Hydrolysis of L-Arginine – Chemical and Enzymatic Catalysis

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Abstract

The depletion of fossil feedstocks and growing concern over CO$_2$ emissions has led to the search for an alternative source for bulk functionalized chemicals. The N-ergy project has the ultimate aim of utilizing agricultural waste streams as the main raw material for the simultaneous production of ethanol and bulk nitrogen-functionalized chemicals. One of the intermediate steps in the projected process is the conversion of L-arginine to L-ornithine. The hydrolysis of L-arginine to L-ornithine and urea can be chemically catalysed or biocatalysed by L-arginase. The hydrothermalysis of L-arginase in alkaline conditions led to the production of L-ornithine and numerous secondary products. The maximum L-ornithine yield obtained was of 15.2% at 150°C with initial pH adjusted to 12.0 with sodium hydroxide. The addition of a solid basic catalyst, NaY zeolite, showed limited catalytic effect even at high concentrations. Bacillus subtilis arginase (EC 3.5.3.1) was successfully immobilized in three different epoxy-activated supports – Sepabeads EC-HFA, Sepabeads EC-EP and Eupergit C 250 L. After one hour of incubation at room temperature in the presence of each support no arginase was detected in the supernatant. The covalent-binding to the three tested supports did not lead to a significant increase in arginase’s thermal stability and led to a 40%-60% decrease in catalytic activity.

Key words: L-arginine hydrolysis; NaY zeolite; arginase; enzyme immobilization.

Introduction

Oil and other fossil fuels still are the main sources for energy, transport fuels and (bulk) carbon-based chemicals, but the need for an alternative is undeniable. The depletion of fossil feedstocks, the increasing oil and transport fuels prices, together with the growing concern over climate changes and other consequences of CO$_2$ emissions, has led to the search for a cheap and environmentally friendly alternative.

Focusing on the production of chemicals, the replacement of fossil feedstocks with CO$_2$ neutral biomass offers a wide array of advantages: it’s a renewable resource which is currently being produced in large amounts; it’s a relatively cheap resource; the use of biomass instead of fossil fuels would considerably reduce greenhouse gases emissions. In the long term, after the complete exhaustion of fossil resources reservoirs, biomass will be the only available raw material for the bulk production of organic carbon-based chemicals.

Bio-refinery can be defined as the fractionation of biomass into components that, after further transformation and separation, can be used as final end products. When producing chemicals through bio-refinery two options can be considered: the development of “new” chemicals or the production of chemicals similar to the traditionally obtained from the petrochemical industry. While the first option would provide chemicals and materials with unique structures and properties, in the short to mid-term it is expected that there will be a stronger investment in the development of existing bio-based bulk chemicals which can profit from existing infrastructures. These structures – utilized in the production of bulk chemicals and materials based on fossil fuels – are already highly optimized and do not require large capital injections.

The factor that allows biomass to be a competitive alternative is the absence of functionalized substances in oil derivatives used in the production of functionalized chemicals. The introduction of a functional group in simple molecules, such as ethylene, often implies high production costs and dangerous working conditions. Comparatively, in the biomass mixture it is possible to find a variety of already functionalized components, considerably reducing the heat necessary to introduce the functionality. Additionally, these molecules have chemical compositions and structures similar to the desired products, considerably reducing the number of conversion steps required. A perfect example is the use of amino acids as precursors for nitrogen-containing bulk chemicals. This concept is explored in the N-ergy project, in which this study is integrated.
The ultimate aim of the N-ergy is the combined production of ethanol and nitrogen containing chemicals, utilizing biomass as the main raw material. Initially, a fermentation step performed by recombinant species should convert agricultural waste products to ethanol and cyanophycin granule peptide (CGP). CGP is a nonribosomally synthesized biopolymer, which consists of equimolar amounts of arginine and aspartic acid arranged as a polyaspartate backbone, with arginine residues linked to the β-carboxyl group of each aspartate by its α-amino group\(^2\). In nature this polymer is synthesized by most cyanobacteria and is accumulated as granules in the cells cytoplasm.

