A study on the applicability of L-aspartate α-decarboxylase in the biobased production of nitrogen containing chemicals

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β-Alanine could serve as an intermediate in the biobased production of nitrogen containing chemicals from L-aspartic acid. Following the biorefinery concept, L-aspartic acid could become widely available from biomass waste streams *via* the nitrogen storage polypeptide cyanophycin. Since α-decarboxylation of L-aspartic acid is difficult to perform chemically, the applicability of *Escherichia coli* L-aspartate α-decarboxylase (EC 4.1.1.11) (ADC) for the production of β-alanine was studied. With an increasing activity up to 90 °C and maintaining its activity upon storage for 24 hours at 60 °C, ADC showed a remarkably high thermostability. ADC has an optimum at pH 7.5 and starts to lose activity upon storage below pH 6. An inhibiting effect by β-alanine was not observed. Immobilization on Sepabeads EC-EP and EC-HFA epoxy supports did not result in an increased thermostability, but did improve operational stability. Nonetheless, enzyme inactivation occurs during catalysis, probably caused by irreversible transamination of the catalytically essential pyruvoyl group.

Introduction

Global warming, depletion of fossil resources and geo-political instability are forcing us to make the transition towards CO_2 neutral, sustainable and economically stable alternatives for our naphtha based society. In this respect, biomass has great potential. At the moment of writing, the emphasis is on the application of biomass as a feedstock for energy and transport fuels. However, the application of biomass as a feedstock for functionalized bulk chemicals receives far less attention. This is remarkable considering the enormous amounts of functionalized compounds (*e.g.* amino acids, organic acids, and lignins) that are generated as side streams of the continuously growing biofuel production.[1]

Nowadays, most nitrogen containing bulk chemicals (*e.g.* 1,4-butanediamine and acrylamide) are produced from naphtha. In these processes, large amounts of energy and ammonia are used for the ammoxidation of olefins.[2] Following the biorefinery concept, it would be more energy efficient to start from functionalized compounds that already have nitrogen incorporated, such as amino acids, instead of starting from naphtha's plain hydrocarbons.[3] Bearing this in mind, our attention has been drawn to the polypeptide cyanophycin (CGP).

CGP consists of a poly(L-aspartic acid) backbone with L-arginine branches in equimolar amounts and is naturally produced in cyanobacteria as a nitrogen storage polymer.[4] Heterologous expression in industrially relevant bacteria[5–8] opened up the possibility to produce CGP in considerable amounts. Even more interestingly, recent work on *Saccharomyces cerevisiae*[9,10] has opened possibilities for the simultaneous production of CGP and ethanol from amino acid rich waste streams from the agro-industries.[11–13]

Since CGP is insoluble under physiological conditions, it can be easily isolated[14,15] which together with its defined composition, its integrated production and its high nitrogen content make CGP a potential feedstock for the production of nitrogen containing bulk chemicals.[13]

Our research is focused on the development of integrated processes for the conversion of CGP's hydrolysate, L-aspartic acid and L-arginine, towards existing nitrogen containing bulk chemicals. Starting from L-aspartic acid, we envision β-alanine (3-aminopropionic acid) as intermediate in the production of nitrogen containing bulk chemicals such as acrylamide and acrylonitrile (Fig. 1).

In this route, L-aspartic acid is decarboxylated at the α-position yielding β-alanine and carbon dioxide. Examples of non-enzymatic methods for the α-decarboxylation of L-aspartic acid and analogues such as L-glutamic acid are rather limited.[16,17] For this reason we focus on the applicability of the enzymatic α-decarboxylation of L-aspartic acid by *E. coli* L-aspartate α-decarboxylase (EC 4.1.1.11) (ADC) leading to β-alanine as the sole product.[18] The only study known to the authors that investigated the applicability of ADC concerns a patent on the conversion of D/L-aspartic acid to D-aspartic acid and β-alanine using whole cell biocatalysis.[19]

When considering the application of an enzyme, the temperature and pH dependent activity and stability are crucial. Previously, the optimal pH (pH 6.5–7.5) and temperature (55 °C) for ADC were determined.[18,20] However, this information is not very detailed and apart from the conditions for optimal activity, also information about the temperature and pH dependent stability of ADC is required.

