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A green-by-design bioprocess for L-carnosine production integrating enzymatic synthesis with membrane separation†

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L-Carnosine (L-Car, β-alanyl-L-histidine) is a bioactive dipeptide with important physiological functions. Direct coupling of unprotected β-Ala (β-alanine) with L-His (L-histidine) mediated by an enzyme is a promising method for L-Car synthesis. In this study, a new recombinant dipeptidase (*Sm*PepD) from *Serratia marcescens* with a high synthetic activity toward L-Car was identified by a genome mining approach and successfully expressed in *Escherichia coli*. Divalent metal ions strongly promoted the synthetic activity of *Sm*PepD, with up to 21.7-fold increase of activity in the presence of 0.1 mM $MnCl_2$. Higher temperature, lower pH and increasing substrate loadings facilitated the L-Car synthesis. Pilot biocatalytic syntheses of L-Car were performed comparatively in batch and continuous modes. In the continuous process, an ultrafiltration membrane reactor with a working volume of 5 L was employed for catalyst retention. The dipeptidase, *Sm*PepD, showed excellent operational stability without a significant decrease in space–time yield after 4 days. The specific yield of L-Car achieved was 105 g_{Car} $g_{catalyst}^{-1}$ by the continuous process and 30.1 g_{Car} $g_{catalyst}^{-1}$ by the batch process. A nanofiltration membrane was used to isolate the desired product L-Car from the reaction mixture by selectively removing the excess substrates, β-Ala and L-His. As a result, the final L-Car content was effectively enriched from 2.3% to above 95%, which gave L-Car in 99% purity after ethanol precipitation with a total yield of 60.2%. The recovered substrate mixture of β-Ala and L-His can be easily reused, which will enable the economically attractive and environmentally benign production of the dipeptide L-Car.

Introduction

L-Carnosine (L-Car) is a β,α-dipeptide composed of β-alanine (β-Ala) and L-histidine (L-His) and is abundantly present in mammalian skeletal muscles and brain tissue. As a natural biologically active compound, L-Car has many important physiological functions *in vivo*, including anti-oxidation, anti-glycosylation, pH buffering and free hydroxyl radical elimination through complexation with transition metal ions such as zinc or copper.[1] Therefore, L-Car is used widely in medicine, cosmetics, food additives and other fields.

Chemical synthesis of L-Car has been extensively reported. In these methods, activation of the carboxyl group of β-Ala is usually required, and the amino group of β-Ala needs to be protected to avoid the formation of unwanted by-products because of the poor specificity of the reaction.[2] A commonly used industrial route of L-Car synthesis is shown in Scheme 1a.[3] In this route, the amino group of β-Ala is protected by using *o*-phthalic anhydride as a protective agent, and after the reaction, the phthaloyl protecting group is deprotected by hydrazine, a highly toxic reagent. Moreover, the tedious reaction steps, severe reaction conditions and high-energy consumption make this process environmentally unfriendly, which should be replaced by other methods.

Enzymatic synthesis of dipeptides has become a popular research topic because of the excellent selectivity and environmental friendliness of enzymes.[4] Lipases and proteases have been widely used to synthesize various short peptides in organic solvents.[5] However, research on enzymatic synthesis of L-Car mediated by a lipase or protease has not been reported. Aminopeptidases are commonly used for the synthesis of dipeptides.[6] Many studies on aminopeptidase-catalysed synthesis of L-Car have been reported, using an activated derivative of β-Ala, such as β-alaninamide (Scheme 1b)[7] or β-alanine methyl ester (Scheme 1c)[8] as the acyl donor and unprotected L-His as the

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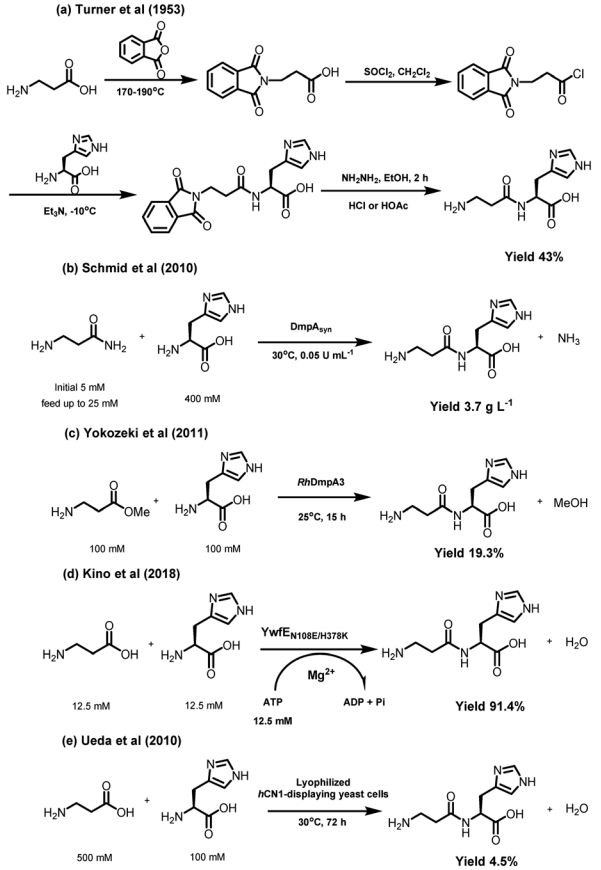

