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Characterization of the Individual Collagenases from *Clostridium histolyticum*†

Michael D. Bond and Harold E. Van Wart*

ABSTRACT: The six collagenases (α, β, γ, δ, ϵ, and ζ) from *Clostridium histolyticum* isolated in the preceding paper [Bond, M. D., & Van Wart, H. E. (1984) *Biochemistry* (first paper of three in this issue)] have been characterized in detail. The molecular weights determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis range from 68 000 to 125 000. Isoelectric focusing experiments demonstrate that the isoelectric points of the collagenases are in the 5.35–6.20 range. These experiments also reveal that the subspecies of α- and γ-collagenases (α_1 vs. α_2 and γ_1 vs. γ_2) have different isoelectric points but the same molecular weights. Microheterogeneity is also observed for the β- and ϵ-collagenases. The amino acid compositions of all six collagenases have been determined, and analysis for neutral sugars and hexosamines shows that none of the enzymes have a significant carbohydrate content. Zinc and calcium are the only metals that copurify with the collagenases. The purified enzymes contain approximately 1 mol of zinc/mol of protein and a calcium content that varies from about 2 mol/mol for α-collagenase to about 7 mol/mol for β-collagenase. All of the collagenases are 5–10 times more active against gelatin than collagen. The α-, β-, and γ-collagenases are significantly less active toward the synthetic peptide substrates examined than the δ-, ϵ-, and ζ-collagenases. This property, taken together with data on the stabilities and amino acid compositions of these enzymes, strongly supports their assignment to two distinct classes. This establishes clearly that *C. histolyticum* does, indeed, produce more than one different type of collagenase.

Over the last 30 years, numerous studies have been carried out in which one or more of the collagenases (EC 3.4.23.3)[1] from *Clostridium histolyticum* have been isolated [Bond & Van Wart (1984a) and references cited therein]. Most of what is presently known about *Clostridial* collagenases has come from the early pioneering studies of Mandl, Seifter, Harper, and their associates. Unfortunately, these enzymes have re-

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[1] Abbreviations: collagenase, *Clostridium histolyticum* collagenase; FALGPP, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-proline; FALGPA, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine; PZ, *p*-phenylazobenzyloxycarbonyl; [$^{14}CH_3$]collagen, [*methyl*-^{14}C]collagen; [$^{14}CH_3$]gelatin, [*methyl*-^{14}C]gelatin; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; cpm, counts per minute; nkat, nanokatal.

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ceived relatively little attention recently. Ironically, general interest in *Clostridial* collagenases has remained high because of their potent hydrolytic action on connective tissue, a property that has made them the reagent of choice for tissue dissociation experiments (Seglen, 1976). Also, there has developed an ever increasing awareness of the need for a detailed understanding of the catabolism of connective tissue by these and more specific collagenases. Thus, there is a clear need for a detailed characterization of these enzymes.

In spite of the early interest in *Clostridial* collagenases, relatively few attempts have been made to establish their basic biochemical properties. Moreover, there seems to be little agreement concerning those basic properties that have been examined. For example, the molecular weights of these collagenases have variously been reported to be as low as 66 000 (Keil, 1979) and as high as 147 000 (Soru & Zaharia, 1972). The amino acid compositions of several enzymes have been reported, but there is unacceptable agreement between these analyses. While these collagenases have long been assumed to be zinc metalloenzymes that require calcium ions for activity, they have never been purified to homogeneity by procedures designed to prevent adventitious metal ion contamination. Furthermore, there have been no direct measurements of the metal contents of highly purified enzymes, and the true identity of any catalytic metals and their stoichiometry have yet to be convincingly established.

In the preceding paper (Bond & Van Wart, 1984a), we have described the purification to homogeneity of six individual collagenases. The observation of multiple collagenases in this system has been made by previous authors (Grant & Alburn, 1959; Mandl et al., 1964; Yoshida & Noda, 1965; Kono, 1968; Kesselring et al., 1970; Peterkofsky & Diegelman, 1971; Lwebuga-Mukasa et al., 1976), and a great deal of speculation has been put forth regarding the relationship between the individual enzymes. However, in the absence of extensive characterization, it has been impossible to firmly establish whether or not any of the different enzymes that have been isolated are indeed different species or are simply derived from a common precursor by proteolysis. Here, we report the results of a detailed characterization of these enzymes that delineates their basic properties and establishes unambiguously that *Clostridium histolyticum* does indeed produce more than one type of collagenase.

