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Improvement of β-glucosidase Activity of *Olea europaea* Fruit Extracts Processed by Membrane Technology

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Abstract: The β-glucosidase from olive fruit is of particular interest compared to the ones from other sources because it has shown to have high specifity to convert the oleuropein into dialdehydes, which have antibacterial activity and are of high interest for their application in the food and pharmaceutical fields. The enzyme is not yet commercially available and advanced clean and safe technologies for its purification able to maintain the functional stability are foreseen. The purification of this protein from fruit extracts has been already tempted by electrophoresis but either enzyme deactivation or high background with unclear profiles occurred. In this work, fruit extracts obtained from the ripening stage that showed the highest enzyme activity have been processed by diafiltration and ultrafiltration. Asymmetric membranes made of polyamide or polysulphone having 50 and 30 kDa molecular weight cut-off, respectively, were tested for the diafiltration process. Ultrafiltration membranes made of polyethersulfone with 4 kDa molecular weight cut-off were used to concentrate the diafiltered permeate solutions. The efficiency of the separation processes was evaluated byenzyme activity tests using the hydrolysis of *p*-D-nitrophenyl-β-D-glucopyranoside (pNPGlc) as reaction model. Qualitative and quantitative electrophoresis were applied to analyze the composition of protein solution before and after the membrane separation; in addition dot blot and western blot analyses were applied to verify the presence of β-glucosidase in the processed fractions. The overall results showed that the β-glucosidase functional stability was preserved during the membrane operations and the removal of 20 kDa proteins allowed to increase the specific activity of the enzyme of about 52% compared to the one present in the initial fruit extract.

Keywords: *diafiltration, ultrafiltration, diavolume, olea europaea, β-glucosidase, enzyme specific activity*

1. Introduction

In the *Oleaceae* family a β-D-glucoside glucohydrolase EC 3.2.1.21 referred to as β-glucosidase, is a key enzyme in many processes such as the defense against pathogens[1], the physiological role during fruit ripening[2,3] and, restricted to olive tree, the influence in the foodstuff quality resulting from industrial processing of fruits[4,5]. In addition, the products of enzymatic hydrolysis of natural substrate oleuropein are well known as a pharmacologically active molecule[6]. As a general rule, this enzyme family catalyzes the hydrolysis of glycosidic linkages in aryl and alkyl β-glucosides and cellobiose as natural substrates[7]. However, the assay of activity of β-glucosidase toward the synthetic substrate *p*-D-nitrophenyl-β-D-glucopyranoside (*p*NPGlc) was widely employed in higher plants[8,9]. In the olive tree the *p*NPGlcsubstrate was employed to identify β-glucosidase behavior during fruit ripening[10] and in response to the injury[11]. Drupe tissues exhibit higher activity respect those in leaf tissues (data not shown) and for this reason, mesocarp tissues of fruit wererevealed as the eligible materials to get a move on olive protein purification methods that will preserve biological activity.

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On the basis of authors records, the β-glucosidase enzyme in *Olea europaea* has been characterized by *in situ* activity studies[12], but it has not yet been purified and it is not commercially available.

Notoriously leaf and fruit tissues of olive tree (*Olea europaea* L.) are recalcitrant to the common methods for protein extraction due to the presence, at cellular level, of non protein compounds that severely interfere with the extraction in aqueous buffers. When proteins are extracted by directly homogenizing olive leaf in aqueous buffers and then precipitated by organic solvents, polyphenols and other contaminants are co-purifiedwith the proteins, consequently the resultant brownish pellet, due to polyphenols oxidation, is hard to be dissolved[13]. These contaminants interfere also with electrophoresis separation of proteins and subsequent process of purification of class of proteins. High quality protein preparation from olive leaf was obtained by means of a non aqueous extraction that allowed good electrophoresis profiles of proteins[14,15]; on the other hand it caused the irreversible loss of biological activities. By contrast, extracts of leaf proteins using an aqueous sodium borate buffer (pH 9.0) showed high biological activities for the enzymes[10] but poor profiles on SDS-PAGE gels, revealing several bands with high background[16].

