Simple enzymatic procedure for L-carnosine synthesis: whole-cell biocatalysis and efficient biocatalyst recycling

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Summary

β-Peptides and their derivatives are usually stable to proteolysis and have an increased half-life compared with α-peptides. Recently, β-aminopeptidases were described as a new enzyme class that enabled the enzymatic degradation and formation of β-peptides. As an alternative to the existing chemical synthesis routes, the aim of the present work was to develop a whole-cell biocatalyst for the synthesis and production of β-peptides using this enzymatic activity. For the optimization of the reaction system we chose the commercially relevant β,α-dipeptide L-carnosine (β-alanine-L-histidine) as model product. We were able to show that different recombinant yeast and bacteria strains, which overexpress a β-peptidase, could be used directly as whole-cell biocatalysts for the synthesis of L-carnosine. By optimizing relevant reaction conditions for the best-performing recombinant Escherichia coli strain, such as pH and substrate concentrations, we obtained high L-carnosine yields of up to 71%. Long-time as well as biocatalyst recycling experiments indicated a high stability of the developed biocatalyst for at least five repeated batches. Application of the recombinant E. coli in a fed-batch process enabled the accumulation of L-carnosine to a concentration of 3.7 g l⁻¹.

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Introduction

β-Peptides or mixed β,α-peptides that contain β-amino acid residues are highly interesting molecules for pharmaceutical research and drug development. They also occur as natural products with high biological activities. Examples include L-carnosine, microcystin, pantothenic acid and paclitaxel. Synthetic β-amino acid-containing peptides have generally a long half-life as they are highly resistant against proteolytic degradation (Frackenpohl et al., 2001). Due to their high stability, the development of such peptides could lead to novel pharmaceutically relevant substances that function as stable peptidomimetics and specific inhibitors (Kritzer et al., 2004). One of these peptides, the dipeptide L-carnosine (β-alanine-L-histidine), occurs in mammalian tissues (Bonfanti et al., 1999). Although its biological function is still unknown (Aldini et al., 2005), studies have shown anti-oxidant and oxygen-free radical scavenger functions of L-carnosine (Decker et al., 2000).

The synthesis of peptides is mostly accomplished by chemical or enzymatic means (Guzman et al., 2007). Chemical peptide syntheses can be divided into synthesis in solution and solid-phase synthesis and are used for the routine production of peptides in the range of about 2–50 residues (Kimmerlin and Seebach, 2005). Especially for the synthesis of small peptides, alternative enzymatic synthesis approaches are used to overcome limitations in chemical synthesis, such as lack of specificity and the high environmental burden (Tramper, 1996; Guzman et al., 2007). Enzyme-catalyzed reactions give access to a large number of peptides synthesized by different proteases under various conditions (Stehele et al., 1990; Bordusa, 2002; Yokozeki and Hara, 2005; Li and Kanerva, 2006; Heck et al., 2007; Salam et al., 2008) and found industrial interest already in 1988 (Thorbeck, 1988). A common approach in the biocatalysis of enzyme-catalyzed peptide formation is the application of a kinetically controlled process (Jakubke et al., 1985) as illustrated in Fig. 1.

Biocatalysis might be either performed by enzymes in solution or encapsulated in whole cells. Both approaches have been used extensively in industry for the production of various chemicals (Schmid et al., 2002; Ishige et al., 2003).
Fig. 1. Schematic reaction in kinetically controlled peptide synthesis. An acylated enzyme intermediate (R-CO-Enz) reacts either with water (hydrolysis) or with another amino acid (aminolysis). Adapted from Bordusa (2002).

The recently identified enzyme class of β-aminopeptidases (Geueke et al., 2005; 2006) catalyses the hydrolysis of unnatural β- and mixed β,α-peptides (Heck et al., 2006) and also the reverse synthesis reaction (Heck et al., 2007). In the present work, we developed a whole-cell biocatalyst using this new enzymatic activity for the synthesis and production of the β,α-peptide L-carnosine as shown in Fig. 2. Here, we showed that harvested cells could be used directly as whole-cell biocatalysts for the synthesis of L-carnosine, thereby avoiding time- and material-intensive protein purification. By optimizing the synthesis conditions, we were able to increase the yield of product per β-alanine-amide (H-β-Ala-NH₂). These conditions enabled the accumulation of high L-carnosine titres in a fed-batch process under constant concentrations of H-β-Ala-NH₂. Thus, the successful use of this new whole-cell biocatalyst for L-carnosine production might present an alternative route to the chemical synthesis procedures (Vinick, 1981; Hanselmann, 2002).

