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α-Cyano-4-hydroxycinnamic Acid Affinity Sample Preparation. A Protocol for MALDI-MS Peptide Analysis in Proteomics

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We present a new MALDI sample preparation technique for peptide analysis using the matrix α-cyano-4-hydroxycinnamic acid (CHCA) and prestructured sample supports. The preparation integrates sample purification, based on the affinity of microcrystalline CHCA for peptides, thereby simplifying the analysis of crude peptide mixtures. Enzymatic digests can thus be prepared directly, without preceding purification. Prepared samples are homogeneous, facilitating automatic spectra acquisition. This method allows preparation of large numbers of samples with little effort and without the need for automation. These features make the described preparation suitable for cost-efficient high-throughput protein identification. Performance of the sample preparation is demonstrated with in situ proteolytic digests of human brain proteins separated by two-dimensional gel electrophoresis.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) has become an important analytical tool in protein research due to its ability to analyze femtomole amounts of peptide mixtures in short time.[1−6] The implementation of MALDI-TOF-MS peptide analysis in current proteomic research (see reviews, refs 7−10) presents a challenge to the technique with respect to high sample throughput combined with high detection sensitivity. These demands put stress on sample purification, enrichment, and preparation for MALDI-MS, which must be reproducible, cost-efficient, and yielding MALDI samples suitable for automatic analysis.

It has been shown that spectra reproducibility is improved when the sample diameter falls below 250 μm, because a large portion of the sample is simultaneously irradiated by the desorption/ionization laser.[11,12] Such samples are well suited for automatic data acquisition because only one or a few sample positions have to be investigated to obtain a mass spectrum that is representative of the sample. Preparation of such samples requires, however, that the samples have been purified and highly enriched prior to dispensing onto the sample support.

Small reversed-phase columns integrated at the outlet of pipet tips have proven to be efficient for peptide sample purification and analyte enrichment in dilute samples.[11,13] With small column bed volumes, analyte loss due to surface adsorption is minimized. The purified sample can be eluted onto the MALDI sample support in a relatively small volume, leading to increased detection sensitivity by analyte enrichment. For preparation of many samples, however, reversed-phase purification is inefficient with regard to cost and time consumption.

We recently reported a novel prestructured sample support for MALDI-MS.[12] The sample support has a strongly solvent-repellent (lyophobic) surface, equipped with an array of hydrophilic spots acting as sample anchors. Each sample droplet deposited onto a prestructured sample support contacts one anchor. The droplets are repelled by the lyophobic surface but adhere to the anchors by hydrophilic interactions. Upon solvent evaporation, the samples are concentrated onto the anchors. If the matrix concentration is kept sufficiently low and a suitable solvent chosen, sample crystallization occurs solely on the anchor spots. Analyte molecules are thus concentrated only to the small area defined by the anchor, resulting in a sensitivity increment by 1 order of magnitude compared to conventional stainless steel supports. A micrograph of a section of a prestruc-

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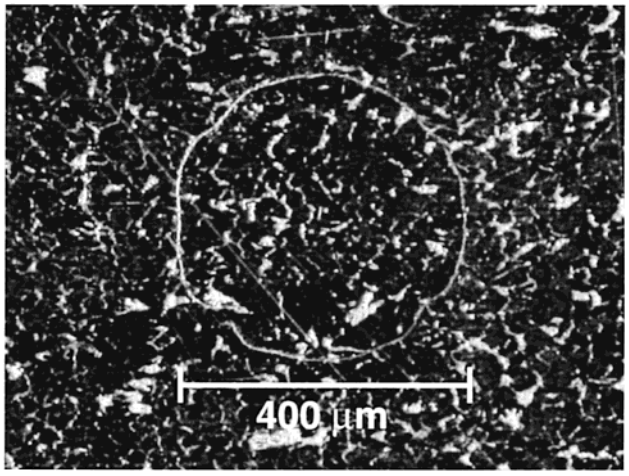

Figure 1. Micrograph of a section of a prestructured sample support. The stainless steel surface of the sample support is covered by a 5-μm-thick coating of a Teflon-like composite material providing a strongly lyophobic surface. A 400-μm hydrophilic sample anchor is provided by an interruption in the surface coating.

tured sample support, harboring a 400-μm hydrophilic anchor is shown in Figure 1.