Materials and Methods

Materials. All purified collagenases and chromatographic fractions are those described in the preceding paper (Bond & Van Wart, 1984a). Alkaline phosphatase, β-glucosidase, β-galactosidase, β-glucuronidase, and carbonic anhydrase were obtained from Worthington Chemical Co., and neuraminidase (type VI) was from Sigma Chemical Co. PZ-Pro-Leu-Gly-Pro-D-Arg and PZ-Pro-Leu were obtained from Tridom Chemical Co. A lectin kit consisting of lectins identified as *Arachis hypogaea*, *Bandeirea simplicifolia* I and II, *Concanavalia ensiformis*, *Dolichos biflorus*, *Glycine max*, *Maclura pomifera*, *Ricinus communis* I, *Triticum vulgaris*, and *Ulex europaeus* I was obtained from E-Y Laboratories, Inc.

Protein Determination. Protein concentration was determined either by the method of Bradford (1976), by the method of Whitaker & Granum (1980), or gravimetrically (Van Wart & Lin, 1981).

Enzymatic Assays. Assays for activity toward [$^{14}CH_3$]-collagen, FALGPA, and FALGPP were carried out as described earlier (Bond & Van Wart, 1984a). Assays toward [$^{14}CH_3$]gelatin were carried out as for [$^{14}CH_3$]collagen with collagen that had been heated at 90 °C for 10 min and cooled to 23 °C prior to the assay. The rate of hydrolysis of PZ-Pro-Leu-Gly-Pro-D-Arg was determined colorimetrically by the procedure of Wunsch & Heidrich (1963) with PZ-Pro-Leu as a hydrolysis standard.

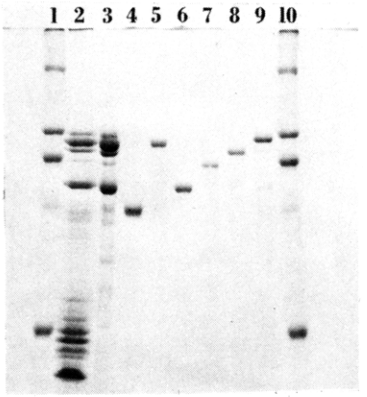

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide electrophoresis gel showing impure and purified collagenases: (lanes 1 and 10) molecular weight standards (myosin, β-galactosidase, phosphorylase *a*, bovine serum albumin, and ovalbumin); (lane 2), crude enzyme; (lane 3), Sephacryl S-200 fraction A; (lanes 4–9) α–, β–, γ–, δ–, ε–, and ζ–collagenases, respectively.

Gel Analyses. Unless stated otherwise, polyacrylamide gel electrophoresis experiments were carried out with 7.5% acrylamide gels as described by Weber & Osborn (1969). To prevent proteolysis of samples during denaturation (see Results), 10 mM EDTA was added to the denaturing buffer and samples were heated at 100 °C for 5 min. isoelectric focusing experiments were carried out at 8 °C on 5% polyacrylamide gels by using an LKB Model 2117 Multiphor System with LKB pH 3.5–10 or pH 6–8 ampholytes. All experiments were run at constant power. Isoelectric points were determined with an LKB surface electrode.

Amino Acid Analyses. Samples for amino acid analyses were dissolved in constant-boiling 6 N HCl containing phenol (0.2% v/v) and dithioglycol (0.2% v/v), degassed extensively, and sealed in hydrolysis tubes under high vacuum. After being heated at 110 °C for 24 h, the samples were dried by lyophilization and analyzed with a Dionex Model D-300 amino acid analyzer equiped with a Hewlett-Packard Model 3390A integrator. Cys was determined as cysteic acid after oxidation with performic acid as described by Moore (1963). Trp was not determined.

Carbohydrate Analyses. Neutral sugars and hexosamines were determined by modifications of the methods of Dubois et al. (1956) and Gatt & Berman (1966), respectively. *D*-(+)-galactose was used as a standard for the neutral sugars and *D*-galactosamine for the hexosamine determination.

