Exploring the "Intensity Fading" Phenomenon in the Study of Noncovalent Interactions by MALDI-TOF Mass Spectrometry

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The difficulties to detect intact noncovalent complexes involving proteins and peptides by MALDI-TOF mass spectrometry have hindered a widespread use of this approach. Recently, "intensity fading MS" has been presented as an alternative strategy to detect noncovalent interactions in solution, in which a reduction in the relative signal intensity of low molecular mass binding partners (i.e., protease inhibitors) can be observed when their target protein (i.e., protease) is added to the sample. Here we have performed a systematic study to explore how various experimental conditions affect the intensity fading phenomenon, as well as a comparison with the strategy based on the direct detection of intact complexes by MALDI MS. For this purpose, the study is focused on two different protease-inhibitor complexes naturally occurring in solution, together with a heterogeneous mixture of nonbinding molecules derived from a biological extract, to examine the specificity of the approach, i.e., those of carboxypeptidase A (CPA) bound to potato carboxypeptidase inhibitor (PCI) and of trypsin bound to bovine pancreatic trypsin inhibitor (BPTI). Our results show that the intensity fading phenomenon occurs when the binding assay is carried out in the sub-μM range and the interacting partners are present in complex mixtures of nonbinding compounds. Thus, at these experimental conditions, the specific inhibitor-protease interaction causes a selective reduction in the relative abundance of the inhibitor. Interestingly, we could not detect any gaseous noncovalent inhibitor-protease ions at these conditions, presumably due to the lower high-mass sensitivity of MCP detectors. (J Am Soc Mass Spectrom 2007, 18, 359–367) © 2007 American Society for Mass Spectrometry

The capacity of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to detect intact noncovalent biomolecular complexes was demonstrated by Karas and Hillenkamp and their coworkers only a few years after they developed MALDI [1, 2], however, it has not found widespread use up to now.

Several major effects have been described to result in nonnative conditions for the noncovalent complexes: MALDI matrix [3–5], sample preparation [4, 6, 7], crystal morphology [4, 8, 9], pH of the solution [6, 10–12], organic solvent [7, 8], ionic strength [13, 14], matrix/analyte ratio [15, 16], speed of solvent evaporation [4], and sample concentration [9, 15]. The effect of some instrumental parameters in the detection of complexes has also been studied; extraction delay time, positive/negative ion mode, linear/reflector and acceleration mode were found to be of minor importance [7, 17], whereas laser pulse energy [8, 17, 18] and the number of laser shots (i.e., "first shot phenomenon") [4, 8, 13, 19, 20] was reported to be a decisive factor in a number of cases.

Also, analyses by electrospray ionization (ESI) [21] are not carried out at physiological conditions as only solutions of very low ionic strength can be analyzed. However, ESI generates "colder" ions than MALDI and it keeps the sample in aqueous "biological" environment before ionization. For these reasons, ESI has been used in numerous studies to detect noncovalent complexes [22].

Relatively few cases have been reported where specific intact noncovalent complexes were successfully observed with MALDI. Besides the frequent dissociation of noncovalent complexes due to the experimental conditions employed, a further complication for their study by MALDI MS is the presence of nonspecific aggregates, i.e., "cluster ions" [23, 24]. Thus, the speci-

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ficity of binding should be always verified by competitive and comparative assays after the addition of known ligands and nonbinding molecules. Following these control experiments, the interaction is confirmed when no cluster ions are observed or when the "specific" complex is far more abundant than "nonspecific" aggregates.

Within this context, one of our groups recently introduced an alternative strategy to detect the presence of noncovalent complexes in solution by MALDI MS [25]. With this strategy, a reduction in the relative abundance of peptide ligands, visualized as a reduction of their relative intensity in the mass spectra, can be observed when their receptor protein is added to the MALDI sample; accordingly, we named this approach intensity fading (IF-) MALDI-TOF MS [25]. This is equivalent to immunological procedures to identify epitopic peptides by their specific removal by antibodies [26, 27]. We have extended it to a wide range of biological interactions (i.e., protein-protein, protein-nucleic acid, and protein-organic compounds) [25] and, more recently, as a promising approach to screen ligands in heterogeneous biological extracts (i.e., protease inhibitors) [28, 29].

However, no systematic study of the influence of various experimental conditions on the "intensity fading" phenomenon has been conducted until now. With this aim, here we report a study to (1) investigate how various experimental conditions and instrumental parameters affect the "intensity fading" phenomenon, and (2) to elucidate if "intensity fading MS" approach is a truly advantageous strategy by comparison with the direct detection of intact noncovalent complexes by MALDI TOF MS. For this purpose, we have selected the noncovalent complexes formed between two different enzymes of intermediate size and two proteic inhibitors (representative of small protein and peptide ligands), all of them well characterized: the pairs bovine carboxypeptidase A (CPA)/potato carboxypeptidase inhibitor (PCI) complex [30] and trypsin/bovine pancreatic trypsin inhibitor (BPTI) complex [31]. We have explored the "fading" behavior of these protease inhibitors (in the low mass range) interacting with the enzymes as well as the observation of the entire specific complex (in the high mass range), with several MALDI matrices (in the presence or absence of ammonium salts). Various sample preparation procedures yielding different crystal morphologies were also investigated.

