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The behaviour of the β-glucosidase, a key enzyme in the ripening and defence response in the olive fruit (*Olea europaea* L.)

Coordinator
Prof. Aldo Musacchio

Tutor
Prof.ssa Silvia Mazzuca

Candidate
Dr.ssa Antonia Spadafora

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Introduction

The research context

Olive (*Olea europaea*) is one of the established and largest crop in term of foodstuff production in Mediterranean countries, such as Spain, Italy, Greece. Starting from the last century, the intensive olive crop has been extended in other Mediterranean countries (e.g. Turkey, Tunisie, Maroc), and exported in several climate lands (e.g. California, Australia), thus its further expansion worldwide seems to be the tendency in the next future.

The reason of this achievement moved from the recognition of positive effects on the human health of olive food, especially virgin olive oil, a noteworthy component of the Mediterranean diet. In particular, many studies have shown that diet supplemented with olive oil daily, reduced the frequency of cardiovascular diseases, offered benefits in terms of colon cancer prevention and showed anti-inflammatory activities. However all these effects are the result of the higher levels of olive antioxidant compounds, particularly phenols [1-4].

Among olive phenols a bitter phenol glucoside, oleuropein [5], is largely accumulated in leaf and fruit and plays a key role in constitutive defence against pathogens [6] as well as in fruit ripening processes [7]. In particular, when olive tissues are damaged, an enzyme specifically hydrolyses oleuropein, producing highly reactive molecules [8].

The antioxidant and antimicrobial activities of oleuropein derivative molecules against herbivores and insect attacks has been demonstrated *in planta* [9] as well as against bacterial strains *in vitro* [10, 11] and they are recognized as pharmacologically active molecules [12].

Also during fruit ripening the same enzyme activity is involved in the progressive degradation of oleuropein, and in the release of glucose and the aglycones molecules, with the consequent physiological debittering of fruit tissues [13-15].

This enzyme involved in the reaction is the β-glucosidase (E.C. 3.2.1.21) belongings to the Gluco Hydrolase enzyme family 1 (GH 1, http://www.cazy.org/fam/GH1.html). The main characteristics of these enzymes are reported in Tab. 1.

Tab. 1. Main characteristics of β-glucosidases.

Mechanism	Retaining
Catalytic Nucleophile/Base	Glu (experimental)
Catalytic Proton Donor	Glu (experimental); absent in plant myrosinases
3D Structure Status	Fold: (β/α)
Clan	GH-A
Relevant Links	CAZypedia HOMSTRAD InterPro PFAM PRINTS PROSITE
Statistics	CAZy Entries (1206); GenBank/GenPept (1996); Swissprot (391); 3D (23); PDB (91); cryst (2)
Taxonomy	Archaea (41); Bacteria (860); Eukaryota (305)
3D Display	GH1_3D

β-glucosidases catalyze the hydrolyis of glycosidic linkages in aryl and alkyl β-glucosides and cellobiose (Fig. 1) and occur ubiquitously in plants, fungi, animals and bacteria. Since β-glucosides and β-glucosidases are ubiquitous in the living world, one expects to find structural and catalytic properties shared by all β-glucosidases. In fact a review reported that almost all β-glucosidases have subunit molecular weights of 55 to 65 kDa, acidic pH optima (pH 5-6) and an absolute requirement for a β-glycoside as substrate [16].

β-glucosidases from different orders and kingdoms appear to differ in their specificities for the aglycone linked to the glucosyl group by a β-glycosidic bond.

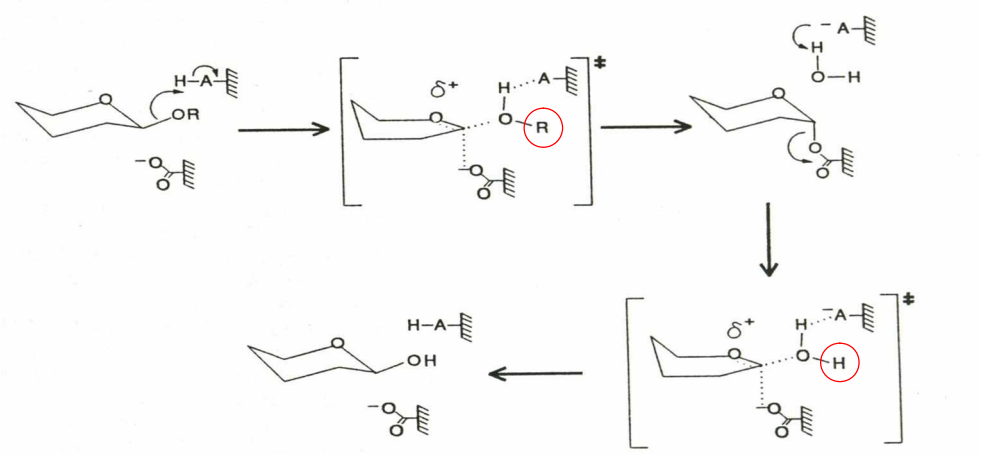

Fig. 1. Hydrolysis of the glycosidic bond. In most cases, this reaction is performed by two catalytic residues of the enzyme: a general acid (proton donor) and a nucleophile/base.

In the past decade, considerable progress has been made on the molecular biology and biochemistry of β-glucosidases. Some data have been available on very different organisms (plants, fungi, bacteria and humans) and/or related to specific problems (biomass conversion, cyanogenisis, host-parasite interactions, Gaucher's disease). Therefore, it is of interest to underline that research on β-glucosidases has significant implications both scientific and economic. In humans, one β-glucosidase, commonly known as glucocerebrosidase, catalyzes the degradation of glucosylceramide in the lysosome; the deficiency of the enzyme leads to an inheredited disease, Gaucher's disease [17].

β-glucosidases of cellulolytic organisms have been the subject of much past and ongoing research. These enzymes are expected to be targets for genetic engineering to design and select β-glucosidases for specific applications. In fact, fungal and bacterial β-glucosidases appear as natural candidates for engineering an ideal β-glucosidase to be used in the conversion of cellulose to glucose in industrial scale.

Plant β-glucosidases have been known for over 170 years since the description of the action of emulsin (almound β-glucosidases) on amygdalin, the cyanogenic β-D-gentiobioside of almonds, by Liebig e Wöhler in 1837 [18]. In plants, β-glucosidases have been implicated in several key metabolic events and

growth-related responses [16]. They range from defence against some pathogens and herbivores through the release of coumarins, thiocyanates, terpenes and cyanide to the hydrolysis of conjugated phytohormones (e.g., glucosides of gibberellins, auxins, abscisic acid and cytokinins) and to the fruit ripening processes.

Plant β-glucosidases have been investigated also for the specificity of their reaction sites through the crystallography and the deduced 3D structures. The omodimers of polypeptide chains arranged in a fold (β/α) structures (Fig. 2). From these data, the absolutely request of aminoacid residues of Glu 358 and Asp 374 are necessary for enzyme activity.

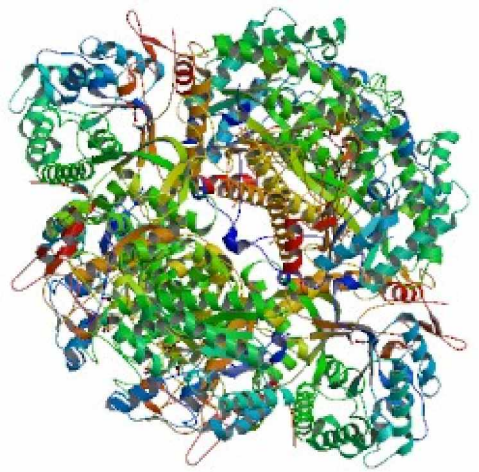

Fig. 2. The 3D structure of a β/α fold β-glucosidase from higher plant.

O. europaea tissues also contain large amounts of β-glucosidase which specifically hydrolyses oleuropein [7, 9]. The detected changes in β-glucosidase activity and in its products of enzymatic hydrolysis at different stages of fruit ripening [7] are strictly related to products quality [19-22]. In fact, good-tasting table olives and olive oil are greatly influenced by the phenolic compounds which are present in the fruit tissues. Thus, debittering of green olives is a major challenge in the industrial processing of fruit. In this context, investigations have been performed to test the efficiency of the enzymatic hydrolysis of oleuropein by the purified β-glucosidase from almond [23] compared with the enzyme from

the crude extract of olive fruit [9-10]. More recently a bioreactor with the immobilized recombinant β-glycosidase from the archaeon *Sulfolobus solfataricus* has been utilized to obtain high reactive molecules from oleuropein cleavage [24]. The results obtained from these investigations indicate that heterologous enzymes were not able to produce highly reactive dialdehydes from oleuropein, which strictly requests olive β-glucosidase.

The research aims

Aim of the present research is to investigate, by *in situ* β-glucosidase activity assay and by biochemical analyses

i. the mechanisms which regulate the enzyme expression and its activity on oleuropein which leads to the sweetening of the ripe olive during fruit ripening;
ii. the timing of enzyme activity and oleuropein content in fruit tissue following a mimed pathogen attack (Fig. 3) in two *cultivars* (Fig. 4) showing a different susceptibility against olive fly infestation [25].

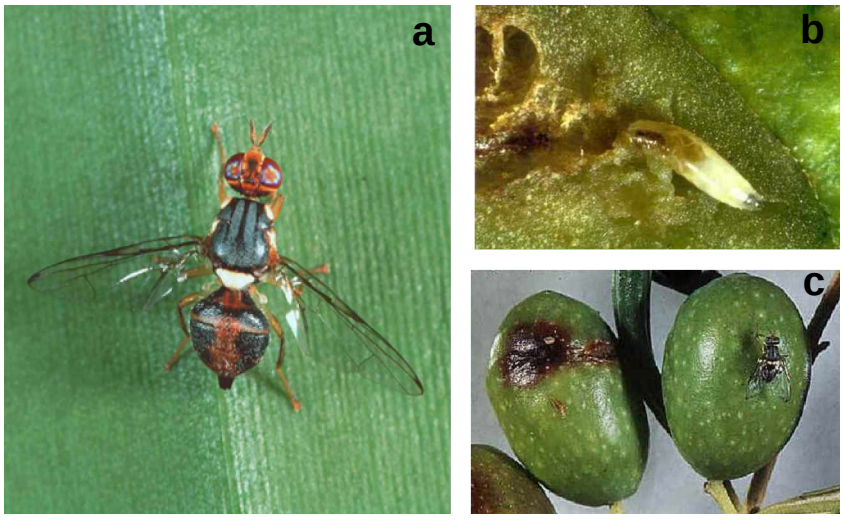

Fig. 3. Fly female (*Bactrocera oleae*) during the attack on olive fruit. By the ovipositor, located in the end part of her abdomen (**a**), the fly oviposes the eggs in tissues; developing larvae eat tissues and make deep tunnels trough the pulp (**b**) altering the integrity and the quality of fruits (**c**).

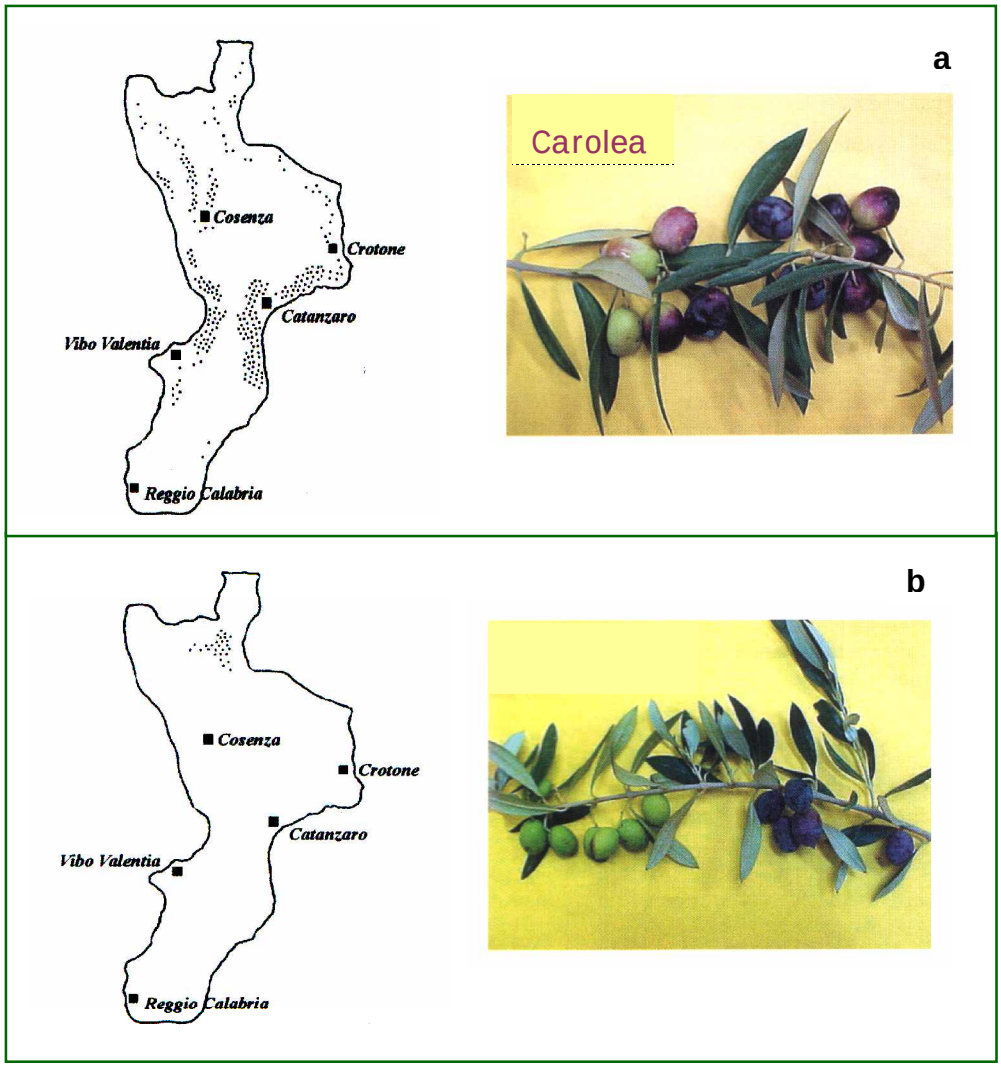

Fig. 4. Spread of the *cultivars* Carolea (**a**) and Cassanese (**b**).

The obtained results have been also useful to determine at which ripening stage the fruit extracts show the highest enzyme activity; these extracts have been thereafter processed by membrane technology to fractionate and purify β-glucosidase (Fig. 5).

In this context the purified enzyme was used to develop a bio catalytic membrane reactor to achieve the oleuropein cleavage able to produce the bioactive molecules.

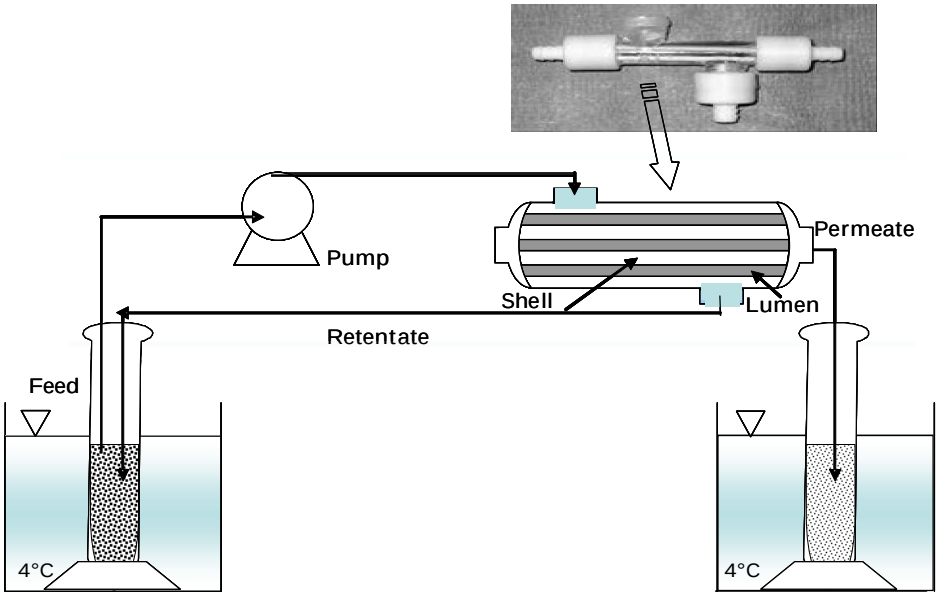

Fig. 5. Schematic draw of membrane diafiltration system.

Research items

Cell and tissue localization of β-glucosidase during the ripening of olive fruit (*Olea europaea*) by *in situ* activity assay

The cytological localization has been performed by the hydrolysis of X-Glc, a chromogenic synthetic substrate developing an insoluble blue precipitate in the cells. A strong reaction was detected within nucleus, chloroplasts and cytoplasm oil droplets. The enzymes kept in chloroplasts and oil droplets showed high specificity for the exogenous oleuropein respect to those toward X-Glc in the *in situ* competitive assay, thus indicating that two different oleuropein-degradative-β-glucosidases are present in these cell compartments.

Following the fruit ripening, significant variations in the number and distribution of reactive cells in mesocarp tissues have been observed. In fact, in immature fruits enzyme activity was not detectable in the outer mesocarp cells, whereas rare nuclei showed positive reaction in the inner mesocarp cells. Instead,

in green mature fruits a great number of reactive cells were found distributed in the whole mesocarp. The β-glucosidase activity appears preferentially localized in the outer mesocarp cells where a high activity was observable at the level of chloroplasts. Finally in black fruits numerous reactive cells were located only in the inner mesocarp close by the woody endocarp, whereas the outer mesocarp cells are devoted to polymeric anthocyanins accumulation. Thus, β-glucosidase activity level also decreased during fruit ripening.

These results clearly showed that the variations of oleuropein-degradative-β-glucosidases activity during ripening seems to be related to a different competence of single mesocarp cells to synthesize the enzyme isoforms.

Oleuropein-specific-β-glucosidase activity marks the early response of olive fruits (*Olea europaea*) to mimed insect attack

Two *cultivars* of *Olea europaea* showing different susceptibilities to the fly infestation have been taken into account: a) *cv* Carolea, characterized by a high susceptibility to *Bactrocera oleae* infestation and b) *cv* Cassanese showing a low susceptibility. In both *cultivars*, the histochemical assay for β-glucosidase showed that, within 20 min after the injury, a strong enzyme activity was present in the damaged tissues. Thereafter a progressive enzyme inactivation occurred starting from tissues around the boundary of the injury and the enzyme activity disappeared after 60 min. The loss of activity decreased and stopped after 3 h, whereas the active cells limit reached the distance of 300 $\pm$ 50 μm from the edge of injury. Biochemical analyses showed that in injured fruit extracts the β-glucosidase activity rapidly increased within 20 min from the injury, thereafter it decreased, reaching values comparable with those in sound fruits. Following the damage, the oleuropein contents did not change significantly in the high susceptible *cultivar*, while it rapidly decreased in the *cultivar* showing a low

susceptibility. These results strongly suggest that the olive fruit susceptibility toward the fly infestation could be related to the ability of the oleuropein-degradative-β-glucosidase to produce high reactive molecules in the damaged tissues. As a consequence of injury also a strong peroxidase activity was thereafter detected. This interesting pathway suggest that also this enzyme was involved in the following defence response.

Improvement of β-glucosidase activity of *Olea europaea* fruit extracts processed by membrane technology

The purification of olive β-glucosidase is of high interest for its 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. Fruit extracts obtained from the mature green phase of ripening, showing the highest enzyme activity, were 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 by enzyme 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 process allowed to 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 assay and 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 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.

Immunolocalization of β-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity *in situ*

The new method we performed results from the merging of the classic *in situ* enzyme activity assay together with western blot technique. The results either at low than at high magnification can be easily detectable light microscopy.

β-glucosidase was immobilized by physical method in asymmetric capillary membranes made of polysulphone having 30 kDa *cut-off*. Membranes sections processed with *in situ* assay showed a blue uniform staining by the insoluble reaction product. In order to verify if the colour distribution was not due to product diffusion, but corresponded to the presence of the immobilized enzyme, an immunolocalization method to localize β-glucosidase specifically was also developed. As result of the antibody recognition, black spots were localized inside the membrane.

The results obtained by polyclonal antibody against β-glucosidase and the synthetic substrate clearly showed a coherent correlation between the catalytic activity and the sites of enzyme immobilization.

References

1. H.K. Hamdi, R. Castellon (2005). Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. *Biochemical and Biophysical Research Communications*, **334** (3): 769-778.

2. A. Keys, A. Menotti, M.J. Karvonen (1986). The diet and 15-year death rate in the Seven Countries Study. *American Journal of Epidemiology*, **124**: 903-915.

3. Willett W.C. (1990). Diet and coronary heart disease. *Monographs in Epidemiology and Biostatistics*, **15**: 341-379.

4. World Health Organization (1990). Diet, nutrition, and the prevention of chronic diseases. Report of a WHO Study Group. *WHO Technical Report Series*, 797.

5. C. Soler-Rivas, J.C. Espin, H.J. Wichers (2000). Oleuropein and related compound. *J. Sci. Food Agric.*, **80**: 1013-1023.

6. M.J. Amiot, A. Fleuriet, J.J. Macheix (1989). Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry*, **28**: 67-69.

7. R. Briante, M. Patumi, S. Limongelli, F.C. Febbraio, A. Vaccaio, F. Di Salle, R. La Cara, M. Nucci (2002). Changes in phenolic and enzymatic activities content during fruit ripening in two Italian *cultivars* of *Olea europaea* L. *Plant Sci.*, **162**: 791-798.

8. A.D. Bianco, A. Piperno, G. Romeo, N. Uccella (1999). NNMR experiments of oleuropein biomimetic hydrolysis. *J. Agric. Food Chem.* **47**: 3665-3668.

9. K. Konno, C. Hirayama, H. Yasui, M. Nakamura (1999). Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defence in the privet tree. *Proc. Natl. Acad. Sci.* U.S.A., **96**: 9159-9164.

10. G. Bisignano, M.G. Laganà, D. Trombetta, S. Arena, A. Nostro, N. Uccella, G. Mazzanti, A. Saijia (2001). *In vitro* antibacterial activity of some aliphatic aldehydes from *Olea europaea* L. FEMS *Microbiol. Lett.*, **198**: 9-13.

11. A. Kubo, C.S. Lunde, I. Kubo (1995). A multichemical defence mechanism of bitter olive *Olea europaea* (Oleaceae). Is oleuropein a phytoalexin precursor? *J. Chem. Ecol.*, **11**: 251-263.

12. G. Bisignano, M.G. Laganà, D. Trombetta, S. Arena (2001). *In vitro* antibacterial activity of some aliphatic aldehydes from *Olea europaea* L. FEMS *Microbiol*. *Lett*., 198-199.

13. J.R. Morello, M.P. Romero, M.J. Motiva (2004). Effect of maturation process of the olive fruit on the phenolic fraction of drupes and oils from Arbequina, Farga and Morrut *cultivars*. *J*. *Agric*. *Food Chem*., **52**: 6002-6009.

14. M. Brenes Balbuena, P. Garcia, A. Garrido Fernandez (1992). Phenolic compounds related to the black color formed during the processing of ripe olives. *J*. *Agric*. *Food Chem*., **40**: 1192-1196.

15. D. Ryan, K. Robards, S. Lavee (1999). Changes in phenolic content of olive during maturation. *Int*. *J*. *Food Sci*. *Technol*., **34**: 265-274.

16. A. Esen (1993). β-Glucosidases: Biochemistry and Molecular Biology. Edited by A. Esen. *American Chemical Society*, Washington, 1-14.

17. G. Grabowski, A. Berg-Fussman, M. Grace (1993). Molecular biology and enzymology of human acid β-glucosidase. In β-Glucosidases: Biochemistry and Molecular Biology. Edited by A. Esen, *American Chemical Society*, Washington, 66-77.

18. J. Liebig, F. Wöhler (1837). *Annalen*, **22**: 11-14.

19. J.R. Morello, M.J. Moltiva, M.J. Tovar, M.P. Romero (2004). Changes in commercial olive oil (*cv* Arbequina) during storage with special emphasis on phenolic fraction. *Food Chem*., **85**: 357-364.

20. F. Angerosa, N. D'Alessandro, F. Cornara, G. Mellerio (1996). Characterization of phenolic and secoiridoid aglycones present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. *J*. *Chromatogr*. *A*., **736**: 195-203.

21. G. Cianfardini, V. Marsilio, B. Lanza, N. Pozzi (1994). Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation. *Appl*. *Environ*. *Microbiol*., **60**: 4142-4147.

22. V. Marsilio, B. Lanza, N. Pozzi (1996). Progress in table olive debittering: degradation *in vitro* of oleuropein and its derivatives by *Lactobacillus plantarum*. *J*. *Am*. *Oil Chem*. *Soc*., **73**: 593-597.

23. R. Capasso, A. Evidente, C. Visca, L. Gianfreda, M. Maremonti, G. Greco (1996). Production of glucose and bioactive aglycone by chemical and enzymatic hydrolysis of purified oleuropein from *Olea europaea*. *Appl*. *Biochem*. *Biotechnol*., **61**: 365-377.

24. R. Briante, F. La Cara, F. Febbraio, R. Barone, G. Piccialli, R. Caroll, P. Mainolfi, L. De Napoli, M. Patumi, G. Fontanazza, R. Nucci (2000). Hydrolysis of oleuropein by recombinant β-glycosidase from hyperthermophilic archeon *Sulfolobus solfataricus* immobilised on chitosan matrix. *J*. *Biotechnol*., **77**: 275-286.

25. N. Iannotta, L. Perri, C. Tocci, F. Zaffina (1999). The behaviour of different olive cultivars following attacks by *Bactrocera oleae* (Gmel.). *Acta Horticulturae*, **474**: 545-548.

RESULTS AND DISCUSSION

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Cell and tissue localization of β-glucosidase during the ripening of olive fruit (*Olea europaea*) by *in situ* activity assay

Silvia Mazzuca *, Antonia Spadafora, Anna Maria Innocenti

Laboratorio di Citofisiologia Vegetale, Dipartimento di Ecologia, Cubo 6b Ponte Bucci, Università della Calabria, 87030 Rende (CS), Italy

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Abstract

Olea europaea is an endemic tree in the Mediterranean basin of large economic interest for both oil and fruits production. One of the main problems to solve for improving the fruit quality is olive debittering. Thus we aimed to study, at cytological level, the β-glucosidases involved in the enzymatic degradation of oleuropein, a bitter defence phenol glucoside. This work represents the first insight on the *in situ* localization of β-glucosidase activity in the mesocarp tissue of olive during fruit ripening. The cytological localization is performed by the hydrolysis of X-Glc, a chromogenic synthetic substrate developing an insoluble blue precipitate in the cells. A strong reaction is detected within nucleus, chloroplasts and cytoplasm oil droplets. The enzymes kept in chloroplasts and oil droplets showed high specificity for the exogenous oleuropein respect to those toward X-Glc in the *in situ* competitive assay, thus indicating that two different oleuropein-degradative-β-glucosidases are present in these cell compartments. Following the fruit ripening, significant variations in the number and distribution of reactive cells in mesocarp tissues are observed. In fact in immature fruits the reactive cells are very few and located in the inner mesocarp zone; thereafter, in green mature fruits a great number of reactive cells are found distributed in the whole mesocarp. Finally in black fruits numerous reactive cells are located only in the inner mesocarp close by the woody endocarp, whereas the outer mesocarp cells are devoted to polymeric anthocyanins accumulation. These results clearly showed that the variations of oleuropein-degradative-β-glucosidases activity during ripening are due to changes in the competence of single mesocarp cells to synthesize the enzyme isoforms.

Keywords: *Olea europaea*; β-Glucosidase (EC 3.2.1.21); Fruit ripening; *In situ* enzyme activity; X-Glc; Oleuropein

1. Introduction

Olea europaea is an endemic plant of the Mediterranean basin and has been cultivated both oil and fruit production with considerable efforts to improve the fruits quality. In this context, one of the most crucial problems to solve is olive debittering. In fact, in olive leaf and fruit a bitter phenol glucoside, oleuropein [1], is accumulated as a defence mechanism against phytophatogens [2]. In particular, when olive tissues are injured by phatogens or by mechanical damage, an enzyme specifically hydrolyses oleuropein producing highly reactive molecules [3]. The antioxidant and antimicrobial activities of oleuropein derivative molecules against herbivores and insect attacks has been demonstrated *in planta* [4] as well as against bacterial strains *in vitro* [5,6]. The enzyme involved in this reaction is the β-glucosidase (E.C. 3.21.1.21) belongings to the glucohydrolase enzyme family 1 (GH 1); many components enzymes have been identified in plants where they play important roles in growth, development, detoxification, ripening and defence [7]. *O. europaea* tissues also contain large amounts of β-glucosidase which specifically hydrolyses oleuropein [8,4]. Also during fruit ripening the β-glucosidases are involved in the progressive degradation of oleuropein, and in the release of glucose and the aglycones molecules, with the consequent physiological debittering of fruit tissues [9–11]. The detected changes in β-glucosidase activity and in its products of enzymatic hydrolysis at different stages of fruit ripening [8] are strictly related to products quality [12–15]. In fact good-tasting table olives and olive oil are greatly influenced by the phenolic compounds which are present in the fruit tissues. Thus debittering of green olives is a major challenge in the industrial processing of fruit. The most commonly employed methods involve dilute NaOH solution or with low concentration of NaCl solutions. The NaOH treatments hydrolyse oleuropein into its derivatives and produces sugar.

