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TEMPERATURE AND ENZYMIC CONTROL OF MALATE METABOLISM IN BERRIES OF *VITIS VINIFERA*

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Key Word Index—*Vitis vinifera*; Vitaceae; grapes; malic acid metabolism; enzymes; temperature dependence.

Abstract—The specific activities of grape enzymes concerned with malic acid metabolism were determined at various stages of berry development under two temperature regimes. It was found that the lower acidity levels occurring in high temperature grapes at maturity are due to a changed pattern in acid breakdown rather than to reduced malic acid accumulation. The differences in acid disappearance cannot be explained by a shift in relative enzyme activities towards malate consumption with rising temperature.

INTRODUCTION

The negative correlation between acid concentrations in grapes and high seasonal temperatures is of considerable interest to viticulturists [1–4]. Malic acid concentration varies more than tartaric acid [1,5] and research in recent years has focused on the enzymes and biochemical reactions concerned with malic acid synthesis and breakdown [6–8]. The formation of malic acid in grapes is considered to be mainly due to β-carboxylation of phosphoenolpyruvate (PEP) by PEP-carboxylase (EC 4.1.1.31) and degradation occurs via NADP-dependent malic enzyme (EC 1.1.1.40), followed by respiration of the resulting pyruvate [7,9,10]. In addition, part of the malic acid can be converted to sugars by PEP-carboxykinase (EC 4.1.1.32) and subsequent 'reversal' of glycolysis [8,9,11]. The role of temperature in regulating these reactions, however, is not clear.

Based on different temperature responses of PEP-carboxylase and malic enzyme, Lakso and Kliewer [7] concluded that an optimal tendency for malic acid accumulation should exist between 20° and 25°, a temperature range which indeed results in high acid content in grapes at maturity [1–3,5]. However, even though the enzymes were extracted from grape berries which were rapidly accumulating acid, the sp act of malic enzyme was at all temperatures greater than PEP-carboxylase activity, which suggests that additional mechanisms mediating acid metabolism may be operating *in vivo*. Furthermore, the results of earlier work concerning temperature influence on acid concentrations in grapes show that high temperature affects malic acid accumulation considerably less than it does the subsequent rate of degradation [1,4].

The present paper describes changes in sp act of the relevant enzymes at various stages of berry development under warm (30/25°) and cool (20/15°) conditions and the results are compared with data obtained from grape leaves of three different age groups. This provides information concerning the coarse control of malic acid metabolism in grape berries as opposed to its regulation in leaf tissue which does not exhibit the peculiar pattern of seasonal acid and sugar fluctuations distinctive of maturing fruits.

RESULTS

The difference in temperature, not only affected the acid and sugar levels of the grapes, but also had considerable bearing on berry growth, as has been reported previously (Fig. 1 and ref. [2]). While the influence on

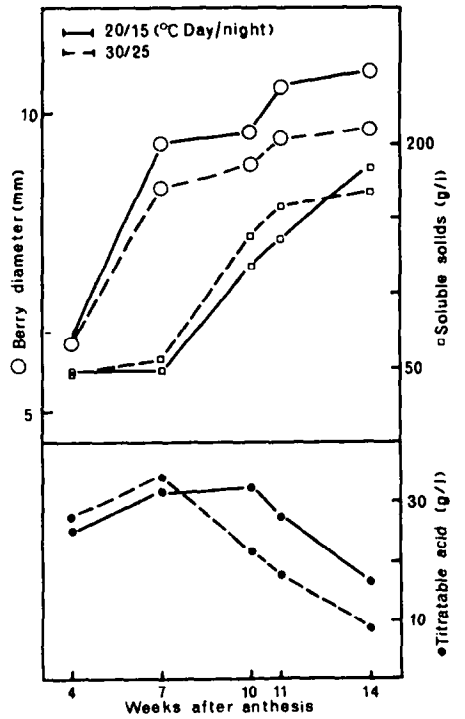

Fig. 1. Effect of temperature on grape berry diameter and acid/sugar concentrations (for details see Experimental Section).

