Construction and Enzyme-activity Assessment of *L*-Asparaginase Mutants

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【ABSTRACT】 AIM: To construct nine novel *L*-asparaginase mutants and study their enzyme-activity. METHODS: The mutants were constructed using overlap extension PCR according to the principle of alanine-scanning mutagenesis. The enzyme-activity was detected by Nessler's method. RESULTS: The DNA sequencing showed that the mutagenesis was consistent with the theoretical prediction. The enzyme-activity assay demonstrated that each mutant possessed enzyme activity equal to the original enzyme. CONCLUSION: Through gene modification, epitop of *L*-asparaginase was changed without activity loss. These results provide foundation for further study of the structure-function relationship of *L*-asparaginase.

【KEY WORDS】 *L*-Asparaginase; Site-directed mutagenesis; Overlap extension PCR; Alanine-scanning

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L-Asparaginase (*L*-ASP, *L*-asparagine aminohydrolase, EC. 3. 5. 1. 1) is widely used in the treatment of acute lymphoblastic leukemia (ALL, mainly in children), acute myelomonocitic leukemia, lymphosarcoma and melanosarcoma, for its ability to catalyze the deamination of *L*-asparagine (*L*-Asn) to *L*-aspartic acid (*L*-Asp). Its antitumor activity is based on the fact that certain tumor cells, especially ALL tumor cells, have nutritional dependence on the external supply of Asn. In contrast, normal cells are protected from Asn-starvation during *L*-ASP therapy due to the ability to produce Asn[1, 2]. The enzyme is currently employed within the framework of combination chemotherapy schedule which achieves a treatment response about 90% and long-term remission in the majority of patients[3, 4]. However, serious side effects often happen during *L*-ASP therapy. The hypersensitivity reaction, the main restriction to *L*-ASP clinical application, is caused by antigenicity for the reason that *L*-ASP is a foreign protein with high antigenicity.

Studies indicated that two predicted peptides, ^{192}TPARKHTS^{199} and ^{261}KNGTAV^{266} exhibited stronger antigenicity than other peptides[5]. The former involves three continuous alkaline residues Arg-Lys-His, and the latter contains three polar residues Lys-Asn and Thr. Thus, an interactive site with macromolecules probably formed because of the asymmetric distribution of charges on the enzyme molecular surface[6, 7]. In addition, study on protein structure-function relationship showed that Lys was essential in the antibody-antigen interaction. Therefore, we selected these polar residues as mutagenic sites. These two peptides were not involved in the active site of the enzyme, so the mutants shouldn't lose the activity.

In Silence's study, the charged residues in these two antigenic peptides of *L*-ASP were systematically converted to alanine according to the principle of alanine-scanning mutagenesis[8]. We selected alanine as the replacement residue because it eliminates the side chain beyond the β-carbon and does not alter the main-chain conformation (as can glycine or proline), nor does it impose extreme electrostatic or steric effects. Furthermore, alanine is the most abundant amino acid and frequently appears in both buried and exposed positions of all varieties of secondary structures. Alanine-scanning site-di-

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rected mutagenesis can be used to assess the residues' individual function and characterize the role of the residues[9].

Site-directed mutagenesis is a powerful tool for analyzing protein structure-function relationship. Techniques designed to introduce mutations contain mainly the oligonucleotide-directed mutagenesis system, box-mutagenesis and mutagenesis using overlap extension PCR (OE-PCR)[10,11]. OE-PCR can generate site-directed mutagenesis through three PCRs only. This mutagenesis technique does not rely on available restrictive sites and multiple subcloning steps. In addition to point mutations, insertions and deletions can be obtained using this method independent of the targeted sequences. So OE-PCR is widely used to generate site-directed mutagenesis. In this paper, nine novel mutants were obtained using OE-PCR.

