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Self-Renaturing Enzymes: Design of an Enzyme-Chaperone Chimera as a New Approach to Enzyme Stabilization

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ABSTRACT: Molecular chaperones in aqueous-organic mixtures can broaden the utility of biocatalysis by stabilizing enzymes in denaturing conditions. We have designed a self-renaturing enzyme-chaperone chimera consisting of penicillin amidase and a thermophilic chaperonin that functions in aqueous-organic mixtures. The flexible linker separating the enzyme and chaperone domains was optimized and the design was extended to incorporate a chitin binding domain to facilitate immobilization of the chimera to a chitin support. The initial specific activity of penicillin amidase was not compromised by the enzyme-chaperone fusion or by immobilization. The total turnover number of immobilized chimera for amoxicillin synthesis in aqueous-methanol mixtures was 2.8 times higher after 95 h than the total turnover number of the immobilized penicillin amidase lacking a chaperone domain. Similarly, in 32% methanol the soluble chimera was active for over three times longer than the enzyme alone. This approach could easily be extended to other enzyme systems.

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KEYWORDS: chaperone; antibiotic synthesis; protein folding; fusion protein; organic solvent

Introduction

Enzymes have many proven applications and hold great promise for expanded use in synthesis because of their remarkably high chemo- and stereoselectivities in chemically challenging reactions (Pollard and Woodley, 2007; Schoemaker et al., 2003). However, a common shortcoming of enzymes as practical catalysts is their limited operational stability, particularly in the presence of organic co-solvents. Water-miscible organic co-solvents can increase the solubility of nonpolar substrates, suppress hydrolysis reactions, and shift reaction selectivity towards an otherwise unstable product (Castro and Knubovets, 2003; Schmid et al., 2001). One strategy for improving enzyme stability in general is to immobilize the enzyme (Grazu et al., 2005; Miyazaki et al., 2005), which offers the additional advantages of enabling continuous operation of the enzyme reactor and reuse of the enzyme, recycling of substrates, and steady removal of products to alleviate product degradation and inhibition (Bornscheuer, 2003; Polizzi et al., 2007). Immobilization has also been employed to increase enzyme stability in organic co-solvents (Fernandez-Lafuente et al., 2001); however, a common drawback of most immobilization methods is a concomitant reduction in the enzyme's intrinsic activity (Fernandez-Lafuente et al., 2001; Kranz et al., 2007; Wehtje et al., 1993).

Chaperonins are proteins that stabilize other proteins under stress (Hendrick and Hartl, 1993), both in vivo and in vitro. For example, the chaperonin from *Thermococcus* sp. strain KS-1 was co-immobilized with malate dehydrogenase to increase the thermal-stability of the immobilized enzyme (Kohda et al., 2006). However, this approach to enzyme stabilization presents the challenge of achieving close proximity between the immobilized enzyme and chaperone. With regard to solvent stability, the single-subunit Group II chaperonin from *Methanocaldococcus jannschii* (rTHS) has been shown to preserve enzyme activity while performing batch and fed-batch reactions in water-miscible organic co-solvent mixtures (Bergeron et al., 2008), raising the possibility of combining enzyme immobilization and proximity to the rTHS to produce a stable biocatalyst for synthetic applications in aqueous-organic solutions.

In the present study, we describe a new approach to enzyme immobilization/stabilization in which an enzyme-chaperone chimera is engineered to attach a functional chaperone domain (rTHS) to the enzyme of interest (the model penicillin amidase, or PGA), creating a single protein

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with biocatalytic activity and protein refolding capability. This self-renaturing enzyme was further fused to a chitin binding domain to enable simple and effective immobilization. Previous chaperone fusions have been generated for increased expression of aggregation-prone proteins (Furutani et al., 2005); however, to date, there are no reports of chaperone-protein fusions for in vitro applications such as biocatalyst stabilization. This is thus the first report of an enzymatically active fusion protein functioning as both a chaperone and an enzyme.