To our knowledge, procedures for the high purification of β-glucosidase extract from olive tissues preserving the biological activities are still not reported.

The use of membrane operations to separate complex mixtures of labile macromolecules offers an attractive alternative since they are able to preserve thebiological stability.

In the last decade, there has been considerable interest in developing membrane systemsfor the purification of complex protein mixtures for biotechnological, food and biomedical application[17]. The reliability of using affinity-ultrafiltration[18] and diafiltration for high resolution protein fractionation has been largely demonstrated[19-29]. The configurations that have mainly been used for protein fractionation are ultrafiltration with total recycle, batch or discontinuous diafiltration, continuous single-stage ultrafiltration[27].

Traditionally, ultrafiltration has been employed for size based separation of protein mixtures where the ratio of the protein molecular mass is at least around 7 ~10[28]. To achieve better purification of similarly sized biomolecules, considerable research has taken place focusing on the operating and physicochemical conditions to attain higher selectivity[20,21,23,30].

Diafiltration allows removing effectively small components from the retained species by washing them out. In this process, water or buffer solution is added to the retentate during the filtration, with the membrane- permeating species being removed from the feed as this excess fluid is filtered through the membrane. The diavolume coefficient, which is equal to the total volume of the wash buffer divided by the initial feed volume, is a suitable parameter used to evaluate diafiltration process[31,32].

A major challenge in the protein purification process is to verify the protein stability before and after each separation step the enzyme activity has been revealed as a fine quality tester to evaluate this parameter[33]. A purification step that would reduce the initial activity to lower than 80% will not be considered suitable, since it will dramatically influence the overall costs of the process.

The aim of this work was to identify appropriate membrane operations and process conditions able to improve the purity of β-glucosidase present in fruit extracts as well as to save its catalytic properties.

To achieve this goal, the best ripening period that produced high concentration of β-glucosidase in the selected olive orchard, the preparation of fruit extracts containing β-glucosidase and their processing by diafiltration and ultrafiltration were investigated.

The diafiltration of olive fruit extracts was carried out through polyamide 50 kDa and polysulphone 30 kDa. The diafiltered permeate solutions were concentrated using 4 kDa polyethersulfone ultrafiltration membrane.

The processing by membrane technology allowed to

improve the β-glucosidase purity in the fruit extracts by improving also its specific activity.

2. Materials and Methods

2.1. Plant Materials

The Italian cultivar Carolea of *O. europaea*, was chosen for the experiments. During two consecutive harvest seasons (2003 and 2004) olive fruits, grown in the Calabria region, were randomly picked by hand from the established groves at the following different stages of fruit ripening: *i)* green immature fruit with woody endocarp, *ii)* green mature fruit, *iii)* green-brown fruit.

2.2. Preparation of β-glucosidase Fruit Extract

Olives at different stage of ripening were collected from a selected orchard in two consecutive harvesting seasons. Fruits (n=5) were washed with distilled water and immediately frozen in liquid N_2 and then destoned using a mortar and pestle. Typically, 1 g fresh pulp (n = 6 different sample for each sampling time) was ground in liquid N_2 using a mortar and pestle. The obtained frozen powder was further ground to a fine powder by the aid of quartz sand and then transferred on ice in 10 mL tubes and resuspended in 12.5 mL 0.1 M borate buffer, pH 9.0, 6% (w/v) PVP (poly-vinil pirrolidone), 1% (w/v) b-mercaptoethanol, 1.0 mM PMSF (phenylmethylsulfonylfluoride) according with the procedures described by Briante *et al.*[10] The suspension was shaken gently for 1 h at 4°C and centrifuged in a minifugue at 27000 g for 1 h. The upper oil phase was carefully removed and the aqueous phase, representing a protein extract and the enzyme enriched phase, was filtered on paper and stored at -80°C for further analyses.

