Isolation of Isolectins from *Vitis vinifera* L. Cv. Chardonnay Grape Berries

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A lectin fraction from Chardonnay grape juice has been isolated by affinity chromatography on a column of *p*-aminophenyl *β*-D-glucoside-derivatized agarose. The lectin fractions agglutinate rabbit and human erythrocytes without serological specificity. None of the usual monosaccharides, glycosides, or glycoproteins inhibit the hemagglutinating activity. Erythroagglutination is only inhibited by nitrophenyl glycosides, *p*-nitrophenyl *β*-D-glucoside being the strongest inhibitor. In SDS−PAGE in the presence of 2-mercaptoethanol and gel filtration HPLC, the lectin fraction gave a single band or peak corresponding to M_r 13.2−11.9 kDa, thus indicating it to be a monomer. Three bands were observed by isoelectric focusing with p*I* values of 4.1, 4.4, and 4.9. The isolectins seem to be glycoproteins since they are bound on a concanavalin A−Sepharose column.

Keywords: *Lectin; glycoprotein; must; erythroagglutination; affinity chromatography*

INTRODUCTION

Plant lectins are proteins mostly extracted from the *Leguminosae* seeds where they are abundant (Howard et al., 1971; Rüdiger, 1977; Iglesias et al., 1981; Perez, 1995; Zhu et al., 1996; Duverger and Delmotte, 1997; Moreira et al., 1997). They have the particularity to recognize and agglutinate red blood cells. Some are very specific against blood groups (Yariv et al., 1967; Matsumoto and Osawa, 1969; Blacik et al., 1972; Poretz et al., 1974; Mbadiwe and Agogbua, 1978; Moreira and Ainouz, 1981) and are well-characterized in terms of molecular weight, subunits, and sugar specificity (for review, see Franz, 1988). Some of them were shown to be glycoproteins (Iglesias et al., 1982; Perez, 1995; Duverger and Delmotte, 1997). However, few lectins have been purified from fruits (Kilpatrick et al., 1983; Peumans et al., 1991), and to our knowledge, occurrence and isolation of lectins from grape berries has not yet been reported. Such compounds could be responsible for the foam properties of Champagne wines. Indeed, among the foam-active compounds already identified in Champagne wines, proteins have been shown to play an important role (Malvy et al., 1990; Maujean et al., 1990; Brissonnet and Maujean, 1991, 1993), since their tensioactive properties constitute a stabilizing agent of foam as has been previously reported for beer (Roberts, 1975). Thus, the aim of the present study was to characterize, for the first time, the presence of a phytohemagglutinin in *Vitis vinifera* L. cv. Chardonnay grape berries in order to investigate its possible tensioactive properties.

MATERIALS AND METHODS

Samples. Chardonnay grape berries were hand-harvested and pressed in September 1995. Musts were treated by 5 g/hL SO_2 to minimize oxidation phenomenon. After settling (24 h at 15 °C) the grape juices were then centrifuged (8000*g*, 10 min). Clear supernatants were then filtered through a 0.45-*μ*m filter (Sartopure GF, Sartorius) before concentration and dialysis against distilled water according to the procedure of Marchal et al. (1996). Samples were then freeze-dried (Serail CS5L) and stored at −20 °C before use.

Ion-Exchange Chromatography. Samples were subjected to a prepurification step by ion-exchange chromatography prior to hemagglutination. In our study, two columns were used. The first one was a strong cation exchanger (SP 40 HR Protein Pak, Waters), equilibrated with 100 mM phosphate/citrate buffer, pH 4, containing 0.02% NaN_3. The second one was a strong anion exchanger (Q Sepharose Fast Flow, Pharmacia) equilibrated with 100 mM triethanolamine-HCl buffer, pH 8, containing 0.02% NaN_3. In both cases, proteins were eluted by increasing the concentration in NaCl from 0 to 80 mM by 45-min steps of 20 mM each. Then, a linear gradient was used from 80 to 800 mM NaCl. In both cases, the length of the columns was the same (5 × 150 mm). Detection of proteins was monitored at 280 nm (UV detector Spectra-Physics 100). Flow rates were 0.8 mL/min. All the fractions were collected, concentrated with a 10-kDa MWCO membrane (Centriprep-10, Amicon), and desalted on a Biogel P2 (Biorad) column (5 × 800 mm) equilibrated in ultrapure water (Milli-Q, Millipore) and then freeze-dried before use.

