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Chymopapain C, an Immunosuppressive Protease: I. Partial Purification and Characterization[1] (37991)

JAMES A. CLAGETT,[2] SEI TOKUDA,[3] AND WARREN E. ENGELHARD[4]

In a previous report, we demonstrated that a chymopapain-rich fraction of papaya latex produced a potent immunosuppression (1). Our objective in this study was to isolate and to characterize the protein responsible for the immunosuppression. We report a method of preparation of a protease from papaya latex and compare some of its enzymic properties to commercial chymopapain and papain. This protease, tentatively named chymopapain C, was isolated by a modified method of Jansen and Balls (2), employing carboxymethyl cellulose columns (CMC). We will report in a separate communication that chymopapain C has immunosuppressive properties (3).

Materials and Methods. Carica papaya latex was kindly provided by Wallenstein Laboratories, Long Island, N. Y. Chymopapain (code PCYP)[5] and papain (2X) were purchased from Worthington Biochemical Corp., Freehold, N. J.

Isolation. All enzyme preparative procedures were carried out at 6°C.

Step 1. Three hundred grams of *Carica papaya* latex was extracted by stirring in one liter of 0.2 *M* citrate–phosphate buffer pH 5.0 for 24–48 hr and filtered through celite (Hyflo-Super Cell). The extract was brought to pH 1.8–2.0 and the precipitate formed removed by centrifugation at 4,000*g* for 3 hr. The supernatant was dialyzed against 0.075 *M* HCl for 4–6 days.

Step 2. The dialyzed supernatant from step 1 was brought to pH 3–4 and enough solid NaCl was added to make a 50% saturated solution. The precipitated material was removed by centrifugation and the supernatant brought to 100% saturation by the addition of more solid NaCl. After lowering the pH to 2.0, the solution was allowed to stand for 24–48 hr. The precipitate that formed was collected by centrifugation, dissolved in and dialyzed against water, and lyophilized.

Step 3. CM cellulose chromatography—the lyophilized precipitate from step 2 was loaded on a CMC column (2.5 × 40 cm) that had been equilibrated with 0.1 *M* citrate–0.2 *M* phosphate buffer, pH 5.0. The same buffer containing additives of 0.1 *M* KCl and 0.5 *M* KCl was used for stepwise elution as shown in Fig. 1. Because of its immunosuppressive activity, the protein eluted in Peak C was selected for further characterization.

Enzyme assays. Proteolytic activity was assayed by a modified case in digestion method of Kunitz (4) as described by Ebata and Yasunobu (5). For papain ac-

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[2] Present address: James A. Clagett, Department of Microbiology & Periodontics, University of Washington, Schools of Medicine & Dentistry, Seattle, WA 98195.

[3] Recipient of a Research Career Development Award (K04–GM–14893) from the National Institute of General Medical Sciences. Reprint request should be addressed to Dr. Sei Tokuda, Department of Microbiology, School of Medicine, University of New Mexico, Albuquerque, NM 87131.

[4] Deceased.

[5] Papain 2X and commercial chymopapain (PCYP) were designated papain and chymopapain throughout.

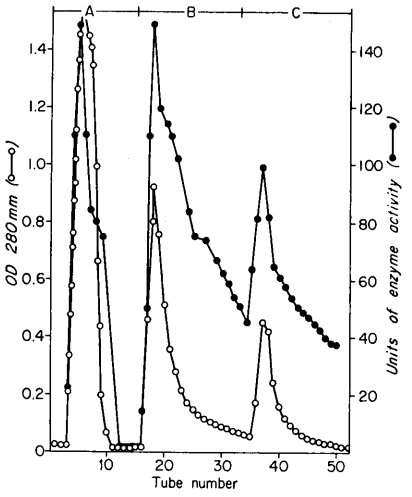

FIG. 1. Isolation of chymopapain C by ion exchange chromatography. After salt and acid precipitation, the supernatant material was separated on a carboxymethyl cellulose column (2.5 × 40 cm) which was equilibrated with 0.1 *M* citrate–0.2 *M* phosphate buffer, pH 5.0. The materials contained in Peaks A, B and C were eluted with equilibration buffer, equilibration buffer plus 0.1 *M* KCl and equilibration buffer plus 0.5 *M* KCl, respectively.

tivity, α-benzoyl-DL-arginine *p*-nitroaniline (BAPA) was used as the substrate (6). One unit of enzyme activity on casein substrate has been expressed as a change of 1 OD unit per min per mg protein. The enzyme activity towards BAPA was expressed as μm *p*-nitroaniline released per min per mg protein.

