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A single step purification for autolytic zinc proteinases

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ABSTRACT

We describe a novel single-step method for the purification of stromelysin-1 catalytic domain (SCD) via immobilized metal affinity chromatography under denaturing conditions that inhibit proteolytic activity followed by on-column refolding and spontaneous autolysis of the fusion peptide to yield pure, active stromelysin-1 catalytic domain. The methodology provides a general approach for the rapid purification of large quantities of zinc proteinases.

The metal-dependent proteases encompass several large enzyme families, including the metzincin superfamily to which the matrix metalloproteinases (MMPs) belong. Disregulation of MMP expression and function is implicated in numerous pathophysiological processes,[1–6] and a number of potent inhibitors of MMP activity have been developed and evaluated as clinical candidates.[7] The matrix metalloproteinases also serve as important model systems for the study of structure–activity relationships in enzymatic catalysis, due to their highly variable substrate specificities despite a high degree of sequence homology. Despite their physiological and biophysical importance, the study of the MMP proteins is hindered by protein availability. Protein misfolding, the formation of insoluble inclusion bodies, and autolysis all contribute to low and inconsistent isolated protein yields. Here, we report a robust, facile approach to MMP isolation and purification that overcomes these significant limitations.

MMPs are expressed physiologically as preproproteins consisting of three domains—an N-terminal propeptide domain of roughly 80 residues, a large catalytic domain, and a C-terminal hemopoexin-like domain. The N-terminal prodomain contains a critical 'cysteine switch' responsible for chelation of the catalytic zinc in the zymogen form.[8] In vitro, MMPs are typically activated by the organomercurial aminophenyl mercuric acetate, although the mechanism by which this activation occurs remains obscure.[8–10] Stable constructs lacking the N-terminal prodomain and thus requiring no activation have been expressed.[11] The next approximately 180 amino acids encode the catalytic domain, containing structural calcium and zinc ions, as well as the catalytic zinc.[12] Although the C-terminal hemopoexin-like domain is present in all MMPs except MMP-7 the domain is rarely included in recombinant MMP constructs since it is proteolytically cleaved after MMP activation.[13–17]

In the course of our studies on substrate specificity, we required a robust, reproducible expression and purification protocol for MMP sufficient to provide 100 mg quantities of pure protein. Although several purification protocols have been reported,[11,18–21] most are limited by the requirement for specialized equipments (ultracentrifuges and/or FPLC), cost, and/or scale. Solid-phase IMAC procedures have been described for His_6-tagged variants of proMMP-7[22] and proMMP-13,[23,24] but no reports of immobilized metal affinity chromatography (IMAC) purification of a catalytic domain exist. Obviation of organomercurial activation would be of clear value, especially for purification on the scale proposed here. Furthermore, the majority of protein isolated from IMAC proprotein purification is inactive due to misfolding, necessitating a second inhibitor- or substrate mimetic affinity purification to isolate active enzyme.[23]

To overcome these limitations in the expression and purification of this important class of proteins we have developed a novel purification strategy that relies on metal affinity purification of inactive, partially denatured MMP, followed by refolding to yield active protein. The approach is reliable, highly reproducible and scalable.

A vector plasmid encoding amino acid residues 95–268 was constructed, representing the stromelysin catalytic domain and the final five residues of the prodomain to preserve the autolytic cleavage site. A plasmid containing stromelysin-1 c-DNA was a generous gift from Dr. L. Jack Windsor. The PCR primers 5′-TGC GGATCCCCTGATGTTGGTCAC TTC-3′ and 5′-ATT CTCGAGCTATCAG GGATCCGGGGGAGGTCC-3′ were used to amplify the desired sequence; a stop codon was introduced with the reverse primer. The amplified sequence was digested with BamH1 and XhoI and ligated with T4 ligase into the pET28 vector (Novagen, Darmstadt

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Germany), introducing an N-terminal His_6-fusion peptide. The sequence was confirmed by DNA sequencing, and the construct was transformed into BL-21 (DE3) Gold cells (Stratagene, La Jolla CA) for expression.

A culture of transformed *Escherichia coli* was grown at 37 °C in terrific broth (TB) media supplemented with 30 mg L^{-1} of kanamycin to $OD_{600\ nm}$ 0.6–0.8; protein expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 6–8 h. During expression, a 23 kDa protein appeared within 2 h, corresponding to the mass expected for the soluble catalytic domain (SCD) plus vector-derived fusion peptide. Under these conditions, the protein expressed exclusively as inclusion bodies. Following cell harvesting and lysis, the insoluble debris was resuspended in a bind buffer of 6 M urea, 250 mM NaCl, and 20 mM Tris–HCl, pH 7.9 (20 mL per liter of growth) supplemented with 2% SDS (w/v) and shaken overnight at 37 °C to solubilize the inclusion bodies.

