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Glass-chip-based sample preparation and on-chip trypic digestion for matrix-assisted laser desorption/ionization mass spectrometric analysis using a sol–gel/2,5-dihydroxybenzoic acid hybrid matrix

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A glass-chip-based sample preparation method for matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) analysis of tryptic digests of proteins and intact cells is described. A MALDI matrix, 2,5-dihydroxybenzoic acid (2,5-DHB), was hybridized with sol–gels to generate a sol–gel-derived material. Taking advantage of the characteristics of sol–gels, the sol–gel-derived material readily adhered to the surface of a glass chip through covalent bonding. Only one step of sample preparation, deposition of the sample solution on the glass chip, was required before MALDI-MS analysis. Because 2,5-DHB was homogeneously dispersed on the sol–gel network structure, good spot-to-spot reproducibility was obtained in MALDI analysis using this approach and the analyte signals were uniform throughout the chip. The modified glass chips were robust and effective for at least 1 week. This glass-chip-based matrix preparation method provides a straightforward approach to developing techniques for analyzing the on-chip enzymatic digestion of proteins and intact cells of microorganisms. Cytochrome C and *Escherichia coli* were used as analytes to demonstrate the feasibility of this approach. The products of the on-chip enzymatic digests were identified through protein database searches. Copyright © 2004 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a very powerful tool for the analysis of a wide range of analytes.[1–5] The method of sample preparation and the choice of matrix for MALDI-MS are crucial factors for obtaining reliable results.[6–13] For example, the dried-droplet,[6] fast evaporation,[7,8] two-layer,[9,10] and thin-layer[11] approaches to sample preparation for MALDI-MS have been developed to improve mass resolution and sensitivity.

Previously, we presented a new sample preparation method for MALDI-MS analysis[14–16] that uses a sol–gel/2,5-dihydroxybenzoic acid (2,5-DHB) hybrid material to assist the desorption/ionization of analytes. A droplet of the sol–gel/2,5-DHB hybrid material is initially applied on Parafilm to generate a homogeneous deposition of matrix. The sample solution is then applied on the top of the matrix for MALDI analysis. Analyte signals were found to be homogeneous using this sample deposition technique. One of the characteristics of sol–gels is that they readily adhere to the surface of a glass chip through covalent bonding. Thus, a chip-based matrix can be developed by applying a thin film of sol–gel/MALDI matrix hybrid material homogeneously on the surface of a glass chip. Sample solutions are then simply deposited on a modified chip whenever MALDI-MS analysis is performed. This approach can reduce the time required for sample preparation.

Conventionally, in-solution enzymatic digests are employed for the enzymatic cleavage of proteins, which generally takes several hours to complete. On-target (or on-chip) enzymatic digestion has been demonstrated, however, to be an alternative method that reduces the reaction time, usually requiring only 10–15 min for a digestion reaction.[17,18] The MALDI matrix is then added to the digestion products on the sample target before MALDI-MS analysis. The MALDI-MS analysis results are combined with protein database searches for protein characterization and identification. MALDI fingerprint mass spectra of intact cells from microorganisms have been used widely to characterize different species of microorganisms.[3,19–22] The results for on-target intact-cell digests can provide further information for the identification of microorganisms. However, the analyte signals may only be found from specific 'sweet spots' when using conventional MALDI preparation methods. Use of glass chips previously coated with sol–gel/2,5-DHB for on-chip digestion is an alternative sample preparation method for MALDI analysis to reduce the 'sweet spots' problems. Thus, we here report a straightforward method for the on-chip tryptic digestion and analysis of proteins, with both cytochrome C and intact *E. coli* cells as examples, using a glass-chip-based matrix sample preparation method and MALDI-MS analysis.

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EXPERIMENTAL

Reagents

2,5-Dihydroxybenzoic acid (2,5-DHB) and cytochrome C were obtained from Sigma (MO, USA). Prometryn and ammonium hydrogen carbonate were obtained from Riedel de Haën (Deisenhofen, Germany). Melittin was obtained from Fluka (Buchs, Switzerland). Hydrogen peroxide was purchased from Janssen (Geel, Belgium), sulfuric acid from Pharmaco (Brookfield, CT, USA), tetraethoxysilane (TEOS) from Acros (NJ, USA), and hydrochloric acid from Merck (Darmstadt, Germany). *Escherichia coli* (*E. coli*) was a gift kindly provided by Professor Y.-K. Li (Department of Applied Chemistry, National Chiao Tung University, Taiwan).

