Analytical Biochemistry 537 (2017) 72–77

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# IEF peptide fractionation method combined to shotgun proteomics enhances the exploration of rice milk proteome

Marcello Manfredi [a, b, 1], Jessica Brandi [c, 1], Eleonora Conte [b], Paolo Pidutti [c, 2], Fabio Gosetti [a], Elisa Robotti [a], Emilio Marengo [a, *], Daniela Cecconi [c]

[a] Department of Sciences and Technological Innovation, University of Piemonte Orientale, Viale T. Michel 11, 15121, Alessandria, Italy
[b] ISALIT S.r.l., Novara, Italy
[c] Department of Biotechnology, Proteomics and Mass Spectrometry Lab, University of Verona, Strada le Grazie 15, 37134, Verona, Italy

ARTICLE INFO

*Article history:*
Received 30 June 2017
Accepted 27 August 2017
Available online 30 August 2017

*Keywords:*
Milk
Proteome
Isoelectrofocusing
Shotgun proteomics

ABSTRACT

We conducted a proteomics study in order to detect the proteomic method which provides the most complete characterization of the proteins of rice milk. In particular, we compared the results obtained from LC-MS/MS after protein precipitation with acetone or TCA, as well as the results obtained from LC-MS/MS after protein prefractionation based on SDS-PAGE (GeLC-MS/MS) or ProteoMiner™ technology (ProteoMiner-LC-MS/MS), and after peptide prefractionation based on IEF (pIEF-LC-MS/MS). A total of 158 protein species have been detect in rice milk. The physical-chemical analysis and classification of the identified proteins were also reported. In particular, we showed that pIEF-LC-MS/MS method led to a significant increase in the proteome coverage, allowing the identification of a total of 96 proteins of milk rice. This study demonstrates the utility of a prefractionation step based on pIEF before the shotgun proteomic analysis and offers an in-depth insight into the rice milk proteome.



## Introduction

Nowadays a growing number of consumers opt for plant-based milk substitutes for medical reasons (such as lactose intolerance and cow's milk allergy) or as a lifestyle choice [1]. Also, in countries where mammal milk is scarce and expensive, plant milk substitutes serve as a more affordable option. Technologically, plant milk substitutes are suspensions of dissolved and disintegrated plant material in water, resembling cow's milk in appearance [2]. In particular, they are manufactured by extracting the plant material in water, separating the liquid, and formulating the final product into palatable and nutritionally adequate plant milk, which can offers a sustainable alternative to dairy products. Among the different types of plant milks there is the milk extracted from rice.

Rice milk is made from boiled rice (both brown and white rice versions are available), and it is often supplemented with flavour enhancers, like brown rice syrup and vanilla to help it taste more like cow's milk. The different brands of rice milk have about the same number of calories as 2% cow's milk, but about half the fat and different carbohydrate and protein content. In addition unlike other plant-based dairy substitutes, rice milk has little or no fiber. Notably, rice milk represents a better alternative than soy milk for infants allergic to cow's milk [3]. However, despite the known benefits and potential of rice milk, a detailed characterization of its protein content is still lacking.

To obtain the in-depth exploration of milk rice proteins is therefore necessary to apply a proteomic approach, which can be gel based or gel free. As concerning gel-free proteomics the dominating techniques are represented by combination of prefractionation methods and LC-MS/MS. Indeed in shotgun proteomics, prefractionation at protein or peptide level is a common practice which reduces sample complexity and increases the chances of identifying low-abundant proteins. In particular, and thanks to its high capability, resolving power and well-established protocols, the prefractionation by isoelectric focusing (IEF), combined with MS identification, represent a really effective strategy [4]. In this study, in order to provide the most complete characterization of the proteins contained in the rice milk we have evaluated and compared different proteomic methods. In particular, we initially compared the results obtained from LC-MS/MS after protein

* Corresponding author.
*E-mail address:* emilio.marengo@uniupo.it (E. Marengo).
[1] These authors contributed equally to this work.
[2] Current address: VeronaLab s.r.l., Via Alessandro Volta 8, 37062 Dossobuono di Villafranca, Verona, Italy.

http://dx.doi.org/10.1016/j.ab.2017.08.021
0003-2697/

precipitation with acetone or TCA. Then we evaluated the results obtained from LC-MS/MS after a prefractionation step. In particular, we analyzed the results obtained by protein prefractionation based on SDS-PAGE (GeLC-MS/MS), protein prefractionation based on ProteoMiner™ technology (ProteoMiner-LC-MS/MS), and peptide prefractionation based on IEF (pIEF-LC-MS/MS). Here, we demonstrate that pIEF-LC-MS/MS method led to a significant increase in the proteome coverage and allowed us to identify a total of 96 protein species of milk rice.

## Material and methods

### *Protein precipitation by trichloroacetic acid or acetone*

Acetone precipitation of rice milk proteins was performed by adding four sample volumes of ice-cold 100% acetone to 1 ml of rice milk (1 mg/ml concentration), vortexed briefly, and incubated at −20 °C overnight. Subsequently, the sample was centrifuged at 14 000 g at 4 °C for 15 min. The supernatant was carefully aspirated and the protein pellets allowed to air dry at room temperature. While, as concerning the precipitation of rice milk proteins by trichloroacetic acid (TCA), it was performed by adding 15% TCA to 1 ml of rice milk, vortexing and incubating at 4 °C for 2 h. The sample was centrifuged at 14 000 g at 4 °C for 15 min and the supernatant was carefully aspirated. The pellet was washed twice with 100% ice-cold acetone. The final pellets were allowed to air dry at room temperature.