Estimation of protein. Protein was determined by the method of Lowry *et al.*, (7).

Molecular weight determination. Gel filtration (8) and sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (9) were employed to determine the molecular weight of chymopapain C.

Urea starch gel electrophoresis. The micro-technique of Marsh, Joliff and Payne (10) was used.

Digestion of human IgG immunoglobulin. This experiment was designed to answer two questions about chymopapain C: does it cleave human IgG and if so, do the cleavage fragments resemble those of chymopapain or papain? Chromatographically purified human IgG (11) was digested with papain or chymopapain C as described elsewhere (11). The digestion was followed by immunoelectrophoresis (IEP) as described by Scheidegger (12) and was developed with a rabbit antiserum specific for human IgG. Fc fragments were isolated by DEAE–cellulose chromatography (13) from a chymopapain C digestion of IgG, a process which occurred over a 28 hr span. The Fc fragments were concentrated by vacuum dialysis, and IEP analysis was performed by using rabbit anti-human IgG antiserum. After finding that the Fc preparation was devoid of Fab or intact IgG, an N-terminal analysis was completed on the Fc fragments by employing the Dansyl method of Gray (14) and polyamide thin-layer chromatographic sheets to identify the dansylamino acids as described by Woods and Wang (15).

Results. The data in Table I describe the isolation of chymopapain C from papaya latex. There was no detectable enzyme activity in any of the precipitates formed during the isolation procedures. Purification was achieved when the enzyme preparation was placed on CMC column and eluted with increasing salt (Fig. 1). All the eluted materials (Peaks A, B, and C) had detectable proteolytic activity. The three fractions were distinctly different in elution characteristics because of the different molarities of KCl required for elution. Peaks B and C were

TABLE I. Isolation of Chymopapain C.

Fraction	Protein (g)	Specific activity	Recovery of enzyme activity
Supernatant (pH 2.0)	41.3	0.64	26,400
Supernatant (50% sat. NaCl)	37.4	0.67	25,000
Supernatant (100% sat. NaCl)	29.1	0.60	17,200
Peak "C" CMC column	9.5	1.72	15,300

TABLE II. Differential Activity of Chymopapain C on Two Substrates.

Enzyme	Enzyme activity on casein	Enzyme activity on BAPA	Activity of casein/BAPA
Papain 2X	4.0[a]	1380[b]	2.9×10^{-3}
Chymopapain (PCYP)	2.0	12	1.6×10^{-1}
Chymopapain C	1.7	12	1.4×10^{-1}

[a] *Conditions of assay.* Enzyme preparations of papain, chymopapain and chymopapain C were diluted in 0.1 *M* phosphate buffer, pH 7.2, to contain 0.8–2.4 mg protein ml^{-1}. One half ml of the enzyme solution was mixed with 0.5 ml of 0.75 *M* mercaptoethanol (ME) in phosphate buffer, pH 7.2. The mixture was incubated at 37°C for 1 hr, and 9 ml of phosphate buffer, pH 7.2 was added. Various quantities of enzyme solution were added to a 1% casein (Isoelectric Casein, Difco Co., Detroit, Mich.) solution in 0.1 *M* phosphate buffer, pH 7.2 containing 0.018 *M* ME. The mixture was incubated at 35°C for 20 min, and 3 ml of 5% trichloroacetic acid solution was added to stop the reaction. Each sample was centrifuged at 1,000*g* for 10 min. The optical density (OD) was determined at 280 nm in the spectrophotometer. Control samples were prepared by adding the trichloroacetic acid to the casein solution followed by an aliquot of the most concentrated enzyme solution (50 μg).

