Immobilization of L-aspartate oxidase from *Sulfolobus tokodaii* as a biocatalyst for resolution of aspartate solutions

Paola D'Arrigo,*[abc] Chiara Allegretti,[a] Andrea Fiorati,[a] Luciano Piubelli,[bd] Elena Rosini,[bd] Davide Tessaro,[ab] Mattia Valentino[bc] and Loredano Pollegioni[bd]

Received 25th July 2014,
Accepted 5th October 2014

1. Introduction

L-Amino acid oxidase (LAAO, EC 1.4.3.2), a flavoenzyme containing non-covalently bound flavin adenine dinucleotide, catalyzes the stereospecific oxidative deamination of L-amino acids to α-keto acids, ammonia and hydrogen peroxide (Fig. 1). LAAOs purified from snake venoms are the best-studied members of this family of enzymes; several LAAOs from bacterial and fungal sources have also been reported (for a review, see ref. 1). In analogy to the well-known biotechnological applications of D-amino acid oxidase (DAAO, EC 1.4.3.3),[2,3] LAAOs have important potential biotechnological applications, in particular, as part of biosensors and as catalysts in biotransformations.[4] DAAO and LAAO activities can be exploited for the production of optically pure amino acids by resolution of D,L-racemic mixtures. Enantiomerically pure proteinogenic and synthetic amino acids are of increasing interest for the fine chemical and pharmaceutical industries: unnatural α-amino acids (such as naphthyl and fluorinated substituted amino acids, *i.e.* D-Phe for the antibiotics ampicillin and amoxicillin) are used in drug discovery for the synthesis of combinatorial libraries and for the *de novo* synthesis of peptides and are also used as additives in the food industry and as valuable pharmaceuticals (*e.g.* D- and L-DOPA and D-2-naphtylalanine). Unluckily, the use of LAAO was hampered by the difficulty to produce suitable amounts of this enzyme from both the original and the recombinant sources.[1] The conversion of *N*-ε-CBZ-L-lysine to CBZ-L-oxylysine, a precursor of the anti-hypertensive drug ceronapril, represents the first attempt to use microbial LAAO activity (whole cells of *Providencia alcalifaciens*).[5] More recently, *Rhodococcus* sp. AIU Z-35-1 LAAO has been employed for the resolution of D,L-mixtures of citrulline, Gln, homoserine, *N*-ε-acety-Lys and Arg,[6] and crude extracts of *A. fumigatus* have been used for the racemic resolution of Ala, Phe and Tyr: nevertheless, an optically pure product was obtained only for D-Ala.[7] LAAO from the bacterium *R. opacus* DSM 43250 was also used for the

[a] *Dipartimento di Chimica, Materiali e Ingegneria Chimica "Giulio Natta", Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milano, Italy.*
E-mail: paola.d'arrigo@polimi.it; Fax: +39 02 23993180; Tel: +39 02 23993075

[b] *The Protein Factory, Politecnico di Milano, ICRM CNR Milano, and Università degli Studi dell'Insubria, Via Mancinelli 7, 20131 Milano, Italy*

[c] *CNR – Istituto di Chimica del Riconoscimento Molecolare (ICRM), Via Mancinelli 7, 20131 Milano, Italy*

[d] *Dipartimento di Biotecnologie e Scienze della Vita, Università degli Studi dell'Insubria, Via J.H. Dunant 3, 21100 Varese, Italy*

