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# Fractionation of peptides in proteomics with the use of pI-based approach and ZipTip pipette tips

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**Abstract**

The aim of the work was to explore the utility of the in-solution isoelectric focusing (sIEF) fractionation method. That method was proved to be the alternative separation method of mixtures of protein tryptic digests in proteomics. Analysis of the identification of peptides was performed with the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). For that research, previously designed the miniaturized multi-chamber fractionation sIEF device (75 μl volume for each chamber) based on polyacrylamide gel membranes with immobilines technology was utilized. To evaluate the efficiency and accuracy of sIEF fractionation combined with MS/MS peptides identification, bovine serum albumin (BSA) digest and mixture of five proteins digest were used.

First, fractionation of bovine serum albumin digest sample was performed using sIEF method. Studies performed for that simple mixture of peptides proved the ability of the sIEF device to focus peptides mostly in one chamber. Additionally performed the correlation analysis between $pI_{calc}$ and $pI_{exp}$ values for identified peptides proved the possibility to obtain experimentally useful high correlation. That information was found to have a potential value for construction of additional constraint during false positives evaluation process among identified proteins. Next, studies on the sIEF fractionation were combined with the evaluation of practical use of ZipTip pipette tips to fractionate peptides in the case of simple mixture of proteins. For this, five proteins digest samples were used. The analysis without prior any fractionation enabled to identify very limited number of proteins. The significant improvement was obtained when one used sIEF alone or with combination with ZipTips fractionation prior to MS analysis. The proposed approach based on in-solution isoelectric focusing proved to be an efficient and accurate alternative fractionation method of protein digests and can be considered as the first useful dimension in two-dimensional proteomics separations. Moreover, analytical information from that pI-based fractionation method can be considered as the additional source of database matching constraint. It can also be a valuable tool for analytical and bioinformatic studies of peptides fractionation in proteomics.
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*Keywords:* In-solution isoelectric focusing (sIEF); pI value; Peptides; Proteomics; ZipTip pipette tips

## 1. Introduction

Today, the most widely used procedure for analyzing complex protein mixtures is two-dimensional gel electrophoresis [1–4]. While this approach

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has achieved the highest resolving power of any method to date, the method suffers from a number of factors [5,6]. But it is well known that efficient separation is required prior to mass spectrometry analysis and bioinformatics database searching enabling the correct identification of proteins [7]. The high-resolution separation techniques like multidimensional chromatography (including especially ion exchange chromatography (IEC) with reversed phase liquid chromatography (RPLC) [8–13], but also size-exclusion chromatography (SEC) with RPLC [14], and RPLC with capillary zone electrophoresis (CZE) [15]) coupled to mass spectrometry instruments are currently intensively developed and tested. Also alternative proteome analysis strategies based on peptide separations, such as in-solution isoelectric focusing (sIEF) [16–19] and capillary isoelectric focusing (cIEF) [20,21] as well as chromatofocusing [22–24], are studied and developed for both protein identification and expression studies.

Preparative isoelectric focusing analogously to the first step in 2D-PAGE, can be performed on an IPG strip, in a tube gel, or in solution. pH gradient in this method is created with the use of ampholytes in the certain pH range or with the certain immobilines mixture immobilized into polyarylamide gel. Stable pH gradient is generated, when voltage is applied across the focusing cell making the possibility to separate the proteins or peptides according to their isoelectric points. However, in case of proteins, some of them can have a tendency to aggregate and precipitate during focusing [5]. On the other hand, preparative solution isoelectric focusing devices have been employed just for the fractionation of proteins. For that case, Rotofor [24–27], multicompartment electrolyzer [18,28], microscale solution isoelectrofocusing device (μsol-IEF) [17,29,30], off-gel isoelectric focusing [31,32] or multicompartment electrolyzer with polyacrylamide gel beads [33] are used. Another pI-based method—chromatofocusing has been utilized as well in proteomics [24], but again for fractionation of proteins. Hence, despite the wide use of isoelectric focusing-based methods for protein fractionation, reports on the separation of peptides are rather limited and include either cIEF [20,21] or sIEF [16]. However, the physical and chemical properties of peptides, derived from enzymatic digestion, are less diverse than those of the original proteins. Indeed, most peptides resulting from enzymatic digestion are readily soluble in water or water/organic mixtures.

The main goal of that work was to explore the in-solution isoelectric focusing method based on polyacrylamide gel membranes with immobilines technology. Utility of that pI-based method as the alternative separation method (also combined with ZipTip pipette tips fractionation) for simple and complex mixtures of protein tryptic digests was tested and examined. The potential of the sIEF fractionation method for the evaluation of proteomics data was also demonstrated.

