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Letter

Production of L-carnitine from D-carnitine by dried cells of *Agrobacterium* sp. 525a [1]

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Abstract

D-Carnitine was converted into L-carnitine with dried cells of *Agrobacterium* sp. 525a grown on D-carnitine medium. During the conversion reaction, NAD^+ concentration decreased. *o*-Phenanthroline inhibited the NAD^+ degrading activity in dried cells, and the formation of L-carnitine increased 2.8 times. About 190 mM of L-carnitine was converted from 0.5 M of D-carnitine under the best conditions.

Keywords: Bioconversion; L-Carnitine; D-Carnitine; *Agrobacterium*; Carnitine dehydrogenase

1. Introduction

Agrobacterium sp. utilizes D-carnitine as sole source of carbon and nitrogen. D-Carnitine dehydrogenase was induced during growth on D-carnitine, and was purified and characterized [1]. Hanschmann and Kleber reported the conversion of D-carnitine into L-carnitine with stereospecific carnitine dehydrogenases [2]. In the previous paper, we also reported the conversion of D-carnitine into L-carnitine by partially purified D- and L-carnitine dehydrogenase, and described the fundamental reaction conditions of conversion [3].

In this Letter, we describe the conversion reaction by dried cells of *Agrobacterium* sp.

2. Experimental

2.1. Materials

L-Carnitine hydrochloride was purchased from Sigma, St. Louis, USA. D-Carnitine hydrochloride used for the cultivation and conversion reaction was a gift from Ajinomoto, Tokyo, Japan. L-Carnitine dehydrogenase from *Alcaligenes* sp. was a gift from Asahi Chemical Industry, Tokyo, Japan. All other reagents were commercial products of analytical grade.

2.2. Microorganisms and culture conditions

Agrobacterium sp. 525a was used. The culture medium at pH 7.0 contained 0.5% D-carni-

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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tine hydrochloride, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% yeast extract. Culture was done at 30°C for 48 h with reciprocal shaking.

2.3. Preparation of dried cells

Agrobacterium sp. 525a was collected by centrifugation at $10{,}000 \times g$ for 20 min at 5°C, and washed with 0.85% KCl. The washed cells paste was spread onto petri dish and dried with an electric fan at room temperature for overnight. The cells were further dried in vacuo under P_2O_5 and stored at −20°C before use.

2.4. Conversion reaction by dried cells

Standard reaction mixture (6.0 ml) contained 100 mM Tris–HCl buffer (pH 8.5), 0.1 M D-carnitine which was adjusted to pH 7 by the addition of NaOH solution previously, 5 mM NAD^+, 1 mM glutathione, and 100 mg of the dried cells at 30°C under shaking. An aliquot of the reaction mixture (0.5 ml) was added to 0.5 ml of 1.0 M HCl at different time intervals.

2.5. Enzymatic determination of L-carnitine and NAD^+

The concentration of L-carnitine was enzymatically determined with L-carnitine dehydrogenase [3]. The concentration of NAD^+ in reaction mixture of L-carnitine conversion was determined with alcohol dehydrogenase [4].

3. Result and discussion

As described in our previous paper [3], optimum pH of the conversion reaction by partially purified enzymes was 8.5. In this experiment, the effect of pH on the conversion reaction by dried cells was examined using various buffers. The highest accumulation of L-carnitine was obtained by using glycine–NaOH buffer, pH 9.0. Final pH of the reaction mixture with glycine–NaOH buffer, pH 9.0, decreased to 8.5. So we selected Tris–HCl buffer, pH 8.5, as the buffer in reaction mixture. The reaction temperature was examined at 10, 20, 30 and 37°C. The highest accumulation of L-carnitine was obtained at 30°C. Accumulation of L-carnitine at 37°C decreased to 25% in comparison with the accumulation at 30°C. It is suggested that the carnitine dehydrogenases are not so stable for temperature. Various NAD^+ concentrations were tested to obtain the highest formation of L-carnitine. Effective concentration of NAD^+ on L-carnitine formation were found at 5–20 mM NAD^+. The conversion of L-carnitine from various concentration of D-carnitine was examined. The maximal amount of L-carnitine accumulation was about 70 mM from 0.5 M of D-carnitine, and the maximal yield was about 50% on a molar basis at 0.1 M of D-carnitine. By using the partial purified carnitine dehydrogenases, the maximal amount of L-carnitine accumulation was 108 mM from 0.5 M of D-carnitine, and the maximal yield was about 64% on a molar basis at 0.1 M of D-carnitine [3]. Hanschmann and Kleber reported that by using highly purified D- and L-carnitine dehydroge-