[b] *Conditions of assay.* Substrate concentration 1.12×10^{-3} *M*; pH 6.25, 0.05 *M* citrate buffer; 0.001 *M* EDTA and 0.015 *M* Me.

tested for their immunosuppressive activity in mice and it was found that peak C possessed maximum activity. Therefore, this peak was selected for further characterization.

In order to test for papain activity in the Peak C preparation, papain (2X), chymopapain and Peak C (denoted chymopapain C) were tested on two substrates, casein and BAPA. As shown in Table II by the ratio of casein-to-BAPA activities, chymopapain and chymopapain C did not possess any papain activity. However, chymopapain C and chymopapain were similar in enzymatic

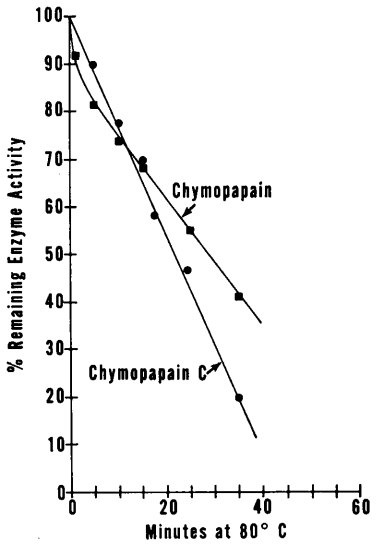

FIG. 2. Thermal half-life determinations of chymopapain (■—■) and chymopapain C (●—●) utilizing BAPA as substrate.

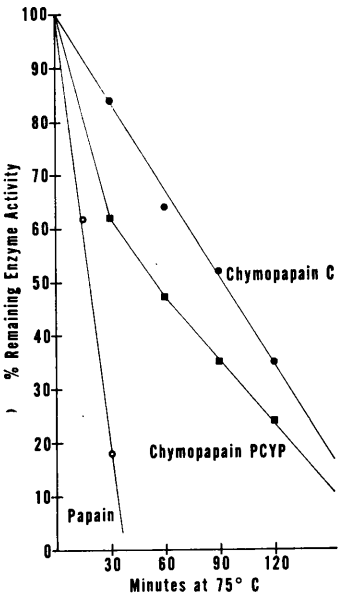

FIG. 3. Thermal half-life determinations of chymopapain (■—■), chymopapain C (●—●), and papain (○—○) with 1% casein as substrate.

activity as evidenced by their casein-to-BAPA ratio of 1.4×10^{-1} and 1.6×10^{-1}, respectively.

In order to determine whether chymopapain C and chymopapain are different enzymes, thermal inactivation studies were performed. The enzymes were assayed using BAPA as substrate, chymopapain C had a $T_{½}$ of 22 min and denatured linearly throughout the inactivation period (Fig. 2). On the other hand, chymopapain had a $T_{½}$ of 29 min and denatured in a biphasic manner. This strongly suggested that the commercial chymopapain contained at least two enzymes. Papain, under the conditions of

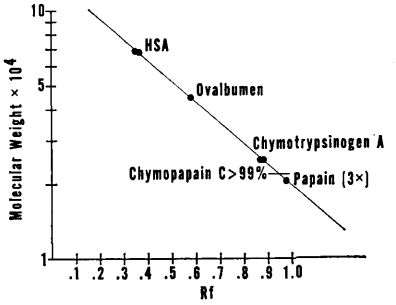

FIG. 4. The determination of the molecular weight of chymopapain C by SDS polyacrylamide gel electrophoresis. The major component of chymopapain C (>99% of the protein) had a Rf of 0.945 as shown by the slash mark.

