JULY 1998
VOLUME 46, NUMBER 7

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

© Copyright 1998 by the American Chemical Society

Enzymatic Release of Antioxidants for Human Low-Density Lipoprotein from Grape Pomace

Anne S. Meyer,*,[†] Signe M. Jepsen,[†] and Nina S. Sørensen[‡]

Department of Biotechnology and Department of Biochemistry and Nutrition, Technical University of Denmark, 2800 Lyngby, Denmark

Enzyme-assisted release of phenolic antioxidants from grape pomace from wine production was examined. The enzymes used were Grindamyl pectinase from *Aspergillus niger* and Celluclast from *Trichoderma reesei*. Total phenols released ranged from 820 to 6055 mg/L gallic acid equivalents (GAE) and varied in response to enzyme type, time of enzyme treatment, particle size of the pomace, and type of extraction solvent employed. The yield of total phenols was correlated to the degree of plant cell wall breakdown of grape pomace ($r > 0.6$, $P < 0.01$). Grindamyl pectinase catalyzed degradation of grape pomace polysaccharides ($P < 0.001$), whereas Celluclast did not. Reduction of the particle size of grape pomace to 125−250 μm increased the enzymatic polysaccharide hydrolysis and the recovery of phenols. The grape pomace extracts significantly retarded human low-density lipoprotein oxidation in vitro. When evaluated at 3.0 μM GAE, phenolic extracts of Grindamyl pectinase treated pomace of small particle size (125−250 μm) appeared to release more active antioxidant phenols than the other types of enzyme treatments tested ($P < 0.05$).

Keywords: *Pectinase; cellulase; phenols; plant cell wall degradation; LDL oxidation*

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) is believed to play an important role in the pathogenesis of atherosclerosis and coronary heart disease (Steinberg et al., 1989; Steinberg, 1988). Dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis and prevent coronary heart disease (Esterbauer et al., 1992; Kinsella et al., 1993). Grapes and wine, notably red wine, contain a large array of phenolic compounds that have been shown to effectively inhibit human LDL oxidation in vitro (Frankel et al., 1993, 1995; Meyer et al., 1997; Teissedre et al., 1996). This ability of wine phenolics to protect LDL from oxidation has been suggested to be a possible mechanism explaining the "French paradox" (Frankel et al., 1993). The paradox refers to the epidemiological finding that in certain parts of France, where wine consumption is high, coronary heart disease mortality is low despite a high intake of saturated fats and relatively high plasma cholesterol levels in the population (Renaud and de Lorgeril, 1992).

Grape pomace is the press residue remaining when grapes are processed for wine-making. The pomace consists of pressed skins, disrupted cells from the grape pulp, seeds and stems. The wine industry produces very large amounts of grape pomace, amounting to ∼5−7 million tonnes per year as estimated from an annual use of 43 million tonnes of grapes for wine production (Jackson, 1994). A number of processes have been suggested for utilization of this pomace, including production of anthocyanins, citric acid, ethanol, and grape seed oil (Hang, 1988; Mazza, 1995). However, at

* Address correspondence to this author at the Department of Biotechnology, Bldg. 221, Technical University of Denmark, 2800 Lyngby, Denmark [fax (+45) 45 88 49 22; e-mail AM@ibt.dtu.dk].
[†] Department of Biotechnology.
[‡] Department of Biochemistry and Nutrition.

S0021-8561(97)01012-1 CCC: $15.00 © 1998 American Chemical Society
Published on Web 06/04/1998

present, grape pomace is mainly used as cattle feed or for soil conditioning or it is trucked away to disposal sites (Mazza, 1995). Grapes constitute one of the major sources of phenolic compounds among different fruits (Macheix et al., 1990), and grape seeds and skins are particularly rich in phenols (Bourzeix et al., 1986; Singleton and Esau, 1969). Even after contact with the fermenting wine, grape pomace has a high content of phenols with potential antioxidant activity (Fumiyiwa and Ough, 1982; Larrauri et al., 1997). Grape pomace therefore represents a potentially valuable source of phenolic antioxidants that may have technological applications as food additives and possible nutritional benefits.

Polyphenols extracted from air-dried grape pomace, particularly from red grape skin fractions (*Vitis vinifera* var. Cencidel), were shown to exert antioxidant activity in an ethanolic linoleate test system (Larrauri et al., 1996, 1997). However, there are no reports on the potential antioxidant activity of grape pomace phenols toward human LDL.

The purpose of this work was to enhance extraction of phenolic compounds from grape pomace by use of plant cell wall degrading enzymes. To optimize such an enzymatic extraction procedure, we examined the influence of various enzyme process parameters (enzyme type, enzyme hydrolysis time, and substrate particle size) on the cell wall breakdown and on the recovery of phenols. We also evaluated how effective selected, enzyme-treated phenolic extracts were at inhibiting human LDL oxidation in vitro.

MATERIALS AND METHODS

Grape Pomace. Red grape pomace (*V. vinifera* var. Merlot) was obtained fresh from Rutherford Brothers Winery, Napa, CA. The pomace was freeze-dried, flushed with nitrogen, and kept frozen (−30 °C) until use.

Chemicals and Enzymes. Catechin, gallic acid, and 3,5-dinitrosalicylic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Acetone, Folin−Ciocalteu phenol reagent, and sodium potassium tartrate were obtained from Merck (Darmstadt, Germany). Glucose was from AnalaR BKH Ltd. (Poole, U.K.). Grindamyl pectinase from *Aspergillus niger* was obtained from Danisco Ingredients (Brabrand, Denmark). Celluclast from *Trichoderma reesei* was from Novo Nordisk A/S (Bagsværd, Denmark).

