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

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Aspartate ammonia-lyases (AALs) catalyze the non-oxidative elimination of ammonia from L-aspartate to give fumarate and ammonia. In this work the AAL coding gene from *Pseudomonas fluorescens* R124 was identified, isolated, and cloned into the pET-15b expression vector and expressed in *E. coli*. The purified enzyme (PfAAL) showed optimal activity at pH 8.8, Michaelis-Menten kinetics in the ammonia elimination from L-aspartate, and no strong dependence on divalent metal ions for its activity. The purified PfAAL was covalently immobilized on epoxy-functionalized magnetic nanoparticles (MNP), and effective kinetics of the immobilized PfAAL-MNP was compared to the native solution form. Glycerol addition significantly enhanced the storability of PfAAL-MNP. Inhibiting effect of the growing viscosity (modulated by addition of glycerol or glucose) on the enzymatic activity was observed for the native and immobilized form of PfAAL, as previously described for other free enzymes. The storage stability and recyclability of PfAAL-MNP is promising for further biocatalytic applications.

Introduction

Aspartate ammonia-lyase (AAL, EC 4.3.1.1, often referred as aspartase) catalyzes the elimination of ammonia from L-aspartic acid (Asp), or addition of ammonia onto fumarate.[1] AALs play an important role in microbial nitrogen metabolism, and they have been widely studied and applied since the 70s to produce Asp in large quantities, which is an important compound in the artificial sweetener production, in the pharmaceutical industry, and in the enzymatic production of L-alanine being utilized in parenteral nutrition and as food additive.[2,3] Although the AAL of *Pseudomonas fluorescens* was isolated,[4,5] cloned and sequenced earlier,[6] the first extensively studied and applied AAL originated from *Escherichia coli*.[7] Aided by recombinant technologies, many AALs were identified and characterized since then.[6,8–16] All AALs are homotetrameric enzymes consisting of four identical active sites forming from sidechains of multiple monomeric chains of ~52 kDa size.[1,2] The reaction mechanism of AALs has been studied with several methods, such as site-directed mutagenesis, chemical modification and computational modeling.[1,17] Based on these studies, a serine residue present in the conformationally flexible SS-loop acts as the catalytic base in abstraction of the *pro-R* proton of Asp.[1] The opening-closing movement of this SS-loop is the most important feature of AALs, since the closed state of the SS-loop contributes to substrate binding and safeguards the substrate from the solvent during the catalysis.[1] Although this reaction mechanism seems to be highly conserved in the whole aspartase/fumarase superfamily, the so far identified aspartases exhibit different features regarding their regulation and other enzymatic properties.[18] The AAL from *E. coli* (AspA) shows high substrate specificity towards Asp and allosteric activation with its substrate, and requires divalent metal ions for enzymatic activity above pH 7.[19] On the other hand, the AAL isolated from *Bacillus* sp. YM55-1 (AspB) is more stable, exhibits higher activity at elevated temperatures, does not need Mg^{2+} for its activity and is not allosterically activated.[2,10] AspB was the only AAL so far that could be applied in the production of *N*-substituted aspartic acids.[9] Furthermore, engineering of AspB enabled the redesigned mutants to catalyze asymmetric addition of ammonia to substituted acrylates, affording enantiopure aliphatic, polar and aromatic β-amino acids that are valuable building

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blocks for the synthesis of pharmaceuticals and bioactive compounds.[20] Recently, the computer-aided redesign of AspB resulted in novel C–N lyases with cross-compatibility of non-native nucleophiles and electrophiles enabling the production of a wide range of unnatural amino acids with excellent conversion, regioselectivity and enantiotopic selectivity at industrially relevant scale.[21]

Because enzyme immobilization may increase the range of conditions where the enzyme can be applied, and also may enhance enzyme activity, selectivity or specificity,[22] it is understandable that immobilization of the industrially relevant AALs has been carried out with several methods already. The first approaches captured AAL expressing whole cells enabling the synthesis of Asp due to the high half-life of the immobilized whole-cell biocatalysts.[3] Purified AALs for industrial production of Asp have been immobilized on various supports such as silica-based or weakly basic anion-exchange carriers.[23] AALs were immobilized on epoxy (Eupergit® C) and amine (MANA-agarose) supports,[3] or entrapped in polyacrylamide gel[24] and polyvinyl alcohol (LentiKats®).[3] Furthermore, AAL has been co-immobilized with a transaminase on amino-epoxy ReliZyme® support and used for the production of L-phenylalanine.[25]

Magnetic nanoparticles (MNPs) attracted attention in various fields of biotechnology as suitable carriers for enzyme immobilization.[26,27] MNPs have several advantageous features such as high surface area and low mass transfer resistance-resulting in high reactivity-, and simple isolation of the immobilized biocatalyst from the reaction mixture. However, magnetic nanoparticles may lose their beneficial properties in the case of poor stability of the enzyme coating, since the stripping MNPs may show little or no activity.[22,27,28] Because MNPs have not been investigated as carriers for AAL immobilization yet, our study aimed the covalent attachment onto epoxy-activated MNPs as an efficient method for the immobilization of AALs and characterization of the resulting biocatalyst.

