Enzymatic tools for peptide ligation and cyclization

Development and applications

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CHAPTER 3

Thymoligase: Design of a Substrate-Tailored Peptiligase Variant for the Efficient Synthesis of Thymosin-α₁

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Abstract

Thymosin-α₁, an acetylated 28 amino acid long therapeutic peptide, is exceptionally challenging to synthesize via conventional chemical methods. Enzymatic coupling of unprotected peptide fragments in water offers great potential for a more efficient synthesis of peptides that are difficult to synthesize. Based on the design of a highly engineered peptide ligase, we developed a fully convergent chemo-enzymatic peptide synthesis (CEPS) process for the production of thymosin-α₁ via a 14-mer + 14-mer fragment condensation strategy. Using structure-inspired enzyme engineering, the thiol-subtilisin variant peptiligase was tailored to recognize the respective 14-mer thymosin-α₁ fragments in order to create a clearly improved biocatalyst, termed thymoligase. Thymoligase catalyzes peptide bond formation between both fragments with very high efficiency (>94 % yield) and is expected to be well applicable to many other ligations in which residues with similar characteristics (e.g. Arg and Glu) are present in respective positions P1 and P1’. The crystal structure of thymoligase was determined and shown to be in good agreement with the model used for the engineering studies. The combination of solid phase peptide synthesis (SPPS) of the 14-mer fragments followed by their thymoligase-catalyzed ligation at gram scale resulted in a significantly increased, two-fold higher overall yield (55 %) of thymosin-α₁, as compared to those typical for existing industrial processes.
Introduction

Owing to their outstanding biological selectivity and efficacy, exploiting peptides as therapeutics has become increasingly popular. Their therapeutic potential addresses the full scope of medical disorders with currently over 60 peptides approved in major markets and over 150 peptides in active development today. The peptide therapeutics market is expected to reach about $25 billion by 2020 and its growth is accompanied by an increased demand for new and more efficient processes for their production at industrial scale. The manufacture of long peptides using conventional chemical strategies is still very challenging and the technology used has remained almost unchanged since Merrifield developed the solid phase approach in the 1960s. Solid-phase peptide synthesis (SPPS) is characterized by an exponential decrease in crude yield and purity as peptide length increases: coupling and deprotection steps become less efficient and purification from accumulating by-products becomes increasingly difficult. In contrast, fragment condensation processes are intrinsically more efficient, since short peptide fragments can be produced in high yield and purity. However, chemical ligation of peptide fragments is still a significant challenge, mainly due to the low solubility of the protected fragments and potential epimerization of the C-terminal amino acid upon activation. To overcome some of these difficulties the application of chemo-enzymatic peptide synthesis (CEPS), a combination of conventional SPPS for the production of unprotected peptide fragments and an enzymatic epimerization-free coupling of the fragments in water represents a promising strategy. Hence, enzyme-mediated ligation technologies, e.g. the use of sortases, butelase-1, trypsiligase and subtilisin variants such as subtiligase or peptiligases, have recently gained increased attention for a more cost-efficient synthesis of medium-sized or long peptides.

A well-known peptide that is difficult to synthesize using conventional methodologies is thymosin-α₁, an acetylated 28-mer therapeutic peptide (Ac-SDAAVDTSSEITTKDLKEKKEVVEEAEN-OH, Mₚ = 3108.32 g/mol) with immunoregulating activity. After administration, thymosin-α₁ elicits a variety of immune system responses and is being used for a range of medical applications (e.g. treatment of hepatitis B and C). It is approved in more than 30 countries and mainly marketed in China. Triggered by the market demand several solid- and solution phase strategies have been developed during the past decades. However, these repeatedly resulted in low overall yields (approx. 25%). The synthesis of thymosin-α₁ is especially hampered by the large number of protecting groups required (20 in total) and its tendency to form β-sheets.

Approaches for the production of the highly acidic peptide in pro- or eukaryotic expression systems as a cost-effective alternative resulted in low titers (<30 mg/L of cell culture) and even more difficult isolation and purification procedures compared to full SPPS.

The feasibility of a chemo-enzymatic approach is critically dependent on the availability of an enzyme capable of efficiently forming a peptide bond between two peptide fragments. A successful example of a kinetically controlled peptide ligation strategy is the use of a highly engineered, stabilized and Ca²⁺-independent thiol-
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substrilisin variant termed peptiligase.[12,13] Using peptiligase, a peptide fragment that is C-terminally activated as a carboxamidomethyl(Cam)-ester (acyl donor) can be coupled to a peptide fragment with a free nucleophilic N-terminus (acyl acceptor) in high yield. However, when using engineered proteases such as peptiligase, hydrolytic side-reactions may occur, including hydrolysis of the acyl donor ester moiety and a very slow hydrolysis of the final product. Hence, minimization of these side-reactions is required in order to maximize product yield.

Earlier engineering studies already indicated that peptiligase can serve as a template for engineering variants with dedicated selectivity and improved performance.[13] When we tested the use of peptiligase for a [14+14]-mer coupling strategy to synthesize thymosin-α₁ (Figure 1A), a low coupling yield (<20%) and significant hydrolysis of the acyl donor ester was observed (Figure 4). Thus, in order to obtain a dedicated biocatalyst for this purpose, we followed a structure-based enzyme design approach. Ultimately, enzyme engineering yielded the improved peptiligase variant, thymoligase, that allowed the efficient synthesis of thymosin-α₁ using a [14+14]-mer fragment condensation approach.

Results and Discussion

Substrate-Tailored, Structure-Inspired Enzyme Engineering.

To explore the chemo enzymatic synthesis of thymosin-α₁ we initially tested peptiligase in a [14+14]-mer fragment coupling under conditions similar to those reported for the synthesis of the incretin mimetic drug exenatide.[13] These initial experiments resulted in the formation of thymosin-α₁ with very low efficiency, with a yield (acyl donor substrate converted to synthetic product) below 20% (Figure 4). In addition, the S/H ratio (ratio between desired synthetic product and hydrolyzed acyl donor) was also very low. In earlier mutagenesis studies substitution M222G was introduced to reduce sensitivity towards oxidation[29] and to reduce steric crowding in the active site of peptiligase (Ptl)[13] with the aim of improving the coupling efficiency. Indeed, the introduction of this mutation (giving variant Ptl G) improved the synthetic yield to 33% (Figure 2A), which was, however, still unsatisfactory.

