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Purification to homogeneity and characterization of major fatty acid ethyl ester synthase from human myocardium

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Non-oxidative metabolism of ethanol via fatty acid ethyl ester synthase is present in those extrahepatic organs most commonly damaged by alcohol abuse DEAE-cellulose chromatography of human myocardial cytosol at pH 8 0 separated synthase I, minor and major activities, eluting at conductivities of 5, 7 and 11 mS, respectively The major synthase was purified 8900-fold to homogeneity by sequential gel permeation, hydrophobic interaction, and anti-human albumin affinity-chromatographies with an overall yield of 25% SDS-PAGE showed a single polypeptide with a molecular mass of 26 kDa and gel permeation chromatography under nondenaturing conditions indicated a molecular mass of 54 kDa for the active enzyme The purified enzyme catalyzed ethyl ester synthesis at the highest rates with unsaturated octadecanoic fatty acid substrates (V_{max} = 100 and 65 nmol/mg/h for oleate and linoleate, respectively) K_m values for oleate, linoleate, arachidonate, palmitate and stearate were 0 22 mM, 0 20 mM, 0 13 mM, 0 18 mM and 0 12 mM, respectively Thus, human heart fatty acid ethyl ester synthase (major form) is a soluble dimeric enzyme comprised of two identical, or nearly identical, subunits (M_r = 26000)

Alcohol, Fatty acid ethyl ester synthase, (Human myocardium)

1. INTRODUCTION

Alcohol-induced heart muscle disease is a common disorder, characterized by physiological changes, such as heart failure and dysrhythmias [1] and also by biochemical abnormalities such as accumulation of triacylglycerides and decreased myocardial β-oxidation of fatty acid [2,3]. Fatty acid ethyl esters were recently identified in heart, and these neutral lipids were shown to accumulate in vivo after being synthesized in the myocardium [4,5]. Importantly, in isolated rabbit heart mitochondria, these ethyl esters induce mitochondrial dysfunction in vitro [6], a finding that establishes a link between alcohol intake and mitochondrial damage, a hallmark of alcohol-induced heart muscle diseases. Thus, these compounds provide a link between ethanol ingestion and the subsequent development of myocardial dysfunction.

Fatty acid ethyl ester synthase, the enzyme(s) which catalyzes synthesis of these esters, exists in two forms in rabbit myocardium and they can be separated from each other by chromatography on DEAE-cellulose [7]. The major form in rabbit accounts for 67% of the activity, and the purified enzyme catalyzes ethyl ester synthesis at the greatest rates when the lipid substrate is an unsaturated octadecanoic fatty acid.

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The human organs most commonly injured by alcohol (brain, pancreas, liver and heart) also contain fatty acid ethyl ester synthase [8,9]. However, even though each enzyme form is eluted at the same conductivity as the minor and major synthases found in rabbit myocardium, their relative proportions are reversed. In the present work, we have purified to homogeneity and partially characterized the major fatty acid ethyl ester synthase from human myocardium. These studies serve as an important first step for clinical studies examining the genetic predisposition to alcohol-induced end-organ damage.

2. MATERIALS AND METHODS

2 1 *Materials*

All reagents were the highest commercially available grade [^{14}C]Oleic acid (59.9 Ci/mol), and [^{3}H]oleic acid (5.7 Ci/mol) were purchased from Amersham Ethyl [^{3}H]oleate was synthesized in 70% yield by acid-catalyzed esterification of [^{3}H]oleic acid in ethanol [7]

2 2 *Enzyme assay*

Rates of fatty acid ethyl ester synthesis were determined following the procedure described elsewhere [7]

2 3 *Homogenates*

Human heart (patients with no pathological record) taken at autopsy (within hours post-mortem) was placed in ice-cold 1 mM 2-mercaptoethanol (BME), 10 mM Tris, pH 8 0, and all subsequent procedures were performed at 4°C Ventricular myocardium (180–200 g) was trimmed free of fat and connective tissue, minced and homogenized for 50 s in 10 vols of cold 1 mM BME, 10 mM Tris, pH 8 0, with a Polytron PT-20 homogenizer at half-maximal