Metal Analyses. All metal analyses were carried out by atomic absorption spectroscopy with a Perkin-Elmer Model 5000 instrument. Before analysis, all enzyme samples were dialyzed for 24 h against 6 L of metal-free 1 mM Tris, pH 7.5, and then for 8 h against 2 L of metal-free water, pH 7.0. Most metals were measured by using an air–acetylene flame, but a graphite furnace was used to analyze for Fe^{2+}, Cu^{2+}, Ni^{2+}, and Mn^{2+}. Calibration curves were made from Fisher certified standards.

Results

The protein banding pattern observed when the six collagenases and two of the impure chromatographic fractions from the preceding paper (Bond & Van Wart, 1984a) are subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis is shown in Figure 1. Lanes 2 and 3 contain the

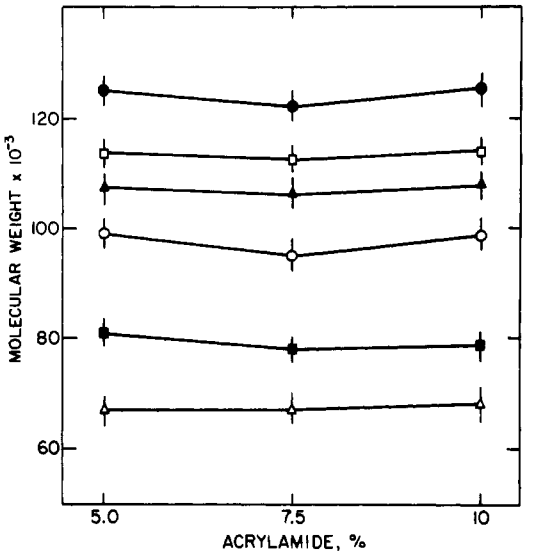

FIGURE 2: Variation in the apparent molecular weight of (●) ζ-, (□) β-, (▲) ϵ-, (○) δ-, (■) γ-, and (△) α-collagenases determined from sodium dodecyl sulfate gel electrophoresis experiments as a function of gel porosity.

crude starting preparation and Sephacryl S-200 fraction A, respectively. Both of these samples have six protein bands in the M_r 60 000–130 000 range, which correspond to the six purified collagenases that are present individually in lanes 4–9. The lower molecular weight bands most evident in the crude starting material are impurities. Each of the purified collagenases is completely homogeneous and free from both low molecular weight contaminants and other collagenases.

By use of the mobilities of the protein molecular weight standards (Figure 1, lanes 1 and 10) for calibration, the molecular weights of the α-, β-, γ-, δ-, ϵ-, and ζ-collagenases are found to be 68 000, 115 000, 79 000, 100 000, 110 000, and 125 000, respectively (Weber & Osborn, 1969). The molecular weights of the α_1 and α_2 subspecies of α-collagenase are identical with each other, as are the γ_1 and γ_2 subspecies of γ-collagenase. The molecular weights obtained for all collagenases are the same whether or not 2-mercaptoethanol is present during denaturation and electrophoresis. Moreover, the same molecular weights are obtained if the enzymes are reduced and alkylated prior to electrophoresis, indicating that they all consist of a single polypeptide chain.

It has been reported that sodium dodecyl sulfate gel electrophoresis is not a suitable method to determine the molecular weights of *Clostridial* collagenases because the mobilities of the enzymes vary anomalously with the porosity of the gel (Lwebuga-Mukasa et al., 1976). To investigate this, the molecular weights of all six collagenases were determined from sets of experiments carried out with gels that contained 5.0, 7.5, and 10.0% acrylamide. The molecular weights obtained are plotted as a function of the acrylamide concentration of the gel in Figure 2. The values shown are the averages and thee error bars the standard deviations for three determinations. This figure shows that the molecular weights obtained are, within experimental error, independent of the gel porosity.

In our early gel electrophoresis experiments in which EDTA was omitted from the denaturing buffer, we encountered great difficulty obtaining reproducible band patterns. For certain samples, bands would appear in the low molecular weight region at the expense of those in the high molecular weight region. This could not be attributed to proteolysis of the samples before the start of the gel experiment because the samples involved had high enzymatic activities. After an extensive series of experiments designed to track down the source of this problem (not shown), we have been able to establish that if EDTA is not included in the denaturing buffer, proteolysis will occur during heating to an extent determined by how impure the sample is and how much calcium is present. It is likely that it is the source of many of the conflicting reports on these enzymes.

