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Automated, Rapid Solid-Phase Proteolytic Cleavage and Sample Preparation for Proteomics

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The development of robotic sample preparation systems has allowed the throughput of protein analysis to be accelerated. However, one of the rate-limiting steps in preparing protein samples for analysis by mass spectrometry is their proteolytic cleavage. Robots have been successfully used for the in-gel tryptic digestion of proteins as well as the desalting, concentration, and loading of protein digests onto matrix-assisted laser desorption/ionization (MALDI) targets. The advantages of these instruments are fast and stable sample processing and precise spotting. It seems reasonable to use these advantages for the entire protein sample preparation in a single instrument. In this paper, we describe a method to achieve this goal by performing protein sample concentration, all the digestion chemistry steps, and MALDI target loading on ZipTips using a rapid and fully automated method.

KEY WORDS: automation, proteomics, mass spectrometry, proteolytic cleavage.

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In order to understand the myriad of interactions that occur between the proteins that participate in cellular control networks, large numbers of protein complexes will have to be analyzed and their molecular components determined. Such a significant undertaking will only be possible if sample preparation and analysis are automated. Protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF) fingerprinting of peptides derived from the proteolytic cleavage of proteins is a widely used method in proteomics. The development of robotic sample preparation systems has allowed the throughput of protein analysis to be accelerated.[1] However, one of the rate-limiting steps in preparing protein samples for analysis by mass spectrometry is their proteolytic cleavage.

Several approaches to reduce cleavage times, which rely on immobilized trypsin, have been developed.[2] Blackburn and Anderegg[3] pumped proteins through a microcolumn packed with Poroszyme-immobilized trypsin and obtained digestion times of around 10 min. Vecchione et al.[4] inserted a Poroszyme-immobilized trypsin column into a multidimensional LC-MS system for the detection of errors in the biosynthesis of human fibrinogen. They achieved short digestion times of less than 20 min. Similar results were achieved by Xue et al.[5] in experiments with multichannel microfabricated chips. Similar devices were introduced by Gao et al.[6] and Wang et al.[7] Promising technology was demonstrated by Ekstrom et al.,[8] who developed a microchip immobilized enzyme reactor (IMER) for online sample preparation.

Another approach to accelerate the digestion reaction is to concentrate proteins on polymeric beads. Aguilar et al.[9] reported the use of C-4 and C-18 microbeads for protein concentration followed by on-bead enzymic digestion. The aim of their research was to determine the binding domains of proteins to RP-HPLC sorbents. Qian et al.[10] carried out sequential enzymatic cleavages of peptides and proteins bound to immobilized metal ion beads on a MALDI target followed by direct MS analysis. Doucette et al.[11] concentrated proteins by adsorption onto POROS R2 beads

with subsequent digestion. Lee et al.[12] performed digestion of β-casein on a polyurethane membrane which served as a support in MALDI MS experiments.

Robots have been successfully used for high-throughput sample preparation.[1] The advantages of these instruments are fast and stable sample processing and precise spotting. It seems reasonable to use these advantages for the entire protein analysis. In this paper, we describe a method to achieve this goal by performing all the digestion chemistry steps in ziptips using a rapid and fully automated method.

EXPERIMENTAL

Materials and Sample Preparation

Water (HPLC grade), methanol (MeOH, HPLC grade), and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Ottawa, Canada). Ammonium bicarbonate, hydrochloric acid (HCl), myoglobin (horse heart), lysozyme (chicken egg white), bovine serum albumin (BSA), and α-cyano-hydroxycinnamic acid (CHCA) were obtained from Sigma (St. Louis, MO). Trypsin (sequencing grade) was from Roche Diagnostic (Laval, Canada). Iodoacetomide (IAM) was purchased from Fluka Biochemika (Buchs, Switzerland). Dithiothreitol (DTT, ULTROL grade) was from Calbiochem (San Diego, CA). ZipTip Pipette Tips were obtained from Millipore (Bedford, MA). DTT (10-mM solution) and IAM (50-mM solution) were prepared in filtered 50-mM ammonium bicarbonate. Trypsin (25 µg) was dissolved in 10% acetonitrile in water, to which 20 µL of 100-mM HCl was added. This trypsin solution was diluted 1:1 v/v by addition of 50-mM ammonium bicarbonate directly prior the experiments.