Experimental

Materials

The MALDI matrices sinapic acid (SIN), 6-aza-2-thiothymine (ATT), 2,6-dihydroxyacetophenone (DHAP), α-cyano-4-hydroxycinnamic acid (CHCA), 2-(4-hydroxyphenylazo)benzoic acid (HABA), and the structural isomers 2,5- and 2,6-dihydroxybenzoic acid (2,5- and 2,6-DHB) were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Ammonium acetate, ammonium citrate, and ammonium dihydrogen phosphate were purchased from Fluka (Buchs, Switzerland). Universal pH indicator strips, pH 0.0 to 2.5, 2.5 to 4.5, and 4.0 to 7.0 were purchased from Merck KGaA (Darmstadt, Germany). Trypsin (modified/sequencing grade) was purchased from Promega (Madison, WI). Carboxypeptidase A modified from bovine pancreas was purchased from Boehringer Mannheim (Ingelheim, Germany). The solvents acetonitrile (ACN) and acetone were analytical or LiChrosolv grade (Merck, Darmstadt, Germany). Leech carboxypeptidase inhibitor (LCI), potato carboxypeptidase inhibitor (PCI) were obtained as previously described [32, 33]. Aprotinin (BPTI) bovine was recombinant, expressed in *Nicotiana* (tobacco) (Sigma-Aldrich, St. Louis, MO). All aqueous solutions were prepared using Milli-Q water filtered with a 0.2 μm membrane filter (Millipore, Bedford, MA). Extract from *Hirudo medicinalis* was supplied by the group of Professors H. Fritz and C. Sommerhoff (Chirurgischen Klinik Innenstadt, Ludwig-Maximilians-Universitat, Munich, Germany). *Hirudo medicinalis* extract was dissolved in deionized water at a concentration of 20 mg/mL. These solutions were centrifuged at 8000 *g* for 10 min, and the supernatant was processed by size-exclusion chromatography Superdex Peptide HR 10/30 (Amersham Biosciences, Barcelona, Spain). The chromogenic substrate N-(4-methoxyphenylazoformyl)-Phe-OH was obtained from Bachem (Weil am Rhein, Germany).

Sample Preparation

Lyophilized samples of LCI, PCI, Aprotinin, and CPA were dissolved in 20 mM ammonium acetate at the desired molar concentration. Trypsin was dissolved in Milli-Q water at the desired molar concentration. The size-exclusion chromatography fraction of *Hirudo medicinalis* (essentially enriched with proteins in the range of 3 to 5 kDa) was lyophilized and dissolved in Milli-Q water. Possible inhibitory activity of the selected fraction was determined by measuring the inhibition of the hydrolysis of the chromogenic substrate N-(4-methoxyphenylazoformyl)-Phe-OH by carboxypeptidase type A at 350 nm.

Nonbinding control samples were diluted to yield ion abundances similar to that of the protease inhibitor assayed in parallel; 0.5 μL of each solution (i.e., protease inhibitor, nonbinding molecules, and protease or ammonium salt) were mixed and incubated for 3 min at room temperature. (1) For the dried-droplet sample preparation method, the following solutions were prepared: 10 mg/mL SIN, ATT, α-CHCA, 2,5-DHB, DHAP, and 2 mg/mL HABA, in ACN mixed with either water 20 mM ammonium acetate, 20 mM ammonium citrate, or 20 mM ammonium dihydrogen phosphate 1:4 (vol/vol). For the MALDI analysis, 1.5 to 2 μL of sample and 3 to 4 μL of matrix solution were mixed into a 0.5 mL tube and 0.5 μL of this mixture was deposited on a

Table 1. Intensity fading assays for protease inhibitors (PCI, BPTI) and detection of the intact complexes in presence of their target proteases (CPA, trypsin)

	CPA-PCI interaction		Trypsin-BPTI interaction
MALDI matrix[a]	Intensity fading of PCI[b]	Detection of the intact non-covalent complex[c]	Detection of the intact noncovalent complex
SIN (dried droplet)	+	+	+
SIN (thin layer)	−	nt	nt
α-CHCA (dried droplet)	+	+	+
α-CHCA (thin layer)	−	−	nt
DHAP (dried droplet)	+	+	+
ATT (dried droplet, large crystals)	−	−	+/−
ATT (dried droplet, micro crystals)	+	+	+/−
2,5-DHB (dried droplet)	−	−	+/−
2,6-DHB (thin layer)	−	−	nt
HABA (dried droplet)	+	+	+

