Investigation of Calmodulin–Peptide Interactions Using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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In this report, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used to study the binding interactions between calmodulin and two target peptides (melittin and substance P). Various matrix conditions were tested and the less acidic matrix DHAP and THAP were found to favor the survival of the intact calcium–calmodulin as well as the calmodulin–peptide complexes. However, the application of direct MALDI-MS to detect the intact complexes turned out to be very difficult due to the dissociation of the complexes and the formation of nonspecific aggregates. In contrast, the specific binding of the target peptides to calmodulin could be easily deduced using intensity-fading (IF) MALDI-MS. Compared with the nonbinding control, clear reduction in the ion abundances of the target peptides was observed with the addition of calmodulin. Relative binding affinities of different peptides towards the protein could also be estimated using IF-MALDI-MS. This study may extend the application of IF-MALDI-MS in the analysis of noncovalent complexes and offer a perspective into the utility of MALDI-MS as an alternative approach to study the peptides binding to calmodulin. (J Am Soc Mass Spectrom 2009, 20, 576–583) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Calmodulin (CaM) is an acidic ubiquitous calcium-binding protein in eukaryote cells and engages in many important biological processes, such as cell division, protein phosphorylation/dephosphorylation, metabolic pathways, and signal transduction [1, 2]. Generally, calcium binding to calmodulin leads to conformational changes of the protein and enables the protein to bind and activate a number of target enzymes. However, these target enzymes are usually too large to study, and short peptide sequences derived from their CaM-binding domains are mostly used for studies of the CaM-target protein interactions [3]. Moreover, many natural occurring peptides (e.g., melittin) and endogenous neuropeptides are also known to exert their biological effects through interactions with calmodulin [4, 5]. Therefore, studies on the peptide binding to calmodulin have attracted considerable interests because such interactions may not only provide the molecular basis that underlies their bioactivities but also help to understand the mechanisms of CaM binding to protein [2, 3].

Several methods have been employed to study the binding interactions between calmodulin and its target peptides, including gel electrophoresis [4], fluorescence [5], X-ray crystallography [6], NMR [7], calorimetry [8], surface plasmon resonance [9], and dynamic light scattering [10]. However, most of these methods require large quantities of samples or fluorescent labeling of calmodulin. Soft ionization mass spectrometry has shown promising applications in the analysis of noncovalent complexes due to its low sample consumption, fast analysis time, and no need of labeling [11–13]. Electrospray ionization mass spectrometry (ESI-MS) has long been recognized as the method of choice to study noncovalent interactions because the intact complexes can survive the gentle ionization process and be maintained in the gas phase [11–13]. To date, binding interactions of several target peptides with calmodulin have been studied using ESI-MS [14–20]. The detailed information on the calmodulin–peptide interactions could also be obtained by coupling ESI-MS with other techniques (e.g., cross-linking and hydroxyl radicals) [21, 22]. In addition, new technique such as laser-induced liquid beam ionization/desorption mass spectrometry (LILBID-MS) has also been developed for the analysis of noncovalent complexes and applied to study the calmodulin–peptide interactions [23].

Although Matrix-assisted laser desorption/ionization (MALDI) is also regarded as a soft ionization technique, it has been less frequently used in the analysis of

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noncovalent complexes than ESI-MS [11, 24]. The main drawbacks of MALDI-MS are the crystallization before analysis and the necessity of laser irradiation, which may destroy the specific interactions presented in the solution. Meanwhile, nonspecific aggregates are often formed during the MALDI process. However, MALDI-MS still has several intriguing advantages over ESI-MS, such as high sensitivity, salt tolerance, easy automation, and spectrum evaluation. And many efforts have been devoted to the application of MALDI-MS for the detection of noncovalent complexes (see reference [24] for a review). Most of the previous studies focused on the detection of the intact complexes using direct MALDI-MS. However, in these cases, special precaution must be taken throughout the procedure because various experimental conditions (e.g., matrix solutions and instrumental parameters) may result in the dissociation of the intact complexes and the formation of nonspecific aggregates. Until now, no general procedure has been established to detect the intact noncovalent complexes using direct MALDI-MS. Recently, Aviles and coworkers have developed an alternative strategy to detect the presence of noncovalent complexes in solution using intensity-fading (IF-) MALDI-MS [25–27], which was based on a selective reduction of the ion abundance of the specific ligands after the addition of their receptor protein. The strategy was similar to the immunological procedures for identification of the epitopic peptides for antibodies introduced by the Downard group [28–31] and has shown promising applications to detect several biological interactions [25–37]. Nevertheless, it should be noted that a nonbinding control is usually necessary to assess the specificity of binding assay in both direct MALDI-MS and IF-MALDI MS analysis [24–37].