After extraction, cyanophycin should be converted to nitrogen-functionalized chemicals. In a first step, the polymer is completely hydrolyzed to its monomers: aspartic acid and arginine. After separation, both amino acids should undergo further transformations until the desired N-functionalized chemicals are obtained. Figure 1 illustrates the different steps in the conversion of biomass to the target products. The focus of the present study will be on the arginine route, which can yield 1,4-butanediamine (a building block for nylon-4,6) and valuable secondary products such as ammonia and urea.

In order to be converted to 1,4-butanediamine, arginine has to undergo two transformations: the hydrolysis of arginine to ornithine and urea; and the decarboxylation of ornithine to 1,4-butanediamine and carbon dioxide. Both steps can be catalyzed chemically or enzymatically. The two catalysis methods applied to the first step are explored and compared during this study.

The hydrolysis of arginine to ornithine can be chemically catalyzed in the presence of strongly acid or alkaline conditions\(^3,4\). The acid hydrolysis is a slow process even at high temperatures – Murray et al.\(^3\) obtained a conversion of 98% of L-arginine to L-ornithine after 120 hours of heating at 176°C in the presence of 6 N hydrochloridic acid. The acid catalyzed reaction leads solely to the formation of L-ornithine, no undesirable by-products are produced. Alkaline catalyzed hydrolysis can be performed faster and at lower temperatures, with the disadvantage of the formation of by-products. For each 5.5 equivalents of L-arginine that is converted to L-ornithine and urea, 1 equivalent is converted to citrulline and ammonia. The citrulline is further hydrolyzed to ornithine but at a slow rate. Other by-product comes from the reversible conversion of ornithine to its lactam (3-amino-piperid-2-one) at high temperatures under strong alkali concentration. Despite the existence of these side-reactions high yields of L-ornithine have been obtained. The heating at 110°C for 24 hours with the pH adjusted to 12 with ammonia\(^3\) resulted in a yield of 90% L-ornithine with 8% 3-amino-piperid-2-one and 1% citrulline.

Other than traditional acid and basic catalysis, other techniques have potential to produce interesting results. The use of zeolites as heterogeneous catalysts would allow the easier incorporation of the hydrolysis of arginine reaction in an industrial process. Today, the most relevant application of zeolites as solid catalysts is the use of acid Y zeolites in the petroleum industry\(^5\), although recent work has focused on the use of basic Y zeolites in the treatment of toxic compounds\(^6\). Concerning the catalysis of the arginine hydrolysis reaction, no work with zeolites can be found in literature.

The hydrolysis of L-arginine to L-ornithine and urea is biocatalyzed by L-arginase (L-arginine amidinohydrolase, EC 3.5.3.1). This is accomplished by the cleaving of the
guanidinium group from arginine which yields urea, a small nitrogen rich molecule. Thus, arginase plays a fundamental role in the nitrogen metabolism. It is widely spread through the evolutionary spectrum and can be find in significantly distinct organisms such as bacteria, yeast, plants and animals. Arginase is a multimeric (trimeric according to the latest studies’) metallo-enzyme comprised of identical or near identical sub-units. Each monomer has a binuclear manganese spin-coupled cluster located in the active site that is profoundly involved in the reaction mechanism.

Naturally, an industrial setting would require the immobilization of the enzyme, which would allow the reutilization of the biocatalyst. In literature, various methods for arginine immobilization have been explored with mixed results. However, usually these studies are focused on the use of arginase has an arginine detection tool.

Due to its multimeric structure, arginase is a good candidate for immobilization by covalent bond. The binding of this enzyme to a support can play an important role in avoiding dissociation of the enzyme by keeping the sub-units together and consequently enhancing the enzyme stability. Covalent epoxy-activated supports have additional advantages that make them ideal for protein immobilization on an industrial scale, these supports: are very stable during storage and also when suspended in neutral aqueous media; are easy to handle before and during immobilization procedures; are able to directly form very stable covalent linkages with different protein groups (amino, thiol, and phenolic ones) under very mild experimental conditions.