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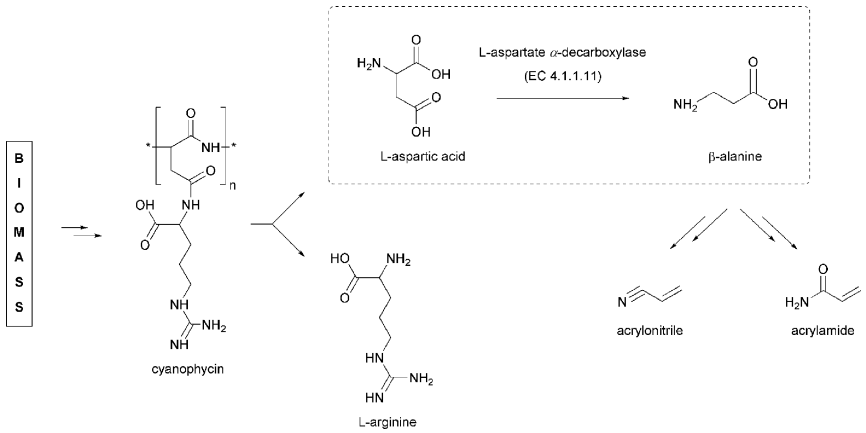

Fig. 1 Route from cyanophycin towards nitrogen containing bulk chemicals with the enzymatic α-decarboxylation of L-aspartic acid as an intermediate step.

On an industrial scale immobilization of enzymes is desirable, because this enables reuse and overcomes problems with product recovery. Additionally, immobilization might enhance the enzyme's stability towards the stressed conditions of an industrial process.[21] Due to their ease of operation and their robustness under physical stress, epoxy activated Sepabeads are suitable supports for immobilization on an industrial scale.[22,23]

In this paper, we present the results of a study on the applicability of *Escherichia coli* L-aspartate α-decarboxylase for the production of β-alanine from L-aspartic acid. For the soluble enzyme, the temperature and pH dependent activity and stability and the effect of product inhibition were investigated. ADC was immobilized on two different types of Sepabeads supports: the relatively hydrophobic epoxy support EC-EP and the relatively hydrophilic amino-epoxy support EC-HFA. The thermal and operational stability of the immobilized and soluble enzyme were compared.

Results and discussion

Product inhibition

Williamson and Brown[18] did not observe product inhibition for ADC, based on an assay with 1 mM L-aspartic acid and 0.250 mM β-alanine. In the current study, the inhibiting effect of β-alanine was investigated within a range of different combinations of β-alanine and L-aspartic acid concentrations. Using SigmaPlot 10.0 (Systat Software, Inc.) in combination with Enzyme Kinetics Module 1.3 (Systat Software, Inc.), a Michaelis–Menten competitive inhibition model was fitted to the obtained specific activities.

A first observation of the results clearly revealed that β-alanine does not have an inhibiting effect in the observed concentration range (results not shown). Subsequently, the Michaelis–Menten competitive inhibition model returned an extremely high and insignificant product inhibition constant. Although an inhibiting effect at higher concentrations of β-alanine can not be ruled out, it can safely be assumed that with conversions at higher concentrations of L-aspartic acid inhibition by β-alanine can be neglected.

Fitting a Michaelis–Menten model without inhibition returned a Michaelis–Menten constant (K_M) of 0.240 ± 0.011 mM and a maximum activity (V_{max}) of 640 ± 8 nmol min^{-1} mg^{-1}.

Temperature dependent activity and stability

The results of the temperature dependent activity and stability study are remarkable. Instead of a temperature optimum, ADC showed an increasing specific activity from 224 nmol min^{-1} mg^{-1} at 25 °C to 4390 nmol min^{-1} mg^{-1} at 90 °C (Fig. 2, 0 h incubation). The absence of an optimum contradicts previous findings that report a temperature optimum at 55 °C with half-maximal activities at 26 °C and 78 °C.[18,20]

