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Production and properties of threonine aldolase immobilisates

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ABSTRACT

Dichiral β-hydroxy-α-amino acids are a highly valuable class of compounds from which pharmaceutically active intermediates for the synthesis of e.g. β-sympathomimetic drugs can be obtained. In lieu of laborious multi-step "classical" organic synthesis, biocatalysis using threonine aldolases (TAs) opens up a way to synthesise β-hydroxy-α-amino acids in one step. Although enzyme kinetics, stereospecificity as well as substrate specificity were and are matters of investigation, there is a lack of investigations addressing enzyme stability, which is crucial if the reaction is thought to be transferred to an economical scale.

Hence methods to immobilise the L-low specificity threonine aldolase of *Escherichia coli* (L-TA) were studied. After extensive screening the entrapment of the enzyme into a porous network of orthosilicate appeared to be the most promising method.

1. Introduction

Threonine aldolases (TAs) and the related serine hydroxymethyltransferases (SHMT) [1,2] are a group of enzymes that are able to catalyse formation and cleavage of the C_2—C_3-bond in β-hydroxy-α-amino acids like threonine [3,4]. While the physiological role of TAs is to cleave threonine yielding acetaldehyde and glycine (Scheme 1), it is the reverse reaction that is attracting interest of synthetic organic chemists, since it would allow to synthesise stereopure β-hydroxy-α-amino acids and it derived molecules like 1,2-aminoalcohols from simple starting materials (an aldehyde and an amino acid) [5–7].

By using this biocatalytic approach highly valuable chiral building blocks needed to synthesise pharmaceutically active substances like antibiotics as well as β-sympathomimetic drugs (Fig. 1) would be accessible in a more straightforward and environmentally benign way than the currently used laborious multi-step synthesis making use of "classical" organic chemistry [8].

However this straightforward and promising biocatalytic approach still suffers from drawbacks such as unsatisfactory diastereoselectivity of TAs and the achievable product yield. As a consequence those issues are matter of current investigations [10]. But although product yield and stereoselectivity are important performance criteria of a biocatalytic process, it often is the stability of the enzyme determining whether a biocatalytic route becomes economically viable or not. Hence investigating methods and ways to maximise stability of an enzyme under process conditions is as important as improving stereoselectivity and yield. However in case of TAs investigations on improving stability of those enzymes are rather scarce, which prompted us to study ways to improve the stability of TAs.

The most common strategy to improve stability is immobilisation [11,12] which can also be used to make an enzyme separable from the reaction mixture and hence reusable, given the activity of the enzyme is retained. Furthermore immobilisation enables to run a process continuously as the immobilised enzyme can be used as a fixed bed.

But although numerous immobilisation methods have been developed so far [13], no universal principles for selecting the appropriate method for a given enzyme have yet been identified, making selection of the most appropriate immobilisation method for a particular enzyme still a matter of empirical work [14]. Consequently different immobilisation methods needed to be studied in order to identify suitable methods for stabilising threonine aldolases (TAs).

Among known TAs the low-specificity TA of *Escherichia coli* (L-TA) appeared to be the most promising one to be studied for immobilisation as it is well studied and reported to catalyse synthesis of amino alcohols and hydroxy amino acids with good stereoselectivity (values obtained by Baer et al. for *o*-chlorophenylserine: conversion > 95%, enantioselectivity > 99%, diastereoselectivity with d.r. (threo/erythro) = 80:20) making it one of the promising candidates to become commercially interesting [15].

2. Results and discussion

The low-specificity L-threonine aldolase of *E. coli* (L-TA) is a homotetrameric enzyme [16] for which inactivation (16% loss in

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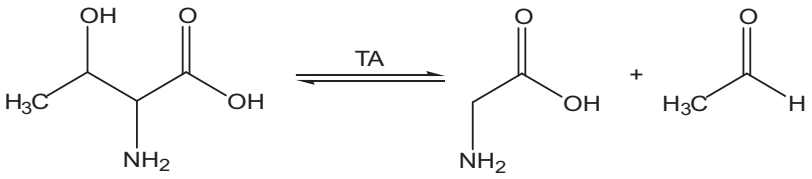


Scheme 1. Threonine aldolase catalysed cleavage of threonine to give glycine and acetaldehyde.

