Enzyme and Microbial Technology 73–74 (2015) 20–28

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

# Peptide synthesis in neat organic solvents with novel thermostable proteases

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ARTICLE INFO

*Article history:*
Received 9 October 2014
Received in revised form 24 February 2015
Accepted 3 March 2015
Available online 11 March 2015

*Keywords:*
Thermostable
Thermophile
Protease
Subtilase
Organic solvent
Peptide synthesis

ABSTRACT

Biocatalytic peptide synthesis will benefit from enzymes that are active at low water levels in organic solvent compositions that allow good substrate and product solubility. To explore the use of proteases from thermophiles for peptide synthesis under such conditions, putative protease genes of the subtilase class were cloned from *Thermus aquaticus* and *Deinococcus geothermalis* and expressed in *Escherichia coli*. The purified enzymes were highly thermostable and catalyzed efficient peptide bond synthesis at 80 °C and 60 °C in neat acetonitrile with excellent conversion (>90%). The enzymes tolerated high levels of *N*,*N*-dimethylformamide (DMF) as a cosolvent (40–50% v/v), which improved substrate solubility and gave good conversion in 5+3 peptide condensation reactions. The results suggest that proteases from thermophiles can be used for peptide synthesis under harsh reaction conditions.



## 1. Introduction

The discovery of novel peptide receptors and of bioactive peptides that modulate receptor- and enzyme activity have resulted in a growing importance of peptides for the pharmaceutical, food and cosmetics industries [1–4]. Synthesis of bioactive peptides can be performed chemo-enzymatically by enzymatic coupling of peptide fragments obtained by solution- or solid-phase peptide synthesis (SPPS). For these conversions, a protease is applied in the synthetic direction [5]. In kinetically controlled coupling reactions, an activated acyl donor reacts with the enzyme and forms an acyl-enzyme intermediate that provides the N-terminal segment of the peptide product. If selectivity at the N-terminus is too restricted, substrate mimetics or strong leaving groups may be applied [6]. The intermediate undergoes an aminolytic reaction with the amine terminus of a nucleophilic peptide that becomes the C-terminal segment of the product. If these coupling reactions are carried out in an aqueous environment, the nucleophile needs to act in competition with water to cleave the acyl-enzyme intermediate and form a peptide bond; otherwise hydrolysis of the acyl-enzyme by water will occur. The influence of enzyme properties and reaction conditions on synthesis/hydrolysis ratio's in aqueous systems is well explored for other applications of hydrolytic enzymes in coupling reactions [7,8]. Solely synthetic product without acyl donor hydrolysis should also be obtained when water is omitted from the reaction mixture, i.e. by using neat organic solvents [9,10]. This obviously requires stability of the enzyme during preparation and use of water-free protein [11].

Enzymatic coupling of peptide fragments in organic solvents requires a broad-substrate-range enzyme that is stable and active in the absence of water. The industrial serine protease from *Bacillus licheniformis* (subtilisin A, subtilisin Carlsberg, Alcalase) has been used with success in peptide synthesis in neat organic solvent [12,13] with substrates carrying multiple side-chain protective groups to enhance solubility [14]. Peptide solubility may also be enhanced by increasing the reaction temperature and/or by the addition of an adequate cosolvent, such as dimethylformamide (DMF). Many enzymes show high thermal stability in anhydrous medium [15], but chemo-enzymatic peptide synthesis in neat organic solvent systems is generally performed at modest reaction temperatures, i.e. 25–37 °C [10,14,16–19]. The use of higher temperatures is rare (50 °C [20]). DMF can be added to increase substrate solubility in enzymatic coupling reactions [21,22], but it may negatively affect enzyme activity and, dependent on the substrate, the activity of subtilisin in neat DMF may be strongly reduced [23]. Protein engineering has been used to increase aqueous DMF

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http://dx.doi.org/10.1016/j.enzmictec.2015.03.003
0141-0229/

resistance [24–26], but it remains to be established if such variants are active in peptide synthesis in anhydrous media mixed with DMF.

Enzymatic coupling at elevated temperatures in anhydrous medium with DMF as cosolvent would combine benefits of suppressed hydrolysis by the absence of water and increased substrate solubility due to high temperature and the presence of DMF. Since solvent stability and enzyme thermostability are often correlated [27,28], we decided to explore subtilisin-related enzymes from thermophilic organisms for peptide coupling under anhydrous conditions. In this paper, we report the identification of two highly thermostable proteases and their synthetic performance in peptide synthesis at high temperature in acetonitrile–DMF solvent mixtures.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Restriction enzymes were from New England BioLabs (Beverly, MA, USA), the PCR system was supplied by Finnzymes (Vantaa, Finland), and the LigaFast ligase system and trypsin were from Promega (Madison, WI, USA). Sypro Orange dye was from Molecular Probes (Life Technologies, Carlsbad, CA, USA). All chemicals and subtilisin A type VIII (Carlsberg, Alcalase) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Organic solvents were dried over 3 Å 4–8 mesh molecular sieves, activated by heating at 200 °C. Peptide amides were obtained from Sigma–Aldrich and Bachem (Bubendorf, CH). C-terminal carbamoylmethyl (Cam, glycolamide) esters of peptides were synthesized by SPPS as reported by de Beer et al. [29].

