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Processing of the Papain Precursor

PURIFICATION OF THE ZYMOGEN AND CHARACTERIZATION OF ITS MECHANISM OF PROCESSING*

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Thierry Vernet‡§, Henry E. Khouri¶‖, Pierre Laflamme¶, Daniel C. Tessier‡, Roy Musil‡, Barbara J. Gour-Salin¶, Andrew C. Storer¶, and David Y. Thomas§

From the ‡Genetic Engineering Section and ¶Protein Engineering Section, Biotechnology Research Institute, National Research Council of Canada, Montréal, Québec H4P 2R2, Canada

The precursor of the cysteine protease papain has been expressed and secreted as propapain from insect cells infected with a recombinant baculovirus expressing a synthetic gene coding for prepropapain. This 39-kDa secreted propapain zymogen molecule is glycosylated and can be processed *in vitro* into an enzymatically active authentic papain molecule of 24.5 kDa (Vernet, T., Tessier, D. C., Richardson, C., Laliberté, F., Khouri, H. E., Bell, A. W., Storer, A. C., and Thomas, D. Y. (1990) *J. Biol. Chem.* 265, 16661-16666). Recombinant propapain was stabilized with Hg^{2+} and purified to homogeneity using affinity chromatography, gel filtration, and ion-exchange chromatographic procedures. The maximum rate of processing *in vitro* was achieved at approximately pH 4.0, at a temperature of 65 °C and under reducing conditions. Precursor processing is inhibited by a variety of reversible and irreversible cysteine protease inhibitors but not by specific inhibitors of serine, metallo or acid proteases. Replacement by site-directed mutagenesis of the active site cysteine with a serine at position 25 also prevents processing. The inhibitor ^{125}I-*N*-(2S,3S)-3-*trans*-hydroxycarbonyloxiran-2-carbonyl-L-tyrosine benzyl ester covalently labeled the wild type papain precursor, but not the C25S mutant, indicating that the active site is accessible to the inhibitor and is in a native conformation within the precursor. Based on biochemical and kinetic analyses of the activation and processing of propapain we have shown that the papain precursor is capable of autoproteolytic cleavage (intramolecular). Once free papain is released processing can then occur in trans (intermolecular).

Proteolytic enzymes are ubiquitous in biological systems. Their physiological roles range from protein degradation within specialized cellular compartments to precise cleavage of protein precursors (Neurath, 1984). Proteases are often synthesized as higher molecular weight precursors, zymogens or proproteases. There are a variety of mechanisms by which different protease precursors can be activated to functional proteases (Kassell and Kay, 1973).

Of the major classes of proteases, the best characterized precursors are trypsinogen of the serine protease family (Kunitz and Northrop, 1936) and pepsinogen (Herriott, 1938) of the aspartyl protease family (for a review see Desnuelle (1986)). The former is activated following limited proteolysis by other proteases, whereas the activation of the latter is by an autocatalytic mechanism, being intramolecular below pH 3 and mainly intermolecular at higher pH values (Al-Janabi *et al.*, 1972). The structural basis of inactivation-activation is known for these precursors. The three-dimensional structure of pepsinogen has been elucidated and has revealed that the N-terminal 44 residues of the pro region interact with the protease active site via salt bridges which are disrupted at low pH (James and Sielecki, 1986). A similar type of interaction between the activation segment (pro region) and the active site has been recently reported for procarboxypeptidase B (Coll *et al.*, 1991). Precursors of cysteine proteases are comparatively poorly understood and no three-dimensional structures of cysteine protease precursors have been reported so far. Autocatalytic conversion of precursors of cysteine proteases including those of cathepsin B (Felleisen and Klinkert, 1990) and cathepsin L (Smith and Gottesman, 1989) have been reported. Processing of the precursor of the vacuolar plant cysteine protease aleurain, on the other hand, requires the activity of another cysteine protease (Holwerda *et al.*, 1990).

The canonical member of the cysteine protease family, papain (EC 3.4.22.2) is a 24-kDa plant endoprotease which has a wide substrate specificity and which contains an essential thiol group in its active site. Despite extensive structural and enzymatic characterization of mature papain (for a review see Brocklehurst *et al.*, 1987; Baker and Drenth, 1987) very little is known about its precursor form. Cloning of a cDNA encoding papain has revealed that the enzyme is synthesized as a precursor that includes a 133-amino acid N-terminal extension[1] (Cohen *et al.*, 1986). Such a high molecular weight precursor form of papain has not been observed in *Carica papaya* latex despite earlier reports of the presence of a catalytically inactive form of papain (Brocklehurst and Kierstan, 1973; Kierstan *et al.*, 1982). This, in addition to the difficulties in expressing the functional recombinant molecule in *Escherichia coli* (Cohen *et al.*, 1990; Vernet *et al.*, 1989), has impaired further biochemical characterization of the precursor. We have recently overcome this obstacle by expressing the functional papain precursor using the baculovirus/insect cell system. The initial translation product of the prepropa-

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§ To whom correspondence should be addressed: Genetic Engineering Section, Biotechnology Research Inst., NRCC, 6100 Royalmount Ave., Montréal, Québec H4P 2R2, Canada. Tel.: 514-496-6154; Fax: 514-496-6213.

