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Production of crystallizable human chymase from a *Bacillus subtilis* system

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Abstract **A *Bacillus subtilis* strain deficient in seven extracellular proteases was used to produce human mast cell chymase and is a viable expression system for serine proteases and other classes of proteins. Chymase is produced at 0.3–0.5 mg/l and is purified by three chromatography steps. Two crystal forms of PMSF-treated chymase were optimized. The first is C2 with $a = 47.94$ Å, $b = 85.23$ Å, $c = 174.18$ Å, $\beta = 96.74°$, and diffracts to at least 2.1 Å, while the second is P212121, with cell dimensions $a = 43.93$ Å, $b = 58.16$ Å, and $c = 86.09$ Å, and a diffraction limit of approximately 1.9 Å. The first crystal form has either three or four molecules/asymmetric unit, while the second has one molecule/asymmetric unit.**

Key words: Protease; Expression; Angiotensin; Mast cell; X-ray diffraction

1. Introduction

Human chymase is a mast cell serine protease with chymotrypsin-like specificity [1,2]. The enzyme is initially synthesized as an inactive precursor but is ultimately stored in, and released from, the secretory granules of mast cells as a mature enzyme [3–5]. Processing of the precursor appears to involve removal of a signal peptide followed by DPP1-mediated removal of the Gly–Glu propeptide in a heparin-dependent reaction [6,7]. Human chymase has been implicated in a variety of physiological and pathological processes including vasoactive peptide processing [8,9], tissue remodeling [10], activation of proIL-1β [11], and release of latent growth factors from the extracellular matrix [12]. While the parent chymotrypsin shows a relaxed substrate specificity, with clear preferences observed only for the P1 substrate position, chymase is a much more selective enzyme. Specificity for Phe, Tyr, or Trp at P1 remains, but is then limited by strict requirements for the extended substrate-binding site. For example, human chymase activates angiotensin I to angiotensin II by cleavage of a Phe–His bond, but does not degrade angiotensin II by clipping a Tyr–Ile bond, as do chymotrypsin, and chymases from other species [9]. To date, only one *chymase* gene has been identified in humans [13], although a Cys-to-Ser variation at position seven of the mature enzyme has been reported [14]. This is the first report of crystallization of human chymase. An atomic resolution structure of human mast cell chymase will provide useful information for understanding this enzyme's substrate specificity requirements and may shed light on the details of activation by DPP1, and the stabilizing role of heparin within the mast cell granule.

Production of milligram quantities of pure chymase was a prerequisite for the structural studies. Although a variety of expression systems have proven useful for serine proteases, many are unsuitable for one reason or another. For example, *Escherichia coli* can produce serine proteases to high yield, but many are then found inextricably complexed with the periplasmic inhibitor, ecotin [15,16]. Other reported expression systems for human chymase involve substantial downstream processing to recover limited amounts of active enzyme, e.g. refolding and proteolytic processing of an insoluble fusion protein [17] or proteolytic activation of secreted prochymase with cell extracts [6] or DPPI [7]. The *B. subtilis* system described herein yields mature chymase, ideally suited for crystallization because of the lack of glycosylation. *B. subtilis* has been successful at producing other serine proteases (B.F.S., unpublished results), and is a heretofore untapped resource as an expression system.

2. Methods and results

2.1. Expression

E. coli XL-2 Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r}) Amy Cam^{r}]} (Stratagene) or MM294 (*endA1 thi-1 hsdR17 supE44*) [18] were used as hosts for the vector constructions. Bacteria were transformed and plasmid DNA was purified as described elsewhere (B.F.S., manuscript in preparation).