The immobilization of enzymes in epoxy-activated supports usually follows a two step mechanism: first a rapid mild physical adsorption between the protein and the support is produced, and secondly the covalent reaction between adsorbed protein and epoxy groups occurs. In order to adsorb proteins during incubation at high ionic strengths, commercial epoxy supports are fairly hydrophobic, in hydrophilic supports (e.g., agarose) this preliminary physical hydrophobic adsorption is not possible. The remaining epoxy groups may be easily blocked after the protein immobilization, to stop any kind of undesired covalent support-protein reaction. Even though other immobilization techniques inside porous supports can increase the enzyme operational stability by preventing any intermolecular process (proteolysis, aggregation) and by preserving the enzyme from interactions with external interfaces (air, oxygen, immiscible organic solvents, etc.), these techniques do not necessarily increase the conformational stability of the enzyme. This kind of stability should be achieved if the immobilization of each enzyme occurs through several residues. This way, all the residues involved in immobilization preserve their relative positions and the enzyme is unaffected by conformational changes promoted by heat, organic solvents, or any other distorting agents. Thus, multipoint covalently immobilized enzymes should become more stable than their soluble counterparts or than randomly immobilized derivatives.

Three epoxy supports will be tested for arginase immobilization: Sepabeads® EC-EP, Sepabeads® EC-HFA and Eupergit C 250 L. All supports are microporous, epoxy-activated, acrylic polymer matrix spherical beads. Sepabeads® EC-EP is a highly activated support functionalized with short chain epoxy groups while the Sepabeads® EC-HFA support is functionalized with epoxy groups on a longer, more complex, spacer. The standard grade beads have a diameter of 150-300 µm with an average pore diameter of 30-40 nm and a specific gravity of 1.13 g/ml. The epoxy group density on the beads is around 100 µmol/(g of wet support). Eupergit C 250 L is activated similarly to Sepabeads® EC-EP, having on its surface a dense monolayer of reactive and stable epoxy groups (200 µmol/(g of dry support)). The bead diameter is 100-250 nm with an average pore diameter of 100 nm.

The main goal of this study is to establish an industrial viable method of converting L-arginase to L-ornithine. With this objective in mind both approaches are studied: the chemically catalyzed hydrolysis of L-arginine and the hydrolysis of L-arginine catalyzed by the enzyme arginase. Concerning the chemical catalysis, the effects of pH and temperature in the yield of ornithine are studied. NaY zeolite, a heterogeneous catalyst, is also tested. All research on the chemically catalysis of the conversion of arginine is focused on the basic hydrolysis. The acid catalyzed reaction, regardless of being cleaner, is considerably slower and requires very high temperatures, being inadequate for a large scale process. Regarding the biocatalysis, the hydrolysis of L-arginine to L-ornithine and urea catalyzed by Bacillus subtillis arginase is studied. In this case, the main objective is to investigate the effect of immobilization in epoxy-activated supports on the performance of the enzyme. Different commercially available supports are tested for optimum stability/activity. After selecting the best performing enzyme preparation, the chemical and enzymatic hydrolysis of arginine are compared.
Materials and Methods

Chemical Catalysis

Materials. All reagents were of analytical grade.

Hydrothermalolysis experiments. A solution of 25 mM L-arginine in Milli-Q water was prepared. Portions of this solution were adjusted to pH 11 and 12 with 0.1 M sodium hydroxide. Volumes of 60 ml of the three solutions were heated in Parr pressurized stirred reactors at 110°C, 125°C and 150°C for a total of 9 distinct experimental conditions. The reactors were heated accordingly to a pre-programmed temperature gradient. 3 ml samples were taken from each reactor through a dip tube at 0, 1, 2, 18 and 20 hours. The reaction was stopped by immediately placing the samples on ice. The experiments were conducted in duplicate. The samples were stored in the fridge (5˚C) until dabsyl chloride derivatization and HPLC analysis.

Zeolite catalysis experiment. Different quantities of NaY zeolite were added to solutions of 25 mM L-Arginine in Milli-Q for final concentrations of 0.5 g/l sol, 1 g/l sol, 2 g/l sol, 5 g/l sol. Volumes of 30 ml of the four suspensions and a blank solution with no zeolite added were heated in Parr pressurized stirred reactors (utilizing a glass liner) at 125°C accordingly to a pre-programmed temperature gradient. 3 ml samples were taken from each reactor through a dip tube at 0, 1, 2, 18 and 20 hours. The reaction was stopped by immediately placing the samples on ice. The experiments were conducted in duplicate. The samples were stored in the fridge (5˚C) until dabsyl chloride derivatization and HPLC analysis.

