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Peptiligase, an Enzyme for Efficient Chemoenzymatic Peptide Synthesis and Cyclization in Water

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Abstract: We describe a novel, organic cosolvent-stable and cation-independent engineered enzyme for peptide coupling reactions. The enzyme is a variant of a stable calcium-independent mutant of subtilisin BPN′, with the catalytic Ser212 mutated to Cys and Pro216 converted to Ala. The enzyme, called peptiligase, catalyzes exceptionally efficient peptide coupling in water with a surprisingly high synthesis over hydrolysis (S/H) ratio. The S/H ratio of the peptide ligation reaction is correlated to the length of the peptide substrate and proved to be >100 for the synthesis of a 13-mer peptide, which corresponds to >99% conversion to the ligated peptide product and <1% hydrolytic side-reaction. Furthermore, peptiligase does not require a particular recognition motif resulting in a broadly applicable and traceless peptide ligation technology. Peptiligase is very robust, easy to produce in *Bacillus subtilis*, and its purification is straightforward. It shows good activity and stability in the presence of organic cosolvents and chelating or denaturing agents, enabling the ligation of poorly soluble (hydrophobic) or folded peptides. This enzyme could be useful for the (industrial) synthesis of diverse (pharmaceutical) peptides. In addition, peptiligase is able to efficiently catalyze head-to-tail peptide cyclization reactions.

Keywords: calcium-free peptide ligase; chemoenzymatic peptide synthesis; peptide cyclization

Introduction

There is a large and growing interest in pharmaceutical peptides over the past decades, with about 60 approved peptide drugs currently on the market and some 140 candidates in different phases of clinical development. By 2018, the worldwide peptide drugs market is expected to reach $18 billion.[1] The importance of peptides is also recognized in the nutritional and cosmetic industries.[2] Despite the high demand for peptides, their large-scale manufacturing remains challenging.[3]

Complete solid phase or solution phase synthesis of long peptides usually leads to accumulation of by-products and cumbersome purification. Furthermore, chemical peptide segment condensation can cause racemization, especially at the coupling site, and requires fully protected peptides, which are difficult to dissolve or purify due to their hydrophobicity. Protection and deprotection steps also reduce the overall synthetic yield and require the use of auxiliary chemicals, reducing process economy.

Chemoenzymatic peptide synthesis (CEPS), wherein chemically synthesized peptide segments are ligated enzymatically, is potentially one of the most cost-efficient technologies for the synthesis of medium-sized and long peptides (20–50 amino acids).[4] As compared to chemical segment coupling, CEPS offers several notable advantages, i.e., protection of the side-chain functionalities is not necessary, racemization is absent, and it is more environmentally friendly. Nevertheless, CEPS is currently rarely applied in academia and industry since it is not broadly applicable and suffers from severe hydrolytic side-reactions. An-

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other available coupling method is native chemical ligation[5] which, however, relies on the presence of certain amino acids in the peptide sequence, e.g., an N-terminal Cys, and is not scalable due to the instability of peptide thioesters.

CEPS is usually carried out by using (hydrolytic) proteases in the reverse direction.[6] Since peptide bond synthesis is thermodynamically unfavourable in the presence of water, the use of neat organic solvents with very low water activity can be beneficial for peptide coupling. For instance, the industrial serine protease Alcalase has been applied for peptide synthesis under nearly anhydrous conditions.[7] However, proteases exhibit very low activity in neat organic solvent,[8] and the peptide substrates are often poorly soluble both when side chains are (partially) protected. An alternative approach would be performing coupling reactions in water. Here, a peptide that is C-terminally activated as ester (acyl donor) is coupled to a peptide N-terminal amine (acyl acceptor), and the enzyme or reaction system is designed to kinetically favour formation of the ligation product. There are some effective ligating enzymes known from nature, such as sortase and butelase,[9] but they require specific recognition sequence motifs. Another approach is the use of substrate mimetics[10] and a protease. However, synthesis of the required highly activated peptide esters is challenging and some amino acids may undergo side reactions, such as Cys, Met, Trp, His and Tyr.[11]

