Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 416–425
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

The diversity of pathogenesis-related proteins decreases during grape maturation

Sara Monteiro [a,b,*], Maria A. Piçarra-Pereira [b,c], Virgílio B. Loureiro [b], Artur R. Teixeira [b], Ricardo B. Ferreira [a,b]

[a] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal*
[b] *Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisboa, Portugal*
[c] *Escola Superior Agrária, Instituto Politécnico de Castelo Branco, 6001-909 Castelo Branco, Portugal*

Received 24 July 2006; received in revised form 9 October 2006
Available online 26 December 2006

Abstract

It was recently shown that wines contain typically a huge diversity of structurally similar polypeptides that exhibit a high degree of homology to pathogenesis-related (PR) proteins. This observation suggested the existence of one or a few precursors in mature grapes, common to most or all the wine PR proteins. Limited proteolysis and chemical modification of the precursor(s) during fruit ripening and winemaking could then generate the large number of distinct wine polypeptides. However, the patterns of PR proteins extracted from grape berries regularly harvested from the onset of development until maturity did not confirm the previous hypothesis. Two different methodologies, involving 2-D immunoblotting and a combination of FPLC cation/anion exchange chromatographies with 1-D immunoblotting, indicate that the total concentration of PR proteins is increased but its diversity is reduced from the early stages of berry development until maturity. These results indicate that PR proteins are synthesized in a wide variety of forms from the early stages of grape development, eliminating the hypothesis previously formulated on the existence of one or few precursors common to the wine proteins.

Keywords: *Vitis vinifera*; Vitaceae; Grapevine; Wine; Grape berries; PR proteins

1. Introduction

Pathogenesis-related (PR) proteins are generally considered as plant defence proteins, functioning in preventing or limiting pathogen multiplication and/or spread. Fourteen different classes of structurally and functionally unrelated proteins have been considered, numbered PR-1 to PR-14 (van Loon, 1999), some of which have been detected in grapevine (*Vitis vinifera*) (Ferreira et al., 2004). These include PR-5 (thaumatin-like proteins and osmotins), which are thought to create transmembrane pores and have therefore been termed permatins; PR-2 (β-1,3-glucanases) and PR-3, -4, -8 and -11 (chitinases), which attack β-1,3-glucans and chitin, respectively, components of the cell walls in most higher fungi (Ferreira et al., 2004).

PR proteins accumulate in grapes during the growing season (Tattersall et al., 2001). They are synthesized in healthy grape berries in a developmentally dependent manner as a normal part of the ripening process, with véraison (the French term used by viticulturalists to denote the inception of ripening) apparently being the trigger for PR gene expression (Van de Rhee et al., 1994; Ferreira et al., 2002). There is a significant increase in total grape protein content after véraison, with only a small number of proteins being synthesized in significant amounts during ripening (Tattersall et al., 1997). The two most prominent soluble proteins accumulated in grapes during ripening have been identified as chitinase and thaumatin-like proteins (Robinson and Davies, 2000), with chitinase alone

* Corresponding author. Fax: +351 213653238.
E-mail address: smonteiro@isa.utl.pt (S. Monteiro).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.11.014

being reported to account for half of the soluble protein in ripe grapes (Waters et al., 1998).

In addition, PR proteins are also induced in pre-véraison berries in response to environmental and stress-related stimuli, such as wounding, chemical elicitors, pathogen attack or abiotic stress, by the expression of specific PR genes (Jacobs et al., 1999; Robinson and Davies, 2000; Monteiro et al., 2003a,b). Taken together, these processes modulate the levels and proportions of the PR proteins in grapes, so that the environmental conditions prevailing during vegetative growth determine the precise pattern of major polypeptides that accumulate in mature berries (Monteiro et al., 2003a,b).

Given the physiological role attributed to PR proteins, genetically modifying vines in order to overexpress these proteins has been reported to obtain plants with enhanced resistance to pathogens (Ferreira et al., 2004). However, due to their inherent resistance to proteolytic attack and to the low pH values characteristics of musts and wines, vinification may be considered as a purification strategy for the grape PR proteins (Ferreira et al., 2002). The consequent accumulation of the grape PR proteins in wines is a technological nuisance because they greatly affect the clarity and stability of wines (Ferreira et al., 2002).