Scheme 1 Typical L-Car synthesis by chemical or enzymatic methods.

acyl receptor. The aminopeptidase catalysed synthesis of L-Car is a kinetically controlled reaction with various by-products such as H-β-Ala-β-Ala-His-OH and poly-β-Ala-OMe(NH_2) formed during the process, and enzymatic hydrolysis of L-Car occurs with extended reaction times. The reported highest titre of L-Car was only 19.3 mM in these processes.[8] Moreover, the relatively expensive L-His was used in excessive loadings, making this process uneconomical.

In vivo, L-Car is synthesized from β-Ala and L-His by a specific synthetase (EC 6.3.2.11) *via* an ATP-dependent pathway, but the sequence of L-Car synthase from mammals has not been reported.[9] In 2018, Kino *et al.* obtained an L-amino acid ligase YwfE from *Bacillus subtilis*, which catalysed the synthesis of L-Car by coupling β-Ala with L-His in an ATP-dependent manner, as observed for L-Car synthetase (Scheme 1d).[10] The product titre reached 11.4 mM at a substrate loading of 12.5 mM when using the best mutant N108E/H378K. The addition of an equal molar amount of ATP made the process impractical.

Peptidases catalysing L-Car degradation have been reported. Two mammalian-derived dipeptidases that catalyse the hydrolysis of L-Car are identified as tissue-specific carnosinase (CN1, EC 3.4.13.20) and cytoplasmic non-specific carnosinase (CN2, EC 3.4.13.18).[11] CN1 has a narrow substrate specificity for aminoacyl histidine (Xaa-His), whereas CN2 is a broad-spectrum dipeptidase. The carnosinase catalyses reversible hydrolysis of L-Car to generate β-Ala and L-His. In 2010, Ueda *et al.* synthesized L-Car from nonprotected β-Ala and L-His by employing the yeast cell surface-displayed carnosinase CN1 as a catalyst in an organic-aqueous biphasic system (Scheme 1e),[12] which provided a new enzymatic approach to synthesize L-Car. However, in this report, the activity of the carnosinase CN1 expressed on the surface of yeast cells was rather low. The loading of lyophilized cells was as high as 80 g L^{-1}, while the titre of L-Car produced was only 4.5 mM after a 72 h reaction in a system composed of 500 mM β-Ala and 100 mM L-His in aqueous buffer and a triple volume of isooctane. The specific yield was only 12.7 mg_{Car} g_{cell}^{-1}. The low activity of the catalyst severely limits the development of this process.

In addition, several L-Car hydrolases derived from prokaryotes have also been reported, including peptidase V (PepV) derived from *Lactobacillus delbrueckii*,[13] β-alanyl dipeptidase (BapA) derived from *Pseudomonas* sp.,[14] aminoacyl-histidine dipeptidase (*Va*PepD) derived from *Vibrio alginolyticus*[15] and peptidase D (*Ec*PepD) derived from *Escherichia coli* (*E. coli*).[16] Among them, PepV and BapA specifically catalyse the hydrolysis of β-alanyl dipeptides (β-Ala-Xaa), whereas PepDs specifically hydrolyze aminoacyl Xaa-His. In this paper, we identified a highly active dipeptidase (*Sm*PepD) from *Serratia marcescens* by a genome mining approach using the reported sequences of prokaryotic L-Car hydrolases as leading templates. The resultant dipeptidase *Sm*PepD chosen among 20 candidates was characterized biochemically and synthetic reaction conditions were optimized systematically. Under the optimized reaction conditions, *Sm*PepD could efficiently catalyse the coupling of unprotected β-Ala with L-His, forming L-Car in one step with very high productivity. An ultra-filtration membrane bioreactor (UF-MBR) was applied to realize the continuous synthesis of L-Car. By combining with nanofiltration technology to isolate L-Car produced from the excess substrates β-Ala and L-His, the final product L-Car was successfully harvested in kilogram scale with a purity up to 99%.

