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

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Summary

β-Peptides and their derivates 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 (Stehle *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 (Thorbek, 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.*,

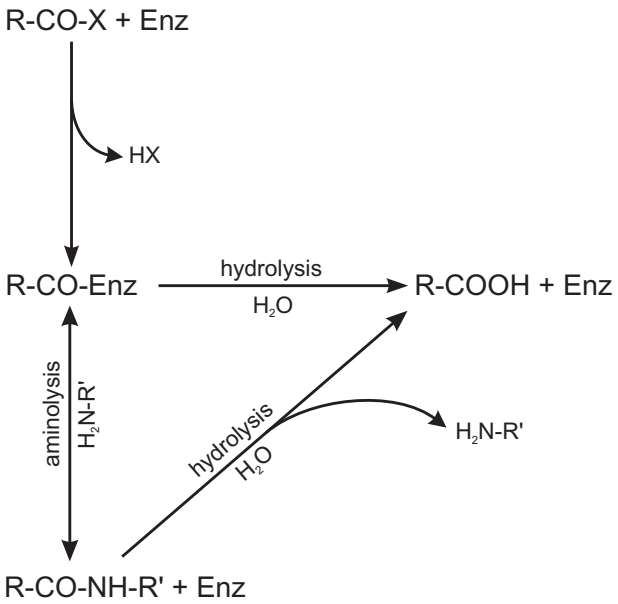

Fig. 1. Schematic reaction in kinetically controlled peptide synthesis. An acylated enzyme intermediate (R-CO-Enz) reacts either with water (hydrolysis) or with another amino acid (aminolysis). Adapted from Bordusa (2002).

2005). The decision whether a free enzyme or a whole-cell biocatalyst is used depends on a large variety of parameters including enzyme stability and cost (Woodley, 2006). The use of whole cells in a biotransformation can be relatively simple, as the enzyme of interest is naturally encapsulated, but can often be limited due to reduced enzyme activities compared with the free enzyme or unspecific side reactions. Thus, the biocatalytic reaction step has to be thoroughly evaluated in order to develop a high-performing and ultimately economically attractive solution, which can be the basis for the development of an industrial process (Schmid *et al*., 2001).

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-NH_2). These conditions enabled the accumulation of high L-carnosine titres in a fed-batch process under constant concentrations of H-β-Ala-NH_2. 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 xenopeptidilytica*, 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 $g_{totalProtein}^{-1}$ for 3-2W4 BapA and 215 U $g_{totalProtein}^{-1}$ for DmpA in *E. coli*, respectively, 42 U $g_{totalProtein}^{-1}$ 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 $g_{totalProtein}^{-1}$, compared with native 3-2W4 BapA expression in *P. pastoris* (data not shown). Adaptation of the *dmpA* codon usage ($DmpA_{syn}$)

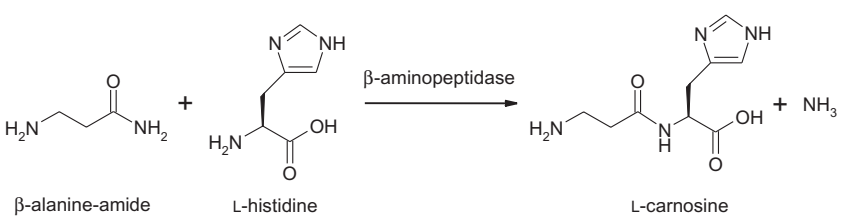

Fig. 2. Scheme of enzymatic biosynthesis of L-carnosine.

Table 1. Peptidase activities of recombinant hosts.

Strain name	Hydrolytic activity[a] of whole cells (U g_{CDW}^{-1})	Hydrolytic activity[a] of crude extract (U g_{CDW}^{-1})	Ratio whole cells/crude extract (%)	Activity of L-carnosine synthesis of whole cells (U g_{CDW}^{-1})	Yield[b] L-carnosine/ H-β-Ala-NH_2 (%)
E. coli BapA	1 ± 0	3	36	0.2 ± 0.1	18 ± 2
P. pastoris $BapA_{syn}$	3 ± 1	19	17	0.1 ± 0.1	2 ± 1
E. coli DmpA	42 ± 4	110	38	12 ± 2	19 ± 2
E. coli $DmpA_{syn}$	71 ± 5	171	42	29 ± 2	23 ± 2
P. pastoris DmpA	4 ± 1	22	19	0.5 ± 0.2	12 ± 2

a. Hydrolytic activity is specified as the hydrolysis of H-β-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.

to *E. coli* resulted in a further increase in specific hydrolytic activity to 285 U $g_{totalProtein}^{-1}$.