In our previous study, the use of prestructured sample supports was, however, limited to water-soluble matrixes (2,5-dihydroxybenzoic acid was used for peptide analysis). Furthermore, very pure samples were required because contaminants are enriched on the anchors as well as analytes. The motivation for the current study was to find a preparation protocol for the water-insoluble matrix α-cyano-4-hydroxycinnamic acid (CHCA) compatible with the prestructured sample supports and integrating sample purification in the procedure.

EXPERIMENTAL SECTION

Prestructured Sample Supports. In the previous study, the sample support was coated by lyophobic Teflon, carrying an array of gold spots acting as hydrophilic sample anchors. In the present study, a new sample support design was used. The sample supports were coated with a ~5-μm-thick layer of a Teflon-like composite material equipped with an array of 384 circular interruptions of 400-μm diameter, at which the underlying stainless steel surface acts as sample anchors (AnchorChip 400/384, Bruker Daltonik, Bremen, Germany). The new surface modification gives the same results as the old but is more robust affording multiple reuse of the sample support.

Standard Peptide Stock Solution. The following peptides (peptides 1−8) were purchased from Bachem: human angiotensin I and II, substance P methyl ester, neurotensin clip 1−11 and 1−13, ACTH clip 1−17 and 18−39, and somatostatin. A stock solution containing 1 pmol/μL of each of the peptides 1−6 and 2 pmol/μL of peptides 7 and 8 in 35% acetonitrile (ACN), 0.1% TFA was prepared following the quantity specifications provided by the manufacturer.

Tryptic Digests of Gel-Separated Proteins from Human Brain. Coomassie G250-stained large-format 2D gels[14] of human brain total protein extract were a gift from Prof. J. Klose, Humboldt-University, Germany. Cylindrical gel samples of 1-mm diameter were excised and destained by incubation with 400 μL of 25% 2-propanol for 30 min. The destained gel samples were dried in a Speedvac for 10 min, followed by addition of 5 μL of digestion buffer (5 mM DTT, 5 mM *n*-octyl glucopyranoside (*n*-OGP), 20 mM Tris, pH 7.8) containing 12 ng/μL modified porcine trypsin (sequencing grade, Promega). After overnight incubation at 37 °C, 5 μL of 0.4% TFA, 5 mM *n*-OGP was added and the incubation was extended for 1 h at room temperature. To obtain sufficient volume for comparison of sample preparation methods, an additional 5 μL of 0.2% TFA, 5 mM *n*-OGP was added. Samples were stored at −20 °C prior to MALDI sample preparation.

CHCA Surface Affinity Preparation. Thin layers of CHCA crystals were prepared on all anchors on the sample plate. This was done by dispensing 100 μL of CHCA solution (100 g/L CHCA in 90% acetone, 0.005% TFA (v/v)) near one of the short edges of the support. A Teflon rod was then placed horizontally across the sample plate surface, in contact with the matrix solution, which then spread out across the length of the rod creating a liquid film between the rod and the sample support. The rod was then drawn across the sample support, dragging the matrix solution along. Each hydrophilic anchor adsorbed a small volume of matrix solution resulting, after solvent evaporation, in a thin layer of crystalline CHCA. The procedure and a micrograph of a prepared sample is shown in Figure 2 a. If it is not desired to prepare matrix spots on all anchors of the support, a small number of samples may be prepared by using a narrow pipet tip (GELoader, Eppendorf). The pipet tip is immersed in CHCA solution (1 g/L, 99% acetone, 0.2% TFA), aspirating a volume of the solution by capillary force. Upon touching an anchor with the pipet tip, a small volume of matrix solution is deposited. Aqueous peptide samples were acidified by addition of trifluoroacetic acid (TFA) to a final concentration of minimum 0.2% (v/v). A 2-μL sample was deposited onto the CHCA matrix layer (Figure 2b). After 2 min, the remaining liquid was removed by absorption onto a paper tissue (Figure 2c). In some cases, on-target washing resulted in improved spectra quality. Washing was performed by immersing the sample support in a solution of 0.1% TFA for a few seconds, after which the remaining liquid was removed by a stream of air. Note that for good performance it is important that the samples do not contain organic solvents, which may partially dissolve the matrix crystals. Also, negative results were obtained when samples were acidified by 5−10% formic acid instead of TFA, probably for the same reason.

