

# Short Technical Reports

## Large-Scale Purification of a Stable Form of Recombinant Tobacco Etch Virus Protease

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### ABSTRACT

*Tobacco etch virus NIa proteinase (NIa-Pro) has become the enzyme of choice for removing tags and fusion domains from recombinant proteins in vitro. We have designed a mutant NIa-Pro that resists autoproteolytic inactivation and present an efficient method for producing large amounts of this enzyme that is highly pure, active, and stable over time. Histidine-tagged forms of both wild-type and mutant NIa-Pro were overexpressed in E. coli under conditions in which greater than 95% of the protease was in the insoluble fraction after cell lysis. An inclusion body preparation followed by denaturing purification over a single affinity column and protein renaturation yields greater than 12.5 mg enzyme per liter of bacterial cell culture. NIa-Pro purified according to this protocol has been used for quantitative removal of fusion domains from a variety of proteins prepared for crystallization and biochemical analysis.*

### INTRODUCTION

Advances in recombinant protein technology over the past decade have led to the routine use of affinity tags to aid protein purification for applications including protein engineering and structural biology. Proteases are frequently used to remove these tags following purification, yielding native protein for subsequent applications. Factor Xa, commonly used for this purpose, is relatively inefficient, nonsequence specific, and cannot be recombinantly expressed. In contrast, tobacco etch virus NIa proteinase (NIa-Pro) recognizes a specific heptapeptide sequence, E-X-X-Y-X-Q↓S/G, and is active under a wide range of conditions and in the presence of various protease inhibitors (3,4,6,8,9).

In nature, NIa-Pro is the 27-kDa proteolytic portion of the 49-kDa NIa polypeptide found as an aggregate with

the 54-kDa NIb polypeptide in nuclear inclusion bodies in infected plant cells (2,7). Purification of NIa-Pro in large quantity has proved difficult because of the limited solubility of the protein. Inclusion bodies purified from infected plant tissue with subsequent separation of the 49- and 54-kDa polypeptides did not yield significant amounts of active NIa-Pro (5,7). Native purification of a histidine-tagged recombinant NIa-Pro from *E. coli* similarly produced low quantities of protease because the majority of the recombinant protein resides in inclusion bodies (7). An additional problem with NIa-Pro is that it contains an internal self-cleavage site that, when proteolyzed, produces a truncated protease with significantly reduced activity (7). Autoproteolysis during preparation and long-term storage thus reduces the yield of fully active protease.

To address these issues, a single amino acid change that inhibits autoproteolysis without affecting substrate cleavage activity, Ser219Asn (S219N), was introduced at the self-cleavage site within NIa-Pro. Furthermore, a protocol was developed for the denaturing purification and efficient renaturation of active NIa-Pro from inclusion bodies in *E. coli*. NIa-Pro purified using this protocol has been utilized to process a variety of fusion-tagged proteins that have been subsequently used for biochemical and crystallographic studies (1,10,11).

### MATERIALS AND METHODS

#### Cloning

A wild-type NIa-Pro gene was generated by PCR using pTL-5495 (ATCC, Manassas, VA, USA) as the template. The 5' primer 5TEVP1, 5'-CATCAGC-GGGCCATGGCTGAAAGCTTGTTT-AAG-3', changes the N terminus of NIa-Pro from the wild-type Gly-Glu-Ser to Met-Ala-Glu-Ser and contains an *Nco*I restriction site for cloning. The 3' primer 3TEVH1, 5'-CTGATGCACG GATCCTCATTAATGGTGATGGTG-ATGGTGCAATTGCGAGTAGATTC-ACTG-3', encodes a Leu-His6 tag and a *Bam*HI restriction site for cloning. The mutant NIa-Pro gene was generated by sequential PCRs. For the first reaction, pTL-5495 was used as the template

