Vol. 192, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1993 Pages 15-21

Enhancement of the Stability and Activity of Aspartase By Random and Site-Directed Mutagenesis

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Received February 8, 1993

Enzymatic generation of mutant libraries for random mutagenesis of aspartase gene from E.coli J_2 was made. A mutant enzyme with 4-fold increase in aspartase activity was found. It is stable at pH7.5-9.0 (wild-type , pH7.0-8.0); heat stability and α-helicity are higher than those of the wild-type. By using site directed mutagenesis, the aspartase was activated by replacement of Lys-126 with an arginie residue. The mutation produced functional alterations without appreciable structure changes. The optimum pH for the mutant enzyme is 8.5. The stable pH range is 7.0-9.0. Heat stability is higher than that of the wild-type one; Activity of the mutant enzyme is about 5-fotd as much as that of wild type one.

Aspartase (L-aspartate ammonia, EC4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and NH_4^+. It is an important enzyme in industy, which is maimly used to produce L-aspartic acid . In recent years, the requirement for L-aspartic acids is increasing all over the world. Thus, scientists pay more attention to the research on it in order to meet the need of theory research and production.

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Abbreviations used: MR, mutant(random); MSD, mutant(site-directed); SABC, SIN-AMERICAN BIOTECHNOLOGY CO.

0006-291X/93 $4.00

Previously, we showed that aspartase purified from E.coli AS1.881 cells is composed of four identical subunits and has a molecular weight of 193,000. The aspartase gene and its nucleotide sequence(1) from E.coli AS1.881 used in Chinese industry has been obtained. Here we describe results about enhancement of its stability and activity by random and site-directed mutagenesis.

MATERIALS AND METHODS

Materials. E.coli AS1.881 was provided Institute of Micro biology Academia Sinica, Beijing; E.coli J_2 containing plasmid carrying aspartase gene was stored at our lab. An otigionucleotide primer was synthesized using a System 1 plus DNA Synthesizer by the method of solid phosphoramidiate in the Institute of Cell Biology, Shanghai.

Other reagents were purchased from SABC Company, China.

Enzymatic generation of mutant libraries in vitro for random mutagenesis of the aspartase gene. This was carried out as (2).

Site-directed mutagenesis. The Lys-126 mutant was constructed directly on the double-stranded plasmid using the method described by Ming,H.(3). The plasmid pBR322 containing the aspartase gene from E.coli J_2 was as the target of site -directed mutagenesis with an otigonucleotide of 21 bases in tengeth. E.coli J that has not aspartase activity was transformed with the above mutant plasmid. Because replacement of Lys-126 with an arginine had been introduced MnlI restriction site, thus mutant was confirmed by restriction site and DNA sequence analysis.

Enzyme preparation. Wild-type aspartase was purified as described in Ref(4). The procedures for the mutant enzyme were essentially the same as those for wild-type (Table 1). Polyacrylamide gel electrophoresis was used to identify purification of two enzymes.

Activity measurement and protein determination. These were carried out as Ref(5).

Amino acid composition analysis. This was made in HITACHI 835-50 Amino Acid Analyzer (Table 2).

RESULTS AND DISCUSSION

Purifications of both wild-type and mutant enzymes

The mutants were screened by the method of cell enzyme activity, and indentified by restriction site and DNA sequencing. During the purification, the denatured protein was removed by nucleoprotamine, $(NH_4)_2SO_4$ fraction, DEAE-sephadex A-50, Sephadex G-200 column chromatography. The results were shown in Table 1. Both wild-type and mutant enzymes were homogeneous by judgement of polyacrylamide gel electrophoresis.

CD spectra of both wild-type and mutant enzymes

CD spectra of both wild-type and mutant enzymes were measured in order to compare the structural differences . The mutants had almost the same spectra as the wild-type enzyme (Fig.1), and gel filtration showed that it had the same molecular weight as that of the wild-type one. These results indicate that there are no obvious differences in secondary and quaternary structures between the wild-type and mutant enzymes.