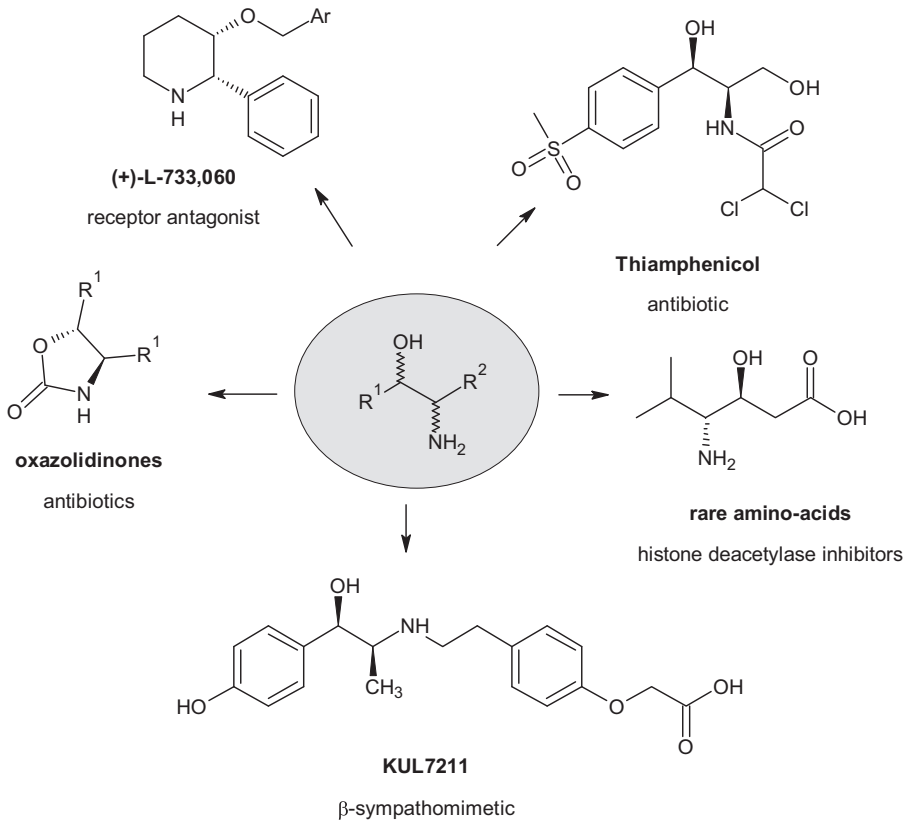


Fig. 1. Pharmaceutically active products based on dichiral intermediates having a 1,2-aminoalcohol moiety.

activity in 6 weeks) was observed even if the enzyme was stored at 4 °C indicating that immobilisation is clearly required in order to maintain enzyme activity under reaction conditions ($T \sim 30$ °C) as well as over normal periods of storage.

In general there are four basic methods for enzyme immobilisation: (1) adsorption onto a carrier, (2) entrapment into a gel or porous media, (3) covalent attachment onto a carrier and (4) crosslinking of enzyme crystals and aggregates (CLEA, CLEC) [17,18].

2.1. Adsorption onto carriers

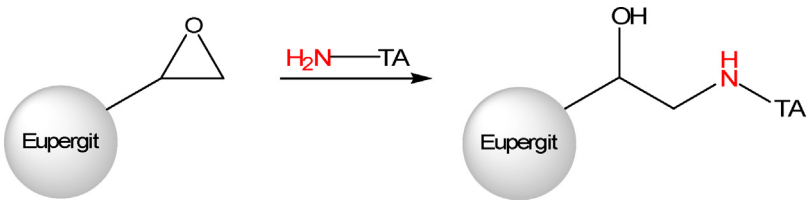
Adsorption makes use of the ionic interactions between the enzymes surface and a carrier substrate. However investigations on adsorption of L-TA onto different materials ($CaCO_3$, Al_2O_3, Alginit) [19,20] having ion exchange properties showed that this method is not a promising way to obtain L-TA immobilisates, as adsorption yields did not exceed 1% of the original activity (Table 1). Only in the case of the styrene-divinylbenzene based commercial ion exchange resin Amberlite XAD 2 [21] a significant amount (Table 1) of enzyme remained on the carrier.

In order to make sure that poor adsorption properties instead of other reasons caused the low enzyme activity after fixation onto the adsorbent, the L-TA was eluted with sodium phosphate buffer at pH = 7.0. From the resulting enzyme activity in the supernatant which was >93% in all cases, it was concluded that in fact the poor adsorption behaviour of L-TA was responsible for the low activity found for L-TA immobilised by adsorption.

These results show that binding the enzyme via ionic interaction is not efficient in case of L-TA as the overall charge of the enzyme at process conditions of pH = 8.0 does not appear to be sufficient to permanently link the enzyme to the carrier. Hence alternative immobilisation methods were investigated.