Affinity Chromatography. *(a) Chromatography on p-Aminophenyl β-D-Glucoside Agarose (Sigma).* The column (4 × 70 mm) was equilibrated in 50 mM sodium acetate, pH 4, containing 0.02% NaN_3. Elution was performed first with the above buffer containing 1 M NaCl and then with 20 mM carbonate buffer, pH 10, containing 0.02% NaN_3.

(b) Chromatography on Immobilized Stroma in Polyacrylamide Gel. Stroma were prepared from rabbit red blood cells, included in a 7.5% polyacrylamide gel, and packed into a column (60 × 150 mm) according to the procedure of Betail et al. (1975). Equilibration and elution buffers were the same as described in part a.

(c) Chromatography on Concanavalin A−Sepharose (Sigma). The con A column (10 × 150 mm) was equilibrated in 100 mM sodium acetate buffer, pH 5, containing 0.3 M NaCl, $CaCl_2$, $MnCl_2$, $MgCl_2$ (1 mM each), and 0.02% NaN_3. Elution was carried out first with the above buffer and then with buffer containing 5 or 300 mM α-methylmannoside.

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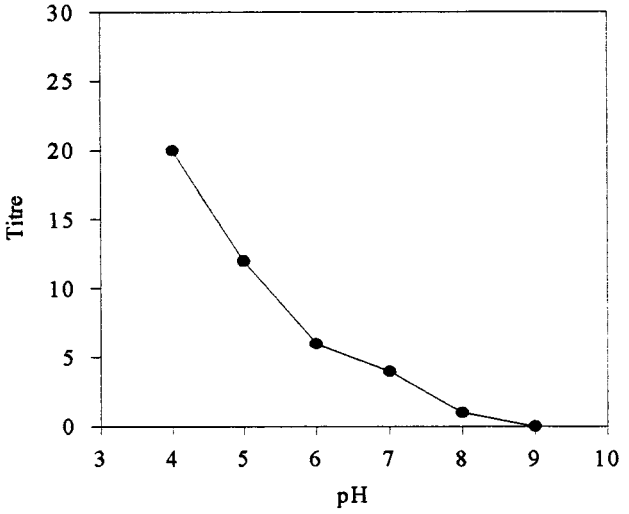

Figure 1. Effect of pH on hemagglutination by *V. vinifera* L. cv. Chardonnay lectin.

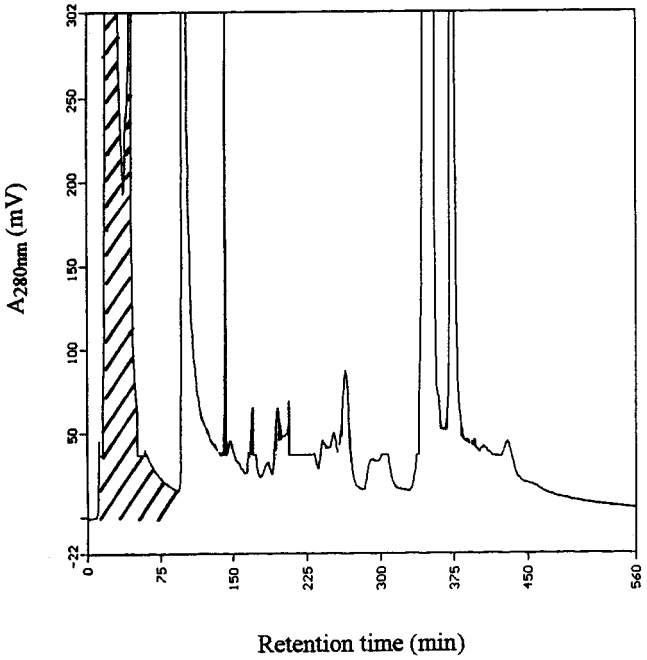

Figure 2. Fractionation of grape juice Chardonnay proteins using SP column. The dashed peak contains erythroagglutinating activity.