Enzymatic Catalysis

Materials. Bacillus Subtillis arginase was supplied as a solution with the K-LARGE commercial kit for L-arginine/urea/ammonia detection from Megazyme International Ireland Ltd. Sepabeads® EC-EP and Sepabeads® EC-HFA were supplied by Resindion Srl (Mitsubishi Chemical Corporation). Eupergit® C 250 L was supplied by Sigma-Aldrich. Other reagents were of analytical grade.

Preparation of arginase stock solution. An arginase stock solution was prepared diluting 0.8 ml of enzyme solution to a total volume of 5.5 ml with Milli-Q water. Sodium azide was added (0.05% mass) to inhibit microbial growth. The solution was filter sterilized and samples were collected for Bradford and activity assays.

Arginase activity in the solution was determined by monitoring the hydrolysis of 1 ml of 100 mM L-arginine at pH 9.5 and 37°C. At different times, 10 μl samples were taken from the reaction solution. The samples were immediately quenched with 150 μl of 1 M acetic acid and stored in the fridge (5˚C) until fluorescamine derivatization and HPLC analysis to determine L-arginine and L-ornithine concentrations.

Immobilization of arginase in different epoxy-activated supports. To 0.5 ml of arginase stock solution were added to 7.5 ml of filter sterilized sodium phosphate buffer pH=8.0 (10 mM for Sepabeads EC-HFA; 0.8 M for Sepabeads EC-EP and Eupergit C 250 L). After gentle mixing, the solution was added to a sterile 10 ml test tube containing 1 g (dry weight) of support, the tube was immediately placed on the rotator at slow rotation speed and the clock was started. The immobilization was carried for 24 hours with 60 μl samples of the supernatant being collected at 0, 1, 2, 4, 6, 23 and 24 hours. 30 μl of each sample were immediately utilized for an activity assay, while the remaining of the sample was stored in the fridge (5˚C) for posterior Bradford assay. The immobilizations was conducted in duplicate and in the presence of a blank containing the same enzyme solution/buffer ratio but no support added.

Arginase activity in the solution was determined by monitoring the hydrolysis of 100 μl of 100 mM L-arginine at pH 9.5 and 37°C. Samples were taken, quenched, stored and analyzed as previously described.

Washing of support beads. After incubation, the suspensions were filtered using a sintered glass filter. The filtrate was rinsed with 8 ml of 50 mM sodium phosphate buffer (pH=8.0) and filtrated once more. The filtered support was collected on a 10 ml sterile test tube, to which was added 8 ml of sterile 50 mM sodium phosphate buffer (pH=8.0), and placed on the rotator at slow rotation speed for 45 minutes. After this washing step, the filtering/rinsing procedure was repeated and the dry support with the immobilized arginase was stored in the fridge (5˚C). After each filtration step a 1 ml sample of the supernatant was collected for posterior analysis.

Blockage of the remaining epoxy groups. Portions of the three supports containing the immobilized arginase were submitted to a similar blockage procedure. 0.5 g of each
support were placed in a 10 ml sterile test tube to which were added 4 ml of 3 M glycine in 50 mM sodium phosphate buffer (pH=8, filter sterilized). The suspensions were place in the rotator at slow rotation speed. After 18h the blocked supports were washed and stored accordingly to the previously described procedure.

**Immobiled arginase activity assay.** Arginase activity in the support beads was determined by monitoring the hydrolysis of 1 ml of 100 mM L-arginine in 50 mM triethanolamide/HCl buffer (with 1mM MnCl) at 37°C. 25 mg of support were used for each assay; these portions were pre-incubated in the triethanolamide/HCl buffer for 5 minutes also 37°C. Samples were taken, quenched, stored and analyzed as previously described.

