Partial Purification and Some Properties of Alcohol Acyltransferase from Strawberry Fruits

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The enzyme system concerning volatile ester formation in strawberry *Fragaria ananassa* × *Duchessne* var. Chandler was studied. Protein with alcohol acyltransferase activity was purified about 29-fold from Chandler strawberry fruits by ammonium sulfate fractionation, gel filtration, and anion-exchange chromatography. The enzyme activity had a pH optimum of 8.0 and an optimum temperature of 35 °C. The apparent M_r estimated by gel filtration was 70 000. The enzyme was tested for its preference in using different acyl-CoAs and alcohols. Maximum activity was obtained using acetyl-CoA and hexyl alcohol as substrates. A clear correlation was observed between substrate preference and volatile esters present in strawberry var. Chandler.

INTRODUCTION

Esters are qualitatively and quantitatively one of the most important classes of volatiles in fruit aroma (Dirinck et al., 1977; Macku and Jennings, 1987; Pérez et al., 1992). The main components are those formed from low molecular weight fatty acids and low molecular alcohols. Alcohol acyltransferase (AAT) plays a major role in the biosynthesis of volatile esters, not only in fruits but also in the process of fermentation by microorganisms. This enzyme catalyzes the transfer of an acyl moiety from acyl-CoA onto the corresponding alcohol.

Ester formation by microorganisms has been the subject of many studies (Nordstrom, 1961; Lanza et al., 1976; Howard and Anderson, 1976; Sarris and Latrasse, 1985), and two alcohol acetyltransferases have been identified and characterized in *Cladosporium cladosporioides* and brewers' yeast (Yamawaka et al., 1978; Yoshioka and Hashimoto, 1981). More recently, an AAT has been purified from *Neurospora* sp. (Yamauchi et al., 1989). Nevertheless, there are very few results on the biochemical aspects of ester formation in fruits. There are some published papers related to flavor precursors (Salunke and Do, 1976), mainly focused on amino acid metabolism (Myers et al., 1970; Tressl and Drawert, 1973; Drawert and Berger, 1982), but there is not much information available regarding enzymes implicated in these biosynthetic pathways. Yamashita et al. (1976) reported the formation of esters from aldehydes that were incubated with whole strawberry fruits. Ueda and Ogata (1977) found that formation of volatile esters in banana fruit was a coenzyme A dependent reaction. The esterification of added alcohols in separated cells of banana, strawberry, and melon has been reported (Ueda and Ogata, 1976), although as far as we are aware, only an alcohol acetyltransferase from banana (Harada et al., 1985) has been previously described in fruits.

In a previous paper (Pérez et al., 1992) we studied the changes in the volatile components of strawberries var. Chandler during ripening and free amino acid composition in relation to aroma biogenesis. In this study we have focused our attention on the enzymatic aspects of flavor biogenesis. The present paper describes the purification method and some properties of an AAT enzyme that is involved in the biosynthesis of volatile esters in strawberry fruits.

EXPERIMENTAL PROCEDURES

Materials. Mature fresh fruits of *Fragaria ananassa* × *Duchessne* var. Chandler were obtained from Cooperativa Agrícola de Lucena del Puerto, Huelva, Spain.

Preparation of Crude Extract. Mature strawberry fruits (250 g) were cut into slices (2 mm thick) and infiltrated with 500 mL of a 0.7 M glycerol solution containing 0.7% pectinase, 0.5% potassium dextran sulfate, and 50 mM mercaptoethanol (ME). After 1 h of agitation at 25 °C and filtration through two layers of gauze, the separated cells were collected by centrifugation at 3500*g* for 7 min and washed twice with 50 mM Tris-HCl, pH 8.0, 50 mM ME. Cells were suspended in the latter buffer containing 0.1% Triton X-100 and disrupted by sonic oscillation (Sonics Model 375 W) at 0–4 °C for 3 min. The resulting homogenate was centifuged at 12000*g* for 30 min. The supernatant was considered the crude extract.

Purification. All procedures were performed at 0–4 °C. Proteins with AAT activity were purified as follows.

