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Biochemical and Catalytic Properties of Three Recombinant Alcohol Acyltransferases of Melon. Sulfur-Containing Ester Formation, Regulatory Role of CoA-SH in Activity, and Sequence Elements Conferring Substrate Preference

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Alcohol acyltransferases (AAT) play a key role in the biosynthesis of ester aroma volatiles in fruit. Three ripening-specific recombinant AATs of cantaloupe Charentais melon fruit (Cm-AAT1, Cm-AAT3, and Cm-AAT4) are capable of synthesizing thioether esters with Cm-AAT1 being by far the most active. All proteins, as well as AAT(s) extracted from melon fruit, are active as tetramers of around 200 kDa. Kinetic analysis demonstrated that CoA-SH, a product of the reaction, is an activator at low concentrations and an inhibitor at higher concentrations. This was confirmed by the addition of phosphotransacetylase at various concentrations, capable of modulating the level of CoA-SH in the reaction medium. Site-directed mutagenesis of some amino acids that were specific to the Cm-AAT sequences into amino acids that were consensus to other characterized AATs greatly affected the selectivity of the original protein and the number of esters produced.

KEYWORDS: ***Cucumis melo*; alcohol acyltransferases; sulfur-containing esters; aroma volatiles; site-directed mutagenesis**

INTRODUCTION

Fruit aroma is a complex mixture of a wide range of compounds among which volatile esters represent a major contribution to the odor of many fruits [apple and pear (*1*), banana (*2*), pineapple (*3*), and strawberry (*4*)]. In cantaloupes a large majority of compounds identified were alcohols, aldehydes, ketones, esters, and sulfur-containing compounds (*5*, *6*). Sulfur-containing esters often contribute to the typical aromatic note of melons. For instance, ethyl (methylthio)acetate, has been considered by Buttery et al. (*7*) to be responsible for a specific note of honeydew melons (*Cucumis melo* var. *inodorus*). Several authors have identified a number of sulfur compounds in the aroma profile of muskmelon (*C. melo* var. *reticulatus*) that include mainly sulfur esters (*8*−*10*). Some sulfur-containing esters such as ethyl (methylthio)acetate, ethyl 3-(methylthio)-propanoate, and 3-(methylthio)propyl acetate have high odor values and strongly contribute to the overall aroma perception of ripe fruit (*11*, *12*), and ethyl 3-(methylthio)propanoate has a typical aroma note of melon (*8*).

Esters are synthesized from alcohols and acyl-CoAs through the action of alcohol acyltransferases (AATs) (*13*). AAT activity has been measured in a number of fruits [banana (*14*), apple (*15*), strawberry (*16*), melon (*17*), and grape (*18*)]. On some occasions, the catalytic properties of the enzyme(s) have been determined after partial purification (*19*). In recent years genes encoding AATs have been isolated and characterized from cultivated strawberry (*20*), wild strawberry and banana (*21*), melon (*22*), apple (*23*), and grapes (*18*). In melon at least three genes encoding AATs have been characterized that show fruit-specific and ethylene-dependent expression (*24*). Proteins encoded by the three genes show differential substrate preference and are, therefore, considered to be participating in different notes of the aroma of melon. However, so far, none of the recombinant proteins have been tested for their capacity to produce sulfur esters despite the strong importance of these compounds in the odor of melon and other fruits. The only

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demonstration of the capacity of AATs to synthesize sulfur-containing esters arises from studies on protein extracts of intact fruit. Shalit et al. (*25*) showed production of 3-(methylthio)-propyl acetate by protein from crude extract of melon.

In this paper, we have produced recombinant proteins by expressing the three Cm-AAT genes in the yeast and have purified the proteins by affinity chromatography. This has allowed the study of the biochemical characteristics of the three recombinant proteins. Because no information was available on the proteins and genes participating in the biosynthesis of sulfur-containing esters, we have first evaluated the capacity of the three Cm-AAT proteins to produce such compounds. Other biochemical characteristics of the proteins have been determined, particularly the molecular weight and kinetics properties. Large variations exist in the evaluation of the size of the protein going from 400 kDa in melon (*17*) to 70 kDa in partially purified AAT of strawberry (*16*) and to 53−58 kDa in various recombinant AATs (*20*, *23*, *26*−*28*). So far, the kinetic properties of fruit AATs, whether native or recombinant, have been studied without considering a feedback role of the CoA-SH liberated by the reaction. In this work, we demonstrate that CoA-SH can have stimulatory or inhibitory effect in vitro depending on the concentration, and this greatly influences the kinetic properties of the enzymes. Our previous data (*24*) had demonstrated the crucial role of a single amino acid, threonine, in enzyme activity. Here we extend our study to the search of the role of amino acids that are specific to each of the melon sequences as compared to other active plant AATs. Site-directed mutagenesis has been used to replace these amino acids by their consensus counterparts to evaluate the impact of these amino acids on the specificity and substrate preferences of the enzymes.

MATERIALS AND METHODS

Expression of Cm-AAT in Yeast and Extraction and Purification of the Recombinant Proteins. All *Cm-AAT* cDNAs were cloned in the pYES2.1 TOPO-TA cloning vector for regulated protein expression in yeast as described in El-Sharkawy et al. (*24*). Cells were collected by centrifugation (1800*g*, 10 min at room temperature) from 50 mL of yeast cultures induced with galactose and resuspended in buffer A (50 mM sodium phosphate pH 7.5, 10% v/v glycerol, 0.3 M NaCl) containing 2 mM β-mercaptoethanol. The cells were mechanically ground in liquid nitrogen for 2 min and stored at −80 °C until needed. To extract AAT enzyme, the powder was thawed and centrifuged at 45000*g* for 20 min at 4 °C. The crude extract obtained was purified by an affinity column designed to purify polyhistidine-tagged proteins (BD Talon Metal Affinity, BD Biosciences), according to the manufacturer's protocol. Proteins were quantified according to the method of Bradford (*29*).