Table 1
Screening of immobilisation procedures for L-threonine aldolase of *Escherichia coli* and obtained yields.

Enzyme	Technique	Support	Residual activity (%)
L-TA *E. coli*	Inclusion	Alginate beads	5–12
		Orthosilicates	≤ 30
		Superabsorber Favor®	–
	Adsorption	Amberlite XAD-2	<6
		Al_2O_3	<1
		$CaCO_3$	<1
		Cellulose	<1
		Alginit	<1
	Covalent attachment	Eupergit® C	<1
		Chitosan	5



Scheme 2. Direct covalent attachment of enzyme onto Eupergit® C.

2.2. Covalent attachment onto a carrier

The use of carriers bearing reactive groups on its surface capable of reacting with e.g. enzyme amino groups enables the covalent attachment of an enzyme onto a carrier. In comparison to immobilisation products obtained from equilibrium dependent ionic interactions, covalent attachment of enzymes onto a carrier yields stable immobilised enzymes with preserved activity [22].

As a starting point immobilisation of L-TA using Eupergit® C was tested. The material is commercially available and well known for efficient covalent enzyme immobilisation [23,24] However, Eupergit® C proved not to be suitable for L-TA from *E. coli* as immobilisation yields (based on activity) did not exceed 1%. The observed immobilisation yields are contrary to results obtained in comparative experiments with L-TA from *T. maritima* for which activity based immobilisations yields reached 47% applying the same conditions (Scheme 2). The latter is in good accordance with the values reported by Fu et al. (52% immobilisation yield) [25]. The reasons for the observed differences in immobilisation yields may be attributable to catalytically important surficial amino groups that are present and accessible for reaction in the *E. coli* L-TA but not in the *T. maritima* L-TA. However further research is needed to fully understand the observations made.

As a consequence alternative carriers needed to be sought. An interesting alternative to Eupergit® C is chitosan, a non-toxic and inexpensive biopolymer [26]. The polyaminosaccharide is accessible to functionalisation with glutaraldehyde thus placing aldehyde moieties on the surface with the aim of establishing covalent bonds with surficial amino groups (ε-amino- or *N*-terminal-amino groups) of L-TA [27].

To achieve immobilisation of L-TA two strategies were pursued: (i) linkage of L-TA to chitosan pre-treated with glutaraldehyde and (ii) cross-linking the enzyme with non-pre-treated chitosan in solution to which the aldehyde is added. In addition, direct cross-linking of L-TA using only glutaraldehyde (CLEA, CLEC approach) was also tested. In fact none of the strategies proved to be satisfactory. While linking the enzyme to chitosan resulted in approx. 5% residual activity, irrespective of the method applied, enzyme cross-linking experiments were found to lead to full enzyme deactivation. The observed maximal immobilisation yield (Table 1) implies that glutaraldehyde, fixed to chitosan or not, affects catalytically important amino groups crucial for enzyme activity [28].

In addition, also physical interaction between enzyme and the carriers surface may have contributed to the observed low immobilisation yields. In some cases this phenomenon could be avoided or its extent reduced by increasing the length of the spacer group connecting carrier and enzyme [29]. On the other hand the immobilisation tests with glutaraldehyde and Eupergit® C (glycidol) clearly indicate that the enzyme is not sufficiently tolerant towards reactive groups and that the essential amino groups in the active site experience modification resulting in enzyme inactivation. Therefore no further experiments were conducted with respect to a potential deactivating physical interaction.

2.3. Entrapment into a matrix

From the observations that neither adsorption nor covalent attachment of L-TA onto a carrier resulted in immobilisates with satisfactory activity, it becomes clear that successful immobilisation requires methods not modifying catalytically important functional groups of the enzyme or resulting in full or partial denaturation and thus retaining enzyme activity. An immobilisation technique from which neither effects on the active site nor negative effects on protein integrity were to be expected, is entrapment of the enzyme into a matrix among which alginate [30] and orthosilicate gels [31] were studied.

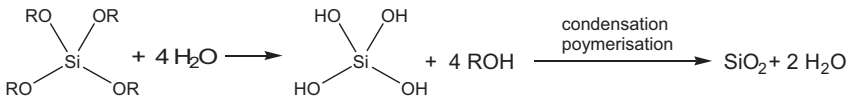
Alginate encapsulation provided immobilisation yields in the range of 10% (Table 1). However entrapment into orthosilicates proved to be the immobilisation procedure of choice as up to 30% of residual enzyme activity were retained after immobilisation. Thus the L-TA immobilisates produced with this technique showed the highest activity of all immobilisates investigated in this study.

