Publikation II

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Purification of recombinant hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* expressed in *E. coli*: Comparison of wild-type and genetically modified proteins.

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Abstract

Two enzymes, hydantoinase (HyuH) and L-*N*-carbamoylase (HyuC), are required for the biocatalytic production of natural and unnatural, optically pure L-amino acids starting from D,L-5-monosubstituted hydantoins using the so called 'hydantoinase-method'. For the preparation of immobilized enzymes, which omit several drawbacks of whole cell biocatalysts, purified or at least enriched HyuH and HyuC have to be provided. In order to simplify existing purification protocols several genetically modified derivatives of HyuH and HyuC from *Arthrobacter aurescens* DSM 3747 have been cloned and expressed in *E. coli*.

A fusion protein consisting of maltose-binding protein (MalE) and HyuH resulted in an enhanced solubility of the hydantoinase, which easily forms inclusion bodies. On the other hand the fusion protein could easily be purified with high yield (76%) by just one chromatographic step (amylose resin) and the complex purification protocol of the wild-type enzyme could therefore be simplified and shortened significantly. Interestingly, the specific activity of the MalE-HyuH fusion protein was as high as the one of the wild-type enzyme despite the molecular mass was doubled. A second modification of HyuH carrying a histidine-tag was efficiently bound to a metal affinity matrix but inactivated completely during elution from the column at either low pH or in the presence of imidazole.

In the case of HyuC an aspartate-tag has been added to the biocatalyst to allow an integrated purification-immobilization procedure since this enzyme is immobilized efficiently only via its carboxylic groups. The diminished isoelectric point of the Asp-tagged HyuC resulted in a simplified purification procedure. Compared to the wild-type enzyme expressed in *E. coli* HyuC-Asp_6 was shifted off the elution range of the contaminating proteins and higher purification factors were obtained even in the capturing step. In contrast to HyuH, it was possible to purify a L-*N*-carbamoylase carrying a histidine-tag to apparent homogeneity using immobilized metal affinity chromatography. Therefore, the existing three step purification protocol was reduced to one chromatographic step and the yield of this relatively unstable protein enhanced remarkably.

Keywords: Hydantoinase; L-*N*-carbamoylase; fusion protein; purification; histidine fusion; aspartate fusion; maltose binding protein fusion.

1 Introduction

The use of tag-technology is becoming increasingly popular for the simple and inexpensive production of industrial enzymes. For this, mainly poly-His tags are applied and meanwhile tailor-made metal chelate supports for certain enzymes are under preparation [1].

The tag-technology was applied for two enzymes, Hydantoinase (HyuH) and L-*N*-carbamoylase (HyuC), which are used in the so called 'hydantoinase-method' for the biocatalytic production of natural and unnatural, optically pure L-amino acids starting from D,L-5-monosubstituted hydantoins [2]. The enzymes have previously been cloned [3, 4] and immobilized [5] and a production process (mini-plant technology) using a cascade of the two enzymes in combination with other steps including the racemization of the remaining substrate enantiomer and product separation, is under development. To omit the severe drawbacks exhibited by whole cell biocatalysts, HyuH and HyuC have to be immobilized and therefore purified or at least enriched HyuH and HyuC have to be provided in sufficient amounts and purity.

In order to simplify the existing purification protocols for the wild-type enzymes, several genetically modified derivatives of HyuH and HyuC from *Arthrobacter aurescens* DSM 3747 have been created, cloned and expressed in *E. coli*. Additionally, an aspartate tag has been added to HyuC in order to allow an integrated purification-immobilization procedure since this enzyme is immobilized efficiently only via its carboxylic groups.

In detail, fusion proteins of the hydantoinase have been created (i) to provide high amounts of the hydantoinase required for the immobilization experiments and for the continuous production of amino acids using the mini-plant-technology, (ii) to simplify the existing purification protocol, which, for the wild-type hydantoinase from *Arthrobacter aurescens*, consists of three chromatographic steps [6] and most important (iii) to diminish the tendency to form inclusion bodies found for the wild-type hydantoinase expressed in *E. coli*. Fusions of the hydantoinase (HyuH) to the maltose binding protein [7] (MalE-HyuH) and to the chitin binding domain via an intein linker [8] (HyuH-intein-CBD), and a histidine-tagged [9, 10] hydantoinase (HyuH-His_6) have been prepared.

Genetically modified L-*N*-carbamoylase has been created mainly because of two reasons: (i) for the optimization of the immobilization procedure using carbodiimide as an cross-linking agent, an aspartate-tagged [11] protein (HyuC-Asp_6) has been produced and (ii) a histidine-tagged protein (HyuC-His_6) has been cloned and expressed to simplify the purification procedure, which, for the wild-type enzyme consists of three steps [4].

In the present paper, the procedures developed for the purification of the MalE-HyuH fusion protein and the aspartate- and histidine-tagged L-*N*-carbamoylase are described. The purification protocol of HyuC-Asp_6 is compared with the protocol used for the purification of the wild-type L-*N*-carbamoylase.

2 Experimental

2.1 General

Unless otherwise stated all reagents were of analytical grade and purchased from Fluka AG (Buchs, Switzerland) and all chromatographic media, columns, and instruments for chromatography (FPLC, Äkta-Explorer) were purchased from Pharmacia (Freiburg, Germany).

Hydantoins and *N*-carbamoyl amino acids were synthesized according to the literature [12] [13]. The solutions of salts and acids were prepared in deionized water purified with the MilliQ-system (Millipore Corp., Bedford, MA, USA). Centrifugation was carried out using a Sorvall RC-5B superspeed centrifuge (DuPont Instruments, Nauheim, Germany). Restriction enzymes were purchased from New England Biolabs (Schwalbach, Germany).

2.2 Expression of *malE-hyuH* and purification of the fusion protein.

The construction of the *malE-hyuH* fusion is based on a protein purification system developed by New England Biolabs and is described elsewhere [3]. In the resulting plasmid pAW211, a rhamnose inducible promoter from *E. coli* controls the expression of *hyuH* fused to the *malE* gene of *E. coli*, which encodes maltose-binding protein. *E. coli* JM109 pAW211 was grown in 2xYT broth [14] supplemented with 100 μg/ml of ampicillin to OD_{600} ~ 0.3 at 30°C and the expression was induced by addition of 0.2 % □-D-rhamnose. After 4 h, cells from 400 ml culture were harvested and resuspended in 12.5 ml buffer A (20 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 1 mM $MnCl_2$, 0.02 % NaN_3). After cell disruption using a french pressure cell (Aminco, SLM Instruments, IL, USA) the extract was clarified by centrifugation at 9000 x *g* for 30 min at 4 °C and loaded onto a amylose resin (New England Biolabs, Schwalbach, Germany). The affinity chromatography was performed as described in the manufacture's instructions.

2.3 Cloning, expression of *hyuC-His$_6$* and h*yuC-Asp$_6$* and purification of the tagged proteins.

2.3.1 Bacterial strains, plasmids, media and culture conditions:

E. coli JM109 [15] was used as a host for the cloning and the expression of the *hyuC* gene from *Arthrobacter aurescens* DSM 3747 [16]. *E. coli* JM109 was either grown in LB liquid medium or on LB-agar plates [17], both supplemented with 100 μg/ml ampicillin. Plasmid pAW29 is a pUC18 derivative containing a 3.4 kb *Nar*I fragment from pAW16 [3]. Plasmid pBW1 contains the *hyuC* gene fused to 6 histidine codons at the C-terminal end. It was constructed by PCR amplification of the *hyuC* gene from pAW29 using the primers S1066 (5´-AGA ACA TAT GAC CCT GCA GAA AGC G-3´) and S1135 (5´-AAA AGG ATC CCC GGT CAAGTG CCT TCA-3´). The amplified fragment was cleaved with the restriction enzymes *Nde*I and *Bam*HI and inserted downstream the *rhaBAD* promotor in the vector pJOE2775 [18] which was cut with the same restriction enzymes. Plasmid pBW2 contains the *hyuC* gene fused to 6 aspartic acid codons at the C-terminal end. It was constructed by cleaving pBW1 with the enzymes *Bam*HI and *Hin*dIII and inserting the complementory oligonucleotides S1294 (5´-GAT CCG ATG ATG ATG ATG ATG ATT GA-3´) and S1295 (5´-AGC TTC AAT CAT CAT CAT CAT CAT CG-3´). Induction of the *rhaBAD* promotor and cultivation of recombinant *E. coli* were performed as described elsewhere [4].

2.3.2 Purification of the his-tagged L-*N*-carbamoylase:

A) Preparation of crude cell extracts

Cells were grown to a final OD_{600} of approximately 5. 2.7 g harvested cell wet mass were resuspended in 30 ml 20 mM Tris HCl, 100 mM NaCl, pH 8.0 (buffer B), centrifuged at 4000 x g for 10 min and resuspended in the same buffer to give a final volume of 3 ml. This suspension was disrupted two times using a 3 ml french pressure cell (Aminco, SLM Instruments, IL, USA) at 6°C. Cell debris was removed by centrifugation (30 min, 48000 x g).

B) Equilibration of metal affinity resin

1 ml of "Talon metal affinity resin" (Clontech Laboratories, Inc. CA, USA) was washed with 10 ml buffer B, centrifuged and resuspended in 6 ml of the same buffer containing 0.02% NaN_3.

C) Purification protocol

0.46 ml of the supernatant obtained in section A were mixed with the Talon resin and incubated over night at 4°C. The resin was collected by centrifugation at 700 x g for 5 min and filled in a spin column. After washing with 15 ml of buffer B the elution was performed with 5 ml of buffer B containing additionally 100 mM imidazole. In each step 1 ml fractions were collected and analyzed. Fractions containing the purified enzyme were pooled and dialyzed against 2 l of 0.2 M Tris HCl buffer, 0.1 mM $MnCl_2$, 0.02% NaN_3, pH 7.0 using a dialysis tube (exclusion M_r 15,000, Serva Electrophoresis GmbH, Germany).

2.3.3 Purification of HyuC-Asp$_6$

HyuC-Asp$_6$-was purified according to the method published previously for the purification of the wild-type L-*N*-carbamoylase expressed in *E. coli* [4].

2.4 Determination of enzymatic activity and protein concentration

Activities of hydantoinase and L-*N*-carbamoylase were measured according to May et al. [6] and Wilms et al. respectively [4].

Protein concentrations were determined by the method of Bradford [19], using bovine serum albumin as a protein standard.

2.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE separation of proteins was performed according to the method of Laemmli [20] using the twin mini gel set purchased from Biometra (Göttingen, Germany).

3 Results

3.1 Purification of the hydantoinase by fusion to maltose binding protein (MalE-HyuH)

The MalE-HyuH fusion protein was purified to apparent homogeneity (Fig. 1, lane 7) by affinity chromatography. A high yield of activity of 76 % was achieved (Table 1). In contrast to the MalE-HyuH fusion other fusion proteins like HyuH-intein-CBD and HyuH-His$_6$ were mainly obtained as inclusion bodies as in the case of the wild-type hydantoinase [3]. HyuH-His$_6$ was inactivated during elution from a metal affinity column at either low pH or in presence of imidazole (data not shown).

3.2 Purification of recombinant L-*N*-carbamoylase

By recombinant DNA-technology, six aspartate or histidine residues have been tagged to the C-terminus of the L-*N*-carbamoylase from *Arthrobacter aurescens*. The modified proteins have been expressed in *E. coli* and purified.

3.2.1 Aspartate-tagged versus wild-type L-*N*-carbamoylase

Wild-type L-*N*-carbamoylase has been purified previously from recombinant *E. coli* using a purification protocol, which consisted of three steps: (i) cell disruption using a french pressure cell, (ii) capturing using a packed bed Streamline DEAE column, and (iii) a final polishing step using MonoQ [4]. For comparison, the Asp_6-tagged L-*N*-carbamoylase has been purified according to the same protocol (Table 2).

The specific activity of the aspartate-tagged L-*N*-carbamoylase was not affected by the fusion. Both, HyuC-Asp_6 and wild-type L-*N*-carbamoylase purified from *E. coli* [4] exhibited the same specific activity of 5.0 U/mg. As can be seen from Figure 2, the elution profiles of the final polishing step using anion exchange chromatography on MonoQ differed for the wild-type and the aspartate-tagged L-*N*-carbamoylase. At constant pH, the additional negative charges originating from the aspartate-tag resulted in a stronger interaction with the anion exchanger. The salt concentration necessary for the elution of the protein was therefore shifted from 0.3 – 0.4 M to 0.5 – 0.63 M sodium chloride. As judged by SDS-polyacrylamide gel electrophoresis the protein obtained was nearly pure (Fig. 3).

Table 1: Purification of the MalE-HyuH fusion protein from *E. coli* JM109 pAW211.

