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THE EXTRACTION AND PURIFICATION OF LIPOGENIN

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SUMMARY

A method for the extraction and purification of lipogenin has been described. By means of alcohol fractionation, chromatography, electrophoresis, and mannanase treatment an approx. 1000-fold purification was achieved. The purified material contained some 30 % carbohydrate, mainly mannan, and 70 % of a peptide.

INTRODUCTION

The presence of a factor termed lipogenin, its occurrence and mechanism of action *in vitro* and *in vivo* have been described[1-3]. Lipogenin has been found in mammalian liver and in yeast. It increases the rate of incorporation of labeled acetate into fatty acids and into cholesterol in the livers of fasted rats both *in vivo* and *in vitro*. It has no effect on fatty acid synthesis in fed animals. The purification of lipogenin is described here.

EXPERIMENTAL AND RESULTS

Assay

Lipogenin preparations were assayed in liver homogenates from fasted rats as previously described[1,2]. The stimulation of incorporation of labeled acetate into fatty acids was roughly proportional to the quantity of lipogenin used and about 10 times the amount incorporated in the controls. Higher concentrations of lipogenin were inhibitory. Aliquots of fractions for assay were lyophilized to determine the quantity of soluble material, and the amount required to give a 10-fold stimulation was measured. Determinations of the individual sugars were carried out according to DISCHE[4]. Reducing sugars were assayed by the dinitrosalicylic acid reaction[5], total carbohydrates by the anthrone method[6]. Total nitrogen was estimated by the micro-Kjeldahl method, and amino nitrogen by the micro biuret reaction of ZAMENHOF AND CHARGAFF[7]. Amino acid analyses were carried out in an automatic amino acid analyzer. Electrophoresis was carried on a Geon 426 (the Goodrich Chemical Company) bed contained in a lucite block.

Extraction and purification of lipogenin

Preparation of the crude extract: 65 kg of fresh baker's yeast was mixed with 200 l of water until a thin homogeneous slurry was obtained. The yeast suspension was then passed through a steam-heated tube 90 cm long inclined at an angle of about 20°. The flow was adjusted so that the slurry remained for 2 min in the heated tube and the temperature of the emerging liquid was 95°. The slurry was then cooled rapidly and centrifuged in a basket centrifuge at 3500 × *g*. The precipitate was discarded and the clear supernatant was concentrated *in vacuo* to approximately one-fourth its original volume at 30°.

Alcohol fractionation: The concentrated crude extract was cooled to about 30° and an equal volume of cold absolute ethanol was added slowly with stirring. After several hours of settling, the precipitate was sedimented by centrifugation at 5000 × *g*. It was washed once with 50 % aqueous ethanol. The supernatant fluid was discarded since it contained no appreciable amounts of lipogenin.

The precipitate was dissolved in approximately eight volumes of distilled water, the solution was concentrated in a flash evaporator to remove remaining alcohol and centrifuged for 20 min at 15000 × *g*. A floating fatty layer and a dark greyish sediment were discarded since they were found to be inactive. The aqueous solution contained 55 g of material.

To the lipogenin solution absolute ethanol was slowly added with stirring to a concentration of 25 %. After 4 h, the precipitate which contained approx. 10 % of the lipogenin was sedimented by centrifugation at 5000 × *g*. It was not processed further. More ethyl alcohol was then added to the supernatant until a concentration