Biotransformation performance of the whole-cell biocatalysts

The potential of the β-aminopeptidases DmpA and 3-2W4 BapA for β-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 β,α-dipeptide L-carnosine. As shown in Table 1, the five strains were tested for hydrolytic activities on H-β-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* ($DmpA_{syn}$) with 71 ± 5 U g_{CDW}^{-1} and for 3-2W4 BapA from *P. pastoris* ($BapA_{syn}$) with 3.3 ± 0.9 U g_{CDW}^{-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-β-Ala-NH_2 and L-histidine to L-carnosine was examined. To investigate the peptidase-catalyzed synthesis of L-carnosine, 10 mM H-β-Ala-NH_2 in 100 mM Na_2CO_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^{-1} (hydrolytic activity on H-β-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-β-Ala-NH_2 of 23 ± 2% was found to be for *E. coli* $DmpA_{syn}$. Slightly lower yields were obtained from recombinant *E. coli* DmpA (19 ± 2%) and 3-2W4 BapA (18 ± 2%), whereas a significantly lower L-carnosine formation was observed for *P. pastoris* DmpA (12 ± 2%) and $BapA_{syn}$ (2 ± 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* $DmpA_{syn}$, 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* $DmpA_{syn}$ 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; Bordusa, 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

100 mM Na_2CO_3 buffer at 25°C supplemented with 10 mM H-β-Ala-NH_2 and 50 mM L-histidine using *E. coli* $DmpA_{syn}$ 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^{-1}, 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^{-1} (4.52 mmol l^{-1} h^{-1}), whereas the lowest rate was determined for 0.05 U ml^{-1} (1.11 mmol l^{-1} h^{-1}), 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^{-1}. More specifically, using 0.2 U ml^{-1} 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-NH_2 was also observed under all conditions. The highest yield of total peptide synthesis (L-carnosine, H-β-Ala-β-Ala-NH_2, H-β-Ala-β-Ala-β-Ala-NH_2, H-β-Ala-β-Ala-OH and H-β-Ala-β-Ala-His-OH) was 49% of the consumed H-β-Ala-NH_2 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.

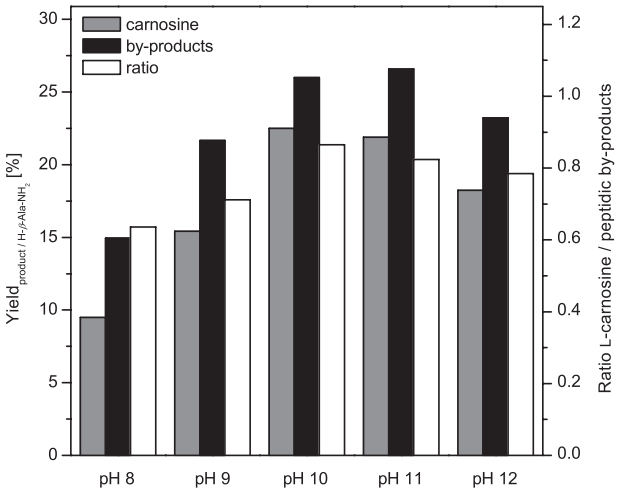

Fig. 3. Impact of pH on L-carnosine synthesis. Yields of product L-carnosine (grey) and of peptidic by-products (black) are given in per cent of H-β-Ala-NH_2 as substrate. Maximal yields were obtained after 90 min of incubation for pH 10 and 11, 105 min for pH 9 and 120 min for pH 8 and 12. The ratio of L-carnosine to the peptidic by-products (white) illustrates the synthesis performance in dependence to the pH. Summations of yields are not equal to 100% due to simultaneous hydrolysis of substrate and products to H-β-Ala-OH. Reaction conditions: 100 mM Na_2CO_3 buffer, 10 mM H-β-Ala-NH_2, 50 mM L-histidine, 25°C, 0.05 U ml^{-1}. Protein expression was induced for 4 h.