Purification Step	Total Protein [mg]	Specific Activity [U/mg]	Total Activity [U]	Yield [%]	Purification-factor
Crude extract	20.9	1.4	29.4	100	1.0
Amylose resin	1.75	12.8	22.5	76	9.1

Table 2: Purification of the Asp_6-tagged L-*N*-carbamoylase from *E. coli* pBW2

Purification Step	Total Protein [mg]	Specific Activity [U/mg]	Total Activity [U]	Yield [%]	Purification-factor
Crude extract	100	0.4	40.0	100	1.0
Streamline DEAE	9.1	0.9	8.2	21	2.3
MonoQ	1.1	5.0	5.5	14	12.5

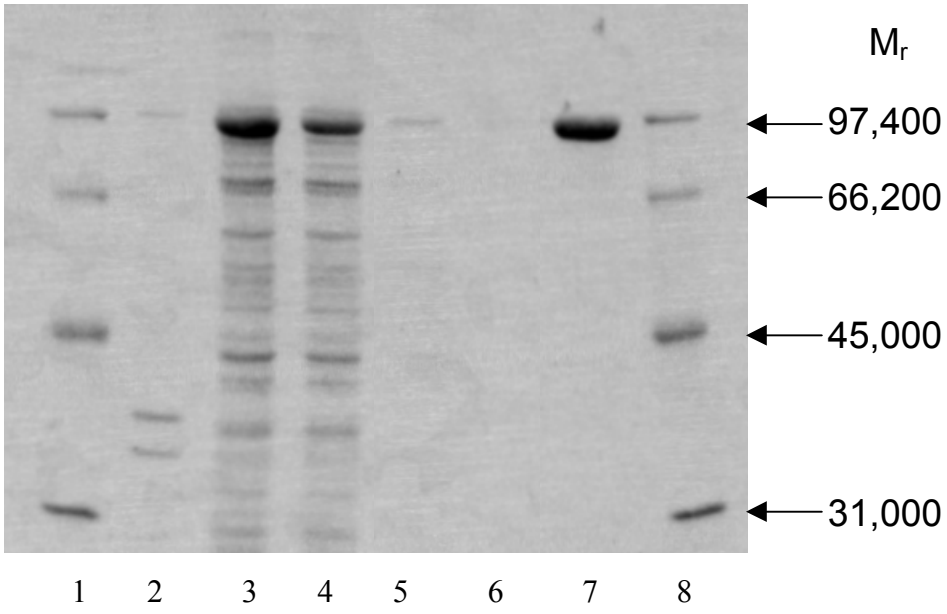

Figure 1: SDS-PAGE of MalE-HyuH fusion protein from *E. coli* JM109 pAW211. Standard proteins carbonic anhydrase (M_r 31,000), ovalbumin (M_r 45,000), serum albumin (M_r 66,200), phosphorylase B (M_r 97,400) (lanes 1 and 8), crude extract (insoluble protein, lane 2), crude extract (soluble protein, lane 3), break-through fraction of amylose resin (lane 4), washing step 1 (lane 5), washing step 2 (lane 6), and active fractions of MalE-HyuH fusion protein (M_r 88,000) eluted from amylose resin (lane 7).

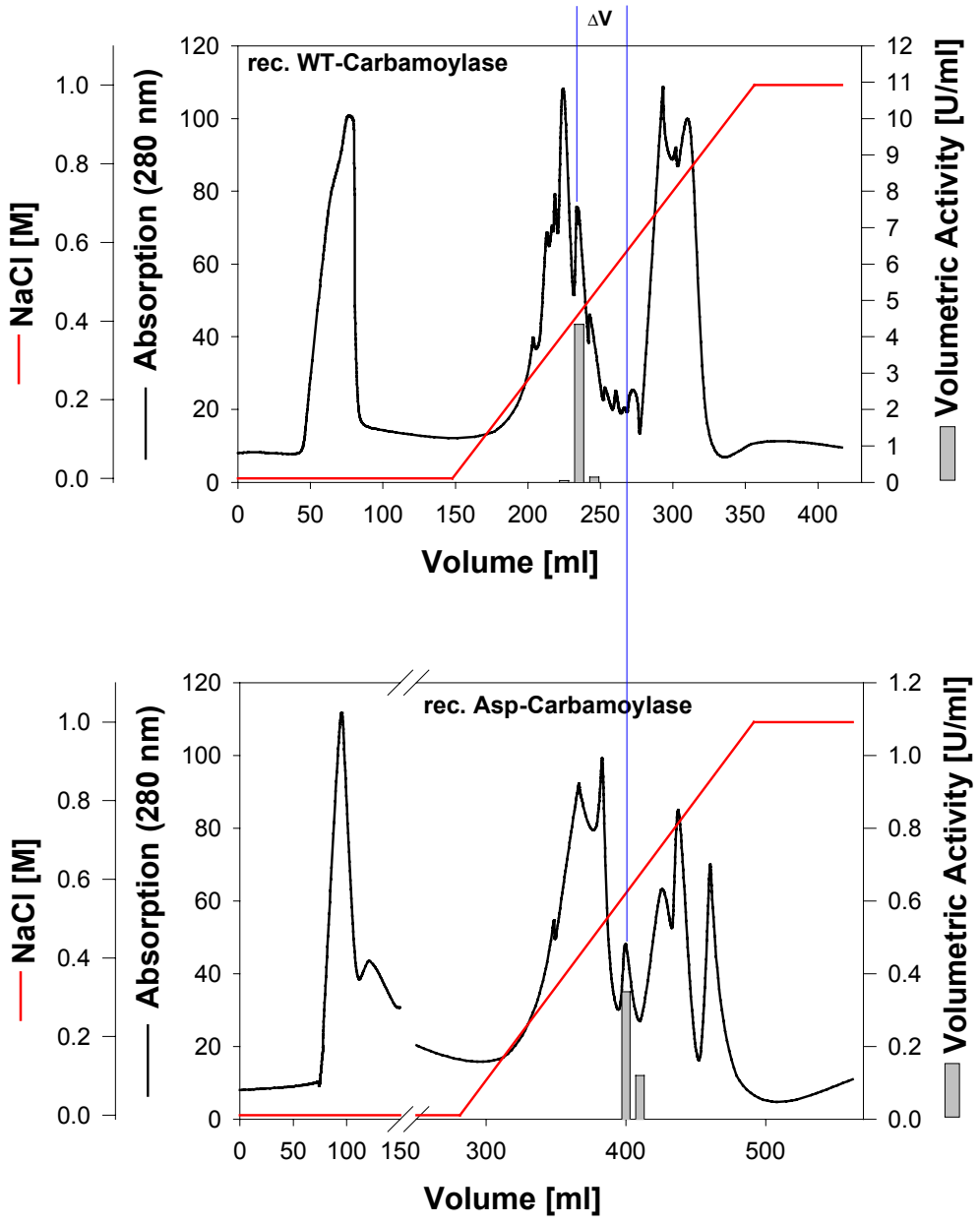

Figure 2: Comparison of elution chromatograms of recombinant wild-type L-*N*-carbamoylase (top) and recombinant Asp_6-tagged L-*N*-carbamoylase (bottom). Final polishing step on MonoQ 16/10 using 0.05 M Tris-buffer, pH 7.0 containing 1.0 mM $MnCl_2$. The active fractions of tagged and wild-type L-*N*-carbamoylase are shown in grey bars.

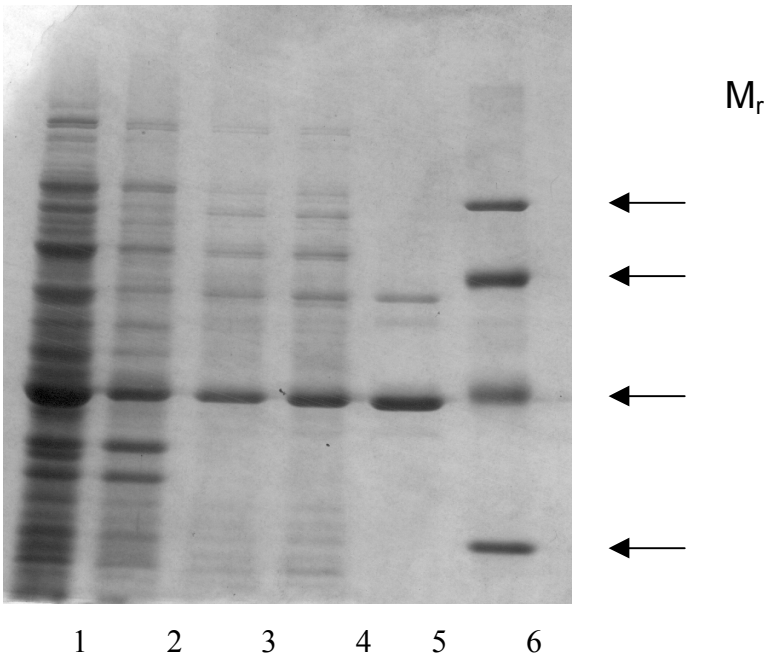

Figure 3: SDS-PAGE of Asp_6-tagged L-*N*-carbamoylase (HyuC-Asp_6) expressed in *E. coli* JM 109 pBW2. Crude cell extract (lane 1), break-through fraction of Streamline DEAE (lane 2), active fractions eluted from Streamline-DEAE (lane 3), after ultrafiltration (lane 4), active fractions eluted from MonoQ 16/10 (lane 5), and standard proteins (lane 6). M_r of HyuC-Asp_6 44,000.

3.2.2 Histidine-tagged L-*N*-carbamoylase

The histidine-tagged L-*N*-carbamoylase was purified from *E. coli* by metal affinity chromatography. In contrast to the histidine-tagged hydantoinase it was possible to elute bound His_6-HyuC using imidazole as a chelating agent. The specific activity of HyuC-His_6 was only slightly higher than the wild-type and the HyuC-Asp_6 fusion. The purification factors of HyuC-Asp_6 (Table 2) and HyuC-His_6 (Table 3) are approximately 3.5 times higher than that of the wild-type enzyme expressed in *E. coli* [4] indicating a lower expression level of the tagged enzymes.

Table 3: Purification of the His_6-tagged L-*N*-carbamoylase from *E. coli* pBW1.

Purification Step	Total Protein [mg]	Specific Activity [U/mg]	Total Activity [U]	Yield [%]	Purification-factor
Crude extract	11.89	0.5	6.3	100	1.0
Talon	0.58	5.9	3.5	55	11.3

As can be seen from Fig. 4, the histidine-tagged L-*N*-carbamoylase was electrophoretically pure. It can be seen from the figure as well (lane 2, break-through fraction) that the binding capacity of the column used was not sufficient to bind the fusion protein completely.

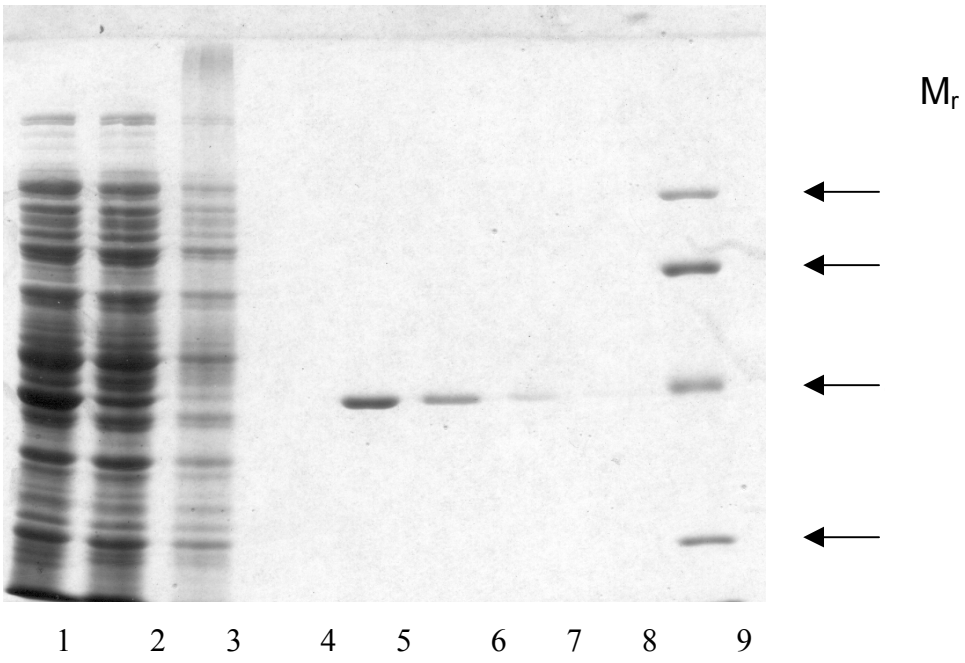

Fig. 4: SDS-PAGE of His_6-tagged L-*N*-carbamoylase (HyuC-His_6) expressed in *E. coli* JM 109 pBW1. Crude extract (lane 1), break-through fraction of Talon-column (lane 2), washing fractions (lanes 3 and 4), elution of active HyuC-His_6 with 20 mM Tris HCl, 100 mM NaCl, 100 mM imidazole, pH 8.0 (lanes 4, 5, 6, 7 and 8; M_r of active protein 44,000), and standard proteins (lane 9).

4 Discussion

The enzymes, hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* play an important role in the process development for the production of optically pure amino acids from 5-monosubstituted hydantoins. During the last years this process is becoming increasingly interesting for industrial application. Early developments, which used whole cells as biocatalysts are now being replaced by isolated immobilized enzymes mainly because (i) products of whole cell biotransformations are usually colored and contaminated with metabolites deriving from cell lysis resulting in tedious procedures for downstream processing of the products, (ii) the intermediate product, i. e. L-*N*-carbamoyl amino acid is easily transported out of the cell but not taken up, resulting in reduced yields and (iii) the L-*N*-carbamoylase is an relatively unstable enzyme but is absolutely required for the synthesis of optically pure amino acids in the case of *Arthrobacter* enzymes since the hydantoinase is

not stereospecific. To provide sufficient amounts of isolated enzymes several genetically modified proteins carrying different fusions at either the N- or the C-terminus have been prepared. A MalE-HyuH fusion protein was found to be superior to all other tagged hydantoinases and to the recombinant wild-type enzyme with respect to solubility and simplicity of purification. Additionally, the activity was enhanced by the MalE fusion as can be seen from the comparison of the specific activities of the wild-type hydantoinase purified from *Arthrobacter aurescens* DSM 3745 (13 U/mg) [6] and MalE-HyuH (Table 1). The MalE fusion resulted in approximately a doubling of the molecular mass of the protein and therefore the specific activity of MalE-HyuH should be half the specific activity of the wild-type enzyme. The reasons for the enhanced specific activity are not yet known.