Sample Preparation, Enzyme Hydrolysis, and Extraction of Phenols. Freeze-dried grape pomace was milled for 30 s under nitrogen in an IKA-Universal mill model H20 (Jahnke & Kunkel, Staufen, Germany). Particle size distribution of crushed pomace was analyzed using a four-sieve sieving tower (Endecotts Filters Ltd., London, U.K.) with aperture sizes of 1000, 500, 250, and 125 μm, respectively. For enzymatic hydrolysis grape pomace samples (2.0 g) were incubated under nitrogen in 0.1 M acetate buffer pH 5.0 (20.0 mL) at 40 °C for various lengths of time with different enzyme additions. The selected hydrolysis conditions 40 °C and pH 5.0 were based on evaluations of temperature and pH activity curves for the enzymes as given on the enzyme suppliers' data sheets. The enzymes employed were selected after preliminary trials (data not shown). Individual and interactive effects on plant cell wall degradation and phenol yield of the factors incubation time, enzyme type (Grindamyl pectinase, Celluclast), and enzyme concentration were tested in a full factorial 2^3 design (Montgomery, 1991). To study also the influence of particle size of the grape pomace, the enzyme hydrolysis was optimized further in a 2^4 factorial design (Montgomery, 1991). Enzymes were dosed on the basis of enzyme/substrate ratio (E/S), that is, the total mass of the enzyme preparation relative to the dry mass of grape pomace. E/S ratios of 0−10% corresponding to enzyme concentrations from 0 to 1% (w/w) of the total reaction volume were used. All investigations were carried out in two parallel, identical systems. One was used for extraction [70% acetone (Kallithraka et al., 1995) or pure water, 3 min solvent contact time] and quantification of phenols. The other was used for evaluation of the degree of plant cell wall degradation. Prior to analyses enzyme hydrolysis was halted by heat (100 °C, 10 min).

Table 1. Yield of Phenols As Affected by Crushing of Grape Pomace Prior to Extraction with 70% Acetone

	total phenols GAE[a] (mg/L)
whole grape pomace	241.9 ± 13.7
crushed grape pomace[b]	2634.2 ± 128.0

[a] GAE, gallic acid equivalents. [b] Crushed for 30 s in an IKA laboratory mill.

Phenol Quantification. The concentration of total phenols in enzyme-hydrolyzed pomace extracts was determined by the Folin−Ciocalteu procedure (Singleton and Rossi, 1965). Total phenols were expressed as milligrams per liter gallic acid equivalents (GAE).

Analysis of Plant Cell Wall Degradation. The extent of cell wall hydrolysis of pomace was evaluated by measuring total water soluble carbohydrates liberated from the sample (Slominski et al., 1993).

Isolation of Human LDL. Plasma was obtained from the blood of healthy, female volunteers (The University Hospital, Copenhagen, Denmark), frozen in liquid nitrogen, and stored under nitrogen at − 80 °C until use. LDL was isolated from the plasma by density gradient ultracentrifugation (Princen et al., 1992). After determination of protein concentration according to the method of Lowry et al. (1951), LDL was diluted to a standard protein concentration of 0.25 mg/mL for the oxidation assay.

Inhibition of Human LDL Oxidation. Antioxidant activities of enzyme-hydrolyzed grape pomace extracts were determined by direct monitoring of conjugated diene hydroperoxide formation (234 nm) during copper-induced oxidation of human LDL (37 °C, 40 μM $CuSO_4$) (Esterbauer et al., 1989; Princen et al., 1992). Immediately prior to assay, the grape pomace extracts were diluted with doubly distilled water and tested at equimolar concentrations of 3.0 and 4.6 μM GAE. After replicate analyses, antioxidant activity was calculated as prolongation of induction time for LDL oxidation as compared to LDL oxidation without phenolic extracts added (Esterbauer et al., 1989).

Statistical Analyses. The computer program Modde (Umetri AB, Umeå, Sweden) was used to aid the statistical design of factorial experiments and to fit and analyze the data by multiple linear regression. Differences in antioxidant activities were tested by one-way analysis of variance (Minitab Statistical Software, Addison-Wesley, Reading, MA). Correlation coefficients of phenol yield versus degree of plant cell wall degradation were determined by linear regression analysis. The statistical significance of the correlations were tested by the dose−response F test (Berry and Lindgren, 1996).

RESULTS

Effect of Pomace Crushing on Phenol Yield. The level of total phenols was 242 mg/L GAE when whole grape pomace was extracted with a conventional 70% acetone extraction procedure without enzyme treatment (Table 1). When the pomace was crushed by milling (30 s in an IKA-Universal mill) prior to extraction, the level of total phenols increased by >10-fold to 2634 mg/L GAE (Table 1). Crushed pomace was therefore used as a substrate for enzyme hydrolysis in all subsequent experiments. The particle size distribution of the crushed pomace is shown in Table 2.

Effect of Enzyme Type, Enzyme Dosage, and Time of Enzyme Hydrolysis on Yield of Phenols.

Table 2. Proximate Particle Size Distribution of Crushed Grape Pomace[a]

particle size (μm)	distribution (%)
>1000	2.6
500–1000	17.3
250–500	40.9
125–250	31.8
<125	7.4

[a] Crushed for 30 s in an IKA laboratory mill.

Table 3. Effects of Enzyme Type and Time of Enzyme Hydrolysis on Plant Cell Wall Degradation and Yield of Phenols

expt no.	Celluclast (E/S)[a] %	Grindamyl (E/S)[a] %	time (h)	carbohydrate[b] (mg/mL)	phenols[c] (GAE mg/L)
1	0	0	1.0	0.561	2677
2	10	0	1.0	0.761	2773
3	0	10	1.0	0.961	3072
4	10	10	1.0	0.861	3017
5	0	0	48.0	0.666	1944
6	10	0	48.0	0.763	2080
7	0	10	48.0	1.278	2243
8	10	10	48.0	1.346	2514
9	5	5	24.5	1.268	2528
10	5	5	24.5	1.068	2405
11	5	5	24.5	1.155	2664

[a] E/S, enzyme/substrate ratio of enzyme dosage. [b] Yield of soluble carbohydrate, mg/mL glucose. [c] GAE, gallic acid equivalents.