‖ Present address: Merck Frosst Canada Inc., 16711 Trans-Canada Hwy., Kirkland, Québec, H9H 3L1, Canada.

[1] The numbering of amino acid residues of the precursor of papain has been described previously (Vernet *et al.*, 1990); the pro region amino acids are designated with negative numbers starting at the pro region papain junction (Asn^{-1}/Ile^{1}).

pain gene expressed from the polyhedrin promoter is composed of three functional domains which become separated post-translationally by independent proteolytic cleavage events: the 26-residue N-terminal signal peptide which is removed during secretion of the protein, the 107-residue N-glycosylated pro region, and the 212-residue mature papain. Characterization of insect cell culture supernatant containing the recombinant protein has shown that the 39-kDa glycosylated papain precursor is inactive and that it can be activated *in vitro* (Vernet *et al.*, 1990). The activation process generates active mature papain with a molecular mass of 24.5 kDa and whose enzymatic properties are indistinguishable from those of papain isolated from *C. papaya*, the natural source (Ménard *et al.*, 1990).

In this paper we describe the characteristics of the papain precursor. Using a combination of site-directed mutagenesis, with kinetic and biochemical studies we show that the processing of the precursor of papain can result from an autocatalytic intramolecular and, once mature papain is released, from an intermolecular proteolytic event.

EXPERIMENTAL PROCEDURES

Enzyme Assay—The standard enzymatic assay was performed at room temperature in 0.1 M phosphate buffer, pH 6.0, 2 mM cysteine, 1 mM EDTA, and either 5 or 50 μM of the synthetic substrate, carbobenzoxyl-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide hydrochloride. The requirement of cysteine for precursor processing was determined by omitting cysteine from the assay mix. The reactions were initiated by adding the enzyme sample. Fluorescence of the 7-amino-4-methylcoumarin product was monitored for about 2 min in a Cary 2200 spectrometer fitted with a fluorescence attachment and measured at an excitation wavelength of 380 nm with a type GG455 cut-off filter in the emission beam.

Mutant Construction and Expression of Recombinant Papain Precursor—Uracil-containing single-stranded DNA (Kunkel, 1985) from the transfer plasmid IpDC176 (Tessier *et al.*, 1991) which contains the honeybee melittin signal peptide fused to propapain (proP)[2] was used as template for *in vitro* site-directed mutagenesis (Zoller and Smith, 1982). The replacement of the active site cysteine at position 25 in mature papain by a serine (C25S mutant) was done using the oligonucleotide: 5′-GGCAGCTGTGGCTCGAGTTGGGCTTTCTC-3′, which also creates a diagnostic *Xho*I restriction site. The resulting plasmid construction (IpDC201) was sequenced in the region surrounding the site of the mutation (Sanger *et al.*, 1977). A mixture of transfer plasmid IpDC201 DNA and *Autographa californica* (AcNPV) genomic DNA was used to co-transfect *Spodoptera frugiperda* (Sf9) insect cells (Summers and Smith, 1987). The recombinant baculovirus was isolated as previously described (Vernet *et al.*, 1990) and was designated as Ac(melproP C25S). Construction of the recombinant baculovirus Ac(melproP), which expresses the wild type papain precursor fused to the melittin signal sequence, has been described elsewhere (Tessier *et al.*, 1991). For the production of the recombinant proteins, Sf9 insect cells were grown in serum-supplemented medium and washed 2 h post-infection under conditions previously described (Vernet *et al.*, 1990) except that the osmolarity of the Grace's medium used to wash and resuspend the cells was adjusted to 380 mosm with 2 ml of sterile 80% glycerol/liter. The secreted propapain was harvested 48 h post-infection.

Purification of Recombinant Propapain—Purification steps were performed at room temperature. Culture medium containing the secreted papain precursor was collected by centrifugation of the cell culture at 1,500 × *g*. The precursor was stabilized by the addition of $HgCl_2$ to the cell culture supernatant and to all the buffers to a final concentration of 1 mM. In a typical preparation, 1 liter of culture medium was concentrated 10-fold by ultrafiltration using an Amicon Diaflo S1Y10 spiral cartridge. The concentrated medium was loaded onto a lentil lectin column (100 × 10 mm, inner diameter) which had previously been equilibrated with 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl (buffer A). The column was washed with 10-bed volumes of buffer A. The papain precursor was eluted using 10-bed volumes of 0.2 M α-methylmannopyranoside in buffer A. The eluted fraction was then concentrated 30-fold using Centriprep-10 concentrators (Amicon) and subjected to gel filtration chromatography on a Superose 12 HR 10/30 column which had previously been equilibrated with 20 mM Tris-HCl, pH 7.0 (buffer B). The fractions containing the papain precursor were pooled and subjected to ion-exchange chromatography on a Mono Q HR 5/5 column which had been previously equilibrated with buffer B. The column was washed with 5-bed volumes and the bound proteins were eluted with 1 M KCl in buffer B. The papain precursor was found entirely in the wash fraction as determined by our immunodetection procedure. This was performed as follows: protein fractions were treated with 0.1 μM E-64 and transferred to nitrocellulose (Western blot procedure) from SDS-PAGE (Laemmli, 1970). Detection used rabbit antibodies raised against denatured papain and then a secondary antibody, goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad), as described previously (Vernet *et al.*, 1989). Protein concentrations were determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as the standard.