**Thermal stability of immobilized arginase.** Multiple 25 mg portions of each of the three supports containing the immobilized enzyme (with and without blockage of the remaining epoxy groups) were incubated in 600 μl of 50 mM triethanolamide/HCl buffer (with 1mM MnCl) at 60°C. Periodically, a preparation was withdrawn and the remaining activity of the immobilized arginase was immediately assayed at 60°C as previously described. Preparations of each support were assayed after 1, 2, 4, 6, 16 and 24 hours of incubation. A comparable set of preparations containing the soluble enzyme was also incubated and assayed following the same protocol.

**Results and Discussion**

**Chemical Catalysis**

**Hydrothermolysis experiments.** The alkaline hydrothermolysis of L-arginine was followed for 20 hours at different temperatures. Previous studies on this reaction under similar pH and temperature conditions reported high L-ornithine yields. Murray *et al.*, for example, obtained a yield of 90% L-ornithine at pH=12 and T=110°C. Yet, the various works found in literature utilize experimental setups considerably different from the one that was employed in the present study. Similar thermal degradation experiments were performed in sealed evacuated tubes, with the reaction being stopped at certain point by cooling the tubes. The experimental setting now employed is closer to an industrial scale process, the reactions are carried in pressurized reactors and samples are regularly taken through a dip tube.

According to what is described by Warner *et al.*, temperature positively influences arginine hydrolysis (figure 2). The higher conversion values were detected at 150°C where, after 20 hours of reaction, the high temperature nullifies the pH effect, with concentrations of remaining arginine reaching 6%. The influence of pH is also clear, especially at lower temperatures. Although the adjustment of the initial pH to 11 with sodium hydroxide has almost no effect on the rate of consumption, the adjustment to pH=12 leads to a decrease of arginine concentration of 25% at 110°C and 125°C after 20h of reaction. This effect was also previously described by Warner *et al.*

The influence of pH in ornithine formation is concordant with its influence in arginine degradation: for every temperature higher pH leads to a higher ornithine yield (figure 3). However, in this case, the effects of temperature are not as linear. The maximum yield, 15.2%, was obtained at 125°C pH=12.
Figure 3. Time course of L-ornithine formation under different experimental conditions: (A) 110°C; (B) 125°C; (C) 150°C. The percentages are based on the total concentration of amino acids (L-arginine and L-ornithine) in the reaction mixture at t=0.

Comparing arginine consumption with ornithine yield, it becomes clear that secondary products are being formed. The complex HPLC chromatograms and NMR spectra obtained reinforce the idea that multiple products are present in the reaction mixture. Probable side-products are: citrulline, formed directly from arginine; 3-aminopiperid-2-one, formed from the lactamization of ornithine; 1,4-butanediamine, formed from the decarboxylation of ornithine. The decrease of ornithine concentration between 18h and 20h at 150°C suggests that ornithine is being converted to another product.

After identification of the different peaks on the HPLC chromatogram, the presence of citrulline and 3-aminopiperid-2-one in the reaction mixture was established. Still, citrulline appears only in small amounts and the presence of the lactam in reasonable amounts is not enough to explain the substantial disparity between arginine conversion and ornithine yield.

Despite not being detected during HPLC analysis, the hypothesis of 1,4-butanediamine formation can’t be neglected. 1,4-butanediamine is a relatively volatile product, and there is a possibility that it escapes as a gas at the moment a sample is being collected. This supposition is supported by the presence of foul odor felt during sample collection (1,4-butanediamine is known for its strong odor). However, the comparison of $^1$H-NMR spectra (not shown) appears to demonstrate that there is no 1,4-butanediamine formed during the experiments. The $^1$H-NMR spectrum of 1,4-butanediamine shows a distinct peak at 2.6 ppm that clearly is not present on the $^1$H-NMR spectrum for the reaction mixture (samples for NMR analysis are collected only after the reactors cool to room temperature, which guarantees that this product is in liquid state). Thus, new and more precise analytical methods should be employed to follow the thermohydrolysis of arginine reaction in order to clarify which compounds are being formed and in what amounts.

During analysis no urea was detected. This is easily explained as at temperatures above 100°C in alkaline conditions urea is almost immediately hydrolyzed to ammonia and carbon dioxide. Considerable amounts of ammonia were detected through HPLC analysis. Despite being less valuable than urea, ammonia market prices at the moment (August 2008) are extremely high, reaching $600/tonn. Bearing in mind that each tonne of arginine as the potential to produce 195 kg of ammonia, it could be of financial interest to recuperate this secondary product.