2.3. β-glucosidase Assay and Protein Content in the Fruit Extracts, Permeate and Retentate Solutions

The β-glucosidase activity toward pNPGlc (*p*-D-nitrophenyl-β-D-glucopyranoside) was evaluated in the fruit extracts and in processed solutions (permeate and retentate) at 37°C by measuring the increase in absorbance at 405 nm of the reaction medium composed by 200 mM Na-phosphate buffer adjusted to pH 4.6. The linear coefficient to calculate the concentration of the reaction product was measured by calibration curve made with standard solutions of *p*-nitrophenol (Sigma-Aldrich) and corresponded to 14.0 $M^{-1}cm^{-1}$. The enzyme specific activity was expressed as mmoles of *p*-nitrophenol produced per minute at 25°C per mg of proteins (mmol/min·mg).

Protein content in the various solutions was determined by Bradford spectrophotometric assay[34].

Membranes before experiments were rinsedwith ultrapure water to eliminate additives used to preserve the membrane.

The variability, reported in all the figures, for β-glucosidase activity and mass protein content is due to the variability present in the different assayed fractionation.

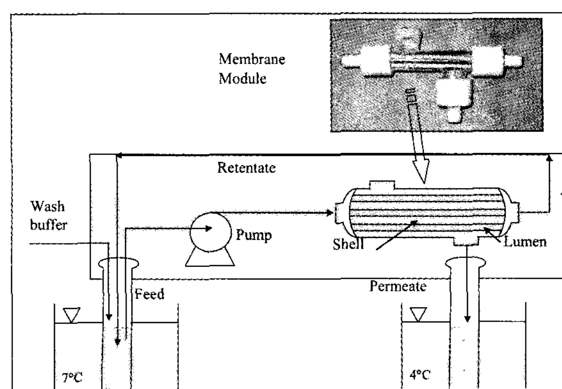

Fig. 1. Schematic draw of membrane diafiltration system.

2.4. Membrane Equipment

A schematic draw of the membrane diafiltration equipment is shown in Fig. 1. Polyamide capillary membranes of 50 kDa nominal molecular weight cut-off (NMWCO) (PA 50 kDa) and polysulphone of 30 kDa (PS 30 kDa) were used. The structure of this kind of membranes is asymmetric with the selective layer on the lumen side and the sponge layer on the shell side. The lab-made membrane modules were prepared by assembling the capillary membranes inside a pirex glass

cylinder. The PA 50 kDa were assembled in modules of 1.2 cm I.D., 22 cm long, while the PS 30 kDa were assembled in modules of 1.2 cm I.D and 5.5 cm long. The internal membrane surface area was $6.9 \cdot 10^{-3}$ m^2 for PA 50 kDa and $4.60 \cdot 10^{-4}$ m^2 for PS 30 kDa. A Masterflex pump (Cole Parmer) was used to supply the feed solution to the module and to recirculate the retentate solution along the lumen circuit, during diafiltration with PA 50 kDa membranes, while an Ismatec multichannel pump (Cole-Parmer) was used during diafiltration with PS 30 kDa membrane. The experiments were carried out by mantaining the permeate solution and the membrane module at 4(±1)°C while the feed solution was mantained at 6(±1)°C. The permeate was collected from the shell side. The wash buffer used for diafiltration of protein extract from *Olea europaea* was 0.1 M borate buffer pH 9.

Experiments were carried out at flow rate ofabout 55 mL/min (0.09 m/sec) during diafiltration with 50 kDa PA membranes and 0.96 mL/min (0.005 m/sec) during diafiltration with 30 kDa PS membranes.

Depending on the diafiltration coefficient, the concentration of proteins permeated through the membrane and collected in the permeate can be very low. In order to be able to detect proteins in these samples by electrophoresis the permeate solution was concentrated by flat membranes made of polyethersulphone having 4 kDa NMWCO (NADIR, Germany). The permeate concentration was carried out in a dead-end cell.