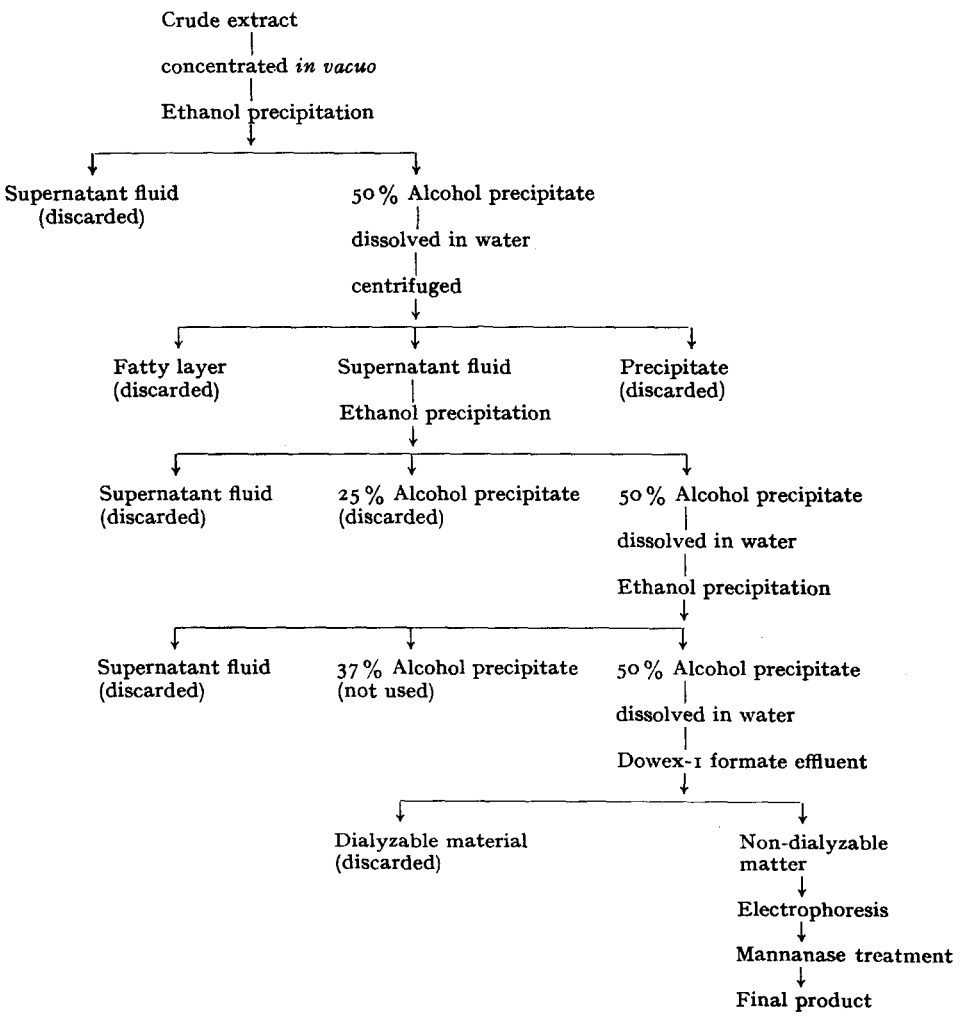
of 50 % was obtained. The material which precipitated was centrifuged, washed with cold 50 % aqueous ethanol and lyophilized. The residue amounted to 17 g and contained the greater part of the lipogenin activity.

The lyophilized residue from the previous step was dissolved in 200 ml distilled water. Absolute ethyl alcohol was added slowly with stirring to 37 % concentration. The precipitate which was formed was allowed to settle and then sedimented at 10000 × *g*. It was dissolved in water and lyophilized to yield 10.5 g (precipitate No. 1). Approximately one-fourth of the original amount of lipogenin was contained in this fraction, but it was not processed further.

Ethanol was added with stirring to the supernatant to 50 % alcohol concentration. The precipitate was centrifuged, washed with 50 % aqueous ethanol, dissolved in water and lyophilized to yield 4.0 g (precipitate No. 2). This fraction contained

TABLE I

PURIFICATION SCHEME



the main portion of lipogenin activity. The supernatant containing 2.7 g of solute was discarded.

Chromatography: A column of Dowex-1 in the chloride form 3.5 × 45 cm was treated with 0.2 M ammonium formate until free of chloride ions and washed with water. Precipitate No. 2 was dissolved in water and passed through the Dowex column. The column was eluted with water and nearly all the lipogenin activity was recovered in the first 600 ml. The slightly turbid eluate was concentrated and lyophilized to yield 2.3 g of residue.

