

Double Fractionation by Polyacrylamide Gel Electrophoresis (DF-PAGE): new method for quantitative proteomics studies

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ABSTRACT

In order to increase the possibilities of detecting low abundance proteins in complex mixtures by proteomics techniques, it is essential to use protein or peptide separation methods to reduce the complexity of the biological sample. However, despite the wide use of several electrophoretic techniques, the usefulness of zone electrophoresis for the fractionation of complex peptide mixtures had not been previously investigated. In this work, a new method was established for proteomics studies, called Double Fractionation by PAGE (DF-PAGE). It combines protein fractionation by SDS-PAGE, in-gel enzymatic hydrolysis and peptide separation by SDS-free PAGE; the latter applied for the first time for fractionation and simplification of complex peptide mixtures. Then, it was necessary to design, for the case of DF-PAGE, a new batch buffer system for the selection of acid peptides ($pI \leq 5.5$). DF-PAGE allowed the identification of a greater number of proteins than PAGE-SDS and isoelectric focusing in solution. Its application to the characterization of the active principle of the VA-MENGOC-BC® vaccine allowed the identification of 67 proteins previously undetected with traditional techniques. A series of proteins differentially modulated by the antitumor peptide CIGB-552 in the HT-29 cell line of colon adenocarcinoma were also identified by DF-PAGE, which contributed to the characterization of the molecular bases of the action of said peptide. This work granted the Annual Award of the National Academy of Sciences of Cuba for the year 2015.

Keywords: Proteomics, electrophoresis, peptide fractionation, DF-PAGE, SDS-free PAGE, membrane proteins, VA-MENGOC-BC, CIGB-552

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RESUMEN

Doble Fraccionamiento por Electroforesis en Geles de Poliacrílida (DF-PAGE): nuevo método para estudios de proteómica cuantitativa. Para aumentar las posibilidades de detección de proteínas de baja abundancia en mezclas complejas mediante técnicas de proteómica, es esencial utilizar métodos de separación de proteínas o péptidos para reducir la complejidad de la muestra biológica. Sin embargo, a pesar del amplio uso de varias técnicas electroforéticas, no se había investigado con anterioridad la utilidad de la electroforesis de zona para el fraccionamiento de mezclas complejas de péptidos. En el presente trabajo se estableció un nuevo método para estudios de proteómica, denominado Doble Fraccionamiento por PAGE (DF-PAGE). Este combina el fraccionamiento de proteínas por SDS-PAGE, la hidrólisis enzimática en el gel y la separación de péptidos por PAGE sin SDS, esta última aplicada por primera vez para el fraccionamiento y la simplificación de mezclas complejas de péptidos. Además, fue necesario diseñar para el DF-PAGE un nuevo sistema discontinuo de soluciones tampón para la selección de péptidos ácidos ($pI \leq 5.5$). El DF-PAGE permitió identificar un mayor número de proteínas que la PAGE-SDS y la focalización isoelectrica en solución. Su aplicación a la caracterización del principio activo de la vacuna VA-MENGOC-BC permitió identificar 67 proteínas previamente no detectadas con las técnicas tradicionales. También se identificaron mediante el DF-PAGE una serie de proteínas moduladas diferencialmente por el péptido antitumoral CIGB-552 en la línea celular HT-29 de adenocarcinoma de colon, lo cual contribuyó a la caracterización de las bases moleculares de la acción de dicho péptido. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2015.

Palabras clave: Proteómica, electroforesis, fraccionamiento de péptidos, DF-PAGE, PAGE en ausencia de SDS, proteínas de membrana, VA-MENGOC-BC, CIGB-552

Introduction

The structural and functional heterogeneity of proteins and the systematic discovery of new post-translational modifications that vary their physical, chemical and biological properties demand a constant development of analytical proteomics methods. The fractionation and the simplification of the biological sample, increase the possibility of identifying low abundance proteins (LAPs)[1-4]. LAPs generally regulate the response of the biological system and therefore have been shown to be of therapeutic interest for the development of new drugs or the identification of diagnostic markers.

