Pergamon 0031-9422(95)00708-3 Phytochemistry, Vol. 41, No. 3, pp. 691–697, 1996

0031-9422/96 $15.00 + 0.00

ROLE OF CHLOROPHYLLASE IN CHLOROPHYLL METABOLISM IN OLIVES CV. GORDAL

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(*Received in revised form* 12 *August* 1995)

Key Word Index—*Olea europaea*; Oleaceae; olive; enzyme; pigments; chlorophyllase; chlorophylls; chlorophyllic pigments.

Abstract—During the development of the olive two stages in chlorophyll evolution can be distinguished: one of synthesis when the fruit is in the growth phase, and a degradative stage which begins when the fruit is completely developed. Although the enzyme chlorophyllase is present in olives throughout their development cycle, its activity reaches a maximum at both the beginning and end of the vegetative growth phase. During these two periods chlorophyll synthesis prevails, so the two maxima in enzyme activity are associated with the involvement of chlorophyllase in chlorophyll synthesis. Small amounts of chlorophyllides *a* and *b* have been detected during the initial growth period of the fruit, which coincides with a phase of great chlorophyll synthesis. Later, synthetic and degradative mechanisms may overlap, making the detection of dephytylated compounds impossible. At the same time allomerized chlorophyllic derivatives—13^2-hydroxychlorophyll *a*, 13^2-methoxychlorophyll *a*, 13^2-methoxychlorophyll *b*, 15^1-methoxylactone-chlorophyll *a* and 15^1-methoxylactone-chlorophyll *b*—can be detected, suggesting the presence of chlorophyll oxidase.

INTRODUCTION

Although the disappearance of chlorophylls during fruit ripening has been the subject of research for many years, the process remains to be elucidated. While the loss of chlorophylls has been attributed to the rupture of the chloroplast and/or the activity of chlorophyllase, neither of these possible causes can be easily demonstrated [1]. Different hypotheses postulate that chlorophyllase may intervene in both the synthesis and the catabolism of chlorophyll, although the synthetic function is more commonly associated with enzyme *in vivo* and the catabolic function *in vitro*. Other enzymes involved in the degradation of chlorophylls include chlorophyll oxidase [2,3], Mg-dechelatase [4,5] and a dioxygenase [6] apparently responsible for the oxygenolytic cleavage reaction of the porphyrin ring. Consequently, it has been argued that the disappearance of chlorophyll is associated with oxidative enzyme systems and that chlorophyllase intervenes only in an early stage of the process [7]. Although monitoring of the compounds resulting from chlorophyll degradation is complicated, great advances are currently being made in the identification of chlorophyll catabolites in senescent barley leaves [6,8,9] and in senescent rape cotyledons [10–12].

Chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) is present in fruits during the phases of growth and senescence. Abdul-Baki [13] found a maximum in chlorophyllase activity in fruits of the lima bean, coinciding with the maximum content of chlorophylls. This maximum activity was higher than that found when catabolism began or when there was a loss of chlorophylls. In an analogous study on tomatoes, Minamide and Ogata [14] found maximum chlorophyllase activity in the colour-break stage (48 days after flowering), after which activity rapidly decreased. Other authors, however, have demonstrated that both chlorophyllase activity and chlorophyll degradation increase during leaf senescence [15,16] as well as in ethylene-treated fruits exposed to air [1,17–20].

Amir-Shapira *et al.* [21] detected chlorophyllides in the skin of senescent citrus fruits and found that these were much more abundant when the fruits had been treated with ethylene. Purvis and Barmore [17] found that the level of chlorophyllase activity did not change when ethylene treatment was ceased and remained constant even when the degradation of chlorophylls stopped. The latter authors concluded that, although ethylene is necessary to induce chlorophyll degradation, its function is not restricted to the induction of chlorophyllase activity. The relationship between ethylene and chlorophyllase is not clear from the results of Baardseth and von Elbe [22], who were unable to demonstrate any increase in the rate of degradation of chlorophylls in spinach in the presence of ethylene.