* Corresponding author. Tel.: +39 0984 492967; fax: +39 0984 492986.
E-mail address: s.mazzuca@unical.it (S. Mazzuca).

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Conversely, oleuropein increased the salt diffusivity and was extracted from tissues when olives were immersed in the NaCl solution. Following both treatments a lactic fermentation takes place that included yeast [16]. There is good evidence that the chemical treatments could be substituted by a microbiological procedure that would provide both debittering and fermentation steps by using oleuropeinolytic *Lactobacillus plantarum* strains [15]. In fact, the *L. plantarum* strains are able to hydrolyse oleuropein by means of a bacterial β-glucosidase reaction with the formation of aglycone and sugar. This treatment results in olives with better taste than alkali or salt treatments, due to the higher sugar and protein contents in the final products. In this context, investigations have been performed to test the efficiency of the enzymatic hydrolysis of oleuropein by the purified β-glucosidase from almond [17] compared with the enzyme from the crude extract of olive fruit [4,5]. More recently an immobilized recombinant β-glycosidase from the archaeon *Sulfolobus solfataricus* has been tried [18]. In spite of the extensive investigations we lack data about the enzyme behaviour *in vivo* during olive fruit ripening. In our opinion knowledge of the tissue and cell localizations of β-glucosidase might be essential to understand the mechanisms which regulate the enzyme activity toward the oleuropein during fruit ripening that it determines the sweetening of the ripe olive. This information could be also useful to purify the enzyme from olive fruit and for the further technological applications on olive foodstuff processing.

The aim of the present research is the *in situ* localization of β-glucosidase activity in olive fruit tissues by using the synthetic substrate X-Glc specific for the GH 1 enzyme family [19,20]. The *in situ* assay was performed at different stages of fruit development, from anthesis to ripening; clarification of enzyme activity levels in tissue and/or cell compartment was sought.

2. Materials and methods

2.1. Plant materials

O. europaea fruits cv. *Carolea*, grown in the Calabria region were randomly hand picked every two weeks from June to November from two established orchards. The experiments were carried out on fruits at three developmental stages: (i) green immature fruit without woody endocarp (50 days after anthesis), (ii) green mature fruit with woody endocarp (120 days after anthesis), (iii) green-brown fruit (160 days after anthesis). In Table 1 the relationship between developmental stages of fruits and days after anthesis is reported.

2.2. β-Glucosidases in situ assay

For each ripening stage ($n = 5$) fruits were freshly sectioned at 4 °C, using a vibratome (Leica VT1000E, Germany), at of 80 μm thickness. Median sections were immediately placed in the minivials containing 1 ml of detection buffer, adapted for β-glucosidase according to Jefferson [21], containing 60 μM X-Glc (5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde, Sigma, St. Louis), 50 mM phosphate buffer pH 6.5, 1 mM potassium ferricianide, 1 mM potassium ferrocianide, 10 mM EDTA pH 8.0. After the enzymatic hydrolysis by endogenous the synthetic substrate X-Glc, glucosidase developed an insoluble blue precipitate on the site of reaction, thus localizing the enzyme in the cells. For each sample three seed sections treated with detection buffer without the X-Glc represented the controls. Sections were incubated in a wet chamber at 37 °C in the dark for 30 min, 1 h, 2 h and 3 h. The reaction was stopped in cold phosphate buffer and the sections were mounted with glycerol on slides for optical microscopy. Images were acquired by Leitz Dialux epifluorescent microscope (EB) equipped with a Charge-Coupled Device (CCD) camera. Image analysis on five sections for each fruit ($n = 25$ for each ripening stage) was performed by QWin™ Image System Software (Leica). The percentages of reactive cells per section were evaluated as dyed cells/total cells. For each section about 100 cells were scored.

2.3. Substrate competition assay

Mature green fruits ($n = 5$), were freshly sectioned as described above. Median sections were immediately placed in a minivials containing the detection buffers. Purified oleuropein, from green mature fruit, was dissolved in 80% methanol and 10 μl of solution was immediately added in three different detection buffers at the final concentration of 18, 30 and 60 μM oleuropein, respectively. Sections were immersed in the buffers and incubated for 1 h at 37 °C. Images were acquired and analysed, as above described. The assay was replicated three times

2.4. β-Glucosidase activity in fruit extracts

The β-glucosidase activity in fruit extract was performed according to Briante et al. [8]. Typically, 1 g fresh pulp of fruits ($n = 5$ for each ripening stage) was ground in liquid N_2 using a mortar and pestle. Tissue powder was suspended 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

Table 1
The relationship between developmental stages of fruits, days after anthesis and the β-glucosidase activity in mesocarp tissues during one harvesting season

Developmental stages of fruit[a]				
	Immature green with no woody endocarp	Immature green with woody endocarp	Mature green	Green-brown
Days after anthesis	50	80	120	160
β-Glucosidase[b] U/g fresh weight	0.31 ± 0.02	1.35 ± 0.1	1.20 ± 0.1	0.60 ± 0.05

[a] For the determination of the developmental stage $n = 30$ fruits for each stage were randomly screened.
[b] Results are the mean of three independent replicates. For each replicate at least of $n = 5$ fruits were used.

(phenylmethylsulfonyl fluoride). The suspension was shaken gently for 1 h at 4 °C and centrifuged in a minifugue at 27,000 × *g* for 1 h. The upper oil phase was carefully removed and the aqueous phase, representing the active enzyme enriched phase, was filtered on paper and used in the enzyme assay. The β-glucosidase activity against pNPGlc (*p*-D-nitrophenyl-β-D-glucopyranoside) [22] was evaluated at 37 °C, by measuring the increase in absorbance at 405 nm of the reaction medium composed of 200 mM Na-phosphate buffer adjusted to pH 4.6. The linear coefficient used to calculate the concentration of the reaction product was measured by a 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 g of fresh weight (mmol/min g).

3. Results

3.1. Cytological aspects of fresh and fixed tissues of olive mesocarp

Longitudinal sections of olive fruit mesocarp of both resin embedded samples, were stained with 0.1 % Safranin solution (Fig. 1a) whilst unfixed samples sectioned by a vibratome (Fig. 1b) were compared. In the parenchyma cells of fixed samples a well structured nucleus is visible together with a highly vacuolated cytoplasm, containing one or a few large oil bodies and clusters of small oil droplets (Fig. 1a, arrow). In fresh tissues sections the parenchyma vacuolated cells show prominent and translucent oil bodies very similar in both shape and dimension to those in fixed tissue. Clusters of small droplets are also observable in the cytoplasmic enclaves (Fig. 1b, black arrows). Thus the only difference detected, at the cytological level, between fixed and fresh tissue, is the higher number of chloroplasts per cell in fresh tissues, certainly due to the higher section thickness (Fig. 1b, white arrow). The above observations indicate that fresh samples can be used for the *in situ* localization of β-glucosidase activity, since they preserve both the enzymatic activity and the cell structures.

3.2. In situ localization of β-glucosidase activity

Longitudinal sections of the outer and inner mesocarp of olive fruit, after *in situ* β-glucosidase assay, are shown in Fig. 1c and d, respectively. The blue precipitate, resulting from X-Glc hydrolysis, shows the endogenous β-glucosidase localization.

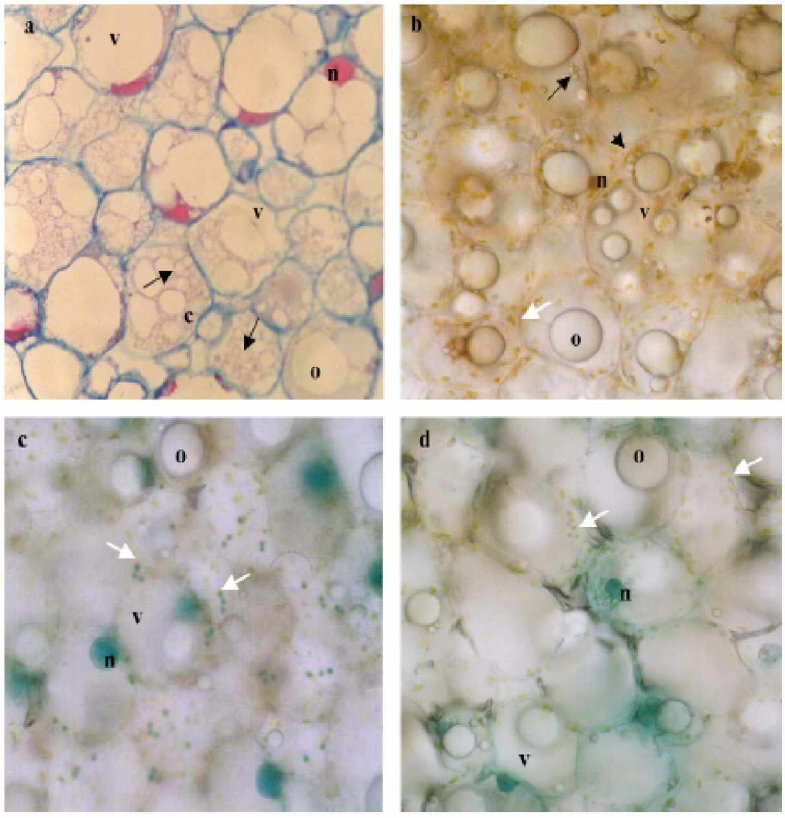

Fig. 1. Cyto-histological aspects of olive mesocarp from cv. *Carolea* green fruits (a) 3 μm resin embedded section of 2,5% gluteraldeyde fixed tissue and stained with 0.1 % Safranin O solution; (b) 80 μm fresh section obtained by the vibrotome; (c and d) sections after the *in situ* assay of β-glucosidase. The insoluble products of the X-Glc precipitate in the cells of the whole mesocarp. The sites of reaction were identified as blue spots inside the nuclei and chloroplasts in the mesocarp immediately below the epidermis (c), in the nuclei and in the oil droplets in the inner mesocarp (d). The black arrows indicate the oil droplets, the white arrows indicate the chloroplasts. v = vacuole, *n* = nucleus; c = cytoplasm; o = oil body. 150× (a–d).

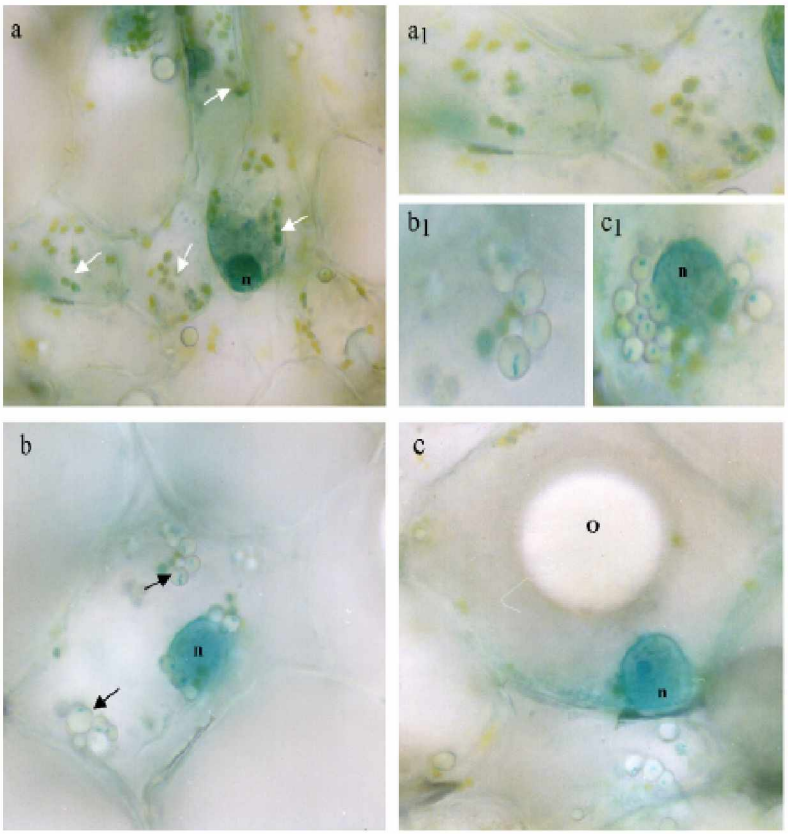

Fig. 2. Cellular localizations of β-glucosidase activity in the mesocarp of olive green fruit after the *in situ* assay. (a) Cells in the mesocarp below the epidermis showing a strong enzyme activity in the chloroplasts (white arrows; see image details in a_1), at level of the nucleus and in the cytoplasm enclave around it; (b and c) cells in the inner mesocarp which exhibit the β-glucosidase activity in the nucleus and in the core of each small oil droplets (black arrows; see image details in b_1 and c_1). *n* = nucleus, o = oil body. 400× (a–c and c_1); 800× (a_1 and b_1).

Clear differences are noted by comparing the outer and the inner mesocarp parenchyma cells. Indeed the outer mesocarp cells exhibit a strong reaction both in nuclei and chloroplasts (Fig. 1c), whereas in the inner mesocarp cells only reactive nuclei are present (Fig. 1d). Details of enzyme localization in the cells of outer and inner mesocarp are shown in Fig. 2. Reactive chloroplasts, within cytoplasm enclaves encircling the nucleus of outer mesocarp cell are shown in Fig. 2a (white arrows). In particular, enzyme localization is observable inside the chloroplasts in Fig. 2a1. Regarding the parenchyma of the inner mesocarp, the enzyme reaction is observable in the cells nuclei (Fig. 2b) and in the core of each small oil droplet in cytoplasmic clusters (Fig. 2b1 and c1).

3.3. Oleuropein-degradative-β-glucosidases localization

Longitudinal sections of outer and inner mesocarp of olive fruit after the *in situ* competitive assay are shown in Fig. 3. Exogenous oleuropein just at the concentration of 18 μM, three times less than synthetic substrate, strongly inhibits X-Glc hydrolysis in the chloroplasts of the outer mesocarp cells (Fig. 3a) and in the small cytoplasm oil droplets of the inner mesocarp cells (Fig. 3b). Notably both in outer and inner mesocarp cells the nuclei remain reactive (Fig. 3a and b). Altogether these results indicate that at least two different β-glucosidases, are highly specific to oleuropein, they remain stable in oil droplets and chloroplasts. On the contrary, the activity in nuclei seems to be due to a non-specific β-glucosidase which shows a higher specificity toward X-Glc in comparison to the oleuropein. The cell compartments of oleuropein and its degradative-β-glucosidases are reported in Fig. 3. In the outer mesocarp cell the oleuropein is stabilised in the vacuole of cells, the enzyme is located inside the chloroplasts. A β-glucosidase precursor protein is also in the cytoplasm, before going in the chloroplast organelle (Fig. 3c); alternatively, in the cell of the inner the oleuropein degradative-β-glucosidase is localized inside the core of oil droplets and the oleuropein is stay in the vacuole compartment (Fig. 3d).

3.4. β-Glucosidase activity distributions during the olive fruit ripening

The histo-anatomical pattern of olive fruit collected 50 days after anthesis is portrayed in Fig. 4a (see figure caption for

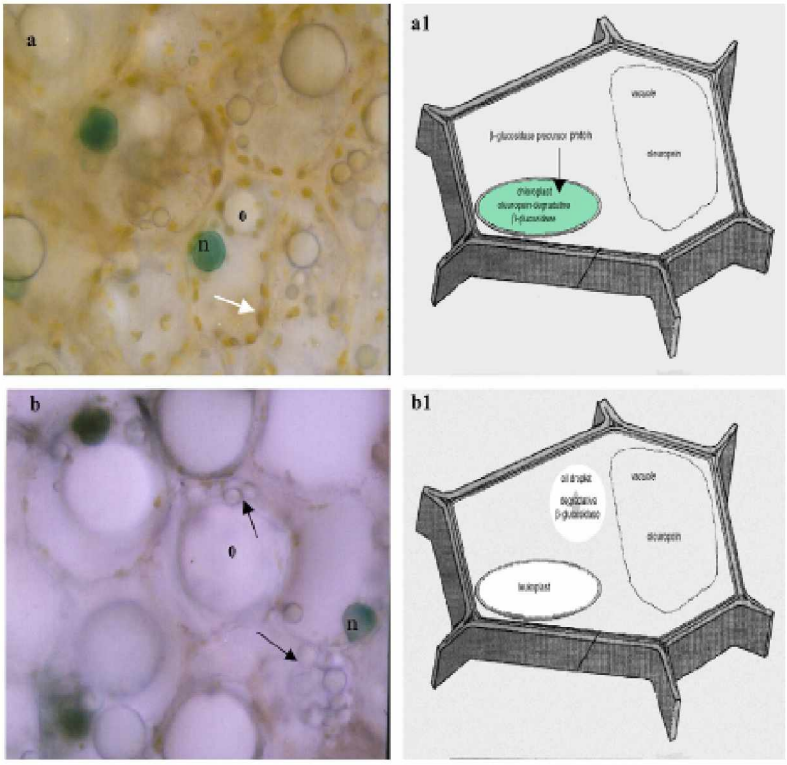

Fig. 3. Cell localizations of β-glucosidase activity subsequent to the competitive *in situ* assay by adding 18 μM exogenous oleuropein (a) in the cells of the outer mesocarp and (b) in the cells of inner mesocarp. The white arrows indicate chloroplasts, the black arrows indicate small oil droplets; models of oleuropein-degradative-β-glucosidase and oleuropein compartimentations in the cells the outer mesocarp tissues (c) and in the inner mesocarp tissues (d). 800× (a and b). Drawings showed the cell compartimentation of oleuropein and its degradative-β-glucosidase in the cell of the outer mesocarp (a1) and in the cell of the inner mesocarp (b1).

details). The β-glucosidase activity, localized at the nuclear level, is evident both in the cells of developing embryo (see arrow in Fig. 4b) and in the endocarp cells around the embryo sac (Fig. 4c). At this stage enzyme activity was not detectable in the outer mesocarp cells, whereas rare nuclei show positive reaction in the inner mesocarp cells (Fig. 5a and a1).

From 80 to 120 days after anthesis, the endocarp became completely lignified and in there was a substantial increase in the number of labelled cells. The β-glucosidase activity appears preferentially localized to the outer mesocarp cells (Fig. 5b and b1) where a high activity is observable at the level of chloroplast resolution. At the beginning of browning process (about 160 days after anthesis), β-glucosidase activity was not detectable in the purple cells of the outer mesocarp that show polymeric anthocyanin accumulation (Fig. 5c). Thus, in line with the cytological data, β-glucosidase activity level also decreased during fruit ripening (Table 1).

As can be seen in Fig. 5c1, in the mesocarp cells of mature fruit the nuclei are still positive in the assay, whereas the chloroplasts did not shown any reactivity. However in the inner mesocarp, β-glucosidase activity is still detectable in the core of small oil droplets (data not shown). Note that β-glucosidase activity is never present in fruits epidermis cells, whatever the stage of ripening considered.

4. Discussion

This research is the first report on *in situ* detection of β-glucosidase in the mesocarp of *O. europaea* fruit during both development and ripening. The mesocarp of olive fruit consists of parenchyma in which highly vacuolated cells increase their volume during both fruit ripening and oil production [23]. They show a structured nucleus and a cytoplasm containing chloroplasts, large oil bodies as well as clusters of small oil droplets. In unfixed tissues we localized β-glucosidase activity in chloroplasts, which is strictly related to the cell position in the fruit mesocarp. In fact, in the outer mesocarp cells, subjected to light, we observed numerous blue reactive chloroplasts whereas, in inner mesocarp cells enzyme activity in these organelles was not detected. The plastids of inner mesocarp cells lack chlorophyll pigments, suggesting a relationship between β-glucosidase activity and photosynthetic competence. The absence of β-glucosidase activity in the plastids in the cells of epidermis seems to confirm this hypothesis, since in this tissue chlorophyll biosynthesis and chloroplast differentiation are genetically repressed. Our results are in line with those obtained in *Polygonum tinctorium* where β-glucosidase was localized in the chloroplast stroma of leaf and its tissues specific expression has been strongly related to

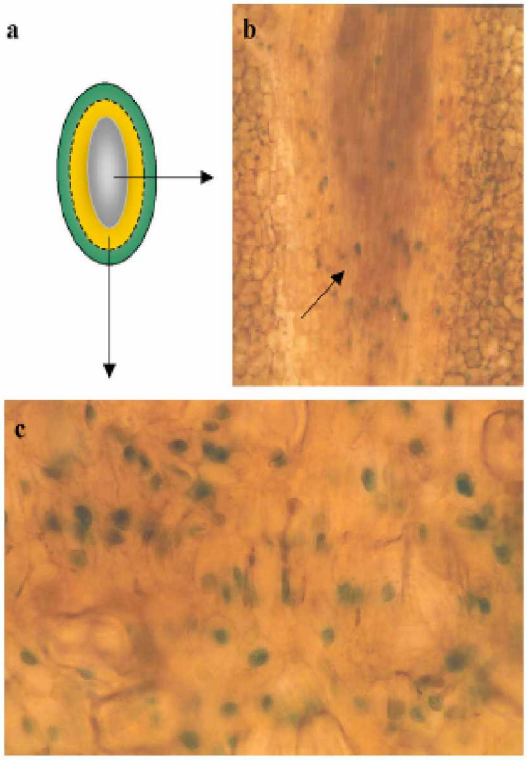

Fig. 4. Localizations of β-glucosidase in immature green fruit at 50 days after anthesis. (a) A fruit scheme having not wooded endocarp: the thin mesocarp (green), the developing endocarp (orange), the immature seed (grey); (b) embryo axis (em) and embryo teguments (emt) showing the β-glucosidase activity at level of nuclei; (c) developing endocarp tissue with strong β-glucosidase activity within the nuclei. 80× (b); 150× (c).

photosynthesis [24]. Since, oleuropein is contained in the vacuoles of olive mesocarp cells [25], the detection of β-glucosidase activity in mesocarp cell chloroplasts, clearly indicates that enzyme and substrate are kept in different cell compartments. However many cases have been described in plants where the enzyme and its substrates are localized in different cell compartments or tissues [4,7]. For example in *Sorghum bicolour*, the β-glucosidase is localized in the chloroplast stroma whereas the substrate is in the leaf epidermal cells [26]; in *Trifolium repens* the substrate limarin is localized in the vacuole, while the enzyme is present in the cell wall [27]. Also in *Ligustrum*, a genus of the Oleaceae family, an oleuropein-degradative-β-glucosidase activity has been detected in purified chloroplast fractions of leaf extracts [4]. The authors suggested that chloroplast β-glucosidase is related to leaf defence mechanism by storing and releasing a toxic chemical, which in Oleaceae is the oleuropein. The damage of cells and tissues by pests bring in contact β-glucosidase with oleuropein with the consequent release of toxic aglycones which either deterred herbivores or inhibited the entry, growth and spread of phytophathogens [4,7,28]. Our findings suggest that the oleuropein degradative-β-glucosidase is expressed in two different isoforms, able to segregate in two different cell compartments. They are also differentially expressed in the cells of the outer and inner mesocarp tissues. Since the GH 1 enzyme family is encoded by nuclear genes [7], the chloroplast isoform must moves from the cytoplasm, where it is synthesized, into the chloroplast organelle by the cleavage of the transit peptide sequences located in the N-terminus of the β-glucosidase protein precursor [29]. Conversely, the enzyme localized in the core of each oil droplet, very probably, belongs to fragments of endoplasmic reticulum trapped during the droplet formation. In fact, the X-Glc is routinely applied as a marker of glycosidase activity of endoplasmic reticulum in eukaryotes [30–32]. Thereafter the enzyme activity is lost when droplets fuse with each other into a large oil body. The β-glucosidase isoforms together with oleuropein, provided a constitutive pest control system. In fact, under normal physiological conditions, the localization of oleuropein degradative-β-glucosidases is in two cell compartments, separated from the oleuropein, a mechanism exists whereby oleuropein may be protected enzymatic breakdown thereby safeguarded the cells from toxic derivative products.

During fruit developing and ripening, histological distributions of enzyme activity undergoes significant changes. At 50 days after anthesis the β-glucosidase activity is very probably related to the growth and development of the embryo as well as to the lignifications process of the endocarp cells. In fact, the major lignin precursor, coniferyl alcohol, derived from the coniferin a 4-*O*-coniferyl glucoside after the hydrolysis by β-glucosidase [33]. In green mature fruits (120 days after anthesis) the greatest number of reactive cells were found distributed in the whole mesocarp. Finally, in black fruits (160 days after anthesis) numerous reactive cells were located only in the inner mesocarp, close by the woody endocarp, whereas the outer mesocarp cells were devoted to the synthesis of polymeric anthocyanins that are physiologically accumulated in black mature olive [10,34]. Since β-glucosidase activity could not be found in chloroplasts and oil droplets, it seems reasonable to suppose that at this stage of fruit ripening, the oleuropein-degradative-β-glucosidases was not expressed in tissues and the residual activity detected might be due to non specific β-glucosidases (Table 1). As general rule our results agree with the previous observations on the enzymatic activity changes during fruit ripening [18]. The authors identified a β-glucosidase whose activity trend during ripening appeared to be linked to oleuropein degradation. In fact, the levels of β-glucosidase activity was high in green fruit, when the oleuropein amount was highest, and low in black fruit when the oleuropein concentration declined. The enzyme *in situ* assay indicated that β-glucosidase activity changes during ripening are due to the different competence of single mesocarp cells to synthesize the two oleuropein-degradative-β-glucosidase isoforms, whose histological distributions appear strongly related to the fruit ripening stage.

In our view, all these results clearly point out that in green fruits the debittering, due to the hydrolysis of oleuropein, does not occurs because enzyme and substrate do not come in contact because they are in the separate compartments of cells, despite the respective concentrations in tissues are very high. On the contrary, in the green-brown fruits the debittering is

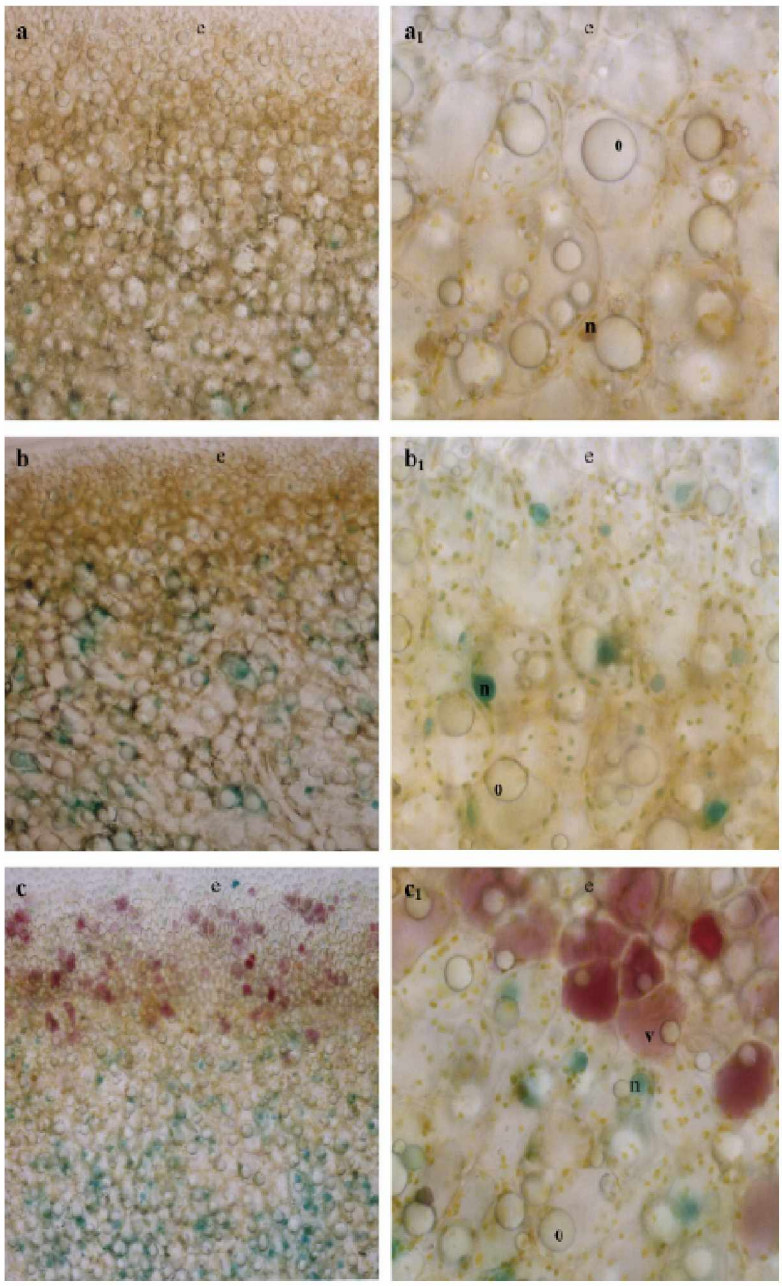

Fig. 5. Histochemical localizations of β-glucosidase in the mesocarp at different stages of fruit ripening. (a and a_1) green immature fruit collected at 50 days after anthesis; (b and b_1) green mature fruit collected at 120 days after anthesis; (c and c_1) browning fruit collected at 160 days after anthesis. e = epidermis; *n* = nucleus, o = oil body; v = vacuole. 80× (a–c); 150× (a_1–c_1).

allowed by the damage of cell structures, due to the senescence of tissues, that brings in contact β-glucosidase and oleuropein, despite the concentration of enzyme is very low. The hydrolysis of oleuropein continues during the black maturation up to when the substrate is completely hydrolysed and the enzyme is not express.