Using a simple three step purification protocol it was possible to provide sufficient amounts of nearly pure HyuC-Asp_6 for our ongoing immobilization experiments. Since the only method applicable for the immobilization of the L-*N*-carbamoylase by covalent binding is the carbodiimide-mediated cross-linking of carboxyl-groups presented by the enzyme and amino-groups provided by a carrier, a stronger interaction between carrier and protein is expected with the tagged protein. An enhanced number of bonds possibly formed between carrier and enzyme may lead to a higher operational stability of the immobilized biocatalyst as found in the case of covalently immobilized trypsin [21].

5 Conclusions

From the literature it is known that fusions of proteins or tags to recombinant enzymes can be used either to enhance the solubility of the enzyme or to simplify the respective purification procedure [22]. To test whether this methodology is applicable to hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* DSM 3747 which are used for the production of optically pure L-amino acids from racemic D,L-5-monosubstituted hydantoins, several genetically modified versions of the enzymes have been produced for the first time. When compared to the wild-type enzyme expressed in *E. coli*, the solubility of a MalE-HyuH fusion protein was indeed enhanced and it was possible to purify the fusion protein in just one chromatographic step. In contrast, HyuH-intein-CBD fusion was predominantly formed as inclusion bodies [3]. A histidine-tagged hydantoinase showed also a high formation of inclusion bodies and was inactivated during elution from the column at either low pH or in the presence of imidazole. Therefore, histidine-tagged hydantoinase and HyuH-intein-CBD fusion protein are not considered as enzyme source for further experiments.

For the first time, fusion proteins of the L-*N*-carbamoylase from *Arthrobacter aurescens* have been cloned and expressed in *E. coli* and purified. In comparison to the purification of the

wild-type protein expressed in *E. coli* both, histidine- and aspartate-fusions resulted in simplified purification protocols.

In our ongoing research, the enzymes are overexpressed during high-cell density cultivation. The purified enzymes are immobilized and used in a continuous production process to produce optically pure L-amino acids using the hydantoinase-method.

6 Abbreviations

C	L-*N*-carbamoylase
CBD	chitin binding domain
Hyu	hydantoin utilizing protein
hyu	hydantoin utilizing gene
H	hydantoinase
MalE	maltose binding protein
malE	gene of maltose binding protein
OD	optical density

7 References

[1] P. Armisén, C. Mateo, E. Cortés, J. L. Barredo, F. Salto, B. Diez, L. Rodés, J. L. García, R. Fernández-Lafuente, J. M. Guisán, *J. Chromatogr. A 848* (1999) 61.

[2] M. Pietzsch, C. Syldatk, in K. Drauz, H. Waldmann (Eds.): *Enzyme Catalysis in Organic Synthesis*, VCH-Verlag, Weinheim 1995, p. 409.

[3] A. Wiese, R. Mattes, J. Altenbuchner, *in prep.* (1999).

[4] B. Wilms, A. Wiese, C. Syldatk, R. Mattes, J. Altenbuchner, M. Pietzsch, *J. Biotechnol. 68* (1999) 101.

[5] M. Pietzsch, H. Oberreuter, B. Petrovska, K. Ragnitz, C. Syldatk, in A. Ballesteros, F. J. Plou, J. L. Iborra, P. Halling (Eds.): *Stability and Stabilization of Biocatalysts, Vol. 15*, Elsevier Science, Amsterdam 1998, p. 517.

[6] O. May, M. Siemann, M. Pietzsch, M. Kiess, R. Mattes, C. Syldatk, *J. Biotechnol. 61* (1998) 1.

[7] C. V. Maina, P. D. Riggs, A. G. Grandea, B. E. Slatko, L. S. MOran, J. A. Tagliamonte, L. A. McReynolds, C. D. Guan, *Gene 74* (1988) 365.

[8] S. Chong, F. B. Mersha, D. G. Comb, M. E. Scott, D. Landry, L. M. Vence, F. B. Perler, J. Benner, R. B. Kucera, C. A. Hirvonen, J. J. Pelletier, H. Paulus, M. Q. Xu, *Gene 192* (1997) 271.

[9] S. K. Sharma. (1994). US 365,994, Pharmacia & Upjohn Company, MI, USA.

[10] E. Hochuli, H. Dobli, A. Schacher, *J. Chromatogr. 411* (1987) 177.

[11] J. Y. Zhao, C. F. Ford, C. E. Glatz, M. A. Raugvie, S. M. Gendel, (1990) 273.

[12] T. Suzuki, K. Igarashi, K. Hase, K. Tuzimura, *Agric. Biol. Chem. 37* (1973) 411.

[13] G. R. Stark, D. G. Smyth, *J. Biol. Chem. 238* (1963) 214.

[14] J. Sambrock, E. F. Fritsch, T. Maniatis, *Molecular cloning - A laboratory manual*, Cold Spring Harbour Laboratory Press, USA 1989.

[15] C. Yanisch-Perron, J. Viera, J. Messing, *Gene 33* (1984) 103.

[16] C. Groß, C. Syldatk, F. Wagner, *Biotechnol. Techniques 1* (1987) 85.

[17] S. E. Luria, J. N. Adams, R. C. Ting, *Virology 12* (1960) 348.

[18] N. Krebsfänger, F. Zocher, J. Altenbuchner, U. T. Bornscheuer, *Enzyme Microb. Technol. 22* (1998) 219.

[19] M. M. Bradford, *Anal. Biochem. 72* (1976) 248.

[20] U. K. Laemmli, *Nature 227* (1970) 680.

[21] R. M. Blanco, J. J. Calvete, J. M. Guisán, *Enzyme Microb. Technol. 11* (1989) 353.

[22] N. Sheibani, *Prep Biochem Biotechnol 29* (1999) 77.

Publikation III

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Immobilization of the hydantoin cleaving enzymes from *Arthrobacter aurescens* DSM 3747

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Abstract

The immobilization procedure of the two industrially important hydantoin cleaving enzymes - hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* DSM 3747 - was optimized. Using different methods (carbodiimide, epoxy activated carriers) it was possible to immobilize the crude hydantoinase from *Arthrobacter aurescens* DSM 3747 to supports containing primary amino groups with a yield of up to 60%. Immobilization on more hydrophobic supports such as Eupergit C and C 250 L resulted in lower yields of activity, whereas the total protein coupled remained constant.

All attempts to immobilize the crude L-*N*-carbamoylase resulted in only low activity yields. Therefore, the enzyme was highly purified and used in immobilization experiments. The pure enzyme could easily be obtained in large amounts by cultivation of a recombinant *E. coli* strain following a three step purification protocol consisting of cell disruption, chromatography on Streamline DEAE and Mono Q. The immobilization of the L-*N*-carbamoylase was optimized with respect to the coupling yield by varying the coupling method as well as the concentrations of protein, carrier and carbodiimide. Using 60 mM of water-soluble carbodiimide nearly 100% of the enzyme activity and protein could be immobilized to EAH Sepharose 4B.

Keywords: Hydantoinase; L-*N*-carbamoylase; immobilization; stabilization

Abbreviations: DEAE: Diethylaminoethyl; HPLC: High performance liquid chromatography; IMH: 5-Indolylmethylhydantoin; *N*-CTrp: *N*-carbamoyl tryptophan; TCA: trichloroacetic acid.

1. Introduction

The “hydantoinase method” can be used for the production of a variety of proteinogenic and non-proteinogenic amino acids in optically pure form [Pietzsch and Syldatk, 1995]. Depending on the enzymatic system, either D- or L-amino acids can be produced with 100% theoretical yield. For this hydrolytic reaction, two enzymes are involved in the stereoselective cleavage of D,L-5-monosubstituted hydantoins and *N*-carbamoyl amino acids, respectively.

In contrast to the enzymes used in the preparation of D-amino acids starting from hydantoins, which are usually absolutely D-specific, the hydantoinase has only limited enantioselectivity in case of the enzymes obtained from *Arthrobacter aurescens* DSM 3747 and 3745 for a range of interesting products [May et al., 1998]. Therefore, the key step in the synthesis of optically pure products is represented by the second enzyme, an *N*-carbamoyl-L-amino acid amidohydrolase (L-*N*-carbamoylase) which therefore is absolutely necessary, in contrast to the D-process. Unfortunately, in resting cell biotransformations using *Arthrobacter aurescens* DSM 3747, especially this second enzyme was found to be relatively unstable, which can be explained by the fast proteolysis observed for this enzyme [Siemann et al., 1993b]. Whole cell biocatalysts therefore are not reusable and a production process economically not feasible with the wild type strain.

For this reason, the immobilization and stabilization of the hydantoinase and the L-*N*-carbamoylase were of major importance for the development of a process for the production of unnatural L-amino acids from D,L-5-monosubstituted hydantoins.

In a previous contribution, we were able to show that both isolated enzymes (crude preparations) could significantly be stabilized by several immobilization methods. However, so far the yield of enzymatic activity immobilized to the support was too low to fulfill industrial requirements [Pietzsch et al., 1998].

In the present paper, we report on our results obtained during the optimization of covalent coupling methods of the hydantoinase and the L-*N*-carbamoylase. The influence of the support, the protein concentration and the crosslinking agent (including glutaraldehyde which was recently used for the immobilization of a recombinant D-*N*-carbamoylase [Nanba et al., 1999]) on the yield of activity are discussed. For the L-*N*-carbamoylase, both, the crude wild-type as well as the recombinant enzyme purified after expression in *Escherichia coli* [Pietzsch et al., 2000; Wilms et al., 1999] were used for the optimization experiments.

2. Materials and Methods

General: All chemicals were of analytical grade and purchased from Fluka Chemie AG, Buchs, Switzerland. Immobilization supports were a gift from Röhm GmbH (Darmstadt, Germany: Eupergit C, Eupergit C 250 L) or purchased from Pharmacia Biotech (Freiburg, Germany: EAH-Sepharose 4B).

Enzyme preparation: A) To obtain the hydantoinase and the L-*N*-carbamoylase wild type enzymes, *Arthrobacter aurescens* DSM 3747 was cultivated in a 100-l-bioreactor under conditions as previously reported [Syldatk et al., 1990] using 0.3 g l^{-1} *N*-3-methylene-D,L-IMH as inducer. Cell disruption was carried out continuously under optimized conditions according to the results presented elsewhere [Bunge et al., 1992] using a cooled (-20°C) 600 ml disintegration cell and the Dyno-Mill KDL (Willy A. Bachofen, Basel, Switzerland). Unleaded glass beads (480 ml, diameter 0.3 mm) were agitated at an agitator speed of 2500 rpm. An ice-cooled suspension of bio-wet-mass (30% w/v) suspended in 0.2 M TRIS-buffer, containing 1 mM $MnCl_2$, pH 7.0 was pumped three times through the cell with a flow rate of 80 ml mi*N*$^{-1}$ (crude extract). The enzyme fraction used for the immobilization experiments was obtained by ammonium sulfate precipitation at 4°C. Therefore a precooled, saturated $(NH_4)_2SO_4$-solution (105 ml) was continuously added with a flow rate of 0.34 ml min^{-1} (Watson Marlow, Falmouth, GB) to the crude extract (70 ml) while mechanical stirring (Heidolph, Kelheim, Germany). The hydantoinase and the L-*N*-carbamoylase precipitated at a $(NH_4)_2SO_4$-concentration of 60%. After centrifugation (Beckman, GB) of the suspension with 24700 $\times$ g for 20 min at 4°C the supernatant was decanted and the pellet was stored at -20°C.

B) To obtain the pure recombinant L-*N*-carbamoylase, *E. coli* W3110 pAW178-2 was cultivated in 400 ml batch cultures in LB-medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) containing 0.1 mg ml^{-1} ampicillin according to the results presented elsewhere [Wilms et al., 1999]. At OD_{500} = 0.5 the enzyme activity was induced by adding rhamnose (2 g l^{-1}). Cell disruption of an ice-cooled suspension of 5 g of bio-wet-mass suspended in 11 ml of 0.1 M TRIS-buffer, containing 0.1 mM $MnCl_2$, pH 7.0 was carried out using a French pressure cell (AMINCO, Urbana, Illinois). The enzyme fraction for the immobilization experiments was obtained by two chromatography steps. The crude extract (13 ml) was diluted with 10 ml of 10 mM TRIS buffer, pH 7.0 and applied to a Streamline DEAE column (Pharmacia, Freiburg, Germany) (column volume: 20 ml) equilibrated with buffer A (50 mM TRIS, pH 7.0) and eluted by applying a linear gradient between buffer A and B (50 mM TRIS, 1 M NaCl, pH 7.0) within 200 ml. The active fractions were collected (54 ml) and concentrated by ultrafiltration (Filtron, Northborough, USA) (30 kDa). The concentrate (20ml)

was diluted with 20 ml 10 mM TRIS buffer, pH 7.0 and applied to a Mono Q column (Pharmacia, Freiburg, Germany) equilibrated with buffer A and eluted by applying a linear gradient between buffer A and B within 200 ml. The active fractions were collected (volume 10 ml; protein concentration 3,4 g l^{-1}; enzyme activity 4,3 U ml^{-1}) and stored in portions of 1 ml at - 20°C.

Preparation of Eupergit C (-NH_2) and Eupergit C 250 L (-NH_2): For the immobilization via the carbodiimide method [Verhoeven et al., 1994], NH_2-groups were introduced into Eupergit C and Eupergit C 250 L. Ammoniolysis of 5 g dry carrier was carried out in closed containers for 48h at 45°C using 40 ml aqueous ammonia (2.5%). Afterwards the excess of NH_3 was washed off with deionized water until the pH was neutral again. Finally the modified carrier was equilibrated with phosphate buffer (0.1 M, pH 6.5) and stored at 4°C.