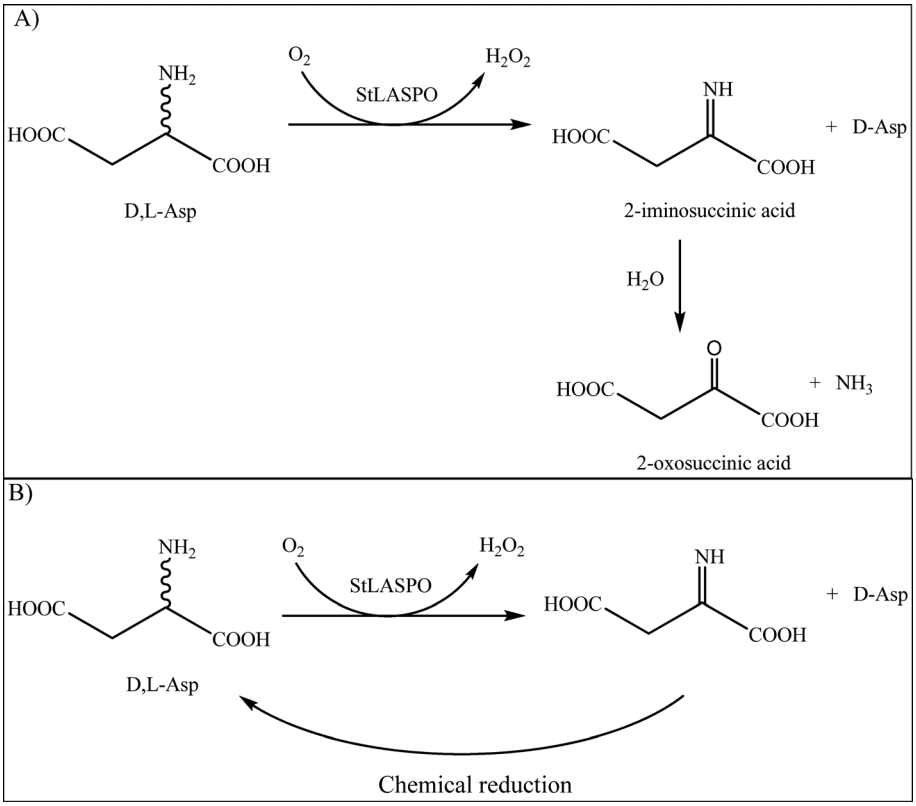

Fig. 1 Kinetic resolution of D,L-aspartic acid. (A) Reaction catalyzed by StLASPO on D,L-aspartic acid: oxidation of the L-isomer yields pure D-aspartate and oxaloacetate (2-oxosuccinate). Catalase is used frequently to eliminate hydrogen peroxide, thus halving the overall amount of oxygen consumed by the oxidase. (B) Chemo-enzymatic process for D-aspartate quantitative obtainment starting from racemic D,L-aspartic acid.

racemic resolution of 7 mM D,L-Phe and D,L-Leu and 3,4-dihydroxyphenyl-L-alanine (L-DOPA), the precursor of the melanine pigment, of all cathecolamine neurotransmitters, and of hormones and one of the main drugs for treatment of Parkinson's disease. By employing a batch biotransformation based on a membrane reactor, complete oxidation of L-DOPA was reached for three consecutive cycles performed by adding fresh L-DOPA to the same reaction mixture without discharge of the products.[8] Altogether, LAAOs have not been extensively exploited for biotechnological applications because of the difficulties in their expression as recombinant proteins.[1] In order to obtain substantial amounts of a catalyst, as well as to improve its biochemical properties by protein engineering, the recombinant production of the protein of interest is essential.[9,10]

A suitable alternative to LAAO is represented by L-aspartate oxidase (LASPO, EC 1.4.3.16) which catalyzes the oxidative deamination of L-aspartate and L-asparagine. From a structural point of view, LASPO is substantially different from the "classical" LAAOs: it belongs to the succinate dehydrogenase/fumarate reductase family of flavoproteins and is not evolutionarily related to the classical LAAO members.[1] Each LASPO monomer is formed by a single polypeptide chain (comprising 472 residues) that folds into three distinct domains: a FAD-binding domain, a capping domain, and a helical domain.[1,11] In particular, LASPO from the thermophilic archaea *Sulfolobus tokodaii* (StLASPO) was efficiently produced as a recombinant protein in *E. coli* in the active form as holoenzyme (up to 9% of the total proteins in the crude extract and up to 13.5 mg L^{-1} in the fermentation broth).[11,12] This monomeric (52 kDa) recombinant flavoenzyme shows the classical properties of FAD-containing oxidases but also possesses distinctive features that make it attractive for biotechnological applications: i) high thermal stability (it is fully stable up to 80 °C) and high temperature optimum, ii) stable activity in a broad range of pH (7.0–10.0), iii) weak inhibition by the product (oxaloacetate) and by the D-isomer of aspartate, and iv) tight binding of the FAD cofactor. Indeed, steady-state measurements highlighted a low $K_{m,O2}$ (0.3 mM); this property significantly distinguishes StLASPO from the *E. coli* counterpart.[13,14]

Altogether, StLASPO represents an appropriate novel biocatalyst for the production of D-aspartate: a very low amount of StLASPO (9 U) allowed the resolution of a racemic mixture of D,L-aspartate (final ee $>$ 99.5%).[12] In fact, a straightforward enzymatic method to convert an α-amino acid to the corresponding α-keto acid is based on the use of a highly enantioselective amino acid oxidase: the enzyme specifically oxidizes one enantiomer only based on its enantioselectivity (*e.g.*, the L-form – (*S*) enantiomer – is oxidised when LAAO is employed) to the imino acid that rapidly deaminates to the corresponding α-keto acid; see the reaction

scheme in Fig. 1A. A full deracemization is then obtained using non-selective inorganic reducing agents (*i.e.* sodium borane) that convert the α-imino acid intermediate into the D,L-amino acid racemate:[15] after a few cycles of enzymatic oxidation of the L-isomer and chemical reduction of the imino acid, the D-amino acid only accumulates (Fig. 1B). Aspartate is used in the pharmaceutical industry, for parenteral nutrition, as food additive and in sweetener manufacturing.