Flow rates were 0.16 mL/min. Detection of proteins was monitored continuously at 280 nm using a UV detector (Shimadzu SPD 2A).

Gel Filtration Chromatography. This was performed on a AIT Ultrogel SEC 2000 column (7.5 × 250 mm) previously equilibrated with 100 mM PBS, pH 6.8. The flow rate was 0.7 mL/min. Detection of proteins was monitored continuously at 220 nm using a UV detector (Waters 490E). Molecular weight standards, purchased from Sigma, were ovalbumin (43 000), carbonic anhydrase (30 000), α-chymotrypsinogen (23 000), lysozyme (14 400), and Urtica dioïca agglutinin−UDA (8 500) (a gift from Prof. Debray). All buffers were filtered through a 0.45-μm Gelman filter before use.

Analytical Gel Electrophoresis. SDS−PAGE was performed according to the procedure of Laemmli (1970), on 6.7% T, 2.7% C slab gels. Coloration was done with Brilliant Coomassie Blue R250 (Sigma). Low molecular weight and peptide calibration kits (Pharmacia) were used for calibration. Isoelectric focusing was performed on a 5% T, 3% C gel (125 × 250 × 0.25 mm) containing 1% Pharmalyte 2.5-5 (Pharmacia), 8 M urea, and 0.2% dithiothreitol (Fluka). Ultrathin layer

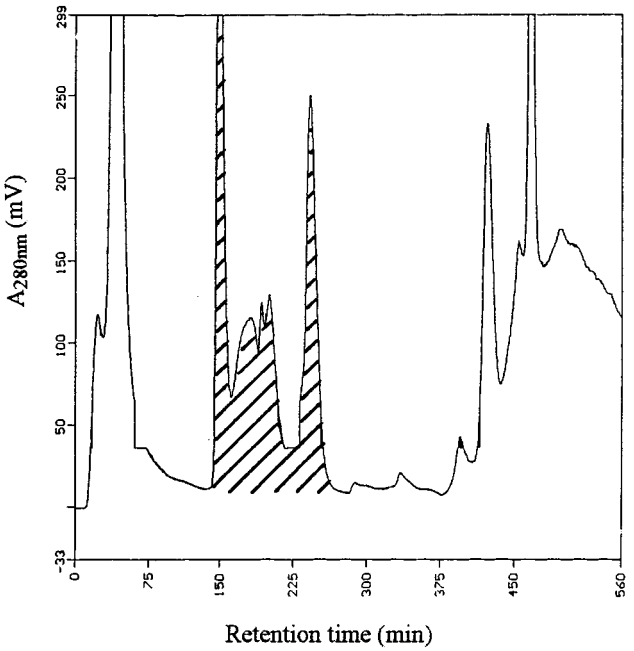

Figure 3. Fractionation of grape juice Chardonnay proteins using Q Sepharose Fast Flow column. Dashed peaks contain erythroagglutinating activity.

Table 1. Hemagglutination−Inhibition Assay of *V. vinifera* L. Cv. Chardonnay Crude Extract or Positive Ion-Exchange Fractions by Various Sugars[a]

inhibitors	minimum inhibitory (mM)[b]
pNO_2phe-β-D-Gal	3
pNO_2phe-β-D-Glc	3
pNO_2phe-β-D-Man	12
pNO_2phe-β-D-GlcNAc	25
pNO_2phe-α-D-Gal	25
pNO_2phe-α-D-Glc	25
oNO_2phe-β-D-Gal	20
oNO_2phe-β-D-Glc	20
pNO_2phenol	>100
pNO_2phenyl−acetate	>60