Table 4. Multiple Linear Regression Coefficients Describing the Release of Phenols by 70% Acetone Extraction[a]

term	tegression coefficient	P^b	95% confidence limit
constant	2537.82	5.69×10^{-12}	±66.99
Celluclast	55.88	0.136	±78.55
Grindamyl	171.63	1.30×10^{-3}	±78.55
time	−344.89	1.67×10^{-5}	±78.55

[a] As estimated from data in Table 3. [b] $P < 0.05$ indicates significance at 95% level.

The effects of Celluclast, Grindamyl pectinase, and time of enzyme treatment on plant cell wall degradation and the release of phenols from crushed grape pomace were tested in a two-level full factorial experimental design (2^3 design). This design comprised eight different combinations of parameters with three center points. As expected, the yield of total phenols varied in response to the different treatments and ranged from 1944 to 3072 mg/L GAE in the extracts (Table 3). Only addition of Grindamyl pectinase (10% E/S) had a significantly positive effect on the phenol yield ($P < 0.01$) (Table 4). Addition of Celluclast (10% E/S) did not influence the extraction yield of phenols, but long enzyme treatment time, 48 versus 1 h, had a significantly negative effect ($P < 0.001$) on the phenol recovery (Table 4). This negative effect of time indicates that phenols were degraded during the long enzyme treatment.

In accordance with the results for phenol yields, Grindamyl pectinase had a positive effect on the plant cell wall breakdown as evaluated from the release of soluble carbohydrates ($P < 0.05$; Table 5). Neither Celluclast addition nor the time of enzyme treatment influenced the plant cell wall degradation (Table 5), and there were no significant interactions among the parameters tested.

Influence of Particle Size of Grape Pomace on Enzymatic Release of Phenols. Most of the crushed grape pomace had particle sizes between 125 and 500 μm with 32% (w/w) from 125 to 250 μm and 41% (w/w) from 250 to 500 μm (Table 2). The particle size was shown previously to be a major determinant for enzymatic degradation of other lignocellulosic substrates, where the smaller size of substrate particles increased the surface available for enzyme attack and in turn enhanced enzymatic breakdown of the plant cell wall material (Düsterhöft et al., 1993). We therefore determined the influence of size reduction of pomace particles on the enzymatic cell wall degradation and release of phenols. This was done by testing the polysaccharide breakdown and phenol yield from 125 to 250 μm grape pomace particles versus coarser particles of 500–1000 μm in an optimized 2^4 factorial experiment composed of 16 different enzyme treatments with 4 centerpoints. On the basis of the findings that a very long enzyme treatment time affected the phenol yield negatively (Table 4), we narrowed the enzyme treatment time to 1 or 8 h. Furthermore, the release of phenols by 70% acetone extraction was compared to extraction with pure water.

Table 5. Multiple Linear Regression Coefficients of Plant Cell Wall Hydrolysis As Evaluated by Release of Soluble Carbohydrate (Milligrams per Milliliter of Glucose) in Response to Enzyme Factors[a]

term	regression coefficient	P^b	95% confidence limit
constant	0.97	6.33×10^{-7}	±0.32
Celluclast	0.03	0.639	±0.16
Grindamyl	0.21	0.017	±0.16
time	0.11	0.137	±0.16

[a] As estimated from data in Table 3. [b] $P < 0.05$ indicates significance at 95% level.

In this optimized experimental design the highest levels of phenols ranged from 5100 to 6000 mg/L GAE in the 70% acetone extracts and from 1200 to 1300 mg/L in the water extracts (Table 6). Thus, in general, extraction with 70% acetone after enzymatic hydrolysis gave 4–5 times higher yields of phenols than extraction with pure water. As expected, the yields of phenols varied markedly in response to the different treatments (Table 6).

The particle size of the grape pomace affected significantly the phenol yield in both 70% acetone and water extracts (Tables 6–8). Reduction of the particle sizes from 500–1000 to 125–250 μm thus increased release of phenols by 56% on average in 70% acetone extracts ($P < 0.001$) and by 27% on average in water extracts ($P < 0.001$). The particle size also significantly affected the enzymatic degradation of the grape pomace polysaccharides ($P < 0.001$) (Table 9). The negative regression coefficient for particle size signifies that small particles (125–250 μm) released significantly higher amounts of carbohydrates, that is, were degraded more efficiently by the enzymes. Within the time interval tested (1 vs 8 h), there was no effect of enzyme treatment time on the phenol yield in either 70% acetone or water extracts (Tables 7 and 8). Neither did the time of enzyme treatment affect the enzymatic breakdown of pomace plant cell wall polysaccharides (Table 9). In accordance with the earlier results (Table 5), addition of Grindamyl pectinase resulted in enhanced plant cell wall degradation ($P < 0.001$) but did not enhance the recovery of phenols to a statistically significant degree in either 70% acetone extracts or water extracts (Tables 7 and 8). Nevertheless, the optimal combination for the maximum phenol yield was small particle size and 8 h treatment