## 2. Experimental

### 2.1. Materials

#### 2.1.1. Reagents

Bovine serum albumin (minimum 96%, electrophoresis, pH 7), chicken egg ovalbumin (O 4757), bovine milk β-lactoglobulin (minimum 90%, PAGE), bovine milk β-casein (minimum 90% as β-casein, electrophoresis) and equine skeletal muscle myoglobin (95–100%) were purchased from Sigma–Aldrich (St. Louis, MO) and used without further purification. Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), γ-methacryloxypropyltrimethoxysilane, trypsin, ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), were also obtained from Sigma–Aldrich (St. Louis, MO). Immobiline II pK 3.6, pK 4.6, pK 6.2, pK 7.0, pK 8.5 and pK 9.3 were products of Amersham Biosciences (Piscataway, NJ). Acrylamide and methylenebisacrylamide, urea and tris(hydroxymethyl)aminomethan were from Pharmacia Biotech (Uppsala, Sweden). IEF anode and cathode buffers, ion exchange membranes and PowerPac 3000 power supply were obtained from Bio-Rad (Hercules, CA).

#### 2.1.2. In-solution isoelectric focusing (sIEF) device

In-solution isoelectric focusing device was made according to [16] in polycarbonate plate (78.4 mm × 105 mm), and the main fractionation part of that device contained eight parallel milled channels with 12 fractionation chambers in each row. Two supporting blocks for electric connection between the

anolyte and catholyte reservoirs and the sIEF device (73 mm × 27 mm × 15.4 mm, 30 ml volume each) were machined also in polycarbonate plates. Each reservoir contained Pt wire (electrodes). The holes for anion exchange membranes (from the side of anolyte reservoir) or cation exchange membranes (from the side of catholyte reservoir) positioned and supported by rubbers from both sides separated sample from the anolyte or catholyte. Eighty-eight glass O-rings (4 mm i.d., 3 mm long) were glued with epoxy glue into the channels of the main part of the device to create eight rows with 12 fractionation chambers (width: 2.8 mm; length: 6.0 mm; depth: 4.5 mm; volume: 75 μl volume each). Immobiline gel membranes of specific pH values were prepared according to the Amersham Biosciences protocol “Isoelectric Membrane Formulas for IsoPrime Purification of Proteins, Protocol Guide” [34]. Rubbers (2.5 mm × 3.0 mm × 4.5 mm) put into the middle of the chambers supported the casting of the gels into glass O-rings and their polymerization. After polymerization for 1 h at 50 °C, rubbers were removed and gels were washed with water three times (20 min each) in an excess of water. To facilitate gel binding to the inner tube, glass surface was treated with γ-methacryloxypropyltrimethoxysilane prior to gel polymerization.

### 2.2. Methods

#### 2.2.1. Protein digestion

Bovine serum albumin or mixture of five proteins was digested according to a standard protocol (Promega Corporation, Madison, WI). Briefly, appropriate amount of protein was denatured in a solution containing 7 M urea, 50 mM Tris and 3 mM DTT at 60 °C for 60 min. After denaturation, the mixture was allowed to cool and IAA was added to a final concentration of 15 mM, and placed in the dark for 30 min at room temperature. After dilution with 50 mM ammonium bicarbonate until urea concentration was below 1 M, trypsin was added at an enzyme:protein ratio of 1:50 (w/w). Incubation at 37 °C was performed overnight.

#### 2.2.2. In-solution isoelectric focusing (sIEF) fractionation

For the in-solution isoelectric focusing fractionation of bovine serum albumin and digest of five proteins (BSA, ovalbumin, β-lactoglobulin, β-casein and myoglobin) into 12 fractions for each sample, the pH values of the 11 gel membranes were 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.5, 8.5, 9.0 and 9.5, respectively. In the case of BSA sample, 0.1 mg/ml was loaded into one row of the sIEF device. In the case of five proteins samples, two samples were prepared. For one of the experiments (sample 1), concentrations of each protein in the range of one order of magnitude were used (0.08 mg/ml of BSA, 0.17 mg/ml of ovalbumin, 0.26 mg/ml of β-lactoglobulin, 0.35 mg/ml of β-casein and 0.44 mg/ml of myoglobin). For the second experiment (sample 2), the concentration of proteins differed in the range of four orders of magnitude (80 μg/ml of BSA, 8 μg/ml of ovalbumin, 0.8 μg/ml of β-lactoglobulin, 80 ng/ml of β-casein and 8 ng/ml of myoglobin). To prevent overheating, the samples were focused sequentially starting with lower voltage and increasing the voltage in time. The following voltage program was used: 100 V for 30 min, 200 V for 30 min, 500 V for 60 min, 1000 V for 60 min and 2000 V until the completion of the IEF process evaluated on the basis of the current value lower than 200 μA was obtained. After focusing, 12 fractions were simultaneously transferred using a 12-channel digital pipette (Labnet International, Woodbridge, NJ) into 0.2 ml tubes.