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^{-1} h^{-1} (25°C), 1.9 mmol l^{-1} h^{-1} (30°C) and 3.1 mmol l^{-1} h^{-1} (37°C) followed the increase of temperature. The average yield of 20.5 $\pm$ 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 $DmpA_{syn}$ expression, the course of the reaction was followed at different times after induction. The course of enzyme expression [hydrolytic activity on pNA per g_{CDW} (U g_{CDW}^{-1})] over time increased in correlation to the induction times of 1 h (12 U g_{CDW}^{-1}), 2 h (23 U g_{CDW}^{-1}), 3 h (36 U g_{CDW}^{-1}), 4 h (73 U g_{CDW}^{-1}) and 6 h (214 U g_{CDW}^{-1}). All following reactions on L-carnosine for-

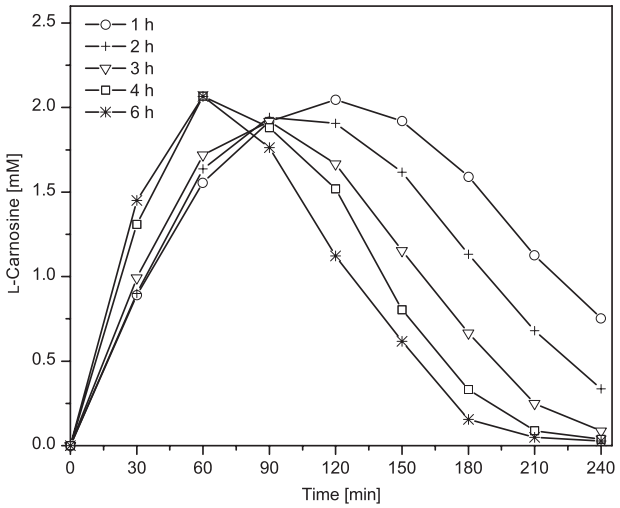

Fig. 4. Impact of specific cell activity by variation of induction times on L-carnosine synthesis. Reaction conditions: 100 mM Na_2CO_3 buffer, 10 mM H-β-Ala-NH_2, 50 mM L-histidine, 30°C, pH 10, 0.05 U ml^{-1}.

