Identification of a cDNA encoding an active asparaginyl endopeptidase of *Schistosoma mansoni* and its expression in *Pichia pastoris*[1]

Conor R. Caffrey[a], Mary A. Mathieu[a], Alan M. Gaffney[a], Jason P. Salter[a], Mohammed Sajid[a], Kimberley D. Lucas[a], Christopher Franklin[a], Matthew Bogyo[b], James H. McKerrow[a,*]

[a] *Tropical Disease Research Unit at the Department of Pathology, UCSF, VAMC, 4150 Clement St.-113B, San Francisco, CA 94121, USA*
[b] *Department of Biochemistry and Biophysics, UCSF, San Francisco, CA 94121, USA*

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Abstract Asparaginyl endopeptidases, or legumains, are a recently identified family of cysteine-class endopeptidases. A single gene encoding a *Schistosoma mansoni* asparaginyl endopeptidase (a.k.a. Sm32 or schistosome legumain) has been reported, but by sequence homology it would be expected to yield an inactive product as the active site C197 had been replaced by N. We now describe a new *S. mansoni* gene in which C197 is present. Both gene products were expressed in *Pichia pastoris*. Autocatalytic processing to fully active C197 Sm32 occurred at acid pH. In contrast, N197 Sm32 was not processed and this is consistent with the hypothesis that C197 is essential for catalysis. This was confirmed by mutation of N197 to C and re-expression in *Pichia*. The availability of recombinant active Sm32 allows detailed analysis of its catalytic mechanism and its function(s) in the biology of this important human parasite.

Key words: Asparaginyl endopeptidase; Legumain; Sm32; Recombinant expression; *Schistosoma*

1. Introduction

Asparaginyl endopeptidases or legumains are a novel family of cysteine-class endopeptidases with members reported in plants [1], mammals [2] and in the parasitic blood fluke *Schistosoma* spp. [3]. Asparaginyl endopeptidases are proposed to belong to the CD clan of cysteine endopeptidases along with caspases, clostripain and gingipains [4]. They have a specificity for an asparaginyl residue in the P_1 position of the substrate (terminology of Schechter and Berger [5]) for hydrolysis and possess an approximately 15 kDa extension C-terminal of the catalytic domain [1–3]. In plants, legumains process seed precursor proteins to their final forms [6,7]. In mammals, the related asparaginyl endopeptidases have a key function in the initial processing of antigens for MHC class II presentation [8] and in the inhibition of osteoclast formation and bone resorption [9].

The asparaginyl endopeptidase of *S. mansoni* (a.k.a. Sm32) is an important immunodiagnostic antigen for schistosomiasis [10]. A single Sm32 gene in both the *S. mansoni* and *S. japonicum* species has been identified [3,11,12]. However, on the basis of sequence homology with other asparaginyl endopeptidases, the product of this Sm32 gene would not be proteolytically active as the crucial active site cysteine residue at position 197 had been replaced by an asparagine (N197 Sm32) [4]. We now identify a second distinct gene for Sm32 containing the requisite C197 residue (C197 Sm32). Using the *Pichia pastoris* protein expression system, we have expressed recombinant, active C197 Sm32. We further show by site-directed mutagenesis (N197C) that N197 Sm32 is rendered proteolytically active and demonstrate that autocatalysis at acid pH leads to production of mature, active Sm32, accompanied by removal of the C-terminal extension and possibly of the N-terminal pro-domain.