with primers 5TEVP1 and TEVPSN, 5'-GAGTTGAGTTGCTTCTTTGAC-TGGCTGAAAGGGTTCTTCAGGT-TTGTTTCATGAAAACCTTTGTGGC-3', to introduce the S219N mutation at the internal self-cleavage site. The resultant PCR product mixture was used as the template in a second PCR using the 5' primer 5TEVP1 and the 3' primer 3TEVH1. PCR products were ligated into the pET15b expression vector (Novagen, Madison, WI, USA) using the *Nco*I and *Bam*HI sites transformed into *E. coli* DH5 $\alpha$ <sup>TM</sup> (Life Technologies, Rockville, MD, USA) competent cells, and ampicillin-resistant colonies were selected. The resulting plasmids, pT-PWT and pTPSN, encoding polypeptides with molecular weights of 28.6 kDa, were sequenced for accuracy and then transformed into BL21 (DE3) (Novagen) competent cells for expression.

#### Protease Expression and Purification

NIa-Pro was expressed in BL21 (DE3) cells grown at 37°C in Luria Broth supplemented with 100  $\mu$ M ampicillin until cultures reached an absorbance (600 nm) of approximately 1.0. Expression was induced by adding 400  $\mu$ M isopropyl- $\beta$ -D-thiogalactoside (IPTG) followed by vigorous aeration at 37°C for 4–5 h. Cell pellets from each liter of culture were harvested by centrifugation, resuspended in 25 mL buffer containing 50 mM Tris-HCl, pH 8.0, and 300 mM NaCl, and stored at -80°C. Cell lysis and native purification of NIa-Pro were performed as described (7) using nickel affinity chromatography (Ni-NTA Superflow; Qia-gen, Valencia, CA, USA), except 10% glycerol, 300 mM NaCl, and 5 mM 2-mercaptoethanol were included in all buffers, and the Ni-NTA column was washed with buffer containing 10 mM imidazole and eluted with buffer containing 400 mM imidazole. For inclusion body purification, cell suspensions were thawed in cool water, and the buffer was adjusted to a final concentration of 500  $\mu$ g/mL lysozyme (Sigma, St. Louis, MO, USA), 200  $\mu$ g/mL DNase I (Roche Molecular Biochemicals, Indianapolis, IN, USA), 50  $\mu$ g/mL PMSF, 10  $\mu$ g/mL leupeptin (Roche Molecular Biochemicals), 20 mM

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**Table 1. Purification Yields from Native and Denaturing NIa-Pro Protocols<sup>a</sup>**

NIa-Pro	Method	<i>n</i> <sup>b</sup>	Total Protein In Ni-NTA Load (mg)	Total Ni-NTA Pure Protein (mg)	Active NIa-Pro <sup>c</sup> (mg)	% yield <sup>d</sup>
TPWT	Native	1	977	4.08	<0.41	<0.04
	Denaturing	5	80	32.25 ± 4.12	9.07 ± 2.90	0.86
TPSN	Native	1	946	3.67	<0.37	<0.04
	Denaturing	7	81	31.79 ± 5.07	9.87 ± 2.21	0.96

<sup>a</sup>Yields are reported as milligrams protein per liter cell culture.  
<sup>b</sup>*n* is the number of independent preparations.  
<sup>c</sup>Active NIa-Pro was not assayed for the native preparations; milligrams of active NIa-Pro are estimated to be less than 10% of the total Ni-NTA-pure protein. Data reported for the denaturing preparations reflect the yield from the initial dialysis of the Ni-NTA eluate.  
<sup>d</sup>% yield is reported as the percent active NIa-Pro obtained from total cellular protein.

MgSO<sub>4</sub>, and 2 mM CaCl<sub>2</sub>. Cell suspensions were incubated for 30 min with agitation at 4°C and then lysed by three freeze-thaw cycles. Triton<sup>®</sup> X-100 was added to a final concentration of 1%, and the samples were vortex mixed for 1 min. The inclusion bodies were recovered by centrifugation for 20 min at 10 000× *g*. Pellets were stored at -80°C.