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

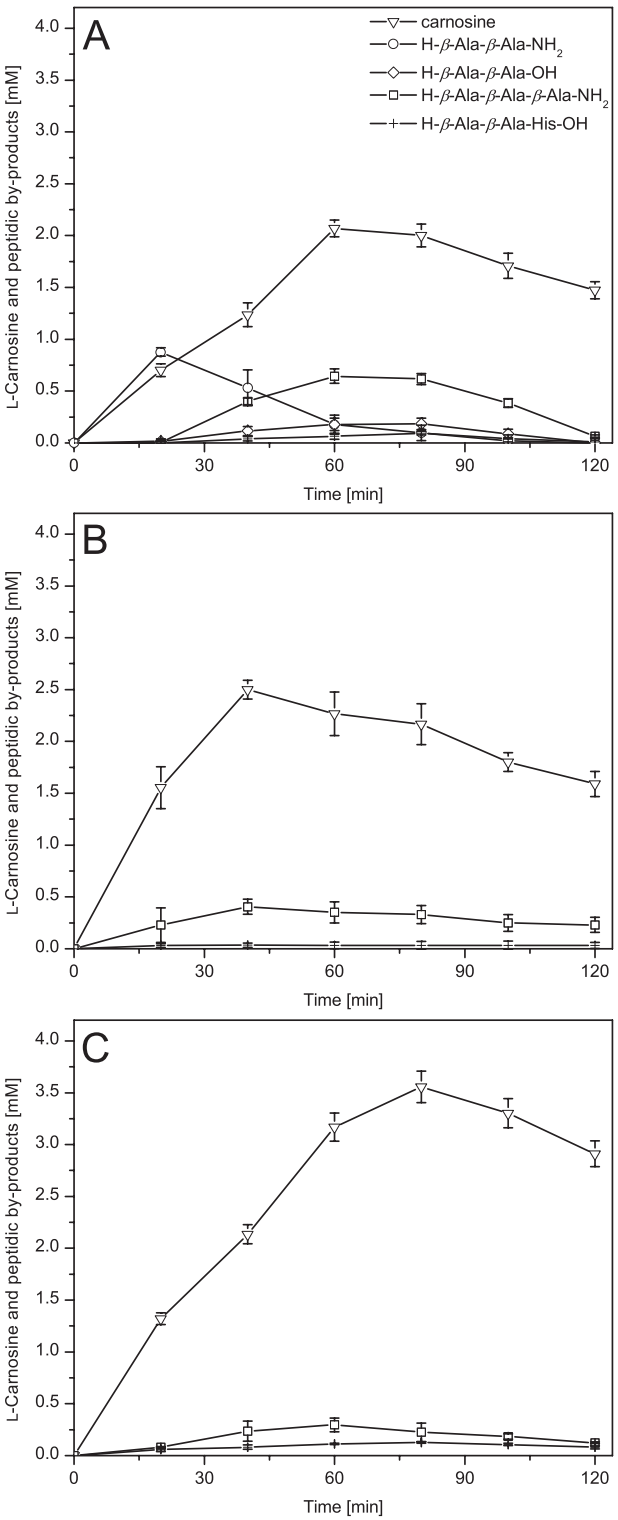

Fig. 5. Impact of substrate concentrations. L-Carnosine synthesis using (A) 10 mM H-β-Ala-NH_2 and 50 mM L-histidine, (B) 5 mM H-β-Ala-NH_2 and 250 mM L-histidine, and (C) 5 mM H-β-Ala-NH_2 and 400 mM L-histidine. Standard errors of three independent experiments were determined using Origin Plot and the Gaussian law of error propagation. Reaction conditions: 100 mM Na_2CO_3 buffer, 30°C, pH 10, 0.05 U ml^{-1}. Protein expression was induced for 4 h.

of the educts. The maximum yield (71 ± 3% L-carnosine, 89% total peptidic products) was obtained by using an H-β-Ala-NH_2 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-NH_2 and 250 mM L-histidine) and 21 ± 2% (48% total peptidic products) for a fivefold excess of L-histidine (10 mM H-β-Ala-NH_2 and 50 mM L-histidine) respectively. Increased concentrations of the acyl donor (10 mM H-β-Ala-NH_2 and 50 mM H-β-Ala-NH_2) 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-NH_2) 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-NH_2 could be detected in the total amount of formed peptidic products. Significant hydrolytic activity was only observed after depletion of H-β-Ala-NH_2.

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 Na_2CO_3 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

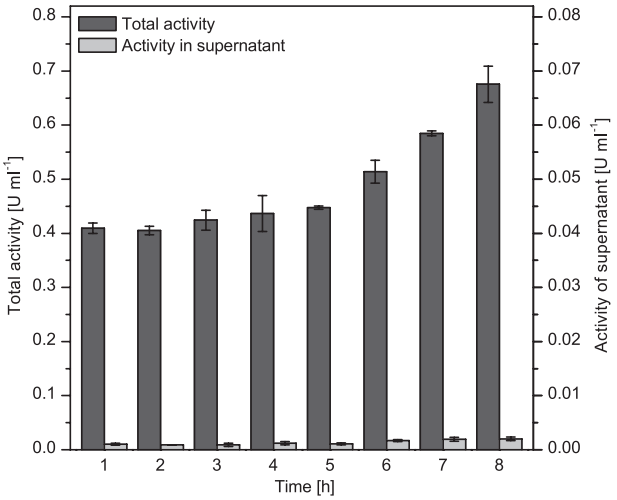

Fig. 6. Stability of whole-cell biocatalysts. Cells were incubated in 100 mM Na_2CO_3 buffer at pH 10 for 8 h. Every hour the cell suspension (dark grey) and supernatant (light grey) activity was measured. Reaction conditions: 100 mM Na_2CO_3 buffer, 30°C, pH 10, 0.4 U ml^{-1}.

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,

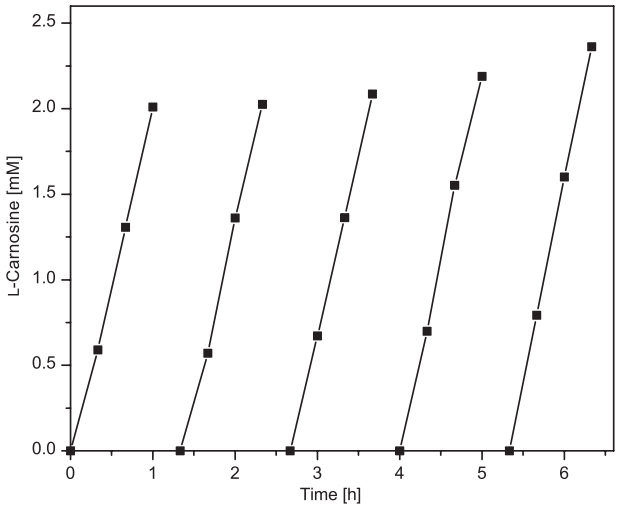

Fig. 7. Whole-cell biocatalyst performance in sequential batches for L-carnosine synthesis. Repetitions of L-carnosine synthesis for five times using harvested cells from the previous batch. Reaction conditions: 100 mM Na_2CO_3 buffer, 10 mM H-β-Ala-NH_2, 50 mM L-histidine, 30°C, pH 10, 0.05 U ml^{-1}. Protein expression was induced for 4 h.