Materials and Methods

Reagents

Enzymes for DNA manipulation were purchased from New England Biolabs (Waltham, MA). Oligonucleotides were custom ordered from Operon (Huntsville, AL), and DNA plasmids for gene expression were ordered from Novagen (San Diego, CA). All reagents, chemicals, and enzymes were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

Plasmid Construction

The codon optimized rTHS gene and the L2-4-CBD gene were purchased from DNA 2.0 (Menlo Park, CA). Sequences, shown in the Appendix, were subcloned into the respective chimera plasmids. *Escherichia coli* (ATCC 11105) genomic DNA was prepared using DNeasy Tissue Kit (Qiagen, Valencia, CA) and used as a template for PCR. The PGA coding region of *E. coli* was amplified using oligonucleotides shown in Table I. PCR fragments were cloned into TOPO sequencing vectors (Invitrogen, Carlsbad, CA) and sequences were verified by DNA sequencing (DNA Sequencing Facility, UC Berkeley). Flexible linkers L1-1, L1-2, and L1-4 (Table I) were purchased as complimentary single-stranded oligonucleotides (Operon), annealed at 95°C for 1 h, and cloned into TOPO sequencing vectors. Low-melt agarose was used for DNA extraction and ligation of linkers.

Point mutations to the rTHS sequence to remove restriction sites SacII and HindIII, as well as deletion of the stop codon, and insertion of a PGA stop codon, were performed using a QuickChange Site Directed Mutagenesis kit (Stratagene, La Jolla CA). Changes to the restriction sites on the chitin binding domain (CBD) were implemented with PCR using primers listed in Table I. Changes in incorporated proteins and linkers were carried out by restriction digests of respective restriction sites and ligations into a pET30a vector (Novagen).

Expression and Purification of Soluble and Immobilized Chimeras

All enzymes and chimeras were subcloned into a pET30a vector (Novagen) and expressed in *E. coli* strain BL21(DE3) at 37°C under kanamycin resistance and induced with 1 mM IPTG. Upon induction, cells were grown overnight at 30°C

Table I. Primers used for the design of protein constructs.

Primer	Sequence	Restriction sites (5′/3′)	Application
PGA_F	GGAATTCCATATGGAGCAGTCGTCAAGTGAGATAAAGAT	NdeI	*pac* gene
PGA_R	TCCCCGCGGTCTCTGAACGTGCAACACTTCCT	SacII	
PGA_M	GCAGCTCTGTTGCCACGC		Sequencing
PGA_F2	GCGATCGTCCTGTGCTTGC		Sequencing
CBD_F	GGGGTACCGCTTGGCAGGTCAACACAGC	KpnI	CBD for PGA_CBD
L1-1F	TCCCCGCGGTCAGGTGGTGGCGGATCGGGTACCCC	SacII/KpnI	Linker L1-1
L1-1R	GGGGTACCCGATCCGCCACCACCTGACCGCGGGGA	KpnI/SacII	
L1-2F	TCCCCGCGGTCAGGTGGTGGCGGTTCAGGCGGTGGCGGATC GGGTACCCC	SacII/KpnI	Linker L1-2
L1-2R	GGGGTACCCGATCCGCCACCGCCTGAACCGCCACCACCTGA CCGCGGGGA	KpnI/SacII	
L1-4F	TCCCCGCGGTCAGGTGGTGGCGGTTCAGGCGGAGGTGGCTC TGGTGGCGGTGGCTCTGGCGGTGGCGGATCGGGTACCCC	SacII/KpnI	Linker L1-4
L1-4R	GGGGTACCCGATCCGCCACCGCCAGAGCCACCGCCACCAG AGCCACCTCCGCCTGAACCGCCACCACCTGACCGCGGGGA	KpnI/SacII	
PGA-SF	GAAGTGTTGCACGTTCAGAGATAACCGCGGTCAGG		PGA TAA insertion
PGA-SR	CCTGACCGCGGTTATCTCTGAACGTGCAACACTTC		
THSNS_F	GCGACATGGGCGGTGATGAATTTGGATCCGAATTCG		rTHS TAA deletion
THSNS_R	CGAATTCGGATCCAAATTCATCACCGCCCATGTCGC		
THSHF	GTGAACAGCTGGCTGTTAAGGCTTTCGCAGATGCA		HindIII site removal
THSHR	TGCATCTGCGAAAGCCTTAACAGCCAGCTGTTCAC		
THSSF	GGAAACCAAACTGATCCGGGGTGTGGTTATTGATAAA		SacII site removal
THSSR	TTTATCAATAACCACACCCCGGATCAGTTTGGTTTGG		