In Vitro Activation of the Recombinant Papain Precursor—All reactions were performed in 1.5-ml Eppendorf tubes incubated in a thermoregulated water bath. Precursor processing was assessed as the amount of mature active papain released under the various experimental conditions employed. The activity of papain was determined using 20-200-μl aliquots by the spectrofluorometric assay described above. The effects of temperature on processing was investigated using a standard reaction mixture (SRM) containing approximately 50 nM of precursor, 20 mM cysteine, 50 mM NaOAc buffer, pH 4.0 in a final volume of 1 ml. For the determination of the initial rate of activation as a function of pH, reaction mixtures containing 20 mM cysteine and 25 mM of either HCl/KCl buffer (pH 2.5–2.8), citrate buffer (pH 3–5), or phosphate buffer (pH 5–7.5) were incubated at 50 °C for 0–15 min. The time course of activation as a function of cysteine concentration was determined using the SRM described and an incubation temperature of 60 °C.

Activation of the mixture of C25S and wild type papain was performed using the SRM described above. The effect of chymotrypsin on processing of the C25S and wild type papain precursor was determined at 60 °C using the SRM described above except that the pH was adjusted to 7.0. Chymotrypsin was inactivated by the addition of an excess of PMSF (100 μg/ml) prior to the assay of papain enzymatic activity.

Determination of the rate of processing as a function of concentration of the precursor was performed as follows. Various concentrations of the precursor, ranging from approximately 10 to 65 nM were incubated with 20 mM cysteine and 25 mM NaOAc buffer, pH 3.86, at 50 °C for 0–10 min. Quantitation of active papain released at various times of incubation was performed by spectrofluorometry as described above.

Inhibition of Papain Precursor Processing—A crude solution of about 100 nM of papain precursor was preincubated for 30 min at room temperature with various inhibitors (see figure legends for concentrations used). The mixture was then equilibrated with 100 mM NaOAc, pH 4.0, or with 100 mM Tris-HCl, pH 7.0 (for human cystatin C). The mixture was preincubated with a variety of inhibitors in the absence or in the presence of 2 or 20 mM cysteine for 20 min at 60 °C (see figure legends for specific conditions) except for the experiment with cystatin C (Dalboge *et al.*, 1989) that was incubated overnight. The amount of mature papain released was measured by spectrofluorometry and Western blot analysis as described above.

Affinity Labeling of the Papain Precursor—The synthesis of the *N*-(2S,3S)-3-*trans*-ethoxycarbonyloxiran-2-carbonyl-L-tyrosine benzyl ester was based on the method of Tamai *et al.* (1981). This compound was selectively hydrolyzed using pig liver esterase to produce the *N*-(2S,3S)-3-*trans*-hydroxycarbonyloxiran-2-carbonyl-L-tyrosine benzyl ester. Details of the synthesis, purification, and characterization of these compounds will be reported elsewhere.[3] EpsTyrOBzl was labeled with ^{125}I using immobilized lactoperoxidase reagent (Bio-Rad). The labeled inhibitor was incubated for 30 min at room temperature with either crude Hg^{2+}-free preparations of the wild type or C25S mutant

[2] The abbreviations used are: proP, propapain; 2-PDS, 2,2′-dithiodipyridine; AcNPV, *A. californica* polyhedrosis virus; E-64, 1-[[(L-*trans*-epoxysuccinyl)-L-leucyl]amino]-4-guanidino)butane; EpsTyrOBzl, *N*-(2S,3S)-3-*trans*-hydroxycarbonyloxiran-2-carbonyl-L-tyrosine benzyl ester; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, N^{α}-tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; SRM, standard reaction mixture; NaOAc, sodium acetate.

[3] B. J. Gour-Salin, P. Lachance, R. Ménard, M.-C. Magny, and A. C. Storer, manuscript in preparation.

TABLE I

Purification of propapain

The conversion of the precursor into mature active papain and the measurement of papain activity by spectrofluorometry were performed as described under "Experimental Procedures." Propapain was purified from 1 liter of medium.