In this study, the aspartate ammonia-lyase from *Pseudomonas fluorescens* R124 (PfAAL) was identified, expressed, and purified. The purified PfAAL has been covalently immobilized on magnetic nanoparticles (MNPs) via epoxy groups. The amino-coated carrier was functionalized with glycerol diglycidyl ether providing the active epoxide functions and with glycidol providing a hydrophilic diol function to ensure an ambient heterofunctional surface for enzyme immobilization.[29–31] The enzyme has been characterized in its native and immobilized form. PfAAL turned out to be a stable, highly efficient enzyme, being applicable in further biocatalytic studies such as characterization of the efficiency of novel magnetically agitated batch reactors.[32]

Results and Discussion

Our primary goal was to identify a novel and effective aspartate ammonia-lyase which has high expression levels in bacterial fermentation and shows excellent kinetic properties, thus can be a promising candidate for biocatalytic conversions. The gene of the aspartate ammonia-lyase investigated in this study was identified in *Pseudomonas fluorescens* R124, wherefrom we characterized three MIO-enzymes (a phenylalanine-2,3-amino-mutase, a tyrosine/phenylalanine/histidine ammonia-lyase, and a histidine ammonia-lyase).[33] The ammonia-lyases exhibited promising catalytic properties in the ammonia elimination reactions, therefore we decided to investigate the aspartate ammonia-lyase of this *Pseudomonas* strain as well. Thus, the aspartate ammonia-lyase of *P. fluorescens* R124 was cloned, expressed, and purified to investigate the immobilization on MNPs and to characterize the behavior of the native and the immobilized enzyme under different conditions in the ammonia elimination from L-aspartic acid (Figure 1).

Identification and substrate specificity of aspartase from *Pseudomonas fluorescens* R124

The coding sequence of PfAAL was identified with the PA-AspA sequence from *Pseudomonas aeruginosa* PAO1 (UniProt ID: Q9HTD7).[11] The BLAST result showed 89% identity and 93% similarity to the query sequence (see ESI Figure S1). The sequence from the genomic DNA was cloned into pET-15b vector with NdeI and BamHI restriction sites to result in an *N*-terminal His_6-tagged enzyme (see ESI Figure S2). The enzyme was expressed in *E. coli* Rosetta cells, purified with NiNTA, dialyzed against 50 mM TRIS buffer (pH 8) and frozen in aliquots at −80 °C until further use (see ESI Figure S3). The purified PfAAL was tested with a selection of substrates showing ammonia elimination activity only with L-aspartic acid but not

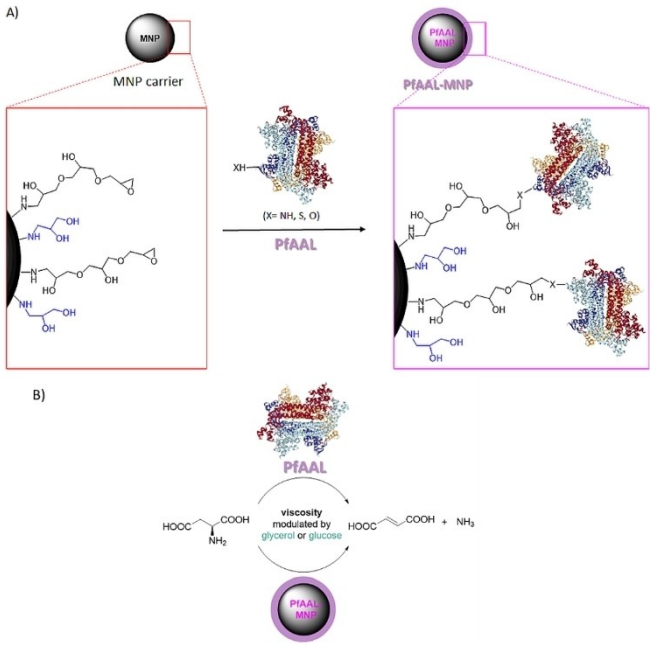

Figure 1. The aspartate ammonia-lyase from *Pseudomonas fluorescens* R124 (PfAAL) was (A) immobilized on epoxy-activated magnetic nanoparticles (MNP) and was (B) tested for production of fumaric acid from L-aspartic acid in its native and covalently immobilized form. The effect of viscosity on the activity the two forms of PfAAL was studied at various glycerol or glucose concentrations.

with L-phenylalanine, L-3,4-dihidroxyphenylalanine, L-alanine, L-histidine, L-lysine, L-glutamic acid, L-tyrosine, L-serine, L-glycine, L-proline, or L-tryptophan. Based on these results it can be concluded that PfAAL has high substrate specificity for Asp, which is common for most aspartases. Only a few exceptions with broader substrate scope have been observed, e.g. the aspartase from *Escherichia coli* accepted L-malic acid, L-asparagine and aspartame (L-Asp-Phe-methyl ester) as substrate.[34] Aspartase from *Pseudomonas aeruginosa* was reported to have ammonia-lyase activity for L-phenylalanine as well.[11] Protein engineering approaches could widen the substrate scope of aspartate ammonia-lyases: e.g. the K327N mutant of *Escherichia coli* aspartase accepted L-aspartic acid-α-amide as substrate.[35] A structure-guided redesign of aspartase from *Bacillus* sp. YM55-1 significantly enhanced the substrate scope towards non-native nucleophiles and electrophiles.[20,21]

Characterization of the purified PfAAL

Aspartases are usually most active in alkaline reaction conditions with a pH optimum between pH 7–9 for L-aspartic acid.[36] Although AAL from *Pseudomonas fluorescens* was investigated among the first reported aspartases,[4–6] there is no information about its pH tolerance and pH optimum. The bacterial aspartases from *E. coli*[11] and *Pseudomonas aeruginosa*[35] had pH optimum values of 8.0 and 8.5, respectively. Thus, the enzyme assays were performed in 100 mM Tris-HCl buffer supplemented with 3 μM $MgCl_2$.[5,37] Our experiments showed, that PfAAL has a similar pH profile as observed for the other bacterial aspartases (Figure 2), with a pH optimum at 8.8 and relative activities above 70% between pH 8.0 and 9.4.