Results and discussion

Identification and characterization of new dipeptidases for L-Car synthesis

Genes of several reported specific L-Car dipeptidases, including cytoplasmic non-specific carnosinase from *Homo sapiens* (*h*CN2), PepV from *Lactobacillus delbrueckii* (*Ld*PepV), dipeptidase PepD from *Vibrio alginolyticus* (*Va*PepD) and *E. coli* (*Ec*PepD), were cloned and solubly expressed in *E. coli*. Human carnosinase *h*CN1 was also cloned, but not successfully expressed in a soluble form in either *E. coli* or *Pichia pastoris*. The recombinant dipeptidases were purified and the activities of hydrolysis and synthesis of L-Car were measured. As shown in Table 1, three dipeptidases catalysed not only the hydrolysis of L-Car to β-Ala and L-His, but also the reverse reaction, *i.e.*, synthesis of L-Car. Among them, human *h*CN2 showed the highest hydrolytic and synthetic activity, but further screening of new dipeptidases with higher activity failed using *h*CN2 as a

Table 1 Activity of several L-Car dipeptidases

Sources	Enzymes	Hydrolytic act. (μmol min^{-1} mg^{-1})	Synthetic act. (μmol min^{-1} g^{-1})
Homo sapiens	*h*CN2	1417 ± 56	62.4 ± 4.8
Vibrio alginolyticus	*Va*PepD	144 ± 7	15.0 ± 4.9
Escherichia coli	*Ec*PepD	179 ± 20	6.7 ± 1.7
Enterobacter	*En*PepD	240 ± 5	12.2 ± 5.1
Erwinia tasmaniensis	*Et*PepD	224 ± 13	15.7 ± 1.0
Serratia marcescens	***Sm*PepD**	**2245 ± 249**	**107 ± 8**

The substrate solutions were adjusted with a sodium hydroxide (NaOH) aqueous solution to pH 8.0. Conversion was maintained within 10–20%.

leading template. Among the prokaryotic L-Car dipeptidases, *Ec*PepD showed a higher hydrolytic activity, whereas *Va*PepD afforded a higher synthetic activity, indicating that the activities toward L-Car hydrolysis may not be completely consistent with their synthetic activities.

Using the amino acid sequence of the prokaryotic dipeptidases as a guide, new dipeptidases with an obvious activity toward L-Car synthesis were identified from the genome database. Three more candidates (*En*PepD, *Et*PepD and *Sm*PepD) with higher activities are shown in Table 1. Among them, a dipeptidase *Sm*PepD derived from *Serratia marcescens* showed the highest synthetic activity for L-Car synthesis, despite having only 62% identity to the leading enzyme *Va*PepD. The hydrolytic and synthetic activities of *Sm*PepD toward L-Car were 2245 and 107 μmol min^{-1} $g_{protein}^{-1}$, respectively, which are 14.6 and 6.1 times higher when compared with those of *Va*PepD.

The kinetic parameters of recombinant *Sm*PepD and *Va*PepD catalysed coupling of β-Ala and L-His were determined. As shown in Table 2, the k_{cat} and K_m of *Sm*PepD toward the substrate β-Ala are much higher than those of *Va*PepD, while the catalytic efficiency constant (k_{cat}/K_m) of *Sm*PepD (3.05 min^{-1} M^{-1}) toward β-Ala is slightly lower than that of *Va*PepD (3.98 min^{-1} M^{-1}). In contrast, the k_{cat} of *Sm*PepD toward L-His is 8.1 times that of *Va*PepD, while the K_m of *Sm*PepD toward L-His is only 1.4 times that of *Va*PepD. As a result, the catalytic efficiency constant (k_{cat}/K_m) of *Sm*PepD (442 min^{-1} M^{-1}) toward L-His is 6.4 fold higher than that of *Va*PepD (69.4 min^{-1} M^{-1}). Thus, *Sm*PepD showed better performance in the synthetic reaction when compared with *Va*PepD because of the higher catalytic efficiency of *Sm*PepD toward L-His.

The reported dipeptidase *Va*PepD belongs to the M20 metal peptidase subfamily (M20C), with five metal-binding sites and two substrate-binding sites, as well as two zinc ions bound in the active center.[17] Some metal ions, especially transition divalent metal ions, such as Co^{2+}, Ni^{2+} and Mn^{2+}, have a strong stimulation effect on the hydrolytic activity of some aminoacyl-histidine dipeptidases.[15,18] These metal ions are predicted to also promote L-Car synthesis catalysed by *Sm*PepD. Thus, the effect of metal ions and EDTA as a metal chelating agent on the L-Car synthesis activity of *Sm*PepD was examined. As shown in Table 3, transition divalent metal ions and alkaline earth metal ions, such as Mn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+} and Mg^{2+}, stimulated the synthesis activity of *Sm*PepD, and the enzyme activity was highest in the presence of Mn^{2+}. The reaction rate increased by one order of magnitude (10-fold) with the addition of only 0.01 mM $MnCl_2$ into the reaction mixture (Table S4†). When added with 0.1 mM $MnCl_2$, *Sm*PepD showed the highest activity with a 22.7-fold increase in activity when compared with the reaction without the addition of metal ions. Increasing the $MnCl_2$ concentration further caused a decrease in the *Sm*PepD activity. Addition of Zn^{2+} did not promote the synthesis activity, and no inhibition was observed either. The presence of 0.1 mM Cu^{2+} caused a complete loss of *Sm*PepD activity. Addition of 1.0 mM EDTA also resulted in no enzyme activity. In our work, although only synthetic activity was examined, the activation effect of transition metal ions was consistent with previous reports.[15,18] Furthermore, *Sm*pepD was proved to be a metal-dependent dipeptidase, because the enzyme activity of *Sm*PepD was completely lost by adding 1.0 mM EDTA, a metal chelating agent. Menard *et al.* speculated that the role of metal ions might be the stabilization of the bridging water molecule, resulting in formation of hydroxide ions.[19] However, the varied binding energy of metal ions with different ligands (side chains) of *Sm*PepD may result in different catalytic performances. The rearrangement of