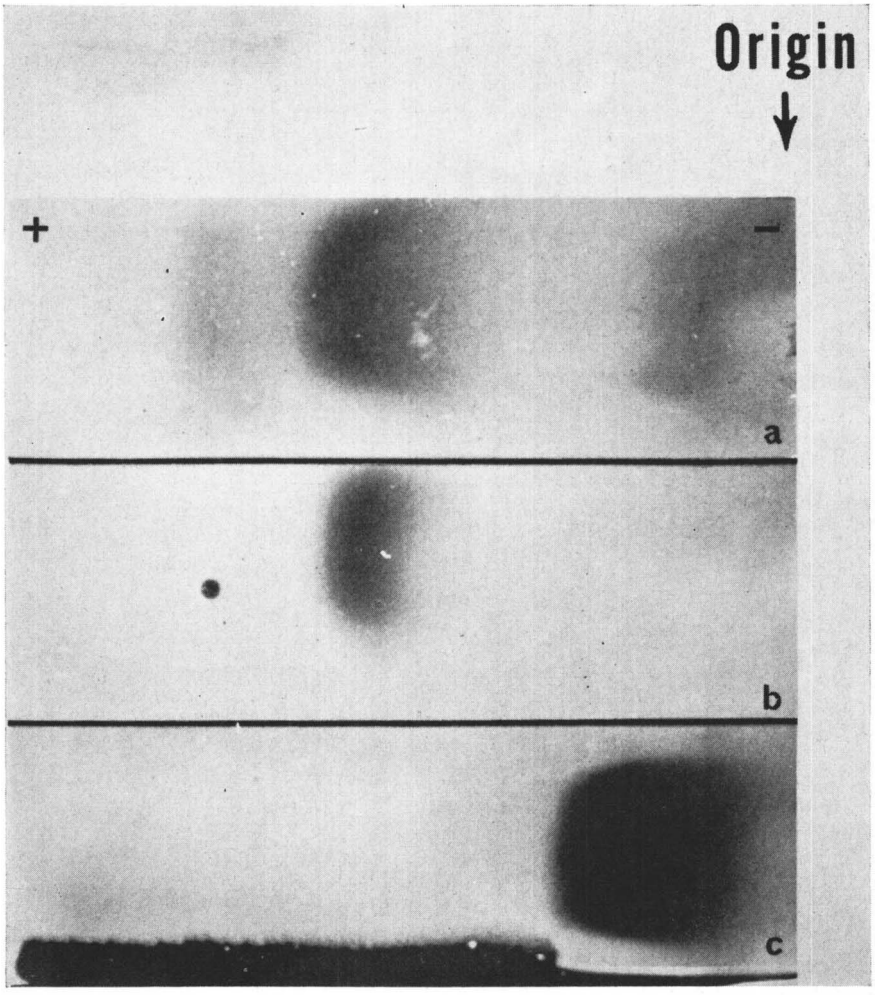

FIG. 5. Photographs of urea starch gel of chymopapain C (a), papain (b) and commercial chymopapain (c).

this assay, was too rapidly inactivated to give accurate values and the data were not included in Fig. 2. In similar thermal inactivation experiments, shown in Fig. 3, using a casein substrate, chymopapain C had a $T_{½}$ of 90 min whereas chymopapain and papain had a $T_{½}$ of 52 and 19 min respectively (Fig. 3). As observed previously, chymopapain was inactivated in a biphasic manner.

Experiments to determine the molecular weight of chymopapain C papain C had a major component (>99% by analysis of gel scans) with a molecular weight of 22,000 and a minor component with a molecular weight of 32,000 (Fig. 4). Gel filtration data on Sephadex G-100 confirmed that chymopapain C had a molecular weight of 22,000.

The thermal inactivation data suggested that chymopapain C was probably homogeneous with respect to its enzymatic activity. However, the homogeneity of the preparation was investigated by urea starch gel electrophoresis as shown in Fig. 5. Chymopapain C had one major and two minor components. The major component of chymopapain C had mobility similar to that of papain and the most cathodal component migrated similar to chymopapain.

