E-mail address: d.craik@imb.uq.edu.au (D.J. Craik).
[1] Present address: CSIRO Livestock Industries, Queensland Bioscience Precinct, St Lucia, Queensland 4067, Australia.

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.07.023

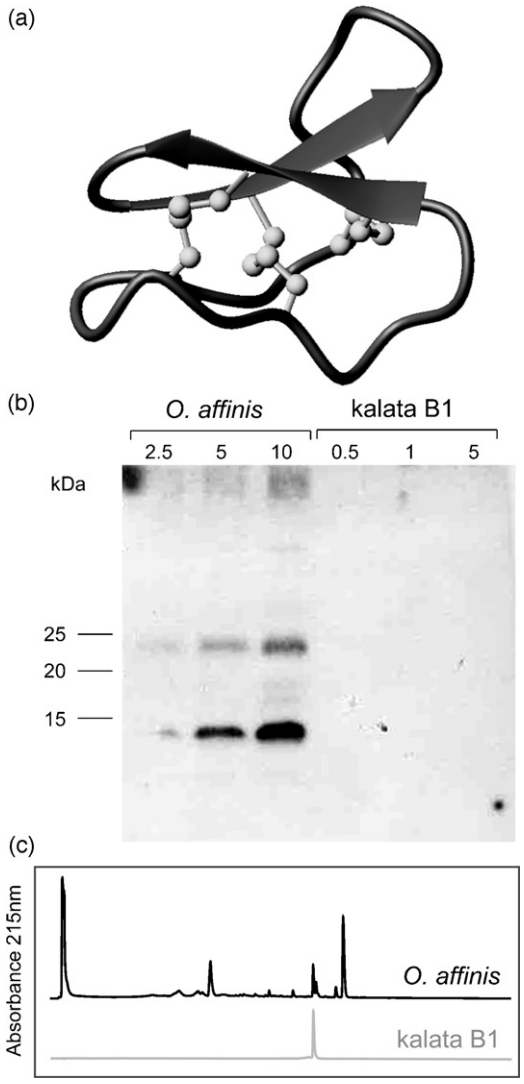

Fig. 1. Structure and characterisation of cyclotides: (a) Representation of the three-dimensional structure of the cyclotide kalata B1 (PDB ID: 1NB1). The six cysteine residues that make up the three disulfide bonds at the core of the protein are shown in ball-and-stick format. (b) Immunoblot analysis of *O. affinis* leaf extracts (2.5–10 mg/mL) and purified kalata B1 (0.5–5 μg) using polyclonal antibodies raised against kalata B1. The antibodies do not detect kalata B1 (2892 Da) but in the *O. affinis* extracts identify a protein with a similar molecular weight to the kalata B1 precursor (12 kDa) as well as another higher molecular weight protein, that is possibly an oligomer. (c) RP-HPLC analysis of *O. affinis* leaf extracts (100 μg) and pure kalata B1 (23 μg). An easily identifiable protein in the *O. affinis* extract elutes at the same time as kalata B1.

potentially be applied to the analysis of other 'recalcitrant' plant peptides.

2. Experimental

2.1. Extraction and purification of kalata B1 from O. affinis

Kalata B1 was extracted from the aerial parts of *O. affinis* plants and purified by RP-HPLC as previously described [18]. The purity of the final product was examined by analytical RP-HPLC under isocratic conditions using a Phenomonex Jupiter C18 column (300 Å, 150 mm × 2.00 mm, 5 μm, with SecurityGuard filter) at a flow rate of 0.3 mL/min and by MALDI-TOF MS as described below.

2.2. Characterisation of kalataB1 by immunoblotting and RP-HPLC

Polyclonal antibodies were raised against kalata B1 conjugated to keyhole limpet hemocyanin in rabbits. Purification of the immune serum on protein-A sepharose (Sigma–Aldrich) and immunoblot analysis was carried out as described in Ref. [19]. For immunoblotting *O. affinis* leaf extracts were prepared in 50% ACN, 0.1% TFA (see below). Aliquots of the extracts standardised for total protein concentration and aliquots of purified kalata B1 were fractionated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane (Amersham Biosciences). The membrane was first incubated with kalata B1 antibodies (2 mg/mL diluted 4000-fold) and then horseradish peroxidase-conjugated goat antibodies directed against rabbit IgG (diluted 10,000-fold; Sigma–Aldrich). An enhanced chemiluminescence kit (Amersham Biosciences) was used to visualise antibody binding.

Aliquots of purified kalata B1 (23 μg) and the *O. affinis* extract (100 μg) were analysed by RP-HPLC using a Phenomonex Jupiter C18 column (300 Å, 150 mm × 2.00 mm, 5 μm, with SecurityGuard filter) at a flow rate of 0.3 mL/min. Protein was eluted using a 5–80% gradient of 90% CH_3CN, 0.05% TFA in 0.05% TFA over 75 min and monitored by UV at 215 nm.

2.3. Expression of kalata B1 in Nicotiana benthamiana leaves

Agrobacterium-mediated transient expression of the cyclotide precursor in *N. benthamiana* leaves was carried out as previously described [17]. Infiltrated leaf material was collected at 2–4 dpi, immediately snap frozen in liquid nitrogen and stored at −80 °C prior to analysis.

2.4. Extraction of plant material for MALDI-TOF MS

Leaf tissue was snap frozen in liquid nitrogen and ground to a fine powder in a pre-chilled mortar and pestle. The ground material was used immediately or stored at −80 °C until required. Ground leaf material was combined with water, 0.1% TFA (v/v) or 50% ACN, 0.1% TFA (v/v) and homogenised using a mortar and pestle or, if conducted in a 1.5 mL tube, vortexed vigorously. Particulate matter was pelleted by centrifugation of the homogenate at 17,000 × *g* for 10 min at 4 °C. The soluble supernatant was collected for subsequent analysis.

2.5. Standardisation of protein concentration

Total protein concentration in the soluble leaf extracts was determined using the bicinchoninic acid (BCA) protein assay (Pierce). The assay is based on colourimetric detection of proteins in solution and was conducted with reference to a bovine serum albumin standard (0.025–1 mg/mL). A mean value of three readings was used to calculate total protein concentration. The extracts were adjusted to the desired concentration using the same solvent in which they were extracted.