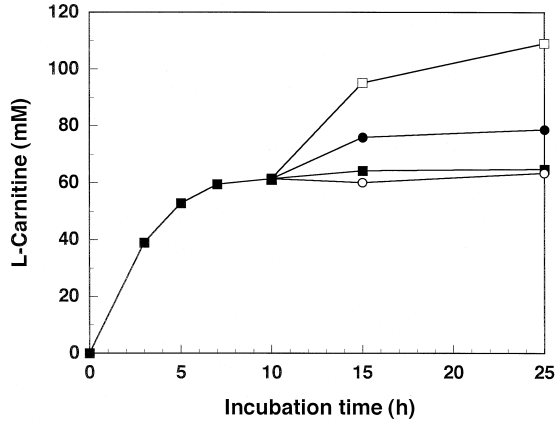

Fig. 1. Effect of re-addition of NAD^+ and dried cells on the formation of L-carnitine. The reaction was carried out at 30°C as described in the text except that the concentration of D-carnitine was 0.3 M. NAD^+, dried cells, and both were added to the reaction mixture at 10 h. (■) No addition of NAD^+ and dried cells, (○) addition of dried cells, (●) addition of NAD^+, (□) addition of both.

Table 1
Disappearance of NAD^+ during the conversion reaction. The reaction was carried out as described in the text except that the concentration of D-carnitine was 0.3 M

Incubation time (h)	Residual NAD^+ (mM)	L-Carnitine formation (mM)
0	5.0	0
3.0	0.27	37.9
5.0	0.02	43.4
10.0	0	51.9
25.0	0	51.4

nase from *Agrobacterium* sp. almost 50% of 50 mM of D-carnitine could be converted into L-carnitine [2].

In all experiments described above, the accumulation of L-carnitine stopped at about 10-h incubation. In order to clear the cause of the stoppage of L-carnitine accumulation, the experiment in which dried cells and NAD^+ were added again at 10-h incubation was done (Fig. 1). The addition of dried cells had no effect for the increase of L-carnitine accumulation. The accumulation of L-carnitine increased about 1.2-fold by the addition of NAD^+. The addition of both, dried cells and NAD^+, was effective for the increase of L-carnitine, and 100 mM of L-carnitine was accumulated at 25-h incubation.

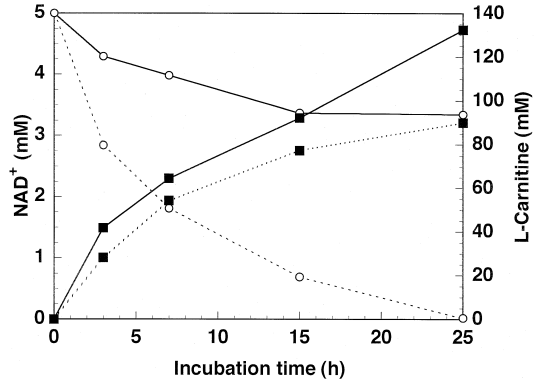

Fig. 2. Formation of L-carnitine from D-carnitine by partially purified D- and L-carnitine dehydrogenases under the addition of *o*-phenanthroline. The reaction was carried out at 30°C as described in Ref. [3] except that the concentration of D-carnitine was 0.3 M. Solid line: addition of 1 mM *o*-phenanthroline, dashed line: no addition of *o*-phenanthroline. (□) NAD^+, (■) L-carnitine.