In this paper, we reported for the first time the application of MALDI-MS to detect the noncovalent interactions between calmodulin and its target peptides. Two typical calmodulin-binding peptides with distinct dissociation constants (Kd) were used in the present study (Table 1). Melittin is a 26-residue peptide from bee venom and can form a high-affinity complex with calcium-saturated calmodulin (Kd $\approx 3\ \text{nmol} \cdot \text{L}^{-1}$) [4]. The calmodulin-melittin complexes have been widely used as a typical model for calmodulin–peptide interactions [3, 10, 14–16, 21–23]. substance P is an endogenous neuropeptide with 11 amino acids, which is considered to have the smallest number of residues in a peptide that could bind to calmodulin with micromolar Kd value (Kd $\approx 2\ \mu\text{mol} \cdot \text{L}^{-1}$) [38]. In addition, the peptide bradykinin with no affinity to calmodulin, was chosen as a nonbinding control [5]. Herein, the binding interactions of calmodulin with the two target peptides were studied using both direct MALDI-MS and IF-MALDI-MS, and the influences of several experimental conditions on the detection of the specific calmodulin–peptide interactions were also examined.

Table 1. Peptides used in the study

Peptide	Amino acid sequence	Mw (Da)
Bradykinin (Bdk)	RPPGFSPFR	1060.2
Substance P (SP)	RPKPQQFFGLM	1347.6
Melittin (Mel)	GIGAVLKVLTTGLPALISWIKRKRQQ	2846.5

Experimental

Materials

Calmodulin from bovine testis, melittin from honey bee venom, substance P, and bradykinin were purchased from Sigma (St. Louis, MO) and used without further purification. The MALDI matrices sinapic acid (SA), 2,6-dihydroxyacetophenone (DHAP), and 2,4,6-trihydroxyacetophenone (THAP) were purchased from Fluka (Buchs, Switzerland) while 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Sigma (St. Louis, MO). Calcium chloride ($CaCl_2$), potassium chloride (KCl) and ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid (EGTA) were obtained from Fluka (Buchs, Switzerland). Ultra purity water was prepared through a Milli-Q preparation system (Millipore, Milford, MA). Acetonitrile (HPLC grade) was from Fisher Chemicals (Fair Lawn, NJ).

Sample Preparation

Calmodulin was dissolved in Milli-Q water at a desired concentration, containing 15 mM $CaCl_2$ to ensure the calcium saturation of calmodulin. Stock solutions of peptide were prepared in Milli-Q water, and diluted to desired concentrations before tests. The MALDI matrices DHB, CHCA, SA, DHAP, and THAP were prepared in the solvent acetonitrile/water (1:2, vol/vol) at a concentration of 10 mg/mL. EGTA was dissolved using ammonium hydroxide to neutral pH, and diluted in Milli-Q water to form a solution containing 300 mM EGTA.

Binding experiments were performed by mixing 1 μL of calmodulin solution with 1 μL of peptide mixture containing the calmodulin-binding peptide(s) and the nonbinding control. The resulting mixture was incubated for 5 min at room temperature. In the IF-MALDI-MS analysis, control sample was prepared by mixing 1 μL of peptide mixture with 1 μL of solution containing the same salt concentration as in the calmodulin solution and the amount of nonbinding peptide was adjusted to yield ion abundances similar to that of the calmodulin-binding peptide before the tests. An aliquot of 1 to 2 μL of the sample solution was mixed with 2 to 4 μL of matrix solution, and 1 μL of the resulting mixture was deposited on a stainless steel target and dried in a stream of cold air.

Mass Spectrometry

MALDI-TOF MS was performed on a Voyager DE-STR instrument (Applied Biosystems, Framingham, MA) equipped with a 337 nm pulsed nitrogen laser. For detection of the intact protein complexes (direct MALDI-MS analysis), the mass spectrometer was operated in positive ion linear mode with an accelerating voltage of 25 kV and delayed extraction time was set to 400 ns. For detection of the peptides (IF-MALDI-MS analysis), the instrument was operated in reflector mode by applying an accelerating voltage of 20 kV and the delayed extraction time was optimized and set to 100 ns. Laser intensity was adjusted according to the matrices and the species of interest (protein or peptide) and set slightly higher than the desorption-ionization threshold except where otherwise stated. In the IF-MALDI-MS analysis, the instrumental parameters were kept constant during the acquisitions of the control mass spectrum and those obtained after the addition of the protein. Mass spectra were acquired by accumulating 300 to 500 single laser shots over each sample spot unless otherwise stated. Five duplicates were performed to check the reproducibility and the variation of different measurements was less than 9% in this study. External calibrations were carried out using either a protein mixture containing horse heart myoglobin and cytochrome *c* (both from Sigma) or a standard peptide mixture provided by the manufacturer.