2. Materials and methods

2.1. Cloning and sequencing of C197 Sm32, and site-directed mutagenesis

Poly(A)$^+$ mRNA was isolated (Pharmacia) from 6-week-old *S. mansoni* worms of mixed sex and converted to single-stranded cDNA using AMV Reverse Transcriptase as described by the manufacturer (Life Technologies). PCR amplification of the C197 Sm32 gene was accomplished with primers designed to encompass the pro- and C-terminal regions of the endopeptidase: forward: 5′-ATACTCGAGAAAAGACAATTAGATACAAATTATGAAGTATCC-3′ and reverse: 5′-AATGCGGCCGCTTAACCGCAAATTTTTATGATTGCT-3′. For eventual cloning into the *P. pastoris* expression vector pPIC Zα A, the forward primer incorporated *Xho*I endonuclease and Kex 2 endopeptidase sites (both underlined) immediately 5′ of the gene-specific sequence. The reverse primer incorporated a transcription termination codon and a *Not*I site (both underlined) immediately 3′ of the gene-specific sequence. Amplification reactions were performed with Pwo polymerase (Boehringer) with 35 cycles of 94°C, 55°C and 72°C, each for 1 min. PCR products were purified (Qiagen) and digested with *Xho*I and *Not*I. pPIC Zα A was similarly digested and ligated to the PCR products. For sequencing, the same PCR products were amplified using the above PCR conditions and cloned into pCR-Blunt II-TOPO according to the manufacturer's (Invitrogen) instructions. A unique *Afl*III restriction site was found for C197 Sm32 (see below) and was used to distinguish such clones from those of N197 Sm32. The signal peptide sequence of C197 Sm32 was obtained from individual Blunt II-TOPO clones that had been generated using a forward primer directed to the signal peptide (5′-ATGATGCTATTCTCTTTATTTCTTA-3′) and the above-described reverse primer. PCR conditions to generate such clones were as described above. The entire open reading frame (ORF) of C197 Sm32 was sequenced in both directions. To generate the N197C mutant Sm32, site-directed mutagenesis was performed by sequential PCR steps [13] using the forward primer 5′-TTATATTGAAGCATGTGAATCAGG-3′ (bases encoding the mutation are underlined) and the appropriate anti-parallel reverse primer. This mutation resulted in the same *Afl*III site as found in C197 Sm32. All recombinant plasmids were propagated in DH5α cells (Life Technologies) and purified (Qiagen).

*Corresponding author. Fax: (1)-415-750 6947.
E-mail: jmck@cgl.ucsf.edu

[1] The EMBL Nucleotide Sequence Database accession number for C197 Sm32 is AJ250582.

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2.2. Expression of Sm32 in P. pastoris

Procedures to express Sm32 in the X33 strain of *P. pastoris* were as detailed by the manufacturer (Invitrogen). Briefly, recombinant pPIC Zα A (10 μg) was linearized with *Sac*I and purified. *P. pastoris* was electroporated at 1.5 kV and 129 Ω in electroporation cuvettes (2 mm gap; BTX). *Pichia* colonies growing under zeocin (Invitrogen) selection were picked for expansion first in 10 ml of yeast extract–peptone–dextrose medium containing 100 μg/ml zeocin and then in 200–400 ml of the same medium. Expression of recombinant protein was induced by incubation of *Pichia* for 48 h in buffered-minimal medium containing 1% methanol as the sole carbon source. Culture medium was clarified through a 0.45 μm filter, lyophilized and stored at 4°C.