Table 6. Influence of Substrate Particle Size, Time of Enzyme Hydrolysis, and Extraction Medium on Plant Cell Wall Degradation and Release of Phenols

expt no.	particle size[a] (μm)	time (h)	Celluclast (E/S)[b] %	Grindamyl (E/S)[b] %	carbohydrates[c] (mg/mL)	phenols aq[d] GAE[e] (mg/L)	phenols act[f] GAE[e] (mg/L)
1	125−250	1	0	0	0.931	1055	4991
2	500−1000	1	0	0	0.436	925	3888
3	125−250	8	0	0	0.885	1126	4615
4	500−1000	8	0	0	0.380	873	3484
5	125−250	1	0	10	1.526	1186	5126
6	500−1000	1	0	10	1.112	995	3807
7	125−250	8	0	10	1.314	1137	6055
8	500−1000	8	0	10	0.946	879	3242
9	125−250	1	10	0	0.895	1183	5530
10	500−1000	1	10	0	0.749	1062	3094
11	125−250	8	10	0	0.931	1227	5180
12	500−1000	8	10	0	0.532	831	3269
13	125−250	1	10	10	1.375	1301	5193
14	500−1000	1	10	10	0.961	1046	3107
15	125−250	8	10	10	1.547	1211	5355
16	500−1000	8	10	10	0.996	821	3013
17	250−500	4.5	5	5	0.739	925	3323
18	250−500	4.5	5	5	0.729	972	3121
19	250−500	4.5	5	5	0.799	929	3228
20	250−500	4.5	5	5	0.749	846	2838

[a] Grape pomace particle size. [b] Enzyme dosage (as in Table 3). [c] Soluble carbohydrate, mg/mL glucose. [d] Extraction solvent pure water. [e] GAE, gallic acid equivalents. [f] Extraction solvent 70% acetone.

Table 7. Multiple Linear Regression Coefficients Describing the Yield of Phenols by 70% Acetone Extraction in Response to Enzyme Hydrolysis Parameters[a]

term	regression coefficient	P^b	95% confidence limit
constant	4072.95	2.47×10^{-14}	±311.50
particle size	−946.31	3.56×10^{-5}	±348.26
time	−32.69	0.844	±348.26
Grindamyl	52.94	0.750	±348.26
Celluclast	−91.69	0.583	±48.26

[a] As estimated from data in Table 6. [b] $P < 0.05$ indicates significance at 95% level.

Table 8. Multiple Linear Regression Coefficients Describing the Release of Phenols by Pure Water Extraction in Response to Enzyme Process Parameters[a]

term	regression coefficient	P^b	95% confidence limit
constant	1026.50	9.46×10^{-18}	±40.10
particle size	−124.63	3.47×10^{-5}	±44.83
time	−40.50	0.073	±44.83
Grindamyl	18.38	0.394	±44.83
Celluclast	31.63	0.153	±44.83

[a] As estimated from data in Table 6. [b] $P < 0.05$ indicates significance at 95% level.

Table 9. Multiple Linear Regression Coefficients Describing the Release of Water Soluble Carbohydrates (Milligrams per Milliliter of Glucose) as a Function of Enzyme Process Parameters[a]

term	regression coefficient	P^b	95% confidence limit
constant	0.93	4.75×10^{-15}	±0.06
particle size	−0.21	1.74×10^{-5}	±0.07
time	−0.03	0.407	±0.07
Grindamyl	0.25	1.63×10^{-6}	±0.07
Celluclast	0.03	0.405	±0.07

[a] As estimated from data in Table 6. [b] $P < 0.05$ indicates significance at 95% level.

with Grindamyl pectinase (10% E/S), which resulted in 6055 mg/L GAE with 70% acetone extraction (experiment 7, Table 6). Celluclast was unable to degrade pomace of both particle sizes (Table 9), and in accordance herewith did not affect the release of phenols by either 70% acetone or water extraction (Tables 7 and 8). There were no interactions among any of the parameters investigated. On the basis of the data obtained (Table 6), the release of phenols was found to be positively, linearly correlated to the degree of plant cell wall breakdown with correlation coefficients $r = 0.62$ ($P < 0.01$) for 70% acetone extracts and $r = 0.68$ ($P < 0.01$) for pure water extracts (Figure 1).

Antioxidant Activity toward LDL Oxidation. The antioxidant activities of water extracts from six different treatments (experiments 1, 2, 5, 6, 9, and 10, Table 6) were compared at equimolar phenol concentrations of 3.0 and 4.6 μM GAE. Antioxidant activities were assessed by measuring the inhibition of human LDL oxidation by direct spectroscopic monitoring of conjugated diene hydroperoxides in an in vitro assay initiated by copper (40 μM $CuSO_4$, 37 °C) (Esterbauer et al., 1989; Princen et al., 1992). The chosen phenolic extracts represented both non-enzyme-treated pomace samples and different combinations of enzyme treatments with Grindamyl pectinase (10% E/S) and Celluclast (10% E/S) on both small (125−250 μm) and large (500−1000 μm) grape pomace particles, but all treated for 1 h. Catechin was used as an antioxidant control compound (Frankel et al., 1995).

The average induction time for copper-mediated LDL oxidation without phenolic extracts added was ~18 min. All of the pomace extracts strongly protected LDL from oxidation as measured by prolongation of this induction time (lag phase) of the formation of conjugated dienes (Table 10). For all extracts tested, the antioxidant activity was higher at 4.6 μM as compared to at 3.0 μM, indicating a dose-dependent antioxidant effect with respect to concentration of phenols. When evaluated at 3.0 μM GAE, the grape pomace extracts delayed conjugated diene formation by 22−62 min. At 4.6 μM GAE, the delay was >2 h (from 146 to 169 min; Table 10). The most significant differences in antioxidant activities were found when the extracts were compared at 3.0 μM GAE. When evaluated at this concentration, the extract obtained from Grindamyl pectinase treated grape pomace of small particle size (125−250 μm)

Table 10. Antioxidant Activities of Grape Pomace Extracts toward in Vitro Human LDL Oxidation[a]

enzyme treatment[b]	pomace particle size (μm)	antioxidant activity[c] (min) at 3.0 μM GAE[d]	antioxidant activity[e] (min) at 4.6 μM GAE[d]
none	125−250	47.1[bc]	152.9[b]
none	500−1000	49.4[bc]	169.3[b]
Grindamyl	125−250	62.2[b]	160.7[b]
Grindamyl	500−1000	45.0[c]	159.7[b]
Celluclast	125−250	38.1[c]	145.7[b]
Celluclast	500−1000	21.9[d]	154.7[b]
catechin[f]		110.4[a]	>300[a]

[a] Antioxidant activities are given as average net prolongation of induction time for conjugated diene hydroperoxide formation. Results in the same column followed by different roman superscript letters are significantly different at $P < 0.05$. [b] Selected aqueous extracts of experimental runs shown in Table 6 (expt no. row-wise: 1, 2, 5, 6, 9, 10). [c] Pooled SD 5.5 min. [d] GAE, gallic acid equivalents. [e] Pooled SD 15.0 min. [f] Antioxidant control compound.

