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Evidence for the presence of a cytosine deaminase that does not catalyze the deimination of creatine

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The presence of a cytosine deaminase, which shows no activity on creatinine, was first noted in *Alcaligenes denitrificans* ssp. *denitrificans* J9 and *Arthrobacter* sp. J11. DEAE-Sephacel column chromatography of cell-free extracts of these microorganisms gave a single peak for cytosine deaminase activity, and no fraction contained creatinine deiminase activity. The DEAE-Sephacel active fractions from *A. denitrificans* ssp. *denitrificans* J9 were resolved into two fractions showing cytosine deaminase activity upon Sephacryl S-200 column chromatography; the larger enzyme was termed cytosine deaminase I and the smaller one cytosine deaminase II. Both cytosine deaminases did not deaminate creatinine but did deaminate 5-fluorocytosine rapidly. The molecular masses of cytosine deaminases I and II were estimated to be 200 and 37 kDa, respectively.

Cytosine deaminase; Creatinine deiminase; Cytosine; Creatinine; 5-Fluorocytosine; Pyrimidine degradation

1. INTRODUCTION

We reported that creatinine is converted to *N*-methylhydantoin in various microorganisms [1,2]. The wide distribution of creatinine-deiminating microorganisms [2,3] is likely due to the wide occurrence of cytosine deaminase (EC 3.5.4.1) (unpublished), which might play an important role in the salvage synthesis of pyrimidines [4–6]. Accordingly, it seemed unnatural that *N*-methylhydantoin was not uniformly detected as a degradation product from creatinine in the cultivation broth of all the microorganisms tested previously [2]. The possible important role of pyrimidine salvage in cell physiology leads to the conceivable idea that two cytosine deaminases exist that differ in their substrate specificity; one degrades creatinine but the other does not.

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Recently we obtained three soil isolates that degrade creatinine to sarcosine via only creatine; they formed neither *N*-methylhydantoin nor creatinine deiminase on cultivation with creatinine [7]. This paper deals with the partial purification and substrate specificity of the two cytosine deaminases, that showed no activity toward creatinine at all, from one of the microorganisms, *Alcaligenes denitrificans* ssp. *denitrificans* J9. *Arthrobacter* sp. J11 has also been reported to possess a cytosine deaminase free from creatinine deiminase (EC 3.5.4.21) activity.

2. MATERIALS AND METHODS

A. denitrificans ssp. *denitrificans* J9 and *Arthrobacter* sp. J11 were used: cultivation and enzyme purification were carried out essentially according to the same procedures as those used for the purification of sarcosine oxidase from *A. denitrificans* ssp. *denitrificans* J9 [7]. Creatinine deiminase was assayed as in [2], and cytosine deaminase was similarly assayed with cytosine as

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substrate instead of creatinine. 1 unit of enzyme was defined as the amount catalyzing the formation of 1 μmol product per min under the assay conditions. The molecular mass of cytosine deaminase was determined by high-performance gel permeation chromatography (Hitachi 655-30, Tokyo) on a TSK-G 3000 SW column (0.75 × 60 mc; Toyo Soda, Tokyo) and Sephacryl S-200 gel chromatography using the standard proteins used previously [7,8]. Protein was determined by the method of Lowry et al. [9] with bovine serum albumin as a standard.

3. RESULTS

Table 1 lists the effects of the medium composition on the formation of cytosine deaminase and creatinine deiminase in *A. denitrificans* ssp. *denitrificans* J9 and *Arthrobacter* sp. J11. Cytosine deaminase was considered to be formed in a rather constitutive way in *A. denitrificans* ssp. *denitrificans* J9: cytosine deaminase was similarly formed under all the cultivation conditions tested, with or without cytosine or creatinine. On the other hand, no creatinine deiminase was detected with these isolates under any of the conditions tested.