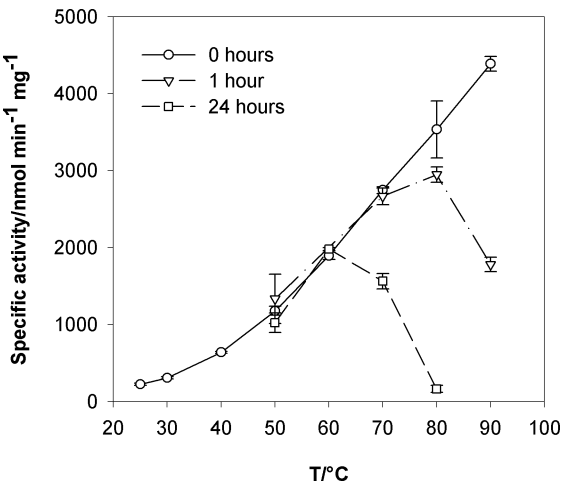

Fig. 2 Temperature dependent activity and stability of ADC. ADC solutions were incubated in 50 mM sodium phosphate buffer pH 7.0 for 0, 1, and 24 hours at the given temperatures. Directly after incubation, L-aspartic acid was added up to 1.5 mM and the specific decarboxylase activity was determined at the same temperature. The error bars represent standard deviation; the depicted lines are a guide to the eye.

The temperature dependent stability of ADC was determined by performing activity assays at different temperatures after one and 24 hours of incubation at the same temperature. ADC was stable for one hour up to 70 °C (Fig. 2, 1 h incubation) and for 24 hours up to 60 °C (Fig. 2, 24 h incubation).

Although pyruvoyl-dependent L-aspartate α-decarboxylases from other sources have been isolated and studied, the thermostability was not determined in these cases.[24,25] It is difficult to make a justified comparison with other pyruvoyl-dependent decarboxylases, because although they are all activated *via*

autocatalytic serinolysis and share similar decarboxylation mechanisms, they share little structural similarity.[26,27] It is, however, notable that the thermostability of *E. coli* ADC is comparable to that of pyruvoyl-dependent *S*-adenosylmethionine decarboxylase from *Sulfolobus solfataricus* (stable at 50 °C for 16 hours)[28] and L-arginine decarboxylase from *Methanococcus jannachii* (50% activity after 20 minutes at 121 °C),[29] both hyperthermophiles.

pH Dependent activity and stability

The pH optimum for ADC was found at pH 7.5 (Fig. 3, 0 h incubation). This observations is in agreement with the results of Williamson and Brown.[18] Twenty-four hours of incubation revealed that ADC was stable under alkaline conditions, but lost activity below pH 6 (Fig. 3, 24 h incubation).

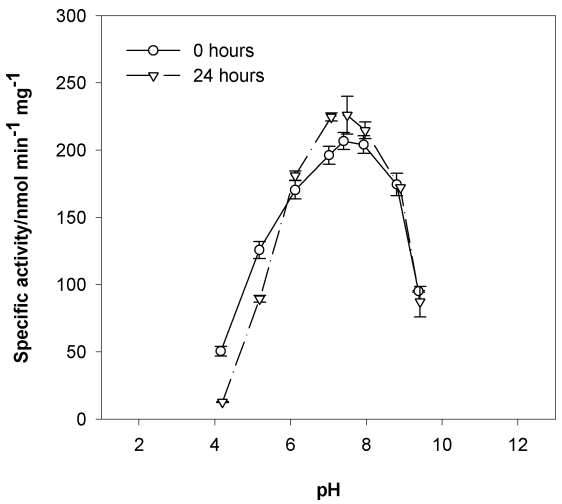

Fig. 3 pH Dependent activity and stability of ADC. Directly or after 24 hours of incubation at 30 °C, the specific activity of ADC solutions with different pH values was determined after addition of L-aspartic acid up to 1.5 mM. To maintain a constant ionic strength, a three component buffer was used.[30] The error bars represent the standard deviation; the depicted lines are a guide to the eye.

The optimum at pH 7.5 and the instability at acidic conditions have their impacts on the application of ADC in the production of β-alanine. It would be beneficial if ADC could be applied in a buffer-free process, since addition of buffer agents introduces salts to the reaction mixture that most probably need to be removed before further conversion of β-alanine.

A buffer-free solution of 50 mM L-aspartic acid (close to saturation) will have a pH of approximately 3, which will increase when L-aspartic acid is converted to the less acidic β-alanine. However, assuming that the released carbon dioxide will dissipate, the pH of the reaction mixture will only start to rise above pH 6 at 95% conversion.[31] Furthermore, buffering the reaction mixture using continuous or fed-batch process designs, in which the presence of previously formed, more basic β-alanine results in a less acidic environment, will never lead to the optimum of pH 7.5. Therefore, addition of a buffer agent to the reaction mixture will be necessary to achieve and maintain the highest activity for ADC.