In case of orthosilicate gels, the three-dimensional network around the entrapped enzymes is formed by acid-catalysed hydrolysis of alkyl orthosilicates, namely tetraethyl/tetramethyl orthosilicate (TEOS/TMOS), and triethoxy/trimethoxymethyl orthosilicate (TRIEMOS/TRIMMOS), resulting in silanols which in turn undergo condensation polymerisation (Scheme 3).

SEM studies revealed that the resulting silicates exhibit a highly porous structure as shown in Fig. 2 depicting TEOS entrapped L-TA. The latter image proves that there are sufficient number of pores providing enough space enabling unhindered transfer of the substrate and product to and from the enzyme, respectively.

But although an immobilisation method for *E. coli* L-TA could be identified, the observed immobilisation yield of 15–27% shows that the method still needs optimisation.

One reason for the loss in enzyme activity in the course of TEOS immobilisation is the fact that hydrolysis of TEOS not only yields the silicate gel matrix encapsulating the enzyme but also an alcohol (in case of TEOS, ethanol) being a potential enzyme denaturant. From the reaction equation (Fig. 3) it becomes evident that per mol of alkyl orthosilicate 4 mol of the respective alcohol are formed. As a consequence the resulting concentration of the alcohol formed reaches a level high enough to impair enzyme activity. In case of immobilisation making use of TEOS, the concentration of ethanol formed during the polycondensation reaction was determined to be 14.5 mol/l, which is three times higher than the concentration at which ethanol caused inactivation of L-TA could be observed



Scheme 3. Schematic illustration of the hydrolysis and condensation reaction of alkyl orthosilicates.

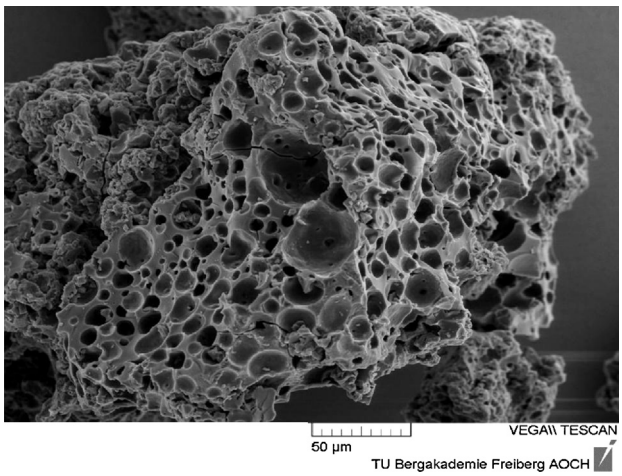


Fig. 2. SEM image of TEOS immobilised L-TA.

(4.2 mol/l, Fig. 3). Experiments with non-immobilised enzyme confirmed these findings. After exposing L-TA to the same amount of ethanol present during immobilisation a residual activity of 24% was obtained compared to 15–27% found for the TEOS immobilisate.

Consequently ethanol needs to be removed from the reaction mixture for which purpose two approaches were found to be viable: (1) ethanol removal by vacuum evaporation [32] and (2) ethanol extraction with organic solvents.

Attempts to remove the alcohol by vacuum evaporation resulted in increased polycondensation rates in the course of preparing the sol solution. The almost instantaneous solidification prevented the enzyme from being fully entrapped into the gel causing lower immobilisation yields.

However the method could still be improved by switching from TEOS to TMOS (tetramethyl orthosilicate). Using the latter, methanol instead of ethanol is released during immobilisation towards which L-TA is more stable (Fig. 3). The rather disadvantageous ecopotential of methanol was considered negligible in view of the amounts released in the course of the production of a catalyst material, in particular in view of the higher stability of L-TA towards methanol.

A more straightforward alternative to evaporation of the alcohol formed during alkylorthosilicate hydrolysis under reduced pressure is extraction of the by-product using a water immiscible organic solvent. The results of experiments making use of this method are depicted in Fig. 3. The presence of organic solvents in the reaction mixture used for immobilisation even is beneficial as it enables controlling pore size distribution in the resulting porous silicate matrix [33]. Studies within the working group identified *n*-hexane, cyclohexane, *n*-octane and *n*-decane as most promising candidates [34].

