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Glycosidase activities in sound and rotten grapes in relation to hydrolysis of grape monoterpenyl glycosides

by

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Activités glycosidases dans les raisins sains et les raisins pourris en relation avec l'hydrolyse des monoterpénylglycosides

Résumé : Les activités β-D-glucopyranosidase, α-L-arabinofuranosidase et α-L-rhamnopyranosidase ont été étudiées dans le raisin, au cours de la maturation ainsi que dans les fruits mûrs, sains et atteints de pourriture grise. Ces activités enzymatiques augmentent au cours de la maturation. A maturité, dans les raisins sains, les activités β-glucosidase et α-arabinosidase sont les plus importantes. La contamination des baies par la pourriture grise entraîne une diminution de l'activité β-glucosidase, tandis que les autres activités augmentent. L'action d'une préparation enzymatique obtenue à partir d'une culture de *Botrytis cinerea* sur des terpénylglycosides de synthèse montre que les β-terpénylrutinosides sont fortement hydrolysés, tandis que les terpényl 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides le sont très faiblement.

Key words: berry, variety of vine, maturation, Botrytis, flavour, carbohydrate, monoterpenyl glycoside, enzyme, glycosidase, inhibition.

Introduction

The occurrence of glycosidically bound forms of monoterpenols in Muscat grapes and other aromatic cultivars has been established (WILLIAMS *et al.* 1982; GÜNATA *et al.* 1985). These forms, which are generally more abundant than the free, flavouring ones, occur as disaccharide glycosides: 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides and 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides (rutinosides). As previously shown, monoterpenols can be released from these precursors by enzymic hydrolysis (CORDONNIER and BAYONOVE 1974; GÜNATA 1984; ARYAN *et al.* 1987). More recently, it has been demonstrated that enzymic hydrolysis of grape monoterpenyl disaccharide glycosides is a sequential reaction requiring three glycosidases, firstly α-L-arabinofuranosidase (EC 3.2.1.55) or α-L-rhamnopyranosidase (EC 3.2.1.40) and secondly β-D-glucopyranosidase (EC 3.2.1.21) (GÜNATA *et al.* 1988). Accordingly, it was of interest to study these enzymes in grapes where they occur naturally. In addition, as it is known that *Botrytis cinerea*, a very common fungus on rotten grapes, produces a certain number of enzymes of enological interest (DUBOURDIEU *et al.* 1983; GRASSIN and DUBOURDIEU 1987), the production of these glycosidases by this mold was studied and their hydrolytic action towards grape monoterpenyl glycosides was tested.

Materials and methods

Grape samples

Grapes of cv Muscat of Alexandria were harvested at appropriate intervals during maturation, from the Compagnie des Salins du Midi, Domaine de Jarras, Aigues-

Table 1

Glycosidase activities in berries of Muscat of Alexandria during the maturation period · Activity as nmol p-nitrophenol liberated from corresponding substrate per min and per g of fruit

Activités glycosidases dans les baies de raisin de Muscat d'Alexandrie au cours de la maturation · Activité exprimée en nmol de p-nitrophénol libéré à partir du substrat correspondant par min et par g de baie

Harvest date	Sugars (g/l)	Total acidity (meq/l)	Glycosidase activity		
			β-Glucosidase	α-Arabinosidase	α-Rhamnosidase
23.07	88	396	3.8	0.3	—
12.08	146	165	15.3	0.6	—
01.09	192	80	16.1	1.7	0.4

Mortes vineyard. The other grape cultivars were harvested at maturity from the vine collections of the Agronomy Research Centre (I.N.R.A.) in Montpellier. Sound and rotten berries were separated, immediately immersed in liquid nitrogen and kept at −20 °C until use.

Enzyme samples

Grape crude enzymic extract: The frozen berries were deseeded and ground in a Dangoumau ball grinder in liquid nitrogen. The powder obtained was treated with acetone to obtain an acetone powder which was then used for the preparation of crude enzymic extract. All details have been described elsewhere (BIRON *et al.* 1988).

Botrytis cinerea crude enzymic extract: A strain of *B. cinerea* isolated from grapes (Laboratory of Botany, E.N.S.A., Montpellier) was inoculated in a grape juice (200 ml) prepared from sound, ripe berries of Muscat of Alexandria and sterilized at 110 °C for 15 min. An aerobic culture was performed at 20 °C in Roux flasks. After 10 d of development, when the mycelium had covered the whole surface of the juice, the medium was centrifuged (10,000 *g*, 2 °C, 10 min) and the supernatant was dialysed against 5 l of 0.1 M acetate buffer, pH 4.2, for 12 h. After straining through cheesecloth and nitrocellulose membrane (0.45 µm), the filtrate was concentrated to 3.5 ml in an Amicon cell (molecular weight membrane cut-off 10^4 daltons). Controls for glycosidase activity determinations were run as above with sterilized and non-inoculated grape juice.