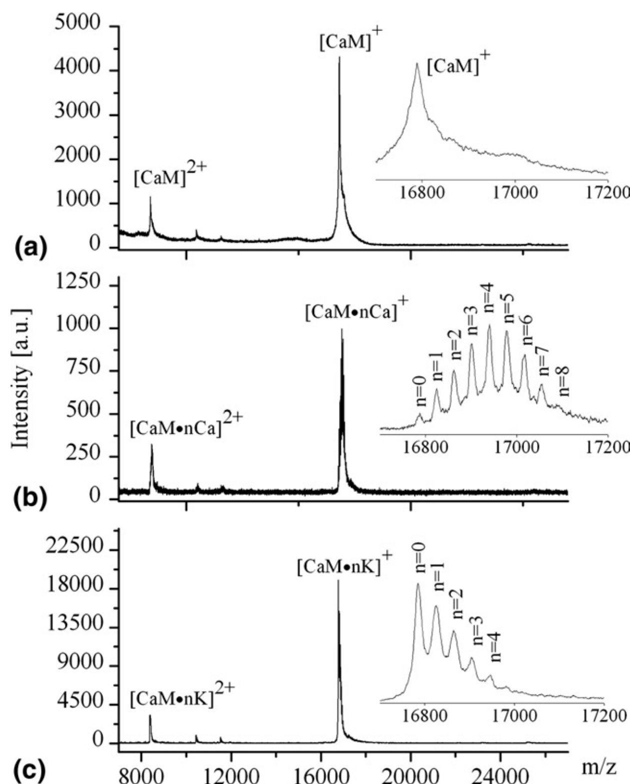

Figure 1. MALDI mass spectra of solutions containing 5 μM calmodulin and 2500 μM calcium chloride using the matrices SA (**a**) and DHAP (**b**); and solutions containing 5 μM calmodulin and 2500 μM potassium chloride using the matrix DHAP (**c**). CaM represent calmodulin.

Results and Discussion

MALDI-MS Analysis of the Calcium Binding to Calmodulin

Since the binding interactions between calmodulin and many target peptides (including melittin and substance P in this study) are largely calcium-dependent [4–7, 14, 15, 38], initial experiments were conducted to study the calcium-binding to calmodulin using direct MALDI-MS. Solutions containing calmodulin and enough calcium (15 mM in this study) were analyzed under different matrix conditions, and representative mass spectra were shown in Figure 1. For the three acidic matrices (DHB, CHCA, and SA), no peaks for the calcium-calmodulin complexes were observed (Figure 1a). The predominant peak at *m/z* 16,789 $\pm$ 8 Da, corresponds to the apo-calmodulin (theoretical *m/z* = 16,792 Da). However, when the less acidic matrices DHAP and THAP were used, peaks corresponding to the calcium–calmodulin complexes were readily observed, with the major peak for $[CaM + 4Ca - 8H + H]^+$ (Figure 1b). The peak pattern of the calcium–calmodulin complexes was similar to those obtained using ESI-MS [14, 16, 39]. These observations were consistent with the fact that there are four calcium-specific binding sites in calmodulin. However, further confirmation of the specific binding of calcium to calmodulin is still necessary because nonspecific protein-metal adducts are often observed in MALDI-MS analysis [40].

Here equal number of moles of potassium chloride were added into the calmodulin solution instead of calcium chloride to discriminate between specific and nonspecific binding since a previous study has suggested that potassium ions bind to calmodulin in a nonspecific manner [41]. Addition of potassium ions did result in the observation of the potassium–calmodulin complexes in the MALDI mass spectra. As shown in Figure 1c, peaks for the potassium–calmodulin complexes were readily observed using the DHAP matrix, with a pattern similar to those of the nonspecific protein-metal adducts recently reported [40]. However, the peaks pattern of the calcium–calmodulin complexes (Figure 1b) was far distinct from that of the potassium–calmodulin complexes, suggesting a specific binding of calcium to calmodulin. The results obtained using the matrix THAP were less pronounced; however, the differences between the peak pattern of calcium–calmodulin complexes and that of potassium–calmodulin complexes were also indicative of a specific binding of calcium ions to calmodulin (see Supplementary Material, which can be found in the electronic version of this article).