[a] Other sugars without inhibitory activity at 100 mM concentration are glucose, glucosamine, *N*-acetylglucosamine (GlcNAc), α-methyl-D-glucoside, galactose, methylgalactose, galactosamine, *N*-acetylgalactosamine (GalNAc), mannose, α-methyl-D-mannoside, lactose, lactulose, D-fucose, L-fucose, saccharose, fructose, raffinose, xylose, arabinose, threalose, cellobiose, melibiose, galactose-α-1,4-galactose, chitobiose, chitotriose, *N*-acetylchitobiose. Carbohydrate or glycoproteins without inhibitory activity at 10 mg/mL are mannan, fetuin, asialofetuin, bovine thyroglobulin, porcine thyroglobulin, human serotransferrin, bovine serotransferrin, human lactotransferrin, and bovine lactotransferrin. [b] Required for complete inhibition of two hemagglutinating units of lectin in the presence of trypsinized rabbit erythrocytes.

acrylamide gel was stained with Brilliant Coomassie Blue G250 (Sigma).

Determination of Protein Concentration. This was determined using a nitrogen chemiluminescence detector (Antek 7000 N) with glycine as a standard and 6.25 as a nitrogen factor conversion.

Hemagglutinating Activity. Human erythrocytes type A, O, B, and AB from healthy donors were obtained from the Centre de Transfusion Sanguine (Reims, France). Rabbit blood was drawn from animals from the Laboratoire de Physiologie Animale (Université de Reims, France), in a 3% trisodium citrate physiological solution as an anticoagulant. Hemagglutinating activity of the crude extract and of ion-exchange fractions was assayed in glass plates since, surprisingly, no agglutination was observed in microtiter U plates (Nunc, Denmark). The agglutinating activity was tested using either a 10% (v/v) suspension of native red blood cell in PBS (pH 7.4), trypsin-treated erythrocytes (10 mg/mL packed erythrocytes at 37 °C for 2 h), or neuraminidase-treated erythrocytes (0.1 U/mL of packed erythrocytes at 37 °C for 4 h). The titer was

Table 2. Purification of Lectin from *V. vinifera* L. Cv. Chardonnay

stage	volume (mL)	protein (mg/mL)	total protein (mg)	specific titer[a]	purification (fold)	yield (%)
1. concentration by ultrafiltration	3	67	200	0.23	1	100
2. ion-exchange chromatography[b]						
SP	41	1.8	73.8	2.22	9.65	36.9
Q Sepharose	120	1.5	180	2.67	11.59	90
3. affinity chromatography[c]	10	0.0325	0.325	61	265	0.16

[a] The specific titer is defined as the hemagglutination titer divided by the protein concentration (mg/mL) of the assayed solution. [b] 0.2 g of crude extract applied to the column. [c] Proteins from peaks giving positive agglutination were applied to the column.

defined as the inverse of the end point dilution giving positive agglutination.

Sugar Inhibition Assay. Sugar inhibition assays were carried out using trypsin-treated erythrocytes with crude grape juice extracts. Results were expressed as the minimal concentration of sugars required to induce total inhibition of two hemagglutinating doses. Various sugars and glycoproteins were dissolved in PBS in order to have 100 mM or 10 mg/mL stock solution.

Effect of pH on Hemagglutinating Activity. It was carried out between pH 4.0 and 9.0 according to the procedure of Zenteno et al. (1995).

RESULTS AND DISCUSSION

Presence of a Hemagglutinating Activity in Chardonnay Must. Chardonnay grape juice presents lectinic activities. No hemagglutination was detected with native red blood cells, but treatment of cells with trypsin or neuraminidase reveals a hemagglutinating activity with a slight preference for trypsinized erythrocytes. Agglutination assays carried out with rabbit or trypsinized human red blood cells with A, B, O, or AB type exhibit the same minimal hemagglutinating dose (1.56 mg of whole protein/mL). When neuraminidase erythrocytes were used, 3.12 mg of whole protein/mL was required. This activity seems to be blood group unspecific. The hemagglutinating activity decreased gradually when increasing the pH values from pH 4.0 to 9.0 (Figure 1). At this value, no hemagglutinating activity could be observed. However, when hemagglutination was assessed with whole proteins from grape juice, no inhibitory sugars were found except nitrophenyl glycosides.