#### 2.2.3. ZipTip pipette tips fractionation

ZipTip pipette tips for sample preparation (Millipore, Billerica, MA) were used for desalting and concentrating of peptides prior to MALDI-TOF/TOF-MS analysis according to the producer guidelines. Using Biohit Proline 12-channel pipettor (SciDynamics, Adelphia, NJ) it was possible to process 12 sIEF fractions at the same time. Utilizing ZipTips fractionation of the peptides for each sIEF fraction, ZipTips were also used as the second dimension to simplify the peptides mixtures in the case of five proteins digest sample. That fractionation was performed with the use of eight different elution solutions (containing 5, 10, 15, 20, 25, 30, 40 and 50% acetonitrile in 0.1% of TFA in water solution). Hence, having 12 fractions from sIEF and eight fractions from ZipTips fractionation for each sIEF fraction, finally 96 fractions were collected prior to mixing with matrix and spotting on MALDI target plate.

#### 2.2.4. MALDI-TOF/TOF-MS

Both MS and MS/MS data were obtained with MALDI-TOF/TOF-MS (4700 Proteomics Analyzer, Applied Biosystems, Framingham, MA) equipped with a 2 m long flight tube and a $N_2$ laser. Forty shots per sub-spectrum and 25 sub-spectra were used per spot. The laser intensity for MS spectra was 5800, and for MS/MS was 6700. The ion source and ion mirror were both operated at 20 kV, and mass resolution was maximized at 3.75 KDa, attaining 14,000. MS and MS/MS spectra were collected in the range 800–5000 *m/z* ratio and 10–1500 *m/z* ratio, respectively. MS/MS analysis was conducted using a TOF/TOF instrument. Precursor ions were separated in a 40 cm long linear time-of-flight (operating with an acceleration voltage of 8 kV) using a double-sided timed ion selector. The selected precursor ions were decelerated as they entered the floating collision cell. The collision energy was set to 1 keV, and the collision gas was atmosphere. The precursor and the resulting fragments then entered the second source region of the TOF/TOF. When the complete collection of ions was in the second source region, a 14 kV pulse was applied. The TOF/TOF instrument was equipped with a high-repetition laser operated at 200 Hz. The effective diameter of the laser spot was approximately 200 μm.

For MALDI experiments, the samples were mixed (1:1, v/v) with 14 mg/ml solution of α-cyano-4-hydroxycinnamic acid (CHCA) suspended in acetonitrile:water (50:50, v/v) with the addition of 0.1% of TFA. Dried-droplet deposition of the sample was achieved by placing a small droplet of the sample onto the surface of a 2 in. × 2 in. stainless steel MALDI target.

#### 2.2.5. Data analysis

Theoretical digests of bovine serum albumin, ovalbumin, β-lactoglobulin, β-casein and myoglobin were performed using PeptideMass (http://us.expasy.org/tools/peptide-mass.html). Isoelectric points of the peptides were calculated using p*K* values for amino acids [35]. In the case of experiments with BSA and five proteins digest samples, MS and MS/MS spectra were searched to identify peptides using Mascot Daemon software (http://www.matrixscience.com). Mascot search parameters included carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modifications as well as peptide mass tolerance (±100 ppm) and fragment mass tolerance (±0.2 Da).

