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Sequence Coverage Enhancement Using Magnetic Nanoparticles in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometric Protein Analysis

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Magnetic nanoparticles (MNPs) treated with phosphoric acid were used to improve sequence coverage in protein identification by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). Sample solution of tryptic peptides from proteins was mixed with the MNPs, and the MNPs were separated from the supernatant using a magnet. MALDI mass spectra obtained separately from the supernatant and the MNPs were distinctly different and complementary to each other. Combination of the two spectra led to a significantly increased sequence coverage.

Key Words : Matrix-assisted laser desorption/ionization (MALDI), Sequence coverage, Magnetic nanoparticles (MNPs), Phosphoric acid

Introduction

In proteomics research, matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF-MS) peptide mass mapping is commonly used for protein identification. A cellular protein is separated, by HPLC or two-dimensional electrophoresis for example, and hydrolyzed usually by trypsin. Analysis of the resulting peptides leads to identification of the protein. In MALDI-TOF-MS analysis, some of the peptides in the tryptic digest are suppressed by the more readily desorbed and ionized peptides. As a result, the peptide mass map and the protein sequence coverage is incomplete. This is not a problem for protein identification, because accurate mass information for only a few peptides is enough for a unique protein assignment. However, it is often necessary to analyze proteins present at low levels in a cell. In such cases, the mass spectrum may not provide enough information and increasing the number of peptide peaks detected becomes important for improved sequence coverage and definitive protein identification.[1] Moreover, the newly detected peptides might contain key information, such as post-translational modification, relevant to functional proteomics studies.

One approach to improve sequence coverage is to obtain mass spectrum in the negative ionization mode using matrix with added phosphoric acid as well as in the normal positive mode. For example, recently Sanaki, T. *et al*. achieved improved sequence coverage by combining positive and negative ionization mode data.[2]

Recently, nanoparticles have been used for various applications in peptide and protein analyses.[3-12] Generally, the applications involve either selection or enrichment of particular peptides via surface modification of nanoparticles or repeated digestion using enzyme bound to nanoparticles. Zhang *et al.* made funtionalized MALDI plate using 4-mercaptophenylboronic acid on gold nanoparticles for on-plate selective enrichment of glycoproteins.[3] Kim *et al.* used polymer nanofilbers to immobilize trypsin for reuse.[4] Lee *et al.* used commercial magnetic beads with aminophenylboronic acid immobilized to enrich glycoproteins.[5] Yang *et al.* achieved high stability of enzymes by immobilizing on nanodiamond.[6] Kong *et al.* used carboxylated diamond nanoparticles to separate hydrophilic and hydrophobic proteins by affinity capture.[7] Jeng *et al.* immobilized enzyme to magnetic nanoparticles for easy separation from solution.[8] Kumarathasan *et al.* purified rat plasma proteins using commercially available weak cation-exchange magnetic beads and analyzed proteins bound to the beads discarding the supernatant.[9]

In this paper, we propose to use magnetic nanoparticles treated with phosphoric acid (MNP-PA) to obtain separate mass spectra from the supernatant and from the nanoparticles after mixing peptide solution with MNP-PA. Peptides are separated into two broad groups using magnetic nanoparticles, based on non-covalent interaction between the peptides and the nanoparticle surface, and both groups are mass spectrometrically analyzed for complementary results. The iron-based magnetic nanoparticles we used are easy to prepare, disperse and separate from solution using a magnet. MNP-PA can be used to obtain peptide peaks from tryptic digest of proteins that were not detected without the use of magnetic nanoparticles. Significant enhancement in sequence coverage was achieved from several model proteins.

Experimental Section

Materials. Trifluoroacetic acid (TFA), iron (III) chloride hexahydrate, iron (II) chloride tetra hydrate, ammonium hydroxide, ammonium bicarbonate, α-cyano-4-hydroxycinnamic acid (CHCA), β-casein, bovine serum albumin (BSA), hemoglobin, human fibrinogen, lysozyme, ovalbumin, poly-lysine and TPCK-treated trypsin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethanol was from Baker. Phosphoric acid (PA) was from Duksan Pure Chemicals

(Ansan, Korea). Acetonitrile (ACN) was from Daejung Chemical (Siheung, Korea). Water was purified with a Millipore water purification system (Bedford, MA, USA).