Restriction sites within primers are underlined and labeled in the appropriate column.

and harvested by centrifugation. Cells harboring soluble enzymes were resuspended in 50 mM Tris, pH 7, and cells with immobilized chimeras were resuspended in 50 mM Tris/HCl pH 8.5, 500 mM NaCl, 0.1% Triton X-100. Cells were lysed by three passages through a French pressure minicell at 9000 psi and chimera proteins were purified from cell lysate. Soluble chimeras and recombinant PGA were purified as described previously (Hunt et al., 1990) using 0.5 M ammonium sulfate, hydrophobic interaction chromatography, and anion exchange chromatography.

Immobilized chimeras were purified from cell lysate. Cell lysate was exposed to 135 mg of chitin beads (New England Biolabs) washed in 50 mM Tris/HCl, pH 8.5, 500 mM NaCl, 0.1% Triton X-100 and incubated with the beads at 4°C with constant stirring overnight (Nagao et al., 2004). The mixture was then centrifuged briefly and the chitin beads were washed a minimum of five times. Purity of the immobilized enzyme was verified and quantified using SDS–PAGE after extended SDS (5% w/v) and heat (90°C) exposure (15 min). Protein content was measured by gel densitometry using Scion imaging software (Frederick, MD) and was used to determine differences in protein loading onto the beads. Over 95% of both PGA-CBD and Chimera-CBD that was initially present in the cell lysate bound to the chitin beads. The molar amount of enzyme produced per g wet cell weight for each enzyme and each fermentation was consistent within 10%.

Inactivation of Soluble Chimera and PGA

Activity of recombinant PGA and soluble chimeras in aqueous buffer was determined by measuring the change in absorbance at 405 nm upon the addition of 90 μM 6-nitro-3-(phenylacetamido) benzoic acid (NIPAB) (Alkema et al., 1999) to 10 μM enzyme in 50 mM Tris/HCl buffer (pH 7) containing 5 mM $CaCl_2$. Reactions proceeded for 1 h and were run at least in triplicate. Irreversible enzyme inactivation upon exposure to 35% v/v MeOH was determined by incubating 2 μM enzyme in 50 mM Tris/HCl pH 7, 5 mM $CaCl_2$, and 1 mM ATP in 35% MeOH at 37°C. Twenty-microliter aliquots were removed at various incubation times, diluted 50× and assayed for activity in aqueous conditions as described above. One hundred percent relative activity represents the enzyme activity when no MeOH was present in the inactivation solution. Enzyme inactivation in 32% v/v methanol (MeOH) was determined by monitoring the change in absorbance at 405 nm of samples containing 2 μM enzyme, 1 mM ATP, 5 mM $CaCl_2$, and 90 μM NIPAB in 32% v/v MeOH, 50 mM Tris/HCl pH7 at 37°C.

Packed Bed Reactions for 5-ANB and Amoxicillin Production

Chimeras immobilized onto chitin beads were washed as described above and loaded onto a 1-mL polypropylene syringe containing a 0.5-cm diameter Whatman filter (Florham Park, NJ). The bed volume (approximately 50 μL) for each reactor was varied to keep the total amount of enzyme constant and changed by less than 10% from one reactor to the next. Each reactor was prepared from a fresh preparation of immobilized enzyme. The packed reactor was attached to a syringe pump, and 50 mM Tris/HCl, pH 8, was pumped through the reactor at 0.5 mL h^{-1} for 1 h prior to the start of a reaction. For each reaction, substrate solution was pumped through the reactor and the effluent was collected and sampled at various reaction times. Reactions were run at 37°C and fresh substrate was prepared every 10–15 h.

