Utilization of Volatile Fatty Acids in Ruminants. VI. Purification of Acetyl-Coenzyme A Synthetase from Mitochondria of Lactating Goat Mammary Gland

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A procedure for purification of acetyl-CoA synthetase from mitochondria of lactating goat mammary gland is described. The method employs $(NH_4)_2SO_4$ fractionation and one chromatographic step using calcium phosphate gel. A 190-fold purification was achieved. The approximate molecular weight is 66,500 as determined by sucrose density gradient centrifugation. Polyacrylamide gel electrophoresis of the enzyme gives one protein band. However, preincubation in 0.5 *M* KCl or 7 *M* urea results in two protein bands. When the enzyme was pretreated in 1% sodium dodecyl sulfate, polyacrylamide gel electrophoresis gave six protein bands. These data suggest that the enzyme exists in multimolecular forms. Antibodies to acetyl-CoA synthetase were obtained by immunization of rabbits with the purified enzyme. Using these antibodies and ^{131}I, the amount of acetyl-CoA synthetase in mitochondria from goat mammary gland was estimated by radioimmunoassay to be 11.09 $\mu g/g$ of tissue.

We have reported that acetyl-CoA synthetase from mitochondria of lactating bovine mammary gland could be purified extensively using DEAE-cellulose, Tris-HCl buffers, and KCl gradients followed by adsorption chromatography using calcium phosphate gel (Qureshi and Cook, 1975). Acetyl-CoA synthetase from goat mammary gland mitochondria could not be purified using gel filtration techniques (Sephadex G-100 or G-200 and Bio-Gel P-100 or P-200) or ion exchange techniques (TEAE-cellulose, DEAE-cellulose, or carboxymethylcellulose). However, the enzyme could be extensively purified using adsorption chromatography on calcium phosphate gel.

Acetyl-CoA synthetase was thus purified from mitochondria of lactating goat mammary glands taken 5 weeks postpartum and a radioimmunoassay for the enzyme was developed. The results of these studies are reported herein.

EXPERIMENTAL PROCEDURE

Enzyme Purification. Acetyl-CoA synthetase was purified from mitochondria of lactating goat mammary gland as described by Qureshi and Cook (1975) except that the two steps employing DE-23 cellulose and DE-52 cellulose were omitted. Enzyme activity and protein were determined as previously described (Cook et al., 1969). Mitochondria were suspended in 0.13 *M* KCl and then frozen and thawed three times. Enzyme activity was measured after the third thaw. The preparation was then centrifuged at 20,000 *g* and the supernatant (mitochondrial extract) was taken for further purification.

Gel Electrophoresis. Polyacrylamide disc gel electrophoresis was carried out as described by Davis (1964). Gels were stained with Coomassie Blue. In all of the experiments the buffer was 0.025 *M* Tris-HCl–0.20 *M* glycine (pH 8.2). Electrophoresis was for 1 hr at 3 mA/gel. The enzyme protein was treated as follows prior to electrophoresis: (1) control, no treatment; (2) incubation in 0.5 *M* KCl for 1 hr; (3) incubation in 7 *M* urea at 37° for 3 hr; the buffer and gels contained 7 *M* urea; (4) incubation in 1% sodium dodecyl sulfate (SDS) for 3 hr at 37 or 60 or 80°; the buffer and gels contained 1% SDS; (5) incubation in 7 *M* urea at 37° for 2 hr and then incubation in 7 *M* urea–1% SDS for an additional 2 hr at 37°; the buffer and gels contained 1% SDS but not urea.

Molecular Weight Determination. The molecular weight of acetyl-CoA synthetase was determined by the method of Martin and Ames (1961). A linear sucrose gradient of from 5 to 20% was employed. Bovine albumin was used as a reference. The centrifugation was for 15 hr at 50,000 rpm (246,000*g*). Eight drops per fraction were collected. Bovine serum albumin was determined by measuring absorption at 210 nm. The fractions containing enzyme protein were assayed for enzyme activity.

