

Evaluation and comparison of four protein extraction protocols for mono- and two-dimensional electrophoresis in *Mytilus galloprovincialis*

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Abstract

In this study, four protein extraction protocols from *Mytilus galloprovincialis* were evaluated with the aim to identify the most practical, efficient and reproducible method. Four extraction protocols frequently used for mussels and organic matrices were selected and compared. The methods were based on the use of: i) TRIZOL reagent; ii) Lysis buffer; iii) phenylmethanesulfonyl fluoride; iv) trichloroacetic acid-acetone. Protein concentration was measured by the Bradford method. Three specimens of mussels were studied and the analysis was conducted in triplicate for each of the four protocols. Results indicated that the four methods could extract significantly different protein profiles. The highest number of protein spots resolved in 2DE gels and the best reproducibility was obtained using trichloroacetic acid-acetone protocol. Results afforded the selection of a suitable extraction protocol to be used for ecotoxicoproteomics studies from mussels and for other proteomic studies conducted by particularly complex tissues such as *Mytilus galloprovincialis*.

Introduction

In recent years proteomics has been used for fish and fish products with several aims. Analysis of the water-soluble muscle proteins from aquaculture and wild fish was used to show the fish breeding effects on seafood quality (Monti *et al.*, 2005; Carrera *et al.*, 2007). Proteomics allowed to elucidate the influence of internal organ colonization by *Moraxella* spp. in organs of *Sparus aurata* (Addis *et al.*, 2010) and has been considered as a tool for species identification in seafood products, producing interesting results (Piñeiro *et al.*, 1999, 2001; López *et al.*, 2002; Pepe *et al.*, 2010, 2012; Chen and Huang, 2011). Bivalves, and mussels

in particular, are good indicators of marine and estuarine pollution, and so they have been widely used in biomonitoring programs in many UE countries (Apraiz *et al.*, 2006).

An accurate and reproducible extraction of proteins is a critical step for proteomics analysis. The criteria for the assessment of protein extraction quality include protein yield, compatibility with IEF, protein spots resolution in a 2DE gel, matched protein spots in replicate gels and reproducibility (Wu *et al.*, 2013).

The aim of this work was to evaluate the efficacy and the reproducibility of four protein extraction protocols for *Mytilus galloprovincialis*. The extraction methods were selected among the most frequently used for mussels and other experimental animal tissues.

Materials and Methods

Three specimens of *Mytilus galloprovincialis* were studied for quality assessment of each selected protein extraction protocol. Each step of the analysis was conducted in triplicate. The flesh (muscle, digestive gland, gills, mantle and intra-valvular liquid) of each *M. galloprovincialis* specimen, were reduced into homogeneous powder under liquid nitrogen and divided into four aliquots (1 g each) for protein extraction. Proteins were extracted using the four following protocols: i) TRIZOL reagent (Lee and Lo, 2008; Young and Truman, 2012; Wu *et al.*, 2013); ii) Lysis buffer (Molloy *et al.*, 1998; Lopez *et al.*, 2002; Chen and Huang, 2011; Zhu *et al.*, 2012); iii) Phenylmethanesulfonyl fluoride (PMSF) (Pepe *et al.*, 2010); iv) Trichloroacetic acid-acetone (Shrader *et al.*, 2003; McDonagh and Sheehan, 2007; Keyvanshokoh *et al.*, 2009).

TRIZOL reagent extraction

The ground sample was re-suspended in 1 mL TRIZOL reagent and centrifuged at 12,000 g for 5 min at 4°C. The supernatant was added with 200 µL chloroform before being shaken vigorously for 3 min and precipitated at room temperature for 3 min. This mixture was then centrifuged at 12,000 g for 15 min at 4°C and the upper aqueous phase was discarded. Then, 300 µL of absolute ethyl alcohol was added to the lower organic phase and the mixture was allowed to stand for 3 min at room temperature before being centrifuged at 2000 g for 5 min at 4°C. The phenol/ethanol supernatant was precipitated for at least 30 min at room temperature by the addition of 750 µL isopropanol prior to centrifugation at 14,000 g for 10 min at 4°C. The supernatant was removed, and the pellet was washed twice using 1 mL ethanol (v/v 95%) and centrifuged at 14 000 g for 10 min at 4°C. The pellets were solubilised in the lysis buffer (7 M urea, 2 M thiourea, 4% m/v CHAPS,

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Key words: Proteomics; Protein extraction; *Mytilus galloprovincialis*.