Production of 5-amino-2-nitrobenzoic acid (5-ANB) was achieved by flowing 90 μM NIPAB, 5 mM $CaCl_2$, and 1 mM ATP through the reactor at 100 μL h^{-1}. Aqueous substrate was used for the first 3 h of the reaction to assure that the enzyme was fully active and inactivation was initiated by introducing 32% v/v MeOH into the substrate solution. Effluent was collected in sealed microcentrifuge tubes and the absorbance of samples was measured periodically at 405 nm.

The substrates 6-aminopenicillanic acid (6-APA) and p-hydroxy-phenyl glycine methyl ester (POHPGME) were used to produce amoxicillin. 1 mM 6-APA and 1.2 mM POHPGME in 50 mM Tris/HCl, 5 mM $CaCl_2$, 1 mM ATP, and 30% v/v MeOH were pumped through the column at a flow rate of 30 μL h^{-1}, effluent was collected into sealed containers, and samples were taken periodically and prepared for HPLC analysis. Fifty-microliter samples were diluted fourfold into 20 mM HEPES, pH 7 (which quenched the reaction) and filtered through Microcon YM30 centrifuge filters at 12,200*g* for 7 min. The filtrate of each centrifuged sample was frozen at −20°C for no more than 4 days until HPLC analysis was performed. HPLC analysis was carried out on a Waters HPLC setup (Waters Corp., Milford, MA) as described previously (Bergeron et al., 2009) with an Altima HP C18 AQ column (Alltech, Deerfield, IL).

Results and Discussion

Soluble Chimera for Linker Length Optimization

Three soluble chimeras were constructed, each consisting of a fusion of rTHS and PGA separated by a flexible linker. The PGA β-subunit is much larger than the α-subunit and unfolds first (Ignatova et al., 2005). For this reason, rTHS was fused to the β-subunit at the C-terminal end, as the N-terminus was necessary for processing of the pac gene spacer peptide (Nagao et al., 2004). Each fusion protein was designed as shown in Figure 1 and described in the Materials and Methods Section. Chimeras containing flexible linkers of variable $(GGGGS)_n$ repeat lengths (Trinh et al., 2004) (designated L1-1, L1-2, and L1-4) were engineered in *E. coli*, and purified as reported for *E. coli* PGA (Hunt et al., 1990). Linker L1-1 consisted of one repeat length $(GGGGS)_1$, L1-2

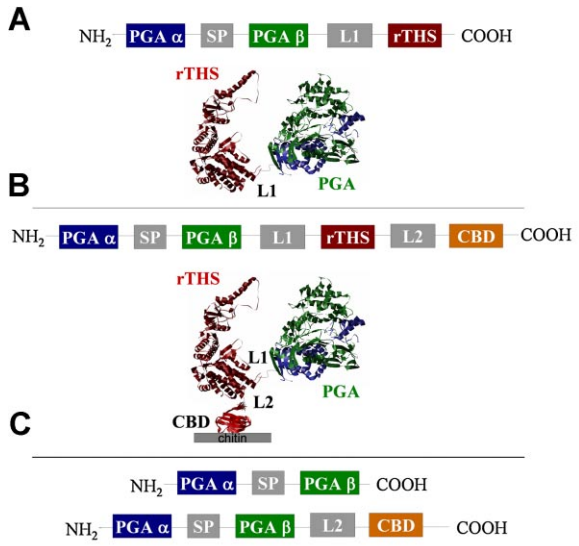

Figure 1. Structural designs of enzyme-chaperone fusions. **A**: Soluble chimeras: L1 represents the flexible linker inserted between the β-subunit of PGA and the rTHS domain. SP represents the spacer peptide present in the PGA *E. coli* pac gene. **B**: Immobilized chimera: Linker L2, $(GGGGS)_4$, separates the rTHS domain from the chitin binding domain (CBD). **C**: Enzyme controls.

contained two repeats $(GGGGS)_2$, and L1-4 had four repeats $(GGGGS)_4$. PGA was produced recombinantly in the same system using protocols of Nagao et al. (2004). In aqueous solution the biocatalytic activity of the chimera with the longest linker, L1-4, was equivalent within error to that of recombinant PGA, and the chimera activity decreased as the linker length decreased (Table II).