Since organic solvents are known enzyme denaturants, only enzyme compatible ones, i.e. with no or little effect on enzyme activity, are suitable for this approach. Hence various organic solvents have been screened. After 18 h of incubation with the respective solvent at water/solvent ratios typically used for silicate gel formation, residual activities were determined as given in Fig. 3. After it had turned out that the presence of an organic solvent not only had no detrimental effect on enzyme activity (with the exception of 25% ethanol in water), but it also became evident that 8 out of 13 tested solvent/water mixtures displayed a considerable (up to 41%) promoting effect on the observed enzyme activity. The underlying reasons for these observations are not yet fully understood and hence the matter of forthcoming investigations.

As can be seen from Fig. 3 there are opposing effects in dependence of the water/solvent ratio. While *n*-decane promotes threonine cleavage with increasing amounts of solvent present, the opposite is observed for *n*-hexane, for which the aldolase catalysed reaction already profits from a rather low fraction of the organic solvent. On the other hand water miscible solvents such as ethanol remain ineffective if concentrations were kept below 5% (i.e. 0.9 mol/l in the case of ethanol). At higher values the L-TA is inactivated. Hence minimising the concentration of alcohols formed during alkyl orthosilicate hydrolysis through extraction is mandatory, regardless of the nature of the alcohol formed.

Consequently the solvent to be chosen should have an excellent extraction capacity for the alcohol formed while not impairing pore size distribution. BET measurements proved that none of the solvents investigated (for the solvents tested see Fig. 3) affected pore sizes significantly. Hence *n*-hexane was selected as it is very effective in extracting water soluble alcohols from an aqueous reaction solution.

Optimisation efforts led to a maximum enzyme loading of 50 U/g carrier depending upon the crude enzyme extract.

The TMOS immobilisate obtained with the optimised immobilisation method showed no observable loss in activity upon storage for ten weeks at 4 °C, whereas the non-immobilised enzyme lost up to 16% of its activity already after 6 weeks.

In the course of investigating the loading capacity of different sol/enzyme combinations protein leaching of the immobilisates was examined (Fig. 4). For this purpose the immobilisate was

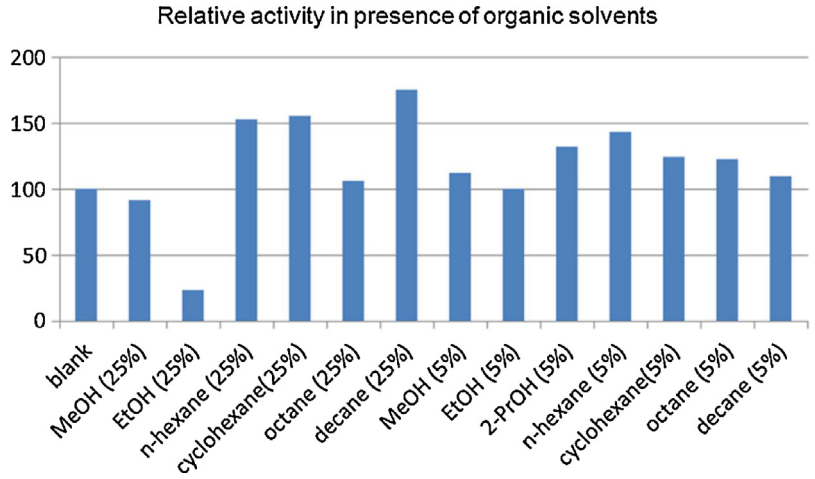
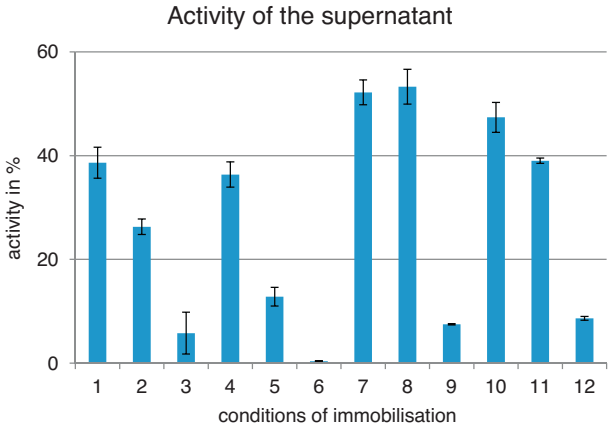


Fig. 3. Relative activity [%] of native enzyme after 18 h of incubation in different organic solvent/water mixtures with organic solvent contents of 5 and 25%.



experiment	TEOS (v/v)	TRIEMOS (v/v)	TMOS (v/v)	enzyme solution
1	0,5	0,5	0	10
2	1	0	0	10
3	0	0	1	10
4	0,5	0,5	0	1
5	1	0	0	1
6	0	0	1	1
7	0,5	0,5	0	3
8	1	0	0	3
9	0	0	1	3
10	0,5	0,5	0	5
11	1	0	0	5
12	0	0	1	5

Fig. 4. Residual enzyme activities (relative activity based on native enzyme activity) in supernatants of different immobilisates.

suspended in a buffer/glycerol/PLP solution and the supernatant was tested for aldolase activity (Fig. 4). Again immobilisates produced by using TMOS for immobilisation showed the best results.