Zeolite catalysis experiment. This experiment was performed with the objective of replicating or improving the results previously obtained for ornithine yield, utilizing a catalyst suitable for an industrial process. In literature, no comparable work is found on the arginine hydrolysis reaction with zeolites or other similar materials. The reaction was followed for 20 hours with addition of NaY zeolite in different concentrations: 0.5 g/l, 1 g/l, 2 g/l, 5 g/l and no zeolite added.

As seen in figure 4, the effects of the zeolite in the reaction are limited even at high concentrations of 5 g/l. Nonetheless, there is a mild catalytic effect, the best results, 11.2%, are obtained with 5 g/l at 18 hours. It should be noted that concentrations this high (5 g/l) of zeolite are probably unsuitable for application in an industrial process.
Figure 4. Effect of different NaY zeolite concentrations on the L-arginine thermohydrolysis reaction. The percentages are based on the total concentration of amino acids (L-arginine and L-ornithine) in the reaction mixture at t=0.

The zeolite utilized also showed to be the impractical, even at laboratory scale, due to its accumulating on the side walls of the glass liners, being extremely difficult to remove.

Enzymatic Catalysis

Characterization of the arginase stock solution. The exact protein concentration and activity in the Bacillus subtilis arginase stock solution where determined by Bradford and activity assays respectively. The Bradford assay gave a protein concentration of 4.1 mg/ml. This value together with the soluble enzyme activity assay, which reported an activity of 325 U/ml, gives a specific activity of 80 U/mg-protein in the arginase stock solution. One unit is defined as one mol of L-ornithine produced per minute at pH=9.5 and 37°C. The value of specific activity obtained is only 9% of the value obtained by Nakamura et al. for purified Bacillus subtilis arginase (858 U/mg). This low value can not be explained by the relatively low concentration of manganese ions. The Mn²⁺ concentration in the stock solution is close to 7 mM, well above the concentration suggested by Nakamura et al. for full enzyme activation (3 mM). A possible explanation can be the use of more aggressive purification methods that damages the enzyme’s structure.

Figure 5. SDS-PAGE gel of the original arginase solution. Lane (A) corresponds to a 100X dilution while lane (B) corresponds to a 200X dilution. Lane (M) contains the molecular markers identified with the corresponding molecular weights in Daltons.

The purification methods employed were not specified by the supplier.

A SDS-PAGE gel of the original arginase solution was prepared in order to verify its purity (figure 5). The run was done in the presence of a reducing agent, guarantying that the enzyme is dissociated to its sub-units. A strong band is observed slightly below 38,000 Da. Due to the presence of reducing agents, this band should correspond to the monomeric units of arginase and is in accordance with the range of values reported for the molecular weight of monomers of other bacterial arginases, 31,000-34,000 Da. This value also suggests that the enzyme purified by Nakamura et al. was probably in the trimeric form, as a molecular weight of 115,000±5,000 Da was obtained by this author for the purified native arginase. The only other bands found on the arginase lanes are located around 70,000 Da and 110,000 Da (only observed in the 100X dilution lane). These bands should correspond to the dimeric and trimeric forms of the enzyme, showing that arginase is not fully dissociated in the presence of the reducing agent. This leads to the conclusion that the arginase solution supplied is highly pure.

Immobilization of arginase in epoxy-activated supports. The arginase covalent immobilization in Sepabeads EC-HFA, Sepabeads EC-EP and Eupergit C 250 L epoxy-activated supports was followed for 24 hours. The immobilization of the enzyme in the
three different supports was clearly successful. After one hour no protein was found in the supernatant of all suspensions (figure 6), indicating that all enzyme is bound to the supports. The analysis of the supernatant activity (results not shown) shows comparable results.