Immobilization of the crude hydantoinase and L-N-carbamoylase to Eupergit C (-NH_2), Eupergit C 250 L(-NH_2) and EAH Sepharose 4B: The pellet from enzyme preparation A was resuspended in 150 ml deionized water containing 1 mM $MnCl_2$. After centrifugation at 24700 × g for 20 min at 4°C, the clarified supernatant containing both enzymes was diluted with deionized water containing 1 mM $MnCl_2$ to a final protein concentrations of 0.6 (Eupergit C (-NH_2), Eupergit C 250 L(-NH_2)) and 1.2 g l^{-1} (EAH Sepharose 4B). 8 ml of the diluted enzyme solution were added to 1 g of wet carrier (Eupergit C (-NH_2), EAH Sepharose 4B) or 0.5 g of dry Eupergit C 250 L (-NH_2). While manually shaking from time to time 500 µl of a *N*-(dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) stock solution in deionized water (1.7 M, pH 5.0, adjusted with 1% HCl) were added dropwise to the respective ice-cooled suspensions which were afterwards shaken overhead for 18h at 4°C. The immobilized enzymes were filtered off and washed 3 times each with 20 ml 0.1 M TRIS containing 0.5 M NaCl, pH 8.5. Before storage at 4°C, the immobilized enzymes were washed 3 times with 20 ml 0.1 M TRIS, pH 8.5.

Immobilization of the crude hydantoinase and L-N-carbamoylase to Eupergit C and Eupergit C 250 L: The pellet from enzyme preparation A was resuspended in 30 ml of a 1 M phosphate buffer, pH 7,5 and centrifuged at 24700 × g for 20 min at 4°C. After diluting the clarified supernatant to a final concentration of 3 g l^{-1}, 4 ml and 6 ml, respectively, were added to 1 g of dry Eupergit C and Eupergit C 250 L. The suspension was gently shaken by horizontal rotation for 20 h at 4°C. To hydrolyze excess oxirane groups, the immobilized enzyme was filtered off and washed 3 times each with 25 ml of 0,1 M TRIS buffer, pH 8,5 before storage at 4°C for at least 48 h prior to use.

Immobilization of the pure recombinant L-N-carbamoylase to Eupergit C(-NH_2), Eupergit C 250 L(-NH_2) and EAH-Sepharose 4B: The thawed Mono Q fraction (enzyme preparation B) was diluted 1:80 with deionized water containing 1 mM $MnCl_2$. Varying amounts of the respective carrier were added to 4 ml of the diluted enzyme solution. For the investigation of the influence of the EDC concentration 444 µl of a 10 mM EDC stock solution, 1 ml of a 100 mM EDC stock solution and 444 µl, 545 µl, 651 µl, 762 µl, 878 µl of a 500 mM EDC stock solution were added dropwise to each portion (final concentrations 1, 10, 20, 50, 60, 70, 80, 90 mM). The suspensions were shaken overhead for 17 h at 4°C. To hydrolyze excess activated esters, the immobilized enzymes were filtered off and washed 3 times each with 25 ml of 0,2 M TRIS buffer containing 0,5 M NaCl, pH 8,5. For storage, the immobilized enzymes were washed 3 times each with 25 ml 0.1 M TRIS buffer, pH 8.5 and stored at 4°C.

Analytical methods: Protein concentrations were determined according to the method of Bradford [Bradford, 1976]. The test kit was purchased from BioRad (Munich, Germany). HPLC analysis was carried out as described elsewhere [May et al., 1998]. Retention times: Trp: 10 min; IMH: 15.4 min; *N*-C-Trp: 17.3 min.

Determination of the activity of free enzymes: 50 µl of the enzyme solution were added to 800 µl of the preincubated (37°C) substrate solution (0.4 g l^{-1} IMH or 0.4 g l^{-1} *N*-C-L-Trp, respectively, in 0.1 M TRIS-buffer, pH 8.5). The reaction was stopped after 5 min by adding 400 µl TCA, (12 %) and substrate as well as product concentrations were analyzed by HPLC after centrifugation.

Determination of the activity of the immobilized enzymes: 800 µl of the preincubated substrate solution (see above) were added to 100 mg of wet immobilized enzyme and shaken for 10 min at 37°C. The reaction was stopped by adding of 400 µl TCA and the conversion was analyzed by HPLC after centrifugation.

3. Results and Discussion

For the immobilization of proteins, several methods including adsorptive, covalent and inclusion techniques have been investigated and published [Fágáin, 1997; Tischer, 1995]. Usually, covalent immobilization is of major interest because the bonds formed are much more stable than those formed by electrostatic interactions or by adsorption. Because the carrier can not be regenerated, for industrial purposes it is required that covalently bound biocatalysts have a high stability.

For the hydantoin cleaving enzymes from *Arthrobacter aurescens*, covalent coupling to different types of Eupergit (via oxirane groups) and via amino groups to EAH-Sepharose and to modified Eupergit C and C 250 L (using water-soluble carbodiimide, see Table 1 for a characterization of the various supports used) was investigated and the procedure of immobilization was optimized. In order to provide a technically useful method the crude hydantoinase and L-*N*-carbamoylase obtained after cell disruption and ammonium sulfate precipitation were used as starting materials for the immobilization procedures at first.

Table 1: Functional groups and corresponding immobilization methods for different types of supports

Support	**Functional group**	**Density of groups** $\mu mol\ g^{-1}$ **dry support**	**Immobilization method**
Eupergit C		> 600[a]	Oxirane
Eupergit C 250 L		>200[a]	Oxirane
modified Eupergit C	$-NH_2$	530[b]	Carbodiimide
modified Eupergit C 250 L	$-NH_2$	370[b]	Carbodiimide
EAH Sepharose 4B	$-NH_2$	440[b]	Carbodiimide

[a] data from supplier [Röhm, 1995]

[b] measured according to [Halling and Dunhill, 1979]

Covalent coupling of the **hydantoinase** using carbodiimide resulted in coupling yields between 10% (Eupergit C ($-NH_2$)) and 60% (EAH Sepharose 4B) (Figure 1). The lower yield obtained with modified Eupergit C and C 250 L in comparison to EAH Sepharose 4B may be explained by the more hydrophilic matrix of the Sepharose based support. The direct immobilization of the crude hydantoinase to epoxy activated Eupergit C and C 250 L led to activity yields of 20 % and 90 %, respectively. Obviously, Eupergit C 250 L is favorable for the immobilization of the L-*N*-carbamoylase in active form, which may be explained by the larger pore size reported for this carrier [Röhm, 1995].

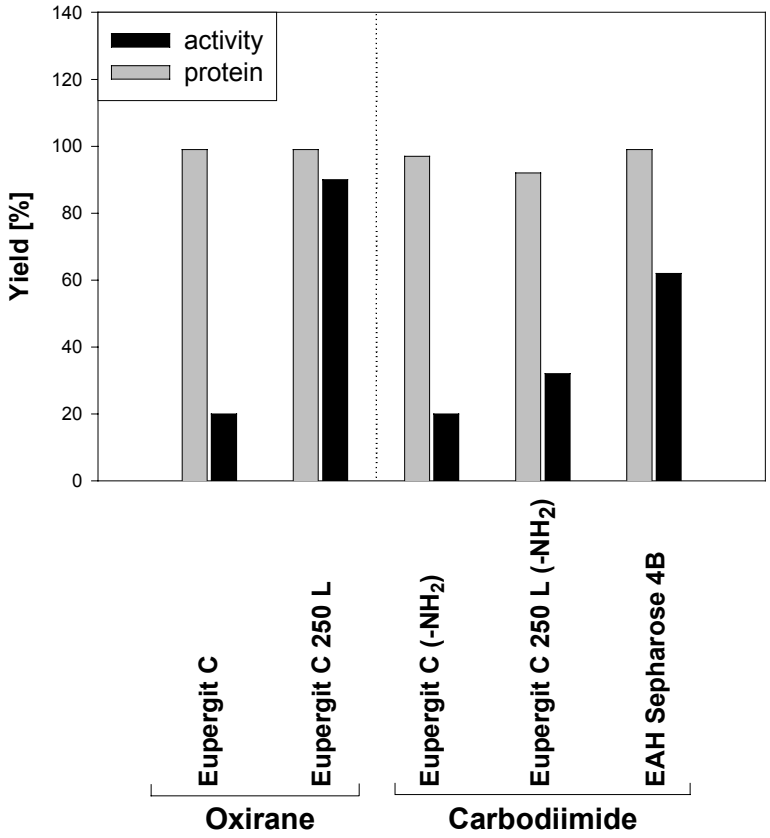

Figure 1: Immobilization of L-hydantoinase from *Arthrobacter aurescens* DSM 3747. Influence of different supports and methods on the yield of activity.

For the wild-type **L-*N*-carbamoylase** it was previously reported that the covalent coupling via oxirane groups which are known to react with amino, thiol and hydroxy groups of the enzyme resulted in only low activity yields [Pietzsch et al., 1998]. During the present optimization, it turned out that crosslinking of the L-*N*-carbamoylase with preactivated supports carrying aldehyde groups (e. g. glutaraldehyde method according to [Bryjak et al., 1993]) resulted in an almost complete loss of activity, too (results not shown). Only the carbodiimide method - crosslinking carboxylic acid groups of the enzyme and amino groups of a carrier - was applicable for the immobilization of the L-*N*-carbamoylase.

However, initial experiments of covalently coupling the wild-type L-*N*-carbamoylase by carbodiimide resulted in low activity yields (Fig. 4). In order to investigate whether the reasons for these results were related to contaminating proteins or to a generally inactivating modification of amino acid residues essential for activity, the recombinant L-*N*-carbamoylase

expressed in *E. coli* was purified [Wilms et al., 1999] and the pure enzyme was used for the immobilization experiments. Interestingly, coupling via carbodiimide resulted in significant amounts of active L-*N*-carbamoylase bound to the carrier. Also, it was proven for the purified recombinant enzyme that coupling via glutaraldehyde was not possible, even if pure enzyme was used. Therefore, covalent coupling of the L-*N*-carbamoylase seems only to be possible via carboxylic groups.

In order to optimize the activity yield for the carbodiimide-mediated immobilization of the purified L-*N*-carbamoylase, the influence of the concentration of *N*-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), the protein to carrier relation and the type of support were investigated. For direct comparison of the oxirane and the carbodiimide coupling results, amino groups were introduced to Eupergit C and Eupergit C 250 L by reaction with aqueous ammonia. As shown in Figure 2 the activity yield versus EDC-concentration plot shows an typical saturation profile with a slight decrease of activity yield at higher EDC concentrations. At a protein concentration of 0.04 g l^{-1} the optimum concentration of EDC was determined to be between 50-60 mM. Almost 20% of the initial activity were immobilized.

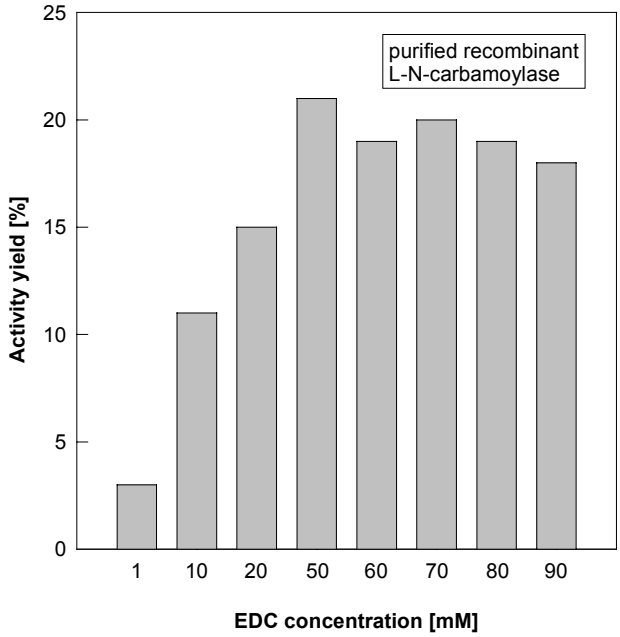

Figure 2: Immobilization of the purified, recombinant L-*N*-carbamoylase from *E. coli*. Dependence of the activity yield on the carbodiimide concentration after adsorption of the L-*N*-carbamoylase to modified Eupergit C 250 L (Eupergit C 250 L (-NH_2). Protein concentration 0.04 g l^{-1}, reaction volume 4 ml, 0.5 g wet carrier.

The activity yield could be further enhanced by variation of the protein to carrier relation. As can be seen from Figure 3 the activity yield of the purified L-*N*-carbamoylase coupled to Eupergit C 250 L (-NH_2) increased from 20 to approximately 30% at higher surface to protein ratios. Interestingly, 90% of the total protein amount were bound to the carrier during these experiments but only 30% were active. The reasons for this difference are still unknown, but may be caused by the formation of multiple layers of the enzyme and by adsorption effects accompanied with conformational changes of the adsorbed protein which may result in changed catalytic efficacy or even complete inactivation [Soderquist and Walton, 1980; Yongli et al., 1999].

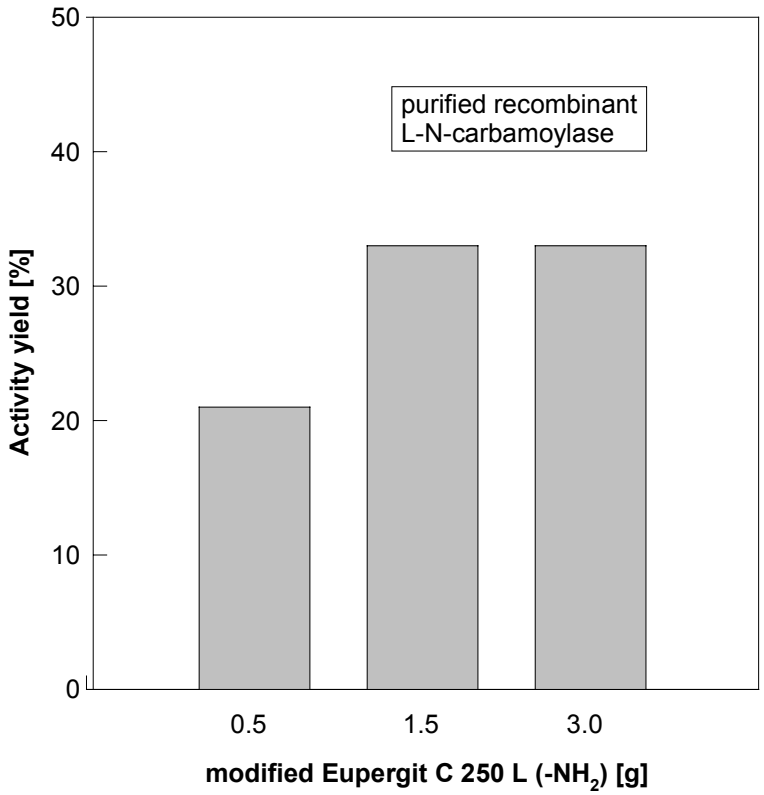

Figure 3: Immobilization of the purified L-*N*-carbamoylase from recombinant *E. coli* using water-soluble carbodiimide (60 mM EDC): Influence of the protein to carrier relation on the activity yield. Protein concentration 0.04 g l^{-1}, reaction volume 4 ml.