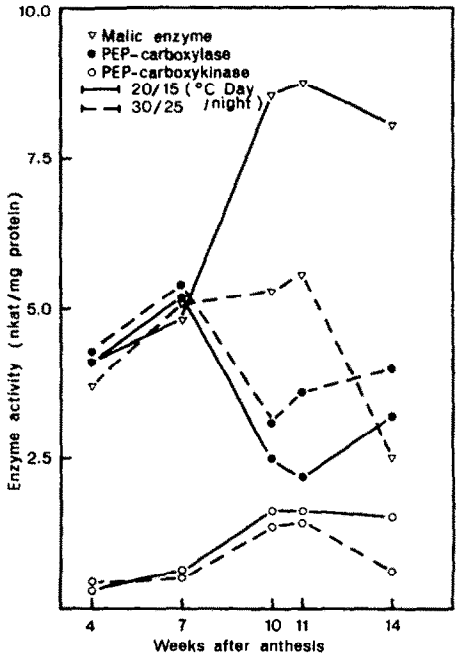

Fig. 2. Specific activities of enzymes concerned with malic acid metabolism in grapes under two temperature conditions at various stages of berry development (the values in Figs. 2, 3 and Table 1 are means of 3 independent extracts, assayed at 25°).

growth is very pronounced during the first stage of berry development, acid and sugar concentrations seemed not greatly different at this time. Towards the end of the period between the second and third harvest the berries changed colour, which is a visual indication of the beginning of acid decrease and sugar accumulation. During the following periods the final differences in size and sugar concentrations developed, whereas the acid levels did not diverge any further.

The sp act of the enzymes, which are concerned with malic acid metabolism, e.g. PEP-carboxylase, malic enzyme and PEP-carboxykinase were determined at the corresponding points in berry development (Fig. 2): PEP-carboxylase activity increased during the first period of berry growth, then fell to *ca* 50% at the time of colour change and recovered to some extent eventually, irrespective of temperature. PEP-carboxykinase showed an almost complementary pattern with highest activities at the time of rapid sugar accumulation. Malic enzyme exhibited activities comparable to those of PEP-carboxylase as long as the berries were green. With the onset of ripening, malic enzyme at both temperatures reached higher values, with nearly double sp act under cooler conditions. Near maturity the rates of the malic acid degrading reactions under warm conditions were relatively low, whereas in the cold, malic enzyme and PEP-carboxykinase maintained close to maximum values.

In contrast to these curves, the enzymes initiating the reactions of glycolysis and of the hexose monophosphate shunt—phosphofructokinase and glucose-6-phosphate (G-6-P) dehydrogenase, respectively—followed very different patterns during berry development (Fig. 3). Phosphofructokinase at both temperatures maintained a steady value throughout all stages. G-6-P dehydrogenase was very high at first in the 30/25° environment, the activity then falling to values comparable to those of the continuously declining rates in the cold climate.

For comparison the respective enzymic activities were assessed in grape leaves. Of all the enzymes assayed, only leaf-malic dehydrogenase (EC 1.1.1.37; NAD) showed an activity in the range of the values obtained from berries: nkat/mg protein, respectively, 285 (leaf), 250 (fruit, warm), 310 (fruit, cool). Malic dehydrogenase remained within ± 10% of the above results, irrespective of the developmental stage of the tissue. The other leaf enzymes had activities 2–5 times lower than those of the corresponding enzymes in the berry (Table 1). G-6-P dehydrogenase again seemed to be more important in young and growing parts. Phosphofructokinase, although exceptionally constant throughout development in berries, showed decreasing activity with increasing size in the leaf. The drop in PEP-carboxylase activity to *ca* 50% with age occurred in both fruits and leaves. Malic enzyme was the most consistent enzyme in the leaf. Higher temperature during development had little effect on NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42), an enzyme which may be involved in malic acid catabolism, and maximum sp act occurred in young material: 2.5–1.6–0.8 (leaves) and 4.7–4.0–3.2–3.1–2.7 nkat/mg protein (berries, cool).

Negligible activity of the presumably mitochondrial NAD-dependent isocitrate dehydrogenase was detected in these extracts. Centrifugation at low speed in the first run of the extraction neither increased the activity of this enzyme, nor that of the equally low α-ketoglutarate dehydrogenase.

Malate inhibits grape (cv. Carignane) PEP-carboxylase [12], half maximum activity being observed at *ca* 35 mM. Our own results confirm the susceptibility of PEP-carboxylase towards this inhibitor, since about 20 mM sodium malate was required for the same effect in Cabernet Sauvignon extracts. The malate concentration needed for a 50% inhibition of grape phosphofructokinase in our assays proved to be as high as 55 mM. However, as with other plants [13], citrate was a much more potent inhibitor of this enzyme, 4 mM sodium citrate giving a 50% inhibition.