The acitivty of mutant enzymes

The activities of the mutant enzymes obtained by random and site-directed mutagenesis were respectively 4-fold and 5-fold as much

Table 1. Purification of the wild-type and mutant enzymes

Enzyme	Procedures	Volume (ml)	Amount of protein (mg)	Specific activity (u/mg)	Activity units (u)	Recovery yield (%)	Purification times
Wild-type	Crude enzyme	39	960.57	83.95	80641.44	------	------
	Nucleoprotamine	43	706.49	167.80	118549.80	147	2.00
	Heat	39	164.58	1166.63	192003.41	238	13.89
	DEAE-Sephadex A-50	75	13.50	1874.22	25302.02	31.38	22.33
	Sephadex G-200	25	2.49	8974.48	22364.40	27.73	106.90
Mutant enzyme	Crude enzyme	40	770	379.07	291885.09	------	------
(Random Mutage-	Nucleo proatamine	40	611.4	566.92	346613.55	118.75	1.496
nesis)	50%$(NH_4)_2SO_4$	40	354.2	869.85	308100.92	105.56	2.295
	Heat	36.5	192.36	2579.26	496146.02	169.98	6.801
	35%$(NH_4)_2SO_4$	10	124.7	2747.63	342629.49	117.39	7.248
	DEAE-Sephadex A-50	60	6.165	35509.14	218913.82	75.00	93.67
Mutant enzyme	Crude enzyme	42	335.58	300.87	177669.75	------	------
(Lys126→Arg126)	50%$(NH_4)_2SO_4$	40	216.00	1038.87	224395.92	126.3	3.42
	80%$(NH_4)_2SO_4$	10	67.20	3895.25	261760.93	147.3	12.95
	DEAE-Sephadex A-50	30	1.40	46266.56	64541.85	36.33	153.98

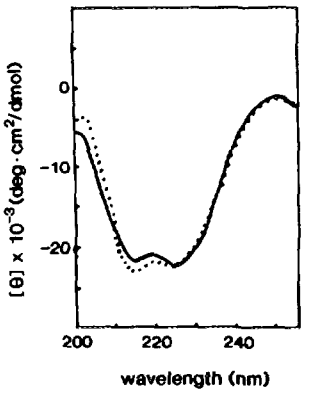

Fig. 1. CD spectra of aspartases from wild-type, site-directed (solid line) and random mutagenesis (broken line) strains. The calculated α-helicity is as follows, The aspartase obtained by wild-type and site-directed, 29.3%. The aspartase obtained by random mutagenesis, 36.3%.

as that of the wild-type one under standard assay conditions(see Table 1, 3). The enhancement of former activity may be owing to the change of amino acid composition (see Table 2) and the new spatial conformation of active site is more reasonable. When aspartase was modified with maleic anhydride and o-methyl-isourea, respectively, rapid inactivation took place. According to the conservation region (MGHQKGEYQYL) for different resources of aspartase, we putative that Lys-126 may be ratative to aspartase activity. Because of similar properties of Lys and Arg, the enzyme structure will not show obvious changes after mutation. But alkalinity of Lys is lower than that of Arg, which can

Table 2. Amino acid composition analysis of the wild-type and its mutant

Amino acid residues	Mutant	Wild-type	Amino acid residues	Mutant	Wild-type
Asp+Asn	57	55	Ile	30	33
Thr	25	26	Leu	40	43
Ser	20	22	Tyr	16	16
Glu+Gln	56	51	Phe	13	13
Gly	33	35	Lys	27	27
Ala	41	42	His	9	9
Cys	11	11	Arg	19	15
Val	41	43	Pro	22	20
Met	17	16			

Table 3. At pH7.0, the kinetic properties of the wild-type and mutant enzymes

Enzyme	Km(M)	Vm(nM×L×min)
wild-type(w)	0.016	39.850
mutant (random) (mr)	0.100	159.360
mutant (site-directed) (msd)	0.120	196.30

Vm(mr)/Vm(w) ≈ 4.
Vm(msd)/Vm(w) ≈ 5.

lead to the change of the optimum pH. The results showed that the mutant enzyme was activated by replacement of Lys-126 with an arginine residue.