Under optimized conditions, the influence of the type of support, varying in the basic matrix and the density of reactive groups (see Table 1) was investigated. As can be seen from Figure 4 and Table 2, the activity yield could be significantly enhanced by immobilizing the

purified, recombinant enzyme from *E. coli*. Although the density of amino groups presented by the carrier is higher for modified Eupergit C (NH_2) (see Table 1) the enzyme was coupled to EAH Sepharose with nearly quantitative yield of activity. On the other hand, Eupergit based supports are obtained by bead polymerization of the monomers methacrylamide, N,N´-methylene-bis-methacrylamide, glycidyl methacrylate and allyl glycidyl ether. Thus, Eupergit supports are in general more hydrophobic than EAH Sepharose. Obviously the interactions between the hydrophilic EAH Sepharose and the L-*N*-carbamoylase are favorable for the retention of enzymatic activity.

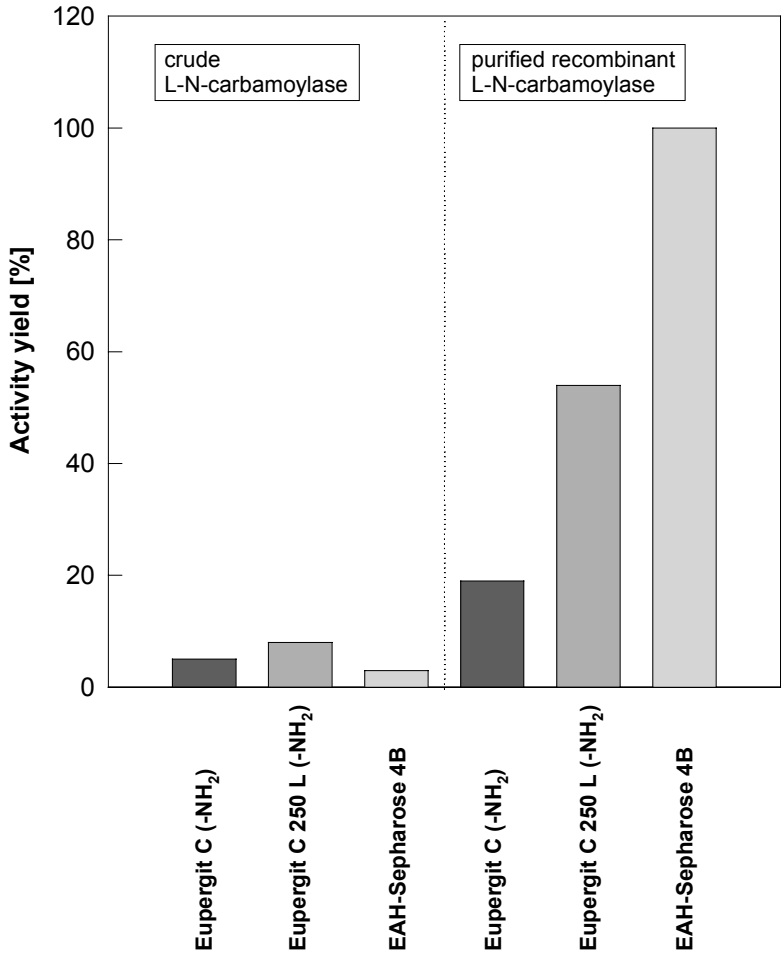

Figure 4: Immobilization of the L-*N*-carbamoylase (crude enzyme from *Arthrobacter aurescens* DSM 3747 and purified enzyme from recombinant *E. coli*): Comparison of different supports with respect to the activity yield after immobilization via water-soluble carbodiimide (60 mM EDC, 0.04 mg ml^{-1} protein concentration).

Table 2: Specific activity, activity yield and protein yield after immobilization of the hydantoinase and the L-*N*-carbamoylase by the carbodiimide method

Enzyme	Support	Specific activity [U g^{-1}]	Activity yield [%]	Protein [mg g^{-1}]	Protein yield [%]
Hydantoinase	EAH Sepharose 4B	0.2	49	3.23	95
Hydantoinase	Eupergit C (-NH_2)	0.14	15	3.63	48
Hydantoinase	Eupergit C 250 L (-NH_2)	0.11	12	5.63	73
L-*N*-Carbamoylase	Eupergit C (-NH_2)	0.005	19	0.25	99
L-*N*-Carbamoylase	Eupergit C 250 L (-NH_2)	0.014	54	0.24	98
L-*N*-Carbamoylase	EAH Sepharose 4B	0.032	100	0.25	99

4. Conclusions

Whereas the immobilization of the hydantoinase from *Arthrobacter aurescens* turned out to be relatively simple in performance, the immobilization of the L-*N*-carbamoylase required a lot of optimization experiments. Since this enzyme catalyzes the stereospecific conversion of the *N*-carbamoyl amino acid produced by the unspecific hydantoinase, it is essential for the production of optically pure L-amino acids. As could be shown by the immobilization of the purified recombinant enzyme, the activity yield of the L-*N*-carbamoylase was deteriorated by contaminating proteins. Furthermore, covalent immobilization of the L-*N*-carbamoylase was only possible via the carbodiimide method, i. e. coupling via enzymes carboxylic groups to supports carrying primary amino groups. Additionally, a hydrophilic carrier such as EAH Sepharose was favorable to immobilize the L-*N*-carbamoylase in active form. Using the optimized protocol, the activity yield could be enhanced from 4% to approximately 100%. Coupling via amino groups by water-soluble carbodiimide had to be carried out at a concentration of between 50 and 60 mM of EDC to ensure a sufficient coupling yield without loss of activity, which might be caused by crosslinking of enzyme molecules prior to the covalent coupling to the support.

Although the high activity yields obtained in the present paper and the operational stabilities observed in a previous one [Pietzsch et al., 1998] represent important improvements towards an industrially feasible biocatalyst, the specific activities obtained so far still are too low to fulfill economical requirements. Since the proteins could almost completely be immobilized in all experiments, there should be some reason for the differences between the activity yields and the coupling yields. Further experiments should reveal, if there is an

influence of initial adsorption processes on the amount of active proteins, which are then fixed by covalent bonds. In continuing experiments, genetically modified proteins have been created and purified [Pietzsch et al., 2000]. For example, a L-*N*-carbamoylase carrying an aspartate-tag has been prepared in order to investigate if an *N*-terminal fusion of carboxylic groups to the wild-type enzyme is accompanied with a positive influence on the amount of actively coupled enzyme during carbodiimide-mediated immobilization.

References

Bradford, M. M. (1976). “A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protei*N*-dye binding.” *Anal. Biochem.,* **72**, 248-254.

Bryjak, J., Trochimczuk, A., and Noworyta, A. (1993). “Effect of polymer matrix on penicilin acylase immobilization on copoymers of butyl acrylate and ethylene glycol dimethacrylate.” *J. Chem. Tech. Biotechnol.,* **57**, 73-78.

Bunge, F., Pietzsch, M., Müller, R., and Syldatk, C. (1992). “Mechanical disruption of *Arthrobacter* sp. DSM 3747 in stirred ball mills for the release of hydantoi*N*-cleaving enzymes.” *Chem. Eng. Sci.,* **47**, 225-232.

Fágáin, Ó. (1997). *Stabilizing protein function*, Springer-Verlag, Berlin.

Halling, P. J., and Dunhill, P. (1979). “Improved nonporous magnetic supports for immobilized enzymes.” *Biotechnology and Bioengineering,* **21**, 393-416.

May, O., Siemann, M., Pietzsch, M., Kiess, M., Mattes, R., and Syldatk, C. (1998). “Substrate-dependent enantioselectivity of a novel hydantoinase from *Arthrobacter aurescens* DSM 3745: Purification and characterisation as new member of cyclic amidases.” *J. Biotechnol.,* **61**, 1-13.

Nanba, H., Ikenaka, Y., Yamada, Y., Yajima, K., Takano, M., Ohkubo, K., Hiraishi, Y., Yamada, K., and Takahashi, S. (1999). “Immobilization of thermotolerant *N*-carbamyl-D-amino acid amidohydrolase.” *J. Molec. Catal. B: Enzymatic,* **6**, 257-263.

Pietzsch, M., Oberreuter, H., Petrovska, B., Ragnitz, K., and Syldatk, C. (1998). “Immobilization of hydantoin cleaving enzymes from *Arthrobacter aurescens* DSM 3747 - Effect of the coupling method on the stability of the L-*N*-carbamoylase.” In: *Stability and Stabilization of Biocatalysts* (Ballesteros, A., Plou, F. J., Iborra, J. L., andHalling, P., eds.), Elsevier Science, Amsterdam, 517-522.

Pietzsch, M., and Syldatk, C. (1995). “Hydrolysis and formation of hydantoins.” In: *Enzyme Catalysis in Organic Synthesis* (Drauz, K., and Waldmann, H., eds.), VCH-Verlag, Weinheim, 409-431.

Pietzsch, M., Wiese, A., Ragnitz, K., Wilms, B., Altenbuchner, J., Mattes, R., and Syldatk, C. (2000). “Purification of recombinant hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* expressed in *Escherichia coli*: Comparison of wild-type and genetically modified proteins.” *J. Chromatogr. B,* **737**, 179-186.

Röhm. (1995). “Eupergit: Immobilization of Enzymes on Eupergit C and Eupergit C 250 L.” *Product information*.

Siemann, M., Syldatk, C., and Wagner, F. (1993b). “Detection and comparison of strains with selective L-hydantoin cleaving activity using polyclonal antibodies.” *Biotechnol. Tech.,* **7**, 361-366.

Soderquist, M. E., and Walton, A. G. (1980). “Structural changes in proteins adsorbed on polymer surfaces.” *J. Colloid Interface Sci.,* **75**, 386-397.

Syldatk, C., Mackowiak, V., Höke, H., Groß, C., Dombach, G., and Wagner, F. (1990). “Cell growth and enzyme synthesis of a mutant of *Arthrobacter* spec. (DSM 3747) used for the production of L-amino acids from D,L-5-monosubstituted hydantoins.” *J. Biotechnol.,* **14**, 345-362.

Tischer, W. (1995). “Immobilization of enzymes.” In: *Enzyme Catalysis in Organic Synthesis* (Drauz, K., and Waldmann, H., eds.), VCH-Verlag, Weinheim, 73-87.

Verhoeven, M., Cahalan, P., Cahalan, L., Hendriks, M., and Foache, B. (1994). "Biomolecule immobilization with carbodiimide." 0 608 095 A1, Medtronic, Inc., USA.

Wilms, B., Wiese, A., Syldatk, C., Mattes, R., Altenbuchner, J., and Pietzsch, M. (1999). “Cloning, nucleotide sequence and expression of a new L-*N*-carbamoylase gene from *Arthrobacter aurescens* DSM 3747 in *E. coli*.” *J. Biotechnol.,* **68**, 101-113.

Yongli, C., Xiufang, Z., Yandao, G., Nanming, Z., Tingying, Z., and Xinqi, S. (1999). “Conformational changes of fibrinogen adsorption onto hydroxyapatite and titanium nanoparticles.” *J. Colloid Interface Sci.,* **214**, 38-45.

Publikation IV

Kerstin Ragnitz, Christoph Syldatk, and Markus Pietzsch (2000) Optimization of the immobilization parameters and operational stability of immobilized hydantoinase and L-N-carbamoylase from *Arthrobacter aurescens* for the production of optically pure L-amino acids. Enzyme Microb. Technol., submitted.

Optimization of the immobilization parameters and operational stability of immobilized hydantoinase and L-N-carbamoylase from *Arthrobacter aurescens* for the production of optically pure L-amino acids.

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Abstract

The immobilization parameters were optimized for the hydantoinase and the L-N-carbamoylase from *Arthrobacter aurescens* DSM 3747 or 3745, respectively. To optimize activity yields and specific activities for the immobilization to Eupergit C, Eupergit C 250 L, and EAH-Sepharose wild-type, recombinant and genetically modified (´tagged´) enzymes were investigated concerning the influence of the protein concentration, the kind of support and the immobilization method. For both enzymes, the use of the recombinant proteins resulted in enhanced specific activities especially when using a hydrophilic support for immobilization such as Sepharose. In the case of a genetically modified hydantoinase carrying a His_6-tag, affinity coupling led to a loss of activity of higher than 80%. Both enzymes were significantly stabilized by immobilization: Eupergit C 250 L (NH_2)-immobilized hydantoinase and EAH-Sepharose-immobilized L-N-carbamoylase showed half-life times of 14000 and 1000 hours, respectively. Together with specific activities of the immobilized enzymes of 2.5 U/g wet carrier (hydantoinase) and 10 U/g wet carrier (L-N-carbamoylase) the newly developed biocatalysts are sufficient to fulfill industrial requirements.

