JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Alcohol Acyltransferase from Olive Fruit

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Alcohol acyltransferase catalyzes the esterification of volatile alcohols with acyl-CoA derivatives to produce volatile esters typically present in the aroma of some fruits. This enzyme was detected in extracts from the pericarp tissues of ripe olive fruits using hexanol and acetyl-CoA as the substrates. Alcohol acyltransferase showed a very low activity level in these fruits, with an optimum pH value at 7.5 and high K_m values for hexanol and acetyl-CoA. The substrate specificity of this enzyme for various alcohols was also studied. The involvement of the studied enzyme in the biogenesis of the volatile esters present in the aroma of virgin olive oil was discussed.

KEYWORDS: ***Olea europaea*; Oleaceae; olive; fruit; oil aroma; alcohol acyltransferase**

INTRODUCTION

Volatile esters are major components of the aroma of many fruits such as strawberries, bananas, and apples, sometimes being the main compound responsible for the flavor that the customers appreciate in them (*1*, *2*).

It is well-known that these compounds are synthesized by the enzyme alcohol acyltransferase (AAT, E.C. 2.3.1.84), which catalyzes the esterification of a volatile alcohol with an acyl-CoA moiety to produce a volatile ester and free CoA-SH. The volatile ester biosynthesis was first described in bananas (*3*, *4*). These studies were completed with in vivo experiments carried out by Yamashita et al. (*5*) in strawberries, in which aldehydes were found to be the precursors of some volatile esters. Ueda and Ogata (*6*) reported the CoA dependence of AAT, which was purified and characterized from banana fruits almost a decade later (*7*). Several AATs from different sources have been characterized since then, resulting in a high degree of correlation between the AAT activity level and the organoleptic quality of the products studied (*8*, *9*). More recently, microarray techniques have been used to determine the expression pattern of the AAT gene in strawberry fruits (*10*). Alcohol acyltransferase protein displayed homology toward some hypersensitivity proteins and other acyltansferases from tobacco or *Arabidopsis*. Consensus sequences from these enzymes allowed the cloning of two newer AAT genes from cantaloupe fruits (*11*). The expression of these AAT clones in microbial hosts permitted a complete characterization of their substrate specificity that was in good agreement with the volatile ester composition of these fruits. However, in the case of apples and strawberries, there were important discordances between substrate specificity and ester composition that have been explained on the basis of substrate availability (*9*, *12*).

Among the different groups of volatile compounds present in virgin olive oil, esters account only for 1 to 2% of the total. This fraction mainly consists of acetyl derivatives of ethanol, butanol, hexanol, and 3(*Z*)-hexenol, which are compounds with high perception thresholds (*13*) and confer positive attributes to the final oil product (sweet, green, and fruity notes).

EXPERIMENTAL PROCEDURES

Plant Material. Olive (*Olea europaea*) fruits were harvested from 30-year-old trees located in an orchard near Sevilla (Spain). These trees were supplied with drop irrigation and fertirrigation during the whole reproductive period (April−November).

Preparation of Enzyme Extracts. The olive fruits were rinsed with tap water and distilled water before endocarp removal. Then, an acetone powder was prepared from the fleshy pulp as described by Salas and Sánchez (*14*). This whitish powder was stored at −20 °C in flasks endowed with silica gel desiccator. In these conditions the enzyme activities were stable for several months. The enzyme extracts were prepared by homogenization of 1.5 g of olive pulp acetone powder in 50 mL of 50 mM HEPES pH 7.5, 7 mM 2-mercaptoethanol, 0.05% Triton X-100 using an ice-cooled glass homogenator. The resulting suspension was centrifuged at 20000*g* for 10 min, the pellet was discarded, and the supernatant was fractionated by ammonium sulfate precipitation. The cut from 30 to 60% of ammonium sulfate saturation, containing most of the extracted protein, was resuspended in 6 mL of 25 mM HEPES pH 7.5, 0.05% ascorbate, and 50% glycerol and was assayed for AAT.

Enzyme Assay. The enzyme AAT was assayed by a modification of the method reported by Fellman et al. (*9*) based on the determination of the free CoA concomitantly produced with volatile esters by AAT. The assay mixture contained 50 mM HEPES pH 7.5, 20 mM $MgCl_2$, 20 mM hexanol, 0.4 mM acetyl-CoA, and enzyme extract equivalent to 50−100 μg of protein in a final volume of 0.5 mL. This mixture was incubated at 30 °C for 10 min, and then a volume of 25 μL 20 mM of 5,5-dithio-bis-nitrobenzoic acid (DTNB) dissolved in 0.1 M HCO_3Na was added. Once the color was developed, a process that takes about 10 min, the absorbance at 407 nm of the assay mixture was measured. AAT activity was calculated by comparing the absorbance of samples containing hexanol and blanks with the same composition without the alcohol substrate. The molar extinction coefficient of the chromophorous compound formed by DTNB and CoA-SH at 407 nm was experimentally determined as 10 400.