Step	Total protein	Specific activity[a]	Total activity	Purification	Recovery
	mg	$\mu M \cdot s^{-1} \cdot mg^{-1}$	$\mu M \cdot s^{-1}$	*-fold*	%
Crude	185	0.17	31.45		100
Ultrafiltration[b]	64	0.39	24.96	2.3	79
Lentil lectin	0.89	14.36	12.78	84	41
Ultrafiltration[c]	0.50	19.78	9.89	116	31
Superose 12	0.16	24.64	3.94	145	12
Mono Q	0.06	48.53	2.91	285	9

[a] Following *in vitro* activation.
[b] On a spiral ultrafiltration unit fitted with an S1Y10 cartridge.
[c] Using a Centriprep filter.

papain precursor or with the mature papain derived from the recombinant wild type papain precursor. The mixture was then resolved by SDS-PAGE and analyzed by Western blot analysis as described above. The Western blot was autoradiographed using a Kodak X-Omat AR film for 48 h.

RESULTS

Expression and Purification of the Recombinant Papain Precursor—A synthetic gene encoding prepropapain (Vernet *et al.*, 1989) has been previously transferred into a recombinant baculovirus Ac(preproP). Insect cells (Sf9) infected with Ac(preproP) accumulate and secrete functional papain precursor (Vernet *et al.*, 1990). The amount of propapain secreted into the culture medium has recently been increased about 5-fold by replacing the authentic signal sequence of the papain precursor by the signal sequence of the honeybee melittin precursor to generate the recombinant baculovirus Ac(melproP) (Tessier *et al.*, 1991). This recombinant was used in the present work. The culture medium of Sf9 cells infected with Ac(melproP) was collected no later than 48 h post-infection so as to minimize proteolysis of the precursor by insect cell proteases released by cell lysis (Vernet *et al.*, 1990). To further reduce proteolysis of the precursor during purification, 1 mM $HgCl_2$ was added to the cell supernatant. Efficient concentration of the protein was achieved by ultrafiltration and the secreted *N*-glycosylated papain precursor (Vernet *et al.*, 1990) was eluted from lentil lectin-Sepharose by α-methylmannopyranoside (Table I). Mature papain which was released during the initial steps of the purification procedure was removed by a gel filtration step followed by concentration with a Centriprep filtration device. Lectins which co-eluted with the precursor fraction were eliminated by binding to the anion-exchanger Mono Q. The papain precursor was found in the wash fraction from this column. The purification was 285-fold, with a 9% recovery (Table I). The homogeneity of the preparation was verified by SDS-PAGE and silver staining (data not shown).

In Vitro Activation of the Papain Precursor—We initially determined the optimal conditions for the *in vitro* activation of the papain precursor by systematic alterations of the reaction mixture. The highest rates of activation were observed at pH values between 3.5 and 4.0. At pH values lower than 3.5 the rate of processing apparently decreased. This may reflect the instability of the precursor and of papain at low pH values. The activation rate is reduced about a hundred-fold when the activation is performed at pH 6 or higher (Fig. 1*A*).

The effect of temperature on the rate of activation was investigated at pH 4 (Fig. 1*B*). For temperatures ranging from 30 to 65 °C the increase in the rate of activation is exponential (Fig. 1*B*), for each 10 °C interval there was an approximate 2.5-fold rate increase. At temperatures above 65 °C the precursor activation rate decreased (data not shown). Again this may be the result of thermal inactivation of the precursor or papain. A more complete analysis of the relative thermal stability of the precursor and mature forms of papain will be presented elsewhere.[4]

The requirements for reducing conditions during the activation process was also investigated using crude preparation of propapain in the absence of Hg^{2+}. In the absence of cysteine the total activity recovered was approximately 60% of that found in the presence of 20 mM cysteine. This may indicate that a fraction of the propapain molecules are in an oxidized state incompatible with *in vitro* processing. To verify this hypothesis we have added 20 mM cysteine to a reducing agent-free mixture 15 min after the start of the reaction. This addition resulted in a sudden increase of activity. The maximal level of activity obtained under these conditions is comparable to that obtained when 20 mM cysteine was present at the beginning of the reaction (Fig. 1*C*).

We selected the following standard conditions for complete activation of the papain precursor: incubation at 60 °C for 15–30 min at pH values between 3.7 and 4.2, in the presence of 20 mM cysteine.

The Active Site Cysteine 25 Is Required for Precursor Processing—The requirement of a functional papain active site for the processing of the zymogen was tested using site-directed mutagenesis. The codon for the active site cysteine at position 25 was replaced with a serine codon. This effect is not believed to grossly destabilize the precursor structure. Indeed, the levels of propapain secretion from insect cells of the wild type and C25S mutant precursor were found to be comparable (data not shown). In addition, the 24.5-kDa papain molecule released upon cleavage with chymotrypsin is resistant to further protease degradation, another indication that the protein has a compact structure and is correctly folded (see below and Fig. 6*B*). The size of the 39-kDa mutant precursor was maintained in the *in vitro* processing assay, and no papain activity was detected by spectrofluorometry (Fig. 2).

Cysteine Protease Inhibitors Prevent Precursor Processing—We have investigated the effects of molar excess of reversible or irreversible protease inhibitors on papain precursor processing (Fig. 3). A crude Hg^{2+}-free preparation of approximately 100 nM papain precursor was preincubated with the inhibitors, and the mixtures were subsequently incubated under the standard activation conditions. The effect

[4] T. Vernet, D. C. Tessier, H. E. Khouri, and D. Altschuh, submitted for publication.