Conflict of interest: the authors declare no potential conflict of interest.

Received for publication: 9 July 2015.
Revision received: 12 January 2015.
Accepted for publication: 13 January 2015.

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Licensee PAGEPress, Italy
Italian Journal of Food Safety 2015; 4:4534
doi:10.4081/ijfs.2015.4534

65 mM DTT) and then incubated for 3 h at room temperature. The homogenate was centrifuged at 12,000 g for 10 min and the supernatant was applied to electrophoresis.

Lysis buffer extraction

Proteins were extracted by suspending the powdered samples in Lysis buffer (7 M urea; 2 M thiourea; 40 mM Tris; 4% w/v CHAPS; 65 mM DTT). The mixture was shaken vigorously at room temperature for 2 h, centrifuged at 14,000 g for 30 min and the supernatant was used for electrophoresis.

Phenylmethanesulfonyl fluoride-based method

In this method, the powdered tissue was dipped in 2 mL of 10 mM Tris-HCl buffer at 4°C, pH 7.2, supplemented with 5 mM PMSF (phenylmethanesulfonyl fluoride) and centrifuged at 15,000 g at 4°C for 20 min. The supernatant was then recovered, filtered using Ultrafree CL (0.22 µm) filters, and stored at -20°C until analysis by electrophoresis.

Trichloroacetic acid-acetone extraction

The trichloroacetic acid-acetone extraction, based on the most frequently used extraction protocol for experimental animal samples, was applied with some modifications. The powdered sample was re-suspended in cold buffer (10% w/v TCA in acetone contained 0.07% w/v DTT), precipitated at -20°C overnight and centrifuged at 15,000 g for 30 min at 4°C. The supernatant was discarded and the pellet was re-suspended two times with cold acetone containing 0.07% w/v DTT, kept at -20°C for 1 h and centrifuged at 15,000 g for 30 min at 4°C. The pellet was solubilized in the lysis buffer (7 M

urea, 2 M thiourea, 4% m/V CHAPS, 65 mM DTT and 0.2% W/V Bio-lyte buffer), the suspension was incubated for 3 h at room temperature, centrifuged at 12,000 g for 10 min and the supernatant was applied to electrophoresis.

Protein concentration was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard. Proteins (50 µg) were separated on a 12.5% (w/w) polyacrylamide gel (10 cm x 8 cm x 0.75 mm) at 10°C with 25 mA/gel constant current until the dye front reached the bottom of the gel, according to Hochstrasser *et al.* (1988). Gels were stained for 50 min with Coomassie Brilliant Blue R-250 and destained with MilliQ grade water.

The first dimensional electrophoresis (isoelectric focusing, IEF) was carried out on non-linear wide-range immobilized pH gradients (pH 3-10; 7 cm long IPG strips; GE Healthcare, Uppsala, Sweden) using the Protean i12™ IEF System (Biorad). Analytical-run IPG-strips were rehydrated with 200 µg of total proteins in 125 µL of rehydration buffer and 0.2% (v/v) carrier ampholyte for 12 h, at 50 mA at 20°C. The strips were then focused according to the following electrical conditions at 20°C: 250 V for 15 min, 4000 V for 1 h, 4000 V until a total of 15000 V was reached. For preparative gels 200 µg of total proteins were used. After focusing, analytical and preparative IPG strips were equilibrated for 15 min in 6 M urea, 30% (V/V) glycerol, 2% (w/V) SDS, 0.05 M Tris-HCl, pH 6.8, 1% (w/V) DTT, and subsequently for 15 min in the same urea/SDS/Tris buffer solution but substituting the 1% (w/V) DTT with 2.5% (w/V) iodoacetamide. The second dimension was carried out on 12 % (w/w) polyacrylamide gels (10 cm x 8 cm x 0.75 mm) at 25 mA/gel constant current and 10°C until the dye front reached the bottom of the gel, according to Hochstrasser *et al.* (1988). Gels were stained for 50 min with Coomassie Brilliant Blue R-250 and destained with MilliQ grade water.

Results

Results showed that Lysis buffer, trichloroacetic acid-acetone, TRIzol reagent and PMSF based extractions could extract significantly different protein profiles from *Mytilus galloprovincialis*. In particular, protein concentration measured by the Bradford method showed the best yield and reproducibility for trichloroacetic acid-acetone (Table 1).

