Immobilization of the Aspartate Ammonia-Lyase from *Pseudomonas fluorescens* R124 on Magnetic Nanoparticles: Characterization and Kinetics

Pál Csuka, Zsófia Molnár, Veronika Tóth, Ali Obaid Imarah, Diána Balogh-Weiser, Beáta G. Vértessy, and László Poppe*
Contents

1 Identification of the aspartate ammonia-lyase gene of *Pseudomonas fluorescens* R124 ................................................................. 2

2 Cloning of the PfAAL gene .......................................................................................................................... 3

3 Purification of the expressed PfAAL ....................................................................................................... 4

4 Characterization of native PfAAL......................................................................................................... 5

4.1 Kinetic characterization of the native PfAAL ..................................................................................... 5

4.2 Thermal behavior of the native PfAAL ............................................................................................ 7

5 Immobilization of PfAAL on epoxy-functionalized MNP ................................................................. 8

5.1 Protein leaching from PfAAL-MNP biocatalyst during reaction .................................................... 8

5.2 Treatment of the PfAAL-MNP biocatalysts for end-capping of the unreacted epoxides ................. 9

6 Characterization of PfAAL-MNP biocatalyst..................................................................................... 9

6.1 Kinetic characterization of the PfAAL-MNP biocatalyst................................................................. 9

6.2 Thermal behavior of PfAA-MNP ..................................................................................................... 11

6.3 Stability of PfAAL-MNP biocatalyst at various pH ..................................................................... 12

7 Viscosity of the various reaction media.............................................................................................. 13
Identification of the aspartate ammonia-lyase gene of *Pseudomonas fluorescens* R124

The coding nucleotide sequence for the investigated aspartate ammonia-lyase was found by the Tblastn tool on the NCBI Whole genome shotgun sequence contigs (WGS) database using the protein sequence of AspA from *Pseudomonas aeruginosa* PAO1 (UniProt ID: Q9HTD7) as query within the WGS of *Pseudomonas fluorescens* R124 (taxid:742713). The resulted hits had sequence similarities between 37-97%. The sequence for AAL in *Pseudomonas fluorescens* R124 WGS showed 89% identity to the query sequence (Figure S1).

| Q9HTD7   | PFAAL   | Query Sequence | Reference Sequence |
|----------|---------|----------------|--------------------|
| 1        | NISIMSSASFRTEIIDLGELEVPQAQYYGIQTLRWNNFLGSVPIS | 46 | 50 |
| 47       | PKLWALAMVKQAAADNRQGLPDKHAISEACARILRGDFHEQFV | 96 | |
| 51       | PKLWGLAMVKQAADANRELGHISHEAKHAISEACARILRGDFHEFV | 100 | |
| 97       | DMICQGAGGTSTNVMNANVEVANIALEAMGHTKGEKYLHPNNDVMNMAST | 146 | |
| 101      | DMICQGAGGTSTNVMNANVEVANIALEAMGHTQGEKYLHPNNDVMNMAST | 150 | |
| 147      | DAYPTAIRLGLLLGLHGTDILASLDLSIQAFAAKGVEFAQVLKGRQOLQDA | 196 | |
| 151      | DAYPTAERLGLLGLGDHALASSLDLSIQAFAAKGEFHNVLKGRQOLQDA | 200 | |
| 197      | VMPLTQDEHFAFATTLCEDDLRLRLAPELLTEVNVLGTAIGTCAADPG | 246 | |
| 201      | VMPLTQDEHRAFATTMCEDDLRLKTLAPELTELNVLGTAIGTCAADPR | 250 | |
| 247      | YQKLAVERALAISGQPFLPAADILIEATSDMCAFVFSLGMLRKTAVLSKI | 296 | |
| 251      | YQKAVQRLAISGQPFLPAADILIEATSDMCAFVFSLGMLRKTAVLSKI | 300 | |
| 297      | CNDLRLLSGGPRTGINEINLPPROPGSSIMPQKVNPVEAVNQFAFI | 346 | |
| 301      | CNDLRLLSGGPRTGINEINLPPROPGSSIMPQKVNPVEAVNQFAFI | 350 | |
| 347      | GNDLALTLAAEGQQLQNVMEPLIAYKTFSDIRLLQANANDMLREHCIT | 396 | |
| 351      | GNDLALTMAEQQGLQNVMEPLIAFKILDLSIRLLQRAMDMLREHCVG | 400 | |
| 397      | TANVERCHELVESQGLVTALNYIYENSTRIKALESGRLVEVLRE | 446 | |
| 401      | TANFARCELVESQGLVTALNYIYENSTRIKALESGRLVEVLRE | 450 | |
| 447      | FKLIDEATLADILPELLNPNIAPRLIPRA | 474 | |
| 451      | ELLDLEAMILRPENMIAPRLVPLKA | 478 | |

**Figure S1.** Alignment of the target AAL from *Pseudomonas fluorescens* R124 with the reference sequence of AspA from *Pseudomonas aeruginosa* PAO1.
2 Cloning of the PfAAL gene

The primers (Integrated DNA Technologies BVBA; Leuven, Belgium) for cloning the AAL of *P. fluorescens* R124 were designed to contain Ndel and BamHI restriction cleavage sites (in bold and underlined).