## 3. Results and discussion

### 3.1. Initial testing of the sIEF device with BSA tryptic digest

A tryptic digest of bovine serum albumin was used for initial testing of the sIEF device. The digest was loaded into all 12 chambers of a specific row, and then an increasing stepped voltage was applied for focusing. Fractionation of BSA digest was performed in sIEF device with the polyacrylamide gel membranes comprising precisely designed pH values as following: 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.5, 8.5, 9.0 and 9.5, respectively. The peptides identified in 12 fractions are listed in Table 1 and exemplary MS spectra for three fractions collected are depicted in Fig. 1. Identification of that protein was done with Mascot Daemon software based on MS/MS spectra acquired with MALDI-TOF/TOF-MS instrument. Twenty-four peptides were identified based on MS/MS analysis: 16 peptides were found only in one chamber, five peptides in two chambers, two peptides in three chambers and one peptide in four chambers. Generally, the steeper the titration curve at the pI, or the higher the value of d*z*/dpH, where *z* is charge of a peptide, the sharper the peptide will be focused. However, it can be considered as true to certain extent. As it is seen in Table 1, peptides with values of d*z*/dpH higher than about 1.3 are found in one chamber (even with one exception for peptide MPCTEDYLSLILNR with d*z*/dpH = −1.3438, which was found in two chambers). Peptides with lover values of d*z*/dpH already were identified based on MS/MS spectra in more than one chamber. However, still it can be noted that mostly identifications of peptides based on MS/MS spectra were found in one chamber. Additionally, the correlation analysis between $pI_{calc}$ and $pI_{exp}$ values was executed for identified peptides. Since the peptides were separated on the basis of isoelectric focusing phenomenon, estimated experimental pI values of individual peptides could be obtained based on their chamber location after completion of focusing. Assuming average values of $pI_{exp}$ of two

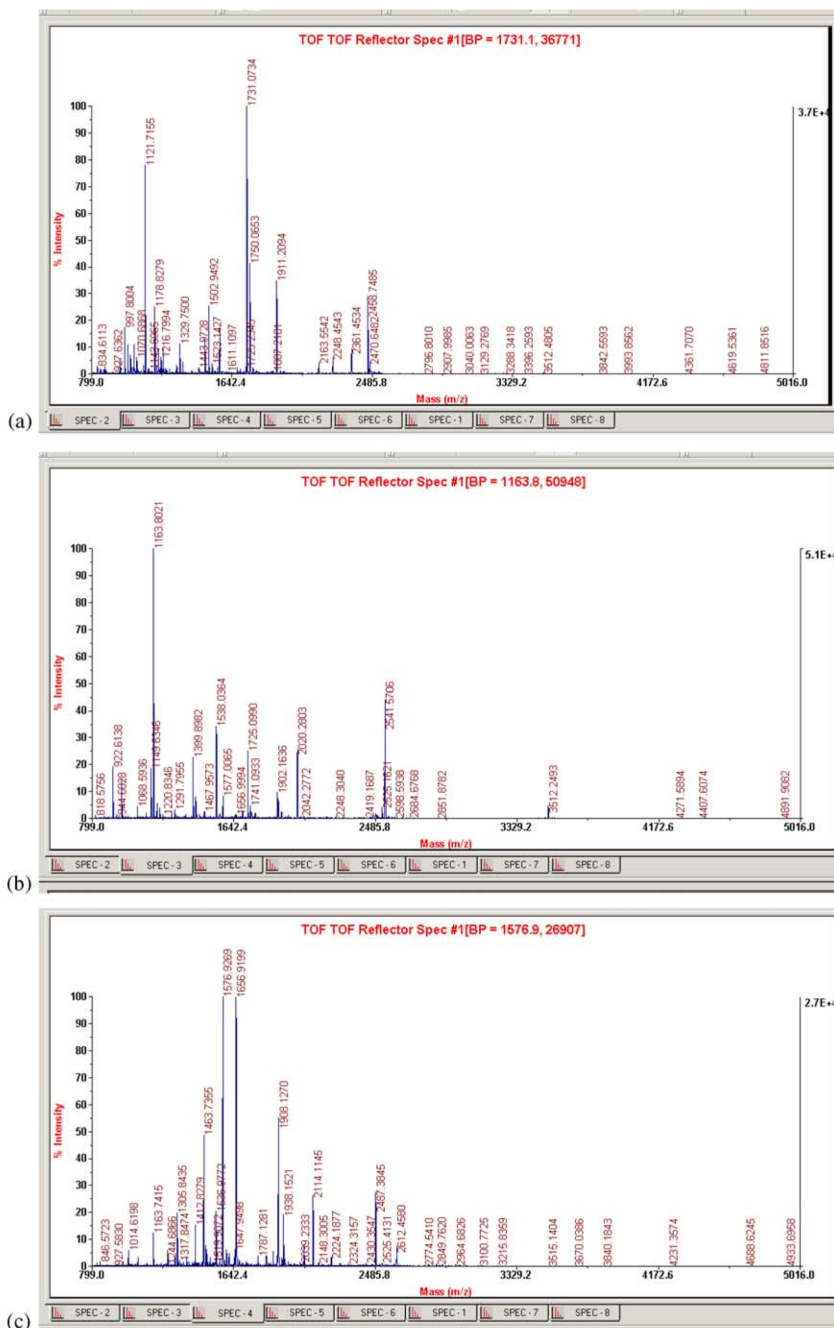


Fig. 1. Exemplary three MS spectra for three consecutive fractions obtained with the use of sIEF fractionation method: (a) fraction 2; (b) fraction 3; (c) fraction 4.