As a note, in developing embryo and endocarp, as well in all mesocarp tissues, a clear nuclear reactivity was due to glucohydrolase activity at the level of the endoplasmic reticulum connected to the nuclear membrane [30,32]. Thus this result seem to confirm the specificity of *in situ* X-Glc hydrolysis as a marker of endoplasmic reticulum also in the cells of olive fruit mesocarp.

In conclusion, our results provide evidence that the commitment of single mesocarp cells to synthesize β-glucosidase is able to modulate either fruit ripening or the availability of oleuropein

aglycones in the defence response. The localization of the oleuropein degradative-β-glucosidases in the chloroplast and in the oil droplets of mesocarp of green mature fruit, could provide useful information to achieve safe and optimized procedures for the enzyme extraction and purification. Purified olive β-glucosidase, with high specificity for oleuropein, could be applied successfully in an alternative industrial process devoted to the debittering of table olives and olive oil.

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References

[1] C. Soler-Rivas, J.C. Espin, H.J. Wichers, Oleuropein and related compound, J. Sci. Food Agric. 80 (2000) 1013–1023.
[2] M.J. Amiot, A. Fleuriet, J.J. Macheix, Accumulation of oleuropein derivatives during olive maturation, Phytochemistry 28 (1989) 67–69.
[3] A.D. Bianco, A. Piperno, G. Romeo, N. Uccella, NNMR experiments of oleuropein biomimetic hydrolysis, J. Agric. Food Chem. 47 (1999) 3665–3668.
[4] K. Konno, C. Hirayama, H. Yasui, M. Nakamura, Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 9159–9164.
[5] G. Bisignano, M.G. Laganà, D. Trombetta, S. Arena, A. Nostro, N. Uccella, G. Mazzanti, A. Saija, In vitro antibacterial activity of some aliphatic aldehydes from *Olea europaea* L., FEMS Microbiol. Lett. 198 (2001) 9–13.
[6] A. Kubo, C.S. Lunde, I. Kubo, A multichemical defense mechanism of bitter olive *Olea europaea* (Oleaceae). Is oleuropein a phytoalexin precursor? J. Chem. Ecol. 11 (1995) 251–263.
[7] A. Esen, β-Glucosidases, in: A. Esen (Ed.), β-Glucosidases: Biochemistry and Molecular Biology, American Chemical Society, Washington, 1993, pp. 1–14.
[8] R. Briante, M. Patumi, S. Limongelli, F. Febbraio, C. Vaccaro, A. Di Salle, F. La Cara, R. Nucci, Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L., Plant Sci. 162 (2002) 791–798.
[9] J.R. Morello, M.P. Romero, M.J. Motiva, Effect of maturation process of the olive fruit on the phenolic fraction of drupes and oils from Arbequina, Farga and Morrut cultivars, J. Agric. Food Chem. 52 (2004) 6002–6009.
[10] M. Brenes Balbuena, P. Garcia, A. Garrido Fernandez, Phenolic compounds related to the black color formed during the processing of ripe olives, J. Agric. Food Chem. 40 (1992) 1192–1196.
[11] D. Ryan, K. Robards, S. Lavee, Changes in phenolic content of olive during maturation, Int. J. Food Sci. Technol. 34 (1999) 265–274.
[12] J.R. Morello, M.J. Moltiva, M.J. Tovar, M.P. Romero, Changes in commercial olive oil (cv Arbequina) during storage with special emphasis on phenolic fraction, Food Chem. 85 (2004) 357–364.
[13] F. Angerosa, N. D'Alessandro, F. Cornara, G. Mellerio, Characterization of phenolic and secoiridoid aglycones present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry, J. Chromatogr. A 736 (1996) 195–203.
[14] G. Cianfardini, V. Marsilio, B. Lanza, N. Pozzi, Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation, Appl. Environ. Microbiol. 60 (1994) 4142–4147.
[15] V. Marsilio, B. Lanza, N. Pozzi, Progress in table olive debittering: degradation in vitro of oleuropein and its derivatives by *Lactobacillus plantarum*, J. Am. Oil Chem. Soc. 73 (1996) 593–597.
[16] C.M. Papoff, A. Fleuriet, M.J. Amiot, J.J. Macheix, Influence of some biotechnhological combinations on the sensory quality of Manna green table olives, Ind. Alim. 35 (1996) 375–381.
[17] R. Capasso, A. Evidente, C. Visca, L. Gianfreda, M. Maremonti, G. Greco, Production of glucose and bioactive aglycone by chemical and enzymatic hydrolysis of purified oleuropein from *Olea europaea*, Appl. Biochem. Biotechnol. 61 (1996) 365–377.
[18] R. Briante, F. La Cara, F. Febbraio, R. Barone, G. Piccialli, R. Caroll, P. Mainolfi, L. De Napoli, M. Patumi, G. Fontanazza, R. Nucci, Hydrolysis of oleuropein by recombinant β-glycosidase from hyperthermophilic archeon *Sulfolobus solfataricus* immobilised on chitosan matrix, J. Biotechnol. 77 (2000) 275–286.
[19] J.C. Gebler, D.E. Trimbur, R.A.J. Warren, R. Aebersold, M. Namchuk, S.G. Withers, Substrate-induced inactivation of a crippled β-glucosidase mutant: identification of the labelled amino acid and mutagenic analysis of its role, Biochemistry 34 (1995) 14547–14553.
[20] R. Gossrau, Tetrazolium methods for the histochemical investigation of hydrolases, Histochemistry 58 (1978) 203–218.
[21] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901–3907.
[22] Z. Zheng, K. Shetty, Solid-state bioconversion of phenolics from cranberry pomace and role of lentinus edodes (-glucosidase, J. Agric. Food Chem. 48 (2000) 895–900.
[23] B. Rangel, K.A. Platt, W.W. Thomsom, Ultrastructural aspects of the cytoplasmic origin and accumulation of oil in olive fruit (*Olea europaea*), Physiol. Plant. 101 (1997) 109–114.
[24] Y. Minami, H. Takao, T. Kanafuji, K. Miura, M. Kondo, I. Hara-Nishimura, M. Nishimura, H. Matsubara, β-Glucosidase in the Indigo plant: intracellular localization and tissue specific expression in leaves, Plant Cell Physiol. 38 (1997) 1069–1074.
[25] M.B. Bitonti, A. Chiappetta, A.M. Innocenti, I. Muzzalupo, N. Uccella, Funzionalità e distribuzione dei biofenoli nella drupa di *Olea europaea* L., OLIVO OLIO 1/2 (1999) 20–29.
[26] S.S. Thayer, E.E. Conn, Subcellular localization of dhurrin β-glucosidase and hydroxynitrile lyase in the mesophyll cells of sorgum leaf blades, Plant Physiol. 67 (1981) 617–622.
[27] P. Kakes, Function and variation of the β-glucosidase linamarase in natural population of *Trifolium repens*, in: A. Esen (Ed.), β-Glucosidases: Biochemistry and Molecular Biology, American Chemical Society, Washington, 1993, pp. 1–14.
[28] S. Mazzuca, N. Uccella, β-glucosidase releasing of phytoalexin derivatives from secobiophenols as defence mechanism against pathogenic elicitors in olive drupes, Acta Hort. 586 (2002) 529–531.
[29] M. Mieszczak, U. Klahre, J.H. Levy, G.J. Goodall, W. Filipowicz, Multiple plant RNA binding proteins identified by PCR: expression of cDNAs encoding RNA binding proteins targeted to chloroplasts in Nicotiana plumbaginifolia, Mol. Gen. Genet. 234 (1992) 390–400.
[30] J.P. Horwith, A histochemical substrate for β-glucosidase, J. Med. Chem. 7 (1964) 574.
[31] A.O. Banjo, Interactions between the rough endoplasmic reticulum and the nuclear membrane during increased proliferation of the endoplasmic reticulum, Afr. J. Med. Med. Sci. 12 (1983) 145–154.
[32] E.I. Philipp, W.W. Franke, T.W. Keenan, J. Stadler, E.D. Jarasch, Characterization of nuclear membranes and endoplasmic reticulum isolated from plant tissue, J. Cell Biol. 68 (1976) 11–29.
[33] D.P. Dharmawardhana, B.E. Ellis, J.E. Carlson, A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin, Plant Physiol. 107 (1995) 331–339.
[34] G. Vlahov, Gli antociani polimeri delle olive nere. Loro determinazione in funzione del processo di maturazione, Ind. Aliment. 29 (1990) 11–14.

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Dear Dr Mazzuca
Thank you for your contribution to our journal *Agricultural Sciences in China*

Your manuscript:≤ Oleuropein-specific-β-glucosidase activity marks the early response of olive fruits (*Olea europaea*) to mimed insect attack) (au-2007-01330), has been evaluated by two reviewers of the journal with the following suggestions for your amendments.

Your manuscript after revised followed those suggestions will be accepted quickly. And also, you can point out your different ideas about the suggestions.

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The effects of oleuropein-specific-β-glucosidase on early defense reaction to mining insect puncturing damage with a micro-needle on olive fruits of two different susceptible varieties were studied. The research topic focuses on olive fruit production practice in local area to investigate the biochemical changes on fruit after insect attack mainly using the histochemistry methods.
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Reviewers 2:

The paper has presented an in situ β-glucosidase activity detection in olive fruits after mechanically punctured by needle to mime the fly infestation in order to elucidate the early defense response of olive fruit to insect injury. It is meaningful and the paper is suggested to adopt to publish, but there still remain some points to discuss:

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Oleuropein-specific-β-glucosidase activity marks the early response of olive fruits (*Olea europaea*) to mimed insect attack

S. Mazzuca[1]*, A. Spadafora[1], F.F. Chiappetta [1], A. Parise[2], E. Perri[2] and A.M. Innocenti[1].

Authors address:

[1] Laboratorio di Citofisiologia Vegetale, Dipartimento di Ecologia, Cubo 6b Ponte Bucci, Università della Calabria, 87030 Rende (CS), Italy

[2] C.R.A. Centro di Ricerca per l'Olivicoltura e l'Industria Olearia, Contrada Li Rocchi 87030 Rende, Italy

Key words: β-glucosidase (EC 3.2.1.21), oleuropein, *Olea europaea*, *Bactrocera oleae* infestation, cultivar resistance

* *Author to whom correspondence should be addressed*

e-mail: s.mazzuca@unical.it

Phone number 39 0984 492967

Fax number 39 0984 492986

Abstract. Olive fruits might be seriously deteriorated by pre- and post-harvested damage, due to the attack of insects, such as *Bactrocera oleae* which strongly alters both table olives and olive oil quality. Defence response in olive fruits injured both by pathogens and by mechanical damages, the enzyme β-glucosidase specifically hydrolyses the oleuropein, producing highly reactive molecules. *In situ* detection of β-glucosidase activity in olive fruit tissues following the injury, miming the *Bactrocera oleae* punctures is reported. The assay has been performed in two cultivars showing different susceptibilities to the fly infestation. In both cultivars, the histochemical assay for β-glucosidase showed that, within 20 min after the injury, a strong enzyme activity can be observed in the damaged tissues. Thereafter a progressive enzyme inactivation occurred starting from tissues around the boundary of the injury and the enzyme activity disappeared during 60 min after injuring. The loss of activity decreased and stopped after 3 h, whereas the active cells limit reached the distance of 300 $\pm$ 50 μm from the edge of the injury. Biochemical analyses showed that in injured fruit extracts the β-glucosidase activity rapidly increased within 20 min from the injury, thereafter it decreased, reaching values comparable wit those in sound fruits. Following *B. oleae* damage, the oleuropein contents did not change significantly in the high susceptible cultivar, while it rapidly decreased in the cultivar showing a low susceptibility. Our results strongly suggest that the olive fruit susceptibility toward the fly infestation could be related to the ability of the oleuropein-degradative-β-glucosidase to produce the high reactive molecules in the damaged tissues. As a consequence of injury a strong peroxidase activity was thereafter detected. This interesting feature suggest that also this enzyme could play a key role in the following defence response.

INTRODUCTION

Olive (*Olea europaea*) is one of the established and largest crop in term of foodstuff production in Mediterranean countries, such as Spain, Italy, Greece. Starting from the last century, the intensive olive crop has been extended in other Mediterranean countries (e.g. Turkey, Tunisie, Maroc), and exported in several climate lands (e.g. California, Australia), thus its further expansion worldwide seems to be the tendency in the next future. The reason of this achievement moved from the recognition of positive effects on the human health of olive food, especially virgin olive oil. Many studies have shown that diet supplemented with olive oil daily, reduced the frequency of cardiovascular diseases, offered benefits in terms of colon cancer prevention and showed anti-inflammatory activities. However all these effects are the result of the higher levels of olive antioxidant compounds, particularly phenols, in the blood (Keys *et al*., 1986; Willett, 1990; WHO Report, 1990). Unfortunately many problems affect the olive grove with consequences on the product quality; namely the olive fruits are often seriously deteriorated by pre- and post-harvest damage, due to the attack of phytophagous insect, such as the larvae of the olive fly *Bactrocera oleae.* As it is well known, while adults of olive fly feed on nectar, honey dew, and other opportunistic sources of liquid or semi-liquid food that they found on leaves (Rice, 2003), the developing larvae, from eggs oviposed in fruit tissue by the *Bactrocera oleae* females, eat fruit tissues and they makes the deep tunnels trough the pulp, reaching the stone (Varela and Vossen, 2003). This parasite infestation, causing large damages in fruits, it alters the quality parameters of the olive oil inducing a decreasing of phenol fraction and of antioxidant activity (Iannotta *et al*., 1999a). Thus the integrate pest management for *B. oleae* in olive culture assumed increasing importance and economic relevance. Some biochemical methods (mass trapping, sexual confusion), together with earlier harvesting, were able to limit the parasite infestation and offered an alternative way to the use of chemicals (Iannotta *et al*., 1994). However, olive cultivars shown a different

degree of susceptibility to infestation which is ranging from high susceptibility (more than 10% of infested fruits), moderate susceptibility (3% of infested fruits) and resistance (less than 0.25 % of infested fruits), that are not influenced by culture conditions, thus suggesting that resistance or susceptibility have a source genetically determined (Iannotta *et al*., 1999b).

In spite of the extensive researches on pest management, very few information is available on the pattern of constitutive defence in *Olea europaea*. The defence molecules in olive are the phenols synthesized and accumulated in fruit tissues during growth and ripening (Amiot *et al*., 1989). The major component among these phenols is the phenolic secoiridoid β-glucoside oleuropein, the bitter molecule of olive fruit (Soles-Rivas 2000). This compound is responsible for many features, such as antioxidant and antimicrobial activities (Bisignano *et al*., 2001), and has been evoked as defence molecules mechanism against herbivores and insect attacks (Baidez *et al*., 2007; Kubo *et al*., 1993).

In particular, when olive tissues are injured by phatogens or by mechanical damage, β-glucosidase belongings to the glucohydrolase enzyme family 1 (Esen 1993), specifically hydrolyses oleuropein producing highly reactive molecules (Bianco *et al*., 1999). *O. europaea* fruits contain large amounts of β-glucosidase which specifically hydrolyses oleuropein (Briante *et al*., 2002; Konno *et al*., 1999). Previously, by *in situ* assay, we localized in the cell of green olive fruit mesocarp two oleuropein-degradative-β-glucosidase isoforms in the chloroplasts and in the oil droplets, respectively (Mazzuca *et al*., 2006). Since, oleuropein is stored in the vacuoles of mesocarp cells (Bitonti *et al*., 1999), the enzymes and its substrate are kept in different cell compartments. This feature provides a constitutive pest control system as in the sound fruits oleuropein may be protected from enzymatic breakdown, thus safeguarding cells from toxic derivative products. In contrast, the injury by pest destroyed the tissues and of consequence β-glucosidase, contacting oleuropein, released toxic molecules which either deterred or inhibited the entry, growth and spread of pest (Mazzuca

and Uccella, 2002; Konno *et al*., 1999; Kubo *et al*., 1993). Thus, the β-glucosidase play a key role in defence mechanism by storing and releasing the toxic chemicals from oleuropein.

However, reaction of cells and tissues in olive fruits following pest attack are at this time unknown. Aim of the present work, by using the *in situ* assay with the synthetic substrate X-Glc, specific for the β-glucosidase (Jefferson *et al*., 1987), is to define the enzyme behaviour and the oleuropein contents in olive fruit tissues submitted to injuries, miming the *Bactrocera oleae* punctures. We have chosen the mimed attack instead the fly punctures, because it allows to control strictly the timing of enzyme activity in tissues and to increase the number of synchronous punctures necessary to produce great effects that can be detected by biochemical analyses. Besides, to our knowledge, the ovipositor does not secret any molecule, then the response of tissue to the puncture by the microneedle is consistent with that made by the female fly. The enzyme and substrate behaviours will be studied in two cultivars showing different susceptibilities to the fly infestation in order to establish the physiological significance in the defence response and in the different susceptibility to pest, which are genetically determined in both selected cultivars. Finally peroxidase activity will also be determined in injured fruit tissues since this antioxidant enzyme is considered as important factors in fruit defence mechanisms (Valentines *et al*., 2005; Keck *et al*., 2002; Saniewski *et al*., 1992).

MATERIALS AND METHODS

Plant materials and fruit injuring

Among the traditional crops growing in the of South of Italy (Calabria) the *cv* Carolea, characterized by a high susceptibility to *Bactrocera oleae* infestation (10.25% infestation, 12.87 % sterile punctures) and *cv* Cassanese showing a low

susceptibility (3.62% infestation, 13.75 sterile punctures) against the same pathogen, were selected. For both cultivars sound green fruits were harvested at the end of October, when the rising of fly infestation generally occurs, from five different groves. For in situ assays each olive has been fixed on the support of a vibratome (Leica, VT1000E, Germany) and fruit mesocarp was injured up to 1-2 mm in depth by using a micro-needle (200 μm diameter). The depth of the injury has been checked by means of the red dyed sign made on the micro-needle and operating under a 10X magnification lens. Then, fruits were sectioned and processed for *in situ* assay of β-glucosidase and peroxidase activities immediately and at 10, 20, 60, 120 and 180 min subsequent to the injury. For biochemical analyses, each fruit was injured approximately 30 times with a group of 10 micro-needles as above described, and frozen in liquid nitrogen at the same time intervals than in situ assay. Frozen sound fruits were considered as the control.

In situ assay of β-glucosidase activity

Sound and injured fruits (n=5 for each time after injuring), were freshly sectioned by vibratome, thus the destruction of enzyme activities, and other deleterious effect due to fixing and embedding procedures are thus avoided. Besides, during the sectioning, fruits were fluxed with N_2 and the temperature was maintained at 4 °C, in order to prevent tissue oxidation and possible enzymes degradation. Starting from epidermis the serial sections each of 80 μm in thickness were obtained. Sections were immediately placed in the minivials with 1 ml of detection buffer, adapted for glucosidase (E.C. 3.2.1.21) from Jefferson (1987), containing 60 μM X-Glc (5-brome-4-chloro-3-indolyl-β-Dglucopyranosyde, Sigma, St Louis), 50 mM phosphate buffer pH 6.5, 1 mM potassium ferricianide, 1mM potassium ferrocianide, 10 mM EDTA pH 8.0. The synthetic substrate X-Glc, after enzymatic hydrolysis by endogenous β-glucosidase, develops, after few minutes, an insoluble blue product that precipitates on the site of reaction

thus localizing the enzyme in the cells and tissues; the sections treated with the detection buffer without the X-Glc were considered as the control of the assay. Reaction reaches the stady state after 1 h of incubation in a wet chamber at 37 °C, and it was stopped by cold phosphate buffer and the sections were mounted with glycerol on slides for optical microscopy and digitalized by Leitz Dialux EB microscope equipped with a CCD camera. Image analysis on five sections for each fruit (n=25 for each time) was performed by QW^{TM} in Image System Software (Leica). The time-dependent behaviour of enzyme activity in the damage tissues was evaluated by measuring the distances from the edge of injury to the onset of dyed cells at each time interval.

In situ assay of peroxidase activity

As a substrate for peroxidase (POX, EC.1.11.1.7), 3,3′-diaminobenzidine tetrahydrochloride (DAB) was widely used in biology (Archibald, 1992; Benayoun *et al*., 1981). It can form an insoluble brown polymeric non-droplet precipitate in the sites of reactions. Tissue sections from sound and injured fruits, obtained as above described, were incubated in 1 mg/ml DAB (SIGMA FAST) at 25 °C in the dark for 1-2 h (modified from Alvarez *et al*., 1998). After that, sections were mounted on slides for visualization of brown precipitates by light microscopy. Simultaneous localization of β-glucosidase/POX activities was obtained by processing the DAB-treated sections with the *in situ* assay for β-glucosidase.

β-glucosidase activity in fruit extracts

The β-glucosidase activity in injured and sound fruit extract was performed according to Briante *et al*., (2002). Typically, 1 g frozen pulp of fruits (n = 5 for each time interval) was ground in liquid nitrogen using a mortar and pestle. Tissue powder was suspended in 12.5 ml 0.1 M borate buffer, pH 9.0, 6% (w/v)

PVP (poly-vinil pirrolidone), 1% (w/v) β-mercaptoethanol, 1.0 mM PMSF (phenylmethylsulfonyl fluoride). The suspension was shaken gently for 1 h at 4 °C and centrifuged in a minifugue at 26.000 g for 1 h. The upper oil phase was carefully removed and the aqueous phase, representing the active enzyme enriched phase, was filtered on paper and used in the enzyme assay. The β-glucosidase activity against *p*NPGlc (*p*-D-nitrophenyl-β-D-glucopyranoside) was evaluated at 37°C, by measuring the increase in absorbance at 405 nm of the reaction medium composed of 200 mM Na-phosphate buffer adjusted to pH 4.6. The linear coefficient used to calculate the concentration of the reaction product was measured by a 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 g of fresh weight (mmol/min g). Since sound green fruits show very high enzyme activity that remains constant for many days in the lab conditions, we have considered as control the mean enzyme levels found in the sound fruit samples, prior to performe the biomimed assay. The values are reported as the dotted lines for both cultivars in the Fig 4.

Determination of oleuropein contents in injured fruits

10 g of olives pulp (pericarp and mesocarp) were homogenized in methanol (3x20 ml). The methanolic fraction was washed with n-exane (3x5ml) and evaporated under reduced pressure. The residue was dissolved in 2 ml of methanol and 10 µl of this solution were analyzed using an Agilent 1100 (Waldbronn, Germany) HPLC fitted with a C-18, reverse-phase (5 µm) column (25 cm l. x 4 mm i.d.) equipped with an Agilent UV photodiode spectrophotometer. Oleuropein was detected at 280 nm at 25°C. The flow rate was 1ml/min; the mobile phase used was 1% formic acid in water (A) vs methanol (B) for a total running time of 45 min. Quantisation of oleuropein was

performed by external calibration curve by using methanolic solutions of commercial oleuropein as external standard.

RESULTS

Longitudinal sections of olive fruit mesocarp sectioned by a vibratome after the *in situ* β-glucosidase assay are shown in Fig. 1. Although the cytological details are not excellently appreciable, the *in situ* assay is a good compromise between cell integrity and enzyme activity maintenance in the unfixed tissues. In the parenchyma cells well structured nuclei were visible together with a highly vacuolated cytoplasm, containing large oil bodies and clusters of small oil droplets (Fig. 1a). The blue precipitate, resulting from X-Glc hydrolysis, showed the endogenous β-glucosidase localization; more than 80 % of cells were positive to the assay; a strong activity in all nuclei has been observed; the lack of activity in some cells, that appear in Fig. 1a, it derives from the position of the nuclei in the non focused plains, thus they are not visible. These effects are more appreciable in the tissue details in the Fig. 1b in which all cells, immediately below the epidermis, exhibited a strong reaction also in chloroplasts, while the nuclei have been not always detectable. (Fig. 1b, see arrows). The cells of epidermis did not react all the time. In the Fig. 2 the microphotographs of injured fruit sections at different times are reported. The punctures have destroyed the tissues and injuries appeared as the hole in the tissue transversal sections. Immediately after injury, as shown in Fig. 2a, most cells on the boundary (80 % average) showed blue spots, suggesting that an efficient cleavage of the synthetic substrate occurred (Fig. 2b). After 10 min from injury, X-Glc hydrolysis occurred only in cells located 1 ± 0.10 mm from hole edge (Fig. 2 c,d). At this time the rate of loss of X-Glc hydrolysis was 50 μm min^{-1}, whereas 1 hours later the rate was reduced to 28 μm min^{-1}. 3 hours after the rate of reduction reached

10 μm min^{-1} and the detection of X-Glc hydrolysis was deplaced at 2.05 ± 0.65 mm from the edge of injury (Fig. 2e, f). At cellular level, just after injury, the chloroplasts appeared colourless, suggesting that the oleuropein-degradative-β-glucosidase, inside them, did not hydrolyse the synthetic substrate and the blue precipitates were observed only in the nuclei and diffused in the cytoplasm of damaged cells (Fig. 3b, see arrows). The levels of β-glucosidase activity in fruit extracts, expressed as enzyme units/fresh weight, at the different times from the injury are reported in Fig. 4. Extracts from the high susceptible *cv* Carolea showed an higher enzyme activity (1.2 U/g fresh weight) than those found in the *cv* Cassanese extracts (0.9 U/g fresh weight). However as consequence of injury in both cultivars the enzyme activity showed a similar kinetics. In fact, within 20 minutes from the injury the β-glucosidase activity rapidly increased of 0.4 and 0.5 enzyme units in the extracts of *cvs* Carolea and Cassanese respectively, thereafter within 3 hours it decreased, reaching the comparable values than those in sound fruits. As general rule, the oleuropein contents in the sound fruit extracts of the high susceptible cv Carolea were higher (20-26 μg/g fresh weight) with respect to the contents detected in the low susceptible cultivar (14-16 μg/g fresh weight). In the damaged tissue extracts immediately after the injury, the oleuropein content was still higher in the *cv* Carolea with respect to those in *cv* Cassanese (Fig. 5). During the first 10 min after the injury, the oleuropein levels decreased very rapidly in the low susceptible cultivar, whereas they did not change appreciably in the *cv* Carolea. Therefore in the *cv* Cassanese, the oleuropein contents decreased progressively lasting from injury and reaching a minimum values (2.8-0.1 μg/g fresh weight) after 120 minutes. On the contrary, 60 min from the injury, in the extracts of the high susceptible cultivar the oleuropein contents was higher (13.2-0.4 μg/g fresh weight) than the one found after 20 min (8.2-0.2 μg/g fresh weight), and this values remained constant in the extracts up to 3 hours after the injury. In the Fig. 6 the microphotographs of damaged tissue sections following the combined β-glucosidase/POX *in situ* assay

are shown. The insoluble brown precipitates, visible in the figures, revealed the presence of a strong POX reactions in tissue in the boundary injury just after 10 minutes (Fig. 6a). The strong POX reactions occurred in damaged tissues, when β-glucosidase activity start to decrease (Fig. 6b, c). No differences in POX behaviours were found between the two cultivars analysed.