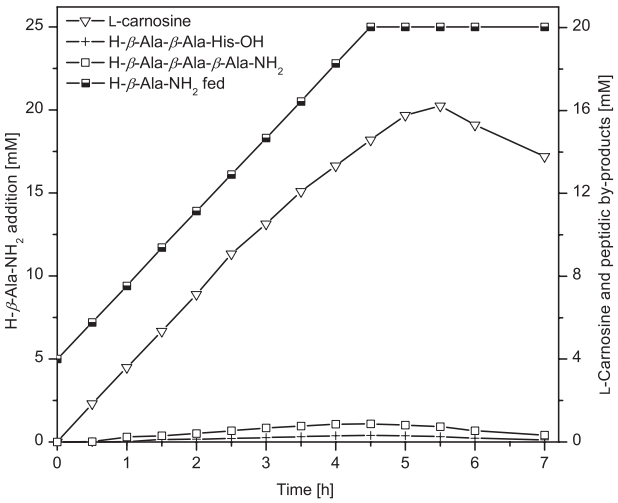

Fig. 8. Fed-batch process for L-carnosine production. Initial reaction conditions: 100 mM Na_2CO_3 buffer, 5 mM H-β-Ala-NH_2 (constant for 4.5 h), 400 mM L-histidine, 30°C, pH 10, 0.05 U ml^{-1}. Protein expression was induced for 4 h.

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-β-Ala-NH_2, 400 mM L-histidine, pH 10, 30°C, and 0.05 U ml^{-1}). Constant H-β-Ala-NH_2 concentrations were achieved by applying a constant H-β-Ala-NH_2 feed of 4.44 mmol l^{-1} h^{-1} calculated from the H-β-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-β-Ala-NH_2 depletion was 3.7 g l^{-1}, which corresponds to a yield of 65% on H-β-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-β-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-β-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-β-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* $DmpA_{syn}$ 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

compared with the synthesis procedure using the free enzyme. Further optimization, such as substrate (L-histidine) recovery, coinstantaneous product precipitation, and feed regulation by integrated pH control, or strain development by directed membrane permeabilization for accelerated biocatalytic activity (Ni and Chen, 2004), are possible options for further reaction optimization. The results presented in this work clearly show the high potency of this new enzymatic activity for the synthesis and production of β-peptides. Due to the wide substrate spectrum of 3-2W4 BapA (Heck *et al*., 2007), the extension of our reaction set-up using this enzyme may offer a platform for the biocatalytic production of pharmaceutically interesting α,β-, β-peptides and derivates.

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$_{syn}$ 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$_{syn}$ were used for constitutive intracellular overexpression. Plasmids pODmpA, pGODmpA and p3BapA encoded the native sequences of the L-aminopeptidase-D-amidase (DmpA) from strain *O. anthropi* and the β-peptidyl aminopeptidase (3-2W4 BapA) from strain *S. xenopeptidilytica*. Plasmids pODmpA$_{syn}$ and pG3BapA$_{syn}$ 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. Adaption 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*$_{syn}$ and *bapA*$_{syn}$ were generated and cloned by GenScript Corp. (Piscataway, USA). *dmpA*$_{syn}$ 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*$_{syn}$ 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 $\mu = 0.1$ 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 Na_2CO_3 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 Na_2CO_3 buffer (pH 10) containing the two substrates H-β-Ala-NH_2 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-

Table 2. Recombinant hosts for whole-cell biocatalysis.

Strain name	Gene coding for	Expression plasmid	Gene codon optimized	Host strain	References
E. coli BapA	3-2W4 BapA	p3BapA	No	*E. coli* BL21(DE3) pLysS	Geueke *et al.* (2006)
P. pastoris BapA$_{syn}$	3-2W4 BapA	pG3BapA$_{syn}$	Yes	*P. pastoris* SMD1168H	This study
E. coli DmpA	DmpA	pODmpA	No	*E. coli* BL21(DE3) pLysS	Heck *et al.* (2006)
E. coli DmpA$_{syn}$	DmpA	pODmpA$_{syn}$	Yes	*E. coli* BL21(DE3)	This study
P. pastoris DmpA	DmpA	pGODmpA	No	*P. pastoris* SMD1168H	This study

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 ($\varepsilon = 10\ 400\ M^{-1}\ cm^{-1}$).

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 filtrated water was used as eluent (solvent A). Typically 10 μl of sample was injected on a Nucleodur Pyramid 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-NH_2 and L-histidine from VWR International GmbH (Darmstadt, Germany) and the peptidic by-products H-β-Ala-OH, H-β-Ala-β-Ala-NH_2, H-β-Ala-β-Ala-β-Ala-NH_2, H-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-petides, 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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