MALDI matrix was prepared by dissolving 10 mg of CHCA in 1 mL of water/acetonitrile (1:1 v/v) mixture.

Instrumentation

On-ZipTip digests were performed on the Investigator ProMS Robot (Genomic Solutions). Samples were spotted onto a 96-position MALDI target and analyzed on a Hybrid QqTOF Mass Spectrometer QSTAR (Applied Biosystems/SCIEX)[13,14] equipped with the research grade UV-MALDI source.[15,16]

RESULTS AND DISCUSSION

BSA, myoglobin, and lysozyme were chosen as test samples for the experiments. These proteins were dissolved in water at a concentration 500 fmol/µL.

Digestion Protocol

The protein samples were concentrated and enzymatically cleaved using the automated ZipTip method outlined below.

- bind protein to beads by aspirating and dispensing 20 µL of sample; repeat twice
- aspirate 30 µL of DTT
- allow 10 min for reduction
- dispense liquid from the tip
- wash ZipTip with 25 µL of water
- aspirate 30 µL of IAM
- allow 10 min for alkylation
- wash ZipTip with 25 µL of water
- wash ZipTip with 30 µL of 60% MeOH, 5% formic acid
- condition ZipTip with 30 µL of 5% MeOH, 5% formic acid
- wash ZipTip with 25 µL of water
- aspirate 20 µL of trypsin
- allow 10 min for digestion
- wash ZipTip with 25 µL of water
- elute peptides from ZipTip into a clean 200-µL tube with 3 µL of 60% MeOH, 5% formic acid
- mix 3 µL of MALDI matrix with eluted sample
- spot 2 µL of this mixture onto a MALDI target plate

Five to ten minutes was shown to be sufficient to obtain reasonable protein cleavage. Spectra were submitted to Mascot Peptide Fingerprint Search at Matrix Science (London, UK) public website. Seventy-two peptides were identified and 75% sequence coverage was achieved for the spectrum of 500 fmol/µL BSA digest. These results are demonstrated in Figure 1 and Table 1.

Similar results were obtained for lysozyme and myoglobin of the same concentration; 82% and 80% sequence coverage were achieved for these samples, respectively.

Application to a Mixture of Proteins

In order to test the applicability of this method to more complex samples, BSA, myoglobin, and lysozyme 500-nM solutions, 30 µL of each, were mixed in a 200-µL tube and subjected to automated digestion with the optimized protocol (reduction time is 10 min, alkylation time is 10 min, digestion time is 10 min). A mass spectrum of the digest is shown in Figure 2. The procedure is therefore applicable to protein mixtures.

Digestion of HSP90

To prove the applicability of the method to proteins of biological interest, human heat shock protein 90