Immobilized enzymes are currently the subject of considerable interest because they facilitate industrial applications by improving reusability and, in turn, by reducing the costs associated with production and purification of the enzyme.[16] For a review on the properties of immobilized thermophilic enzymes, see ref. 17. It should be pointed out that the enzyme reuse is often an essential prerequisite in order to establish an economically viable enzyme-catalyzed process. The possible alterations in enzyme properties related to the effect of the immobilization procedure on the protein structure, as well as the impact of partition effects and diffusional limitations, have been reported in detail in ref. 18.

In this work, StLASPO was immobilized on various matrices and the immobilized recombinant flavoenzyme has been employed in an efficient kinetic resolution of a racemic mixture of D,L-aspartate. Since StLASPO is a very stable flavoenzyme (see above), this investigation aims to improve the recovery/reuse of the biocatalyst. The solid supports which have been evaluated are SEPABEADS® EC-EP/A, SEPABEADS® EC-EP/S, Relizyme™ EP403, Relizyme™ EP113, Relizyme™ HA403/S R, Relizyme™ HA113, and Eupergit® C. The best results in terms of immobilization yield and volumetric activity (U per g support) have been obtained with the amino support Relizyme™ HA403/S R and with the epoxy support SEPABEADS® EC-EP/S, the former showing a higher L-aspartate conversion yield when reused for five times. Also cross-linked enzyme aggregates (CLEA)[19,20] were prepared from StLASPO by using glutaraldehyde as a cross-linking agent: the CLEA-StLASPO preparation also exhibited high activity and stability.

2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich. All solvents were of analytical grade. Catalase from beef liver (20 mg mL^{-1}, 65 kU per mg protein) and horseradish peroxidase were purchased from Roche. Relizyme™ HA403/S R and SEPABEADS EC-EP/S supports were supplied by Resindion (Milano, Italy).

2.2. Enzyme preparation

StLASPO was overexpressed in *E. coli* cells and was purified to >95% purity by the procedure described in ref. 12. The purified StLASPO was stored in 20 mM Tris–HCl buffer at pH 7.5 and 10% glycerol. Before use, it was dialyzed against 0.05 M sodium phosphate buffer, pH 7.5, at 4 °Cfor24h. The activity of the final solution was 2 U mL^{-1}, corresponding to 1.3 U per mg of protein.

2.3. Activity assays

The StLASPO activity was assayed by measuring the initial rate of production of hydrogen peroxide with a coupled peroxidase/dye assay. The colored product produced by the horseradish peroxidase from H_2O_2 and 4-aminoantipyrine was detected spectrophotometrically at 505 nm (ε = 6.58 mM^{-1} cm^{-1}) at 37 °C.[12] One unit (U) is defined as the amount of enzyme that catalyses the degradation of 1 μmol of L-aspartate per minute. The standard assay mixture contained 10 mM L-aspartate in 50 mM sodium pyrophosphate buffer (pH 8.0), 1.5 mM 4-aminoantipyrine, 10 mM phenol, 20 μM FAD, 2.5 U of horseradish peroxidase, and 30 μg (≈0.04 U) of StLASPO in a total volume of 1 mL.

For the assessment of pH dependence of the rate of oxidation of L-aspartate by the various StLASPO preparations, the reaction was performed at a pH value of 8.0, 9.0 or 10.0 using the "magic buffer" composed of 33 mM Tris–HCl, 33 mM Na_2CO_3, and 33 mM H_3PO_4 (final concentration).[21]

For the assessment of the effect of hydrogen peroxide on StLASPO stability, a 1.5 mg mL^{-1} enzyme preparation was mixed in 20 mM Tris–HCl at pH 7.2 and 10% glycerol with 1 or 10 mM H_2O_2 at 50 °C (and as a control in the absence of H_2O_2). Aliquots were withdrawn at different times (up to 96 hours) and assayed with the oxygen consumption method on 10 mM L-aspartate as a substrate (at 37 °C):[22] residual activity was expressed as percentage of the one determined before incubation.

2.4. StLASPO immobilized on Relizyme™ HA403/S R resin

The Relizyme™ HA 403/S R resin (200 mg) was treated with 10 mL of 0.125% glutaraldehyde solution in water for 2.5 h in a rotating device at 18 °C. The glutaraldehyde solution was then removed by centrifugation, and the resin was washed three times with 0.1 M phosphate buffer at pH 7.5. The buffer was then removed, and 1 mL of dialyzed StLASPO (2 U) and 1 mL of 0.1 M phosphate buffer at pH 7.5 were added to the activated support. The mixture was incubated in a rotatory shaker for 24 h at 18 °C: the residual enzymatic activity in the solution was assayed by the coupled peroxidase/dye assay and was close to zero. The resin was then washed with 50 mM phosphate buffer at pH 7.5 and stored at 4 °C in the same buffer. The immobilized enzyme showed an activity of ≈10 U per g matrix corresponding to 100% immobilization yield.