B. subtilis WB751 (*trp Δisp-1 ΔnprA ΔaprE Δepr Δbpf Δmpr*-Cam^{r} *ΔnprB-Ery*^{r} *Δvpr*), a strain deficient in seven extracellular proteases and one intracellular protease, was used for the production of human mast cell chymase C7S. This strain was constructed by deleting the *vpr* gene [19] from *B. subtilis* WB600 (*trp Δisp-1 ΔnprA ΔaprE - Δepr Δbpf Δmpr*-Cam^{r} *ΔnprB*-Ery^{r}) [20]. To accomplish this, two ≈1.2 kb PCR fragments, corresponding to either a 5′- or 3′-portion of the *vpr* gene, were generated by Taq polymerase (Perkin-Elmer) with the primers GATATCCAGAATTCCACAAACTCTATCTATATTTTGTATACGAA and GCCGTTCGGATCCTCAATCACTTATGAGGTAACAGCGACAACGCCTTCTGACATGGC, or the primers GATACGTGGATCCTTAAGCCTGATATTTCCGCGCCAG and GTATCCCTGCAGGAGTCCTCGTATGAAGCTGTTCGG (ABI 394 DNA/RNA Synthesizer, Perkin-Elmer), respectively, using chromosomal DNA from *B. subtilis* BG2036(*ΔnprA ΔaprE*) (a generous gift from Genencor International, Inc.) as a template. The two PCR generated fragments (*Eco*RI–*Bam*HI and *Bam*HI–*Pst*I) were inserted in pBluescript SK+ (Stratagene) with the *kanamycin* gene (≈1.4 kb *Eco*RI–*Sal*I fragment) from pJM114 [21]. The deletion of amino acids 272–451 of the Vpr protease and the introduction of stop codons in all three frames after amino acid 271 were verified by DNA sequencing (ABI 373A DNA Sequencer, Perkin-Elmer). The resultant vector was used to transform *B. subtilis* WB600 cells. Clones with the vector integrated into the chromosome were selected by plating on Luria agar plates with 10 μg/ml kanamycin. Integration at the *vpr* gene locus was determined by screening the chromosomal DNA of the transformants using PCR primers that flank the *vpr* gene. Positive

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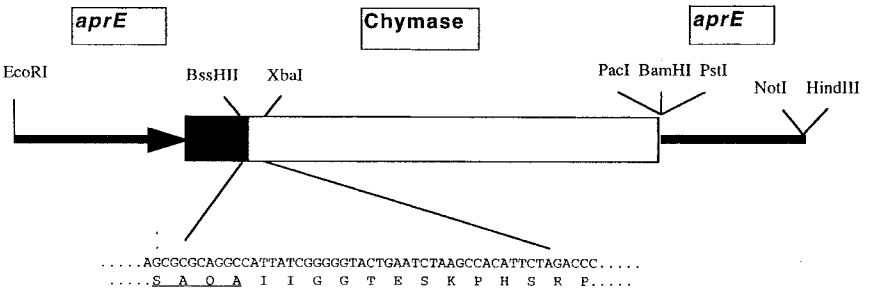

Fig. 1. A schematic representation of the expression cassette used to produce human mast cell chymase C7S in *B. subtilis*. Expression is driven by the *B. subtilis aprE* promoter [28] shown as the thick black arrow. The *chymase* gene is represented by the white box and downstream is the transcription terminator from the *B. amyloliquefaciens aprE* gene [29] shown as a thick black line. The DNA and protein sequence of the *aprE* signal sequence (underlined) fused to the mature *chymase* gene is shown below the schematic figure. Amino acids 9–30 of the signal sequence are derived from the *B. amyloliquefaciens aprE* gene.

clones were grown in Luria broth for 6–14 h then used to inoculate another 15 ml of fresh Luria medium (no kanamycin selection). The re-inoculation process was repeated for up to 2 weeks. Colonies that eventually lost the kanamycin resistance were screened by PCR in order to select clones where the native *vpr* gene was replaced with the deleted version.

A DNA fragment of the human *chymase* gene [5] was generated by PCR with the primers GGGAATATTATCGGGGGTACCGAATG-CAAGCCACATTCTAGACCCTACA and GATCGTTAATTAAT-TTGCCTGCAGGATCTGGTTGATCCAGGG using a cDNA library constructed from ascites tissue as a template [22]. The ≈700 bp *Xba*I–*Pac*I gene fragment was ligated into the *Bss*HII and *Pac*I sites of the *B. subtilis* expression vector pBNppt (B.F.S., manuscript in preparation) using a *Bss*HII–*Xba*I synthetic DNA linker. This linker also substitutes the cysteine residue with serine at amino acid seven of the mature chymase since it was discovered that native human chymase with a free thiol group is not effectively produced in prokaryotic systems (J.M.C. and B.F.S., unpublished results). The entire sequence of the gene was verified by sequencing both DNA strands. A schematic representation of the expression–secretion construction is shown in Fig. 1.