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1/1                                                 31/11
ATG ATG CTA TTC TCT TTA TTT CTT ATT AGC ATC TTA CAT ATT CTA TTA GTC AAA TGT CAA
M   M   L   F   S   L   F   L   I   S   I   L   H   I   L   L   V   K   C   Q
61/21                                               91/31
TTA GAT ACA AAT TAT GAA GTA TCC GAT GAA ACT GTT AGT GAT AAT AAT AAA TGG GCT GTA
L   D   T   N   Y   E   V   S   D   E   T   V   S   D   N   N   K   W   A   V
121/41                                              151/51
TTA GTA GCT GGA TCT AAT GGT TAT CCC AAT TAT AGA CAT CAA GCA GAT GTC TGT CAT GCT
L   V   A   G   S   N   G   Y   P   N   Y   R   H   Q   A   D   V   C   H   A
181/61                                              211/71
TAT CAT GTA TTA CGT TCA AAG GGT ATA AAA CCT GAG CAT ATT ATC ACA ATG ATG TAT GAT
Y   H   V   L   R   S   K   G   I   K   P   E   H   I   I   T   M   M   Y   D
241/81                                              271/91
GAT ATC GCT TAT AAT TTG ATG AAT CCA TTT CCT GGG AAA CTT TTT AAT GAT TAT AAC CAT
D   I   A   Y   N   L   M   N   P   F   P   G   K   L   F   N   D   Y   N   H
301/101                                             331/111
AAA GAT TGG TAT GAA GGA GTG GTG ATA GAT TAT CGC GGT AAA AAA GTC AAC TCG AAA ACT
K   D   W   Y   E   G   V   V   I   D   Y   R   G   K   K   V   N   S   K   T
361/121                                             391/131
TTT CTG AAA GTT TTG AAG GGA GAT AAA AGC GCT GGT GGG AAA GTT TTG AAA AGT GGA AAA
F   L   K   V   L   K   G   D   K   S   A   G   G   K   V   L   K   S   G   K
421/141                                             451/151
AAT GAT GAT GTA TTC ATA TAC TTC ACT GAT CAT GGT GCA CCG GGT CTA ATT GCT TTC CCT
N   D   D   V   F   I   Y   F   T   D   H   G   A   P   G   L   I   A   F   P
481/161                                             511/171
GAT GAT GAA TTA TAT GCT AAA CAA TTT ATG TCA ACA TTG AAA TAC TTA CAT AGT CAC AAA
D   D   E   L   Y   A   K   Q   F   M   S   T   L   K   Y   L   H   S   H   K
541/181                                             571/191
CGT TAT TCA AAA TTG GTG ATT TAT ATT GAA GCA TGT GAA TCA GGT TCC ATG TTC CAA CGA
R   Y   S   K   L   V   I   Y   I   E   A   C   E   S   G   S   M   F   Q   R
601/201                                             631/211
ATA TTA CCG TCG AAT CTA AGT ATT TAT GCG ACT ACA GCT GCT AGT CCA ACT GAA TCT AGT
I   L   P   S   N   L   S   I   Y   A   T   T   A   A   S   P   T   E   S   S
661/221                                             691/231
TAT GGT ACA TTT TGT GAT GAT CCA ACA ATA ACT ACT TGT CTG GCT GAT TTA TAC TCA TAC
Y   G   T   F   C   D   D   P   T   I   T   T   C   L   A   D   L   Y   S   Y
721/241                                             751/251
GAC TGG ATT GTT GAC TCA CAA ACA CAT CAT TTA ACA CAA CGA ACA CTC GAT CAA CAG TAT
D   W   I   V   D   S   Q   T   H   H   L   T   Q   R   T   L   D   Q   Q   Y
781/261                                             811/271
AAA GAG GTT AAA CGT GAA ACG AAT CTT AGT CAT GTT CAG AGA TAT GGT GAT ACG AGA ATG
K   E   V   K   R   E   T   N   L   S   H   V   Q   R   Y   G   D   T   R   M
841/281                                             871/291
GGT AAA TTA CAC GTT AGT GAA TTT CAA GGA AGT CGA GAC AAG TCT TCA ACT GAG AAC GAT
G   K   L   H   V   S   E   F   Q   G   S   R   D   K   S   S   T   E   N   D
901/301                                             931/311
GAA CCT CCA ATG AAA CCA AGA CAT TCC ATC GCT TCG AGA GAT ATT CCA TTG CAT ACT CTA
E   P   P   M   K   P   R   H   S   I   A   S   R   D   I   P   L   H   T   L
961/321                                             991/331
CAT CGT CAA ATA ATG ATG ACC AAT AAT GCA GAA GAC AAA AGC TTT CTG ATG CAA ATT CTT
H   R   Q   I   M   M   T   N   N   A   E   D   K   S   F   L   M   Q   I   L
1021/341                                            1051/351
GGT TTG AAA CTG AAG AGA AGA GAT CTC ATT GAG GAT ACT ATG AAA TTA ATA GTG AAA GTA
G   L   K   L   K   R   R   D   L   I   E   D   T   M   K   L   I   V   K   V
1081/361                                            1111/371
ATG AAT AAT GAA GAA ATA CCA AAT ACC AAG GCA ACT ATC GAT CAA ACA TTG GAT TGT ACA
M   N   N   E   E   I   P   N   T   K   A   T   I   D   Q   T   L   D   C   T
1141/381                                            1171/391
GAA TCA GTC TAT GAA CAG TTC AAA AGT AAA TGT TTC ACA CTA CAA CAG GCT CCA GAA GTT
E   S   V   Y   E   Q   F   K   S   K   C   F   T   L   Q   Q   A   P   E   V
1201/401                                            1231/411
GGA GGG CAC TTT TCT ACT CTT TAC AAT TAT TGT GCA GAT GGT TAT ACA GCT GAA ACG ATC
G   G   H   F   S   T   L   Y   N   Y   C   A   D   G   Y   T   A   E   T   I
1261/421
AAT GAA GCA ATC ATA AAA ATT TGC GGT
N   E   A   I   I   K   I   C   G
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Fig. 1. Nucleotide and deduced amino acid sequence of C197 Sm32. Active site histidine and cysteine residues are presented in bold; potential glycosylation sites are underlined and arrows [14] indicate the putative N-termini of the pro-domain, mature protease and C-terminal extension.