(experiment 5, Table 6) exhibited the highest antioxidant activity, prolonging the induction time by 62.2 min. This induction time was 3.4 times longer than the induction time of copper-catalyzed LDL without antioxidants added. In contrast, Celluclast (10% E/S) treated grape pomace samples of both small and coarse particle size did not exhibit enhanced antioxidant activities as compared to non-enzyme-treated pomace control samples. Rather, the Celluclast (10% E/S) treated coarse particle extract had a significantly lower antioxidant activity at 3.0 μM GAE than all other extracts (Table 10). Pure catechin was consistently more active than the grape pomace extracts. The results indicate that treatment of grape pomace of small particle size (125−250 μM) with Grindamyl pectinase released more potent antioxidant phenolics than the other types of enzyme treatments tested.

DISCUSSION

In plant cells, phenolic compounds may accumulate in the vacuoles or in the cell walls. During the wine-making process mainly soluble phenolic compounds present in the vacuoles of the grape plant cells are extracted, apparently leaving a large amount of phenols associated with the cell walls behind. In this study, we tested the hypothesis that degradation of grape pomace by plant cell wall hydrolyzing enzymes could enhance extraction of antioxidant phenolic compounds. The data obtained demonstrate that the enzyme preparation Grindamyl pectinase was efficient in degrading the grape pomace polysaccharides and thus released antioxidant phenolic compounds from the pomace. Furthermore, the phenols released by Grindamyl pectinase treatment of small grape pomace particles (of a diameter from 125 to 250 μm) seemed to be more active in inhibiting oxidation of human LDL in vitro than those obtained without enzyme treatment or after treatment with Celluclast.

The plant cell walls of grape pomace are composed of cellulose, hemicelluloses, pectin, and lignin, arranged in a complex network. Lignin is the major phenolic constituent of cell walls (Tucker and Mitchell, 1993) and constitutes ~38−40% of the total grape pomace mass (Saura-Calixto et al., 1991; Valiente et al., 1995). Lignin is composed of hydrophobic polymers derived from *p*-coumaryl, coniferyl, and sinapyl alcohols (Brett and Waldron, 1996). Lignin is deposited together with tannins (procyanidins), simpler flavonoids, and hydroxycinnamic acids (mainly *p*-coumaric acid and ferulic acid), but due to the nonenzymic nature of the polymerization, the pattern is irregular (Brett and Waldron, 1996). The hydrophobicity of the lignin matrix strengthens the hydrogen bonds between adjacent polysaccharides. In turn, this increases the strength of the cellulose−hemicellulose network in the cell wall. Lignin may be covalently linked to polysaccharides via sugar residues or via phenolic acids esterified to polysaccharides (Macheix et al., 1990; McDougall et al., 1996). Of these phenolic acids ferulic acid (4-hydroxy-3-methoxycinnamic acid) and *p*-coumaric acid (4-hydroxycinnamic acid) are considered the most important. Ferulic acid was recently reported to be an antioxidant for human LDL oxidation in vitro (Meyer et al., 1998; Nardini et al., 1995).

Grindamyl pectinase was previously shown to be efficient in releasing free and esterified ferulic acid from sugarbeet pulp (Micard et al., 1994), and this trait was our main objective for including this enzyme preparation in our work on grape pomace. According to the manufacturer, Grindamyl pectinase mainly contains pectinolytic activities but also exhibits cellulase and hemicellulase activities. The enzyme preparation has been optimized for extraction of juice from fruit mash, including grapes. The dominant structural cell wall polysaccharide in the grape pomace skin fraction is most likely cellulose (~50%) (Lecas and Brillouet, 1994). Celluclast is an enzyme preparation produced from *T. reesei* and contains mainly cellulolytic activities, although hemicellulase and pectinase activities are also present. We therefore tested the ability of Celluclast to hydrolyze grape pomace polysaccharides to facilitate release of phenols. Under the experimental conditions employed in our study, however, Celluclast was unable to degrade the cell wall polysaccharides in grape pomace and did not enhance the extraction of phenols (Tables 7−9).

It is well-known that in plant cell walls lignin and cellulose combine to produce a material which is very resistant to chemical and biological degradation (Brett and Waldron, 1996; Düsterhöft et al., 1993). Furthermore, as mentioned above, the presence of lignin strengthens the hydrogen bonds in the surrounding cellulose−hemicellulose network (Brett and Waldron, 1996). Cellulose alone may also form highly ordered crystalline structures, which are generally resistant to enzymatic breakdown. Furthermore, in plant cell walls containing lignin, the accessibility of hydrolytic enzymes may be hindered by lignin and low molecular weight phenolic compounds forming covalent linkages with sugar residues, thus "shielding" the substrate from enzymatic degradation (Düsterhöft et al., 1993). Also, enzymatic hydrolysis of plant cell wall material may be retarded by nonproductive adsorption of enzymes to lignin or polysaccharides in the wall matrix (Converse et al., 1990). These factors may explain the low activity of Celluclast on grape pomace.