2.6. Preparation of kalata B1 calibration solutions

A stock solution (0.5 mg/mL) of kalata B1 was prepared by dissolving lyophilised kalata B1 in Milli Q water and accurately determining the concentration using the UV absorbance of the solution at 280 nm ($\varepsilon = 6050\ M^{-1}\ cm^{-1}$; [20]). A stock solution (0.4 mg/mL) of sunflower trypsin inhibitor-1 (SFTI-1) was prepared in Milli Q water and diluted similarly to the kalata B1 standards as follows. A set of standard solutions ranging in concentration from 1 to 400 μg/mL of kalata B1 was prepared by

dilution of the stock solution in 50% ACN, 0.1% TFA. The standard solutions were, in turn, diluted 10-fold in either solvent or plant extract and combined in a 1:1 ratio (v/v) with the internal standard solution, consisting of the Calibration Mixture 2 (Sequazyme Peptide Mass Standards Kit; Applied Biosystems). The internal standard mixture was prepared as a 1/200 or 1/400 dilution of the concentrated solution provided by the manufacturer in 50% ACN, 0.1% TFA. The 1/400 dilution had a final concentration of 125 fmol/μL angiotensin I, 125 fmol/μL ACTH(1–17), 93.75 fmol/μL ACTH(18–39), 187.5 fmol/μL ACTH(7–38) and 218.75 fmol/μL of insulin (bovine).

2.7. Preparation of MALDI matrix

A saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA; Sigma–Aldrich) was prepared by dissolving the matrix in 50% ACN, 0.1% TFA with 5 mM ammonium phosphate to a final concentration of 5 mg/mL. The solution was vortexed thoroughly, sonicated in a water bath for several minutes, and centrifuged at 18,000 × *g* for 10 min at room temperature. The supernatant was used in the preparation of samples for MALDI-TOF MS.

2.8. Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry

An aliquot of each of the kalata B1 solutions in solvent or extract containing the internal standard was combined 1:1 with the CHCA matrix and mixed thoroughly. When quantification was not required the extracts were mixed directly with the matrix (1:1, v/v). Aliquots (0.6 μL) of the mixtures were spotted on a 192 well plate (Applied Biosystems) and air dried. Mass analysis was carried out in positive ion reflector mode on the 4700 Proteomics Analyzer (Applied Biosystems) using a 200 Hz frequency tripled Nd:YAG laser operating at 355 nm. Fifty spectra at each of 30 randomly selected positions were accumulated per spot between 1000 and 5000 Da using an MS positive ion reflectron mode acquisition method. Calibration of the instrument was carried out using the same Calibration Mixture 2 used as the internal standard. Data were analysed on the accompanying 4000 series Explorer Software.

2.9. Data analysis

Baseline correction and Gaussian smoothing was applied to each mass spectrum. Calibration curves were constructed using the summed peak area of kalata B1 (analyte) relative to an internal standard protein. The first three isotopic peaks (S/N > 5) of each protein signal were used in the calculations. Average values were determined from three replicate analyses unless otherwise stated and are presented ± standard deviation. The relative area was not calculated when either the analyte or internal standard mass peaks were not detected above S/N > 5 in all three replicates. Linear regression analysis was performed in GraphPad Prism, which was also used to determine unknown sample concentrations by fitting the linear equation $y = mx + c$.

3. Results and discussion

This study describes a MALDI-TOF MS based approach for the quantification of backbone-cyclised peptides known as cyclotides in plants. Despite the difficulty in identifying cyclotides using gel electrophoresis or by immunoblotting it is possible to detect them in crude leaf extracts using mass spectrometry. MALDI-TOF mass spectrometry is an extremely sensitive technique for the analysis of proteins by mass. As a result of its tolerance to salts and ability to analyse complex mixtures, MALDI-TOF MS is regularly used in the detection and sequencing of proteins, most notably as part of large-scale proteomics initiatives [11–14]. The robustness of the technique has been well demonstrated by its use to analyse whole bacterial cells for taxonomic identification [21], proteins and peptides *in situ* [22–24] and for metabolite profiling [25]. Yet despite these advantages MALDI-TOF MS is not commonly employed for the quantification of peptides in complex mixtures, such as plant tissue extracts. Concerns with poor spot-to-spot reproducibility and variations in signal ion intensities have limited the adoption of MALDI-TOF MS as a quantitative tool [26–28]. Nevertheless, quantitative peptide MALDI quantification has been applied to study of biological molecules including oligosaccharides [29], oligonucleotides [30] and peptides [31–34]. We show here that MALDI-TOF MS can be a sensitive and robust technique for the quantitative analysis of peptide production in plants. The cyclotide kalata B1 was detected down to 0.025 μg/mL (5.2 femtomole) both as the pure native peptide and when part of heterogenous plant extracts. A direct correlation between the concentration of kalata B1 and its response by MALDI-TOF MS was observed when standardised relative to peptides comparable in mass to the analyte. Without the calibration provided by this internal standard the response of kalata B1 was more variable ($r^2 = 0.9094$). The ease and robustness of the approach in quantifying cyclotides suggests that it could be successfully adapted for the quantification of other plant peptides not amenable to 'standard' proteomics techniques.

3.1. Identification of cyclotides from plant extracts using MALDI-TOF MS

Soluble extracts prepared from leaf tissue in 50% ACN, 0.1% TFA were analysed by MALDI-TOF MS in α-cyano-4-hydroxycinnamic acid (CHCA). As shown in Fig. 2, a series of masses in the range expected for cyclotides (2800–3400 Da) is observed in extracts from *O. affinis* leaves. Eight of the masses correspond to the most abundant of 17 cyclotides that have previously been characterised in the aerial portions of the plant [8], including the cyclotide kalata B1, and are labelled using information from CyBase, a repository of cyclotide gene and protein sequences [35]. The small amount of material required to detect a majority of the circular peptides (10–200 ng of total protein per spot) indicated that MALDI-TOF MS can provide a rapid and sensitive means of screening plants for cyclotide production. We wanted to test whether it was possible to use this rapid screening procedure for quantitative studies in order to compare kalata B1 production in different plants and under different conditions.