Table 1
The peptides identified in 12 fractions after the sIEF fractionation for bovine serum albumin (BSA) digest sample

| Peptide identified | $pI_{exp}$ | $pI_{calc}$ | dz/dpH | No. of chambers with the indicated peptide |
|---|---|---|---|---|
| DAIPENLPPLTADFAEDKDVCK | 3.96 | 3.75 | −3.5928 | 1 |
| ECCHGDLLECADDR | 4.10 | 3.75 | −3.1297 | 1 |
| LKPDPNTLCDEFKADEK | 4.44 | 4.25 | −2.8110 | 1 |
| EYEATLEECCAK | 4.09 | 3.75 | −2.3535 | 1 |
| VHKECCHGDLLECADDR | 4.80 | 4.75 | −2.2596 | 1 |
| NECFLSHKDDSPDLPK | 4.66 | 4.25 | −1.8964 | 1 |
| TCVADESHAGCEK | 4.65 | 4.75 | −1.7346 | 1 |
| LFTFHADICTLPDTEK | 4.54 | 4.75 | −1.6991 | 1 |
| LKPDPNTLCDEFK | 4.56 | 4.75 | −1.5893 | 1 |
| TVMENFVAFVDK | 4.37 | 4.25 | −1.3472 | 1 |
| MPCTEDYLSLILNR | 4.37 | 4.75 | −1.3438 | 2 |
| LVNELTEFAK | 4.53 | 4.25 | −1.3408 | 1 |
| DAFLGSFLYEYSR | 4.37 | 3.75 | −1.3393 | 1 |
| LSQKFPK | 10.00 | 9.33 | −1.1585 | 3 |
| QEPERNECFLSHK | 5.50 | 6.25 | −0.9983 | 1 |
| FKDLGEEHFK | 5.45 | 6.00 | −0.9210 | 2 |
| SLHTLFGDELCK | 5.30 | 6.00 | −0.7863 | 2 |
| HLVDEPQNLIK | 5.32 | 5.25 | −0.7444 | 3 |
| LCVLHEK | 6.75 | 6.75 | −0.5951 | 1 |
| KVPQVSTPTLVEVSR | 8.75 | 8.87 | −0.2335 | 4 |
| LKECCDKPLLEK | 6.18 | 6.25 | −0.2062 | 1 |
| RPCFSALTPDETYVPK | 6.07 | 6.25 | −0.1610 | 1 |
| YLYEIAR | 6.00 | 6.00 | −0.1379 | 2 |
| LGEYGFQNALIVR | 6.00 | 6.00 | −0.1379 | 2 |

immobiline gel membranes that bracketed the chamber for peptides found in more than one chamber, the correlation coefficient for the relationship of $pI_{exp}$ in the function of $pI_{calc}$ is 0.9697 (Fig. 2). It indicates yet another important property of the pI-based fractionation system used. Namely, there is a possibility to achieve additional information regards the fractionated peptides—their isoelectric point value. That information has a potential value for construction of additional constraint during false positives evaluation process and generally database searching. Comparing results obtained using protein mass

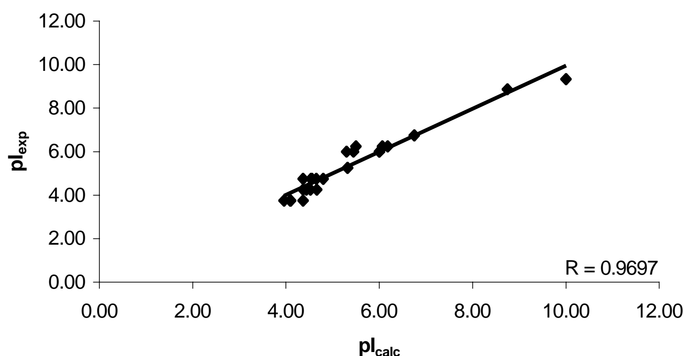


Fig. 2. The relationship between $pI_{exp}$ and $pI_{calc}$ for peptides identified in the case of bovine serum albumin (BSA) digest sample.