Extraction of AAT from Fruit Tissues. One gram of mesocarp tissue was ground mechanically in liquid nitrogen during 2 min in the presence of 1 mL of extraction buffer (250 mM Tris-HCl, pH 7.5, 1 mM DTT), and the protein crude extract was stored at −80 °C until needed. After thawing and centrifugation at 45000*g* for 20 min at 4 °C, the supernatant was desalted using Sephadex G-25 columns (Amersham Biosciences) and eluted with buffer B (50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 1 mM DTT).

AAT Enzyme Activity Assay. Cm-AAT activity of recombinant proteins was assayed in 500 μL total volume containing 70 μL of protein (10−15 μg of purified recombinant protein), 2 mM alcohol, and 250 μM acyl-CoA and adjusted to a final volume with buffer B. Activity of native melon AAT was measured after molecular sieving as described below using the same protocol as for recombinant proteins except that 300 μL of each fraction was used. The mixture was incubated at 30 °C for 20 min. Immediately after reaction, 5 μL of the internal standard (methyl benzoate, 0.5 μL L^{-1}) was added into the mixture. Volatile compounds other than sulfur-containing esters were extracted with 250 μL of pentane and vortexed for 1.5 min, and the pentanolic phase was concentrated. The quantification of these compounds was done for gas chromatography (GC); 1 μL of concentrated pentanolic phase was injected into the GC equipment with a flame ionization detector (GC-FID). The GC-FID equipment was made up of a Hewlett-Packard gas chromatograph (model 5890) equipped with an HP-Innowax cross-linked polyethylene glycol column (30 m × 0.25 mm × 0.25 μm). Injector and detector temperatures were 250 °C. The oven temperature was programmed from 40 °C (1 min) to 60 °C (1 min) at a rate of 2 °C min^{-1} and finally to 190 °C (5 min) at a rate of 10 °C min^{-1}. Nitrogen was used as a carrier gas at 100 kPa. Compounds were identified by comparison of retention times with those of authentic standards. Sulfur-containing esters were analyzed with a QP2010 GC/MS (Shimadzu Co. Ltd., Kyoto, Japan) equipped with a 30 m × 0.25 mm × 0.5 μm fused silica CP-SIL 8 CB LB/MS capillary column (Varian). Injection of 1 μL of extract was made in splitless mode (purge open after 30 s) at 240 °C. The He carrier constant velocity was 35 cm s^{-1}. The oven temperature was held at 40 °C for 2 min and then ramped to 230 °C at 6 °C min^{-1}. The mass spectra were recorded at the rate of 2 scans/s over the mass range from 29 to 300 (*m*/*z*). It was verified that all esters expected from the enzymatic reaction corresponded exactly to the retention time of authentic standards. In the case of sulfur-containing esters additional identification was performed by mass spectrometry. Quantification was performed by calculating the response factors by injection of known amount of standards and internal standard (methyl benzoate).

Determination of Molecular Weight. Molecular weights were determined using a fast protein liquid chromatograph (FPLC, Waters 650E, Millipore) by gel filtration on a Sephacryl S-200 HR column. The column was pre-equilibrated and developed with Tris-HCl 50 mM, pH 7.5, 10% (v/v) glycerol, and 1 mM DTT at a flow rate of 1 mL min^{-1}.

Kinetic Analysis and Effects of CoA-SH. For the determination of K_m and V_{max} for acetyl-CoA (the preferred acyl-CoA for all proteins), reactions were carried out at pH 7.5 and 30 °C with acetyl-CoA concentrations varying from 0 to 6 mM (Cm-AAT1), from 0 to 12 mM (Cm-AAT3), and from 0 to 2 mM (Cm-AAT4). The alcohol cosubstrate was maintained at constant saturating concentration (4 mM). The determination of K_m and V_{max} for alcohols was carried out under the same conditions in the presence of the preferred compounds (*E*-2-hexenol for CmAAT1, benzyl alcohol for Cm-AAT3, and cinnamoyl alcohol for Cm-AAT4). Concentration of alcohols varied from 0 to 6 mM, whereas acetyl-CoA was maintained at saturation concentrations (4 mM for Cm-AAT1, 8 mM for Cm-AAT3, and 2 mM for Cm-AAT4). The effect on enzyme activity of 0−5 mM of CoA-SH was studied in the same conditions of assay as described above. Kinetic studies in the presence of either low (1 or 5 μM) or high (1 or 2.5 mM) concentrations were performed as described in the previous paragraph. To lower the concentration of the product of the reaction, CoA-SH, variable units (0, 0.5, 1.0, and 2.0) of phosphotransacetylase (EC 2.3.1.8), and 4 mM acetylphosphate were added to the reaction medium using the mutated MT1 VA65 recombinant protein, which is capable of synthesizing ethyl butanoate from ethanol and butanoyl-CoA but cannot accept acetyl-CoA to from ethyl acetate.

Site-Directed Mutagenesis. Protein sequences of a number of AAT for which enzymatic activity has been tested after expression in *Escherichia coli* or yeast have been aligned using the ClustalX program (*30*). Site-directed mutagenesis has been performed using the QuickChange (Stratagene, San Diego, CA) PCR-based method with Cm-AAT genes cloned in the pYES2.1 TOPO-TA cloning vector. The mutated clones were then transferred into *Saccharomyces cerevisiae* as described in El-Sharkawy et al. (*24*).

