Optimization of the Electrophoresis Tricine-SDS-PAGE for Simultaneous Detection of Protein and Peptide in Artisanal Cheese

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Abstract: The objective of this paper was to optimize an electrophoretic methodology (Tricine-SDS-PAGE) to monitor the degree of caseins hydrolysis during the maturation time of artisanal cheese. A cheaper and easier method was obtained using small samples and micro-quantities of reagents. Simultaneous detection of proteins and peptides (100 kDa to 1 kDa) in the same gel was another advantage of the method. Initially, protein extraction was performed with 2 mg of lyophilized cheese dissolved in 1.0 mL of sample electrophoretic buffer for 1 h under stirring. After that, the Eppendorf tubes of the samples were kept at -4 °C for 4 h with additional centrifugation at 5,433 ×g for 2 min. This defatting process using centrifugation/refrigeration promoted a good separation of proteins and peptides from the fat layer. After this step, 30 µL of the supernatant of the protein extracts was applied to the electrophoresis gel. The results revealed a clear image of protein and peptides in the polyacrylamide gels and allowed an excellent response of the distribution of casein bands (α , β and κ) and the exposure peptide in cheese. The utilization of artisanal cheese as a pilot study of molecular protein analysis could be helpful for further correlation of the fingerprint of protein-peptide profile with taste quality.

Key words: Artisanal cheese, electrophoresis, maturation time, proteolysis.

1. Introduction

The stage of cheese maturation involves many physical, chemical and biochemical reactions. The time can vary from a few days to more than two years depending on the type of cheese. Proteolysis is the most complex and important primary event that occurs during cheese ripening and it plays a vital role in the development of texture and flavor. Hydrolysis of caseins leads to the formation of large and intermediate-sized peptides, which are degraded into smaller peptides by enzymes produced by starter culture and non-starter bacteria or fungi [1]. This will result in peptides of differentiated molecular mass as already described by studying artisanal cheeses using

proteomic analysis [2]. It is important to emphasize that others authors [2] used a very expensive proteomic and peptidomic methodology of analysis as well as the laborious procedure of protein extraction. Many studies show the application of capillary electrophoresis, liquid chromatography coupled with Tandem mass-spectrometry (MS/MS), and ultra-performance liquid chromatography to study proteolysis in cheese [3]. It is very important to say that electrophoresis in gel of polyacrylamide is a simple technique that can be also used to study proteolysis of cheese. Three kinds of electrophoresis could be applied for this study, SDS-PAGE [4], Native PAGE [5] and Tricine-SDS-PAGE [6]. However, using only the procedure with Tricine-SDS-PAGE [6], it was possible to identify peptides with molecular weight up to 1 kDa. The

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objective of this work was to show the application of Tricine-SDS-PAGE electrophoresis technique with the modification of procedure method of sample preparation, which uses micro-quantities of samples and buffer. The simplicity of the defat step and protein extraction is also a new strategy.

2. Materials and Methods

The cheese samples were purchased from three different producers of the state of Minas Gerais in Brazil. Electrophoresis Tricine-SDS-PAGE [5] was utilized for identification of protein-peptide profiles of cheese matured during two different times (17 d and 60 d). After freeze-drying, 2 mg of crushed cheeses was solubilized during 1 h under agitation with electrophoretic buffer pH 6.8 with 4% SDS, 12% glycerol, 2% mercaptoetanol and 0.01% Coomassie blue G250, using Eppendorf tube. Two different extracts of protein and peptide extracts were obtained: with and without fat. The one with fat was applied directly in the electrophoresis. In order to obtain the

protein-peptide extract without fat, the fatty extract was kept under -18°C during 4 h and further centrifuged $5,433 \times g$ during 1 min. The purified protein-peptide extract has been removed from the upper layer of fat with a micropipette.