Ion-Exchange Chromatography of Chardonnay Grape Juice. As compounds such as polyphenols inevitably present in grape juices and wines can also interact with the active site of the lectin, whole proteins were subjected to fractionation using ion-exchange chromatography. Agglutination of erythrocytes was positive only with proteins recovered at the void volume of the column when a strong cation exchanger was employed (Figure 2). When the fractionation was done with a strong anion exchanger, four peaks showed lectinic activities (Figure 3). Last peaks contain a lot of polyphenolic compounds.

Crude extracts as well as positive ion-exchange collected fractions were submitted to hemagglutination−inhibition assays with various sugars (Table 1). None of the usual glycosides or glycoproteins with N- or O-linked glycans were able to induce partial inhibition of the agglutinating activity. Nevertheless, nitrophenyl glycosides gave interesting results. *p*NO_2phe derivatives were previously shown to inhibit hemagglutinating activity from other plants (Perez, 1995; Zenteno et al., 1995) or yeast lectins (Al-Mahmood et al., 1988, 1991). These compounds were shown to be often the best inhibitors. Rüdiger (1977) used such affinity adsorbents, but this author showed that the interaction was not really biospecific. In our study, strongest inhibitions

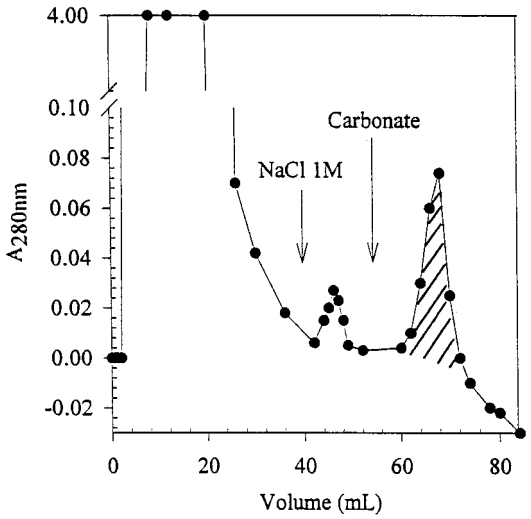

Figure 4. Purification of the *V. vinifera* lectin by affinity chromatography on a *p*-aminophenyl β-D-glucoside−agarose column. Crude extract (100 mg) in 2 mL of equilibration buffer was applied on a *p*-aminophenyl β-D-glucoside−agarose column. Different eluant were applied as indicated in the figure. The dashed peak shows erythroagglutinating activity.

were obtained with *p*NO_2pheGlc and *p*NO_2pheGal which completely inhibit hemagglutination at 3 mM. *o*NO_2phe derivatives showed slighter inhibition. β-NO_2-phenyl glucoside and galactoside are better inhibitors than the corresponding α-derivatives suggesting that the lectins preferentially interact with this anomeric configuration. *p*NO_2pheGlcNAc shows the poorest inhibition in the nitrophenyl derivatives series. Moreover, inhibition assays with *p*-nitrophenol and *p*-nitrophenol acetate suggested that the carbohydrate moiety is really implicated and that the aglycone adjacent does not cause any inhibition of the agglutination.

Purification of the *V. vinifera* Lectin by Affinity Chromatography. According to the preceding results, we prepared a *p*-aminophenyl β-D-glucoside−agarose column and chromatographed all the peaks with hemagglutinating activity obtained by ion-exchange chromatography as well as the crude grape juice extract. Quantification of the lectin was done according to the purification steps indicated in Table 2. Figure 4 set out the fixation of the lectin to the affinity column straight from the crude extract. The lectin was eluted from the affinity matrix by increasing the pH of the eluent to pH 10 (20 mM carbonate buffer). The ion-exchange column treatment seems to induce a 10-fold increase in total agglutination activity (Table 2). We do not have a suggestion about the removal of a substantial amount of an inhibitory substance. The yield of protein is 0.16% from the crude grape juice extract.