Table 2
The comparison of the results obtained using protein mass fingerprinting (PMF) with the results achieved with MS/MS ion search mode (PPS) for peptides from bovine serum albumin (BSA) digest sample

| PMF | PPS | IEF pH range | $pI_{calc}$ |
|---|---|---|---|
| AEFVEVTK | – | 3.83–4.67 | 4.53 |
| CCAADDKEACFAVEGPK | – | 3.83–4.67 | 4.32 |
| CCTESLVNR | – | 5.33–6.17 | 6.00 |
| CCTKPESER | – | 5.33–6.17 | 6.14 |
| DAFLGSFLYEYSR | DAFLGSFLYEYSR | 3.33–4.17 | 4.37 |
| DAIPENLPPLTADFAEDK | – | 3.33–4.17 | 3.77 |
| DAIPENLPPLTADFAEDKDVCK | DAIPENLPPLTADFAEDKDVCK | 3.33–4.17 | 3.96 |
| DAIPENLPPLTADFAEDKDVCK | – | 3.83–4.67 | 3.96 |
| DLGEEHFK | – | 3.83–4.67 | 4.65 |
| DTHKSEIAHR | – | 6.33–7.67 | 6.92 |
| ECCHGDLLECADDR | ECCHGDLLECADDR | 3.33–4.17 | 4.10 |
| ECCHGDLLECADDR | – | 3.83–4.67 | 4.10 |
| ETYGDMADCCEK | – | 3.33–4.17 | 3.92 |
| EYEATLEECCAK | EYEATLEECCAK | 3.33–4.17 | 4.09 |
| FKDLGEEHFK | FKDLGEEHFK | 5.33–6.17 | 5.45 |
| FKDLGEEHFK | FKDLGEEHFK | 5.83–6.67 | 5.45 |
| HLVDEPQNLIK | HLVDEPQNLIK | 4.33–5.17 | 5.32 |
| HLVDEPQNLIK | HLVDEPQNLIK | 4.83–5.67 | 5.32 |
| HLVDEPQNLIK | HLVDEPQNLIK | 5.33–6.17 | 5.32 |
| HPEYAVSVLLR | – | 6.33–7.67 | 6.75 |
| HPEYAVSVLLRLAK | – | 8.33–9.17 | 8.76 |
| KQTALVELLK | – | 8.33–9.17 | 8.59 |
| KVPQVSTPTLVEVSR | KVPQVSTPTLVEVSR | 8.33–9.17 | 8.75 |
| KVPQVSTPTLVEVSR | KVPQVSTPTLVEVSR | 6.33–7.67 | 8.75 |
| KVPQVSTPTLVEVSR | KVPQVSTPTLVEVSR | 8.83–9.67 | 8.75 |
| KVPQVSTPTLVEVSR | KVPQVSTPTLVEVSR | 9.33–(–) | 8.75 |
| LCVLHEK | LCVLHEK | 6.33–7.67 | 6.75 |
| LFTFHADICTLPDTEK | – | 3.83–4.67 | 4.54 |
| LFTFHADICTLPDTEK | LFTFHADICTLPDTEK | 4.33–5.17 | 4.54 |
| LGEYGFQNALIVR | LGEYGFQNALIVR | 5.33–6.17 | 6.00 |
| LGEYGFQNALIVR | LGEYGFQNALIVR | 5.83–6.67 | 6.00 |
| LKECCDKPLLEK | LKECCDKPLLEK | 5.83–6.67 | 6.18 |
| LKPDPNTLCDEFK | – | 3.83–4.67 | 4.56 |
| LKPDPNTLCDEFK | LKPDPNTLCDEFK | 4.33–5.17 | 4.56 |
| LKPDPNTLCDEFKADEK | LKPDPNTLCDEFKADEK | 3.83–4.67 | 4.44 |
| LSQKFPK | LSQKFPK | 8.33–9.17 | 10.00 |
| LSQKFPK | LSQKFPK | 8.83–9.67 | 10.00 |
| LSQKFPK | LSQKFPK | 9.33–(–) | 10.00 |
| LVNELTEFAK | LVNELTEFAK | 3.83–4.67 | 4.53 |
| LVNELTEFAK | – | 4.33–5.17 | 4.53 |
| MPCTEDYLSLILNR | MPCTEDYLSLILNR | 3.83–4.67 | 4.37 |
| MPCTEDYLSLILNR | MPCTEDYLSLILNR | 4.83–5.67 | 4.37 |
| MPCTEDYLSLILNR (Met) | – | 3.83–4.67 | 4.37 |
| NECFLSHK | – | 6.33–7.67 | 6.75 |
| NECFLSHKDDSPDLPK | NECFLSHKDDSPDLPK | 3.83–4.67 | 4.66 |
| QEPERNECFLSHK | – | 5.33–6.17 | 5.50 |
| QEPERNECFLSHK | QEPERNECFLSHK | 5.83–6.67 | 5.50 |
| QNCDQFEK | – | 3.83–4.67 | 4.37 |
| QTALVELLK | – | 5.33–6.17 | 6.00 |
| RHPEYAVSVLLR | – | 8.83–9.67 | 9.61 |
| RHPEYAVSVLLR | – | 9.33–(–) | 9.61 |