To discover mutations that would drastically improve performance in the synthesis of thymosin-α₁, we examined structural models of peptiligase. For this, we constructed a hybrid model using YASARA with pdb files 1GNV[30] (calcium-independent subtilisin BPN') and 1SBN[31] (subtilisin-eglin complex). A fragment of thymosin-α₁ [7-19] was modelled into the substrate binding region and the effects of additional mutations were examined using YASARA.[13] Based on this model, we identified residues that are in close contact with the substrate and aimed to introduce stabilizing interactions between the substrate and the respective binding pockets of peptiligase.
For an efficient thymosin-α1 [14+14]-mer coupling reaction, the enzyme is required to accept the positively charged 14Lys sidechain of the acyl donor in the S1 pocket as well as the negatively charged 15Asp sidechain of the acyl acceptor in the S1’ pocket (Figure 1B). However, inspection of the structural model of peptiligase suggested this to be problematic, especially as the S1 pocket is characterized by its hydrophobic nature that will not readily accept polar amino acids. Similarly, the 15Asp of the nucleophilic fragment will poorly fit the S1’ pocket. Thus, our main design efforts focussed on tailoring the S1 and S1’ pockets for the acceptance of charged residues and, in addition, on fine tuning the enzyme by engineering the S2’ pocket to bind 16Leu (Figure 1B). The major residues shaping the S1 pocket are S156 and S166, therefore suggesting these to be targeted for mutagenesis. Similarly, L217 is prominent in the S1’ pocket. Mutations to consider include the introduction of a negatively charged residue at position S166 (S166D or S166E) [32] and a positively charged residue at position L217 (L217R) [13], which could both lead to the formation of ion pairs between the substrate and the enzyme (14Lys/S166D and 15Asp/L217R, Figure 2C). In addition, polar amino acids in position S156 could also provide ionic interactions with the substrate’s 14Lys sidechain.

Enzyme variants were obtained using site-directed mutagenesis followed by heterologous expression in B. subtilis and subsequent His-tag purification (>80% SDS-PAGE purity). The enzyme variants were screened for the [14+14] thymosin-α1 coupling efficiency using crude 14-mer substrates, with only 1.2 eq. of the acyl acceptor fragment used. Earlier studies on engineering the S1’ pocket revealed that mutation L217R enhances the coupling efficiency of small model peptides bearing an aspartic- or glutamic acid in position P1’. [13] In fact, we found that the introduction of mutation L217R into the Ptl M222G mutant (giving Ptl GR) resulted in a 2-fold increased S/H ratio in the [14+14] thymosin-α1 coupling, while retaining the activity observed with Ptl G (Figure 2A). Further combining mutations M222G and L217R with a negatively charged amino acid residue in the S1 pocket (S166D) resulted in an even more active variant with double the product formation rate (Ptl GRD). However, although for this variant the S/H ratio is not improved compared to peptiligase M222G (Figure 2A) the combined effects of retained S/H ratio and improved product formation result in a significantly
increased effective yield (Figure 2B). In contrast, mutation S166E (Ptl GRE) resulted in an increased S/H ratio, but no improvement in activity and no increased effective yield was observed (Figure 2A and B).

Figure 2. A) Result of testing of peptiligase variants for the thymosin-α1 [14+14]-mer coupling efficiency. Variants bear different combinations of mutations M222G, L217R, S166D/E and A225N. Product formation (%) and S/H ratios after 60 min are shown. Couplings were performed in aqueous solution (1 M phosphate pH 8.3, 3.5 mM tris(2-carboxyethyl)phosphine (TCEP), room temperature) using 0.7 μM of enzyme, crude Ac-Thm(1-14)-Cam-Leu-OH (14.3 mM) and crude H-Thm(15-28)-OH (17.1 mM). Errors are depicted as the SEM and the arithmetic mean is shown. The best performing variant is marked with a star (*). B) Effective yields after 60 min are shown. The effective yield represents as a virtual measure of coupling efficiency. Effective yields are calculated by multiplying the amount of product formation after 60 min (%) with the S/H ratio (% (e.g. an S/H ratio of 11.5 equals 92 %). C) Homology model of thymoligase GRDN. The enzyme backbone is shown in grey, crucial residues of the S1 pocket are shown in green and those of the S1’ pocket in magenta. Enzyme-bound partial thymosin-α1 (ITTKDL) is indicated in orange and hydrogen-bonds between the substrate and the enzyme are given in yellow. Nitrogen atoms are colored in blue, oxygen in red and hydrogens in grey/white.

With a variant in hand that shows clearly improved performance (Ptl GRD), we focused on further improving the S/H ratio. To this end, we addressed mutations at position A225, which is close to the S1’ pocket. Substitutions at this position can have positive effects, as was observed during the engineering of other peptiligase variants, such as omniligase-1. [33,34] Especially substitution A225N was shown to drastically improve
the S/H ratio of different ligases in coupling reactions with short model peptides (unpublished results) and was therefore incorporated into the Ptl GR, GRD and GRE scaffolds. Indeed, the addition of mutation A225N increased both the activity and the S/H ratio of all variants, therefore resulting in an improved effective yield of the coupling (Figure 2B). In particular, Ptl GRDN (M222G L217R S166D A225N) exhibited an exceptionally high coupling efficiency (77%) and a more than seven-fold increase of the S/H ratio compared to Ptl GRD (Figure 2A). The homology model of Ptl GRDN revealed two hydrogen bonds between S166D/14K and L217R/15D, which could potentially lead to an improved overall performance of this variant (Figure 2C). Peptiligase M222G L217R A225N, which does not have an additional negative charge in the S1 pocket, also performed extraordinarily well, with conversions similar to Ptl GRDN and an even slightly higher S/H ratio. However, the higher overall performance of Ptl GRDN emphasizes again the importance of mutation S166D for an efficient ligation (Figure 3C). The best enzyme from the stepwise rational engineering approach, i.e. Ptl GRDN, was chosen as the starting point for further optimization of the S1 pocket. Ptl GRDN carries a negative charge at the bottom of the S1 pocket (due to S166D) and we examined the effect of substitutions at position S156, located in proximity to position S166. Wells et al. showed that subtilisin BPN’ carrying negatively charged residues on positions S156 and S166 is able to form ion pairs with positively charged acyl donors and the effects on binding appeared to be additive. For example, a net charge of -2 (S166D; S156E/D) could possibly improve the binding of positively charged amino acids in the S1 pocket.