Enzyme assays

Glycosidase activities in crude enzymic extracts were determined by the method of BIRON *et al.* (1988) using different synthetic substrates: p-nitrophenyl (pNP)-β-D-glucopyranoside, pNP-α-L-rhamnopyranoside, pNP-6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside (pNP-β-rutinoside) obtained from Sarsynthèse (France) and pNP-α-L-arabinofuranoside (Sigma, USA). For inhibition study by D-glucono-δ-lactone (Sigma, USA), various amounts of this compound were added to an enzymic extract obtained from sound Muscat of Alexandria berries. Controls were made up with no addition of D-glucono-δ-lactone.

Hydrolysis of the grape monoterpenyl glycosides by *B. cinerea* crude enzymic extract was studied using thin layer chromatography. The principal grape monoterpenyl glycosides, i e. geranyl, neryl, linalyl-6-O-α-L-arabinofuranosyl and rhamnopyranosyl-β-D-glucopyranosides, synthesized in our laboratories (VOIRIN *et al.* 1988), were

used as substrates. Each glycoside (4 mM) was dissolved in 100 mM acetate buffer (pH 4.2) and was incubated (40 °C, 36 h) with 100 µl of *B. cinerea* crude enzymic solution. Each sample (10 µl) was then subjected to thin layer chromatography analysis at different intervals of incubation (GÜNATA *et al.* 1988). Controls were run with heat inactivated enzymic extract (95 °C, 30 min).

Gluconic acid determinations

Gluconic acid levels in grape crude enzymic extracts were determined by isotachophoresis techniques (CHAUVET 1981).

Results and discussion

Glycosidase activities during grape maturation

As shown in Table 1, α-arabinosidase and β-glucosidase activities increased during grape maturation, confirming previous results (ARYAN *et al.* 1987). The greatest increase was that of β-glucosidase which reached more than 4-fold the green stage level. α-Rhamnosidase activity was detected only at maturity and at much lower levels than the two others.

Glycosidase activities in mature grapes and *Botrytis cinerea* crude enzymic extract

In sound grapes, β-glucosidase, α-arabinosidase and α-rhamnosidase activities were present in all the cultivars (Table 2), both in Muscat and non-Muscat fruits, in agreement with previous results (ARYAN *et al.* 1987). When compared to the others, β-glucosidase activity was highest. It may seem surprising that glucosidase activities are higher in some non-Muscat varieties which are very poor in monoterpenyl glycosides (GÜNATA *et al.* 1985). This would tend to indicate that these activities may be involved in the hydrolysis of other grape glycosides (RIBÉREAU-GAYON 1964; CHEYNIER and RIGAUD 1986).

In rotten grapes, α-arabinosidase and α-rhamnosidase activities increased considerably when compared with the corresponding sound ones. This was observed in all cultivars (Table 2). The highest increase was for α-rhamnosidase. In some samples (Grenache, Sirah, Cinsaut), there was 5 times as much of this activity as in sound grapes. The high levels of these two enzymes are undoubtedly related to the infection of grape berries by molds. In addition of *B. cinerea*, which is the principal fungus, many species such as *Penicillium* and *Aspergillus* develop on grapes (RIBÉREAU-GAYON 1970; RADLER and THEIS 1972; LE ROUX *et al.* 1973; SAPIS 1978; KOVAC and LAVOLLAY 1983; KOVAC and VZBASKI 1983). The latter fungus was reported to produce α-arabinosidase (WAIBEL *et al.* 1980; KAJI 1984) and α-rhamnosidase (KAMIYA *et al.* 1967; ONO 1980). Production of α-arabinosidase was also reported for *B. cinerea* in media other than grapes (KAJI 1984). A culture of this fungus was developed on a grape juice previously sterilized by heating with no glycosidase activities. After 10 d of *Botrytis* growth, β-glucosidase activity was 9.9 nmoles p-nitrophenol (pNP)/min/ml of culture, α-arabinosidase was 7 nmoles pNP/min/ml and α-rhamnosidase 1.2 nmoles pNP/min/ml. These results show the capacity of *B. cinerea* to synthesize the three enzymes.

In contrast to α-arabinosidase and α-rhamnosidase, β-glucosidase activity was considerably lower in rotten grapes than in sound ones. This is possibly due to the gluconic acid produced by *Botrytis* and the other fungi (DITTRICH *et al.* 1974; HOLBACH and WOLLER 1976; CHAUVET 1981). At the usual pH values of musts and wines, this acid would be expected in equilibrium with its lactone (MCCLOSKEY 1974). Gluconolactone is

Table 2

Glycosidase activities in sound (S) and rotten (R) mature berries of different grape varieties · Activity as nmol p-nitrophenol liberated from corresponding substrate per min and per g of fruit

Activités glycosidases dans les baies saines (S) et les baies pourries (R) de différentes variétés de raisin à maturité · Activité exprimée en nmol de p-nitrophénol libéré à partir du substrat correspondant par min et par g de baie