Step 1. $(NH_4)_2SO_4$ was added to the crude extract from strawberry tissue, and the fraction obtained at 20–70% saturation was collected and desalted on a Sephadex G-25 (Pharmacia) column, using 20 mM Tris-HCl, pH 8.0, buffer containing 1 mM dithiothreitol (DTT), 12 mM ME, and 10% ethylene glycol (conditioning buffer).

Step 2. The desalted $(NH_4)_2SO_4$ pellet was applied to a Sephacryl S-200 (65 cm × 1.6 cm, Pharmacia) column and eluted with the conditioning buffer.

Step 3. Active fractions from gel filtration chromatography were pooled and applied to a DEAE-cellulose DE-52 (7 cm × 3.2 cm, Whatman) column equilibrated with the conditioning buffer. A stepwise elution was run with the latter equilibrium buffer containing 0.0 (80 mL), 0.15 (120 mL), and 0.5 M NaCl (80 mL), respectively.

Assay of AAT Activity. The standard assay mixture consisted of 5 mM Tris-HCl buffer, pH 8.0, 0.25 mM acetyl-CoA, 0.05 mM DTT, 20 mM butyl alcohol, 5 mg of BSA, and the appropriate volume of enzyme solution (10–50 μL) in a total volume of 0.5 mL (Harada et al., 1985). The mixture was incubated at 35 °C for 30 min in a 11-mL sealed vial. The vial was then transferred into an automatic headspace sampler (Hewlett-Packard 19395 A), where a 15-min equilibrium time at 80 °C was set to allow the produced ester to enter the gas phase. The reaction product, butyl acetate, was determined by GLC in a gas chromatograph equipped with FID and a stainless steel FFAP (2 m × 2 mm) column at 120 °C. The amount of ester was calculated from a calibration curve in the range 3–750 nmol. One unit of AAT activity was defined as the formation of 1 μmol of butyl acetate/min.

Protein Determination. Protein was measured according to the method described by Bradford (1976), using the Pierce

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Coomassie protein assay reagent with crystalline BSA as the standard protein.

K_m, V_{max} Determination. For the determination of apparent K_m and V_{max} for acetyl-CoA, reactions were carried out at pH 8.0 and 35 °C and acetyl-CoA concentrations were varied from 20 to 300 μM, while butyl alcohol was maintained at constant saturating concentration (20 mM). In the same way, butyl alcohol concentrations were varied from 1 to 20 mM and acetyl-CoA was maintained at constant saturating concentration (300 μM) for the determination of apparent K_m and V_{max} for butyl alcohol. Data were plotted as a Lineweaver–Burk graph.

M_r Determination. M_r was determined using a fast protein liquid chromatograph (FPLC) by gel filtration on a Superose 12 HR 10/30 (10 × 300 nm, Pharmacia) column. Catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), and chymotrypsin (25 kDa) were used as marker proteins. The column was pre-equilibrated and developed with 20 mM sodium phosphate buffer, pH 7.0, 0.5 M NaCl, 10% glycerol, flow rate of 0.35 mL/min, and detection at 280 nm.

RESULTS AND DISCUSSION

Enzyme Activity in Cell-Free Extract. The high level of esterase activity, enzyme which catalyzes the reverse reaction of AAT, in all fruit tissues, is the main difficulty for AAT extraction. Both activities, the esterifying activity and the esterase activity, have been previously reported in separated cells from strawberry (Ueda and Ogata, 1976). This would explain why no ester formation has been detected when homogenized strawberry tissue is incubated with different alcohols (Yamashita et al., 1975). We have found that to obtain AAT activity from strawberry fruit, 0.7% pectinase (Macerocyme R-10) should be added to the extraction solution. Macerocyme has been reported to be essential for obtaining good alcohol acetyltransferase activity in a cell-free extract of banana fruit (Ueda and Ogata, 1977), since esterase was eliminated during Macerocyme treatment and the subsequent washing process. Harada et al. (1985) also used 0.8% Macerocyme in the purification method of banana alcohol acetyltransferase.