To determine whether the cytosine deaminases of these isolates show creatinine deiminase activity, cell-free extracts of *A. denitrificans* ssp. *denitrificans* J9 and *Arthrobacter* sp. J11 were subjected to DEAE-Sephacel chromatography after extensive dialysis. As expected, no fraction showed any creatinine deiminase activity. On the other hand, cytosine deaminase activity was eluted as a single peak in both cases (not shown). These results might suggest that these isolates do not convert creatinine to *N*-methylhydantoin at all, due to a lack of a cytosine deaminase such as that which acts on creatinine actively to form *N*-methylhydantoin in various cytosine deaminase-forming microorganisms (unpublished).

Regardless of their microbial origins, cytosine deaminases show many similarities as far as has been investigated with various cytosine deaminase-forming microorganisms (unpublished). The cytosine deaminases of the present two isolates were eluted with a concentration of KCl in the elution buffer similar to that for the elution of the cytosine deaminases of other microorganisms (not shown). For further comparison of these cytosine deaminases which do not degrade creatinine with those which degrade creatinine efficiently, DEAE-Sephacel fractions containing the cytosine deaminase from *A. denitrificans* ssp. *denitrificans* J9 were pooled and concentrated, and then subjected to Sephacryl S-200 column chromatography. Creatinine deiminase activity was not detected uniformly in any fractions. However, the cytosine deaminases were completely separated in-

Table 1

Effect of the medium composition on formation of cytosine deaminase and creatinine deiminase in *A. denitrificans* ssp. *denitrificans* J9 and *Arthrobacter* sp. J11

Microorganism	Cytosine deaminase or creatinine deiminase (mU/mg)			
Medium[a]:	Creatinine	Cytosine	Polypeptone	NH_4Cl
A. denitrificans spp. *denitrificans* J9	3.5 (0)[b]	4.4 (0)	5.5 (0)	NT[c] (0)
Arthrobacter sp. J11	15.0 (0)	2.8 (0)	NT (0)	NT (0)

[a] The media contained one of the compounds indicated as the main nitrogen source. The compositions of the polypeptone medium [10] and creatinine and NH_4Cl media [2] were given previously. An equal amount of cytosine was substituted for creatinine in the cytosine medium

[b] Values in parentheses represent creatinine deiminase activity

[c] Not tested

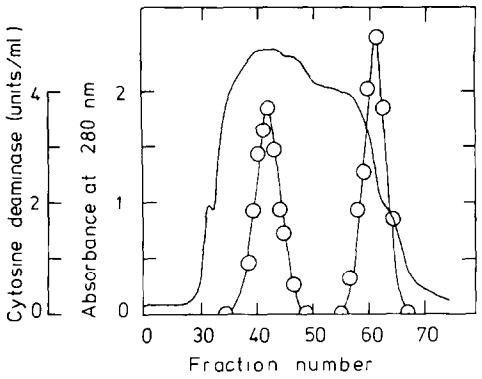

Fig.1. Sephacryl S-200 column chromatography of the cytosine deaminase of *A. denitrificans* ssp. *denitrificans* J9. DEAE-Sephacel active fractions containing cytosine deaminase were combined and concentrated to ~3 ml with an Amicon membrane filter apparatus (Amicon, Danvers) equipped with a YM 10 membrane, and then applied to a Sephacryl S-200 column (1.6 × 80 cm) previously equilibrated with 0.02 M Tris-HCl (pH 7.5) buffer containing 0.1 mM dithiothreitol and 0.25 M NaCl, and eluted with the same buffer. Fractions (3.0 ml) were collected and assayed for cytosine deaminase and creatinine deiminase activities. No fractions contained creatinine deiminase activity. (——) Absorbance at 280 nm. (○) Cytosine deaminase activity.

to two peaks (fig.1). The molecular masses of these two cytosine deaminases were estsimated to be 200 and 35 kDa through comparison of their elution volumes with those of standard proteins. The occurrence of a small cytosine deaminase of bacterial origin was first confirmed in *A. denitrificans* ssp. *denitrificans* J9. Baker's yeast cytosine deaminase might be the only small cytosine deaminase with a molecular mass of 34 kDa [5]. The small cytosine deaminase of *A. denitrificans* ssp. *denitrificans* J9 was further confirmed to have a molecular mass of 37 kDa on gel-permeation chromatography of DEAE-Sephacel fractions containing the cytosine deaminase on a TSK-G 3000SW column (Toyo Soda) previously calibrated with standard proteins. Similarly, the *Arthrobacter* sp. J11 cytosine deaminase was roughly estimated to have a molecular mass of 37 kDa on a TSK-G 3000 SW column of a cell-free extract (the retention time of the cytosine deaminase was determined from the activity profile obtained on assaying every 30 s fraction in both runs).

