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The Lipid Metabolism in Castor beans

(I) The activity of thiokinase in germinating castor beans

By

Yoshiichi TAKEUCHI, Takahiro KUSHIGE,
Yōichi KANEI and Yosaburo IWASA

Laboratory of Nutrition Chemistry, College of Agriculture,
University of Osaka Prefecture

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Introduction

Ricinus communis L. seeds (castor bean) contain 45–50% of oil, and a point of striking contrast on fatty acid composition of the oil to the most other vegetable and animal fat and oil is the presence of very large proportion (about 90%) of hydroxy fatty acid (12-hydroxy-octadec-*cis*-9-enoic acid, ricinoleic acid). The accurate determination of the fatty acid composition have been carried by many investigators in spite of the presence of a number of difficulties in determination[1,2,3,4]. The fatty acid composition of germinating and developing seeds have been determined through the change in their growth in our laboratory and by YAMADA in recent years[5].

It was found, consequently, that the seeds which are in early stage of germinating and developing periods will serve to understand the mechanism of metabolism of hydroxy fatty acid in castor beans. Therefore the experiment on the metabolism of fatty acid in seeds was started in enzymic level by useing the enzyme fractions from germinating seeds in early stage.

Our attempt was pointed to fatty acid activating enzyme, thiokinase, at the beginning, because it was assumed that there are some substrate specificity of the enzyme and we need enough information about the enzyme for the experiment will be done in later. In reference to thiokinase,

$$RCO_2^- + \overline{CoA}SH + ATP^{\equiv} \overset{Mg^{++}}{\rightleftharpoons} RCOS\overline{CoA} + AMP^{=} + P-Pi^{\equiv}$$

it was described that thiokinase from beef and others have not remarkable substrate specificity by Kornberg concenred with long chain fatty acid[6] and by WAKIL with short chain one[7]. HAREL reported, on the other hand, there are two forms of thiokinase, that is, active- and inactive- form. The inactive thiokinase can be activated by preincubation with intermediates of T. C. A. cycle, for example, malic acid[8]. With respect to thiokinase of castor bean YAMADA and STUMPF reported in 1964 that active thiokinase is localised on microsomes and that the developing seeds extract have capacity to synthesize ricinoleic acid from acetate-C^{14}, however the germinating seeds extract is devoid of the capacity[9].

From the experiment on the activity of thiokinase from germinating castor beans some results of the change of the activity during their germination, the influence of pH and temperature on the activity and the substrate specificity have been reported in the present paper.

Experimental

Seedlings

Castor been seeds were sterilized by 0.1% solution of Uspulun for 10 minutes and washed with water and germinated under enough moisture and fresh air in the dark at 37°C on a wire gauze. The germinated seeds were divided to 5 grades by their germinating days and their appearences as shown in fig. 1. After 1 to 2 days of germination, 1st stage, crack are obseaved on seed coat. After 3 to 4 days of germination, 3rd stage, border between radicle and embryo can be observed, and shape of embryo become in flat. After 5 to 6 days, 5th stage, cotyledon are separated from embryo. The thiokinase activity was determined on these seeds in 3 groups of different stages.

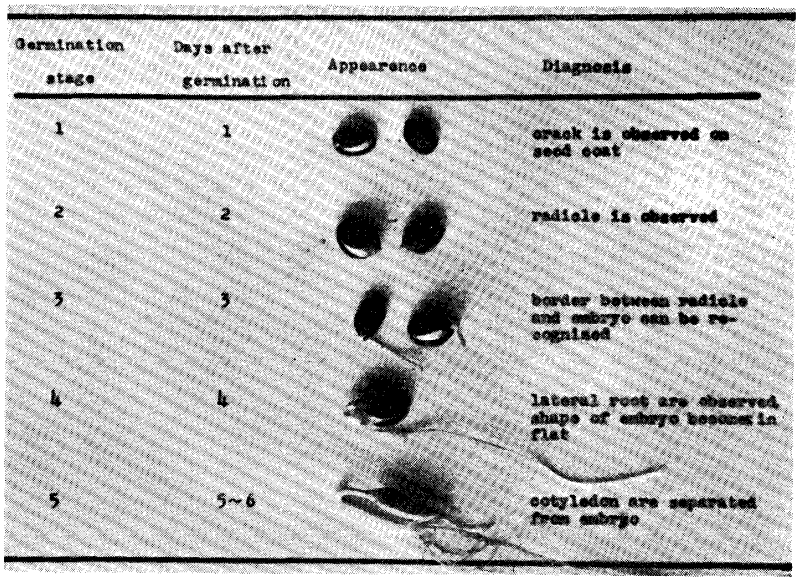

Fig. 1. The germinating stage definition and its appearence durring the germination of castor bean.