2.5. StLASPO immobilized on SEPABEADS® EC-EP/S resin

The dialyzed StLASPO preparation (0.5 mL, 1 U) was added to 60 mg of SEPABEADS® EC-EP/S resin and to 0.3 mL of 1.25 M potassium phosphate buffer at pH 8.0. The mixture was incubated in a rotatory shaker for 18 h at 25 °C: at the end of incubation, approximately 50% of the enzymatic activity was present in solution suggesting that half of the added

enzyme has been immobilized. The immobilized StLASPO was stably bound to the support: no enzyme was released when the SEPABEADS-StLASPO support was incubated with 20 mM potassium phosphate at pH 8.0 and left rotating for an additional hour. The immobilized enzyme showed an activity of 8.3 U per g dry matrix.

2.6. Precipitation of StLASPO as CLEA

The precipitation of StLASPO and the CLEA formation were conducted by adding 4.5 mL of precipitating solution (*i.e.* 60% w/v ammonium sulphate in 0.1 M sodium phosphate buffer at pH 7.5) and 20 μL (0.11 mmol) of glutaraldehyde (50% w/v in water, 5.6 M, d 1.208 g mL^{-1}) (final 0.4% (v/v) corresponding to 200 μmol) to 0.5 mL of dialyzed enzyme solution (2 U mL^{-1}, 14.8 nmol).[23,24] The mixture was incubated on a rotatory shaker for 2.5 h at room temperature: no residual enzymatic activity was detected in the solution. Then, the mixture was centrifuged and the residue was washed twice with 0.1 M phosphate buffer at pH 7.5, centrifuged and decanted. The CLEA-StLASPO was stored at 4 °C in 0.05 M phosphate buffer at pH 7.5. The CLEA-StLASPO showed an activity of 4 U per g wet CLEA.

2.7. Resolution of D,L-aspartate by free StLASPO

The setup of the experimental conditions to resolve a 50 mM D,L-aspartate solution by using free StLASPO was performed by adding 0.3 U of StLASPO to the reaction medium at pH 10.0 (final volume 1.0 mL). The reaction was carried out in the 25–80 °C temperature range under shaking (using a thermomixer set at 600 rpm). Samples were collected at different time intervals for D- and L-aspartate concentration measurements. Subsequently, the same reaction was performed under the same conditions at 50 °C at different pH values (in the 8–13 range).

2.8. Resolution of D,L-aspartate by immobilized StLASPO

All reactions were performed at 70 °C in a thermomixer set to 600 rpm; the addition of 2 μL of catalase did not affect the time course of conversion.

Concerning the SEPABEADS-StLASPO matrix, the resolution of D,L-aspartate was carried out by adding 0.5 mL of the racemic 50 mM amino acid solution (in water, prepared and adjusted to pH 10.0 with 1 M NaOH, final concentration of the amino acid 37.7 mM) and 200 μL of water to the immobilized enzyme (60 mg of support containing ≈0.5 U of StLASPO).

In the case of Relizyme-StLASPO, 0.5 mL of a 50 mM D,L-aspartic solution (adjusted to pH 10.0) and 200 μL of water were added to the immobilized enzyme (≈2 U). For the scaling-up of the reaction, 2 g of Relizyme-StLASPO (corresponding to 20 U) were added to 5 mL of 200 mM D,L-aspartic acid and 5 mL of water at pH 10.0, under constant air flux and stirring.

Concerning the CLEA-StLASPO preparation, 1.1 U of immobilized enzyme were added to 0.5 mL of a 50 mM D,L-aspartate solution in "magic buffer" and adjusted to the desired pH (8.0, 9.0 or 10.0) by adding 1 M NaOH.

In the case of the recycling of immobilized StLASPO, at the end of each cycle, the matrices were collected by centrifugation, washed several times with water and stored in the starting buffer.

2.9. Chiral HPLC analysis of aspartate racemic mixtures

The OPA-NAc reagent was prepared by dissolving 4 mg of *ortho*-phthalaldehyde (OPA) in 300 μL of methanol, followed by the addition of 250 μL of 0.4 M borate buffer at pH 9.4. To this mixture, 15 μL of 1 M *N*-acetylcysteine (NAc) in 1 M NaOH were added, and then the solution was diluted with 435 μL of distilled water. At fixed time intervals, 10 μL aliquots were withdrawn from the reaction mixture and diluted with 40 μL of distilled water. 10 μL of this solution were derivatized with 25 μL of OPA-NAc reagent as described in ref. 25, diluted with 350 μL of HPLC eluent and analyzed by HPLC chromatography in a Zorbax SB-Aq column (150 × 4.6, 5 μm, Agilent Technologies; eluent: 100 mM sodium acetate buffer, pH 5.2, isocratic flux of 0.6 mL min^{-1}; detection at 340 nm). The separation allowed us to distinguish and quantify the L- and D-isomers of aspartate and thus to calculate the enantiomeric excess (ee); R_t (D-aspartate): 7.5 min, R_t (L-aspartate): 8.8 min.