Data Analysis. Each experiment was performed at least in triplicate. Results are expressed as the mean value ± standard error (SE).

RESULTS

Differential Capacity of the Three Recombinant AATs To Produce Sulfur-Containing Esters. Figure 1 shows that all proteins were capable of synthesizing the two thioether esters, 3-(methylthio)propyl and 2-(methylthio)ethyl acetates, with an activities ranging from about 10 to 60 pkat (mg of protein)$^{-1}$.

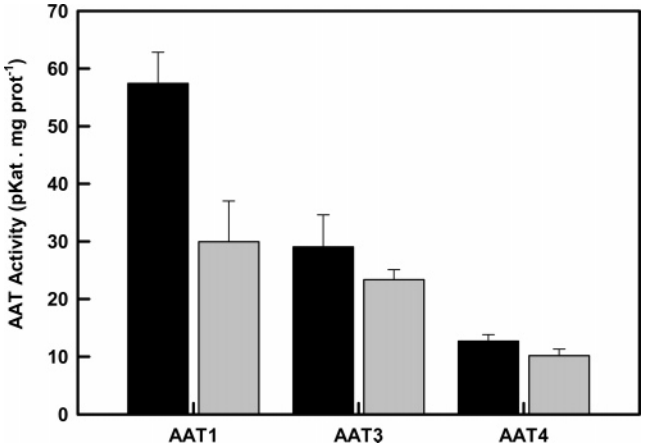

Figure 1. Activity of thioether ester formation of the three recombinant AATs of melon. Activity for 3-(methylthio)propyl acetate is represented in black bars and that for 2-(methylthio)ethyl acetate in gray. Values represent the mean ± SE of three replicates.

Table 1. Effect of Adding Variable Units of Phosphotransacetylase (0, 0.5, 1.0, and 2.0 Units) in the Reaction Medium on the Activity of the Cm-AAT1 Mutated Recombinant Protein (MT1 VA65) for Butanoyl-CoA (2 mM) and Ethanol (4 mM)

units of phosphotransacetylase	activity[a] [pkat (mg of protein^{-1})]
0	2156 ± 45
0.5	3012 ± 237
1.0	4474 ± 180
2.0	traces

[a] Values represent the mean of three replicates ± SE.

Cm-AAT1 was about twice more active than Cm-AAT3 and 3−6 times more active than Cm-AAT4.

Molecular Mass. The molecular mass, estimated by molecular sieve chromatography on the basis of enzymatic activity, was around 200 kDa for all three purified recombinant Cm-AATs. The apparent molecular mass of the native AAT in crude extracts of melon fruit was estimated at 196 kDa (data not shown). Under denaturizing conditions (SDS-PAGE), the molecular mass of the three recombinant proteins was estimated at 51 kDa (data not shown), therefore indicating that the native proteins are tetramers.

Effect of CoA-SH on Activity. Preliminary experiments aimed at determining the optimum concentration of substrates showed an inhibition of AAT activity at high levels of acyl-CoA. Because the product of the reaction is often involved in this type of process, the effects of various concentrations of CoA-SH were assessed. Results in **Figure 2** indicate that CoA-SH stimulated the activity of the Cm-AAT4 protein up to 50 μM concentration. The optimum concentration was 2.5 μM where activity was increased by 400% as compared to the control. Above 50 μM, CoA-SH significantly reduced AAT activity by 20% at 500 μM and by 80% at 5000 μM. Kinetic studies, with acetyl-CoA as a substrate, were performed in the presence of low and high concentrations of CoA-SH. For Cm-AAT4 taken as an example, 1 or 5 μM concentration of CoA-SH stimulated activity through an increase of the V_{max} of the reaction clearly visible on direct kinetics graph (**Figure 3A**) without affecting the K_m as shown in the double-reciprocal plot (**Figure 3B**). At 1 and 2.5 mM concentrations of CoA-SH, the V_{max} of the reaction was unaffected (**Figure 3C**) but the K_m was increased, characteristic of a competitive inhibition with a K_i estimated at 0.96 mM (**Figure 3D**).

Effect of Removal of CoA-SH Using Phosphotransacetylase. During the enzymatic reaction, CoA-SH is released and possibly accumulates at inhibitory concentrations that may affect AAT activity. To modulate the level of CoA-SH in the reaction medium, a phosphotransacetylase was used, which is capable of performing the following reaction: CoA-SH + phosphoacetate = acetyl-CoA + P_i. The addition of phosphotransacetylase at variable concentration affects AAT activity (**Table 1**). At high concentrations of phosphotransacetylase (2.0 units), activity was abolished, consistent with the role of CoA-SH as an activator but further demonstrating that CoA-SH is absolutely required for activity. At lower concentrations (0.5 and 1.0 unit), AAT activity was stimulated, thus confirming the inhibitory role of CoA-SH at high concentrations.

