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Clotting of Mammalian Fibrinogens by Papain: A Re-examination

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Supporting Information

ABSTRACT: Papain has long been known to cause the gelation of mammalian fibrinogens. It has also been reported that papain-fibrin is insoluble in dispersing solvents like strong urea or sodium bromide solutions, similar to what is observed with thrombin-generated clots in the presence of factor XIIIa and calcium. In those old studies, both the gelation and subsequent clot stabilization were attributed to papain, although the possibility that the second step might be due to contaminating factor XIII in fibrinogen preparations was considered. I have revisited this problem in light of knowledge acquired over the past half-century about thiol proteases like papain, which mostly cleave peptide bonds, and transglutaminases like factor XIIIa that catalyze the formation of ε-lysyl-γ-glutamyl cross-links. Recombinant fibrinogen, inherently free of factor XIII and other plasma proteins, formed a stable gel when treated with papain alone. Sodium dodecyl sulfate—polyacrylamide gel electrophoresis revealed that the intermolecular cross-linking in papain-fibrin leads to γ-chain dimers, trimers, and tetramers, just as is the case with thrombin-factor XIIIa-stabilized fibrin. Mass spectrometry of bands excised from gels showed that the cross-linked material is quite different from what occurs with factor XIIIa, however. With papain, the cross-linking occurs between γ-chains in neighboring protobrils becoming covalently linked in a “head-to-tail” fashion by a transpeptidation reaction involving the α-amino group of γ-Tyr1 and a papain cleavage site at γ-Gly403 near the carboxy terminus, rather than by the (reciprocal) “tail-to-tail” manner that occurs with factor XIIIa and that depends on cross-links between γ-Lys406 and γ-Gln398.

The fact that papain can convert mammalian fibrinogen into fibrin clots has been known for some time.1,2 As is the case with thrombin, papain releases peptide material and new glycine end groups are exposed.3 The situation is similar to what occurs with certain snake venom enzymes in that the newly exposed glycine amino groups are quantitatively only half what occurs with thrombin.5 Past reports also showed that fibrin generated by papain is insoluble in dispersive solvents like 5 M urea, 1 M sodium bromide (pH 5.3), or 0.1 M monochloroacetic acid, consistent with the formation of intermolecular cross-links, just as occurs with thrombin-generated fibrin in the presence of factor XIII and calcium.6 When these observations were first made, the details of how factor XIIIa stabilized fibrin were still unclear. Some investigators felt that factor XIII was acting as a transpeptidase (a term used interchangeably with transamidase, but in both cases implying α-peptides).6 Seen in this light, the covalent cross-linking of papain-fibrin was attributed to papain acting as a transpeptidase catalyzing the incorporation of glycine end groups into neighboring fibrin units.5 It was known that cysteine-activated papain could catalyze the replacement of one participant in a peptide bond by closely related ones;7 the fact that both papain and factor XIII were sulfhydryl enzymes seemed to bolster the case. When it was subsequently demonstrated that factor XIIIa-induced cross-linking involves the ε-amino groups of lysine side chains8,9 being joined with side-chain carboxamides of glutamines,10,11 notions of transpeptidation involving newly exposed glycol end groups were quickly abandoned, and interest in papain-fibrin interactions vanished. As far as can be determined, there have been no reports on the subject since.

Although the broad class of sulfhydryl enzymes called transglutaminases is frequently termed “papain-like", papain itself, one of the most intensively studied enzymes with regard to substrate preferences,12–18 has never been reported to catalyze formation or breakage of bonds between amino acid side chains. Indeed, the distinction that, although there are kinetic and mechanistic similarities between thiol proteases (EC 3.4.22.2) and transglutaminases (EC 2.3.2.13), there are “obvious differences in substrate specificities” is often emphasized.19 An often cited key structural difference is a conserved tryptophan residue deemed essential for catalysis in transglutaminases but replaced with a glutamine in thiol proteases like papain.20,21