Chromatography under denaturing conditions was performed at 4°C using 10 mL Ni-NTA Superflow resin for each liter of cell culture. The column was equilibrated with 10 column volumes (cv) equilibration buffer containing 6 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM Tris-HCl, pH 8.0. Inclusion body pellets were resuspended in buffer containing 6 M guanidine-HCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM Tris-HCl, pH 8.0, and incubated in a 65°C bath to dissolve the inclusion bodies. Samples were centrifuged for 20 min at 15 000× *g*, and the supernatant was loaded onto the column by gravity flow. The column was washed with 4 cv equilibration buffer and then 6 cv wash buffer containing 6 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM Tris-HCl, pH 6.3. To elute the protein, a buffer containing 6 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM Tris-HCl, pH 4.5, was applied to the column and one 0.3-cv fraction was collected. Flow was stopped for a 5-min incubation, then was resumed, and four additional 1-cv fractions were collected. Fractions containing NIa-Pro, as determined by

SDS-PAGE, were pooled, adjusted to pH 8.5 with 10 N NaOH, and dialyzed for 4–8 h into storage buffer containing 100 mM Tris-HCl, pH 8.5, 500 mM NaCl, 50% glycerol, 5 mM dithiothreitol (DTT), and 0.5 mM EDTA. The supernatant containing active renatured NIa-Pro was separated from precipitated NIa-Pro by centrifugation for 20 min at 15 000× *g*, and both were stored at -80°C. The precipitated NIa-Pro was redissolved in equilibration buffer and dialyzed into fresh storage buffer to obtain additional pools of active protease.

NIa-Pro preparations were quantified using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. NIa-Pro from the native preparation was not assayed for activity due to significant contamination with other proteins. The yield of active enzyme in these samples was estimated to be less than 10% of the total nickel affinity-purified protein. For the denaturing preparation, the active NIa-Pro yield is reported as milligrams of NIa-Pro obtained from two cycles of renaturation as described.

## Activity Assays

A 17-kDa substrate with the sequence I-E-G-R-P-A-A-E-N-L-Y-F-Q<sup>↓</sup>G-M-G-D-V-L-S at the N terminus contains both a factor Xa cleavage site (underlined sequence with cleavage after R) and a NIa-Pro cleavage site (bold

letters with cleavage at the arrow). When cleaved with commercially available TEV protease (Life Technologies), this substrate yields a 15-kDa peptide. Factor Xa (43 kDa) and NIa-Pro (28.6 kDa) cleavage activities were compared in 30-μL reactions containing 250 μM substrate, 2.33 μM Factor Xa (New England Biolabs, Beverly, MA, USA) or 0.86 μM NIa-Pro, and 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 2 mM CaCl<sub>2</sub>. Reactions were incubated at 23°C for 6 h. Samples were analyzed by electrophoresis in 15% SDS-PAGE gels and stained with Coomassie<sup>®</sup> Blue. NIa-Pro activity was assayed in 30-μL reactions containing 100, 250, or 500 μM substrate, 0.86 μM NIa-Pro, and 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 0.5 mM EDTA. Reactions were incubated at 30°C for 1 h. Samples were analyzed by electrophoresis in 15% SDS-PAGE gels, stained with Coomassie Blue, and the resultant protein bands were quantified using a gel documentation system (Eastman Kodak, Rochester, NY, USA). The net intensities of full-length and cleaved substrate bands were used to calculate a specific activity as picomoles substrate cleaved per femtomole NIa-Pro at each substrate concentration. Self-cleavage activity was assayed under the same buffer conditions in 40-μL reactions containing 6.5 μM NIa-Pro and incubated at 4°C, -20°C, and -80°C. After 0.5, 1, 2, and 5.5 weeks, samples were analyzed by

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electrophoresis in 15% SDS-PAGE gels and quantitated as described above. The net intensities of full-length and truncated NIa-Pro bands were used to calculate the percent NIa-Pro cleaved at each time point.