On the contrary, protein stability was improved by a decrease in the linker length. Irreversible enzyme inactivation in 35% v/v MeOH at 37°C was determined as a function of incubation time. After various exposure times to 35% MeOH, relative activity was measured by dilution into aqueous substrate, 6-nitro-3-(phenylacetamido) benzoic acid (NIPAB), allowing reversibly unfolded enzyme to refold. PGA inactivated most rapidly, indicating that all chimeras provided some stability, and the chimera L1-1 was the most stable enzyme (Fig. 2A). No aggregation was observed on the timescale of inactivation and less than 5% activity remained for all samples after 15 min of exposure to 35% MeOH when activity was assayed in the presence of MeOH (not shown), verifying that refolding occurred upon dilution into aqueous substrate.

The MeOH concentration was reduced to 32% v/v in order to follow the loss of native enzyme directly by monitoring product formation over the lifetime of the enzyme. Enzymes were incubated with substrate, NIPAB, and the hydrolyzed product, 5-amino-2-nitrobenzoic acid (5-ANB), was followed spectrophotometrically at 405 nm. While all chimeras showed greater stability than the native enzyme, chimera L1-1 retained activity after 15 h

Table II. Properties of PGA and chimera enzymes.

	Specific activity[a] (μmol min^{-1} μmol^{-1})	Enzyme loading[b] (nmol g^{-1})
PGA	8.8 ± 0.3	—
L1-1	5.0 ± 0.2	—
L1-2	8.0 ± 0.2	—
L1-4	8.7 ± 0.7	—
PGA-CBD	21.9 ± 3.3	120 ± 22
Chimera-CBD	20.9 ± 2.2	117 ± 16

[a]Initial specific activities of soluble and immobilized chimeras (μmol 5-ANB min^{-1} μmol^{-1} enzyme) pictured in Figure 1.
[b]Enzyme loadings of immobilized chimeras (nmol enzyme g^{-1} chitin beads).