MALDI-TOF-MS. Mass spectra of positively charged ions were recorded on a Bruker Scout 384 Reflex III instrument (Bruker Daltonik) operated in the reflector mode. A total of 100 single-shot spectra were accumulated from each sample. The total acceleration voltage was 25 kV. The XMASS 5.0 and MS Biotools software packages provided by the manufacturer were used for data processing. For the tryptic digests, known autoproteolysis products were used for internal calibration.

Database Searching. For protein identification, human protein sequences in the SwissProt database, release no. 39, were searched using the Mascot Software (Matrix Science Ltd.). The probability score calculated by the software was used as criterion for correct identification, with the additional criterion that a minimum of three peptides more were required to match the highest ranking sequence entry, compared to the next unrelated

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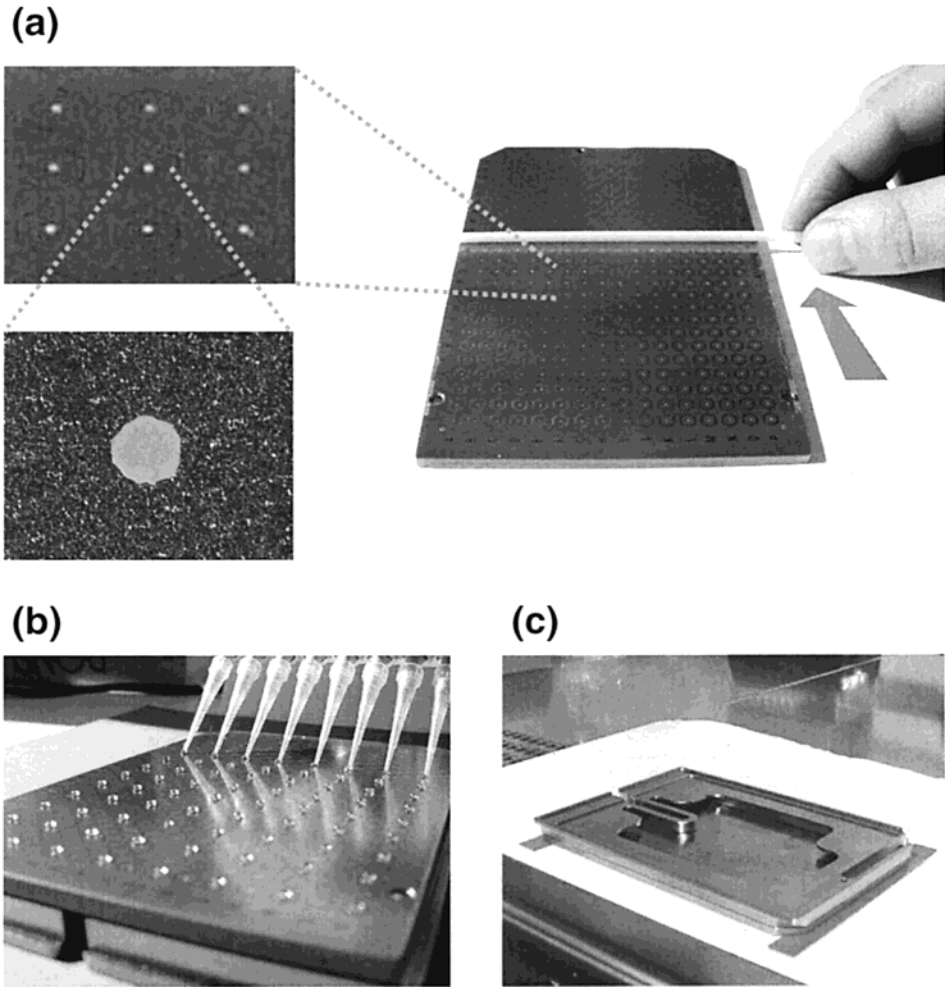