## RESULTS

Following induction of NIa-Pro expression in *E. coli*, native preparations yield minimal quantities of the protease (Figure 1, lane 4 and Table 1) (7). The NIa-Pro detected in high quantity within total cell lysate is concentrated in inclusion bodies, suggesting that virtually all of the enzyme is expressed as insoluble aggregate (Figure 1, lanes 1–3). Expression was explored at a variety of temperatures ranging from 20°C to 37°C; even at low temperatures, which generally favor the soluble expression of proteins, the majority of the NIa-Pro resided in the insoluble fraction. Nickel affinity column purification under de-

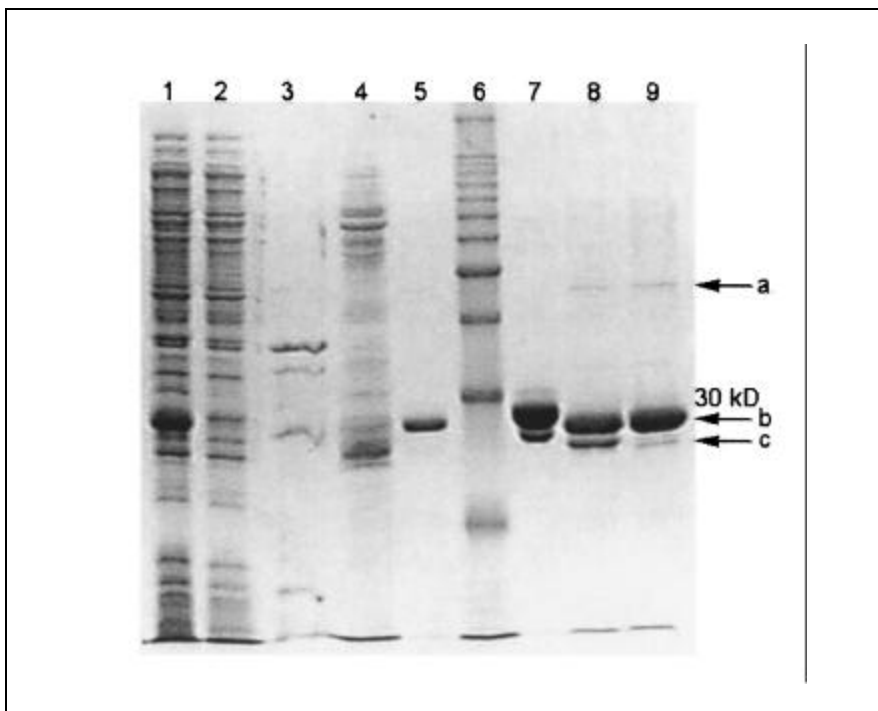
**Table 2. NIa-Pro Renaturation Yields<sup>a</sup>**

	TPWT	TPSN
Ni-pure protein (mg)	32.25 ± 4.12	31.79 ± 5.07
First renaturation <sup>b</sup> :		
total yield (mg)	9.07 ± 2.90	9.87 ± 2.21
% Ni-NTA pure protein renatured	27.9	31.0
Two renaturation cycles <sup>c</sup> :		
total yield (mg)	12.68 ± 3.25	13.62 ± 2.49
% Ni-NTA pure protein renatured	39.3	42.8

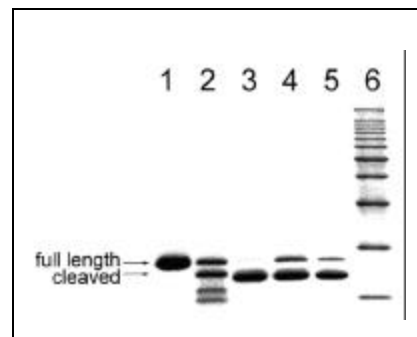
<sup>a</sup>Yields reported as milligrams protein per liter cell culture.  
<sup>b</sup>The first renaturation data are the yields of soluble, active NIa-Pro from the initial dialysis of the Ni-NTA elutions, adjusted to pH 8.5.  
<sup>c</sup>Data reported for two renaturation cycles are the total yields of soluble, active NIa-Pro from the initial dialysis of the Ni-NTA elutions, adjusted to pH 8.5, plus one subsequent renaturation from the dialysis pellet.