Table 2 Kinetic parameters of *Va*PepD and *Sm*PepD for L-Car synthesis

	β-Ala[a]			L-His[b]		
Enzyme	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [min^{-1} M^{-1}]	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [min^{-1} M^{-1}]
*Va*PepD	225 ± 57	0.90 ± 0.03	3.98	8.8 ± 1.6	0.60 ± 0.07	69.4
*Sm*PepD	2580 ± 230	6.36 ± 0.77	3.05	12.4 ± 3.4	5.46 ± 0.42	442.4

Synthetic activities were determined in 0.2 mL Tris-HCl (50 mM, pH 8.0) at 30 °C and 1000 rpm. Various substrate concentrations and appropriate purified *Sm*PepD were used. Conversion was controlled within 10–15%. [a] 60 mM L-His with 0–4 M β-Ala. [b] 4.0 M β-Ala with 0–80 mM L-His.

Table 3 Effects of metal ions and EDTA on the activity of dipeptidase *Sm*PepD

Metal ions or additives	Relative activity[a] [%]	
	0.1 mM	1.0 mM
Control	100 ± 2	100 ± 2
Mn^{2+}	2274 ± 2	2127 ± 4
Ni^{2+}	251 ± 2	196 ± 3
Co^{2+}	228 ± 1	463 ± 6
Ca^{2+}	173 ± 4	247 ± 4
Mg^{2+}	149 ± 4	175 ± 1
Li^{+}	126 ± 2	147 ± 2
Zn^{2+}	101 ± 10	100 ± 5
Fe^{3+}	97 ± 7	n.d.[b]
EDTA	25 ± 7	n.d.[b]
Cu^{2+}	n.d.[b]	n.d.[b]

Synthetic activities were determined in 0.2 mL Tris-HCl (50 mM, pH 8.0) containing 2.0 M β-Ala, 100 mM L-His and 0.5 mg mL^{-1} purified *Sm*PepD with or without the metal ions. Reaction conditions: 30 °C and 1000 rpm. [a] The activity measured without any additive was taken as 100%. [b] n.d.: not detected.

ligands in the active site of *Sm*PepD, which is ideal for Mn^{2+} but less optimal for Cu^{2+}, may lead to the weaker binding of Cu^{2+} and the poorer enzymatic activity. Nevertheless, thorough understanding of the differences in binding and synthetic performance due to the binding of various metal ions with active-site residues still requires further studies of crystal structures and all-atom molecular dynamics simulations of the enzyme.

Optimization of enzymatic L-Car synthesis

Dipeptidase catalysed direct coupling of β-Ala and L-His is a reversible reaction. In a reaction system containing Tris-HCl buffer (50 mM, pH 8.0), 2.0 M β-Ala, 100 mM L-His and 300 U L^{-1} *Sm*PepD incubated at 30 °C, the concentration of L-Car at equilibrium was 7.52 mM and K_{eq} was calculated to be 1.98. The use of Tris-HCl salt may be unnecessary because the high concentrations of β-Ala and L-His used, with isoelectric points of 6.9 and 7.6, respectively, afford a suitable buffering effect at the reaction pH (8.0). To test this, a neat aqueous phase system without any buffering salt gave an enzymatic reaction process that was close to the system containing Tris-HCl, with an equilibrium concentration of 7.62 mM L-Car (Table S5†).

Temperature and pH effects on the *Sm*PepD activity were also examined. *Sm*PepD showed the highest activity at 50 °C, which is 6.7 times higher than the activity at 30 °C (Fig. S9†). The enzyme was activated in an alkaline environment with the highest activity observed at pH 8.0 (Fig. S10†). The thermostability of *Sm*PepD was measured. Surprisingly, strong activation was observed when *Sm*PepD was incubated at 30–50 °C. In the initial period of incubation, the residual activity of *Sm*PepD was significantly higher than its initial value, and later the residual activity decreased with the inactivation of *Sm*PepD (Fig. S11†). At 30 °C and 40 °C, *Sm*PepD showed excellent thermal stability with half-lives of 96 h and 84 h, respectively, whereas at 50 °C *Sm*PepD was relatively unstable with a half-life of only 6 h.