DISCUSSION

We have investigated the damage provide by the microneedles in olive fruit pulp, miming the injury made by the ovipositor, positioned in the terminal part of abdomen of the *Bactrocera oleae* females that depose the eggs in the fruit tissues. To our knowledge, the ovipositor does not secret any molecule, rather cause the spillage of the cellular juice in which the molecules, that we are investigating, are contained. Then, the response to mechanical injury has been suitable to understand what happens in tissues during early phase of fly infestation. Following the fly punctures the active molecules, contained in small droplets inside the cell tissue localized all around the oviposition hole, prevent other females from ovipositing on the same fruit by acting as the bioactive phytoalexins (Scalzo *et al*., 1994). Olive groves, however, showed different susceptibility toward olive fly infestation; in fact, under the same crop conditions, some olive cultivars showed lower levels of infestation than other ones, despite the equal number of fly punctures (Iannotta *et al*., 1999b; Iannotta *et al*., 2007). This indicated that fruit susceptibility or resistance to infestation are genetically determined and that the resistance source must be produced in tissues consequently to the injury. The reported results in this research indicated that the olive susceptibility toward the fly infestation is strictly related to the ability of an endogenous β-glucosidase to hydrolyzed the oleuropein in the damaged tissues, thus producing the defence reactive molecules. In fact, following the mimed fly attack, the *cv* Carolea, highly susceptible to fly infestation, showed a low

efficiency in the oleuropein cleavage. On the contrary in the low susceptible *cv* Cassanese, the oleuropein hydrolysis occurred very fast causing a significant decrease in the amount of this phenols in fruit extracts, just few minutes after the injury. Interestingly, at the same time the β-glucosidase activity increased both in Carolea and in Cassanese fruit extracts, but with an opposite effect on oleuropein cleavage. Very probably, in damaged tissues of the low susceptible fruit, the oleuropein derivative chemicals might be more concentrated than in high susceptible fruit. However, it is well known that egg dispersion strategy of olive fruit fly strictly dependent on fruit chemicals. In particular, both oleuropein and its hydrolytic derivatives exhibit a strong chemotactile repulsive effect, mainly to (*E*)-2-hexenal. Other compounds, such as β-3,4-dihydroxyphenylethanol and other oleuropein derivatives, which exert a strong chemotactile repulsion, have been identified either in fresh olive juice or in olive mill waste water (Scalzo *et al*., 1994, Mazzuca and Uccella, 2002). The mimed insect attack assay allowed to analyse the timing of β-glucosidase activity inside the tissue and cells at the edge of the injury following the cytological localization of β-glucosidase activity by the hydrolysis of X-Glc, the chromogenic synthetic substrate developing an insoluble blue precipitate in the cells. Recent data showed that in the outer olive fruit mesocarp, immediately below the epidermis, a strong reaction is present within nucleus and chloroplasts. The enzymes kept in chloroplasts has been recognized as an oleuropein-degradative-β-glucosidase by the X-Glc in the *in situ* competitive assay; whereas, in nuclei, has been suggested the activity of a β-glucosidase non-specific for oleuropein (Mazzuca *et al*., 2006). Consequently, the reported results obtained by enzyme *in situ* assay, strongly indicate that, in the early defence response against the fly injury, an oleuropein-degradative-β-glucosidase has been involved. In fact, immediately after the puncture in the cells localized around the injury, the nuclei showed a dense blue dye, which was absent in chloroplasts. This pattern could be explained assuming that the β-glucosidase highly specific for oleuropein, was not able in the damaged tissues to

hydrolyse the synthetic substrate because it was engaged in the hydrolysis of its natural substrate which was made available by tissues breakdown. However there is the need of further analyses to identify, in *cv* Carolea injured tissues, the factors affecting the oleuropein enzymatic hydrolysis from which the susceptibility to infestation could be strongly dependent. Another interesting aspect, related to defence mechanism in olive fruit, was the tissue browning observed *in situ* by the activity of the browning-related enzyme POX with its specific substrate. Namely within three hours after injury a clear increasing of the browning potential was found in the damaged tissues. It is particularly worth of note the relationship between β-glucosidase and POX activities which appear to be complementary. In fact, following the early response of olive tissues to mimed insect injury, we observed that the β-glucosidase activity decreased as soon as a progressive strong POX reactions appeared in the damaged tissues. These findings is in line with the literature data, namely it is well known the POX is considered as important factors in fruit defence mechanisms and browning (Valentines *et al*., 2005; Keck *et al*., 2002; Saniewski *et al*., 1992). Thus the great increase of POX activities, oxidizing endogenous phenolic substances to quinones that thereafter polymerized to polyphenols, might be responsible for the intensive staining of damaged tissues. Furthermore, POX activities are also related to the production of cellular substances (e.g. flavanoids, lignin) involved in the early defence against fly infestation. However in this case, no direct link related to the different susceptibility of cultivars to fly infestation has been found. All together our results provide evidence that in olive fruit the early defence against fly injury is mediated by the activity of β-glucosidase and POX which act synergically producing bioactive molecules. Their role, being played during different sequence time, underline that both of them can be considered as important factors in olive fruit reaction to damage. However only the ability of the oleuropein-degradative-β-glucosidase to cleave its substrate can be considered as a marker of olive cultivar with different degree of susceptibility to fly infestation.

CONCLUSIONS

An interesting implication from our research is that the different susceptibility to the fly attack depends on the release of bioactive molecules from the β-glucosidase/oleuropein reaction in the damaged tissues, with the purpose to prevent the puncture and the oviposition. This does not depend on the levels of enzyme and substrate in tissues but it depend from the ability of enzyme to cleave the oleuropein and/or from the availability of oleuropein to undergo the cleavage. On this bases, resistance competence of cultivars is produced. From this, the active molecules should be very concentrated in the cell juice of resistant cultivar, while they would be in little amount in that of the susceptible cultivars. Our results allow to suppose that the production of these molecules is maximum within 20 minutes from the attack and gradually decreases up to reach an equilibrium within the three hours from the attack. The smaller number of infected punctures in the resistant cultivar in comparison to that susceptible ones, could perhaps point out that these molecules can inhibit the development and the spread of the larvae, that take place few days after oviposition. In fact, as it is known (Konno *et al*., 1999), molecules from oleuropein acts as cross-linking of proteins, thus decreasing the nutritive quality of tissues and causing the death of larvae. This hypothesis seems a valid start point for further researches

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REFERENCES

Amiot M.J., Fleuriet A., Macheix J.J. 1989. Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry*, **28**, 67-69.

Archibald F.S. 1992. A New Assay for Lignin-Type Peroxidases employing the Dye Azure B. *Applied and Environmental Microbiology*, 3110-3116.

Báidez A.G., Gómez P., Del Río J.A., Ortuño A. 2007. Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. *Journal of Agriculture Food Chemistry*, **55**, 3373-3377.

Benayoun J., Catesson A.M., Czaninski Y. 1981. A cytochemical study of differentiation and breakdown of vessel end walls. *Annals of Botany*, **47**, 687-698.

Bianco A.D., Piperno A., Romeo G., Uccella N. 1999. NNMR experiments of oleuropein biomimetic hydrolysis. *Journal of Agriculture Food Chemistry*, **47**, 3665-3668.

Bisignano G., Laganà M.G., Trombetta D., Arena S., Nostro A., Uccella N., Mazzanti G., Saijia A. 2001. *In vitro* antibacterial activity of some aliphatic aldehydes from *Olea europaea* L. *FEMS Microbiology Letters*, **198**, 9-13.

Bitonti M.B., Chiappetta A., Innocenti A.M., Muzzalupo I., Uccella N. 1999. Funzionalità e distribuzione dei biofenoli nella drupa di *Olea europaea* L., *OLIVO & OLIO* **1/2**, 20-29.

Briante R., Patumi M., Limongelli S., Febbraio F., Vaccaro C., Di Salle A., La Cara F., Nucci R. 2002. Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L., *Plant Science*, **162**, 791-798.

Esen A. 1993. β-Glucosidases. In: A. Esen, ed, β-*Glucosidases*: *Biochemistry and Molecular Biology*, American Chemical Society, Washington. 1-14.

Iannotta N., Noce M.E., Ripa V., Scalercio S., Vizzarri V. 2007. Assessment of susceptibility of olive *cultivars* to the *Bactrocera oleae* (Gmelin, 1790) and *Camarosporium dalmaticum* (Thüm.) attacks in Calabria (Southern Italy). *Journal of Environmental Science Health B.*, **42**, 789-793.

Iannotta N., Perri, E., Sirianni R., Tocci C. 1999a. Influence of *Colletotrichum gloeosporioides* (Pezing) and *Camarosporium dalmatica* (Thum) attacks on olive oil quality. *Acta Horticulturae*. (*ISHS*), **474**, 573-576

Iannotta N., Perri L., Tocci C., Zaffina F. 1999b. The behaviour of different olive cultivars following attacks by *Bactrocera oleae* (Gmel.) *Acta Horticulturae* (*ISHS*), **474**, 545-548.

Iannotta N., Perri L., Rinaldi R. 1994. Control of the olive fly by mass trapping in Calabria. *Acta Horticulturae* (*ISHS*), **356**, 411-413.

Jefferson R.A., Kavanagh T.A., Bevan M.W. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal*, **6**, 3901-3907.

Keck M., Richter S., Suarez B., Kopper E., Jungwirth E. 2002. Activity of peroxidise in plant material infected with *Erwinia amylovora*. *Acta Horticulturae* (*ISHS*), **590**, 343-350.

Keys A., Menotti A., Karvonen M.J. 1986. The diet and 15-year death rate in the Seven Countries Study. *American Journal of Epidemiology*, **124**, 903-915.

Konno K., Hirayama C., Yasui H., Nakamura M. 1999. Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree, *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 9159-9164.

Kubo A., Lunde C.S., Kubo I. 1995. A multichemical defense mechanism of bitter olive *Olea europaea* (Oleaceae). Is oleuropein a phytoalexin precursor? *Journal of Chemical Ecololgy*, **11**, 251-263.

Lo Scalzo R., Scarpati M.L., Verzegnassi B., Vita G. 1994. *Olea europaea* chemicals repellent to *Dacus oleae* females. *Journal of Chemical Ecology*, **20**, 1813-1823.

Mazzuca S., Spadafora A., Innocenti A.M. 2006. Cell and tissue localization of β-glucosidase during the ripening of olive fruit (*Olea europaea*) by *in situ* activity assay. *Plant Science*, **171**, 726-733.

Mazzuca S., Uccella N. 2002. β-glucosidase releasing of phytoalexin derivatives from secobiophenols as defence mechanism against pathogenic elicitors in olive drupes. *Acta Horticulturae* (*ISHS*), **586**, 529-531.

Rice R. 2003. Bionomics of the olive fruit fly, *Bactrocera* (*Dacus*) *olea*. *University of California Plant Protection Quarterly*. 1-5. http://www.uckac.edu/ppq/PDF/00July.pdf

Saniewski M., Urbanek H., Puchalski J. 1992. Wound-induced phenolic metabolism in scales of *Hippeastrum* x hybr. *Acta Horticulturae* (*ISHS*), **325**, 303-306.

Soler-Rivas C., Espin J.C., Wichers H.J. 2000. Oleuropein and related compound, *Journal of Science Food Agriculture* (*ISHS*), **80**, 1013-1023.

Valentines M.C., Vilaplana R., Usall J., Larrigaudière C. 2005. Involvement of enzymatic browning and peroxidase activity as resistance mechanism in "Golden delicious" apples. *Acta Horticulturae (ISHS)*, **682**, 2041-2048.

Varela L., Vossen P. 2003. Olive fruit fly. University of California Cooperative Extension - Sonoma County.
http://cesonoma.ucdavis.edu/HORTIC/olive_fly/olive_fruit_fly.pdf

Willett W.C. 1990. Diet and coronary heart disease. *Monographs in Epidemiology and Biostatistics*, **15**, 341-379.

World Health Organization.1990. Diet, nutrition, and the prevention of chronic diseases. Report of a WHO Study Group. *WHO Technical Report Series*, 797

Figure captions

Figure 1.
Microphotographs of olive fruit sections after the *in situ* assay for β-glucosidase (E.C. 3.2.1.21). The insoluble products of the X-Glc precipitated in the cells of the whole mesocarp (a). The sites of reaction were identified as blue spots inside the nuclei and chloroplasts in the mesocarp immediately below the epidermis (b); 40 x (a); 150 x (b).

Figure 2
Microphotographs of olive fruit sections following the *in situ* assay for β-glucosidase activity, at different times from the mimed fly attack. The mechanical injury was made by the micro needle 200 μm in diameter and processed for the β-glucosidase *in situ* assay a, b) immediately after injury; c, d) 10 minutes later; e, f) 3 hours later. The bars indicate the distances from the edge of injury to the onset of dyed cells. The arrows indicate the inactive cells. 40 x (a, c, e); 100 x (b, d); 150 x (f).

Figure 3
Details of cellular localizations of β-glucosidase activity in the fruit sections immediately after the injury. (a) Cells at the edge of the injury show dense blue colour in nuclei, but not inside the chloroplasts (see arrows). (b) The colour diffuse very rapidly from the nuclei to the cytoplasm. 150 x

Figure 4
β-glucosidase activity in olive fruits extracts of the two cultivars Carolea (a) and Cassanese (b) at different times after the mimed injury. Enzyme activity was evaluated toward the synthetic substrate *p*NPGlc at pH 4.6. Values are the mean of four independent replicates. The dotted lines indicated the mean enzyme activity values in the sound fruit populations, of both cultivars, prior to performe the biomimed attack.

Figure 5
Oleuropein amount in the olive fruit extracts at the different times after the injury in Carolea (grey bars) and in Cassanese (white bars) cultivars. Values are the mean of four independent replicates.

Figure 6
Microphotographs of olive fruit sections following the combined *in situ* assay for β-glucosidase/peroxidase activity (POX, EC.1.11.1.7), at different times from the mimed fly attack. The mechanical injury was made by the micro needle 200 μm in diameter and processed for the POX *in situ* assay a) 10 min after injury; b) 60 minutes later; c) 3 hours later. As a substrate for peroxidase, 3,3′-diaminobenzidine tetra hydrochloride (DAB) was used. It can form an insoluble

brown polymeric non-droplet precipitate in the sites of reactions. Simultaneous localization of β-glucosidase/POX activities was obtained by processing the DAB-treated sections with the *in situ* assay for β-glucosidase. 100 x.

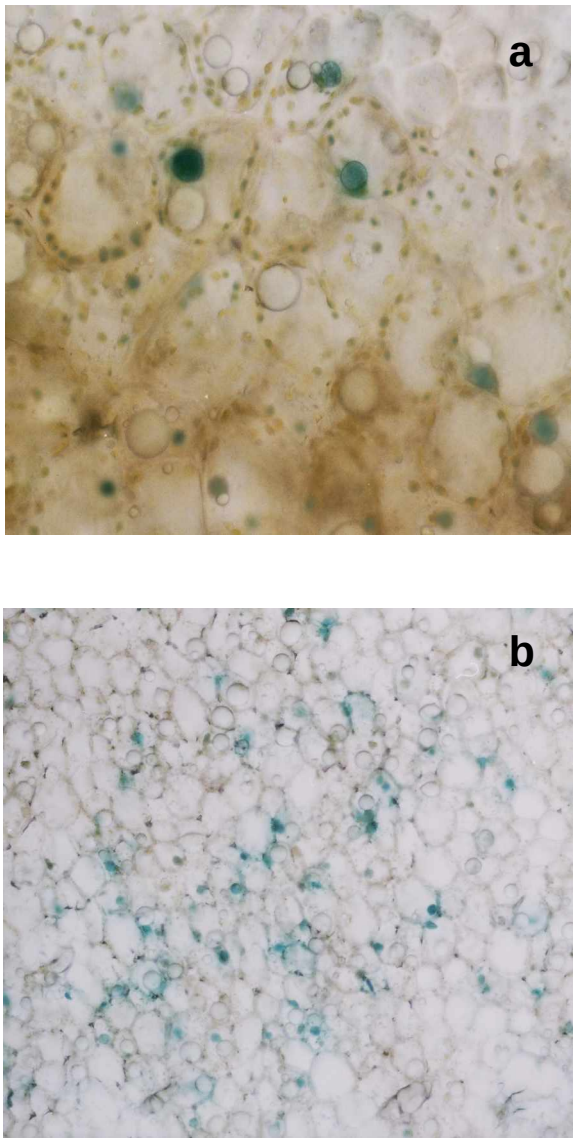


Figure 1

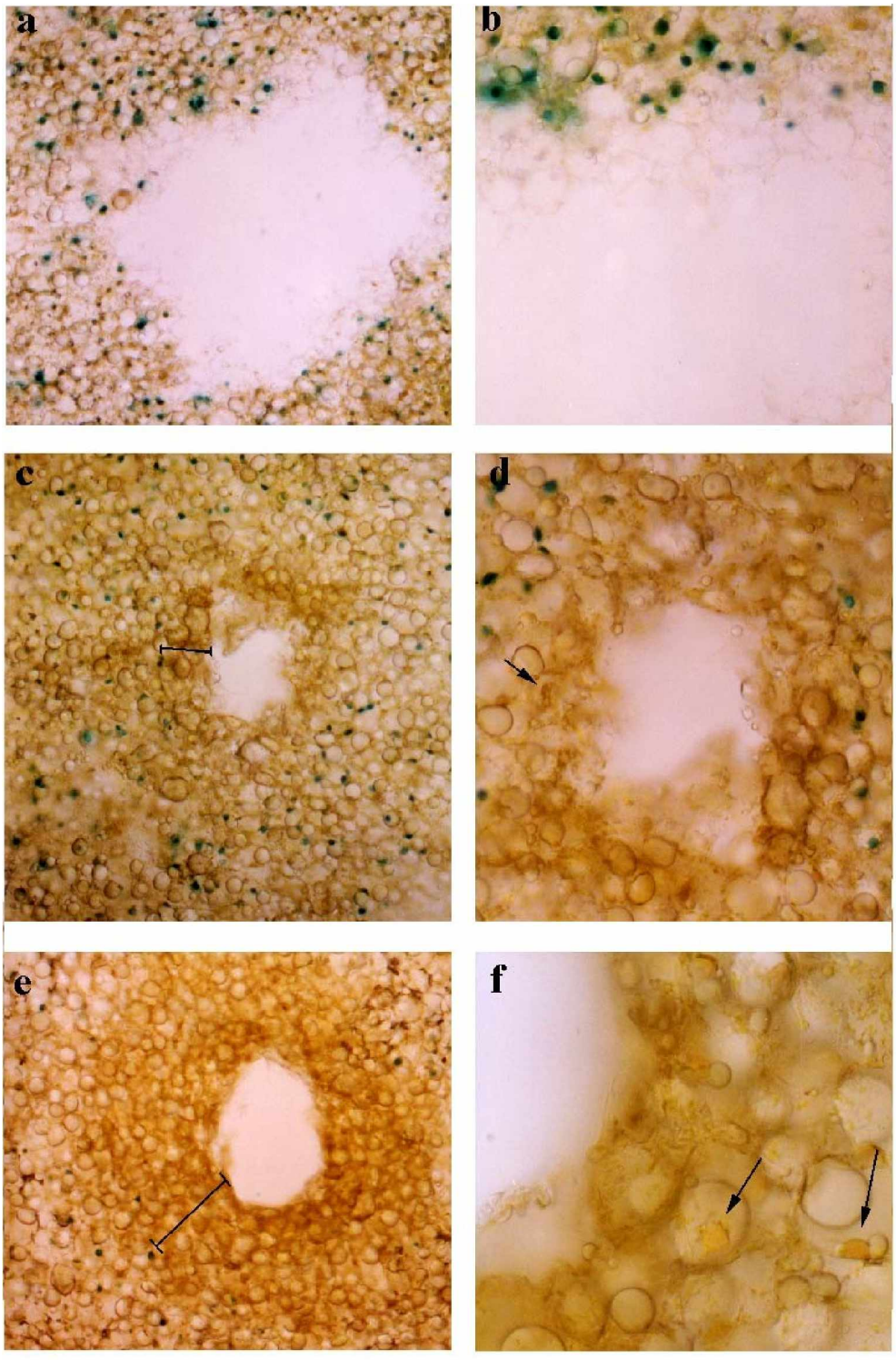
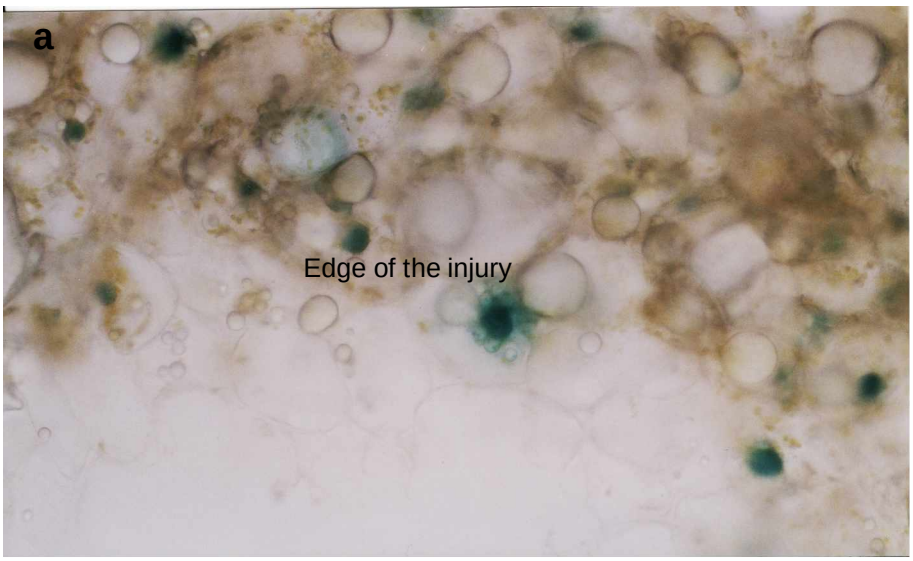


Figure 2



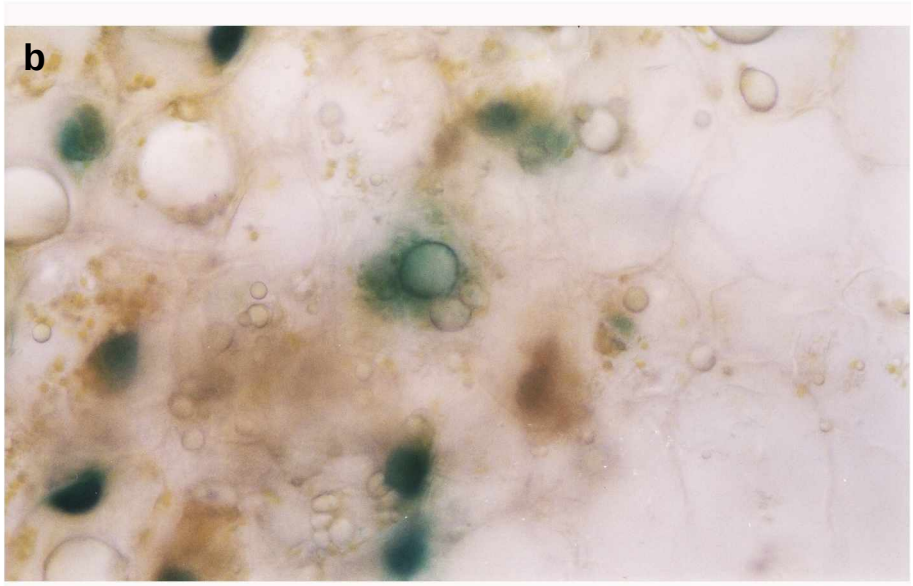


Figure 3

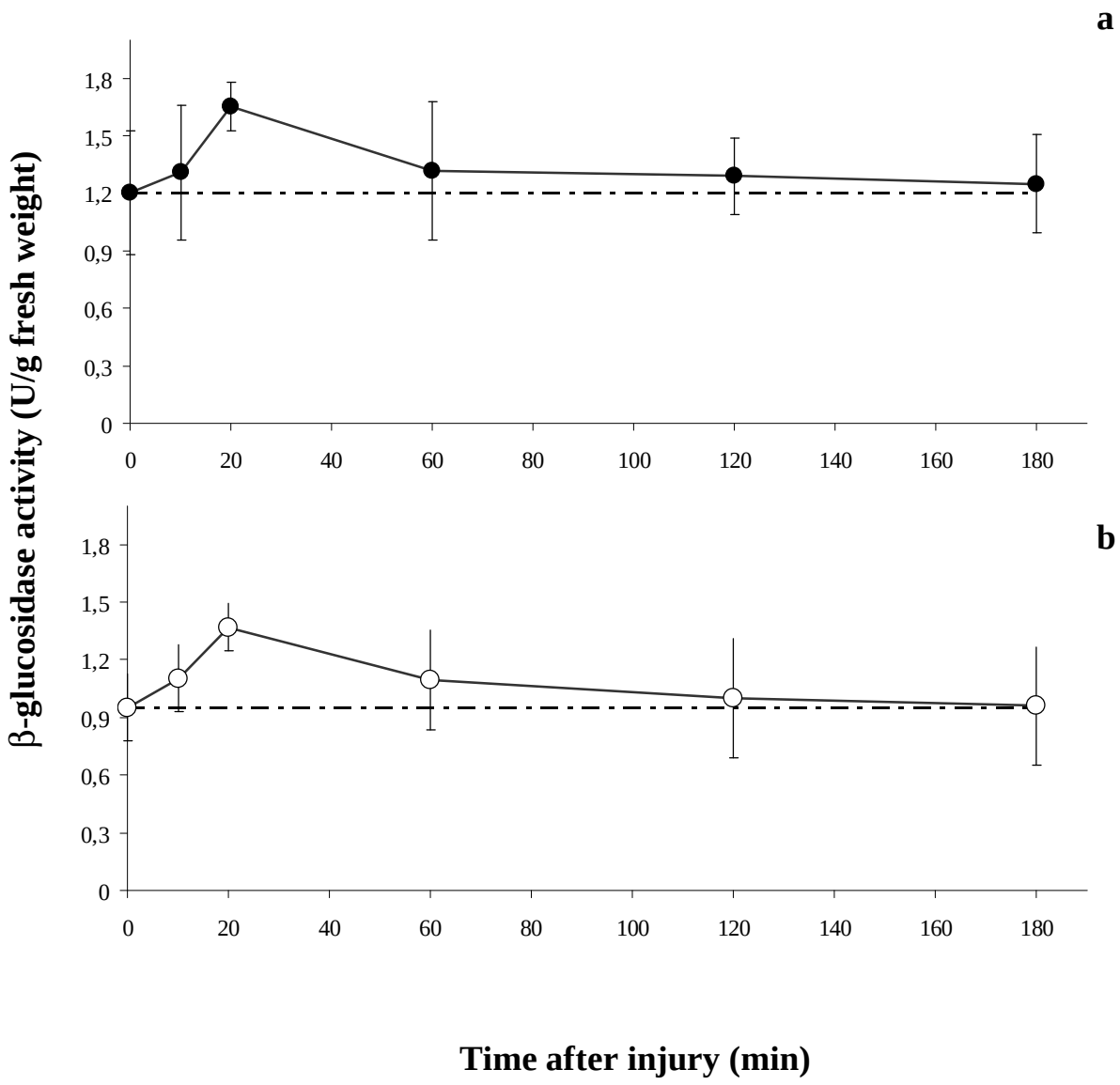

a
b
β-glucosidase activity (U/g fresh weight)
1,8
1,5
1,2
0,9
0,6
0,3
0
0
20
40
60
80
100
120
140
160
180
Time after injury (min)

Figure 4

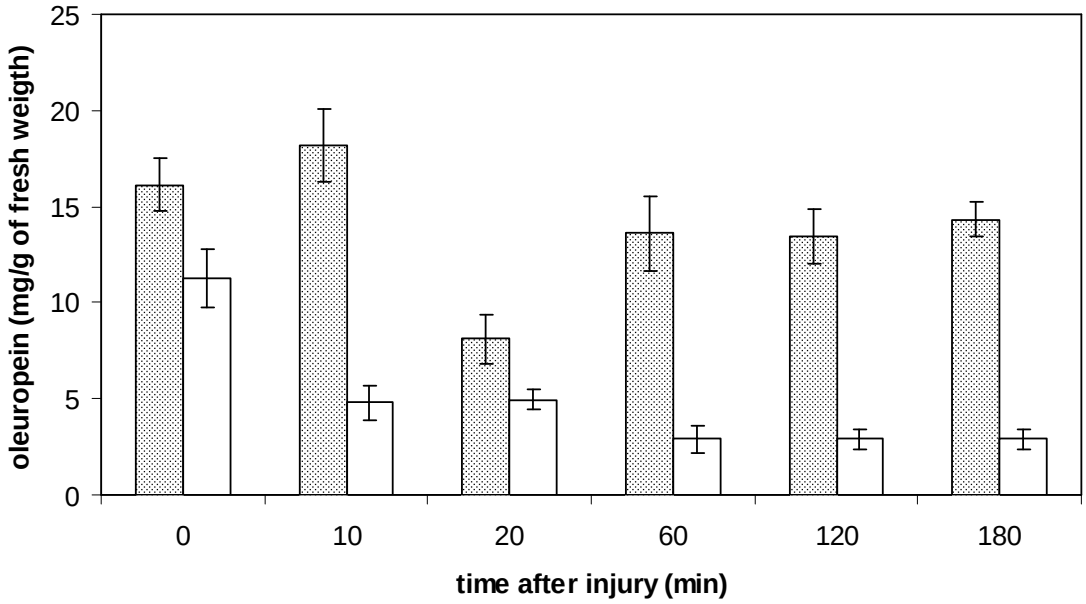

25
20
15
10
5
0
oleuropein (mg/g of fresh weigth)
0
10
20
60
120
180
time after injury (min)

Figure 5

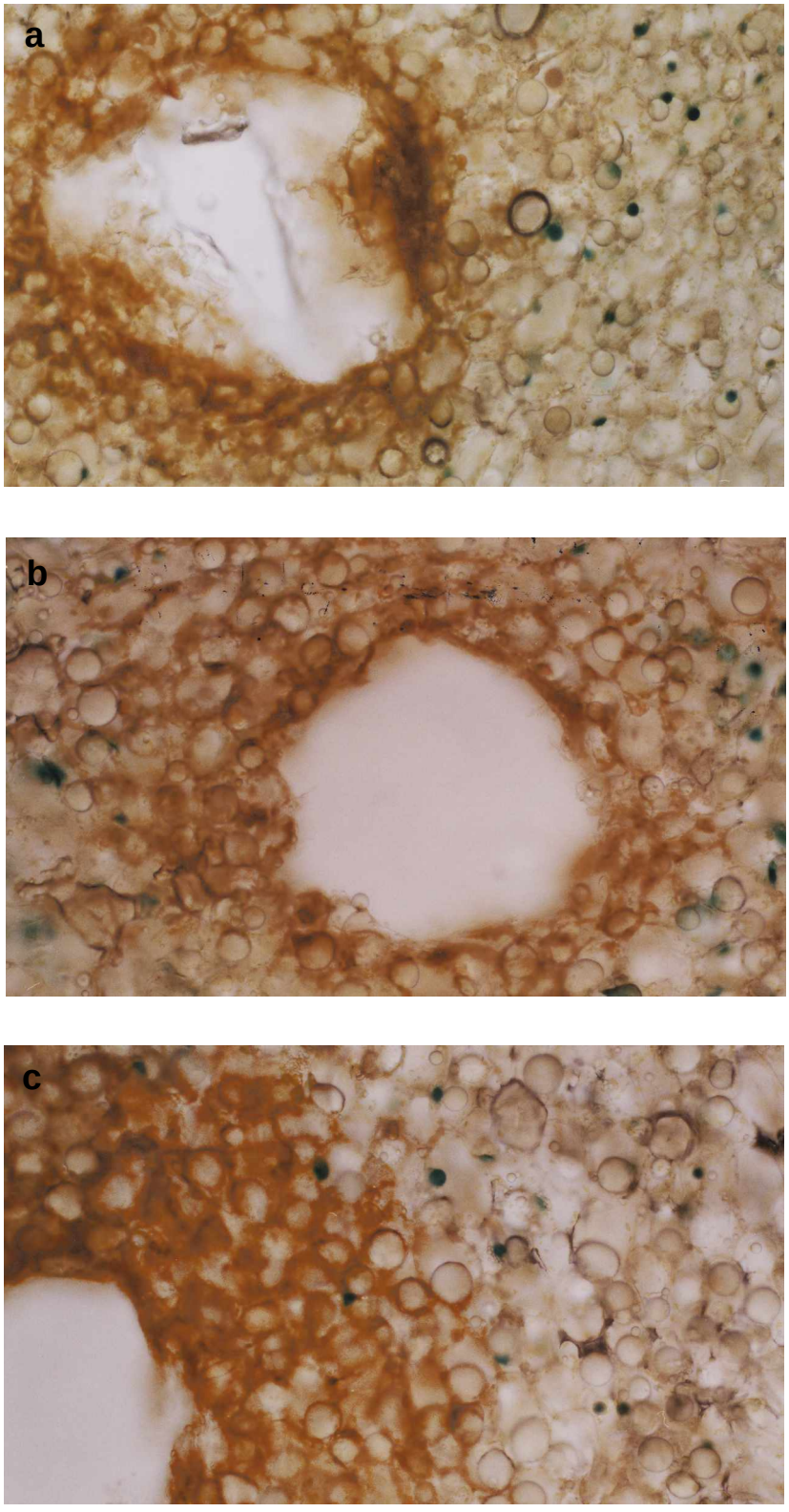


Figure 6

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

R. Mazzei, L. Giorno†, A. Spadafora*, S. Mazzuca*, and E. Drioli

Institute on Membrane Technology, CNR-ITM C/o University of Calabria, Via P. Bucci 17/C, 87036 Rende (CS), Italy
*Department of Ecology, University of Calabria, Via P. Bucci 6/B, 87036 Rende (CS), Italy
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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.