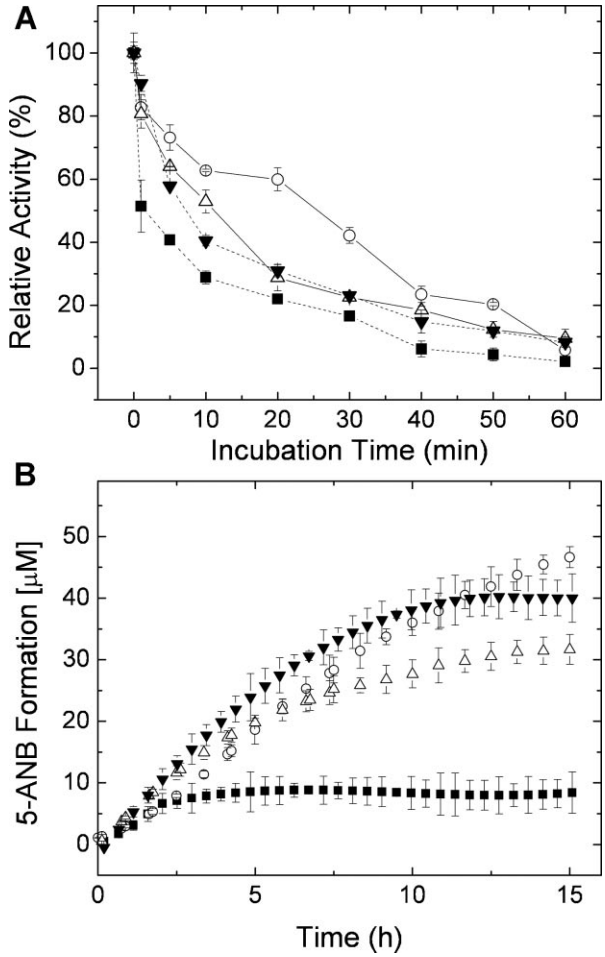

Figure 2. Stability of recombinant PGA (solid squares) and soluble chimeras (open circles: L1-1, open triangles: L1-2, closed triangles: L1-4) in aqueous-methanol solutions. **A**: Restoration of PGA activity upon incubation in 35% v/v MeOH, 37°C, and dilution into aqueous substrate solution. **B**: Product formation profiles for the production of the hydrolysis product, 5-ANB, as a function of reaction time in denaturing conditions. The reaction was performed in the presence of 32% v/v MeOH, 37°C, for all soluble chimeras and recombinant PGA. Error represents standard deviation of triplicate data sets.

in 32% MeOH, whereas PGA and the other two chimeras were inactivated (Fig. 2B). In the case of PGA, the product concentration was nearly level in less than 5 h. Therefore, despite its reduced activity in aqueous conditions, the chimera with the shortest linker length, chimera L1-1, produced the most product over the lifetime of the enzyme due to its increased stability in 32% MeOH.

The difference in stability between PGA and the chimeras is more evident in Figure 2B than in Figure 2A. This can be explained by considering that under the refolding conditions of Figure 2A, all reversibly unfolded PGA has refolded back into its native form (with or without rTHS), whereas under the denaturing conditions of Figure 2B, only the chimeras are able to refold. Hence the stabilizing effect of the rTHS, and the corresponding difference in the activity of the PGA, is much more pronounced in Figure 2B. These results indicate that the rTHS domain is refolding the PGA domain rather than stabilizing the native state. In addition, ATP and calcium were required to maintain optimal stability of chimera L1-2 (not shown), providing further evidence that the rTHS is actively refolding the enzyme.

Immobilized Chimera for Continuous Flow Reactor

Chimera L1-1 was extended to include a chitin binding domain (CBD) for facile immobilization to chitinaceous supports. The linker L1-1 was chosen for the design of an immobilized chimera to maximize the stabilizing influence of the rTHS domain. The CBD was connected to the C-terminus of the soluble chimera L1-1 via the linker L2, $(GGGGS)_4$, (Fig. 1) to produce Chimera-CBD. A chimera protein lacking rTHS in which PGA was fused directly to L2 and CBD (PGA-CBD) was created and used as a control (Nagao et al., 2004).

PGA-CBD and Chimera-CBD were expressed in *E. coli* BL21(DE3) cells, purified, and immobilized to chitin beads. The loadings and specific activities in aqueous solution of the immobilized enzymes are included in Table II. Immobilized enzyme reactors were packed with either Chimera-CBD or PGA-CBD to produce a bed volume of ~50 μL. Each immobilized enzyme was over twofold more active than soluble PGA, which could be due to the different treatments prior to immobilization (i.e., multi-step purification versus direct binding from the cell lysate). Nonetheless, immobilization through the CBD did not result in a compromising loss of PGA activity.

The stability of Chimera-CBD was first investigated by performing hydrolysis reactions of NIPAB in 32% v/v MeOH, 37°C. The total turnover number (TTN) of Chimera-CBD was 2.1-fold greater than PGA-CBD (Fig. 3A). The initial activities were similar; however, Chimera-CBD exhibited a longer lifetime relative to PGA-CBD in 32% MeOH.

The presence of MeOH for the enzymatic production of amoxicillin increases both the selectivity and yield of amoxicillin (Fernandez-Lafuente et al., 1998); however, the stability of PGA in aqueous-MeOH mixtures limits the MeOH concentration that can be used. Previously, rTHS was used to stabilize PGA in 30% MeOH, resulting in an increase of amoxicillin produced (Bergeron et al., 2009). For this study, substrates 6-aminopenicillanic acid (6-APA) and p-hydroxy-phenyl glycine methyl ester (POHPGME) were reacted in the presence of 30% v/v MeOH, 37°C for 96 h (60 reactor average-residence times) and amoxicillin production was recorded. The TTN of Chimera-CBD increased by nearly threefold relative to PGA-CBD (Fig. 3B). Furthermore, after 43 residence times, negligible activity remained for PGA-CBD while Chimera-CBD appeared fully active for up to 60 residence times.