In retrospect, it might reasonably be supposed that the second step in the generation of cross-linked fibrin by papain in those early studies was in fact due to factor XIII contaminating fibrinogen preparations, especially because, in unrelated experiments, papain was subsequently shown to activate factor XIII.22 In the earlier experiments, which were greatly hampered in that sodium dodecyl sulfate—polyacrylamide gel electrophoresis
(SDS–PAGE) had not yet been introduced, efforts to eliminate factor XIII activity from fibrinogen depended on heat treatment of various durations.66 It was subsequently shown that removing all vestiges of factor XIII from fibrinogen preparations is a very challenging proposition.23,24

The study presented here was undertaken to resolve the matter of papain-generated fibrin and its covalent stabilization. Initially, attempts were made to remove factor XIII from plasma-derived fibrinogen preparations by various means, SDS–PAGE being used to monitor both the effectiveness of the purification processes, as reflected by fibrin generated by thrombin in the presence of calcium and cysteine, and the nature of the fibrin generated by papain. In all cases, the papain-fibrin exhibited γ-chain cross-linking even when thrombin-calcium-cysteine treatment of the same material did not.

In the end, the possibility of the involvement of factor XIII was ruled out unequivocally by the use of recombinant fibrinogen made from the milk of transgenic cows, a protein that never in its existence was in the presence of factor XIII25 but exhibited the same pattern of cross-linking seen for other papain-generated fibrins.

Experiments also confirmed that papain mimics thrombin in the release of peptide material from fibrinogen, consistent with the exposure of the fibrin A knob that allows knob-hole polymerization. Thus, gelation is readily inhibited by the A-knob-surrogate peptide, GPRPam.

Other interesting details about the process emerged, including the fact that the cross-links in papain-fibrin are limited to γ chains. In this regard, the earliest detectable event when papain is added to fibrinogen is the removal of the αC domains, yielding an entity equivalent to fragment X. As a result, α chains are not involved in the cross-linking of papain-fibrin. In the wake of α-chain degradation, γ-γ dimers appear. However, these dimers appear not to be cross-linked in the same way as those generated by factor XIIIa. In particular, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry showed that trypsin-treated γ-γ dimers eluted from SDS gels differ from thrombin-factor XIIIa-treated controls in the composition of cross-linked tryptic peptides. Ironically, it turns out that the papain catalysis does in fact involve transpeptidation.

Meanwhile, another example of a thiol protease clotting fibrinogen has been discovered.26,27 The liver fluke Fasciola hepatica produces an enzyme, cathepsin L, that behaves very much like papain in its action toward fibrinogen, a gel with covalently cross-linked units being produced. In that case, the authors, apparently unaware of the older papain-fibrin reports, thought their observations to be the first of a thiol protease being able to gel fibrinogen. Interestingly, they also reported that in the case of the liver fluke enzyme, GPRP does not inhibit the gelation of fibrinogen, a significant difference from what we have found with papain. We now report, also, that another closely related cathepsin, the commercially available cathepsin S, degrades fibrinogen in a fashion that superficially resembles the action of papain but does not cause gelation or cross-linking.

### MATERIALS AND METHODS

**Fibrinogen.** Most of the very early experiments on papain-fibrin were conducted with horse2 or bovine fibrinogen.28 The phenomenon appears to be general for mammalian fibrinogens, however, and all of the work reported here was conducted on human fibrinogen. All told, fibrinogens prepared by seven different procedures were studied: (a) a crude cold-ethanol fractionation method,28 (b) crude fibrinogen chromatographed on DEAE,29,30 (c) plasminogen-free fibrinogen purchased from CalBiochem, (d) GPR-affinity-purified fibrinogen,31,32 (e) DEAE peak 1 fibrinogen purchased from Enzyme Research Laboratories (South Bend, IN),29 (f) cold-ethanol-fractionated fibrinogen prepared from “factor XIII-deficient” plasma purchased from Enzyme Research Laboratories, and (g) recombinant fibrinogen from transgenic cows33 (a generous gift from W. H. Velander, University of Nebraska, Lincoln, NE).

For the purpose of comparison, many of the experiments reported here utilized relatively crude fibrinogen isolated from outdated blood bank plasma by the cold ethanol fractionation procedure.28 These preparations, which are 95% clottable and well-known to contain contaminating factor XIII, were used as a reference material exhibiting conventional cross-linking. Purer fibrinogens lacking or deficient in factor XIII were subsequently compared side by side with the crude fibrinogen to assess the degree of thrombin- and papain-generated fibrins under various conditions. In some experiments, fibrinogen lacking factor XIII was clotted with thrombin in the presence of recombinant factor XIIIa, another generous gift of W. H. Velander.