Effect of Site-Directed Mutagenesis on Activity. Previous experiments had shown that a cDNA clone, Cm-AAT2, with strong homology to Cm-AAT1 was unable to form volatile esters unless the 268-alanine was replaced by threonine, which is present in all active AATs (*24*). Conversely, replacing the 268-threonine by alanine in Cm-AAT1 resulted in almost complete abolition of ester volatile formation. By further comparing the amino acid sequence in silico of eight active AATs with the melon sequences, it appeared that some amino acids were unique to the melon AATs (**Figure 4**). By comparing the Cm-AAT1 sequence with other AATs, it was found that the Cm-AAT1 sequence has an aromatic amino acid, phenylalanine at the 49 position (F49), whereas all other AATs have an aliphatic amino acid in the corresponding position (**Figure 4A**). The replacement of F49 by leucine (L) in the Cm-AAT1 sequence resulted in several changes in AAT activity (**Figure 5A**). The most remarkable one is a change in stereoisomer preference for the hexenol substrate with a strong increase in the capacity of forming *Z*-2-hexenyl acetate (no. **6** in **Figure 5**) and concomitant reduction of the formation of *E*-2-hexenyl acetate (**7**). There was also a decrease in the formation of hexyl hexanoate (**29**) and a strong decrease or abolition of the synthesis of butyl, hexyl, heptyl, *Z*-3-hexenyl, and 2-phenylethyl acetates and 2-phenylethyl hexanoate (**2**−**4**, **8**, **15**, and **30**). Overall, the mutation has reduced the range of esters being produced. Most of the AAT sequences, including Cm-AAT1, contain a valine (V), leucine (L), or isoleucine (I) residue at the 65 position (**Figure 4B**), except Ban-AAT and Cm-AAT3 that harbor an alanine (A). This position is located just upstream of a consensus sequence. Replacing V65 by A in Cm-AAT1 (**Figure 4B**) reduced the synthesis of many esters (**Figure 5B**) except ethyl butyrate (**22**), which was strongly increased. Therefore, the mutated Cm-AAT1 gained specificity by showing very high preference for the formation of ethyl butyrate, and at the same time the synthesis of many other esters produced by the native Cm-AAT1 protein was reduced (**Figure 5B**). Conversely, the mutation of A61 into V in Cm-AAT3 (**Figure 4B**) greatly extended the range of esters synthesized (**Figure 5C**). The mutated protein produced much less of benzyl acetate (**14**) but instead synthesized hexyl hexanoate (**29**) at a very high rate, and many esters that were either not produced or produced at a low rate are now synthesized at significant rates. Among these are hexyl acetate (**3**), heptyl acetate (**4**), *Z*-2-hexenyl acetate (**7**), *E*-2-hexenyl acetate (**8**), hexyl propanoate (**20**), and 2-phenylethyl propanoate (**21**). The Cm-AAT4 sequence compared to other AATs has an amino acid that is unique to the sequence: Q135 (**Figure 4C**). When Q135 was replaced by L in Cm-AAT4 so as to resemble all other AATs, there was a strong change of substrate preference (**Figure 5D**). Whereas the native Cm-AAT4 had strong preference for forming cin-

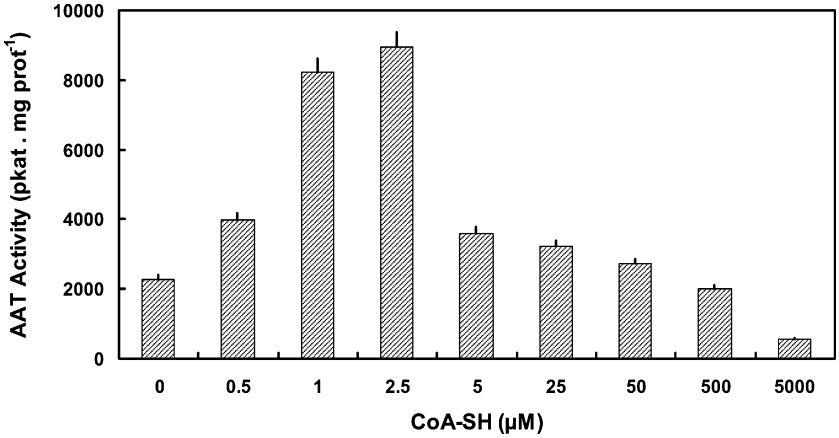

Figure 2. Effect of free CoA-SH on activity of Cm-AAT4. Values represent the mean ± SE of three replicates.