Enzyme preparation

Enzyme fractions were prepared according to the method of YAMADA and STUMPF[9]. Germinated seeds were carefully divided into 3 parts based on their germination stages, and their seed coats were removed. As shown in Fig. 2, 50 grams of fresh seeds were washed several times with distilled water and were then homogenized for 7 minutes in a Waring Blender with 3 volumes of a medium consitsing of 10 volumes of 0.3 M-sucrose and 1 volume of M-potassium phosphate at pH 7.1. The homogenate was sqeezed through several layers of cheescloth and the milky filtrate was centrifuged in a Model Marusan Superor Refrigirated Centrifuge at 500 × g for 10 minutes, and the supernatnat was further centrifuged at 12,000 × g for 30 minutes. The pellet was suspended in 7 ml. of sucrose phosphate buffer. The particurate fraction contained plastid and mitochondria. The supernatant was further fractionated into a soluble protein fraction and a microsomal fraction by centrifuging in a Model 55 Hitachi ultracentrifuge at 100,000 × g for 60 minutes. The microsomal fraction was suspended in 5 ml. of sucrose phosphate buffer. The soluble protein fraction was obtained in 100–120 ml. as supernatant.

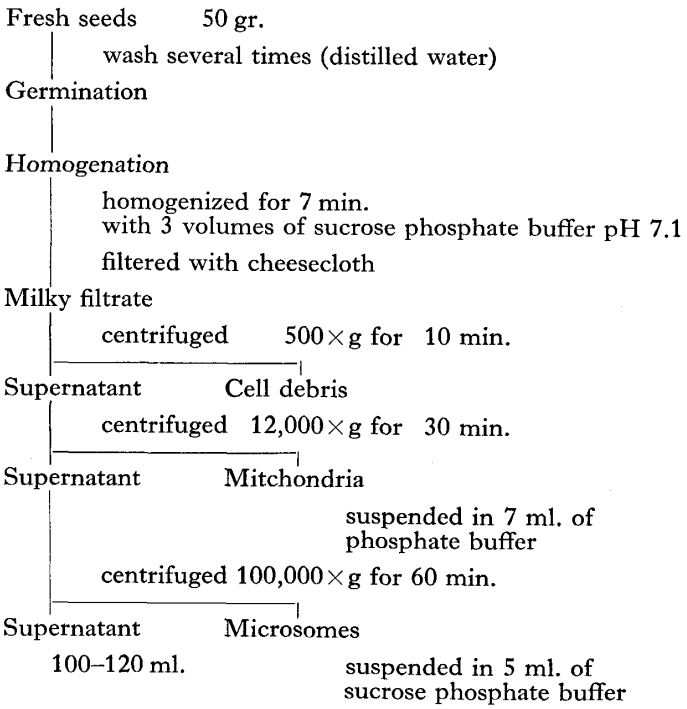

Fig. 2. The procedure for the preparation of thiokinase solutions from germinating castor beans.

Substrates

Ricinoleic acid was separated gaschromatographically, the ester that was methylated with methanolic hydrochloric acid after saponification of castor oil was separated by gaschromatography to yield pure methyl ricinolate (the gaschromatographic condition: Yanagimoto GCG-II, D.E.G.S. C–22 column 2 m. 6 mm. O.D., 215°C, H_2). Then pure ricinoleic acid was obtained after saponification of the ester. Steraric acid was prepared in the same way mentioned above from commercial stearic acid (KATAYAMA CHEMICALS CO. LTD., pure reagent). Unsaturated fatty acid was obtained by the treatment of commerical oleic acid (KATAYAMA's, same grade) with Pb salt alcohol.