Preparation of Magnetic Nanoparticle-phosphoric Acid System. Fe_3O_4 magnetic nanoparticles were prepared by co-precipitating Fe^{2+} ($FeCl_2{\cdot}4H_2O$) and Fe^{3+} ($FeCl_3{\cdot}6H_2O$) ions in ammonia solution. 2.0 g of $FeCl_2{\cdot}4H_2O$ and 5.4 g of $FeCl_3{\cdot}6H_2O$ (1:2 molar ratio of Fe^{2+} and Fe^{3+}) were dissolved in 100 mL distilled water to give a final iron ion concentration of 0.3 M. Chemical precipitation was initiated by adding 75 mL of NH_4OH solution (28-30%) at 25 °C with vigorous stirring. pH was about 10. The solution containing the precipitate was heated at 80 °C for 30 min and centrifuged at 17000 rpm for 30 min. The supernatant was removed, and the precipitate was washed several times with ethanol and thoroughly dried in an oven at about 80-100 °C.[10] Black particles produced showed a strong magnetic property. Dried magnetic nanoparticles aggregated to some extent were ground to fine powder with mortar and pestle. Before use, a portion of the Fe_3O_4 nanoparticles was dispersed in 1% phosphoric acid in acetone at 40 mg/mL concentration and vortex-mixed for 30 min.[13]

MNP-PA Treatment of Tryptic Peptides. Five model proteins (BSA, β-casein, hemoglobin, lysozyme, and ovalbumin) were used. Each protein was dissolved at 1 mg/mL concentration in 20 mM ammonium bicarbonate solution and denatured by heating at 90 °C for about 10 min. After cooling, an aliquot of trypsin solution was added so that the protein/trypsin weight ratio is 5/2. Digestion was allowed to

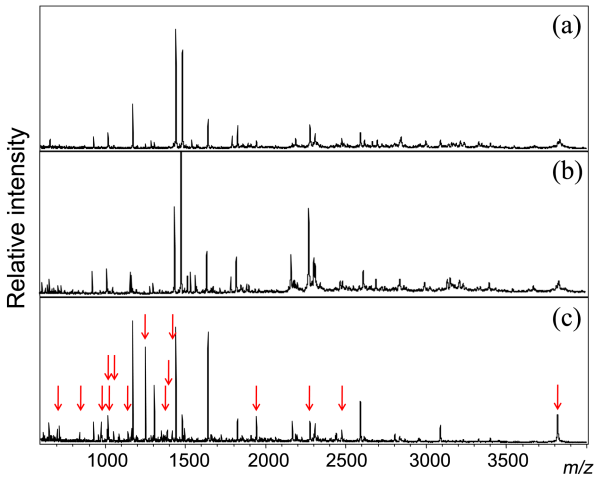

Figure 1. MALDI mass spectra from BSA tryptic digest. (a) control, (b) from supernatant after MNP-PA treatment, (c) from MNP-PA.