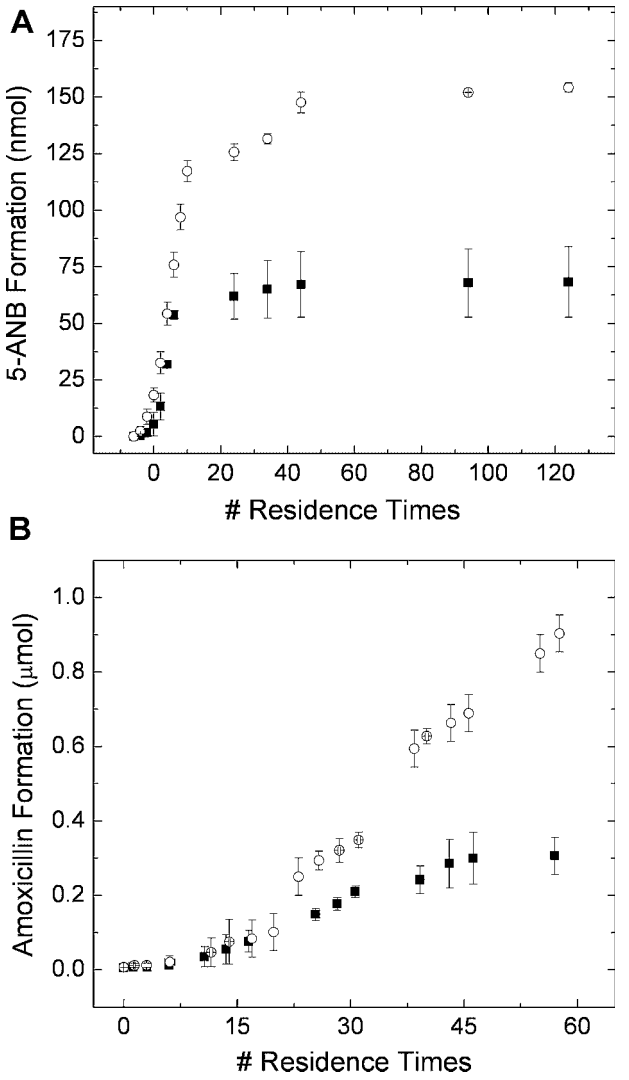

Figure 3. Product formation versus number of residence times for immobilized enzyme packed-bed reactors packed with PGA-CBD (closed squares) or Chimera-CBD (open circles). **A**: Hydrolysis of NIPAB in 32% MeOH, 37°C. The flow rate of the substrate solution was 100 $\mu L\,h^{-1}$. **B**: Synthesis of amoxicillin in 30% MeOH, 37°C at a flow rate of 30 $\mu L\,h^{-1}$.

Conclusions

In summary, enzyme-chaperone fusions can be an effective means of creating reusable, stable, and active immobilized enzymes that are functional in the presence of denaturing co-solvents. We have exploited the simplicity, robustness, and functionality of a single-subunit thermophilic molecular chaperone by constructing enzyme-chaperone chimeras that adequately perform reactions in the presence of methanol–water mixtures in both soluble and immobilized form. This approach can be extended to include other enzymes in which denaturing conditions are necessary to perform desired reactions.

We thank David L Shis for assistance in work leading to the preparation of the chimera proteins.

