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SHORT COMMUNICATION

No peptide left behind: the "out of range" recovery in IPG–IEF fractionation

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Abstract IEF is often used in multidimensional shotgun proteomics and the narrow range of 3.5–4.5 is the recommended pH interval for the fractionation of tryptic peptides. Usually, even if IEF is performed in IPG strip with a narrow range pH, the entire sample must be loaded onto the strip, including the "out of IPG range" peptides. We describe a simple protocol to recover at least a part of these missing peptides and show that this recovery significantly influences the overall fractionation result, increasing the number of the identified proteins and the protein coverage.

Keywords Peptide IEF · Narrow range IPG strip · Peptide fractionation

High sample complexity is one of the major challenges in shotgun proteomics. Sample pre-fractionation is mandatory to reduce peptide mixture complexity and consequently to improve the number of protein identifications. Among the peptide fractionation techniques, IEF is often used in multidimensional MS/MS workflows (Cargile et al. 2004; Millioni et al. 2012; Eriksson et al. 2008; Hörth et al. 2006).

In peptide IPG–IEF, resolving power depends on (1) the length of strip, (2) the pH gradient it holds, and (3) the number of fractions generated. Narrow range IPG strips with a pH range of 3.5–4.5 are becoming increasingly popular for peptide IEF (Slebos et al. 2008; Essader et al. 2005; Pernemalm and Lehtiö 2013; Eriksson et al. 2008; Pernemalm et al. 2009; Lengqvist et al. 2011).

The rationale behind using this narrow pH range is that tryptic peptides with no missed enzymatic cleavage sites are expected to cluster around a *p*I value of 4 due to the limited presence of lysines and arginines (Cargile et al. 2004). This implies that the majority of peptides from a complex trypsin-digested proteome will focus in this region. Therefore, a length of 24 cm (the higher commercially available) and a 3.5–4.5 pH range should be the preferable characteristics for an IPG strip to obtain the highest resolution in the separation of a tryptic peptide complex mixture. Moreover, for an optimized protocol, it is important to decide the number of fractions to be collected on the basis of the amount of loaded peptides. By our estimation (Millioni et al. 2013), a quantity of sample with a minimum yield of 20 µg of peptides per fraction may indicate a threshold at which the quantity that is not recovered after the fractionation and cleanup procedures probably starts to become negligible compared to the total amount of sample that is recovered, therefore increasing the efficiency of IEF.

During the initial stage of IPG–IEF, voltage and current should be limited, respectively, to about 150 V and 50 µA per strip to avoid Joule heating due to the possible presence of salt ions in the sample. As the run proceeds, the salt ions migrate towards the electrodes, resulting in decreased conductivity and allowing high gradient voltages to be

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applied (up to 300 V/cm). If the voltage is not increasing during the IEF, probably the salt content of the sample is higher than 10 mM. Standard protocols suggest to place one paper pad between on each end of the IPG strip and the electrodes. The replacement of these paper bridges, where ions accumulate, allows removing excess salt ions and the continuation of IEF under more suitable conditions. During the IEF, also peptides with a *p*I at a pH value higher or lower than the one included in the IPG strip range will leave the strip and probably accumulate on paper bridges. In fact, even if a narrow range IPG strip is used, the entire sample must be loaded onto the strip, including the "out of IPG range" peptides. The aim of this short communication is to investigate if at least a part of these missing peptides is recoverable from the paper bridges and if this recovery significantly influences (or not) the overall fractionation performance. Our hypothesis is that the paper bridges may have a triple utility: (1) create the connections between the strip and the electrodes, (2) collect and allow the removal of the salt excess, (3) collect and allow the recovery of peptides with a *p*I at a pH value higher or lower than the one included in the IPG strip.

To test this hypothesis, we performed peptide IPG–IEF of a trypsin-digested cellular lysate (120 µg) using the Offgel Fractionator (Agilent, CA) for three times. A 24 cm long IPG strip (GE Healthcare) with a 3.5–4.5 pH range was used. An electrode pad was wetted with 4.8 % glycerol (v/v) solution and put at each end of the strip. Peptides were focused until 60 kVh was reached. After focusing, the buffer in each well (150 µl) containing the diffused peptides was carefully collected. The Offgel allows to obtain 24 fractions. Starting from the more acidic fraction, we merged the fractions in groups of 4, getting 6 pools and obtaining about 20 µg of peptides per fraction. As a deviation from the standard procedure, the anodic and cathodic paper bridges were not thrown away, but rather collected in two clean eppendorf tubes. We added 150 µl of 50 % AcN and 0.1 % formic acid for 15 min to each tube and, after vortexing, the solutions were collected. The six pools, as well as the anodic and cathodic recovery solutions, were dried under vacuum, resuspended in 0.1 % formic acid, cleaned using C18 columns (Sep-Pack), eluted with 60 % acetonitrile and dried in a SpeedVac system. This procedure allows to remove the IEF contaminants, such as residual mineral oil, salts and glycerol prior to MS/MS analysis.