1 Materials and methods

1.1 *Materials*

The recombinant *E. coli* pKA/CPU210009 containing the *asnB* of *L*-ASP was previously constructed and preserved in our lab[12]. *E. coli* JM109 was preserved in our lab. Pfu DNA polymerase and dNTP were purchased from Shenergy Biocolor Biological Science & Technology Company. Bgl Ⅱ, Kpn Ⅰ and T4 DNA ligase were products of MBI Fermentas. Protein standard markers were obtained from TaKaRa Biotechnology Co. Ltd. DNA gel purification kit was from V-gene Biotechnology Co., Ltd.. All primers were synthesized by Shanghai Genebase Gene-Tech Co., Ltd..

1.2 *Site-directed mutagenesis*

The mutated genes were generated by OE-PCR[13]. Primers used for site-directed mutagenesis are listed in Tab.1.

Tab.1 Complementary primers used to generate the mutagenesis

Mutants	Primers	Sequences
Forward primer	a	5′TAC CCA C GG TAC C GA CAC GAT G 3′
		Kpn Ⅰ
Reversed primer	d	5′CTG ATT GA A GAT CT G CTG GAT CTG C 3′
		Bgl Ⅱ
192～199 peptide nucleotide sequence		5′ACC CCG GCA **CGT AAG CAC** ACC AGC3′
		Arg Lys His
L-ASPm Ⅰ/R195A K196A	b Ⅰ	3′G GTT GCA TGG GGC CGT CGT CGT CGT 5′
H197A	c Ⅰ	5′A GCA GCA GCA ACC AGC GAT ACG C 3′
L-ASPm Ⅱ/K196A H197A	b Ⅱ	3′GCA TGG GGC CGT GCA CGT CGT TGG 5′
	c Ⅱ	5′G GCA CGT GCA GCA ACC AGC GAT ACG C 3′
L-ASPm Ⅲ/R195A K196A	b Ⅲ	3′GCA TGG GGC CGT CGT CGT GTG TGG 5′
	c Ⅲ	5′G GCA GCA GCA CAC ACC AGC GAT ACG C 3′
L-ASPm Ⅳ/R195A H197A	b Ⅳ	3′GCA TGG GGC CGT CGT TTC CGT TGG 5′
	c Ⅳ	5′G GCA GCA AAG GCA ACC AGC GAT ACG C 3′
261～266 peptide nucleotide sequence		5′**AAA AAC GGC ACT** GCA GTA3′
		Lys Asn Gly Thr
L-ASPm Ⅴ/K261A	b Ⅴ	3′GAC CGT TGG CGG CGC CGT TTG CCG 5′
	c Ⅴ	5′CC GCC GCG GCA AAC GGC ACT GCA G 3′
L-ASPm Ⅵ/N262A	b Ⅵ	3′CGT TGG CGG CGC TTT CGT CCG TGA 5′
	c Ⅵ	5′CC GCG AAA GCA GGC ACT GCA GTA G 3′
L-ASPm Ⅶ/G263A	b Ⅶ	3′TGG CGG CGC TTT TTG CGT TGA CGT 5′
	c Ⅶ	5′CG AAA AAC GCA ACT GCA GTA GTG C 3′
L-ASPm Ⅷ/T264A	b Ⅷ	3′CGG CGC TTT TTG CCG CGT CGT CAT 5′
	c Ⅷ	5′AA AAC GGC GCA GCA GTA GTG CGT T 3′
L-ASPm Ⅸ/K261A N262A	b Ⅸ	3′AC CGT TGG CGG CGC CGT CGT CGT CGT CG 5′
G263A T264A	c Ⅸ	5′CG GCA GCA GCA GCA GCA GTA GTG CGT TC3′

Primers c Ⅰ～c Ⅸ were forward primers and primers b Ⅰ～b Ⅸ were reversed primers. Amino acids that were mutagenized to Ala are represented in boldface. Sites of mutagenesis are underlined. Letters in box are sequences recognized by restrictive enzymes

The recombinant plasmid pKA/CPU210009 was extracted and used as the template for PCR 1 and PCR 2, which produced fragment AB and CD using primers a, b and c, d, respectively. Primers b and c containing mutagenic sites were partially complementary to each other. Therefore, these two DNA fragments had overlap ends and could serve as a primer for generating mutated genes (ADm) in PCR 3. All the PCR products were recovered from electrophoresis gel using DNA gel purification kit. The nine mutants named *L*-ASPm Ⅰ ~ Ⅸ were obtained using mutagenic primer-pairs b Ⅰ/c Ⅰ ~ b Ⅸ/c Ⅸ.