Sample preparation on sol–gel-modified glass chips

Preparation of the sol–gel-derived 2,5-DHB material is described elsewhere.[14] Briefly, the sol–gel derivative was prepared by reacting 2,5-DHB (59 mg or 88.5 mg) in a starting sol solution consisting of TEOS (4.5 mL), water (1 mL), and hydrochloric acid (0.01 M, 0.4 mL). The mixture was stirred for ca. 1 day at room temperature (ca. 27°C) before use.

The sol (0.15 mL) was spin-coated on the surface of a glass chip (1.8 cm × 1.8 cm × 0.15 mm). The glass chips were pretreated by soaking in a solution of hydrogen peroxide and sulfuric acid (1:2, v/v) for 30 min to remove impurities, and then were washed with water and methanol. The sol solution was spin-coated on the surface of the glass chip using a spin coater at 3000 rpm for 1 min. To locate the sample spots conveniently on the glass chip during analysis, a marker pen was used to draw four longitudinal lines and another four horizontal lines at equal intervals on the reverse side of the glass chip, which generated 16 cross-points on the chip. The glass chip was attached to a sample target using double-sided carbon tape (Ted Pella). Sample solutions were deposited on individual crosses on the glass chip. After the volatile organics had evaporated, the sample target was placed in a mass spectrometer for analysis.

On-chip tryptic digestion

A sol solution (0.5 mL) was deposited on a glass chip and spin-coated at 2000 rpm for 1 min. The glass chip was aged for 30 min under ambient conditions and then was fixed on a sample target using double-sided carbon tape. A mixture of cytochrome C (250 μg/mL, 0.3 μL) and trypsin (250 μg/mL, 0.3 μL) dissolved in 25 mM NH_4HCO_3 (pH 7.7) was deposited directly on this modified glass chip. Similarly, a mixture of *E. coli* cells (1 mg/mL, 0.3 μL) and trypsin (1 mg/mL, 0.5 mg/mL, or 0.25 mg/mL) was deposited for on-chip digestion. The sample target was placed in an enclosure at a temperature held at ca. 35 ± 1°C. When the volatile organics on the glass chip had evaporated (ca. 10 min), 0.3 μL 1% trifluoroacetic acid (TFA) was added to the sample to stop the reaction. Melittin (0.1 mg/mL, 0.2 μL) was then added to the sample as an internal standard before placing the sample target into a mass spectrometer. Because of the presence of a relatively large amount of trypsin used in the digestion, the ion peaks of trypsin autolysis, such as those at *m/z* 1020.50 and 2163.05, were also used for internal mass calibration. When the volatile solvents evaporated from the digest sample, 2,5-DHB and the digestion products tended to crystallize at the periphery of the sample spot. The analyte signal was readily obtained along this periphery.

Database searches were conducted using the NCBI (National Center for Biotechnology Information) database[23] with the aid of Biotools software (Bruker Daltonics, Germany).

Instrument

The experiments were performed using a Biflex III (Bruker Daltonics, Germany) time-of-flight mass spectrometer. The mass spectrometer was equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target with a capacity to load 384 samples simultaneously. The accelerating voltage was set to 19 kV.

RESULTS AND DISCUSSION

Glass chips coated with the sol–gel-derived 2,5-DHB (10 mg/mL) film were used as the substrate for sample loading. Each glass chip was capable of loading 16 samples for MALDI-MS analysis. A sample containing prometryn (100 ng) was deposited at each of these 16 crosses (as described in Experimental) to examine the homogeneity of the MALDI matrix deposition on the chip. Figure 1 displays the MALDI mass spectra of prometryn (MH^+ at *m/z* 242) obtained from the 16 crosses on the glass chip and their corresponding labeling. The MH^+ ion of prometryn was readily found at each of the different sample deposition locations, which suggests that the MALDI matrix deposition on the chip is quite uniform. Spot-to-spot reproducibility was obtained although the analyte intensity may vary with the locations of different spots; the coefficient of variation (CV) is about 63% based on the calculation of the analyte signal (at *m/z* 242) to noise ratio in each mass spectrum, as shown in Fig. 1. Of course it is not possible to provide a comparable figure of merit for conventional MALDI preparations (e.g., dried-droplet) that are characterized by 'sweet spots' isolated randomly in areas providing little or no response.