**Papain.** Two different preparations of papain were purchased from Sigma-Aldrich. One was a suspension in 50 mM sodium acetate buffer (pH 4.6); more than 95% of the enzyme in these suspensions is present as an inactive dimer until it is reduced by an exogenous agent like cysteine. The other was freeze-dried crystalline material. Fibrinogen was gelled by both preparations, but in the interest of day-to-day reproducibility, the following procedure was adopted. A stock solution of freeze-dried crystalline papain was prepared in water (10 mg/mL), and small aliquots (10 μL) were pipetted into Eppendorf tubes for storage at −75 °C. When needed, a tube was thawed and diluted with 1.0 mL of a 5 mM cysteine/0.15 M NaCl/0.05 M imidazole mixture (pH 7.0) and then further with the same buffer depending on the experiment.

With regard to further dilution, proteolysis of the papain-fibrin can be strictly limited by keeping the papain concentration sufficiently low, typically ~1 μg/mL. Under these conditions, papain-generated fibrin clots can survive indefinitely, whereas at higher enzyme concentrations, a progressive digestion occurs that very much parallels digestion by various other proteases, including plasmin or trypsin.

**Other Enzymes.** Human thrombin was purchased from Enzyme Research Corp., and plasmin was obtained from Chromogenix; bovine trypsin was obtained from Sigma. A recombinant version of the human thiol proteinase, cathepsin S, was purchased from Novoprotein Scientific, Inc. (Summit, NJ). Recombinant factor XIIIa was the generous gift of W. H. Velander.

**Chromogenic Peptide Substrates.** Chromogenic peptides substrates for thrombin (D-Phe-Pip-Arg-pNA) and plasmin (D-Val-Leu-Lys-pNA) were obtained from Chromogenix and for papain (pGlu-Phe-Leu-pNA) from Sigma-Aldrich.

**Turbidity Measurements.** The course of gelation of thrombin- or papain-treated fibrinogen was monitored with a Helios spectrophotometer set at λ = 350 nm or, alternatively, with a microtiter plate reader at λ = 420 nm.

**SDS–PAGE.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted on a Bio-Rad instrument with 8 cm × 10 cm slab gels. Gels were stained with Coomassie Blue; in some cases, bands were excised, reduced and alkylated, and subjected to in situ proteolytic digestion followed by mass spectrometry.33

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Mass Spectrometry. Mass spectrometry was performed at the University of California at San Diego Biomolecular and Proteomics Mass Spectrometry Facility. Electrospray ionization MS in combination with commercial searching software was used to identify peptides from the α, β, and γ chains of fibrinogen, as well as any contaminating proteins. Additionally, a library of all consecutive sequences (6–40 residues) and their theoretical masses was constructed from the three chains of human fibrinogen (including minor forms of α and γ chains), thrombin, factor XIII, trypsin, and keratin (a common contaminant in MS analyses). Account was taken of known carbohydrate locations and carboxylation of all cysteine residues; care was also taken to watch for common side effects, including oxidation of methionines, carbamoylation of lysines, and alkylation of histidines. A second library with assigned theoretical masses was made of all possible pairs of cross-linked fragments from the amino-terminal region of the γ chain (residues 391–411) with themselves and with possible fragments from the amino terminus (γ1–15). In the end, MALDI-TOF spectra proved to be especially useful in identifying differences in the nature of cross-linked peptides from papain-fibrin compared with those generated in factor XIIIa-engendered controls.

Phylogenetic Tree. A sequence-based phylogenetic tree of papain and various sulfhydryl cathepsins was generated by the progressive method.34

RESULTS

Gelation of Fibrinogen by Papain. When fibrinogen is treated with low concentrations of papain, a substantial lag phase occurs, after which gelation ensues and a durable clot survives (Figure 1A). At higher concentrations of papain, gelation occurs more quickly but the clot is lysed (Figure 1B). In general, clots formed by papain are less sturdy than control clots formed under comparable conditions with thrombin. The gelation is completely abolished by sulfhydryl inhibitors, including mercury compounds and iodoacetamide. It is also blocked by the synthetic peptide A knob GPRPam (Figure S1 of the Supporting Information).

