



# L-Aspartate oxidase from *Sulfolobus tokodaii*: immobilization studies

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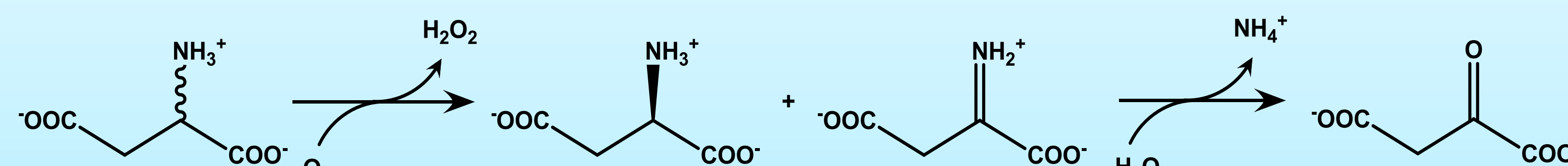
## Introduction

L-Amino acid oxidases (LAOs, EC 1.4.3.2) catalyze the stereospecific oxidative deamination of L-amino acids to  $\alpha$ -keto acids, ammonia and hydrogen peroxide.<sup>1,2</sup> In our labs L-aspartate oxidase from the thermophilic archaea *Sulfolobus tokodaii* (StLASPO, EC 1.4.3.16) has been efficiently produced as recombinant protein in *E. coli* in the active form as holoenzyme<sup>3</sup> and fully characterized.

The enzyme is active only on L-aspartate and on L-asparagine and it's absolutely stereoselective. StLASPO shows a remarkable stability: up to 80 °C in the 7 – 10 pH range. Plotting the activity (on L-aspartate) against temperature in the 25 – 95 °C range, an increase with no evidence for any plateau or decrease up to 80 °C was apparent. The activity of the enzyme as a function of pH shows a bell-shaped behavior: it is maximal at about pH 10.

## Kinetic resolution of D,L-aspartate

In a previous study StLASPO was used for the resolution of a 50 mM solution of D,L-aspartate, and the full resolution was reached in 4 hours in the 37-70 °C and pH 10-11 range using 0.3 U StLASPO/mL.



The kinetic resolution of D,L-aspartate was now used for evaluating the performance of the immobilized enzyme and was carried out by adding 0.5 mL of a 50 mM D,L-aspartic solution (in water, adjusted to pH 10.0), 200  $\mu$ L of water and 2  $\mu$ L of catalase to the StLASPO immobilized on resin. This reaction was performed at 70 °C on a thermomixer set to 600 rpm.

For CLEA preparation the assay was performed by adding 0.5 mL of a 50 mM D,L-aspartic solution in “magic buffer” (160 mM Tris–HCl, 160 mM Na<sub>2</sub>CO<sub>3</sub>, 160 mM H<sub>3</sub>PO<sub>4</sub>, 0.65 mM potassium chloride and 1% glycerol) padjusted to the desired pH (8, 9 or 10) and 1  $\mu$ L of catalase to the CLEA-StLASPO preparation (1.12 U); the reaction was performed at 70 °C on a thermomixer set to 600 rpm.

## Immobilization on Relizyme™ HA403/SR

Cycle number	Time (h)	e.e. (%)
1	1	100
2	1	100
3	1	100
4	1	87
	2	100
5	1	77
	2	100

The resin Relizyme™ HA 403/SR (250 mg), which has amino moieties, was treated with 10 mL of 0.125% glutaraldehyde solution in water for 2.5 h on a rotating device at 18 °C. The glutaraldehyde solution was then removed and the resin was washed with 0.1 M phosphate buffer at pH 7.5.

After that, 1 mL of StLASPO (2 U) was added to the resin, with 1 mL of 0.1 M phosphate buffer at pH 7.5 and the mixture was incubated on a rotatory shaker for 24 h at 18 °C.

StLASPO was immobilized with a full yield in terms of activity.

The resin was then washed with 50 mM phosphate buffer at pH 7.5, and stored at 4 °C in the same conditions.

The immobilized enzyme showed an activity of 8 U/g matrix

The influence of D,L-aspartate concentration on enzymatic (2 U/118 mg resin) resolution was also evaluated, by adding 0.5 mL of a D,L-aspartic solution at different concentration (from 50 to 500 mM) at pH 10.0.