On-chip enzymatic digestion of proteins was then investigated using a modified glass chip that had been coated previously with the sol–gel/2,5-DHB (10 mg/mL) hybrid material. The mixture of trypsin and cytochrome C was deposited on the glass chip while maintaining the temperature at ca. 35 ± 1°C. No extra MALDI matrix was required after digestion; the chip was ready for immediate MALDI-MS analysis. Figure 2 shows the MALDI mass spectrum of the on-chip tryptic digest of cytochrome C. The ions at *m/z* 1168.92, 1296.79, 1433.72, 1561.97, and 1633.62 were identified by protein database searches as being peptide residues from cytochrome C. The probability-based Mowse score was 96. The peaks denoted with asterisks were from the trypsin autolysis products. Notably, after on-chip digestion, a white band containing a mixture of the digest product and 2,5-DHB had formed along the periphery of the sample deposition spot. MALDI mass spectra were readily obtained along this white ring. This phenomenon was not observed in the experiment to obtain the mass spectra shown in Fig. 1, which was not incubated in the same way as that required to obtain

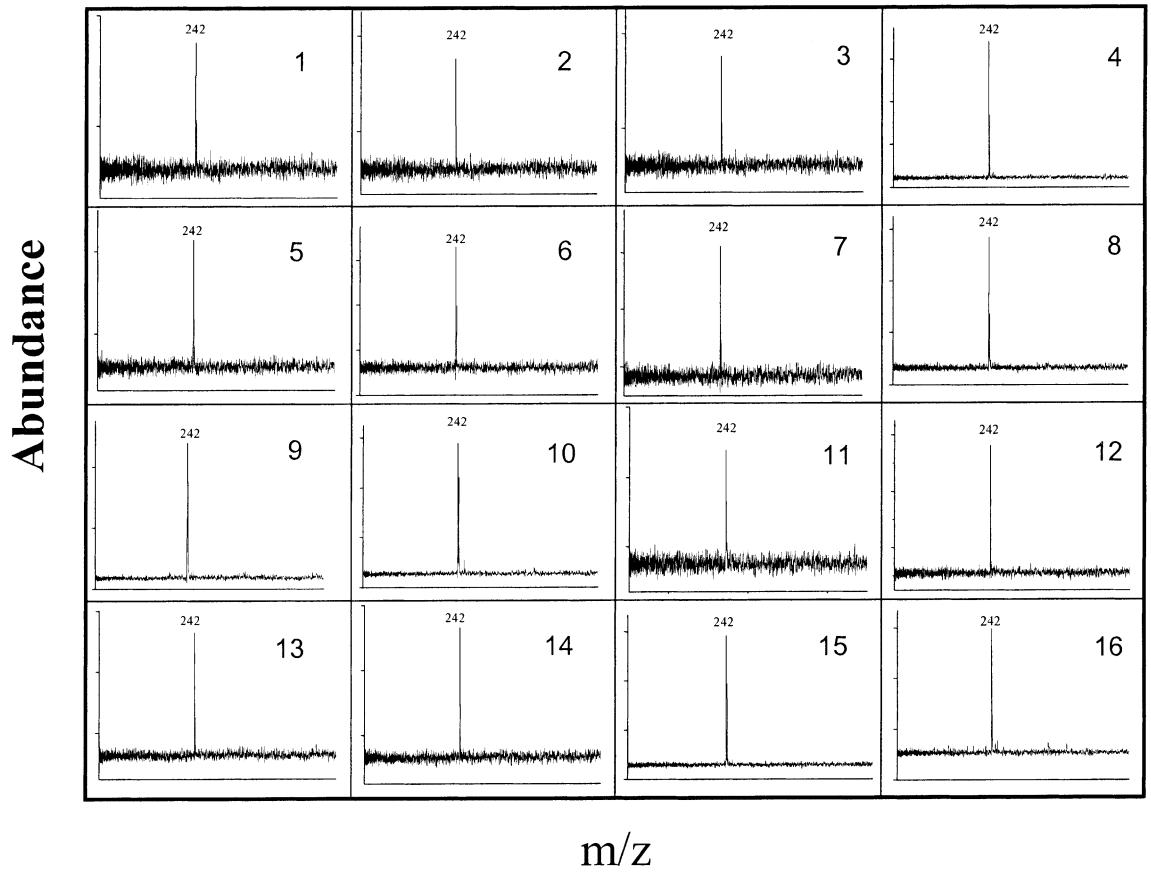

Figure 1. MALDI mass spectra of prometryn (100 ng) obtained from each of the 16 crosses on a glass chip coated with a sol–gel/2,5-DHB (10 mg/mL)-derived film.