3.2. Calibration of the peptide signal using an internal standard

MALDI-TOF MS is not commonly employed for the quantification of peptides in complex mixtures, such as plant tissue extracts, because of the inherent heterogeneity of the matrix-sample preparation, which leads to poor reproducibility of the signal both within

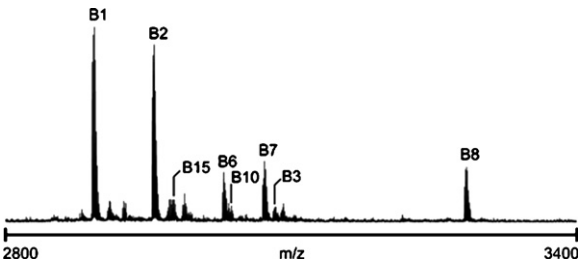

Fig. 2. Identification of cyclotides present in *O. affinis* leaves by MALDI-TOF MS. On the basis of their masses eight kalata cyclotides were identified in the extract.

and between samples spots [36,37]. To improve reproducibility we calibrated the kalata B1 mass signal against an internal standard peptide introduced into the sample prior to mass analysis. Although a related cyclotide, kalata B2, has previously been shown to serve as a reliable internal standard for the quantification of kalata B1 [33] it was not suitable for quantitative studies in *O. affinis* because it occurs naturally and in high abundance in the plant. Instead we used a peptide with a mass similar to kalata B1 but otherwise structurally unrelated to it as the internal standard, thereby enabling the quantitative analysis of cyclotides in multiple plant species. In this study, we adopted the sample preparation methodology of Colgrave et al. [33], as it was found that matrix selection and application methods were critical to the production of uniform co-crystals and that the data acquisition method could overcome problems of poor spot-to-spot reproducibility and signal degradation.

A mixture of peptides with a mass range corresponding to cyclotides was used in the construction of a calibration curve for the cyclotide kalata B1. The internal standard consisted of the commercially available Calibration Mixture 2 from Applied Biosystems, which includes five peptides: angiotensin I (*m*/*z* 1296.69), ACTH(1–17 clip) (*m*/*z* 2093.09), ACTH(18–39 clip) (*m*/*z* 2465.20), ACTH(7–38 clip) (*m*/*z* 3657.93) and bovine insulin (*m*/*z* 5730.61). Stock solutions of kalata B1 were prepared from protein extracted from *O. affinis* and purified by RP-HPLC. Protein concentration was determined using UV absorbance at 280 nm. Dilutions of the stock solution in 50% ACN, 0.1% TFA were mixed 1:1 with an aliquot of the internal standard solution made up in the same solvent, combined with CHCA and spotted onto a MALDI sample plate for mass analysis. To decrease the variation arising from sample spot heterogeneity, multiple spectra (50) were acquired at multiple positions (30) randomly selected from each spot and accumulated into a single spectrum. Calibration curves were generated by plotting the area of the kalata B1 peaks relative to the area of the internal standard peptides, across a range of different kalata B1 concentrations. Relative area was calculated using the area of the first three peaks in the isotopic cluster of the analyte and the internal standard protein as shown schematically in Fig. 3(a).

As shown in Fig. 3(b–d) a good linear correlation, as defined by $r^2 \geq 0.99$ [34,38], was observed between the concentration of kalata B1 and the relative area of the kalata B1 peaks when peptides similar in molecular mass to the cyclotide were used for internal calibration. Linearity across two orders of magnitude of analyte concentration (0.025–5.0 μg/mL) was achieved. At kalata B1 concentrations >5.0 μg/mL saturation of the signal was observed. Without reference to an internal standard the correlation between the absolute area and peptide concentration was weak, especially at high kalata B1 concentrations, as shown in Fig. 3(e). The molecular mass of the internal standard was a determining factor in the efficacy of the calibration; as the difference between the molecular mass of kalata B1 [M+H$^+$ = 2892] and the internal standard proteins increased, the linearity between the relative area and concentration of kalata B1 decreased. Consequently, a highly linear relationship was observed when the area of kalata B1 was standardised relative to the ACTH(18–39) peptide fragment [M+H$^+$ = 2466.7], and this decreased only slightly when plotted relative to the ACTH(1–17) peptide fragment [M+H$^+$ = 2094.5]. Similarly, when kalata B1 was standardised relative to the ACTH(7–38) peptide fragment [M+H$^+$ = 3657.9] with mass ~767 Da higher than that of kalata B1, a good correlation between the relative area and peptide concentration was observed with an r^2 value of >0.99 (data not shown). However, once the mass difference between the analyte (kalata B1) and the internal standard exceeded ~1 kDa (~30% analyte mass), as with angiotensin I [M+H$^+$ = 1297.5], the corre-