In comparison to the free enzymes, temperature and pH-optima were increased by 10°C and one pH unit, respectively, after immobilization. The pH- and temperature optima of the hydantoinase (L-N-carbamoylase) were determined to be pH 8.5 - 10 (pH 9.5) and 45 -60°C (60°C).

In order to provide sufficient amounts of biocatalyst for the process development in mini plant scale, a 50 fold scale-up of the optimized immobilization procedure was carried out for both enzymes. Because of the overlapping optima, both immobilized enzymes can be operated together in one reactor.

Keywords: Immobilization; hydantoinase; L-N-carbamoylase, *Arthrobacter aurescens*; carbodiimide

Abbreviations

c_p	protein concentration
CTrp	carbamoyl tryptophan
IMH	indolylmethylhydantoin
Trp	tryptophan

Introduction

Beside their function as building blocks of proteins, many natural and unnatural amino acids are valuable compounds in food- [1, 2], pharmaceuticals [3, 4] and agro-industry. The industrial production of amino acids can be done either by extraction, fermentation, total chemical synthesis or enzymatic synthesis. Fermentation and extraction methods are used extensively but are restricted to the production of proteinogenic amino acids. For the chemo-enzymatic production of optically pure amino acids, there are several well established processes [2, 5] employing for example transaminases, dehydrogenases or aminoacylases.

Racemic 5-monosubstituted hydantoins are important precursors for the enzymatic production of D- and L-amino acids by the „hydantoinase method“ [6, 7]. The asymmetric bioconversion consists of three reaction steps leading to optically pure products with 100% theoretical yield. Two enzymes are involved in the stereoselective cleavage of D,L-5-monosubstituted hydantoins and N-carbamoyl amino acids, respectively. The hydantoinase from *Arthrobacter aurescens* DSM 3747 and 3745 shows only limited enantioselectivity for a range of interesting products [8]. Therefore, the key step in the synthesis of optically pure products is represented by the second enzyme, an N-carbamoyl-L-amino acid amidohydrolase (L-N-carbamoylase) which is absolutely stereospecific. Unfortunately, this enzyme was found to be relatively unstable in resting cell biotransformations using *Arthrobacter aurescens* DSM 3747, which can be explained by the fast proteolysis observed for this enzyme [9].

For this reason, the immobilization and stabilization of the hydantoinase and the L-N-carbamoylase were of major importance for the development of a process for the production of unnatural L-amino acids from D,L-5-monosubstituted hydantoins.

In a previous contribution, we were able to show that the activity yields as well as the operational stabilities of the free enzymes could be significantly improved by immobilization. However, the specific activities obtained were still to low to fulfill economical requirements for an industrially feasible biocatalyst [10]. With the expression [11] and the purification [12] of the recombinant enzymes in *Escherichia coli*, the starting conditions for the immobilization experiments and optimization were improved in terms of enzyme availability and purity.

In the present paper, we report on results obtained during the optimization of the immobilization parameters for the covalent coupling of the hydantoinase and the L-N-carbamoylase. The influence of the support and the influence of the protein concentration were investigated. Beside the wild-type and recombinant enzymes, genetically modified hydantoinase and L-N-carbamoylase were used for the experiments.

Materials and methods

General

All chemicals were of analytical grade and purchased from Fluka Chemie AG, Buchs, Switzerland. Immobilization supports were a gift from Röhm GmbH (Darmstadt, Germany: Eupergit C, Eupergit C 250 L) or purchased from Pharmacia Biotech (Freiburg, Germany: EAH Sepharose 4B) and Clontech (Palo Alto, USA: TALON® columns).

Enzyme preparation:

(A) To obtain the hydantoinase and the L-N-carbamoylase wild-type enzymes, *Arthrobacter aurescens* DSM 3747 was cultivated under conditions as reported previously [13]. Cell disruption was carried out under optimized conditions according to the results presented elsewhere [14]. The enzyme fraction (c_P = 6 g l^{-1} , specific activity hydantoinase: 0,3 U mg^{-1}; specific activity carbamoylase: 0,2 U mg^{-1}) used for the immobilization experiments was obtained by ammonium sulfate precipitation as described previously [10].

(B) After cultivation of *Escherichia coli* W3110 pBW30 and 31, respectively, according to Wilms [11] the pure recombinant hydantoinase (with and without his_6-tag (c_P=1,2 g l^{-1}, specific activity: 6 U mg^{-1}), respectively) was obtained after two chromatography steps as described previously [8].

(C) To obtain the pure recombinant L-*N*-carbamoylase, *Escherichia coli* W3110 pAW178-2 was cultivated according to the results presented elsewhere [15] and the enzyme fraction was purified using two chromatography steps (c_P=0.7 g l^{-1}; specific activity: 3 U mg^{-1}), previously described by Pietzsch et al. [12].

Preparation of supports

For the immobilization via the carbodiimide method [16], NH_2-groups were introduced into Eupergit C and C 250 L, previously described by Ragnitz et al. [10].

Immobilization

The immobilization of crude hydantoinase and L-N-carbamoylase and the recombinant enzymes from *Escherichia coli* to Eupergit C (-NH_2) and Eupergit C 250 L (-NH_2) and EAH Sepharose 4B were carried out according to the carbodiimide method and the oxirane method, respectively, as described in a previous paper [10]. For the optimization a standard procedure for the immobilization was established: 4 ml of the respective enzyme solution were added to 3 g of wet carrier. After addition of 545 µl of a EDC stock solution (500 mM) the suspensions were shaken overhead for about 18 h at 4°C. The immobilizates were filtered off and washed 3 times each with 20 ml 0.1 M TRIS containing 0.5 M NaCl, pH 8.5.

Before storage at 4°C, the immobilized enzymes were washed 3 times with 20 ml 0.1 M TRIS, pH 8.5.

Immobilization of the crude recombinant hydantoinase (with His_6-tag and without) to Talon® column: 1 ml of the crude extract obtained after cell disruption by french press and centrifugation at 24700 g was applied to a Talon column equilibrated with 50 mM TRIS containing 300 mM NaCl, pH 8.0. The suspensions were shaken overhead for 5 min at 4°C and afterwards centrifuged for 2 min at 5000 rpm. The immobilizates were washed with 1 ml 50 mM TRIS containing 300 mM NaCl, pH 8.0 while shaking overhead for 5 min at 4°C.

Determination of protein concentration

Protein concentrations were determined according to the method of Bradford [17]. The test kit was purchased from BioRad (Munich, Germany).

Determination of the activity of free enzymes

50 µl of the enzyme solution were added to 800 µl of the preincubated (37°C) substrate solution (0.4 g l^{-1} IMH or 0,4 g l^{-1} N-C-L-Trp, respectively, in 0.1 M TRIS-buffer, pH 8.5). The hydantoinase reaction was stopped after 5 min by adding 40 µl TFA and the carbamoylase reaction by adding 400 µl TCA (12%). Substrate as well as product concentrations were analyzed by HPLC after centrifugation. HPLC analysis was carried out as described elsewhere [8]. Retention times: Trp: 10 min; IMH: 15.4 min; *N*-C-Trp: 17.3 min.

Determination of the activity of immobilized enzymes

800 µl of the preincubated substrate solution (see above) were added to 100 mg of wet immobilized enzyme and shaken for 10 min at 37°C. The reaction was stopped by adding of 40 µl TFA and 400 µl TCA (12%), respectively and the conversion was analyzed by HPLC (see above) after centrifugation.

Scale-up of immobilization of hydantoinase and L-N-carbamoylase

A) Hydantoinase: 410 ml of enzyme preparation A (c_P = 8.5 mg ml^{-1}; specific activity: 0.13 U mg^{-1}) were added to 360 g of wet modified Eupergit C 250 L (-NH_2). After addition of 55.9 ml of a *N*-(dimethylaminopropyl)-*N´*-ethylcarbodiimide (EDC) stock solution in deionized water (500 mM, pH 5.0 adjusted with 1% HCl) to the ice-cold suspension, immobilization took place while shaking overhead for 17 at 4°C. The immobilizate was filtered off and washed 3 times each with 1 l of 0.1 M TRIS-buffer containing 0.5 M NaCl, pH 8.5.

B) L-*N*-Carbamoylase: 180 ml of enzyme preparation C (c_P = 70 mg ml^{-1}; specific activity: 3.7 U mg^{-1}) were added to 152 g of wet EAH Sepharose 4B. After addition of 26 ml of a EDC

stock solution in deionized water (500 mM, pH 5.0 adjusted with 1% HCl) to the ice-cooled suspension immobilization took place while shaking overhead for 18 h at 4°C. The immobilizate was filtered off and washed 3 times each with 1 l 0f 0.2 M TRIS-buffer containing 0.5 M NaCl, pH 8.5. The immobilizates were stored at 4°C.

Biochemical characterization of the immobilized enzymes

To determine the effects of temperature and pH on the activity of the immobilized hydantoinase (from *Arthrobacter aurescens* DSM 3747) and L-N-carbamoylase (from *Escherichia coli* W3110 pAW 178.2), enzyme assays were performed as follows:

Determination of pH-optima:

The immobilized enzymes were assayed under the standard conditions (see above) except that the following buffers were used: 100 mM potassium phosphate, pH 5.5 – 8.0; 100 mM TRIS, pH 7.0 - 10.0 and 100 mM potassium carbonate, pH 9.5 – 10.5.

Determination of temperature optima:

After pre-incubation of the corresponding substrate solutions at the respective temperatures in the range between 25 and 70°C, 800 µl were added to 100 mg of wet immobilized enzyme and the enzyme assay was carried out as described above.

Determination of the operational stability of the immobilizates:

A) Continuously operated packed bed reactor: The operational stability of the immobilized hydantoinase and L-N-carbamoylase was determined after packing XK 16/20 columns (Pharmacia) with 22 ml immobilized hydantoinase or 2 ml immobilized L-*N*-carbamoylase, respectively. The standard substrates (0.4 g l^{-1} IMH or 1 g l^{-1} N-C-L-Trp, respectively, in 0.1 M TRIS-buffer, pH 8.5) were pumped from a thermostated vessel to the bottom of the reactor at constant flow rates.

B) Continuously stirred tank reactor (CTSR): Using the standard substrate solutions for each enzyme, the reaction was carried out with 29 g immobilized hydantoinase and 2 ml immobilized carbamoylase, respectively, in a CSTR at 37°C under a stream of nitrogen gas while the pH was controlled at 8.5 with 0.1 M NH_3 and 0.1 M HCl, respectively. The substrate solution was added with a flow rate of 0.3 ml/min and the reaction volume was held constant at 150 ml. The immobilizate was retained in the vessel using a metal sieve with a pore size of 200 µm (Spörl, Sigmaringendorf, Germany). Samples were taken periodically for the calculation of the stability based on the conversion.

Results and discussion

Influence of protein concentration, carrier and immobilization method on the enzyme activity

In order to obtain an industrially feasible biocatalysts, the immobilization methods previously described [10, 18] were optimized for the hydantoinase and the L-N-carbamoylase from *Arthrobacter aurescens* DSM 3747 or 3745. To optimize activity yields and specific activities, wild-type, recombinant and genetically modified (´tagged´) enzymes were investigated, partly after purification. For the **L-*N*-carbamoylase**, the only applicable method for covalent coupling proceeded via carbodiimide-activation of carboxylic groups of the enzyme In order to enhance the specific activity, the relation of protein to carrier was varied. As can be seen from Figure 1, there was an increase of specific activity up to 7 U per gram wet carrier. Unfortunately, the activity yield decreased with increasing specific activities. Since the total amount of bound protein remained almost constant, there seems to be an optimal loading of enzyme to be coupled to the carrier. It was shown by additional experiments that the loss of activity already occurred during the adsorption of the L-N-carbamoylase and was independent of the subsequent crosslinking with carbodiimide (results not shown). Despite the exact reasons for the observed loss in activity yield are unknown so far, one can speculate that there is an optimal enzyme loading of the support. Enzyme above this optimal amount is adsorbed and immobilized only in inactive form.

In order to provide sufficient amounts of immobilized L-N-carbamoylase for the use in a mini plant, the optimal coupling conditions were scaled up by a factor of 50. As a result, 150 g of EAH Sepharose immobilized L-N-carbamoylase were obtained with the same specific activity as in the small-scale experiments. In conclusion it was possible to linearly scale-up the immobilization conditions of the L-N-carbamoylase.

Since it was only possible to immobilize the L-N-carbamoylase via carboxylic groups, a genetically modified enzyme carrying a N-terminal Asp_6-tag was investigated. Using the $(Asp)_6$-tagged carbamoylase, at a protein concentration of 0.1 mg ml^{-1}, a specific activity of 3.8 U per gram wet carrier and an activity yield of 83% was obtained. This result is comparable to the result obtained with the recombinant wild-type enzyme.

As concluded in a previous paper, the immobilization of the wild-type **hydantoinase** turned out to be relatively easy [10]. However, the specific activities obtained so far were too low for an industrial application. In order to increase the specific activities, different preparations of the hydantoinase were investigated: the crude wild-type hydantoinase, the crude recombinant wild-type enzyme after expression in *Escherichia coli* and the genetically

modified enzyme expressed in *Escherichia coli* (His_6-tag). The influence of the protein concentration of the various hydantoinase preparations on the specific activity and activity and coupling yield is shown in Figures 2 and 3. For the two enzyme preparations and carriers a comparable dependence of the three parameters on the protein concentration was observed as seen for the carbamoylase, i. e. increasing specific activity with protein concentration and decreasing coupling yield. Optimal results were obtained for the coupling of the purified recombinant hydantoinase to EAH Sepharose 4B. Specific activities as high as 2.7 U per gram wet carrier were obtained (Figure 5B). Interestingly, after storage of the immobilizates in buffer containing manganese chloride (1 mM), the specific activities were increased up to 5fold. A similar hyper-activation was previously reported by May *et al.* [19] for the free enzyme. The reasons for this phenomenon are still unknown.