2.5. Electrophoresis

Proteins present in the collected samples (feed, permeate and retentate) were analyzed by one-dimensional SDS-PAGE according to Laemmli[35] in two different apparatus: Bio-Rad mini-Protean II apparatus and a 10 ~ 15% PhastGel™ gradient using buffer strips. For the first procedure, Laemmli buffer system was used to cast 6% stacking and 12,5% resolving gel, except that the resolving gel contained 12,5% glycerol and the final concentration of the resolving gel buffer (Tris-HCl, pH 8,8) was 0.75 M rather than original 0.0375 M. After denaturation at 95°C for 3 min, proteins were resolved at 200 mV.

For the second procedure, an 8/1 μL sample applicator was used (Amersham Biosciences, UK). The gel has a continuous 10 to 15% gradient gel zone with 2% crosslinking. The buffer system in PhastGel SDS Strip is composed of 0.20 M Tris-glicine, 0.20 M Tris and 0.55% SDS, pH 8.1.

Sample preparation: to final volume, 2.5% SDS (Sigma-Aldrich) and 5% β-mercaptoethanol (Sigma-Aldrich) were added and heated at 100°C and then 0.01% of bromophenol blue (Sigma-Aldrich) was added. Each sample was loaded onto separate lane of the gel containing 1 μL of sample. The gels were stained with silver and then distained with 3.7% Tris-HCl, and 1.6% sodium tiosulphate. The solution for preserving the gels contained 10% glycerol.

The gel images captured by scanner were analysed by Image Quant TL Software (Amersham Biosciences, UK), which permitted to identify band molecular weights (MW) and concentration.

2.6. Western Blotting

Western blot analysis of feed, permeate and retentate after SDS-PAGE electrophoresis was performed using a polyclonal anti-β-glucosidase as the primary antibody, kindly supplied by Dr Y. Minami[36]. The proteins electroblotted on membrane were blocked overnight at room temperature with 3% BSA in TBST (20 mM Tris-HCl, pH7.5; 0.8% mM NaCl; 0.1% Tween 20) and then incubated with the primary antibody for 1 h at room temperature at a dilution of 1:2000. After washing in TBST, membranes were incubated with secondary antibody (anti rabbit alkaline phosphatase, AP, conjugate) at a dilution of 1:3000 for 1 h at room temperature. The detection was performed using the alkaline phosphatase detection system with the anti-biotin NBT/BCIP (4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) reagent kit (Roche). The antibody recognized a protein band at 65 kDa molecular weight as a putative olive β-glucosidase[36].

The β-glucosidase from almond (Sigma, St Louis, USA) was used as standard purified enzyme.

2.7. Dot Blot

The protein samples of feed, permeate and retentate samples are spotted through circular templates directly onto the nitrocellulose membrane (Hybond ECL, Amersham) and allowed to dry. Then, the membrane is incubated with 3% BSA in TBST (20 mM Tris-HCl, pH 7.5; 0.8% mM NaCl; 0.1% Tween 20) for 30 minutes. The membrane is incubated with the primary antibody (polyclonal anti-β-glucosidase) for 1 h at room temperature at a dilution of 1:2000. After washing in TBST, membrane was incubated with secondary antibody (anti rabbit alkaline phosphatase, AP, conjugate) at a dilution of 1:3000 for 1 h. The detection was performed following the procedures described for western blot. The β-glucosidase from almond (Sigma, St Louis, USA) was used as standard purified enzyme.

3. Results and Discussion

In this section, the results of the experiments carried outto identify the maturation stage at which β-glucosidase showed the maximum activity in the olive fruit and the processing ofextracts from this stage by diafiltration and nanofiltration will be presented and discussed.

3.1. Optimal Ripening Stage Expressing High β-glucosidase Activity in Olive Fruit

Results from enzyme activity assay during fruit ripening are consistent with a gradually increase of enzyme activity in green maturation phase, corresponding approximately from 60 to 180 days after anthesis (Fig. 2). In fact, the enzyme activity found in fruit having not wooded endocarp was very low, while it increased in the pulp of stoned immature green fruits and reached the maximum at green maturation phase. Subsequently, a significant decrease of enzyme activity was detected in fruits undergone to black maturation.

