SHORT COMMUNICATION

Three-Layer Matrix/Sample Preparation Method for MALDI MS Analysis of Low Nanomolar Protein Samples

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A robust and sensitive sample preparation method is presented for matrix-assisted laser desorption ionization (MALDI) mass spectrometric analysis of low nanomolar concentrations of proteins containing high amounts of common salts and buffers. This method involves the production of densely packed sub-micrometer matrix crystals by depositing a matrix solution on top of a matrix seed-layer prepared on a MALDI target. A sub-microliter aliquot of analyte solution is then directly added to the top of the matrix crystals to form a thin-layer. α-Cyano-4-hydroxycinnamic acid (4-HCCA) is used as matrix and demonstrated to give better performance than other commonly used matrices, such as 2,5-dihydroxybenzoic acid (DHB), 2-(4-hydroxy-phenylazo) benzoic acid (HABA), or sinapinic acid. This three-layer method is shown to be superior to the other MALDI sample preparation methods, particularly for handling low nanomolar protein solutions containing salts and buffers. (J Am Soc Mass Spectrom 2006, 17, 780–785) © 2006 American Society for Mass Spectrometry

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) is well recognized as the technique for rapid analysis of protein samples containing high amounts of salts and buffers. With the use of a modern MALDI instrument, the success of MALDI in analyzing real world samples is largely dependent on sample preparation. It is thus not surprising that there are a number of matrix and sample preparation methods being developed. They include dried-droplet [1], vacuum drying [2], crushed-crystal [3], slow crystal growing [4], active film [5, 6], pneumatic spray [7], electrospray [8], fast solvent evaporation [9, 10], sandwich [11, 12], and two-layer method [13]. In addition, numerous methods have been developed for protein concentration and purification purposes; for example, on-target sample cleanup or employing reversed-phase microbeads in pipette tips [12], chemically modified MALDI target plates [14–16], or pretreatment with surfactants [17], to name a few. However, sample cleanup procedures can often result in sample loss or require more expensive and complicated setup.

We have demonstrated earlier that the two-layer method can provide much improved performance in analyzing complex protein and peptide mixtures [18], compared to the conventional dried-droplet method [1], and the fast evaporation method [9]. The two-layer method involves the use of fast solvent evaporation to form the first layer of small matrix crystals, followed by deposition of a *mixture* of matrix and analyte solution on top of the crystal layer. With this method, the matrix and analyte solution conditions for preparing the second layer can be readily altered and fine-tuned for specific applications [18–20]. This method has been demonstrated to be useful for direct analysis of bacteria cell extracts, milk proteins, proteins extracted out of copper or Coomassie-stained gels, and protein/peptide samples containing up to 1% SDS [18–20].

One class of samples commonly encountered in protein mass spectrometric labs that were found to be difficult to handle by using the two-layer method is low nanomolar protein solutions containing high amounts of salts and buffers. Protein concentration and salt contents can affect the efficiency of protein incorporation into matrix crystals. Using the two-layer method to prepare the matrix/analyte co-crystals for this type of samples, the protein concentration in the co-crystals appears to fall below the sensitivity of the MALDI technique for detection. We have developed a three-layer matrix/sample preparation method to handle this type of samples. This method involves the casting of a thin matrix-crystal layer, followed by deposition of another layer of matrix to produce a densely packed, thicker matrix layer. An aliquot of protein solution is directly deposited onto the thick matrix layer. Using this method, the proteins appear to incorporate into the matrix crystals in a sufficient concentration to be detected by MALDI. In this paper, the three-layer matrix/

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sample preparation method is presented and compared to other methods. Several examples are given to illustrate the application of this method to handle real world samples.