naturing conditions enabled recovery of significant amounts of NIa-Pro from the insoluble fraction. Furthermore, this material is of greater purity than that produced under soluble conditions

(Figure 1, lanes 4 and 5). In samples containing 4.5 µg total protein, a single contaminant (Figure 1, lanes 8 and 9, arrow a) is detected in NIa-Pro from the denaturing protocol, indicating greater than 95% purity. NIa-Pro purified in this way was renatured by dialysis to remove urea, resulting in approximately 10 mg active wild-type (TPWT) and S219N (TPSN) forms of the enzyme per liter of cell culture (Table 1). A second renaturation of the dialysis pellet yielded additional active enzyme for a total yield of greater than 12.5 mg renatured active NIa-Pro per liter of cell culture (Table 2). Approximately 30% of the affinity-purified protein was renatured after the first dialysis, and a total



**Figure 1. Fifteen percent SDS-PAGE gel of purification samples from native and denaturing preparations of NIa-Pro.** Lanes 1–5, TPSN purifications: lane 1, total cellular lysate; lane 2, clarified lysate; lane 3, resuspended inclusion body pellet (in 6 M GuHCl); lane 4, concentrated Ni-NTA pure sample from native purification; lane 5, renatured TPSN from denaturing purification. Lane 6 contains a 10-kDa protein ladder (Life Technologies). Lanes 7–9 contain approximately 4.5 µg NIa-Pro: lane 7, NIa-Pro control (Life Technologies); lane 8, renatured TPWT from denaturing purification; lane 9, renatured TPSN from denaturing purification. Arrows a–c indicate significant bands: a, the major contaminant in denaturing NIa-Pro preparations; b, full-length NIa-Pro; and c, truncated NIa-Pro produced by self-cleavage activity.



**Figure 2. Fifteen percent SDS-PAGE gel of factor Xa and NIa-Pro substrate cleavage reactions.** Lane 1 contains full-length substrate. Lanes 2–5, substrate proteolysis reactions: lane 2, factor Xa; lane 3, NIa-Pro control; lane 4, TPWT; and lane 5, TPSN. Lane 6 contains a 10-kDa protein ladder. Substrate was incubated for 6 h at 23°C with 2.33 µM factor Xa or 0.86 µM NIa-Pro.

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of 40% was renatured after two dialysis cycles (Table 2). The average concentration of renatured preparations was approximately 1.0 mg/mL with a maximum solubility of 1.5 mg/mL (data not shown). Average concentrations of samples dialyzed directly out of elution buffer at pH 4.5 were approximately 0.3 mg/mL (data not shown), indicating that adjusting to pH 8.0–8.5 is critical for efficient refolding.

Substrate cleavage by Nla-Pro produces a single product, whereas factor Xa proteolysis yields the desired polypeptide plus two additional degradation products (Figure 2). There also appears to be more unreacted substrate remaining after incubation with factor Xa than with Nla-Pro, despite the use of almost three times as much enzyme in the factor Xa reaction. TPWT and TPSN have specific activities similar to each other and to the commercially available Nla-Pro at all substrate concentrations tested (Figure 3), demonstrating that refolding does not compromise enzyme activity. Full activity is also further evidence of the purity of these preparations.

Both the commercially available Nla-Pro and purified TPWT contain significantly more truncated enzyme than TPSN in the final preparation (Figure 1, lanes 7–9, arrows b and c). To assess self-cleavage activity, we utilized a C-terminal histidine tag to bind only full-length peptide to the Ni-NTA column. Final TPWT preparations contain approximately 20% truncated peptide (Figure 4A,  $t = 0$ ) indicating self-cleavage activity during protease renaturation at 4°C. Self-cleavage continues during enzyme storage at 4°C or -20°C (Figure 4, A and B), potentially reducing the proportion of fully active enzyme in a Nla-Pro stock over time. The self-cleavage activity of TPWT is similar to that of the control protease at 4°C and -20°C. In contrast, TPSN exhibits reduced self-cleavage activity at 4°C and none at -20°C (Figure 4, A and B). None of the Nla-Pro samples tested exhibited significant self-proteolysis at -80°C during our 5.5-week study (data not shown).