Results and discussion

Expression performance of the recombinant hosts

Escherichia coli and Pichia pastoris in high cell density fermentations

Two alternative enzymes, DmpA from Ochrobactrum anthropi and 3-2W4 BapA from Sphingosinicella xenopardilytica, were previously applied for the synthesis of β- and mixed β,α-peptides including L-carnosine (Heck et al., 2007). Here, both genes were expressed in Escherichia coli using dmpA as a native as well as a codon-optimized version (dmpA_syn), while 3-2W4 bapA was cloned only as native gene. Additionally, Pichia pastoris was used to express dmpA as native gene and 3-2W4 bapA as codon-optimized version (bapA_syn). The total set of the five constructed expression strains (see Experimental procedures) was used for enzyme production during high cell density fed-batch fermentations under glucose limitation to prevent by-product formation.

The expressed β-peptide hydrolysing activities were determined with a spectrophotometric assay that followed the hydrolysis of β-alanine-para-nitroanilide (H-β-Ala-pNA) using cell-free extracts after sonification. Specific hydrolytic activities of 21 U/g_total protein for 3-2W4 BapA and 215 U/g_total protein for DmpA in E. coli, respectively, 42 U/g_total protein for DmpA in P. pastoris were determined. Optimization of the codon sequence of 3-2W4 bapA (BapA_syn) for expression in P. pastoris resulted in a sixfold increase in activity of 60 U/g_total protein, compared with native 3-2W4 BapA expression in P. pastoris (data not shown). Adaptation of the dmpA codon usage (DmpA_syn)
to *E. coli* resulted in a further increase in specific hydrolytic activity to 285 U g\textsubscript{protein}\textsuperscript{-1}.

### Biotransformation performance of the whole-cell biocatalysts

The potential of the \(\beta\)-aminopeptidases DmpA and 3-2W4 BapA for \(\beta\)-peptide synthesis in solution has been shown in previous studies using pure enzymes (Heck *et al.*, 2007). Since protein purification and protein recycling in solution is cumbersome, the recombinant strains were characterized as whole-cell biocatalysts for the production of \(\beta\),\(\alpha\)-dipeptide L-carnosine. As shown in Table 1, the five strains were tested for hydrolytic activities on H-\(\beta\)-Ala-pNA as whole cells and compared with the activities of their crude extracts. Importantly, substrate hydrolysis using whole cells was observed for all strains. For DmpA the highest hydrolytic activity was found for *E. coli* (DmpA\textsubscript{syn}) with 71 \(\pm\) 5 U g\textsubscript{CDW}\textsuperscript{-1} and for 3-2W4 BapA from *P. pastoris* (BapA\textsubscript{syn}) with 3.3 \(\pm\) 0.9 U g\textsubscript{CDW}\textsuperscript{-1}. The observed reduced hydrolytic activity of whole cells compared with crude extract (Table 1) is presumably caused by transport limitations across the cellular membrane, which is more prominent for *P. pastoris*. The average ratio of whole-cell to crude extract activity in *E. coli* was 39\%, while the average ratio for the *P. pastoris* strains was only 18\%, suggesting that the differences in membrane structure influence substrate uptake. We assume that the whole cells are non-living under the here used assay conditions, which is supported by the fact that glucose addition did neither impact enzymatic activity nor product yield and thus an active transport mechanism of the substrate/product is unlikely.