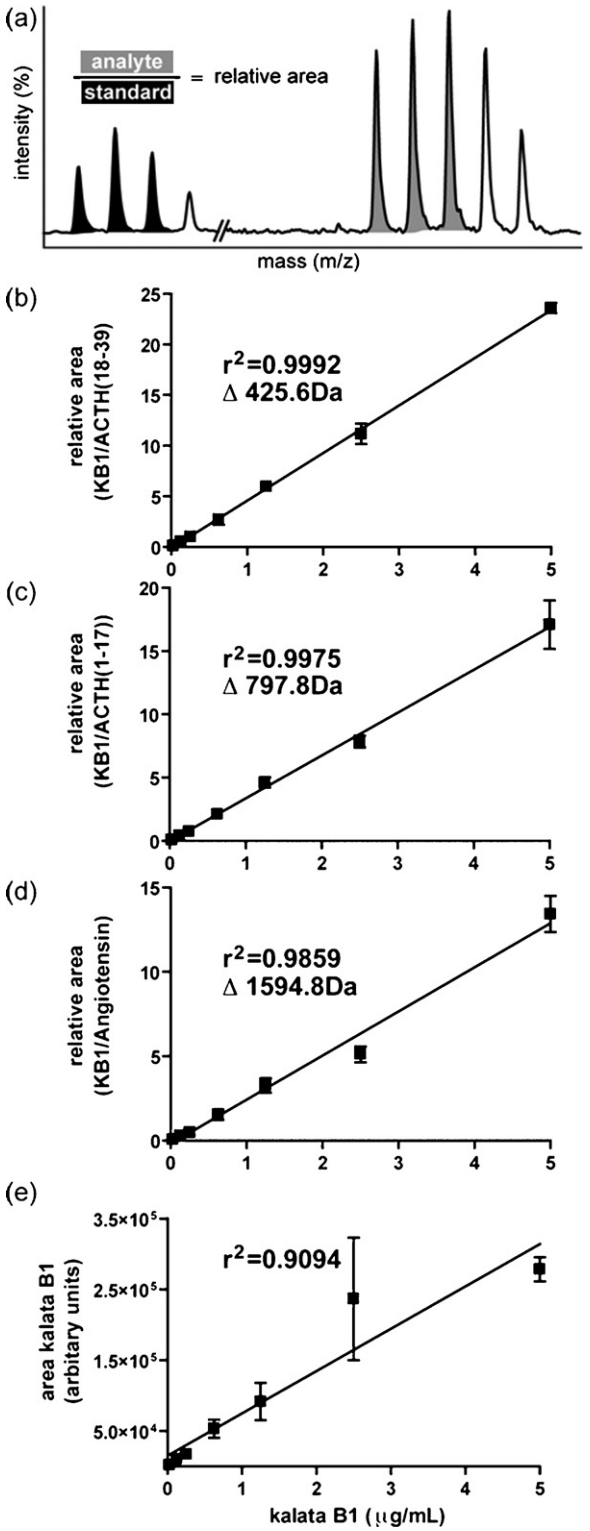

Fig. 3. Calibration of peptide concentration using different internal standards: (a) Schematic representation of the isotopes used to calculate the relative area of the kalata B1 peptide. (b–e) Kalata B1 calibration curves generated in solvent. The relative area of the kalata B1 mass signal was calculated with reference to different peptides in the calibration mixture; (b) ACTH(18–39) (c) ACTH(1–17) and (d) angiotensin I. (e) A plot of kalata B1 peak area without reference to an internal standard. Each point represents the average of three replicates ± SD. Correlation coefficients (r^2) and the difference in mass between kalata B1 and the internal standard protein used are labelled on each plot.

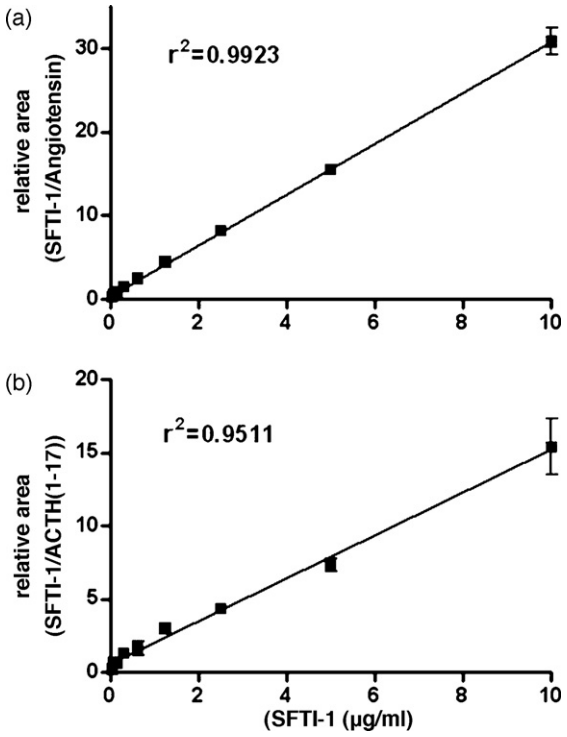

Fig. 4. Application of the quantification method to the unrelated cyclic peptide sunflower trypsin inhibitor-1 (SFTI-1). The relative area of the SFTI-1 mass signal was calculated with reference to different peptides in the calibration mixture: (a) ACTH(1–17) and (b) angiotensin I.

lation between relative area and concentration broke down. The ACTH(18–39) peptide fragment was used as an internal standard for subsequent kalata B1 calibration curves. The general applicability of the quantitative approach for cyclic peptides was tested by examining the potent Bowman-Birk inhibitor sunflower trypsin inhibitor-1 (SFTI-1), which contains a head-to-tail cyclic backbone and a single disulfide bond [39,40]. Fig. 4 shows the linear relationship between the concentration of SFTI-1 and the relative area of the SFTI-1 peaks using peptides similar in molecular mass for internal calibration. Linearity ($r^2 \geq 0.99$) across two orders of magnitude of analyte concentration (0.039–10.0 μg/mL) was achieved using angiotensin I, which differs in mass by 216.2 Da, *i.e.* within 15% of the analyte molecular weight. The correlation between analyte concentration and relative area was decreased ($r^2 \sim 0.95$) however, when SFTI-1 was compared to the ACTH(1–17) peptide fragment. The mass difference between these two peptides was 580.8 Da or ~38% of the analyte molecular weight. These findings support the hypothesis that an unrelated peptide may be used as an internal standard, but it must be similar (±30%) in mass to the analyte.

The use of internal standards for the calibration of mass signals is not new but has predominantly been restricted to isotopic variants of the analyte or structurally related analogues [26,41–43], a requirement that has limited the applicability of the procedure to the subset of analytes for which appropriate internal standards are available and to more highly purified samples. The results presented here indicate that within a restricted mass range, peptides structurally unrelated to the analyte can be used as internal standards. By using a peptide calibration mixture rather than a specific internal standard it may be possible to extend the effective mass range of quantification, allowing multiple peptides within an extract to be quantified simultaneously.