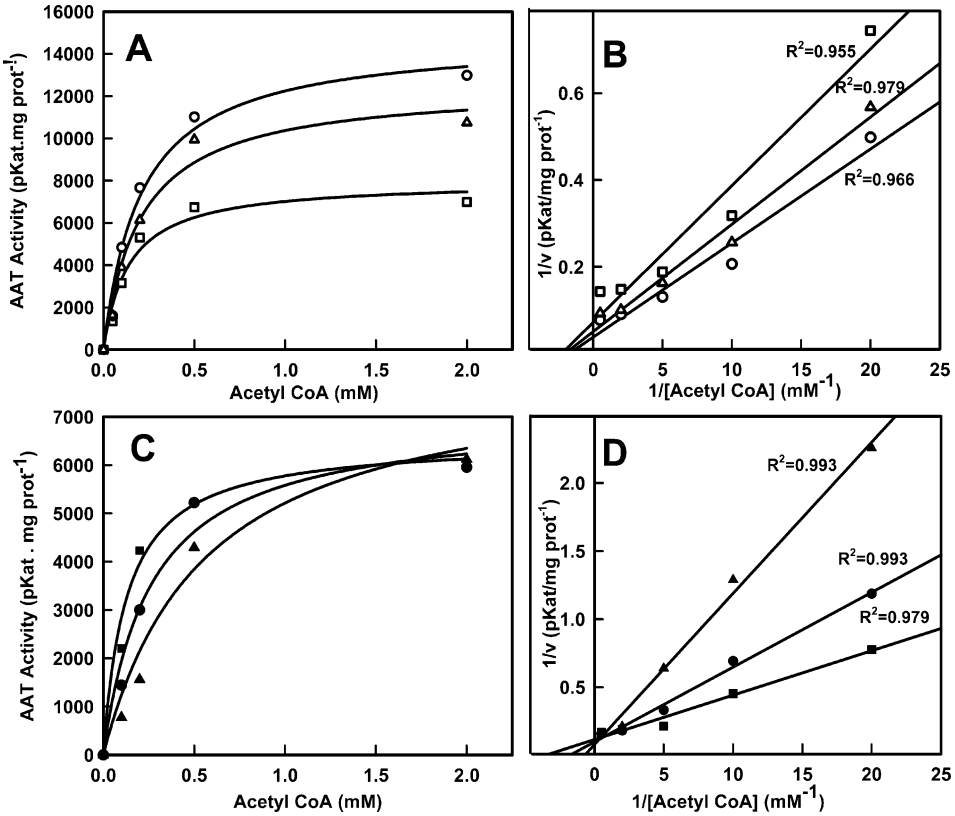

Figure 3. Kinetic analysis of CoA-SH inhibition and stimulation of Cm-AAT4 activity. The data are represented in direct kinetics (**A**, **C**) and double-reciprocal form (**B**, **D**). Panels **A** and **B** represent noncompetitive activation by several concentrations of CoA-SH (squares, 0 μM; circles, 1.0 μM; triangles, 5.0 μM) toward acetyl-CoA. [Values of kinetic parameters are, respectively, K_m = 150, 200, and 170 μM; V_{max} = 7990, 12500, and 11400 pkat (mg of protein)$^{-1}$]. Panels **C** and **D** represent competitive inhibition by several concentrations of CoA-SH (squares, 0 mM; circles, 1.0 mM; triangles, 2.5 mM) toward acetyl-CoA. [Values of kinetic parameters are, respectively, K_m = 150, 280, and 560 μM; V_{max} = 7990, 7500, and 8100 pkat (mg of protein)$^{-1}$]. 1/v is plotted versus 1/[acetyl-CoA] at fixed concentration (2 mM) of cinnamyl alcohol. Each value is the mean of three measurements.

namoyl acetate (**16**), the mutated form synthesized preferentially *Z*-2-hexenyl acetate (**6**), geranyl acetate (**13**), benzyl acetate (**14**), and butyl hexanoate (**27**). On the other hand, Cm-AAT4, contrary to Cm-AAT1 and Cm-AAT3, but similarly to SAAT, VAAT, and Rh-AAT1, has a L residue at the 339 position instead of A. Mutation of L339 into A in Cm-AAT4 (**Figure 4D**) gave stronger activity for most of the short-chain acetates (**4**−**8**), with *E*-2-hexenyl acetate (**7**) being now the ester produced at the highest rate (**Figure 5E**). Another mutation, consisting in the replacement of I59 of Cm-AAT4 by V (**Figure 4B**), did not result in any significant changes in substrate preference (not shown).

DISCUSSION

We demonstrate here that the alcohol acyltransferases of melon are capable of synthesizing sulfur-containing esters (thioether esters). Cm-AAT1 is by far the most active. The observed activities [10−60 pkat (mg of protein^{-1})] are much lower than those observed with the preferred substrates of the three recombinant enzymes, but are in the same order of magnitude as for many other compounds (*24*). Thioether esters are a set of volatile substances that are probably derived from the amino acid methionine and that are very important in the aroma profile of many varieties of melon fruit (*11*). Buttery et al. (*7*) reported that one of the typical aroma notes in *C. melo*