Moreover, the additional negative charge could electrostatically affect the adjacent N155, which plays a key role in transition state stabilization. Hence, the effect of the introduction of a second ion pair (i.e. S156D/E) at the S1 subsite was investigated (Figure 3A). Consequently, serine at position 156 was mutated to different polar and charged amino acids (S156X – X: E, Q, D, N, T). Non-polar amino acids served as a negative control (S156X – X: A, L). In contrast to a negative charge being generally beneficial in position 166, the effects of an additional negative charge at position 156 appeared varied. A three-fold decrease in product formation with an unaltered S/H ratio was observed for the variant with two aspartic acid residues in the S1 pocket (S166D; S156D). On the other hand, the combination of S166D and S156E is well tolerated. Only a small reduction in product formation was observed and the S/H ratio actually doubled as compared to the starting variant Ptl GRDN, however, not resulting in an increased effective yield (Figure 3C). The best mutation introducing a polar group at position 156 was S156N (Figure 3A). Despite the lack of a second charge in the S1 pocket, Ptl GRDN S156N fully retains its activity while clearly improving the S/H ratio almost three-fold. As expected, creating a smaller and more hydrophobic pocket by incorporating S156A resulted in a reduced overall performance compared to the starting variant (Figure 3A and C). The same effect was observed when a branched polar residue was introduced at position S156 (S156T). This suggests that small polar, non-charged sidechains at position 156 are a prerequisite for a high catalytic efficiency. Charges are tolerated, but not generally beneficial. Surprisingly, creating a larger S1 pocket (S156A) resulted in a higher S/H ratio and retained activity as compared to the peptiligase variant bearing serine in position 156. Therefore, an ionic interaction
of the substrate with the amino acid at position 156 seems to be beneficial, but not essential for retaining enzyme activity and obtaining high S/H ratios in the [14+14] thymosin-α, coupling.

In addition to engineering the S1 and S1’ pocket, an attempt was made to further improve the S/H ratio by varying position F189, located in the hydrophobic S2’ pocket. In the case of the thymosin-α, [14+14] coupling strategy hydrophobic 16Leu is present at position P2’. Hence, we examined the substitutions with other apolar amino acids (F189X – X: W, Y, H, V, A, I, L) that may increase the hydrophobic interactions between the enzyme and the substrate. The results clearly indicated that aromatic amino acids in position 189 exhibit a positive overall effect resulting in a remarkably increased S/H ratio, whereas small hydrophobic amino acids (V, A, I, L) led to less than 50% product formation (Figure 3B) and in general considerably reduced overall performance (Figure 3D) compared to Ptl GRDN. The aromatic rings form a more defined, rigid hydrophobic surface in the S2’ pocket and could play a role in π-stacking stabilizing interactions. Beside the hydrophobic nature of their sidechains the residues that are also hydrogen bond donors exhibit a higher overall performance (W>Y>H>F, Figure 3B). Ptl GRDN F189W (Ptl GRDNW) shows the highest product formation rate and S/H ratio, resulting in a high effective yield of the ligation (Figure 3D).

**Figure 3.** Result of testing the peptiligase variants library GRDN (M222G, L217R, S166D, A225N) S156X A) and F189X B) for the thymosin-α, [14+14]-mer coupling efficiency. Product formation (%) and S/H ratios after 60 min are shown. Coupling reactions were performed in aqueous solution (1 M phosphate pH 8.0, 3.5 mM TCEP, room temperature) using 0.35 μM of enzyme, crude Ac-Thm(1-14)-Cam-Leu-OH (7.15 mM) and crude H-Thm(15-28)-OH (8.55 mM). A slightly lower screening pH (8.0 vs. 8.3) was chosen as compared to the conditions used in Figure 2A. In addition, the concentration of enzyme and substrates were halved compared to the conditions given in Figure 2. These sub-optimal conditions were used to clearly visualize the activity and efficiency differences of the peptiligase variants. Errors are depicted as the SEM and the arithmetic mean is shown. The best hit is marked with a star (*). C) and D) Effective yields after 60 min are shown. The effective yield represents a virtual measure of coupling efficiency. Effective yields are calculated by multiplying the amount of product formation after 60 min (%) with the S/H ratio (%).
Finally, we tested whether mutations S156N and F189W had an additive effect if combined into the Ptl GRDN backbone. Indeed, Ptl GRDN F189W S156N, termed thymoligase, noticeably outperformed the best variant so far, i.e. Ptl GRDNW. A 10% higher coupling yield and a 35% higher S/H ratio was achieved (Figure 4).

The comparison of peptiligase variants from different stages unequivocally demonstrates the successful design of this powerful ligase, enabling an efficient \([14+14]\)-mer thymosin-\(\alpha\) fragment condensation. Thymoligase features a more than 5-fold higher reaction yield and a more than 20-fold increased preference for peptide bond formation over hydrolysis compared to peptiligase (see Figure 4). The effective yield of the ligation could be increased more than 10-fold (Figure 4B). In particular, designing the S1 (S166D and S156N) and S1´ (L217R) pockets by introducing electrostatic interactions with the substrate P1 and P1´ positions resulted in the successful tailoring of thymoligase towards charged substrates. F189W and A225N also represent key mutations, which resulted in an improved product formation and a significant increase of the S/H ratio (see Figure 4).

\[\text{Figure 4. Overall comparison of the evolutionary stages of the development from peptiligase to thymoligase. Results of the screening of several variants for the thymosin-}\alpha\text{-}[14+14]\text{-mer coupling efficiency are shown. A) Product formation (%)}\text{ and S/H ratios after 60 min are shown. Screening conditions are identical to the ones depicted in Figure 3. Errors are depicted as the SEM (standard error of mean) and the arithmetic mean is shown. The best hit (thymoligase) is marked with a star (*). Due to altered reaction conditions compared to those in Figure 2A, deviating values for product formation (%) and S/H ratio were obtained for peptiligase, Ptl G, Ptl GR and Ptl GRDN. The screening conditions were identical to the ones shown in Figure 3 B) Effective yields after 60 min are shown. The effective yield represents a virtual measure of coupling efficiency. Effective yields are calculated by multiplying the amount of product formation after 60 min (%) with the S/H ratio (%).}\]

\[\text{X-Ray Crystal Structure Determination}\]

In order to verify the accuracy of the models used, the three-dimensional structure of thymoligase was determined by X-ray crystallography (pdb code: 5OX2). This revealed structural conformity between the model of thymoligase used and its experimentally determined structure. The structural alignment between the homology model of thymoligase and its corresponding crystal structure has a root-mean square deviation.
(RMSD) of 0.4 Å over 266 aligned residues with 95.1% sequence identity. The general fold of subtilisin is well preserved in thymoligase and the mutations that are incorporated in the substrate binding clefts only lead to little structural change of the local topology, without disturbing the overall fold. Therefore, the assessment of our models docked with a partial substrate seemed to be suitable for a rational design approach.

