Exploration and Research on Relieving Feedback Inhibition for Aspartate Kinase Form *Corynebacterium Pekinense* by Using Site-Saturation Mutagenesis and High Throughput Screening

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Abstract. This research investigated the aspartate kinase (AK) of *Corynebacterium pekinense* (CpAK). The amino acid residue A297, which is closely related to feedback inhibition, was selected for site-directed mutagenesis, high-throughput screening, and enzyme kinetic analysis. In this research, the enzyme activity of mutant A297K AK was enhanced. The maximum reaction rate of A297K AK increased by 9.55 times compared with that of the wild-type CpAK (WT AK). In addition, Km value increased and n value decreased. The enzymologic properties are characterized as follows: optimum reaction temperature increased from 25℃ to 30℃; the optimum pH remained to be 8.0; and the stability of A297K AK under the optimal conditions extended from 3.5 h to 5 h. Feedback inhibition of low concentrations of threonine and lysine and the synergistic feedback inhibition of both amino acids were successfully relieved.

Introduction

Aspartate kinase (AK) is the first rate-limiting enzyme [1. 2] of amino acids of the aspartate family during microbial fermentation. Feedback inhibition by the end-product of the aspartate pathway leads to low yield of amino acids, especially methionine, of the aspartate family [3]. Fermentative production of methionine remains unachievable [4], and the use of microbial fermentation to produce methionine is currently a widespread concern.

The AK gene of *Corynebacterium glutamicum* (CgAK) is used as template for the AK gene of *Corynebacterium pekinense* (CpAK), which is our research object. The amino acid sequences of CpAK and CgAK are 99% similar, and these enzymes demonstrate similar feedback inhibition mechanism [5]; research development on CpAK relies on the crystal structure of CgAK [6. 7]. This study aims (1) to eliminate or weaken the synergistic feedback inhibition of end-product of the aspartate pathway by using CpAK [8], to obtain a high-dynamic CpAK, (2) to provide a reference for microbial fermentation employed to yield methionine [9], and (3) to provide insights into structural transformation of proteins by exploring the mechanism of synergistic feedback inhibition.

Materials and Methods

Materials

Plasmid extraction kit was purchased from Sangon; PCR extraction kit was purchased from TaKaRa; Dpni digestive enzymes was purchased from Fermentas; Non-denaturing nickel column was purchased from GE; SDS was purchased from Sigma; PVDF Membrane was purchased from BIO-RAD; Denatured protein electrophoresis Marker was purchased from TaKaRa; Non-denaturing electrophoresis Marker was purchased from Life Technologies.

Application of Bioinformatics Methods to Determine The Mutation Site

By using the CgAK crystal structure 3aaw as template, we selected one of the binding sites for threonine inhibitors, and the highly conserved residue A279 was selected for subsequent investigations following multiple sequence alignment. The inhibitors of CpAK feedback inhibition were removed through saturated site-directed mutagenesis of the A297 site of CpAK.

Site-Saturation Mutagenesis

The design primer Primer 5.0 was used to induce mutation during PCR amplification. The PCR products were electrophoresed on 1% agarose gel to verify whether the target gene was considerably amplified.

Primers:5'-GCACGGAAAACCTTCGCNNNCTCGCCTGGC-3';5'-GATAAGCCAGGCGAGNNNGCGAAGGTTTTC-3'

Sequencing and High-Throughput Screening of Mutants

A successful transformation A single colony was transferred into 96-well plates for activation and induction of fermentation. Screening activity was conducted to determine significantly improved mutants for sequencing.

Native-PAGE, SDS-PAGE and Western Blot Verification

Native-PAGE was used to verify the molecular weight of the intact protein. SDS-PAGE was employed to verify whether the structure of CpAK and CgAK is similar to a known structure and whether both structures contain α and β subunits. Western blot analysis was conducted to determine the molecular weight of the recombinant protein.

Kinetic Analysis of AK

Ten experimental groups were investigated in AK enzyme kinetics research. The final concentration of L-aspartic acid substrate was adjusted to 0.5, 1, 3, 5, 7, 9, 10, 12, 14, and 16 mmol/L. In each group, a control experiment (without substrate) and three parallel experiments were performed. The specific activity of AK in each group was measured and calculated. Data on kinetic parameters of AK protein were analysed before and after mutation using nonlinear analysis combined with the tendency of respective results and the Hill formula, $V=V_{max}(S^n)/(K^n+S^n)$.