These results indicate that the carnitine dehydrogenase activities are partially lost, and that the NAD^+ concentration level is decreased. So, we determined the concentration of NAD^+ in reaction mixture during the conversion reaction (Table 1). As expected, NAD^+ concentration was rapidly decreased, and no NAD^+ was at 10-h incubation. Simultaneously, the accumulation of L-carnitine stopped at 10-h incubation.

Table 2
Effect of various metal ion and chemicals on the decrease of NAD^+ and the formation of L-carnitine

Metal salt or chemical (1.0 mM)	Residual NAD^+ (mM)	L-Carnitine formation (mM)
None	0.0	68.2
$MgCl_2$	0.0	36.1
$CaCl_2$	0.0	33.7
$CoCl_2$	0.0	22.0
$ZnSO_4$	0.0	8.9
o-Phenanthroline	4.51	130.3
KCN	0.0	64.2
N,*N*-Diethyldithiocarbamate	0.66	62.5
EDTA	0.36	58.2
Semicarbazide	0.01	50.9
NaN_3	0.0	40.1
Hydroxylamine	0.0	33.1
α,α′-Dipyridyl	0.16	26.0
Iodoacetamide	0.0	21.6

Metal salt or chemical was added to the conversion reaction mixture at 1 mM.
The reaction was carried out for 15 h as described in the text except that the concentration of D-carnitine was 0.3 M.

Table 3
Effect of *o*-phenanthroline concentration on the decrease of NAD^+ and the accumulation of L-carnitine

o-Phenanthroline (mM)	Residual NAD^+ (mM)	L-Carnitine formation (mM)
0	0	35.9
0.5	1.1	99.2
1.0	2.8	109.9
2.0	3.8	112.6
3.0	3.9	121.8
5.0	3.9	120.7
10	3.6	32.3

The reaction was carried out for 15 h as described in the text except that the concentration of D-carnitine was 0.3 M.

The same phenomena was observed in the conversion reaction with partially purified carnitine dehydrogenases [1]. The NAD^+ degrading activity might be contained in the dried cells of *Agrobacterium* sp. 525a. In order to take out the NAD^+ degrading activity, we examined the heat treatment for dried cells. However, the NAD^+ degrading activity was more stable for heat treatment than carnitine dehydrogenase activity. Then, we examined the several metal compounds and some chemicals to inhibit the NAD^+ degrading activity during the conversion reaction (Table 2). There was no effect on the inhibition of NAD^+ degrading activity by the addition of metal compound, however, only *o*-phenanthroline protected the disappearance of NAD^+ during the conversion reaction. And the accumulation of L-carnitine increased at about 2 times. The effect of *o*-phenanthroline on the conversion reaction with the partially purified D- and L-carnitine dehydrogenases was also examined (Fig. 2). The addition of *o*-phenanthroline to the conversion reaction was effective for the disappearance of NAD^+, and the accumulation of L-carnitine increased about 1.5 times. It is concluded that the addition of *o*-phenanthroline was more effective in the dried cells-reaction than in the partially purified enzyme-reaction. The effect of the concentration of *o*-phenanthroline on the accumulation of L-carnitine in dried cells reaction was examined (Table 3). The effective concentration of *o*-phenanthroline on L-carnitine formation was found at 1.0 to 5.0 mM *o*-phenanthroline. The high concentration of *o*-phenanthroline also protected the disappearance of NAD^+ but inhibited the accumulation of L-carnitine. We guess that the degradation reaction of NAD^+ is catalyzed by NAD^+ pyrophosphatase or NAD^+ nucleosidase, and that *o*-phenanthroline inhibits these enzymes. However, *o*-phenanthroline was not listed as an inhibitor of these enzymes [5]. Further studies on the mode of action by *o*-phenanthroline is not yet done.