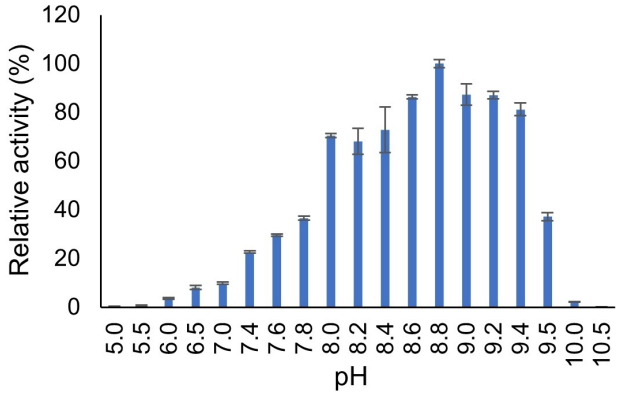

Figure 2. Effect of the pH on the ammonia elimination from L-aspartic acid with native AAL from *Pseudomonas fluorescens* R124.

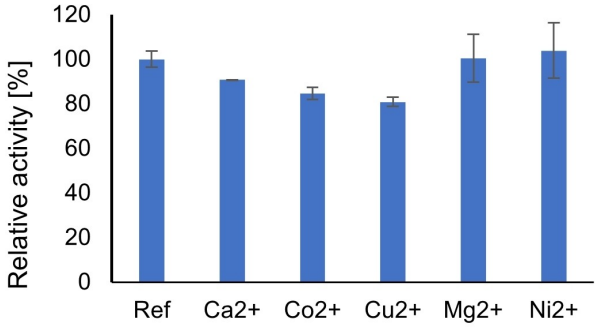

Figure 3. Effect of divalent cations (100 μM) on the relative ammonia-lyase activity of the native aspartase from *Pseudomonas fluorescens* R124. Activity of PfAAL (3 μg mL^{-1}) with L-aspartic acid (15 mM) was measured in 50 mM Tris buffer (pH 8.8) at 30 °C as reference.

Divalent cations play an important role in the catalytic action of several ammonia-lyases influencing both the substrate binding and the catalysis itself.[19,38] Thus, the catalytic performance of the purified and dialyzed PfAAL was investigated in the presence of different divalent cations (Figure 3). No significant effect was experienced in the presence of Ni^{2+} and Mg^{2+}. However, the enzymatic activity decreased with 10–20% in the presence of the Ca^{2+}, Cu^{2+} or Co^{2+}.

Immobilization of PfAAL on magnetic nanoparticles (MNPs)

Inspired by our previous studies on immobilization of different enzymes on MNPs as useful carriers for the enzymes and cofactors[29,39–42] covalent immobilization of PfAAL has been performed on MNPs etched by aminopropyl(trimethoxy)silane and activated by glycerol diglycidyl ether and glycidol in 1:1 molar ratio (Figure 1). Glycidol was used as a thinning component in surface functionalization. Applying components providing groups being inert for covalent bond formation during the surface modification will affect the surface density of the reactive functional groups, the microenvironment in the vicinity of immobilized enzyme, and the stabilizing secondary interactions between the carrier and the enzyme; thereby influencing significantly the specific biocatalytic activity.[43] The effect of end-capping the unreacted epoxy groups after the immobilization has been investigated as well. For this reason, the PfAAL-MNPs were treated with either ethanolamine or glycine after the immobilization. Because the treatment with these agents resulted in decrease of the biocatalytic activity (ESI 5.2), the PfAAL-MNPs were employed without end-capping for the further studies.

The immobilization of purified PfAAL onto this epoxy-modified MNPs (5 mg mL^{-1}) was performed at various enzyme concentrations (30, 50, and 500 μg mL^{-1}), followed by activity tests with the immobilized biocatalyst and with the residual activity of the supernatant after the immobilization.[44] The highest specific activity without any detectable enzyme activity in the residual supernatant was obtained at 30 μg mL^{-1} PfAAL concentration. Although at 50 μg mL^{-1} enzyme concentration no residual activity was detectable in the supernatant, the specific activity of the MNP-bound PfAAL was 70% – as compared to the 30 μg mL^{-1} case – indicating decreased apparent activity due to restricted enzyme mobility caused by the enhanced steric crowd. This effect was even more pronounced when immobilization was performed at 500 μg mL^{-1} enzyme concentration, because only 10% of specific activity of the MNP-bound PfAAL could be obtained while 10% of the initial activity remained in the supernatant

(see Table 1). Under the best condition (30 μg mL^{-1} PfAAL concentration) a PfAAL-MNP preparation was obtained with 6 μg PfAAL per mg biocatalyst. The stability of the immobilized PfAAL was excellent, no significant loss in the activity of the immobilized biocatalyst could be detected after 10 cycles of test reaction (Figure 4).

Our tests after 3 h reaction time revealed protein leaching comparable to the detection limit (< 2% of the protein content of the PfAAL-MNP biocatalyst, see Table S1 in ESI), therefore subunit dissociation cannot be the major reason of the slight enzyme inactivation.

The immobilized AAL showed similar trends of pH dependence as the native enzyme (ESI Figure S17) and the presence of divalent cations had similar effect on the immobilized enzyme (ESI Figure S18) as on the native form as well. The temperature dependence on PfAAL-MNP (ESI Figure S17) was the most dissimilar to the behavior of the native form because the loss of activity occurred more abruptly. We assume that the shearing forces and particle collisions during shaking influence the dissociation of the tetrameric enzyme on the surface of MNPs more seriously as in native solution.

Table 1. Immobilization efficiency determination by assaying the supernatant's PfAAL activity with 10 mM L-Asp.