Protein engineering has been used to tailor more broadly applicable proteases for peptide coupling in water. Wells and co-workers[12] constructed subtiligase, a double mutant derived from the *Bacillus amyloliquefaciens* subtilisin BPN′. The catalytic serine residue was mutated to a cysteine, and a proline near the active site was converted to an alanine to reduce steric crowding. The hydrolytic activity of this mutant is significantly reduced compared to the wild-type, since the introduced cysteine forms a thioester intermediate with the acyl donor, which is more prone to the amine nucleophilic attack leading to the ligation product. Unfortunately, the average ligation yield is 66%,[12a] even when using a ten-fold excess of the acyl acceptor,[12b] making this technology economically non-viable. Elliott[13] reported another approach by using a mutant of *Streptomyces griseus* protease B (SGPB) for peptide coupling. Here, the active site catalytic serine is mutated to an alanine and the reaction proceeds through a proposed histidine-involved acyl-enzyme intermediate. The stability of both enzymes was improved by introducing additional mutations, yielding stabiligase and streptoligase, respectively.[14] However, in both cases, an enzyme bound cation is crucial for the stability, which restricts their application. Alternative broadly applicable, robust and cation-free enzymes that catalyze peptide coupling reactions without (hydrolytic) side reactions are highly desired. Since such enzymes have not been discovered, a stabilized and cation (calcium) independent variant of subtilisin BPN′ (Sbt149)[15] was chosen in our study as the starting point for protein engineering. This variant has a deleted calcium binding domain (deletion of 9 amino acids, number 75 to 83) and an additional 18 stabilizing mutations, including one disulfide bridge. We herein report that by introducing the Ser212Cys and the Pro216Ala mutations into this robust enzyme scaffold, a new subtilisin variant is obtained, termed peptiligase, that catalyzes efficient peptide ligation in water.

Results and Discussion

The gene encoding peptiligase was constructed by site-directed mutagenesis and cloned into the *Escherichia coli/Bacillus subtilis* shuttle vector pBS42. Production of the enzyme was performed in *B. subtilis* strain DB104 which lacks native extracellular neutral and serine proteases.[16] Peptiligase is translated as a precursor and needs to be processed to obtain the mature protein. It was reported that a ‘helper’ protease, i.e., native subtilisin, is required for processing of subtiligase, due to its reduced protease activity.[12] In the present study, we observed that peptiligase can perform the autoproteolytic removal of the *pro*-sequence without any additional subtilisin, which suggests that the retained proteolytic activity of this enzyme is sufficient for its maturation. Secreted peptiligase was purified from the culture medium by ammonium sulfate precipitation and ion-exchange chromatography. The yield from 1 liter of cell culture was approximately 20 mg (70% pure on SDS-PAGE gel). The thermostability of the purified peptiligase was determined using the thermofluor assay. The apparent transition temperature ($T_{m,app}$) of 66 °C indicates that the mutated enzyme well preserves the thermostability of its parent subtilisin.

As anticipated, peptiligase is cation-independent. In a standard peptide ligation reaction consisting of the activated carboxamidomethyl (Cam) ester Ac-Phe-Ile-Glu-Trp-Leu-OCam as acyl donor and H-Ala-Phe-NH_2 as the amidated acyl acceptor none of the metal cations tested (Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}; 10 mM concentration) displayed a remarkably stimulatory or inhibitory effect on the activity of peptiligase. The influence of the metal chelator EDTA (10 mM) on the enzymatic activity was also negligible. In addition, the thermostability of peptiligase was unaffected by metals or EDTA (Table 1). The pH-dependence was determined in MES and Tricine buffers with pH values ranging from 6.5–8.85. The optimal pH value (highest synthesis/hydrolysis ratio) was pH 8.0. When a higher pH was used, chemical hydrolysis of the pep-

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Table 1. Effect of metal ions and chelating agent EDTA on the (Ac-Phe-Ile-Glu-Trp-Leu-OCam + H-Ala-Phe-NH_2) coupling activity and thermostability of peptiligase. Details are given in the Experimental Section.

	Activity [mU/mg]	$T_{m,\ app}$ [°C]
control	26	66.0
Ca^{2+} (10 mM)	20	65.5
Mg^{2+} (10 mM)	26	65.0
Mn^{2+} (10 mM)	30	64.5
Ni^{2+} (10 mM)	22	62.0
EDTA (10 mM)	26	66.5

tide ester was more profound. At lower pH, the amine acyl acceptor is unreactive due to protonation.