Besides enzyme leaching mechanical stability of an immobilisate is an important criterion especially if the biocatalytic reaction catalysed by the immobilised enzyme is done in a stirred tank. In order to evaluate the mechanical stability of the porous immobilisate particle sizes were investigated using a Beckmann Coulter particle analyser. Fig. 5 shows a slight increase in particle size from d_{50} = 155.8 μm to d_{50} = 207.9 μm within a period of 14 h. This increase is explained by the swelling behaviour of the silicate.

Table 2
Comparison of biocatalytic conversions with native enzyme and immobilised enzyme.

Compound	L-TA	Conversion (%)	d.r. (threo/erythro)	ee (threo)
3a	Native enzyme	50	63:37	>99%
3a	Native enzyme	12	46:54	>99%
3a	Immobilised enzyme	7	67:33	>99%
3b	Native enzyme	70	80:20	>99%
3b	Native enzyme	20	77:23	>99%
3b	Immobilised enzyme	17	60:40	>99%

2.4. Biocatalytic conversion

After the successful immobilisation of L-TA its applicability in biocatalytic reactions yielding dichiral compounds in one step needed to be tested. A reaction known to be catalysed by L-TA is the reaction of benzaldehyde (**1a**) and *o*-chlorobenzaldehyde (**1b**) yielding phenylserine and *o*-chlorophenylserine respectively [15] (Scheme 4). A comparison of the conversion as well as the diastereomeric and enantiomeric purity of the products obtained from reactions using native and immobilised L-TA is shown in Table 2.

From Table 2 it can be seen that for both aldehyde starting materials the conversion obtained with immobilised enzyme is lower than the one observed with native enzyme over the same period of time. On the other hand diastereoselectivity is enhanced for **1a** and it is lower for **1b**. This behaviour is owed to the nature of the surface of the silicatic entrapment material. Through intramolecular hydrogen bonds the inner surface of the immobilisate is rather hydrophobic. Consequently both – the aldehydes and the aldol condensation products – will display a tendency of being adsorbed to the silicatic matrix. Thus, the observed rate of conversion is apparently lower when compared to the rate observed in reactions using non-immobilised L-TA since a part of the product is not in solution but adsorbed to the silicatic matrix.

Consequently the tendency of the substrate to adsorb to the silicatic matrix will determine the rate of its transfer to the enzyme. This interpretation is supported by experiments investigating adsorption of hydrophobic molecules such as benzaldehyde (**1a**) onto the silicate matrix. For **1a** it was found that it is extracted from the aqueous solution and adsorbed to a large extent (up to 68%) by the silicatic matrix. The same effect could be observed in experiments using zeolites which are well known to form intramolecular hydrogen bonds rendering the inner surfaces of these