SDS-PAGE and two-dimensional electrophoresis allowed a better evaluation and comparison of the efficacy of the four extraction protocols. The comparison confirmed differences also in the yields and in the purity of protein patterns. The method gave the maxi-

mum reproducibility, the better compatibility to IEF and the most protein spots resolved in 2DE gels for *M. galloprovincialis*.

Discussion

Results showed that the four methods used for protein extraction from *M. galloprovincialis* produce different data. Since in the present study, proteins were extracted from the whole mollusc, and not from specific parts, the high potentiality of the extraction method is demonstrated. The TCA acetone protocol determined the best results in all replications in terms of reproducibility, yield, high protein definition in SDS-page and good compatibility to IEF (Figure 1). Better results in comparison with the other extraction protocols, both in number and resolution of the spots were shown also at 2DE analysis.

If compared with the other methods, the TCA acetone extraction protocol requires more time and is more complex. The TRIzol based is an easier technique and allowed good results for both SDS-page and IEF even if the yield in 2DE analysis was lower than that obtained with TCA acetone. The PMSF method, generally used in proteomics studies of fish muscle

(Pepe *et al.*, 2010), showed a good yield but poor reproducibility as SDS-page showed high definition only in one replication. Using the IEF procedure difficulties in current passage (Figure 1) and as a consequence less spots were pointed out in 2DE images. This may be due to a poor compatibility between a very simple extraction method and a heterogeneous and complex sample as *Mytilus galloprovincialis*. Lysis buffer, even though it is one of the most common protein extraction method used for tissues for its rapid execution (Molloy *et al.*, 1998; Lopez *et al.*, 2002; Chen and Huang, 2011; Zhu *et al.*, 2012), showed a low reliability and reproducibility. Moreover, the protein extract, at the visual examination, was non homogeneous, dark and matt.

Conclusions

Four extraction methods were evaluated and compared for the whole proteome analysis of *Mytilus galloprovincialis*. The extraction of the whole mussels, and not of single tissue, allowed a global vision of the proteome and therefore provided a better chance to identify proteins of interest. On the whole the best results in terms of yield, reproducibility and

Table 1. Protein concentration (µL) of the extracts measured by the Bradford method.

Protocols	Extracts		
	First sample	Second sample	Third sample
1 TRIzol	2.9	0.29	2.2
2 Lysis buffer	20.5	58.8	17.67
3 PMSF	21.1	0.27	0.21
4 TCA-acetone	25.8	23.8	20.4

PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid. Values are expressed as µg/µL.

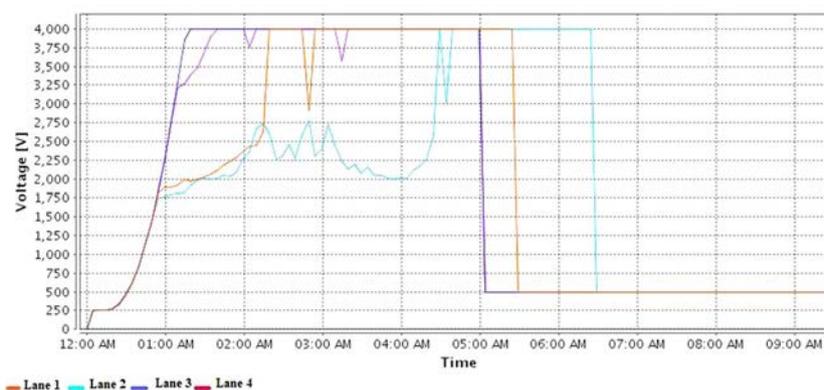


Figure 1. Isoelectric focusing showed difficulties in current passage for TRIzol and Lysis buffer extracts. Phenylmethanesulfonyl fluoride and trichloroacetic acid-acetone extracts displayed the best compatibility to isoelectric focusing. Lane 1, TRIzol; lane 2, Lysis buffer; lane 3, phenylmethanesulfonyl fluoride; lane 4, trichloroacetic acid-acetone.

accuracy were obtained employing the TCA-acetone extraction protocol. Results from this study contribute to increasing the information on quality and reproducibility of protein extraction techniques from complex food matrices, such as *M. galloprovincialis*, since the availability and reliability of protocols which allow a correct data interpretation is retained of the utmost importance for future proteomic work on mussels.

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