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Immobilization of thermotolerant *N*-carbamyl-D-amino acid amidohydrolase [1]

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Abstract

N-Carbamyl-D-amino acid amidohydrolase (DCase), in which amino acid residues were substituted by mutation, followed by the selection based on thermotolerance, showed improved thermostability, by 5° or 10°C, compared to the native DCase. These DCases were immobilized on a macroporous phenol formaldehyde resin, Duolite A-568, and the immobilized thermotolerant enzymes showed higher activity than the immobilized native DCase. From the results of repeated batch reactions, the half-lives of the activities of immobilized thermotolerant DCase, in which Leu was substituted for Pro 203, and immobilized native DCase were 104 and 58 times, respectively. It was revealed that the higher thermotolerance enabled the immobilized enzymes to be more stable in reactions. A reductant, dithiothreitol, also stabilized the enzymes in reactions. Compared with soluble DCase, immobilized DCase was somewhat stable, and its activity was optimum at a lower pH.

Keywords: Immobilization; *N*-Carbamyl-D-amino acid amidohydrolase; Thermotolerant

1. Introduction

Immobilized enzymes are beneficial because they facilitate operation and product isolation. Therefore, such enzymes have been widely used for industrial purposes, such as in the food and pharmaceutical industries [1–3].

In order to improve the production of D-amino acids, which are available intermediates for the preparation of physiologically active peptides and β-lactam antibiotics such as semisynthetic penicillins and cephalosporins, we have studied the application of a bioreactor for enzymatic reactions. In previous papers, we reported the screening of *N*-carbamyl-D-amino acid amidohydrolase (DCase), which can hydrolyze *N*-carbamyl-D-amino acids to D-amino acids, ammonia and carbon dioxide, cloning and overexpression of its gene from *Agrobacterium* sp. KNK712 [4], and immobilization of the enzyme [5]. This overexpressed DCase seemed to exhibit higher activity than other previously re-

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

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ported ones [6–15] but its stability was not enough when the immobilized enzyme was used in repeated reactions. To resolve this problem, we have improved the DCase by means of gene mutation, and obtained some thermotolerant DCases, in which an amino acid was substituted [16].

In this paper, we describe the effect of the thermostability of the improved DCase on the stability of the immobilized enzyme in repeated reactions and the characteristics of the immobilized enzyme.

2. Experimental

2.1. Microorganisms

Escherichia coli JM109 carrying recombinant plasmid pAD108, pAD402, pAD404, pAD406 or pAD416 was reported by Nanba et al. [4] and Ikenaka et al. [16]. Each plasmid has a DCase gene which codes a native or mutant DCase of *Agrobacterium* sp. KNK712. The numbers of the plasmids are also the names of the DCases.

2.2. Media and culture conditions

Recombinant *E. coli* JM109 was aerobically cultured in 2YT medium (1.6% of tryptone, 1% of yeast extract, and 0.5% of NaCl, pH 7.0) containing 100 μg/ml of ampicillin in a 2-l Sakaguchi flask at 37°C, for 16 h.

2.3. Chemicals

N-Carbamyl-D-*p*-hydroxyphenylglycine was prepared from the corresponding amino acid [17]. Duolite A-568 (Rohm and Haas) was employed as a support for immobilization.

Other chemicals used in this work were the best available commercial products.

2.4. Preparation and immobilization of DCases

Native (108) and four kinds of mutant DCases (402, 404, 406, and 416) were used for immobilization (see Table 1 for the positions of the mutations and the amino acid substitutions in each mutated enzyme). Cells of *E. coli* JM109 (pAD108),JM109 (pAD402),JM109 (pAD404), JM109(pAD406), and JM109 (pAD416) were harvested from 500 ml of cultured broth by centrifugation ($3000 \times g$, 10 min, 4°C), suspended in 50 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM dithiothreitol, and then disrupted by sonication. The cell debris was removed by centrifugation ($18\,000 \times g$, 10 min, 4°C), and then the supernatant was obtained as the enzyme solution. Duolite A-568 was washed with 1 M NaCl, water, then with 0.1 M potassium phosphate buffer, pH 7.0, and equilibrated with the same buffer for 18 h at room temperature, after which the wet equilibrated resin was prepared by filtration. The wet resin and dithiothreitol (final concentration, 5 mM) was added to the enzyme solution, followed by stirring at 4°C for 20 h under nitrogen sealing. For adsorption, the weight of the wet resin and that of protein in the enzyme solution were in the ratio of 1 to 0.04. After adsorption, the resin was washed three times with a 5-fold volume of 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol, crosslinked with a 5-fold volume of 0.2% of glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol at 4°C for 10 min, washed 3 times with a 5-fold volume of 0.1 M potassium phos-