The detection of dephytylated chlorophyll derivatives during table olive fermentation [23], as well as the drastic loss of chlorophyllic pigments during virgin olive oil

extraction [24], suggests that chlorophyllase is present in the fruits and is activated during the processing procedures. Once the methodology for the measurement of chlorophyllase had been adapted by separating the substrate and product using HPLC, the enzyme was demonstrated to be present in olives, and the kinetic parameters defining it were characterized [25, 26].

The present study reports the results of experiments conducted to examine the changes that occur in both chlorophyll concentration and chlorophyllase activity during development and ripening of olives of the Gordal variety. The aim of the study was to establish a relationship between the presence of the enzyme and the biosynthesis and catabolism of chlorophylls.

RESULTS AND DISCUSSION

Development of the fruit and changes in moisture with season

Figure 1 shows the changes in moisture content and in fruit weight as a function of the harvesting date. Three stages can be clearly distinguished. The first of these is a period of rapid growth and covers the first four weeks. In this period the fruit increases its moisture content from 71 to 76%. The second stage, between weeks 5 and 14, is characterized by a fluctuation in the moisture content, which increases and decreases slowly within quite tight limits (76–79%). The third stage is one of stabilization, the moisture content remaining at around 75%. The moisture content of ripe Gordal olives is considerably higher than that of other olive varieties (57.5% in Hojiblanca and 68.8% in Manzanilla) [27]. Since high moisture content is directly related to low oil content, Gordal variety is used for table consumption rather than for oil extraction.

The fruit weight increases until about the third week of September (week 14), reaching a maximum of some 10 g. The weight then remains practically constant until the end of ripening. Stabilization of the weight coincides with stabilization of the moisture content and very nearly coincides with the period of maximum fatty acid synthesis [28]. The point of inflection which marks this stabilization also marks the transition from the growth phase to the ripening phase.

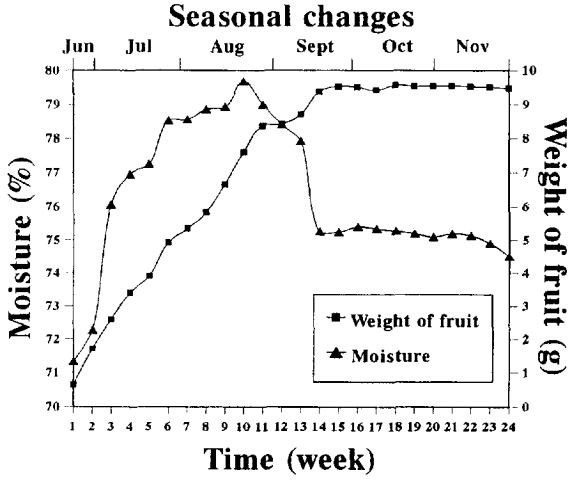

Fig. 1. Changes in moisture content and fruit weight during development and ripening of Gordal variety olives.

Changes in chlorophylls with season

Table 1 shows the concentrations of the individual chlorophyll pigments found in the fruits in relation to the harvesting date. The dates cover the periods of growth and ripening. Although the table includes the concentrations of chlorophyllides *a* and *b*, as well as the allomerized forms—13^2-hydroxychlorophyll *a* (13^2-OH-chl *a*), 13^2-methoxychlorophyll *a* (13^2-MeO-chl *a*), 13^2-methoxychlorophyll *b* (13^2-MeO-chl *b*), 15^1-methoxylactone-chlorophyll *a* (15^1-MeO-lactone-chl *a*) and 15^1-methoxylactone-chlorophyll *b* (15^1-MeO-lactone-chl *b*)–the involvement of the former in the metabolism of chlorophylls will be described later; this section deals exclusively with the changes in chlorophylls *a* and *b*. Figure 2 illustrates the changes in the concentrations of chlorophylls *a* and *b* during the development cycle of the olive. In general terms, these are parallel, although at certain times they differ to a greater or lesser extent.