3.3. MALDI-TOF MS analysis of kalata B1 in plant extracts

To quantify cyclotide production *in planta* it was necessary to confirm that the correlation between kalata B1 and the internal standard in solvent persisted in plant extracts. Calibration curves were prepared using *O. affinis* and *N. benthamiana* leaf extracts to enable both endogenous and transgenic cyclotide production to be analysed. The concentration of the *N. benthamiana* extract was standardised to 1 mg/mL total protein but the *O. affinis* extract had to be used at a much lower concentration to avoid saturation of the signal by endogenous levels of cyclotides. To allow for variation in endogenous cyclotide production, two calibration curves were constructed using *O. affinis* extracts, adjusted to 0.02 and 0.05 mg/mL total protein, respectively. In the absence of kalata B1 no other peaks were detected in the same mass range in the *N. benthamiana* extracts (not shown).

As seen in Fig. 5(a–c) the concentration of kalata B1 in both the *N. benthamiana* and *O. affinis* extracts was directly proportional ($r^2 > 0.99$) to the relative area of the cyclotide peaks analysed by MALDI-TOF MS. This relationship was maintained over two orders of magnitude (0.025–2.5 μg/mL kalata B1) in the extracts, confirming that even in complex mixtures kalata B1 can be quantified reproducibly. The *O. affinis* calibration curves were adjusted for

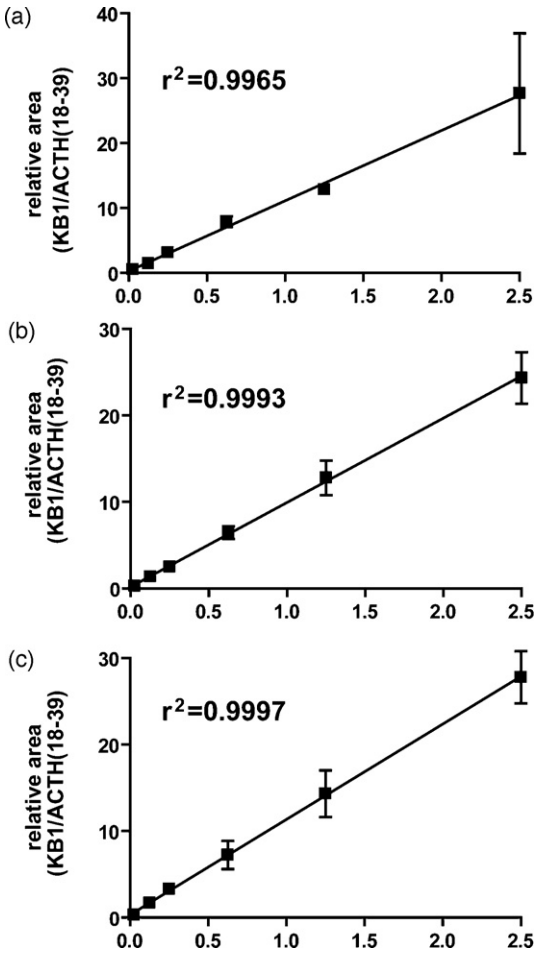

Fig. 5. Kalata B1 calibration curves prepared in leaf extracts. Calibration curves were prepared in *O. affinis* leaf extracts standardised to 0.05 mg/mL (a) and 0.02 mg/mL (b) of total protein and in *N. benthamiana* leaf extracts standardised to 1 mg/mL(c) of total protein. The area of kalata B1 was calculated relative to the ACTH(18–39) peptide fragment, which was added to each sample as part of a calibration protein mixture. Each point represents the average of three replicates ± SD. Correlation coefficients (r^2) are indicated.

Table 1
Reproducibility and accuracy of quantification

Theoretical concentration (μg/mL)	Mean of measured concentration (μg/mL)	Reproducibility CV (%)	Inaccuracy (%)
O. affinis 0.05 mg/mL			
2.5	1.882	17.3	24.7
0.625	0.537	8.2	14.1
0.125	0.105	11.0	16.0
Average		12.2	18.3
O. affinis 0.02 mg/mL			
2.5	2.143	4.2	14.3
0.625	0.656	2.9	5.0
0.125	0.133	18.2	6.4
Average		8.4	8.5
N. benthamiana 1 mg/mL			
2.5	2.580	16.0	3.2
0.625	0.720	3.4	15.2
0.125	0.140	22.0	12.0
Average		13.8	10.1

endogenous levels of kalata B1 by subtracting the relative area of kalata B1 in a control sample from each of the data points.

The reproducibility of the procedure was assessed by measuring the amount of kalata B1 in control samples containing known concentrations of the peptide. Samples were prepared independently in both *O. affinis* and *N. benthamiana* extracts containing 0.125, 0.625 and 2.5 μg/mL of kalata B1 plus an endogenous control (for the *O. affinis* extracts). The concentration of kalata B1 in the samples was estimated by fitting the relative area values to the linear equations derived from the calibration curves in Fig. 5. A comparison of the theoretical and experimentally measured kalata B1 concentrations is given in Table 1. The method achieved average accuracy of ≥90% for quantification of kalata B1 in samples prepared in the *O. affinis* (0.02 mg/mL) and *N. benthamiana* extracts and >80% and in the *O. affinis* (0.05 mg/mL) extracts. Intra-assay coefficients of variation (CV) were on average <15%. To assess sample-to-sample robustness three sets of quality controls were prepared from three independent *N. benthaminana* extracts and each extract was prepared and analysed in triplicate. At each concentration analysed the inter-assay CV values were <10%, which is comparable to or lower than the level of variation accepted in other recent quantitation studies [25,44].