[a]SIN, sinapic acid; α-CHCA, α-cyano-4-hydroxycinnamic acid; DHAP, 2,6-dihydroxyacetophenone; ATT, 6-aza-2-thiothyamine; HABA, 2-(4-Hydroxyphenylazo)benzoic acid; 2,5-DHB and 2,6-DHB, 2,5- and 2,6-dihydroxybenzoic acid.
[b]Intensity fading MS assays in a heterogeneous mixture of non-binding molecules. The relative intensity of protease inhibitor was measured in the presence or absence of protease. + indicates that the relative abundance of inhibitor decreased by at least a factor of 10 in the presence of protease; - indicates no significant decrease in the relative abundance of inhibitor in the presence of protease. The heterogeneous mixture consisted of more than 50 nonbinding molecules obtained from a leech extract.
[c]Direct detection of the intact noncovalent complex between protease and inhibitor. The concentration of the interacting proteins was ~15- to 20-fold increased with respect to that used for intensity fading experiments. Results are indicated without non-binding molecules in the mixture. + indicates a 2-fold or higher relative abundance of the protease-inhibitor complex than that of the non-specific cluster ions formed by homo-aggregates of protease or inhibitor; - indicates the absence of protease-inhibitor complex or a similar relative abundance of the protease-inhibitor complex and nonspecific cluster ions formed by homo-aggregates of inhibitor or protease; +/− indicates ambiguous results due to a large shot-to-shot variability; nt means not tested.

stainless steel target and dried at room-temperature. (2) Thin-layer preparation: SIN, α-CHCA and 2,5-DHB were dissolved in acetone at a concentration of 20 mg/mL; 2,6-DHB was dissolved in acetone at a concentration of 100 mg/mL. A 5 μL aliquot of the matrix solution was spotted onto the target. A thin microcrystalline layer of matrix remained after fast evaporation of solvent; 0.5 μL of the sample solution was deposited onto the thin matrix layer. After solvent evaporation at room temperature, the sample was washed as follows: 2 μL of Milli Q water were added on the sample and removed after a few seconds.

MALDI-TOF Mass Spectrometry

MALDI mass spectra were obtained using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser, a gridless ion source, delayed-extraction (DE), and a 2 GHz digitizer. The instrument was operated in either reflector or linear mode by applying an accelerating voltage of 20 kV except where otherwise stated. Delayed extraction was used and the delay time was set according to the molecular weight of the analytes (protease inhibitor or complex) to optimize resolution of its molecular ion. Mass spectra were acquired by averaging 300 to 600 shots (three different positions into each spot and 100 to 200 shots per position). Laser pulse energy was adjusted according to the various MALDI matrices. All subsequent mass spectra acquisitions were performed by applying the same laser fluence, including control mass spectrum and after the addition of target molecules.

Results

"Intensity Fading MS" in Different Experimental Conditions

A broad variety of experimental conditions were tested to study the specific binding of CPA to PCI, as determined by the "intensity fading MS" approach, when added to a heterogeneous mixture of more than 50 unknown nonbinding molecules derived from a natural extract of the leech *Hirudo medicinalis*. The only available information about these nonbinding molecules was the absence of carboxypeptidase A inhibitory activity (measured by classical spectrophotometric assays) (data not shown).

The relative intensity of PCI at *m/z* 4298, after the addition of its specific interacting partner, CPA (~34.200 Da), was studied using the matrices SIN, DHAP, α-CHCA, ATT, 2,5-DHB, and HABA, following the "dried-droplet" MALDI sample preparation. On the other hand, the MALDI matrices 2,6-DHB, SIN, and α-CHCA were used with the "thin-layer" sample preparation. Results are summarized in Table 1. "Dried-droplet" sample preparation is clearly better than the "thin-layer" preparation for observing the "fading" phenomenon. Figure 1a shows the mass spectra for increasing amounts of CPA added to the complex mixture. A clear gradual fading of the relative intensity of PCI (0.6 pmol) is observed reaching almost a complete reduction of the signal when a 1:1 M ratio of CPA:PCI is employed. The other peaks are virtually unaffected by the addition of the protease. A similar fading phenomenon was observed using α-CHCA as