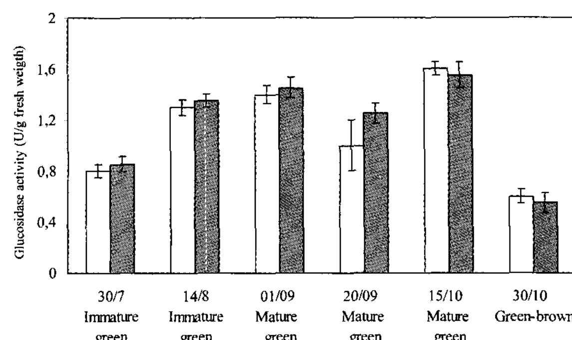

Fig. 2. β-glucosidase activity in olivefruits extracts at different ripening stages during two different harvest seasons: 2003 (□), 2004 (▨). Enzyme activity was evaluated toward the synthetic substrate pNPGlc at pH 4.6.

Considering the long sampling intervals (from July 2003 to November 2004) and consequent fruit growth differences and the variation of environmental conditions, the reproducibility of β-glucosidase activity resulted very good (Fig. 2). In other olive cultivars, Briante *et al.*[10] showed an equivalent trend of β-glucosidase activity levels during ripening, suggesting that, in general, in olive the green maturation is the stage of ripening at which the enzyme expression reaches its maximum. However, we found mean activity values for each sampling time higher (e.g. 1.4 units per g of fresh weight) than those reported for the other cultivars (e.g. 0.8 units of fresh weight)[10]. Recently, evidences that these significant differences might be due to different enzyme isoforms have been reported[12].

On the basis of these results, fruit extracts from green maturation stagewere produced and downstream purified by membrane processes.

3.2. Processing of Fruit Extracts by Diafiltration

3.2.1. Diafiltration Through Polyamide 50 kDa Membrane and Permeate Concentration by Ultrafiltration

60 mL of initial extract solution were diafiltered through PA 50 kDa using 0.1 M borate buffer pH 9. Afterwards, the enzyme activity of the collected fractions and qualitative analysis by electrophoresis (feed, retentate and permeate) were measured versus diafiltration volume (diavolume: total wash buffer volume/ini-

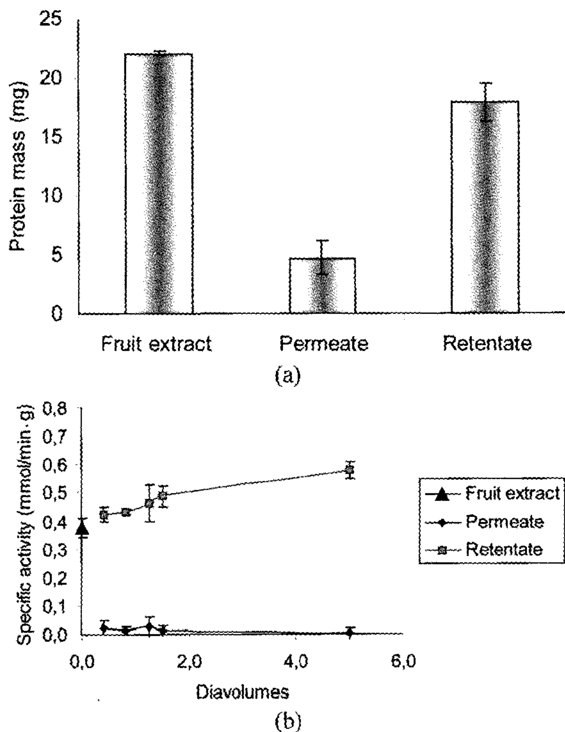

Fig. 3. Protein mass (a) and β-glucosidase activity (b) in solutions from diafiltration through PA 50 kDa membrane of fruit extract from *Olea europaea.*

tial feed volume). The amount of protein in the initial solution and in the processed fractions was determined by Bradford assay. As shown in Fig. 3a, the mass in the initial solution was 22.09 (± 0.2 mg) while in the final permeate and retentate solutions was 4.78 (± 0.4 mg) and 17.96 (± 0.4 mg), respectively.