Temperature is an important factor that affects the equilibrium of reversible reactions. The K_{eq} constants of the enzymatic L-Car synthesis between 30 and 50 °C were measured. The enzymatic reactions at different temperatures were compared. When 2 M β-Ala, 100 mM L-His and 300 U L^{-1} *Sm*PepD were loaded, the L-Car synthesis reactions at 30 °C and 40 °C reached their equilibria within 48 h, and the equilibrium concentration of L-Car increased from 7.62 mM at 30 °C to 8.48 mM at 40 °C. At 50 °C, however, due to the instability of *Sm*PepD at this temperature, the L-Car synthesis reaction stopped within 6 h with the same enzyme dose, resulting in only 4.21 mM L-Car (Fig. S13†). Supplements of the biocatalyst were necessary to drive the reaction toward equilibrium. The equilibrium concentration of L-Car at 50 °C was estimated to be approximately 8.79 mM. Therefore, increasing the temperature resulted in an increase in the K_{eq} calculated from 2.01 at 30 °C to 2.37 at 50 °C (Table S6†), implying that the synthesis of L-Car is an endothermic reaction. Taking into consideration the K_{eq} of the reaction and the thermostability of *Sm*PepD, 40 °C appears to be the most suitable reaction temperature.

Changing the pH affects not only the ionization state of the amino acid substrates but also the equilibrium of the L-Car synthesis. Therefore, we determined the *Sm*PepD activity and the equilibrium concentration of L-Car at pH values between 7.0 and 9.0 at 40 °C (Table S7†). On the one hand, as the pH increased, the equilibrium concentration of L-Car decreased. On the other hand, *Sm*PepD was more active at pH 8.0 by 3.9 and 1.2 times when compared with the activities observed at pH 7.0 and 9.0, respectively. Considering both the synthetic efficiency of the biocatalyst and the reaction K_{eq}, pH 8.0 was chosen as the most suitable reaction pH.

The solubility of β-Ala measured in water is very high, reaching 579 g L^{-1} (*ca.* 6.5 M) at 40 °C, whereas that of histidine is relatively low with a value of 34.7 g L^{-1} (*ca.* 224 mM). According to Le Chatelier's principle, increasing the concentrations of β-Ala and L-His can promote effectively the reaction equilibrium in the direction of the L-Car synthesis, thereby increasing the titre of L-Car. Obviously, increasing the β-Ala concentration was more advantageous for promoting the L-Car synthesis, as shown in Table 4. When the concentrations of β-Ala and L-His were increased to near saturation (6500 mM and 180 mM, respectively), the equilibrium concentration of L-Car increased up to 62.5 mM (*ca.* 14.1 g L^{-1}), which is 13.9-fold higher than the highest recorded value.[12]

The effect of Mn^{2+} addition on the progress of L-Car synthesis was investigated because Mn^{2+} significantly improves the *Sm*PepD activity. With the addition of 0.1 mM $MnCl_2$, the enzymatic reaction of the L-Car synthesis reached equilibrium in only 2 h under conditions of 2 M β-Ala, 100 mM L-His, 75 U L^{-1} *Sm*PepD (*ca.* 0.5 g L^{-1} lyophilized cell free extract), pH 8.0

Table 4 L-Car production at various substrate concentrations

Molar ratio of β-Ala to L-His	β-Ala [mM]	L-His [mM]	L-Car [mM]
1 : 2	50	100	0.19 ± 0.01
1 : 1	100	100	0.43 ± 0.12
2 : 1	200	100	0.90 ± 0.17
5 : 1	500	100	2.19 ± 0.07
10 : 1	1000	100	4.40 ± 0.02
20 : 1	2000	100	8.48 ± 0.07
10 : 1	2000	200	16.3 ± 0.7
Saturated	6500	180	62.5 ± 0.5

Reactions were performed in a 0.2 mL system containing varied concentrations of substrates using a 1500 U L^{-1} lyophilized cell free extract of *Sm*PepD. Reaction conditions: 40 °C and 1000 rpm for 24 h.

and 40 °C, while under similar conditions without $MnCl_2$, the titre of L-Car after an 8 h reaction was only 0.9 mM (Fig. 1). Compared with the reaction without Mn^{2+}, the *Sm*PepD activity in the practical synthesis increased by as much as 117 times upon the addition of 0.1 mM $MnCl_2$. Furthermore, when the substrate loadings were increased to saturating concentrations, the reaction at 40 °C reached equilibrium in 8 h with 0.1 mM $MnCl_2$ and the same dose of catalyst (0.5 g L^{-1} lyophilized cell free extract) (Fig. S14†).