The protein precipitates obtained by acetone and TCA precipitation methods were resuspended in adequate buffer for further analysis and then stored at −20 °C. Protein concentration of samples was evaluated by Bradford assay (Bio-Rad). Aliquots of 100 μg of acetone or TCA-precipitated proteins were directly subjected to LC-MS/MS, or to the different prefractionation methods.

### *GeLC-MS/MS analysis*

For the GeLC workflow, 100 μg of TCA-precipitated proteins were diluted in Laemmli buffer and boiled for 5 min before being separated on a 12% acrylamide SDS-PAGE gel (8 cm × 8 cm, 1.5 mm thick). Proteins were visualized by Coomassie Blue staining. Each lane was cut into 12 homogenous slices that were washed in 100 mM ammonium bicarbonate for 15 min at 37 °C followed by a second wash in 100 mM ammonium bicarbonate, acetonitrile (1:1) for 15 min at 37 °C. All slices were reduced, alkylated, and *in-gel* digested overnight with trypsin [5]. Tryptic peptides were extracted and dried down by using a SpeedVac concentrator.

### *ProteoMiner™-LC-MS/MS analysis*

The proteomic prefractionation of TCA-precipitated proteins by ProteoMiner™ technology (Bio-Rad) was performed by following the manufacturer's recommendations and employing the reagents provided by the kit. Briefly, after addition of 1 ml of sample to the column and continuous agitation for 3 h, proteins saturated their binding sites and were eliminated during washing steps. The remaining unbound proteins were washed four times from the column. The bound proteins were finally eluted by using 100 μL of 8 M urea, 2% CHAPS, 5% acetic acid, this step was repeated four times. Elute proteins of each sample were then precipitated by acetone, pelleted by centrifugation at 14,000 g for 15 min at 4 °C, and resuspended in 100 mM ammonium bicarbonate. Sample was reduced, alkylated and then an aliquot of 100 μg of proteins were subjected to *in-solution* digestion as already described [6].

### *pIEF-LC-MS/MS analysis*

For the pIEF workflow, 100 μg of 15% TCA-precipitated proteins, was reduced, alkylated, and digested in-solution according to [5]. Briefly, the protein pellet was dissolved in 50 mM ammonium bicarbonate solution and digested with trypsin (Promega; 1:50) overnight at 37 °C. The resulting peptides were desalted using C18 spin columns (Thermo Scientific Pierce) according to the manufacturer's instructions. For IEF, dried peptides were dissolved in 450 μL of 8 M urea, 0.2% ampholine pI 3–10 (Bio-Rad), and then applied to 18 cm IPG strip (pH 3–10; GE Healthcare) by passive overnight rehydration. Isoelectric focusing (IEF) of peptides was carried out with a Protean IEF Cell (Bio-Rad), with a low initial voltage and then by applying a voltage gradient up to 10,000 V with a limiting current of 50 mA/strip. The total product time x voltage applied was 75,000 Vh for each strip and the temperature was set at 20 °C. After pIEF, IPG strips were manually sliced by putting the strip on top of graph paper and striping the gel from the plastic into 16 pieces (1 cm each). Peptides were then extracted by sequentially incubating the gel slices for 20 min each with 0.1% TFA, 99.9% water; 0.1% TFA, 49.9% acetonitrile, 50% water; and 0.1% TFA, 99.9% acetonitrile [7]. Each of these three steps were performed by agitation for 10 min on a vortex and then by sonication for 10 in an ultrasonic water bath.

### *Protein identification by LC-MS/MS analysis*

Peptides obtained from the different approaches were dissolved in 20 μL 5% ACN with 0.1% FA; triplicate injections of 5 μL each of the peptide solution were then analyzed by LC-MS/MS. The samples were analyzed on a micro-LC (Eksigent Technologies, Dublin, CA, USA) interfaced to a 5600 + TripleTOF mass spectrometer system (AB Sciex, Concord, Canada) equipped with a DuoSpray Ion Source and a CDS (Calibrant Delivery System). The LC column was a Halo Fused C18 with a pre-column ProteCol C18G. The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 μL $min^{-1}$ at an increasing concentration of solvent B from 2% to 40% in 30 min. The injection volume was 4.0 μL and the oven temperature was set at 40 °C. For identification purposes, the mass spectrometer analysis was performed using a mass range of 100–1500 Da (TOF scan with an accumulation time of 0.25 s), followed by a MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle). The ion source parameters in electrospray positive mode were set as follows: curtain gas ($N_2$) at 25 psig, nebulizer gas GAS1 at 25 psig, and GAS2 at 20 psig, ionspray floating voltage (ISFV) at 5000 V, source temperature at 450 °C and declustering potential at 25 V.

### *Protein database search*

The DDA files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston, MA, USA). Trypsin as digestion enzyme and 2 missed cleavages was specified, the instrument was set to ESI-QUAD-TOF and the following modifications were specified for the search: carbamidomethyl cysteins as fixed modification and oxidized methionine as variable modification. A search tolerance of 0.08 Da was specified for the peptide mass tolerance, and 10 ppm for the MS/MS tolerance. The charges of the peptides to search for were set to 2 +, 3 + and 4 +, and the search was set on monoisotopic mass. The Swiss-Prot *Oryza sativa* reviewed database containing rice proteins (version 2014_07, containing 3993 sequence entries) was used and a target-decoy database search was performed. False Discovery Rate was fixed at 1%.