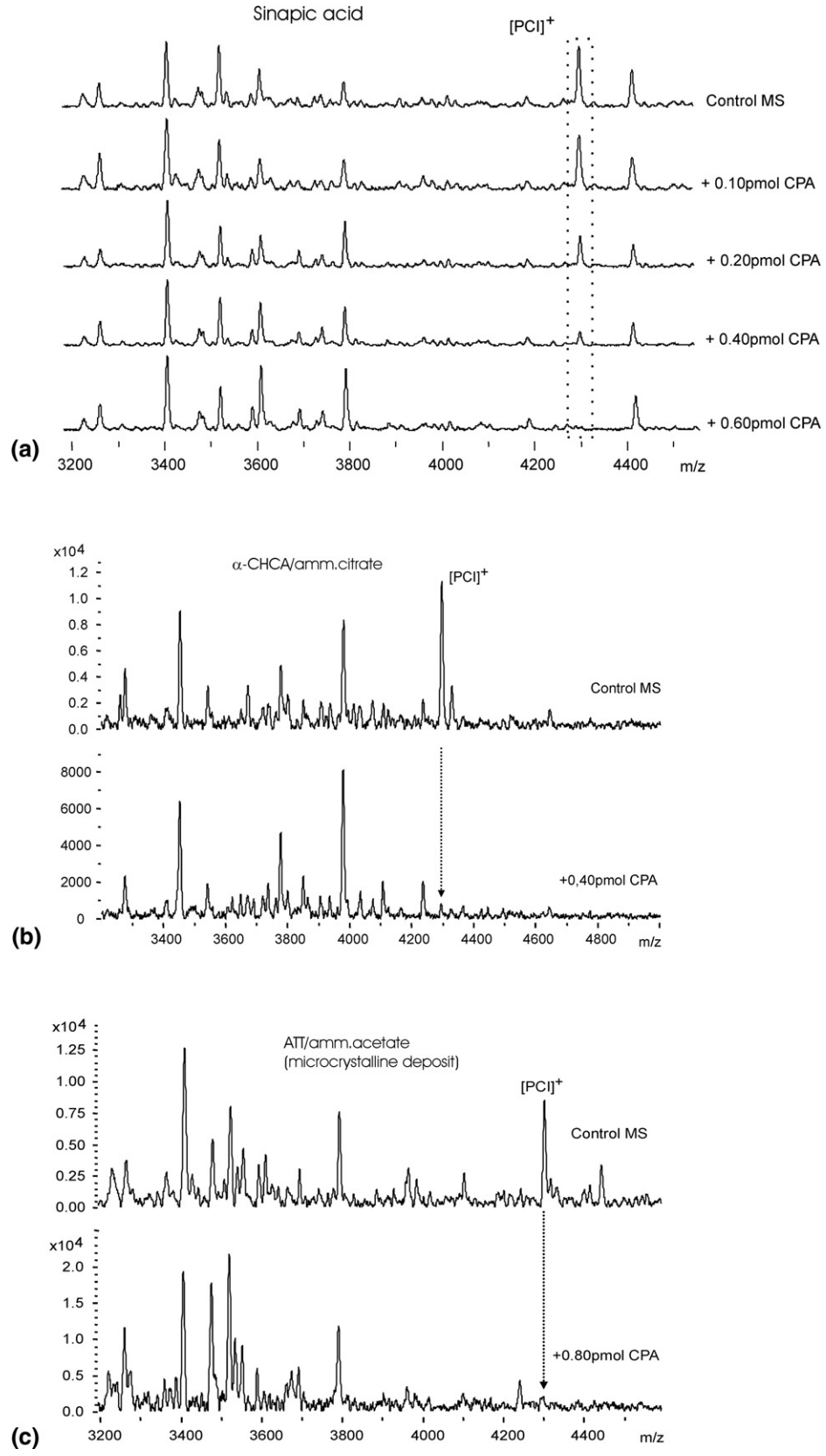

Figure 1. MALDI-TOF mass spectra of a complex mixture containing nonbinding peptides obtained from extract of leech and 0.6 pmol of potato carboxypeptidase inhibitor (PCI) in the presence or absence of carboxypeptidase A (CPA). The peak corresponding to $[PCI]^+$ at *m/z* 4296 is indicated by dotted lines. Only the mass range displaying the $[PCI]^+$ peak and its adjacent peaks are shown. Mass spectra were obtained using the dried-droplet preparation method and (**a**) sinapic acid, (**b**) α-cyano-4-hydroxycinnamic acid (CHCA) with ammonium citrate, (**c**) 6-azathiothymine (ATT) with ammonium acetate, as MALDI matrices.

Table 2. pH[a] values of the matrix solutions

MALDI matrices	20% ACN/water	20% ACN/ 20mM ammonium citrate	20% ACN/ 20mM ammonium acetate	20% ACN/ 20mM ammonium dihydrogen phosphate
SIN	2.5	4	4	3.5
α-CHCA	2-2.5	3-3.5	3.5	3.0
DHAP	4.0	5.0	5.5	4.5
ATT	3.5	4.5	4.5-5	4.0
2,5-DHB	1.5	3.0	3.0	2.5
HABA	3.5-4	5.0	5-5.5	4.5

[a]pH values were measured with universal pH indicator strips (pH ranges 0.0-2.5; 2.5-4.5; 4.0-7.0).