Covalent Cross-Linking of Papain-Fibrin. The formation of covalent cross-links between fibrin units was monitored by the appearance of slower-moving material on SDS gels. Unreduced samples tend to accumulate in the lower mesh stacking gel (Figure S2A of the Supporting Information), reduced samples exhibit bands at approximately the same positions as conventional γ–γ dimers and, to a lesser degree, γ–trimers and γ–tetramers, which are formed by factor XIIIa after thrombin clotting of fibrinogen35 (Figure S2B of the Supporting Information). Papain-generated cross-linked fibrin is calcium-independent as evidenced by the appearance of these bands in the presence of 10 mM EGTA or 10 mM EDTA.

Inhibition by GPRPam. The gelation of papain-treated fibrinogen is inhibited by minimal amounts of GPRPam (Figure 2A), inhibition being complete at concentrations as low as 0.4 mM when the fibrinogen concentration is in the range of 1 mg/mL, similar to what is observed with fibrins generated by snake venoms like batroxobin (Figure 2B). The kinetics of inhibition of papain-fibrin at lower GPRPam concentrations differ from those of batroxobin, however, in that the competition of surrogate knob-hole interaction is never overcome (Figure 2A). It is likely that during the sustained competition between native sites and synthetic peptide knobs, papain continues to digest regions of fibrinogen needed for polymerization and that polymerization itself protects against that progressive attack.

The cross-linking of papain-fibrin is dependent on gelation as shown by the absence of higher-molecular weight polymers and γ–γ dimers when GPRPam is present (Figure S2A,B of the Supporting Information).

Factor XIII-Deficient Fibrinogens. All six fibrinogens used in this study were gelled by the action of papain, and
concomitantly (Figure S3B of the Supporting Information), all six exhibited cross-linking as determined by the appearance of higher-molecular weight material on SDS gels (Figure S3A of the Supporting Information). Unchromatographed (crude) fibrinogen, which is known to contain factor XIII, showed a typical pattern of γ-chain dimers and α-chain polymers when it was clotted with thrombin, calcium, and cysteine (Figure S3C of the Supporting Information). By comparison, only one of five highly purified preparations showed any sign of cross-linking (the affinity-purified fibrinogen) under the same conditions (lane C in Figure S3C of the Supporting Information).

**Time Course of Limited Papain Digestion.** Recombinant human fibrinogen was subjected to limited papain digestion, and samples were taken for SDS gels periodically to establish the course of events. The first observable change under these conditions (papain, 1.25 μg/mL, fibrinogen, 1.5 mg/mL) is due to proteolytic attack on α chains, leading to a lower-molecular weight entity that is the equivalent of the well-known plasmin-generated fragment X (Figure 3A). Gel formation is paralleled by the appearance of a band on reducing gels at approximately the same position as γ-γ dimers generated by factor XIIIa, although, consistently, the bands appear very slightly larger than the usual type. In due course, bands equivalent to γ trimers and tetramers also appear (Figure 3B), just as is observed with factor XIIIa cross-linking after thrombin treatment.35

**Identification of Gel Bands.** Electrospray ionization mass spectrometry was used to identify the bands eluted from reduced SDS gels. The lowest-molecular weight band is denoted dimer in Figure 3B. Tryptic peptides covering the region spanned by residues 12—252 of the α chain were identified, consistent with a molecular weight of about 28000 as observed on SDS gels.

The γ- and β-chain bands yielded tryptic peptides covering virtually all of those chains. The band corresponding to the γ-dimer gave rise to tryptic peptides covering most of the γ chain, although small amounts of α- and β-chain peptides were also detected, likely because of lower-molecular weight material trailing on the gel. Bands corresponding to γ trimer and γ tetramer had tryptic peptide compositions that were the same or very similar to that of the γ-γ dimer.