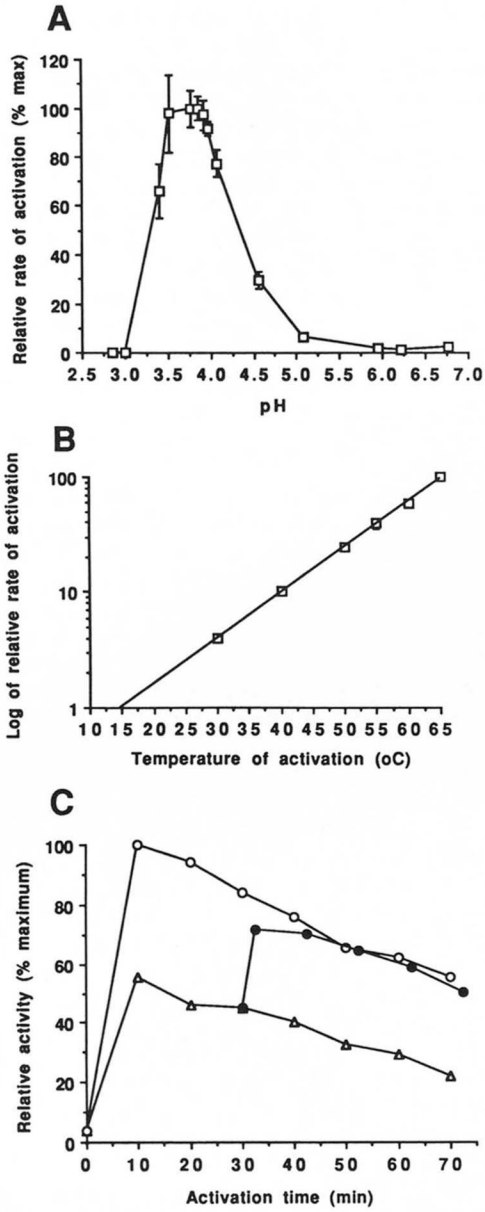

FIG. 1. **Determination of the activation conditions for the papain precursor.** *A*, pH profile of precursor activation. The initial rate of activation of the papain precursor was determined for pH values ranging from 2.8 to 6.8 in conditions described under "Experimental Procedures." The standard deviations of the rates are presented. *B*, temperature profile of precursor activation. The rate of papain precursor activation was determined under conditions described under "Experimental Procedures." The log value of the rates was plotted against the temperature, and the data points were fitted by linear regression. The standard deviations of the rates are represented and mostly contained within the *squares* indicating the data points. *C*, time course of activation. Activation was performed as described under "Experimental Procedures" in the absence (*open triangles*) or in the presence of 20 mM Cys added at the start of the reaction (*open circles*) or 15 min after the start of the reaction (*closed circles*).

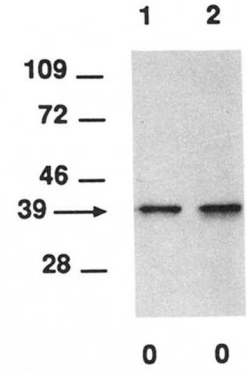

FIG. 2. **Effect of C25S mutation on the papain precursor.** Western blot of crude preparation of C25S papain precursor mutant prior to (*lane 1*) or following (*lane 2*) *in vitro* activation at 60 °C, pH 4.0, for 20 min in the presence 20 mM cysteine. Molecular masses of standards (kDa) are indicated in the *left margin*, and the *arrows* designate the molecular masses (kDa) of the proteins detected.

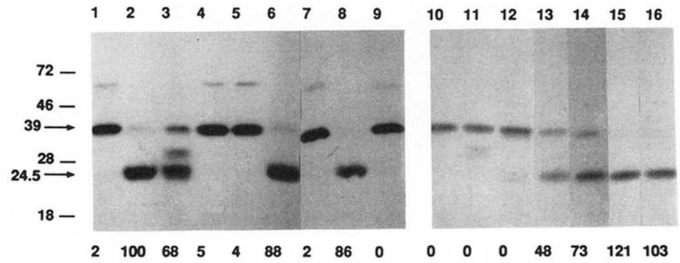

FIG. 3. **Effect of the protease inhibitors on the processing of propapain.** Western blot of the wild type secreted papain precursor following *in vitro* incubation and activation in the presence of molar excess of different protease inhibitors. No activation (*lanes 1* and *10*), activation of an approximatively 100 nM solution of precursor in the absence of inhibitor (*lane 2*), preincubation and activation in the presence of 2 mM Cys (*lane 3*), 2 mM $HgCl_2$ and no Cys (*lane 4*), 2 mM $HgCl_2$ and 2 mM Cys (*lane 5*), 20 mM Cys (*lane 6*), 1.5 μM 2-PDS and without Cys (*lane 7*), 1.5 μM 2-PDS (*lane 8*), 50 μM E-64 (*lane 9*), 3.8 μM recombinant human cystatin C at pH 7.0 (*lane 11*), 50 μg/ml TLCK (*lane 12*), 100 μg/ml TPCK (*lane 13*), 100 μg/ml phenylmethylsulfonyl fluoride (*lane 14*), 0.1 μM pepstatin (*lane 15*), 1 mM EDTA (*lane 16*). *Numbers below* the *columns* indicate the protease activity following *in vitro* activation expressed as the percentage of activity relative to the activity recovered in the absence of inhibitor (*lane 2*). Molecular masses of standards (kDa) are indicated in the *left margin*, and the *arrows* designate the molecular masses (kDa) of the detected proteins.