3. Results and Discussion

All samples with fat, obtained from three different cheese producers at two different times of maturation (17 d and 60 d), showed a gel with a spread staining (Fig. 1). This could be explained by the high amount of fat present in these cheese protein extracts. As a solution to solve this problem, additional steps in the methodology were included. Initially, the same procedure of obtaining the protein extract was utilized as following, 2 mg of crushed and freeze-dried cheese was solubilized in electrophoretic buffer and submitted to vigorous agitation during 1 h at room temperature. The introduction of two steps in this procedure gave as a result a polyacrylamide gel showing a completely clear image, first by submitting

Fig. 1 Tricine-SDS-PAGE electrophoretic profiles of fatted protein-peptide extracts obtained from artisanal cheeses of three different producers (1, 2 and 3).

them to 1 h incubation at -4°C , in the same Eppendorf tubes of 2.0 mL and with further centrifugation at $5,433 \times g$, during 1 min. The clear protein extract separated from small fat plates can be seen in the Eppendorf tubes for all cheeses of the three producers (Fig. 2). Recently, molecular studies involving cheese analysis have shown the use of laborious steps for sample purification as described for the analysis of a peptide (3.5 kDa) by LC-MS/MS [7], while other works show the formation of peptides arising from cheese ripening by electrophoresis Urea-PAGE [8] or SDS-PAGE [9]. The association of Tricine-SDS-PAGE and the samples without fat

shows the quality of this result and that the method has been optimized. The deffated extracts were applied in polyacrylamide gel electrophoresis Tricine-SDS-PAGE (concentrations of acrylamide of 16.5%, 10% and 4% were used, respectively, in the separating gel, spacing gel and application gel) and a gel with a clear aspect was obtained (Fig. 3). The results related to the cheese ripening process show different protein and peptide profiles among the three producers, probably due to the specific ripening conditions of each one. Genomic analysis, nuclear magnetic resonance (NMR) and immunochemical analysis are currently being used for proteolysis studies

Fig. 2 Eppendorf tubes with the separation of fat layer from the protein-peptide extract of cheese from three different producers (1, 2 and 3).

Fig. 3 Tricine-SDS-PAGE electrophoretic profiles of defatted protein-peptide extracts obtained from artisanal cheeses of three different producers (1, 2 and 3).

in cheese. These results refer to the genomic analysis, monitoring of transcriptional mechanism activity of genes [10], proton transverse of water/oil relaxation [11, 12] and immunochemical quantification of milk proteins [9], respectively. The disadvantage of the above-mentioned methods is related to the need for a large financial investment in equipment acquisition [7, 10-12] when compared to the Tricine-SDS-PAGE electrophoresis method. Another scientific group working with soybean proteins has also used Tricine-SDS-PAGE method, however a complicate and long time for pure extracted was utilized [13]. Moreover, the electrophoretic analysis utilized in this paper has three advantages: (a) detection of protein breakdown and peptide formation in the same gel; (b) simple and alternative method of defatting; (c) micro-quantities utilization of reagent and sample. The excellent result obtained can be observed in Fig. 3. A total integrity of the three caseins (α , β and κ) and no appearance of peptides were observed in the peptide/protein extracts. The lack of casein hydrolysis can be observed by the high staining in the two different days of maturation (17 d and 30 d) of the cheese obtained from producer 2 (Fig. 3). In the cheese produced by producers 1 and 3 it was observed a reduction of staining of casein only after 60 d of maturation for producer 1 and for 17 d and 60 d for producer 3 (Fig. 3). Concerning peptide production, cheese of producer 1 has shown 11 kDa peptide (17 d) and 9 kDa peptide (60 d), in Fig. 3. The fingerprint variations among the producers show the need for standardization of the maturation process. Concerning the field of cheese production, it can be suggested that gel electrophoresis can serve as a database of images that together with process data will guarantee the quality of the cheeses. Regarding the methodological aspects, the utilization of micro-quantities of reagents and the simple procedure for fat removal are advantages concerning economic aspects and environmental preservation. Finally, this pilot study with artisanal cheese shows that utilization of this

method could be applied in the future to characterize the quality of all sorts of cheeses. The most important advantage of this work is the simplicity of the method of sample preparation together with the utilization of micro-quantities amounts of samples and reagents, when compared with other methodologies, chromatographic, electrophoretic, genomic and immunochemical [7-10].

4. Conclusions

This paper shows a contribution in the adjustment of a methodology of low cost for simultaneous analysis of protein and peptides using electrophoretic Tricine-SDS-PAGE as analytical tool. This contribution is related to low quantities of reagents and samples utilized for protein and peptide extraction and by the fast and simple procedure of defatting. This methodology may be useful for standardization and guarantee the quality of the cheese either by visualization of the diminishing of caseins content or by the appearance of new peptide, as a consequence of hydrolysis.

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