Substrates	Muscat of Alexandria		Muscat of Frontignan		Merlot		Carignane		Grenache		Baroque		Ugni blanc		Sirah		Cinsaut		Cabernet Sauvignon	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
pNP-α-L-rhamnopyranoside	0.4	0.8	0.4	1.0	1.0	1.3	0.5	2.3	0.2	3.0	1.0	4.7	0.7	2.6	0.3	2.3	0.2	1.5	0.2	1.1
pNP-α-L-arabinofuranoside	1.7	3.6	0.5	0.9	1.3	3.9	2.9	3.2	3.5	7.5	1.5	5.0	1.1	1.7	1.3	2.6	0.7	0.9	1.6	2.9
pNP-β-D-glucopyranoside	15.5	4.0	13.8	2.5	14.3	5.5	18.9	7.0	8.0	5.3	13.1	8.7	16.8	5.0	20.3	6.4	10.0	5.0	16.2	4.1
pNP-β-rutinoside	2.5	—	1.9	0.7	0.8	0.3	4.9	0.5	1.2	—	1.1	—	2.3	0.3	1.4	0.6	0.6	0.3	3.5	0.3

a very strong inhibitor of fungal (BLONDIN *et al.* 1983; DEKKER 1986), bacterial (AÏT *et al.* 1982) and plant (HEYWORTH and WALKER 1961) β-glucosidases and its corresponding acid was present in enzymic extracts as prepared above. Determinations of gluconic acid in the extracts from infected berries indicated levels of between 50 and 200 mg/l (Table 3), while no gluconic acid was detected in extracts from sound berries. Moreover, when increasing amounts of gluconolactone were added to a medium containing grape β-glucosidase, there was a gradual decrease of the enzyme activity (Table 4). With 200 mg/l of gluconolactone, β-glucosidase inhibition reaches 29 %. This accounts for the results found in rotten grapes. The usual levels of gluconic acid in grape juices and wines are between 0.5 and 3 g/l (MCCLOSKEY 1974; CHAUVET 1981; FREGONI *et al.* 1986) and sometimes higher when acetic bacteria are present in grey mold (CHAUVET 1981); 5 g/l of gluconolactone totally inhibited grape β-glucosidase activity (Table 4). For α-arabinosidase and α-rhamnosidase activities we checked that gluconolactone had no effect, even at high levels (2 g/l).

Table 3

Gluconic acid levels in crude enzymic extracts of rotten berries of different grape varieties

Teneur en acide gluconique dans les extraits enzymatiques bruts de baies pourries de différentes variétés de raisin mûr

Cultivars	Gluconic acid (mg/l)
Muscat of Alexandria	75
Muscat of Frontignan	50
Merlot	200
Carignane	125
Grenache	50
Baroque	65
Ugni blanc	50
Sirah	60
Cinsaut	100
Cabernet Sauvignon	50

The enzymic activity towards pNP-β-rutinoside which possesses the same sugar moiety as some monoterpenyl glycosides (WILLIAMS *et al.* 1982) decreased notably or completely disappeared in rotten berries (Table 2). This is probably due to β-glucosidase inhibition by gluconolactone, since complete hydrolysis of this substrate requires α-rhamnosidase and β-glucosidase (GUNATA *et al.* 1988).

Table 4

Effect of gluconolactone concentration on grape β-glucosidase activity

Incidence de la concentration en gluconolactone sur l'activité β-glucosidase du raisin

Gluconolactone concentration (mg/l)	Relative activity (%)
10	97
200	71
1 000	30
2 000	12
5 000	0

Action of *Botrytis cinerea* crude enzymic extract on monoterpenyl glycosides

Major grape monoterpenyl arabinosyl and rhamnosylglucosides were subjected to hydrolysis by *B. cinerea* crude enzymic extract. After 24 h of incubation, monoterpenyl β-rutinosides were completely hydrolysed with the appearance of rhamnose and glucose, whereas monoterpenyl arabinosylglucosides were slightly affected, even after 36 h of incubation. This may be due to low affinity of *Botrytis* α-L-arabinofuranosidase towards grape monoterpenyl arabinosylglucosides. It could also be caused by the presence of inhibitors in the crude enzymic extract. Further studies will be carried out on the hydrolytic activities and other properties of purified *B. cinerea* glycosidases.

Summary

β-D-glucopyranosidase, α-L-arabinofuranosidase and α-L-rhamnopyranosidase activities were studied in grapes, both during maturation and in sound and rotten mature fruits. Enzymic activities increased during maturation. At maturity, in sound grapes, β-D-glucopyranosidase and α-L-arabinofuranosidase activities were the highest ones. Berry infection by fungi results in a decrease in β-glucosidase activity, while the others increase. The effect of an enzymic extract from a culture of *Botrytis cinerea* towards synthetic terpenyl glycosides showed that hydrolysis was strong for β-terpenyl rutinosides and weak for β-terpenyl 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides.

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