### 2.2. Bacterial strains and plasmids

Genomic DNA of *Thermus aquaticus* Y51MC23 and the expression strain *Escherichia coli* C43(DE3) were obtained from Lucigen (Middleton, WI, USA). *Deinococcus geothermalis* (DSM11300) cells were purchased from DSMZ (Braunschweig, Germany). Cloning plasmids included pBADMycHisA(NdeI), which is pBADMyc (Life Technologies) with the NcoI site mutated to an NdeI site, and pET28a(+) from Novagen (Merck KGaA, Darmstadt, Germany).

### 2.3. Enzyme production

The gene coding the putative protein ZP_03495941 from *T. aquaticus* (*Taq*Sbt) was amplified from genomic DNA with a modified start codon to include an NdeI restriction site and a modified stop codon to incorporate a Gln codon preceding the added HindIII restriction site. An optimized touch-up PCR protocol for GC-rich sequences was performed to obtain PCR product (Supplemental material Table S1). Purified PCR products were ligated into the NdeI and HindIII restriction sites of the pET28a(+) vector using LigaFast. The gene coding for the YP_60447 protein (*Dg*Sbt) was amplified in a similar way from *D. geothermalis* cells and ligated into the NdeI and HindIII restriction sites of the pBADMycHis(NdeI) vector using the same ligation protocol. Ligation mixtures were transformed to chemically competent *E. coli* C43(DE3) cells and constructs were confirmed by sequencing (GATC, Konstanz, Germany).

For the production of proteases the recombinant plasmids pET28a(+)-TAQ and pBADMycHisA(NdeI)-DG were introduced in *E. coli* C43(DE3) and transformants were grown at 37 °C for 16 h on Luria Bertani (LB) agar plates containing an antibiotic (50 μg/mL kanamycin or 100 μg/mL ampicillin). Colonies were inoculated into 5 mL of LB broth containing 0.1% glucose and antibiotic. After 16 h of incubation at 37 °C, 0.1% (v/v) of the cultures were inoculated into 1 L of Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, 17 mM $KH_2PO_4$ and 72 mM $K_2HPO_4$) containing the appropriate antibiotic. For *Taq*Sbt protease production the medium was supplemented with 1% glucose and protease synthesis was induced in the late log phase with 0.3 mM IPTG, followed by continued growth at 37 °C for 24 h with shaking at 200 rpm. In the case of *Dg*Sbt production, cultures were grown at 37 °C until they reached the late log phase, cooled down and induced at 17 °C with 0.25% L(+)-arabinose. Growth was continued for 48 h at 17 °C with shaking at 200 rpm.

The protocol for protease processing and isolation was modified from the literature [31] to a one-step protocol. Cells were harvested (4 °C, 15 min, 6000 × *g*), suspended in 50 mM Hepes·NaOH, pH 7.5, containing 10 mM $CaCl_2$, and disrupted by sonication. For autocatalytic processing and purification the total cell lysate was subjected to heat treatment for 12 h at 65 °C in the same Hepes buffer (Supplemental material Fig. S1). During this step most proteins except for the protease are either degraded by proteolysis or precipitated by heat denaturation. After removal of denatured protein by centrifugation (4 °C, 45 min, 30,000 × *g*), the supernatant was concentrated with an Amicon YM10 ultrafiltration membrane (Millipore, Billerica, MA, USA), followed by buffer exchange to 50 mM Hepes·NaOH, pH 7.5, containing 10 mM $CaCl_2$. Protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard. The protocol yielded up to 25 mg of *Dg*Sbt protease and 50 mg of *Taq*Sbt protease from a 1 L culture. The processed proteins gave a single band of 28 kDa on an SDS-polyacrylamide gel (estimated purity >80%). The proteins were stored at −80 °C.