of the inhibitors on the precursor was visualized by Western blot analysis and the relative amount of active mature papain released was measured by spectrofluorometry (see "Experimental Procedures").We first tested known inhibitors of cysteine proteases. The presence of 20 mM cysteine stimulates processing (Fig. 3, compare *lanes 2* and *3*) as expected. In the presence of 2 mM cysteine, the processing reaction was incomplete after 20 min of incubation and a transient immunoreactive species of about 30 kDa was observed (Fig. 3, *lane 3*). This species may represent a processing intermediate since it is converted to the 24.5-kDa species upon prolonged incubation (data not shown). Reversible covalent cysteine protease inhibitors such as Hg^{2+} (Fig. 3, *lane 4*) and 2-PDS (Fig. 3, *lane 7*) were found to prevent processing very efficiently. This effect was fully reversed by the addition of a molar excess of cysteine (Fig. 3, *lanes 6* and *8*). Equimolar amounts of cysteine were not sufficient to reverse the inhibition with Hg^{2+} (Fig. 3, *lane 5*). The low molecular weight irreversible cysteine protease inhibitor E-64 completely prevents processing (Fig. 3, *lanes 9*). 13.3-kDa human cystatin C (for a review see Barrett *et al.* (1986)) is a more efficient cysteine protease inhibitor near physiological pH.[5] At pH 4.0 the rate of papain precursor processing and activation in the presence of cystatin C was low, but the conversion was completed after 90 min at 60 °C (data not shown). At pH 7.0 a molar excess of human cystatin C prevented complete processing even after overnight

[5] P. Berti, personal communication.

incubation where complete processing of the precursor takes place in the absence of inhibitor. Under those conditions two minor immunoreactive species of about 30 and 33 kDa accumulated in the reaction medium but no active papain could be detected (Fig. 3, *lanes 11*).

Inhibitors having different specificities were also tested. Under the experimental conditions used, TLCK fully inhibited processing (Fig. 3, *lane 12*), whereas TPCK (Fig. 3, *lane 13*) and phenylmethylsulfonyl fluoride (Fig. 3, *lane 14*) only partially reduced the amount of mature papain produced. The acid protease inhibitor pepstatin (Fig. 3, *lane 15*) and the metalloprotease inhibitor EDTA (Fig. 3, *lane 16*) did not affect processing.

Affinity Labeling of the Papain Precursor—The inhibition of the processing of the papain precursor by cysteine protease inhibitors may result either from a direct effect on the precursor or from inhibition of traces of mature active papain possibly involved in trans-processing of the precursor (see below). In papain, inhibition by E-64 (Hanada *et al.*, 1978) is accomplished by the formation of a covalent link bond between the sulfur atom of the active site cysteine and the C-2 atom of the inhibitor with concomitant opening of the epoxide ring (Varughese *et al.*, 1989). Based on the structure of E-64 and inhibition studies carried out by Tamai *et al.* (1981) an analogue of E-64, EpsTyrOBzl, was designed as an easily radiolabeled affinity probe. The precursor (Fig. 4, *A* and *B*, *lanes 1*) and mature papain (Fig. 4, *A* and *B*, *lanes 2*) are labeled by the inhibitor whereas the C25S precursor mutant is not (Fig. 4, *A* and *B*, *lanes 3*). Thus cysteine 25 of the precursor is accessible for reaction with a specific reagent.