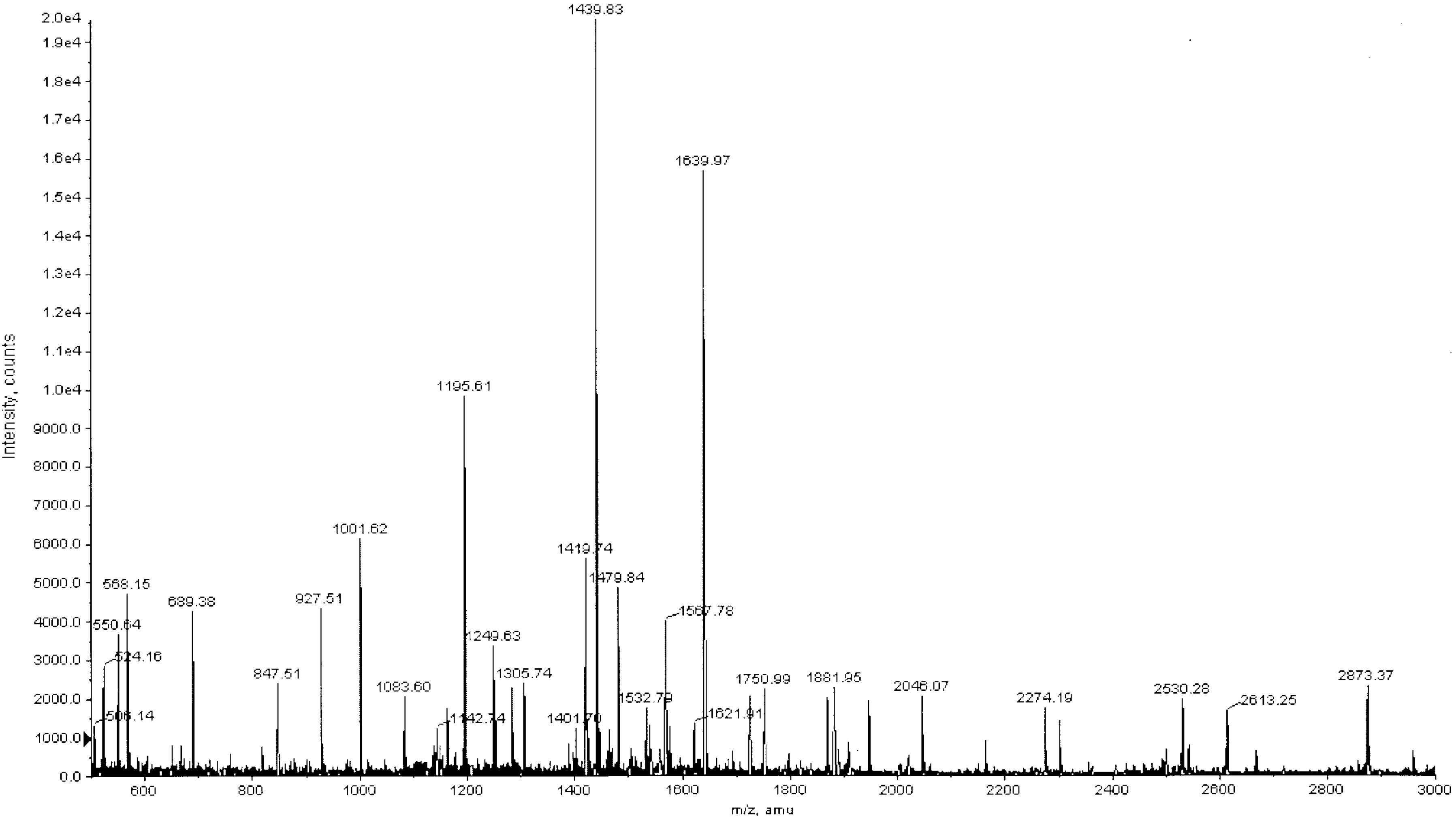

FIGURE 1

Mass spectrum of BSA digest (500 fM). Digestion time = 10 min.