3. Results

3.1. Bioconversion of D,L-aspartate using free StLASPO

A previous study established that for free StLASPO the highest activity was achieved at 65 °C and at pH 10.5.[12] In order to identify the best conditions for the resolution of a 50 mM solution of D,L-aspartate, we analyzed the effect of temperature on the conversion performed at pH 10.0. Full resolution was reached in that case in 4 h in the 37–70 °C range using 0.3 U StLASPO mL^{-1}: the fastest conversion was apparent at 50 °C (see the time course of conversion in Fig. 2a).

Concerning the effect of pH, under similar experimental conditions and at 50 °C, the fastest resolution apparently occurred in the 10–11 pH range (see Fig. 2b). These results show that free StLASPO can be used as a biocatalyst in kinetic resolution of racemic solution of aspartate: the main drawback is represented by the impossibility to reuse the enzyme for additional cycles, and this has a negative impact on the cost of the overall process.

To test the sensibility of StLASPO to hydrogen peroxide, free StLASPO was incubated at pH 7.2 and 50 °C with 0, 1 or 10 mM hydrogen peroxide and the enzymatic activity on L-aspartate was assayed with the oxygen consumption method. As shown in Fig. 3, StLASPO stability is not affected by hydrogen peroxide: after 96 h of incubation, 82 ± 1% of the initial enzymatic activity is maintained in the presence of 1 or 10 mM H_2O_2 *vs.* 86 ± 3% in the absence of hydrogen

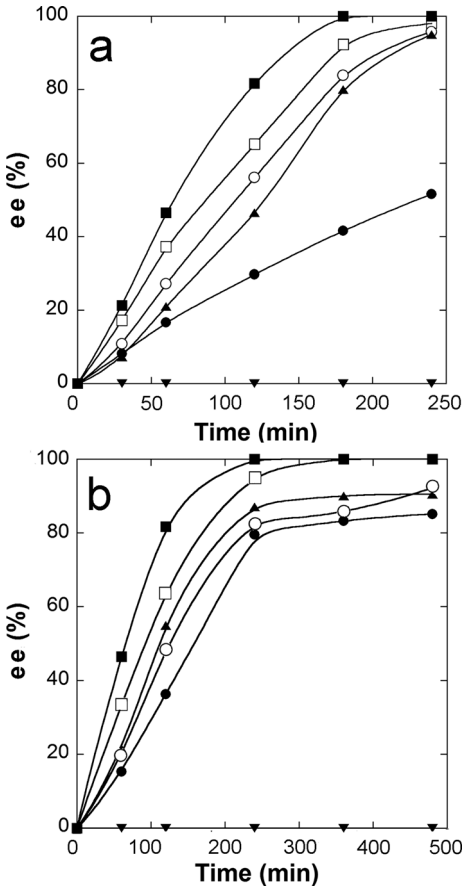

Fig. 2 (a) Effect of temperature on the kinetic resolution of D,L-aspartate by free StLASPO. Conditions: 1 mL of 50 mM D,L-aspartic acid at pH 10.0, 0.3 U of StLASPO, at 700 rpm. Symbols used: (●) 25 °C, (○) 37 °C, (■) 50 °C, (□) 60 °C, (▲) 70 °C, and (▼) 80 °C. The values are the average of at least three measurements; error bars are not shown since error was <5%. (b) Effect of pH on the kinetic resolution of D,L-aspartate. Conditions: 1 mL of 50 mM D,L-aspartate solution adjusted at different pH values, 0.3 U of StLASPO, 50 °C, at 700 rpm. Symbols used: (●) pH 8.0, (○) pH 9.0, (■) pH 10.0, (□) pH 11.0, (▲) pH 12.0, and (▼) pH 13.0. The values are the average of at least three measurements; error bars are not shown since error was <5%.

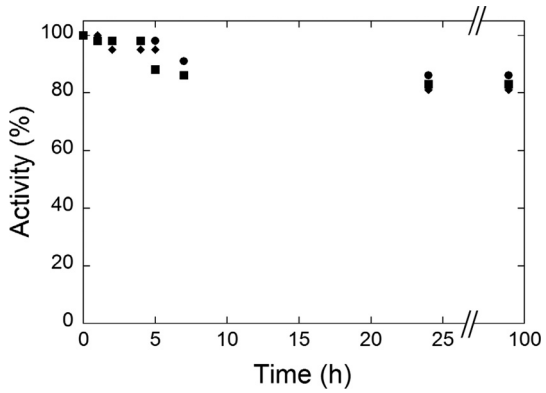

Fig. 3 Effect of hydrogen peroxide on the stability of free StLASPO. Conditions: StLASPO (1.5 mg mL^{-1}) was incubated in 20 mM Tris–HCl buffer, pH 7.2, and 10% glycerol at 50 °C in the presence of 1 mM (■) or 10 mM (♦) hydrogen peroxide. As a control, the activity in the absence of H_2O_2 is also reported (●). Aliquots were withdrawn at different times and assayed with the oxygen consumption method on 10 mM L-aspartate as a substrate (at 37 °C): residual activity was expressed as percentage of the one determined before incubation. The values are the average of at least three measurements; error bars are not shown since error was <5%.