the mass spectrum in Fig. 2. Presumably, 2,5-DHB is washed out of the sol–gel film because of the basicity of the digestion buffer and the high incubation temperature, causing free 2,5-DHB and the products of protein digestion to aggregate together along the periphery of the sample deposition spot.

In order to reproducibly obtain the white ring along the sample deposit, a higher amount of 2,5-DHB is recommended to dope into the sol–gel (15 mg/mL). Figure 3 shows the MALDI mass spectrum of the on-chip tryptic digest of cytochrome C (50 fmol), which was the detection limit for this approach. The ions at *m/z* 1168.62, 1296.73, 1712.89, and 1840.92 were identified by protein database searches as being peptide residues from cytochrome C. The probability-based Mowse score was 72. The white band containing 2,5-DHB and

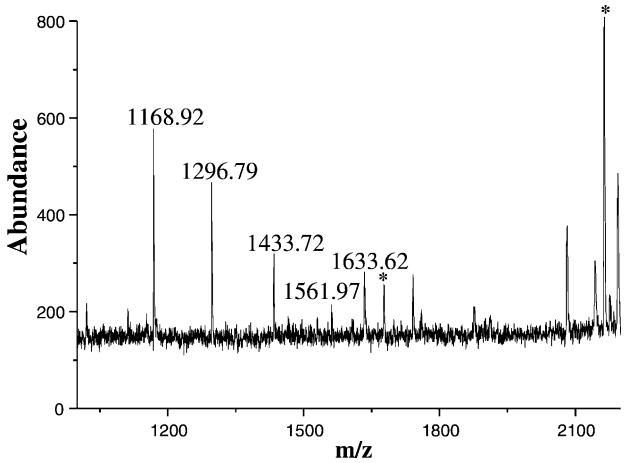

Figure 2. MALDI mass spectrum of the on-chip tryptic (75 ng) digest of cytochrome C (75 ng). The peaks denoted with asterisks are the autolysis residues from trypsin. The chip was coated with a sol–gel/2,5-DHB (10 mg/mL)-derived film.

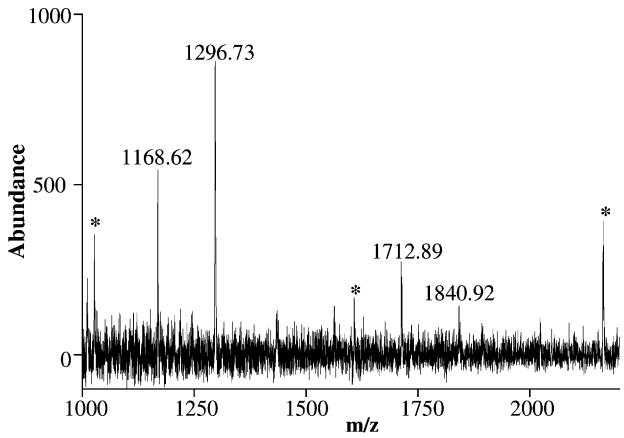

Figure 3. MALDI mass spectrum of the on-chip tryptic (1875 pg = 84 fmol) digest of cytochrome C (375 pg = 50 fmol). The peaks denoted with asterisks are the autolysis residues from trypsin. The chip was coated with a sol–gel/2,5-DHB (15 mg/mL)-derived film.