TABLE 1

Peptides found in the spectrum of BSA digest by Mascot Peptide Mass

Start–end	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
24–28	656.35	655.34	655.34	0.00	1	RDTHK
25–34	1193.60	1192.60	1192.59	0.00	1	DTHKSEIAHR
25–36	1468.77	1467.76	1467.76	0.01	2	DTHKSEIAHRFK
35–44	1249.62	1248.61	1248.61	0.00	1	FKDLGEEHFK
66–75	1163.63	1162.62	1162.62	–0.00	0	LVNELTEFAK
89–100	1419.70	1418.69	1418.69	0.00	0	SLHTLFGDELCK
89–105	1946.02	1945.01	1945.01	0.00	1	SLHTLFGDELCKVASLR
101–105	545.31	544.30	544.33	–0.03	0	VASLR
101–117	2004.90	2003.89	2003.84	0.05	1	VASLRETYGDMADCCEK
123–138	1901.94	1900.93	1900.86	0.07	1	NECFLSHKDDSPDLPK
139–151	1576.78	1575.77	1575.76	0.01	0	LKPDPNTLCDEFK
156–160	665.37	664.36	664.37	–0.01	1	KFWGK
156–167	1573.84	1572.84	1572.85	–0.01	2	KFWGKYLYEIAR
157–160	537.27	536.26	536.27	–0.01	0	FWGK
157–167	1445.75	1444.74	1444.75	–0.01	1	FWGKYLYEIAR
161–167	927.49	926.48	926.49	–0.00	0	YLYEIAR
161–168	1083.59	1082.59	1082.59	–0.00	1	YLYEIARR
169–183	1888.93	1887.92	1887.92	0.00	0	HPYFYAPELLYYANK
184–197	1747.77	1746.76	1746.70	0.06	0	YNGVFQECCQAEDK
198–204	758.42	757.41	757.42	–0.00	0	GACLLPK
198–209	1388.74	1387.73	1387.73	–0.00	1	GACLLPKIETMR
198–211	1645.88	1644.87	1644.87	0.00	2	GACLLPKIETMREK
198–211	1661.90	1660.89	1660.86	0.03	2	GACLLPKIETMREK
205–209	649.30	648.29	648.33	–0.04	0	IETMR
205–211	906.48	905.47	905.46	0.01	1	IETMREK
205–218	1620.88	1619.87	1619.87	0.01	2	IETMREKVLTSSAR
212–218	733.41	732.40	732.41	–0.01	0	VLTSSAR
212–220	1017.58	1016.57	1016.57	–0.00	1	VLTSSARQR
219–222	572.34	571.34	571.36	–0.02	1	QRLR
221–228	975.53	974.52	974.53	–0.01	1	LRCASIQK
221–232	1464.77	1463.76	1463.77	–0.00	2	LRCASIQKFGER
223–232	1195.59	1194.58	1194.58	–0.00	1	CASIQKFGER
229–232	508.36	507.35	507.24	0.11	0	FGER
233–241	1001.59	1000.58	1000.58	0.00	1	ALKAWSVAR
233–245	1457.75	1456.74	1456.85	–0.11	2	ALKAWSVARLSQK
236–241	689.37	688.36	688.37	–0.00	0	AWSVAR
242–248	847.50	846.49	846.50	–0.00	1	LSQKFPK
242–256	1750.98	1749.97	1749.97	0.00	2	LSQKFPKAEFVEVTK
249–263	1692.93	1691.92	1691.93	–0.01	1	AEFVEVTKLVTDLTK
257–266	1153.63	1152.62	1152.69	–0.07	1	LVTDLTKVHK
267–280	1749.75	1748.74	1748.66	0.08	0	ECCHGDLLECADDR
286–299	1684.84	1683.83	1683.81	0.02	1	YICDNQDTISSKLK
298–309	1532.79	1531.78	1531.77	0.00	1	LKECCDKPLLEK
300–309	1291.64	1290.63	1290.59	0.04	0	ECCDKPLLEK
319–340	2458.20	2457.19	2457.17	0.02	1	DAIPENLPPLTADFAEDKDVCK
347–359	1567.75	1566.74	1566.74	0.01	0	DAFLGSFLYEYSR
347–360	1723.84	1722.84	1722.84	–0.00	1	DAFLGSFLYEYSRR
360–371	1439.81	1438.80	1438.80	–0.00	1	RHPEYAVSVLLR
361–371	1283.71	1282.70	1282.70	–0.00	0	HPEYAVSVLLR
361–374	1595.90	1594.89	1594.92	–0.03	1	HPEYAVSVLLRLAK
387–401	1795.85	1794.84	1794.82	0.02	1	DDPHACYSTVFDKLK
402–412	1305.72	1304.71	1304.71	–0.00	0	HLVDEPQNLIK
421–433	1479.80	1478.79	1478.79	0.00	0	LGEYGFQNALIVR
421–436	1899.89	1898.89	1899.00	–0.11	1	LGEYGFQNALIVRYTR
437–451	1639.94	1638.93	1638.93	–0.00	1	KVPQVSTPTLVEVSR
438–451	1511.85	1510.85	1510.84	0.01	0	VPQVSTPTLVEVSR
452–459	817.48	816.48	816.48	–0.01	1	SLGKVGTR
469–482	1724.84	1723.83	1723.83	0.01	0	MPCTEDYLSLILNR
483–489	898.47	897.47	897.47	–0.01	0	LCVLHEK
483–495	1539.82	1538.82	1538.81	0.00	1	LCVLHEKTPVSEK
483–498	1868.03	1867.03	1867.02	0.00	2	LCVLHEKTPVSEKVTK
496–507	1466.76	1465.75	1465.70	0.05	1	VTKCCTESLVNR
499–507	1138.55	1137.54	1137.49	0.05	0	CCTESLVNR
508–523	1880.93	1879.92	1879.91	0.01	0	RPCFSALTPDETYVPK
529–544	1907.93	1906.92	1906.91	0.00	0	LFTFHADICTLPDTEK
548–557	1142.71	1141.70	1141.71	–0.00	1	KQTALVELLK
548–561	1632.99	1631.98	1632.01	–0.03	2	KQTALVELLKHKPK
549–557	1014.61	1013.60	1013.61	–0.01	0	QTALVELLK
549–561	1504.91	1503.90	1503.91	-0.01	1	QTALVELLKHKPK