In the next step, the application of the two enzymes in whole cells for the coupling of H-\(\beta\)-Ala-NH\textsubscript{2} and L-histidine to L-carnosine was examined. To investigate the peptidase-catalyzed synthesis of L-carnosine, 10 mM H-\(\beta\)-Ala-NH\textsubscript{2} in 100 mM Na\textsubscript{2}CO\textsubscript{3} buffer (pH 10) with 50 mM L-histidine was reacted at 25°C using a whole-cell equivalent activity of 0.05 U ml\textsuperscript{-1} (hydrolytic activity on H-\(\beta\)-Ala-pNA) and followed L-carnosine formation by HPLC. L-Carnosine formation could be observed for all five biocatalysts (Table 1). Among all strains the highest L-carnosine yield based on H-\(\beta\)-Ala-NH\textsubscript{2} of 23 \(\pm\) 2\% was found to be for *E. coli* DmpA\textsubscript{syn}. Slightly lower yields were obtained from recombinant *E. coli* DmpA (19 \(\pm\) 2\%) and 3-2W4 BapA (18 \(\pm\) 2\%), whereas a significantly lower L-carnosine formation was observed for *P. pastoris* DmpA (12 \(\pm\) 2\%) and BapA\textsubscript{syn} (2 \(\pm\) 1\%). These results might again be explained by differences in membrane composition of *P. pastoris* compared with *E. coli*, which is supported by the finding that the enzyme activities for L-carnosine synthesis using crude extracts of *E. coli* and *P. pastoris* were highly similar. In principle, transport limitation could cause an accumulation of L-carnosine in the cell, which potentially would benefit hydrolysis of L-carnosine. Beyond this, expression experiments (data not shown) have shown that the expression system for the most efficient strain, *E. coli* DmpA\textsubscript{syn}, facilitated an accumulation of c. 10\% of the total DmpA in the periplasm, and thus a reduced interference by the cytoplasmic membrane might be supposed.

Among the above tested strains, the recombinant *E. coli* DmpA\textsubscript{syn} performed best with respect to the activity of whole-cell L-carnosine production and was therefore selected for further investigations.

### Optimization of L-carnosine synthesis

In the applied reaction system (Fig. 2) under kinetic control (Fig. 1), competing hydrolysis of the acyl-enzyme leads to low L-carnosine yields (Table 1). The ratio of hydrolysis to aminolysis can be influenced by varying different reaction parameters affecting the activity and stability of the enzyme (Jakubke *et al.*, 1985; Stehle *et al.*, 1990; Bordsua, 2002; Lombard *et al.*, 2005; Salam *et al.*, 2008). Thus, the influence of several reaction parameters on the biocatalyst, such as temperature, activity, intracellular enzyme concentration, pH and substrate concentrations, were investigated to characterize the reaction conditions and to enhance the efficiency of peptide synthesis. The initial conditions were set to pH 10 in a

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**Table 1.** Peptidase activities of recombinant hosts.

| Strain name | Hydrolytic activity\(^a\) of whole cells (U g\textsubscript{CDW}\textsuperscript{-1}) | Hydrolytic activity\(^a\) of crude extract (U g\textsubscript{CDW}\textsuperscript{-1}) | Ratio whole cells/crude extract (%) | Activity of L-carnosine synthesis of whole cells (U g\textsubscript{CDW}\textsuperscript{-1}) | Yield\(^b\) L-carnosine/ H-\(\beta\)-Ala-NH\textsubscript{2} (%) |
|-------------|---------------------------------|---------------------------------|-------------------------------|---------------------------------|-------------------------------|
| *E. coli* BapA | 1 \(\pm\) 0 | 3 | 36 | 0.2 \(\pm\) 0.1 | 18 \(\pm\) 2 |
| *P. pastoris* BapA\textsubscript{syn} | 3 \(\pm\) 1 | 19 | 17 | 0.1 \(\pm\) 0.1 | 2 \(\pm\) 1 |
| *E. coli* DmpA | 42 \(\pm\) 4 | 110 | 38 | 12 \(\pm\) 2 | 19 \(\pm\) 2 |
| *E. coli* DmpA\textsubscript{syn} | 71 \(\pm\) 5 | 171 | 42 | 29 \(\pm\) 2 | 23 \(\pm\) 2 |
| *P. pastoris* DmpA | 4 \(\pm\) 1 | 22 | 19 | 0.5 \(\pm\) 0.2 | 12 \(\pm\) 2 |

\(^a\) Hydrolytic activity is specified as the hydrolysis of H-\(\beta\)-Ala-pNA over time followed spectrophotometrically in an activity assay. 