**Time Course of Sustained Papain Digestion.** Treatment of fibrinogen with higher concentrations of papain (papain, 10 μg/mL; fibrinogen, 2 mg/mL) gave rise to a series of products corresponding to well-known fragments X, Y, D, and Eαβ (Figure S4 of the Supporting Information). Significantly, unlike the situation with plasmin degradation, no D dimer survives papain digestion under these conditions (Figure S4 of the Supporting Information).

**Preparative Scale Papain-Fibrin.** On several occasions, substantial batches of papain-fibrin were prepared for various exploratory characterizations. Full details are provided for two such preparations in the Supporting Information, one made from plasma-derived fibrinogen and another from recombinant fibrinogen. The aims and general results for these are described briefly here.

**Partially Dispersible Papain-Fibrin.** It was necessary to demonstrate that there is a strict correspondence with the indisperbility of the papain-fibrin and the appearance of cross-linked γ chains on SDS gels. To this end, a substantial batch of papain-fibrin (30 mg) was subjected to dispersal in 1 M NaBr (pH 5.3) to separate out soluble and partially soluble entities from the totally insoluble material. The soluble portion was passed over a Bio-Gel A15 column in the presence of NaBr, and four pools were collected (Figure S5 of the Supporting Information).

Gel electrophoresis showed that there was a clear correlation between fibrin being indisperse in 1 M NaBr (pH 5.3) and the abundance of cross-linked material. The fraction most soluble in 1 M NaBr (pH 5.3), as indicated by its trailing position on the A15 column, had the smallest amount of γ-γ dimer, and the totally insoluble material exhibited the most.

**Purification of γ-γ Dimers from Papain-Fibrin.** A substantial batch of papain-fibrin (30 mg) was reduced and alkylated in 6 M guanidine (pH 8.3) and passed over a Sephadex G-150 column equilibrated with 4 M guanidine (Figure S6 of the Supporting Information). The earliest eluting fractions were richest in the γ dimer, γ trimer, and γ tetramer, although some β-chain material was also present. Repassage over a second G150 column yielded much purer γ dimers and trimers and tetramers. γ-γ dimer material that eluted from SDS gels of this fraction were especially useful for authenticating the nature of cross-linking.

MALDI-TOF Analysis of “γ-γ Bands” Eluted from SDS Gels. Three different fibrinogen preparations were analyzed according to the following regimen. First, each was divided into two equal portions, one to be treated with papain and the other with thrombin, calcium, and factor XIIIa. After fibrin gel formation had reached completion, samples were treated with iodoacetamide in strong urea/SDS solutions and heat-treated at 100 °C for 10 min to kill all residual enzyme activity, after which they were reduced with mercaptoethanol and run on polycrylamide gels. Bands at the γ-γ dimer positions were excised, destained, and subjected to in situ digestion with trypsin. Both kinds of digest yielded an extensive array of predicted peptides from the γ chains (Table S1 of the Supporting Information).

Significantly, factor XIIIa-generated material contained two peaks that were absent from the digests of papain-fibrin: one at 3545.5 amu corresponding to singly cross-linked carboxy-terminal peptides (residues 392—411 and 392—406) and the other a peak at 3998 amu corresponding to reciprocally cross-linked peptides (two cross-linked peptides of residues 392—411) (Figure 4A,B).

Attention was then shifted to peaks that appeared in the tryptic digests of papain-fibrin that were absent in control preparations. Prominent among these was a peak with a mass equal to 1880.1 amu (Figure 5A,B). A search of our fragment mass database revealed that a possible match was a joined pair of peptides effected by a hypothetical transpeptidation reaction between a putative preferred papain cleavage site after γ-Gly403 and the α-amino group of another γ chain, γ-Tyr1 (Figure 6). The solution was authenticated by MS2 analysis of a 1880 amu peak in which exact matches for fragments covering the full stretch of the
ligated sequence LTIGEGQQHHLGYVATR were identified (Figure 7).