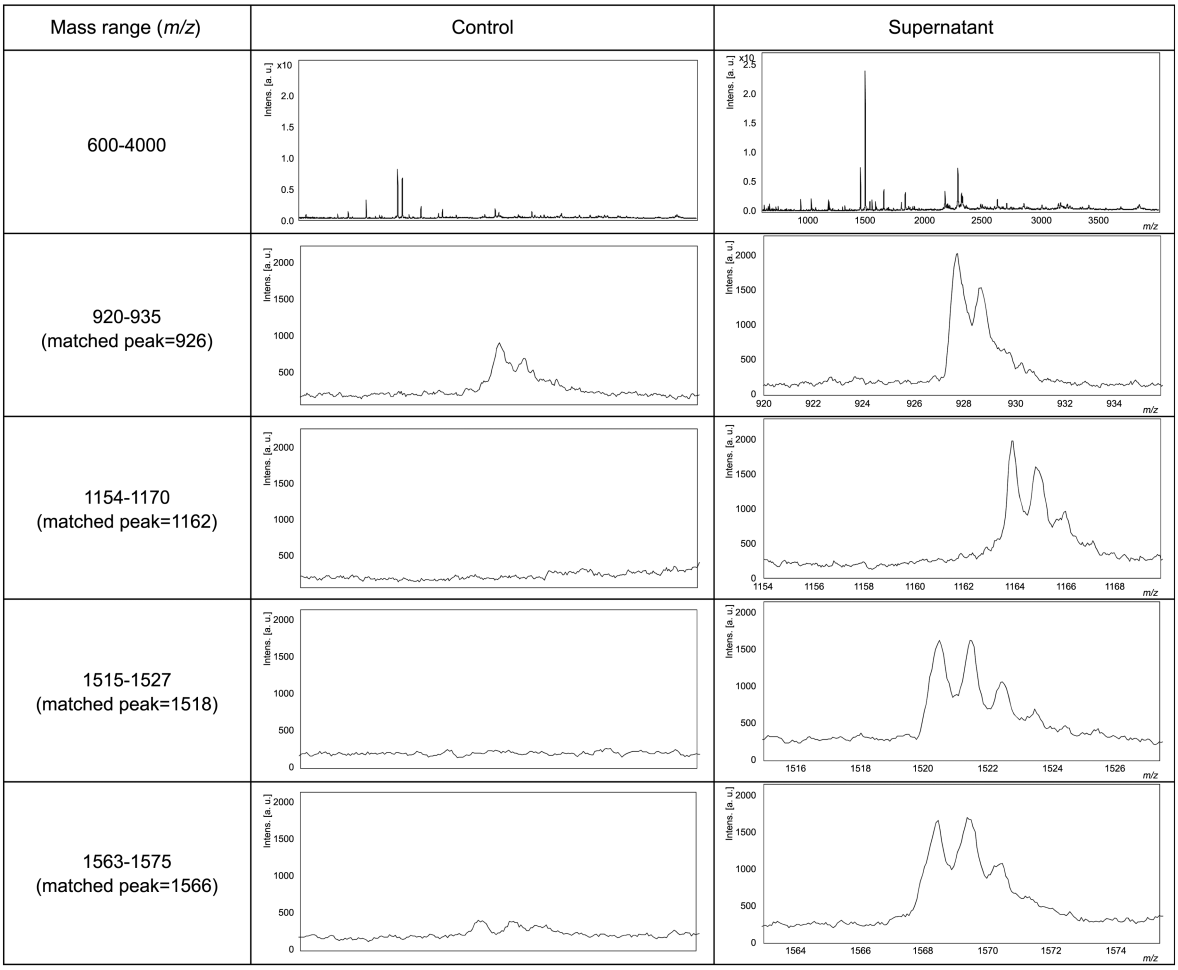

Figure 2. Signal enhancement in the supernatant from several BSA tryptic peptides.

Typically, 10 μL of the MNP-PA suspension was added to 50 μL of the tryptic digest, and the mixture was vortex-mixed for 30 min. The mixture was centrifuged briefly to collect material on the wall of the tube. Then, while holding a magnet on the outer wall of the sample tube to collect the MNPs, the supernatant was transferred to another tube using a micropipette. MNPs remaining in the sample tube were washed several times with distilled water. This procedure separated the peptides into two groups, one group remaining in the supernatant and another bound to the MNPs (MNP-PA).

MALDI-TOF-MS Analysis. Matrix solution was prepared by dissolving CHCA in 50% ACN/50% distilled water containing 0.1% TFA at 10 mg/mL concentration. 1 μL of the matrix solution was mixed with 1 μL of the sample solution (supernatant or MNP-PA dispersed in 1 μL distilled water), and 1 μL of the mixture was loaded onto the MTP Anchor Chip var-384 plate. After drying under vacuum, MALDI mass spectra were obtained using Bruker AutoflexII MALDI time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). A total of 50 shots on the positive ion linear mode were averaged.

Results and Discussion

We tested the efficacy of the method using five proteins BSA, β-casein, hemoglobin, lysozyme, and ovalbumin. Figure 1 shows mass spectra from control BSA tryptic peptides without MNP-PA treatment (a), from supernatant after mixing with MNP-PA (b), and from MNP-PA (c). Mass spectrum from the supernatant in Figure 1(b) was generally similar to the control (Fig. 1(a)); however, ionization efficiency was significantly enhanced. Good mass spectrum was obtained at lower laser power, and the S/N ratio was improved.