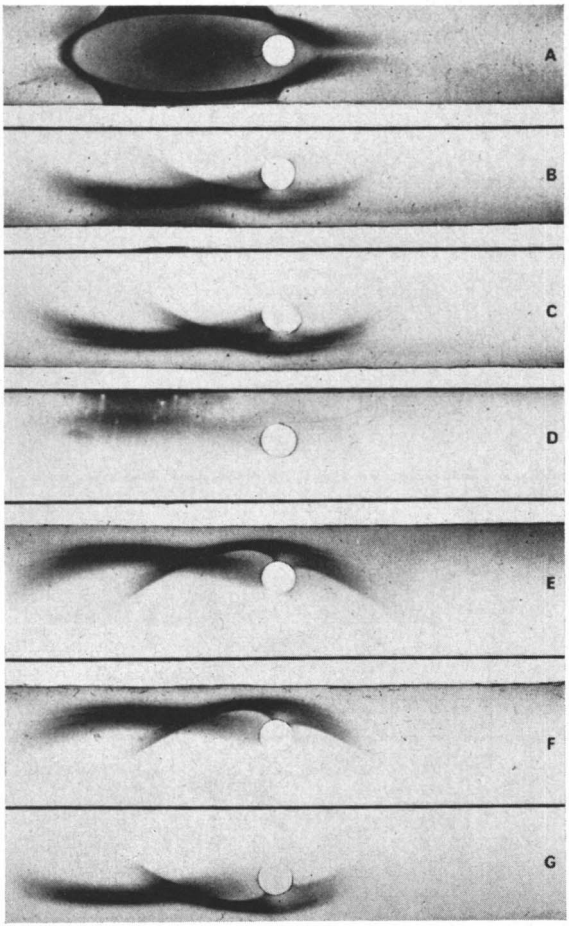

FIG. 6. Immunoelectrophoretic analysis of chymopapain C and papain digests of human IgG. Undigested human IgG (Panel A) served as control. Panels B, C, and D show the papain digestion profile at 2.25, 4, and 28 hr respectively. Panels E, F, and G show the chymopapain C digestion profile at the corresponding times.

Human IgG was digested by chymopapain C in order to compare the fragments of IgG with those produced by papain digestion. The IEP patterns in Fig. 6 show that the untreated human IgG preparation remained stable and homogeneous (well A). In wells B, C and D were the 2.25, 4.0 and 28 hr papain digests of human IgG immunoglobulin; at 2.25 and 4.0 hr, Fab and Fc fragments were visible. By 28 hr only human IgG, which was papain resistant, or Fab remained. By contrast, at 2.25, 4.0 and 28 hr (wells E, F and G) chymopapain C made only limited cleavages of the IgG molecule resulting in the formation of Fab and Fc; possibly some intact γG remained which was not chymopapain C-sensitive.

On further separation on DEAE cellulose, Fab and IgG components were eluted from the column with the 0.01 *M* phosphate buffer as shown by the IEP pattern denoted Fab in Fig. 7. The 0.3 *M* fraction eluate, denoted as Fc, showed a homogeneous precipitin arc after IEP, thereby indicating that little γG or Fab was present in the Fc preparation. The Fc protein was then analyzed for the predominant aminoterminal amino acid by the Dansyl–Edman procedure. Although no quantitation was attempted, the only N-terminal detected in the Fc material was threonine. Fc fragments produced by a 16-hr papain digestion served as controls; the major and minor N-terminals were leucine or isoleucine and threonine, respectively. These data support the IEP analysis in Fig. 6, where only limited proteolysis of IgG by chymopapain C was detected after 28 hr digestion resulting in the production of Fc and Fab.

Discussion. We have described an isolation scheme for preparing chymopapain C from papaya latex. Advantage has been taken of the acid stability and high salt solubility of chymopapains. Carboxymethyl cellulose column chromatography by stepwise elution with phosphate–citrate buffer system with increasing KCl concentrations was used to fractionate the Jansen–Balls (2) preparation.

The data describing the differential activities of chymopapain C would be compatible with the hypothesis that chymopapain C differs significantly from either chymopapain or papain. First, the method of preparation of chymopapain C selected for proteinases that were acid stable. Papain is acid labile (16) and hence should have been removed by the preparative procedures. The differential activities on BAPA, casein and human IgG strongly suggest that no papain activity was present in chymopapain C. On the other hand, chymopapain C and chymopapain were identical in their behavior with respect to enzymic activities on the same substrates.