Intramolecular and Intermolecular Processing of the Papain Precursor—The requirement for a functional active site for processing of the precursor may result either from an intramolecular or intermolecular processing mechanism or a combination of both. An intramolecular mechanism would be characterized by a zero-order reaction where the rate of precursor processing would be independent of the precursor concentration. An intermolecular reaction would have a higher order of reaction. Thus we have measured the initial rate of precursor processing as a function of precursor concentration. The initial rate of precursor processing was determined by measuring the activity of mature papain released over time. Reaction conditions (pH 3.86, 50 °C, and low precursor concentration) were chosen to allow for the acquisition of sufficient data points for the precise determination of the initial rate of precursor processing. Within the first 10 min of activation the increase in papain activity appears to be linear for precursor concentrations ranging from approximately 10 to 65 nM. A plot of (activation rate)/[precursor] *versus* [precursor] was fitted by linear regression (Fig. 5). Two conclusions can be drawn from this experiment. First, when the concentration of precursor tends toward zero, the extrapolated rate is not null and this finding is consistent with an intramolecular event. Second, when the precursor concentration increases, the ratio of the rate of activation to precursor concentration increases suggesting that the processing reaction can also follow an intermolecular mechanism. We can compare the relative importance of the intramolecular and intermolecular processing from the data in Fig. 5. For example it can be calculated that the relative rates of the two types of processing are equal at a concentration of precursor of 110.8 nM. Below this concentration the intramolecular processing reaction predominates over the intermolecular one. An independent experiment was designed to confirm that processing of the papain precursor results from a combination of intramolecular and intermolecular proteolytic reactions. In this experiment equal amounts of wild type precursor and C25S precursor mutant were mixed and incubated together under standard activation conditions. In control experiments the wild type precursor was fully converted to mature active papain after 15 min of incubation (Fig. 6*A*, *lane 2*), whereas no detectable processing of the C25S mutant is observed even after 60 min of incubation (Fig. 6*A*, *lane 4*). The equimolar mixture of both precursors (Fig. 6*A*, *lane 5*) was incubated for up to 60 min and aliquots analyzed at the times indicated. After 15 min of incubation when it was anticipated that the wild type precursor would be fully processed and that the C25S precursor would be unprocessed, two species of 39 and 24.5 kDa were observed in an approximately equal concentration (Fig. 6, *lane 6*). Upon prolonged incubation the residual precursor concentration decreased more slowly as the concentration of the fully processed species continued to increase (Fig. 6*A*, *lanes 7* and *8*). This experiment shows that the papain precursor (in this case the C25S mutant) can be processed in trans by the mature active papain. Under the conditions of this experiment, the rate of the intramolecular processing reaction appears to be greater than the rate of the intermolecular reaction.

To investigate the specificity of the intermolecular processing event we have incubated the papain precursor with the serine protease chymotrypsin. The temperature and pH conditions of the reaction were selected so as to limit the autoprocessing of the wild type precursor and to favor the activity of chymotrypsin. The C25S precursor mutant was not modified by prolonged incubation with an equal molar ratio of

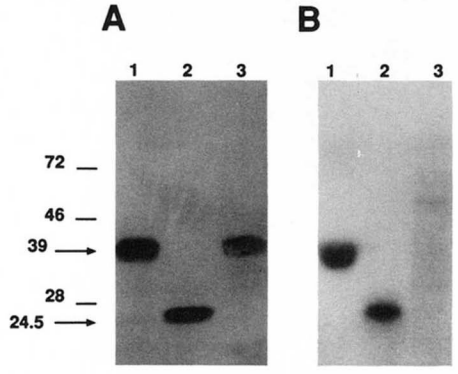

FIG. 4. **Binding of ^{125}I-EpsTyrOBzl to the precursor of papain.** Molecular masses of standards (kDa) are indicated in the *left margin*, and the *arrows* designate the molecular masses (kDa) of the detected proteins. *A*, Western blot of recombinant wild type papain precursor (*lane 1*), recombinant mature papain (*lane 2*), and recombinant C25S papain precursor (*lane 3*) incubated with ^{125}I-EpsTyrOBzl. *B*, autoradiography of the Western blot from *panel A*.

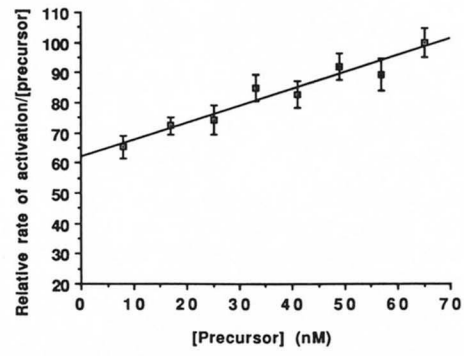

FIG. 5. **Plot of the initial rate of papain precursor processing *versus* precursor concentration.** The calculated data points have been fitted by linear regression. The standard deviation of the relative rates of processing/[precursor] is represented.

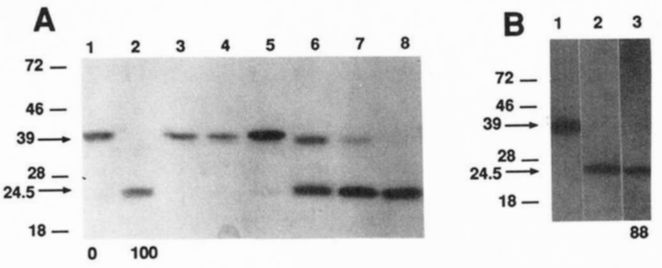

FIG. 6. **Processing of the papain precursor in the presence of exogenous proteases (trans-processing).** *A*, processing of wild type papain precursor in the presence of C25S precursor mutant. Western blot of the wild type papain precursor (*lanes 1* and *2*), C25S mutant papain precursor (*lanes 3* and *4*) or mixture of both precursors (*lanes 5–8*) prior to *in vitro* incubation (*lanes 1*, *3*, and *5*), 15 min (*lanes 2* and *6*), 30 min (*lane 7*), or 60 min (*lanes 4* and *8*) after the start of the incubation. The incubation was performed as described under "Experimental Procedures." *B*, effect of chymotrypsin on the papain precursor. Western blot of the C25S mutant papain precursor (*lanes 1* and *2*) and wild type precursor (*lane 3*) before (*lane 1*) and after 15 min (*lanes 2* and *3*) of incubation in the presence of an excess of chymotrypsin. The reaction conditions are described under "Experimental Procedures." *Numbers below* the *columns* indicate the protease activity (percentage of activity of the activated wild type precursor, *A*, *lane 2*) following *in vitro* activation. Molecular masses of standards (kDa) are indicated at the *left margin*, and the *arrows* designate the molecular masses (kDa) of the detected proteins.