DISCUSSION

The development of the experimental grapes in the growth cabinets at different temperatures (Fig. 1) followed a pattern described for fruits of the same cultivar (Cabernet Sauvignon) under similar conditions [1,2]. During the stage of acid accumulation the titratable acid content tended to be slightly higher under hot conditions (Fig. 1 and ref. [4]), but the higher values are probably due to increased tartaric acid concentrations rather than to elevated malic acid content [1]. Since tartaric acid is believed to be an oxidation product of glucose [14,15], a faster turnover of glucose via the hexose monophosphate shunt could possibly account for intensive tartaric acid formation in young and growing tissue (9, 16, Table 1), particularly under hot conditions (Fig. 3). The subsequent decrease in acid components during ripening on the other hand, has previously been observed to be due mainly to a decline in malic acid [3,5].

At the onset of ripening, low rates of CO_2-fixation have been found in grape berries [10] and the substantial drop in PEP-carboxylase activity during that period is

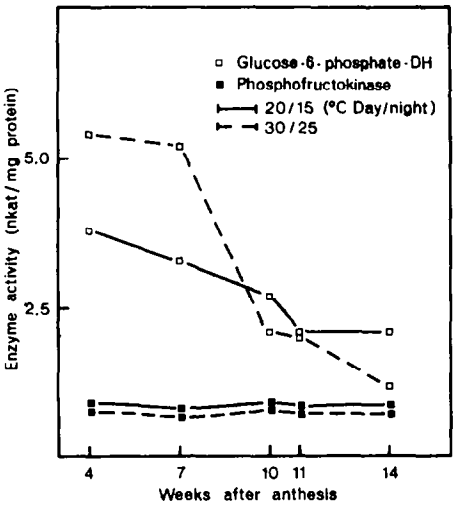

Fig. 3. Specific activities of enzymes catalyzing irreversible reactions in glycolysis and hexose monophosphate shunt, respectively, at five points of grape berry growth under two temperature regimes.

consistent with this observation. The activity of PEP-carboxykinase, the enzyme initiating gluconeogenesis in grapes [8,9,11], simultaneously increased about threefold. Temperature had only a slight effect on the sp act of both these enzymes (Fig. 2).

In the case of malic enzyme, the activity per mg protein diverged considerably between the two temperatures. It has to be borne in mind, however that the assays were run at 25° in both cases and that the enzymic activity within the intact berries kept at 30° is likely to be higher than the obtained values by a factor of about 1.3 and lower by *ca* 0.65 at 20° [7]. If we adjust our results using the above factors, the actual malic enzyme activities during ripening under either condition were very similar, while for example, PEP-carboxylase and PEP-carboxykinase activities would be *ca* twice as high at 30°. This indicates that malic acid is metabolized via malic enzyme at a given rate virtually independent of the average ambient temperature, provided that the ratio between NADP and NADPH remains the same. An increase in activity of the pentose phosphate pathway would thus reduce the rate of malate decarboxylation and such a regulatory effect may be involved as well.

Gluconeogenetic transformations of malic acid involve a reversal of carbon flow in glycolysis which, in other tissue, was found to be accomplished by inhibiting the relevant irreversible reaction—phosphorylation of fructose-6-phosphate (F-6-P)—and bypassing it via fructose-1,6-diphosphatase in the reverse direction [13]. Plant phosphofructokinase is sensitive to ATP, inorganic phosphate, PEP and citrate levels [13]. Addition of 1–5 mM citrate inhibited the grape enzyme by 4–74%, ten times higher concentrations of malate yielding about the same effect. Since the overall concentration of malate in grapes can be as high as 150 mM, inhibitory malate levels could easily be present at the site of enzymic action.

In *Vicia faba* a number of carboxylic acids activate sucrose phosphate synthase [13], an enzyme believed to be responsible for sugar accumulation in grapes [20]. Malate and/or citrate may therefore not only mediate glycolysis, but also direct the incoming sugars towards accumulation.

Sacher [18] has suggested that the permeability of membranes increases with progressing fruit maturity. Although the mechanisms of these changes in tonoplast permeability, which would permit malic acid efflux from the vacuole are not known, our observations and other data concerning temperature effects on grape acids [1,2,20], indicate that the timing of this process could be crucial in determining the final acid level.