Radioimmunoassay for Acetyl-CoA Synthetase. Antibodies to the enzyme were obtained by immunization of rabbits with the purified protein. Rabbits were injected subcutaneously with 2 ml containing 0.5 mg of acetyl-CoA synthetase diluted 1:1 with complete Freund's adjuvant. Injections were followed 3 weeks apart at the same dose level but using Freund's incomplete adjuvant. Blood was taken after the third injection. Anti-rabbit γ-globulin was obtained by immunization of goats with rabbit γ-globulin.

The procedures described by Niswender et al. (1969) for radioiodination of protein and for the radioimmunoassay were followed. A 1:50 dilution of the rabbit serum gave maximum binding of ^{131}I-labeled acetyl-CoA synthetase.

RESULTS AND DISCUSSION

A summary of the purification of acetyl-CoA synthetase is shown in Table I, and a typical chromatogram is shown in Figure 1. It was found that no purification of the $(NH_4)_2SO_4$ fraction could be achieved using gel filtration employing Sephadex G-100 or G-200 and Bio-Gels P-100 and P-200. The enzyme could not be purified using carboxymethylcellulose. Unlike the cow enzyme (Qureshi and Cook, 1975) the goat enzyme could not be purified using TEAE-cellulose or DEAE-cellulose. However, the goat enzyme could be extensively purified using $CaPO_4$ gel. A 190-fold purification was achieved (Table I).

The molecular weight of the enzyme was estimated to be approximately 66,500 (Figure 2). This is similar to the cow enzyme which has a molecular weight of approximately 63,000.

Polyacrylamide gel electrophoresis of the enzyme gave one protein band (Table II). However, when the enzyme was pretreated with 0.5 *M* KCl or 7 *M* urea, two protein bands were observed. When the enzyme was preincubated in 1% SDS at 37° for 3 hr, five protein bands were observed. However, the slower moving protein band separated into two distinct protein bands when the enzyme was preincubated in 1% SDS at 60° or at 80° prior to electrophoresis. Thus, under these conditions six protein bands were observed.

These studies using KCl or urea indicate that the enzyme consists of two different molecular species. However, treatment with urea followed by SDS results in the for-

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Table I. Purification of Acetyl-CoA Synthetase from 108 g of Wet Mitochondria Extracted from 957 g of Mammary Gland Tissue

Fraction	Vol, ml	Protein, mg	Act., units	Sp act., units/mg	Recovery, %	Purification, -fold
Mitochondria (after third thaw)	756	7085	23,664	3.3	100	1.0
Mitochondrial extract	238	2285	26,843	11.7	113	3.5
$(NH_4)_2SO_4$ ppt	48	724	12,597	17.3	53	5.2
$CaPO_4$ gel (sum of enzyme recovered from 8 columns)		10.2	6,528	640.0	28	193.0

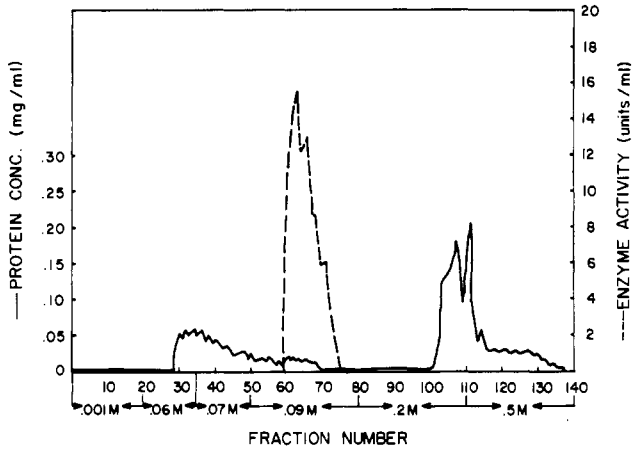

Figure 1. Chromatography of acetyl-CoA synthetase on calcium phosphate gel. The column was eluted with a stepwise gradient of increasing concentration of potassium phosphate buffer (pH 7.0).