Unexpectedly, in the crystal structure a nonapeptide (VEEDHVAHA) derived from the prosequence of thymoligase (residues 68'-76'; prime symbols indicate the profragment) was observed in the substrate-binding region and occupied sites S4-S2 but left position S1 empty (Figure 1 and see experimental section: Figure 7A). The nonapeptide has several hydrogen bonds to main chain atoms of thymoligase, namely 76'N-O100, 75'O-127N, 75'N-127O, 74'O-102N, 74'N-102O, 72'O-104N, 72'N-131O, 71'O-104N, 71'O-105N, 71'O-105Ser and 71'O-103Gln (see experimental section: Figure 6). The first two amino acids (V'68-E'69) of the peptide, visible in the electron density, exhibit interactions with two symmetry-related thymoligase molecules and these two amino acids are found near the S2'pocket of one of these molecules (16Leu in the peptide model). The sidechain of His75' (P3) has a hydrogen bond to Oδ₁ of Asn109 of a symmetry related molecule. Compare with other subtilisin-propeptide complexes indicated that the main chain atoms of residues 71'-76' (DHVAHA) of the nonapeptide overlay well with the propeptide observed in the crystal structure of a profragment-subtilisin BPN' complex (pdb code: 1SPB) but deviate at residues 68'-70' (VEE), while His75' also has a different conformation. Moreover, the main chain atoms of the nonapeptide also overlap with the stabilized subtilisin prodomain in 3CNQ. This prodomain extends across the (modified) active site of the S221A mutant by addition of four residues at its C-terminus (from 68'-79' VEEDKLYRALSA, in which H72'K, A74'Y, H75'R and Y77'L are mutated).

When the 4D-1E fragment of thymosin-α₁ (bold in Ac-SDADVDSSEITTKDLKKEKKKEVVEAEN-NH₂) was modeled in the active site of thymoligase, with 11I-16L in the P4-P2' subsites, it appeared that residues P4-P2 of thymosin-α₁ (ITT) and of the propeptide (AHA) had the same position, but the peptide conformations may be different beyond this region (Figure 7C). The mutations S156N, at the S1 cleft rim, and S166D, at the bottom of the S1 site, create a hydrogen bond between Nδ₁ of N156 and Oδ₁ of D166 (2.9 Å). The walls of the S1 pocket are shaped by G118, G119 and backbone atoms of L117 on one side, and N146 on the other side. The Ne of 14Lys would perfectly fit in this modified S1 pocket and would be capable of making a salt bridge to O62 of D166, similar to the observed hydrogen bond between the Ne of 14Lys and O62 of D166 in the model of peptiligase GRDN. However, an irregular, disordered structure of substrate residue 14Lys in models of different peptiligase variants suggests that there is not a clearly defined orientation of this residue.

Generally, the inspection of the thymoligase crystal structure can explain its improved performance in thymosin-α₁ synthesis. Two important amino acids shaping the most discriminating nucleophile binding pocket S1' for peptide ligation are M222 and L217. Mutation M222G was expected to enhance the size of the
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...pocket and thereby to widen the range of amino acids that is accepted. Indeed, the S1’ pocket is much widened in the crystal structure while the φ/ψ angles of residue M222G are not changed. Mutation A225N stabilizes the α-helix in which it is located with the formation of extra hydrogen bonds, also to the sidechains of N123 and S125 and the main chain of 221O. As a result, the S1’ pocket has become more polar so that the carboxylate sidechain of 15Asp of the thymosin-α1 acyl acceptor fragment is better accommodated. Mutation L217R increases the size of the S1’ pocket even more by folding back the R217 sidechain to the β-strand and making a hydrogen bond with 216O. Unexpectedly, the hydrophobicity of the S1’ pocket is not changed by this substitution, and the C62 of L217 overlaps with the C6 of L217R. The important thymoligase F189W mutation in the S2’ pocket, which should accommodate 16Leu, has no influence on the local backbone conformation, and the six-membered ring of the W189 sidechain superimposes well on the wild type F189 sidechain. However, the larger surface of the Trp sidechain enlarges a platform that can contribute to hydrophobic contacts with the peptide 16Leu sidechain and therefore enhance the coupling efficiency.

In spite of the overall good agreement between the model used for designing the mutations and the crystal structure of thymoligase, there were some deviations. For example, the striking effect of the A225N mutation was originally suspected to be due to binding of water between His67 and Asn225, but these are 1.6 Å more apart in the model as compared to the crystal structure (4.8 Å). In addition, the conformation of Cys221 in the model is different from the conformation observed in the crystal structure and in 1GNV and 1SBN. In the model, the sulfur atom is directed towards M222G while in the crystal structure it is directed between P1 and P1’, which more closely reflects an active conformation. In contrast, some active site backbone changes in the crystal structure are smaller than expected.