matrix (Figure 1b). In the case of the less acidic MALDI matrix (see Table 2) ATT, two different matrix crystal morphologies were observed. At the rim of the sample spot, thin macrocrystalline long needles were prominent, whereas the center was covered by a homogeneous microcrystalline layer of matrix (particularly with ammonium acetate). Surprisingly, the relative signal intensity of PCI was *not* reduced in the presence of CPA when the macrocrystalline needles were analyzed (with and without ammonium salts) (data not shown). By contrast, a marked reduction of the relative signal intensity of PCI was detected in the microcrystalline layer (Figure 1c). For the long needles, a high spot-to-spot signal variability was observed; at some positions, the relative signal intensity of PCI was significantly reduced from the spectra within the first few laser shots. But, at increasing laser fluence or number of laser shots, the inhibitor was unaffected, i.e., having the same relative signal intensity as that of the control spectrum. Similar long needles were observed at the rim of the spot when the matrix 2,5-DHB was used. Also for this matrix, the relative signal intensity of PCI was unaffected by the addition of CPA, even in the presence of a 2-fold molar excess of the protease (data not shown). It should be noted that neither the specific CPA-PCI complex (m/z ~38,500) nor the free CPA (m/z ~34,200) were detected in these experiments at the corresponding mass range in the mass spectra, regardless of the matrix and the experimental conditions conceived.

The same experiments were performed using SIN, α-CHCA, and 2,6-DHB as matrices and the "thin-layer" as sample preparation method. With these conditions, the addition of CPA did not affect the relative signal intensity of its natural ligand PCI (data not shown).

Variation of the laser fluence had no effect on the fading phenomenon. We did not observe an equivalent of the "first-shot phenomenon" for the "intensity fading MS" approach, i.e., the relative signal intensity of the ligand was reduced to the same extent after exposure to 200 subsequent laser shots on the same position of the spot (except for macrocrystalline structures of ATT). Switching from positive linear to positive reflectron mode had no influence on the observed fading.

Intensity Fading MS Versus Direct Detection of Intact Noncovalent Complexes by MALDI-TOF MS

From a simple mechanistic point of view, the signal fading of the protease inhibitor ion should be a direct consequence of the preservation and detection of the intact noncovalent complex formed between the target protease and its specific inhibitor. Therefore, both strategies should be equally affected by the same experimental conditions. However, our results indicate that this is not true and that analyte concentration plays a key role, affecting in a different way both strategies. The detection of the intact noncovalent complexes assayed in this work (CPA-PCI and trypsin-BPTI) could only be observed when the overall amount of the analytes were increased ~15- to 20-fold with regard to those applied in the intensity fading experiments.

Detection of the intact CPA-PCI complex in the mass spectrum. The formation of the noncovalent complex between CPA and PCI was also tested using seven different matrices and two different MALDI sample preparation methods, i.e., "dried-droplet" and "thin-layer" method. As summarized in Table 1, the complex was detected using SIN, α-CHCA, DHAP, HABA as well as the microcrystalline surface of ATT, whereas no complex could be observed using either 2,5-DHB or the thin long needles of ATT (Figure 2).

The specificity of the assay was investigated by adding another protease inhibitor (BPTI) with no affinity for CPA in solution, as a nonbinding control molecule. The preservation of the specific binding between CPA and PCI was strongly dependent upon the type of MALDI matrix. Only the CPA-PCI complex is observed with SIN matrix (Figure 3a), whereas ATT matrix results in the exclusive formation of the nonspecific complex between CPA and BPTI (Figure 3b). Note that although we exclusively detected the specific CPA-PCI complex with SIN matrix, or the nonspecific CPA-BPTI with ATT matrix, there was no detectable fading of the m/z ion corresponding to PCI or BPTI, respectively, in these experimental conditions. In the case of the DHAP