Table 1
Enzyme thermostability

Enzyme	Amino acid substitution	Thermostability (°C)
108 (native)	none	61.8
402	His 57 → Tyr	67.3
404	Pro 203 → Leu	68.0
406	Pro 203 → Ser	66.5
416	Val 236 → Ala	71.4

Solutions of JM109(pAD108), JM109(pAD402), JM109(pAD404), JM109(pAD406), and JM109(pAD416), see Section 2, were incubated at temperatures from 56°C to 74°C at 3°C intervals for 10 min. The remaining activity was determined under the standard assay conditions, and thermostability was expressed as the temperature at which 50% of the activity was lost.

Table 2
Immobilization of DCases

Enzyme	Specific activity			Activity yield (%)
	Loaded enzyme (u/mg)	Immobilized enzyme (u/g)		
		before GA	after GA	
108 (native)	2.43	41.8	10.8	9.6
402	2.31	37.8	15.1	16.4
404	2.02	36.8	11.5	12.8
406	2.34	36.9	11.1	11.9
416	2.51	44.5	14.6	14.6

GA: glutaraldehyde.

phate buffer, pH 7.0, containing 10 mM of dithiothreitol at 4°C, and then filtered.

2.5. Enzyme assay

Soluble DCase and immobilized DCase were assayed at 40°C and pH 7.0 by the methods of Nanba et al. [4,5].

One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of D-*p*-hydroxyphenyl-glycine at the rate of 1 μmol/min under the assay conditions.

To determine the effects of temperature and pH on the activity or stability of immobilized DCase, standard assays were performed as follows. The reaction was started by the addition of 200 mg of wet immobilized enzyme to the assay mixture comprising 4430 μmol of *N*-carbamyl-D-*p*-hydroxyphenylglycine, 3000 μmol of potassium phosphate buffer, pH 7.0, and 486 μmol of dithiothreitol, in a total volume of 30 ml. After 10 min incubation with stirring at 50°C, the reaction was stopped by the addition of 1 ml of 5 M H_2SO_4. The D-*p*-hydroxyphenylglycine formed was analyzed by high-performance liquid chromatography as described previously [4].

2.6. Analytical methods

All analytical methods were described in a previous paper [4].

2.7. Repeated batch reactions

As described in a previous paper [5], using *N*-carbamyl-D-*p*-hydroxyphenylglycine as a substrate solution, batch reactions were carried out repeatedly in a stirred reactor at 40°C under a stream of nitrogen gas while the pH was controlled at 7.0 with 2 M HCl. At 10 and 60 min, samples were taken for the calculation of the activity based on the product amount of 60 min minus that of 10 min, the reaction being continued for 23.5 h in total. After the removal of

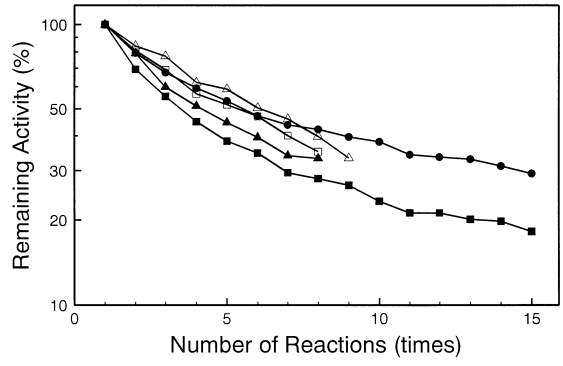

Fig. 1. Stability of immobilized DCases on repeated batch reactions without a reductant. Repeated batch reactions were carried out without a reductant as described under Section 2 using immobilized 108 (■), 402 (▲), 404 (●), 406 (□), and 416 (△) DCase. The remaining activity was expressed as a percentage of the activity in the first reaction.

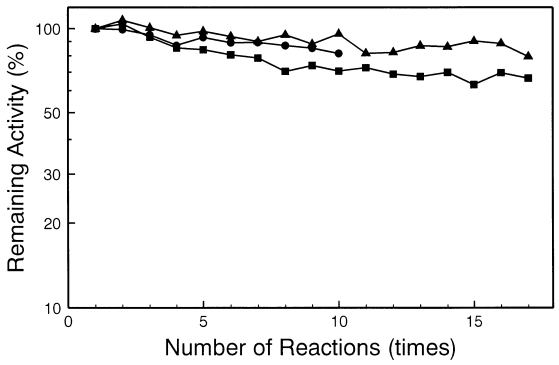

Fig. 2. Stability of immobilized DCases on repeated batch reactions with a reductant. Repeated batch reactions were carried out with 5 mM dithiothreitol as described under Section 2 using immobilized 108 (■), 404 (▲), and 416 (●) DCase. The remaining activity was expressed as a percentage of the activity in the first reaction.

reaction mixture, the reaction was repeated in the same manner.

