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Review

Pros and cons of peptide isolectric focusing in shotgun proteomics

Renato Millioni[a,b,*], Cinzia Franchin[b,c], Paolo Tessari[a], Rita Polati[d], Daniela Cecconi[d], Giorgio Arrigoni[b,c]

[a] Department of Medicine, University of Padova, Via Giustiniani 2, 35121 Padova, Italy
[b] Proteomics Center of Padova University, VIMM and Padova University Hospital, Via G. Orus 2/B, 35129 Padova, Italy
[c] Department of Biomedical Sciences, University of Padova, Viale G. Colombo 3, 35131 Padova, Italy
[d] Proteomics and Mass Spectrometry Laboratory, Department of Biotechnology, University of Verona, Strada le Grazie 15, 37134 Verona, Italy

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ABSTRACT

In shotgun proteomics, protein mixtures are proteolytically digested before tandem mass spectrometry (MS/MS) analysis. Biological samples are generally characterized by a very high complexity, therefore a step of peptides fractionation before the MS analysis is essential. This passage reduces the sample complexity and increases its compatibility with the sampling performance of the instrument. Among all the existing approaches for peptide fractionation, isoelectric focusing has several peculiarities that are theoretically known but practically rarely exploited by the proteomics community. The main aim of this review is to draw the readers' attention to these unique qualities, which are not accessible with other common approaches, and that represent important tools to increase confidence in the identification of proteins and some post-translational modifications. The general characteristics of different methods to perform peptide isoelectric focusing with natural and artificial pH gradients, the existing instrumentation, and the informatics tools available for isoelectric point calculation are also critically described. Finally, we give some general conclusions on this strategy, underlying its principal limitations.

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1. Introduction

In shotgun proteomics peptide fractionation is a common practice to reduce sample complexity and consequently increase the chances of identifying low-abundant proteins, which are the best candidates to become important diagnostic markers. The most commonly used fractionation technique is the multidimensional protein identification technology approach (MudPIT), where peptides are separated by strong cation exchange (SCX) and reversed phase (RP) chromatography [1], prior to their identification by mass spectrometry (MS). However, protein and peptide fractionations using electrophoresis are also a common practice in proteomics, and thanks to its high capability, resolving power,

* Corresponding author at: Department of Medicine, University Hospital of Padova, Italy. Tel.: +39 049 821 2172.
E-mail address: millionirenato@gmail.com (R. Millioni).

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and well-established protocols, isoelectric focusing (IEF) separation, combined with MS identification, has become one of the most widely used approaches [2].

The idea of combining IEF with liquid chromatography–mass spectrometry (LC–MS) was initially proposed to improve the two-dimensional gel electrophoresis (2-DE) technology: protein mixtures were firstly separated by IEF, using conventional IPG strips that were then cut into sections. Proteins from each section were in-gel digested with trypsin, and the resulting peptides analysed by LC–MS/MS [3,4]. Loo et al. [5] developed a method called "virtual 2D" where, after protein focusing, trypsin digestion is performed directly in the gel pieces obtained by cutting the IPG strip. Each gel piece is mixed with a solution of α-cyano-4-hydroxycinnamic acid and MALDI-MS/MS analysis is performed directly on the matrix-treated IPG strips, avoiding the step of peptide extraction. For both these approaches, the exploitation of MS as second dimension improved sensitivity, resolution, and accuracy compared with traditional 2-DE protocols. However, beside these advantages, these methods inherited the main disadvantages of the 2-DE technology, mainly related to IEF protein separation step, and in particular to its limitations in the analysis of hydrophobic proteins. Moreover the presence of contaminants derived from the strip (such as the ampholytes, CHAPS, and urea) and poorly compatible with mass spectrometry requires cleaning procedures prior to MS analysis.

Since 2004, the proteomics field has witnessed a strong and progressive increase of peptide IEF application in shotgun proteomic approaches [6]. The main advantage of the shotgun approach based on peptide IEF separation coupled to LC–MS is the possibility to perform successful analyses also on small or hydrophobic proteins that are difficult to be analysed with a protein IEF approach. For example, from the membrane fraction of *Staphylococcus aureus*, about 5000 unique peptides, representing more than 850 proteins and the 30% of the predicted proteome, were identified and quantified in a single experiment by a shotgun peptide IEF approach [7].