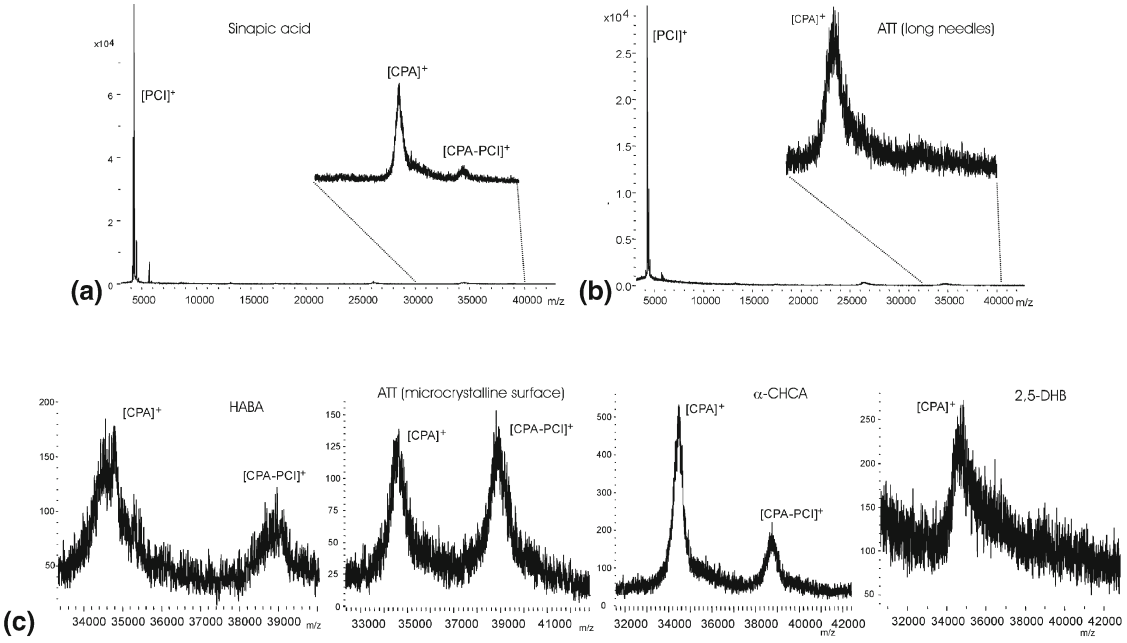

Figure 2. MALDI-TOF mass spectra of a sample containing 7 pmol of PCI and 10 pmol of CPA. Dried-droplet sample preparation with sinapic acid (**a**) and ATT (**b**) as matrices was used. Inset displays amplified regions corresponding to free CPA and the specific CPA-PCI complex. (**c**) MALDI-TOF mass spectra (same mixture and molar ratios as in (**a**) and (**b**) but shifted to the high *m/z* region) showing free CPA and the specific complex CPA-PCI. HABA, ATT, α-CHCA, and 2,5-DHB were used as matrices and dried-droplet as sample preparation.

matrix, neither the specific CPA-PCI complex nor the nonspecific one between CPA and BPTI could be detected; the mass spectrum was dominated by the three unligated compounds (data not shown). For HABA, the addition of BPTI led to reduction of the free CPA signal, thereby hampering the possibility of detecting any complex (data not shown). The effect of time-delayed extraction in linear and reflector mode was also studied, increasing from 120 to 500 ns, showing insignificant changes in the spectra.

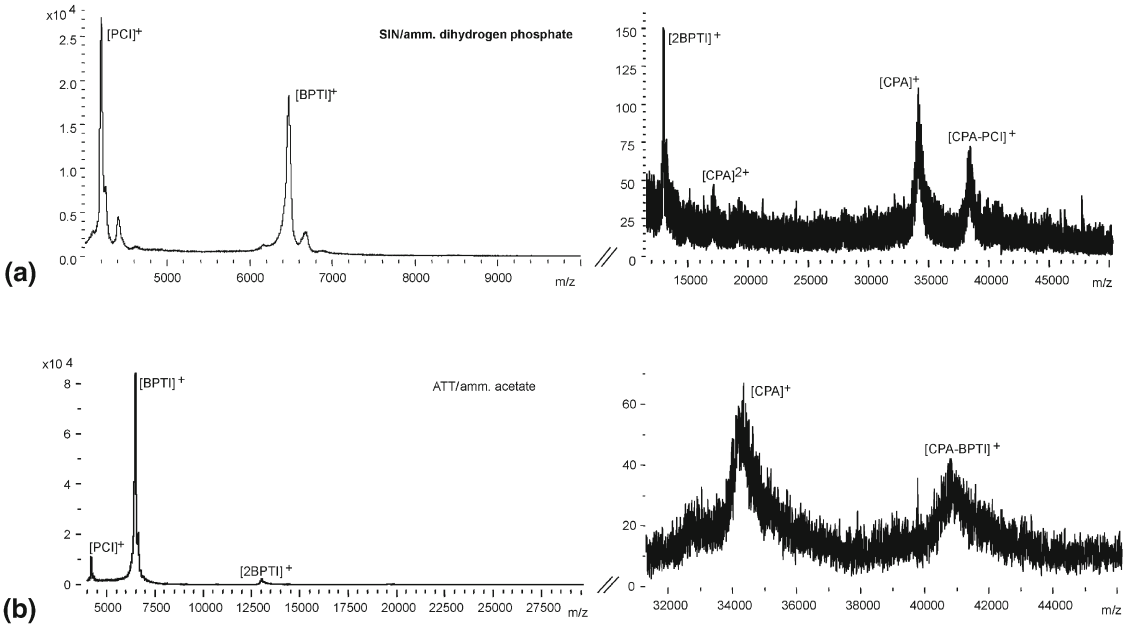

Figure 3. MALDI-TOF mass spectra of a sample containing equimolar amounts of PCI, BPTI, and CPA (20 pmol). Dried-droplet sample preparation with sinapic acid/ammonium dihydrogen phosphate (**a**) and ATT/ammonium acetate (**b**) as matrices was used.