Enzyme conc [μg mL^{-1}]	$U_{spec,enz}$ [mM min^{-1} mg^{-1} enzyme]	Supernatant activity [%]	Y_{immob} [%]
500	0.107	9	91
50	0.864	0	100
30	1.191	0	100

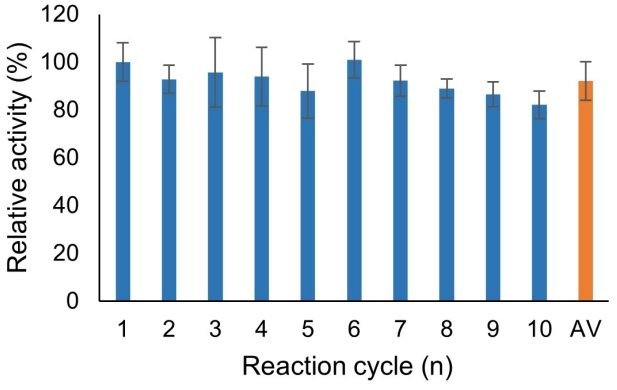

Figure 4. Stability of immobilized AAL from *Pseudomonas fluorescens* R124. Ammonia-lyase activity from L-aspartic acid was measured in 10 cycles of test reaction (30 °C, 15 min; each) without significant loss in activity. 'AV' corresponds to the average of 10 cycles.

Table 2. Kinetic parameters of native and immobilized PfAAL.

Enzyme type	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [mM^{-1} s^{-1}]
Native	5.1 ± 0.3	129.7 ± 1.9	25.3
Immobilized	18.4 ± 1.2	20.1 ± 0.5	1.1

Kinetic characterization of native and immobilized PfAAL

The kinetic behavior of the PfAAL was investigated both in the native and immobilized form of the enzyme. The purified, native PfAAL catalyzed the ammonia elimination from Asp with a K_M value of 5.1 mM at pH 8.8 (Table 2). The saturation curve was hyperbolic, and the Hill coefficient analysis gave a Hill constant of 1.0, indicating that PfAAL obeys Michaelis-Menten kinetics (see Figures S4–S6 in ESI).

The Michaelis-constant of PfAAL is somewhat higher than that of other aspartases, such as AspA from *E. coli* W (K_M = 1.2 mM) or thermostable aspartase from *Cytophaga* sp. (K_M = 1.09 mM), but lower than that of AspB from *Bacillus* sp. YM55-1 8 (K_M = 28 mM).[10,14,36] Although allostery is quite common for aspartases,[14,36] PfAAL showed no signs of cooperativity in the ammonia elimination from Asp. PfAAL turned out to be an efficient enzyme having a turnover number (129.7 ± 1.9 s^{-1}) almost as high as of *E. coli* aspartase (180 s^{-1}). All other reported wild-type aspartases have lower turnover number in the ammonia elimination of Asp.[14,45,46]

Multipoint covalent immobilization onto a solid support can significantly enhance the stability of biocatalysts and MNPs are advantageous carriers for enzyme immobilization because of their high surface-to-volume ratio and magnetic properties enabling easy separability, as well as their good chemical and mechanical stability.[29,42] However, the immobilization of enzymes changes their effective kinetic parameters. In the case of PfAAL-MNP, significant alteration of the effective kinetic parameters of the biocatalyst was observed: the affinity towards Asp (K_M) decreased 3.6-fold while the catalytic velocity (k_{cat}) decreased 6.4-fold (Table 2). The altering effect of immobilization can be explained rigidification of PfAAL by the random covalent bonds to the MNP surface lowering the conformational mobility of the tetrameric enzyme.

Storage stability of the immobilized biocatalysts

Next, the storability of the PfAAL-MNP biocatalyst was tested in Tris buffer (pH 8.8, 50 mM) and in the same buffer supplemented with 10 v/v% glycerol or 10 v/v% PEG400, at 4 °C and 30 °C (Figure 5A). Glycerol is a commonly used protective compound for the stabilization of proteins.[47] PEG400 was also chosen as potential stabilizing agent due to its protein stabilization properties in the structural studies of AspB.[2,48,49]

The residual activity of the PfAAL-MNP samples in these media monitored for 4 days revealed no significant difference in the activity drop at 30 °C or 4 °C storage temperature: after the first day the residual activity was around 30% which after the fourth day dropped below 10%. The storage buffer supplemented with 10 v/v% PEG400 showed similar activity decrease at 4 °C as the one without additives. Surprisingly, the biocatalyst lost its activity at 30 °C in storage buffer with 10 v/v% PEG400 even after the first day. The Tris buffer complemented with 10 v/v% glycerol provided the best protective effect for PfAAL-MNP during storage resulting in significantly higher residual activities than the other two storage media. Biocatalysts stored

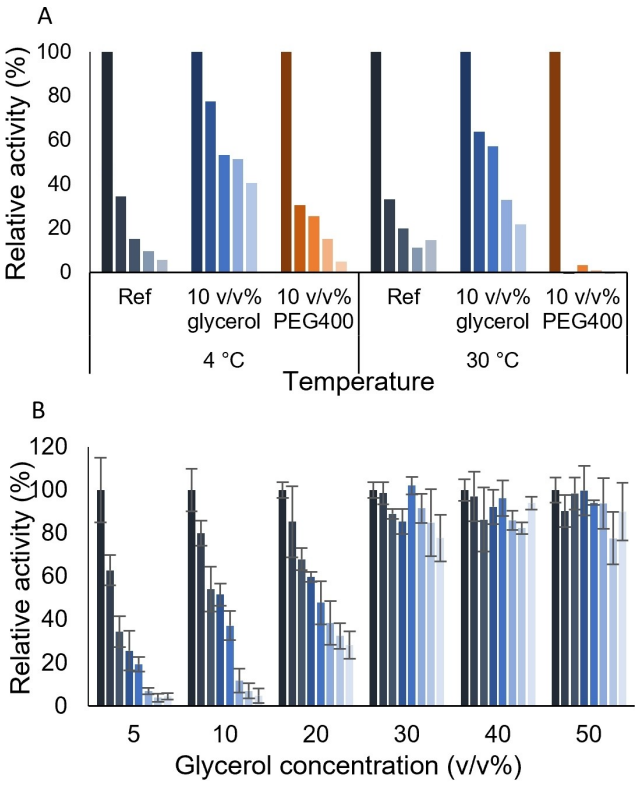

Figure 5. Effect of the storage conditions (A) and the glycerol amount (B) on the relative residual ammonia-lyase activity of the PfAAL-MNP. A) Preliminary series at two different temperatures (4 °C or 30 °C) with single sampling in Tris buffer: Ref (without additive, colored with grey), in Tris buffer supplemented with 10 v/v% glycerol (blue) and 10 v/v% PEG400 (orange). Bars appear in the following order: initial activity-100%, and relative activities measured after 1, 2, 3 and 4 days, respectively. B) Effect of the glycerol amount in Tris buffer on the relative residual ammonia-lyase activity of the PfAAL-MNP after different periods of storage: ▪: initial, ▪: 1 d, ▪: 2 d, ▪: 3 d, ▪: 4 d, ▪: 7 d, ▪: 10 d, ▪: 14 d.