1.3 *Cloning of mutants*

The mutated genes were digested with Bgl Ⅱ/Kpn Ⅰ, then ligated into the plasmid pKA/CPU210009 digested with the same restriction enzymes. The recombinant plasmids were transformed into *E. coli* JM109. The positive clones were identified by PCR. The site-directed mutagenesis were further verified by sequencing. The obtained mutants were designated as *E. coli* pKA/CPU210009-*L*-ASPm Ⅰ ~ Ⅸ.

1.4 *Enzyme-activity assay and SDS-PAGE assay*

The positive mutants, as well as wild-type strain were cultured in LB liquid medium supplied with 100 μg/mL ampicillin at 37 ℃ for 12 h. The seed cultures were inoculated into fermentative medium (5%, *v*/*v*) for expression under the same condition for additional 18 h. Then the bacterial cells were harvested by centrifugation at 8 000 r/min for 5 min.

The cells were suspended in 0.1 mol/L borate buffer (pH 8.4), containning 40 mmol/L *L*-Asn, and the reaction mixture was incubated at 37 ℃ for 15 min. The reaction was stopped with 1 mL of 50% trichloroacetic acid. The amount of NH_4^+ was determined by the Nessler's method[14]. A unit (U) of *L*-ASP was determined as the amount of enzyme that catalyzes 0.04 mol/L *L*-asparagine liberating 1 mmol of ammonia at 37 ℃ for 1 min.

The bacteria were subjected to SDS-PAGE for the analysis of expression products. The gels were stained with Coomassie Brilliant Blue R-250.

2 Results

2.1 *Site-directed mutagenesis*

PCR products were analyzed in 1.5% agarose gel and the bands with correct size were clearly shown in Fig. 1. Taking *L*-ASPm Ⅰ for example, the fragments of 337 bp and 394 bp were AB Ⅰ and CD Ⅰ, respectively. Both of the mutated OE-PCR products of *L*-ASPm Ⅰ and wild-type *L*-ASP were 721 bp. The mutagenesis of *L*-ASPm Ⅰ ~ Ⅸ were verified in gel electrophoresis analysis (Fig. 2).

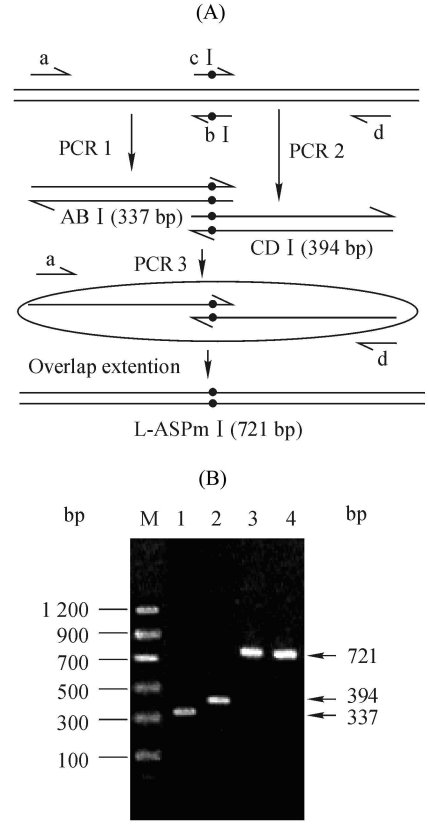

Fig. 1 *L*-ASPm Ⅰ constructed by OE-PCR

(A) Summary of mutagenesis strategy for *L*-ASPm Ⅰ by OE-PCR; (B) Gel electrophoresis analysis of *L*-ASPm Ⅰ

