Natural Science Report, Ochanomizu University, Vol. 12, No. 2, 1961
お茶の水女子大学 自然科学報告 第12巻 第2号

Higher Fatty Acids Dehydrogenase

VII. On the Coenzyme of Porcine Liver Higher Fatty Acids Dehydrogenase

Hiroyasu Fukuba and Mieko Hori

Laboratory of Nutrition Chemistry, Faculty of Home Economics,
Ochanomizu University, Tokyo, Japan

Introduction

Previously, the author investigated the presence and some charactors of higher fatty acids dehydrogenase of soy beans, and the results obtained were as follows :[1-6]

1) The activity of this enzyme was only recognized at the ripining and germinating stages of beans and not at the resting stage.

2) Higher fatty acids of saturated series, such as myristic, palmitic, and stearic acids, unsaturated series, such as oleic and linoleic acids, and hydroxy acids, such as 12-hydroxy-, 9, 10-dihydroxy-, and 9, 10, 12-trihydroxy-stearic acids were found to be used as the substrate of this enzyme.

3) After the action of this enzyme, the products were extracted with ethyl ether and oxidized to ozonide with O_3 and then to mono- and di-carboxylic acids with $KMnO_4$. This dicarboxylic acid was chromatographed with silicic acid. It was found that, when myristic and stearic acids were dehydrogenized with this enzyme, nonanedioic acid was produced as dicarboxylic acid, while, palmitic acid was submitted, heptanedioic acid was obtained.

4) It was also found that this enzyme required DPN as a coenzyme. In the previous papers, this conclusion was brought out from the experiment only with the Thunberg's tube method. As shown in Fig. 1, from the direct determination of DPNH produced by the enzyme action, this point was further confirmed.

5) From the results that the action of this enzyme was greatly inhibited by the addition of *p*-chloromercuribenzoate, monoiodo- and monochloro-acetic acids, the -SH group would be concerned as the prothetic group of this enzyme.

6) As Annau[7,8] pointed out, the crude preparation of this enzyme could take lecithin as the substrate, but, following the purification, this was not. So, it was assumed that the enzyme capable to dehydrogenize the free acid and the one to the esterified acid was the separate one.

On this enzyme of the animal sourse was investigated by Lang [9–11], Champougny-Clément and Le Breton[12–14], and Koyanagi[15] and so others. As the coenzyme of this, muscle adenylic acid (Lang) and vitamin B group, such as pantothenic acid, pyridoxine, and nicotinic acid (Champougny-Clément and Le Breton), and riboflavin (Koyanagi), were reported. In the paper of Champougny-Clément *et al.*, the difference between the time required for the decoloration of media added the coenzyme and this of midia without the coenzyme was very small. It was thought dangerous to decide the action of these vitamins as the coenzyme from such a slight difference.

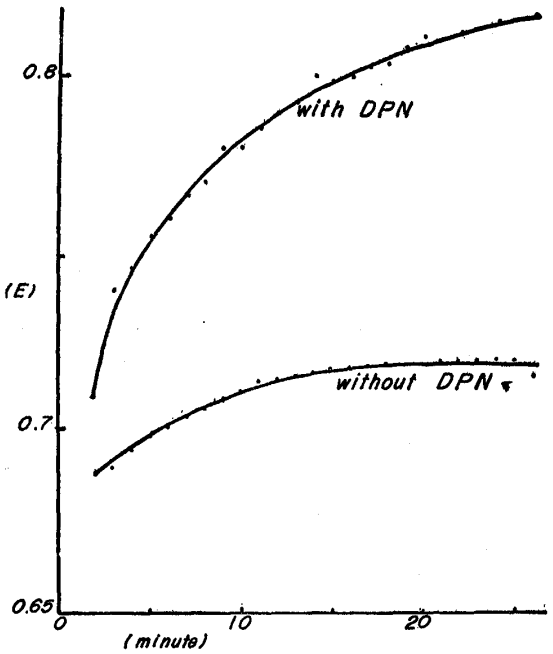

Fig. 1. Role of DPN as coenzyme of higher fatty acids dehydrogenase of soy beans
Composition of reaction media:

Enzyme solution (partially purified)	0.2 ml
Substrate (M/1,000 Na-palmitate)	0.1 ml
Buffer solution (pH 8.0)	9.5 ml
DPN (100 γ/ml)	0.2 ml

(For the medium without DPN, 0.2 ml of buffer solution were further added instead of DPN solution.)