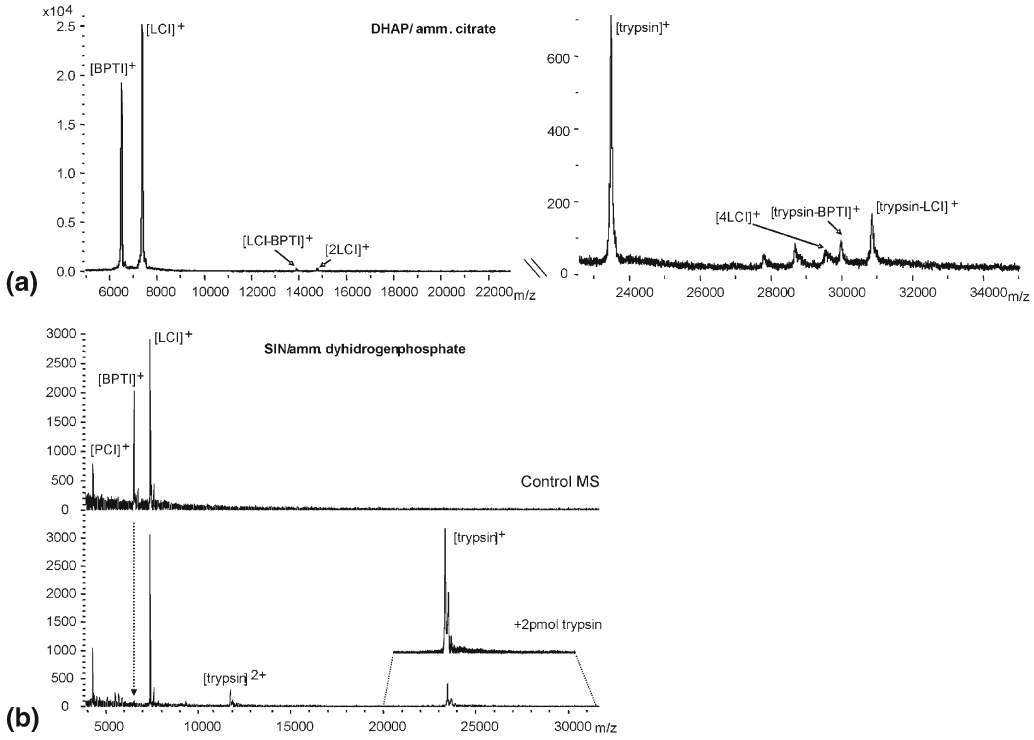

Figure 4. MALDI-TOF mass spectrum of a sample containing equimolar amounts of BPTI, LCI, and trypsin (15 pmol) (**a**). DHAP/ammonium citrate was used as matrix and dried-droplet as sample preparation. MALDI-TOF mass spectra of a simple mixture containing PCI and LCI as nonbinding control proteins and 0.8 pmol of BPTI in the absence (top) and presence (bottom) of trypsin (**b**). SIN with ammonium dihydrogen phosphate was used as matrix and dried-droplet as sample preparation. Dotted arrow indicates the signal of the ligand.

Detection of the intact trypsin-BPTI complex in the mass spectrum. The noncovalent complex between trypsin and its specific natural inhibitor BPTI (1:1 M ratio) was also tested using the seven different matrices and "dried-droplet" method for MALDI sample preparation. As summarized in Table 1, the intact complex was detected using SIN, DHAP, α-CHCA, HABA (with and without ammonium salts) as matrices, whereas a high spatial signal variability was observed when ATT and 2,5-DHB were used.

A carboxypeptidase inhibitor, LCI, showing no affinity for trypsin in solution, was added to the mixture as nonbinding control molecule (1:1 M ratio). The nonspecific complex between trypsin and LCI was observed with all matrices. For DHAP, this unspecific complex was even more abundant than the naturally occurring trypsin-BPTI complex (Figure 4a). Note again, the absence of signal fading for both the specific (BPTI) and nonspecific (LCI) inhibitors in presence of trypsin. This is in contrast with the intensity fading MS experiments performed in the sub-picomole range with the same analytes, plus another nonbinding molecule like PCI (Figure 4b). Here, the BPTI ion specifically faded after the addition of its target protease, trypsin, whereas LCI and PCI signals are virtually unaffected. However, as stated also in the previous section for CPA-PCI interaction, we could not observe the intact trypsin-BPTI complex in its corresponding mass range of the mass spectrum.

Discussion

The "intensity fading MS" methodology has the ability to detect biomolecular interactions (i.e., protease-protease inhibitor in this work) in heterogeneous mixtures by a simple comparison of MALDI mass spectra. It is based on the phenomenon that signals of ligands can become strongly suppressed in MALDI mass spectra when they are engaged in complex formation with a receptor protein. In the present study, we have investigated the "intensity fading" phenomenon using a broad variety of MALDI matrices in combination with two preparation methods (dried-droplet and thin-layer) to explore and optimize the conditions to implement it as a screening methodology to detect biomolecular interactions (protease-protease inhibitor in our case) in heterogeneous biological mixtures, and verify whether it is a truly advantageous strategy compared with the direct detection of intact complexes by MALDI mass spectrometry.