[14+14] Fragment Ligation using Thymoligase on Gram Scale

The solid-phase synthesis of both [14]-mer fragments was improved at gram scale to 84% yield and 73% HPLC purity (net peptide content 64%) for the acyl donor Cam-ester fragment and 75% crude yield and 88% HPLC purity (net product content 90 %) for the acyl acceptor fragment, respectively (for details of the synthesis see experimental section and supporting information). After the discovery of thymoligase and the improved SPPS of the respective 14-mer fragments on larger scale, we successfully demonstrated the scalability of the [14+14] thymosin-α1 fragment condensation. Using thymoligase (0.0002 molar equivalents) under optimized reaction conditions (1 M phosphate, pH 8.3, 3.5 mM TCEP, room temperature) the crude acyl donor fragment Ac-Thm(1-14)-Cam-Leu-OH was coupled to only 1.2 eq. of the crude acyl acceptor fragment H-Thm(15-28)-OH on gram scale. To the dissolved acyl acceptor fragment (1.2 eq.) a total amount of 1 g crude Cam-ester fragment was dosed over time (200 mg every 30 min), corresponding to a final crude substrate concentration of more than 250 g/L. The addition of TCEP to the reaction mixture was crucial, since the enzyme was significantly more active under reducing conditions. Quantitative conversion of the acyl
donor fragment after 4 hours of reaction time resulted in >94% product yield (Figure 5A). Less than 6% of ester hydrolysis was observed (Figure 5A), corresponding to an S/H ratio of more than 16. The rate determining step of the reaction appeared to be the dissolution of the Cam-ester fragment. The dissolved part of the Cam-ester fragment dissolved, it was immediately and predominantly converted to thymosin-\(\alpha_1\) product, thus limiting the amount of ester hydrolysis. The crude HPLC purity of thymosin-\(\alpha_1\) in the reaction mixture could be increased from 62% to 78% by precipitation of the product at pH 3.8 and subsequent washes with a buffer solution (100 mM ammonium acetate, pH 3.8, 0°C). A single RP-HPLC purification of the crude reaction mixture resulted in over 98% HPLC purity (Figure 5B). The net peptide content of the highly enantiopure product was determined to be 82.4% (C.A.T., Tübingen, Germany). A clearly improved separation of impurities from the product peak during HPLC purification was achieved compared to straight-through SPPS of the 28-mer and the overall yield of the CEPS approach was calculated to be 55%, which is more than twice as high in comparison to yields using straight-through SPPS. In addition, using doped reversed phase (DRP) HPLC purification (Zeochem DRP 120 C5 / 10 \(\mu\)m column material, gradient: 5-8% acetonitrile in 40 min) we further reduced the solvent consumption during HPLC purification by more than 50% while retaining the product purity and overall yield.

**Figure 5.** A) HPLC chromatogram \((\lambda = 220\text{ nm})\) of the crude reaction mixture of a \((14+14)\)-mer thymosin-\(\alpha_1\) condensation on gram scale using thymoligase. Product formation was 94% with only 6% of Cam-ester hydrolysis. CEPS was performed at room temperature (20°C) in aqueous solution (1 M potassium phosphate pH 8.3, 1 mg mL\(^{-1}\) TCEP; 1 g Ac-Thm(1-14)-Cam-Leu-OH with 1.2 eq. of the amine fragment H-Thm(15-28)-OH were used. B) Zoom into the HPLC spectrum \((\lambda = 220\text{ nm})\) of thymosin-\(\alpha_1\) after a single HPLC purification.
Conclusion

Chemo-enzymatic peptide synthesis appears to be a viable and scalable strategy for the efficient and cost-optimized production of thymosin-α₁ using a [14+14]-mer ligation strategy on gram scale (>94% coupling yield). During several generations of structure-inspired enzyme engineering we discovered thymoligase, an enzyme that features a more than 5-fold higher catalytic efficiency and a more than 20-fold increased S/H ratio compared to its parent enzyme peptiligase. Thymoligase was specifically tailored for the thymosin-α₁ [14+14] coupling by introducing selected key mutations in the respective pockets, i.e. to accept positively and negatively charged amino acid residues in position P₁ and P₁'. We expect thymoligase to be well applicable to many other ligations in which residues with similar characteristics (e.g. Arg and Glu) are present in the respective positions P₁ and P₁'. The quality of the homology models used for the design approach was examined by comparison to the 3D-structure obtained by X-ray crystallography. This indicated good overall agreement, but local deviations are observed, especially in the binding of water molecules to mutated sidechains that influenced synthetic performance. Using the optimized enzyme scaffold of thymoligase for the [14+14]-mer fragment condensation we were able to synthesize thymosin-α₁ in a significantly increased yield (55%), more than twice as high as compared to those typical for existing industrial processes. Due to an improved separation of impurities from the product peak compared to straight-through SPPS the product was obtained in over 98% purity after only a single HPLC purification. Therefore, this process could lead to very significant reduction of manufacturing costs. In addition, the CEPS strategy does not only provide a promising avenue for the synthesis of thymosin-α₁, but can also be used to produce several other linear (e.g. the incretin mimetic exenatide) and cyclic therapeutic peptides. Therefore, CEPS represents a powerful and broadly applicable tool for the large-scale production of peptide therapeutics.
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Experimental Section

Associated content

The Supporting Information of this article is available free of charge on the Organic & Biomolecular Chemistry publications website at DOI: 10.1039/C7OB02812A.

Enzyme Engineering: Computational Approach

Models of peptiligase with the protease inhibitor eglin bound to the active site were generated based on a hybrid model, which was constructed with YASARA using the pdb files 1GNV\(^{30}\) and 1SBN\(^{31}\) as templates. Further mutations were incorporated using a YASARA script and a fragment of thymosin-\(\alpha_{1}\) [7-19] modelled into the substrate binding region. The YASARA script applies the desired sidechain mutations and allows the surrounding residues to adapt to the mutations. After the new mutations were introduced into the three-dimensional structure of the protein, the new mutations were optimized by 6 repetitive cycles of energy optimization with stepwise dead-end elimination (DEE) optimization based on rotamers, followed by a local energy minimization in water. The volume that is energy optimized starts at 7 Å from the mutated residues and increases with 1 Å every cycle, finally resulting in an energy optimization of the entire enzyme scaffold. The numbering of mutations is based on subtilisin BPN’\(^{39}\).

Construction of Peptiligase Variants.

Peptiligase variants were prepared by Quikchange site-directed mutagenesis using pBE-S-peptiligase vector as the template plasmid\(^{12}\). The *E. coli*-B. subtilis shuttle vector pBE-S (Takara Bio Inc.) included the *aprE* promoter sequence, the peptiligase secretion signal peptide and the sequence followed by a C-terminal hexahistidine tag and a terminator sequence. DpnI-digested and purified plasmids were transformed to competent *E. coli* TOP10 and transformants were plated on LB-agar plates containing 100 \(\mu\)g/mL ampicillin. The mutant genes were confirmed by DNA sequencing.