The same samples were also submitted to affinity chromatography on a column prepared with stroma from rabbit red blood cells included in a polyacrylamide gel. Such an affinity adsorbent was used for the purification of lectins which were not inhibited by either

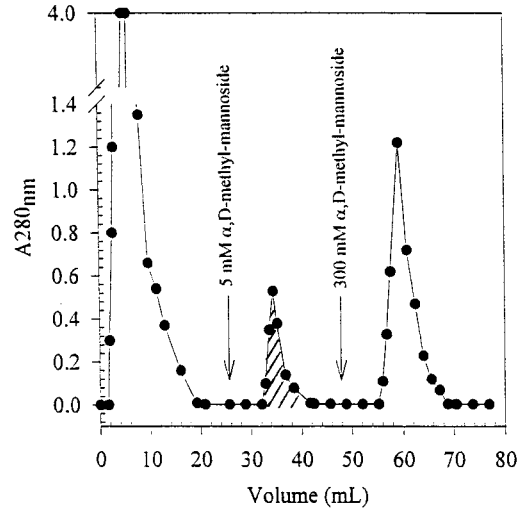

Figure 5. Affinity chromatography of glycoproteins from *V. vinifera* grape juice on a con A−Sepharose column. The crude extract (100 mg) in 2 mL of equilibration buffer was applied on a con A−sepharose column. Different concentrations of α-methyl-D-mannoside were applied as eluant buffer. The dashed peak shows erythroagglutinating activity.

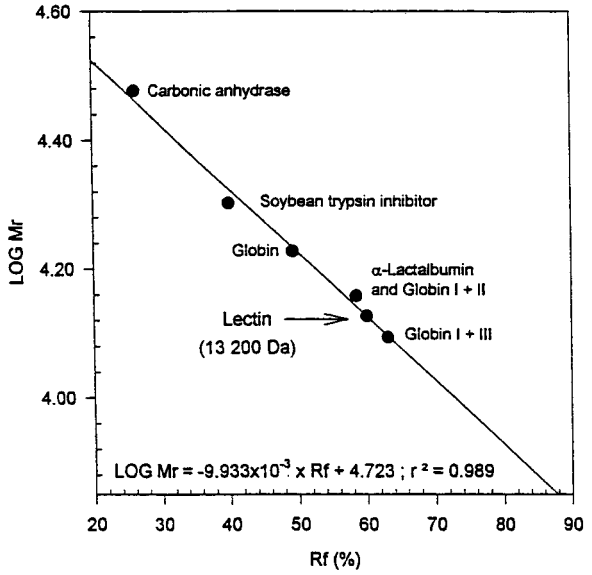

Figure 6. Calibration curve of the analytical SDS−PAGE. M_r of the lectin was calculated from the calibration curve equation. Markers used from the low molecular weight kit were carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400) and from the peptide calibration kit globin (16 949), globin I + II (14 404), and globin I + III (10 700).

glycosides or glycoproteins (Ochoa and Kristiansen, 1978, 1982).

By elution with 20 mM carbonate buffer, the lectin was also eluted from this affinity matrix with the same yield as that obtained with the *p*-aminophenyl *β*-D-glucoside−agarose column (data not shown). Purified *V. vinifera* lectin migrated as a single band on SDS−PAGE. The relative mobility (R_f) of the lectin was 60.78%, corresponding to a M_r of 13 200 Da according to the calibration curve (Figure 6). The M_r of the native protein determinated by Ultragel SEC 2000 column was 11 900 Da (Figure 7).

Isoelectric focusing (IEF) of the purified lectin shows three bands with p*I* values of 4.1, 4.4, and 4.9 (Figure 8) which could represent isolectins. When the crude