To test whether peptiligase tolerates high concentrations of organic solvents, peptide ligation reactions were performed in the presence of various amounts of water-miscible cosolvents, including tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO). Reaction mixtures again contained the Ac-Phe-Ile-Glu-Trp-Leu-OCam acyl donor and H-Ala-Phe-NH_2 as acceptor. Conversion of substrate and product formation were estimated by HPLC.

As shown in Figure 1, the enzyme exhibited moderate tolerance towards THF, and a reasonable residual

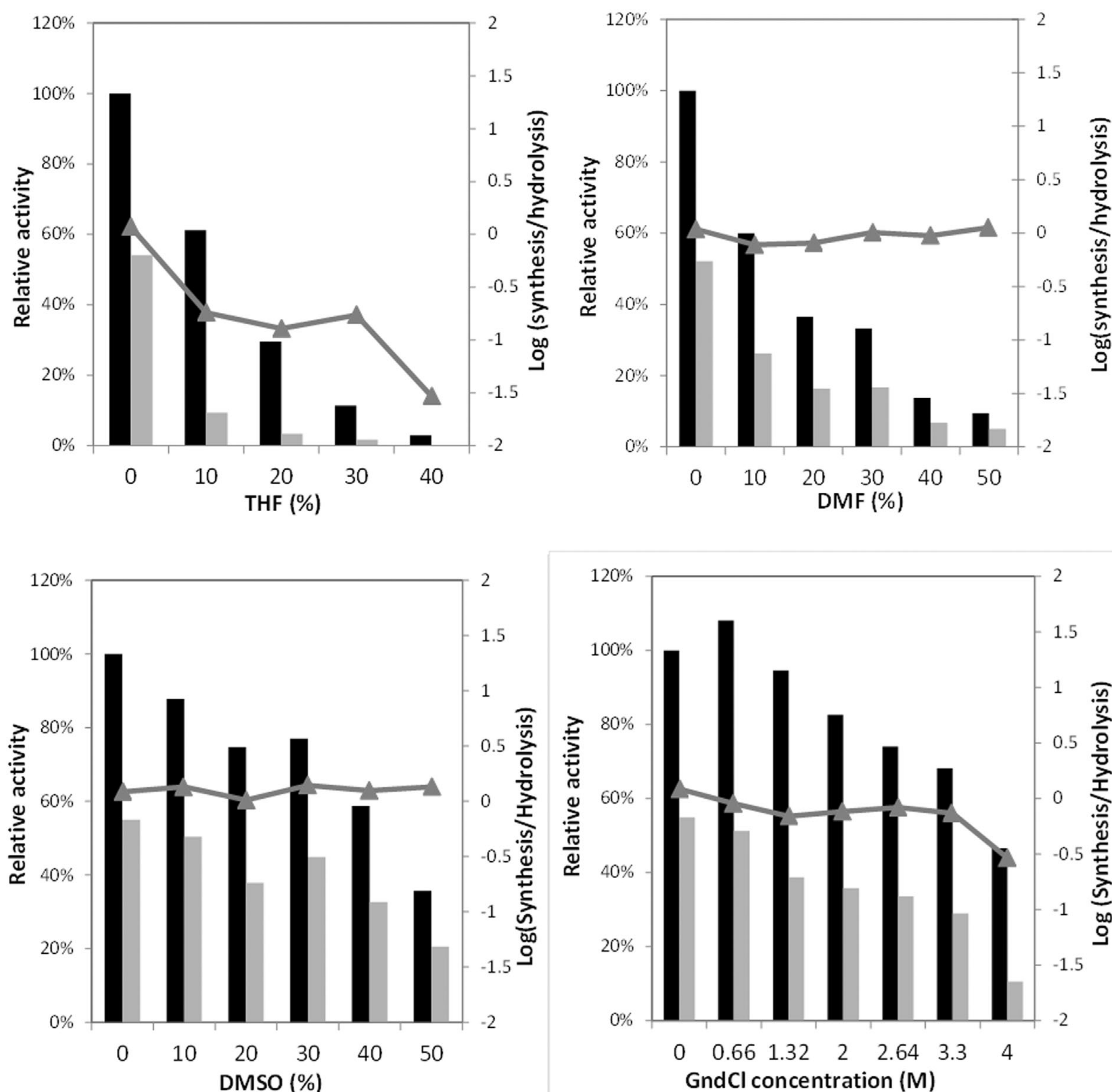

Figure 1. Effect of organic solvents and denaturing agent on peptiligase-catalyzed peptide coupling of Ac-Phe-Ile-Glu-Trp-Leu-OCam and H-Ala-Phe-NH_2. Black bars: relative total activity; grey bars: relative synthetic activity; grey triangles: log value of synthesis/hydrolysis ratio. Details are given in the Experimental Section.