2.2. Fermentation and purification

Chymase-producing *B. subtilis* was grown in 4 l of baffled shake flasks (Bellco) at 37°C, 270 r.p.m. A rich medium [23] was used for production and consisted of 3% (w/v) tryptone (Difco), 2% (w/v) yeast extract (Difco), 3% (w/v) glucose, 150 mM potassium phosphate, pH 7.4, 10.0 μg/ml neomycin. An inoculum (1 l) was grown overnight and

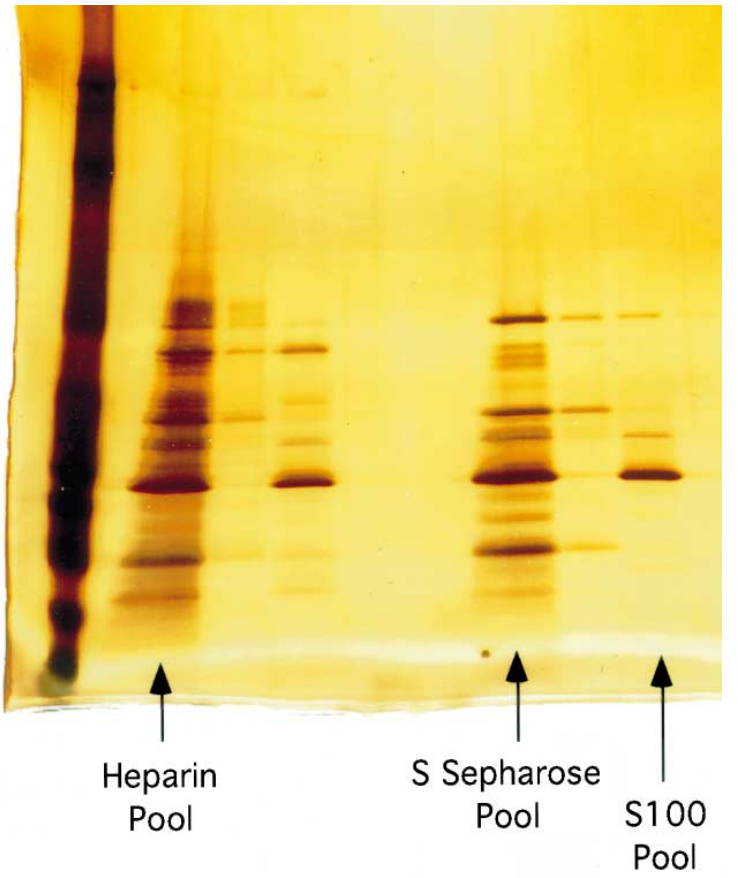

Fig. 2. A silver-stained 4–20% SDS-PAGE gel showing chymase at various purification steps. Chymase is the major protein band seen in the lane containing the pool from the S-100 chromatography step.

150 ml was used to seed each of six flasks containing 1 l of media. Cultures were grown for 6 h and harvested by centrifugation at 7200 r.c.f. The supernatant was filtered by passage through a 0.2 μm Sartobran-PH capsule filter (Sartorius, Edgewood, NY). Approximately 0.3–0.5 mg of purified chymase was produced per liter of fermentation broth, as determined by activity.