Incubation

The incubation mixture contained some components indicated as Table 1. The pH

Table 1. The concentrations of each components in the incubation mixture for the measurment of thiokinase activity

Components	Concentration
CoA	1.8×10^{-1} μM/ml.
ATP	2.5 〃
Cysteine	1.5×10 〃
NaF	2.5×10 〃
$MgCl_2$	7.5 〃
Ricinoleic acid*	9.8×10^{-1}~10.1 〃
NH_2OH	5×10^{2} 〃
Enzyme solution	0.5~0.9 ml.

Total .....4.0 ml. Temperature ...37°C Incubation.....1 hr.

* 0.05 M Tris buffer, pH 7.4, ammonium salt. Stearic acid 9.8 μM/ml. and unsaturated acid 10.2 μM/ml. were adopted as substrate in place of ricinoleic acid

of the mixture was at 8.0.

Measurement of thiokinase activity

Thiokinase activity was measured by the modified hydromaxate method (KORNBERG and PRICER)[6]. The hydroxamic acid in the incubation mixture was extracted by 5 ml. of ether for 3 times and washed with water. After distilation of the ether 10 ml. of the ferric chlorid (HILL's reagent A)[10] was added to the residual solution. The concentration of acyl-SCoA was determined by means of spectrophotometer at 520 mμ. Specific activity and total activity were calculated from following equations:

$$\text{Specific activity} = \frac{\text{acyl-SCoA } (\mu\text{M})}{\text{reaction mixture (ml.) protein (mg.) reaction time (hr.)}}$$

$$\text{Total activity} = [\text{specific activity}]\ [\text{total protein (mg.) of each enzyme fraction}]$$

The concentration of protein of the enzyme solution was determined by BIURET's method[11] after removement of sucrose from the enzyme solution by dialysis. Lipase activity was determied according to Ory *et al.*[12]

Results and Discussion

Intracellular localization of thiokinase of germinating castor bean

Distribution of the active thiokinase on the fractions prepared by centrifuging of germinating castor bean was shown in Table 2. With the enzyme preparations from germinating castor bean in 3rd stage, the microsomal fraction indicated the most remarcable thiokinase activity. This fact accorded with a result which YAMADA and STUMPF obtained on developing castor bean. The activity of the mitochondrial fraction was more intense than that of the supernatant fraction.

Table 2. Intracellular localization of enzymes of germinating castor bean

	Volume of fraction ml.	Protain* content mg/ml.	Specific activity μM/mg. hr. ml.	Total activity S. A.** mg. ml.
Mitochondaria	7	12.4	0.526	45.66
Microsomes	5	2.4	1.96	23.48
Supernatant	120	5.2	0.187	116.68

Enzyme .....Prepared from germinating castor bean in 3rd stage.
Substrate .....Ricinoleic acid 9.8×10^{-1} μM/ml.
Incubation.....37°C 1 hr.

* Protein contents indicated in mg. of protein per ml. of enzyme solution of each fractions.
** S.A....Specific activity.

The change of thiokinase activity during the germination

The change of thiokinase activity during the germination stage of castor bean was shown in Table 3. It was observed that thiokinase from every germinating stage of castor beans activated fatty acids. In comparison with each fractions, the activity of the microsomal fraction was in the most conspicuous and a considerable difference was not observed between the mitochondrial and the supernatant fractions. The greatest activity was observed in the microsomal fraction in early stage of germination. The concentration of protein (mg./ml.) was minor in the microsomal fraction.

Table 3. The change of thiokinase activity during the germination stage of castor bean

Fraction	Germination stage	Acyl-SCoA produced μM./ml./hr.	Concentration of protein* mg./ml.	Specific activity μM./ml.mg. prot. hr.
Mitochondria	1	0.296	0.70	0.423
	3	0.815	1.55	0.526
	5	0.980	1.47	0.667
Microsomes	1	0.490	0.28	1.751
	3	0.587	0.30	1.957
	5	0.534	0.52	1.027
Supernatant	1	0.082	1.12	0.076
	3	0.218	1.17	0.187
	5	0.097	0.43	0.225

Substrate.....Ricinoleic acid
Measurement condition and concentration of components were described in Table 1.
* Concentration of protein indicated that of the incubation mixture