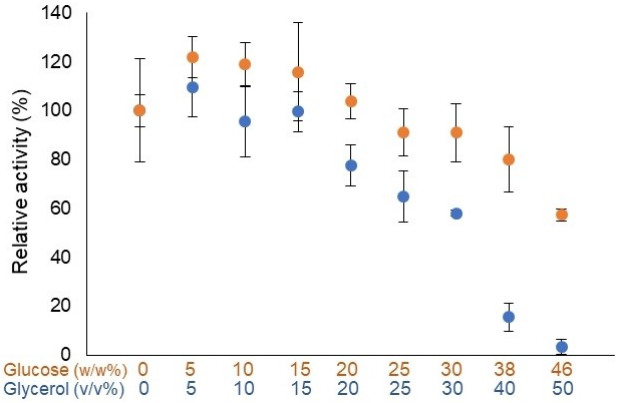

Figure 6. Effect of growing viscosity on the relative ammonia-lyase activity of the PfAAL-MNP in Tris buffer supplemented with glycerol (blue) and with glucose (orange).

at 4 °C in the presence of 10 v/v% glycerol had 80% residual activity after the first day, and 40% after the fourth day. As glycerol proved to be an effective protective agent for the storage of PfAAL-MNP, next the glycerol concentration was studied further aiming to achieve the optimal storage stability (Figure 5B). The residual activity of PfAAL-MNP in Tris buffer supplemented with glycerol between 5 and 50 v/v% were investigated for a 14-day long period at 4 °C. The low glycerol contents (5 and 10 v/v%) could not preserve PfAAL-MNPs which lost almost all their activity until the end of the 14 days. The 20 v/v% glycerol content provided enhanced protection, preserving about 30% of the initial activity after 14 days. The biocatalysts stored in Tris buffer supplemented with 30 v/v% to 50 v/v% glycerol preserved about 80% of the initial activity of PfAAL-MNPs even after 14 days.

Next, the biocatalytic activity of freshly prepared PfAAL-MNP was investigated in the presence of viscosity increasing compounds, such as glycerol and glucose (see Figure 6, for the composition of the used solution see Section 7 in ESI). It was shown that a small increase in viscosity (from 0.8 to 1.3 mPas, up to approx. 15% glycerol or glucose content) had no significant effect on the activity of the immobilized PfAAL, or even resulted in a slight enhancement of the activity. However, further increase of viscosity (from 1.5 to 5.7 mPas, corresponding to approx. 20–50% glucose or glycerol content) resulted in a significant and gradual decrease of activity correlating with the increase of viscosity of the reaction medium. The activity of PfAAL in 20 v/v% glycerol was about 80% of the initial activity, while above 40 v/v% glycerol concentration the relative activity dropped under 10%. These observations motivated us to further investigate the activity of immobilized enzymes as a function of the medium's viscosity.

Effect of the glycerol content on the kinetics of native and immobilized PfAAL

During catalysis, some enzymes undergo large conformational changes as they progress through the catalytic cycle, solvent viscosity results in friction against proteins in solution, and this should result in decreased motion, inhibiting catalysis in motile enzymes.[50] Kramers' theory has been used to describe the effect of viscosity on the behavior of protein reactions where conformational changes are involved, stating that the reaction rate depends linearly on the viscosity of the solution.[51] Glycerol is a commonly used protective compound for the short and long term storage of enzyme solutions, but because of its high viscosity it can influence the enzymatic activity if it is present during the reaction as well. Based on our preliminary studies we aimed to study the effect of viscosity on the activity of the native and immobilized form of PfAAL. The correlation between the increasing viscosity and decreasing maximum reaction rate has been described for native enzymes, but in the case of immobilized enzymes, this effect has been examined only quite scattered.[50] Since the movement of the flexible SS-loop is essential for the catalytic function of aspartate ammonia-lyases, the PfAAL is an excellent candidate to investigate this effect.

Thus, the kinetic behavior of native and immobilized PfAAL was studied in the presence of increasing amounts of glycerol (Table 3). Expectedly, the turnover number of PfAAL decreased

Table 3. Kinetic parameters of native and immobilized PfAAL in media with different glycerol amounts.

Glycerol [v/v%]	K_M[a] [mM]		k_{cat}[b] [s^{-1}]		k_{cat}/K_M [$mM^{-1} s^{-1}$]	
	nat.	immob.	nat.	immob.	nat.	immob.
0	5.1	18.4	129.7	20.1	25.3	1.1
5	4.1	22.6	103.7	17.7	25.6	0.8
15	2.2	14.1	58.7	11.8	26.5	0.8

[a] Relative error is under 10%. [b] Relative error is under 5%.

significantly with the increasing viscosity in its native as well as in its immobilized form. Even a relatively small increase in the viscosity at 5 v/v% glycerol amount caused 20% decrease in the enzymatic activity, and by 15 v/v% glycerol only 60% of the original reaction rate was achieved. This effect can be fully attributed to the increasing viscosity hindering the structural movements of the protein. Notable, that the K_M values dropped significantly as well which besides the change of viscosity can be rationalized at least partially by the drop of polarity of the bulk medium. The kinetic characterization of PfAAL-MNP at different concentrations of glycerol (0; 5; 15 v/v%; see Figures S6–S8 in ESI) showed that immobilization of PfAAL on MNPs resulted in decreased turnover number and affinity as compared to the native form of the enzyme due to the enhanced mass transfer limitations and possible rigidification of the enzyme (Table 3).