Immobilization of L-aspartate α-decarboxylase

It is proposed that protein immobilization on epoxy supports follows a two-step mechanism: (i) first the enzyme is adsorbed to the surface of the support and (ii) subsequently the protein's nucleophilic groups (amino, thiol, or hydroxyl groups) react with the epoxy groups on the support to form covalent bonds.[32] Following this mechanism, the hydrophobicity of the support plays a major role in the binding performance of the support. For this reason it was decided to compare the immobilization of ADC on the relatively hydrophobic epoxy support EC-EP and the relatively hydrophilic amino-epoxy support EC-HFA.[33,34] To facilitate adsorption, the recommended buffer concentrations were used: 1 M for EC-EP and 5 mM for EC-HFA.[35]

The residual specific activity of ADC in the supernatant was followed during immobilization. In the case of both supports, the specific activity of ADC in the supernatant disappeared within the hour (results not shown). Comparisons between the two supports made with other enzymes under similar conditions, show a much slower adsorption to EC-EP supports even though four times lower protein concentrations were used (0.9 mg protein g^{-1} support).[33,35] Possibly the size of the enzymes plays a role in the rate of adsorption, since ADC in its tetrameric form of 55 kD[36] is notably smaller than the other enzymes studied (90 to 270 kD).[33,35,37]

After immobilization, both ADC preparations were washed and the amount of enzyme lost through leaching from the supports was determined using Bradford protein assays (results not shown). Using this data, the immobilization yields were calculated and it appeared that ADC was to a greater extent covalently bound to EC-EP (94.8%) than to EC-HFA (88.0%).

Aliquots of EC-EP and EC-HFA were incubated in 3 M glycine at pH 8 to block the remaining epoxy groups in the supports. Since both the substrate and product are amino acids, their amino groups might react with remaining epoxy groups. However, more importantly, these epoxy groups might interact with enzyme residues during catalytic operation, leading to deactivation.[38] Blocking of remaining epoxy groups by incubation in an excess of glycine has become a common step in protocols for protein immobilization on epoxy supports.[39]

After immobilization, the recovered activity (the activity relative to the expected activity) for all ADC preparations was determined (Table 1). The recovered activity was higher for EC-HFA (89%) than for EC-EP (80%). Taking into account the immobilization yields, the difference can be explained by the fact that covalent binding of ADC to EC-HFA occurred to a lesser extent and thus led to less deactivation during immobilization. Blocking of the ADC preparations with glycine appeared to have a negative effect on the recovered activity (Table 1).

Table 1 The recovered activity, the activity relative to the expected activity, of different ADC preparations

ADC preparations	Recovered activity (%)
EC-EP	80 ± 0.5
EC-HFA	89 ± 1.6
EC-EP blocked	76 ± 5.4
EC-HFA blocked	72 ± 0.9

Overall, when compared to studies with EC-EP and EC-HFA applied with other enzymes, the recovered activities for immobilized ADC are quite good.[33,40–42]

Thermostability of L-aspartate α-decarboxylase preparations

Since ADC appears to have a high thermostability, the thermostability of immobilized and soluble ADC was compared by following their residual activity in time at a relatively high temperature of 80 °C to be able to observe deactivation (Fig. 4). The thermostability of EC-HFA preparations is much lower than that of EC-EP preparations and that of soluble enzymes. It is difficult to give a direct explanation for this observation, but the fact is that covalently bound to ADC, EC-EP and EC-HFA only differ in the structure of their spacer arms.

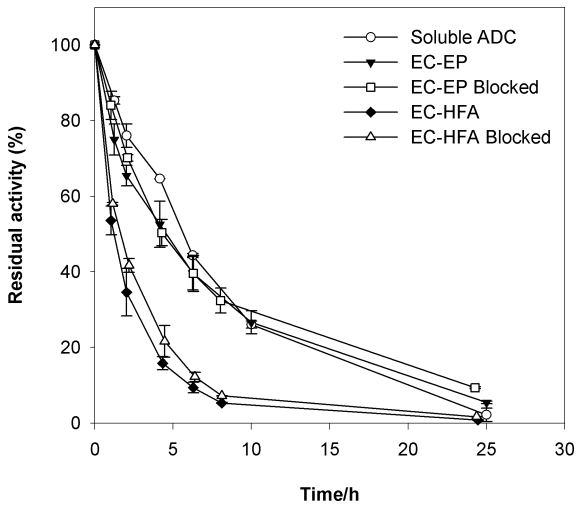

Fig. 4 Thermostability of immobilized ADC compared to soluble ADC. Aliquots of ADC preparations were incubated in 50 mM sodium phosphate buffer pH 7.0 at 80 °C and in course of time the residual activity was determined upon addition of L-aspartic acid up to 1.5 mM. The error bars represent standard deviation; the depicted lines are a guide to the eye.