Table 2 (*Continued*)

| PMF | PPS | IEF pH range | $pI_{calc}$ |
|---|---|---|---|
| RPCFSALTPDETYVPK | – | 5.33–6.17 | 6.07 |
| RPCFSALTPDETYVPK | RPCFSALTPDETYVPK | 5.83–6.67 | 6.07 |
| SHCIAEVEK | – | 5.33–6.17 | 5.38 |
| SLHTLFGDELCK | – | 4.83–5.67 | 5.30 |
| SLHTLFGDELCK | SLHTLFGDELCK | 5.33–6.17 | 5.30 |
| SLHTLFGDELCK | SLHTLFGDELCK | 5.83–6.67 | 5.30 |
| TCVADESHAGCEK | TCVADESHAGCEK | 4.33–5.17 | 4.65 |
| TVMENFVAFVDK | TVMENFVAFVDK | 3.83–4.67 | 4.37 |
| VHKECCHGDLLECADDR | VHKECCHGDLLECADDR | 4.33–5.17 | 4.80 |
| VHKECCHGDLLECADDR | – | 4.83–5.67 | 4.80 |
| VPQVSTPTLVEVSR | – | 5.83–6.67 | 5.97 |
| YLYEIAR | YLYEIAR | 5.33–6.17 | 6.00 |
| YLYEIAR | YLYEIAR | 5.83–6.67 | 6.00 |
| YNGVFQECCQAEDK | – | 3.83–4.67 | 4.14 |
| YNGVFQECCQAEDKGACLLPK | – | 4.33–5.17 | 4.68 |

fingerprinting (PMF) with the results achieved with MS/MS ion search mode (PPS), it was found that the same set of peptides for BSA digest sample is identified on the basis of their pI values and using MS/MS spectra (Table 2). It can be additional confirmatory constraint for unambiguously identified peptides. Taking into account potential inaccuracy regarding experimental pH ranges for each chamber in sIEF device, new pH ranges considered for that part of the research were estimated. This estimation was done on the basis of the average standard deviation value (S.D. = 0.17) obtained for the relationship between $pI_{calc}$ and $pI_{exp}$ values for peptides identified with the use of MS/MS spectra, which were found in one chamber. Using protein mass fingerprinting, 42 peptides were identified (18 only with PMF) and using MS/MS ion search mode, 24 peptides were identified. Only in the case of one peptide (DAFLGSFLYEYSR) identification based on MS/MS spectrum did not agree with identification based on pI value. Six other peptides (FKDLGEEHFK, HLVDEPQNLIK, KVPQVSTPTLVEVSR, LSQKFPK, QEPERNECFLSHK and SLHTLFGDELCK) did not fix correctly their pI values, as well as identified with MS/MS spectra. However, in these cases all of those peptides were found in more than one pH range, and finally one of the identification was classified correctly based on both pI values as well as MS/MS spectra. Only peptide SLHTLFGDELCK did not match correctly pH range, and it was identified with MS/MS spectrum.

### *3.2. Separation of five proteins mixture digest with sIEF–ZipTips fractionation system*

Next, studies on the sIEF fractionation were combined with the evaluation of practical use of ZipTip pipette tips. ZipTips were used here not only to desalt and concentrate the sample prior to MALDI-TOF/TOF-MS analysis, but also to fractionate peptides in the case of simple mixture of proteins. For that analysis mixture of five proteins (bovine serum albumin, chicken egg ovalbumin, bovine milk β-lactoglobulin, bovine milk β-casein and horse skeletal muscle myoglobin) was fractionated by sIEF with the polyacrylamide gel membranes comprising precisely designed pH values as described in Section 2. Fractionated peptides were evaluated with MALDI-TOF/TOF-MS instrument. The analysis was performed for two kinds of samples: proteins mixed at the concentration of the range of one order of magnitude (sample 1), and proteins for which concentration differed in four orders of magnitude (sample 2). The comparison between the numbers of peptides identified on the basis of MS/MS analysis without any fractionation, after sIEF fractionation and after sIEF fractionation combined with ZipTips fractionation was performed (Table 3). The analysis without prior any fractionation enabled to identify four proteins in sample 1 and one protein in sample 2. Four proteins in sample 1 and two proteins in sample 2 were identified where sIEF fractionation prior to MS/MS analysis was utilized. Additional fractionation with ZipTips prior