Figure 2. CHCA surface affinity preparation. (a) Thin layer of CHCA prepared onto 400-μm anchor. (b) A 2-μL acidified sample is deposited onto the crystalline matrix layer. (c) After 3-min incubation, remaining liquid is removed by adsorption onto a paper tissue. Spacers prevent direct contact between matrix crystals and the paper tissue.

candidate. A mass deviation of 30 ppm was tolerated in the searches, and oxidation of methionine residues was considered a possible modification.

RESULTS AND DISCUSSION

The CHCA surface affinity sample preparation resembles the conventional fast evaporation preparation[15] in that an acidified, aqueous sample is deposited onto a microcrystalline surface of CHCA. It differs, however, in one important aspect: sample droplets deposited onto the crystalline matrix layer are not allowed to dry, but are instead removed after a short time in contact with the crystalline matrix layer. The effect of this modification is demonstrated in the following. Figure 3 shows mass spectra obtained from the standard peptide mixture diluted to 10 fmol/μL in 0.2% TFA. Sample aliquots of 2 μL were deposited onto CHCA microcrystalline layers prepared on 400-μm anchors. Remaining sample solution was removed at different time points. One sample was allowed to dry completely. For comparison, one sample was prepared according to the fast evaporation method on a conventional stainless steel support. Already after 10-s incubation (a), most of the peptides could be detected. Between 1- and 3-min incubation (b−d), signals were detected from all sample components, and no significant increase in the overall signal intensity was observed over time. When the sample was left to dry completely onto the matrix-coated anchor (e), strong selective suppression of several peptide signals was the result. Such strong discrimination was, however, not observed for the conventional fast evaporation preparation on stainless steel support (f) in which the sample droplet is spread over a larger surface. Amino acid side-chain oxidation was observed to increase with increasing incubation time and was also observed for the fast evaporation preparation. Peptides 6 (ACTH 1−17) and 8 (ACTH 18−39) contains one methionine and one tryptophan residue each. They were detected with additional signals of +16, +32, and +48 Da, corresponding to partial oxidation of the methionine residue to methionine sulfoxide (+16 Da) and oxidation of tryptophan (+32 Da). For peptide 3 (substance P), containing one methionine residue and no tryptophan, only an additional +16 Da signal was observed. After 3-min incubation, the oxidized forms were dominating. This high degree of oxidation was not observed for samples prepared according to the standard dried-droplet method (data not shown) and appears to be a general feature of sample preparation on microcrystalline CHCA surfaces. The abundance of oxidized species decreases detection sensitivity and impairs protein identification because the possibility of multiple oxidation