Expression and Purification of Peptiligase Variants

For production of the enzyme variants the respective mutants vector was transformed into *B. subtilis* GX4935 (trpC2 metB10 lys-3 ΔaprE ΔoprE), which is a strain deficient in extracellular neutral and serine proteases and was a kind gift from Prof. P.N. Bryan (University of Maryland, USA)\(^{40}\). After overnight incubation on LB-agar plates colonies were picked and grown overnight in 5 mL LB with kanamycin (10 \(\mu\)g/mL) at 37°C in a shaking
Design of a Substrate-Tailored Peptiligase Variant for the Synthesis of Thymosin-α

incubator. 0.6 mL of the culture were added to 30 mL Terrific Broth (TB) medium supplemented with antibiotic (kanamycin 10 μg/mL) and amino acids (100 mg/L Trp, 100 mg/L Met and 100 mg/L Lys). The cells were grown for 48 h at 37°C in a shaking incubator, before being harvested by centrifugation (4°C). The supernatant was concentrated by ultrafiltration (Amicon-centrifugal unit, 10 kDa MW cut-off) in two centrifugation steps. The concentrated medium was then exchanged to buffer A (25 mM Tricine, 0.5 M NaCl, pH 7.5). Talon resin (Clontech) was used for gravity-flow His-tag purification. After washing the resin with MilliQ water (5 column volumes, CV) and equilibration with buffer A (10 CV), the crude enzyme was loaded on the column and washed with 10 CV buffer A. The enzyme was eluted with 3 CV buffer B (25 mM Tricine, pH 7.5, 0.5 M NaCl, 200 mM imidazole). The eluate was concentrated with an Amicon-centrifugal unit (10 kDa MW cut-off) by centrifugation and the buffer was exchanged to the storage buffer (25 mM Tricine, pH 7.5). The protein concentration was determined using UV/VIS spectrophotometry (λ = 280 nm) and the purity was assessed via SDS-PAGE. The purity was estimated to be > 80%. The purified enzyme was flash-frozen in liquid nitrogen and stored at -80°C until further use.

HPLC-MS Analysis

The peptide purity was assessed using an Agilent 1260 Infinity HPLC system coupled with an Agilent 6130 quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) to determine the peptide mass. Separation was performed using ReProSil-Pur C18 5μm, 4.6 x 250 mm (Dr. Maisch, Ammerbuch, Germany) column, eluting with 0.05% (v/v) MSA in a water acetonitrile gradient, with a flow rate of 1 mL/min. As mobile phase a binary mixture of A (water + 0.05% (v/v) MSA) and B (acetonitrile (ACN) + 0.05% (v/v) MSA) was used. A linear gradient from 5 to 98% B in 21 minutes, followed by isocratic 95% B for 5 min was used by default. For optimal results an appropriate gradient was chosen for each sample individually (see Table 1). The purity of peptides was determined by automatically integrating product and impurity peaks of the relevant HPLC spectrum (λ = 220 nm).

HPLC-MS-MS analysis was performed by DSM Resolve (Geleen, The Netherlands).
Table 1. Individual conditions for HPLC-MS analysis of peptide fragments.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Thm(1-14)-Cam-Leu-OH</td>
<td>12 to 30% (v/v) ACN/ MQ-water (0.05% (v/v) MSA) in 21 min; flow 1 mL/min; 40°C column temperature</td>
</tr>
<tr>
<td>H-Thm(15-28)-OH</td>
<td>5 to 14% (v/v) ACN/ MQ-water (0.05% (v/v) MSA) in 30 min; flow 1.5 mL/min; 20°C column temperature</td>
</tr>
<tr>
<td>Ac-Thm(1-28)-OH*</td>
<td>12 to 30% (v/v) ACN/ MQ-water (0.05% (v/v) MSA) in 21 min; flow 1 mL/min; 40°C column temperature</td>
</tr>
</tbody>
</table>

* Method also used for thymoligase library screenings.

Testing of Peptiligase Variants

The ligation reactions were typically performed at 20°C in a 96-well plate. Two different stock solutions were prepared, i) crude Ac-Thm(1-14)-Cam-Leu-OH (7.15 mM) and crude H-Thm(15-28)-OH (8.55 mM, 1.2 eq.) in 1 M potassium phosphate buffer (pH 8.0, 3.5 mM TCEP) and ii) enzyme solution in MiliQ water (0.2 mg/ mL of each peptiligase variant). The reactions were started by adding 190 μL of solution i) to 10 μL of solution ii). Aliquots of 8 μL were quenched in 1 mL acetonitrile (ACN)/ H2O (1:2 (v/v); 0.2% (v/v) methanesulfonic acid (MSA)) after 2.5, 5, 7.5, 10, 15, 30 and 60 min and analyzed by RP-HPLC (λ=220 nm) using a ReProSil-Pur C18 5 μm, 4.6 x 250 mm (Dr. Maisch, Ammerbuch, Germany) column. As mobile phase a binary mixture of A (water+ 0.05% (v/v) MSA) and B (ACN+ 0.05% (v/v) MSA) was used by default. Samples were separated using a gradient of 12 to 30% (v/v) B in A in 21 min with a flow of 1 mL/min and 40°C column temperature. All measurements were performed in triplicate and the arithmetic mean was calculated. Errors are depicted as the SEM (standard error of mean). The product formation was calculated by automatically integrating the HPLC peaks of the Cam-ester, its hydrolysis product and the corresponding coupling product. The synthesis over hydrolysis ratio (S/H ratio) was calculated after every time point by dividing the amount of product by the amount of hydrolyzed Cam-ester. Because some spontaneous hydrolysis occurs while dissolving the substrates before the reaction start, values were corrected for initial chemical background hydrolysis by subtracting the percentage of hydrolysis of blank Ac-Thm(1-14)-Cam-Leu-OH (without enzyme) at t= 2.5 min from each value measured in time. Effective yields are given as a virtual measure of coupling efficiency. They were calculated by multiplying the amount of product formation after 60 min (%) with the S/H ratio (%).

After screening the first variants (Figure 2) the substrate and enzyme concentration was halved, and pH was lowered to pH 8.0 for the second and third screening (Figure 3 and 4) in order to clearly highlight the differences between peptiligase variants. Differences between the enzyme variants are easier to visualize using...
Design of a Substrate-Tailored Peptiligase Variant for the Synthesis of Thymosin-α1

these slightly adapted conditions: 1 M phosphate pH 8.3, 3.5 mM TCEP, room temperature, 0.7 μM of enzyme, crude Ac-Thm(1-14)-Cam-Leu-OH (14.3 mM) and crude H-Thm(15-28)-OH (17.1 mM).