These results suggested that the intact calcium–calmodulin complexes could survive, at least in part, as the less acid matrices DHAP and THAP were used.

However, under the classic acidic matrices conditions (DHB, CHCA, and SA), the protein may be denatured or the amino acids for coordinating with calcium ions may be protonated, resulting in the loss of binding ability for calcium. Further evidences for the survival of the intact calcium–calmodulin could be provided from the IF-MALDI-MS results (see the Discussion section below).

Detection of the Intact Calmodulin–Peptide Complexes using Direct MALDI-MS

The formation of noncovalent complexes between calmodulin and its target peptides (melittin and substance P) was examined using direct MALDI-MS as the first step because most of the previous studies on noncovalent interactions focused on the direct detection of the intact complexes [24, 42–45]. To check the specificity of the assay, the peptide bradykinin with no affinity for calmodulin was added as a nonbinding control (1:1 M ratio).

Solutions containing equal amount of calmodulin (calcium-saturated), target peptide, and nonbinding control were mixed with different matrix solutions and subjected to direct MALDI-MS analysis. Figure 2 shows the MALDI mass spectra obtained from solutions containing the target peptide melittin using the five different matrices. For the acidic matrices DHB, CHCA, and SA, peaks for the apo-calmodulin and its complexes with the target peptide melittin were observed in the spectra (Figure 2a, c, and d). The DHB matrice also resulted in the detection of apo-calmodulin complex with the nonbinding control (Figure 2a). Since the binding interactions of calmodulin with its target peptides are largely calcium-dependent, these apo-calmodulin complexes were most likely to originate from nonspecific aggregates of the peptides with the protein. When the less acidic matrix DHAP and THAP were used, peaks corresponding to the calcium-loaded calmodulin and the complexes with the target peptide melittin were readily observed; however, the nonspecific aggregates of the calcium-loaded calmodulin with the nonbinding control were also detected (Figure 2b and e). Similar results were obtained for solutions containing the target peptide substance P (Figure 3), except that the ion abundances of complexes of apo-calmodulin with substance P were insignificant when the acidic matrices CHCA and SA were used (Figure 3c and d). It should be noted that all the signals for the protein–peptide complexes are rather weak compared with that of calmodulin, and that the signals for complexes with the nonbinding control (if present) were equal or even more abundant than those with the target peptide.

Previous studies have revealed that some instrumental parameters may influence the observation of the specific noncovalent complexes [42–45]. In several cases, the peak for the intact and specific complex is only observed for the first few laser shots onto a fresh sample spot, and the subsequent irradiation of the same position produces dissociated subunits [43–45]. However, that is not the case in this study—the number of laser shots has little impact on the intensity ratios of the