Synthesis of volatile esters was assessed by gas−liquid chromatography by injections of the headspace of the assay vials after 30 min of

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10.1021/jf035433a CCC: $27.50
Published on Web 04/15/2004

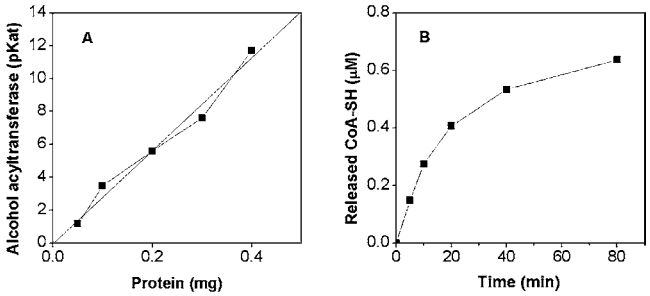

Figure 1. Optimization of the alcohol acyltransferase assay. (**a**) Influence of loaded protein in the assay. (**b**) Influence of reaction time in the release of CoA-SH using hexanol as substrate.

equilibration at 70 °C. A Carbowax 20 M (2 × 2000 mm) packed column (Supelco) was used for volatile separation. Volatile esters were identified by comparison of their retention times with those of the corresponding standards.

Protein Determination. Protein was determined by a modification of the method described by Bradford (*15*) using bovine gamma globulin as the standard.

RESULTS AND DISCUSSION

Volatile esters confer positive attributes such as green, fruity, and sweet notes to olive oil (*16*), and thus it is interesting to promote their synthesis within the olive oil elaboration process. Previous attempts to characterize olive AAT failed to detect any activity in olive fruit pulp crude extracts. However, olive pulp homogenates were able to produce volatile esters from acetic acid and volatile alcohols or aldehydes, implying the sequential action of the acetyl-CoA synthetase, alcohol dehydrogenase, and AAT (*17*). In the present work, the AAT activity was detected and characterized in crude cell-free preparations from olive fruit pulp. Because of the low level of activity in the crude extracts, it was necessary to concentrate the protein as described in the Experimental Section. The AAT activity level detected in the extracts from the pulp of mature olives ranged from 30 to 90 pkat/mg prot. The preparations carried out with green unripe olives yielded inactive extracts, indicating that this enzyme is induced in the latter period of the fruit development, as it occurs in other fruits such as strawberries or cantaloupes (*11*, *18*). Moreover, despite olive AAT being a soluble enzyme, the addition of Triton X-100 in the extraction medium was necessary to obtain active fractions. This confirms that in olive fruit, as in banana, Triton X-100 may protect or alter the enzyme from a coagulated insoluble form into a soluble form that allows its detection in the extracts (*7*). Furthermore, the low activity level of AAT in olive pulp, which was much lower than in other sources such as bananas or apples (*7*, *9*), makes difficult its study and purification. Thus, several attempts to purify this enzyme failed because the activity was lost after its elution from ion exchange or exclusion columns.

Optimization of the Enzyme Assay. The gas chromatography-based method was not used routinely because it provided less sensibility and higher variability than the DTNB-based one, which was not affected by esterases that could be present in the crude extract (*1*). Since that method involved end point determinations, the assay conditions were optimized by studying the influence of the protein concentration and the incubation time on this activity. AAT increased linearly with protein concentration from 0.5 to 1.0 mg/mL (**Figure 1**A). However, an increase in the blank absorbance was observed as well (data not shown), which hampered the assay at high enzyme extract loads. This increment of absorbance could be due to the presence

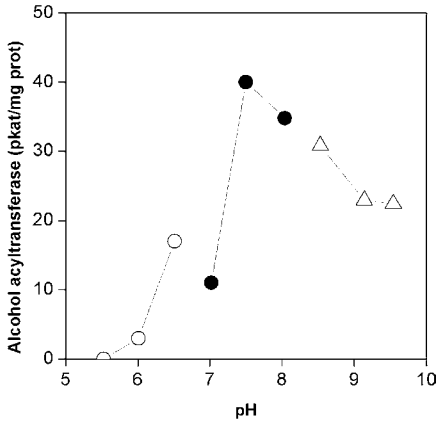

Figure 2. Effect of pH in alcohol acyltransferase from olive fruit. Sodium acetate (-○-), HEPES (-●-), and TRIS (-△-) were used as the assay buffers.

of unspecific acetyl-CoA esterases in the enzyme extract. Therefore, a concentration of protein of 0.2 mg/mL in the enzyme assay was used provided it yielded a good activity level as well as low absorbance blanks. Furthermore, the release of free CoA catalyzed by AAT in function of time displayed a hyperbolic curve, with a linear interval embracing the first 10 min of reaction (**Figure 1B**), and therefore it was the incubation time used in the standard AAT assay.