†Author for all correspondences
(e-mail : l.giorno@itm.cnr.it)

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).

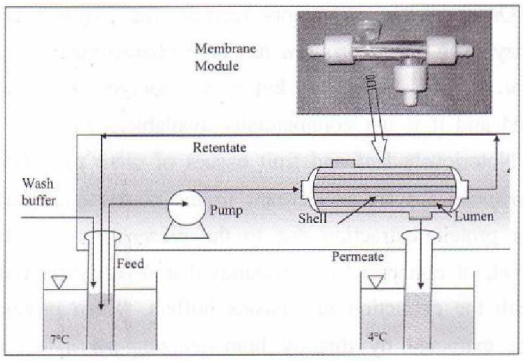

Fig. 1. Schematic draw of membrane diafiltration system.

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.

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.

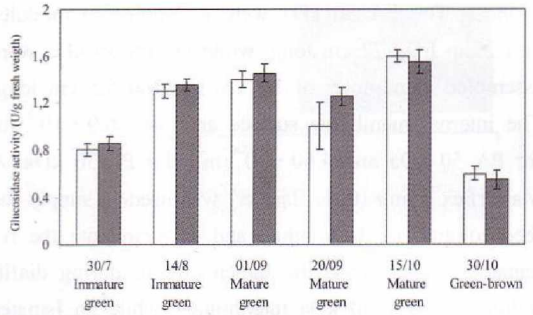

Fig. 2. β-glucosidase activity in olivefruits extracts at different ripening stages during two different harvest seasons: 2003 (□), 2004 (■). Enzyme activity was evaluated toward the synthetic substrate pNPGlc at pH 4.6.

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-

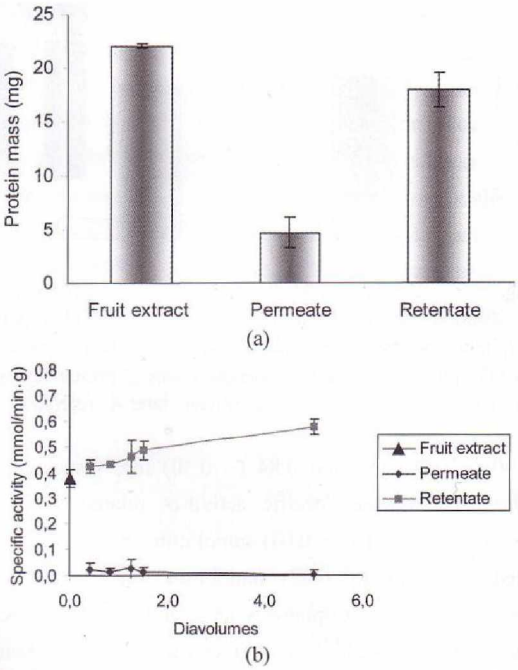

Fig. 3. Protein mass (a) and β-glucosidase activity (b) in solutions from diafiltration through PA 50 kDa membrane of fruit extract from *Olea europaea*.

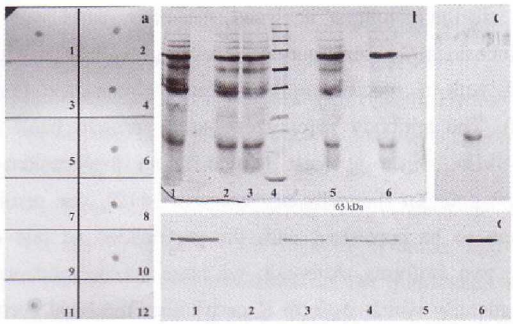

Fig. 4. (a) Dot blots performed by the antibody against b-glucosidase: 1. and 2. crude protein extract from *Olea europaea* 3. and 4. permeate and retentate, respectively after 0.42 diavolumes; 5. and 6. permeate and retentate after 0.83 diavolumes; 7 and 8 permeate and retentate after 1.25 diavolumes; 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.

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 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

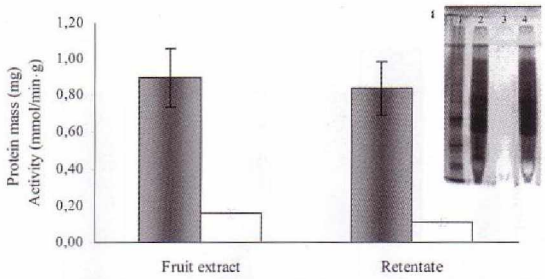

Fig. 5. Protein mass (■) and β-glucosidase activity (□) in solutions from diafiltration through PS 30 kDa polysulphone membrane of fruit extract from *Olea europaea*. a) SDS-page assay: lane 1. marquer; lane 2 protein extract from *Olea europaea* lane 3 permeate lane 4. retentate.

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.

Acknowledgements

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References

1. K. Konno, C. Hirayama, H. Yasui, and M. Nakamura, Enzymatic activation of oleuropein: A protein crosslinker used as a chemical defense in the privet tree. *Proc. Natl. Acad. Sci., USA*, **96**, 9159-9164 (1999).
2. P. Goupy, A. Fleuriet, M. J. Amiot, and J. J. Macheix, Enzymatic browning, oleuropein content, and diphenol oxidase activity in olive cultivars (*Olea europaea* L.). *J. Agric. Food Chem.*, **39**, 92-95 (1991).
3. G. Cianfardini, V. Marsilio, B. Lanza, and N. Pozzi, Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation. *Appl. Environ. Microbiol.*, **60**, 4142-4147 (1994).
4. V. Marsilio, B. Lanza, and N. Pozzi, Progress in table olive debittering: degradation *in vitro* of oleuropein and its derivatives by *Lactobacillus plantarum*. *J. Am. Oil Chem. Soc.*, **73**, 593-597 (1996).
5. R. Materassi, N. Miclaus, and O. Pelagatti, Hydrolysis of oleuropein in yeasts. *Ann. Ist Speriment Elaiotec.*, **5**, 53-65 (1975).
6. A. Saija, D. M. Trombetta, D. Tomaino, R. Lo Cascio, P. Princi, N. Uccella, F. Bonina, and F. Castelli, *In vitro* evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. *Int. J. Pharm.*, **166**, 123-133 (1998).
7. A. Esen, β-Glucosidases. In β-Glucosidases Biochemical and Molecular Biology, *American Chemical Society, Washington*, 1-14 (1993).
8. K. J. Dietz, A. Sauter, K. Wichert, D. Messdaghi, and W. Hartung, Extracellular β-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *J. Exp. Bot.*, **51**, 937-944 (2000).
9. R. G. Cameron, J. A. Manthey, R. A. Baker, and K. Grohamann, Purification and characterization of a β-glucosidase from Citrus sinensis var. Valencia fruit tissue. *J Agric. Food Chem.*, **49**, 4457-4462 (2001).
10. R. Briante, M. Patumi, S. Limongelli, F. C. Febbraio, A. Vaccaio, F. Di Salle, R. La Cara, and M. Nucci, Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L., *Plant Sci.*, **162**, 791-798 (2002).
11. S. Mazzuca and N. Uccella, β-glucosidase releasing of phytoalexin derivatives from secobiophenols as defence mechanism against pathogenic elicitors in olive drupes. *Acta Hort.*, **586**, 529-531 (2002).
12. S. Mazzuca, A. Spadafora, and A M. Innocenti, Cell and tissue localization of β-glucosidase during the ripening of olive fruit (Olea europaea) by in situ activity assay. *Plant Science, in Press* (2006).
13. S. Mazzuca, Research reports of Italian National Research Council "OEVOCAL" n° 97.00138.MZ79 project (2000).

14. W. Wang, M. Scali, R. Vignani, A. Spadafora, E. Sensi, Mazzuca, and M. Cresti, Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electroph, **24**, 2369-2375 (2003).
15. F. Granier, Extraction of plant proteins for two-dimensional electrophoresis. Electroph. **9**, 712-718 (1988).
16. J. L. Garcia, N. Avidan, A. Troncoso, R. Sarmento, and S. Lavee, Possible juvenile-related proteins in olive tree tissues. *Sci. Hort.*, **85**, 271-284 (2000).
17. B. B. Douglas and A. L. Zydney, Effect of Solution on Protein Transport Through Ultrafiltration Membranes. *Biotechnol. Bioeng.*, **64**, 27-37 (1999).
18. E. Klein, Affinity Membranes Their Chemistry and Performance in: Adsorptive Separation Processes. John Wiley and Sons, New York (1991).
19. A. Higuchi, S. Mishima, and T. Nakagawa, Separation of proteins by surface modified polysulphone membranes. *J. Membr. Sci.*, **57**, 175-185 (1992).
20. S. Saksena and A. L. Zydney, Effect of solution pH and ionic strength on the separation of albumin from immunoglobulins. *J. Membr. Sci.*, **43**, 960-968 (1994).
21. R. H. C. M. van Eijndhoven, S. Saksena, and A. L. Zydney, Protein fractionation using electrostatic interaction in membrane filtration. *Biotech. Bioeng.*, **48**, 406-414 (1995).
22. N. Ehsani, M. Parkkinen, and M. Nyström, Fractionation of natural and model egg-white protein solution with modified and unmodified polysulphone UF membranes. *J. Membr. Sci.*, **123**, 105-119 (1997).
23. M. Nyström, P. Aimar, S. Luque, M. Kulovaara, and S. Metsämuuronen, Fractionation of model proteins using their physiochemical properties. *Colloid and Surf.*, **138**, 185-205 (1998).
24. R. Ghosh and Q. Y. Li, Fractionation of BSA and lysozyme using ultrafiltration: effect of gas sparging. *AIChE J.*, **44**, 61-67 (1998).
25. A. L. Zydney, Protein Separation Using Membrane Filtration: New opportunities for Whey Fractionation, *Int. Diary J.*, **8**, 243-250 (1998).
26. E. Drioli and L. Giorno, Biocatalytic Membrane Reactors: Application in Biotechnology and the Pharmaceutical Industry, Taylor & Francis Publisher, London (1999).
27. R. Ghosh, Novel cascade ultrafiltration configuration for continuous, high-resolution protein-protein fractionation: a simulation study. *J. Membr. Sci.*, **226**, 85-99 (2003).
28. R. Ghosh, Y. Wan, Z. Cui, and G. Hale, Parameter scanning ultrafiltration: rapid optimization of protein separation, *Biotech. Bioeng.*, **81**, 673-682 (2003).
29. M. Feins and K. K. Sirkar, Novel internally staged ultrafiltration for protein purification, *J. Membr. Sci.*, **248**, 137-148 (2005).
30. M. Feins and K. K. Sirkar, Highly Selective Membranes in Protein Ultrafiltration, *Biot. And Biong.*, **86**, 603-611 (2004).
31. A L. Zydney, Engineering aspect of Microfiltration/ Ultrafiltration technology, in: Microfiltration and Ultrafiltration: Principles and Application. Marcel Dekker, New York, 391-392 (1996).
32. F. Lipnizki, J. Boelsmand, and R. F. Madsen, Concepts of industrial-scale diafiltration system, Desalin, **144**, 179-184 (2002).
33. S. J. Brewer and H. M. Sassenfeld, Recovery of native protein, in: Protein purification applications, a pratical approach. Oxford University Press, *Tokio*, 99-111 (1990).
34. M. M Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analis. *Biochem.*, **72**, 248-254 (1976).
35. U K Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T$. Nat. **222**, 680-685 (1970).
36. Y. Minami, H. Takao, T. Kanafuji, K. Miura, M. Kondo, I. Hara-Nishimura, M. Nishimura, and H. Matsubara, β-Glucosidase in the Indigo plant: intracellular localization and tissue specific expression in leaves, *Plant Cell Physiol*, **38**, 1069 (1997).

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Immunolocalization of β-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity *in situ*

S. Mazzuca [a], L. Giorno [b,*], A. Spadafora [a], R. Mazzei [b], E. Drioli [b]

[a] *Department of Ecology, University of Calabria, Via P. Bucci 6/B, 87036 Rende, CS, Italy*
[b] *Institute of Membrane Technology, CNR-ITM, C/o University of Calabria, Via P. Bucci 17/C, 87036 Rende, CS, Italy*

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Abstract

A new combined method is reported to localize the sites of enzyme immobilization and to determine its catalytic activity on a polymeric capillary membrane reactor. The useful new method resulted from the merging of the classic *in situ* enzyme activity assay and western blot technique whose both results are easily detectable either at low than at high magnification in light microscopy. β-Glucosidase from olive fruit was selected as enzyme model because of its suitable relevance in the industrial processing of foods, in biotechnology and in pharmaceuticals and for its activity against the synthetic substrate 5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde which develops an insoluble dyed product. The enzyme was physically immobilized within 30 kDa cut-off capillary polysulphone membranes and results obtained by means of a polyclonal antibody against β-glucosidase and the synthetic substrate clearly showed a coherent localization of the immobilization enzyme sites and its activity.

Keywords: Polysulphone capillary membrane; Enzyme immobilization; Enzyme activity *in situ*; Immunolocalization *in situ*; Light microscopy

1. Introduction

Immobilized biocatalysts have widespread applications in areas like organic synthesis, pollution control and diagnostics. Enzymes can be immobilized on different supports like polymeric beads, gels and membranes. Hybrid systems using immobilized enzymes, such as biocatalytic membrane reactors are well described in the open literature.

Membranes possess the distinct advantage of very high surface area for enhanced enzyme loading and can sustain convective flow mode. Immobilization eliminates the need to separate an enzyme from the product solution and allows these expensive compounds to be reused. In addition, the thermal and storage stability of an enzyme may be increased as a result of immobilization [1,2]. These advantages are in addition to those that enzymes possess over conventional catalysts such as high efficiency and regio/stereospecificity [3]. Common immobilization techniques may be grouped in two classes: physical entrapment [4] and chemical immobilization [5]. Chemical immobilization include non-covalent linkage (e.g. adsorption) [6–8] and covalent attachment. While the physical immobilization often helps to preserve the properties of an enzyme and facilitate regeneration, covalent attachment generally contributes a greater degree of immobilization stability [9]. The amount of biocatalyst loaded, its distribution and activity through the support and its lifetime are very important parameters to properly orientate the development of such systems. Usually the amount of immobilized protein is known by mass balance between the initial solution (feed) and the solutions after immobilization process (e.g. retentate and permeate) [10]. The catalytic properties are then measured as observed reaction rate on the basis of the reaction product in the bulk solution as a function of time. The possibility to visualize the enzyme *in situ* and detect its activity *in situ* has not yet fully explored. The use of fluorescence labelling of proteins was used [11] to monitor protein fractionation by ultrafiltration and to control membrane fouling. Sousa et al. [12] evaluated the distribution along hollow fibres membranes of the amount of enzyme immobilized on microporous nylon membrane. The procedure was based on measuring the protein content in membranes pieces sectioned after enzyme

* Corresponding author. Tel.: +39 0984 492050/2134; fax: +39 0984 402103.
E-mail address: l.giorno@itm.cnr.it (L. Giorno).

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immobilization, along the module length, i.e. initial, middle and end of the membrane module, by Lowry assay. Liu et al. [13] proposed a method for the localization of active peroxidase immobilized on microfiltration flat membranes by the use of 3,3′-diaminobenzidine tertahydrochloride (DAB). It can form an insoluble brown polymeric non-droplet precipitate which is strongly osmium-philic when oxidized in presence of H_2O_2. After the reaction with DAB, the membranes pieces, embedded in Epon resin, were cut by ultra-microtome and observed by electron microscopy.

In the present work a new combined method revealing the sites where the enzyme is immobilized within the thickness of ultrafiltration polysulphone membranes and the assessment of its activity *in situ* has been developed.

This method is based on the merged procedures of *in situ* assay developed for histochemistry detection of glucohydrolase activity and the western blotting for immunodetection of target proteins on nitrocellulose membranes [14,15]. The histochemical detection of glycohydrolase enzymes was developed and extensively applied in the *in situ* detection of the expressions of GUS reported gene constructs in the transformed tissues [16–18]. Based on the same procedural conditions the enzyme family 1 of β-D-glucoside glucohydrolase, E.C. 3.2.1.21, which specifically hydrolyzes the synthetic substrate (5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde), it is routinely applied as marker of endoplasmic reticulum and useful as bacterial differentiation [19–21]. In fact following the hydrolysis, the synthetic substrate develops a blue dyed product that precipitates in the site of the reaction; consequently it is possible to visualized the localization of β-glucosidases in the cells by means of the light microscope observation still at low magnifications. Actually, no evidences exist at our knowledge of the performances of the assay on enzyme loaded on the artificial moieties as polymeric membranes, except for its analogous synthetic substrate used for the glucohydrolases detection on acrylamide gels [22]. On the other hand, besides no evidences exist on the classic western blot analysis applied on capillary polysulphone membrane loaded with the target proteins, practically, no significant challenges are expected respect to the nitrocellulose membrane performances toward the effectiveness on the epitope-antibody recognition. For this purpose the native β-glucosidase from olive fruit (*Olea europea*) was chosen as enzyme to immobilize onto the membrane because the well known catalytic activity towards the synthetic substrate able to develop the insoluble dyed products that precipitates on the sites of reaction. In addition the β-glucosidase was selected among other enzymes because of its suitable target for protein engineering to address the food processing and quality enhancement [23,24], biomass conversion in biotechnology [25], as well as substrate processing in pharmaceutical [26]. In particular, olive β-glucosidase activity is linked to many processes such as the defense against pests and pathogens [27], the loss of bitter taste of ripens fruit pulp [28,29] and influence foodstuff quality resulting from industrial processing of olive fruits [30–32]. In addition, the products of enzymatic hydrolysis of its natural substrate oleuropein are well known as pharmacologically active molecules [33].

2. Materials and methods

2.1. Enzyme extraction

Olive green fruits showing high β-glucosidase activity (1.5 ± 0.4 U/mg of proteins) 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 10 kDa (poly-vinyl pyrrolidone), 1% (w/v) β-mercaptoethanol, 1.0 mM PMSF (phenylmethylsulfonylfluoride) according to Briante et al. [34]. The suspension was shaken gently for 1 h at 4 °C and centrifuged in a minifugue at 27,000 × *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. Western blot analysis of enzyme extract after SDS-PAGE electrophoresis was performed using a 1:2000 polyclonal anti-β-glucosidase as the primary antibody, kindly supplied by Minami et al. [35]. The secondary antibody was a 1:3000 anti-rabbit AP conjugate antibody. The detection was made by 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 (Fig. 1). This result is not surprising, because the enzyme monomers of GH1 family have molecular weights between 35 and 65 kDa [22]. The used method allowed to recognize only the monomer of the enzyme, since the SDS-PAGE electrophoresis does not preserve the aggregates forms. Native enzymes in most plants are monomers or dimmers, as

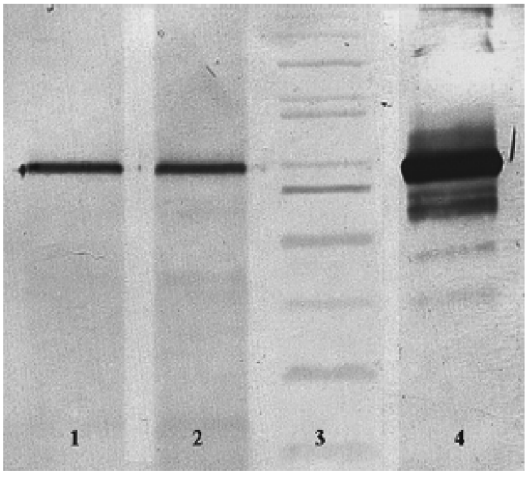

Fig. 1. Western blot of olive tissues extracts after SDS-PAGE in which the polyclonal anti-β-glucosidase antibody recognized a protein band at 65 kDa as a putative olive β-glucosidase (lanes 1 and 2), lane 3: 200–14 kDa markers, lane 4: β-glucosidase from almond (Sigma, St. Louis, USA) was used as standard purified enzyme.

almond β-glucosidase that has a molecular weight of 130 kDa. In analogy, we can suppose that olive β-glucosidase could exist as monomers or higher aggregates forms.

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

2.2. Structure of polysulphone membrane and module assembling

For enzyme immobilization, commercial polysulphone capillary membranes with nominal molecular weight cut-off (NMWCO) of 30 kDa, having inner/outer diameter of 1.08/1.75 mm, were used. The membranes were kindly provided by Romicon Inc. 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 three capillary membranes inside a pirex glass cylinder of 1.2 cm i.d., 5.5 cm long. The internal membrane surface area was 4.25×10^{-4} m^2. Before permeability tests, the membranes were first washed with ultra pure water to remove the water-soluble residues. After this step immobilization procedure with fruit extract was carried out.

2.3. Immobilization of β-glucosidase from olive fruit by cross-flow ultrafiltration

Five millilitres of fruit extract solution was recirculated along the shell side at a flow rate of 1.45 ml/min (axial velocity of 0.028 m/s) and a transmembrane pressure of 0.10 bar. The enzyme solution permeated from shell to lumen. In this way the β-glucosidase present in the extract (65 kDa monomers or higher molecular weight aggregates), entered the spongy layer but could not pass through the thin layer (cut-off 30 kDa). After the cross-flow ultrafiltration was completed the membrane was rinsed in order to remove the amount of the enzyme reversibly adsorbed on the membrane. Rinsing cycles of 5 min each with 0.1 M borate buffer pH 9 were carried out, with a flow rate of 2.38 ml/min, axial velocity of 0.046 m/s and a transmembrane pressure of 0.15 bar. The removal of non-immobilized enzyme was monitored by spectrophotometer analysis in the UV–vis range.

The same immobilization procedures was carried out using pure β-glucosidase from almond.

An Ismatec peristaltic pump (Cole-Parmer) was used to supply the feed solution to the module and to recirculate the retentate stream along the shell circuit. The experiments were carried out by maintaining the feed solution and the permeate at 4 °C and the membrane module at room temperature. The fractions collected after immobilization procedure were 0.8 ml of permeate and 4.2 ml of retentate. Protein content in the various solutions was determined by Bradford spectrophotometric assay [36]. The amount of immobilized protein was calculated by mass balance:

$$\mathrm{Mass}_{\text{immobilized protein}} = \mathrm{Mass}_{\text{feed}} - (\mathrm{Mass}_{\text{retentate}} + \mathrm{Mass}_{\text{permeate}} + \mathrm{Mass}_{\text{washing solution}})$$

2.4. In situ assay activity of β-glucosidase

The three fibers loaded with the enzyme were disassembled from the modules and freshly sectioned at 4 °C by using a vibrotome (Leica), along fibers length (initial, middle and end of the fibers) obtaining the serial sections each of 80 μm in thickness. Longitudinal and cross-sections were immediately placed in the minivials containing 1 ml of detection buffer, adapted for β-glucosidase from Jefferson et al. [16], containing 60 μM of the synthetic substrate (5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde, Sigma, St. Louis), 50 mM phosphate buffer pH 6.5, 1.5 mM potassium ferricyanide, 1.5 mM potassium ferrocyanide, 10 mM EDTA pH 8.0. After enzymatic hydrolysis by immobilized β-glucosidase, the synthetic substrate develops an insoluble blue product that precipitates on the site of reaction thus localizing the active enzyme in the membrane. For each sampling three membrane sections, initial, middle and end of the fibers, were placed in the vials contained the detection buffer without the synthetic substrate as the controls. Sections were incubated in a wet chamber at 37 °C in the dark for 30 min, 1, 2 and 3 h. The reaction was stopped in cold phosphate buffer and the sections were mounted with glycerol on slides for optical microscopy and digitalized by Leitz Dialux EB microscope equipped with a CCD camera. Image analysis on three sections for each membrane replicate ($n = 9$) was performed by QWin^{TM} Image System Software (Leica).

2.5. Immunoblotting on β-glucosidase loaded membrane

Subsequent to the *in situ* detection of β-glucosidase activity the enzyme-loaded membrane sections were processed using a modified protocol drawn from conventional western blot analyses [4]. The membrane sections were embedded in a drop of blocking buffer (pH 7.6, 0.025% Tween, 5% normal serum). After washes the primary polyclonal anti-β-glucosidase antibody was added in blocking buffer for 1 h at room temperature. The antibody dilutions were from 1:2000 to 1:5000. Secondary anti-rabbit antibody gold conjugated (Inalco spa) at the dilution 1:6000 in TBST with 2% normal serum was added and incubated for 1 h at room temperature. For light microscopy analyses, secondary antibodies linkages were visualized on membrane by silver enhancing kit (Sigma, St. Paolo, MO, USA). As control, for each antibody dilution, membranes were processed excluding the incubation with primary anti-β-glucosidase antibody. Same membrane sections were processed for immunoblotting, immediately after cuttings, without to perform the *in situ* activity in order to ascertain the alterations on enzyme distribution within the membrane due to the assay conditions.

3. Results and discussion

3.1. Physical immobilization of β-glucosidase from olive fruit

The immobilization of β-glucosidase was achieved by cross-flow ultrafiltration from shell to lumen through a polysulphone membranes which initial pure water permeability was 263.62

Table 1
Example of samples collected from protein extract ultrafiltration

Sample	Concentration (mg/ml)	Volume (ml)	Mass (mg)
Feed	0.400 (±0.060)	5.000	2.000 (±0.300)
Permeate	0.020 (±0.003)	0.800	0.024 (±0.004)
Retentate	0.380 (±0.057)	4.200	1.630 (±0.240)
Washing solution 1	0.090 (±0.013)	4.000	0.024 (±0.004)
Washing solution 2	0.000	4.000	0.000

(±43.81) l/h m^2 bar. During the protein extract filtration the permeability dropped to 9.41 (±2.81) l/h m^2 bar. This severe decrease was due to the high concentration polarization caused by the low axial velocity 0.028 m/s used in order to preserve the biological activity of the enzyme.

As described in Section 2, the total protein content of the collected samples after ultrafiltration was determinated by Bradford assay.

The amount of immobilized protein for fruit extract was about 0.32 (±0.05) mg and was calculated as mass balance from values shown in Table 1. During immobilization with pure β-glucosidase from almond the amount of immobilized enzyme was 0.03 (±0.002) mg.

In the case of fruit extract immobilization, the mass immobilized does not represent the effective β-glucosidase content but the total protein content remained entrapped in the membrane. Furthermore this information does not indicate where the enzyme is located and how it is distributed through the membrane thickness and along the module length.

3.2. *In situ* assay activity of β-glucosidase

The vibrotome cut is useful for certain difficult materials such as very soft biological specimens or in the case of investigation of some specific activities or molecules that are inactivated and destroyed by fixatives or embedding procedures. The sections obtained can be relatively thick (50–100 μm), however this method is suitable for light microscopy analyses. The enzyme loaded membrane appeared to be easily sectioned and handily manipulated by means of this technique, even though it was not possible to obtain good cutting accuracy of longitudinal and cross-sections of less than 70 μm in thickness. Cutting took place in the physiological buffer thus minimizing damage to loaded enzyme and maintaining the bath temperature of 4 °C. Also, the operating vibrating blade was kept constant with a vibration frequency of 25 Hz (value 2 of the vibration scale of the vibrotome Leica). As shown in Fig. 2(a) and (b) cross and longitudinal vibrotome membrane sections, observed in light stereoscopy, preserved a good structure. The typical structure of this kind of membranes, with the selective layer on the lumen side and the sponge layer on the shell side resulted to be not damaged or deformed in the texture by cutting. The asymmetric membrane structure at electron microscopy level is illustrated in the part (c) of Fig. 2.

After enzyme immobilization, the activity *in situ* was measured by using the synthetic substrate 5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde.