3. Results

3.1. Enzyme thermostability

The thermostabilities of the native and mutated DCases are shown in Table 1. The thermostabilities of the mutated DCases having an amino acid substitution were 4.7–9.6°C higher than that of the native one, and 416 DCase, having the substitution of 236 valine to alanine, was the most stable.

3.2. Immobilization of DCases on Duolite A-568

The native and mutated DCases were immobilized on Duolite A-568 by adsorption and crosslinking with glutaraldehyde (Table 2). The immobilized 402 and 416 DCases exhibited higher activity than that of the native one, and all the mutant DCases were superior in the activity yield to the native one. However, the activity yields ranged from 9.6% to 16.4%, which was not so high.

3.3. Repeated batch reactions

The stability on repeated batch reactions in a stirred reactor was examined. When dithiothreitol was not present in the reaction mixture, all the mutant DCases were more stable than the native one. Among them, the 416 and 404 DCases showed the highest stability (Fig. 1).

Compared with the results in Fig. 1, Fig. 2 shows that the stability of the immobilized en-

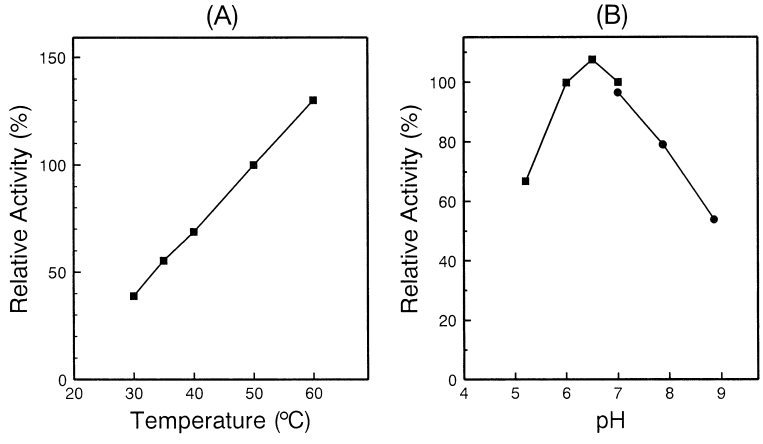

Fig. 3. Effects of temperature and pH on the activity of an immobilized DCase. Immobilized DCase from JM109(pAD416) was used. (A) The immobilized DCase was assayed at various temperatures under the standard assay conditions. (B) The immobilized DCase was assayed under the standard assay conditions except that the following buffers were used: 100 mM potassium phosphate, pH 5.2–7.0 (■); and 100 mM potassium phosphate monobasic–50 mM sodium borate, pH 7.0–8.9 (●). The relative activity was expressed as a percentage of the activity at 50°C and pH 7.0.

zymes was improved when dithiothreitol was present in the reaction mixture.

Based on the results of repeated reactions with dithiothreitol (Fig. 2), the half-lives of the activity were calculated from the regression coefficients. The half-lives of the activity on reaction for 1 to 8 times were 15 times for 108 DCase, 42 times for 404 DCase and 37 times for 416 DCase, and those for 9 to 17 times were 58 times for 108 DCase and 104 times for 404 DCase. From the results shown in Fig. 2 and the calculated half-lives, the mutant 404 and 416 DCases showed almost the same stability, which was superior to that of the native 108 DCase. And the activity loss was greater at the early rather than the late stages of the reaction, this phenomenon being observed also in the case of Fig. 1.

3.4. Effects of temperature and pH on the activity and stability of DCase

With increasing temperature, the activity of immobilized 416 DCase increased, the activity at 50°C being 1.5 times higher than that at 40°C, as shown in Fig. 3A. The slope of the activity of soluble 416 DCase was the same as that of immobilized 416 DCase in the range of 30°C to 60°C, when assayed for 10 min under the standard assay conditions (data not shown).

The optimum pH was found to be about 6.5 (Fig. 3B), although that of soluble 416 DCase was about 7 (data not shown).