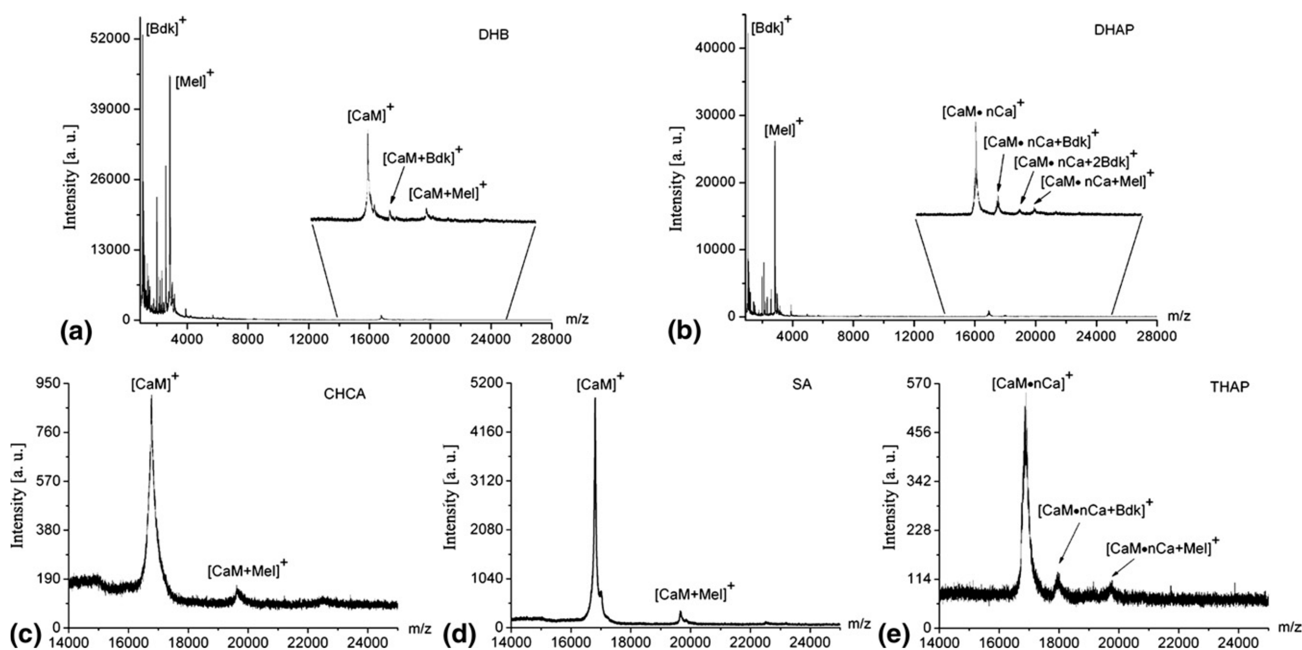

Figure 2. MALDI mass spectra of samples containing equal amounts of calmodulin, melittin and bradykinin (5 μM each) with 2500 μM calcium chloride using the matrices DHB (**a**), DHAP (**b**), CHCA (**c**), SA (**d**), and THAP (**e**). Inset displays amplified regions corresponding to calmodulin and calmodulin–peptide complexes. CaM, Mel, and Bdk represent calmodulin, melittin, and bradykinin, respectively.

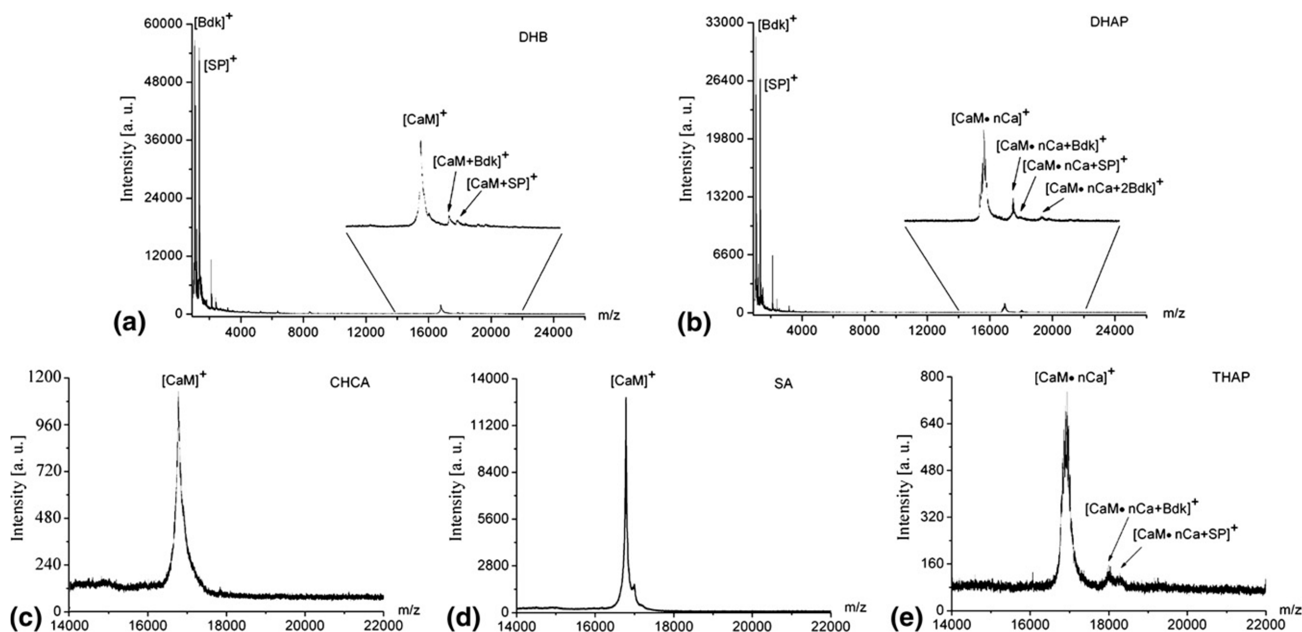

Figure 3. MALDI mass spectra of samples containing equal amounts of calmodulin, substance P and bradykinin (5 μM each) together with 2500 μM calcium chloride using the matrices DHB (**a**), DHAP (**b**), CHCA (**c**), SA (**d**), and THAP (**e**). Inset displays amplified regions corresponding to calmodulin and calmodulin–peptide complexes. CaM, SP, and Bdk represent calmodulin, substance P, and bradykinin, respectively.

complexes to calmodulin. Variation of the laser intensity and the delayed extraction time also show little effect on the relative intensities of the complexes in the spectra.