For further purification of the two cytosine deaminases of *A. denitrificans* ssp. *denitrificans* J9, Sephacryl S-200 active fractions containing the large enzyme (cytosine deaminase I) and the small one (cytosine deaminase II) were separately collected and concentrated, and then subjected to phenyl-Sepharose column chromatography. Both cytosine deaminases were found to be rather hydrophobic; both were eluted with nearly the same composition of the elution buffer (not shown). The highest specific activities for cytosine deaminases I and II obtained at this step were 14 and 4 U/mg protein, respectively, which are comparable to those for bacterial cytosine deaminases ([12,13]; and unpublished) and yeast cytosine deaminase [5], respectively. However, both preparations still appeared to be significantly heterogeneous on SDS-polyacrylamide gel electrophoresis.

After phenyl-Sepharose column chromatography cytosine deaminase I was subjected to partial characterization without further purification. Its K_m value for cytosine was 1.0 mM. The enzyme had an optimum pH of 8–9 and showed relatively high activity even at pH 12. The enzyme was highly specific for cytosine; 5-fluorocytosine and 5-methylcytosine were the only compounds deaminated by the enzyme, at respective rates of 1.2 and 0.1 of that for cytosine, under the conditions tested. Ammonia was formed from none of the following compounds used as substrates: creatinine, guanine, guanosine, 1-methylcytosine, 3-methylcytosine, 4-aminopyrazolo-(3,4-d)pyrimidine-2′-deoxyribose, 3′-cytidine monophosphate, cytidine, 5-aminopyrazolo-(3,4-d)pyrimidine-2′-deoxyribose and adenosine monophosphate.

Cytosine deaminase II showed a substrate specificity similar to that of cytosine deaminase I; cytosine deaminase II also showed no activity toward creatinine.

4. DISCUSSION

Deimination of creatinine has been widely observed in various microorganisms including many cytosine deaminase-forming ones ([2,3]; and unpublished): on the basis of this, cytosine deaminase was generally assumed to be the only enzyme which catalyzes the deimination of creatinine. Cytosine deaminase is required for

pyrimidine salvage and the presence of the enzyme has been reported in various microorganisms [5–7,10]. Here, we first reported cytosine deaminases which showed no activity of creatine deimination. Throughout the purification, creatinine deiminase activity was consistently not detected. This might explain why many microorganisms do not show any detectable formation of *N*-methylhydantoin from creatinine regardless of the wide distribution of cytosine deaminases in microorganisms [2]. *A. denitrificans* ssp. *denitrificans* J9 contains two cytosine deaminases, cytosine deaminases I and II, which differ from each other in their molecular masses. Except for the substrate specificity, the general properties of cytosine deaminase I are remarkably similar to those of known cytosine deaminases and creatinine deiminases ([3,11–13]; and unpublished).

Esders and Lynn [11] suggested the same imino nitrogen functionality in cytosine and creatinine molecules for *Flavobacterium filamentosum* creatinine deiminase, based on the possible tautomeric forms of cytosine. On the other hand, extensive inhibition studies on the deaminases for cytosine, cytidine and adenosine revealed that amino groups were uniformly involved in their deamination [14–16]. With regard to this, two different cytosine deaminases, which either do or do not degrade creatinine, should be carefully investigated as to their requirement of amino or imino groups for the release of ammonia from cytosine or creatinine. The fact that creatinine deiminating activity was always co-detected with high cytosine deaminase activity, as far as has been tested (unpublished), might support the involvement of amino groups but not imino groups in the hydrolysis of either creatinine or cytosine by creatinine-deiminating enzymes.

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