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total activity (of 30%) was observed in the presence of 20% (v/v) THF. However, THF strongly suppressed the nucleophilicity of the acyl acceptor and hydrolysis of the acyl donor became dominant. Only hydrolysis of the acyl donor Cam ester to free acid was observed. In contrast, the S/H ratio was not significantly affected by the addition of either DMF or DMSO. The enzyme also showed good stability in these two cosolvent systems. Notably, the enzyme retained 36% of its activity in the presence of 50% (v/v) DMSO in water, which is a very good mixture for dissolving hydrophobic peptides. Therefore, if required for substrate solubility, DMSO is the preferred organic cosolvent to use in coupling reactions.

A possible issue with peptide ligation reactions, as described by Chang et al.,[14a] is that substrates could adopt a secondary structure reducing the accessibility to the binding pockets of the coupling enzyme. In such cases, denaturing agents like guanidinium hydrochloride (GndCl) or urea can be added to weaken the secondary structure and expose the ligation junction of the substrates. Under extreme denaturing conditions, peptiligase retained significant activity; up to 4M GndCl could be used. Moreover, the S/H ratio was only marginally influenced by the addition of GndCl.

Next, the efficiency of peptide ligation with peptiligase was investigated. The parent enzyme Sbt149 binds the substrate in an extended antiparallel β-sheet conformation, while substrate residues surrounding the scissile bond (peptide groups P4–P3′) are in close contact with their respective binding pockets (subsites S4–S3′).[17] To test whether ligation reactions catalyzed by peptiligase also require both the acyl donor and the acyl acceptor substantially occupying the binding pockets of the enzyme, we used substrates of varying lengths in coupling reactions (Table 2, Table 3 and Table 4). First, single amino acids and dipeptides were coupled *via* a kinetically controlled conversion. Cam-esters are optimal substrates for proteases used as ligases as they mimic a glycine residue.[12] Peptide Cam-esters are easily produced *via* standard solid phase peptide synthesis using conventional resins in high yield and good purity.[18] However, when such amino acids and dipeptides were tested in coupling reactions, the efficiency by peptiligase was poor (Table 2).

Subsequently, a series of longer (5–11 amino acids) acyl donor Cam-esters with the same ligation junctions were tested. The results indicate that peptiligase catalyzes the coupling of longer acyl donors with high efficiency, and that there is a clear correlation between the length of the acyl donor and the S/H ratio of the reaction (Table 3). Virtually no hydrolytic side-reactions were observed at all when an 11-mer Cam-ester was used, i.e., the S/H ratio exceeded 100. This corresponds to a conversion of >99% to product and less than 1% of hydrolysis. Notably, this high yield was obtained using only 1.5 equivalents of acyl acceptor, making this an economically attractive ligation.

Next, we investigated whether increasing the length of the acyl acceptor has a similar beneficial effect (Table 4). The efficiency of peptide ligation was analyzed using a series of acyl acceptors with the same amino-terminal residue and stepwise length extension (1–4 amino acids). While a coupling reaction with alanine amide as nucleophile appeared to be accompa-

Table 2. Peptiligase-catalyzed coupling reactions of amino acids and dipeptides[a].

Acyl donor	Acyl acceptor	Conversion [%]	Synthesis [%]	Hydrolysis [%]	S/H ratio
Cbz-F-OCam	H-A-NH_2	26	3	23	0.13
Cbz-F-OCam	H-AF-NH_2	50	6	44	0.13
Cbz-AF-OCam	H-A-NH_2	53	2	51	0.04
Cbz-AF-OCam	H-AF-NH_2	53	6	47	0.13

[a] Reactions were performed with 1 mM acyl donor and 3 mM acyl acceptor in 100 μL Tricine buffer (100 mM, pH 8.0). Reaction time = 3 h.

Table 3. Peptiligase-catalyzed coupling reactions using acyl donors with varying lengths and H-AF-NH_2 as the amine nucleophile[a].