Although various stages can be distinguished in the changes in the two chlorophylls, at first sight two stand out. The first coincides with the rapid increase in moisture content (weeks 1 and 4), and is characterized by a continuing increase in the concentration of chlorophylls *a* and *b*. This is related directly with the synthesis phase. From then on, there is a systematic decrease in the concentration of chlorophylls that lasts, though discontinuously, until the end of ripening. Consequently, this second stage is longer than the first. The decrease in chlorophyll concentration begins during the later stages of the fruit growth phase and continues during the ripening stage. It may be that during this stage two effects are being manifested throughout: the first a dilution effect resulting from the increase in size of the fruit, and the second a degradation of chlorophyll as a result of the progressive loss of photosynthetic capacity. At certain times both of these effects could overlap. Fruit development, culminating in week 14, is accompanied by an increase in storage parenchyma or pulp and this causes a considerable increase in the weight. This increase is not paralleled by an increase in the surface area of the fruit. Although the net chlorophyll content (milligrams of chlorophyll per fruit) increases while the fruit is in the growth phase, the amount per kilogram dry weight of fruit would decrease. This was tested by picking olives of different sizes, from 2 to 8 g in weight. The chlorophyll content of these fruits was measured and the results expressed in micrograms per fruit (Fig. 2). The changes in the chlorophylls differ according to how the results are expressed, supporting the observations made above.

If the decrease in the concentration of chlorophylls during this period is attributed solely to the dilution

Table 1. Chlorophyllic pigment composition of fruits during development and ripening of Gordal variety olives*†

	Concentration ($mg\,kg^{-1}$)								
	a series‡					*b* series‡			
Time (weeks)	Chl	Chld	OH-chl	MeO-chl	MeO-L	Chl	Chld	MeO-chl	MeO-L
1	147.4	1.18	1.39	0.59	1.40	57.5	nd	0.75	0.30
2	164.9	1.81	2.99	2.81	0.36	64.5	0.59	0.75	0.53
3	166.4	0.85	3.00	3.64	1.89	59.1	nd	1.44	1.03
4	181.1	nd	0.99	0.60	0.53	72.5	0.14	0.46	0.18
5	170.0	1.38	1.91	3.20	2.31	65.1	0.11	0.86	0.94
6	149.7	0.20	0.46	1.96	nd	58.6	0.09	1.86	0.48
7	140.7	0.39	0.35	0.98	nd	54.8	nd	1.65	0.97
8	131.7	0.02	0.21	1.94	nd	51.2	nd	1.74	1.02
9	108.7	nd	0.34	8.10	nd	32.2	nd	2.57	3.34
10	98.8	nd	0.64	6.78	nd	32.3	nd	2.59	1.60
11	99.9	nd	0.30	2.16	nd	33.7	nd	1.83	0.90
12	102.3	nd	0.21	1.20	nd	38.0	nd	1.49	0.17
13	100.4	nd	0.18	2.52	nd	42.2	nd	1.76	0.38
14	96.6	nd	0.15	2.00	nd	38.0	nd	0.87	0.25
15	80.1	nd	nd	1.58	nd	24.8	nd	0.76	0.33
16	71.0	nd	0.16	2.05	nd	22.5	nd	1.22	0.38
17	70.4	nd	nd	1.82	nd	21.5	nd	0.45	0.54
18	61.2	nd	1.46	4.24	0.65	17.8	nd	1.02	0.59
19	42.0	nd	0.35	3.66	0.92	13.1	nd	0.78	1.40
20	22.4	nd	0.25	2.98	4.09	6.5	nd	0.64	1.21
21	13.9	nd	nd	2.95	4.99	3.6	nd	0.56	1.38
22	9.8	nd	0.23	1.19	1.89	3.1	nd	0.17	0.40
23	5.8	nd	nd	0.37	0.70	2.3	nd	0.03	0.14
24	3.3	nd	0.01	0.01	0.03	1.6	nd	0.06	0.02

*Destoned olive basis, average of duplicate analysis of two samples (CV < 10% for all measures).

†Key: chl = chlorophyll; chld = chlorophyllide; OH-chl = 13^2-hydroxychlorophyll; MeO-chl = 13^2-methoxychlorophyll; MeO-L = 13^2-methoxylactone chlorophyll; nd = not detected.

‡*a* series: chlorophyll *a* and derivatives; *b* series: chlorophyll *b* and derivatives.

effect, the ratio of chl *a* to chl *b* should follow a certain pattern. However, the fluctuations detected during the decrease in concentration of both chlorophylls makes the ratio between them also vary. This variation is not attributable to a dilution effect resulting from the growth of the fruit. Although the decrease in concentration of both chlorophylls in this stage is continuous and more or less constant, as is to be expected with a dilution effect, this pattern is not maintained and, furthermore, the decrease in the concentration of chl *b* tends to be somewhat greater than that of chl *a*.