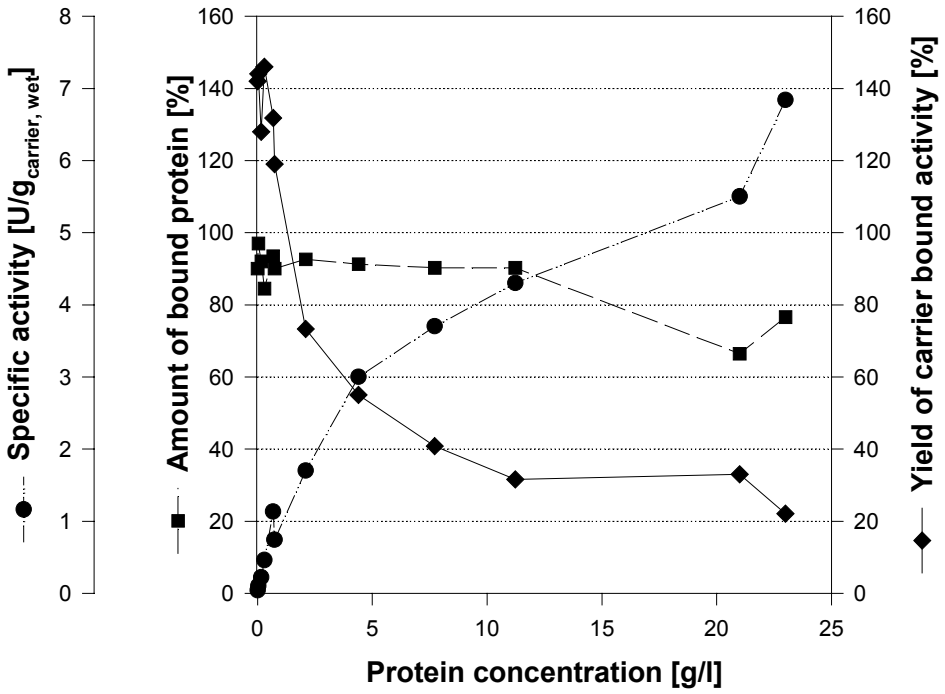

Figure 1: Immobilization of the recombinant carbamoylase to EAH Sepharose 4B (carbodiimide method) - Dependence of specific activity, activity and coupling yield on the protein concentration.

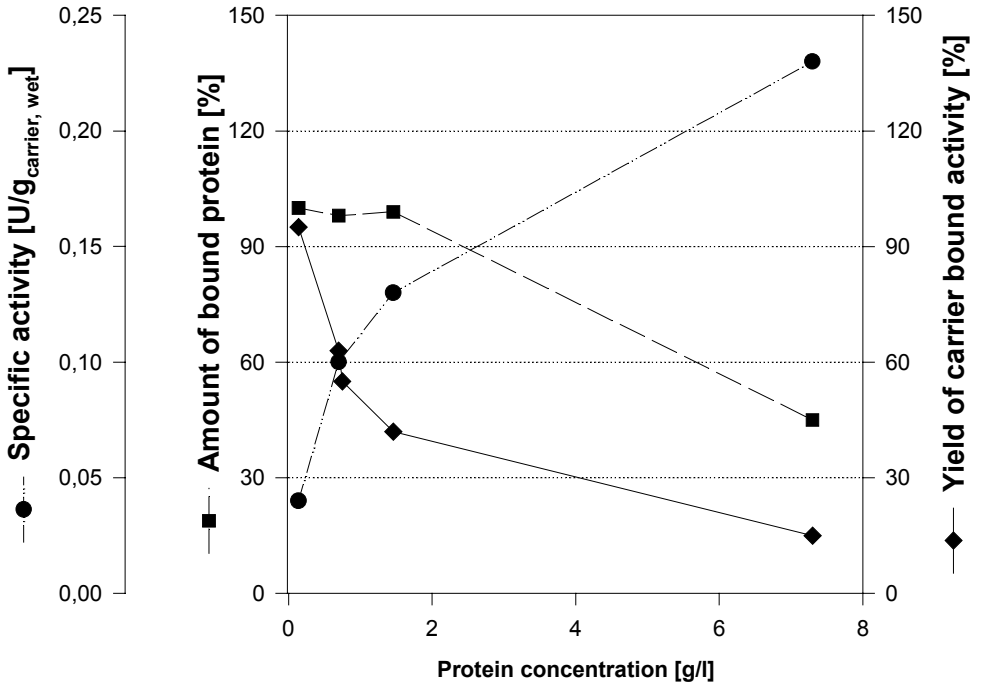

Figure 2: Immobilization of the crude wild-type hydantoinase to Eupergit C 250 L (-NH_2) - Dependence of specific activity and activity and coupling yield on the protein concentration.

Since affinity chromatography represents a versatile tool for the fast and simple purification of recombinant, tagged proteins [20] it should also be suitable for immobilization of the histidine-tagged hydantoinase and L-*N*-carbamoylase to a TALON® column. Therefore, it was investigated whether the are still active after adsorption to the affinity matrix (TALON®). By following the proposed purification and immobilization protocol, only less then 10% of the enzymatic activity – for the hydantoinase and the L-*N*-carbamoylase – were bound to the matrix resulting in specific activities of 0.2 and 0.5 U per gram wet carrier, respectively. Unfortunately, this led to a significant loss of activity for both enzymes.

Nevertheless, storage stability was determined and the immobilized carbamoylase was determined to be stable over a period of about 15 days whereas the immobilized hydantoinase completely inactivated during this time period. To elucidate whether an unspecific adsorption via histidine residues of the hydantoinase lead to the mentioned loss of activity, experiments with the corresponding wild-type enzyme were carried out. Only 2% of the wild-type enzyme were bound in active form to the column and a balance of the activity to 100% was impossible. Applying a pH-gradient to elute the bound protein, there was no elution of active hydantoinase in both cases.

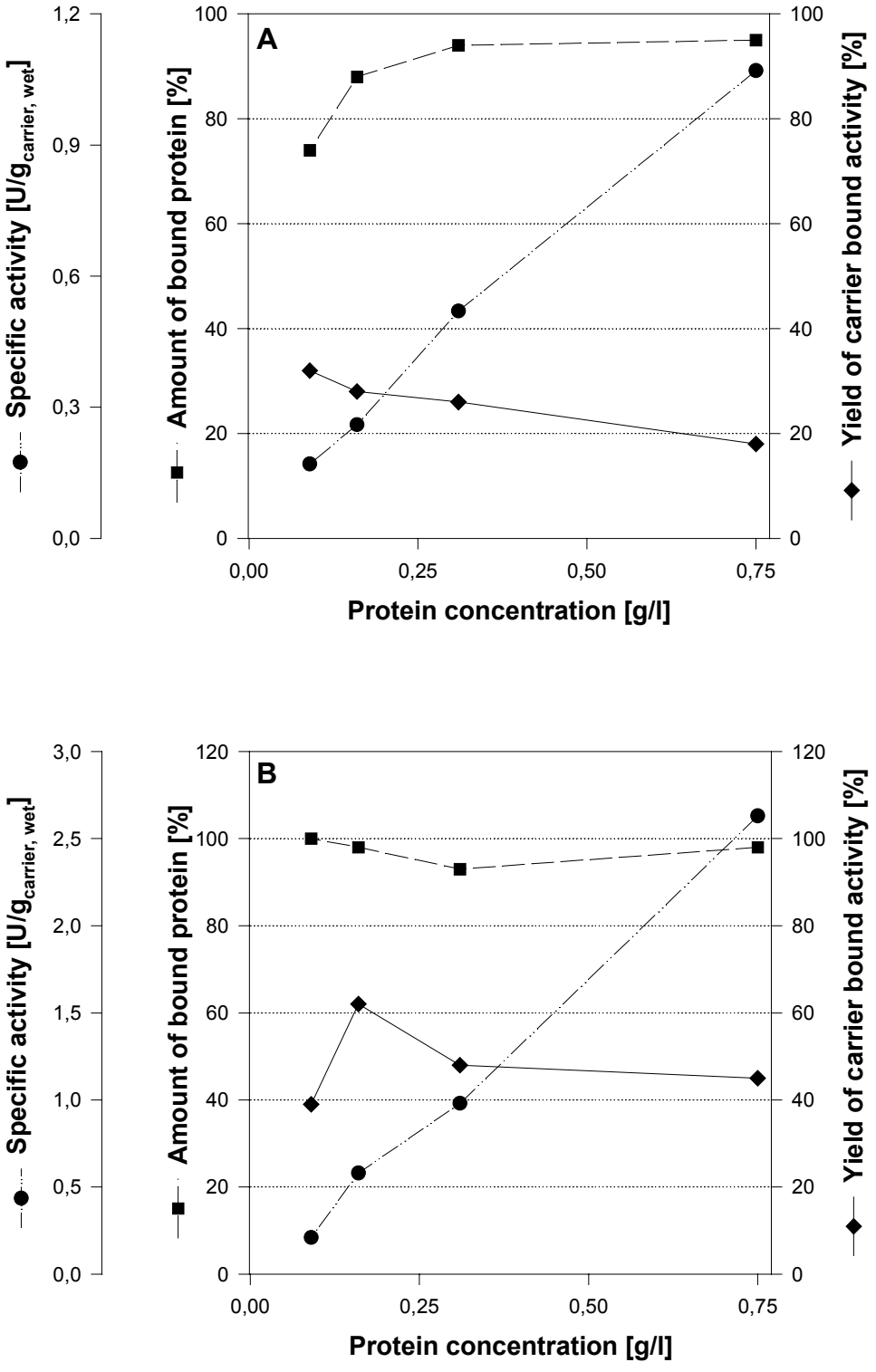

Figure 3: Immobilization of the purified recombinant hydantoinase to A) Eupergit C 250 L (-NH_2) and B) EAH Sepharose 4B - Dependence of specific activity and activity and coupling yield on the protein concentration.

In order to provide sufficient amounts of immobilized hydantoinase for the use in a mini-plant, the conditions for the coupling via the carbodiimide method were scaled up by a factor of 120 leading to 360 g of immobilizate with a specific activity of 1 U per gram wet carrier.

Biochemical characterization of immobilized hydantoinase and L-N-carbamoylase

Upon immobilization, changes in the pH-activity-profile as well as in the temperature-activity-profile may occur [21]. Especially in the case of covalent coupling, changes in the enzyme properties are expected. Therefore immobilized hydantoinase and L-*N*-carbamoylase were investigated for their pH- and temperature optima. As result, the immobilized hydantoinase and as well as L-*N*-carbamoylase exhibit higher pH and temperature optima than the corresponding free enzymes (40 and 50 °C, respectively) [11]. Between 25 and 45°C, the activity of immobilized hydantoinase increased significantly. In the range of 45 up to 60°C the activity remained constant at maximum level and decreased at higher temperatures as shown in Figure 4A. With increasing temperatures from 25 up to 60°C, the specific activity of immobilized L-*N*-carbamoylase increased to a maximum at 60°C and decreased also at higher temperatures (Figure 4B).

The determination of the pH optima (Figure 4B) for the immobilized L-*N*-carbamoylase resulted in a shift from pH 8 for the free enzyme to pH 9.5 for the immobilizate. The hydantoinase showed maximal activity over a pH range from pH 8 to 9.5 (Figure 4A).

Stability of the immobilized enzymes

For the process the operational stability (OS) of the immobilizates is of high importance. The OS of immobilized hydantoinase and L-*N*-carbamoylase was determined in CSTR and packed bed reactors, respectively, using the standard substrates IMH for the hydantoinase and L-N-CTrp for the carbamoylase. Under reaction conditions, both immobilized enzymes were more significantly more stable than the free ones [18]. For the hydantoinase and the L-*N*-carbamoylase half-life times of 14000 and 1000 hours, respectively, were obtained in the case of the packed bed reactor (Figure 5A and B) as well as for the reaction in a continuously stirred tank reactor (Figure 6A and B).