**Primers:**

*Fw_Ndel: 5’ atacatatgtctctccgctgc*

*Re_BamHI: 5’ ataggatctcaggtcttttgac*

The PCR reactions applying 30 cycles were made with Phusion Flash PCR Master Mix (Thermo Fisher Scientific; Waltham, USA) in the recommended reaction mixture as described in the user guide. The annealing temperature was applied 62 °C.

DNA purifications after gel electrophoresis were performed with EZ-10 Spin Column gel extraction kit (BioBasic Canada Inc; Toronto, Canada). Plasmid isolation (BioBasic Canada Inc; Toronto, Canada) was performed from overnight culture with EZ-10 Spin Column Plasmid isolation kit as specified in the protocol of the kit.

DNA ligations were made with pJET1.2/blunt cloning vector kit (Thermo Fisher Scientific; Waltham, USA) as specified in the protocol of the kit. Ligations were performed at 22 °C for 30 min. The results were checked by PCR reaction followed by agarose gel electrophoresis. Sequencing was performed by Macrogen Europe (Amsterdam, Netherlands).

The coding DNA was cloned to the pET15bTEV vector using Ndel/BamHI sites (Thermo Fisher Scientific; Waltham, USA) to produce an N-terminal His 

*T16-tagged protein. Ligation was performed at 18 °C overnight (Figure S2a shows the translated protein sequence of the identified AAL in WGS of *P. fluorescens* R124, Figure S2b depicts the protein sequence of PfAAL expressing from the plasmid construct after the cloning procedure).

![Figure S2. Translated protein sequence of the target AAL from *Pseudomonas fluorescens* R124 within the whole genome shotgun sequence (a) and the protein sequence of His 

*T16-*PfAAL expressing from the pET15bTEV vector.]
Purification of the expressed PfAAL

Figure S3. Purification of His(TEV)-PfAAL by Ni-NTA affinity chromatography. M: Marker; Start: cells before disruption; Pellet: cell debris after disruption; Lys.: cell lysate after disruption; Flowt.: flowthrough; Salt: washing step with low and high salt solutions (LS and HS, 30mM and 300 mM KCl respectively, 100 mM HEPES pH=8.0); 15 mM imidazole in LS; Elution: 250 mM imidazole in LS; 1 M imid.: 1 M imidazole in LS.
4 Characterization of native PfAAL

4.1 Kinetic characterization of the native PfAAL

**Figure S4.** Kinetic curves of the reaction from L-aspartic acid with PfAAL at different glycerol concentrations

**Figure S5.** Hill plot of the reaction from L-aspartic acid with PfAAL indicating regular MM kinetics; Evaluation was performed with MS Excel.
Figure S6. Kinetic evaluation of the reaction from L-aspartic acid with PfAAL without glycerol; Evaluation was performed with Statistica 13 (TIBCO Software Inc, Palo Alto, USA).

Figure S7. Kinetic evaluation of the reaction from L-aspartic acid with PfAAL at 5 v/v% glycerol concentration; Evaluation was performed with Statistica 13 (TIBCO Software Inc, Palo Alto, USA).
Figure S8. Kinetic evaluation of the reaction from L-aspartic acid with PfAAL at 10 v/v% glycerol concentration; Evaluation was performed with Statistica 13 (TIBCO Software Inc, Palo Alto, USA).

4.2 Thermal behavior of the native PfAAL

The thermal behavior of PfAAL activity was investigated with L-aspartic acid (20 mM) in a reaction mixture (1 mL) containing Tris buffer (50 mM, pH 8.8) and PfAAL (3 µg mL$^{-1}$) in a temperature range between 30–60 °C (30; 35; 40; 45; 50; 55 and 60 °C). The enzyme stock solution and the reaction mixture were preincubated separately for 5 min at the desired temperature.
5 Immobilization of PfAAL on epoxy-functionalized MNP

Epoxy-functionalized MNP (5 mg) was suspended and sonicated in Tris buffer (500 µL, 50 mM pH= 8.8) for 15 min, followed by addition of enzyme solution (500 µL, with 60, 100, or 1000 µg mL⁻¹ PfAAL) to the suspension (final enzyme concentrations: 30, 50, or 500 µg mL⁻¹). The suspension was shaken at 600 rpm at 30 °C for 2 hours. Then the PfAAL-MNPs were separated with neodymium magnet and washed twice with 1 mL of Tris buffer (50 mM, pH=8.8). The supernatant activity was determined with measuring fumarate formation by using a Spectronic Genesys 2 UV-Vis spectrophotometer (assay at 240 nm: 500 µL supernatant complemented with 500 µL of 30 mM l-asparatic acid in 50 mM Tris buffer of pH= 8.8).