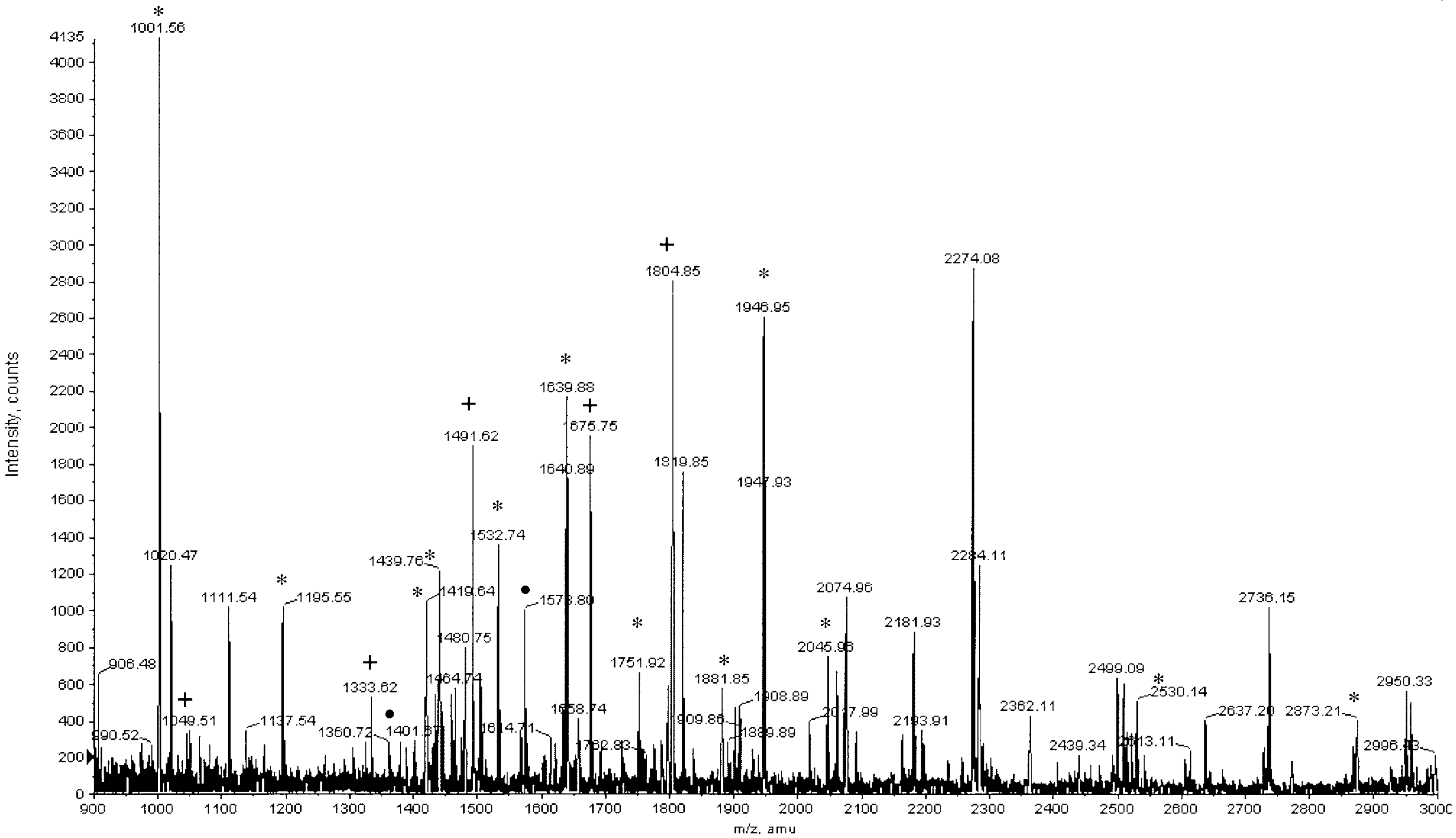

FIGURE 2

Mass spectrum of protein mixture digest: BSA, lysozyme, myoglobin. Digestion time = 10 min.

Labeled peptides: * BSA, +lysozyme, •myoglobin.