\(^b\) Maximal yields on L-carnosine for bacteria strains were obtained after approximately 120 min of incubation, whereas *P. pastoris* strains were incubated for approximately 8 h to obtain maximal yields.
100 mM Na₂CO₃ buffer at 25°C supplemented with 10 mM H-β-Ala-NH₂ and 50 mM L-histidine using E. coli DmpA<sub>syn</sub> cells, in which protein expression was induced for 4 h.

**Effect of biocatalytic activity**

First, three different whole-cell concentrations with total hydrolytic activities on H-β-Ala-pNA of 0.05, 0.1 and 0.2 U ml<sup>-1</sup>, respectively, were tested for L-carnosine synthesis. The formation of L-carnosine correlated linearly with the applied enzyme activity. The rate of L-carnosine formation was the highest for 0.2 U ml<sup>-1</sup> (4.52 mmol l<sup>-1</sup> h<sup>-1</sup>), whereas the lowest rate was determined for 0.05 U ml<sup>-1</sup> (1.11 mmol l<sup>-1</sup> h<sup>-1</sup>), reaching equal L-carnosine yields of approximately 20% after 30 and 120 min respectively. Although application of the highest biocatalytic activity would enhance productivity of the process, for practical reasons, further investigations were performed using the lowest hydrolytic activity of 0.05 U ml<sup>-1</sup>. More specifically, using 0.2 U ml<sup>-1</sup> would increase the reaction rate to values that would be difficult to follow by the here employed analytical procedures. In an industrial process the increase in catalytic activity would be beneficial as it would directly increase the space-time yield.

**Effect of pH**

The effect of pH on the formation of L-carnosine and peptidic by-products was studied in the range of pH 8–12 (Fig. 3). The pH value of the reaction system affected the yield of L-carnosine and peptidic by-products significantly. While maximal hydrolytic activities of DmpA were found to be between pH 8 and 9, L-carnosine formation was most effective at pH 10. The corresponding maximal yield was 22.5% at this condition. The increase in yield on L-carnosine in correlation to the pH is caused by the fact that deprotonated L-histidine acts as effective nucleophile in the formation of peptide bonds, which was clearly observed at a pH value higher than pH 9. pH values higher than 11, however, reduced peptide formation significantly, most likely due to inactivation of the enzyme DmpA, or decreased stability of the amide. Additionally, the formation of the by-products H-β-Ala-β-Ala-His-OH and H-β-Ala-β-Ala-β-Ala-NH₂ was also observed under all conditions. The highest yield of total peptide synthesis (L-carnosine, H-β-Ala-β-Ala-NH₂, H-β-Ala-β-Ala-β-Ala-NH₂, H-β-Ala-β-Ala-β-Ala-β-Ala-NH₂ and H-β-Ala-β-Ala-β-Ala-His-OH) was 49% of the consumed H-β-Ala-NH₂ at pH 10 and 11. The remaining 51% were assumed to be the product of direct substrate hydrolysis, H-β-Ala-OH. Consequently, pH 10 was used throughout the present study resulting in the highest L-carnosine yield.

The results indicate that the whole-cell biocatalyst consists at these harsh conditions of non-viable cells. One might assume that viable cells would maintain an almost constant intracellular pH and thus extracellular variation of pH would have less impact on peptide synthesis.

**Effect of temperature**

The influence of temperature in the range from 25°C to 37°C was studied. The observed increase in L-carnosine formation rates of 1.1 mmol l<sup>-1</sup> h<sup>-1</sup> (25°C), 1.9 mmol l<sup>-1</sup> h<sup>-1</sup> (30°C) and 3.1 mmol l<sup>-1</sup> h<sup>-1</sup> (37°C) followed the increase of temperature. The average yield of 20.5 ± 0.3% was constant under all conditions and was obtained after 45 min at 37°C, 60 min at 30°C and 120 min at 25°C respectively. All subsequent experiments were performed at 30°C to ensure reaction rates that allow good time-resolved data acquisition.