Table 1. Activities (nkat/mg protein) of enzymes extracted from grape leaves of three different age groups

Leaf age Leaf size (av. cm^2)	Young 15	Medium 70	Mature 130
Glucose-6-phosphate dehydrogenase	1.21	1.10	0.94
Phosphofructokinase	0.30	0.22	0.14
Malic enzyme (NADP)	1.30	1.35	1.17
PEP-carboxylase	1.38	1.54	0.79
Malic dehydrogenase	276	304	276

EXPERIMENTAL

Vines of *Vitis vinifera*, cv. Cabernet Sauvignon, were established [21] and transferred into growth cabinets 3 weeks after flowering. They were kept at 30° or 20° during the 16 hr days and at a 5° lower temp for the dark periods (for more details, see ref. [2]). Berries, 20 g per treatment, were taken at random from 10 vines for each of the 5 harvests. For the third point (week 10), the berries were sampled within 4 days after appearance of the first hue of colour. Grape leaves were harvested from an identical batch of vines without fruit in the glasshouse (mean temp. 20°).

Enzyme extractions. Grape enzymes were extracted essentially as described in ref. [8], the extraction medium containing 2 mM EDTA, 5 mM DTT, 10 mM cysteine–HCl, 50 mM KCl and 5% polyethylene glycol (PEG) 4000 in 2.5 vol of cold 0.5 M Tris–HCl buffer, pH 8.2. The PEG 40% pellet was resuspended in 0.1 M Tris–HCl buffer, pH 7.4 (1 ml/4 g fr. wt). Grape leaves (10 g) were ground in liquid N_2 and the frozen powder transferred into 50 ml of cold 0.5 M Tris–HCl buffer, pH 7.4 and with additives as above. The brei was strained through cheese-cloth and the filtrate treated as above.

Enzyme assays. All enzymes, except PEP-carboxykinase, were assayed by following the oxidation–reduction of the respective coenzyme at 340 nm and 25°. Optimum conditions for grape enzymes (buffer, pH, substrate and cofactor concentrations) were determined and the mixtures contained in a total vol of 3 ml (conc mM in parentheses) for: G-6-P dehydrogenase: G-6-P (2); NADP (0.1); $MgSO_4$ (3); *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 7.4 (50); extract 0.1 ml. Phosphofructokinase: F-6-P (2); NADH (0.15); $MgSO_4$ (1); ATP (0.15); 20 μg aldolase; 8 μg glycerol-3-phosphate dehydrogenase–triosephosphate isomerase; extract 0.1 ml. Malic dehydrogenase: oxaloacetic acid (neutralized 0.5); NADH (0.15); piperazine-*N*,*N'*-bis (2-ethanesulfonic acid (PIPES) pH 7 (50); extract 5 ml. Malic enzyme: L-malate (neutralized 4); NADP (0.1); $MgSO_4$ (1); HEPES pH 7.2 (50); extract 0.1 ml. PEP-carboxylase: PEP (1); $NaHCO_3$ (2); NADH (0.15); $MgSO_4$ (1); HEPES pH 8.0 (50); extract 0.1 ml (the extracts contained an excess of endogenous malic dehydrogenase). Isocitrate dehydrogenase: isocitrate (1); NADP (0.10); $MgSO_4$ (1); Tris pH 8 (50); extract 0.1 ml. PEP-carboxykinase activity was assayed at 25° by the $^{14}CO_2$–oxaloacetic acid exchange reaction [8]. The assay mixture contained 5 mM oxaloacetic acid (previously neutralized with NaOH), 15 mM $NaH^{14}CO_3$ (0.4 μC), 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM ATP and 0.3 ml enzyme extract in a total vol of 2 ml

0.1 M PIPES buffer pH 6.6. The reaction was started by the addition of oxaloacetate and after 10 min supplemented with 5 units exogenous malic dehydrogenase and excess NADH (12 mM) and allowed to proceed for another 2 min. The protein was precipitated with 0.2 ml 20% TCA and after addition of two drops of *n*-octanol the mixture was flushed for 30 min with CO_2. An aliquot (1 ml) was mixed with 10 ml Insta-Gel and counted in a scintillation spectrometer. The ATP dependent incorporation of $^{14}CO_2$ into the acid stable fraction was taken as a measure of PEP-carboxykinase activity.

Acid concentrations, soluble solid content and size of the berries. Acids were determined by twice titrating two samples of pressed grape juice to the phenolphthalein end point with 0.1 M KOH. The results are means of these 4 values and are expressed as g/l. tartaric acid. The content of soluble solids in the same samples was estimated refractometrically. The average berry diameter represents a mean of at least 80 berries which were subsequently extracted for enzyme determination.

Protein determination. In order to minimize polyphenolic and detergent interference, aliquots of the extracts were treated according to ref. [22], the washed TCA ppts redissolved in M NaOH and the protein content determined as described in ref. [23].

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