Since the complexes formed between calmodulin and its target peptides are rather stable in solution, the corresponding peaks are expected to be the most intense in the spectra. However, in our measurements, peaks for the intact complexes were always observed with relative low abundances, indicating that the intact complexes were dissociated to a large extent during the sample preparation and/or the desorption/ionization process. On the other hand, the observation of calmodulin complexes with the target peptides was often concomitant with the detection of nonspecific aggregates of calmodulin with the nonbinding control, which made it difficult to distinguish the specific complexes from nonspecific aggregates formed in the gas phase. Overall, direct detection of the intact calmodulin–peptide complexes using MALDI-MS was found to be much more difficult due to the dissociation of the intact complex and the formation of unspecific aggregates during the analysis.

Detection of the Calmodulin–Peptide Interactions using IF-MALDI-MS

The difficulties to detect the intact calmodulin–peptide complexes using direct MALDI-MS prompted us to explore intensity fading (IF)-MALDI-MS to study the noncovalent interactions between calmodulin and its target peptides. Recent studies have suggested that the specific interactions between proteins and their peptide ligands could be deduced using IF-MALDI-MS, namely, introduction of a protein receptor could result in a selective decrease of the ion abundances of its peptide ligands compared with the nonbinding control [25–34].

In this study, peptide mixtures containing the target peptide and the nonbinding control were incubated with the calcium-saturated calmodulin, and then analyzed using different matrices. The resulting MALDI mass spectra were recorded and compared with those obtained from the control samples. When the acidic matrices DHB, CHCA, and SA were used, the incubation with the calcium-saturated calmodulin did not show any change on the relative intensities of its target peptides compared with the nonbinding control (data not shown). However, when the less acidic matrices DHAP and THAP were used, the ion abundances of the target peptides underwent a clear decrease after the addition of calcium-loaded calmodulin compared with the nonbinding control (Figure 4). Close inspections of these results obtained using DHAP and THAP revealed that the nature of the matrix may also play a role in the decrease extent of the target peptide—the ion abundances of the target peptides decreased to a larger extent when THAP was used as the matrix. These results indicated that the binding interactions between calmodulin and its target peptide could survive the sample preparation using the less acidic matrices DHAP and THAP and that the preferable binding of the target peptides to calmodulin could be detected using IF-MALDI-MS. The results also revealed that calmodulin–peptide interactions with Kd values ranging from