peroxide. Because of the insensitivity of StLASPO to hydrogen peroxide, there is no need to add catalase during the bioconversion. Indeed, catalase shows a very low residual activity when incubated at pH 10.0 and 70 °C for 30 minutes (<2% of the activity assayed at neutral pH and room temperature).

3.2. Bioconversion of D,L-aspartate using immobilized StLASPO

Then, immobilization of StLASPO has been investigated in order to increase the competitiveness of the resolution process. With the SEPABEADS® EC-EP/A, Relizyme™ EP403, Relizyme™ EP113, Relizyme™ HA113, and Eupergit® C supports, StLASPO was only partially immobilized, ranging from 8% with Relizyme™ HA113 to 29% with SEPABEADS EC-EP/A. On the other hand, we have focused our attention on the two solid supports named Relizyme™ HA403/S R and SEPABEADS® EC-EP/S that resulted in higher StLASPO immobilization yields in preliminary experiments. These supports are composed of a rigid methacrylic polymer matrix with high physical and chemical stability given by intense cross-linking. They also exhibit the same particle size (100 to 300 nm).

3.2.1 StLASPO immobilized on Relizyme™ HA403/S R. Immobilized StLASPO was used as a biocatalyst for the resolution of the racemic solution of the amino acid aspartate. Relizyme™ HA403/S R is a rigid methacrylic polymer matrix (particle size of 100–300 μm with a mean diameter of 40–60 nm) which is functionalized with amino groups. A pre-activation step is necessary using a bifunctional coupling agent such as glutaraldehyde. As stated in ref. 26, the final effect of glutaraldehyde treatment will be a mixture of cross-linking and chemical modifications. Then, the covalent binding between the enzyme and the derivatized support is performed. Briefly, pure StLASPO was immobilized on activated Relizyme™ HA 403/S R support at pH 7.5 in 100 mM potassium phosphate buffer: the immobilized enzyme showed an activity of ≈10 U per g matrix corresponding to 100% immobilization yield. These conditions should favor ionic exchange of StLASPO prior to covalent immobilization. The enzyme is covalently bound to the matrix since no protein was released when the Relizyme-StLASPO resin was boiled for 3 minutes – as assessed by SDS-PAGE analysis performed on the soluble fraction obtained after centrifugation of the heated resin.

The resolution of 50 mM D,L-aspartate was carried out at pH 10.0 and 70 °C using the immobilized StLASPO (≈2 U). Full oxidation of L-aspartate was obtained in ≤1 hour (ee ≈ 100%) for three sequential cycles, while for the following cycles a

longer reaction time was required (Table 1). This result points to partial inactivation of the immobilized StLASPO when reused for more than 3 times since no StLASPO was released from the immobilized matrix (as demonstrated by SDS-PAGE analysis).

In order to evaluate the influence of D,L-aspartate concentration on the enzymatic resolution, the reaction was carried out by adding the StLASPO immobilized on Relizyme™ HA403/S R resin (2 U) to a solution containing 0.5 mL of D,L-aspartate at different concentrations (from 50 to 500 mM) at pH 10.0 and 200 μL of water. As reported in Table 2, in the presence of 200 mM D,L-amino acid (final concentration 142 mM), 6.6 mg of L-aspartate were fully converted by 2 U of enzyme in 4 h and at 70 °C. The observation that at 500 mM D,L-aspartate concentration the reaction stopped at 21% conversion points to an inhibition of StLASPO activity by the racemic amino acid solution.

3.2.2 StLASPO immobilized on SEPABEADS® EC-EP/S resin. SEPABEADS® EC-EP/S is a methacrylate hydrophobic support functionalized with epoxide groups (131 μmol oxirane content per g wet) which react efficiently with the amino groups of the protein and with a median pore diameter of 10–20 nm and a particle size of 100–300 μm. Immobilization was performed at high ionic strength to allow protein adsorption so that covalent attachment can then take place.[16] Each StLASPO protomer contains 28 lysine residues: analysis of the 3D structure (pdb code 2E5V) shows that three of them (namely K24, K147 and K396) show a very high degree of accessibility. One unit of pure StLASPO was immobilized on 60 mg of SEPABEADS® EC-EP/S matrix: the flavoenzyme was immobilized with a ≈50% yield in terms of activity using 20 mM potassium phosphate buffer at pH 8.0. The covalent linkage between the resin and the enzyme has been verified by the boiling procedure as stated above for the Relizyme support: also in this case, no protein was released.