Other Thiol Proteases. The fact that fibrinogen can be covalently gelled by a thiol protease found in liver flukes26,27 raised the question of whether other members of this cathepsin family might clot fibrinogen. A phylogenetic tree constructed from the amino acid sequences of a variety of endothioproteinases showed that cathepsin S, available commercially, was phylogenetically intermediate between papain and the liver fluke protease (Figure S7 of the Supporting Information). The enzyme was purchased and its action toward fibrinogen compared side by side with that of papain. It was found to cleave fibrinogen in a manner similar to that of papain but with no gelation or cross-linking. It had been reported previously that human cathepsin B cleaves human fibrinogen in a manner similar to that of plasmin.37

**DISCUSSION**

Once it was established that contaminating factor XIII is not the cause of covalent stabilization of papain-fibrin, attention was given to finding the exact nature of cross-linking. Although MS analysis of gel bands confirmed that cross-linking was limited to γ chains, the alleged γ-γ dimers invariably appeared to be slightly larger than those generated by factor XIII, suggesting that the cross-linked sites are not the same in the two kinds of fibrin. In particular, direct comparison of tryptic digests from the two kinds of γ-γ dimers showed that the material cross-linked by factor XIII had both singly and doubly (reciprocally) cross-linked peptides corresponding to residues γ392–411, but these peaks were not present in digests of the γ-γ bands from papain-fibrin.

The observation that γ-γ dimers from papain-fibrin are slightly larger on SDS gels than their counterparts produced by factor XIII was consistent with cross-linking nearer the ends of γ chains and in a manner different from that linking the side chains of Lys406 and Gln398. On the basis of preferences taken from the literature,12−18 a likely papain cleavage point near the carboxyl terminus of the γ chain was identified as the carboxyl side of Gly403. The proposal is that, after gel formation, the mobile amino termini of γ chains on associated fibrin units are near the equally mobile carboxyl termini of neighboring γ chains within associated protofibril bundles (Figure 8). Because this is the case, the fully extended form of the proposed transpeptidated γ-γ dimer is 10 residues longer than that of a singly cross-linked factor XIII γ-γ dimer.

It may be helpful to comment briefly on the known mechanism and specificity of papain-catalyzed transpeptidations. At the outset, a thioacyl intermediate is formed, with a cleaved portion

![Figure 4](image_url)

Figure 4. MS (MALDI) spectra (approximate range of 3400–4100 amu) of tryptic digests of γ-γ dimer bands: (A) thrombin-factor XIIIa fibrin showing a strong peak for a single cross-linked unit (3544 amu) and another for doubly cross-linked units (3997 amu) and (B) a close-up of the same region for papain-fibrin. Arrows denote missing “cross-linked” peaks in papain-fibrin.

![Figure 5](image_url)

Figure 5. MS (MALDI) spectra (approximate range of 1800–2000 amu) of tryptic digests of γ-γ dimer bands: (A) papain-fibrin and (B) thrombin-factor XIIa-fibrin. The arrow denotes the missing 1880 amu peak in thrombin-factor XIIa-fibrin. Other peaks with the same masses in both spectra are labeled.
of the protein or peptide leaving as usual, but when the concentration of some free peptide is sufficiently high, the $\alpha$-amino group of that peptide can outcompete water. In the case of papain-fibrin, it is the association of protofibrils and the juxtapositioning of $\alpha$-amino groups from neighboring molecules that allow the new peptide bond to form (Figure 9).

Many years ago, Schechter and Berger performed an ingenious study in which they mapped the binding site of papain. Using a set of diastereomeric peptide substrates, they identified a line of seven subsites extending on both sides of the active site thiol, each binding a substrate residue. They labeled the binding sites S4$\ldots$S1 and S1$'$$\ldots$S3$'$ and the corresponding substrate residues P4$\ldots$P1 and P1$'$$\ldots$P3$, the numbering in each case proceeding away from the cleavage point (Figure 10). This general description was subsequently borne out by X-ray crystallographic studies. It was also found that papain greatly prefers binding to substrates in cases in which P2 is large and hydrophobic. Moreover, transpeptidation was found to be favored by the provision of peptides with amino-terminal residues that are large and nonpolar. Auspiciously, the amino terminus of the human fibrinogen $\gamma$ chain begins with the sequence Tyr-Val-Ala. Moreover, these regions are necessarily brought into the general neighborhood of the carboxyl terminus of $\gamma$ chains on neighboring molecules during the association of protofibrils on the way to gelation (Figure 8).

