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Protein extraction from Ca-alginate encapsulated plant material for comparative proteomic analysis

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ABSTRACT

The extensive use of encapsulation material in biotechnology drove the need to develop analytical techniques for this type of material. This study focuses on the specific problems of protein extraction from Ca-alginate encapsulated plant material. Proteomics is one of the fast-developing analysis categories, specifically for stress resistance and developmental changes in plant material. Sample preparation is a critical step in a two-dimensional gel electrophoresis proteome approach and is essential for good results. The aim was to avoid preliminary manipulations and get good quality material for comparative proteome analysis technique 2DE. The phenol extraction method and the complex method with preliminary TCA precipitation, SDS buffer and phenol phase were compared with respect to the efficiency and quality of the resulting 2DE gel. The most appropriate method turned out to be the TCA/phenol method with the phenol fractioning technique adapted to the gentian cell suspension. It resulted in a high protein concentration and good quality sample that could be analyzed using the standard separation procedures of 2DE and spectrometric identification with high efficiency. The work presented here confirms the possibility of obtaining a sufficient protein sample for effective proteomic analysis from a small number of capsules.

1. Introduction

Encapsulation of plant material in calcium alginate beads has a number of applications in biotechnology. It is used in bioreactors to immobilize material during the production of secondary metabolites and recombinant proteins [1]. Cultures immobilized in vitro are less exposed to stress and contamination. They are also easier to manipulate, especially when dealing with single cells or small aggregates. The broadest possible use of encapsulation was found in cryopreservation. Encapsulation allows the attainment of a considerably higher level of dehydration than that of a non-encapsulated material. This results in the ability to induce a higher level of stress and freezing tolerance [2]. New opportunities to study the mechanisms of response to stress conditions, mainly based on osmotic dehydration, are therefore opened.

As shown in previous studies [3,4] the encapsulation-dehydration of gentian cell suspensions is the most effective cryopreservation technique for preparation before freezing in liquid nitrogen (LN). The gentian cell suspension is widely used in laboratory analysis. Due to the potential of the embryogenesis, it is an interesting source for the production of this medically significant plant with a high potential for horticulture as well. Understanding the mechanisms of gentian cell suspensions acquiring a resistance to freezing by induction of osmotic dehydration, which takes place in the procedure of encapsulation–dehydration, is the new challenge in the development of the applied techniques.

Proteomic analysis, especially two-dimensional polyacrylamide gel electrophoresis (2DE), remains the best technique that can be routinely applied to expression profiling of large sets of complex protein mixtures for the comparative analysis of responses to biological stimuli. For 2DE the choice of sample preparation protocol is the critical factor influencing isoelectrofocusing (IEF) which in turn affects the gel pattern quality. The choice of method for isolating proteins depends on many factors, including the type of material, the impurities present in the sample and the purpose of the analysis. Plant tissues have a relatively low content of protein with concurrently high concentrations of interfering substances. They are manifested in smearing in the electrophoretic separation of the extracted proteins [5]. For these reasons, the step of protein

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extraction and their purification is the greatest limitation and individual conditions for the material need to be improved [6].

The plant material enclosed in an alginate capsule was previously not used in proteomic studies. However, analysis of the yeast transcriptome permitted development of a technique to extract RNA directly from Ca-alginate beads, indicating that the capsule does not need to be an obstacle in such studies [7]. It is the first such report. In the case of encapsulation in Ca-alginate, an extremely high amount of impurities in the capsule material and possible components of the culture environment that diffuse into the capsule during the incubation carried out, are present. So far, the study of encapsulated material was preceded by the release of cells from alginate shells. These studies were related to the number of cell culture, and bioreactor performance associated mainly with a fermentation process. 1% sodium citrate solution was used at pH 6.0 [8] as well as a more complex buffer containing, in addition to sodium citrate, EDTA and sodium chloride [9]. When there is a need for molecular testing of the condition of the encapsulated plant material, based on the sensitive and easily degrading particles like proteins, this type of manipulation using low pH and high concentrations of ions creates a concern about potential changes in the material.

The aim of this study was to develop a protein isolation technique without the need to dissolve the capsules, which results in good quality material for comparative proteome analysis using 2DE on *Gentiana cruciata* cell suspensions.

2. Materials and methods

2.1. Plant material

Experiments were carried out on embryogenic cell suspensions of *Gentiana cruciata* L. The induction and maintenance of these cultures were performed as previously described [10]. Suspensions were transferred every 7 days to fresh liquid media with the initial density of ca. 5 g tissue in 80 ml medium in a 250 ml conical flask.