Optimum of pH

The influence of pH of incubation solution on the thiokinase activity was shown in Fig. 3. Optimum pH was approximately from 7.5 to 8.0. The activity was decreased gradually in acidic side and sharply in basic side against the change of pH. MAHLER and WAKIL[7] reported concerning short chain fatty acid that the rate of activity of thiokinase which was obtained from beef liver was directly proportional to pH. They explained this fact, as the rate equations for the activation, probably contains a term involving the concentration of OH^-,

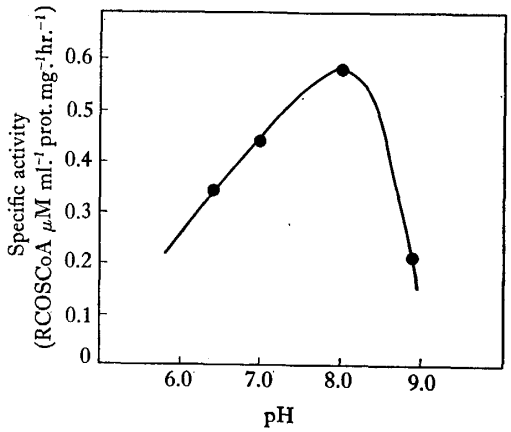

Fig. 3. The influence of pH of incubation solution on the thiokinase activity from germinating castor beans.
enzyme·····mitochondrial fraction prepared from germinating castor bean in 3rd stage.
substrate concentration ··· ricinoleic acid 9.8×10^{-1} μM/ml.
measurement condition ··· It is descrived in Table I 37°C 1hr.

And in their report, if reaction mixture is used in the pH range between 7.0 and 10.0, the rate is decreased rapidly in acidic side compared with that in basic side. The fact could not be observed in our case with castor bean thiokinase.

Optimum temperature

Effect of temperature on thiokinase activity was shown in Fig. 4. The optimum temperature range was between 30° and 37°C. The activity was depressed sharply above 40°C.

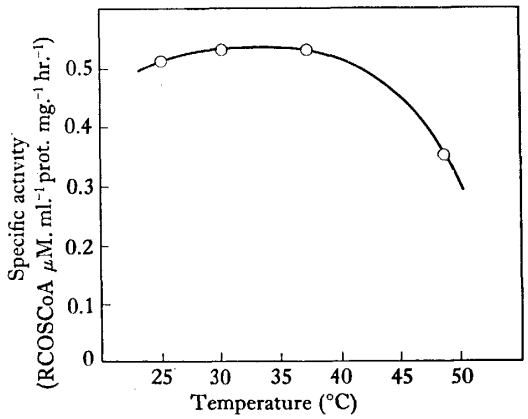

Fig. 4. The influence of the incubation temperature on the thiokinase activity from germinating castor beans.
enzyme……mitochondrial fraction prepared from germinating castor beans in 3rd stage.
substrate concentration…… ricinoleic acid 9.8×10^{-1} μM/ml.
measurment condition……It is described in Table 1.

Substrate specificity

The main component of fatty acid of castor oil is ricinoleic acid[4] which has a double bond at the 9th carbon and a hydroxyl group at the 12th carbon atom from the carboxyl

Table 4. Substrate specificity of thiokinase from germinating castor bean

Substrate	Fraction	Acyl-SCoA produced μM/ml. hr.	Concentration Of protein mg. prot./ml.	Specific acitvity μM./ml. mg. prot. hr.
Ricinoleic acid	Mitchondria	0.476	3.87	0.123
	Microsomes	0.315	0.51	0.618
	Supernatant	0.121	0.87	0.139
Stearic acid	Mitochondria	1.407	3.87	0.364
	Microsomes	1.802	0.51	3.711
	Supernatant	0.519	0.87	0.597
Unsaturated acid	Mitchondria	0.825	3.87	0.213
	Micromsomes	0.374	0.51	0.733
	Supernatant	0.126	0.87	0.145

Substrate concentrations;
Ricinoleic acid.....10.1 μM/ml.
Stearic acid ..... 9.8 μM/ml.
Unsaturated acid.....10.2 μM/ml.
Mesurment codition;
It was descrived in Table 1.

group. We considered whether the thiokinase from castor bean will activate specifically on hydroxy-, unsaturated-, and the other fatty acid. In the point of view, the thiokinase activity on octadecenoic and octadecanoic acids were examined to compare with the activity on ricinoleic acid. The result was shown in Table 4.