Previous studies\textsuperscript{16} showed similarly successful results with Sepabeads EC-HFA, the complete immobilization of different enzymes is observed after 1 to 6 hours. In the case of Sepabeads EC-EP and Eupergit C, the results obtained for arginase immobilization were better than the results reported for other enzymes. Using these supports and similar incubation conditions for covalent immobilization of various enzymes, Mateo et al.\textsuperscript{16} observed an immobilization inferior to 65\% of the enzyme after 25 hours of incubation. After 24 hours, low concentrations of protein are detected on the supernatants of Sepabeads EC-EP and Eupergit C immobilizations, suggesting that arginase might be only temporarily bond to this supports. This apparent release of arginase from both short-chained epoxy group supports is contained in the experimental error margin for this experiment, but the immobilization should be followed for a longer period in order to clarify the results. If the release of arginine from the supports after longer periods of incubation is confirmed, a possible explanation can be damage inflicted to the structure of the beads by mixing or the high concentration of sodium phosphate buffer (0.8 M). This seems unlikely, as the mixing should also affect the Sepabeads EC-HFA beads and previous work with higher concentration of sodium phosphate buffer during incubation did not show similar problems\textsuperscript{12}.

After incubation for 24 hours in the presence of arginase, a washing protocol was applied to the support beads. During this procedure, the supernatants of the filtration steps were analyzed in order to detect any protein that might be released from the beads. The analysis confirmed the presence of small amounts of enzyme in the supernatants of Sepabeads EC-EP and Eupergit C suspensions at the end of incubation time. Moreover, during the first rinse step enzyme was released from the Eupergit C 250 L beads, showing that not all arginase is covalently bound to the support. The protein that is released when the beads are rinsed with 50 mM sodium phosphate buffer has yet to complete the second step of the binding mechanism, and is only physically adsorbed to the support’s surface by hydrophobic interactions. When in contact with a buffer with lower ionic strength (50 mM as opposed to 800 mM), these interactions are weaker and the enzyme is released. The presence of residual, non-covalently bound enzyme after incubation has previously been reported for Eupergit C supports\textsuperscript{13}.

Finally, it should be noted that both Sepabeads supports were easily handled, leading to minimal support loss during the immobilization of arginase in these supports. However, Eupergit C 250 L originates viscous suspensions, making the manipulation of the beads more difficult. This led to a considerable loss of Eupergit C support during the washing steps.

**Recovered activity of immobilized arginase.** The recovered activity exhibited by the enzyme immobilized in the different supports was compared with the soluble form. The support beads and soluble enzyme were assayed in the presence of a 50 mM triethanolamide/acetate buffer (pH=8.0) with 1mM Mn\textsuperscript{2+}.

As expected, the covalent immobilization greatly influences the catalytic activity of arginase (figure 7). The multi-point reaction between arginase and the support deforms the enzyme, altering the shape of its active site and, possibly, affecting manganese uptake. The values of recovered activity obtained for immobilization of arginase in the different epoxy supports – from 43\% to 61\% – are low but acceptable. The results described in literature for immobilization of other enzymes on similar supports are extremely irregular.
Figure 7. Recovered activity of arginase immobilized in different epoxy supports. Percentages are calculated by comparison with the soluble form. The following assumptions were made: only 95% of the enzyme in solution during incubation was permanently immobilized in the Sepabeads EC-EP and Eupergit C supports; 5% of Eupergit C support was lost during the washing steps.

For example, in the case of β-galactosidase values of recovered activity range from 15% to 100% depending on the organism of origin and type of epoxy support utilized.

Despite the rather inconsistent results, it is clear that the treatment with glycine decreases the recovered activity of the immobilized arginase. The opposite results were expected, as glycine was added with the objective of reacting with the epoxy groups that remained free after incubation time. This would stop the covalent-binding reaction, preventing excessive enzyme/support interaction that could destabilize the enzymes’ active site. One possible explanation for the abnormal results is that glycine might directly interact with arginase’s active site, interfering with the catalytic mechanism.

The effect of the type of epoxy-activated support on the activity of immobilized arginase is not clear. Previous studies show that different supports can have considerably different effect on the immobilized enzyme activity. This may be related to the distinct hydrophobicity of the surface of the various supports. The stronger or weaker hydrophobic interactions between the support and the hydrophobic residues can affect the orientation of the enzyme and of its active site, affecting the catalytic activity. The length of the epoxy groups’ spacer arms (longer in Sepabeads EC-HFA) can also be a factor on the orientation of the enzyme.