Final D,L- Asp (mM)	L-aspartate conversion (%)									
	Time (h)									
	0.5	1	2	3	4	5	7	21	42	
35	100	-	-	-	-	-	-	-	-	
70	62	100	-	-	-	-	-	-	-	
110	12	36	100	-	-	-	-	-	-	
140	-	-	-	67	100	-	-	-	-	
180	-	-	-	-	53	69	84	-	-	
360	-	-	-	-	17	21	21	21	21	

## Analytical

At predetermined intervals, 25  $\mu$ L aliquots were withdrawn from the reaction mixture and diluted in 100  $\mu$ L of distilled water. 10  $\mu$ L of this solution were derivatized with 25  $\mu$ L of OPA-NAC reagent, diluted with 25  $\mu$ L of 50 mM eluent and analyzed by HPLC chromatography (column Gemini 5 $\mu$  C18 Phenomenex; eluent: 50 mM sodium acetate buffer pH 5.2 / MeOH 90/10; flux of 0.8 mL/min; detection at 340 nm).

OPA-NAC reagent is a basic aqueous-methanolic solution of ortho-phthalaldehyde and N-acetylcysteine which are able to react with free amine giving a fluorescent derivate.

## Other supports tested

Also other solid supports were evaluated:

Sepabeads EC-EP/A, Relizyme™ EP113, Relizyme™ HA403, Relizyme™ HA113, Eupergit® C, IDA-3 Sepharose® yielded poor results in term of immobilization except for IDA-3 resin: this matrix showed a high immobilization yield but the enzyme was leached out due to the non-covalent interaction with the matrix.

## Immobilization on resin SEPABEADS EC-EP/S

Cycle number	Time (h)	e.e. (%)
1	3	68
	4	100
2	2	66
	4	100
3	2	65
	4	100
4	2	59
	4	81
	6	100
5	2	28
	4	45
	6	73
	8	80
	10	100

One unit of pure StLASPO was added to 60 mg of Sepabeads EC-EP/S, with oxirane moieties, and to 0.3 mL of 1.25 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 8.0.

The mixture was incubated on a rotatory shaker for 18 h at 25 °C: approximately 50% of the enzymatic activity was present in solution suggesting that half of the starting enzyme has been immobilized.

This immobilized StLASPO preparation yielded full conversion of L-isomer of aspartate in 4 hours for 3 cycles. For the following cycles, the full resolution was obtained in a longer time, requiring up to 10 hours in the fifth cycle.

## Immobilization as CLEA

The precipitation of StLASPO as a CLEA<sup>4</sup> was conducted by adding to 0.5 mL of enzyme solution (2.24 U/mL), 4.5 mL of a 60% w/v (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution, in 0.1 M phosphate buffer at pH 7.5 and 20  $\mu$ L of glutaraldehyde.

The mixture was incubated on a rotatory shaker for 2.5 h at room temperature: no residual enzymatic activity was detected in the solution. CLEA-StLASPO was then washed twice with 0.1 M phosphate buffer at pH 7.5 and was stored at 4 °C in 0.05 M phosphate buffer at pH 7.5.

Before each cycle, the CLEA-StLASPO preparation was incubated with 1  $\mu$ L of FAD for 30 min in 0.1 M phosphate buffer at pH 7.5.

Cycle number	Reaction time (h)	e.e. (%) <sup>a</sup> pH 8	e.e. (%) <sup>a</sup> pH 9	e.e. (%) <sup>a</sup> pH 10
1	2	49	32	53
	4	87	100	100
	6	100	-	-
2	2	49	63	66
	4	97	100	100
	6	100	-	-
3	2	69	69	64
	4	73	100	100
	6	100	-	-
4	2	88	54	42
	4	100	100	68
	6	-	-	100
5	2	91	91	40
	4	100	100	69
	6	-	-	95
	7	-	-	100
6	2	0	0	31
	4	0	0	56
	6	0	0	80
	8	0	0	100

In the first cycle, the conversion was fully reached at all pH values tested, in a comparatively shorter time at highest pH. On the contrary, starting from the fourth cycle, the full oxidation of L-aspartate was obtained in a shorter time at pH 8 and 9.

## Conclusions

Due to the very interesting properties of this enzyme, we propose the immobilized StLASPO as an attractive tool for biotechnological applications. We recognize that CLEA technique or Relizyme™ HA403/SR as solid support represent the best conditions for StLASPO immobilization: this step will allow to improve the stability as well as enzyme reusability.

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