Because these papain experiments are very straightforward, it should be possible for others with special resources to confirm certain aspects quickly. For example, laboratories in possession of recombinant fibrinogens in which $\gamma$-Lys406 and/or $\gamma$-Gln398 and Gln399 have been replaced could easily verify that a factor XIIIa-like mechanism is not involved, the prediction being
that those mutated fibrinogens would yield γ-γ dimers after papain treatment, just as occurs with native fibrinogen.

Other Cross-Linking Possibilities. A variety of interchain cross-links are known to occur in many other biological systems, and some, including ubiquitinylation and sumoylation, are due to papain-like enzymes in both their formation and their destruction.32,33,45 In some other cases, isopeptide linkages are known to be formed autokatallytically between lysine and the side chains of aspartic acid or asparagine46,47 or, after an unexpected oxidation reaction, methionine.48,49 We could not find any combinations of peptides in our mass libraries that could accommodate any of these linkages.

A Final Comment. Reports of half a century ago were enthusiastic about papain clotting fibrinogen and stabilizing the clot, to the point where it was suggested that papain might be useful during surgery for persons lacking factor XIII, in which absence the synthetic peptide knob GPRPam (Figure S2A,B),23,24 comparison of six different fibrinogen preparations (DEAE peak 1, Enzyme Research peak 1, GPR affinity purified, recombinant, cold alcohol-purified fibrinogen from factor XIII-deficient plasma, and “crude”, cold alcohol-purified from normal plasma) with regard to purity on SDS gels (Figure S3A), γ-dimer formation resulting from papain treatment (Figure S3B), and γ-dimer formation as a result of thrombin, calcium, and cysteine (Figure S3C), sustained digestion of human fibrinogen by higher concentrations of papain (Figure S4), gel filtration of NaBr-soluble material extracted from papain-fibrinogen on a Bio-Gel A1.5 column (Figure S5), gel filtration of reduced and alkylated papain-fibrin on Sephardex G-150 (Figure S6), and the phylogenetic relationship of various cathepsins to papain based on amino acid sequences (Figure S7). This material is available free of charge via the Internet at http://pubs.acs.org.

ADDENDUM

After the initial submission of this article, the papain experiment was repeated with bovine fibrinogen instead of human fibrinogen. The regions of the γ chain involved in the proposed transpeptidation for the bovine form differ from those of the human form at two amino acid positions: γ-393Thr → Ala and γ-400His → Gln (human numbering). The “γ-γ dimer” band from bovine papain-fibrin was eluted from an SDS gel, digested with trypsin, and subjected to MALDI-TOF. A prominent peak at the predicted mass was observed (1841 amu instead of 1881 amu for the human form), the fragmentation pattern of which corresponded exactly to the bovine transpeptidated sequence (LAIGEQQHQQLGYYVATR).

ASSOCIATED CONTENT

Supporting Information

Predicted and observed masses for γ-γ dimers excised from SDS–polyacrylamide gels of papain-fibrin and (control) thrombin-F13α fibrin (Table S1), inhibition of papain-catalyzed gelation of human fibrinogen by 0.8 mM GPRPam (Figure S1), time course of papain-digested fibrinogen in the presence and absence of the synthetic peptide knob GPRPam (Figure S2A,B), comparison of six different fibrinogen preparations (DEAE peak 1, Enzyme Research peak 1, GPR affinity purified, recombinant, cold alcohol-purified fibrinogen from factor XIII-deficient plasma, and “crude”, cold alcohol-purified from normal plasma) with regard to purity on SDS gels (Figure S3A), γ-dimer formation resulting from papain treatment (Figure S3B), and γ-dimer formation as a result of thrombin, calcium, and cysteine (Figure S3C), sustained digestion of human fibrinogen by higher concentrations of papain (Figure S4), gel filtration of NaBr-soluble material extracted from papain-fibrinogen on a Bio-Gel A1.5 column (Figure S5), gel filtration of reduced and alkylated papain-fibrin on Sephardex G-150 (Figure S6), and the phylogenetic relationship of various cathepsins to papain based on amino acid sequences (Figure S7). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GPRPam, Gly-Pro-Arg-Pro-amide; SDS, sodium dodecyl sulfate; MS, mass spectrometry.

REFERENCES