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setting Homogenate was centrifuged at 14000 × *g* for 30 min and the resulting supernatant was centrifuged at 48400 × *g* for 1 h After each centrifugation, floating fat was removed by aspiration Before purification, the clear supernatant was dialyzed overnight against 1 mM BME, 10 mM Tris, pH 8 0. Protein was estimated by the Bradford method [10]

2.4 *Electrophoresis*

The molecular mass and purity of the enzyme were determined by SDS-PAGE [11] Gels containing 3 2% (stacking gel, pH 6 8) and 10% (separating gel, pH 8 8) acrylamide were run at 150 V (stacking) and 200 V (separating), after which they were fixed and stained with Coomassie brilliant blue and silver [12]. Molecular mass was calculated using polypeptide standards of known molecular mass (phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000); carbonic anhydrase (30000); soybean trypsin inhibitor (20100); and α-lactalbumin (14000))

2.5. *Anti-human albumin antibody affinity resin*

The IgG fraction from rabbits immunized with human albumin (Sigma Chemical Co., St Louis, MO) was coupled for 2 h to CNBr-activated Sepharose 4B (Pharmacia) in 0 1 M $NaHCO_3$, pH 8 3, containing 0 5 M sodium chloride. The resultant resin was treated with 0 2 M glycine, pH 8 0, for 1 h and then washed with 0 1 M sodium acetate, pH 6 0, containing 0 5 M sodium chloride. The Sepharose anti-human albumin antibody conjugate was washed with 1 mM BME, 10 mM Tris, 50 mM sodium phosphate, pH 7.0

3. RESULTS

3.1. *Purification of the major fatty acid ethyl ester synthase*

The human heart soluble fraction (1800 ml) was applied to DEAE-cellulose (5 × 16 cm) in 1 mM BME, 10 mM Tris at pH 8.0. All the activity was bound, and when the absorbance at 280 nm was less than 0.1, the column was developed further with a linear salt gradient running from starting buffer to 400 mm NaCl. As shown in fig.1, fatty acid ethyl ester synthase eluted as 3 peaks of activity, and each was pooled separately.

The major fatty acid ethyl ester synthase pool (450 ml) from DEAE chromatography was precipitated with ammonium sulfate (70% of saturation), dialyzed against 1 mM BME, 10 mM Tris, 50 mM sodium phospohate, pH 7.0, and applied to a Sephadex G-100 column (2.5 × 72 cm). A single peak of activity eluted with 70% recovery of activity and 6-fold purification (data not shown).

Fractions containing enzyme activity greater than 2 nmol/ml/h were pooled and applied at 20 ml/h to octyl-Sepharose CL-4B (1.0 × 6.0 cm) equilibrated with 1 mM BME, 10 mM Tris, 50 mM sodium phosphate, pH 7.0 (fig.2). The resin was washed with 0.1% sodium cholate in the same buffer, and the column was developed further with a linear sodium cholate gradient (0.1–0.25%). The enzyme emerged as a single peak (85% yield) at approximately 0.15% (w/v) sodium cholate. SDS-PAGE of this peak (fig.3) showed a single band of molecular mass 26 kDa, containing only a trace amount of albumin. The fractions eluting from octyl-Sepharose having synthase activity greater than 2 nmol/ml/h were then pooled and applied to an anti-