The signal enhancement led to observation of several new peaks not observed in Figure 1(a). Figure 2 shows, in an expanded scale, the signal enhancement from several BSA tryptic peptides in the supernatant (Fig. 1(b)). The peak at *m/z* 926 was enhanced about 3-fold in S/N. The peaks at *m/z* 1162, 1518, and 1566 were enhanced more than 10-fold; they were below the noise level in the control.

The advantage of using MNP-PA was more evident in the mass spectrum obtained from the MNP-PA portion (Fig. 1(c)). Signal intensity was somewhat decreased in the mass spectrum from the MNP-PA; however, noise was also suppressed facilitating detection of low intensity peaks. Peaks detected from the MNP-PA, but not observed from the control or the supernatant, are marked in Figure 1(c). Figure 3 shows signal enhancement from several peptides. To test the role of PA in the MNP-PA, the BSA tryptic peptides

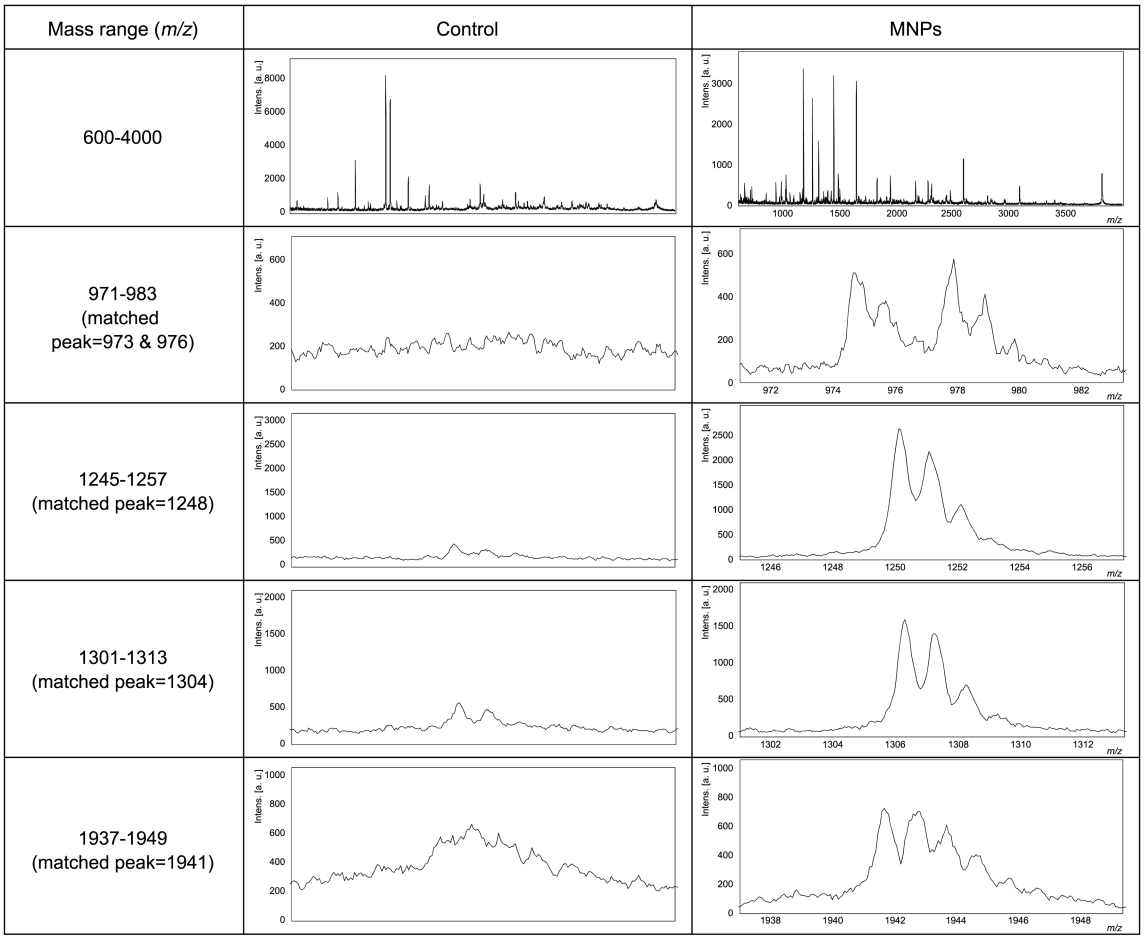

Figure 3. Signal enhancement in the MNP-PA from several BSA tryptic peptides.