Enzymatic Properties of AK

Optimum temperature: Eight experimental groups were reacted for 30 min at 15, 20, 25, 26, 28, 35, 40, 30, 45 and 50℃. Optimum pH: Under optimum temperature, eight experimental groups were separately reacted for 30 min at pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. The maximum relative enzyme activity of the experimental group is set to 100%. Thermal stability: The enzymatic activity of AK was determined by adding 5 mL of the reaction system into the enzyme solution every 1 h. Any change was recorded. The relative enzyme activity determined at 0 h in water bath was set as 100%.

Effects of substrate inhibitors:

The final concentrations of inhibitor in seven experimental groups were 0.2, 1, 5, and 10 mmol/L). The relative enzyme activity in the absence of inhibitor was set to 100%. Three parallel groups were prepared for each concentration.

Results and Discussion

Site Selection of A297

This study used the 3D crystal structure of CgAK as template. Pymol is used to identify amino acid residues near T501 and around the inhibitor (Fig. 1A). One of the highly conserved sites (i.e. A297) after multiple sequence alignment and bioinformatics analysis was selected as research object (Fig.

1B). A297 possibly plays a crucial role on the function of AK if the site was not altered during the evolutionary process.

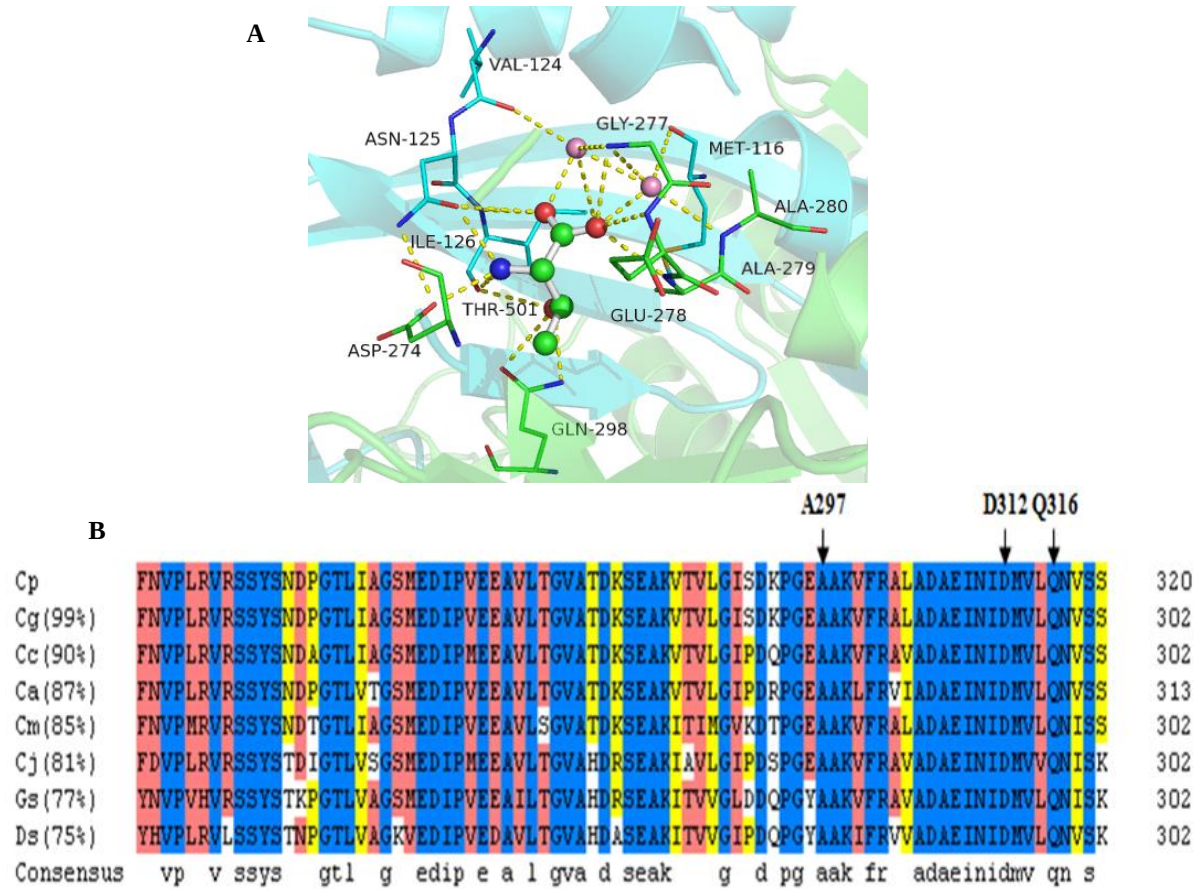

Figure 1. Selection of key site. A: Thr and important amino acid residues around the inhibitor.B: Multiple sequence alignment of CpAK with other members. Cp, *Corynebacterium pekinense*; Cg, *Corynebacterium glutamicum*; Cc, *Corynebacterium casei*; Ca, *Corynebacterium accolens*; Cm, *Corynebacterium maris*; Cj, *Corynebacterium jeikeium*; Gs, *Gordonia sputi*; Ds, *Dietzia sp*. *UCD-THP*.