5.1 Protein leaching from PfAAL-MNP biocatalyst during reaction

The quantitative protein determination was performed with Bradford method with ready to use Coomassie (Bradford) reagent (Thermo Fischer Scientific; Waltham, USA). The sample and the Bradford reagent were used in 1:1 volume ratio. After 10 min incubation the detection was performed at 595 nm with Multiskan™ FC Microplate Photometer (Thermo Fischer Scientific; Waltham, USA). Calibration was made from the purified PfAAL enzyme stock solution in the followed enzyme concentrations: 1; 5; 10; 15; 20; 25 and 30 µg mL⁻¹.

![Figure S10](https://example.com/figure_s10.png)

**Figure S10.** Calibration for protein determination (PfAAL) with Bradford method

Samples were taken from the immobilization solution of PfAAL, from the supernatant after the enzyme immobilization and after 3 h incubation in Tris buffer (1 mL, 50 mM pH= 8.8). Samples were taken in triplicate and analyzed.

| Sample                                 | Absorbance (A₅₉₅) | Protein (µg mL⁻¹) | (%)    |
|----------------------------------------|--------------------|-------------------|--------|
| PfAAL immobilization solution          | 0.4819             | 30                | 100    |
| Supernatant after the immobilization   | 0.0007             | 0.04              | <1     |
| Incubation in reaction buffer after 3 h| 0.0086             | 0.51              | 1.7    |
5.2 Treatment of the PfAAL-MNP biocatalysts for end-capping of the unreacted epoxides

The immobilized PfAAL-MNP preparation (5 mg) was shaken in 5 mM ethanolamine or 5 mM glycine in 1 mL Tris buffer (50 mM pH= 8.8) for 2 h at 600 rpm. After the treatment, the PfAAL-MNP biocatalysts were washed with 1 mL Tris buffer (1 mL, 50 mM pH= 8.8). The activity of the biocatalysts was determined in a reaction with 20 mM L-aspartic acid in 50 mM Tris buffer pH= 8.8.

![Figure S11. End-capping treatment of the PfAAL-MNP biocatalyst with ethanolamine or glycine](image)

6 Characterization of PfAAL-MNP biocatalyst

6.1 Kinetic characterization of the PfAAL-MNP biocatalyst

The PfAAL-MNP biocatalyst (5 mg) was tested in Tris buffer (1 mL, 50 mM pH= 8.8) supplemented with different concentrations of glycerol (0; 5; 15 and 50 v/v%) at substrate concentrations ranging from 0.1 to 60 mM of L-asparatic acid. After the activity tests, the PfAAL-MNP's were washed twice with 1 mL of Tris buffer (50 mM, pH7 8.8) supplemented with the given amount of glycerol. The samples were measured with Thermo Fischer NanoDrop 2000 at 240nm. The kinetic data was evaluated with Statistica 13 using non-linear regression.

![Figure S12. Kinetic curves of the reaction from L-asparatic acid with PfAAL-MNP at different glycerol concentrations](image)
Figure S13. Kinetic evaluation of the reaction from L-aspartic acid with PfAAL-MNP without glycerol; Evaluation was performed with Statistica 13 (TIBCO Software Inc, Palo Alto, USA).

Figure S14. Kinetic evaluation of the reaction from L-aspartic acid with PfAAL-MNP with 5 v/v% glycerol; Evaluation was performed with Statistica 13 (TIBCO Software Inc, Palo Alto, USA).
6.2 Thermal behavior of PfAAL-MNP

The PfAAL-MNP biocatalysts (5 mg) were tested in reactions containing 20 mM L-aspartic acid (50 mM Tris buffer, pH 8.8) at various reaction temperatures (30; 35; 40; 45; 50; 55; 60 °C). Before the activity test, the PfAAL-MNP-containing buffer (0.9 mL) and L-aspartic acid (200 mM in 50 mM Tris buffer, pH 8.8) were preincubated at the desired temperature for 5 min and the reaction was started by addition of the L-aspartic acid solution. The reactions were performed at 600 rpm on orbital shaker at the desired temperature. Samples (10 µL) were taken at 2.5, 5-, and 7.5-min reaction times (during sampling, MNPs in the reaction mixture were sedimented rapidly with a neodymium magnet). The reaction rate was calculated as the average of the rates between 2.5-5 min and 5-7.5 min.