(a) Control _ BSA tryptic digestion (b) After mixing MNPs–PA _ BSA tryptic digestion

Figure 4. Improvement in sequence coverage for BSA.

were mixed with bare MNP without the PA treatment. Signal enhancement observed after MNP-PA treatment was not obtained without the PA treatment either from the supernatant or from the MNP (result not shown).

Figure 4 shows the BSA sequence matched from the peptide mass maps. Only 14% was covered using the control mass spectrum (Fig. 1(a)). The coverage was increased almost 4-fold to 58% using peaks combined from Figure 1(b) and 1(c). The amino acid residues covered only from peaks obtained from the MNP-PA are denoted in bold. The

Table 1. Match results for BSA tryptic peptides

(a) Control

Theoretical *m/z* values	Experimental *m/z* values	Δmass	From-to	Sequence	Intensity	S/N
926.486	926.028	0.458	137-143	YLYEIAR	635	19
1282.703	1282.642	0.061	337-347	HPEYAVSVLLR	441	12
1304.709	1304.535	0.174	378-388	HLVDEPQNLIK	320	9
1438.804	1438.823	-0.019	336-347	RHPEYAVSVLLR	467	14
1478.788	1478.889	-0.101	397-409	LGEYGFQNALIVR	288	10
1638.930	1638.080	-0.150	413-427	KVPQVSTPTLVEVSR	1820	55
1822.892	1823.437	-0.545	484-499	RPCFSALTPDETYVPK	605	22
2471.190	2470.590	0.600	389-409	QNCDQFEKLGEYGFQNALIVR	151	7

(b) Supernatant after MNP-PA treated

Theoretical *m/z* values	Experimental *m/z* values	Δmass	From-to	Sequence	Intensity	S/N
648.326	648.248	0.078	199-204	CASIQK	706	16
648.326	648.248	0.078	181-185	IETMR	706	16
711.366	711.302	0.064	5-10	SEIAHR	525	12
926.486	926.472	0.014	137-143	YLYEIAR	1852	51
1162.623	1162.664	-0.041	42-51	LVNELTEFAK	1644	45
1282.703	1282.765	-0.062	337-347	HPEYAVSVLLR	556	16
1304.709	1304.782	-0.073	378-388	HLVDEPQNLIK	743	20
1438.804	1439.036	-0.232	336-347	RHPEYAVSVLLR	6487	134
1478.788	1478.346	0.442	397-409	LGEYGFQNALIVR	1195	28
1518.739	1519.190	-0.451	115-127	LKPDPNTLCDEFK	1288	25
1566.735	1567.106	-0.371	323-335	DAFLGSFLYEYSR	1300	29
1638.930	1639.277	-0.347	413-427	KVPQVSTPTLVEVSR	3035	69
1822.892	1823.392	-0.500	484-499	RPCFSALTPDETYVPK	1251	38
1849.914	1850.702	-0.788	428-444	SLGKVGTRCCTKPESER	228	8
1849.892	1850.702	-0.810	505-520	LFTFHADICTLPDTEK	228	8
1889.796	1889.933	-0.137	77-93	VASLRETYGDMADCCEK	258	8
1887.988	1888.572	-0.584	65-81	SLHTLFGDELCKVASLR	258	8
1887.920	1888.572	-0.652	145-159	HPYFYAPELLYYANK	258	8
2315.038	2315.358	-0.320	160-180	YNGVFQECCQAEDKGACLLPK	1012	17