When covalently bound, EC-EP has relatively short (two bond lengths), apolar spacer arms, while EC-HFA has relatively long (fifteen bond lengths), polar spacer arms. It has been suggested by Mateo *et al.*[43] and more recently by Magner *et al.*[44] that short spacer arms (two to three bond lengths) are beneficial for the conformational stability of the covalently bound enzyme.

Binding through multiple short spacer arms provides that immobilized enzyme residues are likely to preserve their relative positions in the event of heat induced conformational change. However, the stabilizing effect of covalent immobilization through the short spacer arms of EC-EP does not seem to add up to the already remarkable thermostability of soluble ADC (Fig. 4).

Accordingly, EC-HFA's significantly longer spacer arms provide a higher degree of conformational freedom and, therefore, the enzyme is more susceptible to heat induced conformational changes leading to deactivation. This could explain why ADC immobilized on EC-HFA even shows a lower thermostability than soluble ADC (Fig. 4).

Although immobilization on EC-HFA has a negative effect on the thermostability of ADC, it must be stated that there are examples of enzymes that show an increased thermostability upon immobilization on EC-HFA[35,41] and that compared to EC-HFA the relatively short EC-EP spacer arms are not always beneficial.[33,42,45]

Fig. 4 shows that blocking with 3 M glycine at pH 8 did not have a significant effect on the thermostability of the ADC preparations. While in some studies the blocking of remaining epoxy groups under similar conditions was applied as a standard procedure,[33,40,42,46] Fig. 4 stresses that not every aspect of an immobilization procedure can be generalized for each and every enzyme and some steps could be left out or should be optimized.

Although immobilization on Sepabeads did not lead to an increased thermostability, it was successful in the case of EC-EP in the respect that ADC was converted into a heterogeneous catalyst while retaining its high thermostability. This opens possibilities for application of ADC in continuous processes.

Operational stability of L-aspartate α-decarboxylase preparations

In order to test the operational stability, the reusability of immobilized ADC was tested at 30 °C in 30 minutes batch cycles. After each cycle, the supernatant of the reaction mixtures was discarded and replaced by fresh buffer and the next cycle was started by adding substrate. After four cycles, all ADC preparations showed a decrease in activity of approximately 30% (results not shown).

Activity and Bradford protein assays applied on the discarded supernatant ruled out the possibility of ADC leaching from the supports. Additional to the standard applied filter-sterilization, addition of sodium azide or protease inhibitor (Complete, Roche), degassing of the reaction mixture before and after each cycle to diminish the effect of CO_2 release or different methods and speeds of mixing had no effect on the strong deactivation over the cycles.

Based on these results, it was decided to compare the operational stability of immobilized ADC with that of soluble ADC using batch experiments. For all ADC preparations the amount of β-alanine produced was plotted against the amount of ADC spent, giving the total turnover number (TTN) (Fig. 5).

The linearity of the curves in Fig. 5 together with the observation that ADC is stable when stored under the applied conditions (30 °C, pH 7.5) clearly shows that the inactivation of the enzyme is turnover dependent. Although covalent immobilization on Sepabeads, independent of the type, has an overall positive effect on the operational stability, this improvement is relatively small in relation to the total decrease in activity. The resulting total turnover numbers (TTNs) of $2.39 \times 10^3 \pm 0.04 \times 10^3$ and $3.73 \times 10^3 \pm 0.06 \times 10^3$ for soluble and immobilized ADC, respectively, are relatively low for enzymatic reactions.