Figure S16. Thermal behavior of PfAAL-MNP biocatalyst
6.3 Stability of PfAAP-MNP biocatalyst at various pH

The pH optimum of PfAAP-MNP (5 mg; 6 µg mg\(^{-1}\) PfAAP on MNP) of the ammonia elimination reaction was investigated with 20 mM L-aspartic acid in a reaction mixture (1 mL) containing buffer (50 mM, sodium phosphate for pH 6-7; Tris for pH 7-9; and sodium carbonate for pH 9-10) at 30 °C and at 600 rpm in a thermostated orbital shaker. Samples (10 µL) were taken at 2.5, 5-, and 7.5-min reaction times (during sampling, MNPs in the reaction mixture were sedimented rapidly with a neodymium magnet). The reaction rate was calculated as the average of the rates between 2.5-5 min and 5-7.5 min.

![Figure S17](image_url)  
**Figure S17.** Relative activity of PfAAP-MNP biocatalyst on various pH

6.4 Effect of divalent cations on PfAAP-MNPs

The effect of divalent cations on the activity of PfAAP-MNP (5 mg; 6 µg mg\(^{-1}\) PfAAP on MNP) was investigated in the ammonia elimination starting from 20 mM L-aspartic acid in a reaction mixture (1 mL) supplemented with divalent metal ion chloride (100 µM, Co\(^{2+}\); Mg\(^{2+}\); Cu\(^{2+}\); Ni\(^{2+}\); or Ca\(^{2+}\)) at 30 °C and at 600 rpm in a thermostated orbital shaker. Samples (10 µL) were taken at 2.5, 5-, and 7.5-min reaction times (during sampling, MNPs in the reaction mixture were sedimented rapidly with a neodymium magnet). The reaction rate was calculated as the average of the rates between 2.5-5 min and 5-7.5 min.

![Figure S18](image_url)  
**Figure S18.** Effect of divalent cations at 100 µM concentration on the relative activity of PfAAP-MNPs
7 Viscosity of the various reaction media

Viscosity of the following solutions and suspensions were measured:
A. Tris buffer (pH 8.8, 50 mM) containing glycerol (0; 5; 10; 15; 20; 30; 40; 50 v/v%)
B. Tris buffer (pH 8.8, 50 mM) with soluble PfAAL (3 µg mL⁻¹) containing glycerol (0; 5; 10; 15; 20; 30; 40; 50 v/v%)
C. Tris buffer (pH 8.8, 50 mM) with epoxy-modified MNP (5 mg mL⁻¹) containing glycerol (0; 5; 10; 15; 20; 30; 40; 50 v/v%)
D. Tris buffer (pH 8.8, 50 mM) with PfAAL-MNP (5 mg mL⁻¹ with 6 µg PfAAL mg⁻¹ MNP) containing glycerol (0; 5; 10; 15; 20; 30; 40; 50 v/v%)

![Figure S19. Viscosity of the various media (A-C) with different glycerol amounts](image)

| Glycerol [v/v%] | Viscosity [mPas] |
|----------------|------------------|
|                | A                | B                | C                | D                |
| 0              | 0.7886           | 0.7591           | 0.7718           | 0.8405           |
| 5              | 0.8998           | 1.0051           | 0.9188           | 1.0687           |
| 10             | 1.0481           | 1.0961           | 1.1033           | 1.2044           |
| 15             | 1.2571           | 1.2471           | 1.2895           | 1.3622           |
| 20             | 1.5386           | 1.4714           | 1.6067           | 1.4803           |
| 30             | 2.2752           | 2.2505           | 2.3238           | 1.8874           |
| 40             | 3.5085           | 3.4058           | 3.5776           | 3.0258           |
| 50             | 5.7371           | 5.3673           | 8.1533           | 4.3597           |

Table S2. Viscosity of the various media (A-C) with different glycerol amounts
| Glucose (w/w%) | Viscosity [mPas] |
|---------------|-----------------|
| 0             | 0.794           |
| 5             | 0.906           |
| 10            | 1.031           |
| 15            | 1.203           |
| 20            | 1.348           |
| 38            | 2.227           |
| 46            | 2.842           |

*Table S3. Viscosity of glucose solution at different glucose concentration in Tris buffer (50 mM, pH 8.8)*