Synthesis of Fmoc-Lys(Boc)-O-Glycolic Acid (according to Nuijens et al.\[41\])

2-CTC resin (50 g, 55 mmol) was swollen in dichloromethane (DCM) and washed with dimethylformamide (DMF). The resin was activated with a 15% (v/v) solution of thionyl chloride in DMF by stirring overnight. The resin was washed with DMF and DCM. Glycolic acid (0.11 mol, 2 eq.) was dissolved in DCM and DIPEA (0.22 mol, 4 eq.) was added. The resulting solution was added to the resin and the mixture was stirred for 60 min, before the resin was washed with DCM and unreacted chloride was capped by adding a mixture of DCM/methanol/DIPEA (80/15/5 (v/v/v)). The resin was washed with DCM and DMF, and the ester bond was formed by adding a solution of Fmoc-L-Lys-OH (0.22 mol, 4 eq.), DMAP (22 mmol) and DIC (0.22 mol, 4 eq.). The mixture was stirred for 60 min, the resin was washed with DMF and DCM. Cleavage was performed with 5% TFA (v/v) in DCM (3x 15 min). The filtrates were collected and washed with demineralized water and with brine. The organic phase was collected and dried on sodium sulfate. After washing the solid with DCM, the combined filtrates were concentrated in vacuo to yield a white powder (26 g, 49.4 mmol, 90% yield). The final product was purified via preparative HPLC.

Synthesis of Ac-Thm(1-14)-Cam-Leu-OH

7.2 gram of Fmoc-Leu Wang resin (loading of 0.7 mmol/g) was washed with DCM and DMF. Fmoc deprotection was performed using 20% (v/v) piperidine/DMF (2x 8 min). After washing with DMF, DCM and DMF, Fmoc-Lys(Boc)-O-glycolic acid (10 mmol) was coupled to the resin using HBTU (4 eq.) and OxymaPure (4 eq.) in DMF (45 min). After washing with DMF, DCM and DMF, Fmoc deprotection was performed as described. The following Fmoc protected amino acids were coupled using 4 eq. Fmoc-AA-OH, HBTU (4 eq.), OxymaPure (4 eq.) and diisopropylethylamine (DIPEA; 8 eq.) in DMF for 45 min. After washing with DMF, DCM and DMF, cycles of washing, deprotection and coupling were performed repeatedly to elongate the peptide. In order to overcome severe diketopiperazine formation and to prevent β-sheet formation of the aggregation prone sequence \[^{11}\text{Ile}^{12}\text{Thr}^{13}\text{Thr}^{14}\text{Lys}^{26}\] two pseudoproline building blocks, i.e. Fmoc-L-Asp(tBu)-L-Thr[ψ(Me,Me)Pro]-OH and Fmoc-L-Thr(tBu)-L-Thr[ψ(Me,Me)Pro]-OH, were introduced at positions \[^{6}\text{Asp}^{7}\text{Thr}^{12}\text{Thr}^{13}\text{Thr}\]. In these cases, the coupling time was extended to 90 min. Finally, N-terminal acetylation was performed using a mixture of DMF/OxymaPure/DIPEA/AC₂O (450 mL/1.03 g/9.82 mL/21.3 mL) for 30 min. Cleavage from the resin and sidechain deprotection was performed using a mixture of trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water (95/2.5/2.5, v/v/v) for 120 min. The crude peptide was precipitated using methyl tert-butyl ether (MTBE)/n-heptane (1:1 (v/v)).
followed by drying under reduced pressure at 35°C overnight. Lyophilization of the peptide fragment was omitted in order to reduce the amount of aspartimide formation.

**Ac-Thm(1-14)-Cam-Leu-OH:**

\[
\text{Ac}^1\text{Ser}^2\text{Asp}^3\text{Ala}^4\text{Ala}^5\text{Val}^6\text{Asp}^7\text{Thr}^8\text{Ser}^9\text{Thr}^{10}\text{Ile}^{11}\text{Thr}^{12}\text{Thr}^{13}\text{Lys}^{14}\text{Cam-Leu-OH}
\]

Mol. weight: 1637.71 g mol\(^{-1}\) (C\(_{67}\)H\(_{112}\)N\(_{16}\)O\(_{31}\))

**Synthesis of H-Thm(15-28)-OH**

18.5 g of Fmoc-Asn(Trt) Wang resin (loading of 0.27 mmol/g) was washed with DCM and DMF. Fmoc deprotection was performed using 20% (v/v) piperidine/DMF (2x 8 min). After washing with DMF repetitive cycles using standard SPPS protocols (4 eq. Fmoc-AA-OH, 4 eq. HBTU, 4 eq. OxymaPure, 8 eq. DIPEA, 45 min) were followed to elongate the peptide, with couplings of amino acids 15 to 21 performed for 90 min. In general, a low resin loading and double couplings in a sequence of mostly charged amino acids significantly increased the overall purity. Cleavage from the resin and sidechain deprotection was performed using a mixture of TFA/TIS/phenol (95/2.5/2.5, v/v/v) for 120 min. The crude peptide was precipitated using MTBE/n-heptane (1:1 (v/v)), followed by drying under reduced pressure at 35°C overnight. Lyophilization of the peptide fragment was omitted in order to reduce the amount of deamidation of the C-terminal Asn.