It has been suggested that deactivation of ADC in the presence of substrate occurs *via* decarboxylation-dependent transamination of the catalytically essential pyruvoyl group, resulting in a catalytically inactive alanine residue at the *N*-terminus.[26,47] This suggestion has been based on observations by Smith,[48] who observed an irreversible deactivation of ADC in the presence of L-aspartic acid in combination with the transamination of the pyruvoyl group, as determined by *N*-terminal sequencing. The mechanism behind this 'suicidal' transamination is proposed

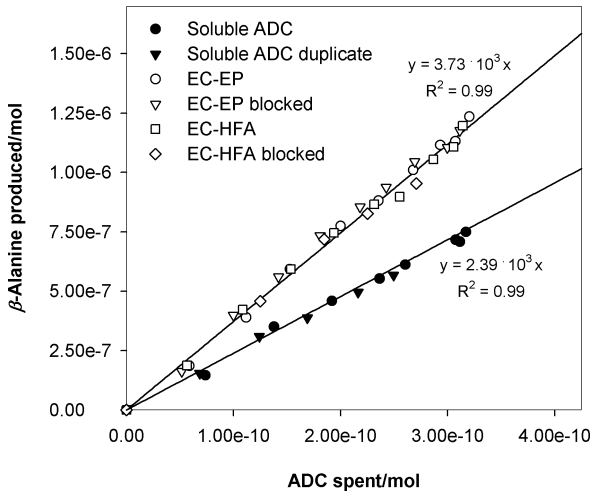

Fig. 5 Comparison of operational stability of different ADC preparations. Using the data obtained from batch experiments (3 mM L-aspartic acid in 50 mM sodium phosphate buffer pH 7.5 at 30 °C), the amount of β-alanine produced was plotted against the amount of ADC spent, grouped by soluble and immobilized ADC. Linear curves were fitted through the data points giving the total turnover numbers (TTNs).

to be comparable to that observed in several pyridoxal phosphate-dependent enzymes[49,50] and has been confirmed by mechanistical studies for *S*-adenosylmethionine decarboxylase, another pyruvoyl-dependent enzyme.[51,52]

Since deactivation caused by transamination of the catalytically essential pyruvoyl group is irreversible, the solution to this problem has to be found in the field of protein engineering. A study in which protein engineering is applied to overcome substrate deactivation in pyruvoyl-dependent enzymes is to date not known to the authors. Nonetheless, McElroy and Robertus[53] showed us that at least the opposite is possible; with help of site-directed mutagenesis, they were able to induce substrate deactivation in pyruvoyl-dependent *Lactobacillus* 30a histidine decarboxylase, where it was not present before.

Conclusion

In this paper, we have investigated the possibilities for the application of the enzyme L-aspartate α-decarboxylase from *E. coli* (ADC) in the biobased production of nitrogen containing chemicals as an alternative to the petrochemical route. ADC has several advantages, such as its perfect selectivity, ease of production, lack of product inhibition and high thermostability under storage conditions. In addition, covalent immobilization of ADC on Sepabeads EC-EP epoxy supports is easy and makes the enzyme slightly more thermostable. However, ADC's low operational stability, probably caused by irreversible transamination of its catalytically essential pyruvoyl group, needs to be addressed before large scale applications become feasible. Although operational stability was improved by covalent immobilization on Sepabeads epoxy supports, protein engineering is the most likely solution to this problem.

Experimental

pRSETA Plasmids containing one copy of the *panD* gene with a *N*-terminal histidine tag, constructed following Schmitzberger *et al.*,[54] were kindly provided by Prof. Alison Smith from the Department of Plant Sciences, Cambridge University, UK.

Z-competent *E. coli* XL1-blue and One Shot chemically competent *E. coli* BL21star(DE3) were obtained from Zymo Research and Invitrogen, respectively.

The Sepabeads EC-EP (epoxide) and EC-HFA (epoxide on a an amino spacer) used in this study are epoxy activated methacrylic beads with pore sizes in the range of 30–40 nm and diameters in the range of 150–300 μm.[55] Both types were kindly donated by Dr Paolo Caimi from Resindion S.R.L. (Mitsubishi Chemical, Milan, Italy).

All buffers and solutions used for procedures involving ADC were prepared with MilliQ water and filter sterilized (0.20 μm) before use. Instant low salt LB agar (Duchefa Biochemie) and instant high salt LB broth (Sigma) were prepared according to protocol, autoclaved and ampicillin stock solution was added up to a concentration of 100 μg mL^{-1}.

Bradford protein assays were performed using Bio-Rad Protein Assay Kit II with BSA standard. All chemicals used in this study were of analytical grade or better and used as received.

Plasmid amplification

Z-competent *E. coli* XL1-blue cells were transformed with the *pRSETA* plasmid according to protocol. After selection on low salt LB agar (16 h, 37 °C), the transformants were grown in high salt LB medium (16 h, 37 °C, 250 rpm). The plasmids were isolated from the cultures using a GenElute HP Plasmid Kit (Sigma) according to protocol and stored at –20 °C.