Appendix

Optimized DNA Sequence Encoding *M. jannaschii* Thermosome

GGTACCGCTATGGCTGGTGCTCCAATTGTTGTTCTG-
CCTCAAAATGTGAAACGTTACGTGGGTCGTGACGCCCA-
GCGTATGAACATCCTGGCGGGTCGTATCATCGCTGAAAC-
CGTGCGTACTACCCTGGGTCCGAAAGGTATGGACAAAAT-
GCTGGTAGACGAACTGGGTGACATTGTGGTGACTAACGA-
TGGCGTGACTATCCTGAAAGAAATGTCCGTGGAACACCC-
AGCCGCTAAAATGCTGATTGAAGTTGCGAAAACCCAGG-
AAAAAGAAGTTGGTGACGGCACCACCACTGCAGTTGTT-
ATCGCAGGTGAGCTGCTGCGCAAAGCGGAAGAACTGCT-
GGATCAGAACATCCACCCGAGCGTGATTATCAACGGTTA-
CGAGATGGCCCGTAACAAAGCGGTTGAAGAGCTGAAAA-
GCATTGCGAAGGAAGTTAAACCGGAAGACACTGAAATG-
CTGAAGAAAATCGCCATGACTTCCATCACCGGTAAAGGC-
GCTGAAAAAGCACGTGAGCAGCTGGCCGAGATCGTAGT-
TGAAGCAGTACGTGCCGTAGTCGACGAAGAAACCGGCA-
AGGTTGACAAAGACCTGATTAAAGTTGAAAAAAAAGAG-
GGCGCACCGATTGAGGAAACCAAACTGATCCGCGGTGT-
GGTTATTGATAAAGAACGCGTCAATCCGCAGATGCCGAA-
GAAGGTTGAAAACGCTAAGATCGCACTGCTGAACTGCC-
CGATCGAGGTGAAAGAAACGGAGACTGACGCTGAAATT-
CGTATCACCGATCCGGCTAAACTGATGGAGTTCATCGAA-
CAGGAAGAGAAGATGATTAAAGATATGGTGGAAAAAAT-
CGCTGCGACCGGCGCCAACGTAGTGTTTTGCCAGAAAG-
GTATCGACGACCTGGCGCAGCACTACCTGGCAAAAAAA-
GGTATCCTGGCTGTGCGTCGCGTTAAGAAATCCGACATG-
GAGAAACTGGCTAAGGCTACCGGTGCCCGTATCGTTACT-
AAAATTGATGATCTGACTCCGGAAGACCTGGGTGAAGC-
CGGTCTGGTTGAGGAGCGTAAAGTTGCTGGTGACGCCAT-
GATCTTTGTGGAACAGTGCAAACACCCGAAAGCCGTCA-
CCATTCTGGCTCGTGGCTCCACCGAGCATGTAGTCGAGG-
AAGTGGCACGCGCTATTGACGACGCGATCGGTGTGGTG-
AAATGCGCGCTGGAAGAAGGCAAAATCGTTGCCGGCGG-
TGGTGCCACTGAGATTGAGCTGGCCAAACGCCTGCGTA-
AATTCGCCGAATCTGTTGCGGGTCGTGAACAGCTGGCTG-
TTAAAGCTTTCGCAGATGCACTGGAAGTGATCCCGCGTA-
CCCTGGCGGAAAACAGCGGTCTGGACCCGATTGATATGC-
TGGTGAAACTGCGCGCTGCACACGAAAAGGAAGGTGGT-
GAAGTCTACGGCCTGGACGTGTTCGAAGGCGAAGTTGT-
GGACATGCTGGAGAAAGGTGTGGTGGAACCGCTGAAGG-
TTAAAACCCAAGCCATTGATTCCGCAACCGAAGCATCTG-
TAATGCTGCTGCGTATTGATGACGTAATCGCAGCGGAAA-
AAGTTAAAGGTGACGAAAAAGGTGGCGAAGGCGGCGA-
CATGGGCGGTGATGAATTTTAAGGATCC.

DNA Sequence Encoding Flexible Linker L2-4 $(GGGGS)_4$ Linked to Chitin Binding Domain (CBD) From *bacillus circulans*

GGATCCTCTGGTGGCGGTGGCTCGGGCGGAGGTGG-
GTCAGGTGGTGGCGGTTCGGGTGGCGGCGGATCAAAGC-
TTGCTTGGCAGGTCAACACAGCTTATACTGCGGGACAAT-
TGGTCACATATAACGGCAAGACGTATAAATGTTTGCAGC-
CCCACACCTCCTTGGCAGGATGGGAACCATCCAACGTTC-
CTGCCTTGTGGCAGCTTCAATAACTCGAG.

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