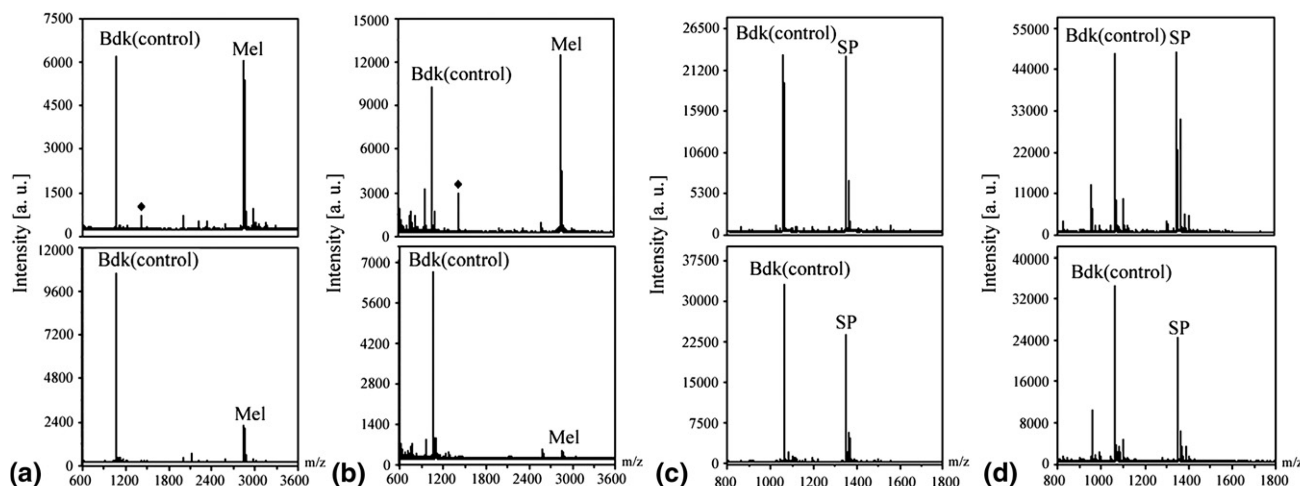

Figure 4. MALDI mass spectra of mixtures of the nonbinding control (bradykinin) with melittin (**a**), (**b**) and substance P (**c**), (**d**) before (upper panel) and after (lower panel) mixing with equal amount of calcium-saturated calmodulin. The matrix DHAP was used for (**a**) and (**c**); and the matrix THAP was used for (**b**) and (**d**). Bdk, Mel, and SP represent bradykinin, melittin, and substance P, respectively. The concentrations of Mel, SP, and $CaCl_2$ in the control samples were 5 μM, 5 μM, and 2500 μM after mixing with the matrix solutions. The peaks marked with ♦ indicate the doubly-charged melittin.

$nmol \cdot L^{-1}$ (e.g., melittin) to $\mu mol \cdot L^{-1}$ (e.g., substance P) could be analyzed using IF-MALDI MS.

The matrix dependency of the fading phenomena is reminiscent of the matrix-dependent observation of calcium-binding to calmodulin as described in a previous section. The less acidic matrices DHAP and THAP were found to favor the survival of the calcium-loaded calmodulin compared with the three acidic matrices (Figure 1). Since these target peptides can form high-affinity complexes with calcium-loaded calmodulin rather than apo-calmodulin, it was reasonable to believe that the specific interactions between the target peptides and calmodulin could be maintained by mixing with the less acidic matrice DHAP and THAP, whereas these interactions may be greatly destroyed by acidic matrices, and thus result in a matrix dependency of the observed fading phenomena.

The specificity of the assay was verified by two control experiments. In one experiment, the protein calmodulin was replaced by horse heart myoglobin, a protein with similar molecular weight to calmodulin. When myoglobin was added into the peptide mixtures, no changes of the relative intensities of peptides were observed (data not shown), suggesting that the decrease of the ion abundances of the target peptides after the addition of calmodulin should be attributed to their interactions with calmodulin. In another control experiment, the chelating reagent EGTA was added to generate calcium-free conditions. Under such conditions, the incubation of the peptide mixtures with calmodulin did not result in any significant change of the relative intensities of the target peptides compared with the nonbinding control (data not shown), indicating either lack or weak binding of the peptides to the calcium-free calmodulin. The results were in agreement with the calcium-dependent binding of these target peptides to calmodulin and further supported the specificity of the observed fading phenomena.

The influences of several instrumental parameters on the intensity-fading phenomena were also investigated. The laser shots and the delayed extraction time showed little effect on the fading phenomena. Switching from reflector mode to linear mode also had no influence on the observed fading. The laser intensity showed a well-known effect on the overall signal intensity, and had an impact on the fading phenomena to some extent. The overall signal intensity increased with the laser intensity up to a point where saturation was reached. Before the signal intensity reach saturation, variation of the laser intensity showed little effect on the intensity-fading phenomena. When the signal intensity reached the saturation point, further increase of the laser intensity made the fading phenomena less pronounced and it was difficult to discriminate the target peptides with the nonbinding control. Besides these instrumental parameters, the influence of analyte concentration on the fading phenomena was also investigated because previous studies suggested that the analyte concentration might have an impact on the observed fading [34]. Peptide mixtures containing the target peptide at desired concentrations were incubated with equal amount of calcium-saturated calmodulin (1:1 M), followed by the addition of matrix solutions. Sample solutions with a final analyte concentration of 1, 5, 10, 15, and 25 μM were analyzed. A clear reduction of the ion abundances of the target peptides was observed when the final analyte concentration was above 5 μM. However, when the analyte concentration was decreased to 1 μM, the reduction of the ion abundances of the target peptides was less pronounced, and the signal-to-noise ratio decreased (data not shown).