Thermal Stability of Immobilized Arginase. The stability of the immobilized arginase derivatives was analyzed by following the residual activity of the enzyme during incubation at 60°C. The activity of arginase immobilized in the three tested supports (with and without blockage with glycine) and of soluble arginase was assayed after periods of incubation at 60°C up to 24 hours. The beads were incubated in the presence of a 50 mM triethanolamide/acetate buffer (pH=8.0) with 1mM Mn²⁺. The addition of manganese ions slows the deactivation of the enzyme (results no shown). In the absence of the metal ion the deactivation is too fast, not allowing accurate comparison of the influence of immobilization in the different supports.

The results obtained for Sepabeads EC-EP and Eupergit C blocked with glycine were inconsistent due to the low remaining activity of arginase immobilized in these supports. Concerning the effect of the other tested supports (figure 8), only the covalent-binding to Sepabeads EC-EP without glycine blockage shows a mild positive effect in arginase thermal stability. The immobilization in this supports leads to a 20% increase in residual activity when compared with the soluble enzyme after 6 hours of incubation at 60°C. The immobilization of arginase in Sepabeads EC-HFA and Eupergit C 250 L appears to have a negative effect on the enzyme’s stability. The deactivation of the immobilized derivatives for both these supports is faster than the deactivation observed for the free enzyme, although the blockage of Sepabeads EC-HFA with glycine seems to lead to an increase in the derivatives stability.

The results are not in accordance with the results reported by Mateo et al. This author claims that the immobilization of different enzymes in all the three tested supports consistently increases the enzymes’ thermal stability.

Figure 7. Evolution of residual activity of arginase immobilized in different epoxy supports compared to soluble arginase during the course of the first 6 hours of incubation at 60°C. The percentages are based on the initial (t=0) enzyme activity.

Conclusion

The results obtained show that the chemical catalysis of arginine hydrolysis is not, at this moment, a suitable method for ornithine production on an industrial scale. Not only were the yields obtained disappointing when compared to the values mentioned in literature, but also the optimization of the reaction would...
require techniques undesirable for an industrial process (use of buffers, constant correction of pH). A potential alternative could be the manipulation of pH through the continuous separation of the different components of the reaction mixture. The high temperatures employed do not allow the recovery of urea, a considerably valuable side product. Nonetheless, the production of ammonia can still provide a substantial source of income in an industrial scale process.

The solid catalyst utilized, NaY zeolite, showed limited catalytic effects even at high concentrations. As an alternative, other materials with catalytic properties could be utilized including: zeolites ion-exchanged with metal ions that generate stronger basic sites (Cs⁺, Rb⁺, K⁺); alkaline earth oxides; diverse heterogeneous superbasic catalysts.

The results obtained for the enzymatically catalyzed reaction were promising, with Bacillus subtilis arginase being successfully immobilized in three different supports suitable for industrial application. The covalent binding to the three tested supports did not show a significant increase in arginase’s thermal stability and the activity of the immobilized derivatives is considerably low when compared with the soluble form. The blockage of the un-reactive epoxy groups with glycine did not show significant increase in stability/activity. Further research should focus on identifying the operational conditions that maximize the production of ornithine and the stability of immobilized arginase, as Bacillus subtilis arginase’s properties have not been the subject of extensive studies.

The results on the chemical and enzymatic catalysis of the hydrolysis of L-arginine can not be objectively compared yet. While the experimental setup utilized to study the chemically catalyzed reaction is similar to a potential industrial process, the work realized on the enzymatic conversion was primarily focused on the immobilization of arginase in industrial suitable supports and not on the optimization of L-ornithine production. Nonetheless, the biocatalysis approach seems to be the most promising. The arginase catalyzed reaction is very clean when compared to the alkali catalyzed reaction, the only secondary product produced being urea, an economically attractive side product that cannot be obtained from the alkali catalyzed conversion of due to the high temperatures employed. The major drawbacks of arginase application to a large scale process should be its dependence on manganese ions and limited availability of the enzyme.

References