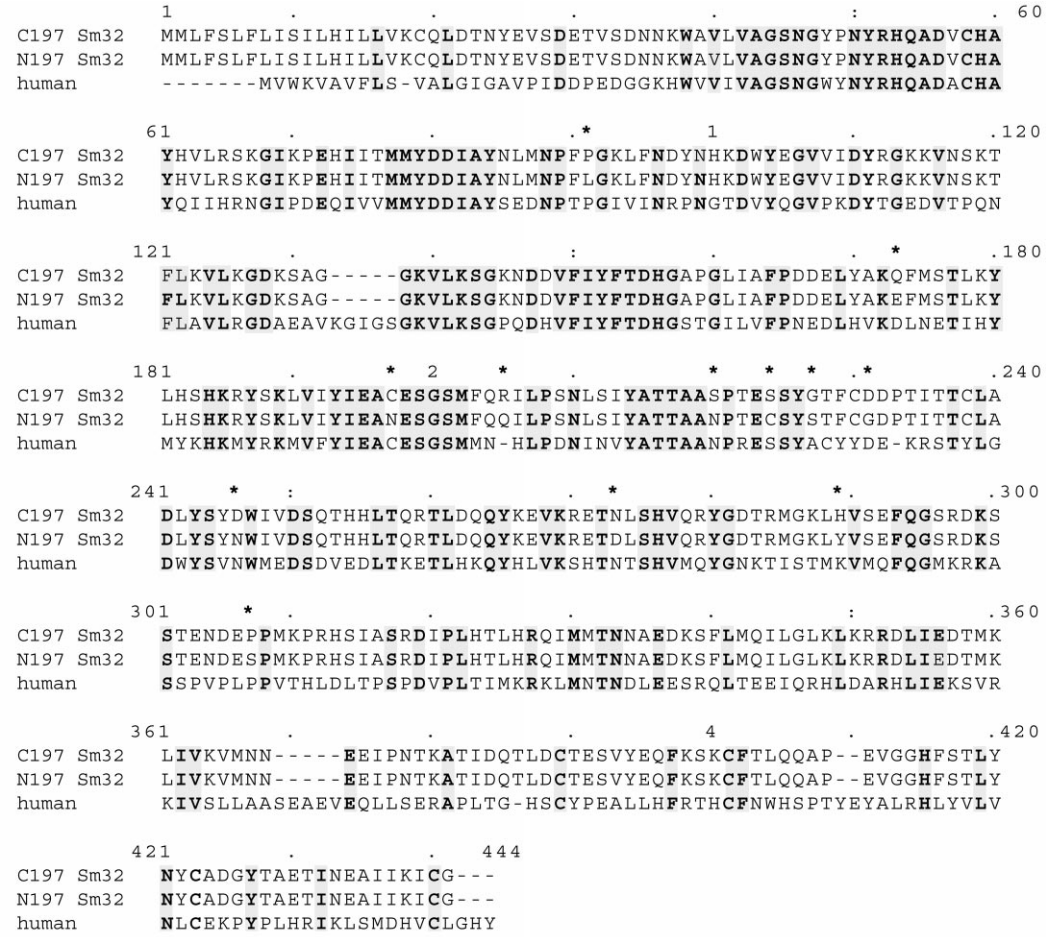

Fig. 2. Amino acid sequence alignment of C197 Sm32 with N197 Sm32 [11] and human legumain [2]. Amino acid residues differing between the two schistosome sequences are indicated by asterisks; areas where all three sequences are identical are shaded.

Lyophilized culture media containing recombinant Sm32 were resuspended in 0.1 M citrate–phosphate, pH 6.8 to a volume one-tenth of that of the original. Complete buffer exchange was accomplished using PD10 desalting columns (Pharmacia). Solutions were concentrated 10 times in Centricon 10 centrifugation units (Millipore) and stored at −20°C.

2.3. Detection and activation of recombinant Sm32, and endopeptidase assays

Recombinant Sm32 was detected by immunoblotting after SDS–PAGE (4–12% gradient gels; Novex) using a monospecific rabbit antiserum developed to recombinant N197 Sm32 expressed in *Escherichia coli* (Ewald Beck, Biochemisches Institut, Universität Giessen, Germany). For activation experiments, enzyme solution was incubated for different time periods at 37°C in the presence of 2 mM dithiothreitol (DTT) and no further additions (pH was measured to be 6.8) or 0.2 M sodium acetate pH 4.5 (pH was adjusted using a one-tenth volume of 2 M sodium acetate pH 4.5). The following endopeptidase inhibitors (final concentrations were 14 μM unless otherwise indicated) were tested for their ability to prevent activation of Sm32: iodoacetic acid (IA; 5 μM), E-64, morpholinourea-Phe-homoPhe-vinyl sulfone phenyl, PMSF (2 mM) leupeptin, pepstatin A and 1,10-phenanthroline (2 mM). Endopeptidase activity was monitored by hydrolysis of benzyloxy (Z)-Ala-Ser-Asn-7-amido-4-methylcoumarin (NMec; custom