Table II. Polyacrylamide Gel Electrophoresis of Acetyl-CoA Synthetase; Relative Mobility and Schematic Presentation of Protein Bands

Pretreatment of protein	No. and relative mobility of protein bands[a]			
None (control)	0.66			
0.5 *M* KCl	0.80	0.82		
7 *M* urea, 37°	0.50	0.53		
7 *M* urea, 1% SDS, 37°	0.55	0.58	0.65	0.68
1% SDS, 37°	0.62	0.65	0.68	0.73
1% SDS, 60°	0.56	0.58	0.62	0.67
1% SDS, 80°	0.56	0.58	0.62	0.67

[a] Relative mobility is the distance the protein band traveled divided by the length of the gel.

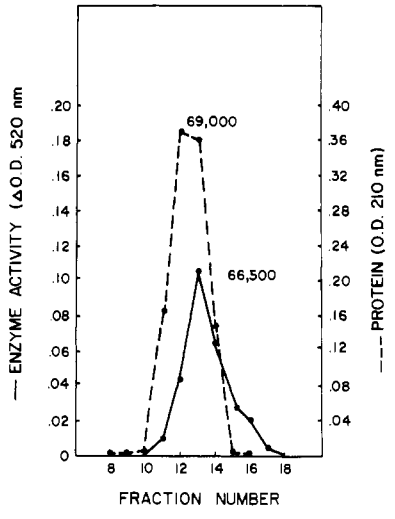

Figure 2. Sucrose density gradient centrifugation of acetyl-CoA synthetase using bovine serum albumin as a reference. Serum albumin was determined by measuring the adsorption at 210 mμ. Acetyl-CoA synthetase was determined by measuring enzyme activity.

Table III. Radioimmunoassay for Acetyl-CoA Synthetase in Mitochondria from Lactating Goat Mammary Gland[a]

Goat no.	Sp act., μmol/hr per mg of protein	Tissue concn, μg of enzyme protein/g of tissue
1	4.61	9.00
2	3.41	8.21
3	6.41	15.55
4	4.05	11.52
		Av 11.09

[a] The measurements were made on the mitochondrial extract from tissue biopsies taken 4 weeks postpartum.

mation of two additional faster migrating molecular species. Treatment with SDS at 37° for 3 hr causes the second protein band to separate into two distinct protein bands. Also, treatment with SDS at 60 or 80° for 3 hr causes the first as well as the second protein band to separate into two distinct protein bands. The results indicate that acetyl-CoA synthetase is made up of at least six different molecular species. However, it may be that the six proteins do not differ in molecular weight. In a separate study we have found that goat mammary acetyl-CoA synthetase contains fucose, glucose, galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid. Sodium dodecyl sulfate may bind to the protein such that the proteins have different charges due to the presence of carbohydrates and thus can be separated into six protein bands using polyacrylamide gel electrophoresis.

With the immunization scheme described above, all rabbits produced antibody to acetyl-CoA synthetase. Compared with normal rabbit serum the globulin fraction from rabbits immunized with injections of 0.5 mg of protein bound up to 28% of [^{131}I]acetyl-CoA synthetase. The addition of 10 to 1000 ng of unlabeled acetyl-CoA synthetase significantly inhibited the binding of labeled enzyme. Although the blood titers were not as high as would be desirable for maximum sensitivity (e.g., 50% binding of [^{131}I]acetyl-CoA synthetase compared to normal rabbit serum), the radioimmunoassay procedure was used to estimate the amount of acetyl-CoA synthetase in the mitochondrial fraction isolated from four different goats. The

results are expressed as micrograms of enzyme protein in mitochondria per gram of mammary tissue (Table III). The enzyme concentrations found for all four goats were of the same order of magnitude and averaged 11.09 μg/g of tissue.

The fact that acetyl-CoA synthetase has been purified and antibodies to the protein have been produced in rabbits will provide an opportunity for extensive characterization of the enzyme. At present the approximate molecular weight of the enzyme is known. The protein has been shown to contain carbohydrates and is a glycoprotein (Stamoudis and Cook, 1975).