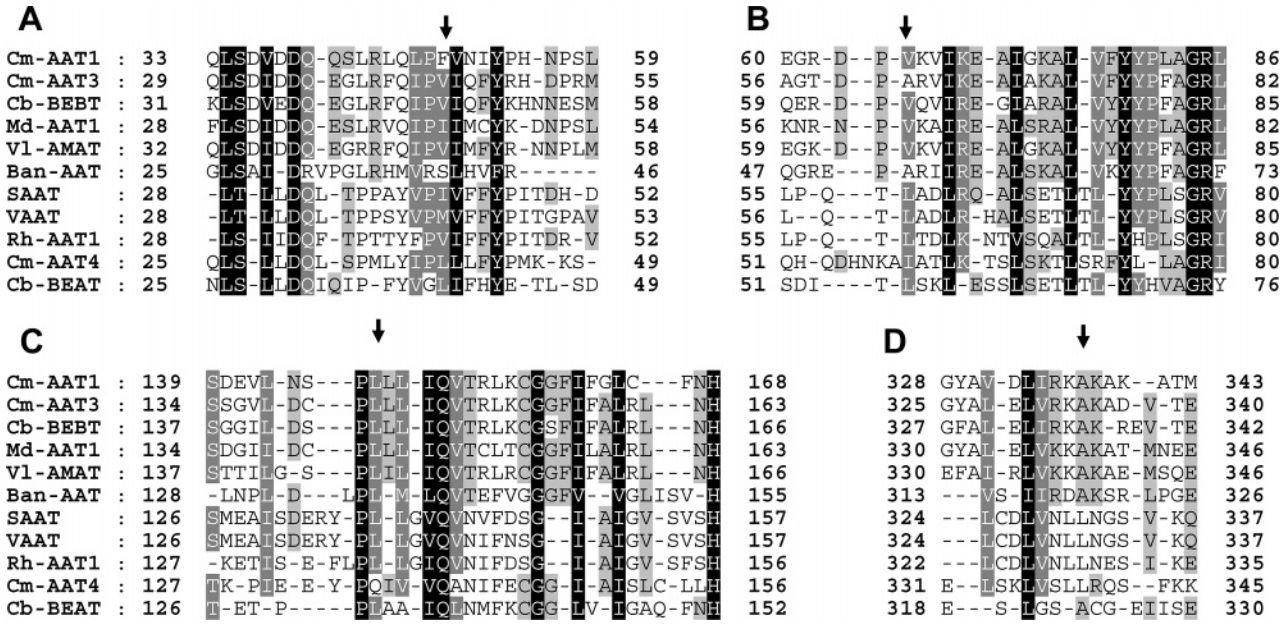

Figure 4. Partial amino acid sequence alignment of the *Cucumis melo* enzymes, Cm-AAT1 (CAA94432), Cm-AAT3 (AAW51125), and Cm-AAT4 (AAW51126) with closely related sequences *Clarkia breweri* Cb-BEBT (AAN09796), *Malus domestica* Md-AAT1 (AAU14879), *Vitis labrusca* Vl-AMAT (AY705388), *Musa sapientum* Ban-AAT (CAC09063), *Fragaria* × *ananassa* SAAT (AAG13130), *Fragaria vesca* VAAT (CAC09062), *Rosa hybrida* Rh-AAT (AAW31948), and *Clarkia breweri* Cb-BEAT (AAF04787) using the ClustalX program. Conserved residues are shaded in black. Dark gray shading indicates similar residues in 8 of 10 of the sequences, and clear gray shading indicates similar residues in 9 of 11 of the sequences. The arrows represent the residues that have been replaced by site-directed mutagenesis: (**A**) F49 replaced by L in Cm-AAT1 (MT1 FL49); (**B**) V65 replaced by A in Cm-AAT1(MT1 VA65); A61 replaced by V in Cm-AAT3 (MT3 AV61) and I59 replaced by V in Cm-AAT4 (MT4 IV59); (**C**) Q135 replaced by L in Cm-AAT4 (MT4 QL135); (**D**) L339 replaced by A in Cm-AAT4 (MT4 LA339).

var. *inodorus* cv. Honeydew was due to a sulfur compound, ethyl (methylthio)acetate. Analyzing the aroma profile of *C. melo* var. *reticulatus*, Jordan et al. (*8*) identified five sulfur compounds: 2-(methylthio) ethanol, 3-(methylthio)propan-1-ol, ethyl (methylthio)acetate, methyl 2-(methylthio)acetate, and ethyl 3-(methylthio)propanoate. In *C. melo* var. *cantalupensis*, Wyllie and Leach (*9*) and Homatidou et al. (*10*) reported a total of nine sulfur compounds. The thioether esters ethyl (methylthio)acetate, 3-(methylthio)propyl acetate, and ethyl 3-(methylthio)propanoate have very high odor values, which indicate that they contribute to the overall aroma perception of ripe fruit (*11*) with 3-(methylthio)propyl acetate and ethyl (methylthio)acetate having a sweet grassy and a fruity grassy odor, respectively (*12*), and ethyl 3-(methylthio)propanoate giving a note of fresh and green melon (*8*). The sulfur compound 2-(methylthio)ethyl acetate is present at substantial concentrations in Charentais melon (*31*) but has a very weak odor (*12*), whereas 3-(methylthio)propyl acetate is the most abundant sulfur-containing ester in Charentais melon (*31*) and strongly contributes to the melon note, as mentioned above. The molecular mass was estimated at about 200 kDa for the three recombinant proteins and 196 kDa for the native AAT extracted from melon fruit. The higher molecular mass of the recombinant AATs can be explained by the fact that they bear a C-terminal V5 epitope (GKPIPNPLLGLDST) and 6 His-tag. Because the molecular mass determined by SDS-PAGE and predicted by sequence analysis was around 50 kDa, it can be concluded that the melon AAT proteins are tetramers. It was verified that all of them were active under the tetrameric from. The presence of polymeric forms of AATs has already been mentioned in oriental sweet melon fruit but, in that case, the molecular mass was estimated at 400 kDa, corresponding to an octomer (*17*). The apparent molecular masses of native ATTs extracted reported for other fruit ranged from 40 kDa in banana (*14*) to 70 kDa in strawberry (*16*), indicating that, contrary to melon, they are monomers. All data on recombinant AATs of fruits and flowers indicate that the proteins have a molecular mass, in both native or denaturizing conditions, between 53 and 58 kDa (*20*, *23*, *26*−*28*). However, the situation of the melon is not unique among the superfamily of BAHD acyltransferases because dimers and trimers are commonly found (*32*).

The K_m values reported in the literature for AATs producing volatile esters in fruits and flowers are highly variable. For instance, the K_m for acyl-CoAs may range from around 0.02 mM (*27*) to around 2 mM (*21*), and the K_m for the alcohols can vary from 0.2 mM (*26*) to 46.5 mM (*20*). The K_m values for the melon AATs are highly different between the three proteins. For acetyl-CoA, K_m values were 1.23 mM for Cm-AAT1, 3.75 mM for Cm-AAT3, and 0.15 mM for Cm-AAT4. Souleyre et al. (*23*) have shown that great variations of K_m were observed with different combinations of acyl-CoAs and alcohols. Our results indicate that CoA-SH can either stimulate or inhibit the reaction depending on its concentration. This has been confirmed both by adding variable concentrations of CoA-SH in the reaction medium (**Figure 3**) and by modulating the level of CoA-SH produced by the reaction using phosphotransacetylase (**Table 1**). Such an effect of CoA-SH had never been reported for the BAHD superfamily. However, a strong stimulation of free CoA-SH has been reported for animal fatty acid synthetase (*33*) and for a plant 6-hydroxymellein synthase (*34*) that are not related to the BAHD superfamily but are involved in the condensation of acetyl- and malonyl-CoAs. The inhibitory effect of CoA-SH at high concentrations is competitive in nature, indicating an interaction of CoA-SH with the acyl-CoA substrate at its enzymatic site. The absence of significant effect on the K_m for alcohols is in agreement with the idea of two-step reactions for the binding of acyl-CoAs and alcohols (*23*). The stimulatory effect of CoA-SH at low concentrations is more difficult to interpret. By analogy to animal fatty acid synthetases (*35*), it can be hypothesized that CoA-SH would facilitate the