Effect of pH. The pH curves reported in previous works for AATs from several sources exhibited maximum activity values in the alkaline range (*7*, *8*, *11*). In contrast, when the synthesis of volatile esters was studied in olive pulp homogenates a slightly acidic optimum pH value of 6.8 was found (*17*). The olive AAT was assayed within a pH range from 5.5 to 9.5, using the buffers sodium acetate, HEPES, and TRIS. The resulting pH curve displayed a maximum at pH 7.5, with a quick drop of activity at acidic pH values (**Figure 2**), being much more similar to that showed by AATs from other fruits. The difference of optimum pH values between both works carried out in olive fruit could be due to the fact that in the first case the participation of other enzymes such as acetyl-CoA synthetase and alcohol dehydrogenase was necessary to produce the volatile esters.

Kinetic Parameters. AATs from other fruits showed K_m values for their substrate alcohols in the millimolar order. Therefore, the K_m value of banana fruit AAT was 0.4 mM for isoamyl alcohol (*7*) and 3 mM for butanol in the case of strawberry (*8*). These numbers were lower than that reported for yeasts, from which K_m values of 30 mM have been reported for isoamyl alcohol (*19*). Moreover, the K_m values of the CoA derivative substrates were usually 10 to 100-fold lower. Thus, they were in the micromolar range in bananas and strawberries (*7*, *8*), and it was 0.2 mM for acetyl CoA in the case of brewer yeast (*19*). Recombinant His-tagged AATs isolated from strawberry and cantaloupe, expressed in *Escherichia coli* or yeast and purified by affinity chromatography, displayed the same magnitude order in their kinetic parameters for both substrates (8.9 mM and 0.1 mM in AAT from strawberry (*10*) and 1.4 mM and 0.09 mM in AAT1 from cantaloupe (*11*) for hexanol and acetyl-CoA, respectively). When the kinetic parameters of olive AAT for hexanol and acetyl-CoA were studied, hyperbolic curves that fit well with the model of Michaelis−Menten were obtained. However, the hexanol curve did not reach saturation even at the highest concentrations used of this alcohol (**Figure 3**) and showed a K_m value for this substrate of 21 mM (**Table 1**). The K_m value for Acetyl-CoA was about 10-fold lower than that corresponding to hexanol, indicating as expected a higher

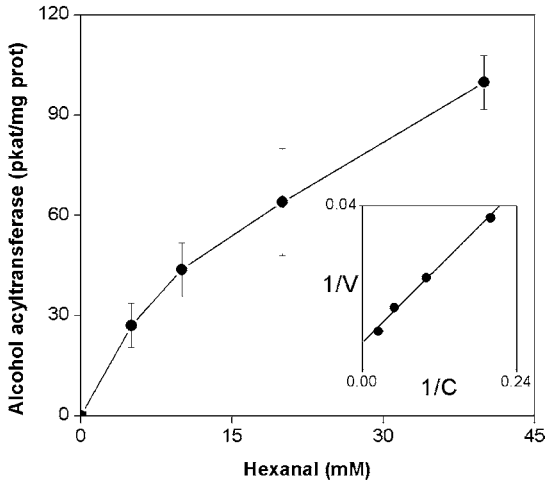

Figure 3. Effect of hexanol concentration on the activity alcohol acyltransferase. The inset shows the double reciprocal plot of the data, which yielded a K_m value of 21 mM and a V_{max} of 128 pkat/mg prot.

Table 1. Kinetic Parameters of Olive Alcohol Acyltransferase for Hexanol and Acetyl-CoA[a]

substrate	K_m (mM)	V_{max} (pkat/mg prot)	R^2
hexanol	21	128	0.9949
acetyl-CoA	2	213	0.9671

[a] Kinetic parameters for Acetyl-CoA were calculated using a hexanol concentration of 20 mM.

affinity of this enzyme for its cofactor (**Table 1**). These K_m values were much higher than those reported for other fruits, being more similar, in the case of hexanol, to the K_m value displayed by the brewers yeast for isoamyl alcohol (*19*). On the other hand, the K_m value of olive AAT for acetyl-CoA (**Table 1**) was also higher than the reported K_m values of AATs from other sources. The high apparent K_m values showed by olive AAT for its substrates could also contribute to the low volatile ester content of olive oil. However, it has to be considered that the kinetic parameters reported in previous works corresponded to total or partially purified enzymes, which could enlarge the differences on apparent kinetic parameters when they are compared with activities measured in nonpurified enzyme extracts.