**Impact of specific cell activity**

To investigate the kinetics of DmpA<sub>syn</sub> expression, the course of the reaction was followed at different times after induction. The course of enzyme expression [hydrolytic activity on pNA per g<sub>CDW</sub> (U g<sub>CDW</sub><sup>-1</sup>)] over time increased in correlation to the induction times of 1 h (12 U g<sub>CDW</sub><sup>-1</sup>), 2 h (23 U g<sub>CDW</sub><sup>-1</sup>), 3 h (36 U g<sub>CDW</sub><sup>-1</sup>), 4 h (73 U g<sub>CDW</sub><sup>-1</sup>) and 6 h (214 U g<sub>CDW</sub><sup>-1</sup>). All following reactions on L-carnosine for-
mation were conducted using the same hydrolytic activity of 0.05 U ml\(^{-1}\). Thus, different amounts of cells, corresponding to their specific hydrolytic activities, had to be applied. As illustrated in Fig. 4, the maximum L-carnosine yield was not affected by the amount of cells used, whereas the activity for L-carnosine synthesis was significantly affected, although volumetric hydrolytic activity of 0.05 U ml\(^{-1}\) was set to be constant in all cases. Periplasmic localization of DmpA increased after 3 h (data not shown) and therefore differences in substrate availability might explain the increased activity after 4 and 6 h of induction. Although an induction time of 6 h resulted in the highest specific activity per g CDW, due to time restrictions we used an induction time of 4 h for all subsequent experiments. The volumetric activity for carnosine synthesis was not affected at a hydrolytic activity of 0.05 U ml\(^{-1}\).

**Impact of substrate concentrations**

Protease-catalyzed peptide synthesis under kinetic control is strongly dependent on the reaction parameters of the educts and the parallel hydrolytic activity of the enzyme (Jakubke et al., 1985). Free enzyme studies of DmpA for L-carnosine formation indicated a much higher \(K_M\) value of approximately 51 mM for L-histidine compared with a \(K_M\) value of approximately 1.5 mM for H-β-Ala-NH\(_2\) (J. Lutz, K. Lang, T. Heck, B. Geueke, L. M. Blank and A. Schmid, in preparation). Therefore, an excess of L-histidine and variation of the ratio of H-β-Ala-NH\(_2\) to L-histidine might affect the L-carnosine yield and thus, the impact of substrate concentration on the synthesis performance was studied.

As illustrated in Fig. 5, the formation of L-carnosine was indeed strongly affected by the ratios and concentrations...
of the educts. The maximum yield (71 ± 3% L-carnosine, 89% total peptidic products) was obtained by using an H-β-Ala-NH₂ concentration of 5 mM and an 80-fold excess of L-histidine (400 mM). Decreased concentrations of L-histidine were associated with lower L-carnosine yields of 50 ± 2% (76% total peptidic products) for a 50-fold excess of L-histidine (5 mM H-β-Ala-NH₂ and 250 mM L-histidine) and 21 ± 2% (48% total peptidic products) for a fivefold excess of L-histidine (10 mM H-β-Ala-NH₂ and 50 mM L-histidine) respectively. Increased concentrations of the acyl donor (10 mM H-β-Ala-NH₂ and 50 mM H-β-Ala-NH₃) at high L-histidine concentrations (250 mM) did not enhance L-carnosine yields (data not shown), but rather a decrease in L-carnosine yields of 42% and 20%, respectively, was observed. These results indicate that the yield for L-carnosine is independent of the substrate concentrations in the range used in this study, hence the ratio of the educts determines the maximum yield.

In summary, a high ratio of acyl acceptor (L-histidine) to acyl donor (H-β-Ala-NH₃) promoted aminolysis, whereas a low ratio promoted hydrolysis. These results are consistent with previous studies for papain-catalyzed peptide synthesis (Stehle et al., 1990) and underline the theoretical aspects in kinetic protease-catalyzed peptide formation (Jakubke et al., 1985).

By increasing the ratio of nucleophile over acyl donor we were able to direct the reaction to the preferred aminolysis resulting in high yields of L-carnosine of 71 ± 3%. Moreover, hydrolysis could significantly be reduced during synthesis of L-carnosine, underlined by the fact that 89% of H-β-Ala-NH₂ could be detected in the total amount of formed peptidic products. Significant hydrolytic activity was only observed after depletion of H-β-Ala-NH₂.