synthesis, Enzyme Systems Products). In our hands, this substrate is degraded by Sm32 approximately twice as fast as the more commonly used Z-Ala-Ala-Asn-NMec (Caffrey, unpublished observation). Assays were performed in black 96-well microtiter plates and contained 5–30 μl enzyme solution preincubated for 10 min in 100 μl 0.1 M citrate–phosphate, 2 mM DTT, pH 6.8. Substrate (stocks in dimethyl sulfoxide) in 100 μl of the same buffer (10 μM) was then added and the reaction allowed to proceed for 20–30 min at room temperature. Fluorescence was measured in a Labsystems Fluoroskan II plate reader at excitation and emission wavelengths of 355 and 460 nm, respectively.

3. Results and discussion

3.1. A second gene encodes Sm32 with cysteine in the active site

Heretofore, only one complete gene sequence has been identified for Sm32 (a.k.a. *S. mansoni* asparaginyl endopeptidase or schistosome legumain) [3,11]. This was predicted to encode an inactive enzyme as the active site C197 had been replaced by an asparagine residue [4]. Consistent with this prediction, our preliminary attempts to obtain endopeptidase activity from an N197 clone expressed in *P. pastoris* had failed. Subsequent isolation and sequencing of a number of clones derived from *S. mansoni* mRNA identified a second gene for Sm32 where C197 was present and the entire ORF is pre-

sented in Fig. 1. C197 Sm32 has 97.2 and 41.0% identity to the previously published N197 Sm32 [11] and human legumain [2], respectively (Fig. 2). C197 Sm32 differs from N197 Sm32 by 12 amino acid residues. Also, C197 Sm32 has two potential *N*-glycosylation sites whereas N197 Sm32 has three, one of which, interestingly, is found at position 197.