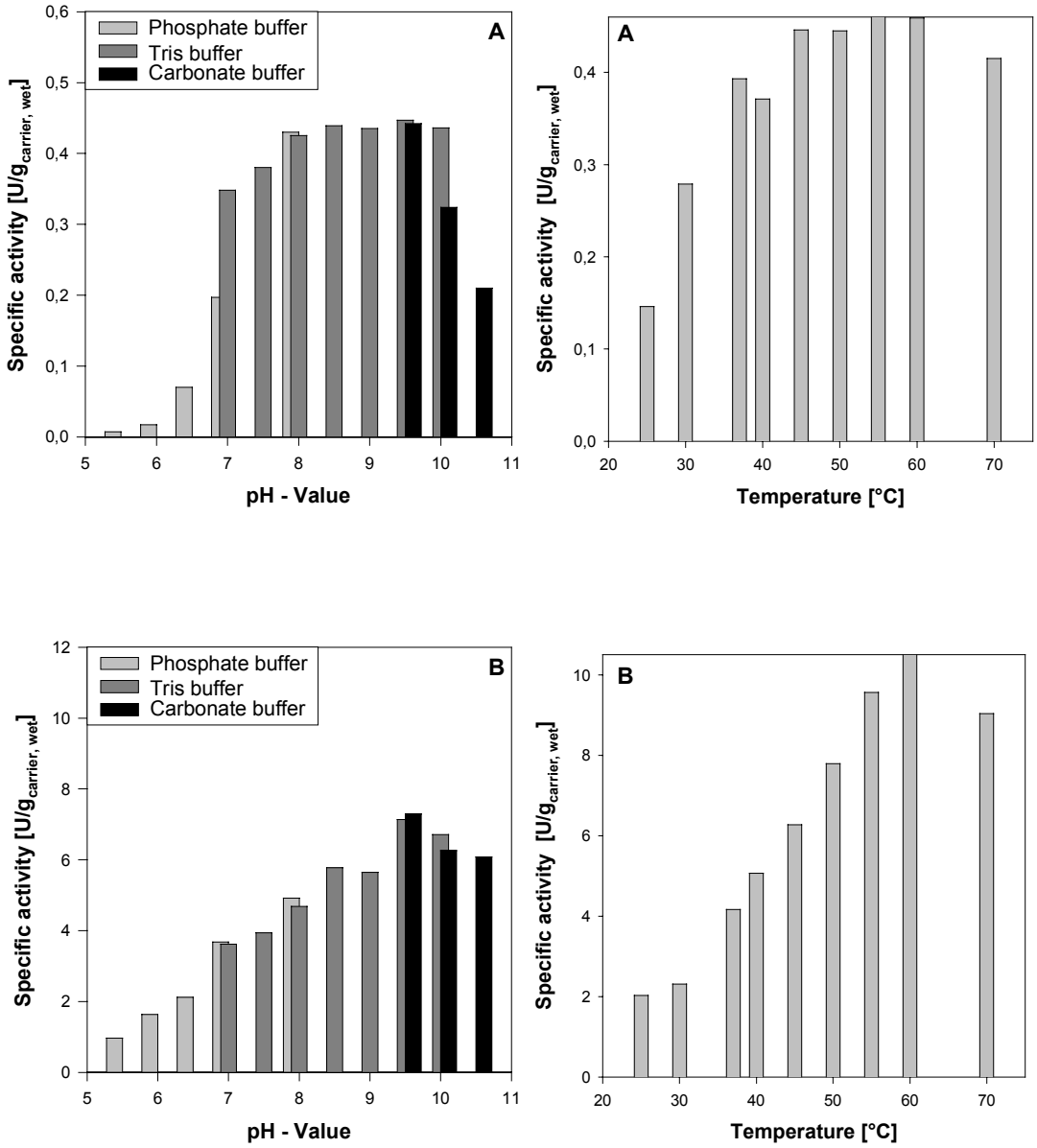

Figure 4: pH- and temperature optima of A) the immobilized hydantoinase (Eupergit C 250 L ($-NH_2$)) and B) carbamoylase (EAH Sepharose 4B).

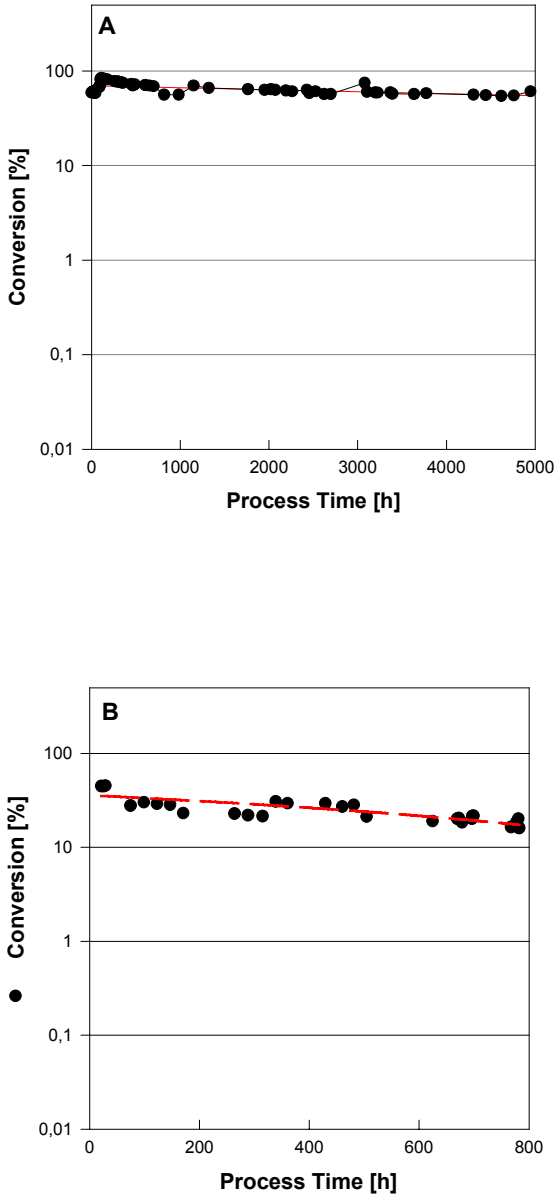

Figure 5: Determination of the operational stability of A) the immobilized hydantoinase (Eupergit C 250 L (-NH_2)) and B) L-*N*-carbamoylase (EAH Sepharose 4B) in a packed bed reactor.

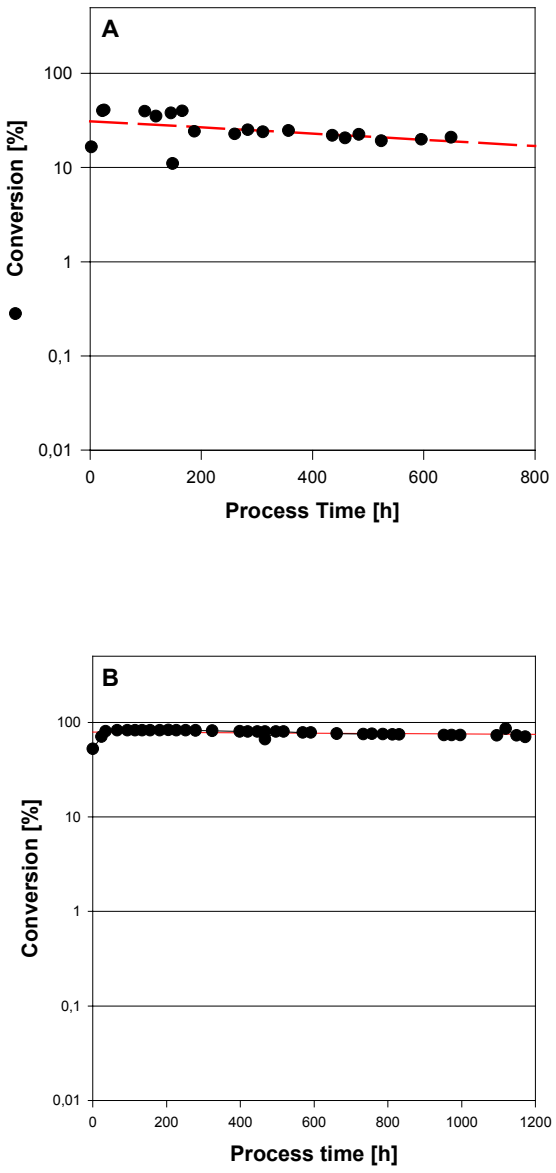

Figure 6: Determination of the operational stability of A) the immobilized hydantoinase (Eupergit C 250 L (-NH_2)) and B) L-*N*-carbamoylase (EAH Sepharose 4B) using a continuous stirred tank reactor.

Conclusions

The optimization of the immobilization parameters for the hydantoinase and the L-*N*-carbamoylase led to industrially feasible biocatalysts in terms of specific activity and operational stability. With the availability of recombinant expressed hydantoinase and L-*N*-carbamoylase, favorable conditions for systematic investigations were created. Based on results presented earlier, the influence of protein concentration was investigated in detail. The specific activity was increased at the expense of activity yield whereas the coupling yield was not affected. Best results for hydantoinase as well as for L-*N*-carbamoylase were obtained using EAH-Sepharose 4B as carrier in comparison to Eupergit C (-NH_2) and Eupergit C 250 L (NH_2), presumably due to the higher hydrophilicity of the matrix.

The use of affinity chromatography for the specific adsorption of recombinant his-tagged hydantoinase and L-*N*-carbamoylase was not successful, but resulted in a loss of activity of about 90%. Biochemical characterization of the immobilized hydantoinases and L-*N*-carbamoylase resulted in equal pH and temperature optima enabling operation in one pot under the same reaction conditions and co-immobilization of both enzymes. Furthermore, small deviations from the optimal conditions have negligible influence on the first reaction step because the immobilized hydantoinase works with maximal activity over a broad pH and temperature range.

By following the optimized immobilization protocol, biocatalysts with specific activities of about 2.5 and 10 U per gram wet carrier and high operational stabilities with half-life times of 14000 and 1000 for the hydantoinase and the L-*N*-carbamoylase, respectively, were obtained.

References

1. Calton, J. C. The enzymatic production of L-aspartic acid. In: *Biocatalytic Production of Amino Acids and Derivatives* (Rozell, J. D., and Wagner, F., eds.), Hanser Publishers, New York, 1992, 3-21.

2. Leuchtenberger, W. Amino acids - Technical production and use. In: *Biotechnology* (Rehm, H.-J., Reed, G., Pühler, A., and Stadler, P., eds.), VCH-Verlag, Weinheim, 1996, 465-502.

3. Bommarius, A. S., Drauz, K.-H., Groeger, U., and Wandrey, C. Membrane reactors for the production of enantiomerically pure alpha-amino acids. In: *Chirality in Industry: The commercial manufacture and application of optically active compounds* (Collins, A. N., Sheldrake, G. N., and Crosby, J., eds.), John Wiley & Sons, New York, 1992, 372-395.

4. Drauz, K.-H. Chiral amino acids: A versatile tool in the synthesis of pharmaceuticals and fine chemicals. *Chimia* 1997, **51**, 310-314.

5. Yagasaki, M., and Ozaki, A. Industrial biotransformation for the production of D-amino acids. *Journal of Molecular Catalysis B: Enzymatic* 1998, **4**, 1-11.

6. Syldatk, C., Müller, R., Pietzsch, M., and Wagner, F. Microbial and enzymatic production of L-amino acids from D,L-monosubstituted hydantoins. In: *Biocatalytic Production of Amino Acids & Derivatives* (Rozzell, D., and Wagner, F., eds.), Hanser Publishers, München, 1992, 129-176.

7. Syldatk, C., Müller, R., Siemann, M., and Wagner, F. Microbial and enzymatic production of D-amino acids from D,L-monosubstituted hydantoins. In: *Biocatalytic Production of Amino Acids & Derivatives* (Rozzell, D., and Wagner, F., eds.), Hanser Publishers, München, 1992, 75-128.

8. May, O., Siemann, M., Pietzsch, M., Kiess, M., Mattes, R., and Syldatk, C. Substrate-dependent enantioselectivity of a novel hydantoinase from *Arthrobacter aurescens* DSM 3745: Purification and characterisation as new member of cyclic amidases. *J. Biotechnol.* 1998, **61**, 1-13.

9. Siemann, M., Syldatk, C., and Wagner, F. Detection and comparison of strains with selective L-hydantoin cleaving activity using polyclonal antibodies. *Biotechnol. Tech.* 1993, **7**, 361-366.

10. Ragnitz, K., Pietzsch, M., and Syldatk, C. Immobilization of the hydantoin-cleaving enzymes from *Arthrobacter aurescens* DSM 3747. *J. Biotechnol.* 2000, submitted.

11. Wilms, B., Wiese, A., Syldatk, C., Mattes, R., Altenbuchner, J., and Pietzsch, M. Cloning, nucleotide sequence and expression of a new L-*N*-carbamoylase gene from *Arthrobacter aurescens* DSM 3747 in *E. coli*. *J. Biotechnol.* 1999, **68**, 101-113.

12. Pietzsch, M., Wiese, A., Ragnitz, K., Wilms, B., Altenbuchner, J., Mattes, R., and Syldatk, C. Purification of recombinant hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* expressed in *Escherichia coli*: Comparison of wild-type and genetically modified proteins. *J. Chromatogr. B* 2000, **737**, 179-186.

13. Syldatk, C., Mackowiak, V., Höke, H., Groß, C., Dombach, G., and Wagner, F. Cell growth and enzyme synthesis of a mutant of *Arthrobacter* spec. (DSM 3747) used for the production of L-amino acids from D,L-5-monosubstituted hydantoins. *J. Biotechnol.* 1990, **14**, 345-362.

14. Bunge, F., Pietzsch, M., Müller, R., and Syldatk, C. Mechanical disruption of *Arthrobacter* sp. DSM 3747 in stirred ball mills for the release of hydantoin-cleaving enzymes. *Chem. Eng. Sci.* 1992, **47**, 225-232.

15. Wilms, B., Hauck, A., Reuss, M., Syldatk, C., Mattes, R., Siemann, M., and Altenbuchner, J. High cell density fermentation for production of L-*N*-carbamoylase using an expression system based on the *E. coli* rhaBAD-promoter. *Enzyme Microb. Technol.* 2000, accepted.

16. Verhoeven, M., Cahalan, P., Cahalan, L., Hendriks, M., and Foache, B. Biomolecule immobilization with carbodiimide., Medtronic, Inc., USA.

17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, **72**, 248-254.

18. Pietzsch, M., Oberreuter, H., Petrovska, B., Ragnitz, K., and Syldatk, C. Immobilization of hydantoin cleaving enzymes from *Arthrobacter aurescens* DSM 3747 - Effect of the coupling method on the stability of the L-*N*-carbamoylase. In: *Stability and Stabilization of Biocatalysts* (Ballesteros, A., Plou, F. J., Iborra, J. L., and Halling, P., eds.), Elsevier Science, Amsterdam, 1998, 517-522.

19. May, O., Siemann, M., and Syldatk, C. Catalytic and structural function of zinc for the hydantoinase from *Arthrobacter aurescens* DSM 3745. *J. Mol. Cat. B: Enzymatic* 1998, **4**, 211-218.

20. Clemmitt, R. H., and Chase, H. A. Facilitated downstream processing of a histidine-tagged protein from unclarified E. coli homogenates using immobilized metal affinity expanded-bed adsorption. *Biotechnol Bioeng* 2000, **67**, 206-216.

21. Kennedy, J. F., and Cabral, J. M. S. (1987). *Enzyme Immobilization*, Verlag Chemie, 1987, Weinheim.