As stated by Uribe et al., if a reaction obeys Kramers' theory, a linear relationship should be observed between the rate constant of the reaction and the solution viscosity.[50] We wanted to investigate, if PfAAL obeys this theory especially in its immobilized form. As the kinetic parameterization of immobilized enzymes in different conditions is rather time consuming, instead of measuring the full Michaelis-Menten kinetics, the initial reactions rates were measured only at high substrate concentration (60 mM Asp) where the turnover number and the initial reaction rate are directly proportional.

When the relative initial reaction rates (v_0/v – where v_0 is the initial reaction rate without glycerol) were plotted against the relative viscosity (η/η_0 – where η_0 is the viscosity of the reaction without glycerol) a good linear correlation could be observed (Figure 7) indicating that Kramers' theory stands for both the native and the immobilized PfAAL. According to our best knowledge this is the first time when this relationship was remarked for an enzyme immobilized on magnetic nanoparticles. As the catalytic mechanism of PfAAL requires a loop movement, the increased frictions against the solution decreased its motion, lowering the reaction velocity and impeding the reaction.

It should be noted as well, that the initial reaction rate dropped more drastically for the native than for the immobilized PfAAL. This can be interpreted by assuming that conformational changes are already hindered in the case of rigidified immobilized enzyme and the hindrance caused by the enhanced viscosity of the medium is less serious than for the free enzyme.

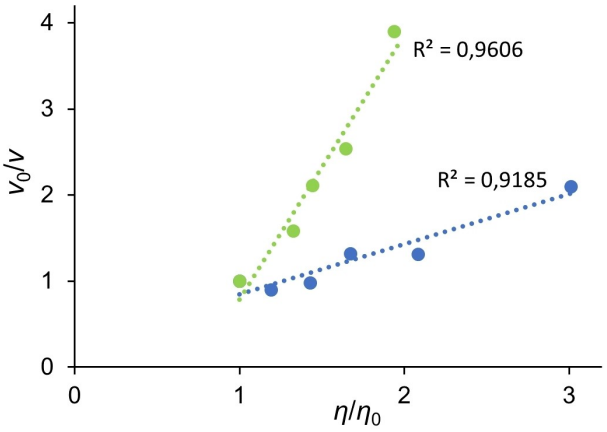

Figure 7. Relative initial reaction rates (fumarate production measured with native and immobilized enzyme in 60 mM Asp) plotted against the relative solution viscosity (0; 5; 10; 15; 20 and 30 v/v% glycerol) for the various forms of PfAAL: ● native PfAAL, ● immobilized PfAAL. The v_o and η_0 refer to the initial reaction rate and viscosity measured without glycerol. Relative error is under 10%.

Conclusion

In this work, we identified, isolated, and cloned the gene of a novel aspartate ammonia-lyase from *Pseudomonas fluorescens* R124 (PfAAL). The enzyme was expressed in *E. coli* and purified with HisTag affinity chromatography. The purified PfAAL proved to be a stable and efficient enzyme in the ammonia elimination of L-aspartate. The enzyme was covalently immobilized on epoxy functionalized MNPs, and the native and immobilized PfAAL was characterized under different conditions. PfAAL had similar kinetic constants than other isolated aspartases but exhibited no allostery. Glycerol proved to be an efficient protective additive for the storage of PfAAL-MNPs. Furthermore, it was shown that the increasing viscosity of the reaction medium impedes the activity of PfAAL-MNP in a similar manner as in the case of the native enzyme.

Experimental Section

General information

All reagents [iron(III) chloride hexahydrate, sodium acetate, polyethylene glycol 400 (PEG 400), and 4000 (PEG 4000), 35% aqueous ammonia solution, (3-aminopropyl)trimethoxysilane (APTMOS), tetraethoxysilane (TEOS), glycerol diglycidyl ether (GDE) and glycidol] and solvents for MNP-carrier synthesis, and the amino acids (L-aspartic acid, L-phenyl-alanine, 3,4-dihidroxy-L-phenylalanine, L-alanine, L-histidine, L-lysine, L-glutamic acid, L-tyrosine, L-serine, L-glycine, L-proline, and L-tryptophane) for substrate profile testing were products of Sigma-Aldrich/Merck (St. Luis, USA/Karlsruhe, Germany).

The primers were purchased from Integrated DNA Technologies BVBA (Leuven, Belgium). The Phusion Flash PCR Master Mix, pJET1.2/blunt cloning vector, Dream Taq Green PCR Master Mix, T4 DNA ligase, Tango 2X buffer, NdeI and BamHI restriction enzymes were purchased from Thermo Fisher Scientific (Waltham, USA). DNA

gel extraction kit and plasmid isolation kit were purchased from BioBasic Canada Inc (Toronto, Canada). Ni-NTA for affinity protein purification was from GE Healthcare (Chicago, IL, USA).

Ammonia-lyase activity measurements of the native enzyme were based on detection of product formation of fumaric acid followed by UV absorbance measurements with Multiskan Sky Microplate spectrophotometer (Thermo Fischer, Waltham USA) with Greiner UV-STar 96 well plate (300 μL; $\varepsilon_{240}=2.0359\ mM^{-1}\,cm^{-1}$) or Genesys 2 UV-Vis spectrophotometer (Spectronic/Thermo Fischer, Waltham USA) with quartz cuvette (1 mL; path 1 cm; $\varepsilon_{240}=2.393\ mM^{-1}\,cm^{-1}$) in thermostated system at 30 °C. The product formation in the immobilized enzyme reactions were detected by Thermo Fischer NanoDrop 2000 spectrophotometer (Thermo Fischer, Waltham USA) in 3 μL volume ($\varepsilon_{210}=1.2486\ mM^{-1}\,mm^{-1}$).