3.2. Expression of Sm32 forms in P. pastoris and activation of C197 Sm32

Both N197 and C197 Sm32 were expressed and detectable in *Pichia* cultures using an anti-N197 Sm32 monospecific rabbit serum (Fig. 3). In fresh preparations of the N197 Sm32, a single immunoreactive protein was detected at 50 kDa (Fig. 3a). For C197 Sm32, predominant protein bands at 50 and 47 kDa were detected with minor species at 42 and 39 (Fig. 3b). Very little hydrolysis ($< 5\%$) of the fluorogenic substrate Z-Ala-Ser-Asn-NMec could be detected in fresh C197 Sm32 preparations (Fig. 4). Significant endopeptidolytic activity was only evident on incubation of C197 Sm32 preparations at pH 4.5 and correlated with the appearance of a final protein product at 34 kDa (Fig. 3b, 2 h time point onwards). Prolonged incubation of C197 Sm32 at pH 6.8 did not evince increased hydrolysis of the fluorogenic substrate from the 5% basal level (Fig. 4) nor conversion of the major 50 and 47 kDa proteins (not shown), thus indicating the acid pH requirement for conversion to the fully active enzymatic form. In contrast to C197 Sm32, N197 Sm32 was incapable of being activated, either at pH 4.5 (Fig. 3) or at pH 6.0 (not shown), and did not hydrolyze Z-Ala-Ser-Asn-NMec (Fig. 4). These results support the prediction based on sequence homology [4] that Sm32 with N rather than C at position 197 would be most likely inactive.

3.3. Activation of Sm32 requires Cys at position 197 and is an autocatalytic event

Confirmation that the endopeptidolytic activity of Sm32 requires Cys at position 197 was obtained by recombinant expression of N197 Sm32 where the active site Asn at 197 had been mutated to a Cys. Like C197 Sm32, the N197C mutant was capable of being activated, but only at pH 4.5 (Figs. 4 and 5) and not at pH 6.8 (not shown). Also, increased

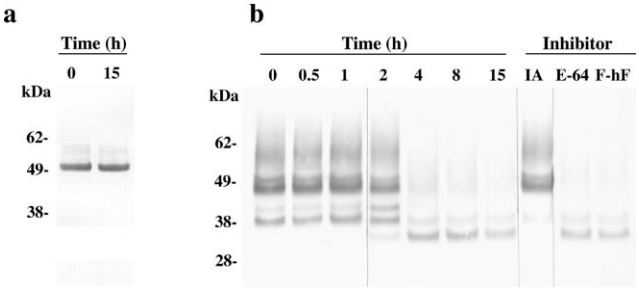

Fig. 3. Conversion of C197, but not N197 Sm32, to the final active enzyme product. As described in Section 2, Sm32 proteins, expressed in *P. pastoris* supernatants, were incubated at 37°C in the presence of DTT at pH 4.5 for different time periods. After SDS–PAGE and blotting onto PVDF membrane, supernatants were reacted with a monospecific anti-N197 Sm32 rabbit serum. a: N197 Sm32 after incubation at pH 4.5 for 0 and 15 h. b: as for a, but with C197 Sm32; inhibitors were incubated for 15 h with C197 Sm32. IA, iodoacetic acid; F-hF, morpholinourea-Phe-homoPhe-vinyl sulfone phenyl.