3.4. Analysis of endogenous cyclotide production

Endogenous levels of kalata B1 in *O. affinis* extracts were quantified by fitting the relative area of the kalata B1 signal from a control sample (no kalata B1 added) to a linear equation derived from the calibration curve. Using this method it was estimated that the 0.02 and 0.05 μg/mL *O. affinis* extracts used to prepare the calibration curves contained 0.030 μg/mL (SD ± 0.009) and 0.057 μg/mL (SD ± 0.014) kalata B1, respectively. Adjusted for total protein concentration this indicates that the extract contained 1.32 ± 0.18 μg kalata B1 per mg of soluble protein. This value is consistent with previous estimates of cyclotide concentration at ∼2 mg/g of dry weight material [45] and highlights the high proportion of these proteins in plants, especially given that kalata B1 is only one of a suite of different cyclotides produced. The abundance of cyclotides in *O. affinis* suggests that they naturally accumulate in plants, consistent with their proposed role in plant defence. Like the cysteine rich defensins, cyclotides may be stored in plant tissue until pathogen attack, when they are either mobilised or consumed by the invading organism [46,47]. Both kalata B1 and kalata B2 inhibit the development of caterpillar larvae [5,6] and achieve this effect by disrupting the luminal tissue within the digestive tract of the insect [48]. Recently cyclotides have also been reported to have nematocidal and molluscicidal activities [49,50], further emphasising their importance as host defence agents. The ability to readily quantify cyclotide production in plants should aid in the identification of the environmental cues that stimulate the production of cyclotides in plants.

3.5. Quantification of transgenic kalata B1 production in N. benthamiana

Transient expression of the kalata B1 precursor in *N. benthamiana* produces cyclic kalata B1 as well as a suite of mis-processed linear peptides [10,17]. The linear peptides correspond in mass to linear kalata B1, with the successive addition of residues from the C-terminal pro-region of the precursor (GLPSLAA). These are represented schematically in Fig. 6(a) using the labels +G, +GL, +GLP and +GLPS. In Fig. 6(b) the species are labelled on a mass spectrum of an extract of *N. benthamiana* leaves 3 days after infiltration

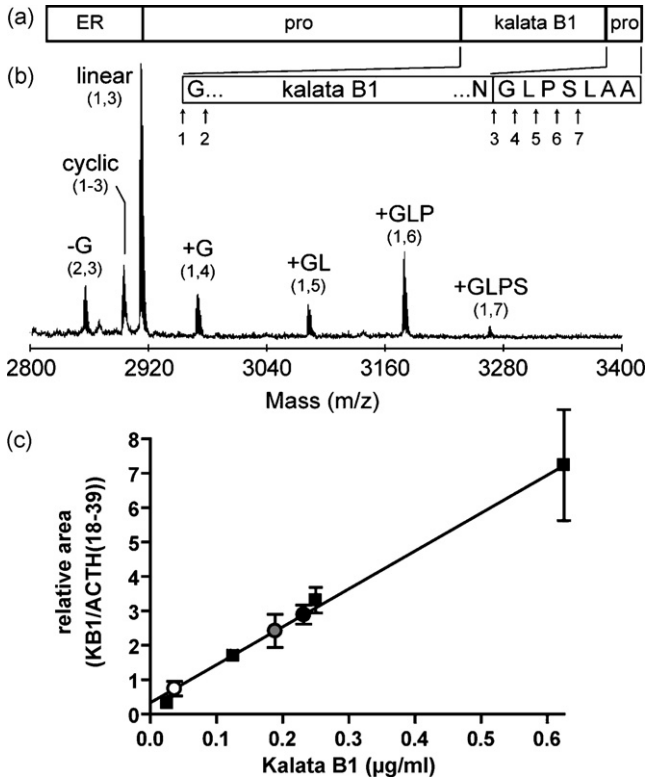

Fig. 6. Quantification of transgenic kalata B1 production in *N. benthamiana*: (a) Schematic representation of the kalata B1 precursor protein which contains an N-terminal endoplasmic reticulum signal sequence and the kalata B1 domain flanked by N- and C-terminal pro regions. Transient expression of the precursor in *N. benthamiana* leaves leads to the production of cyclic kalata B1 plus a range of truncated linear peptides that include residues from the C-terminal pro region (GLPSLAA). These are represented below with the different residues at which the peptides terminate labelled 1–7. (b) Representative mass spectrum of an extract of *N. benthamiana* leaves 3 days after infiltration with the kalata B1 precursor construct. Kalata B1 proteins are labelled according to the residues they lack or have in addition to linear kalata B1. The numbers below each label refer to the termini identified in (a). (e) The amount of cyclic kalata B1 produced in *N. benthamiana* leaves 2–4 days post infiltration (dpi) of the kalata B1 precursor construct was determined by fitting the relative area of kalata B1 in each of the samples onto the *N. benthamiana* calibration curve (black squares) in Fig. 4c (in circles, white = 2 dpi, grey = 3 dpi and black = 4 dpi). Each point represents the average of three replicates ± SD.

with agrobacterium harbouring the kalata B1 precursor. From this spectrum it appears that cyclic kalata B1 is expressed in low abundance in the transgenic plant. To quantify this, the relative area of cyclic kalata B1 in 1 mg/mL extracts of *N. benthamiana* leaves 2–4 days post infiltration (dpi) was plotted onto the *N. benthamiana* calibration curve. As seen in Fig. 6(c) a general increase in kalata B1 concentration is observed over time from 0.04 to 0.23 μg/mL. Despite over-expression of the kalata B1 precursor driven by a 35S constitutive promoter the amount of cyclic kalata B1 produced in *N. benthamiana* from 2 to 4 dpi is ~6–30-fold lower than is produced in *O. affinis*.

The approach described here may be applied to the comparative analysis of very small changes in protein production as is the case for transgenic cyclotide production in *N. benthamiana*. We have demonstrated that it is possible to measure relative differences between sample populations without the need for labelling or other treatments. Overall, transgenic production of kalata B1 in leaves was well below endogenous levels, reaching a maximum of 0.2% of soluble protein 4 days after agroinfiltration. Several factors may have contributed to this lower level of production. The possibility that the kalata B1 precursor was not being expressed in *N. benthamiana* was discounted by immunoblot detection of the precursor protein, which appeared to be expressed at higher levels than in *O. affinis* (data not shown).