The resolution of 50 mM D,L-aspartate was carried out at pH 10.0 and 70 °C with the immobilized enzyme: this reaction yielded full oxidative deamination of the L-isomer of aspartate into 2-oxosuccinic acid in 4 hours for 3 cycles. For the following cycles, the full resolution required a longer time, up to 10 hours in the fifth cycle (Table 3).

Table 1 Results of kinetic resolution of 50 mM D,L-aspartate solution (0.5 mL) using 2 U of StLASPO immobilized on Relizyme™ HA403/S R support at pH 10.0 and 70 °C

Cycle number	Time (h)	ee[a] (%)
1	1	>99.5
2	1	>99.5
3	1	>99.5
4	1	87.0
	2	>99.5
5	1	77.0
	2	>99.5

[a] ee values calculated using the HPLC analysis.

3.2.3. StLASPO immobilized as CLEA. The cross-linked enzyme aggregates (CLEAs) technology is applicable to a wide variety of enzymes as a mean for improving the performance of enzymes under operating conditions.[27–29] It is a very useful method in biocatalysis because it combines purification and immobilization of enzymes into a single operation. Cross-linked StLASPO aggregates were prepared using a two-step procedure: aggregation by precipitation with ammonium sulphate and cross-linking with glutaraldehyde.[23,24] Different cross-linker/precipitation solution ratios have been used: 0.4% (v/v, glutaraldehyde solution/total precipitation solution) was chosen to stabilize towards leaching since the enzyme was fully active even after different bioconversion cycles (see Table 4). In fact, the CLEA-StLASPO preparations obtained using a 0.2% or 0.6% ratio lost a large part of the initial activity after one cycle of bioconversion. About 1.1 units of pure StLASPO were precipitated as CLEA at pH 7.5: the enzymatic activity was 4 U per g wet CLEA. The stability of the CLEA-StLASPO aggregate was tested by the boiling procedure (as stated above): no free protein was released from the preparation.

The resolution of 50 mM D,L-aspartate was carried out at 70 °C using the CLEA-StLASPO preparation, in "magic buffer" adjusted to the desired pH (8.0, 9.0 or 10.0) by adding 1 M NaOH. In the first cycle, a full conversion was reached at all pH values tested and in a comparatively shorter time at the highest pH. Indeed, at pH 9.0, complete oxidation of L-aspartate was obtained in ≤4 hours for 5 cycles.

3.3. Resolution on a semi-preparative scale of D,L-aspartate

The immobilized StLASPO on Relizyme™ HA403/S R resin was incubated under mechanical stirring with 100 mM D,L-aspartic acid (10 mL final volume) at pH 10.0 and 70 °C under constant air stream. The oxidation of L-isomer of aspartate was complete after 24 hours of incubation: noteworthily, the immobilized StLASPO preparation was reused with no lack of activity. In particular, the scale-up of the enzymatic reaction catalyzed by Relizyme-StLASPO produced 66 mg of optically pure D-aspartate in 24 hours using 20 units of StLASPO per cycle.

4. Conclusions

The L-amino acid oxidase class of flavoproteins comprises a group of enzymes sharing similar functional and structural properties that can be divided into two groups:[1] LAAOs possessing a broad substrate specificity and LAAOs which oxidize one or a narrow group of substrates, such as LASPO, L-lysine oxidase, L-tryptophan oxidase, *etc.* Recombinant StLASPO shows distinctive features making it attractive for biotechnological applications:[11,12] a) it is a highly stable flavoenzyme: it is thermostable (*i.e.* fully stable up to 80 °C), shows a high temperature optimum, and is stable in a broad range of pH; b) it presents suitable kinetic properties: it is weakly inhibited by the product 2-oxosuccinic acid and by the D-isomer of aspartate, K_m for dioxygen is pretty low, and the FAD cofactor is stably bound. Indeed, we now

Table 2 Effect of D,L-aspartate concentration on the resolution by StLASPO (2 U) immobilized on Relizyme™ HA403/S R support at pH 10.0 and 70 °C

$[D,L\text{-Asp}]_{solution}$ (mM)	L-Aspartate conversion (ee) Time (h)								
	0.5	1	2	3	4	5	7	21	42
35	>99.5%	—	—	—	—	—	—	—	—
70	62%	>99.5%	—	—	—	—	—	—	—
110	12%	36%	>99.5%	—	—	—	—	—	—
140				67%	>99.5%	—	—	—	—
180					53%	69%	84%	—	—
360					17%	21%	21%	21%	21%

Table 3 Results of kinetic resolution of a 50 mM D,L-aspartate (0.5 mL) solution using 0.5 U of StLASPO immobilized on SEPABEADS® EC-EP/S support at pH 10 and 70 °C

Cycle number	Time (h)	ee[a] (%)
1	3	68
	4	>99.5
2	2	66
	4	>99.5
3	2	65
	4	>99.5
4	2	59
	4	81
	6	>99.5
5	2	28
	4	45
	6	73
	8	80
	10	>99.5

[a] ee values calculated using the HPLC analysis.