Chymase activity was measured by hydrolysis of suc-Val-Pro-Phe-SBzl (Bachem) with 4,4′-dithiodipyridine (Sigma), in assay buffer (30 mM Tris-HCl, 1.0 M NaCl, 0.05% (v/v) Tween 20, 10% (v/v) DMSO, pH 8.0). Beads of Heparin Sepharose CL-6B (Pharmacia, Piscataway, NJ) were added to the filtered harvest supernatant at a ratio of up to 0.5 l of harvest/ml resin and suspended with mild agitation for 1 h. The beads were collected on a 0.8 μm filter (Nalgene), resuspended in 20 mM MES, 150 mM NaCl, pH 5.5, and packed into a 5-cm-diameter column. Chymase was eluted from the column using a gradient of 0.52–2 M NaCl over five column volumes in 20 mM MES, pH 5.5. Fractions which tested positive for chymase activity were pooled, and diluted to a conductivity of 25 mS/cm with 20 mM MES, pH 5.5. The diluted chymase pool was applied to a SP-Sepharose FF column (1.6×10 cm, Pharmacia) equilibrated in 20 mM MES, 0.4 M NaCl, pH 5.5. Chymase was eluted from the column using a gradient of 0.4–1 M NaCl over 10 column volumes in 20 mM MES, pH 5.5. Chymase-rich fractions eluted in a peak centered around 0.7 M NaCl, and were assessed for purity by SDS-PAGE and pooled. The SP-Sepharose FF chymase pool was applied to a Sephacryl S-100 HR (2.6×92 cm, Pharmacia) column equilibrated with 20 mM MES, pH 5.5, with at least 0.4 M NaCl used to prevent background binding. Fractions that tested positive for chymase activity were assessed for purity by SDS-PAGE and pooled (Fig. 2). The identity of chymase was confirmed by Western blot analysis and by N-terminal sequencing of the first 12 residues (data not shown). The S-100 chymase pool was concentrated using a Collodion vacuum dialysis unit (Schleicher and Schuell, Keene, NH) fitted with a 10 kDa membrane. After concentrating to dryness, the chymase was reconstituted with 20 mM MES, pH 5.5, to a concentration greater than 5 g/l (0.2 mM). An aliquot of 200 mM phenylmethylsulfonylfluoride (PMSF) in dimethylsulfoxide (DMSO) was added to the concentrate to at most 5% (v/v) DMSO in the final solution. Approximately 40-fold stoichiometric excess of PMSF to chymase was used.

2.3. Crystallization

PMSF-treated chymase, at a concentration of approximately 5 mg/ml, was subjected to hanging drop vapor-diffusion [24] crystallization experiments using factorial screening [25] kits (Hampton Research, Laguna Hills, CA). Preliminary crystals were obtained in a variety of polyethylene glycol (PEG) and PEG–monomethyl ether (MME) conditions. The pH of these conditions varied from 4.6 to 8.5, and salts such as ammonium sulfate, sodium chloride and sodium acetate also appear to facilitate crystallization. Two crystal forms were optimized. The first, from 20 to 30% PEG MME 2000, 0.1 M sodium acetate, pH 4.6, with 0.2 M ammonium sulfate was characterized and found to be space group C2. The cell constants are $a = 47.94$ Å, $b = 85.23$ Å, $c = 174.18$ Å, $\beta = 96.74°$. There appears to be either three or four molecules in the asymmetric unit, which corresponds to Matthews' coefficients [26] of 2.5 Å^3/Da and 1.9 Å^3/Da, respectively. These crystals diffract to at least 2.1 Å using an Raxis IV image plate system (MSC, The Woodlands, TX), with a Rigaku generator powered at 55 kV×90 mA, and have a useable X-ray lifetime of approximately 15–24 h. The second crystal form is obtained from 20 PEG 4000, 20% 2-propanol, 0.1 M sodium citrate, pH 5.6. These crystals were found to be of an orthorhombic habit, space group P212121, with cell dimensions $a = 43.93$ Å, $b = 58.16$ Å, and $c = 86.09$ Å. The Matthews' coefficient is 2.3 Å^3/Da for one molecule in the asymmetric unit. The crystals diffract to approximately 1.9 Å, and are not unusually sensitive to X-ray damage. The second crystal form provides a better opportunity for structure determination by molecular replacement. To this end, search models have been constructed from a homology model of human chymase, derived, in part, from the related rat mast cell protease II structure (pdb: 3RP2, [27]; 60% identity).

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