The differences in the rate of decrease of the two chlorophylls at certain times and the sudden sharp decrease in week 9 can be interpreted as reflecting metabolic reorganization. The lack of common intermediates in the synthesis of these two chlorophylls could make it necessary to use existing chl *b* to synthesize chl *a*. This implies that the fruit modifies its metabolism according to requirements. The literature makes reference to the preferential degradation of one or other chlorophyll during fruit ripening. It should be noted, however, that the studies published to date [29–31] refer to senescent leaves or ripe fruits and make no reference to earlier stages.

In the later stages of the growth phase (weeks 9–14), the fruit weight increases by *ca* 40%, but the chlorophyll concentration remains constant. This indicates that synthesis of these pigments occurs, counterbalancing the dilution effect produced by the weight increase. In this stage the decrease in the content of chl *a* fluctuates between 0.42 and 3.94%, which implies that its net concentration remains more or less constant. During the same period the concentration of chl *b* increases by between 1.02 and 9.76%. This means a period of considerable accumulation of chl *b*. If, at week 9, chl *b* undergoes a significant decrease in concentration due to the changes that are occurring in the fruit, once the mechanisms that have caused the changes have normalized, those that are involved in the synthesis of the pigment may tend to work more efficiently, restoring the levels of chl *b*. By so doing, a balance can be reached ensuring the efficient functioning of the plant. In this way, the levels of chl *b* would increase to reach a similar ratio which chl *a* as in the weeks preceding week 9. In terms of net

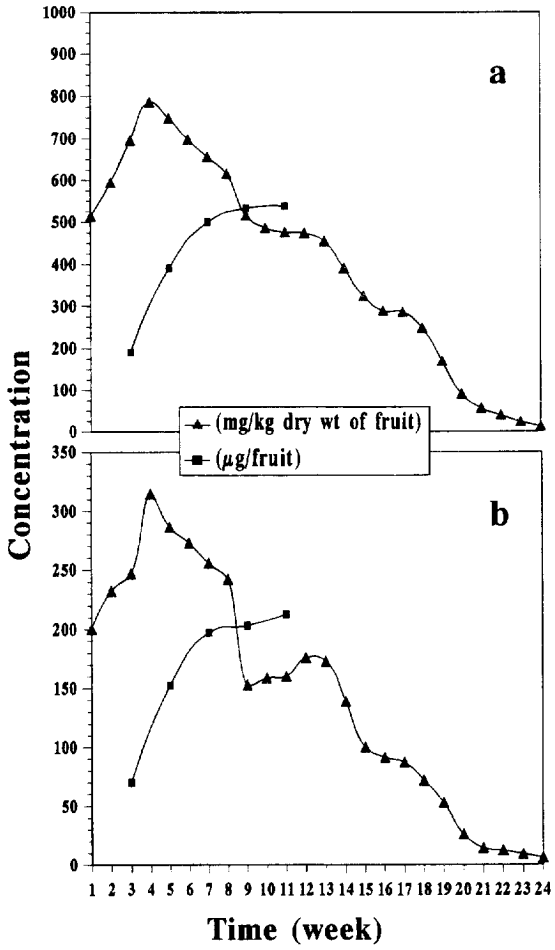

Fig. 2. Changes in chlorophyll concentration in fruits during development and ripening of Gordal variety olives: (a) chlorophyll *a*; (b) chlorophyll *b*.