BARRON and STUMPF[13] have shown that the fatty acid thiokinase in avocado-mesocarp tissue is localized in microsomes. YAMADA and STUMPF[9] have reported that the thiokinase in castor bean tissue is localized in microsomes and the enzyme concerned elongation of carbon chain localized in mitochondria and fatty acid hydroxylase is in supernatant fraction.

As we summerized in Table 4, in our experiments, every enzyme fractions had thiokinase activity to the 3 kinds of substrates, however significant substrate specificity of the enzyme could not be observed to ricinoleic acid. It showed a rare specificity to stearic acid. The microsomal fraction had a greater activity than other two fractions with the substrates.

Thiokinase activity during storage

The reduction in the activity of thiokinase from castor beans during the storage was shown in Fig. 5. The enzyme was prepared from the beans in 3rd stage germination. It is probably considered that the enzyme of supernatant fraction is the most unstable.

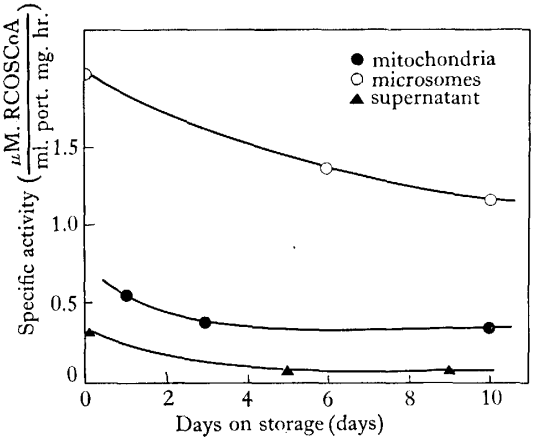

Fig. 5. The reduction in the activity of thiokinase from germinating castor beans during the storage.

enzyme ····· prepared from germinating castor beans in 3rd stage.
substrate ····· ricinoleic acid 9.8×10^{-1} μM/ml.
measurment condition····· It is described in Table 1.

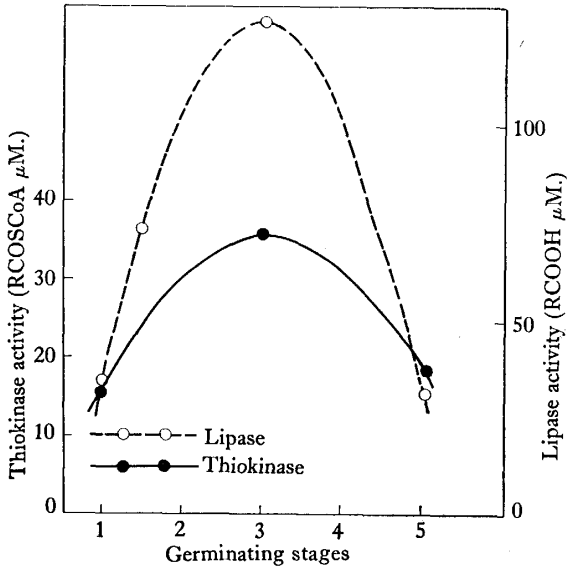

Fig. 6. The inter-relation between the activities of thiokinase and of lipase from germinating castor beans.

Thiokinase activity·····Total activity was calculated from sum of products of specific activity and volume of fractions of mitochondria, microsome and supernatant which were prepared from 10 gr. of germinating castor beans.

Measurment condition is same as in Table I.

Inter-relation between the activity of thiokinase and lipase

The inter-relation between the activity of thiokinase and that of lipase was shown in Fig. 6. As a whole, thiokinase activity runs paralleled with lipase activity during the germination.

Summary

1. The enzyme fractions which were prepared from various stages of germinating castor beans have thiokinase activity.
2. Their specific activity, in general, are apt to be increased with their germination until 3rd stage.
3. The most remarkable activity is observed in microsomal frction.
4. A significant substrate specificity of thiokinase can not been observed with hydroxy-, saturated- and unsaturated fatty acids.
5. For the thiokinase, optimum temperature is at 30–37°C, and optimum pH is approximately at 8.0.

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