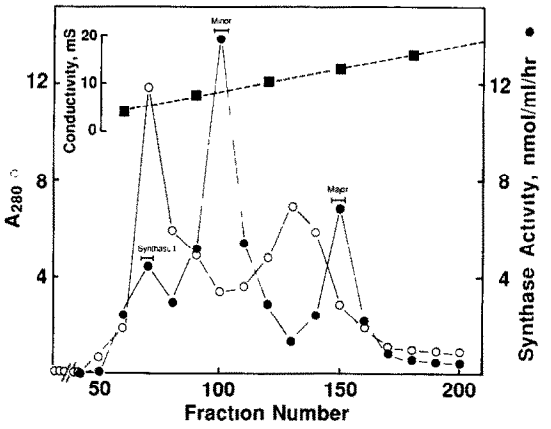

Fig 1 DEAE-cellulose chromatography Enzyme activity from human myocardium was fractionated at 40 ml/h in 1 mM BME, 10 mM Tris, pH 8 0 The column was developed with a linear salt gradient running from buffer to 400 mM NaCl (■). Fractions (6 ml) were collected and monitored for protein (○) and synthase activity (●)

human albumin affinity column (0.9 × 7 cm) equilibrated with 1 mM BME, 10 mM Tris, 50 mM sodium phosphate, pH 7.0. Homogeneous synthase washed through with full retention of enzyme activity, and SDS-PAGE of the preparation demonstrated the presence of a single polypeptide with a molecular mass of 26 kDa (fig.3). To confirm its molecular mass, the enzyme from the anti-human albumin antibody affinity column was then applied to FPLC superose 12 (gel filtration column). Enzymatic activity eluted as a single peak corresponding to a molecular mass of 54 kDa, indicating that the enzyme is a dimer consisting of two 26 kDa subunits (data not shown).

The purification summary of the major human myocardial fatty acid ethyl ester synthase is given in table 1. The enzyme was purified 8867-fold with an

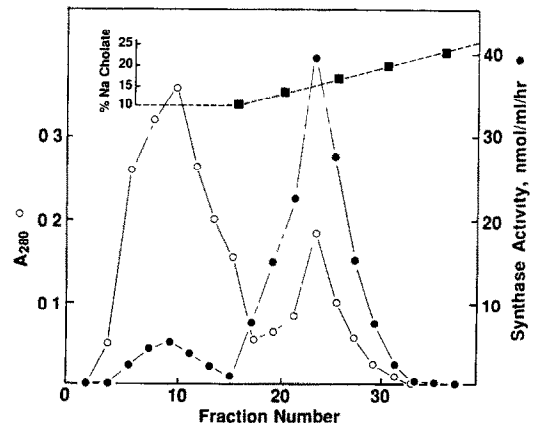

Fig 2 Hydrophobic interaction chromatography. Protein was applied to octyl-Sepharose CL-4B (1 × 6 cm), equilibrated with 0 1% cholate, 1 mM BME, 10 mM Tris, 50 mM sodium phosphate, pH 7 0, and the column was developed with a linear sodium cholate gradient running from 0 1% cholate to 0.25% cholate (■) Fractions (2 ml) were monitored for protein A_{280} (○) and enzyme activity (●)

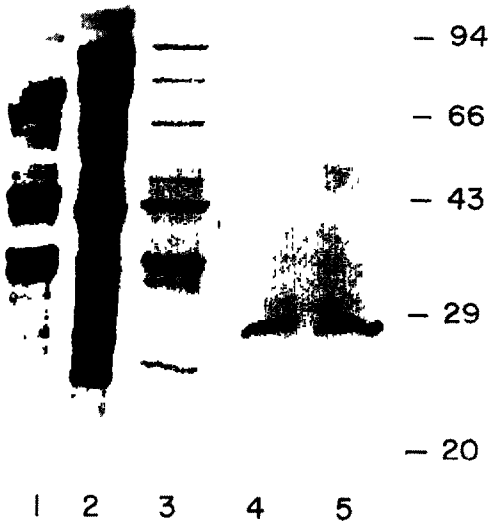

Fig 3 SDS-PAGE of major fatty acid ethyl ester synthase purification (Lane 1) cytosol; (lane 2) DEAE-cellulose, (lane 3) G-100; (lane 4) octyl-Sepharose, (lane 5) anti-human albumin affinity chromatography

overall yield of 25%. Assuming a specific activity of 2660 nmol/mg/h for the pure synthase when assayed in the presence of 0.2 M ethanol and 0.91 mM oleic acid, myocardium contains approximately 30 μg of major fatty acid ethyl ester synthase/g of tissue.