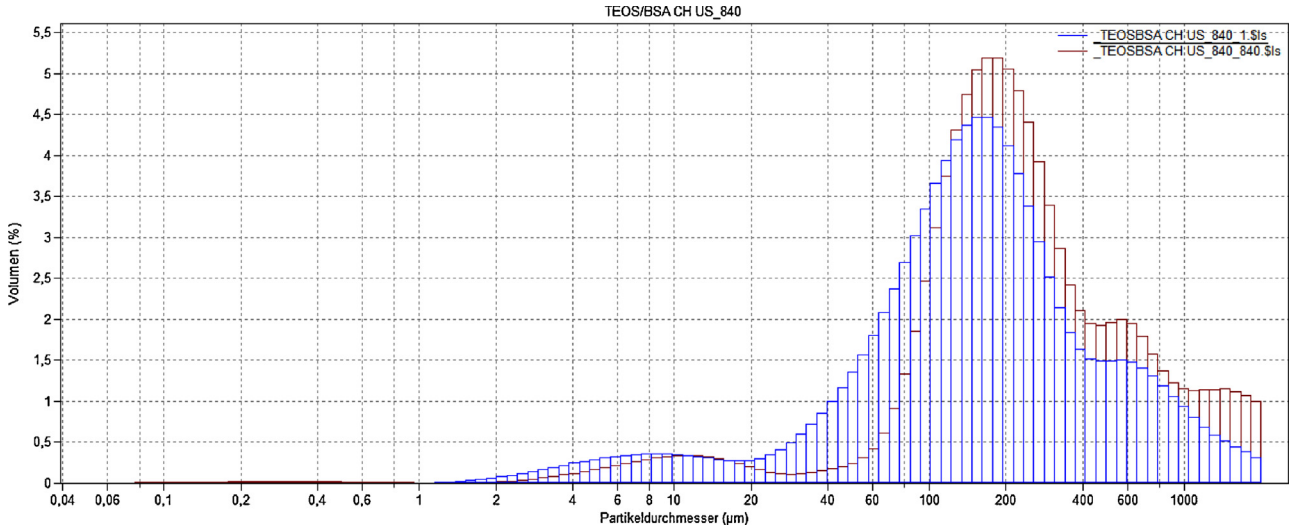
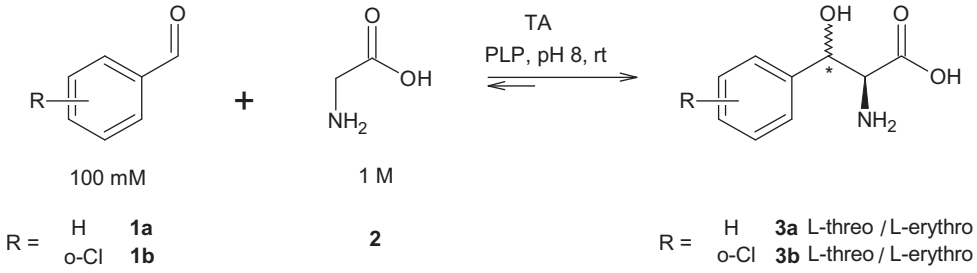


Fig. 5. Exemplified particle size distribution of an immobilisate (Fig. 4, experiment 5) before (blue bars) and after 14 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)



Scheme 4. Biotransformation of glycine with benzaldehyde (**1a**) and *o*-chlorobenzaldehyde (**1b**).

silicates hydrophobic. In particular **1a** was adsorbed almost quantitatively (≥95%) by zeolites [35,36].

The adsorption properties of benzaldehyde on the inner surface of the immobilisate are also suited to explain the changes in diastereoselectivities observed in comparison with the native enzyme. It is obviously the residence time in the system and the time allowed for equilibration which eventually determines product diastereopurity. Thus the enzyme displays only an insufficient capability to discriminate between *threo* and *erythro* product formation. Eventually it is the repeated course of product formation and cleavage which after all leads to the diastereomer distribution observed in the equilibrated state.

On the hydrophobic surfaces there is only a retardation effect observable leading to prolonged residence times. There is no indication for a process taking effect on product stereopurity (**3a**). Moreover, longer residence times provide sufficient time for the reaction equilibrium to establish, and it is this residence time in the immediate proximity of the enzyme which in this way takes effect on diastereomer distribution. Consequently the situation is different for **1b**, where the compound exhibits a higher dipole moment, is more polar than **1a**, adsorbs less effectively and consequently shows higher conversion within the same time frame, yet accompanied by lower diastereoselectivity (d.r. = 60:40).

In order to circumvent these intricacies caused by hydrophobicity of the inner surface of the entrapment material, attempts were made to modify the surface by inserting a methyl group through reacting trialkylmethoxyorthosilicates with TMOS instead of the pure orthosilicate. However, these immobilisates showed a poorer solidification behaviour and a higher leaching potential (Fig. 4) due to less efficient formation of three-dimensional silicate network. For these reasons this option was not further pursued.

3. Conclusion

Threonine aldolases can efficiently be immobilised by entrapment into silicatic matrices. The reaction behaviour of these immobilisates is determined by the hydrophobicity of their inner surfaces. The more hydrophobic the latter, the slower the biocatalytic aldol condensation will proceed. Further activities will be directed towards optimising diastereoselectivity of the L-TA catalysed reaction.

4. Experimental

4.1. Materials

All chemicals and solvents were of appropriate purity and were obtained from commercial sources or synthesised in our laboratories.

L-TA in the form of crude extracts of *E. coli* cells overexpressing L-TA was kindly provided by the research group of Prof. Hummel (University of Düsseldorf) and evocatal [16].

4.2. Instrumentation

Analytical HPLC was carried out with a Dionex Ultimate 3000 system. The separation was achieved on a Chirex® 3126 D-penicillamine chiral column (250 mm × 4.6 mm) from Phenomenex®.