In Fig. 3, cross-sections of fibers at middle level of the module are reported. Similar results were obtained along the fiber length at the beginning and end of the module.

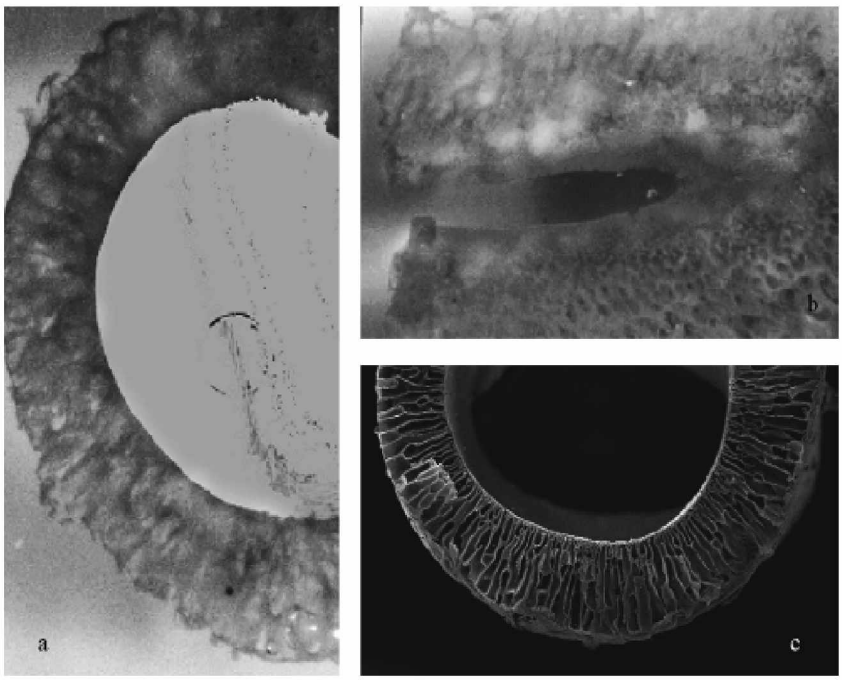

Fig. 2. Eighty-micrometer membranes section obtained by vibrotome and observed in light stereoscopy: (a) cross-section (40×), (b) longitudinal section (60×) and (c) scanning electron microscopic picture of cross-section of 30 kDa polysulphone capillary membrane 30×.

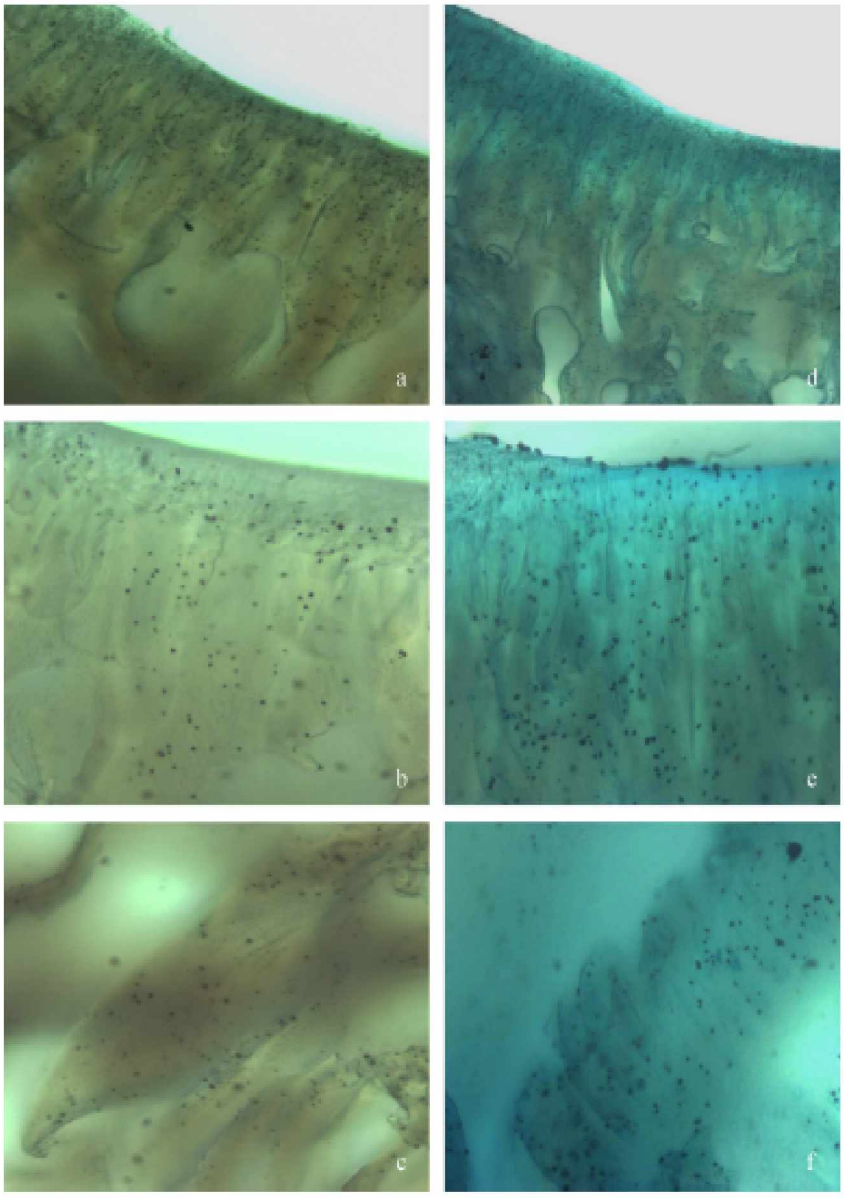

Fig. 3. Microphotographs of β-glucosidase loaded membrane cross-sections (a)–(c) after the immunolocalization with the antibody against β-glucosidase; (d)–(f) after the combined methods of immunolocalization and *in situ* activity assay. (a) 100× of sample on the lumen side; (b) and (c) 400× of samples on the lumen side and shell side, respectively; (d) 100× of sample on the lumen side; (e) and (f) 400× of samples on the lumen side and shell side, respectively.

From microscope pictures in Fig. 3(d)–(f) it can clearly be seen the uniform spreading of the insoluble blue colour of the product obtained by enzymatic hydrolysis of the synthetic substrate which can be supposed the representative site of active β-glucosidase immobilized in the membrane. In order to minimize the product diffusion within the membrane, the detection buffer for β-glucosidase activity assay contained an higher concentration of ferricyanide and ferrocyanide (up to 20%) respect to those in the biology standard assay [16]. The results showed the enzyme was distributed through the membrane thickness along the all module length. Therefore the cross-flow ultrafiltration in the used operating conditions it is confirmed a useful method to immobilize enzyme through the overall membrane.

3.3. Immunoblotting on β-glucosidase loaded membrane

A preliminary analysis of polyclonal antibody against β-glucosidase is performed in the western blot carried out on the electrophoreses proteins under denaturant conditions. The

Fig. 4. Microphotograph of membrane after the immunoblotting control test.

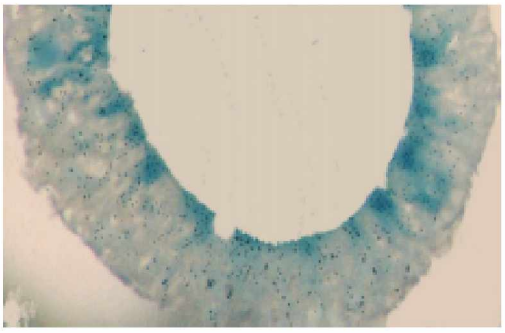

Fig. 5. Cross-section of pure β-glucosidase from almond loaded-membrane, after combined methods of immunolocalization and *in situ* activity assay (40×).

antibody recognizes both β-glucosidase from olive extract and commercial purified almond β-glucosidase, as a single protein band of 65 kDa (Fig. 1), as previously reported in Section 2. The anti-β-glucosidase is specifically against the chloroplastic β-glucosidase from leaves of *Polygonum tinctorium* [35] and, in this case, demonstrated to have high specificity for β-glucosidase from different plant sources. On these bases the immunoblotting on β-glucosidase loaded polysulphone membrane is performed using a protocol drawn from traditional western blot for immunocalization on nitrocellulose membrane [14]. The major modifications made consist in the decrease of detergent concentration (up to 30%) of the blotting solutions and in the employ of floating membrane sections on the drops of solutions which minimized the amount of antibodies required. Immunoblottings from β-glucosidase loaded membranes were performed prior Fig. 3(a)–(c) and after (d)–(f) the *in situ* assay. The microscope observations showed that the antibody recognized the immobilized β-glucosidase since the black spots are localized inside the membrane. The spots size and number, in fact, develop from the amount of the linkages of gold-conjugated secondary antibodies which are silver enhanced to allow the observation of the linkage in light microscopy [15]. A control test was performed by carrying out the immunoblotting procedure on membranes without incubation with primary antibody, in this case no spots were observed (Fig. 4).

The spots appear to be distributed through the thickness, either within the selective layer on the lumen side than in the sponge layer on the shell side. Protein density is higher in the layer next to the lumen since there the polymer density is higher. The *in situ* assay appears to do not influence the spots distribution along the membrane thickness and does not affect the efficiency of epitope-antibody recognition in the membrane section as indicated by the equal amount of spots number and spot size between the two treatments (Fig. 3(a) *versus* (d)). The enzyme localization and its activity *in situ* are considered the results from a combined method of the immunoblotting *in situ* assay, they clearly showed a correlation between the catalytic activity and the sites of enzyme immobilization, confirming that the colour distribution is not due to product diffusion inside the membrane, but corresponded to the presence of the immobilized active enzyme in the site of reaction. These results pointed that the physical immobilization can provide high membrane surface for the enzyme to be immobilized and showed that the biocatalysts can be distributed through all the membrane thickness. The combined method was also verified on membrane loaded with pure β-glucosidase from almond as standard, results are reported in Fig. 5. Also in this case the results showed that the enzyme was immobilized through all the membrane thickness with major presence on the thin layer.

4. Conclusion

A new combined method merged from the classical *in situ* detection of enzyme activity and western blot analysis was applied for the first time to the capillary asymmetric polysulphone membrane reactor to determine simultaneously the enzyme spatial distribution through the membrane thickness and along the membrane module and its activity after the immobilization through the observation in the light microscopy both at low and high magnifications. Results revealed a coherent localization of enzyme and its insoluble product of hydrolysis through the all membrane which evidenced that physical entrapment by cross-flow ultrafiltration allowed the enzyme macromolecules to penetrate through the membrane module preserving the catalytic activity. The described approach can be useful to clarify crucial informations for the optimisation of the enzyme immobilization techniques, for estimating the enzyme content over a non-pure protein starting solution, for measuring intrinsic kinetic properties of immobilized enzymes, for the modelling of membrane reactors and their development at large scale.

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References

[1] D.A. Butterfield, Biofunctional Membranes, Plenum, New York, NY, 1996.
[2] L. Cao, Immobilized enzymes: science or art? Curr. Opin. Chem. Biol. 9 (2005) 217.
[3] G.J. Lye, O.P. Pavlou, M. Roujidi, D.C. Stuckey, Immobilization of Candida cylindracea lipase on colloidal liquid aphrons (CLAs) and development of a continuous CLA-membrane reactor, Biotech. Bioeng. 51 (1996) 69.
[4] S.P. Shukla, K. Modi, P.K. Gosh, S. Devi, Immobilization of horsedarish peroxidase by entrapment in natural polysaccharide, J. Appl. Polym. Sci. 91 (2004) 2063.
[5] S.P. Shukla, S. Devi, Covalent coupling of peroxidase to a copolymer of acrylamide (AAm)-2-hydroxyethyl methacrylate (HEMA) and its use in phenol oxidation, Prog. Biochem. 40 (2005) 147.
[6] E. Pamula, P.G. Rouxhet, Influence of surface properties of carbon fibres on the adsorption of catalase, Carbon 43 (2005) 1432.
[7] F. Rojas-Melgarejo, J.N. Rodriguez-Lopez, F. Garcia-Canovas, P.A. Garcia-Ruiz, Immobilization of horsedarish peroxidase on cinnamic carbohydrate ester, Prog. Biochem. 39 (2004) 1455.
[8] L. Marron-Brignone, R.M. Morélis, P.R. Coulet, Immobilization through adsorption of luciferase on Langmir-Blodgett film. Influence of the hydrophilicity or hydrophobicity of the surface on the enzyme kinetic behaviour, Langmuir 12 (1996) 5674.
[9] R.F. Taylor, Protein Immobilization: Fundamentals and Applications, Marcel Dekker Inc., New York, NY, 1991.
[10] E. Drioli, L. Giorno, Application in Biotechnology and the Pharmaceutical Industry: Biocatalytic Membrane Reactors, Taylor & Francis, London, 1999.
[11] J.P.S.G. Crespo, M. Trotin, D. Hough, J.A. Howell, Use of fluorescence labelling to monitor protein fractionation by ultrafiltration under controlled permeate flux, J. Membr. Sci. 155 (1999) 209.
[12] H.A. Sousa, C. Rodrigues, E. Klein, C.A.M. Afonso, J.G. Crespo, Immobilisation of pig liver esterase in hollow fibre membranes, Enzyme Microb. Technol. 29 (2001) 625.
[13] Z.M. Liu, J. Dubremez, V. Richard, Q. Yang, Z.K. Xu, P. Seta, Useful method for the spatial localization determination of enzyme (peroxidase) distribution on microfiltration membrane, J. Membr. Sci. 267 (2005) 2.
[14] W. Neal Burnette, Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, Anal. Biochem. 112 (1981) 195.
[15] J. Polak, S. Van Noorden, Immunocytochemistry. Modern Methods and Applications, J. Wright and Sons, Bristol, 1986.
[16] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901.
[17] R. Bauer, F. Paltauf, S.D. Kohlwein, Fuctional expression of bacterial β-glucuronidase and its use as a reporter system in the yeast *Yarrowia lipolytic*, Yeast 9 (1993) 71.
[18] U.K. Schmitz, D.M. Lonsdale, R. Jefferson, Application of the β-glucuronidase gene fusion system to *Saccharomyces cerevisiae*, Curr. Genet. 17 (1990) 261.
[19] J.P. Horwith, A histochemical substrate for β-glucosidase, J. Med. Chem. 7 (1964) 574.
[20] S. Amigorena, J.R. Drake, P. Webster, I. Mellman, Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes, Nature 369 (1994) 113.
[21] I. Romano, A. Giordano, L. Lama, B. Nicolaus, A. Gambacorta, *Planococcus rifietensis* sp. nov. isolated from algal mat collected from a sulfurous spring in Campania (Italy), Syst. Appl. Microbiol. 26 (2003) 357.
[22] A. Esen, in: A. Esen (Ed.), β-Glucosidases: Biochemical and Molecular Biology, American Chemical Society, Washington, 1993, p. 1.
[23] Z. Günata, Flavor enhancement in fruit juices and derived beverages by exogenous glycosidases and consequences of the use of enzyme preparations, in: J.R. Whitaker, A.G.J. Voragen, D. Wong (Eds.), Handbook of Food Enzymology, Marcel Dekker Inc., Portland, 2003.
[24] G.R. Fenwick, R.K. Heaney, W.J. Mullin, Glucosinolates and their breakdown products in food and food plants, CRC Crit. Rev. Food Sci. Nutr. 18 (1983) 123.
[25] P. Beguin, Molecular biology of cellulose degradation, Annu. Rev. Microbiol. 44 (1990) 219.
[26] L.W. Wattenberg, Studies on polycyclic hydrocarbon hydroxylases of the intestine possibly related to cancer. Effect of diet on benzopyrene hydroxylase activity, Cancer 28 (1971) 99.
[27] K. Konno, C. Hirayama, H. Yasui, M. Nakamura, Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 9159–9164.
[28] D. Ryan, K. Robards, S. Lavee, Changes in phenolic content of olive during maturation, Int. J. Food Sci. Technol. 34 (1999) 265.
[29] M. Brenes Balbuena, P. Garcia Garcia, A. Garrido Fernandez, Phenolic compounds related to the black color formed during the processing of ripe olives, J. Agric. Food Chem. 40 (1992) 1192.
[30] J.R. Morello, M.J. Moltiva, M.J. Tovar, M.P. Romero, Changes in commercial olive oil (cv Arbequina) during sorage with special emphasis on phenolic fraction, Food Chem. 85 (2004) 357.
[31] V. Marsilio, B. Lanza, N. Pozzi, Progress in table olive debittering: degradation in vitro of oleuropein and its derivatives by *Lactobacillus plantarum*, J. Am. Oil Chem. Soc. 73 (1996) 593.
[32] G. Cianfardini, V. Marsilio, B. Lanza, N. Pozzi, Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation, Appl. Environ. Microbiol. 60 (1994) 4142.
[33] G. Bisignano, M.G. Laganà, D. Trombetta, S. Arena, In vitro antibacterial activity of some aliphatic aldehydes from *Olea europea* L., FEMS Microbiol. Lett. 198 (2001) 9.
[34] R. Briante, M. Patumi, S. Limongelli, F.C. Febbraio, A. Vaccaio, F. Di Salle, R. La Cara, M. Nucci, Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L., Plant Sci. 162 (2002) 791.
[35] Y. Minami, H. Takao, T. Kanafuji, K. Miura, M. Kondo, I. Hara-Nishimura, M. Nishimura, H. Matsubara, β-Glucosidase in the indigo plant: intracellular localization and tissue specific expression in leaves, Plant Cell Physiol. 38 (1997) 1069.
[36] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248.

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β-Glucosidase separation from *Olea europaea* fruit and its use in membrane bioreactors for hydrolysis of oleuropein

Rosalinda Mazzei[a], Lidietta Giorno[a]*, Silvia Mazzuca[b], Antonia Spadafora[b], Enrico Drioli[a]

[a]*Institute on Membrane Technology, ITM-CNR, C/o University of Calabria, Via P. Bucci 17/C, 87036 Rende (CS), Italy*
email: l.giorno@itm.cnr.it
[b]*Department of Ecology, University of Calabria, Via P. Bucci 6/B, 87036 Rende (CS), Italy*

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1. Introduction

β-Glucosidase from olive fruit tissues is a key enzyme in fruit ripening and defence response [1–3]. The purification of olive β-glucosidase is of high interest for its application in the food and pharmaceutical fields. In this work, the use of membrane technology to fractionate and purify protein extract from *Olea europaea* fruit containing β-glucosidase was investigated. The enriched enzymatic fraction was immobilized on polymeric membranes to develop biocatalytic membrane reactors for the hydrolysis of oleuropein, whose products have shown antibacterial activity.

2. Results and discussion

Olea europaea fruit extracts from optimal ripening stage were processed by diafiltration. Asymmetric membranes made of polyamide or polysulfone having 50 and 30 kDa nominal molecular weight cut-off (NMWCO), respectively, were used. The effects of membrane material and cut-off on the separation performance and β-glucosidase stability were studied. Qualitative and quantitative electrophoresis was applied to analyse the composition of protein solution before and after membrane separation process. In general, the membrane treatment allowed improving the purity of the protein extract giving better electrophoretic profile (Fig. 1). The β-glucosidase stability was analysed by catalytic activity tests before and after fractionation, using the hydrolysis of *p*-D-nitrophenyl-β-D-glucopyranoside (pNPGlc) as reaction model. As shown in Fig. 2, the specific activity (mmol min^{-1} mg^{-1}) of the enzyme in the retentate was slightly improved after diafiltration compared to the initial fruit extract solution.

The purified fraction was immobilized on hollow fibre membrane bioreactors. The alternative approach of carrying out the purification and the immobilization in a single step has been explored.

The enzyme-loaded membranes were used to carry out the hydrolysis of oleuropein into glucose and dialdehydes α-β-unsaturated that

*Corresponding author.

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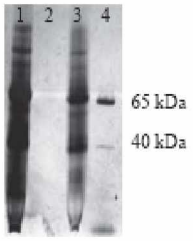

Fig. 1. SDS-page assay: lane 1, protein extract from *Olea europaea* (total volume 2 mL); lane 2, permeate; lane 3, retentate (total volume 2 mL); lane 4, β-glucosidase from almond.

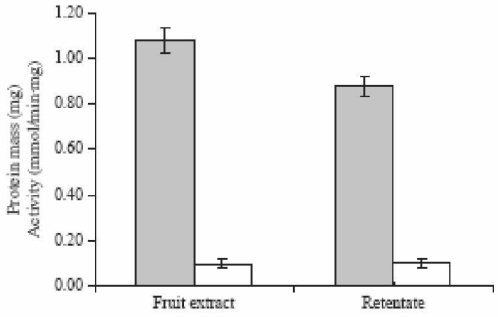

Fig. 2. Protein mass (■) and β-glucosidase activity (□) in solution from diafiltration through polymeric membrane of fruit extract from *Olea europaea*.

show a strong antibacterial, antitumoral and antioxidant activity.

The influence of amount and distribution of immobilized enzyme on the reactor performance have been investigated. A comparison between activity and stability of immobilized and free enzyme has been studied.

3. Conclusions

Preliminary results showed that the purity of fruit extracts was improved by diafiltration. The process allowed obtaining better electrophoresis profiles and slightly improving enzyme activity in retentate solution.

Immobilization of the β-glucosidase from purified solutions as well as from unpurified extracts has been performed. In the latter case, the immobilization permitted to load the enzyme and purify it at the same time.

References

[1] K. Konno, C. Hirayama, H. Yasui and M. Nakamura, Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree, Proc. Natl. Acad. Sci. Unit. States Am., 96 (1999) 9159–9164.

[2] P. Goupy, A. Fleuriet, M.J. Amiot and J.J. Macheix, Enzymatic browning, oleuropein content, and diphenol oxidase activity in olive cultivars (*Olea europaea* L.), J. Agric. Food Chem., 39 (1991) 92–95.

[3] M. Brenes Balbuena, P. Garcia Garcia and A. Garrido Fernandez, Phenolic compounds related to the black color formed during the processing of ripe olives, J. Agric. Food Chem., 40 (1992) 1192–1196.

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A new combined method to localize enzyme immobilized in polymeric membranes and evaluate its activity in situ

Silvia Mazzuca[a], Lidietta Giorno[b]*, Antonia Spadafora[a], Rosalinda Mazzei[b], Enrico Drioli[b]

[a]*Department of Ecology, University of Calabria, Via P. Bucci 6/B, 87036 Rende, Italy*
[b]*Institute on Membrane Technology, ITM-CNR, C/o University of Calabria, Via P. Bucci 17/C, 87036 Rende (CS), Italy*
email: l.giorno@itm.cnr.it; s.mazzuca@unical.it

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Keywords: Enzyme immobilization; Enzyme activity in situ; Immunolocalization; Polymeric membrane

1. Introduction

Biocatalytic membrane reactors using immobilized enzymes is a well-documented technology located at an emerging step in a graph of research efforts vs technology development. The immobilization of enzymes has proven to increase their stability and it has recently been shown that the widely observed inverse relationship between stability and activity is not a general rule.

Various methods are used to immobilize enzymes in membranes and usually the amount of immobilized enzyme is calculated by mass balance between the initial solution (feed) and final solutions (retentate and permeate). The catalytic properties are then measured as observed reaction rate on the basis of the reaction product in the bulk solution. At authors' knowledge, there is not yet published information on where the enzyme is immobilized within the polymeric membrane matrix.

2. Results and discussion

In this study, a new combined method revealing enzyme activity in situ all together the immunolocalization of the sites of immobilization on polymeric membrane has been developed. For this purpose β-glucosidase (from almond and olive fruit) was selected among other enzymes because of its suitable target for protein engineering to address the problems of biomass production in agriculture and forestry, biomass conversion in biotechnology, as well as substrate processing in pharmaceutical. β-glucosidases were immobilized by physical method in asymmetric capillary

*Corresponding author.

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membranes made of polyamide having 50 kDa molecular weight cut-off. The membranes were assembled in a tube-and-shell module in Pyrex of about 3 cm length. The immobilization was carried out by cross-flow ultrafiltration of the enzyme solution from shell to lumen. After the permeate flux reached a steady state value, the system was rinsed with buffer to remove the reversibly adsorbed enzyme. The membranes were disassembled from the module and sectioned by means of a vibrotome (Leica) without any fixing procedure. Longitudinal and cross-section of about 70 μm thickness were prepared. The sections were incubated with a reaction mixture containing 60 μM X-Glc (5-brome-4-chloro-3-indolyl-β-D-glucopyranosyde, Sigma, St Louis) as the synthetic substrate for b-glucosidase, ferricyanide and ferricyanate in the presence of EDTA [1,2]. The blue colour exhibited by the insoluble reaction product, observed in light microscopy, allows evidencing the active enzyme immobilized through the membrane. Results showed a uniform staining of cross and longitudinal membrane sections, which means a distribution of the enzyme in the all membrane. In order to verify the colour distribution was not due to product diffusion, but corresponded to the presence of the immobilized enzyme, an immunolocalization method to specifically localize the b-glucosidase was developed. Enzyme-loaded membrane sections were processed using an adapted protocol drawn from traditional western blot for immunocalization [3]. Polyclonal antibody against β-glucosidase was used. The detection of secondary antibody gold-conjugated was made by a silver enhanced kit for light microscopy [4]. As a result of antibody recognition, black spots were localized inside the membrane. The combined method of in situ activity and immunolocalization clearly showed a coherent correlation between the catalytic activity and the sites of enzyme immobilization (Fig. 1).

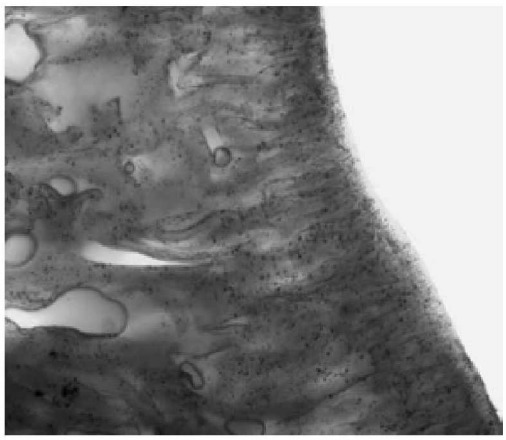

Fig. 1. Combined detection of in situ activity (blue colour) and immunolocalization of β-glucosidase (black spots) within the polysulfone membrane.

3. Conclusions

The method allows identifying the active β-glucosidase distribution within the membrane, which is of crucial importance for the optimisation of immobilization methodologies, the modelling of membrane reactors and their development at large scale. This simple method can be applied to all the enzymes of which is available a substrate able to develop a dyed product and a specific antibody against the enzyme.

References

[1] J.P. Horwith, A histochemical substrate for β-glucosidase. J. Med. Chem., 7 (1964) 574.

[2] R.A. Jefferson, T.A. Kavanagh and M.W. Bevan, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J., 6 (1987) 3901–3907.

[3] W. Neal Burnette, Western Blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem., 112 (1981) 195–203.

[4] J. Polak and S. Van Noorden, Immunocytochemistry. Modern methods and applications, J. Wright and Sons eds, 1986.

Wei Wang[1]
Monica Scali[1]
Rita Vignani[1]
Antonia Spadafora[2]
Elisabetta Sensi[1]
Silvia Mazzuca[2]
Mauro Cresti[1]

[1]Dipartimento di Scienze Ambientali, University of Siena, Siena, Italy
[2]Laboratorio di Citofisiologia Vegetale, Dipartimento di Ecologia, University of Calabria, Rende, Italy

Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds

The purpose of this research is to establish a routine procedure for the application of proteomic analysis to olive tree. Olive leaf tissue is notoriously recalcitrant to common protein extraction methods due to high levels of interfering compounds. We developed a protocol for isolating proteins suitable for two-dimensional electrophoresis (2-DE) from olive leaf. The remarkable characteristics of the protocol include: (i) additional grinding dry acetone powder of leaf tissue to a finer extent, (ii) after extensive organic solvent washes to remove pigments, lipids *etc.*, using aqueous tricholoroacetic acid washes to remove water-soluble contaminants, and (iii) phenol extraction of proteins in the presence of sodium dodecyl sulfate. The final protein preparation is free of interfering compounds based on its well-resolved 2-DE patterns. The protocol can be completed within 3 h, and protein yield is approximately 2.49 mg·g^{-1} of aged leaf. We also evaluated the protocol by immunoblotting with anti-tyrosinate α-tubulin antibody. To our knowledge, this is the first time that a protocol for protein extraction from olive leaf appears to give satisfactory and reproducible results. The protocol is expected to be applicable to other recalcitrant plant tissues and could be of interest to laboratories involved in plant proteomics.

Keywords: Contaminants / Electrophoresis / Immunoblot / Olive leaf / Plant protein extraction
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1 Introduction

2-DE is one of the most efficient and powerful methods to study complex patterns of gene expression at the level of proteins [1, 2]. However, the electrophoretic separation of proteins from plant tissue extracts is often complicated by other nonprotein contaminants indigenous to the plant, such as organic acids, lipids, polyphenols, pigments, terpenes, *etc.* [3]. In our research, high quality protein preparation from olive (*Olea europea* L.) leaf is required for proteomics. Olive leaf tissue is notoriously recalcitrant to common protein extraction methods due to high levels of interfering compounds. When proteins are extracted by directly homogenizing olive leaf in aqueous buffers and then precipitated by organic solvents, polyphenolic and other contaminants will copurify with the proteins, consequently the resultant brownish pellet, due to polyphenolic oxidation, is hard to be dissolved and is not suitable for 2-DE (our observations). To our knowledge, there are no reports on successful 2-DE separation of olive leaf proteins to date. Recently, Garcia *et al.* [4] characterized possible juvenile-related proteins in olive leaf by SDS-PAGE. They extracted leaf proteins using an aqueous sodium borate buffer (pH 9.0), followed by cold methanolic ammonium acetate precipitation. Nevertheless, the leaf protein profiles on SDS-PAGE gels they presented revealed only several bands with a high background, indicating the presence of substantial interfering substances. We tried their protocol but found it did not give good results for protein analysis by 2-DE.