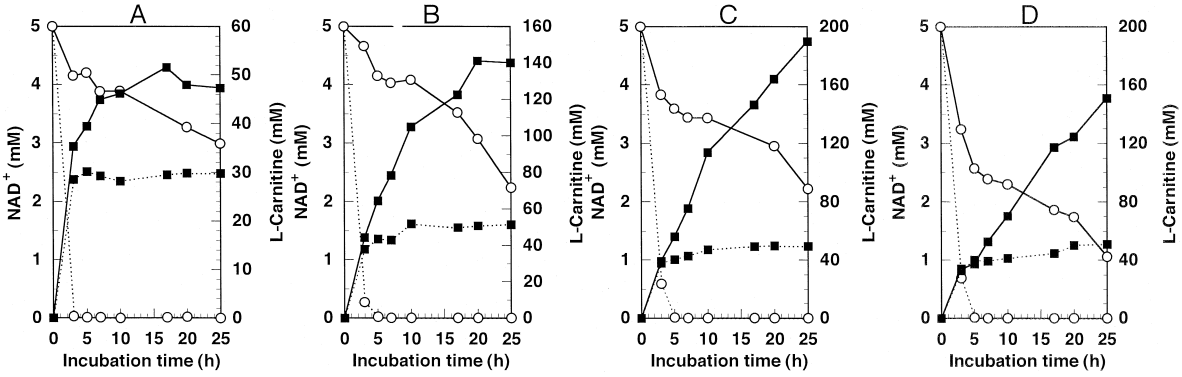

Fig. 3. Formation of L-carnitine from D-carnitine by dried cells-reaction at different concentration of D-carnitine under the addition of *o*-phenanthroline. The reaction was carried out at 30°C as described in the text except that the concentration of D-carnitine was varied as indicated. (A) 0.1 M of D-carnitine, (B) 0.3 M of D-carnitine, (C) 0.5 M of D-carnitine, (D) 1.0 M of D-carnitine. Solid line: addition of 1 mM *o*-phenanthroline, dashed line: no addition of *o*-phenanthroline. (○) NAD^+, (■) L-carnitine.

To confirm the effect of *o*-phenanthroline, the time course of L-carnitine accumulation and NAD^+ disappearance were examined at 0.1, 0.3, 0.5, and 1.0 M of initial D-carnitine concentration (Fig. 3). The accumulation of L-carnitine at 0.3 M of initial D-carnitine concentration was 2.8 times than that with no addition of *o*-phenanthroline, and the conversion rate was about 45%. There was a little effect of *o*-phenanthroline in the conversion reaction of 0.1 M of initial D-carnitine concentration due to the coming to equilibrium at 50 mM of L-carnitine. The accumulation of L-carnitine at 0.5 M of initial D-carnitine concentration was 3.8 times than that with no addition of *o*-phenanthroline, and the conversion rate was about 38%. In the case of 1.0 M of initial D-carnitine, the accumulation of L-carnitine decreased compared to that with 0.5 M of initial D-carnitine concentration, and the concentration of residual NAD^+ was decreased. High concentration of D-carnitine hydrochloride required a large amounts of NaOH to adjust a pH to 7. So, high concentration of Na^+ might inhibit D- and L-carnitine dehydrogenase activities.

In the previous paper, we reported that D-carnitine was converted into L-carnitine by partially purified D- and L-carnitine dehydrogenases from *Agrobacterium* sp. 525a, and that about 60% of initial D-carnitine was converted into L-carnitine and the accumulation of L-carnitine reached at 108 mM. In this Letter, we showed that the dried cells of *Agrobacterium* sp. 525a grown on D-carnitine medium could convert D-carnitine into L-carnitine, and that the addition of *o*-phenanthroline increased the accumulation of L-carnitine. Furthermore, 190 mM of L-carnitine accumulated into the reaction mixture at 0.5 M of D-carnitine. It is very easy to prepare the dried cells of the micrograms than to prepare the partially purified D- and L-carnitine dehydrogenases. So, it is concluded that the dried cells-reaction is better than the enzyme-reaction in the conversion of D-carnitine into L-carnitine.

Acknowledgements

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