TABLE II

PURIFICATION OF LIPOGENIN

Fraction	*Amount (g)*	*Specific activity* (μg)*	*No. of assays*	*Approximate total purification (x)*
Crude		700	39	
First alcohol 50%		35–40	36	20
Second alcohol 25–50%	17	15	4	40
Third alcohol	4	7	5	80
Chromatographic eluate	2.3	3–4	4	160
Electrophoretic band	1.1	1.2	5	480
Mannanase treated		0.7	3	960

* Quantity required for maximal stimulation of acetate incorporation *in vitro*.

Electrophoresis: Geon was treated at room temperature with 0.5 N NaOH, washed with water, suspended in 0.5 N acetic acid, washed with water again and equilibrated with borate buffer (pH 7.8, *I* 0.07). The Geon bed measured 40 × 10 × 0.8 mm. Electric power was applied for 30 min at 3° before the introduction of the sample. The active material obtained from the previous step was submitted to electrophoresis in 250-mg batches. The sample was applied to the middle of the block which was then sealed by a thin plastic sheet. With a potential of 350 V a current of 35–40 mA was obtained. After 15 h the block was sectioned into 1-cm wide bands which were eluted separately with 5-ml portions of distilled water each. The eluates were dialyzed and tested for activity. Lipogenin had migrated approx. 6 cm toward the cathode as a 5-cm wide band. The eluates containing lipogenin were pooled and lyophilized to give 1.1 g of residue. A sample of this material was analyzed. It contained about 59% mannose, 3.5% glucose and 36% amino acids*. All the usual amino acids were present except methionine.

Repetition of chromatography and electrophoresis did not result in further purification.

Mannanase treatment: The composition of lipogenin made it likely that an active component was combined with mannan, a yeast polysaccharide. This polysaccharide could not be hydrolyzed by amylases. Therefore, mannanase, an adaptive enzyme, was prepared from a culture of soil organisms grown in the presence of mannan as sole carbon source. The enzyme was partially purified by means of ion exchange resin and DEAE-cellulose[8,9].

* The author wishes to thank Professor Z. DISCHE of Columbia University for his hospitality and advice in regard to the sugar analysis.

When 9.4 mg of lipogenin and 0.9 mg of a 50-fold purified mannanase in 1 ml of water were incubated at 53° for 1 h, 4.8 mg of dialyzable material was formed. This consisted of a mixture of easily dialyzable sugars and amounted to approx. 60–65 % of the carbohydrate present in lipogenin. The lipogenin activity was found in the non-dialyzable fraction. Electrophoresis of this fraction was carried out at pH 7.0 in borate buffer. Under these conditions lipogenin migrated to the cathode while mannanase moved slightly towards the anode. Elution of the lipogenin activity, dialysis and lyophilization resulted in approx. 4 mg of material containing 30 % carbohydrate, mainly mannan. The purification scheme is summarized in Table I and the properties of the fractions are given in Table II.

DISCUSSION

The stability and mode of action of lipogenin have been described previously[2,3]. A purification procedure is given here which used six steps and yielded a lipogenin preparation purified some 1000 times over the original yeast extract. The lipogenin content at each step was ascertained by assaying an aliquot which in a liver homogenate from fasted rats increased the rate of incorporation of labeled acetate into fatty acids 10–15 fold. The final preparation contained some carbohydrate, probably in the form of a mannose polysaccharide and a polypeptide.

Since the major portion of the carbohydrate in lipogenin preparations can be split off by mannanase, without destroying the biological activity, it appears most likely that the residual carbohydrate is also not required for activity and that the peptide represents the active moiety. Due to the uncertain state of purity of the best material thus far obtained, quantitative amino acid analyses have no particular significance and no connection between the absence of methionine and the biological activity could be established. The molecular weight of lipogenin was not determined, since at this state of purification it would have little meaning.

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