Kilogram-scale preparation of L-Car

A batch reaction for the L-Car synthesis was carried out in a 15 L stirred glass-tank reactor (working volume: 10 L) with a thermostat water jacket, which was operated under the optimized reaction conditions. Substrates β-Ala and L-His were added excessively to ensure their saturation state during the reaction. The pH of the reaction mixture was around 7.5 and not adjusted to simplify the reaction as well as the subsequent product purification procedure. The reaction was initiated by adding 5 g lyophilized cell free extract (*ca.* 750 U) and 1.0 mmol $MnCl_2$, and continued by agitating at 40 °C and 200 rpm. After 6 h of reaction, the L-Car production reached 66.7 mM, giving a space–time yield of 60.3 g L^{-1} d^{-1} and a specific productivity of 30.1 g_{Car} $g_{catalyst}^{-1}$.

Since β-Ala, L-His and L-Car are all water-soluble, it is easy to separate the enzyme *Sm*PepD from the mixture of the product and substrates by using an ultrafiltration (UF) membrane. Considering the high stability of the dipeptidase *Sm*PepD, the UF-MBR was used for the continuous production of L-Car. A batch reaction was carried out initially, and when the L-Car concentration was higher than 50 mM, the continuous reaction was initiated through continuously feeding the saturated substrate solution at a relatively constant flow rate (*ca.* 0.5 L h^{-1}), and the effluent was collected at the same flow rate. The reaction lasted for 4 d. In the continuous reaction phase (Fig. 2, phase II), *Sm*PepD showed high operational stability with a nearly constant reaction rate of about 25 mmol h^{-1} L-Car. The excellent stability of *Sm*PepD in the continuous process may be attributed to the stabilizing effect of the amino acids and dipeptide on the enzyme. The average space–time yield achieved during the continuous reaction was 34.9 g L^{-1} d^{-1} and the resulting specific productivity was 105 g_{Car} $g_{catalyst}^{-1}$.

Since the *Sm*PepD catalysed synthesis of L-Car is a reversible reaction, the relative weight content of L-Car in the continuous reaction effluent was only 2.3% (w/w). It is difficult to separate L-Car from the excess substrates β-Ala and L-His because of their similar properties, including the very close isoelectric points. Membrane technology is an efficient method for product separation. A nanofiltration (NF) membrane was used for the separation and refining of L-Car. The molecular weights of β-Ala, L-His and L-Car are 89, 155 and 226 Da, respectively, so an NF membrane with a molecular weight cut-off (MWCO) of 150 Da was selected. The retention factors (*R*) of these three compounds

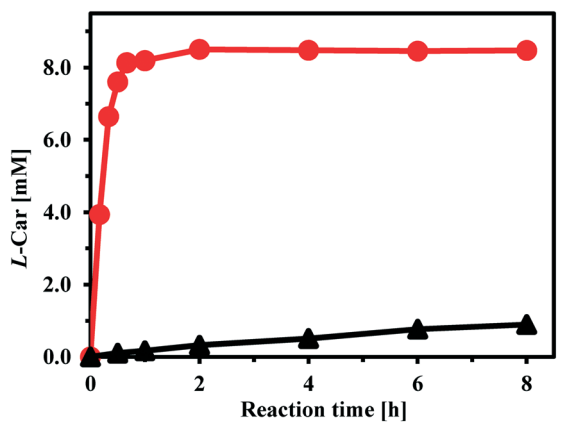

Fig. 1 Reaction process of L-Car synthesis with and without Mn^{2+}. Reactions were performed in 100 mL flasks with 10 mL mixtures containing 2 M β-Ala, 100 mM L-His, and 75 U L^{-1} lyophilized cell-free extract of *Sm*PepD, pH 8.0, magnetically agitated at 40 °C and 600 rpm. Symbols: (●) with 0.1 mM Mn^{2+} ions; (▲) without Mn^{2+} ions.

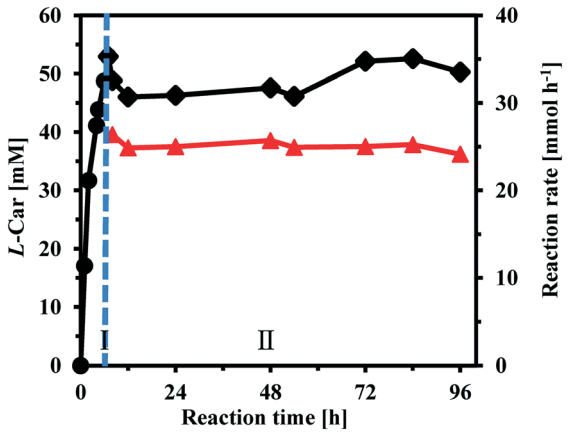

Fig. 2 Progress curves of the L-Car synthesis reaction in a continuous MBR. Phase I: batch reaction; phase II: continuous reaction. Symbols: (●) L-Car produced in batch reaction; (◆) L-Car produced in continuous reaction; (▲) reaction rate. The reaction rate is defined as the amount of L-Car produced by *Sm*PepD per hour.