Acyl donor	Conversion [%]	Synthesis [%]	Hydrolysis [%]	S/H ratio
Ac-DLSKQ-OCam	93	81	12	6
Ac-TSDLSKQ-OCam	85	83	2	41.5
Ac-TFTSDLSKQ-OCam	66	64	2	32
Ac-EGTFTSDLSKQ-OCam	60	60	<1	>100

[a] Reactions were performed with 10 mM acyl donor and 15 mM acyl acceptor in 100 μL Tricine buffer (100 mM, pH 8.0). Reaction time = 3 h.

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Table 4. Peptiligase-catalyzed coupling reactions using amine nucleophiles with varying lengths and Ac-FIEWL-OCam as the acyl donor[a].

Acyl acceptor	Conversion [%]	Synthesis [%]	Hydrolysis [%]	S/H ratio
H-A-NH_2	35	<1	35	<0.01
H-AF-NH_2	59	32	27	1.2
H-AFA-NH_2	25	13	11	1.2
H-AFAY-NH_2	23	14	9	1.5

[a] Reactions were performed with 1 mM acyl donor and 3 mM acyl acceptor in 100 µL Tricine buffer (100 mM, pH 8.0). Reaction time = 1 h.

nied by overwhelming hydrolysis, longer peptide amides served as better acyl acceptors.

These results suggest that occupation of both the S1′ and the S2′ subsites as well as the S1–S4 subsites is a prerequisite for efficient ligation. Thus, the minimal peptide length for an efficient coupling reaction is 4+2, with longer segments giving more efficient ligation, as desired. From the results of an 11+8 coupling reaction, i.e., Ac-Lys-Lys-Lys-Lys-Lys-Lys-Asp-Phe-Ser-Lys-Leu-OCam-Leu-OH+H-Ala-Ala-Pro-Arg-Ala-Ala-Arg-Glu-OH, it became clear that we have not reached the limitations of this technology since the reaction reached 97% conversion to a 19-mer product with only 3% hydrolysis of the ester bond (details in the Supporting Information). Clearly, enzymatic ligation can be used for the synthesis of both medium-sized and long peptides.

To determine whether the synthetic properties of peptiligase are better than those of subtiligase, S/H ratios were determined using various acyl acceptors (Table 5). Sub-optimal (diluted) reaction conditions were used to clearly quantify the amounts of synthetic product and product of the hydrolytic side reaction. Under the same reaction conditions, the S/H ratio of peptiligase is surprisingly higher than that of subtiligase; in most cases the S/H ratio of peptiligase was two-fold higher.

The improved S/H ratio together with the excellent stability render peptiligase a highly promising enzyme for further development of a generally applicable CEPS technology. We are currently investigating the peptiligase-catalyzed synthesis of various pharmaceutical products on a gram scale to determine purified product yield and compare the results to those of conventional synthetic methods.

Besides peptide segment condensation, subtiligase has been used to cyclize linear peptides longer than 13 amino acids in reasonable yield (average of 64%).[19] Macrocylic peptides are a very promising class of pharmaceuticals, mainly due to their high stability in blood serum.[20] We embarked on the cyclization (Figure 2) of linear Microcin J25, the precursor of a 21 amino acid long lasso-peptide with antibiotic properties that is naturally produced by a strain of *E. coli* isolated from human feces.[21] Chemical head-to-tail cyclization of such a long peptide is very challenging and the reaction has to be performed with extremely diluted substrate to prevent polymerization, making it difficult for scale-up.[22] Microcin J25 is hydrophobic and cosolvents such as DMSO are needed

Table 5. P1′ and P2′ substrate scope of peptiligase and subtiligase using Ac-DFSKL-OCam as acyl donor and various acyl acceptors, where Xxx = the proteinogenic amino acid indicated. Best eight results shown[a].

Acyl acceptor	Peptiligase		Subtiligase	
H-Xxx-Leu-Arg-NH_2	Synthesis [b] [%]	S/H ratio	Synthesis [b] [%]	S/H ratio
Ser	58	1.38	40	0.67
Gly	48	0.92	25	0.33
Ala	47	0.89	20	0.25
Asp	21	0.27	6.8	0.07
H-Ala-Xxx-Arg-NH_2	Synthesis [b] [%]	S/H ratio	Synthesis [b] [%]	S/H ratio
Ile	46	0.83	26	0.35
Val	46	0.86	29	0.41
Leu	32	0.46	26	0.34
Met	31	0.38	24	0.28

[a] Reactions were performed with 0.75 mM acyl donor and 1.5 mM acyl acceptor in 1 mL Tricine buffer (100 mM, pH 8.0) and 5 µg of enzyme. Full conversion was reached after 30 min.
[b] Product yields were derived from HPLC analysis of the reaction mixtures.