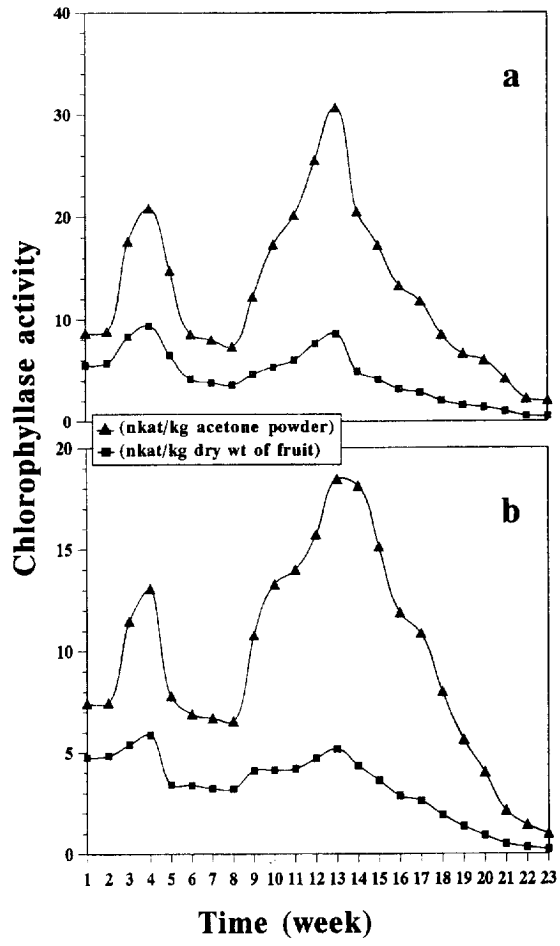

Fig. 3. Changes in chlorophyllase activity in fruits during development and ripening of Gordal variety olives. Substrate: (a) chlorophyll *a*; (b) chlorophyll *b*.

chlorophyll content, this stage involves an appreciable synthesis of chlorophylls *a* and *b* immediately prior to the onset of the ripening phase.

When the fruit reaches its maximum weight and size (from week 14), the decrease in the concentration of the chlorophylls is continuous. Under these conditions the loss of chlorophylls is real and can only be attributed to their metabolic degradation. Between weeks 13 and 16 the losses of chl *b* (47.2%) are again greater than those of chl *a* (36.6%). Even between weeks 16 and 17, when the rate of loss of both pigments is considerably reduced (1.0% for chl *a* and 4.68% for chl *b*) degradation continues to effect chl *b* to a greater extent. This latter stage ends with the disappearance of chlorophylls.

Changes in the activity of chlorophyllase with season

The activity of chlorophyllase, for both chl *a* (Fig. 3a) and chl *b* (Fig. 3b) during olive ripening, expressed in nkat kg^{-1} of acetone powder, demonstrates that the enzyme is present throughout the development cycle of the fruit. Furthermore, its activity shows two marked maxima in two well differentiated periods. The first maximum occurs during the growth phase and coincides with a period of massive synthesis of chlorophyll. The second maximum is in the period when the olive is very nearly completely developed, a time when the rate of synthesis of chlorophyll practically parallels that of the increase in weight of the fruit, and the concentration of the pigments remains almost constant.

The different levels of activity shown by chlorophyllase in the two key moments during fruit development are worth emphasizing. When the substrate is chl *a*, the enzyme activity during the second maximum (when the chlorophyll concentrations stabilize) is 32% higher than that found with the same substrate during the first maximum (when chlorophylls are being synthesized). When the substrate is chl *b* the difference between the activity levels of the two maxima is 29.1%. The higher activity for both chlorophylls in the second maximum is interpreted as indicating the existence of a much more active chlorophyllase when the fruit is more fully developed than when it is in a less well-developed stage. This may be due to an increase in the amount of enzyme present in the fruit and/or to intrinsic regulation of its activity.

Figure 3a and b also includes the changes in enzyme activity for the same substrates as a function of the dry weight of the fruit. It is evident that, although in general terms the comments made earlier apply, there are some notable differences. The levels of activity are at all times lower. Furthermore, not only are the maximum levels of chlorophyllase activity lower, but also the activity found for both chlorophylls in the first maximum is higher than that in the second maximum. However, this does not contradict what has been discussed previously, since in this case the reference taken is the weight of the fruit, which does not remain constant during ripening. In other words this decrease is a fictitious one arising from the dilution of the enzyme activity in a sample with a greater weight.

Different information is obtained on chlorophyllase activity according to the reference material used and this reflects the complex composition of the olive fruit. During the preparation of enzyme powder the acetone washes eliminate the liposoluble components, which represent a greater proportion of the weight of the olive as the fruit develops. Referring the enzyme activity to the amount of enzyme powder used gives a clear description of the rates of activity in the protein concentrate free from fats, water and inhibitors and gives an approximation of the theoretical maximum activity. The second form of expressing the enzyme activity in the non-aqueous portion of the fruit, i.e. in the structures, membranes and organelles of the fruit, gives a closer approximation of the activity under the real conditions in the fruit. Both methods of representing the values are therefore valid, and the information given by each form is complementary.