The combination of isoelectric focusing and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) resulted in the emergence of two-dimensional electrophoresis (2DE)[5]. 2DE became the basic methodological platform for the separation of complex protein mixtures. However, 2DE has limitations as for the separation of highly hydrophobic proteins [6]. In this sense, new analytical techniques have emerged for identifying proteins without previous separation by electrophoretic methods and using the mixture of peptides generated by

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enzymatic digestion. These analytical techniques are based on the combination of selective peptide isolation methods and chromatographic methods based on orthogonal principles [7].

Despite the broad range of methodological tools available in proteomics, none of the current analytical methods has proven to be ideal for the identification of all proteins present in a biological sample. Only the combination of several methodological strategies offers a more complete view of the biological problem under study. Therefore, the development of analytical methods remains a demand in the field of proteomics and an area of active research.

Hence, in the present work we report the development of a new method for proteomics studies combining protein fractionation by SDS-PAGE, protein in-gel digestion and fractionation of peptides by SDS-free PAGE, prior to sample analysis by liquid chromatography-mass spectrometry [8, 9].

The method established was called Double Fractionation by PAGE (DF-PAGE) and combines three orthogonal separation principles: protein fractionation according to their molecular size, peptide fractionation based on their electric charge and molecular size [10], and peptide separation according to their hydrophobic properties. Compared to other proteomics methods employing fractional electrophoretic techniques such as SDS-PAGE [11] and isoelectric focusing in solution (OGE) [12], the DF-PAGE method allows the identification of a greater number of proteins from complex mixtures. This novel method was applied to the characterization of the active principle of the VAMENGOC-BC vaccine [8], and to study of the mechanism of action of the antitumor peptide CIGB552 [13]. The results showed the feasibility of inserting the DF-PAGE method into the proteomics platform.

Results

Design and assessment of the DF-PAGE method for proteomics studies

The DF-PAGE method contains three main steps: i) Protein fractionation by SDS-PAGE; ii) Protein in-gel digestion; and iii) Peptide fractionation by SDS-free PAGE. The peptide fractions obtained by this way are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and proteins are further identified in databases from the peptide fragmentation spectra (Figure 1). The use of SDS-free PAGE for the fractionation of complex peptide mixtures in proteomics studies is a novel element within the development of the DF-PAGE method.

The SDS-free PAGE technique, which combines a batch buffer system (Tris/Glycine) with a 15 % polyacrylamide separating gel, allows the fractionation of peptides according to their migration rates with a fractionation selectivity (percentage of peptides resolved in one of the fractions compared to the total of detected peptides) above 80 %. The separation principle of the SDS-free PAGE technique is based on two orthogonal properties of peptides: electric charge and molecular size. In particular, the electric charge is an essential factor of the separation. This technique can separate peptides that differ in only one unit of electric charge, such as the deamidated peptides and their precursors.

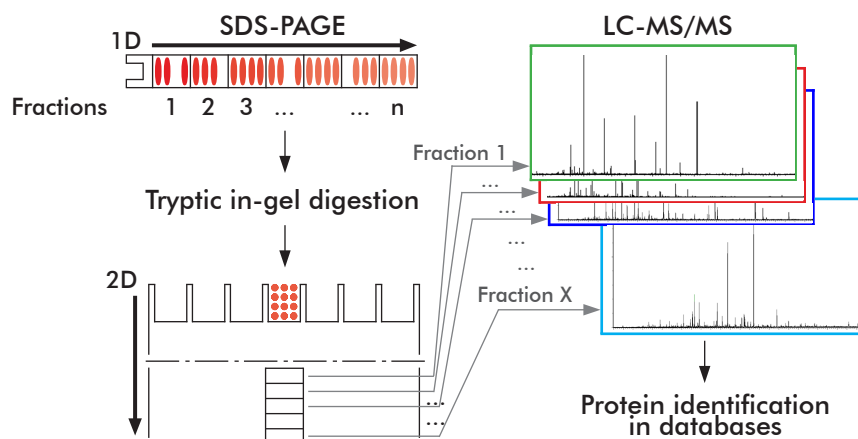


Figure 1. Representative diagram of the double fractionation method by polyacrylamide gel electrophoresis (DF-PAGE). It starts with unidimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (1D PAGE), followed by tryptic in-gel digestion of proteins and 2D SDS-free PAGE, and peptide fractions are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Finally, LC-MS/MS spectra of peptides are compared against databases and protein are identified.