Reduction of the particle size diameter of grape pomace from 500−1000 to 125−250 μm increased the phenol yield significantly ($P < 0.001$; Tables 7 and 8). This increase in phenol yield was a result of better extractability and notably for Grindamyl pectinase an enhanced enzymatic degradation of grape pomace polysaccharides ($P < 0.001$; Table 9). In addition to the nature of the substances associated with the cellulose (as discussed above), a most important factor affecting

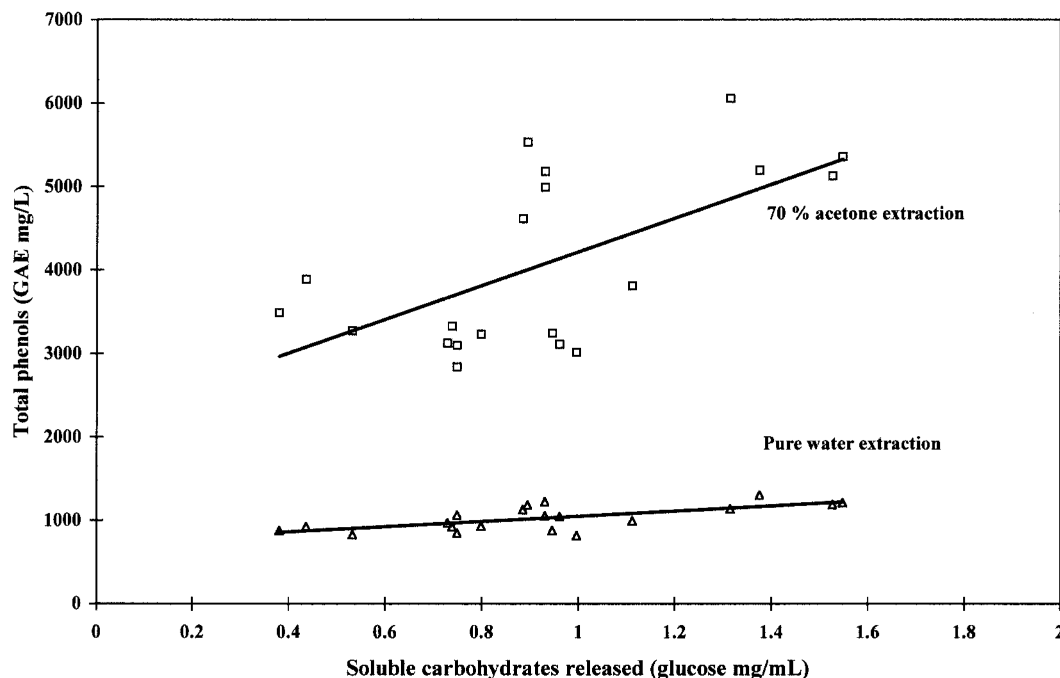

Figure 1. Yield of total phenols versus total soluble carbohydrates released by enzyme treatment. Data were obtained from Table 6. Linear regression equations: 70% acetone extraction, $Y = 2265.0X + 1984.8$ [correlation coefficient, $r = 0.62$ ($P < 0.01$)]; pure water extraction, $Y = 310.3X + 742.1$ [correlation coefficient, $r = 0.68$ ($P < 0.01$)].

the susceptibility of lignocellulosic materials to enzymatic breakdown appears to be the surface area of the substrate available for enzymatic hydrolysis (Converse et al., 1990; Marsden and Gray, 1986). Thus, the observed enhanced polysaccharide hydrolysis catalyzed by Grindamyl pectinase and the higher phenol yield obtained for small pomace particles (125−250 μm) are most likely a result of increased accessibility of the substrate for enzyme attack. This conclusion is also in agreement with data for other lignocellulosic substrates (Düsterhöft et al., 1993).

In general, the porosity of lignocellulosic cell wall material is largely determined by the nature of the hemicellulose fraction (Marsden and Gray, 1986). It might therefore be inferred that enzymatic degradation of hemicellulose substrates in grape pomace would increase pore formation, pore size, and overall substrate porosity and, with that, the recovery of phenols. In the lignin fraction of grape pomace, protein and condensed tannins are linked together in an insoluble lignin−protein−tannin complex (Saura-Calixto et al., 1991; Valiente et al., 1995). Furthermore, in grapes, the procyanidins, of which several were shown previously to inhibit human LDL oxidation in vitro (Teissedre et al., 1996), are known to bind to proteins by hydrophobic interactions, hydrogen bonds, and covalent bonds. The major binding factor appears to be hydrophobic interactions (Teissedre et al., 1996). However, overall, the exact association of phenolic compounds to the cell walls in the skin fraction versus the pulp versus the seeds in grapes (and other fruits) has not been investigated. Thus, very little is known about the location, type of bonding, possible physical entrapment in lignin etc., of phenols in grape pomace.

Grindamyl pectinase degraded the pomace polysaccharides but, when analyzed as a single factor, was unable to enhance significantly the release of phenols, either by 70% acetone extraction ($P = 0.75$, Table 7) or by extraction into pure water ($P = 0.39$, Table 8). In our experimental setup these observations indicate that a possible increase in substrate porosity caused by the action of Grindamyl pectinase was apparently only negligible and, in turn, did not result in a significantly increased release of phenols. However, when including data from both Celluclast and Grindamyl pectinase hydrolysis, a significant correlation was found between the yield of phenols and the degree of polysaccharide breakdown in the plant cell wall material (Figure 1). This positive correlation strongly indicates that enzymatic breakdown of the plant cell wall caused enhanced release of phenols from grape pomace.

All enzyme reactions were carried out in closed containers purged with nitrogen. The initially observed negative effect of the time parameter (Table 4) indicates, however, that the phenolic compounds were degraded during the 48 h long enzymatic hydrolysis. When the enzymatic hydrolysis was tested in a narrower time interval (1 vs 8 h), there was no loss of phenols with time (Tables 7 and 8).