Thermostability of mutant enzymes

The mutant enzyme obtained by random mutagenesis showed the increase of thermostability (Fig. 2). At 30℃, the activity increases gradually with the incubated time, but wild-type enzyme decreases gradually under the same condition. At 40℃, both decrease gradually, but the decrease of wild-type is faster. After being incubate for 45 minutes at 50℃, the wild-type conserves about 17% of its activity, while mutant enzyme conserves about 30% of that. At 60℃, both inactivated quickly after being incubated for 5 minutes. That is to say that the heat stability of mutant enzymy is better than that of wild-type one. Comparison with α-helicity of both wild-type and mutant enzymes, we know α-helicity of mr is higher, i.e., the mutant enzyme increase the rigidity of conformation. This may be the reason of improvement of heat stability. The mutant enzyme obtained by site-directed mutagenesis leads to an increased thermostability as well (Fig. 2). This may be due to an increase of ratio in the Arg to Arg plus Lys and conversion of lysine to arginine residue leads to an increased thermostability(6,7).

The pH stability of mutant enzymes

The optimum pH of mutant enzyme obtained by random mutagenesis is 8.0(wild-type, 8.0). The stable pH range is 7.5-9.0 (wild-type,

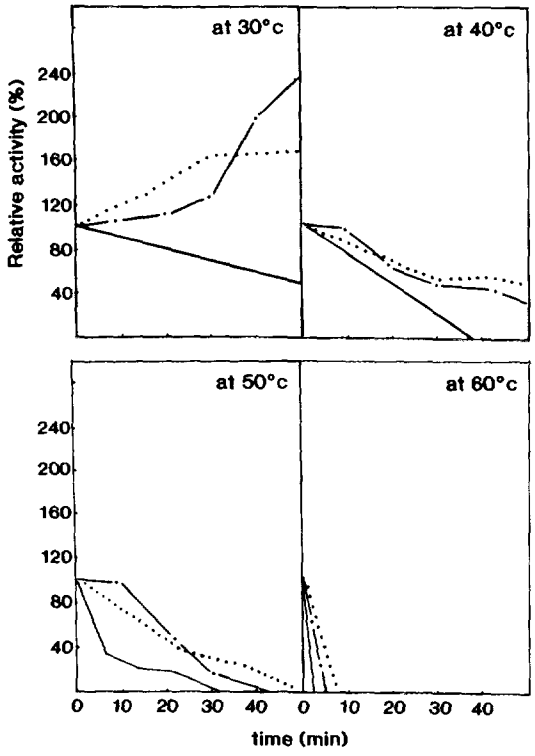

Fig. 2. Thermostability of the wild-type and mutant aspartase.

—·— : site-directed mutagenesis.
····· : random mutagenesis.
——— : wild-type enzyme.

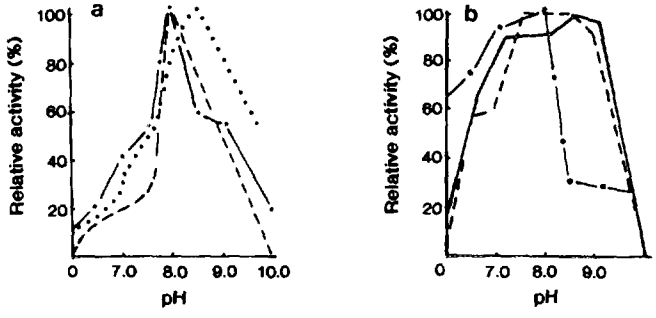

Fig. 3a. The optimum pH of the wild type and mutant enzymes.
———, wild-type, pH8.0.
– – – –, Random mutagenesis, pH8.0.
·····, Site-directed mutagenesis, pH8.5.

Fig. 3b. The pH stability range of the wild-type and mutant aspartase.
—·—·—, wild-type, pH7.0-8.0.
– – –, mutant enzyme by random mutagenesis, pH7.5-9.0.
———, mutant enzymes by site-directed mutagenesis, pH7.0-9.0.

7.0-8.0). The optimum pH of mutant enzyme obtained by site-directed mulagenesis is 8.5. Its stable pH range is 7.0-9.0(see Fig.3). This may be due to the change of Lys to Arg. Owing to lower alkalinity of Lys than that of Arg, there is a change in optimum pH. At pH6.0, both mutant enzymes show negative cooperativity, while they show positive cooperativity at pH8.0.

Our results suggest that two mutant methods produced similar action. There are, however, obvious differences in the properties of the wild-type and mutant enzymes. The molecular basis of these alterations is now being investigated in order to clarify the relationships between the two enzyme types.

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