Experiments

I. On the purification of the enzyme

120 g. of liver were homogenized with the aid of purified sea sand in a mortar, and then extracted with 200 ml. of 0.9% cold NaCl solution. After this, the extract was incubated for 3 minutes at 37°C, and then quickly cooled to 5°C, after this, the solution was centrifuged for 25 minutes at 6000 r.p.m. at 0-5°C. The supernatant solution was fractionally saturated with ammonium salfate. The sediments thus obtained were resolved with 60–80 ml. of phosphate buffer solution (pH 8.0).

Further, this enzyme solution was dialized overnight with distilled water in an ice box.

The enzyme activity of these preparations was determined by the Thunberg's tube method, and the result was cited in Table 1.

Table 1. Higher fatty acids dehydrogenase activity of various fractions of porcine liver extracts.
Composition of reaction media:

Medium No.	I	II	III	IV
Enzyme preparations	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Na-palmitate (M/100)	1.0	1.0	0.0	0.0
Phosphate buffer	1.0	1.1	2.0	2.1
DPN (100 γ/ml)	0.1	0.0	0.1	0.0
Methylene blue (M/2,000)	0.2	0.2	0.2	0.2

Reaction temperature: 37°C

Fractions	Medium No.	Decoloration time (minutes)
A	I	5
	II	10
	III	6
	IV	19
B	I	>120
	II	>120
	III	>120
	IV	>120
C	I	16.5
	II	30.5
	III	43
	IV	55
D	I	37
	II	>120
	III	70
	IV	>120

A; sediment obtained between 0.4 to 0.5 saturation of ammonium salfate
B: sediment obtained between 0.5 to 0.6 saturation of ammonium salfate
C: supernatant fraction obtained after the centrifugation of 0.5 saturation solution
D: fraction obtained after the dialysis of fraction A

From these results, it is assumed that this enzyme would not be present in the sediment obtained by the salting out of 0.5–0.6 saturation of ammonium salfate, and, after the dialysis, in the fraction obtained by the salting out of 0.4–0.5 saturation of this salt, this enzyme action could be recognized and the role of DPN was also noticed.

As the preparations separated by the above cited procedures were still contaminated with some amounts of blood and other coloring materials, the estimation of the decoloration time or the spectrophotometric determination of DPNH produced by this enzyme action was greatly disturbed. Then, the removal of these disturbing ones was found to be necessary. For this, the irrigation of cold 0.9% NaCl solution to liver through the hepatic artery before the homogenization of the organ was carried out.

Porcine liver (1.3–1.5 kg) was irrigated with 2 l of cold 0.9% NaCl solution. By this, the colour of this organ turned to brown from red. After this procedure, the enzyme preparations were prepared with this following the same method as before.

The determination of the protein content of each preparations was done by the colorimetric method with the Follin's reagent.

The results with these preparations were as shown in Table 2.

From the results, the role of DPN in this enzyme system was presumed. The reason for the small difference between the decoloration time of I and III of Fraction B and C would be due to the contamination of emulsified fatty matters even after the dialysis of the

enzyme preparations. But, at present, the trial to find out the procedure for the further purification of the enzyme preparation without loss of the activity was not accomplished.

Table. 2. Higher fatty acids dehydrogenase activity of porcine liver (irrigated with cold 0.9% NaCl solution)

(Reaction media, and reaction temperature were the same as cited in Table 1.)

Fractions	Medium No.	Decoloration time (minutes)
A	II	4.5
	IV	50
B	I	22
	II	>120
	III	48
	IV	>120
C	I	15
	II	>120
	III	20
	IV	>120

Fractions:
A: extract
B: sediment obtained between 0.4 to 0.5 saturation of ammonium salfate
C: fraction obtained after the dialysis of Fraction B

Protein content:
Fraction A: 16800 γ/ml
B: 6360 γ/ml
C: 6480 γ/ml

II. Estimation of the enzyme activity following the increase of DPNH

The determination of the decoloration time owing to the formation of leucomethylene blue from methylene blue by the action of dehydrogenase is the one method for the experiment of enzymology, but this method teaches us only the end point of the reaction and not the proceedings of the reaction. For this purpose, the determination of the adsorption at 340 mμ due to the formation of DPNH from DPN by the action of dehydrogenase was adopted.