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Utilization of Volatile Fatty Acids in Ruminants. VII. Acetyl-Coenzyme A Synthetase. A Glycoprotein

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Acetyl-CoA synthetase purified from mitochondria of lactating cow and goat mammary gland was tested for the presence of carbohydrates. Polyacrylamide gel electrophoresis followed by PAS staining was positive. Sulfuric acid hydrolysis or preincubation with neuraminidase gave a difference in anodic migration on polyacrylamide gel electrophoresis suggesting the presence of *N*-acetylneuraminic acid. The thiobarbituric acid test for neuraminic acid was positive. GLC analysis showed that the cow enzyme contains fucose, glucose, and *N*-acetylneuraminic acid. The goat enzyme contains fucose, galactose, glucose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid. These observations were confirmed using mass spectrometry. The role carbohydrates play in determining structural and catalytic properties of acetyl-CoA synthetase is not clear at this time. Other work in our laboratory has shown that acetyl-CoA synthetase is more active on propionate than on acetate in liver and lung, but is equally active on both substrates in heart and kidney. Also, the enzyme is not active in the nonlactating mammary gland but activity begins to develop near the time of parturition. The activity in the mammary gland decreases with advancing lactation. These differences in substrate specificity and other phenomena may be explained by differences in the carbohydrate composition of the enzymes in different tissues and under different physiological states.

We consider the acetate activation reaction catalyzed by acetyl-CoA synthetase to be an important rate-limiting step in acetate utilization by ruminant tissues (Cook et al., 1969; Quraishi and Cook, 1972). In order to study this reaction further, considerable effort was devoted to purification of the enzyme from mitochondria of lactating ruminant mammary gland (Qureshi and Cook, 1975; Cook et al., 1975). During these studies the apparent aggregation phenomena and difficulties in purification suggested to us that the enzyme might be a glycoprotein.

Consequently, the purified enzyme was tested for carbohydrates and was found to be a glycoprotein. The details of these experiments are reported in this paper.

EXPERIMENTAL SECTION

Enzyme Purification. The enzyme from lactating bovine mammary gland was purified as described by Qureshi and Cook (1975). The enzyme from lactating goat mammary gland was purified as described by Cook et al. (1975).

Enzyme Assay. Acetyl-CoA synthetase activity was determined by the acetate-dependent disappearance of the free sulfhydryl group of coenzyme A as described by Cook et al. (1969). Protein was determined by the method of Lowry et al. (1951).

Polyacrylamide Gel Electrophoresis. The general method of Davis (1964) was used for these studies. The buffer used was 0.025 *M* Tris-HCl–0.20 *M* glycine (pH 8.3). The electrophoresis was carried out with a current of 6 mA/tube for 30 min. After electrophoresis the gel columns were stained for proteins with Coomassie Blue according to the procedure of Chrambach et al. (1967). Staining for glycoproteins was performed according to Hotchkiss (1970) and Kaschnitz et al. (1969).

Determination of Neuraminic Acid. The thiobarbituric acid assay was used to determine neuraminic acid (Warren, 1959). Neuraminic acid was removed from the protein by hydrolysis for 60 min at 80° in 0.1 *N* H_2SO_4.

Gas–Liquid Chromatography. Monosaccharides were determined as the Me_3Si derivatives of the methyl glycosides by GLC as described by Sweeley et al. (1972). The chromatograph was equipped with a flame ionization detector. The column used was 6 ft × ⅛ in. glass packed with Chromosorb W containing 3% OV-1. Nitrogen was used as carrier gas at a flow rate of 30 ml/min. Isothermal GLC was conducted at two different temperatures, 160 and 190°.

Mass Spectrometry. The presence of carbohydrates was confirmed using a LKB-9000 gas chromatograph–mass spectrometer. The ionizing energy was 70 eV. The GLC column was 4 ft × ⅛ in. glass packed with Chromosorb G containing 3% SE-30. The temperature was programmed

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