Detergents were also necessary to extract strawberry AAT. Crude extracts prepared without Triton X-100 showed very low activity levels. This suggests that AAT could be a membrane-bound enzyme, although an indirect effect of this detergent, protecting the enzyme from inactivation as proposed by Harada et al. (1985) for alcohol acetyltransferase from banana, could not be rejected.

Purification. Strawberry AAT was purified about 29-fold by ammonium sulfate fractionation followed by gel filtration and anion-exchange chromatography. Precipitated proteins between 20 and 70% ammonium sulfate saturation represented 35% of initial protein content and 80% of total AAT activity in crude extract. Two main protein peaks and a single peak of activity were obtained by Sephacryl S-200 gel filtration of the desalted ammonium sulfate precipitate (Figure 1). Protein-containing fractions 24–27 were pooled and subjected to an anion-exchange chromatography on a DEAE-cellulose column. The stepwise elution (Figure 2) gave two main protein peaks. The AAT activity was eluted at 0.15 M NaCl (fractions 34–37). The purification procedure is summarized in Table I. Strawberry AAT specific activity values were higher than those reported for alcohol acetyltransferase from banana (Harada et al., 1985) but lower than those reported for AAT from *Neurospora* sp. (Yamauchi et al., 1989).

Partially purified enzyme solution was used for M_r determination on Superose 12, using the FPLC system. Strawberry AAT exhibited an M_r of 70 000 in relation to marker proteins.

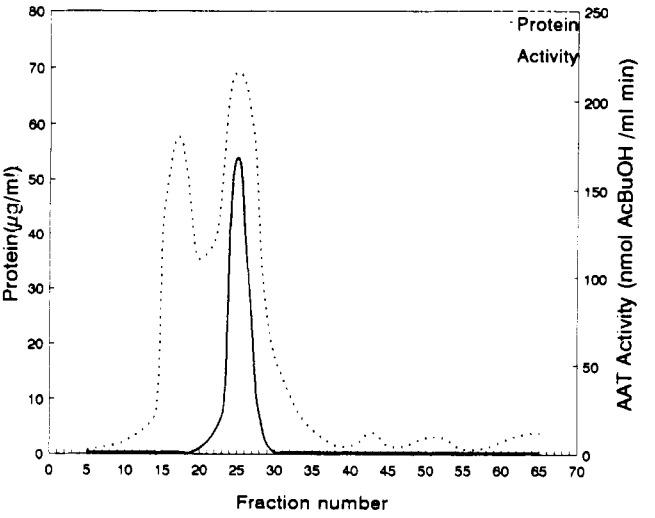

Figure 1. Elution profile of strawberry AAT on a Sephacryl S-200 column. Flow rate was regulated to 14 mL/h, and fractions of 3.5 mL were collected and assayed for protein and AAT activity.

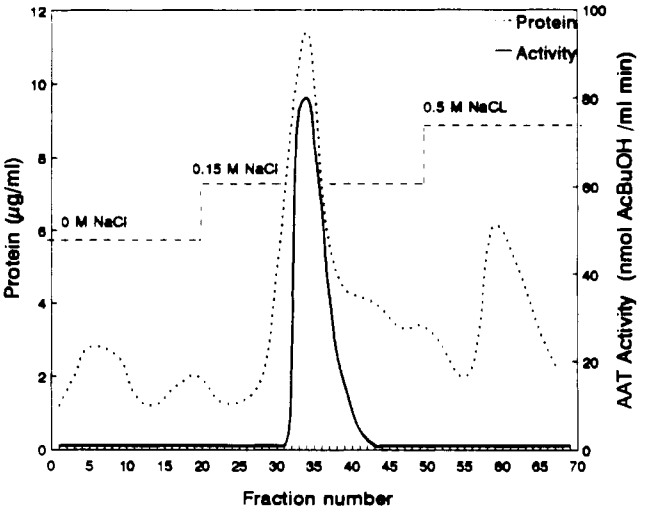

Figure 2. Elution profile of strawberry AAT on a DEAE anion-exchange column. Fractions of 4.1 mL were collected at flow rate of 16.4 mL/h.