Table 1. Continued
(c) MNP-PA

Theoretical *m/z* values	Experimental *m/z* values	Δmass	From-to	Sequence	Intensity	S/N
648.326	648.317	0.009	181-185	IETMR	509	17
648.326	648.317	0.009	199-204	CASIQK	509	17
*700.394	700.449	-0.055	174-180	GACLLPK	265	10
711.366	711.443	-0.077	5-10	SEIAHR	364	14
*840.453	840.552	-0.099	459-465	LCVLHEK	202	9
926.486	926.669	-0.183	137-143	YLYEIAR	468	22
973.451	973.633	-0.182	13-20	DLGEEHFK	451	19
*976.444	976.691	-0.247	99-106	NECFLSHK	427	18
*1010.413	1011.607	-1.194	389-396	QNCDQFEK	288	12
*1014.480	1014.590	-0.110	286-294	SHCIAEVEK	391	16
*1049.485	1049.630	-0.145	564-573	EACFAVEGPK	236	11
*1141.707	1141.945	-0.238	524-533	KQTALVELLK	223	10
1162.623	1162.886	-0.263	42-51	LVNELTEFAK	320	14
*1248.614	1248.963	-0.349	11-20	FKDLGEEHFK	2487	105
1304.709	1305.062	-0.353	378-388	HLVDEPQNLIK	1503	59
*1385.613	1385.986	-0.373	262-273	YICDNQDTISSK	237	8
*1387.564	1387.219	0.345	351-362	EYEATLEECCAK	237	8
*1417.731	1417.479	0.252	274-285	LKECCDKPLLEK	208	8
1438.804	1439.222	-0.418	336-347	RHPEYAVSVLLR	3200	117
1478.788	1479.263	-0.475	397-409	LGEYGFQNALIVR	664	27
1638.930	1639.432	-0.502	413-427	KVPQVSTPTLVEVSR	2957	119
1822.892	1823.354	-0.462	484-499	RPCFSALTPDETYVPK	559	24
*1941.813	1941.767	0.046	240-256	VHKECCHGDLLECADDR	522	28
*1941.970	1941.767	0.046	5-20	SEIAHRFKDLGEEHFK	522	28
*2272.240	2273.499	-1.259	174-194	GACLLPKIETMREKVLASSAR	171	10
*2471.190	2470.526	0.664	389-409	QNCDQFEKLGEYGFQNALIVR	87	6
*3814.138	3815.145	-1.007	188-221	VLASSARQRLRCASIQKFGERALKAWS VARLSQK	79	9

(*: peptides detected only from MNP-PA)

total number of residues in bold is 137, corresponding to about half of the number of residues covered by the combined peaks (Fig. 4(b)). Moreover, the number of residues in bold exceeds the number of residues covered in Figure 1(a). This observation alone demonstrates the advantage of using the MNP-PA system for improved sequence coverage.

Sequence search results are summarized in Table 1 for BSA tryptic peptides showing S/N greater than 6. Similar results were obtained from other proteins. Increased sequence coverage for five proteins is summarized in Table 2. The increase was not significant for a low molecular weight protein, lysozyme (from 72% to 93%). However, about 4-fold increase (from 14% to 58%) was obtained for a high molecular weight protein (BSA), where a large number of tryptic peptides could be suppressed and not observable in the control mass spectrum. MNP-PA treatment increases the S/N ratio and makes more peptides detectable thus increasing the sequence coverage.

The signal enhancement in the supernatant appeared to be due to removal of salt in the sample solution by the MNP-PA. This was tested by adding NaCl to the BSA peptides and proved by observing the signal enhancement after treating the sample with MNP-PA (result not shown). Most dramatic demonstration was obtained using human fibrinogen commercially available with high salt content to prevent aggregation of the proteins. Tryptic peptides from fibrinogen without any treatment were not detected at all (Fig. 5(a)). After removal of salt using Zip-tip, mass spectrum in Figure 5(b) was obtained leading to 5% sequence coverage. When the same tryptic peptides were treated with MNP-PA, mass spectra in Figure 5(c) and 5(d) were obtained from the

Table 2. Enhancement in sequence coverage for five proteins

Protein	Control		After MNP-PA treatment (Sum of supernatant and MNPs)	
	Matched peaks	Coverage (%)	Matched peaks	Coverage (%)
Lysozyme	15	72 %	17	93 %
Hemoglobin	13	54 %	26	87 %
β-casein	2	12 %	7	28 %
Ovalbumin	2	8 %	7	24 %
BSA	8	14 %	37	58 %