determined were 46.5%, 65.7% and 92.3%, respectively, indicating significant differences. Therefore, β-Ala and L-His were efficiently permeated, while L-Car was selectively retained. After enrichment by the NF membrane, the relative content of L-Car was increased to more than 95% (w/w) with an isolation yield of 67.2% after the nanofiltration process. The excess substrates and a minor amount of product in the permeate during the NF membrane separation can be reused easily in a subsequent reaction. The crude product harvested after NF enrichment was further refined through decolourisation with activated carbon and removal of residual $MnCl_2$ by zeolite powder adsorption. After subsequent ethanol precipitation and drying, a purified product of L-Car was afforded with a purity of 98.8%. To sum up, 72.3 g of L-Car was harvested per 10 L of the reaction mixture, with a total isolation yield of 60.2%. The concentration of residual $MnCl_2$ detected was lower than 2 mg $kg_{product}^{-1}$ (ppm) and the specific rotation of the product was +21.0° (c = 3.0, water). Lit.[20] L-Car $[\alpha]_D^{25}$ + 21.9 (c = 3.0, water).

Conclusions

A highly active dipeptidase *Sm*PepD was cloned from *Serratia marcescens* by a genome mining approach. The enzyme can efficiently catalyse the reverse synthesis of L-Car through direct coupling of the unprotected β-Ala and L-His. To the best of our knowledge, this is the first report using a prokaryote-derived dipeptidase for the synthesis of the dipeptide L-Car. The Mn^{2+} ion had a very strong stimulation effect on the synthetic activity of *Sm*PepD. A continuous enzymatic reaction in an UF-MBR was realized, and L-Car of high purity was harvested through NF membrane separation. The whole process is very simple and clean, without the use of any buffer salt and any toxic solvent (except water and edible ethanol), and without any by-products, making the process a highly efficient and green synthesis process for L-Car production.

Experimental

Chemicals and materials

All chemicals and reagents were commercially available and of the highest purity.

UF membrane PT 1812 and NF membrane DK 1812 with MWCOs of 10 000 Da and 150 Da were obtained from Suez Water & Process Technologies (Wuxi) Co., Ltd (China).

E. coli BL21 (DE3) and plasmid pET-28a (+) were used for DNA manipulation and protein expression.

Gene cloning, expression and purification of recombinant enzymes

The genes encoding putative L-Car hydrolases were amplified by the PCR using the corresponding genomic DNAs as the template. The amplified DNA fragment was ligated into the pET-28a (+) vector and transformed into *E. coli* BL21 (DE3). The recombinant *E. coli* was cultured in Luria-Bertani (LB) medium supplemented with 50 μg mL^{-1} of kanamycin at 37 °C till the optical density at 600 nm reached 0.6–0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the final concentration 0.2 mM and cells were cultivated at 16 °C for a further 24 h. The cells were collected and disrupted by ultrasonification. After centrifugation, the cell free extract was loaded onto a Ni-NTA column and eluted by buffers containing different concentrations of NaCl and imidazole. The purity of the recombinant enzymes in the eluent was analysed *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified recombinant enzymes were collected for further assay.

Standard enzymatic assays for hydrolysis and synthesis of L-Car

Enzymatic hydrolysis of L-Car was performed in 0.2 ml Tris-HCl buffer (50 mM, the pH was adjusted with a NaOH solution to 8.0) containing 1.9 M β-Ala and 0.1 M L-Car. After pre-incubation at 30 °C, the reaction was started by adding adequate amounts of purified enzymes and then agitated at 30 °C and 1000 rpm for 5 min. The reaction was quenched by mixing 40 μL of the reaction solution with a 760 μL $HClO_4$ solution (0.1 M, in water) and the concentration of L-His formed was analysed by HPLC. One unit of the enzyme was defined as the amount of enzyme that hydrolysed 1.0 μmol L-Car per minute under the above conditions.

Enzymatic synthesis of L-Car was performed in 0.2 mL Tris-HCl buffer (50 mM, the pH was adjusted with a NaOH solution to 8.0) containing 2.0 M β-Ala and 0.1 M L-His. The reaction was performed as described above for 20 min. After quenching, the concentration of L-Car formed was analysed by HPLC.

For determining the kinetic parameters, the initial rates of the enzymatic L-Car synthesis at different molar ratios of β-Ala to L-His were determined and fitted to the Michaelis–Menten model using the software Origin 9.0.