Table I. Purification of Alcohol Acyltransferase from Strawberry Fruit

purifn yield step	total protein, mg	total act.,[a] units	sp act., units/mg	purifn x-fold	purifn %
crude extract	56.14	3.48	0.0619	1.00	100.0
Ppt 20–70% $(NH_4)_2SO_4$	19.10	2.78	0.1455	2.30	79.8
S-200	3.36	2.11	0.6279	10.13	60.6
DEAE	1.55	1.55	1.8023	29.05	44.5

[a] One unit of alcohol acyltransferase is defined as the amount of enzyme which yielded 1 μmol of butyl acetate/min.

Determination of Optimum Enzyme Quantity and Reaction Time. The AAT from strawberry showed a linear relationship between quantity of protein used and rate of ester production for incubation times lower than 30 min and for enzyme solution volumes equal or lower than 25 μL. Unless otherwise stated, these conditions were used in all experiments.

Effect of pH and Temperature. The pH dependency of the strawberry AAT enzymatic reaction with acetyl-CoA and butanol as substrates was assessed using 20 mM universal buffer (Perrin and Dempsey, 1979). The enzyme exhibited a broad range of pH activity of 5.5 and 9.3 (Figure

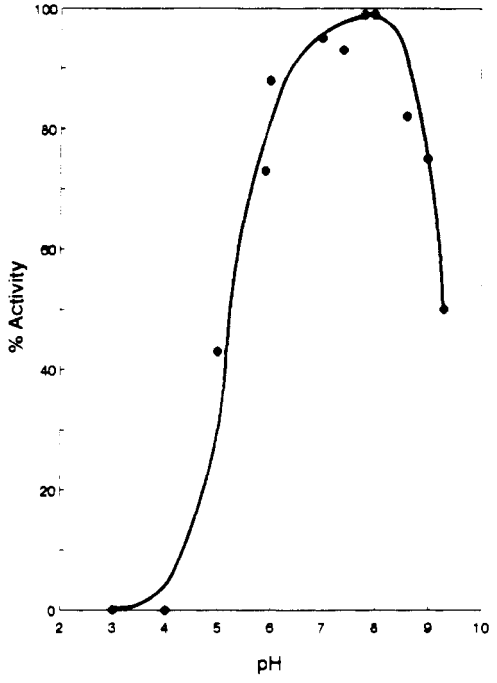

Figure 3. Effect of pH on strawberry AAT activity.

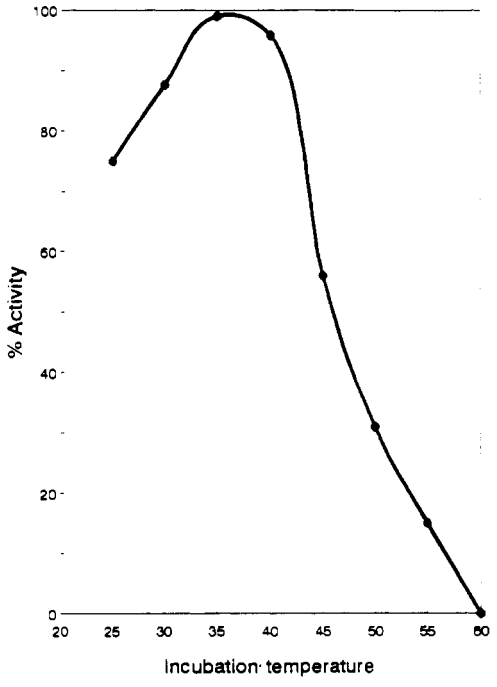

Figure 4. Effect of incubation temperature on strawberry AAT activity.

3). This pH optimum value is similar to those reported in the literature for various alcohol acetyltransferases (Yamawaka et al., 1978; Yoshioka and Hashimoto, 1981; Harada et al., 1985) and that reported for AAT from *Neurospora* sp. (Yamauchi et al., 1989).

The optimum temperature for strawberry AAT activity at pH 8.0 was found to be 35 °C (Figure 4). The enzyme showed 75% of maximum activity at 25 °C and a marked drop in activity above 35 °C.