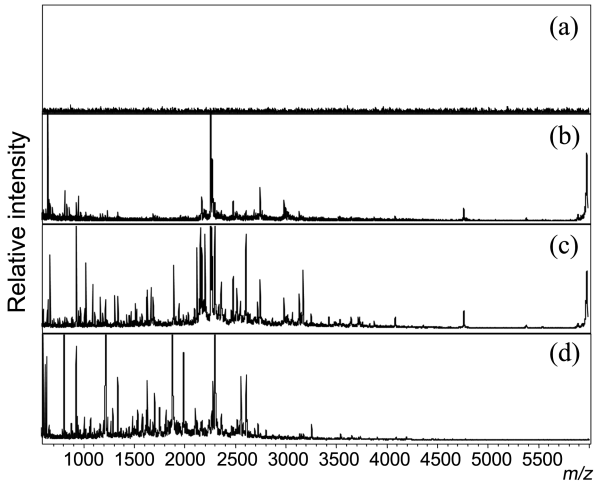

Figure 5. MALDI mass spectra from human fibrinogen. (a) control, (b) after Ziptip treatment, (c) from supernatant after MNP-PA treatment, (d) from MNP-PA.

supernatant and MNP-PA, respectively. The sequence coverage from the supernatant and MNP-PA was 15% and 8%, respectively, resulting in 18% using combined peptide peaks.

The signal enhancement in the MNP-PA was more intriguing. The physical state of the MNP-PA surface is not known. We suspect that the interaction between phosphoric acid and the nanoparticles is not covalent but electrostatic, considering the mild treatment of the nanoparticles with phosphoric acid. The positive charges on the peptide might interact again electrostatically with the negatively charged phosphate groups on the large surface area of the nanoparticles. Partial removal of salt might also occur similarly.

The charge interaction between positively charged peptides and MNP-PA was tested using poly-lysine (average MW, 1000 Da). A distribution was observed from both control (Fig. 6(a)) and the supernatant (Fig. 6(b)) with a maximum intensity around 700 Da. The highest MW detected was about 1400 Da. On the other hand, a different distribution was observed from MNP-PA (Fig. 6(c)) with a maximum intensity around 1100 Da and the highest MW detected around 3700 Da. Figure 6(c) also shows that sodium and potassium adduct peaks were much smaller from MNP-PA than from control or the supernatant. Therefore, the poly-lysine ladder with 128 Da interval was evident from the MNP-PA spectrum.

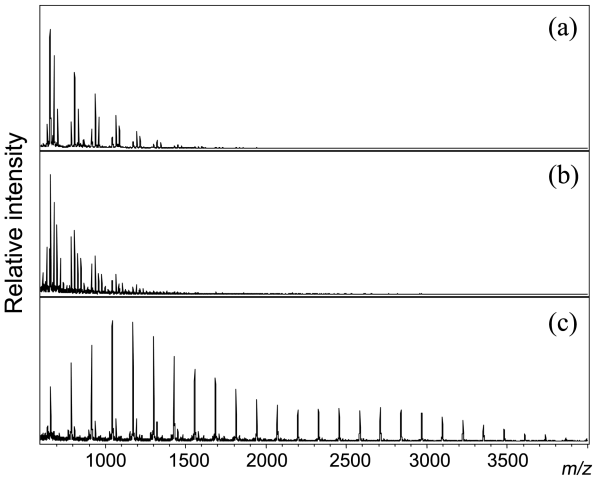

Figure 6. MALDI mass spectra from poly-lysine. (a) control, (b) from supernatant after MNP-PA treatment, (c) from MNP-PA.

This shift to the high MW region implies that peptides with more lysine residues and thus with higher positive charge are more strongly bound to MNP-PA and detected when the MNP-PA portion is subjected to MALDI analysis. This charge interaction might explain detection of new peaks from tryptic peptides in five proteins tested. Charge interaction might also explain the lack of salt adducts in Figure 6(c). Na^+ and K^+ ions bound to the MNP-PA cannot participate in the adduct formation. Desalting effect suggested above (Fig. 1(b), Fig. 5(c)) may also arise from binding of salts in the sample solution to MNP-PA.

Conclusions

Fe_3O_4 magnetic nanoparticles treated with phosphoric acid were used to separate peptides in solution into two portions. Peptides remaining in the supernatant after mixing with the nanoparticles showed enhanced signal probably due to removal of salt by the phosphoric acid on the nanoparticles. Peptides undetected either from the control sample solution or from the supernatant were detected from the nanoparticles. Combination of peptides from the two portions leads to enhanced sequence coverage in the protein analysis by MALDI-TOF-MS.

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