Although many protocols are reported defining optimal conditions for sample preparation from plant tissues prior to IEF [3, 5–9], in the case of olive leaf, the resultant proteins are still contaminated to a degree that interferes with 2-DE. To overcome this problem, we developed a new protocol that attempts to minimize the presence of such compounds as pigments, polyphenols, lipids, polysaccharides, nucleic acids, *etc.* The protocol produces high quality proteins suitable for 1-DE and 2-DE from olive leaf. In this paper, we demonstrate well-separated patterns of olive leaf proteins by 1-DE and 2-DE. In addition, as an example we demonstrate immunoblots of olive leaf extracts probed with anti-tyrosinate α-tubulin antibody.

Correspondence: Dr. Wei Wang, Dipartimento di Scienze Ambientali, University of Siena, Via P A Mattioli 4, I-53100 Siena, Italy
E-mail: mails_wang@yahoo.com
Fax: +39-0577-232860

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2 Materials and methods

2.1 Preparation of dry tissue powder

Olive leaf was ground in liquid N_2 using a mortar and pestle. The powdered tissue was placed in microtubes (0.1–0.2 g tissue powder per 1.5 or 2.0 mL microtubes) and then resuspended in 1.0–2.0 mL cold acetone. After vortexing thoroughly for 30 s, the tubes were centrifuged at 10 000 × *g* for 3 min (4°C). The resultant pellet was washed once more with cold acetone. After the initial two washes, the pellet was transferred into a mortar and allowed to dry at room temperature (*ca.* 20 min). The dried powder was further ground to a finer powder by the aid of quartz sand and then transferred into new microtubes. The fine powder was sequentially rinsed with cold 10% TCA in acetone 3–4 times or until the supernatant was colorless, then with cold aqueous 10% TCA twice, and finally with cold 80% acetone twice. Each time the pellet was resuspended completely by vortexing and then centrifuged as above. The final pellet was dried at room temperature and used for protein extraction, or stored at −80°C for future use. The protocol described above can be scaled up to use with 10–30 mL centrifuge tubes.

2.2 Protein extraction

Phenol extraction of proteins is based on the protocol described before [8], but we carried out it in the presence of SDS (designated as phenol/SDS extraction). About 0.05–0.1 g of the dry powder of leaf tissue was resuspended in 0.8 mL phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO, USA) and 0.8 mL dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-mercaptoethanol) in a 2.0 mL microtube. The mixture was vortexed thoroughly for 30 s and the phenol phase was separated by centrifugation at 10 000 × *g* for 3 min. The upper phenol phase was pipetted to fresh microtubes (0.2 mL for 1.5 mL tube, 0.4 mL for 2.0 mL tube). After phase separation, white SDS complex often appears at the interphase. Be careful not to disturb the interphase by pipetting. If the recovered phenol phase is not clear, pool phenol phase together and centrifuge again. At least 5 volumes of cold methanol plus 0.1 M ammonium acetate was added to the phenol phase and the mixture was stored at −20°C for 30 min. Precipitated proteins were recovered at 10 000 × *g* for 5 min, and then washed with cold methanolic ammonium acetate twice and cold 80% acetone twice. The final pellet was dried and dissolved in a buffer of choice, such as Laemmli buffer [10] or 2-DE rehydration solution (8 M urea, 4% CHAPS, 2% IPG buffer, 20 mM dithiothreitol). For comparison, the dry powder of leaf tissue was extracted with 2-DE rehydration solution, phenol, and dense SDS buffer, respectively. The SDS or phenol extracts were subjected to methanol precipitation as above, and the recovered proteins were dissolved in 2-DE rehydration solution. Protein was quantified by the Bio-Rad protein assay (Hercules, CA, USA) [11] with bovine serum albumin as standard. After protein quantification, protein extracts were supplemented with trace bromophenol blue and resolved by 2-DE.

2.3 Electrophoresis and electroblotting

For SDS-PAGE, the Laemmli buffer system [10] was used to cast 4.75% stacking and 12.5% resolving gel, except that the 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 0.375 M. After denaturation at 95°C for 3 min, proteins were resolved under constant 200 V in a Bio-Rad mini-Protean II apparatus until bromophenol blue reached the bottom of the gel. For mini-2-DE, protein samples were applied in 125 μL of 2-DE rehydration solution by reswelling 7 cm Immobiline DryStrip (pH 3–10 or 6–11, Amersham, Piscataway, NJ, USA) overnight. IEF was performed in Multiphor II system (Pharmacia Biotech) at 10°C, applying 200 V for 1 Vh, 200 to 3500 V for 2800 Vh, and then 3500 V until reaching 8000 or 10 000 Vh. Focused strips were equilibrated using dithiothreitol and iodoacetamide solutions [12] and then positioned on a 12.5% minigel (1 mm thick, cast as described above). Secondary SDS-PAGE was carried out exactly as above. After electrophoresis, proteins were visualized with colloidal CBB G [13], or electroblotted onto polyvinylidene difluoride membrane (Hybond-P; Amersham) [14]. The 1-DE and 2-DE images were processed using Quantity One and PDQUEST software (Bio-Rad), respectively.

2.4 Immunoblotting

The protein blots were blocked with 3% bovine serum albumin in TBST buffer (20 mM Tris-HCl, pH 7.6, 0.8% NaCl, 0.1% Tween 20) and incubated with monoclonal anti-tyrosinate α-tubulin antibody (Sigma, dilution 1:2000) for 2 h at room temperature. A peroxidase-labelled anti-mouse antibody (Amersham, dilution 1:8000) was used as a secondary antibody. The detection system is ECL Plus Western Blotting Reagents (Amersham).

3 Results

Proteins isolated from aged olive leaf by our protocol show less contamination as determined by 1-DE and 2-DE. In 1-DE gel (Fig. 1) about 50 sharp bands of poly-

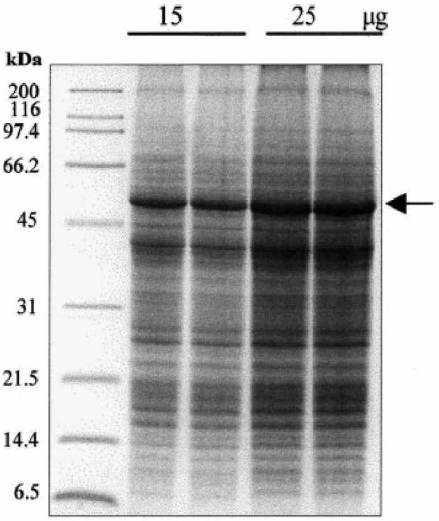

Figure 1. SDS-PAGE separation of olive leaf proteins. About 15 or 25 µg (as indicated) proteins extracted from dry powder of aged olive leaf are resolved using 12.5% polyacrylamide gel and visualized with CBB. The arrow indicates the prominent polypeptide Rubisco (large subunit), characteristic of leaf tissue extracts. The sizes of protein standards are indicated on the left.

peptides ranging from 6.5–200 kDa, estimated by Quantity One software (Bio-Rad), are excellently resolved with a low background; the prominent band of approximately 55 kDa is characteristic of leaf protein fractions of many plant species and represents the large subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) [4, 8, 15, 16]. The protocol also presents a high resolution of olive leaf protein separation in 2-D gel. The pH 3–10 Immobiline DryStrip pattern of olive leaf extract reveals well-resolved polypeptide spots throughout the gel with little streaking, even basic polypeptides appear as round-shaped spots up to pH 9.0 (Fig. 2a). Approximately 160 spots (estimated by PDQUEST software, Bio-Rad) in diversity of p*I*s and molecular masses are visualized by colloidal CBB G staining, which provides approximately a 10-fold increase in sensitivity (with fixation) over methanol/acetic acid based CBB G and R [14], and the prominent spot, Rubisco, is a good land marker on 2-D image (Fig. 2, see arrow). Besides, well-resolved 2-D patterns of olive leaf proteins can also be obtained with pH 6–11 Immobiline DryStrip (Fig. 2b).

We further compare the 2-DE patterns of olive leaf proteins extracted by different procedures on the basis of equal amounts of starting materials. In fact, proteins directly extracted from aged olive leaf in 2-D rehydration solution are not possibly to be resolved by 2-DE, due to the high background resulting from the CBB-staining of interfering compounds (Fig. 3a). With the extensively washed dry powder of leaf tissue, 2-DE patterns of phenol/SDS, phenol and dense SDS buffer extracts look similar (Fig. 3b–d). The phenol/SDS extraction displays a good resolution and gives more spots (156 spots, Fig. 3b) than phenol extraction (145 spots, Fig. 3c) and dense SDS buffer extraction (89 spots, Fig. 3d), whereas the SDS extraction produces considerable horizontal streaking (Fig. 3d). Overall, spot intensities are increased with phenol extraction in the presence of SDS (compare Figs. 3b and c). Besides, spot-to-spot comparison between Figs. 2a and Fig. 3b reveals a reproducibility of

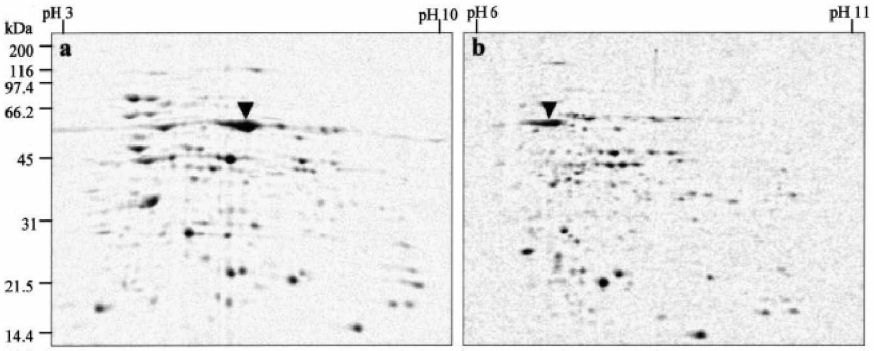

Figure 2. Representative 2-DE patterns of olive leaf proteins. About 100 µg of proteins from dry powder of olive leaf, extracted by phenol/SDS buffer mixture, are resolved by 2-DE (a) with pH 3–10 or (b) pH 6–11 (b) Immobiline DryStrip. The gels were visualized with colloidal CBB G. The arrow indicates Rubisco, the most abundant polypeptide in olive leaf extracts. The sizes of protein standards are indicated on the left.

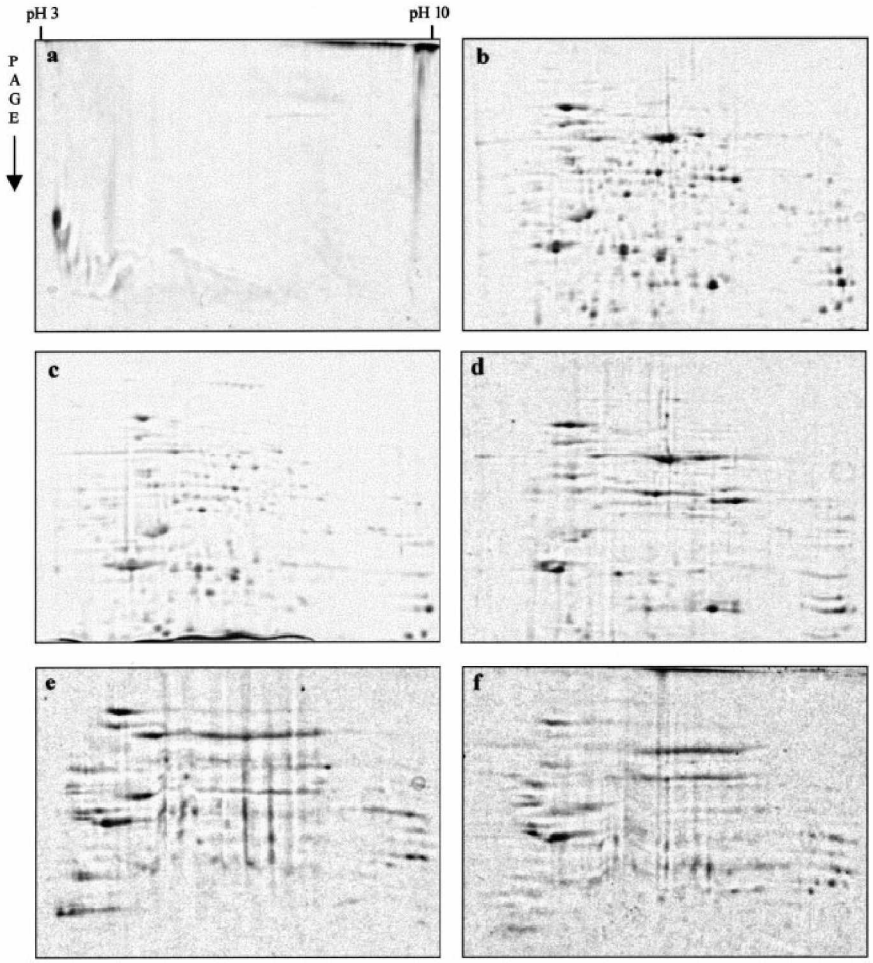

Figure 3. Comparison of 2-DE patterns of olive leaf proteins extracted by different procedures on the basis of equal amounts of starting materials. (a) Proteins are directly extracted from fresh aged olive leaf with 2-D rehydration solution and resolved by 2-DE. Proteins in dry powder of olive leaf are extracted by (b) phenol/SDS buffer, (c) phenol, (d) dense SDS buffer, (e) phenol/SDS buffer, but omitting 10% TCA washes, and (f) 2-DE rehydration solution, respectively.

74% (matched spots/total spots ratio). Considering the long sampling interval (3 months from mid January to mid April) and consequent leaf growth difference, this reproducibility may be acceptable. Furthermore, 2-D rehydration solution extraction of dry tissue powder (Fig. 3e) and omitting 10% TCA washes (Fig. 3f) all result in a poor resolution. Protein yields are maximized with phenol/SDS extraction, followed by dense SDS buffer extraction and phenol extraction (Fig. 4). The above results indicate the efficient removal of interfering compounds prior to electrophoresis by the protocol described here. The protocol can be completed within 3 h. By this protocol, 1.0 g of fresh aged olive leaf typically yields 0.20–0.25 g of dry tissue powder and approximately 2.49 mg protein (with phenol/SDS extraction).

In addition, we evaluate the protocol using immunoblotting. As an example, olive leaf extracts are immunoprobed with anti-tyrosinate α-tubulin monoclonal antibody. Consequently, the tyrosinate α-tubulin in olive leaf appears as a single band of about 55 kDa in 1-DE blot (Fig. 5a), the same size as those from other plants and

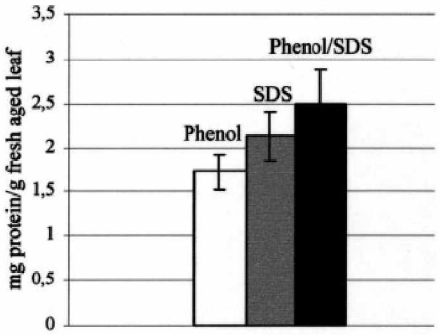

Figure 4. Protein yields of olive leaf from different extraction methods. Proteins in dry powder of aged olive leaf tissue are extracted by phenol/SDS buffer, phenol and dense SDS buffer, respectively. Protein is quantified by the Bio-Rad protein assay. Results (mg protein/g fresh leaf) are the mean values from three independent experiments.

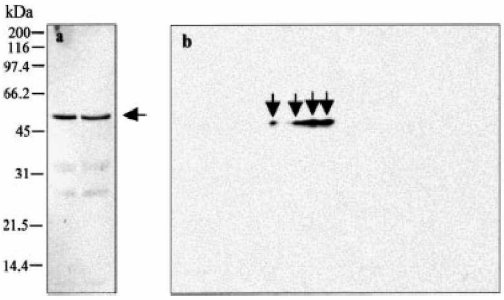

Figure 5. Immunoblotting tyrosinate α-tubulin in olive leaf extracts. Proteins in dry powder of olive leaf are extracted by phenol/SDS buffer mixture, separated with1-DE (a, 10 μg) or 2-DE (b, 25 μg), electroblotted, and probed with anti-tyrosinated α-tubulin. The arrow indicates the band or the isotypes of tyrosinate α-tubulin.

animals [17]; 2-DE immunoblot reveals the presence of at least four isotypes of tyrosinate α-tubulin in olive leaf tissue, with various p/s from 5.0–6.0 (Fig. 5b).

4 Discussion

4.1 Removal of nonprotein contaminants

Sample preparation is one of the most crucial, yet problematic, steps for high-quality resolution of proteins in 2-DE. Most problems can be traced to coextraction of nonprotein cellular components that can affect protein migrations [18]. Plant tissues are rich in compounds that interfere with 2-DE. These interfering compounds, *e.g.*, polyphenols, terpenes, and organic acids, mainly accumulate in vacuole in various soluble forms, and are more abundant in green tissues than in young seedlings or etiolated material [3]. Usually, two main strategies exist for removing these contaminants: (i) removal before protein extraction and (ii) removal after protein extraction. Olive leaf contains high levels of interfering compounds. Direct homogenization of olive leaf tissue in various extraction buffer, followed by organic solvent precipitation, always results in a brownish pellet difficult for further processing. Therefore, in the case of olive leaf, it is necessary to remove thoroughly the contaminants from leaf tissue prior to protein extraction.

Conventional removal of nonprotein contaminants involves the use of organic solvents (*e.g.*, acetone, 10% TCA in acetone) to wash contaminants out of tissue powder [6]. To a great extent the removal efficiency depends on the fineness of tissue powder. Based on our experience, the finer the powder, the more thoroughly the contaminant removal. Thus, rather than conventionally grinding plant tissue in liquid N_2 once, we further grind the dry acetone powder a second time. After the initial two acetone washes, the resultant air-dried tissue powder becomes quite fragile and thereby is easy to be ground to a finer powder. Another remarkable modification of our protocol is that, following 10% TCA/acetone washes, we utilize aqueous 10% TCA to remove water-soluble contaminants, otherwise which would copurify with proteins until the final step and could not be removed by phenol extraction afterwards. In our hands, the additional grinding and the aqueous TCA wash are proven to be very effective in removal of contaminants. After extensive organic solvent washes, the leaf tissue powder can be directly extracted in Laemmli buffer [10] for SDS-PAGE, but at this stage it still contains residual contaminants that would interfere with 2-DE (Fig. 3e). Therefore, phenol extraction is carried out to further minimize the presence of these contaminants in protein extracts.

4.2 Protein extraction

We extract proteins from dry powder of olive leaf with a mixture of phenol and dense SDS buffer. With 30% sucrose, the SDS extraction buffer is heavier than Tris-buffered phenol, so during phase separation the phenol phase is 'pushed' on top, which facilitates to take out the phenol phase. Phenol dissolves proteins (including membrane proteins) and lipids leaving water-soluble substances (carbohydrates, nucleic acids, *etc.*) in the aqueous phase, thus proteins in phenol phase are purified and concentrated together with subsequent methanol precipitation. Another advantage of phenol extraction is that it

minimizes protein degradation often encountered during sample preparation, due to endogenous proteolytic activity [19]. As shown in Fig. 5, anti-tyrosinate α-tubulin antibody detects a major band on 1-DE blot or four major spots on 2-DE blot around 55 kDa, and no degraded products from α-tubulin are detected, indicating that this protocol can effectively inhibit protein degradation *in vitro*. After phenol extraction, we prefer to precipitate proteins with cold methanolic ammonium acetate at −20°C for 30 min to shorten the protocol, since no significant differences in the protein yield and in the electrophoretic profiles are found among 30 min, 2 h and overnight incubation at −20°C (not shown).

SDS is an excellent solubilizing agent, which allows the recovery of membrane-bound proteins. We found that the phenol/SDS extraction is more powerful than dense SDS buffer and only phenol. Besides, the behavior of phase separation of the phenol/SDS mixture depends greatly on the SDS concentration. Under 5% SDS, the mixture separates approximately equal volume of phenol phase and aqueous phase after centrifugation; from 5–7% SDS, the volume of the phenol phase increases, and no phase separation occurs above 8% SDS. In addition, protein losses can be minimized during wash steps when the supernatant is pipetted out of microtubes instead of decanting the supernatant in the case of large centrifuge tubes. Therefore, though a number of wash steps are involved in this protocol, the final protein yield is relatively high (2.49 $mg \cdot g^{-1}$ fresh aged leaf). Unfortunately, the protein concentration in fresh aged leaf is not possibly determined due to the presence of interfering compounds.

4.3 Electrophoresis and immunoblotting

By the protocol described here we always obtained similar results in different batches of olive leaf protein extraction, with the 55 kDa polypeptide (Rubisco) being the most abundant component in 1-DE and 2-DE gels. Phenol/SDS extraction displays a high spot-to-spot match between different 2-DE gels of olive leaf proteins from two independent extractions (Fig. 2a and Fig. 3b). It is needed to note that the changes in spot intensities between Fig. 2a and Fig. 3b probably result form the different leaf growth because the leaves used were collected in mid January and mid April, respectively. Besides, the protocol allows us to present much more numbers of well-resolved polypeptide bands on 1-DE gel than previously reported by Garcia *et al.* [4]. We also noticed that the prominent polypeptide Rubisco is not always available in their leaf extracts [4], although this discrepancy could be due to different extraction methods and different genotypes used.

In the present study, we prefer to run mini-2-DE, with the first-dimensional IEF using 7 cm Immobiline DryStrip resolved in Multiphor II system and the second-dimensional SDS-PAGE using minigel run in a Bio-Rad mini-Protean II apparatus. The advantages of this combination include (i) rapid (3 h IEF, 1 h SDS-PAGE), (ii) convenient for downstream application (*e.g.*, gel stain and transfer), (iii) comparable patterns of gels and blots with 1-DE due to the same size, (iv) less protein loads but without significant loss of resolution, and finally (v) low cost compared to 2-DE using 11 or 18 cm Immobiline DryStrips.

Tubulin exists in all cells as a heterodimer of two similar but nonidentical polypeptides (*ca.* 55 kDa), designated as α and β, and functions as the major building block of microtubules. Both α- and β-tubulins consist of various isotypes and undergo post-translational modifications, including acetylation, tyrosination, detyrosination, polyglycylation, and polyglutamylation [20]. Therefore, we chose α-tubulin as an indicator to evaluate the immunoblots of olive leaf extracts prepared by our protocol. As in tobacco [21] and maize (paper in preparation), in olive leaf tissue α-tubulin is highly post-translationally modified with tyrosination and at least four isotypes of tyrosinate α-tubulin with similar p/s can be resolved on 2-D blot (Fig. 5b).

In summary, by the protocol described here we succeed in isolating high-quality proteins from aged and young leaf (data not shown) of olive trees. The resultant 1-DE and 2-DE gels and immunoblots have a high quality, free of smearing and streaking. The present study shows for the first time the well-resolved 1-DE and 2-DE protein patterns of olive leaf. The major drawback of the protocol is its relatively time-consuming (3 h). It is expected that our protocol could also be applied for other recalcitrant plant tissues, despite that different plant can vary considerably in the amounts and types of interfering compounds they produce.

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5 References

[1] Celis, J. E., Ostergaard, M., Jensen, N. A., Gromova, I., Rasmussen, H. H., Gromov, P., *FEBS Lett.* 1998, *430*, 64–72.

[2] Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., Aebersold, R., *Proc. Natl. Acad. Sci. USA* 2000, *97*, 9390–9395.

[3] Granier, F., *Electrophoresis* 1988, *9*, 712–718.

[4] Garcia, J. L., Avidan, N., Troncoso, A., Sarmento, R., Lavee, S., *Sci. Hort.* 2000, *85*, 271–284.

[5] Cremer, F., Van de Walle, C., *Anal. Biochem.* 1985, *147*, 22–26.

[6] Damerval, C., de Vienne, D., Zivy, M., Thiellemen, H., *Electrophoresis* 1986, *7*, 52–54.

[7] Colas des Francs, C., Thiellement, H., de Vienne, D., *Plant Physiol.* 1985, *78*, 178–182.

[8] Meyer, Y., Grosser, J., Chartier, Y., Cleyet-Marel, J. C. C., *Electrophoresis* 1988, *9*, 704–712.

[9] Flengsrud, R., Kobro, G. A., *Anal. Biochem.* 1989, *17733*–36.

[10] Laemmli, U. K., *Nature* 1970, *222*, 680–685.

[11] Bradford, M., *Anal. Biochem.* 1976, *72*, 248–254.

[12] Görg, A., Boguth, G., Obermaier, C., Posch, A., Weiss, W., *Electrophoresis* 1995, *16*, 1079–1086.

[13] Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., *Electrophoresis* 1988, *9*, 255–262.

[14] Towbin, H., Staehelin, T., Gordon, J., *Proc. Natl. Acad. Sci. USA* 1979, *76*, 4350–4354.

[15] Hubbs, A., Roy, H., *Plant Physiol.* 1992, *100*, 272–281.

[16] Saha, S., Callahan, F. E., Dollar, D. A., Creech, J. B., *J. Cotton Sci.* 1997, *1*, 10–14.

[17] Kreis, T. E., *EMBO J.* 1987, *6*, 2597–2606.

[18] Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, B., Weiss, W., *Electrophoresis* 2000, *21*, 1037–1053.

[19] Schuster, A. M., Davies, E., *Plant Physiol.* 1983, *73*, 809–816.

[20] MacRae, T. H., *Eur. J. Biochem.* 1997, *244*, 265–278.

[21] Del Casino, C., Li, Y.-Q., Moscatelli, A., Scali, M., Tiezzi, A., Cresti, M., *Biol. Cell* 1993, *79*, 125–132.

Summary (in italian)

Introduzione

Considerata l'importanza economica che riveste nella nostra regione la coltivazione dell'ulivo, sia per quanto riguarda la produzione di olive da tavola sia per la produzione di olio, la presente tesi di dottorato é volta a caratterizzare l'attività di una β-glucosidasi endogena, che converte l'oleuropeina, un biofenolo largamente accumulato nei tessuti del frutto, in residui agliconici e glucosio. Tali residui intervengono come diretti responsabili nei meccanismi di difesa contro agenti patogeni ed erbivori ed influenzano le caratteristiche organolettiche e sensoriali del prodotto agroalimentare.

La conoscenza delle dinamiche chimiche e temporali di queste molecole offrirà, quindi, interessanti informazioni da utilizzare nel miglioramento della qualità e della produttività del prodotto agroalimentare, rappresentando per la Calabria un elemento di traino nel settore delle esportazioni di materie prime agricole e dei prodotti derivati.

Le β-glucosidasi (EC 3.2.1.21), appartenenti alla famiglia 1 delle glucoidrolasi (GH 1, http://www.cazy.org/fam/GH1.html), sono enzimi ampiamente diffusi nel regno dei viventi procarioti ed eucarioti. Esse calatizzano l'idrolisi di aril e alchil-β-D-glucosidi come anche di glucosidi in solo mezzo carboidratico, come il cellobiosio. Dal momento che i β-glucosidi e le β-glucosidasi esistono dappertutto nel regno dei viventi ci si aspetta che ci sia una similitudine fra tutte le β-glucosidasi diverse per quel che riguarda la loro struttura e le loro proprietà catalitiche. Infatti, dalla letteratura si sa che quasi tutte le β-glucosidasi hanno più o meno lo stesso peso molecolare compreso fra 55 e 65 kDa, un optimum di pH acido (pH 5-6) ed una assoluta richiesta di β-glucosidi come substrato [1]. Le β-glucosidasi di ordini e regni diversi si

differenziano per la loro specificità all'aglicone che è legato con il gruppo glucoside tramite un legame β-glicosidico.

Le attuali ricerche sulle β-glucosidasi hanno assunto sempre più rilevante interesse scientifico, medico ed economico.

Ad esempio, una β-glucosidasi acida umana (glucocerebrosidasi) ha attualmente acquistato un potenziale terapeutico nel trattamento della sindrome di Gaucher e nei disordini ereditari causati dalla deficienza di questa β-glucosidasi acida localizzata nei lisosomi; una β-glucosidasi citosolica umana è implicata nel metabolismo della piridossina-5'-β-D-glucoside, così come nell'idrolisi dei β-glucosidi ingeriti con alimenti di origine animale e vegetale; applicazioni di sistemi β-glucosidasi/glucosidi cianogenici trovano positivo riscontro nella terapia antitumorale [2].

Tali enzimi hanno un grande interesse in campo economico sia perché le β-glucosidasi di funghi e batteri appaiono come candidati naturali per creare una β-glucosidasi ideale da utilizzare nella conversione della cellulosa in glucosio su scala industriale sia perchè risultano coinvolte nella modulazione delle qualità organolettiche e sensoriali di prodotti agroalimentari.

Le β-glucosidasi delle piante sono conosciute da oltre 170 anni dalla descrizione dell'azione dell'emulsina (β-glucosidasi di mandorlo) sull'amigdalina, una β-D-gentiobiside cianogenica, da parte di Liebig e Wöhler nel 1837 [3]. Solo negli ultimi vent'anni, però, è stato fatto un progresso considerevole nella biologia molecolare e nella biochimica di tali enzimi.