Experimental

Chemicals and Reagents

Bovine lactoferrin (M.W. ~80 kDa), bacteriorhodopsin (M.W. ~27 kDa), trifluoroacetic and formic acid, dithiothreitol (DTT), iodoacetamide, sinapinic acid, 2,5-dihydroxybenzoic acid (DHB), 5-methoxybenzoic acid, 2-(4-hydroxy-phenylazo) benzoic acid (HABA), and α-cyano-4-hydroxycinnamic acid (4-HCCA) were purchased from Sigma-Aldrich-Fluka Canada (Oakville, Ontario, Canada). 4-HCCA was recrystallized from ethanol (95%) at 50 °C before use. Protein stock solutions were made up in distilled water at a concentration of 1 mg/mL. Final protein concentrations and mixing ratios with matrix substances are mentioned for each spectrum in the following sections. All protein solutions were prepared in siliconized polypropylene vials (Rose Scientific, Edmonton, Alberta, Canada) to minimize sample loss by container wall adsorption.

Matrix and Sample Preparation

The three-layer method was developed based on the previously reported two-layer method [13, 18]. To form a very thin first layer, a ~1 μL volume of a 20 mg/mL solution of 4-HCCA in 40% methanol in acetone was deposited onto a MALDI target spot. The drop spreads over the whole target spot and evaporates quickly, leaving a very thin-layer of tiny matrix crystals. The layer was visually checked over with a 30× magnification handheld microscope for cavities and if necessary the first layer formation was redone. As a second layer, a 0.4 μL volume of saturated 4-HCCA in 40% methanol in water was then deposited onto the first layer. The third and last layer was a ~0.3 μL volume of the protein sample solution. It should be noted that basic protein solution had to be acidified before deposition (e.g., adding some 0.1% TFA). Otherwise, the matrix layers would dissolve. As a washing step, a ~0.5 μL volume of distilled water was deposited on top of the third layer and blown off with pressurized air after approximately 30 s. However, it was found that for larger proteins (>30 kDa) washing steps were often not necessary (and sometimes not advantageous because of sample loss) even in the presence of high amounts of salt.

The two- and three-layer methods using HCCA as matrix were compared with the two-layer method of Dai et al. [13] using sinapinic acid as matrix and the dried-droplet method of Spengler et al. [21] using DHB as matrix, and the dried-droplet method of Juhasz et al. [22] using HABA as matrix. In the two-layer method, 1 μL of 6 mg/mL sinapinic acid in 60:40 methanol/acetonitrile was deposited onto the target as a seed layer and left for drying. The protein sample was mixed 1:1 with a saturated solution of sinapinic acid in 30% acetonitrile, 20% methanol, and 50% water. 0.5 μL of the sample/matrix mixture was then deposited onto the seed layer and left for drying. In the dried droplet method using DHB as matrix, the sample was mixed 1:2 with a 20 mg/mL DHB solution in 33% acetonitrile in water. 1 μL of the sample/matrix mixture was deposited onto a target and left for drying. In the dried droplet method using HABA as matrix, the sample was mixed 1:1 with a 1.5 mg/mL HABA solution in 50% acetonitrile/water. 1 μL of the sample/matrix mixture was deposited onto a target and left for drying.

MALDI-TOF MS and Data Processing

MALDI mass spectra were collected on a Hewlett-Packard model G2025A LD-TOF linear time-of-flight system (Hewlett-Packard, Reno, NV) or a Bruker REFLEX III time-of-flight mass spectrometer (Bremen/Leipzig, Germany) operated in linear positive ion mode. For protein analysis, the detection sensitivity of the Bruker instrument is similar to that of the Hewlett-Packard system. Both instruments were equipped with a pulsed nitrogen laser. In general, 50 to 100 laser shots were averaged to produce a mass spectrum. Spectra acquired were reprocessed with the Igor Pro software package (WaveMetrics Inc., Lake Oswego, OR).

Results and Discussion

In MALDI-TOF analysis, there is a subtle difference in sample handling for the detection of relatively high molecular weight analytes (i.e., M.W. > 30 kDa in our TOF instruments), compared to low molecular weight species such as peptides and small proteins. On the one hand, the high mass analyte needs to be isolated with a relatively larger number of matrix molecules in the co-crystals (i.e., a greater matrix to analyte ratio), causing the reduction of analyte concentration in the solid crystals. On the other hand, detection sensitivity of TOF instruments generally start to degrade as the masses of the ions increase, likely due to the reduction of detection efficiency of conventional detectors, such as the multi-channel plate detector. Thus sample preparation methods that work well for highly contaminated peptide or small protein samples may not provide any useful results for protein samples.