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Table 1. Ions observed in the MALDI mass spectrum of cytochrome C tryptic digest and a comparison with the output of the NCBI database

Observed Ion *m/z*	Mr (expt) Da	Mr (calc) Da	Delta Da	Start–End	Miss[a]	Peptide sequence
1168.72	1167.71	1167.61	0.10	28–38	0	TGPNLHGLFGR
1296.79	1295.79	1295.71	0.08	28–39	1	TGPNLHGLFGRK
1433.72	1432.71	1432.77	−0.06	26–38	1	HKTGPNLHGLFGR
1561.97	1560.96	1560.86	0.10	26–39	2	HKTGPNLHGLFGRK
1633.62	1632.62	1632.81	−0.19	9–22	1	IFVQKCAQCHTVEK

[a] Number of missed cleavages.

digest product was easily obtained. This result demonstrates an advantageous feature in using this approach for on-chip digestion combined with MALDI-MS analysis; the digestion product is further concentrated and automatically aggregated with free 2,5-DHB released from the sol–gel film. Thus, extra MALDI matrix is unnecessary and signals from the digestion products can be readily analyzed along the white circles on the chip.

Furthermore, the glass chip coated with sol–gel-derived 2,5-DHB film is quite robust and remains effective at least for 1 week, as determined by performing seven individual on-chip digest reactions at various times. Figure 4 shows the MALDI mass spectra of the on-chip tryptic digests of cytochrome C obtained after 3, 5, and 7 days after the modified glass chip had been produced, using the same batch of modified glass chips as that used to obtain the spectrum in Fig. 2. The modified glass chips were stored in a refrigerator at 4°C. In Fig. 4, the peaks denoted with asterisks are the peptide residues from the trypsin digest product of cytochrome C, while the peaks marked with a 'T' are the trypsin autolysis residues. The peaks corresponding to the tryptic digestion products of cytochrome C obtained from the NCBI database searches are listed in Table 1, while Table 2 summarizes the appearance of these peptide ions in the MALDI mass spectra obtained over several days. The search results based on the probability-based Mowse scores conducted using the NCBI database are significant (Table 2).

In addition, we investigated the use of on-chip enzymatic digestion for analyzing intact bacterial cells. Again, a white circle containing a mixture of bacterial cell trypic digest products and 2,5-DHB formed on the chip, and MALDI-MS analysis was readily conducted along the periphery of the