Activity measurements were performed with Genesys 2 spectrophotometer (Spectronic/Thermo Fischer, Waltham USA) in 1 mL reaction volume. To the substrate solution (900 μL, as specified later) was added the enzyme solution (100 μL, 50 mM Tris pH 8.8 and 30 $\mu g\,mL^{-1}$ PfAAL) and the reaction was measured for 2 min at 30 °C. The activity measurements at 240 nm were performed in triplicates.

Enzyme kinetics with soluble PfAAL was measured with Multiskan Sky Microplate spectrophotometer (Thermo Fischer, Waltham USA) in 300 μL reaction volume. To the substrate solution (270 μL, 50 mM Tris pH = 8.8 containing 0.001–40 mM L-aspartic acid) was added the enzyme solution (30 μL, 50 mM Tris pH 8.8 and 15 $\mu g\,mL^{-1}$ PfAAL) and the reaction was measured for 3 min at 30 °C.

The rheological characterization of different solutions and mixtures were performed with Physica MCR 301 rotational viscometer (Anton Paar, Graz, Austria) coupled with a Peltier device for precise temperature setting. The zero-shear viscosity of the samples was measured with cone-plate (diameter of 25 mm/1°) at 30.0 °C in five parallel determinations (Figure S19 in ESI).

Identification and cloning of PfAAL

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 fluorescens* (UniProt ID: P07346) as query within the WGS of *P. fluorescens* R124 (taxid.:742713). The resulted hits had sequence similarities between 37–97%. The sequence of AAL in *P. fluorescens* R124 WGS showed 89% identity to the query *P. aeruginosa* PAO1 AspA sequence (Figure S1). Primers for the cloning of AAL of *P. fluorescens* R124 were designed to contain NdeI and BamHI restriction cleavage sites. The coding DNA was first cloned into pJET1.2 cloning vector, then into pET-15b expression vector using NdeI/BamHI restriction cleavage sites to produce an *N*-terminal His_6-tagged protein (for sequence details see Figure S2).

Expression and purification of PfAAL

Briefly, PfAAL was expressed in *E. coli* Rosetta BL21 (DE3) cells in autoinduction media (Na_2HPO_4, 6 $g\,L^{-1}$; KH_2PO_4, 3 $g\,L^{-1}$; tryptone, 20 $g\,L^{-1}$; yeast extract, 5 $g\,L^{-1}$; NaCl, 5 $g\,L^{-1}$; glycerol, 7.56 $g\,L^{-1}$; glucose, 0.5 $g\,L^{-1}$; lactose, 2 $g\,L^{-1}$) induced by lactose at 28 °C overnight. After harvesting the cells with an Avanti J-25I centrifuge (Beckman Coulter, Indianapolis, USA; 14000 × *g*, 20 min, 4 °C), the wet cells from the expression culture (1 L) were suspended in lysis buffer (50 mL, 150 mM NaCl, 50 mM HEPES, supplemented with protease inhibitors) in a glass beaker. After disrupting the cells by a Sonoplus HD 2070.2 sonicator (Bandelin Electronic GmbH & Co. KG, Berlin, Germany) for 4 min (75% power setting, 10 s intervals with 30 sec resting phase on ice), separation of the cell debris was performed by centrifugation with an Avanti J-25I centrifuge (Beckman Coulter, Indianapolis, USA; 40 min, 22000 × *g*, 4 °C). PfAAL was purified from the supernatant by gravitational Ni-NTA chromatography following the purification protocol of the manufacturer. Composition of all fractions were verified by SDS-PAGE (Figure S3). Imidazole from the pooled fractions containing PfAAL was removed by dialysis against 10-fold excess volume of TRIS buffer (pH 8.0, 50 mM) for 16 h at 4 °C. The PfAAL samples were stored at −80 °C until activity measurements and immobilization.

Characterization of native PfAAL

The substrate acceptance profile of PfAAL was probed with several L-amino acids (L-aspartic acid, L-phenylalanine, 3,4-dihidroxy-L-phenylalanine, L-alanine, L-histidine, L-lysine, L-glutamic acid, L-tyrosine, L-serine, L-glycine, L-proline, and L-tryptophane). In this series of substrate acceptance test, the reaction mixture (1 mL) containing the L-amino acid (10 mM) and PfAAL (6 $\mu g\,mL^{-1}$) in Tris buffer (pH 8.8, 50 mM) was shaken for 5 days at 30 °C.

The pH optimum of PfAAL in ammonia elimination was investigated with L-aspartic acid (15 mM) in a reaction mixture (1 mL) containing buffer (50 mM, sodium phosphate for pH 5–7; Tris for pH 7–9; and sodium carbonate for pH 9–10.5) and PfAAL (3 $\mu g\,mL^{-1}$).

The effect of different divalent metal ions on the activity of PfAAL was investigated with L-aspartic acid (15 mM) in a reaction mixture (1 mL) containing PfAAL (3 $\mu g\,mL^{-1}$) and Tris buffer (50 mM, pH 8.8) supplemented with divalent metal ion chloride (100 μM, Co^{2+}; Mg^{2+}; Cu^{2+}; Ni^{2+}; or Ca^{2+}) or EDTA (100 μM) at 30 °C.

Kinetics of PfAAL was investigated in Tris buffer (50 mM, pH 8.8) with L-aspartic acid (concentration ranging from 0.01 to 40 mM) at different glycerol concentrations (0; 5 and 15 v/v%). Enzyme kinetics data were evaluated with Statistica 13 (TIBCO Software Inc, Palo Alto, USA) using non-linear regression (Figures S6–S8 in ESI).

All experiments were conducted in triplicate.