## DISCUSSION

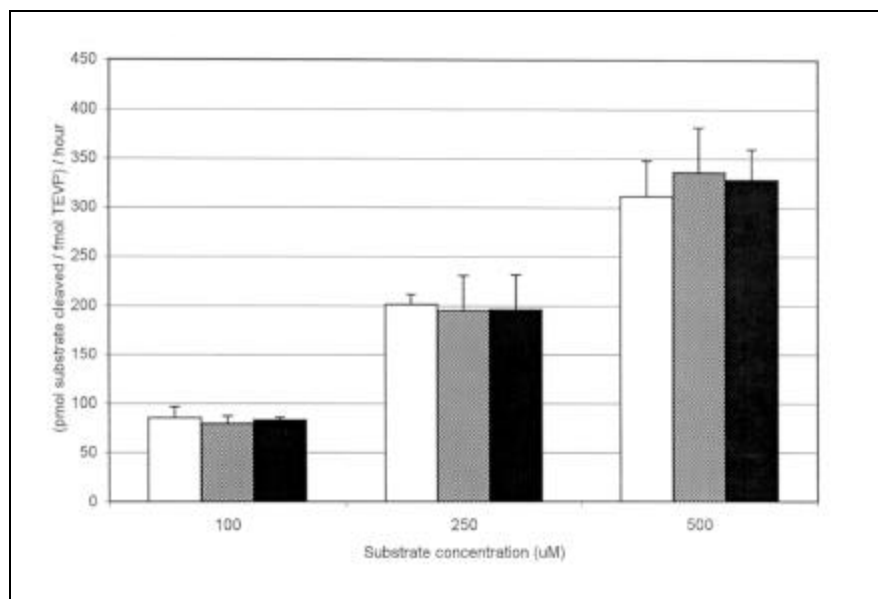
The high specificity of the Nla-Pro

protease for the heptapeptide sequence E-X-X-Y-X-Q↓S/G and its activity in the presence of protease inhibitors and under a wide range of conditions make Nla-Pro an ideal tool for quantitative removal of fusion tags from recombinant proteins. We have shown that *E. coli* strains transformed with wild-type, as well as mutant, histidine-tagged Nla-Pro constructs can be induced to express greater than 95% of the protease in inclusion bodies (Table 1 and Figure 1). This property was exploited to produce milligram quantities of Nla-Pro using an efficient purification and renaturation protocol. A single denaturing affinity column purification of the Nla-Pro inclusion bodies, followed by renaturation of the purified enzyme yields 10 times as much active Nla-Pro as the soluble preparation (7), allowing greater than 12.5 mg stock preparations of greater than 95% pure active Nla-Pro to be prepared per liter of cell culture (Table 2). Improved yields and the ability to denature and renature Nla-Pro without the loss of activity, as shown by comparison to a commercially available Nla-Pro, might also be exploited for crystallographic and biochemical analysis of this protease.