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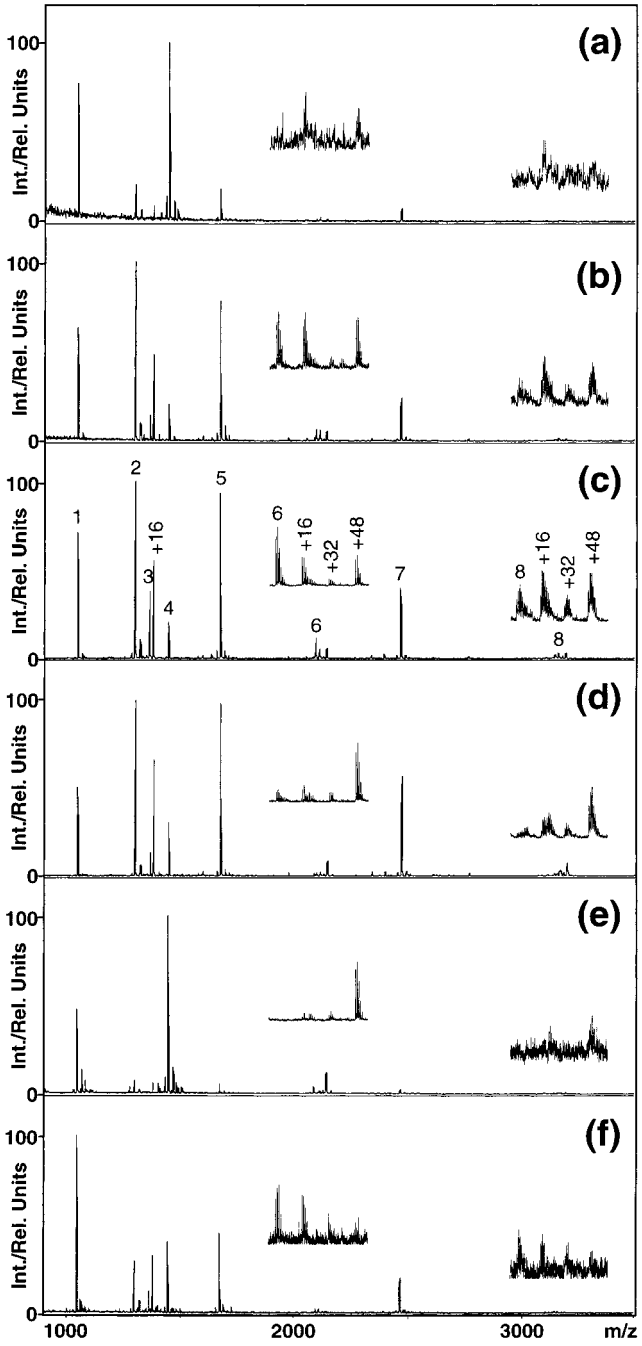

Figure 3. MALDI-TOF mass spectra of a standard peptide mixture in 0.2% TFA: 1, human angiotensin I; 2, angiotensin II; 3, substance P methyl ester; 4, neurotensin 1−11; 5, neurotensin 1−13; 6, ACTH 1−17; 7, ACTH clip 18−39; 8, somatostatin. The concentration was 10 fmol/μL peptides 1−6 and 20 fmol/μL peptides 7 and 8. Aliquots of 2 μL were deposited onto CHCA microcrystalline layers (see Figure 2). Remaining liquid was removed after (a) 10 s, (b) 30 s, (c) 1 min, and (d) 3 min or (e) and left to dry onto the matrix layer. (f) Sample prepared according to the fast evaporation method on a conventional stainless steel support.

has to be considered in the database search. Removing the remaining liquid sample before the solvent evaporated only partially decreased the degree of oxidation.

The apparently strong affinity of microcrystalline CHCA layers for peptides makes it possible to concentrate analytes on the anchor spots without enriching salts. This feature provides a simple and efficient means for sample purification. Figure 4 a

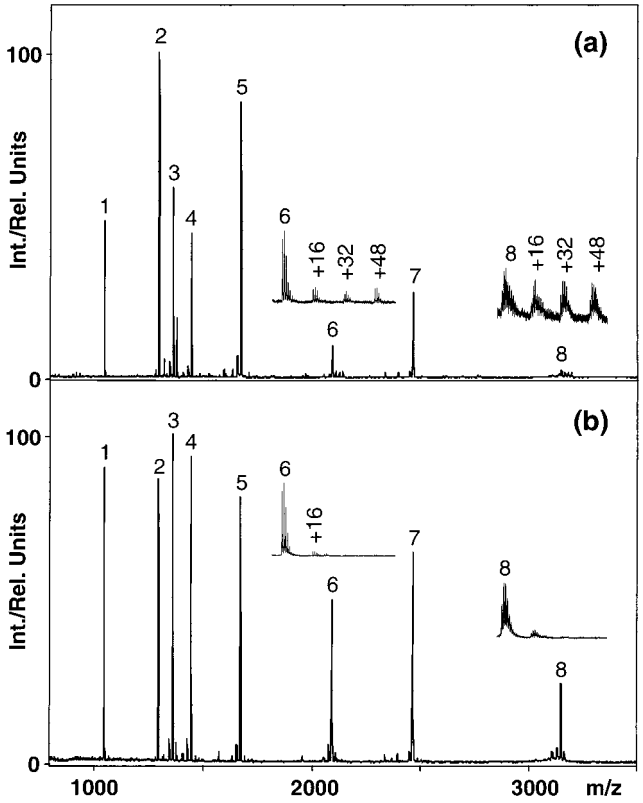

Figure 4. MALDI-TOF mass spectra of a standard peptide mixture in (a) 20 mM Trizma base, 5 mM DTT, 0.2% TFA and (b) 20 mM Trizma base, 5 mM DTT, 0.2% TFA, 5 mM *n*-OGP. Remaining sample liquid was removed after 3-min incubation with the CHCA matrix layer.