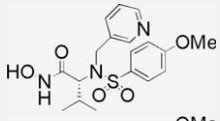
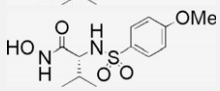
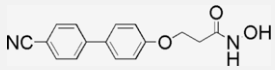
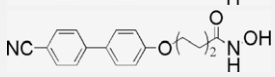
The solution containing protein solubilized from inclusion bodies (360 mg in 17.6 mL) was diluted fourfold with bind buffer to yield a final sodium dodecyl sulfate (SDS) concentration of 0.5% w/v. A 15 mL bed volume of His-bind Resin (Novagen) was charged with 75 mL of 50 mM $CoCl_2$. Co(II) was preferable to Ni(II) due to its increased specificity for His_6-fusions.[25,26] The resin was equilibrated with 45 mL bind buffer and then loaded with protein solution. The solubilized recombinant protein bound efficiently to the resin; no recombinant protein was detected in the flow-through. It is noteworthy that the Novagen resin seems to be uniquely suited for this procedure: attempts to reproduce the protocol using other iminodiacetic acid resins were unsuccessful (data not shown). The column was washed with six bed volumes of bind buffer, 10 bed volumes of wash I (6 M urea, 250 mM NaCl, 5 mM imidazole, 20 mM Tris–HCl, pH 7.9), and 10 bed volumes of wash II (6 M urea, 500 mM NaCl, 20 mM imidazole, 20 mM Tris–HCl, pH 7.9).

The protein was refolded on the resin using a two-step refolding similar to that described for proMMP-13.[23] The resin was washed with five bed volumes of refolding buffer (2.3 M urea, 200 mM NaCl, 10 mM $CaCl_2$, 10 μM $ZnCl_2$, 50 mM Tris–HCl, pH 7.78). The resin was allowed to stand for an additional 20 min before application of five bed volumes of elution buffer (200 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 50 mM Tris–HCl, pH 7.9). The eluate was collected in 10 mL aliquots; aliquots containing protein and absent visible Co(II) were combined to yield a solution containing 10–15 mg L^{-1} of active recombinant SCD lacking the fusion peptide; this material also reacted positively with anti-stromelysin-1 antibody. (Fig. 1) The protein was dialyzed once against a 50X volume of elution buffer to remove residual urea and three times against calorimetry buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$). A circular dichroism spectrum was consistent with previous reports (data not shown). Table 1 summarizes the purification procedure.

Table 1
Protein purification

Fraction	Volume (mL)	Concentration (mg/mL)	Total protein (mg)	Enrichment Factor
Crude lysate	15.2	48.4	736.9	n.a.
Insoluble crude fraction	17.6	20.4	359	2.08
Purified protein	36.0	0.38	13.7	53.8

Table 2
Binding data

Compound	IC_{50} or K_D (Lit)	K_D (Exp)[a,b]
	43 nM[28]	7 nM
	113 nM[28]	43 nM
	100 nM[19]	110 nM
	11 μM[19]	17 μM

[a] Values are means of three or more experiments.
[b] Protein concentrations were determined by the method of Edelhoch[29] using ε_{280} = 27,630 units.

The activity of the recovered SCD was verified by isothermal titration calorimetry (ITC) using previously described inhibitors; binding data are summarized in Table 2.[19,27,28] The observation that ITC titrations uniformly yielded binding stoichiometries ranging from 0.9 to 1.08 confirmed that all recovered protein was active (data not shown).

The solid phase refolding procedure described above evolved from initial attempts at traditional solution phase refolding. MMPs require both Ca(II) and Zn(II) ions for proper refolding. In a solution phase procedure, the His_6-fusion peptide readily chelated these ions, resulting in large protein aggregates. Chelation of the His_6-fusion peptide to the IMAC resin renders it unavailable for cation binding, preventing aggregation.

A second limitation of solution phase refolding is contamination by soluble but misfolded protein that must be removed in subsequent purification steps.[11,17,30] Although contamination by inactive protein is acceptable for high-throughput screens, inactive contamination is a significant problem for applications requiring highly pure protein such as ITC and crystallography. These applications therefore require the previous IMAC procedures to be cou-

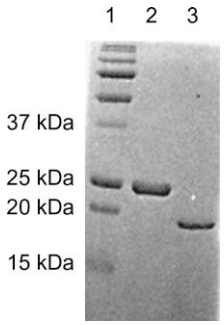


Figure 1. Autolysis of His_6-fusion peptide with refolding. Lane 1, standards; lane 2, purified SCD eluted prior to refolding; lane 3, eluate after solid phase refolding.

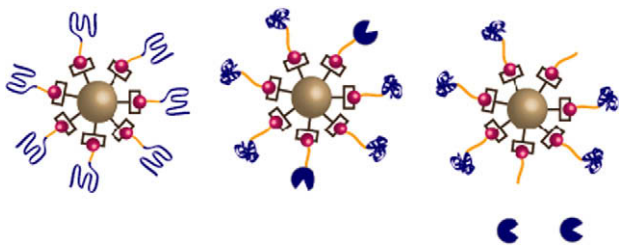


Figure 2. Schematic of solid phase refolding. The protein is purified while denatured (left). Upon refolding, only some of the protein is refolded properly (middle) and the rest is either misfolded or trapped as a protein folding intermediate. Properly refolded enzyme regains activity, autolyzes the fusion peptide tethering it to the resin and elutes from the column.

pled with a successive affinity purification step.[18] Contaminating inactive protein is completely avoided in our novel procedure. Our use of on-column refolding provides an in situ positive control for activity: only functional enzyme capable of autolysis and cleavage of the His_6-tag is recovered. In the absence of proper refolding, misfolded protein remains tethered to the immobilized matrix. A schematic of this process is shown in Figure 2.

Our procedure represents an operationally simple, general, and scalable approach to zinc metalloproteinase purification. The use of IMAC facilitates large scale purification, while in situ refolding ensures recovery of completely active protein. Although the process was validated here with a zinc proteinase, the protocol should be applicable to other metal-dependent proteolytic enzymes.

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