GC–MS separation and analysis were performed on a Varian 431-GC Gas Chromatograph and a 220-MS IT Mass Spectrometer system.

Photometric measurements were conducted on a Jasco V-630 spectrophotometer.

Particle size distribution and the swelling capacity of the immobilisates were investigated using a Beckmann Coulter equipped with a LS 13320 Laser Diffraction, a universal liquid module (ULM) and a sonication control unit.

The SEM images were produced by a Tescan REM/EDT coupled with EDX INCAx-sight from Oxford Instruments and a Cressington Sputter Coater 108anfo.

4.3. Activity assays

The native enzyme activity was determined by a 2 step coupled assay. At first threonine is cleaved by the threonine aldolase to give acetaldehyde and glycine. Parallel to this the evolving acetaldehyde is reduced to ethanol by a yeast alcohol dehydrogenase with simultaneous oxidation of NADH to NAD*. The resulting decrease of absorbance was recorded spectrophotometrically at 340 nm. Experiments were carried out in a standard cuvette with a total solution volume of 1 ml at 30 °C. The solution consisted of 100 μl 1 M buffer pH = 8.0, 20 μl 2.5 mM PLP, 100 μl 0.5 M threonine, 20 μl 10 mM NADH (H_2O), 10 μl threonine aldolase crude extract, 10 μl 3 U/μl ADH and 720 μl H_2O. One unit of TA is defined to catalyse the formation of 1 μmol of acetaldehyde (corresponds to the oxidation of NADH) per min at 30 °C.

The activity of immobilised enzyme was obtained based on the TA catalysed retro-aldol reaction of phenylserine giving glycine and benzaldehyde. The formed aldehyde was extracted with chloroform from the aqueous solution and quantified by means of GC–MS measurements. In this case one unit of TA is defined to catalyse the formation of 1 μmol of benzaldehyde per min at 30 °C. The reaction mixture contained 20 μl 2.5 mM PLP, 100 μl 1 M buffer pH = 8.0, immobilised enzyme, 980 μl 0.05 M phenylserine (dissolved in buffer pH = 8.0). The aqueous phase was extracted with 800 μl chloroform for 1 min and centrifuged for 1 min in order to separate both phases. 1 μl of the organic layer was then injected into the GC system and separated on a Varian column VF-35ms (30 m × 0.25 mm × 0.39 μm). The flow of the carrier gas Helium was 1.0 ml/min and the temperature of the injector was 250 °C. The temperature programme 60 °C (0 min); 60–90 °C with 3.0 °C/min; 90 °C (5 min) gave a total of 15 min and the following retention time for benzaldehyde 9.2 min. The analytes were ionised via EI and the MS acquisition method recorded mass to charge values in the range of 40–150.

4.4. Immobilisation procedures

The immobilisation procedure is composed of two steps, the preparation of the different silica sol stock solutions and the addition of crude enzyme extract and subsequent gelation. The reaction mixture comprises of 0.02 mol of the correspondent silicate (4.5 ml TEOS, 3.95 ml TMOS, 3.98 ml TRIEMOS, 2.85 ml TRIMMOS), 0.4 ml ultrapure H_2O and 1 ml 0.01 M HCl [37]. The initial two phase system is stirred vigorously at 40 °C until a homogenous sol is obtained. According to the different experiments sol stock solution and enzyme solution are mixed together until solidification occurs. The gel is stored for 12 h at 4 °C for ageing purposes. In order to modify the porous structure the sol and enzyme solution are added to an organic solvent (*n*-hexane, cyclohexane, *n*-octane, decane) and mixed well until solidification.

4.5. Biotransformation

The reaction mixture consisted of 1.375 ml of crude protein extract (≥10 U/ml) or water respectively (immobilisates), glycine **2** (3 mmol), aldehyde **1a** (**1b**) (0.3 mmol), 1.375 ml glycerol, 300 μl buffer (1 M, pH = 8.0) and pyridoxal-5-phosphate (50 μM). The different reaction mixtures were stirred at 25 °C for different periods of time. Conversion, diastereo- and enantioselectivity were determined by analytical HPLC. Therefore samples (100 μl) were withdrawn from the reaction mixture and the enzymatic reaction was stopped via acidic denaturation. This was followed by a dilution of 1:10 with a water/methanol mixture to give the final ratio of 25% methanol and 75% water. The solvent consisted of 75% 0.2 mM $CuSO_4 \cdot 5H_2O$ and 25% methanol. Additional measurement parameters were flow rate: 1 ml/min, column oven temperature: 40 °C and detection: variable wavelength detector at 254 nm.

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