Active subtilases were also isolated from the heat-treated centrifuged cell-free extract by precipitation with ammonium sulfate (70% sat. at 4 °C, 30 min stirring at 4 °C). The precipitate was collected by centrifugation (4 °C, 15 min, 30,000 × *g*), washed with 80% acetone to remove remaining lipophilic compounds, re-suspended in 20 mM Hepes·NaOH, pH 7.5, containing 1 mM $CaCl_2$ and 20% glycerol, and used for anhydrous enzyme preparation as described below. If required, glycerol was removed by exchanging the buffer on a HiPrep 26/10 desalting column (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

### 2.4. Preparation of anhydrous enzyme for synthesis reactions

Enzyme in 20 mM Hepes·NaOH, pH 7.5, containing 1 mM $CaCl_2$ and supplemented with sucrose (10 mg/mL) as lyoprotectant, was used for lyophilization. Anhydrous enzyme samples were prepared using several dried solvents in the same way as for isopropanol [30]. Briefly, to obtain isopropanol-precipitated and rinsed enzyme preparations (IPREP), organic solvent was added to the enzyme solution in buffer (20 mM Hepes·NaOH, pH 7.5, containing 1 mM $CaCl_2$ and 20% glycerol) to a ratio of 1:3, 1:6 or 1:9 (v/v, aq/solvent) until formation of a precipitate was observed. After vortexing, the precipitate was collected in a tabletop centrifuge (room temperature, 5000 rpm, 5 min) and remaining liquid was removed. The pellet was twice suspended in the same organic solvent, followed by vortexing and centrifugation. Subsequent drying in a Speedvac at room temperature for 20 min gave a white powder (IPRED) that was active when redissolved in buffer. The dry enzyme was stable for at least one month when stored at room temperature (20–25 °C). The IPRED preparations could be easily suspended in dry solvents, without a strong tendency to aggregate. Anhydrous preparations of subtilisin A were obtained by precipitation with *t*-BuOH as described by Chen et al. [32].

Active site titration with the irreversible serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) was used to determine the concentration of active enzyme present in enzyme samples. To 190 μL of PMSF solution of varying concentration (0–75 μM), prepared by mixing fresh 3 mM stock in ethanol and buffer (100 mM Hepes·NaOH, pH 7.5, with 1 mM $CaCl_2$), 10 μL of enzyme solution (approx. 0.4 mg/mL) was added. The mixture was vortexed and incubated for 10 min at room temperature. The remaining activity was then measured in the standard amidolytic assay as described below.

### 2.5. Enzyme characterization

Homology models of both subtilases were constructed with Yasara [33] based on the aqualysin I structure (PDB: 4DZT, 98% sequence identity) for *Taq*Sbt and based on proteinase K structure (PDB: 2B6N, 59% sequence identity) for *Dg*Sbt.

General proteolytic activities were measured at several temperatures by following the release of soluble sulfanilamide-azopeptides from azocasein in a UV/VIS spectrophotometer at 440 nm, as described previously [34]. Amidolytic activity was quantified by following the release of *p*-nitroaniline from the standard proteolytic substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (*N*-suc-AAPF-pNA) at 40 °C in 100 mM Hepes·NaOH, pH 7.5, containing 1 mM $CaCl_2$, 10% (v/v) DMSO and substrate (0.3–4 mM) [34]. Residual activities of heated samples were assayed after cooling. For thermostability measurements, the thermofluor method [35] was applied as follows. To the PMSF-inhibited subtilase (20 min incubation with 10 mM PMSF) in Hepes buffer (100 mM Hepes·NaOH, pH 7.5, with 1 mM $CaCl_2$) 5 μL of 100× Sypro Orange dye was added in a thinwall 96-well PCR plate. The plate was sealed with Optical-Quality Sealing Tape (Bio-Rad). The samples were heated for 10 min in a CFX 96 real-time PCR system (Bio-Rad) from 20 to 99 °C with a heating rate of 0.5 °C/min. Fluorescence changes were monitored with a charge-coupled device (CCD) camera. The wavelengths for excitation and emission were 490 nm and 575 nm, respectively.

### 2.6. MALDI/TOF/TOF analysis

For mass spectrometry based protein identification, the samples of *Taq*Sbt and *Dg*Sbt were analyzed by SDS-PAGE. After Coomassie staining, bands corresponding to 28 kDa were excised, digested with trypsin as reported previously [34], and analyzed by LC–MS. Mass spectrometry was carried out with a MALDI-TOF/TOF 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in the *m*/*z* range 600–4000. Peptide identification was done using the program ProteinPilot 4.0 (ABsciex, Foster City, CA, USA) against UniProtKB/Swiss-Prot protein sequence database [36] to which the sequences of trypsin and keratin were added. Trypsin specificity and default parameters were used for the search.

For intact protein analysis, samples were prepared using the ultra-thin layer method for MALDI [37] and mass spectra were recorded by MALDI-TOF on the Applied Bio-systems proteomics analyzer mentioned above, which was operated in linear positive ionization mode. Bovine serum albumin was used for calibration.

### 2.7. Enzymatic peptide synthesis

Tests to examine enzymatic synthesis of the dipeptide Cbz-Phe-Phe-$NH_2$ were carried out by mixing the IPRED suspension (10%, w/v) in the desired anhydrous solvent or solvent/cosolvent mixture with the N-terminally protected acyl donor