The MS analyses were performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled online with a nano-HPLC Ultimate 3000 (Dionex–Thermo Fisher Scientific) as previously explained (Menegazzo et al. 2013). All peptides, identified with high or medium confidence, were used to design a static excluding list inserted in the MS method, and all samples were analyzed again under previous conditions but not considering the excluded masses.

Data were analyzed with Proteome Discoverer 1.4 software (Thermo Fischer Scientific) using Sequest as protein search engine. Results obtained from every LC–MS/MS run (from each pool of IEF fractions and from the anodic and cathodic solutions, combining the run with and without the excluding list), were merged by Proteome Discoverer into a single file. Peptides, which could not be unequivocally attributed to a single protein, were grouped into protein families to satisfy the principle of maximum parsimony.

Peptides found in the anodic paper bridges (i.e., with a *p*I < 3.5) were only about twenty (data not shown). Con-

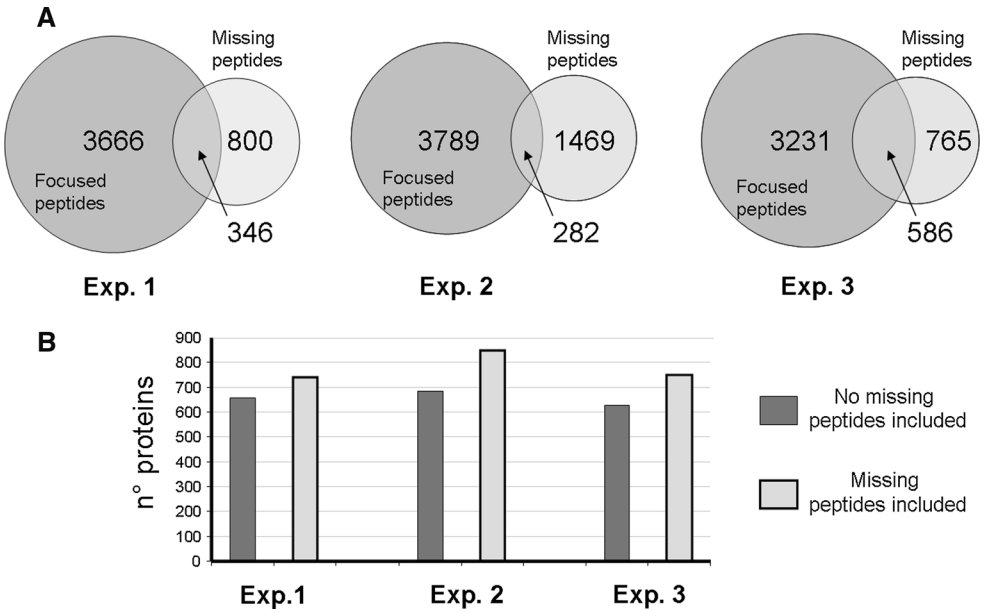

Fig. 1 **a** Venn diagrams show the overlap of focused versus missing peptides in three different experiments. **b** The number of proteins identified by IEF with and without including missing peptides. Proteins with at least two unique peptides were regarded as confident identifications

versely, we identified many unique peptides from the cathodic bridge (i.e., with a $pI > 4.5$) (Fig. 1). Peptides identified in the Offgel fractions and in cathodic bridge were only partially in common. In fact, the great majority of the "out of range" peptides have a *p*I between 4.5–5 and 5.5–6. When data obtained from Offgel fractions and from the cathodic solution were merged by Proteome Discoverer into a single file, we observed an increase of the number of protein identifications (+18 %; *t* test, $p < 0.05$) and of the median protein coverage (+10 %; Chi-square test, $p < 0.01$).

For each experiment, detailed information about peptide and protein identifications is reported in Supplementary data 1. A comparison between the features of identified peptides revealed that the percentage of missed cleavages is higher in the "out of IPG range" peptides (14 %) with respect to focused peptides (7 %). Indeed, peptides with a higher number of lysine and arginine residues tend to migrate towards the basic pH when subjected to IEF. In conclusion, the addition of a simple recovery step of peptides from paper bridges, instead of their discard, can heavily improve the performance of the peptide IPG–IEF offgel standard protocol, in particular when IPG strips with a narrow pH range are used.

Conflict of interest The authors have declared no conflict of interest.

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