Within the Nla-Pro self-cleavage

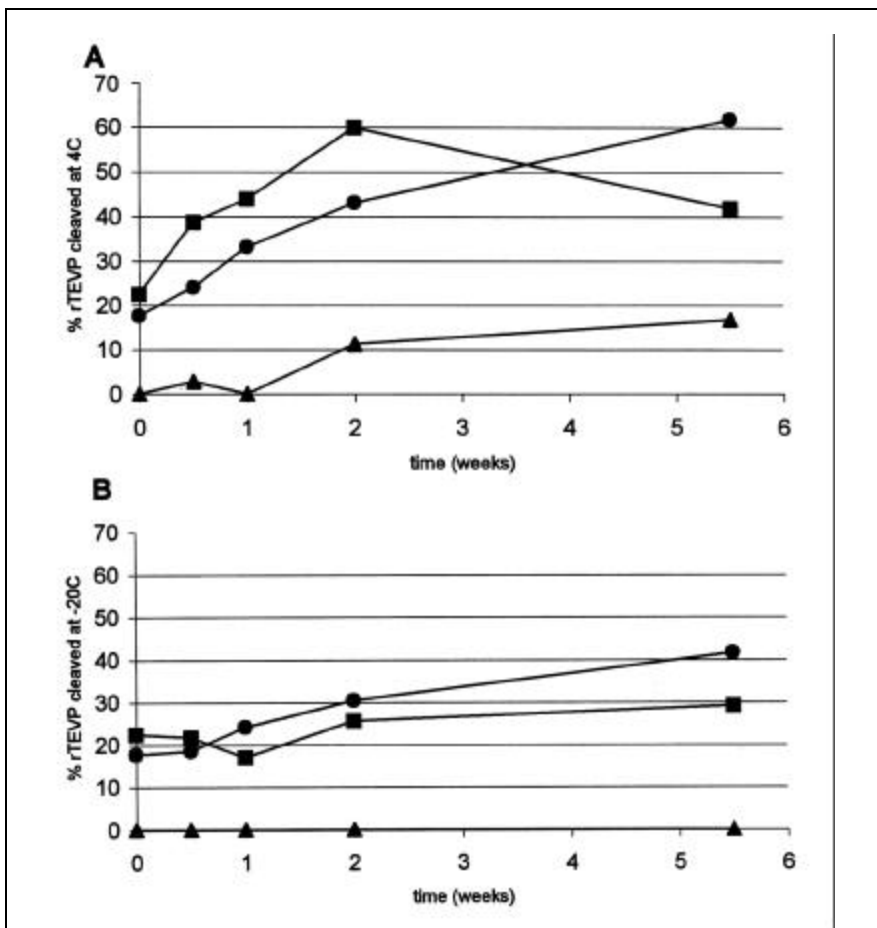
site, G-H-K-V-F-M↓S, Ser219 is the only amino acid matching the consensus substrate cleavage site (7). The S219N mutation in TPSN significantly inhibits self-cleavage activity (Figure 3, A and B), allowing for increased yields of full-length Nla-Pro. It also permits long-term storage (at least six weeks) of Nla-Pro stocks at -20°C and short-term storage (up to six weeks) at 4°C without significant autoproteolysis. The mechanism of self-cleavage was not studied, but a S219A mutant exhibited wild-type levels of self-cleavage activity (data not shown). Parks et al. (7) reported significantly less substrate cleavage activity of the truncated form of Nla-Pro compared to the full-length enzyme. We found comparable specific activities for both wild-type and mutant Nla-Pro with our substrate despite the fraction of truncated polypeptide present in the control and wild-type samples (Figure 2). This effect may be concentration or substrate dependent, and activity with other substrates is currently under investigation.

The significantly greater specificity of Nla-Pro over factor Xa makes this protease a superior tool for many protein applications. Development of a rapid and efficient protocol for the ex-



**Figure 3.** The activity of commercially available Nla-Pro control (white bars,  $n = 3$ ), TPWT (shaded bars,  $n = 6$ ), and TPSN (black bars,  $n = 8$ ) at various substrate concentrations is reported with error bars to reflect the standard deviation. Approximately 0.9 μM Nla-Pro was incubated for 1 h at 30°C with 100, 250, and 500 μM substrate. The net intensities of bands on Coomassie blue-stained 15% SDS-PAGE gels were used to calculate specific activity as the picomoles substrate cleaved per femtomole Nla-Pro in 1 h at 30°C.

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**Figure 4.** The self-cleavage activity of 6.5  $\mu$ M commercially available N1a-Pro control (circles), TPWT (squares), and TPSN (triangles) was monitored for up to 5.5 weeks at 4°C (A) and -20°C (B). Data are reported as percent truncated N1a-Pro at each timepoint.

pression and purification of milligram quantities of recombinant N1a-Pro should also facilitate the routine use of this protease.

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