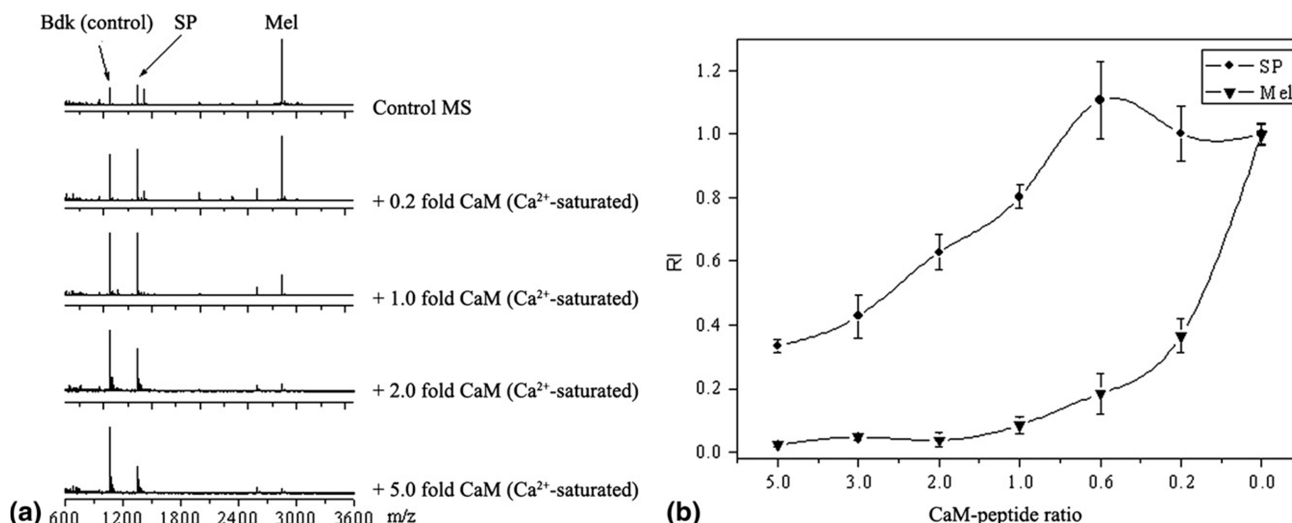

Figure 5. MALDI mass spectra of mixtures of calmodulin-binding peptides melittin, substance P, and a nonbinding control (bradykinin) after the addition of different concentrations of calcium-saturated calmodulin (**a**). Plot of the RI of both melittin and substance P (corrected with the RI of the control) after the addition of calcium-saturated calmodulin with different concentrations (**b**). THAP was used as the matrix. Bdk, Mel, and SP represent bradykinin, melittin, and substance P, respectively. The concentrations of Mel, SP, and $CaCl_2$ in the control samples were 5 μM, 5 μM, and 2500 μM after mixing with the matrix solutions.

In addition to the qualitative identification of target peptides for calmodulin, we also examined the feasibility of IF-MALDI-MS to evaluate the relative binding affinities of different peptides towards calmodulin. For this purpose, equal amounts of melittin and substance P were mixed simultaneously with the nonbinding control, and then incubated with different amounts of calcium-saturated calmodulin. Figure 5a showed the MALDI mass spectra of such mixtures. When a 5 M excess of calmodulin (five equivalents to the target peptides) was added, both melittin and substance P underwent significant decrease. When the concentration of calmodulin was gradually decreased, the signal of melittin was still faded, whereas the signal of substance P with lower affinity for calmodulin was gradually increased. Figure 5b shows a plot representing the relative intensities (RI) of both melittin and substance P molecules (corrected with the RI of the control) versus the calmodulin-to-peptide ratios. It was obvious that the RIs of the two molecules were affected differently by the addition of different amounts of calmodulin. It can be inferred from the plot that melittin shows a higher affinity towards calmodulin than substance P. Thus, clearly IF-MALDI-MS could also provide information on the relative affinities of different peptides towards calmodulin.

Conclusions

The results of this study demonstrate that intensity fading (IF)-MALDI-MS is a promising technology to study the noncovalent interactions between calmodulin and its target peptides. The binding occurrence and the relative affinities between the interacting partners can be rapidly estimated using IF-MALDI-MS. Moreover, only small amounts of materials and no labeling of the protein are acquired for the analysis. The compatibility of MALDI-MS to heterogeneous samples also made it possible to exploit this method to complex mixtures. The approach may be employed to identify the calmodulin-binding domain of a specific protein from its enzymatic digests or to screen peptide ligands for calmodulin from natural extracts. In addition, the method may also find its way to study the interactions between small molecules with the protein calmodulin.

Acknowledgments

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