shows a mass spectrum of the standard peptide mixture diluted to 10 fmol/μL in 20 mM Tris, 5 mM DTT, 0.2% TFA (acidified digestion buffer) after 2-min incubation with the CHCA layer. The quality of the spectrum is similar to that observed in the absence of salt (Figure 3). Phosphate salt (20 mM) in the sample was also investigated and gave similar results (data not shown).

A major factor limiting detection sensitivity for peptide mixtures is analyte loss due to surface adsorption (sample vials, pipet tips, etc.). Nonionic detergents, such as Tween 20 and NP-40, efficient in keeping analytes in solution, are incompatible with MALDI. The nonionic detergent *n*-octyl glucopyranoside, known to promote protein solubilization,[16] is, however, MALDI-compatible and has been previously shown to enhance the MALDI-MS response, especially of larger peptides.[17,18] We therefore investigated whether *n*-OGP is compatible with the CHCA surface affinity preparation. Figure 4b shows a mass spectrum of the standard peptide mixture in the same solution as in Figure 4a, but with the addition of 5 mM *n*-OGP. The addition of *n*-OGP resulted in an increased signal intensity, especially for peptides 6 (ACTH 1−17) and 8 (ACTH 18−39), and more even signal intensities for all peptides. An unexpected observation was that almost no oxidation of peptides 3, 6, and 8 was observed when *n*-OGP was present in the sample solution, thereby eliminating the above-described problem. *n*-OGP was therefore included in the sample buffer for in situ digestion of gel-separated proteins.

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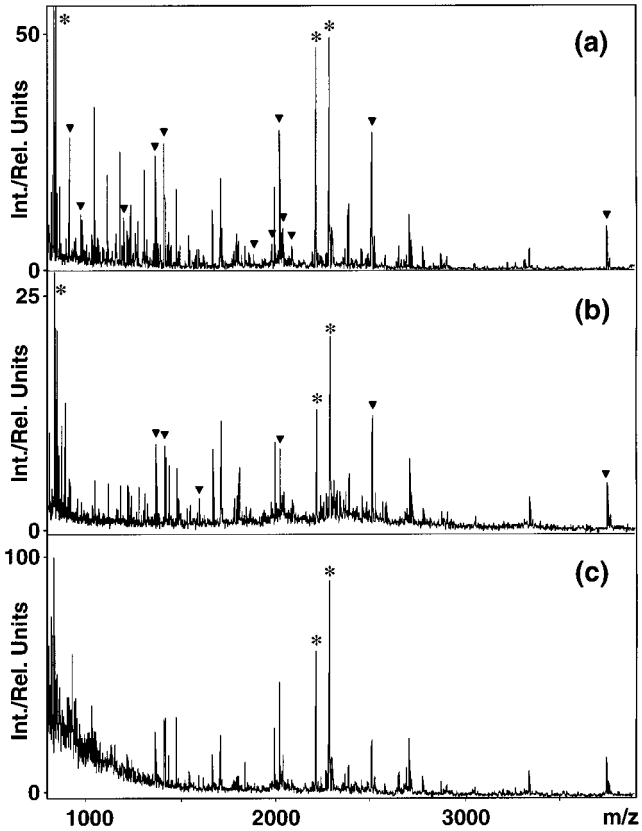

Figure 5. Comparison of sample preparations for the analysis of an in situ proteolytic digest of a human brain protein isolated by 2-DE. (a) CHCA surface affinity preparation on a prestructured sample support, (b) fast evaporation method, and (c) ZipTip-C_{18} preparation with CHCA on a conventional stainless steel support. Peptide signals matched to the identified protein are marked with a triangle. Trypsin autoproteolysis products are indicated with "∗".