We have compared our method with several matrix/sample preparation methods that were reported to be particularly useful for protein analysis by MALDI MS. Figure 1 shows the mass spectra of bovine lactoferrin obtained from these methods with two different amounts of sample loading. Table 1 lists the obtained mass resolution values for the singly charged molecular ion of lactoferrin. The two-layer method of Dai et al. using sinapinic acid [13] and the method of Spengler and coworkers using DHB as matrix substance [21],

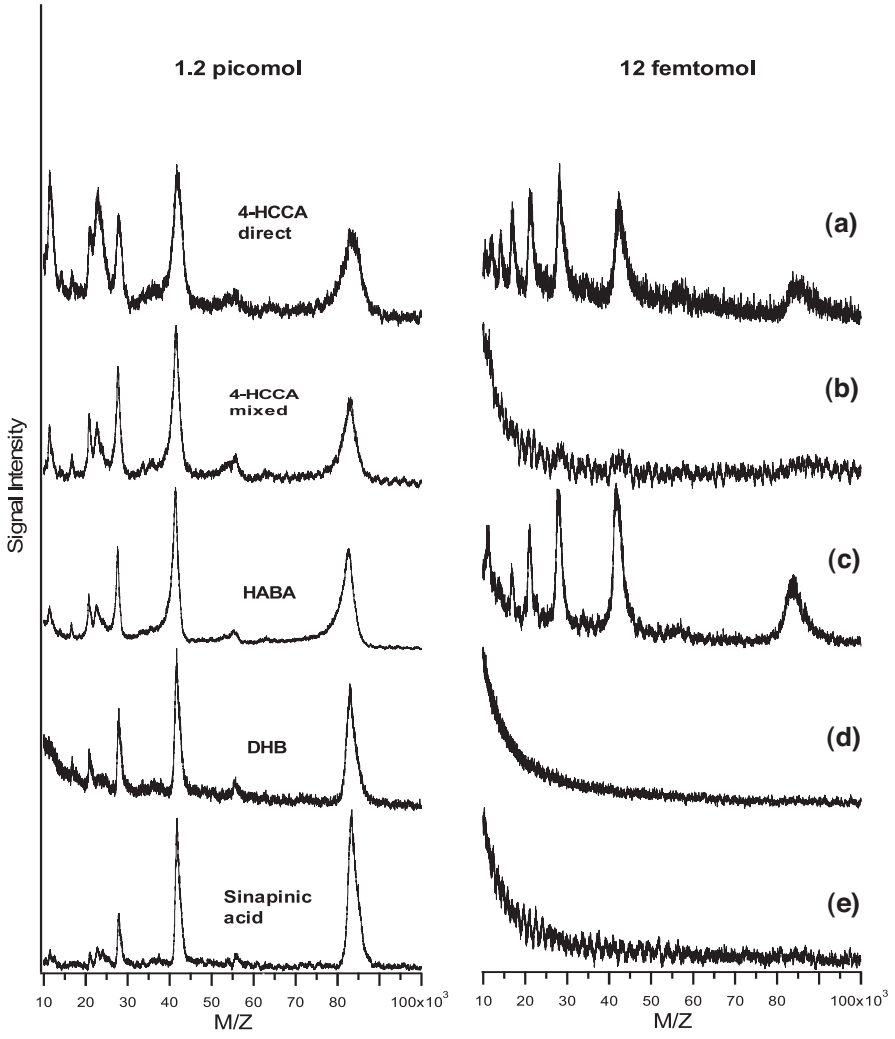

Figure 1. Comparison of the performance of different matrix/sample preparation methods for MALDI-TOF analyses of two concentrations of lactoferrin (M.W. ~80 kDa). (**a**) Three-layer method by direct deposition of the analyte solution onto a 4-HCCA matrix double-layer. (**b**) Two-layer method by deposition of aqueous protein solution mixed with saturated 4-HCCA onto a single 4-HCCA matrix layer. (**c**) Method according to Juhasz et al. [22] using HABA as matrix. (**d**) Method according to Spengler et al. [21] using DHB as matrix. (**e**) Method using sinapinic acid as matrix according to Dai et al. [13].

give the best resolution for this analyte at a sample loading of 1.2 pmol. However, it becomes clear that at low analyte concentration (i.e., with a loading of 12 fmol) only the method of Juhasz et al. using HABA as matrix [22], and our three-layer method using 4-HCCA are suitable for protein detection.