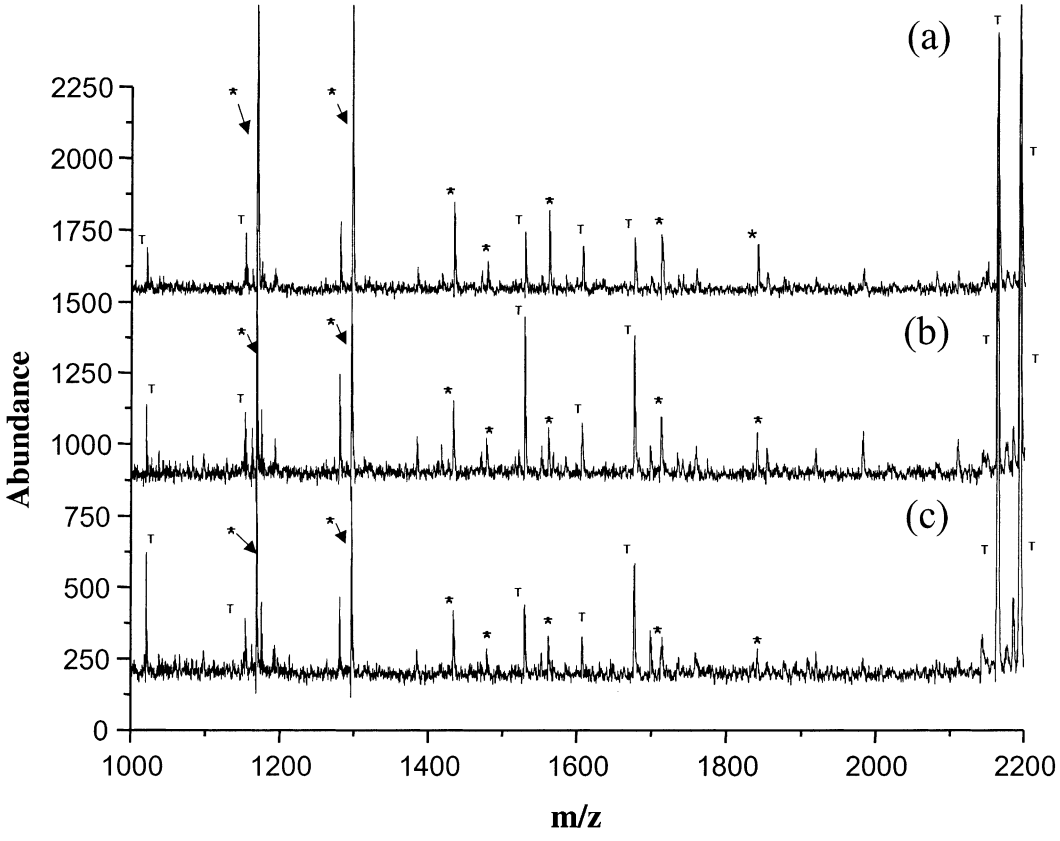

Figure 4. MALDI mass spectra of the on-chip tryptic (75 ng) digests of cytochrome C (75 ng) obtained on (a) the third day, (b) the fifth day, and (c) the seventh day, respectively, after the modified chips had been made, using the same batch of modified glass chips as were used to obtain the spectrum in Fig. 2. The peaks denoted with asterisks are the peptide residues from the tryptic digest product of cytochrome C; the trypsin autolysis residues are marked with 'T'.

Table 2. Summary of the ions observed in the MALDI mass spectra of the on-chip tryptic digest of cytochrome C obtained during the course of 1 week using the same batch of modified glass chips

m/z	1168.72	1296.79	1433.72	1478.80	1561.97	1633.62	1712.89	1841.08	Score[a]
Day 1	+	+	+		+	+			96
Day 2	+	+	+		+	+			110
Day 3	+	+	+	+	+		+		106
Day 4	+	+	+	+	+	+	+	+	197
Day 5	+	+	+	+	+		+	+	125
Day 6	+	+	+	+			+	+	137
Day 7	+	+	+		+		+	+	110

[a] Score is equal to $-10\log(P)$, where P is the probability that the observed match is a random event. Scores greater than 70 are significant ($p < 0.05$).

sample deposition spot. Figures 5(a) and 5(b) show the MALDI mass spectra obtained from the on-chip tryptic digestion of *E. coli* (300 ng) using 75 and 150 ng of trypsin, respectively. Only a few peaks are observed in Fig. 5, which indicates that these amounts of trypsin are insufficient for completion of the digestion reaction in such a short time. A larger amount of trypsin is required to speed up the reaction. Figure 6 shows the MALDI mass spectrum of the on-chip tryptic digest of intact *E. coli* cells (300 ng) obtained when the amount of trypsin was increased. More peaks are observed in this mass spectrum than in Fig. 5. The peaks at *m/z* 1179.57, 1236.56, 1501.73, 1509.78, 1552.73, and 1794.87 correspond to the peptide residues of the trcP protein derived from *E. coli*,[21] with a significant score of 79 based on the NCBI database searches. The peaks denoted with a 'T' correspond to trypsin autolysis residues. The sequence coverage is 32%. The sequence of trcP is listed below.

- **1** MGERIITFSV NQMNKIILNT VTR**QSTKDIS SWKSDKRKVY PSR**LINMGID
- **51** KHCSENNTNI TGVVRQRVFT LIAEDFGIKL DSNAAQSSIT HIVNGNGWFS
- **101** K**KLASLCEGM SRDDKNK**TRE ILENKLADIF FEK**HIDSKID IKNYR**VA

The amino acid sequences shown in bold correspond to the peptide ions observed in the mass spectrum, which is summarized in Table 3. trcP is a protein unique to *E. coli* based on the NCBI protein database searches. Thus, without any time-consuming purification procedures, the bacterial species was identified readily by combining the results obtained upon MALDI-MS analysis of the tryptic digest with protein database searches. However, further confirmation using post-source decay (PSD) or tandem mass spectrometry is required to confirm this result. *E. coli* cells contain many proteins in addition to trcP. Why was only the one protein identified? Possible reasons are that the amount of trcP in *E. coli* cells may be higher than other proteins, and/or the digestion products from other proteins may have poor MALDI efficiencies, resulting in insignificant search scores.