Substrate Specificity. The most abundant volatile alcohols present in olive oil are the six-carbon ones synthesized through the lipoxygenase pathway (*16*), by the successive action of the enzymes lipoxygenase (*20*), hydroperoxide lyase (*21*), and alcohol dehydrogenase (*14*). Thus, olive AAT was assayed with a variety of alcohol substrates that ranged from one to six carbon atoms, including alcohols typically present in the olive oil volatile fraction such as hexanol, 3(*Z*)-hexenol, and 2(*E*)-hexenol (**Table 2**). This enzyme did not display any activity with the short-chained alcohols methanol and ethanol and showed low activities toward butanol and 3-methylbutanol. The maximum activities were obtained when hexanol and 3(*Z*)-hexenol were used as the substrates, although the six-carbon alcohol 2(*E*)-hexenol, the most abundant in olive oil (*16*), was a poorer substrate than its homologous compounds. Olive AAT specificity profile is in agreement with the volatile ester composition of olive oil, and thus hexanol and 3(*Z*)-hexenol, which are the precursors of the preponderant volatile esters of olive oil, were the substrates more efficiently used by this enzyme. Furthermore, the short-chain alcohol ethanol was not a substrate of this enzyme, which indicates that ethyl acetate, an ester

Table 2. Substrate Specificity of the Alcohol Acyltransferase from Olive Fruit Pulp[a]

substrate	alcohol acyltransferase (pkat/mg prot)	relative activity (%)
methanol	nd[b]	0
ethanol	nd[b]	0
butanol	12 ± 6	16
3-methylbuthanol	12 ± 2	16
hexanol	94 ± 20	100
3(*Z*)-hexenol	80 ± 10	86
2(*E*)-hexenol	42 ± 2	45

[a] Data are the means of three determinations ± standard deviation. [b] nd = not detected

commonly detected in olive oil (*16*), could be synthesized through an alternative pathway. Furthermore, the substrate specificity of this enzyme would explain why the ester 2(*E*)-hexenyl acetate was so scarce in olive fruit although the concentration of its precursor was addressed to be the highest among the volatile alcohols (*16*). On the other hand, this specificity profile kept certain similitude to that reported for strawberry fruits, in which butyl- and isoamyl- (3-methylbutyl-) alcohols were poorer substrates than hexanol (*8*, *10*). Recombinant AAT1 from cantaloupe displayed, similarly to the form from olive, high activities toward all C6 alcohols assayed combined with lower activities toward the C4 ones and barely detectable esterification rates for ethanol (*11*).

This specificity profile was in agreement with the in vivo experiment carried out by Olías et al. (*17*), in which the higher rates of ester formation by disrupted olive pulp tissues took place with 3(*Z*)-hexenol, explaining the fact that 3-hexenyl acetate was one of the most abundant volatile esters in olive oil.

Summarizing, the enzyme responsible of the volatile ester formation, AAT, was detected and investigated in olive pulp. The characteristics of this enzyme broadly justify the composition of volatile esters present in olive oil, being responsible for the most positive fruity and sweet notes in the aroma of this product. Data retrieved from this work also indicates that AAT limits the production of volatile esters in olives provided its activity is several times lower than the previous enzymes in the pathway synthesizing C6 volatile compounds: lipoxygenase, hydroperoxide lyase, and alcohol dehydrogenase (*14*, *20*, *21*). This limitation led to the accumulation of the volatile alcohol precursors, which display lower perception thresholds and sometimes are responsible for grassy and rancid negative notes (*16*). Therefore, the increment of AAT activity during the olive oil elaboration process, which implies steps of milling, malaxation, and phase separation (*22*), would produce a double effect of removal of alcohols and production of esters that would be translated in an increase in the organoleptic quality of the final oil. The base of these future improvements could imply either the selection of olive cultivars with a higher AAT activity or modifications in the extraction process such as operating at lower temperatures to prevent enzyme inactivation or the neutralization of the acidic pH values of the olive paste during malaxation to promote a higher esterification activity.

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Received for review December 5, 2003. Revised manuscript received March 5, 2004. Accepted March 14, 2004. This work has been carried out with the support of the E.U. (AIR 3-93-1967), which is gratefully acknowledged.

JF035433A