Verification of Mutant AK Gene Through Nucleic Acid Electrophoresis

Three annealing temperatures were chosen in the experiments, and PCR products of approximately 7000 bp long were observed as bright bands (Fig. 2A). The plasmid was considerably replicated under the action of a high-throughput primer. After the successful sequencing, PCR validation of the strain showed that both the WT and mutant strains displayed bright strips of 1000–2000 bp, consistent with the 1453 bp long strip in AK (Fig. 2B). This finding shows that CpAK gene was successfully imported into the mutant strain. The strain can then be used for continuous research of gene expression.

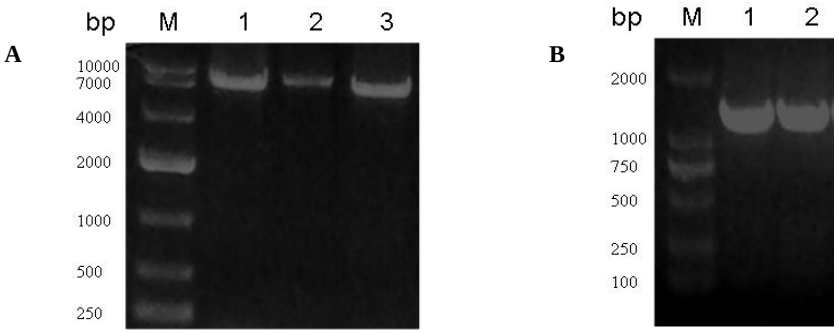

Figure 2 Mutation PCR and AK gene clone. A: The result of mutation PCR.M, DL10000 DNA Marker; 1, the result of PCR reaction at 54°C; 2, the result of PCR reaction at 56°C; 3, the result of PCR reaction at 58°C. B: The result of AK gene clone. M, DL2000 DNA Marker; 1,WT AK; 2,A297K AK.

High-Throughput Screening and Sequencing Results

A competent cell (*E. coli* BL21) was imported after successful digestion of the imported plasmid of the CpAK gene mutant strains. Two mutant strains displaying significantly improved enzyme activity were obtained after high-throughput screening. Sequencing results (Fig. 3) show that the A297 site of the original code for alanine (GCT) mutated into AAA (codon for lysine) and CCA (codon for proline). These two mutants were named A297P AK and A297K AK.

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WT     AAGTAACCGTTCTGGGTATTTCCGATAAGCCAGGCGAGGCTGCGAAGGTT 900
A297K  AAGTAACCGTTCTGGGTATTTCCGATAAGCCAGGCGAGAAAGCGAAGGTT 900
A297P  AAGTAACCGTTCTGGGTATTTCCGATAAGCCAGGCGAGCCAGCGAAGGTT 900
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Figure 3 Sequence alignment before and after the mutation of A297

Verification of AK Native-PAGE, SDS-PAGE and Western Blot

The results of Native-PAGE and Western blot analyses show that the molecular weight of CpAK is approximately 130 kDa (Fig. 4A). SDS-PAGE result shows that the α subunit is approximately 47 kDa and the β subunit is 18 kDa (Fig. 4B). In addition, the structure of CpAK is similar to that of CgAK. Thus, choosing CgAK as template to modify CpAK is reasonable.