Besides, the SDS-free PAGE technique achieves a simplification effect of the peptide mixture since only the negatively charged peptides migrate to the gel and separate into different fractions. Sampling simplification strategies in proteomics methods have the advantage of increasing the chances of detecting LAPs.

The SDS-free PAGE technique was compared to the OGE technique [9], a method for peptide fractionation that has been successfully established in the proteomics methodological platform. For this comparison, tryptic digestion of a fraction enriched in *E. coli* cytosolic proteins was used. LC-MS/MS analysis of the sample without previous SDS-free PAGE processing or OGE allowed the identification of 126 proteins. The effect of peptide fractionation by both methods (SDS-free PAGE or OGE) allowed multiplying 3-4 times the protein identification capacity. The quality of fractionation (assessed from the number of peptides detected in individual fractions compared to total peptides identified) was higher in the SDS-free PAGE technique (86 %, Figure 2) compared to OGE (76 %).

The GeLC method (gel electrophoresis combined with liquid chromatography) is based on protein frac-

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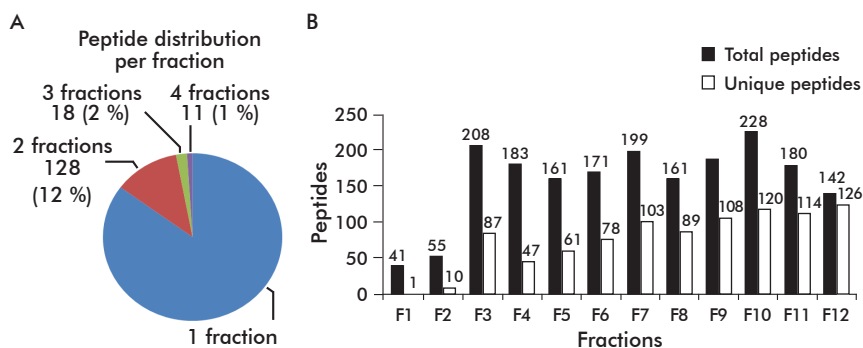


Figure 2. Quality of peptide fractionation by SDS-free PAGE with the discontinuous Tris/glycine buffer solution system. A) Proportion of single peptides in the experiment. B) Number of single peptides by fraction. The lower part of the figure shows the percentage of single peptides by fraction.

tiation by SDS-PAGE combined with the analysis of peptide mixtures by LC-MS/MS [8]. In fact, the DF-PAGE method arises by inserting the peptide fractionation step by SDS-free PAGE in the GeLC method. As a result of inclusion of the peptide fractionation step, the DF-PAGE method compared to the GeLC method increases the number of peptides identified from a fraction enriched in *E. coli* cytosolic proteins. The DF-PAGE method was able to identify all proteins previously identified by the GeLC method and other unidentified 89 proteins. This demonstrated the superiority of the DF-PAGE method compared to GeLC.

Selection of peptides by SDS-free PAGE

As part of the development of the DF-PAGE method, a novel discontinuous system of histidine/MOPS buffer solutions was also assessed for the selection and fractionation of acidic peptides (pI ≤ 5.5). Compared to the classical Tris/glycine system[14], which selects and fractionates peptides in a different pI range (pI ≤ 6.8), the histidine/MOPS system achieved a greater degree of sample simplification without affecting proteome coverage (Table). Moreover, the novel system increases the resolution capacity in the gel zone of higher migration, to resolve peptides with chemical modifications that shift the peptide pI distribution towards more acidic values.

Application of the DF-PAGE method to descriptive proteomics studies

The DF-PAGE method was used to determine protein composition of the active pharmaceutical ingredient of the VA-MENGOC-BC vaccine against *N. meningitidis*. The analysis allowed the identification of 138 proteins from 602 peptides. Prior to this study, the same preparation was characterized by 2DE and selective capture of peptides (SCAPE) methodologies [15, 16]. The 2DE study revealed the presence of 31 proteins, whereas 121 proteins were identified by the SCAPE methodology. After the study using DF-PAGE, the protein catalog describing the vaccine preparation increased from 129 to 196 (Figure 3). In total, 67 proteins were detected for the first time in the sample using the DF-PAGE method, 9 of them were membrane proteins. Some of these last were evaluated by other authors as vaccine candidates against infections of *N. meningitidis* type B.

