Synthesis of Thymosin-\(\alpha_1\) using CEPS: a novel and scalable process

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Chemo-enzymatic peptide synthesis (CEPS), which combines conventional chemical SPPS with an enzymatic, racemization-free coupling of unprotected peptide segments in water, bears great potential to strongly improve peptide manufacturing on industrial scale. A well-known difficult to synthesize peptide via full SPPS is thymosin-\(\alpha_1\)\(^1\), an acetylated 28-mer therapeutic peptide. During the past decades the interest of pharmaceutical companies promoted the development of several solid- and solution phase strategies for the synthesis of thymosin-\(\alpha_1\), repeatedly resulting in low overall yields (generally < 25 %).

A [14+14]-mer chemo-enzymatic segment condensation strategy based on the use of peptiligase\(^2\) was chosen to synthesize this peptide. Enzyme engineering yielded thymoligase, an enzyme capable of catalyzing peptide bond formation between both 14-mer segments with exceptional efficiency (>94 % yield). The hydrolysis of the acyl donor segment (Cam-ester) is minimized to < 6 %.

I) Segment Synthesis via Fmoc SPPS

\[
\text{Ac-}^{\text{Fmoc}}\text{-Asp(4Glu)}\text{-}^{\text{Fmoc}}\text{-Leu-Wang resin (0.7 mmol/g)}
\]

\[
\text{Fmoc: 20 % Fmoc 2x 8 min}
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\[
\text{Coupl: 4 eq. HBTU, Oyana, AcDIEA (8 eq.) in DMF (45 min)}
\]

**Thymosin-\(\alpha_1\) Cam-ester**
- pseudoproline 90 min coupl.
- Fmoc Leu-Wang resin (0.7 mmol/g)

**Thymosin-\(\alpha_1\) amine**
- AA15-Asp(4Glu) 2x 45 min coupling
- Fmoc-Asp(Trt) Wang resin (0.29 mmol/g)

**5.0 scale/ mmol**
- crude yield/ %
- crude HPLC purity/ %

5.0
84
73

**Rational design after inspection of homology models.**

II) Enzyme Engineering: Thymoligase

**Starting point:** Peptiligase\(^4\)
- engineered subtilisin variant
- 6 substrate binding pockets (S1 - S4 & S1' & S2')

**BUT [14+14] thymosin-\(\alpha_1\) coupl. → low yields (15%)**

**Enzyme Engineering**
- e.g. introduction of charged residues
- formation of new H-bonds
- 

**Thymoligase**

- [14+14] coupling crude segments
- 1 M phosphate, pH 8.0
- Enzyme induction

**Product formation after 60 min/ %**
- 5x

**Synthesis/hydrolysis ratio after 60 min**
- 20x

**Conclusion**
- Synthesis of segments optimized (> 70 % crude yield & purity)
- High process productivity (crude substrate conc. > 250 g L\(^{-1}\))
- > 2-fold higher overall yield (55 %) compared to full SPPS
- Final product within originator’s specifications after 1x HPLC purif.

Greener, scalable process for manufacturing thymosin-\(\alpha_1\) with a potential cost-price reduction of over 50 %

Enzyme engineering yielded Thymoligase:
- tailored towards the acceptance of charged residues in pockets S1 (positive) and S1' (negative)
- engineering of hydrogen bonds for better substrate recognition
- 5x higher reaction rate & 20 x higher S/H ratio

Efficient, epimerization-free thymosin-\(\alpha_1\) [14+14] segment condensation in water with 94 % yield coupling efficiency

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\(^1\) A. Toplak, T. Nuijens, P. J. L. M. Quaedflieg, B. Wu, D. B. Janssen, Thymoligase: a potential strategy of over 50 % enzymatic, hydrolysis full of thymoligase.