L-Aspartate α-decarboxylase overexpression

Chemically competent *E. coli* BL21star(DE3) was transformed with *pRSETA*, according to protocol. After selection on low salt LB agar, transformed cells were grown in high salt LB medium (25 °C, 200 rpm). After 16 hours incubation ($OD_{600} = 0.90$), the cultures were used to prepare a 80% glycerol stock, which was stored at –80 °C.

The glycerol stock was used to inoculate 10 mL high salt LB medium. After 4 hours incubation (37 °C, 250 rpm), the culture ($OD_{600} = 0.30$) was used to inoculate 0.5 L high salt LB medium. After 4 hours incubation (37 °C, 250 rpm), the culture ($OD_{600} = 0.30$) was induced with IPTG (1 mM) and incubated for another 16 hours (25 °C, 250 rpm). The cells were harvested in three subsequent centrifugation steps (3000 rpm, 4 °C, 25 min) and stored at –80 °C.

L-Aspartate α-decarboxylase isolation and activation

The pellet with IPTG induced cells was thawed on ice and suspended in 5 mL binding buffer (20 mM imidazole, 500 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4). 5 mg Egg white lysozyme was added and the suspension was kept at room temperature for 15 minutes, while carefully vortexing from time to time. After sonication on ice, 3 times 30 seconds with one minute in between, the suspension was centrifuged (12 000 rpm, 4 °C, 10 min). The supernatant was passed through a syringe filter (0.45 μm) and stored in ice.

A 5 mL HisTrap HP column (GE Healthcare) was operated on an ÄKTA Explorer system. After equilibration (25 mL binding

buffer), 2 mL cell extract was applied to the column. Subsequent to washing (25 mL binding buffer), histidine tagged ADC was eluted with elution buffer (500 mM imidazole and 500 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4). The fractions were collected upon UV detection at 280 nm and stored at 4 °C. The purity and protein content were determined with SDS-PAGE analyses and Bradford protein assays, respectively.

To activate ADC, fractions were filter-sterilized (0.20 μm) and incubated for 16 hours at 50 °C. Finally, the buffer was exchanged with reaction buffer (see ADC activity assay section) using a Millipore Centriprep centrifugal filter (10 kD cutoff). The activated ADC was stored at 4 °C.

L-Aspartate α-decarboxylase activity assay

A solution of 25 μg ADC in 970 μL reaction buffer (1 mM EDTA, 50 mM sodium phosphate buffer, pH 7.0) was incubated for 5 minutes at 30 °C using an Eppendorf Thermomixer (1200 rpm). The activity assay was started by adding 30 μL 50 mM L-aspartic acid solution. At given time intervals, 50 μL samples were taken and quenched with 5 μL of 1 M NaOH and stored at −20 °C.

β-Alanine concentrations were determined using the fluorescamine derivatization method described by Udenfriend *et al.*[56] The samples were thawed at room temperature and to 5 μL of each sample 75 μl of 100 mM borate buffer (pH 9.0) was added. The diluted sample was derivatized by adding 20 μl 1 mg mL^{-1} fluorescamine in acetonitrile, directly followed by vortexing.

The HPLC system used for quantification was based on separation on a NovaPak C18-column (60 Å, 4 μm, 3.9 × 150 mm) at 30 °C, isocratic elution with 1 mL min^{-1} 20% acetonitrile in 20 mM sodium acetate buffer (pH 4.50) and detection with a Jasco 820-FP fluorescence detector ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 460$ nm).

Product inhibition

Assays were performed with 0.050, 0.100, 0.250, 0.600, 1.000, 1.400 and 1.800 mM L-aspartic acid in combination with 0.000, 0.500, 1.000 or 1.500 mM β-alanine. Solutions of 25 μg ADC in 800 μL reaction buffer were incubated for 5 minutes at 30 °C. The activity assays were started by addition of 200 μL β-alanine/L-aspartic acid solution with the desired concentration. Sampling and HPLC analysis were performed as previously described.