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H-Gly-Thr-Pro-Ile-Ser-Phe-Tyr-(Gly)$_3$-Ala-Gly-His-Val-Pro-Glu-Tyr-Phe-Val-Gly-Ile-OCam-Leu-OH

↓ peptiligase, 20 vol% DMSO, phosphate buffer pH = 8.0, 82% cyclic product

cyclo[Gly-Thr-Pro-Ile-Ser-Phe-Tyr-(Gly)$_3$-Ala-Gly-His-Val-Pro-Glu-Tyr-Phe-Val-Gly-Ile]

Figure 2. Head-to-tail cyclization of linear Microcin J25 (21-mer) by peptiligase in 20% (v/v) DMSO (details in the Experimental Section).

in high amounts to solubilize the peptide in aqueous environment, which is well tolerated by peptiligase.

When Cam-Leu-activated linear Microcin J25 (1 mg mL^{-1}) was cyclized with peptiligase using 20% (v/v) DMSO in phosphate buffer, a very good conversion to cyclic product was observed, i.e., 82% of synthetic product and only 18% of hydrolysis of the linear Microcin J25Cam-Leu ester to produce the free acid. No dimerization or polymerization was detected. Moreover, a similar conversion to cyclic peptide (81%) was obtained in a reaction with concentrated substrate (100 mg mL^{-1} in DMSO) dosed to peptiligase in phosphate buffer, thus obtaining a concentrated product solution (50 mg mL^{-1}). Clearly, besides peptide segment condensation, peptiligase can be used to synthesize head-to-tail macrocyclic peptides in an efficient manner.

Conclusions

We have successfully constructed a stable peptide ligase that is able to ligate peptides in water with very high S/H ratios. When longer peptides are coupled, the hydrolytic side-reaction is almost completely suppressed. To the best of our knowledge, this is the first reported broadly applicable and traceless peptide ligating enzyme that is independent of cations and is very robust. The enzyme can be expressed in *B. subtilis* and can be directly isolated from the culture medium since it is secreted and no additional protease is required for maturation. Furthermore, peptiligase is compatible with a variety of additives and cosolvents, has a relaxed specificity in coupling reactions, and does not hydrolyze amide bonds in the main chain of precursor or product peptides. These features endow the enzyme with the catalytic properties required for the synthesis of a variety of medium-sized and long (pharmaceutical) peptides. Moreover, we have shown that peptiligase can be used for the synthesis of a peptide macrocycle, which represents an important upcoming class of pharmaceutical peptides for future medicines. Based on the robust framework of peptiligase, further protein engineering for even higher synthetic performance or broader substrate acceptance is ongoing.

Experimental Section

Construction of Peptiligase Expression Plasmid

The Sbt149 encoding gene with its natural promoter sequence (a kind gift from Prof. P. N. Bryan, University of Maryland, USA) was used as the template to generate the Ser212Cys/Pro216Ala mutant by the megaprimer method. The final PCR product was purified, digested with EcoRI and BamHI, and ligated into the EcoRI/BamHI treated pBS42 vector (DSM8748, DSMZ, Braunschweig, Germany). The ligation mixtures were transformed to competent *E. coli* ER 1821 cells (NEB, Ipswich, MA, USA) and transformants were plated on LB plates containing 34 μg mL^{-1} chloramphenicol. The mutant gene was confirmed by DNA sequencing.