3.2. *Major fatty acid ethyl ester synthase: kinetic properties*

The substrate specificity of the major fatty acid ethyl ester synthase with respect to fatty acid chain length and saturation was next examined. The homogeneous enzyme was incubated with 0.2 M ethanol in the presence of the following ^{14}C-labeled fatty acids: palmitate, stearate, oleate, linoleate and arachidonate. For each fatty acid, saturation kinetics were observed,

Table 1

Purification of major fatty acid ethyl ester synthase

Step	Total protein (mg)	Total activity (nmol/h)	Specific activity (nmol/mg/h)	Purification (x-fold)	Yield (%)
Cytosol[a]	9315	2700	0 3	1	100
DEAE-cellulose	99	1585	15 9	53	59
G-100	11	999	91 0	303	37
Octyl-Sepharose	0 42	638	1520 0	5067	24
Anti-human albumin affinity chromatography	0 25	665	2660 0	8867	25

[a] Major activity in the cytosol was calculated from the proportions of major and minor activities as determined after DEAE-cellulose chromatography

Table 2

Kinetic constants[a] for major fatty acid ethyl ester synthase

Fatty acid	K_m (mM)	V_{max} (nmol/mg/h)
Oleic	0 23	100
Linoleic	0 20	65
Arachidonic	0 13	50
Palmitic	0 18	20
Stearic	0.12	22

[a] Determined at 0 2 M ethanol, 200 mM phosphate, pH 7 2

and there was specificity with respect to fatty acid chain length and saturation (table 2). Maximum rates of ethyl ester synthesis (V_{max}), determined from Lineweaver-Burke plots, were approximately 3-fold greater for linoleate and 5-fold greater for oleate substrates than for stearate and palmitate, i.e. 65 and 100, compared to 22 and 20 nmol/mg/h, respectively. In contrast, binding affinities (K_m) for these fatty acid substrates varied only two-fold (0.12 to 0.23 mM).

4. DISCUSSION

Fatty acid ethyl esters are important metabolites of ethanol, and they are found in highest concentrations in those organs that are damaged by alcohol abuse [8]. These nonoxidative products of ethanol accumulate in heart and can induce mitochondrial damage [6]. Their synthesis is catalyzed by fatty acid ethyl ester synthase as judged by the presence of 3 activity peaks from anion exchange chromatography. The major form of the enzyme has been isolated and characterized in rabbit myocardium [7]. In the present work, these studies have been extended to human myocardium, from which the form eluting at 11 mS has now been purified to homogeneity.

This synthase is a dimer with a subunit molecular mass of 26 kDa, as is the major synthase from rabbit heart. A detailed kinetic study of this enzyme was performed to determine its substrate specificity. The highest rates of ethyl ester synthesis were found with oleic and linoleic acids. The V_{max} for the synthesis of ethyl oleate by this enzyme is 100 nmol/mg/h. The K_m value of the enzyme for all fatty acids is 0.10–0.2 mM.

Previous investigations suggest that alcohol metabolism may be under genetic control and related to the presence of different amounts or types of ethanol-metabolizing enzymes [13,14]. Since no other pathway for alcohol metabolism exists in the heart, fatty acid ethyl ester synthase may be one of the gene products underlying a genetic vulnerability to the effects of alcohol [8]. Recent genetic studies using peripheral human leukocytes have shown that this synthase activity is inheritable in an autosomal recessive pattern for high activity [15,16], a pattern expected for a gene controlling the production of a toxic agent.

It will now be possible to examine this hypothesis by cloning the major synthase to enable restriction fragment length polymorphism studies of families containing an alcoholic proband. Thus, the present work provides a foundation for subsequent protein sequence studies, cloning and genetic studies of the fatty acid ethyl ester synthase system in man. Because it is present in those organs commonly injured by alcohol, establishing a genetic link between alcohol consumption and end-organ damage may now be feasible.

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