Involvement of chlorophyllase in the changes in chlorophyll concentrations

During the growth phase of the fruit, there is an increase in chlorophyllase activity and this reaches maximum levels when the concentrations of pigments in the olive are greatest, during week 4 of monitoring. From then on, chlorophyllase activity decreases and there is a decrease in the rate of chlorophyll synthesis with respect to the rate of fruit growth, as shown by a decrease in pigment concentration. It is possible that the activity of residual chlorophyllase is related to the low levels of chlorophyll synthesis.

The last part of the growth phase (weeks 10–14) is a period in which the concentration of chlorophylls stabilizes. This has been interpreted as being a stage of real synthesis since there is an increase in the chlorophyll content when related to the dry weight of the fruit. In this same period there is a further maximum of chlorophyllase activity, which corroborates the suggestion that this phase is biosynthetic and predisposes the fruit to the subsequent ripening phase. The fact that the increase in the concentration of chl *b* is greater than that of chl *a* can be explained by the difference in the activity of chlorophyllase for the two substrates. By calculating the percentage difference between the activity at each week of this stage and the maximum activity in week 13, it can be shown that, in the case of chl *b*, the activity of chlorophyllase is closer to its maximum activity for a longer period of time than it is in the case of chl *a*. In week 10 the enzyme activity for chl *a* differs by 43.6% from its maximum (week 13), while the activity for chl *b* differs from its maximum by only 28.0%. This difference is similar at weeks 11 and 12. This result suggests that, in the period from weeks 10 to 14, chlorophyllase works faster to synthesize chl *b* than to synthesize chl *a*.

Catabolism of chlorophyllic pigments

The detection of the dephytylated derivatives only in the first phases of olive development may be due to the fact that this period is one of massive synthesis of chlorophylls. Given this massive synthesis, detection is possible, albeit at very low levels, of the biosynthetic intermediates chlorophyllides *a* and *b*. In contrast, once the fruit has reached a certain level of development, synthetic and degradative mechanisms may overlap. The latter may be non-enzymic. Dupont and Siegenthaler [32] suggest that the formation and accumulation of free radicals is the main factor involved in the destruction of pigments in freshly isolated thylakoid membranes ageing *in vitro*.

On the other hand, the detection of 13^2-OH-chl *a*, 13^2-MeO-chl *a*, 13^2-MeO-chl *b*, 15^1-MeO-lactone-chl *a* and 15^1-MeO-lactone-chl *b* would seem to indicate the presence of oxidative enzymes. However, the fact that these compounds, which are associated with intermediates in the degradation of chlorophylls [33], were detected during the first few weeks of pigment synthesis suggested that they were formed as artefacts during the extraction of the pigments, as indicated by Shioi *et al.* [4], rather than naturally. These allomerized compounds can be formed by chemical means when chlorophylls are treated with strongly nucleophilic reagents [34]. To test this possibility a standard amount of chl *a* isolated from fresh spinach leaves was subjected to the procedures used in the present study. The solvents and the extraction and chromatographic systems used did not at any time lead to the formation of the compounds listed above. Their presence in olives of the Gordal variety must, therefore, be attributed to metabolic processes characteristic of the fruit.

The rather puzzling and constant presence of 13^2-OH-chl *a*, 13^2-MeO-chl *a* and 13^2-MeO-chl *b* throughout the growth and ripening process in the olive fruit (at times reaching concentrations of 10–15% that of chl *a*), can only be due to a continuous cycle of restoration of the chlorophyll molecule in photosynthetically active cells. The formation of 13^2-OH-chl *a* in living tissues is associated with the action of chlorophyll oxidase on chlorophylls. The presence of this enzyme has been detected during leaf senescence in *Phaseolus vulgaris* and *Hordeum vulgare* [27], as well as in the thylakoid membranes of young barley chloroplasts [2, 35]. In the present study we detected 15^1-MeO-lactone chl *a* and 15′-MeO-lactone chl *b* during the first weeks of the growth phase of fruits (weeks 1–5) and during the last stages of ripening (weeks 18–20).

