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Purification of recombinant hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* expressed in *Escherichia 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 wild-type enzyme despite that 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. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The use of tag-technology is becoming increasingly popular for the simple and inexpensive production

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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, 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 2×YT 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% α-D-rhamnose. After 4 h, cells from

400 ml culture were harvested and resuspended in 12.5 ml buffer A (20 m*M* Tris–HCl, pH 7.4, 0.2 *M* NaCl, 1 m*M* $MnCl_2$, 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 *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 hyuC–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 six 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 of the *rhaBAD* promotor in the vector pJOE2775 [18] which was cut with the same restriction enzymes. Plasmid pBW2 contains the *hyuC* gene fused to six 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 complementary 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

2.3.2.1. Preparation of crude cell extracts. Cells were grown to a final OD_{600} of approximately 5. A wet mass of 2.7 g of harvested cells were resuspended in 30 ml of 20 m*M* Tris HCl, 100 m*M* NaCl, pH 8.0 (buffer B), centrifuged at 4000 *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, 48 000 *g*).

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

2.3.2.3. Purification protocol. A volume of 0.46 ml of the supernatant obtained in Section 2.3.2.1 were mixed with the Talon resin and incubated overnight at 4°C. The resin was collected by centrifugation at 700 *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 m*M* 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 m*M* $MnCl_2$, 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

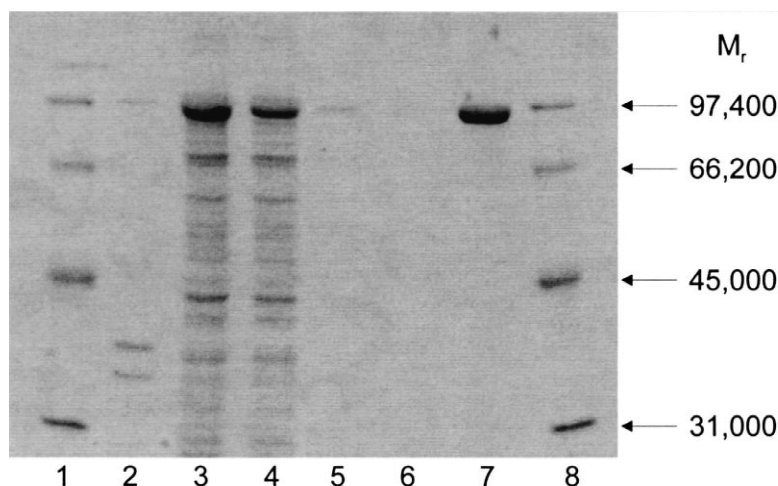

Fig. 1. SDS–PAGE of MalE–HyuH fusion protein from *E. coli* JM109 pAW211. Standard proteins carbonic anhydrase (M_r 31 000), ovalbumin (M_r 45 000), serum albumin (M_r 66 200), phosphorylase B (M_r 97 400) (lanes 1 and 8), crude extract (insoluble protein, lane 2), crude extract (soluble protein, lane 3), breakthrough 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).

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).

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

Purification	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

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 Fig. 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).