The present study showed that phenolic compounds extracted from grape pomace significantly retarded in vitro human LDL oxidation at micromolar phenol concentrations. In particular, it was observed that treatment of small pomace particles (125−250 μm) with Grindamyl pectinase apparently released more potent antioxidants for human LDL than treatment with Celluclast. The equimolar antioxidant activity at 3.0 μM GAE for Grindamyl pectinase treated small particles was also higher than non-enzyme-treated pomace extracts (62.2 versus 47.1−49.4 min, Table 10). There was a clear grouping of the data obtained after variance analysis (one-way anova). However, distinct statistical significance was not strong as the tails of the 95% confidence limits for the antioxidant activity of the Grindamyl pectinase treated small particles overlapped slightly with antioxidant activities obtained for the non-enzyme-treated pomace extracts (Table 10). Contemplation of the data suggests that Grindamyl pectinase contains relevant enzymatic activities for polysaccharide breakdown of grape pomace and release of phenols.

Furthermore, the enzyme preparation may contain activities that (1) directly promote selective release of antioxidant phenols or (2) modify released phenols to more potent antioxidant compounds. In the first case, ferulic acid esterase is a possible activity. Ferulic acid esterase was previously suggested to be present in Grindamyl pectinase, as the amount of free ferulic acid released from sugarbeet pulp was particularly high after hydrolysis by this enzyme preparation (Micard et al., 1994). In the second case, it may be speculated that undeclared glucosidase activities in the enzyme preparation may convert phenolic glucosides into less glycosylated species and in turn affect their antioxidant activity. In a very recent study (Satué-Gracia et al., 1997), the anthocyanin aglycon malvidin was thus found to be a better antioxidant for in vitro human LDL oxidation than its corresponding glucoside malvin. Previous work on wine (Frankel et al., 1995) and fresh grapes (Meyer et al., 1997) has shown that antioxidant activity toward human LDL in vitro is distributed among several different types of phenolic compounds but also that the relative concentrations of phenols in the extracts are critical for the antioxidant activity. It was previously shown that another pectinase preparation produced from *A. niger* (Pectinex Ultra SP-L produced by Novo Nordisk Ferment, Basel, Switzerland) changed the sugar moieties of raspberry anthocyanin glucosides by hydrolysis of β1−2 glucosidic bonds (Jiang et al., 1990). Furthermore, several commercial pectolytic enzyme preparations were demonstrated to possess β-galactosidase activities that catalyzed the hydrolysis of anthocyanin β-galactosides, resulting in color loss in cranberry juice (Wrolstad et al., 1994). Detection of such enzymatic activity by HPLC anthocyanin pigment analysis of the juice gave higher and different ranking of activities as compared to standard assay procedures using *o*- or *p*-nitrophenol monosaccharide derivatives as substrates (Wrolstad et al., 1994). While the presence of β-galactosidase and β-glucosidase activity may cause color degradation in fruit juices, such activity might be beneficial for extraction of potent antioxidants from grape pomace. Better knowledge of the presence of side activities in different enzyme preparations intended for fruit processing is therefore needed to tailor new enzyme applications. Furthermore, additional research is obviously required to understand the effect of enzymes on the phenolic composition after grape pomace hydrolysis. Such understanding may also be relevant in winemaking, in which pectinolytic enzymes are gaining increased use as processing aids (Lao et al., 1996).

The data obtained in the present work may be useful in the commercial exploitation of grape pomace for production of antioxidant concentrates. Grape seed extract, referred to as oligomeric procyanidins (OPC), is already widely sold as a nutritional supplement. However, more work is needed to improve our knowledge of the physiological significance of grape phenolics. Such data may permit an even better targeting of enzymatic action for extraction of phenolic antioxidants with nutritional benefits.

LITERATURE CITED

Berry, D. A.; Lindgren, B. W. *Statistics: Theory and Methods*, 2nd ed.; Duxbury Press at Wadsworth Publishing: Belmont, CA, 1996; pp 608−609.

Bourzeix, M.; Weyland, D.; Heredia, N. A study of catechins and procyanidins of grape clusters, the wine and other by-products of the vine. *Bull. O. I. V.* **1986**, *59*, 1171−1254 (translated from French).

Brett, C.; Waldron, K. *Physiology and Biochemistry of Plant Cell Walls*, 2nd ed.; Chapman and Hall, London, U.K., 1996; pp 4−74.

Converse, A. O.; Ooshima, H.; Burns, D. S. Kinetics of enzymatic hydrolysis of lignocellulosic materials based on surface area of cellulose accessible to enzyme and enzyme adsorption on lignin and cellulose. *Appl. Biochem. Biotechnol.* **1990**, *24/25*, 67−73.

Düsterhöft, E.-M.; Engels, F. M.; Voragen, A. G. J. Parameters affecting the enzymic hydrolysis of oil-seed meals, lignocellulosic by-products of the food industry. *Bioresour. Technol.* **1993**, *44*, 39−46.

Esterbauer, H.; Striegl, G.; Puhl, H.; Rotheneder, M. Continuous monitoring of *in vitro* oxidation of human low-density lipoprotein. *Free Radical Res. Commun.* **1989**, *6*, 67−75.

Esterbauer, H.; Gebicki, J.; Puhl, H.; Jürgens, G. The role of lipid peroxidation and anti-oxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **1992**, *13*, 341−390.

Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

Frankel, E. N.; Waterhouse, A. L.; Teissedre, P. L. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *J. Agric. Food Chem.* **1995**, *43*, 890−894.

Fumiyiwa, O. O.; Ough, C. S. Grape pomace: Possibilities as animal feed. *Am. J. Enol. Vitic.* **1982**, *33*, 44−46.

Hang, Y. D. Recovery of food ingredients from grape pomace. *Process Biochem.* **1988**, *23*, 2−4.