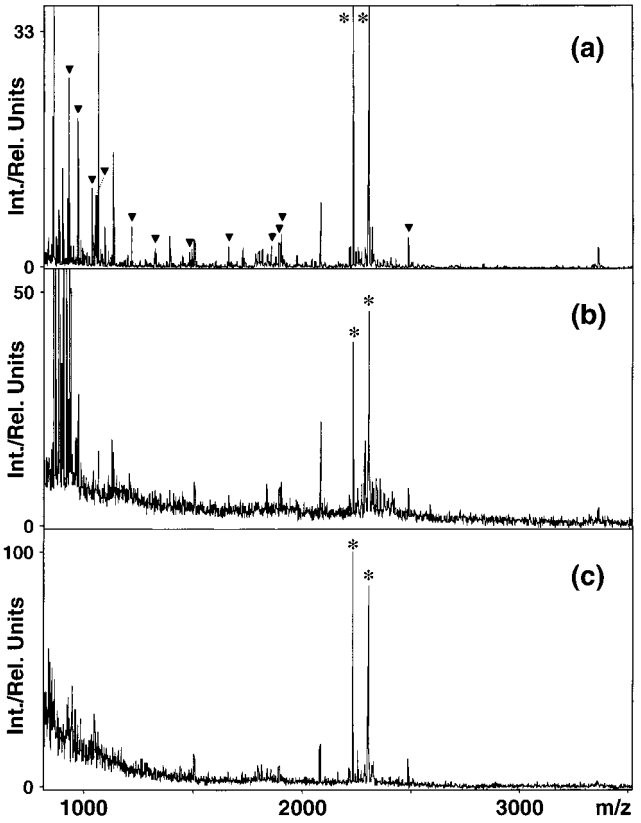

Figure 6. Comparison of sample preparations for the analysis of an in situ proteolytic digest of a human brain protein isolated by 2-DE. (a) CHCA surface affinity preparation on a prestructured sample support, (b) fast evaporation method, and (c) ZipTip-C_{18} preparation with CHCA on a conventional stainless steel support. Figure annotations are the same as for Figure 5.

The method was applied to peptide mixtures obtained from in situ digestion of human brain proteins separated by 2DE. With six samples, a comparison was made with the conventional CHCA fast evaporation method[15] and with CHCA ZipTip-C_{18} sample preparation (preparation according to the manufacturer). In all cases, the CHCA surface affinity preparation yielded the best signal-to-noise ratio and the highest number of detected peptides. The mass spectra obtained from two of the protein digests with the different preparations are shown in Figures 5 and 6. A database search with the peak list obtained from the spectrum in Figure 6 a identified transketolase (Swiss Prot accession number P29401). Thirteen experimental masses matched theoretically calculated tryptic peptide masses of this protein within a tolerated mass deviation of 30 ppm. With the fast evaporation sample preparation (Figure 5b), six detected peptides matched the same protein, also ranking the same protein the highest in a database search, however with a too low confidence to be considered a reliable identification by the search engine. The mass spectrum obtained from ZipTip-C_{18} sample preparation (Figure 5c) resulted in detection of four peptides matching the protein, not sufficient for identification. From the second sample, the CHCA surface affinity preparation (Figure 6a) allowed identification of pyruvate kinase (Swiss Prot accession number P14618) with 12 matching peptides. With the two other preparations (Figure 6b,c), an insufficient number of signals were detected to allow protein identification.

CONCLUSIONS

The described preparation simplifies the analysis of crude peptide mixtures. The omission of purification procedures prior to MALDI sample preparation minimizes analyte loss due to surface adsorption. The preparation facilitates parallel preparation of large numbers of samples and is suitable for direct analysis of in situ proteolytic digests in proteomics. Integration of the technique in other proteomics enabling technology developed at our institute will be described in a separate research article.[19] The current development will be applied in a proteome study of *Arabidopsis thaliana*, undertaken by our laboratory.

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