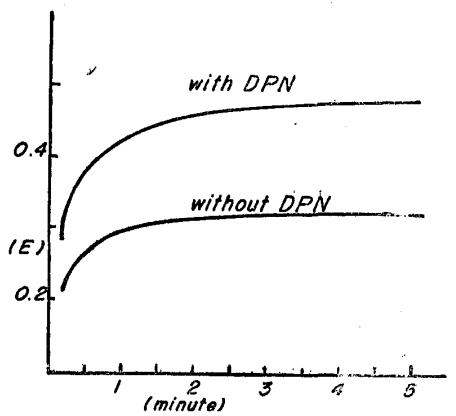

Fig. 2. Role of DPN as coenzyme of higher fatty acids dehydrogenase of porcine liver

Photoelectrospectrophotometer was emyloyed for this determination. In the cuvette of this photometer, enzyme solution (1.0 ml), *M*/1,000 Na-palmitate, (1.0 ml), and buffer solution (pH 8.0) were introduced. Immediately after the addition of

0.6 ml of DPN solution (100 γ/ml), the adsorption at 340 mμ was measured, and as the control of the reaction, the medium which was omitted the substrate and buffer solution was added so as to adjust the final volume to the same as the test medium was used. In this experiment, the turbidity due to the presence of Na-palmitate was found to be the one trouble for the spectrophotometric determination, so, the concentration of this substrate was diminished as compared with that of the Thunberg's tube test. The result were shown in Fig. 2.

Discussion

Higher fatty acids dehydrogenase of animal sourse, as this of plant reported previously, was found to be very feeble one, and to be not able to resist some treatments for purification. After the passage of the enzyme solution through activated almina column or ion exchange resins, the activity was not found in the eluate, and also, whatever the solvent was selected, this was not recovered from the adsorbent without great loss of the activity.

Then, the enzyme preparations cited in the experimental part of this report was obliged to use in this experiment. As the purification of this enzyme was limited at the lower stage, the contamination of some fatty materials and the other dehydrogenase system in the preparations made the interpretation of the results to troublesome one.

Owing to this, even after the dialysis, the difference between the decoloration time of the reaction medium with substrate and that without substrate was still small. But, from the comparison between the time required for the decoloration of the media with DPN and that without DPN, the role of DPN in their enzyme action would be supposed.

At the spectrophotometric determination of DPNH, the turbidity due to the presence of soap in the media was thought to be the fatal phenomenon in these determination, but it was impossible to exclude this soap from the media, then, the concentration of this was diminished to M/1,000 instead of M/100 for the Thunberg's tube test.

From the results cited in Fig. 2, the role of DPN would be again recognized, and from this curve, it was also assumed that this enzyme would loss its activity at the early stage of its action.

Summary

1. Higher fatty acids dehydrogenase of porcine liver was investigated.

2. This enzyme action was significantly recognized in the fraction

which was salted out at 0.4-0.5 saturation about ammonium salfate from the extract obtained from liver with 0.9% cold NaCl solution.

3. This enzyme was found to be so feeble that the purification was obliged to stop at the very primitive stage. Then, the nature of the coenzyme was not definitely determined, but from the experiments, it was assumed that DPN would be employed as the coenzyme in this enzyme.

Literature

(1) Hiroyasu Fukuba and Haruo Komaru, *Nihon Nogei-kagaku Kaishi* (*J. Agr. Chem. Soc. Japan*) **28**, 74 (1954).
(2) Hiroyasu Fukuba, *Ibid.* **31**, 67 (1957).
(3) Hiroyasu Fukuba, *Ibid.* **31**, 69 (1957).
(4) Hiroyasu Fukuba, Nobue Yamazawa, and Tokuko Mitsuzawa, *Ibid.* **31**, 132 (1957).
(5) Hiroyasu Fukuba and Tokuko Mitsuzawa, *Ibid.* **31**, 136 (1957).
(6) Hiroyasu Fukuba and Tokuko Mitsuzawa, *Ibid.* **31**, 151 (1957).
(7) E. Annau, *Enzymologie* IX, 150 (1941).
(8) E. Annau and A. Eperjessy, *Z. Physiol. Chem.* **279**, 66 (1943).
(9) K. Lang, *Z. Physiol. Chem.* **261**, 240 (1939).
(10) K. Lang and H. Meyer, *Z. Physiol. Chem.* **261**, 249 (1939); **262**, 120 (1940).
(11) K. Lang and F. Adickes, *Z. Physiol. Chem.* **263**, 227 (1940).
(12) E. Le Breton and J. Champougny-Clément, *Arch. Soc. Physiol.* **2**, 243 (1948).
(13) J. Champougny-Clément and E. Le Breton, *Compt. Rend. Soc. Biol.*, **141**, 43 (1947).
(14) J. Champougny-Clément and E. Le Breton, *Ibid.* **141**, 450 (1947).
(15) S. Koyanagi and H. Noro, *Nihon Nogei-kagaku Kaishi* (*J. Agr. Chem. Soc. Japan*) **28**, 825 (1954).

(*Received Sept. 1, 1961*)