Jackson, R. S. *Wine Science Principles and Applications*; Academic Press: San Diego, CA, 1994; pp 1−10.

Jiang, J.; Paterson, A.; Piggott, J. R. Effects of pectolytic enzyme treatments on anthocyanins in raspberry juice. *Int. J. Food Sci. Technol.* **1990**, *25*, 596−600.

Kallithraka, S.; Garcia-Viguera, C.; Bridle, P.; Bakker, J. Survey of solvents for the extraction of grape seed phenolics. *Phytochem. Anal.* **1995**, *6*, 265−267.

Kinsella, J. E.; Frankel, E.; German, B.; Kanner, J. Possible mechanisms for the protective role of antioxidants in wine and plant foods. *Food Technol.* **1993**, *47*, 85−89.

Lao, C.; Lopez-Tamames, E.; Buxaderas, S.; de la Torre-Boronat, M. C. Grape pectic enzyme treatment effect on white musts and wines composition. *J. Food Sci.* **1996**, *61*, 553−556.

Larrauri, J. A.; Ruperez, P.; Saura-Calixto, F. Antioxidant activity of wine pomace. *Am. J. Enol. Vitic.* **1996**, *47*, 369−372.

Larrauri, J. A.; Ruperez, P.; Saura-Calixto, F. Effect of drying temperature on the stability of polyphenols and antioxidant activity of red grape pomace peels. *J. Agric. Food Chem.* **1997**, *45*, 1390−1393.

Lecas, M.; Brillouet, J. Cell wall composition of grape berry skins. *Phytochemistry* **1994**, *35*, 1242−1243.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin reagent. *J. Biol. Chem.* **1951** *193*, 265−275.

Macheix, J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990; pp 1−33.

Marsden, W. L.; Gray, P. P. Enzymatic hydrolysis of cellulose in lignocellulosic materials. *Crit. Rev. Biotechnol.* **1986**, *3*, 235−276.

Mazza, G. Anthocyanins in grapes and grape products. *CRC Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 341−371.

McDougall, G. J.; Morrison, I. M.; Stewart, D.; Hillman, J. R. Plant cell walls as dietary fibre: range, structure, processing and function. *J. Sci. Food Agric.* **1996**, *70*, 133−150.

Meyer, A. S.; Yi, O.-S.; Pearson, D. A.; Waterhouse, A. L.; Frankel, E. N. Inhibition of human low-density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes (*Vitis vinifera*). *J. Agric. Food Chem.* **1997**, *45*, 1638−1643.

Meyer, A. S.; Donovan, J. L.; Pearson, D. A.; Waterhouse, A. L.; Frankel, E. N. Fruit hydroxycinnamic acids inhibit human LDL oxidation *in vitro*. *J. Agric. Food Chem.* **1998**, *46*, 1783−1787.

Micard, V.; Renard, C. M. G. C.; Thibault, J.-F. Studies on enzymic release of ferulic acid from sugar-beet pulp. *Lebensm.-Wiss. -Technol.* **1994**, *27*, 59−66.

Montgomery, D. C. *Design and Analysis of Experiments*, 3rd ed.; Wiley: New York, 1991; pp 270−318.

Nardini, M.; Dáquino, M.; Tomassi, G.; Gentili, V.; Di Felice, M.; Scaccini, C. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radical Biol. Med.* **1995**, *19*, 541−552.

Princen, H. M. G.; van Poppel, G.; Vogelezang, C.; Buytenhek, R.; Kok, F. J. Supplementation with vitamin E but not β-carotene in vivo protects low-density lipoprotein from lipid peroxidation in vitro. *Arterioscler. Thromb.* **1992**, *12*, 554−562.

Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

Satué-Gracia, M. T.; Heinonen, M.; Frankel, E. N. Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. *J. Agric. Food Chem.* **1997**, *45*, 3362−3367.

Saura-Calixto, F.; Goñi, I.; Mañas, E.; Abia, R. Klason lignin, condensed tannins and resistant protein as dietary fibre constituents: Determination in grape pomaces. *Food Chem.* **1991**, *39*, 299−309.

Singleton, V. L.; Esau, P. Phenolic substances in grapes and wine, and their significance. *Adv. Food Res. Suppl.* **1969**, *1*, 61−111.

Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagens. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

Slominski, B. A.; Guenter, W.; Campbell, L. D. New approach to water-soluble carbohydrate determination as a tool for evaluation of plant cell wall degrading enzymes. *J. Agric. Food Chem.* **1993**, *41*, 2304−2308.

Steinberg, D. Metabolism of lipoproteins and their role in the pathogenesis of atherosclerosis. *Atheroscler. Rev.* **1988**, *18*, 1−6.

Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witzum, J. L. Beyond cholesterol. Modification of low-density lipoproteins that increase its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915−924.

Teissedre, P. L.; Frankel, E. N.; Waterhouse, A. L.; Peleg, H.; German, J. B. Inhibition of *in vitro* human LDL oxidation by phenolic antioxidants from grapes and wines. *J. Sci. Food Agric.* **1996**, *70*, 55−61.

Tucker, G. A.; Mitchell, J. Cell walls, structure, utilisation and manipulation. In *Biosynthesis and Manipulation of Plant Products*; Grierson, D., Ed.; Blackie Academic and Professional: Glasgow, U.K., 1993; pp 55−103.

Valiente, C.; Arrigoni, E.; Esteban, R. M.; Amado, R. Grape pomace as a potential food fiber. *J. Food Sci.* **1995**, *60*, 818−820.

Wrolstad, R. E.; Wightman, J. D.; Durst, R. W. Glycosidase activity of enzyme preparations used in fruit juice processing. *Food Technol.* **1994**, *48*, 90−98.

Received for review December 1, 1997. Revised manuscript received April 6, 1998. Accepted April 7, 1998.

JF971012F