**H-Thm(15-28)-OH:**

H^15\text{Asp}^{16}\text{Leu}^{17}\text{Lys}^{18}\text{Glu}^{19}\text{Lys}^{20}\text{Lys}^{21}\text{Glu}^{22}\text{Val}^{23}\text{Val}^{24}\text{Glu}^{25}\text{Glu}^{26}\text{Ala}^{27}\text{Glu}^{28}\text{Asn-OH}

Mol. weight: 1659.81 g mol\(^{-1}\) (C\(_{70}\)H\(_{118}\)N\(_{18}\)O\(_{28}\))

**([14+14]-mer CEPS of Thymosin-α\(_1\))**

The crude amine nucleophile fragment H-Thm(15-28)-OH (1.1 g, 0.47 mmol) was dissolved in 5 mL potassium phosphate buffer (1 M, pH 8.3, 3.5 mM TCEP) and 2 mg of thymoligase (0.0002 molar eq.) were added from a stock solution (c= 26 mg/mL). The crude Cam-ester fragment Ac-Thm(1-14)-Cam-Leu-OH (1 g, 0.37 mmol) was dosed over time (200 mg every 30 min). The pH was kept constant at pH 8.3 (titration with acid/base), resulting in a final reaction volume of 7.5 mL. The mixture was stirred at room temperature (20°C) for a total of 4 hours. The reaction progress was monitored by HPLC-MS (λ= 220 nm). The crude reaction mixture was diluted with ACN/H\(_2\)O (3:1 (v/v)) to 100 mL and 50 mL were directly purified by RP-HPLC and freeze-dried. Thymosin-α\(_1\) was obtained in an overall yield of 55%. For purification a Luna
Prep C18 column (10 μm, 200x 51 mm, Phenomenex, Torrance, CA, USA) with a gradient of 16 to 28% ACN/ H2O (0.05% (v/v) TFA) in 42 min and a flow rate of 45 mL/min was used.

### X-ray Crystal Structure Determination

Thymoligase was further purified after the His-tag purification by gel filtration using a Superdex 200 HR10/30 column (GE Healthcare), equilibrated with 20 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer, pH 7.5, containing 150 mM NaCl. The enzyme eluted at a molecular mass of 20 kDa. Thymoligase fractions were pooled and concentrated to 17.5 mg/mL using a Vivaspin-10K filter unit (Sartorius). Dynamic light scattering experiments were performed using a DynaPro Nanostar instrument (Wyatt Technology Corporation, Santa Barbara, CA, USA) at 20°C. Dynamic light scattering data were processed and analyzed using Dynamics software and an apparent molecular mass of 41 kDa was deduced with a polydispersity of 12%.

Crystallization trials were performed in 96-well MRC2 plates (Molecular Dimensions Ltd., Newmarket, UK), using a Mosquito crystallization robot (TTP Labtech Ltd. Melbourn, UK) with commercially available screening matrices (PACT, JCSG+ and Wizard I&II, from Molecular Dimensions). Droplets containing reservoir solution (75-125 nL) and protein solution (125-75 nL) were incubated against 50 μL of each reservoir solution at 21°C. Plate-like thymoligase crystals were obtained from 1.0 M ammonium sulfate, 0.1 M Bis-Tris pH 5.5 and 1% PEG 3350.

Prior to data collection, single crystals were harvested and flash-cooled in liquid nitrogen with addition of 25% glycerol as cryoprotectant. A native dataset to 2.25 Å was collected on beamline P11 at Petra III (EMBL, DESY, Hamburg, Germany) at 100 K. Intensity data were processed using XDS\(^{42}\) and the CCP4 package.\(^{43}\) The space group was \(P2_1\), with unit cell dimensions of \(a = 47.5, b = 40.1, c = 64.3\) Å and \(\beta = 106.0^\circ\). With one monomer of 27.4 kDa in the asymmetric unit, the \(V_M\) is \(2.1\) Å\(^3\) Da\(^{-1}\).\(^{44}\) With a calculated solvent content of 43%. A summary of data collection statistics is given in Table 2.

Molecular replacement was performed using PHASER\(^{45}\) with the thymoligase model obtained by YASARA. ARP/wARP\(^{46}\) was used for automatic building, and the model was refined using REFMAC5\(^{47}\). COOT\(^{48}\) was used for manual rebuilding and map inspection. In Fo–Fc maps, clear electron density was present for a nonapeptide in the active site which was derived from the propeptide of thymoligase.

The quality of the model was analyzed using MolProbity.\(^{49}\) Figures were prepared using PYMOL (Schrödinger LLC). Atomic coordinates and experimental structure factor amplitudes for thymoligase have been deposited in the RCSB Protein Data Bank under accession code 5OX2.
### Table 2. Crystallographic data and refinement statistics. Values in parentheses are for the highest resolution shell.

<table>
<thead>
<tr>
<th>Data Collection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Spacegroup</td>
<td>P2₁</td>
</tr>
<tr>
<td>Unit cell dimensions (Å, °)</td>
<td>a = 47.5, b = 40.1, c = 64.3, β = 106.0</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>45.6 - 2.24</td>
</tr>
<tr>
<td>No total measurements</td>
<td>36626 (3300)</td>
</tr>
<tr>
<td>No unique reflections</td>
<td>10355 (950)</td>
</tr>
<tr>
<td>R_pim (%)</td>
<td>7.1 (35.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>91.6 (92.1)</td>
</tr>
<tr>
<td>Average I/σ</td>
<td>7.8 (2.7)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>3.5 (3.5)</td>
</tr>
</tbody>
</table>

| Refinement              |                  |
| Contents of A.U.        |                  |
| protein                 | 2 chains, residues 1 – 266 and residues 1 - 9 |
| waters                  | 52               |
| other                   | 4 sulfate ions   |
| R/ R_free               | 18.8 / 23.0      |

| Geometry                |                  |
| r.m.s.d. bonds (Å)      | 0.009            |
| r.m.s.d. angles (°)     | 1.4              |
| Ramachandran plot (%)   | 97.8 / 1.5 / 0.7 |
Figure 6. A LigPlot+ diagram\cite{10} of the peptide binding site of thymoligase with orange bonds and residue names and the nonapeptide (VEEDHVAHA) (residues 68'-76', chain P) with purple bonds and residue names. The green dashed lines, with distances, represent the hydrogen bonds between nonapeptide and thymoligase. Hydrophobic contacts made with the nonapeptide are indicated by the spoked arcs.
Figure 7. Peptide binding sites in thymoligase. A) The residues names with prime belong to the nonapeptide in cyan sticks and the others are the hydrogen bonded residues from the protein in green. Hydrogen bond interactions between thymoligase and nonapeptide are indicated with black dashed lines. B) Fo-Fc omit electron density for the nonapeptide in cyan sticks is shown at 3σ in light orange. C) Modeling of thymosin-α. N156 and D166, the catalytic residue C221, N225, R217, and W189 are shown in green sticks. Residues from thymosin-α: E10-17K are shown in salmon sticks and italic letters. The nonapeptide is shown in cyan.
References