Our results show that the "intensity fading" effect occurs under the specific experimental conditions characterized by the low concentration (sub-picomole) of the interacting partners and the complex mixture of internal nonbinding compounds, as well as the use of homogeneous crystal morphology obtained from dried-droplet sample preparation. For the model systems investigated in this work, pH-values of matrix-analyte solutions is not a limitation, but may indeed become a

restriction for systems more susceptible to pH. Larger crystals of ATT and 2,5-DHB matrices as well as thin-layer sample preparations with α-CHCA, 2,6-DHB, and SIN matrices prevented the fading of the protease inhibitors.

The absence of specific intact noncovalent complex ions (as well as the free enzyme) in the mass spectra in the "intensity fading" assays, is a consequence of the poor efficiency of detection of high molecular weight proteins, such as CPA or CPA-PCI complex, by MALDI-TOF MS instruments equipped with conventional microchannel plate (MCP) detectors [34, 35]. Besides sensitivity problems, with MCP there is often the issue that when high molecular weight proteins are present in complex mixture of lower mass ions, the latter turn off channels of the detector by saturation during amplification. Results with improved detection efficiency for high mass ions by superconducting tunneling junction detectors (i.e., cryodetectors) [36] confirm this point.

Given the complex experimental situation in MALDI, a nonoccurrence of the intensity fading does not necessarily mean that complexes are not formed. This is exemplified when the applied overall amount of proteins was increased by a factor of 15 to 20 with regard to those used in the intensity fading assays (i.e., sub-picomole range). We were able to detect the protease as well as the noncovalent complex formed with its specific inhibitor. It is worth mentioning that these analyte concentrations (~10 to 30 pmol/μL) reproduce the experimental conditions reported in most of the previous studies of biomolecular interactions in solution by MALDI [4, 6, 7, 17, 37]. However, when the specific trypsin-BPTI and CPA-PCI interaction was probed by adding inhibitors that are known *not* to interact with the protease in solution, we observed abundant nonspecific protease-inhibitor complexes (e.g., CPA-BPTI and trypsin-LCI complexes in Figure 3b and Figure 4a, respectively) and aggregates in the mass spectra (e.g., the trameric LCI in Figure 4a). This make clear that, at certain analyte concentrations, the formation of gaseous noncovalent complexes by MALDI does not originate exclusively from specific interactions that are preformed in solution, as recently also indicated [17].

Conclusions

The "intensity fading MS" approach is attributable to an exclusive reduction in the relative intensity of peptide/small protein ligands (i.e., protease inhibitors in our case) that can be observed when their target protein (i.e., protease) is added to the MALDI sample. The other peaks (i.e., nonbinding peptides, protein substrates) are virtually unaffected by the addition of the protease. Our results have shown that the "intensity fading" phenomenon shares some common disadvantages associated to the detection of intact noncovalent complexes by MALDI, such as sample preparation and choice of matrix.

However, the detection of the intact noncovalent complexes studied in this work by MALDI-TOF MS was only possible because of the relatively high amounts of interacting partners in the assays, which led to the formation of nonspecific complexes or aggregates (i.e., cluster ions). On the contrary, the "intensity fading MS" strategy is focused in the low mass range (below 10 to 15 kDa), profiting from the best sensitivity in this mass range of standard MALDI-TOF instruments equipped with microchannel plate detectors [35]. This allowed us to work with rather highly complex mixtures of analytes in the sub-picomole range, which, we suggest, preserve at least partially the specific natural occurring bindings in solution and minimize the formation of nonspecific interactions in the gas phase.

Historically, any methodology to study noncovalent interactions in solution based on MALDI mass spectrometry has inevitably suffered from the physical and chemical processes associated to this technology. For this reason, "intensity fading MS" will not replace established affinity-based methodologies coupled to mass spectrometry such as surface plasmon resonance, cross-linking, or affinity purification among others. Even within the soft ionization techniques, ESI has been long recognized as the method of choice for studying noncovalent complexes [21]. However, to date, we strongly believe that the real potential of MALDI mass spectrometry in the study of noncovalent interactions has been underestimated because of our limitation of a complete understanding of all processes underlying this technology. New experimental approaches not conceived until recent years like "intensity fading MS" or possible modifications based on the same principles [28, 36], infrared (IR) MALDI [38, 39], atmospheric pressure (AP) MALDI [40, 41], DIOS-MS [42–44], or new generation of ion detectors [45, 46], may put on the same level MALDI and ESI mass spectrometry in the field of noncovalent interactions.

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