Application of the DF-PAGE method to quantitative proteomics studies

The DF-PAGE method was used to study the protein profile modulated in the HT-29 cell line in response to treatment with the antitumor peptide CIGB-552. Peptide fractionation by SDS-free PAGE was performed with the discontinuous histidine/MOPS buffer solution. The comparative proteomics study was performed using two biological replicates. As an outcome, 3372 peptides corresponding to 868 proteins were identified. Proteins found with a variation rate greater than 1.8 between biological replicates in the statistical analysis of the data were regarded as differentially modulated. A total of 68 proteins changed their expression levels by the action of CIGB-552 peptide. These proteins mostly participate in the modulation of apoptosis, response to oxidative damage, activation of the NF-κB pathway

Table 1. Selection of theoretical tryptic peptides with negative charge at pH 6.8 and 5.5*

Organism	Peptides/proteins (total)	pI ≤ 6.8		pI ≤ 5.5	
		Peptides/proteins	Proteome coverage (%)	Peptides/proteins	Proteome coverage (%)
<i>Escherichia coli</i>	13.8	9.8	98.7	5.7	93.5
<i>Saccharomyces cerevisiae</i>	21.0	14.7	97.8	8.9	92.5
<i>Arabidopsis thaliana</i>	19.2	13.4	99.4	8.0	97.3
<i>Drosophila melanogaster</i>	25.5	17.4	99.3	10.4	97.1
<i>Mus musculus</i>	23.9	16.3	99.5	9.7	97.4
<i>Homo sapiens</i>	24.3	16.4	99.0	9.8	95.6
Average	21.3	14.7	98.9	8.8	95.6

* A theoretical study of proteomes of *E. coli*, *S. cerevisiae*, *A. thaliana*, *D. melanogaster*, *M. musculus* and *H. sapiens* is shown. Only tryptic peptides in the 800-3500 Da mass range were considered.

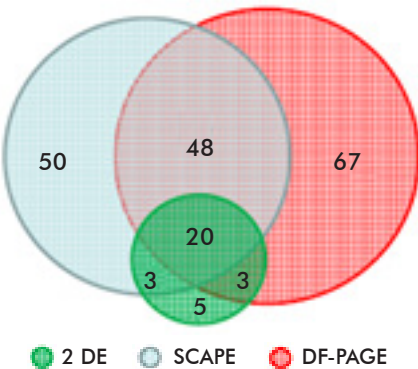


Figure 3. Number of proteins identified in the active pharmaceutical ingredient (API) of the VA-MENGOC-BC® vaccine by the 2DE, SCAPE and DF-PAGE methods.

and anti-inflammatory response [13]. These data are valuable information for understanding the mechanism of action of the CIGB-552 peptide.

Conclusions

As a direct result of this research, a new method for proteomics studies called DF-PAGE was introduced, which increases the number of proteins identified compared to other established methods such as 2DE and the GeLC method. In the context of the method, the SDS-free PAGE technique is developed for the fractionation and simplification of complex peptide mixtures. The discontinuous system of histidine/MOPS buffer solutions designed and established for the SDS-free PAGE technique selects and fractionates peptides with pI lower than 5.5.

The application of the DF-PAGE method to the characterization of the active principle of the VA-MENGOC-BC® vaccine allowed the identification of 67 proteins that had not been previously detected in this vaccine preparation. The method was also applied to the identification of differentially modulated proteins by the action of the antitumor activity peptide CIGB-552 on an HT-29 cell line. The results provided new experimental evidence for the characterization of the molecular mechanism of action of this peptide.

In summary, the DF-PAGE method is an analytical contribution to research in proteomics, which could be further optimized. This method proposes an alternative to solve problems established methodologies still have. Therefore, it is an advantageous methodological alternative for the identification of LAPs for the development of new vaccine and therapeutic candidates.

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