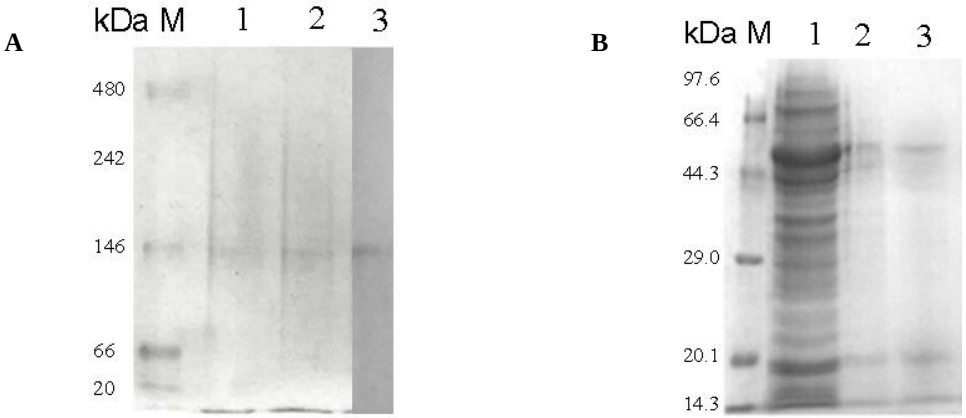

Figure 4 Results of Native-PAGE, SDS-PAGE and Western blot for CpAK. A: The result of Native-PAGE and Western blot. M, Native Mark™; 1, the purified WT AK; 2, the purified A297K AK; 3, Western blot of purified CpAK. B: The result of SDS-PAGE. M, High molecular weight protein marker; 1, Supernatant of induced sample; 2, the purified WT AK, 3, the purified A297K AK.

Analysis of The Enzyme Kinetics of WT and Mutant AK

Table 1 shows the enzyme kinetics of WT and mutant AK. Compared with the maximum reaction rate (V_{max}) of WT AK, that of A297P AK and A297K AK increased by 8.95 and 9.55 times, respectively. Their corresponding Km values were 4.70 and 6.37, which did not significantly change before A297P AK affinity with the substrate as compared with mutant, where the affinity A297K AK-substrate decreased obviously. A297K AK may be mutated to alter the overall structure of CpAK, which is not conducive for substrate binding. The corresponding Hill coefficients n of the mutated enzymes are 1.53 and 1.02, which decreased obviously compared with that of the WT AK 3.58. This result shows that the mutations of enzymes synergistically decreased. In addition, changes in the nature of the residues, such as the binding microenvironment, are detrimental to binding of threonine. In addition, the volume of Lys297 and Pro297 side chain is larger than that of Ala297. Therefore, mutations that interfere with the steric threonine can possibly be introduced into the hydrophobic microenvironment. A297K AK was selected for subsequent characterizations given that its enzyme activity obviously increased.

Tabe. 1 AK kinetics parameters of WT and A297 mutants

Strains	Vmax(U/mg·min)	Km(mmol/L)	n
WT	3.07	4.64	3.38
A297P	27.49	4.70	1.53
A297K	29.33	6.37	1.02

Characterization of the Enzymologic Properties of A297K AK

Optimum pH and Reaction Temperature

The results are shown in Figs. 5A and 5B. The optimum pH of A297K AK was 8.0, which is similar to pH at premutation. After the mutation, the acid resistance of A297K AK is lower than that of WT AK, and its relative activity is less than 10% under weakly acidic conditions. The optimum temperature for A297K AK is 5℃ higher than that in pre-mutation (30℃ vs. 25℃). Beyond 30℃, the rate of decline in enzyme activity in mutated AK is significantly lower than that in WT AK, which exhibits good heat resistance.

Thermal Stability

Fig. 5C shows that the half-life of A297K AK under the optimum reaction temperature and pH is 5h. Thus, the stability of the enzyme is enhanced compared with the control.

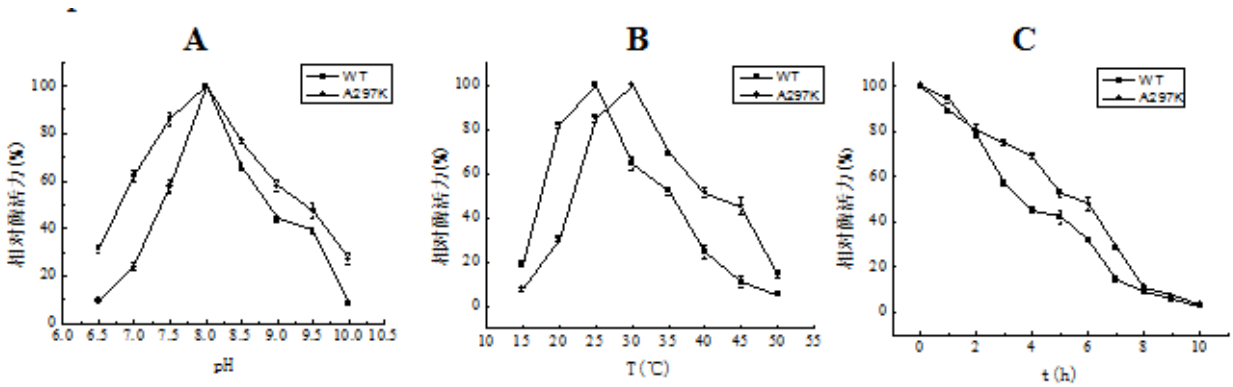

Figure 5 The results of the optimum pH, optimum temperature and thermal stability. A: The effect of different pH on AK enzyme activity of WT and mutant strains. B: The effect of different temperature on AK enzyme activity of WT and mutant strain. C: The stability of WT and mutant strains.