A simple electrophoretic analysis of the wine proteins detects the presence of only a few major polypeptides, ranging in molecular mass from 15 to 30 kDa. However, a more detailed examination of the whole protein fraction, by a combination of techniques including cation/anion exchange chromatographies or two-dimensional electrophoresis, reveals that wines contain, typically, a very large number (many tens and, possibly, many more) of distinct polypeptides, exhibiting similar molecular masses but different electrical charges. Most of these polypeptides are structurally similar and exhibit a high degree of homology to PR proteins (Monteiro et al., 2001).

All these observations suggest the existence of one or a few precursors in the grape, common to most or all the wine proteins, which could generate during grape maturation and/or winemaking all the detected polypeptides by limited proteolysis and/or chemical modification. For this reason, the initial purpose of this work was to locate the presence of such putative precursors at the maturing stage of the grape berry where they first appear, to isolate them and to study the proteolytic process that leads to the structural diversity observed in the wine proteins. To this end, using the total wine protein as a control, the total soluble protein was extracted from grapes at different stages of development, from post-flowering to maturity. This was a surprisingly difficult task due to the high content of secondary metabolites in the berries during most of their developmental stages. Antibodies highly specific for the grape PR proteins were subsequently employed to detect the pattern of synthesis and accumulation of these proteins during berry development.

2. Results

2.1. Specificity of the polyclonal antibodies for the grape PR proteins

Polyclonal antibodies were developed in rabbits against a highly purified 20 kDa polypeptide extracted from Assario wine as previously described (Monteiro et al., 1999). These antibodies were shown to be highly specific for the major 20 kDa Assario wine polypeptide (Monteiro et al., 2001). Two abundant polypeptides from Assario wine were selected and subsequently purified by a combination of FPLC cation/anion exchange chromatographies. These polypeptides (Fig. 1a) are recognized by the anti-20 kDa Assario polypeptide antibodies (Fig. 1b) and were identified by N-terminal sequencing as grape osmotin (Figs. 1a and b, lane 1) and grape thaumatin-like protein (Figs. 1a and b, lane 2; Table 1). In fact, the anti-20 kDa Assario polypeptide antibodies were found to specifically recognize a very wide diversity of structurally similar proteins that are present in grapes and wines, prepared from white or red varieties, regardless of the variety, year or region (Ferreira et al., 2000). This very wide diversity of structurally similar proteins, or protein isoforms, explains the multiple bands visible on the western blot shown in Fig. 1b (Monteiro et al., 2001). N-Terminal sequencing analyses revealed that these are PR proteins with high sequence homologies with osmotin, thaumatin-like protein and chitinase (Monteiro et al., 2001).

2.2. Two-dimensional analysis of the pattern of total grape PR proteins from post-flowering to maturity

After establishing that the anti-wine protein antibodies were highly specific towards the grape PR proteins, our attention moved towards the pattern of synthesis and accumulation of PR proteins during grape development. To this end, *Vitis vinifera* L. (cv Moscatel) grapes were regularly

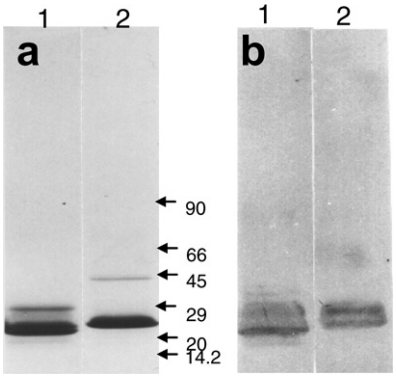

Fig. 1. Identification of the polypeptides recognized by the anti-pathogenesis-related protein antibodies. Selected wine polypeptides (lanes 1 and 2: 100 μg protein in (a) or 50 μg in (b)) were purified by a combination of cation/anion exchange fast protein liquid chromatography, subjected to SDS–PAGE and either stained for total protein (a) or probed with the antibodies (b). Molecular masses of markers are indicated in kDa.