Figure 2 shows the MALDI spectra of lactoferrin with a sample loading of 2 fmol obtained by the HABA method and the new three-layer method. The HABA method gives a slightly better resolution and sensitivity for this relatively pure sample, compared to the three-layer method. However, there are several disadvan-

Table 1. Mass resolution comparison of singly charged species of bovine lactoferrin for different sample/matrix preparations

Label in Figure 1.	Matrix and method	Resolution (FWHM) 1.2 picomole	Resolution (FWHM) 12 femtomole
(a)	4-HCCA, three-layer	~18	~7
(b)	4-HCCA, two-layer	~25	Not detectable
(c)	HABA, ref. 22, dried-droplet	~27	~21
(d)	DHB, ref. 21, dried-droplet	~36	Not detectable
(e)	Sinapinic acid, ref. 13, two-layer	~38	Not detectable

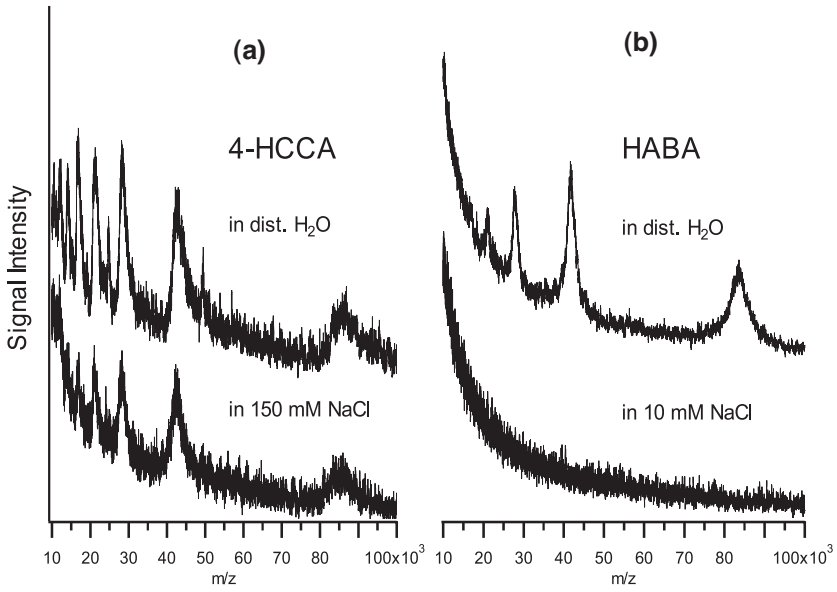

Figure 2. Comparison of 4-HCCA and HABA with different matrix/sample preparation methods. For each experiment, a total protein amount of ~2 femtomole was used. (**a**) Direct depositions of 0.2 μL 10 nM lactoferrin solutions (top: in distilled water, bottom: in 150 mM NaCl) onto a 4-HCCA matrix double-layer. (**b**) 0.2 μL of 10 nM lactoferrin solutions (top: in distilled water, bottom in 10 mM NaCl mixed 1:2 with HABA, 1.5 mg in acetonitrile:methanol:water [40:40:20, by volume]).

tages using HABA as a matrix substance. First of all, direct deposition of the analyte solution onto a HABA matrix layer did not yield any signals and thus it is necessary to premix the matrix with the analyte solution which dilutes the analyte solution. In the three-layer method, analyte solution is directly deposited to the matrix double-layer. Second, HABA has low salt tolerance. As is shown in Figure 2, even at a salt concentration of 150 mM the three-layer method still gives strong signal at the 2 femtomole level without using a washing step. HABA, however, does not give any signal for the same analyte amount in the presence of only 10 mM salt. Third, the double-layer of 4-HCCA withstands several washing steps if necessary; but HABA crystals are too soluble in water and many matrix crystals that contain analyte are easily washed off.