3.6. Analysis of protein processing—relative quantification of transgenic cyclotide production

The processing of transgenically expressed kalata B1 in *N. benthamiana* leaves was monitored from 2 to 4 dpi. From Fig. 6(c) it is evident that the amount of cyclic kalata B1 increased slightly over this time period. To ascertain what changes were occurring in the other linear protein species we measured their relative area at each time point. Fig. 7 shows a plot of the relative area of each of the different cyclotides species from 2 to 4 dpi. The fact that the linear correlation between kalata B1 and the internal standard proteins was maintained over ~1 kDa mass range suggested that within this range the linear correlation would also hold for the closely related proteins.

The quantification data indicate that the amount of the longer species (+GL, +GLP) progressively decreases as the amount of the shorter species (−G, linear, +G) increases. This finding confirms the prediction made from qualitative observations that the linear cyclotide proteins are progressively trimmed from the C-terminus

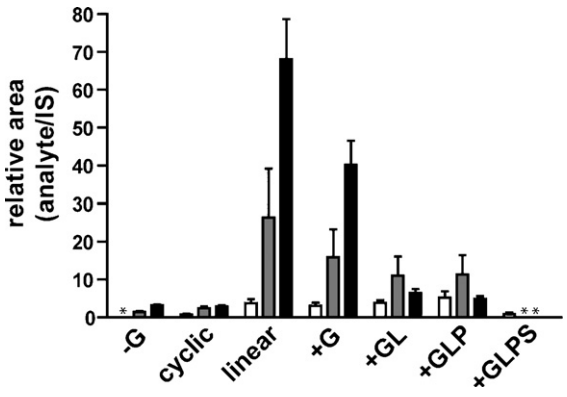

Fig. 7. Comparative analysis of protein processing using MALDI-TOF MS: a plot of the relative area of each of the seven kalata B1 proteins identified in *N. benthamiana* leaves 2 dpi (white), 3 dpi (grey) and 4 dpi (black) of the kalata B1 precursor construct. Labelling of cyclotide species is the same as in Fig. 5. Asterisks indicate that the isotopic cluster of that peptide species could not be detected above S/N of 5:1 in all four replicates.

over time [17]. The increase in the total relative area of all cyclotide species over the 2–4 dpi period also highlights that the trimming process occurs against a background of overall increasing cyclotide protein accumulation, consistent with increasing precursor protein expression over this time (data not shown). This overall increase in peptide production could not be confirmed without reference to an internal standard to normalise the data. The changes observed represent only very small increments in the amount of protein (femtomole), which could not be readily discerned using less sensitive techniques. At higher concentrations, where the relative area of the analyte exceeds the range observed in the *N. benthamiana* calibration curve (Fig. 5c) the data are less accurate but still indicative of overall increases in peptide concentration. The quantitative data suggested that the kalata B1 precursor was being processed more slowly or was being mis-processed in *N. benthamiana*. Aberrant processing of kalata B1 was confirmed by analysing the relative abundance of the different cyclotide species produced in *N. benthamiana*, which were shown to increase at a much faster rate than the circular peptide.

3.7. Applicability of the approach

A number of methods employ mass spectrometry for protein quantification. The most common of these, such as ICAT, iTRAQ and AQUA, use chemically indistinguishable tags to change the mass of a protein and are designed to minimise variations in protein ionisation efficiencies. The procedure used for the quantification of cyclotides has certain of advantages over other MS quantification techniques. Most importantly, it does not require the often extensive sample preparation and fractionation involved in analysing labelled protein populations, such as 2-DG and liquid chromatography, which can exclude peptides. It is also a label-free approach and avoids the error associated with differential labelling efficiencies and side reactions. Furthermore, because a mixture of proteins is used for calibration, the technique is potentially applicable to multiple proteins in a sample at one time and does not require a high investment in the production of specific internal standards. The need to establish a direct correlation between the internal standard and the analyte does, however, disadvantage the method relative to the use of internal standards that are chemically identical to the analyte and therefore have the same ionisation properties. For relative quantification of similar proteins to kalata B1 it is probably not necessary to produce a separate standard curve for each of the proteins in a mixture, as within an approximately 1 kDa (<30% of analyte molecular weight) range, the relationship between the internal standard and the analyte did not vary significantly.

4. Conclusions

The results presented here demonstrate that MALDI-TOF MS can serve as a viable approach for the detection and quantification of peptides from heterogenous mixtures that would otherwise be difficult to analyse by standard proteomic methods. The simple procedure outlined here used an internal standard that is similar to the peptide of interest in mass but structurally unrelated to it. This allowed the analysis of cyclotide production in both endogenous and transgenic contexts and provided a reliable means of addressing questions about the novel biosynthesis of circular peptides. The ability to monitor processing events at high sensitivity (femtomole) provides a platform for future analysis of cyclotide function and biosynthesis under different external treatments that was previously not accessible to biochemical analysis. Given the apparent ease of the approach and its potential for the concomitant analysis

of multiple peptides we expect it will be a valuable contribution to the proteomics toolbox.

Acknowledgements

This work was supported by a grant from the Australian Research Council (ARC). DJC is an ARC Professorial Fellow.