Biocatalyst stability

Optimization of β-aminopeptidase-catalyzed L-carnosine synthesis has led to an improved whole-cell process using recombinant E. coli, obtaining high yields of 71 ± 3% L-carnosine. However, for the applicability of a biocatalytic process it is indispensable to prove stability of the biocatalyst (Schmid et al., 2001). Thus, the biocatalyst’s stability was examined under the harsh reaction conditions.

First, long-time stability of the cells in the reaction buffer was tested. Therefore, cells were harvested after expression and re-suspended after a washing step in 100 mM Na₂CO₃ buffer pH 10 without substrate. The suspension was incubated for 8 h at 30°C. β-Peptide hydrolysing activity on H-β-Ala-pNA of cell-free supernatant and of the cell suspension was tested in a spectrophotometric assay every hour. As illustrated in Fig. 6 the hydrolysis of H-β-Ala-pNA in the supernatant was rather constant at very low activities, less than 0.4% of the total activity. Although cell lysis could occur at these harsh conditions, our results indicate enzymatically active cells for more than 8 h. Moreover, due to constant activities, we suspect that the activity in the supernatant was caused by mechanical stress during sample preparation.

Rise in total activity was observed after more than 5 h of incubation. Since constant activity of the supernatant can be related to intact cell scaffolds, cell lysis could be excluded. Comparison of activities from whole cells and crude extract in our previous results (Table 1) indicated reduced activity due to the membrane barrier; we suppose alterations in the membrane over time. van der Werf and colleagues (1995) has shown that increase in enzymatic activity of whole cells was obtained over time due to permeabilization by Triton X-100. Alkaline pH values have been reported to show an impact on the membrane structure (Padan et al., 2005); our results might be evidence for increased membrane permeabilization over time. However, incubation of biocatalysts for up to 8 h did not cause enzyme loss and thus is not interfering with biocatalytic cell performance for L-carnosine synthesis.

In the next step, stability of the biocatalyst was tested in sequential batch experiments. Synthesis was conducted in 50 ml reaction volumes operated in a sequence of five repeated batches. After every batch, cells were harvested by centrifugation and re-suspended in the same volume of fresh reaction buffer. The time-courses of the five repeated batches for L-carnosine synthesis are presented in Fig. 7. Substrate conversion and productivities increased significantly from the third batch onwards. Rather constant activities were found for the first three batches of 2.02, 2.06 and 2.08 mmol l⁻¹ h⁻¹, respectively.
whereas a significant increase was observed for the fourth (2.23 mmol l$^{-1}$ h$^{-1}$) and fifth (2.37 mmol l$^{-1}$ h$^{-1}$) batch. The increase of activity strongly underlines the argumentation of membrane permeabilization. Besides, it is apparent that loss of enzyme activity did not occur in our experiments and thus the presented biocatalytic system offers a stable system and promising route for L-carnosine synthesis.

**L-Carnosine synthesis in a fed-batch process**

Although our present study indicates the practical value of a whole-cell system for L-carnosine synthesis, commercial production of L-carnosine by whole cells might be limited due to low L-carnosine concentration and thus cause increased costs in downstream processing. To circumvent these limitations, a fed-batch process was established to overcome low L-carnosine titres.

Fed-batch processes were conducted in bioreactors in small volume (200 ml) at optimized synthesis conditions (5 mM H-$\beta$-Ala-NH$_2$, 400 mM L-histidine, pH 10, 30°C, and 0.05 U ml$^{-1}$). Constant H-$\beta$-Ala-NH$_2$ concentrations were achieved by applying a constant H-$\beta$-Ala-NH$_2$ feed of 4.44 mmol l$^{-1}$ h$^{-1}$ calculated from the H-$\beta$-Ala-NH$_2$ conversion rate of previous experiments. A L-histidine feed was not introduced due to solubility limitations above 400 mM.