A relatively large amount of trypsin was required to obtain a complete reaction. These results are consistent with those suggested by the previous study[22] that suggests that the use of a large amount of enzyme is necessary to obtain effective on-chip digestion.

CONCLUSIONS

Combination of glass-chip-based sample preparation with on-chip digestion is a straightforward and promising approach for MALDI-MS analysis of proteins. In particular, MALDI-MS analysis of the products of on-chip enzymatic digestion of intact cells provides an alternative method for characterizing microorganism species that complements the technique of determining the fingerprint mass spectra of intact cells. This approach can be used potentially for the

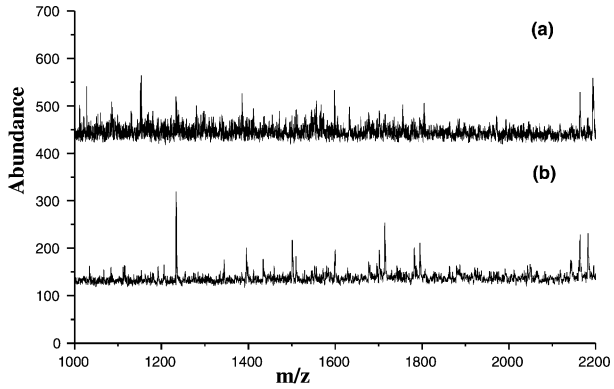

Figure 5. MALDI mass spectra of on-chip digests of *E. coli* cells (300 ng) with (a) 75 ng and (b) 150 ng of trypsin applied to the chip. Trypsin autolysis peaks are marked with asterisks.

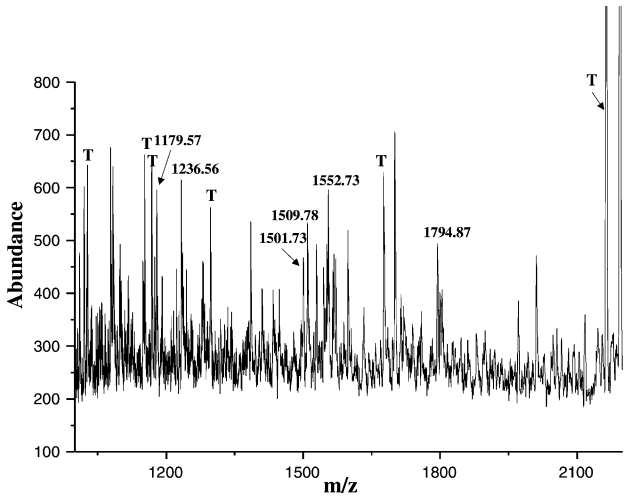

Figure 6. MALDI mass spectrum of the on-chip tryptic (300 ng) digest of *E. coli* cells (300 ng). Trypsin autolysis peaks are marked with 'T'.

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Table 3. Ions observed in the MALDI mass spectra of *E. coli* tryptic digest and a comparison with the peptide residues of trcP protein from the output of the NCBI database

Start–End Sequence	Observed *m/z*	Mr (expt) Da	Mr (calc) Da	Delta Da	Miss[a]	Sequence
24–33	1179.57	1178.56	1178.59	−0.03	1	QSTKDISSWK
24–36	1509.78	1508.77	1508.75	0.02	2	QSTKDISSWKSDK
34–43	1235.56	1234.55	1234.68	−0.12	3	SDKRKVYPSR
102–115	1552.73	1551.72	1551.74	−0.01	2	KLASLCEGMSRDDK
102–117	1794.87	1793.87	1793.88	−0.01	3	KLASLCEGMSRDDKNK
134–145	1501.73	1500.73	1500.80	−0.08	2	HIDSKIDIKNYR

[a] Number of missed cleavages.

rapid identification of microorganisms by combining with the results of tandem mass spectrometry.

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