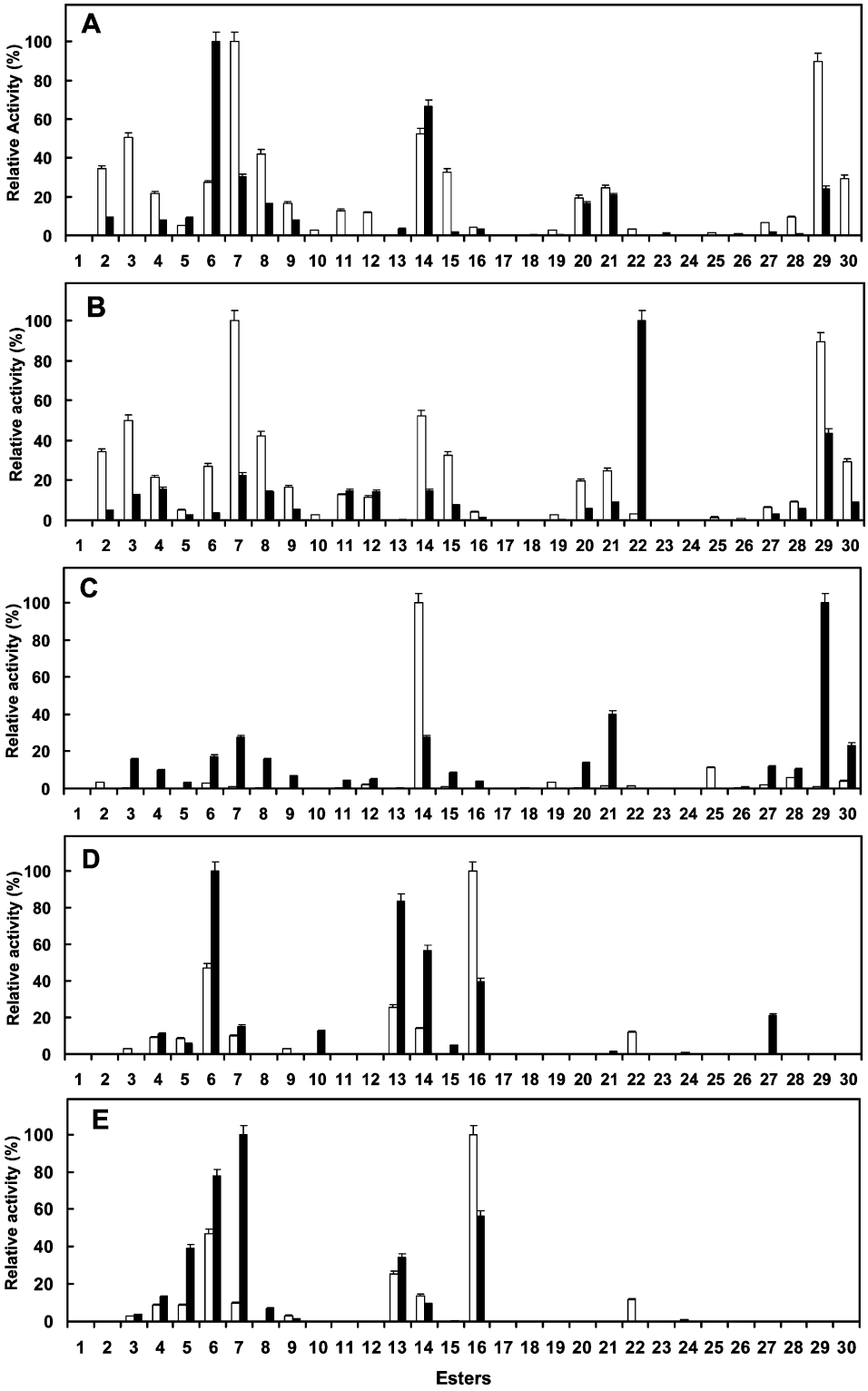

Figure 5. Substrate preference of the original (open bars) and mutated (black bars) recombinant Cm-AAT enzymes toward different alcohols and acyl-CoAs: (**A**) Cm-AAT1 MT1 FL49 mutation; (**B**) Cm-AAT1 MT1 VA65 mutation; (**C**) Cm-AAT3 MT3 AV61 mutation; (**D**) Cm-AAT4 MT4 QL135 mutation; (**E**) Cm-AAT4 MT4 LA239 mutation. Activity was measured after purification of the proteins as described under Materials and Methods. Activity is expressed as percent of the most active substrate: (**A**, **B**) 2490 pkat mg^{-1}; (**C**) 16285 pkat (mg of protein)$^{-1}$; (**D**, **E**) 3961 pkat (mg of protein)$^{-1}$. The numbers at the *X*-axis correspond to the following esters: **1**, ethyl acetate; **2**, butyl acetate; **3**, hexyl acetate; **4**, heptyl acetate; **5**, octyl acetate; **6**, *Z*-2-hexenyl acetate; **7**, *E*-2-hexenyl acetate; **8**, *Z*-3-hexenyl acetate; **9**, *E*-3-hexenyl acetate; **10**, 2-methylpropyl acetate; **11**, 2-methylbutyl acetate; **12**, 3-methylbutyl acetate; **13**, geranyl acetate; **14**, benzyl acetate; **15**, 2-phenylethyl acetate; **16**, cinnamoyl acetate; **17**, ethyl propanoate; **18**, butyl propanoate; **19**, 3-methybutyl propanoate; **20**, hexyl propanoate; **21**, 2-phenylethyl propanoate; **22**, ethyl butanoate; **23**, butyl butyrate; **24**, ethyl 2-methylpropanoate; **25**, 3-methylbutyl 2-methylbutanoate; **26**, ethyl hexanoate; **27**, butyl hexanoate; **28**, 3-methylbutyl hexanoate; **29**, hexyl hexanoate; **30**, 2-phenylethyl hexanoate.

unloading of the inappropriately bound substrates, allowing better access to the enzymatic site of the free substrates. Because, in our case, the effect of low levels of CoA-SH was mainly on the V_{max} for the acetyl-CoA moiety, it is probable that it is the

access to the enzymatic site of this cosubstrate, which would be preferentially facilitated. This assumption is consistent with the fact that an independent binding of the two cosubstrates has been deduced from crystallographic studies of a member of the BAHD superfamily, the vinorine synthase (*36*).

Previous experiments had shown that a cDNA clone, Cm-AAT2, with strong homology to Cm-AAT1 was unable to form volatile esters. Replacing A268 of Cm-AAT2 by T268, which is present in all active AATs, restored Cm-AAT2 enzyme activity, whereas substituting T268 by A268 abolished the activity of Cm-AAT1 (*24*). To identify the functional amino acid residues involved in the Cm-AATs mechanism, we have screened the amino acid sequence in silico of eight characterized AATs with the melon AAT sequences to find the amino acid residues that were unique to the melon AATs. It appeared that at least four amino acid residues were unique to the melon Cm-AATs in general or to a specific melon AAT (F49 in Cm-AAT1; A61 in Cm-AAT3; Q135 and L339 in Cm-AAT4). In addition to the consideration that these amino acids were unique to the Cm-AATs or to a specific Cm-AAT, the importance of these amino acids comes from their position in the protein. By comparing the melon Cm-AATs sequences with the sequences and the predicted structure of vinorine synthase, the first crystallized BAHD superfamily protein (*36*), it appeared that the concerned amino acid residues are located in areas of the protein that are important for AATs activity. As mentioned earlier, a hydrophilic amino acid at the 268 