Preparation of the MNP carrier for immobilization of PfAAL

To produce core MNPs, iron(III) chloride hexahydrate (20.2 g), PEG 4000 (20.2 g) and 54 g sodium acetate were suspended in ethylene glycol (600 mL). After homogenizing the resulted mixture by sonication in an Emmi 20HC ultrasonic bath (Emag AG, Mörfelden-Walldorf, Germany) with full power at 45 kHz for 30 min, the resulting mixture was stirred in a stainless-steel autoclave at 200 °C for 24 h. The formed suspension was washed with distilled water (3 × 200 mL) and with 2-propanol (3 × 150 mL) applying a neodymium magnet enabling decantation of the washing liquids from the MNP suspension, followed by drying in a VDL 23 vacuum drying chamber (Binder GmbH, Tuttlingen, Germany) at room temperature and 100 mbar overnight.

For silica shell formation, the core MNPs (2.5 g) were suspended in a mixture of ethanol (87.5 mL) and distilled water (12.5 mL) and PEG 400 (2.5 g) by sonication in an Emmi 20HC ultrasonic bath with full power at 45 kHz for 30 min. After adding 35% aqueous ammonia solution (6.25 mL) and TEOS (3.75 mL) into the resulted MNP suspension, the resulted mixture was shaken for 24 h at room temperature. After washing the formed silica covered MNPs with distilled water (4 × 15 mL) and ethanol (3 × 10 mL), the product was dried in vacuum as described above.

For amine functionalization, the silica covered MNPs (1.5 g) were suspended in a solution of PEG 400 (675 mg) and ethanol (15 mL) by sonication in an Emmi 20HC ultrasonic bath with full power at 45 kHz for 30 min. After addition of 35% aqueous ammonia solution (120 μL) to the suspension and shaking for 10 min, APTMOS (262 μL) was added, and the suspension was shaken further at room temperature for 24 h. The formed amine-functionalized MNPs were washed with 2-propanol (3×5 mL) and dried in vacuum as described above.

For epoxy functionalization, the amine-functionalized MNPs (150 mg) were suspended in 2-propanol (4 mL) by sonication in an Emmi 20HC ultrasonic bath with full power at 45 kHz for 15 min. After addition of GDE (43.5 μL) and glycidol (17.4 μL, required for 1:1 molar ratio of GDE and glycidol) to the suspension and shaking at 60 °C for 24 h, the formed epoxy functionalized MNPs were washed with ethanol (3×5 mL) and hexane (3×5 mL) and dried in vacuum as described above.

Immobilization of PfAAL onto epoxy functionalized MNP carrier

In a single immobilization within a series of experiments, the epoxy functionalized MNPs (5 mg) were suspended in Tris buffer (500 μL, 50 mM, pH 8.8) by sonication in an Emmi 20HC ultrasonic bath with full power at 45 kHz for 15 min. To the formed suspension was added PfAAL solution (500 μL, with varied enzyme concentration of 60, 100, and 1000 $\mu g\,mL^{-1}$), and the mixture was shaken at 600 rpm at 30 °C for 2 h. The formed PfAAL-MNP biocatalyst was washed with Tris buffer (2×1 mL, 50 mM, pH 8.8) using neodymium magnet for retaining the PfAAL-MNPs during separation. The AAL activity of the supernatants was investigated with a Spectronic Genesys 2 UV-Vis spectrophotometer (see Section 5 in ESI).

Activity and storage tests with the immobilized PfAAL

The enzyme activity of the PfAAL-MNP biocatalyst (5 mg; 6 $\mu g\,mg^{-1}$ PfAAL on MNP; from the immobilization with 30 $\mu g\,mL^{-1}$ PfAAL concentration) was determined in a reaction mixture (1 mL) containing L-aspartic acid (20 mM) in Tris buffer (1 mL, 50 mM, pH 8.8) at 30 °C 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).

For recyclability tests, the reactions described above as activity test were conducted for 15 min in 10 consecutive cycles. Between cycles, the PfAAL-MNP biocatalysts were sedimented with neodymium magnet and washed two times with Tris buffer (1 mL, 50 mM, pH 8.8). Protein leaching after 3 h reaction time has been determined by Bradford method (see Section 5.1 in ESI).

To investigate the effect of the reaction media viscosity on the immobilized PfAAL-MNP, the biocatalysts (5 mg) were tested in reactions containing 20 mM L-aspartic acid (50 mM Tris buffer, pH 8.8) supplemented with different concentrations of glycerol (0; 5; 10; 15; 20; 25; 30; 40 and 50 v/v%) or glucose (0; 5; 10; 15; 20; 25; 30; 38 and 46 w/w%). Before the activity test, the biocatalyst was preincubated for 15 min in the glycerol or glucose supplemented 50 mM Tris buffer.

For the kinetic studies, the PfAAL-MNP (5 mg) were tested in 50 mM Tris buffer (pH 8.8) supplemented with different concentrations of glycerol at substrate concentrations ranging from 0.1 to 60 mM L-aspartic acid. The kinetic data was evaluated using non-linear regression of Statistica 13 (TIBCO Software Inc, Palo Alto, USA) (Figures S12–S15 in ESI)

For storage stability tests, first the initial activity of the PfAAL-MNPs was tested. Then the samples of PfAAL-MNP biocatalyst (5 mg, for each measurement) were stored in Tris buffer (500 μL, 50 mM, pH 8.8) without additive or supplemented with glycerol (5, 10, 15, 20, 30, 40, and 50 v/v%), or with PEG 400 (10 v/v%) at 4 °C or 30 °C. Before the activity tests after the given storage time, the PfAAL-MNP biocatalysts were preincubated at 30 °C for 30 min and washed twice with Tris buffer (1 mL, 50 mM, pH 8.8).

All experiments were conducted in triplicates.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: aspartases · aspartate ammonia-lyases · biocatalysis · enzyme kinetics · magnetic nanoparticles · viscosity

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