Kinetic Parameters. The apparent K_m value of AAT obtained with butyl alcohol as substrate under standard conditions was estimated from a Lineweaver–Burk plot as 3 mM. This value is in good agreement with that reported for acetyltransferase from banana pulp (Harada et al., 1985). Apparent V_{max} was 1.76 nmol/min.

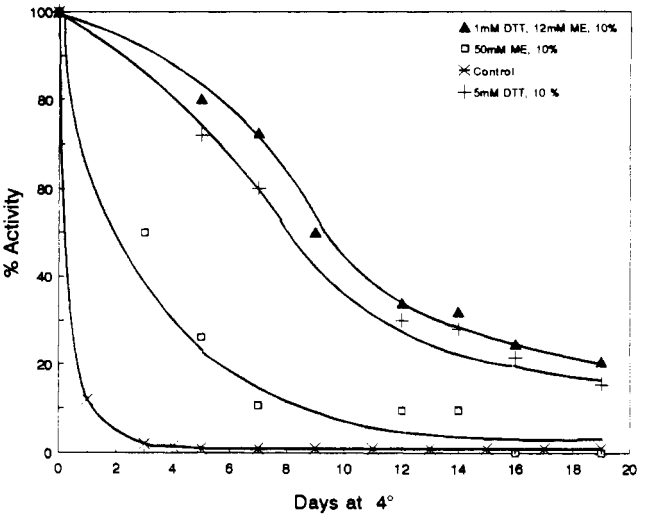

Figure 5. Effect of SH-group protectors and ethylene glycol on strawberry AAT stability.

Table II. Effect of Incubation Time (2 and 24 h) of Different Metal Ions (1 mM) on the Strawberry AAT Activity

	rel AAT act., %	
ion	2-h incubation	24-h incubation
K^+	120.0	113.1
Cu^{2+}	55.0	35.0
Mg^{2+}	120.0	105.4
Hg^{2+}	0.0	0.0
Ca^{2+}	78.0	80.0
Mn^{2+}	100.0	80.0
Fe^{2+}	79.0	61.5
Zn^{2+}	95.2	60.3

In the same way, the apparent K_m value for AAT with acetyl-CoA as substrate was found to be 65 μM, and V_{max} was 2.36 nmol/min. This K_m value is very close to that reported for alcohol acetyltransferase from banana fruit (Harada et al., 1985).

Enzyme Stability. In the absence of SH-group protectors, the partially purified enzyme lost more than 80% of its activity in 24 h at 0–4 °C (Figure 5). This suggests that SH residues could be implicated in the catalytic activity of AAT from strawberry. This was confirmed by using *p*-HMB, a sulfhydryl reagent which caused strong inhibition of the enzyme activity. Sulfhydryl groups have been reported to be important for the activity of various acetyltransferases (Yoshioka and Hashimoto, 1981; Harada et al., 1985) and for different diacylglycerol acyltransferases in higher plants (Cao et al., 1987; Weselake et al., 1991). Two different SH-group protectors were tested, DTT (dithiothreitol) and ME (mercaptoethanol). A combination of both reagents, 1 mM DTT and 12 mM ME, produced the best results in terms of enzyme stability. Addition of 10% ethylene glycol to the latter preparation also improved stability. Under these conditions AAT from strawberry showed a half-life of 10 days at 0–4 °C.

Effect of Metal Ions. The effects of metal ions (1 mM) on strawberry AAT are shown in Table II. The enzyme was totally inactivated by Hg^{2+}; high levels of inactivation also occurred with Cu^{2+} and Fe^{2+}. On the other hand, it has been reported that under certain conditions Mg^{2+} precipitates acyl-CoA from the reaction mixture and thus may interfere with acyltransferase assays (Constantinides and Stein, 1986). Partial inhibition caused by Mg^{2+} has been described in AAT from *Neurospora* sp. (Yamauchi et al., 1989) and alcohol acetyltransferase from brewers' yeast (Yoshioka and Hashimoto,