Salts and buffers are commonly used to provide stability for the proteins under investigation. Direct analysis of protein samples containing salts and buffers clearly has the advantage of minimizing sample loss due to protein aggregation and adsorption during sample workup. The impurity tolerance level of the three-layer method is quite high. For example, the analysis of 20 nM lactoferrin solution contaminated with following common salts, detergents, and buffers are still successful: 1 M Sodium chloride, 0.5% n-Octyl-β-D-glucopyranoside, 20 mM Guanidine HCl, or 0.2% TRIS (neutralized). However, protein samples containing 0.01% SDS or 0.01% CHAPS do not give protein MALDI signals (data not shown).

It should be noted that we have also attempted to deposit protein samples directly onto the first matrix layer, which resembles the fast evaporation method reported by Vorm et al. for peptides [9]. We found that similar detection limits can be achieved for relatively pure protein samples, compared to the three-layer method. However, the use of a single layer of matrix is not as rugged. The thin-layer formed is much more easily washed off when washing steps are applied. In addition, in cases where the sample solution contains slightly basic buffers (pH 7–8) or organic modifiers, the single layer dissolves easily, thus ruining the experiment.

Sample cleaning by using small columns such as a Ziptip before the MS analysis of peptides and small proteins is effective for generating useful MALDI spectra. We have compared our method with the Ziptip technique in their ability to handle low nanomolar protein solutions containing high amounts of salts and buffers and found that our method without prior sample cleanup is better than the Ziptip technique. This is understandable considering that the possibility of sample loss in the sample handling procedure is greater for larger proteins than for small proteins or peptides, most likely due to their size and the greater number of nonspecific binding sites, such as hydrophobic patches, charged groups, and hydrogen bonding sites.

Because of its simplicity and high sensitivity, this three-layer technique has become a routine method in our laboratory as well as service laboratories including the Alberta Cancer Board Proteomics Resource Lab and the Chemistry Department Mass Spectrometry Facility. This method is very useful, especially as a preliminary fast screening method for unknown protein samples. Figure 3 illustrates several examples of analyzing samples from different sources, which could not be ana-