M: DNA Marker; 1: AB Ⅰ; 2: CD Ⅰ; 3: Wild-type *L*-ASP; 4: *L*-ASPm Ⅰ

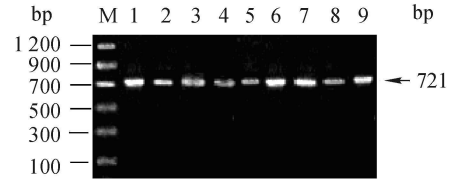

Fig. 2 Gel electrophoresis analysis of OE-PCR products of 9 mutants

M: DNA Marker; 1: *L*-ASPm Ⅰ; 2: *L*-ASPm Ⅱ; 3: *L*-ASPm Ⅲ; 4: *L*-ASPm Ⅳ; 5: *L*-ASPm Ⅴ; 6: *L*-ASPm Ⅵ; 7: *L*-ASPm Ⅶ; 8: *L*-ASPm Ⅷ; 9: *L*-ASPm Ⅸ

2.2 *DNA sequencing*

The DNA sequencing results proved that the mutated genes were consistent with the theoretical prediction (Tab. 2).

Tab. 2 Partial sequencing results of mutants and amino acids replaced by Ala

Strains	Partial sequences	Target residues
Wild-type	571 5′ CGTACCCCGGCACGTAAGCACACCAGCGAT 3′	. . . . . Arg-Lys-His. . . . .
L-ASPm Ⅰ	571 5′ CGTACCCCGGCA**GCAGCAGCA**ACCAGCGAT 3′	. . . . . **Ala-Ala-Ala**. . . . .
L-ASPm Ⅱ	571 5′ CGTACCCCGGCACG**TGCAGCA**ACCAGCGAT 3′	. . . . . Arg-**Ala-Ala**. . . . .
L-ASPm Ⅲ	571 5′ CGTACCCCGGCA**GCAGCA**CACCAGCGAT 3′	. . . . . **Ala-Ala**-His. . . . .
L-ASPm Ⅳ	571 5′ CGTACCCCGGCA**GCA**AAG**GCA**ACCAGCGAT 3′	. . . . . **Ala**-Lys-**Ala**. . . . .
Wild-type	781 5′ GCGAAAAACGGCACTGCAGTAGTGCGTTCT 3′	. . . . . Lys-Asn-Gly-Thr. . . . .
L-ASPm Ⅴ	781 5′ GCG**GCA**AACGGCACTGCAGTAGTGCGTTCT 3′	. . . . . **Ala**-Asn-Gly-Thr. . . . .
L-ASPm Ⅵ	781 5′ GCGAAA**GCA**GGCACTGCAGTAGTGCGTTCT 3′	. . . . . Lys-**Ala**-Gly-Thr. . . . .
L-ASPm Ⅶ	781 5′ GCGAAAAAC**GCA**ACTGCAGTAGTGCGTTCT 3′	. . . . . Lys-Asn-**Ala**-Thr. . . . .
L-ASPm Ⅷ	781 5′ GCGAAAAACGGC**GCA**GCAGTAGTGCGTTCT 3′	. . . . . Lys-Asn-Gly-**Ala**. . . . .
L-ASPm Ⅸ	781 5′ GCG**GCAGCAGCA**GCAGCAGTAGTGCGTTCT 3′	. . . . . **Ala-Ala-Ala-Ala**. . . . .

Bases underlined are according to the target residues and the mutagenic sites are indicated in boldface

2.3 *Results of activity assay and SDS-PAGE*

The comparative enzyme activity of wild-type strain versus mutants was studied parallelly. The enzyme activity of mutants didn′t decrease significantly compared with wild-type strain. These results indicated that the mutated residues were not involved in the active site of the enzyme and not necessary for the catalytic activity. The results provide a foundation for further investigating their structure-function relationship.