Table 4 Results of kinetic resolution of a 50 mM D,L-aspartate (0.5 mL) solution using 1.1 U of StLASPO precipitated as CLEA at different pH values and 70 °C

Cycle number	Reaction time (h)	ee[a] (%)		
		pH 8.0	pH 9.0	pH 10.0
1	2	49	32	53
	4	87	>99.5	>99.5
	6	>99.5		
2	2	49	63	66
	4	97	>99.5	>99.5
	6	>99.5		
3	2	69	69	64
	4	73	>99.5	>99.5
	6	>99.5		
4	2	88	54	42
	4	>99.5	>99.5	68
	6			>99.5
5	2	91	91	40
	4	>99.5	>99.5	69
	6			95
	7			>99.5

[a] ee values calculated using the HPLC analysis.

demonstrate that StLASPO is not sensitive to hydrogen peroxide (Fig. 3): accordingly, the use of catalase during bioconversions is not required.

In order to facilitate the recovery of the reaction products and the reuse of the biocatalyst, immobilization experiments of pure, recombinant StLASPO have been carried out. The best results in terms of immobilization yield have been obtained with the amino support Relizyme™ HA403/S R (100% of StLASPO activity) and in terms of volumetric activity (U per g support) and recycling with both the Relizyme™ HA403/S R and the epoxy support SEPABEADS® EC-EP/S. Because of activation of the Relizyme™ HA403/S R support with glutaraldehyde, both matrices reacted with the free amino groups of the flavoenzyme.

StLASPO was also used as CLEA form: the immobilization *via* precipitation and cross-linking of enzyme molecules with a bifunctional cross-linking agent is a carrier-free method and the resulting biocatalyst is permanently insoluble and essentially maintains 100% of its activity for 5 cycles, with complete conversion in <8 hours. The volume ratio of cross-linker to enzymatic precipitation solution of 0.4% was chosen to stabilize towards leaching. Such a value was considered a moderate concentration of glutaraldehyde: it is sufficient to ensure the activation of most amino groups with one molecule of glutaraldehyde. Indeed, the high recovery in terms of enzymatic activity of CLEA-StLASPO excludes loss of enzyme flexibility due to an excessive cross-linking, as instead we observed at 0.6% volume ratio. Glutaraldehyde is a widely used cross-linker whose reaction with amino groups is still not fully elucidated.[26] The optimization of StLASPO immobilization will require further investigations of the glutaraldehyde-induced production of intra- and inter-molecular enzyme cross-links and a stricter control of the immobilization process. The time course of the CLEA-catalyzed reaction was similar to that obtained with the flavoenzyme immobilized on SEPABEADS, even if slower compared to the Relizyme-immobilized StLASPO (compare Tables 1, 3 and 4). Indeed, the scale-up of the enzymatic reaction catalyzed by Relizyme-StLASPO allowed us to produce 66 mg of D-aspartate (with high ee) in 24 hours using 20 units of StLASPO per cycle. Moreover, it has been previously demonstrated for D-amino acid oxidase that the *in situ* chemical reduction of the imine product allows us to converge to a full conversion into a single enantiomer:[30,31] a similar process is also feasible for the reaction catalyzed by StLASPO on D,L-aspartate (Fig. 1B).

In conclusion, because of the very interesting properties of this enzyme, we propose the immobilized StLASPO as an

attractive tool for biotechnological applications and, together with the set-up of an efficient expression system in *E. coli*,[12] as a complete biocatalytic process for the synthesis of fairly high amounts of enantiopure D-aspartate.

Abbreviations

CLEA	cross-linked enzyme aggregates
DAAO	D-amino acid oxidase (EC 1.4.3.3)
LAAO	L-amino acid oxidase (EC 1.4.3.2)
LASPO	L-aspartate oxidase (EC 1.4.3.16)
StLASPO	*Sulfolobus tokodaii* L-aspartate oxidase
ee	enantiomeric excess

Acknowledgements

This work was supported by grants from Fondo di Ateneo per la Ricerca to L. Pollegioni and L. Piubelli. The authors gratefully acknowledge PRIN 2010–2011 NANO Molecular Technologies for Drug Delivery – NANOMED prot. 2010 FPTBSH and the support from Consorzio Interuniversitario per le Biotecnologie (CIB).

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