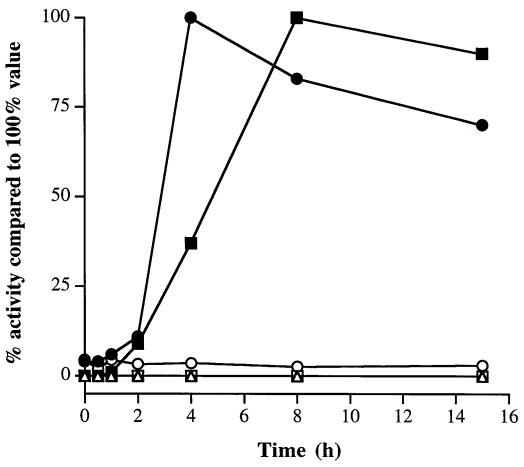

Fig. 4. Conversion to final Sm32 products at acid pH is associated with increased endopeptidolytic activity. As described for the above experiments (Section 2 and Fig. 3) N197, C197 or N197C mutant Sm32 was incubated at pH 6.8 or 4.5 for different time periods for up to 15 h. Endopeptidolytic activity of various Sm32 preparations was measured using the fluorogenic substrate Z-Ala-Ser-Asn-NMec. Incubation at pH 4.5; N197 (△), C197 (●) and N197C mutant (■). Incubation at pH 6.8; C197 (○) and N197C mutant (□).

hydrolysis of Z-Ala-Ser-Asn-NMec was only associated with the appearance of the final protein product which resolved at 37 kDa (Fig. 5, 2 h time point and onwards). Tests with endopeptidase inhibitors formally eliminate the possibility that an endopeptidase(s) other than Sm32 in the *Pichia* culture medium activates Sm32 in *trans*. Only the general cysteine endopeptidase inhibitor IA blocked activation of both C197 (Fig. 3b) and N197C mutant Sm32 (Fig. 5). Importantly, inhibitors of papain-like cysteine endopeptidases such as E-64, morpholino-Phe-homoPhe-vinyl sulfone phenyl (Fig. 3b) and leupeptin (not shown) did not prevent conversion to the final enzyme forms. E-64 has previously been shown to be relatively ineffective against asparaginyl endopeptidase activity in schistosome extracts [15] and pig legumain [2]. Likewise, inhibitors of serine (PMSF and leupeptin), aspartic (pepstatin A) and metallo-endopeptidases (1,10-phenanthroline) were ineffective (not shown).

3.4. The function of the C-terminal extension in Sm32

The immunoblot and enzymatic experiments herein demonstrate that maximal endopeptidolytic activity of C197 and N197C mutant Sm32 is associated with the autocatalytic proc-

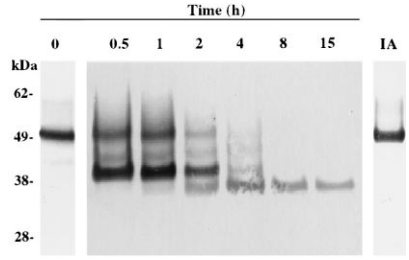

Fig. 5. Mutating N197 to C restores the ability of N197 Sm32 to convert to the final active Sm32 product. Experimental design was as for C197 Sm32 (legend to Fig. 3 and Section 2). IA, iodoacetic acid.

essing of an approximately 50 kDa protein species to about 35 kDa. Consistent with plant and animal legumains [1–3], loss of the C-terminal extension and possibly of the N-terminal pro-domain would account for this conversion. Given the specificity of asparaginyl endopeptidases like Sm32 for Asn at P_1, the processing sites for removal of the N-terminal pro-domain and the C-terminal extension of Sm32 as previously proposed [14] (Fig. 1) must now be re-evaluated. The identification of a 50 kDa Sm32 precursor and a 35 kDa mature protein agrees with the detection of native asparaginyl endopeptidase species in *S. mansoni* extracts [16]. It is not clear, however, whether the conversion to the fully active enzyme in the worm is a *cis* or *trans* event. The C-terminal extension may perform a function similar to that of the N-terminal pro-region of papain-like endopeptidases such as cathepsin B and L [17] by inhibiting the active site until activity is required by the cell.

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