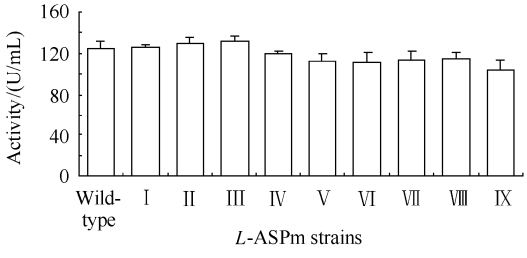

Fig. 3 Comparison of the *L*-ASP activity of wild-type and mutants (n=3)

The SDS-PAGE analysis of the mutants was studied parallelly compared with that of *E. coli* JM109 and *E. coli* pKA/CPU210009. Clearly visible bands were observed with the same molecular weight (34 kDa) as wild-type *L*-ASP (Fig. 4). Gel scanning showed that the content of targeted protein in the total bacterial protein of mutants didn′t decrease markedly compared with that of wild-type strain.

3 Discussion

Studies showed that the active site of *L*-ASP was located in *N*-terminal domain, in which a scarce left-handed β-α-β structure composed the bracket of active center[15, 16]. In this paper, the mutated peptides were not involved in the active site. The results indicated that the mutagenesis of peptides ^{192}TPARKHTS^{199} and ^{261}KNGTAV^{266} apparently didn′t affect *L*-ASP activity and expression. These studies provided foundation for further exploration of the structure-function relationship of *L*-ASP.

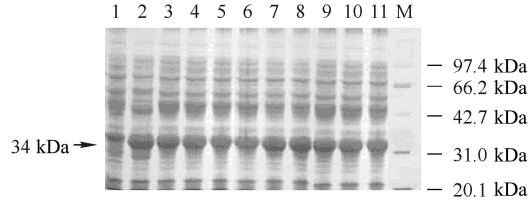

Fig. 4 SDS-PAGE profile of proteins expressed in wild-type *L*-ASP and mutants

1: *E. coli* JM109; 2: Wild-type; 3: *L*-ASPm Ⅰ; 4: *L*-ASPm Ⅱ; 5: *L*-ASPm Ⅲ; 6: *L*-ASPm Ⅳ; 7: *L*-ASPm Ⅴ; 8: *L*-ASPm Ⅵ; 9: *L*-ASPm Ⅶ; 10: *L*-ASPm Ⅷ; 11: *L*-ASPm Ⅸ; M: Protein marker

Antigenicity is the main reason for the toxic effect of foreign protein drug and greatly restricts the application of this kind of bio-tech drug in clinical use. Various methods were used to modify *L*-ASP in order to eliminate its antigenicity[17-19], such as monomethoxy-polyethylene glycol modification, liposome shielding, immobilizing modification and so on. Though these methods countercheck the antigenic determination, the loss of activity was great and this problem was not well solved. In

our work, we modify *L*-ASP on molecular level firstly. The mutated sites were not involved in the active sites of the enzyme, so all the mutants possessed enzyme activity equal to the original enzyme.

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L-门冬酰胺酶突变体的构建及其活性测定

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【摘 要】 目的: 将 *L*-门冬酰胺酶抗原表位区域的两个肽段作为突变对象, 构建 9 个突变体, 并对其酶活力和蛋白表达量与原酶进行比较研究。方法: 根据丙氨酸扫描原理, 应用重叠延伸 PCR 技术构建突变体, 并对突变菌株进行基因测序和酶活力测定。结果: 构建成功的突变菌株均具有酶活力, 且蛋白表达水平不受影响。结论: 首次采用基因修饰, 改变 *L*-门冬酰胺酶抗原表位区域的肽段, 而不影响其酶活力, 为后续研究和探讨 *L*-门冬酰胺酶抗原表位结构与功能的关系提供了基础。

【关键词】 *L*-门冬酰胺酶; 定点突变; 重叠延伸 PCR; 丙氨酸扫描

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