Effect of Substrate Inhibitor on the Activity of Mutated AK

Table 2 shows the results of comparison between A297K AK and WT AK. The WT AK inhibition was caused synergistically by Thr and Lys, whereas Met had little effect. Methionine may have the characteristics of product feedback inhibition in metabolic pathways, but it does not have a direct effect on AK. For A297K AK, the inhibitory effect of low threonine concentration on CpAK was relieved and demonstrated activation effect instead. At the same time, the inhibitory effects of low concentrations of lysine and those of the mixture of lysine and threonine on CpAK weakened.

Tabe.2 The influence of different substrate inhibitors on the enzyme activity of AK from WT and A297K

Enzyme	WT				A297K			
Substrate inhibitor	Relative activity (%) Concentration (mmol/L)							
	0.2	1	5	10	0.2	1	5	10
Control	100	100	100	100	100	100	100	100
Thr	60.28± 0.4	42.97± 0.7	29.45± 1.2	21.33± 0.7	128.46± 1.1	139.03± 0.5	103.75± 0.2	82.36± 1.3
Lys	73.86± 0.2	55.61± 0.9	30.92± 0.6	18.55± 1.4	110.33± 2.1	103.94± 2.1	92.62± 0.9	39.25± 0.4
Met	97.26± 2.0	84.62± 1.2	89.34± 1.1	86.22± 1.2	83.57± 0.8	81.09± 1.7	77.01± 1.4	68.32± 0.9
Thr + Lys	57.52± 1.7	34.51± 1.5	22.76± 0.4	ND	101.47± 0.5	134.59± 1.6	119.27± 1.1	72.58± 1.9
Thr + Met	80.34± 2.4	66.51± 0.9	41.89± 1.2	22.67 ±1.3	94.27± 2.2	73.49± 1.1	48.51± 0.8	30.69± 1.2
Lys + Met	85.47± 1.8	68.44± 0.8	48.26± 1.9	25.44± 2.2	76.41± 1.3	86.49± 0.6	71.35± 1.6	64.36± 0.4
Thr + Lys +Met	59.76± 1. 2	40.51± 0. 3	26.13± 1.4	ND	49.72± 0.8	28.34± 1.1	ND	ND

Conclusion

This study used the crystal structure of CgAK as template and the inhibitor binding pocket of Thr501 as target. Based on the results of multiple sequence alignment and bioinformatics analysis, A297 was selected as the key amino acid residue. Through site-directed saturation mutagenesis and high-throughput screening, two high-activity mutant, namely, A297P AK and A297K AK, were obtained.

Native-PAGE, SDS-PAGE and Western blot analyses were employed to verify that CpAK is overexpressed in *E. coli* BL21. We speculated based on the results that CpAK and CgAK are both heterotetramer.

Enzyme kinetics was used to analyse WT AK, A297P AK and A297K AK. The results show that the V_{max} of A297P AK and A297K AK increased by 8.95 and 9.55 times, respectively, compared with that of WT AK. In addition, the Km value increases, whereas the n value decreases. A297K AK was selected for further analysis after selecting V_{max}, which has improved significantly. Moreover, the results show that the optimum reaction temperature is 30 ℃, which is higher by 5 ℃ than the optimum temperature for WT AK; the optimum pH remained to be 8.0; and the half-life increased to 5 h. The feedback inhibition of low concentrations of threonine and lysine and the synergistic feedback inhibition of both amino acids have been successfully relieved. These changes are caused by the changes in binding pocket in microenvironment. Structural analysis shows that following Ala297 mutation in Lys297, the side chain volume increases; steric hindrance is enhanced. The distance between the nitrogen atom in the R group and oxygen atom in Gln 316 on the same α subunit is reduced to 2.32 Å, and the distance between the nitrogen atom of the R group and oxygen atoms in Ile126 on the β subunit is reduced to 3.29 Å, thus forming a stable hydrogen bond. These changes hinder threonine from binding to the binding pocket, leading to the overall changes in the structure of the enzyme.

Acknowledgements

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