2.2. Encapsulation

Gentian alginate capsules have been prepared in accordance to the previously described technique [11]. Cell aggregates were washed with Ca^{2+} – free Murashige and Skoog Medium (MM) and mixed (1:1) into a bead solution composed of Ca^{2+} – free MM supplemented with 3% (w/v) sodium alginate (Sigma) and 6% sucrose. After 10 min the mixture was dripped into liquid culture medium containing 0.1 M $CaCl_2$ and 6% sucrose, forming beads of about 4 mm in diameter. For the completion of the gelling process, 50 capsules were kept in 50 ml Ca^{2+} -enriched medium for 20 min at room temperature (22 °C ± 2). Samples for the isolation of proteins (5 capsules each) were placed in 2-ml Eppendorf tubes and stored at −80 °C. As a control, 150 mg samples of non-encapsulated fresh cell suspension were collected.

2.3. Protein extraction

Tested extraction procedures with modified steps highlighted and compared to the original methodology, are detailed in Table 1.

2.3.1. Protocol 1 (Phenol extraction)

The standard technique of plant material isolation proposed by Hurkman and Tanaka [12] was tested for its ability to extract proteins from 5 alginate beads with cell aggregates. Samples were pulverized in liquid nitrogen using a pre-chilled pestle and mortar. Powder was suspended in 500 μl of extraction buffer and 500 μl Tris-saturated phenol 7.9 pH (Bio Shop, Canada). The suspension was vortexed for 15 min. Phases were separated by centrifugation at 14,000 rpm for 30 min at 4 °C. The phenolic phase was transferred to fresh tubes and 500 μl of extraction buffer was added once more. The sample was vortexed and centrifuged again in the same conditions, after which the tube with the separated phenolic phase was filled with ammonium acetate (0.1 M) in cold methanol and stored at −20 °C overnight to precipitate. The protein pellet after centrifugation (14,000 rpm; 0 °C) was washed with precipitation solution (−20 °C) and finally with iced acetone (freshly supplemented with 20 mM DTT). The pellet was air-dried and resolved in rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.002% (w/v) bromophenol).

2.3.2. Protocol 2 (TCA/acetone-phenol extraction)

In the attempt to isolate proteins with Wang's method [13] the original procedure was modified. In a preliminary step, material was homogenized in liquid nitrogen as described for Protocol 1. Tubes with transferred powder were filled with 10% TCA/acetone supplemented with 20 mM DTT and incubated for 2 h on ice. The centrifuged pellet was washed with iced acetone containing 20 mM DTT and air-dried for 20 min. For the final extraction pellet was resuspended in lysis buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl pH 8.0, 5% 2-mercaptoethanol) and Tris-saturated phenol pH 7.9 (Bio Shop, Canada) (1:1 v/v). The mixture was vortexed for 10 min, incubated on ice for the next 10 min, and then recovered phenol phase in centrifugation (14,000 rpm at 0 °C). Proteins were precipitated from the phenol phase in fresh tubes by 0.1 M ammonium acetate in acetone during the night. The next steps of centrifugation, washing and dissolving proceeded as in the previous method.

2.4. Protein quantification

The extraction efficacy was evaluated based on the protein concentration determined using the Bradford method, with BSA as the standard [14], utilizing GeneQuant 1300 apparatus (GE Healthcare, UK).

2.5. Two dimensional polyacrylamide gel electrophoresis

The quality of the isolated protein was assessed using images obtained from the 2DE. The first dimension was performed using IPG strips 24 cm pH 3–10 NL (non-linear) (GE Healthcare, UK). A 300 μg sample of protein was loaded to each strip with IEF buffer supplemented on 0.5% (v/v) IPG buffer pH 3–10 NL (GE Healthcare, UK) in final volume of 450 μl. Focusing proceeded using experimentally determined parameters: rehydration 0 V 14 h; 500 V step 1 h; gradient 1000 V 1 h; 8000 V gradient 3 h; 8000 V step 3 h, all at 20 °C.

2.5.1. *Focusing improving

During IEF, desalting was tested if it was indicated. Desalting was performed by placing small squares of filter paper between the electrode and the stripe. Paper on the cathode side was soaked with 20 mM DTT and the one on the anode with double distilled water. It was performed at the first step after rehydration. This step was extended to 4 h with papers changed every hour.

For rehydration and focusing IPGphor III Isoelectric Focusing (GE Healthcare, UK) equipment was used. Prior to the second dimension, IPG strips were incubated in equilibration buffer (6 M urea, 75 mM Tris pH 8.8, 30% glycerol, 2% SDS, 0.002% bromophenol) successively for 10 min in 1% (w/v) DTT and 2.5% (w/v) iodoacetamide. Separation of proteins in the second direction was performed in lab cast 1 mm 12.5% polyacrylamide gels as described (Laemmli 1970). The Ettan DALTsix Large Vertical System (GE Healthcare, UK)