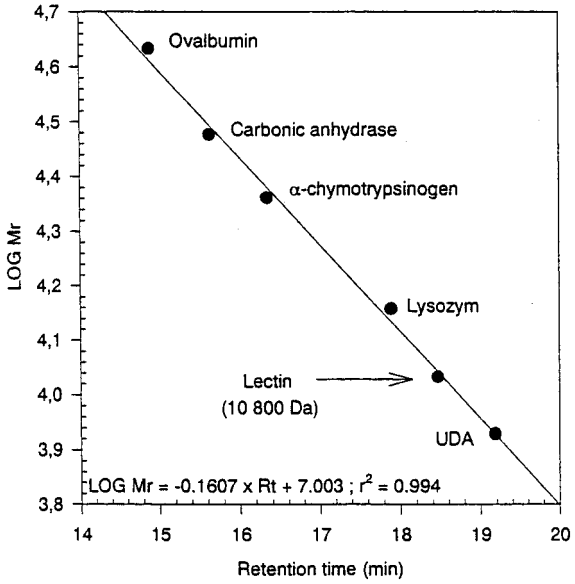

Figure 7. Gel filtration on an Ultragel SEC 2000 column (PBS as running buffer). Marker proteins were ovalbumin (43 000), carbonic anhydrase (30 000), α-chymotrypsinogen (23 000), lysozyme (14 400), and UDA (8 500). M_r of the lectin was calculated from the calibration curve equation.

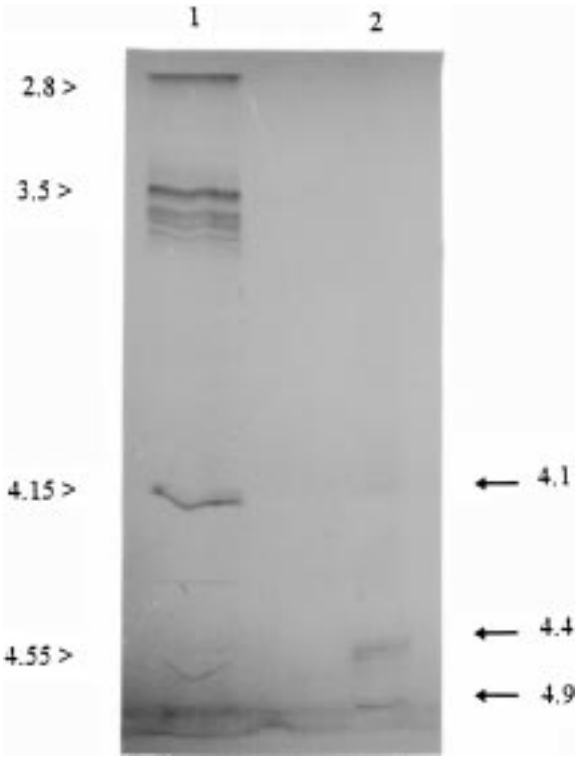

Figure 8. Analytical isoelectric focusing: lane 1, isoelectric point markers; lane 2, lectin isolated with the *p*-aminophenyl *β*-D-glucoside−agarose column (arrows show different isoforms).

grape juice extract was applied to a con A column, all the erythroagglutinating activity was recovered from the column by elution with 5 mM α-methylmannoside showing that the isolectins are glycoproteins with glycans weakly recognized by con A. However, other *V. vinifera* glycoproteins also present in the crude extract were eluted together with the isolectins with 5 mM α-methylmannoside (Figures 5 and 6). This indicates that con A−Sepharose cannot be used as an affinity

support to purify the isolectins from *V. vinifera* L. cv. Chardonnay must in a single step.

CONCLUSION

The isolation of a lectin from *V. vinifera* L. cv. Chardonnay grape berries has been achieved using ion-exchange and affinity chromatography. This lectin seems to be monomeric and presents several isoforms and low M_r. Moreover, with regards to its affinity toward con A, these isolectins could be glycosylated proteins. The isolectins seem to be present in other grape varieties. Indeed, we have found such hemagglutinating activity in a Pinot noir grape variety (data not shown). Erythroagglutining activities were also found in the corresponding wines. Studies are in progress to define the glycoproteinic nature of the *V. vinifera* isolectins: sugar composition and glycan structures as well as their sugar specificity. Their possible tensioactive properties are also under investigation.

ABBREVIATIONS USED

con A, concanavalin A; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; IEF, isoelectric focusing; LCA, *Lens culinaris* agglutinin; Man, mannose; MWCO, molecular weight cutoff; *o*NO_2phe, *o*-nitrophenyl; PBS, phosphate-buffered saline; p*I*, isoelectric point; *p*NO_2-phe, *p*-nitrophenyl; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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