The β-glucosidase activity as a function of diavolume coefficient was also investigated. As shown in Fig. 3b, the specific activity of initial solution was 0.380 (± 0.03) mmol/min · $mg_{protein}$, while in the final permeate and retentate solutions were 0.003 mmol/min · $mg_{protein}$ and 0.580 (± 0.020) mmol/min · $mg_{protein}$, respectively. The specific activity present in the retentate solution increased as a function of diavolume. After diafiltration of 4.5 diavolumes the specific activity in the retentate solution increased of about 52% compared to the initial one. On the other hand, the protein present in the permeate solution did not show any activity.

A qualitative analysis in the initial solutions and in the collected samples was carried out by dot blot and

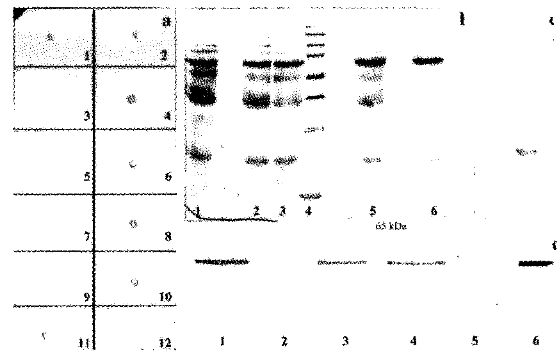

Fig. 4. (a) Dot blots performed by the antibody against b-glucosidase: 1. and 2. crude protein extract from *Olea europaea* 3. and 4. permeate and retentate, respectively after 0.42 diavolumes; 5. and 6. permeate and retentate after 0.83 diavolumes; 7 and 8 permeate and retentate after 1.25 diavolumes; 9. and 10. permeate and retentate after 1.50 diavolumes. 11 β-glucosidase from almond; b) SDS-PAGE : lane 1. protein extract from *Olea europaea* lane 2. retentate after 0.42 diavolumes; lane 3. retentate after 0.83 diavolumes; lane 4. marker BioRad; lane 5. retentate after 1.25 diavolumes; lane 6. retentate after 1.50 diavolumes; c) after concentration by nanofiltration membrane d) Western blots: lane 1. crude protein extract from *Olea europaea* lane 2. permeate after 0.42 diavolumes; lane 3. retentate after 0.42 diavolumes; lane 4. retentate after 0.83 diavolumes; lane 5. permeate after 1.25 diavolumes; lane 6. retentate after 1.25 diavolumes.

electrophoresis (Fig. 4a and 4b). In order to detect the protein in the permeate solutions, it was necessary to concentrate them. The solutions were concentrated using 4 kDa poliethersulfone ultrafiltration membranes. The suitable concentration factor was about 7, i.e. a volume of 20 mL was reduced to 3 mL. In no one permeate solution the band of the β-glucosidase (65 kDa) was present, as demonstrated by the dot blot tests carried out with a specific antibody for β-glucosidase (Fig. 4a). These results confirmed that β-glucosidase did not pass through the membrane of 50 kDa. In fact, as can be seen in Fig 4c only a protein of 20 kDa, as a major representative band, was detected in the permeate solution after concentration. The intensity of β-glucosidase (65 kDa) in the retentate solutions remained constant, in addition the profile of background became more clear during diafiltration process, (Fig. 4b, compare line: 1 initial solution and lanes 2, 3, 5, 6: retentate solutions after 0.42, 0.83, 1.25, 1.50 diavolumes).

In all the solutions analyzed the main band was β -glucosidase asconfirmed by western analysis carried out with a specific antibody for β-glucosidase (Fig. 4d). The antibody recognizesa single protein band of 65 kDa. Since at least two different β-glucosidases were foundin the cells of fruit tissues[12], our results seem to be consistent with the purification of one of the two isoforms. Although we have yet no evidences to identify which isoform is or whether the band corresponded to both enzymes having an equivalent molecular weight, in our view the extraction buffer used, with low ionic strength and without detergents, could be able to extract only the cytoplasmic isoform, since itcould be not effective in breaking the chloroplasts and removing the isoform inside them[1].