position (T or S) is required for the activity of volatile esters formation (*24*). By referring to the structure of the vinorine synthase protein, the 268 amino acid is located within the end of the α9 helix, which is close to the substrate-accessible channel. It can be hypothesized that the 268 located amino acid controls the entry of the substrate(s). The hydrophilic residue in Cm-AAT1 would allow the entry of low molecular weight alcohols to produce volatile esters, whereas, in the native Cm-AAT2, the hydrophobic residue would prevent the entry of low molecular weight alcohols and allow the access of other substrates that are yet unknown, probably resulting in the production of nonvolatile esters. Replacing F49 by L in Cm-AAT1 induced a change in stereoisomer recognition of the preferred substrate and a reduction of the range of esters being produced. This mutation is located in the β2 strand, which is proximate to the catalytic site and the substrate-accessible channel and therefore could differentiate between different steric conformations. Substituting V65 by A in Cm-AAT1 resulted in a strong restriction of substrate specificity. Inversely, replacing A61 by V at the corresponding position in Cm-AAT3 greatly extended the range of substrates accepted by the enzyme. These amino acids have different hydrophobicities, V being more hydrophobic that A. They belong to the α2 helix in a not very well conserved area located at the external surface of the protein, which does not interfere in the polymeric arrangement of the protein. Changing hydrophobicity could slightly modify the overall conformation of the enzyme under either the monomeric or polymeric form. When Q135, an uncharged polar amino acid, was replaced by L, a hydrophobic amino acid, in Cm-AAT4 so as to resemble all other AATs, there was a strong change of substrate preference. The β6 strand in which the Q-135 is located is close to the catalytic site of the protein, which contains His160 in vinorine synthase and in Cm-AAT4. On the other hand, substituting L339 by A in Cm-AAT4 gave higher activity for most of the short-chain acetates. L339 is located in the α10 helix at the periphery of the protein. Lowering hydrophobicity in this region by introducing an A residue may induce conformational changes improving the accessibility to low molecular weight substrates. The expression of mutated proteins in melon fruit through biotechnology may also be considered. However, the nature of the esters synthesized is dependent not only of the substrate specificity of the AATs but also on the availability of the precursors. Besides providing information on the mechanisms of substrate preference, these experiments may have other biotechnological outcomes such as the expression of native or mutated Cm-AAT genes in bacteria or yeasts to generate fermented food with specific aromas.

In conclusion, this paper demonstrates that the three Cm-AATs of melon have differential capacities to synthesize thioether esters, compounds that confer specific aromatic notes to melon fruit. They function under a tetrameric form and are strongly regulated by the product of the reaction, CoA-SH, which has stimulatory or inhibitory effects depending on the concentration. We have also identified some amino acids in the sequence that contribute to the preference for some substrates.

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