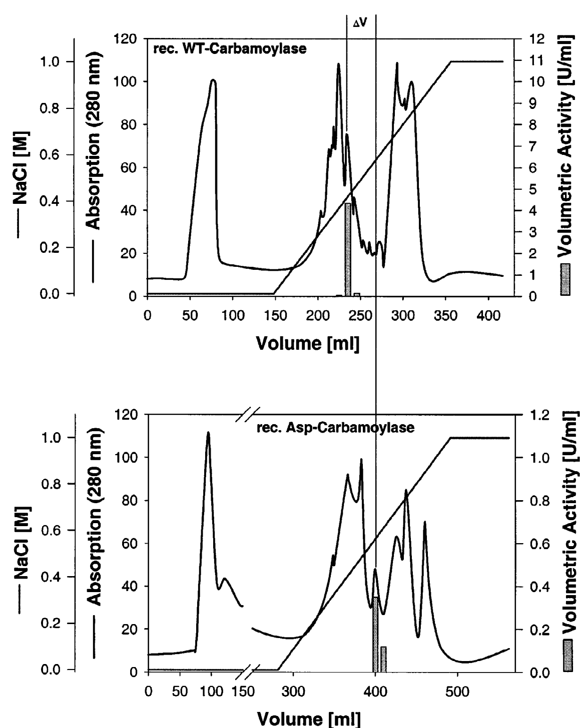

Fig. 2. Comparison of elution chromatograms of recombinant wild-type L-*N*-carbamoylase (top) and recombinant Asp_6-tagged L-*N*-carbamoylase (bottom). Final polishing step on MonoQ 16/10 using 0.05 *M* Tris-buffer, pH 7.0 containing 1.0 m*M* $MnCl_2$. The active fractions of tagged and wild-type L-*N*-carbamoylase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

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–

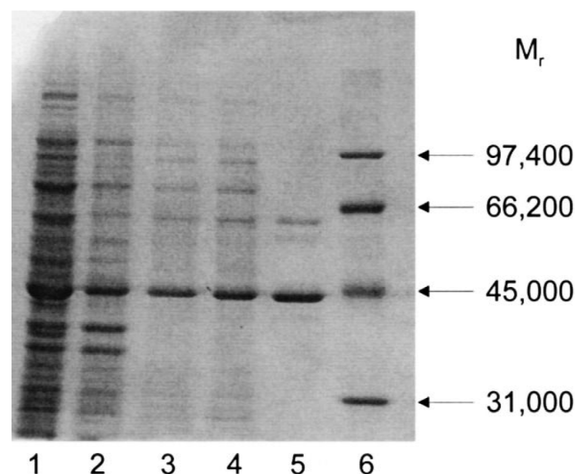

Fig. 3. SDS–PAGE of Asp_6-tagged L-*N*-carbamoylase (HyuC-Asp_6) expressed in *E. coli* JM 109 pBW2. Crude cell extract (lane 1), breakthrough fraction of Streamline DEAE (lane 2), active fractions eluted from Streamline-DEAE (lane 3), after ultrafiltration (lane 4), active fractions eluted from MonoQ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

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

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.

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, breakthrough fraction) that the binding capacity of the column used was not sufficient to bind the fusion protein completely.

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 a 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

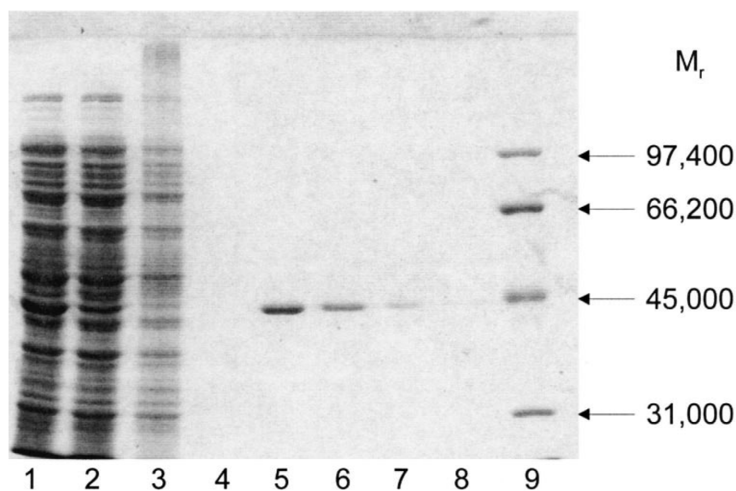

Fig. 4. SDS–PAGE of His_6-tagged L-*N*-carbamoylase (HyuC–His_6) expressed in *E. coli* JM 109 pBW1. Crude extract (lane 1), breakthrough fraction of Talon-column (lane 2), washing fractions (lanes 3 and 4), elution of active HyuC–His_6 with 20 m*M* Tris–HCl, 100 m*M* NaCl, 100 m*M* imidazole, pH 8.0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

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 an 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 over-expressed 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

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