Table III. Substrate Specificity of AAT from Strawberry Fruit: Comparison of Esterification Activity with Different Alcohols, Using Acetyl-CoA as Acyl Donor and Different Incubation Times

ester	incubation time		
	15 min	30 min	60 min
butyl acetate, nmol	14.1	24.3	37.6
amyl acetate, nmol	14.0	22.1	28.0
isoamyl acetate, nmol	12.3	17.5	22.5
hexyl acetate, nmol	23.4	42.4	52.8
3-hexenyl acetate, nmol	2.6	3.9	7.3

Table IV. Substrate Specificity of AAT from Strawberry: Comparison of Esterification Activity with Different Acyl-CoAs, Using Butyl Alcohol as the Cosubstrate and Different Incubation Times

ester	incubation time		
	15 min	30 min	60 min
butyl acetate, nmol	13.1	24.9	34.0
butyl propionate, nmol	1.4	4.6	7.0
butyl butanoate, nmol	11.3	15.8	27.0

1981). However, Howard and Anderson (1976) observed that 6–8 mM magnesium ions stimulated the formation of ethyl acetate from ethanol and acetyl-CoA in microsomal fractions of brewers' yeast. Magnesium ions had no inhibitory effect on strawberry AAT and even promoted activation on enzyme activity. Potassium ions, as reported for alcohol acetyltransferase from banana fruit (Harada et al., 1985), showed marked stimulatory effect.

Substrate Specificity. The study of AAT substrate specificity was the main aim of this work, due to our current interest in flavor biogenesis in fruits. The specificity of strawberry AAT could explain the distribution of different types of esters in its aroma.

To examine the reactivities with acetyl-CoA, equal concentrations of various alcohols (20 mM) were added to the standard assay mixture instead of butyl alcohol (Table III). The enzyme acted on all of the alcohols tested. Different alcohols, primary and secondary, straight-chain and branched, saturated and unsaturated, had been reported to be esterified by whole strawberries (Ueda and Ogata, 1978). Among the different substrates tested, maximum activity was obtained with hexyl alcohol. Unsaturated C_6 alcohol showed a slight esterification rate. Moreover, the enzyme seemed to be more active against straight-chain alcohols than against branched-chain alcohols of the same carbon number. The same preference was observed for alcohol acetyltransferase from brewers' yeast (Yoshioka and Hashimoto, 1981) and in esterification experiments with a disk of banana pulp (Ueda and Ogata, 1978). However, different results have been reported for AAT from *Neurospora* sp. (Yamauchi et al., 1989), which showed maximum activity with isobutyl alcohol. All alcohols tested are present as acetate esters in Chandler aroma. A clear correlation is observed between AAT substrate preference found in this work and the volatile ester composition of strawberry var. Chandler (Pérez et al., 1992). Among all of the acetate esters obtained by enzymatic reaction, hexyl acetate (4.5%) is the most abundant in strawberry aroma, followed by butyl acetate (1.9%) and amyl and isoamyl acetate (1%).

In the same way, equal concentrations (0.25 mM) of different acyl-CoAs were assayed using butyl alcohol as the cosubstrate. Three different acyl-CoAs were tested. Results are shown in Table IV. Acetyl-CoA was found to be the preferred substrate, although butyl butyrate and butyl propionate were also formed. Therefore, this enzyme differs from those reported in the literature since it acts on various acyl-CoAs. Most enzymes described so far acted only on acetyl-CoA, and the only AAT which was able to use various acyl-CoAs as substrate (Yamauchi et al., 1989) did not act on acetyl-CoA.

Although we have not examined this enzyme against higher acyl-CoAs, results obtained could have physiological relevance, since they are in good agreement with the acyl moiety distribution in the volatile esters of strawberry var. Chandler (Pérez et al., 1992). Thus, AAT from strawberry showed less specificity for propionyl-CoA, and propionate esters were detected as minor components (3.5%) in the dynamic headspace of Chandler. In the same way, higher specificity for butyryl-CoA corresponds with the higher amount of butyrates (43.3%) in Chandler aroma.

These data suggest that two main factors could be involved in determining volatile ester composition in strawberry var. Chandler: (1) the availability of the substrates, acyl-CoA and alcohols (Pérez et al., 1992), and (2) the inherent properties of the AAT enzyme (e.g., substrate specificity).

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