chymotrypsin (data not shown) but a 100-fold excess of chymotrypsin over the C25S mutant led to the accumulation of 24.5-kDa mature papain (Fig. 6*B*, *lane 2*). The wild type papain precursor can also be processed by chymotrypsin and the mature papain that is released is enzymatically active (Fig. 6, *lane 3*). We also infer from this result that the wild type and the C25S mutant are equally competent substrates for processing.

DISCUSSION

Large amounts of the cysteine protease papain are readily recovered from the latex of the papaya tree and this has fueled extensive experimental investigation of papain which started late in the 19th century (Wurtz and Bouchut, 1879). However, it is only recently that it was demonstrated (Cohen *et al.*, 1986) that mature papain originated from a precursor molecule (proprotein) of higher molecular weight, a characteristic which is shared with many other proteases belonging to the serine, aspartyl, and metalloprotease families (Kassell and Kay, 1973).

While working on the engineering of the papain molecule (Ménard *et al.*, 1990, 1991) we have found that direct expression of the mature papain molecule is not possible as the pro region is necessary for folding of the protein.[6] Structural manipulation of the papain molecule was eventually achieved using the baculovirus/insect cell system to express a synthetic propapain gene with a melittin signal sequence (Tessier *et al.*, 1991).

The spectrum of inhibition by a variety of agents of the processing of the papain precursor and of the activity of mature papain are similar (Blackburn, 1976), and this confirms the role of the papain active site in the processing reaction, as we have shown by site-directed mutagenesis.

We have demonstrated that the active site of papain can be labeled using an affinity probe, and this indicates that the precursor active site region, and possibly by extension the entire papain molecule within the precursor, has a conformation similar to that of mature papain. The 107-amino acid pro region is large enough to fold as an independent domain, and by analogy with the three-dimensional structure of other protease precursors (James and Sielecki, 1986; Coll *et al.*, 1991) we have hypothesized that the pro region masks the active site within the precursor. However, we have shown that this masking effect allows small molecules to access the active site within the precursor. A similar structural arrangment has been recently described for procarboxypeptidase B (Coll *et al.*, 1991).

Processing of the papain precursor is triggered by low pH and is stimulated by elevated temperatures. These two factors may induce a structural transition required for intramolecular cleavage similar to the one described during processing of pepsinogen to pepsin (Glick *et al.*, 1989). A 30-kDa species has been observed during processing of the papain precursor, and this might indicate that processing occurs in a stepwise fashion. The pro region of the papain precursor is essentially hydrophilic with the exception of two small stretches of hydrophobic residues centered around amino acid positions 86 and 40. Cleavage at the latter site would generate the 30-kDa intermediate molecule. Complete removal of the pro region may be carried out by the active intermediate molecule and by the final product, mature papain, which could then cleave neighboring precursor molecules in an intermolecular reaction. This process would not be particularly favored at pH 4 since the optimal pH of mature papain activity is around pH 6.5 (Ménard *et al.*, 1990), and this would account for the preponderance of the intramolecular reaction at pH 4. Part of the pro region should be exposed to the solvent since the pro region is susceptible to proteolysis by either mature active papain or other proteases. This accounts for the observed intermolecular processing that may be the predominant mechanism of processing at high propapain concentrations. The intermolecular processing pathway may either lead directly to mature papain or it may generate intermediate molecules which are partially processed.

The *in vitro* conditions that we have used for processing and activation of the papain precursor are likely to be very different from those prevailing in the plant cell. There is an abundance of different cysteine proteases in *C. papaya* (Brocklehurst *et al.*, 1987). This may imply that in the plant intermolecular processing by other proteases might predominate. The N-terminal sequence of *in vitro* processed recombinant papain has been shown to be ragged (Vernet *et al.*, 1990). This observation could reflect the contribution of other plant proteases to the final steps in the processing of papain as well as their involvement in the removal of the remaining pro region up to the Ile^1 of mature papain.

We are now testing this model by identification of the initial site of cleavage within the pro region during processing. We are also using mutational analysis to define those residues within the pro region involved in the inactivation of papain and other functions of the precursor. With the exception of the cysteine protease family a three-dimensional structure of a member of each of the four classes of protease precursors has been reported. We are now purifying larger quantities of the papain precursor for the determination of its three-dimensional structure.

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[6] T. Vernet, R. Musil, and D. Y. Thomas, unpublished data.

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