Chemical assay

Samples were analysed using a reversed-phase HPLC, equipped with a chiral column of CROWNPAK® (+) (150 × 4 mm, 5 μm particle size), Daicel Chiral Technologies (China) Co., Ltd. After equilibration with $HClO_4$ solution (pH 1.0, in water) at a column temperature of 25 °C, compounds β-Ala, L-His and L-Car were separated with an isocratic flow of $HClO_4$ solution (pH 1.0) at a constant flow rate of 0.3 mL min^{-1}, detected at 210 nm UV and quantified according to an extraterritorial method. The content of Mn in the product was analysed with an HPLC-inductively coupled plasma mass spectrometer (NexION 2000-(A-10)), PerkinElmer Enterprise Management (Shanghai) Co., Ltd, and the experimental data were provided by the Analytical and Testing Center of East China University of Technology. The details for the retention times of β-Ala, L-His and L-Car are reported in the ESI† (paragraph 5).

Calculation of the equilibrium constant

Enzymatic hydrolysis and synthesis of L-Car were performed simultaneously in a 10 mL system that was magnetically stirred at 600 rpm. Both reactions were conducted under the same conditions except for the substrate concentration. For the hydrolytic reaction, 1.9 M β-Ala and 100 mM L-Car were used, whereas for the synthetic reaction, 2.0 M β-Ala and 100 mM L-His were used. 0.1 g of lyophilized cell free extract (15 U) was added to start the reaction, and extra catalyst was supplemented if necessary, till the concentration of L-Car in the reactions of both directions became identical, that is, the reaction reached equilibrium. Concentrations of β-Ala, L-His and L-Car at equilibrium were determined by HPLC, and the equilibrium constant (K_{eq}) was calculated according to eqn (1).

$$K_{eq.} = C_{Car} \times C_{H_2O}/C_{Ala} \times C_{His} \quad (1)$$

where C_{Car}, C_{H_2O}, C_{Ala} and C_{His} represent the concentrations of L-Car, water, β-Ala and L-His, respectively, at reaction equilibrium.

Batch reaction for the preparation of L-Car

The batch reaction was performed in a 15 L glass-jacked stirred tank reactor. In the reactor, 5.874 kg β-Ala (6.6 M) and 387 g L-His (250 mM) were added, and water was added to dissolve the substrates β-Ala and L-His at 40 °C and 200 rpm, till the volume of the reaction mixture reached 10 L. The reaction was started by adding 5 g lyophilized cell free extract of *Sm*PepD (750 U) and 198 mg $MnCl_2$ (1.0 mmol), stirred at 40 °C and 200 rpm continuously. The samples were withdrawn intermittently for the detection of the L-Car concentration.

Continuous flow reaction based on a UF membrane for the preparation of L-Car

A continuous reaction was performed in a membrane filtration device equipped with the UF membrane PT 1812 (MWCO 10 000 Da), as shown in Scheme S1.† The reaction volume was 5 L. A batch reaction was performed initially in a mixture containing saturated β-Ala and L-His, 0.1 mM $MnCl_2$ and lyophilized cell free extract of *Sm*PepD (750 U). When the L-Car concentration reached 50 mM, the continuous flow reaction began by continuously feeding the saturated solution of β-Ala and L-His (containing 0.1 mM $MnCl_2$), and discharging the reaction mixtures at the same space velocity (0.5 L h^{-1}). The whole reaction process lasted for 4 d.

Product isolation and purification

Portions of the continuous reaction mixture (50 L) were combined with a relative mass content of only 2.3% L-Car. Every 10 L of the reaction mixture was mixed with an equal weight (11.7 kg) of ethanol. Major contents of β-Ala and L-His were precipitated because of their relatively low solubility in an ethanol solution. After filtration, the filtrate was condensed by rotary evaporation to recover the ethanol. The residual solution mixture (5 L) was separated by using an NF membrane (DK 1812, MWCO of 150 Da). A volume-constant pattern was employed during the NF process using water as a leachate solvent at an osmosis rate of 5 L h^{-1}, till the relative mass content of L-Car increased to over 95%.

After nanofiltration, the retentate was collected and 3 g L^{-1} activated carbon was added and stirred at 60 °C for 0.5 h for decolourisation. After cooling, the mixture was filtered and 10 g L^{-1} artificial zeolite powder was added into the filtrate and stirred at 30 °C for 0.5 h to adsorb the undesired Mn^{2+} ions. The mixture was filtered again and the filtrate was vacuum concentrated till a solid sediment was observed. Then a 10-fold volume of ethanol was added slowly into the saturated solution followed by gentle stirring. The mixture was filtered and the filter cake was dried to constant weight.

Conflicts of interest

There are no conflicts to declare.

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