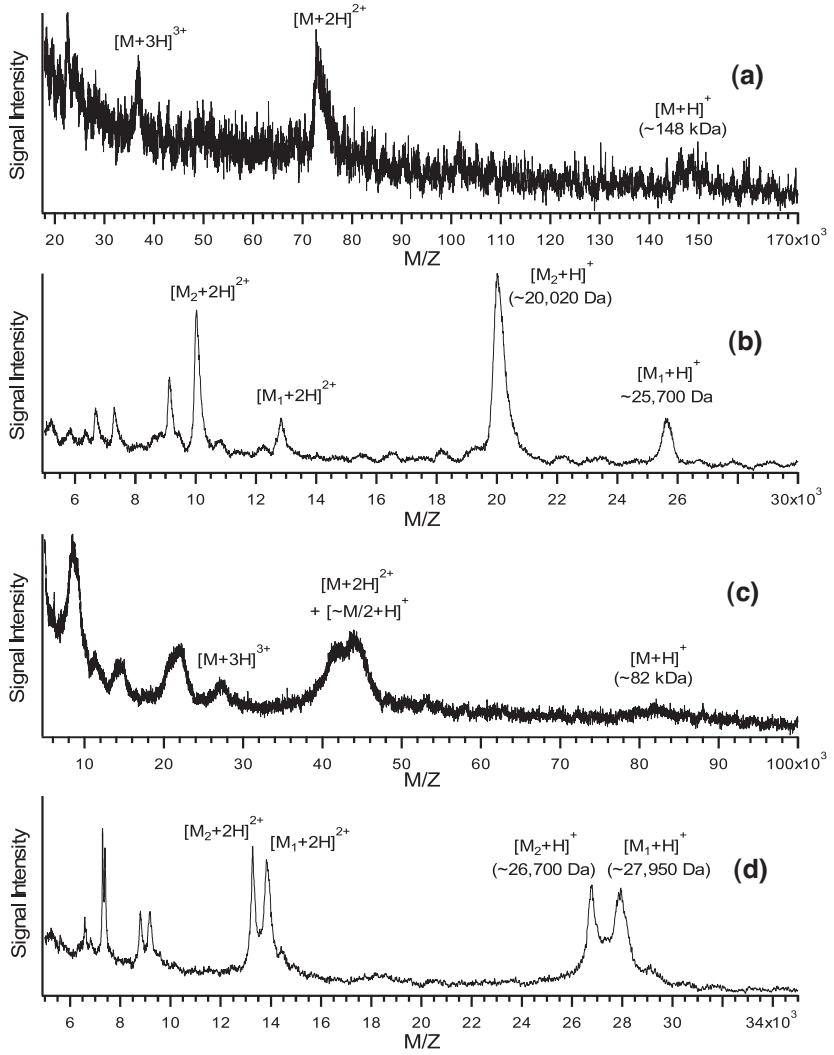

Figure 3. MALDI mass spectra of "difficult" protein samples using the three-layer matrix/sample preparation method. (**a**) Cleaved anti-gal IgG sample in PBS buffer (100 mM phosphate, 150 mM NaCl). (**b**) HRV protein mixture in 300 mM NaCl, 20 mM imidazol, and 0.2% EDTA (pH ~7.5). (**c**) Natural secreted form of human P97 protein (M.W. of deglycosylated form ~82 kDa, gift from Dr. Luis Sojo, ABR Vancouver) in unknown solution conditions. (**d**) Bacteriorhodopsin, a very hydrophobic membrane protein, in aqueous solution.

lyzed previously without extensive purification and/or concentration procedures. Although the protein concentrations for these real world samples were unknown, it was estimated that they were in the low nanomolar range, since none of the other matrix/sample preparation techniques yielded any useful results. Despite the high amounts of buffers and salts, all samples are detectable without applying any washing steps. The attempt to analyze these samples by depositing them only onto the first matrix layer was unsuccessful; the layer dissolved. The slightly thicker matrix layer achieved by the double deposition technique is again an advantage since the layer is able to neutralize slightly basic buffers (pH 7.5–8) right on target and better withstands desolvation. Of course, for samples at higher pH, neutralization will still be necessary before sample deposition.

Comparing the two-layer and three-layer methods, the two-layer method is generally suitable for many applications involving protein solutions with concentrations above 50–100 nM. For these solutions, the two-layer method provides better sensitivity compared to the three-layer method. This is likely due to better incorporation of proteins in matrix crystals when the analyte solution is mixed with the matrix solution as the second-layer solution. In the three-layer method, after the analyte solution is deposited, the matrix layer partially redissolves and then recrystallizes, entrapping the proteins. For high concentration of proteins, the matrix to analyte ratio is likely very low, resulting in reduced protein signals. However, for highly contaminated, low nanomolar protein solutions, the three-layer method, in our hands, is the only viable method to produce protein signals. Another difference is related to

detergent tolerance. While both methods can tolerate high concentrations of salts and buffers, analysis of protein samples containing SDS or CHAPS can only be done by using the two-layer method, thus requiring a protein solution concentration of greater than 50 to 100 nM. Finally, it should be noted that, with both layered methods, the analyte signals can be easily observed without the need of searching for the hot spots which is the case in the dried-droplet method.

In summary, for MALDI analysis of micromolar concentration (>1 μM) of protein samples, a number of matrix/sample preparation methods can be used to generate protein signals. To handle nanomolar concentration of protein samples, the selection of an appropriate matrix/sample preparation method becomes very important. For the analysis of high nanomolar concentration (>50–100 nM) of protein samples, we find that the two-layer method can generate protein signals, even for samples containing high salts, buffers, and detergents including SDS up to about 1%. For the analysis of low nanomolar concentration (<50–100 nM) of protein samples, the three-layer method presented in this work can be used. It can tolerate a high amount of salts, buffers, and some nonionic detergents such as n-Octyl-β-D-glucopyranoside. But the method cannot tolerate even a small amount of ionic detergents like SDS or CHAPS (0.01%).

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