Apart from being an excellent tool for separating complex mixtures of peptides, IEF has also high reproducibility, it is compatible with other techniques for multistep fractionation protocols, and can provide additional information not accessible with the MudPIT approach, thus increasing confidence in protein and PTMs identification. All these points are discussed in the following sections.

2. General properties of peptide IEF and the instruments to perform it

The advantages and limitations of different methods (or approaches) to perform peptide isoelectric focusing with natural and artificial pH gradients are reported in Table 1 and discussed in the following sections.

2.1. CA- and IPG-based IEF

The generation of the pH gradients needed in IEF has for long relied on the use of carrier ampholytes (CA). CA are polycarboxylic polyamino compounds, structurally similar to peptides in terms of size and chemical properties. Commercial CA have been rigorously characterized [8–11]. A mixture of CA with different pI self-organizes in an electric field and creates the pH gradient that can be then used to perform IEF. Tang et al. [12] showed that a high concentration of CA in the capillary IEF (cIEF) process permits the achievement of better resolution but decreases the sensitivity of ESI-MS detection since the ampholyte ions cause suppression of protein or peptide ion intensities. Lamoree et al. [13] also reported that an excess of ampholytes in the capillary effluent leads to electrospray instability and salt deposition in the interface. Another general problem with CA arises when long focusing times are applied since the gradient slowly starts to drift in both directions (but particularly towards the cathode). This in turn leads to a plateau in the middle of the gradient with gaps in the conductivity [11].

To overcome all these problems, immobilized pH gradient (IPGs) were developed in 1982 [14]. IPGs are based on the bifunctional immobilines reagents, which are acrylamide derivatives. Their general structure is CH_2=CH—CO—NH—R, where the R group contains either an amino or a carboxyl group, and forms a series of buffers with different pK values, between 1.0 and 13. Since their reactive end is co-polymerized with the acrylamide matrix, the pH gradients are stable and reproducible also during extended IEF runs. Moreover, the preparation of IPG strips on a plastic backing offers an optimal solution for convenient handling. Nowadays, several companies produce IPG strips of different pH ranges and dimensions. In particular, commercially available strips can be 7, 13, or 24 cm long and all pH ranges (linear and non-linear) between 1 and 13 are available. The length of the strips and the pH range must be therefore chosen based on the sample to be analysed: for example, longer strips (and therefore larger gels in the second dimension) provide higher sample capacity and resolution.

Besides the critical advantages of being more stable and reproducible, compared to the CA-based IEF, the IPG-IEF method allows also to achieve more efficient focusing on both proteins and peptides. However, a benefit of using peptides over proteins is due to their self-focusing ability during IEF in ampholyte-free buffer [15,16]. In fact, IEF of peptides using the CA-IEF approach showed that over 50% of unique peptides could be detected in three or more fractions compared to the 20% obtained using the IPG strip [17]. For this reason, in-solution IEF is mainly useful for preparative separation, while IPG-IEF may be applied both in preparative or analytical steps.

In IPG-IEF, voltage and current should be limited to 150 V and 50 μA per IPG strip during the initial stage (1–2 h) to avoid Joule heating due to 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 (up to 300 V/cm) to be applied. Several changes of the electrode filter paper, where ions are accumulated, help to eliminate the possible salt excess. If the voltage is not increasing during the IEF, probably the salt content of the sample is higher than the recommended 10 mM. Moreover, working at very low ionic strength is useful, since the heat induced by Joule effect, which should provoke convection flows and limit the resolution, is very low and easily contrasted by cooling.

Table 1
Comparison of advantages and disadvantage of different methods to perform peptide IEF (CA: carrier ampholytes; FFE: free flow electrophoresis; cIEF: capillary isoelectric focusing).

Method		Loading capability	Reproducibility	Resolution	Time-saving	Money-saving	User friendliness
CA		++	–	–	+	+	+
IPG	CLASSIC	+	++	++	–	–	–
	OFFGEL	+	++	++	–	––	++
cIEF	CA	––	–	–	+	+	+
	IPG	––	+	+	+	+	+
FFE-IEF		+++	––	–	++	+	–