Temperature dependent activity and stability

Solutions of 25 μg ADC in 485 μL reaction buffer were prepared and incubated for 0, 1 and 24 hours in a water bath at different temperatures. After incubation, the solutions were transferred to an Eppendorf Thermomixer (1200 rpm) and incubated for 5 minutes at the same temperature as the water bath. The activity assays were started by adding 15 μL of 50 mM L-aspartic acid. Sampling and HPLC analysis were performed as previously described.

pH Dependent activity and stability

To maintain a constant ionic strength, a three-component buffer composed of acetic acid (100 mM), MES (100 mM) and triethanolamine (200 mM) was used following Ellis *et al.*[30] A 30 mM L-aspartic acid stock was prepared using the three-component buffer. Aliquots of both the three-component buffer and the L-aspartic acid stock were adjusted to the desired pH values (4, 5, 6, 7, 7.5, 8, 9 and 10) with 37% HCl or 10 M NaOH.

To determine the pH dependent activity, 25 μg ADC in 475 μL of buffer was incubated for 5 minutes at 30 °C in an Eppendorf Thermomixer (1200 rpm). Activity assays were started by adding 25 μL of 30 mM L-aspartic acid stock with a pH similar to that of the respective buffer. To determine the pH dependent stability, the activity assays were preceded by 24 hours incubation in a 30 °C water bath. Apart from using 5 M NaOH for enzyme quenching, sampling and analyses were performed as previously described.

Immobilization of L-aspartate α-decarboxylase

Two different 500 μg mL^{-1} ADC solutions were prepared, one in 5 mM and another in 1.0 M sodium phosphate buffer (pH 7.0). From each type of Sepabeads, 1.000 g dry weight was transferred to a 10 mL screw-cap tube. To EC-EP, 8.0 mL of 1.0 M sodium phosphate ADC solution and, to EC-HFA, 8.0 mL of 5 mM sodium phosphate ADC solution was added. Of each ADC solution, 1.5 mL was transferred to a 2 mL Eppendorf tube to serve as blanks.

All tubes were mounted on a rotator (Cole-Palmer Roto-Torque Heavy Duty Rotator) and incubated for 24 hours at room temperature. In time, 80 μL samples were taken of which 25 μL was directly used in an activity assay and the remainder was stored at 4 °C. The 25 μL was added to 460 μL reaction buffer and after 5 minutes incubation at 30 °C, the activity assay was started by adding 15 μL 50 mM L-aspartic acid solution. Sampling and analyses were conducted as previously described.

After 24 hours of incubation, the supports were filtered over a Büchner-filter, rinsed with 8 mL reaction buffer, resuspended in 8 mL reaction buffer and incubated for another hour at room temperature. After this washing step, the supports were once more rinsed with 8 mL reaction buffer. After weighing the filter-dry supports, 500 mg of each preparation was transferred to a fresh 10 mL screw-cap tube for blocking and the remainder was stored at 4 °C.

The 500 mg aliquots were blocked by incubation on the rotator in 5 mL 3 M glycine (pH 8.0) for 16 hours at room temperature. After incubation, the blocked supports were washed as described above, weighed and stored at 4 °C.

The protein content in the supernatant during immobilization and washing was determined with Bradford protein assays. Residual activities of the enzyme preparations were determined with 30 mg immobilized ADC in the standard activity assay.

Thermostability of L-aspartate α-decarboxylase preparations

Aliquots of soluble and immobilized ADC in 970 μL reaction buffer were incubated in a 80 °C water bath. At various time intervals, the aliquots were transferred to an Eppendorf Thermomixer (80 °C, 1200 rpm). After 5 minutes of incubation, activity assays were started by adding 30 μL 50 mM L-aspartic acid. Sampling and analyses were performed as previously described.

Operational stability of L-aspartate α-decarboxylase preparations

Reusability. Reaction mixtures with 25 mg immobilized ADC were prepared in 970 μL reaction buffer. After 5 minutes of

incubation in an Eppendorf Thermomixer (30 °C, 1200 rpm), activity assays were performed as described earlier. After the activity assay, the reaction mixtures were left for 30 minutes in total. The supernatants were removed and replaced by fresh reaction buffer and the initial reaction rate was determined for three additional cycles in a similar way.

Batch reactions. Reaction mixtures with approximately 25 μg soluble and 25 mg immobilized ADC were prepared in 4.700 mL 50 mM phosphate buffer (pH 7.5) with 0.05% sodium azide. After incubation for 10 minutes in a stirred 30 °C water bath, the activity assays were started by adding 300 μL 50 mM L-aspartic acid. Sampling and analyses were performed as previously described. The total turnover number (TTN) was calculated by dividing the moles of β-alanine produced by the moles of ADC spent.

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