The processed β-glucosidase extract increased the specific activity not only due to removal of non catalytic protein, but also because of removal of inhibitors. In fact, the specific activity (0.580 mmol/min · $mg_{protein}$) obtained considering only the mass present in the retentate was higher than the one (0.460 mmol/min · $mg_{protein}$) evaluated considering the total protein present in the retentate and permeate and it was also higher compared to the specific activity (0.380 mmol/min · $mg_{protein}$) of the initial extract solution.

3.2.2. Diafiltration Through Polysulphone 30 kDa Membrane

In order to identify other intermediate molecular weight fractions, diafiltration through membranes with different membrane cut-off was carried out. Preliminary water permeability tests confirmed that no significant adsorption occurred on the polysulfone membrane.

2 mL of initial extract solution were diafiltered through PS 30 kDa using borate buffer pH 9. Also in this series of experiments diavolume was 4.5. After diafiltration, quantitative analysis for measuring protein mass (by Bradford test) and catalytic activity were carried out. Also in this case, electrophoresis analysis allowed the qualitative identification of protein content. As illustrated in Fig. 5, the amount of protein in the fruit extract (feed) and in the retentate solution were

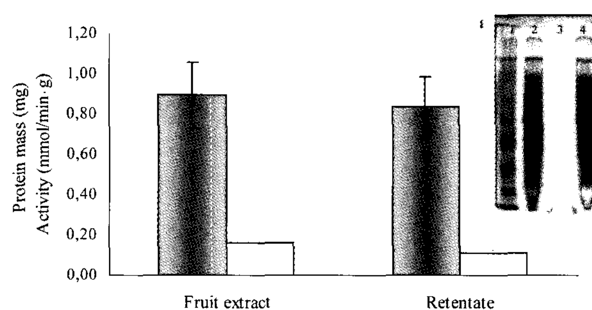

Fig. 5. Protein mass (▩) and β-glucosidase activity (□) in solutions from diafiltration through PS 30 kDa polysulphone membrane of fruit extract from *Olea europaea.* a) SDS-page assay: lane 1. marquer; lane 2 protein extract from *Olea europaea* lane 3 permeate lane 4. retentate.

0.90 (± 0.30) mg and 0.84 (± 0.30) mg, respectively. The β-glucosidase specific activities related to these samples were 0.16 (± 0.03) mmol/min · $mg_{protein}$ for the feed and 0.11 (± 0.02) mmol/min · $mg_{protein}$ for the retentate. The electrophoresis tests of fruit extract, permeate and retentate (Fig. 5a) confirmed these results; in fact, the composition of fruit extract and retentate was not changed. The background in this case remainedconstant and for this reason it was not possible to carry out analysis of western and dot blot.

These results indicated that, although the same diavolume was practised, the protein extract was not purified through PS 30 kDa. This was due to the fact that inhibitors have a molecular weight larger than 30 kDa therefore the 20 kDa proteins detected in the previous experiments must be present as complexes in the native fruit extract.

4. Conclusions

The *Olea europaea* maturation stage, in which β-glucosidase is largely expressed in tissues, was identified in the mature green phase of ripening, according with previous findings. Fruits extract from this stage were prepared and processed by diafiltration and ultrafiltration. The process allowedto separate and to identify a major enzyme form with molecular weight of 65 kDa identified as a putative β-glucosidase by a western blot analysis and enzyme activity assayand a 20 kDa

monomeric protein that in the native extract is combined to form higher molecular weight complexes. The diafiltration resulted a methodology able to guarantee the protein stability. In fact, the purification allowed to increase the specific activity of β-glucosidase from *Olea europaea* extracts during diafiltration with 50 kDa PA membranes up to 52% compared to the initial activity for the measured range of diavolume values in addition better electrophoresis profiles were obtained.

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