Expression and Purification of Peptiligase

Plasmid pBS42-peptiligase was transformed into *B. subtilis* DB104, which is a double mutant (*his nprR2 nprE18 aprΔ3*)[23] strain deficient in extracellular neutral and serine proteases and was a kind gift from Prof. O. Kuipers (University of Groningen, the Netherlands). Transformants grown on an LB plate containing 10 μg mL^{-1} chloramphenicol at 37 °C for 16 h were picked and inoculated into 10 mL of LB broth containing 10 μg mL^{-1} chloramphenicol. After 16 h of incubation at 37 °C, 1% (v/v) of the cultures were inoculated to 1 L rich broth (20 g L^{-1} yeast extract, 5 g L^{-1} tryptone, 6.4 g NaCl, 30 mM phosphates pH 7.6, 10 g L^{-1} glucose, 50 mg L^{-1} Trp, 50 mg L^{-1} Lys, 50 mg L^{-1} Met, 0.06 mM $MnCl_2$). Cultures were grown at 37 °C with shaking for 48 h. Next, the medium was harvested by centrifugation at 6,000 *g* for 20 min at 4 °C. Subsequently, 5 g of $CaCl_2$ were added to the medium to precipitate phosphate salts and the pH was adjusted to 7.5. The precipitate was removed by centrifugation at 6,000 *g* for 20 min, 4 °C. Ammonium sulfate was added to the supernatant to a final concentration of 45 wt%. The mixture was stirred for 1 h at 4 °C to precipitate peptiligase, which was harvested by centrifugation at 8,000 *g* for 30 min at 4 °C. The pellet was washed 4 times with 100 mL 80% acetone, and resuspended in 15 mL water. Resuspended material was centrifuged at 15,000 *g* for 10 min at 4 °C, to remove insoluble material. The supernatant was desalted using a H*i*Prep 26/10 desalting column in buffer (20 mM Tricine, pH 7.5). The desalted proteins were loaded on a 5 mL HiTrap Q HP column. The flow-through containing peptiligase was collected and concentrated. The purity of the protein was analyzed by SDS-PAGE and estimated to be 70%.

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The purified enzyme was flash-frozen in liquid nitrogen and stored at −80 °C until further use.

Expression and Purification of Subtiligase

A gene encoding subtiligase[12] was provided by DNA2.0 (https://www.dna20.com/) and recloned into EcoRI-BamHI digested *E. coli–B. subtilis* shuttle vector pBS42. The plasmid pBS42-S5 was propagated in *E. coli* strain MM294 (DSM5208, DSMZ, Braunschweig, Germany), isolated and validated by sequencing. The validated plasmid was used for transformation of *B. subtilis* DB104. The production and purification protocol was similar to that for peptiligase, but using buffers supplemented with 1 mM $CaCl_2$. The yield of purified subtiligase was 2–5 mgL^{-1} culture.

Thermostability Assays

The fluorescence based thermal stability assay[24] was used to determine apparent melting temperatures of the proteins. For this, 20 μL of protein solution in buffer (20 mM Tricine, pH 7.5) were mixed with 5 μL of 100 x Sypro Orange (Molecular Probes, Life Technologies, San Diego, CA) dye in a thin wall 96-well PCR plate. The plate was sealed with Optical-Quality Sealing Tape and heated in a CFX 96 Real Time PCR System (BioRad, Hercules, CA, USA) from 20 to 99 °C at a heating rate of 1.75 °Cmin^{-1}. Fluorescence changes were monitored with a charge-coupled device (CCD) camera. The wavelengths for excitation and emission were 490 and 575 nm, respectively.

Peptide Ligation Reactions

The ligation reactions were typically performed at 25 °C in 100 mM Tricine buffer (pH 8.0), containing 15 μM peptiligase and different acyl donors and acyl acceptors. The efficiency of the ligation reactions was analyzed by an LC/MS ion-trapping system (Thermo Scientific). The reaction mixtures were loaded on an Alltech C18 3u column (3 μm, 100 mm × 4.6 mm). The solvent system consisted of 0.01% formic acid (eluent A) and 0.08% formic acid in 30% acetonitrile (eluent B). A typical elution gradient was as follows: start with 100:0 A:B for 10 min, in 45 min from 100:0 to 40:60, and from 40:60 to 100:0 from 45 min to 60 min. The analyses were carried out at 25 °C, with concomitant UV detection at 220 nm or 280 nm. For reactions using tryptophan-containing acyl donors, the UV detection was set at 280 nm, and the concentrations of the compounds were calculated based on their peak area. For reactions using tryptophan-free acyl donors, the UV detection was set at 220 nm and the conversion was calculated based on the consumption of the acyl donor substrate. Chemical hydrolysis of the acyl donor was calculated from control experiments.

pH-Ligation Profile

The reaction mixture containing 1 mM Ac-Phe-Ile-Glu-Trp-Leu-OCam, 3 mM H-Ala-Phe-amide, and 15 μM peptiligase were incubated at 25 °C for 1 h. To determine the effect of pH on the ligation efficiency, experiments were carried out over a pH range of 6.0–8.85. The buffers used were MES (100 mM, pH range 6.0–7.0) and Tricine (100 mM, pH range 7.5–8.85). The chemical hydrolysis of the acyl donor was assayed in the absence of the enzyme.