References

[1] R.J. Clark, H. Fischer, L. Dempster, N.L. Daly, K.J. Rosengren, S.T. Nevin, F.A. Meunier, D.J. Adams, D.J. Craik, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 13767.
[2] M.L. Colgrave, D.J. Craik, Biochemistry 43 (2004) 5965.
[3] D.J. Craik, Science 311 (2006) 1563.
[4] M. Trabi, D.J. Craik, Trends Biochem. Sci. 27 (2002) 132.
[5] C.V. Jennings, J. West, C. Waine, D.J. Craik, M.A. Anderson, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 10614.
[6] C.V. Jennings, K.J. Rosengren, N.L. Daly, M. Plan, J. Stevens, M.J. Scanlon, C. Waine, D.G. Norman, M.A. Anderson, D.J. Craik, Biochemistry 44 (2005) 851.
[7] J.P. Mulvenna, J.S. Mylne, R. Bharathi, R.A. Burton, N.J. Shirley, G.B. Fincher, M.A. Anderson, D.J. Craik, Plant Cell 18 (2006) 2134.
[8] M.R.R. Plan, U. Göransson, R.J. Clark, N.L. Daly, M.L. Colgrave, D.J. Craik, ChemBioChem 8 (2007) 1001.
[9] M. Trabi, D.J. Craik, Plant Cell 16 (2004) 2204.
[10] A.D. Gillon, I. Saska, C.V. Jennings, R.F. Guarino, D.J. Craik, M.A. Anderson, Plant J. 53 (2008) 505.
[11] R. Aebersold, M. Mann, Nature 422 (2003) 198.
[12] P. Chaurand, F. Luetzenkirchen, B. Spengler, J. Am. Soc. Mass Spectrom. 10 (1999) 91.
[13] K. Gevaert, J. Vandekerckhove, Electrophoresis 21 (2000) 1145.
[14] J.K.C. Rose, S. Bashir, J.J. Giovannoni, M.M. Jahn, R.S. Saravanan, Plant J. 39 (2004) 715.
[15] U. Göransson, D.J. Craik, J. Biol. Chem. 278 (2003) 48188.
[16] D.C. Ireland, M.L. Colgrave, D.J. Craik, Biochem. J. 400 (2006) 1.
[17] I. Saska, A.D. Gillon, N. Hatsugai, R.G. Dietzgen, I. Hara-Nishimura, M.A. Anderson, D.J. Craik, J. Biol. Chem. 282 (2007) 29721.
[18] D.J. Craik, N.L. Daly, T. Bond, C. Waine, J. Mol. Biol. 294 (1999) 1327.
[19] E. Harlow, D. Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, USA, 1999.
[20] S.C. Gill, P.H. von Hippel, Anal. Biochem. 182 (1989) 319.
[21] J.O. Lay, Mass Spectrom. Rev. 20 (2001) 172.
[22] R.M. Caprioli, T.B. Farmer, J. Gile, Anal. Chem. 69 (1997) 4751.
[23] T. Kondo, S. Sawa, A. Kinoshita, S. Mizuno, T. Kakimoto, H. Fukuda, Y. Sakagami, Science 313 (2006) 845.
[24] L. Li, R.W. Garden, E.V. Romanova, J.V. Sweedler, Anal. Chem. 71 (1999) 5451.
[25] P.D. Fraser, E.M.A. Enfissi, M. Goodfellow, T. Eguchi, P.M. Bramley, Plant J. 49 (2007) 552.
[26] D.C. Muddiman, A.I. Gusev, A. Proctor, D.M. Hercules, R. Venkataramanan, W. Diven, Anal. Chem. 66 (1994) 2362.
[27] A.J. Nicola, A.I. Gusev, A. Proctor, E.K. Jackson, D.M. Hercules, Rapid Commun. Mass Spectrom. 9 (1995) 1164.
[28] L.M. Preston, K.K. Murray, D.H. Russell, Biol. Mass Spectrom. 22 (1993) 544.
[29] G. Jiang, T. Vasanthan, J. Agric. Food Chem. 48 (2000) 3305.
[30] L.K. Zhang, M.L. Gross, J. Am. Soc. Mass Spectrom. 11 (2000) 854.
[31] A. Poljak, C.A. McLean, P. Sachdev, H. Brodaty, G.A. Smythe, J. Neurosci. Res. 75 (2004) 704.
[32] M. Bucknall, K.Y.C. Fung, M.W. Duncan, J. Am. Soc. Mass Spectrom. 13 (2002) 1015.
[33] M.L. Colgrave, A. Jones, D.J. Craik, J. Chromatogr. A 1091 (2005) 187.
[34] H. John, J. Hierer, O. Haas, W.-G. Forssmann, Anal. Biochem. 362 (2007) 117.
[35] J.P. Mulvenna, C. Wang, D.J. Craik, Nucleic Acids Res. 34 (2006) D192.
[36] R.W. Garden, J.V. Sweedler, Anal. Chem. 72 (2000) 30.
[37] P. Önnerfjord, S. Ekström, J. Bergquist, J. Nilsson, T. Laurell, G. Marko-Varga, Rapid Commun. Mass Spectrom. 13 (1999) 315.
[38] P.J.M. Wakkers, H.B.A. Hellendoorn, G.J.O. De Weegh, W. Heerspink, Clin. Chim. Acta 64 (1975) 173.
[39] S. Luckett, R. Santiago Garcia, J.J. Barker, A.V. Konarev, P.R. Shewry, A.R. Clarke, R.L. Brady, J. Mol. Biol. 290 (1999) 525.
[40] M.L.J. Korsinczky, H.J. Schirra, K.J. Rosengren, J. West, B.A. Condie, L. Otvos, M.A. Anderson, D.J. Craik, J. Mol. Biol. 311 (2001) 579.
[41] J. Gobom, K.O. Kraeuter, R. Persson, H. Steen, P. Roepstorff, R. Ekman, Anal. Chem. 72 (2000) 3320.
[42] M.-J. Kang, A. Tholey, E. Heinzle, Rapid Commun. Mass Spectrom. 15 (2001) 1327.
[43] O.A. Mirgorodskaya, Y.P. Kozmin, M.I. Titov, R. Körner, C.P. Sonksen, P. Roepstorff, Rapid Commun. Mass Spectrom. 14 (2000) 1226.
[44] T. Linke, A.C. Ross, E.H. Harrison, J. Chromatogr. A 1123 (2006) 160.
[45] L. Gran, Lloydia 36 (1973) 174.
[46] W.F. Broekaert, F.R.G. Terras, B.P.A. Cammue, R.W. Osborn, Plant Physiol. 108 (1995) 1353.
[47] B. Thomma, B.P.A. Cammue, K. Thevissen, Planta 216 (2002) 193.
[48] B.L. Barbeta, A.T. Marshall, A.D. Gillon, D.J. Craik, M.A. Anderson, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 1221.
[49] M.L. Colgrave, A.C. Kotze, Y.H. Huang, J. O'Grady, S. Simonsen, D.J. Craik, Biochemistry 47 (2008) 9805.
[50] M.R.R. Plan, I. Saska, A.G. Cagauan, D.J. Craik, J. Agric. Food Chem. 56 (2008) 5237.