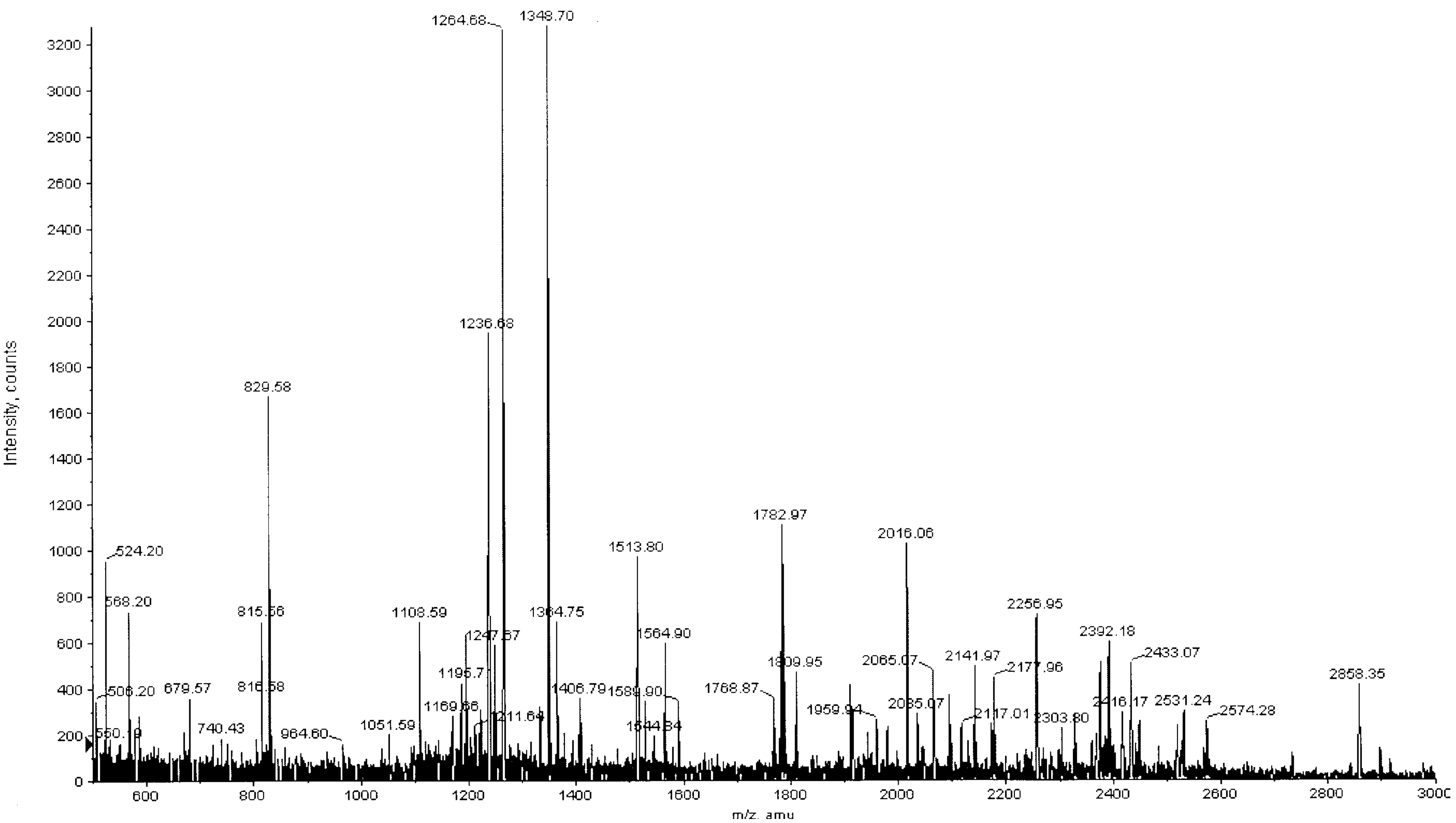

FIGURE 3

Mass spectrum of heat shock protein 90 digest. Digestion time = 10 min.

(3 pmol) was digested in the way described above (Fig. 3). Eighteen peptides in the spectrum were found by Peptide Mass Fingerprint Search engine, and these results were confirmed by performing of MS/MS analysis with the help of Mascot MS/MS search engine.

CONCLUSIONS

The concentration of proteins on the reverse-phase beads accelerate the reaction rates of reduction, alkylation, and enzymatic digestion resulting in short reaction times of 5–10 min. The full time of sample preparation from protein reduction to sample spotting onto MALDI target is about 45 min. The method developed performs fully automated digestion for rapid protein identification and is applicable to low sample concentrations.

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