In Gordal olives the involvement of chlorophyll oxidase may explain the appearance of 13^2-OH-chl *a*, 13^2-MeO-chl *a* and 13^2-MeO-chl *b*, although not the formation of 15^1-MeO-lactone chl *a* and 15^1-MeO-lactone chl *b*, since this would require the participation of another factor allowing it to be formed from chlorophyll or from the 13^2-OH-chl and 13^2-MeO-chl derivatives.

EXPERIMENTAL

Plant material. The study was carried out on olives of the Gordal variety, *Olea europaea regalis* (L.). The fruits were picked from trees in Utrera (Seville, Spain). Olives were picked from the whole perimeter of all 4 olive trees selected for the study. Sampling was always performed between 9 and 10 a.m. and the fruits were picked from branches within arms reach. This procedure was adopted to eliminate any errors arising from differences in the stage of development of the branches, orientation, etc. The experiment began in the middle of June, at which stage the fruits were so small that no stone could be distinguished. Sampling was conducted weekly.

Pigment extraction. This was performed with DMF according to the method of ref. [36]. The technique is based on the selective sepn of components between DMF and hexane. This system yields a soln of pigments free from the fatty matter which is characteristic of these fruits and which would interfere with subsequent sepn and quantification of pigments. In the present study the number of extractions with hexane increased from 1 to 5 as the degree of ripeness increased.

Separation, identification and quantification of pigments. This was carried out by HPLC according to the method of ref. [23], using a reversed phased C_{18} column and an elution gradient with the solvents A: H_2O–ion-pair reagent–MeOH (1:1:8) and B: MeOH–Me_2CO (1:1). The ion-pair reagent was a soln of NBu_4OAc (0.05 M) and NH_4OAc (1 M) in H_2O. Detection was by *A* at 430 nm with a Waters 994 programmable photodiode array detector before quantification. Peaks were identified, comparing their retention times, factors of capacity and absorption spectra with those of the respective authentic standards. 13^2-OH-chl *a*, 13^2-MeO-chl *a* and 13^2-MeO-chl *b* were tentatively identified as has been described in ref. [37].

Chls *a* and *b* were isolated from fresh spinach leaves by pigment extraction with Me_2CO [38] followed by TLC on Silica gel GF_{254} with development using petrol–Me_2CO–Et_2NH_2 (10:4:1) [36].

Preparation of enzyme extracts. Obtaining Me_2CO powder from olives and prepn of the crude enzyme extract is described in ref. [26]. The Me_2CO powder (3 g) was extracted with 90 ml 5 mM Na-Pi buffer (pH 7) containing 50 mM KCl and 0.24% Triton-X 100, with magnetic stirring for 1 hr at 30°. The extract was filtered through gauze and the filtrate centrifuged at 12 000 *g* for 10 min. The supernatant was used as crude enzymic extract.

Enzymic reaction and measurement of activity. The standard reaction mixt. contained *ca* 0.1 μmol substrate dissolved in Me_2CO, 100 mM Tris buffer (pH 8.5) containing 0.24% Triton-X 100 (chlase buffer) and the crude extract of chlorophyllase in a 1:5:5 ratio (total vol. 1.1 ml). The reaction was carried out in 1.5 ml Eppendorf cones at 40° in darkness for 3 hr. The reaction was stopped by freezing the mixt. at −20° until required. The mixt. was centrifuged at 13 000 *g* for 5 min, and an aliquot of the supernatant was injected into a Waters 600 E liquid chromatograph for quantification of the product. Sepn was carried out by HPLC according to the method of refs [23, 26]. The system of gradient elution allows the sepn and quantification of the product formed (chlorophyllides) and of the unreacted substrate. One unit of activity, katal (kat), is defined as the amount of enzyme needed to hydrolyse 1 mol of substrate per sec. Sp. act. was expressed as units per kg of Me_2CO powder.

Acknowledgements—We express our sincere gratitude to CICYT (Spanish Government, ALI-94-0777) for financial support of this research. Thanks are also due to M. Bodineau-Bada for technical assistance.

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