Table 3
Results of identification of five proteins tryptic digest samples mixed in different ratios based on MS/MS spectra and Mascot database searching

| Separation system | Sample 1 | | Sample 2 | |
|---|---|---|---|---|
| | Protein identified | Peptides identified (sequence coverage) | Protein identified | Peptides identified (sequence coverage) |
| MALDI-TOF/TOF-MS | Ovalbumin | 2 (8%) | BSA | 7 (15%) |
| | β-Lactoglobulin | 2 (11%) | | |
| | β-Casein | 2 (6%) | | |
| | Myoglobin | 1 (13%) | | |
| sIEF + MALDI-TOF/TOF-MS | Ovalbumin | 4 (14%) | BSA | 16 (27%) |
| | β-Lactoglobulin | 3 (26%) | Ovalbumin | 3 (11%) |
| | β-Casein | 2 (12%) | | |
| | Myoglobin | 9 (63%) | | |
| sIEF + ZipTips + MALDI-TOF/TOF-MS | BSA | 2 (3%) | BSA | 17 (29%) |
| | Ovalbumin | 4 (13%) | Ovalbumin | 2 (8%) |
| | β-Lactoglobulin | 6 (33%) | β-Lactoglobulin | 4 (19%) |
| | β-Casein | 2 (19%) | Myoglobin | 7 (45%) |
| | Myoglobin | 13 (75%) | | |

to sIEF fractionation provided the identification of five proteins in sample 1 and four proteins in sample 2. The combination of sIEF and ZipTip pipette tips fractionation prior to MALDI-TOF/TOF-MS analysis can increase the possibility to identify more proteins in the mixture containing more than one protein. Results presented in Table 3 confirm that fact and it is seen that in the case of two samples analyzed differing in the range of order of magnitude, the number of peptides identified is improved in comparison to the method of fractionation based on sIEF only or without any fractionation method used. The number of peptides identified with the use of Mascot search tool based on spectra from MALDI-TOF/TOF-MS analysis is limited and challenging in the mixtures of protein digest from more then one protein [36]. Therefore, the effective fractionation prior to MS analysis is necessary. It was proved that sIEF fractionation used prior to MS analysis increased the number of peptides identified on the basis of MS/MS spectra with the use of Mascot search tool. Moreover, the number of peptides and proteins identified increases even more (better sequence coverage), when one uses additionally ZipTips fractionation after sIEF fractionation and prior to MS analysis. Combination of sIEF and additional extension of ZipTips used for fractionation along with desalting and concentration of the sample before MALDI-TOF/TOF-MS, can be considered as the simple and inexpensive additional fractionation method for improving of the analysis of simple proteins mixtures, for example, during the isolation and confirmation of the protein purity studies.

## 4. Conclusions

The proposed approach based on in-solution isoelectic focusing method proved to be useful as an alternative fractionation method of protein digests and can be considered as the first dimension in two-dimensional proteomic separations. It demonstrated the applicability of sIEF in fractionation of BSA digest and mixture of five proteins digest. Additionally, it was proposed to use sIEF in combination with ZipTips fractionation as an easy-to-use and analytically efficient extension of sIEF used previously alone. Those two combined fractionation methods were proved to be useful for five proteins mixture with the concentration of proteins in the same order of magnitude as well as for five proteins mixture differing regarding the concentration in four orders of magnitude. Besides the separation aspects, the sIEF method can be considered as the additional source of database matching constraint used in the evaluation process of proteomics data. Using pI values calculated for identified peptides, it was possible to check

the validity of the database searching, considering the occurrence of peptides in the appropriate fractions in the sIEF device. In-solution isoelectric focusing fractionation method can be treated as an easy-to-use example of pI-based method, which in the next step is supposed to be replaced by pI-based chromatographic method. That last method is nowadays under development and seems to be more flexible with higher recovery of the peptides. Summing up the final conclusion from the approach presented, using simple and complex proteins digest mixtures, practical utility of pI-based method was shown for analytical and bioinformatics studies of peptides fractionation in proteomics.

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