By using this experimental set-up with a constant feed over 4.5 h, an increase of the L-carnosine titre (Fig. 8) was possible. The final concentration after complete H-$\beta$-Ala-NH$_2$ depletion was 3.7 g l$^{-1}$, which corresponds to a yield of 65% on H-$\beta$-Ala-NH$_2$ (totally 25 mM added). Following the course of L-carnosine formation shows a constant reaction rate of L-carnosine for the first 2.5 h, while a steady decrease in the rate is observed afterwards. We assume increased hydrolytic activities on L-carnosine at higher concentrations, which could be underlined by the fact that the final yield of 65% was lower when compared with batch experiments (71%). Also the consumption of the L-histidine concentration over time and thus the decrease in the ratio of L-histidine to H-$\beta$-Ala-NH$_2$ can influence L-carnosine formation. The calculated rate of L-carnosine formation of 3.3 mmol l$^{-1}$ h$^{-1}$ during feeding was slightly higher than during batch experiments (3.1 mmol l$^{-1}$ h$^{-1}$), suggesting that the H-$\beta$-Ala-NH$_2$ concentration dropped below the initial concentration of 5 mM. However, substrate limitation could be excluded due to additional L-carnosine formation after 4.5 h, at which time no additional H-$\beta$-Ala-NH$_2$ was added. Although a decreased yield was observed, the overall process must also be evaluated in the context of downstream processing and thus the here presented fed-batch process with an increased product titre might be suitable for L-carnosine production.

**Conclusions**

We were able to show that harvested cells of *E. coli* DmpA$_{\text{wp}}$ could be used directly as whole-cell biocatalysts for the synthesis of L-carnosine, thereby avoiding time-consuming and material-intensive protein purification. The successful use of this new robust whole-cell biocatalyst in the developed reaction system allows for reduced upstream and downstream processing and thus decreases environmental as well as economic burdens.
Experimental procedures
Plasmids and culture conditions

Recombinant E. coli strains BL21(DE3) and BL21(DE3) pLysS and P. pastoris SMD1168H (Table 2) were used for whole-cell biocatalysis. The recombinant E. coli BL21(DE3) pLysS strains carrying pODmpA (Heck et al., 2007) and p3BapA (Geueke et al., 2006) were used for cytoplasmic overexpression, whereas the E. coli BL21(DE3) strain carrying pODmpA and pG3BapA was used for cytoplasmic/periplasmic overexpression, as transport of the protein to the periplasm was directed by the plasmid-integrated pelB leader sequence. The recombinant P. pastoris SMD1168H strains carrying pGODmpA and pG3BapA were used for constitutive intracellular overexpression. Plasmids pODmpA, pGODmpA and p3BapA encoded the native sequences of the L-aminopeptidase-d-aminidase (DmpA) from strain O. anthrophi and the β-peptidyl aminopeptidase (3-2W4 BapA) from strain S. xenopeptidilytica. Plasmids pODmpA and pG3BapA encoded optimized sequences for DmpA and 3-2W4 BapA. Sequences were optimized mainly for codon usage and GC composition in respect to the corresponding host. Adaptation of dmpA for E. coli resulted in exchange of 210 base pairs (18.6%) and reduced the average GC composition from 60% to 58%; for adaptation of 3-2W4 bapA for P. pastoris 302 base pairs (26.8%) were exchanged which reduced the average GC composition from 67% to 48% respectively. The plasmids pODmpA and p3BapA have been generated in previous studies (Geueke et al., 2006; Heck et al., 2007). Cloning for expression of DmpA in P. pastoris was realized by integration of PCR-amplified dmpA from the existing plasmid pODmpA with primers containing the restriction sites EcoRI and XhoI. The PCR products were restricted with EcoRI and XhoI and cloned into the expression vector pGAPZ A (Invitrogen Corp., Carlsbad, USA). The codon-optimized DNA sequences dmpA and bapA were generated and cloned by GenScript Corp. (Piscataway, USA). dmpA was cloned into the expression vector pET26b(+) (Novagen, Darmstadt, Germany) by using restriction sites MscI and XhoI, whereas restriction sites EcoRI and XhoI were used for cloning bapA into pGAPZ A (Invitrogen Corp.).