Nelle piante, le β-glucosidasi sono coinvolte in una varietà di eventi chiave del metabolismo ed in risposte relative alla crescita. Si passa dall'idrolisi di fitormoni coniugati e, quindi, all'attivazione degli ormoni stessi (ad esempio, glucosidi di gibberelline, auxine, acido abscissico e citochinine) al coinvolgimento nei processi di lignificazione fino alla difesa contro alcuni patogeni ed erbivori attraverso il rilascio di sostanze tossiche (quali cumarine, tiocianati, terpeni e cianidi) ed ai processi di maturazione del frutto.

In particolare, nella famiglia delle Oleaceae una β-glucosidasi endogena specifica per un fenolo secoiridoideo attiva un complesso meccanismo di difesa multichimico i cui prodotti mostrano una spiccata attività antiossidante e antibatterica. La validità dell'applicazione di queste nuove molecole bioattive in campo medico e farmacologico è testimoniata dai tentativi sempre più frequenti di purificare e stabilizzare i prodotti della reazione enzimatica attraverso bioreattori catalitici che utilizzano β-glucosidasi di mandorlo e dall'archeobatterio *Sulfolobus solfataricus* over-espresso in *E. coli*. Questi enzimi, pur dimostrando una buona specificità per il substrato oleuropeina, limitano la produzione dei suoi derivati aldeidici insaturi, gli unici che hanno dimostrato una forte azione antibatterica *in vitro*. Da qui la necessità di disporre dell'enzima β-glucosidasi nativo che troverebbe applicazioni biotecnologiche nella produzione di piante transgeniche resistenti e nella produzione delle molecole ad elevata attività biologica con vaste applicazioni in campo industriale nella produzione degli agroalimenti ed in applicazioni farmaceutiche.

Il sistema β-glucosidasi/oleuropeina nelle Oleaceae

L'ulivo rappresenta una delle colture più importanti, in termini di produzione alimentare, nei paesi mediterranei. La notevole diffusione dell'ulivo dall'antichità ad oggi dimostra l'importanza assunta da questa coltura nell'alimentazione; la ragione di questo successo è dovuta all'individuazione degli effetti positivi sulla salute umana delle olive e, specialmente, dell'olio di oliva. Molti studi hanno mostrato che la dieta integrata quotidianamente con olio di oliva favorisce un abbassamento del colesterolo "cattivo" (LDL) ed un contestuale innalzamento di quello "buono" (HDL) aiutando a prevenire le malattie cardiovascolari; offre, inoltre, benefici in termini di prevenzione di alcune forme tumorali, stimola la mineralizzazione delle ossa e l'assimilazione dei sali minerali di primaria importanza, limita gli effetti dell'invecchiamento cellulare e mostra attività antinfiammatorie [4]. Comunque, tutti questi effetti

sono il risultato di livelli più alti dei composti antiossidanti dell'ulivo, particolarmente fenoli, nel sangue [5-7]. Sfortunatamente molti problemi influenzano la coltivazione dell'ulivo con conseguenze sulla qualità e sulla quantità della produzione. Le avversità dell'ulivo comprendono sia malattie causate da parassiti, quali funghi, batteri, virus e fitoplasmi, che danni provocati da fitofagi (prevalentemente insetti).

L'infestazione di *Bactrocera oleae*, ritenuto il fitofago più pericoloso e dannoso tra tutti i parassiti dell'ulivo, per esempio, causa grossi danni nei frutti e altera la qualità dei parametri dell'olio di oliva, inducendo una diminuzione della frazione fenolica e dell'attività antiossidante ed arrivando, quindi, a condizionare sensibilmente l'entità e la qualità della produzione. Comunque, le *cultivars* di ulivo hanno mostrato un differente grado di suscettibilità all'infestazione il cui range va da alta suscettibilità (più del 10% di frutti infestati), bassa suscettibilità (3% di frutti infestati) e resistenza (meno dello 0.25% di frutti infestati), che non è influenzato dalle condizioni di coltura, suggerendo in questo modo che la suscettibilità o la resistenza hanno una fonte genetica determinata [8].

Malgrado le numerose ricerche sul controllo degli agenti infestanti, poche informazioni sono disponibili sul pattern di difesa costitutivo in *Olea europaea*. Le molecole di difesa nell'ulivo sono i fenoli sintetizzati ed accumulati nei tessuti dei frutti durante la crescita e la maturazione [9].

Molti generi presenti nella famiglia delle Oleaceae sono caratterizzati, infatti, dall'accumulo di metaboliti secondari contenuti nelle cellule e concentrati soprattutto nei tessuti delle foglie e dei frutti in maturazione. Queste molecole sono caratterizzate da gruppi funzionali orto-difenolici. Il principale componente fenolico è il glicoside secoiridoideo, cumarino-simile, conosciuto come oleuropeina. L'oleuropeina è prodotta come composto secondario del metabolismo dei terpeni come precursore di vari indol-alcaloidi. I composti secoiridoidei sono derivati da glucosidi del tipo oleoside che sono caratterizzati dalla funzionalità 8,9-olefinica, una combinazione di residui di acido enolico e residui glucosidici. L'oleuropeina è presente in alte quantità (60-90 mg/g peso

secco) nelle foglie dell'ulivo [10], anche se è stata trovata in tutto l'albero, incluso i frutti [11]. Al momento sia la qualità che la quantità di composti fenolici e secoiridoidei e dei loro derivati risulta essere ben documentata in *O. europaea* [12-14] come anche la variazione dipendente dalla varietà, dalla stagione, dallo stadio di maturazione del frutto e dall'età della foglia [9, 15, 16, 17]. L'oleuropeina è la molecola responsabile del tipico sapore amaro e pungente che caratterizza frutti e foglie delle Oleaceae [18]. Tale principio amaro diminuisce significativamente durante la maturazione del frutto e nella senescenza foliare e deve essere attenuato o eliminato nei prodotti agroalimentari derivanti da essa. Perciò, recentemente l'interesse dell'industria agro-alimentare si è rivolto alla chimica dei composti biofenolici della drupa attraverso lo studio della trasformazione molecolare dell'oleuropeina.

Analogamente ad altri β-glucosidi vegetali, l'oleuropeina è una molecola chiave nel meccanismo di difesa attuato dalle Oleaceae in seguito ad attacco da fitofago o da agente patogeno [19, 20]; tale molecola, inoltre, è responsabile dell'attività antiossidante ed antimicrobica [21]. La molecola originale fenolica di oleuropeina non è efficace contro *S. cervisiae, B. subtilis* ed *E. coli*, eccetto in presenza di β-glucosidasi a pH 7. Infatti, nelle olive verdi è attuato un meccanismo di difesa multichimico in seguito all'attivazione enzimatica da parte di una β-glucosidasi specifica della porzione secoiridoidea dell'oleuropeina che possiede una forte attività alchilante e *cross-linking* proteico. In particolare, quando i tessuti dell'ulivo sono colpiti da patogeni o da danno meccanico, la β-glucosidasi, idrolizza in maniera specifica l'oleuropeina producendo molecole altamente reattive [22]. Bianco ha identificato come nuovi derivati bioattivi dell'oleuropeina un emiacetale agliconico e due aldeidi epimeriche. Queste molecole mostrano una forte azione antibatterica verso un numero di colonie batteriche agenti causali di malattie intestinali e respiratorie umane. I derivati bioattivi dell'oleuropeina sono stati identificati *in vivo* come fitoalessine prodotte in seguito ad attacco patogeno.

In normali condizioni fisiologiche deve attuarsi, quindi, nei tessuti del frutto la regolazione della degradazione dell'oleuropeina. Da diversi studi condotti in numerose piante superiori la β-glucosidasi è stata localizzata in comparti cellulari differenti da quello del suo substrato [23-26] incluse specie appartenenti alle Oleaceae [27] con diversi modelli di compartimentalizzazione. In queste piante il sistema β-glucosidasi/substrati glicosidici è finalizzato alla risposta di difesa dall'attacco di erbivori e agenti patogeni e la loro diversa localizzazione sembra essere fisiologicamente importante, in quanto la maggior parte dei prodotti di idrolisi risulta tossica anche per le cellule.

Scopo della tesi

In tale contesto, scopo del presente lavoro di dottorato è quello di studiare, attraverso saggio *in situ* dell'attività β-glucosidasica e analisi biochimiche

iii. il meccanismo che regola l'espressione dell'enzima e la sua attività nei confronti dell'oleuropeina in relazione allo stadio di sviluppo e maturazione del frutto;
iv. il pattern dell'attività della β-glucosidasi ed il contenuto di oleuropeina nei tessuti del frutto in risposta ad un attacco patogeno simulato in due *cultivars* che presentano una diversa suscettibilità alla mosca [8].

I dati ottenuti sono stati necessari per determinare a quale stadio di maturazione gli estratti dei frutti presentavano la più alta attività enzimatica, in modo da frazionare e purificare gli estratti in cui l'enzima risultava maggiormente espresso mediante tecnologia a membrana.

In questo contesto l'enzima purificato è stato utilizzato per sviluppare un reattore a membrana biocatalitico così da consentire l'idrolisi dell'oleuropeina in grado di produrre molecole bioattive.

Risultati e discussione

Localizzazione della β-glucosidasi durante la maturazione del frutto di ulivo (*Olea europaea*) attraverso saggio *in situ* dell'attività

La localizzazione *in situ* dell'attività β-glucosidasica è eseguita tramite l'idrolisi dell'X-Glc, un substrato cromogenico specifico per la famiglia GH 1. Un'intensa reazione è stata rilevata nel nucleo, nei cloroplasti e nelle goccioline lipidiche del citoplasma. Gli enzimi che si trovano nei cloroplasti e nelle goccioline lipidiche hanno mostrato un'alta specificità per l'oleuropeina esogena rispetto a quella mostrata verso l'X-Glc nel saggio competitivo *in situ*, indicando che in questi compartimenti cellulari sono presenti due diverse β-glucosidasi che degradano l'oleuropeina. Il saggio è stato realizzato a diversi stadi di maturazione del frutto durante i quali sono state osservate significative variazioni nel numero e nella distribuzione delle cellule reattive nei tessuti del mesocarpo. Infatti, nei frutti immaturi (50 giorni dopo l'antesi) l'attività enzimatica non è stata rilevata nelle cellule del mesocarpo esterno, mentre alcuni nuclei del mesocarpo interno hanno mostrato reazione positiva al saggio. Nei frutti verdi maturi (80-120 giorni dopo l'antesi), invece, è stato rilevato un gran numero di cellule reattive distribuite nell'intero mesocarpo, anche se l'attività β-glucosidasica è stata localizzata preferenzialmente nelle cellule del mesocarpo esterno. Infine, nei frutti invaiati (dopo circa 160 giorni dall'antesi) sono state localizzate numerose cellule reattive solo nel mesocarpo interno in prossimità dell'endocarpo legnoso, mentre l'attività non è stata rilevabile nelle cellule del mesocarpo esterno, deputate all'accumulo di antocianine.

Questi risultati hanno mostrato che le variazioni dell'attività delle β-glucosidasi che degradano l'oleuropeina durante la maturazione sono dovute a

cambiamenti nella competenza delle singole cellule del mesocarpo a sintetizzare le isoforme enzimatiche.

Dato che l'oleuropeina è contenuta nei vacuoli delle cellule del mesocarpo di ulivo [28], la scoperta dell'attività β-glucosidasica nei cloroplasti delle cellule del mesocarpo, indica che enzima e substrato sono tenuti in compartimenti cellulari diversi. Il danneggiamento di cellule e tessuti attraverso la puntura degli insetti mette in contatto la β-glucosidasi con l'oleuropeina con il conseguente rilascio di agliconi tossici, che agiscono da deterrenti per gli erbivori o inibiscono l'ingresso, la crescita e la diffusione di fitopatogeni [1, 27, 29].

Tali risultati indicano che nelle drupe verdi la deamarizzazione, dovuta all'idrolisi dell'oleuropeina, non avviene perché enzima e substrato non entrano in contatto, in quanto si trovano in compartimenti separati delle cellule, nonostante le rispettive concentrazioni nei tessuti siano molto alte. Al contrario, nelle drupe verdi-invaiate, la deamarizzazione è causata dal danneggiamento delle strutture cellulari, che consente il contatto tra β-glucosidasi e oleuropeina.

Attività β-glucosidasica oleuropeina specifica in frutti di ulivo (*Olea europaea*) in seguito ad attacco mimato di insetto

Il saggio è stato eseguito in due *cultivars* che mostrano una diversa suscettibilità all'infestazione di *Bactrocera olaea*: Carolea, caratterizzata da un'alta suscettibilità (10.25% di infestazione, 12.87% punture sterili) e Cassanese, che mostra una bassa suscettibilità (3.62% di infestazione, 13.75% punture sterili). In entrambe le *cultivars*, il saggio *in situ* ha mostrato che, nei primi 20 min dal danno, una forte attività enzimatica può essere osservata nei tessuti danneggiati. In seguito si è avuta una progressiva disattivazione dell'enzima a partire dai tessuti intorno al limite del danno e l'attività enzimatica scompare 60 min dopo il danneggiamento. La perdita di attività si è arrestata dopo 3 h, mentre il limite delle cellule attive ha raggiunto la distanza di 300±50

μm dal limite del danno. Analisi biochimiche hanno mostrato che negli estratti dei frutti danneggiati l'attività β-glucosidasica aumenta nei primi 20 min dal danno, successivamente diminuisce, raggiungendo valori comparabili con quelli dei frutti non danneggiati. In seguito al danno di *B. oleae*, il contenuto di oleuropeina non ha subito cambiamenti significativi nelle *cultivars* ad alta suscettibilità, mentre è diminuito rapidamente nelle *cultivars* che mostrano bassa suscettibilità. Tali risultati suggeriscono che la suscettibilità verso l'infestazione della mosca potrebbe essere correlata all'abilità della β-glucosidasi che degrada l'oleuropeina a produrre molecole altamente reattive nei tessuti danneggiati. Come conseguenza del danno è stata rivelata anche una forte attività perossidasica suggerendo che anche questo enzima potrebbe giocare un ruolo chiave nella risposta di difesa.

Purificazione della β-glucosidasica dagli estratti di frutto di *Olea europaea* attraverso tecnologia a membrana

La purificazione della β-glucosidasi da ulivo è di notevole interesse per le sue applicazioni in campo alimentare e farmaceutico. La purificazione di tale proteina è stata già sperimentata ricorrendo all'utilizzo dell'elettroforesi, ma senza ottenere grossi successi. L'uso della tecnologia a membrana per separare molecole labili da estratti complessi offre un'attrattiva alternativa visto che tali metodi consentono di preservare l'attività enzimatica; perciò si è deciso di ricorrere a tale tecnologia per frazionare e purificare gli estratti proteici dei frutti di *Olea europaea* in modo da migliorare la purezza della β-glucosidasi e preservare le sue proprietà catalitiche.

Gli estratti proteici utilizzati per la diafiltrazione sono stati ottenuti da frutti provenienti dallo stadio di maturazione nel quale la β-glucosidasi è maggiormente espressa. Sono state usate membrane asimmetriche costituite da poliammide o polisulfone aventi *cut-off* rispettivamente di 50 e 30 kDa. Le

membrane per l'ultrafiltrazione sono costituite di polietersulfone con *cut-off* di 4 kDa. L'efficienza dei processi di separazione è stata valutata tramite test dell'attività usando il *p*-D-nitrofenil-β-D-glucopiranoside (pNPGlc) come modello di reazione. Sono state eseguite elettroforesi qualitative e quantitative per analizzare la composizione della soluzione proteica prima e dopo il processo di separazione, inoltre sono state effettuate analisi di dot blot e western blot per verificare la presenza della β-glucosidasi nelle frazioni trattate.

Tale processo ha permesso di separare ed identificare una forma enzimatica con peso molecolare di 65 kDa individuata come β-glucosidasi putativa tramite analisi western blot e saggio dell'attività enzimatica e una proteina monomerica che negli estratti nativi si presenta sotto forma di complessi di peso molecolare più alto. La diafiltrazione è risultata una metodologia capace di garantire la stabilità proteica. Infatti, la purificazione ha permesso di preservare la stabilità dell'enzima e di aumentare la sua attività specifica più del 52% durante la diafiltrazione con membrane di PA di 50 kDa confrontata all'attività iniziale.

Le membrane di 30 kDa di PS non sono risultate adatte per la purificazione degli estratti.

Immunolocalizzazione della β-glucosidasi immobilizzata su una membrana capillare di polisulfone e valutazione della sua attività *in situ*

È stato messo a punto un nuovo metodo combinato per localizzare i siti dove è immobilizzato l'enzima e determinare simultaneamente la distribuzione spaziale dell'enzima attraverso la membrana e la sua attività dopo l'immobilizzazione. determinare la sua attività catalitica su un reattore a membrana capillare. Il nuovo utile metodo è risultato dalla fusione del classico saggio *in situ* dell'attività enzimatica e della tecnica del western blot, in cui i risultati sono facilmente rilevabili in microscopia ottica. La β-glucosidasi è stata immobilizzata tramite metodi fisici su membrane capillari di polisulfone aventi

cut-off di 30 kDa. Le sezioni di membrane, processate per il saggio *in situ* con il substrato sintetico 5-bromo-4-cloro-3-indolil-β-D-glucopiranoside, hanno mostrato una colorazione uniforme dovuta ai prodotti di reazione. Per verificare che la distribuzione del colore non fosse dovuta alla diffusione del prodotto, ma corrispondeva alla presenza dell'enzima immobilizzato, è stato sviluppato un metodo per localizzare specificatamente la β-glucosidasi utilizzando un anticorpo policlonale.

I risultati hanno rivelato una coerente localizzazione enzimatica e del suo prodotto insolubile di idrolisi attraverso tutta la membrana che indica che l'intrappolamento fisico, tramite ultrafiltrazione, ha consentito alle macromolecole dell'enzima di penetrare attraverso il modulo della membrana preservando l'attività catalitica. Questo semplice metodo può essere applicato a tutti gli enzimi che hanno a disposizione un substrato capace di sviluppare prodotti colorati e che hanno uno specifico anticorpo.

Tale approccio può essere utile per ottimizzare le tecniche di immobilizzazione enzimatica, per valutare il contenuto di enzima in una soluzione proteica non pura, per sviluppare reattori a membrana su larga scala.

Bibliografia

1. A. Esen (1993). β-Glucosidases: Biochemistry and Molecular Biology. Edited by A. Esen. *American Chemical Society*, Washington, 1-14.

2. G. Grabowski, A. Berg-Fussman, M. Grace (1993). Molecular biology and enzymology of human acid β-glucosidase. In β-Glucosidases: Biochemistry and Molecular Biology. Edited by A. Esen, *American Chemical Society*, Washington, 66-77.

3. J. Liebig, F. Wöhler (1837). *Annalen*, **22**: 11-14.

4. H.K. Hamdi, R. Castellon (2005). Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. *Biochemical and Biophysical Research Communications*, **334** (3): 769-778.

5. A. Keys, A. Menotti, M.J. Karvonen (1986). The diet and 15-year death rate in the Seven Countries Study. *American Journal of Epidemiology*, **124**: 903-915.

6. W.C. Willett (1990). Diet and coronary heart disease. *Monographs in Epidemiology and Biostatistics*, **15**: 341-379.

7. World Health Organization (1990). Diet, nutrition, and the prevention of chronic diseases. Report of a WHO Study Group. *WHO Technical Report Series*, 797.

8. N. Iannotta, L. Perri, C. Tocci, F. Zaffina (1999). The behaviour of different olive *cultivars* following attacks by *Bactrocera oleae* (Gmel.) *Acta Horticulturae*, **474**: 545-548.

9. M.J. Amiot, A. Fleuriet, J.J. Macheix (1989). Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry*, **28**: 67-69.

10. B. Le Tutour, D. Guedon (1992). Antioxidative activities of *Olea europaea* leaves and related phenolic compounds. *Phytochem*, **31** (4): 1173-1178.

11. M. Servili, M. Baldioli, R. Selvaggini, A. Macchioni, G.F. Montedoro (1999). Phenolic compounds of olive fruit: one- and two-dimensional nuclear magnetic resonance characterization of nüzhenide and its distribution in the constitutive parts of fruit. *J. Agric. Food Chem.,* **47**: 12-19.

12. P. Ficarra, R. Ficarra, A. De Pasquale, M.T. Monforte, M.L. Calabro (1991). HPLC analysis of oleuropein and some flavonoids in leaf and bud of *Olea europaea* L. *Farmaco*, **46** (6): 803-15.

13. A.D. Bianco, I. Muzzalupo, A. Piperno, G. Romeo, N. Uccella (1999). Bioactive derivatives of oleuropein from olive fruits. *J. Agric. Food Chem.*, **47**: 3531-3534.

14. C. Savourin, B. Baghdikian, R. Elias, F. Dargouth-Kesraoui, K. Boukef, G. Balansard (2001). Rapid high-performance liquid chromatography

analysis for the quantitative determination of oleuropein in *Olea europaea* leaves. *J. Agric. Food Chem.*, **49** (2): 618-21.

15. D. Ryan, P.D. Prenzler, S. Lavee, M. Antolovich, K. Robards (2003). Quantitative changes in phenolic content during physiological development of the olive (*Olea europaea*) cultivar Hardy's Mammoth. *J. Agric. Food Chem.*, **51** (9): 2532-2538.

16. R. Briante, M. Patumi, S. Limongelli, F.C. Febbraio, A. Vaccaio, F. Di Salle, R. La Cara, M. Nucci (2002). Changes in phenolic and enzymatic activities content during fruit ripening in two Italian *cultivars* of *Olea europaea* L. *Plant Sci.*, **162**: 791-798.

17. M. Esti, L. Cinquanta, E. La Notte (1998). Phenolic Compounds in Different Olive Varieties. *J. Agric. Food Chem.*, **46** (1): 32-35.

18. C. Soler-Rivas, J.C. Espin, H.J. Wichers (2000). Oleuropein and related compound. *J. Sci. Food Agric.*, **80**: 1013-1023.

19. A.G. Báidez, P. Gómez, J.A. Del Río, A. Ortuño (2007). Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. *Journal of Agriculture Food Chemistry*, **55**: 3373-3377.

20. A. Kubo, C.S. Lunde, I. Kubo (1995). A multichemical defence mechanism of bitter olive *Olea europaea* (Oleaceae). Is oleuropein a phytoalexin precursor? *J. Chem. Ecol.*, **11**: 251-263.

21. G. Bisignano, M.G. Laganà, D. Trombetta, S. Arena, A. Nostro, N. Uccella, G. Mazzanti, A. Saijia (2001). *In vitro* antibacterial activity of some aliphatic aldehydes from *Olea europaea* L. FEMS *Microbiol. Lett.*, **198**: 9-13.

22. A.D. Bianco, A. Piperno, G. Romeo, N. Uccella (1999). NNMR experiments of oleuropein biomimetic hydrolysis. *J. Agric. Food Chem.* **47**: 3665-3668.

23. P. Kakes (1993). Function and variation of the β-glucosidase linamarase in natural population of *Trifolium repens*. In β-glucosidases: Biochemistry and Molecular Biology. Edited by A. Esen, *American Chemical Society*, Washington, 143-152.

24. M. Kojina, J.E. Poulton, S.S. Thayer, E.E. Conn (1979). Tissue distribution and enzyme involved in its metabolism in leaves of *Sorghum bicolour*. *Plant Physiol*., **63**: 1022-1028.

25. Y. Minami, H. Takao, T. Kanafuji, K. Miura, M. Kondo, I. Hara-Nishimura, M. Nishimura, H. Matsubara (1997). β-glucosidase in the indigo plant: intracellular localization and tissue specific expression in leaves. *Plant Cell Physiol*. **38**: 1069.

26. S.S. Thayer, E.E. Conn (1981). Subcellular localization of dhurrin β-Glucosidase and hydroxynitrile lyase in the mesophyll cells of *Sorghum* leaf blades. *Plant Physiol.* **67**(4): 617-622.

27. K. Konno, C. Hirayama, H. Yasui, M. Nakamura (1999). Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defence in the privet tree. *Proc. Natl. Acad. Sci.* U.S.A., **96**: 9159-9164.

28. M.B. Bitonti, A. Chiappetta, A.M. Innocenti, I. Muzzalupo, N. Uccella (1999). Funzionalita` e distribuzione dei biofenoli nella drupa di *Olea europae*a L. OLIVO OLIO ½: 20-29.

29. S. Mazzuca, N. Uccella (2002). β-glucosidase releasing of phytoalexin derivatives from secobiophenols as defence mechanism against pathogenic elicitors in olive drupes. *Acta Hort.* **586**: 529-531.

ALLEGATI

OTHER PUBLICATIONS FROM 2004 TO 2007

S. Mazzuca, **A. Spadafora**, D. Filadoro, M. Marsoni, C. Vannoni, M. Bracale, A.M Innocenti (2007). Differential protein expression in *Posidonia oceanica* leaves acclimated to low light. *Journal of Experimental Marine Biology and Ecology*. Resubmitted after revision.

A. Spadafora, D. Filadoro, S. Mazzuca, M. Bracale, M. Marsoni, A.M. Innocenti (2007). 2-DE polypeptides mapping of Posidonia oceanica leaf, a molecular tool for marine environment analyses. *Plant Biosystems*. 142, XXX.

R. Cozza, A. Chiappetta, S. Mazzuca, M. Petrarulo, F. Rende, A. Salimonti, **A. Spadafora**, M.B. Bitonti, A.M. Innocenti (2005). Approccio multifattoriale per la valutazione dello stato di conservazione della praterie di *Posidonia oceanica*. I*nformatore Botanico Italiano* vol 37, 706-708.

ATTENDANCE AT CONGRESS FROM 2004 TO 2007

S. Mazzuca, **A. Spadafora**, D. Filadoro, M. Bracale, M. Marsoni, A.M Innocenti. Proteasome as biomarker for acclimation to light stress in Posidonia oceanica. 102° Congresso Società Botanica Italiana, 26-29 settembre 2007, Palermo (coauthor in oral communication).

S. Mazzuca, **A. Spadafora**, D. Filadoro, M. Bracale, M. Marsoni, A.M Innocenti. The Proteomic approach in Posidonia oceanica population: which protein biomarkers for light stress acclimation? 3RD Mediterranean symposium on marine vegetation. 27-29 March 2007, Marseilles (coauthor in oral communication).

A. Spadafora, D. Filadoro, A. M. Innocenti, E. Perri, S. Mazzuca. Caratterizzazione della β-glucosidasi, un enzima chiave nei meccanismi di difesa contro agenti patogeni ed erbivori in *Olea europaea*. Convegno nazionale "Ricerca ed innovazione per l'olivocoltura meridionale (RIOM)", 30 novembre-1 dicembre 2006, CRA - Istituto Sperimentale per l'Olivicoltura - Rende (CS).

S. Mazzuca, **A. Spadafora**, D. Filadoro, A. Parise, E. Perri, A. M. Innocenti. Localizzazione cellulare e tissutale della β-glucosidasi nella maturazione delle drupe e nel danno meccanico. Convegno nazionale "Ricerca ed innovazione per l'olivocoltura meridionale (RIOM)", 30 novembre-1 dicembre 2006, CRA - Istituto Sperimentale per l'Olivicoltura - Rende (CS).

S. Mazzuca, **A. Spadafora,** D. Filadoro, M. Bracale, M. Marsoni, A.M Innocenti. Protein pattern and 2-DE polypeptides mapping in *Posidonia oceanica* subjected to different environment condition. Marine Genomics an internetional conference, 28 ottobre-1 novembre 2006, Sorrento.

S. Mazzuca**, A. Spadafora**, D. Filadoro, M. Bracale, M. Marsoni, F. Rende, A.M. Innocenti (2005). Proteins extraction from different tissues and organs of *Posidonia oceanica* and polypeptide mapping by two-dimensional

electrophoresis. Mediterranean Seagrass Workshop, 29th May to 3th June 2006, Malta.

A. De Nino, L. Di Donna, S. Mazzuca, R. Dalpozzo, A. Sajjad, G. Sindona, **A. Spadafora** (2005). Determinazione e caratterizzazione di proteine e peptidi in olio extravergine d'oliva mediante elettroforesi su gel e spettrometria di massa MALDI-TOF. XIX Congresso Nazionale di Chimica Analitica. 11-15 Settembre, Polaris, Parco Scientifico e Tecnologico della Sardegna Pula, Cagliari, Sardegna.

S. Mazzuca, **A. Spadafora**, M. Bracale, M. Marsoni, R. Cozza, F. Rende, A. Chiappetta, A.M. Innocenti (2004). Analisi proteomica e morfocitologica in popolazioni di *Posidonia oceanica* in relazione al disturbo ambientale. Convegno Conisma-Aiol, 18-22 ottobre, Terrasini.

S. Mazzuca, **A. Spadafora**, V. Pignataro (2004). Variazione del pattern proteico in popolazioni di *Posidonia oceanica*. Riunione Congiunta Gruppi di Lavoro Biologia Cellulare e Molecolare e Biotecnologie e Differenziamento, 23-25 giugno, Belgirate .

A. Spadafora, W. Wang, S. Mazzuca (2004). Espressione tessuto-specifica e localizzazione sub-cellulare dell'enzima β-glucosidasi in foglie e frutti di *Olea europaea*. Riunione Congiunta Gruppi di Lavoro Biologia Cellulare e Molecolare e Biotecnologie e Differenziamento, 23-25 giugno, Belgirate .