After 16 h incubation, the activities of soluble 416 DCase and immobilized 416 DCase were stable below 40°C, and remained at 25.8% and 40.2% at 60°C, respectively (Fig. 4). The immobilized 416 DCase was stable compared with the soluble enzyme.

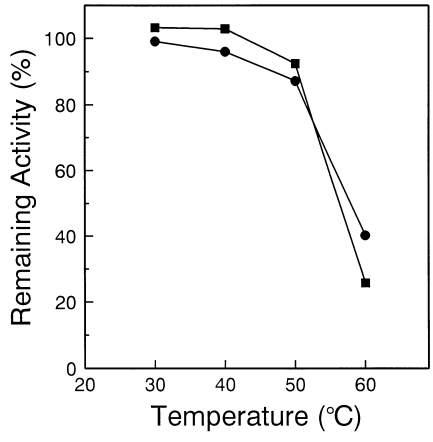

Fig. 4. Effect of temperature on the stability of an immobilized DCase. A solution of JM109(pAD416) containing 10 mM dithiothreitol (■), and 200 mg of immobilized 416 DCase in 30 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol (●) were incubated at various temperatures for 16 h. The remaining activity was determined under the standard conditions. The remaining activity was expressed as a percentage of the activity before incubation.

4. Discussion

We obtained thermotolerant DCases to stabilize immobilized DCases in reactions and to use them practically. They were stable when treated at high temperature, suggesting that the molecular conformations of the enzymes were stabilized by amino acid substitutions. And improvement of the thermostability caused an increase in the activity yield when they were immobilized. From the results, the thermotolerant enzyme seemed to be more resistant to denaturation due to heat, glutaraldehyde treatment or oxidation than the native one. However, the activity yield was not enough, because inactivation by glutaraldehyde was still high, some enzyme remained in the supernatant after adsorption, and the enzyme immobilized on a support did not express complete activity because of the limitations of diffusion of the substrate into macropores.

When immobilized enzymes were used in batch reactions repeatedly, the immobilized thermotolerant DCases showed obviously improved stability compared to that of the native one. It is supposed that the inactivation was caused by heat, oxidation, and the acid used for pH control. Apparently, the conformations of the immobilized thermotolerant DCases were

protected effectively against heat, and the activity seemed to be protected against other forms of denaturation.

The reductant, dithiothreitol, stabilized the activity of the immobilized enzymes effectively, suggesting that oxidation of an SH group is one of the major reasons for the inactivation. Other reductants were also able to stabilize the activity of an immobilized enzyme in reactions, as reported previously [5].

However, even the thermotolerant DCases gradually lost their activity when used in reactions as immobilized enzymes with a reductant and nitrogen. It was supposed that the reason was denaturation or detachment from the support due to insufficient crosslinking with glutaraldehyde and denaturation by the acid used for pH control. Inactivation occurred at early stages of the reaction and the activity gradually became stable at late stages of the reaction, suggesting that an unstable enzyme on the support was easy to denature or detach, and a stable one remained as it was.

Some loss of activity of an immobilized enzyme was observed on 16 h incubation at 50°C or 60°C. This is because of denaturation not only by heat but also by oxidation even with a reductant. When a soluble DCase was incubated with a reductant under nitrogen sealing, it was more stable than without nitrogen (data not shown). An immobilized DCase was somewhat stable compared with the soluble enzyme, suggesting that adsorption on the support and crosslinking with glutaraldehyde stabilized the structure of the enzyme.

The activity of immobilized 416 DCase at 50°C was 1.5 times higher than that at 40°C. An immobilized thermotolerant DCase can be used stably at a higher temperature, at which the enzyme expresses higher activity, so that immobilized enzyme can be used more efficiently. In the range of 30°C to 60°C, the slope for the activity of soluble 416 DCase was the same as that for the immobilized enzyme, suggesting that there was no difference in activation energy between the soluble and immobilized enzymes.

The optimum pH of an immobilized enzyme was lower than that of the soluble enzyme, suggesting that the pH around the enzyme in the macropores of the support was higher than the external pH, because the products of the DCase reaction remained in the macropores.

In this study, it was revealed that an immobilized thermotolerant DCase was more stable than the native one on repeated reactions. And it can be applied to the production of D-amino acids practically. After thermotolerant 416 DCase reported in this paper, we obtained 455 DCase, which showed further improved thermostability [18]. It was also immobilized, and exhibited excellent activity and stability. Further work on the production of this enzyme is necessary. And the mechanism of inactivation in repeated reactions should be clarified in detail.

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