Yet the thermal inactivation data and molecular weight determinations, revealed differences between chymopapain C and chymopapain. The biphasic thermal inactivation of chymopapain could be explained by assuming the presence of chymopapains A and B in this product where one may be

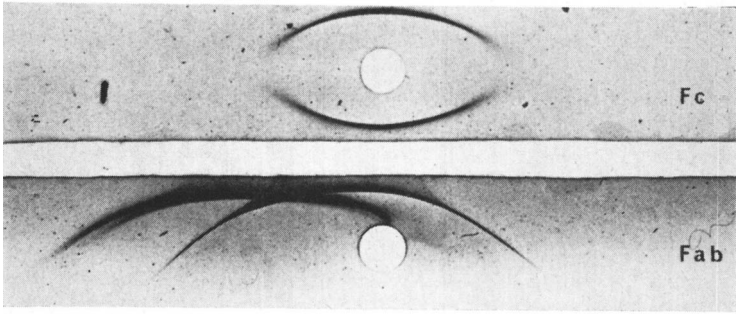

FIG. 7. Immunoelectrophoretic analysis of the Fab and Fc fragments of human IgG. The Fc fragments were eluted by 0.3 *M* phosphate buffer, pH 8.0 (Panel Fc). Intact IgG and Fab fragments were eluted by 0.01 *M* phosphate buffer, pH 8.0 (Panel Fab).

more thermal sensitive and hence, more rapidly inactivated than the other. In part, these findings are in agreement with the data of Ebata–Yasunobu (5), who reported that chymopapain and papain had $T_{1/2}$ values of 75 and 56 min, respectively. Variability between these data for papain may come from our use of mercaptoethanol rather than cysteine for activation. The molecular weight of chymopapain C, as determined by two methods, was 22,000. This differs significantly from the published (17) molecular weights of 35,000 for chymopapains A and B.

The selection of human IgG as a substrate for chymopapain C digestion was designed to answer the question: into which pattern of fragmentation of human IgG does chymopapain C fit? Putnam *et al.*, reported that papain, upon extended incubation, completely digests the Fc fragment while the chymopapains produced two cleavages of the Fc fragment as judged by amino-terminal analysis (18). However, chymopapain C produced only one cleavage as determined by the presence of threonine as the only amino-terminal of the Fc fragment. Clearly, chymopapain C behaved differently than papain on human IgG, but similar to chymopapain.

The question arises whether chymopapain C may be a degradation product chymopapain produced during isolation or a biologically distinct entity present in native latex. It is impossible for us to state that chymopapain C exists in fresh latex. However, we feel that the former is an unlikely possibility. The temperature and the pH of the isolation procedure were not in the appropriate ranges to permit enzymatic activity to occur. Ebata and Yasunobu reported (5) that chymopapains do not undergo significant autolysis at temperatures below 10°C. Whether there is naturally occurring degradation in the papaya plant and in the fresh latex cannot be answered by the data presented in this paper. In this regard, Ota, Moore and Stein (19) showed that adding a reversible inhibitor to stem or fruit bromelain allowed the chromatographic isolation of a single component; however, in the absence of the inhibitor during isolation, autolysis produced multiple proteolytic components. Finkle and Smith (20) concluded that in addition to activity in papain and chymopapain fractions, papaya latex contains proteolytic activity in other fractions as a result of possible autolytic events. Perhaps there is generated a spectrum of biologically distinct enzymatic entities which differ only in size and charge. Our data are consistent with the view that *Carica papaya* contains at least four proteinases: papain, chymopapains A, B, and C.

Summary. This study has demonstrated the presence of a proteolytic enzyme, chymopapain C, in the extract of *Carica papaya*. This enzyme differs significantly from papain in enzymatic activity, thermal half-life and action of human IgG. However, this enzyme is similar to commercial chymopapain in its activity and in its digestion of human IgG, but differs in molecular weight, thermal half-life and relative electrophoretic mobility.

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