All experiments using whole-cell biocatalysts were conducted at 30°C in complex media. LB medium according to Luria and Bertani was used for bacterial cultivations and YPD medium was used for yeast cultivations containing the essential selectable markers. Recombinant strains for whole-cell biocatalysis were grown for 5 h on complex medium, after inoculation from an overnight culture. Expression in E. coli strains was initiated for 4 h by adding 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG), whereas a constitutive promoter was used for yeast overexpression.

High cell density fed-batch fermentations under glucose limitation to prevent by-product formation for determination of expression performance were conducted using minimal medium according to Geueke and colleagues (2006) for E. coli and minimal medium according to Hellwig and colleagues (1999) for P. pastoris. An exponential feed of μ = 0.1 was applied using 70% (w/v) glucose solution.

Whole-cell biocatalysis

Prior to every biocatalytic experiment, recombinant hosts were harvested and re-suspended in 100 mM Na2CO3 buffer (pH 10). In the next step the specific activity was determined by an enzyme assay to ensure defined enzyme amounts during biocatalysis. The corresponding amount of cells were harvested again and re-suspended in 1 ml of buffer. The biocatalysis was performed in 100 mM Na2CO3 buffer (pH 10) containing the two substrates H-β-Ala-NH2 and L-histidine and started by addition of the cells. Biocatalysis was performed either in 14 ml reaction tubes and shaken at 200 r.p.m. during the process or in a bioreactor (RALF Plus 0.5 l, Bioengineering, Wald, Switzerland) at a low stirrer speed (300 r.p.m.). In a fed-batch process a constant sub-
strate feed, corresponding to the substrate consumption, was introduced to realize a steady reaction environment. The reaction was stopped by addition of 2.5% (v/v) trifluoroacetic acid (TFA) to the reaction supernatant after centrifugation.

**Enzyme assay and kinetic measurement**

The activities of DmpA and 3-2W4 BapA were assayed by following the hydrolysis of H-β-Ala-pNA in a spectrophotometer (Infinite M200, Tecan GmbH, Groedig, Austria) at 405 nm for 30 min. The standard reaction mixture contained 5 mM H-β-Ala-pNA, 50 mM Tris (pH 10), 10% (v/v) DMSO, and whole-cell biocatalysts in limiting amounts. One unit is defined as the amount of enzyme that catalyses the formation of 1 μmol pNA per minute (ε = 10 400 M⁻¹ cm⁻¹).

**HPLC analysis**

Quantitative l-carnosine analysis was performed on a reversed-phase HPLC system (Merck Hitachi, Darmstadt, Germany) recording the 210 nm UV signal. Before HPLC, 2.5% (v/v) TFA was added to 400 μl of each sample to stop the enzymatic reaction and to add the ion-pairing reagent. Afterwards the samples were centrifuged for 5 min at 4°C and then 200 μl of the supernatant was diluted fourfold in 0.1% (v/v) TFA water. An isocratic system of 0.1% (v/v) of TFA in de-ionized and filtered water was used as eluent (solvent A). Typically 10 μl of sample was injected on a Nucleodur 100-5 C18 column (250 by 4 mm; Macherey Nagel, Düren, Germany). The compounds were separated by using an isocratic flow of 100% solvent A for 8 min. For the different experiments, the samples were diluted with 0.1% (v/v) TFA water to the concentrations specified.

H-β-Ala-pNA was purchased from Bachem (Weil am Rhein, Germany), l-carnosine from Novabiochem/Merck (Darmstadt, Germany), H-β-Ala-NH₂ and l-histidine from WVR International GmbH (Darmstadt, Germany) and the peptidic by-products H-β-Ala-OH, H-β-Ala-β-Ala-NH₂, H-β-Ala-β-Ala-β-Ala-NH₂, H-β-Ala-β-Ala-β-Ala-His-OH were a gift of EMC microcollections GmbH (Tuebingen, Germany).

According to the nomenclature of Seebach and colleagues (2004) for β-amino acids and β-peptides, H-β-Ala-OH is formally named H-β-hGly-OH. Since H-β-Ala-OH is a long-known chemical with an established name, we decided to use the less formal version of this amino acid and its derivatives.

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