Effect of Organic Solvent and Additives on Peptiligase Activity

The standard reaction mixtures consisted of 1 mM Ac-Phe-Ile-Glu-Trp-Leu-OCam, 3 mM Ala-Phe-amide, 15 μM enzyme and different concentrations of organic solvents, metal ions, chelating agent or denaturing agent in 100 mM Tricine buffer (pH 8.0). The assays were performed at 25 °C. The uncatalyzed chemical hydrolysis of the acyl donor was assayed in the absence of the enzyme.

Synthesis of a 19 Amino Acid Peptide

The coupling reaction was performed using 4 mM acyl donor (Ac-Lys-Lys-Lys-Lys-Lys-Lys-Asp-Phe-Ser-Lys-Leu-OCam-Leu-OH) and 6 mM acyl acceptor (H-Ala-Ala-Pro-Arg-Ala-Ala-Arg-Glu-OH) in phosphate buffer (1 M, pH 8.0). To this reaction mixture, 5 μg of enzyme was added and the mixture (1 mL) was analyzed by LC-MS after 30 min. The Cam-ester starting material was fully consumed and the ratio between the product and hydrolysis peak was 97/3. See the Supporting Information for further details.

Cyclization of Linear Microcin J25 Cam-Leu-Ester

The cyclization reaction was performed with 1 mg linear peptide Cam-Leu ester (H-Gly-Thr-Pro-Ile-Ser-Phe-Tyr-Gly-Gly-Gly-Ala-Gly-His-Val-Pro-Glu-Tyr-Phe-Val-Gly-Ile-OCam-Leu-OH) in 1 mL phosphate buffer (100 mM, pH 8.0) supplemented with DMSO (20 vol%). To this reaction mixture, 10 μg of peptiligase were added and the solution was analyzed by LC-MS after 30 min. Conversion to product was measured by integrating the starting material, product and hydrolyzed Cam-ester peaks. The amount of product after 30 min was 82% and the amount of hydrolysis 18%. The Cam-ester starting material was completely consumed. See the Supporting Information for further details.

In a second reaction, concentrated substrate solution in DMSO (100 mgmL^{-1}) was prepared and dosed in time (20 μL every 15 min) to the enzyme solution (10 μg peptiligase) in 200 μL phosphate buffer (100 mM, pH 8.0). The pH was continuously kept at 8.0 using 5 N aqueous NaOH. The reaction mixture was analyzed by LC-MS after 150 min as described above. The starting material was fully consumed and the amount of product was 81% and hydrolysis 19%.

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ployed by Enzypep B. V. Some of Enzypep's products are based on the use of protease technology.

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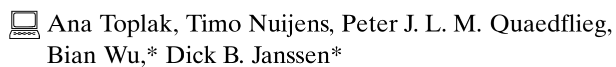

Peptiligase, an Enzyme for Efficient Chemoenzymatic Peptide Synthesis and Cyclization in Water

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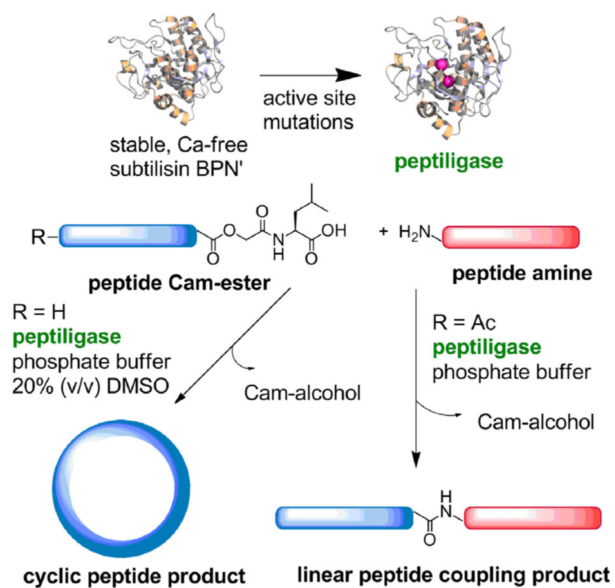

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