Table I: Physicochemical Properties of *Clostridium histolyticum* Collagenases

	ϵ_{280} ($\times 10^{-5}$ M^{-1} cm^{-1})				
	method				
	method 1[a]	2[a]	method 3[a]	M_r	isoelectric point[b]
α_1		1.06	1.31	68 000	5.85
α_2		1.06	1.31	68 000	5.90
β	1.62	1.67	2.21	115 000	5.55, 5.60, 5.75
γ_1		1.19	1.51	79 000	6.10
γ_2		1.19	1.51	79 000	6.20
δ		1.48	1.92	100 000	5.80
ϵ		1.37	2.12	110 000	5.90, 5.95
ζ		1.55	2.40	125 000	5.35

[a] Protein concentration was determined gravimetrically (method 1), by the methods of Whitaker & Granum (1980) (method 2), and by the method of Bradford (1976) (method 3). [b] Multiple listings indicate heterogeneity.

Isoelectric focusing experiments have been carried out on polyacrylamide gels to measure the isoelectric points of the purified enzymes and to provide another measure of their homogeneity. All of the collagenases have isoelectric points in the 5.35–6.20 range. Certain of the collagenases exhibit more than one band on these gels, but all of the bands cited clearly copurify with the collagenase and cannot be attributed to impurities. The δ- and ζ-collagenases both showed a single major band with isoelectric points of 5.80 and 5.35, respectively. The electrofocusing gels revealed that subspecies α_1 and α_2 have slightly different isoelectric points (5.85 vs. 5.90), as do subspecies γ_1 and γ_2 (6.10 vs. 6.20). The most heterogeneous collagenases were β and ϵ. β-Collagenase exhibits discernible bands with isoelectric points of 5.55, 5.60, and 5.75 while ϵ-collagenase shows bands that focus at pH 5.90 and 5.95.

The molar extinction coefficients, ϵ_{280}, of these collagenases have been measured in order to permit the molar activities and metal stoichiometries to be calculated. Since these values depend critically on the protein determination used, three different methods have been employed. The accurate gravimetric method requires a sizeable amount of protein and has only been carried out for β-collagenase, yielding a value of 1.62 $\times 10^5$ M^{-1} cm^{-1}. Protein concentrations were also determined by the dye binding method of Bradford (1976) and the spectrophotometric method of Whitaker & Granum (1980). The Bradford method does not give very good agreement (ϵ_{280} = 2.21 $\times 10^5$ M^{-1} cm^{-1}) with the dry weight method for β-collagenase. However, the Whitaker and Granum method gives excellent agreement for β-collagenase (ϵ_{280} = 1.67 $\times 10^5$ M^{-1} cm^{-1}). The values of ϵ_{280} for all the collagenases calculated by both of the methods just described, together with a summary of their molecular weights and isoelectric points, are listed in Table I.

The specific activities of the collagenases toward collagen, gelatin, FALGPP, FALGPA, and PZ-Pro-Leu-Gly-Pro-D-Arg, expressed as both nanokatals per milligram and nanokatals per nanomole, are summarized in Table II. These activities were calculated by the Whitaker & Granum (1980) method of protein determination. Thus, the values listed differ

Table II: Enzymatic Activities of *Clostridium histolyticum* Collagenases

	[$^{14}CH_3$]collagen[a]		[$^{14}CH_3$]gelatin[a]		FALGPP		FALGPA		PZ peptide[b]	
	nkat/mg	nkat/nm	nkat/mg	nkat/nm	nkat/mg	nkat/nm	nkat/mg	nkat/nm	nkat/mg	nkat/nm
α_1	0.023	0.0016	0.21	0.014	46	3.1	7.3	0.50	81	5.5
α_2	0.022	0.0015	0.20	0.013	46	3.1	7.3	0.50	81	5.5
β	0.017	0.0019	0.095	0.011	32	3.7	5.1	0.59.	58	6.7
γ_1	0.020	0.0015	0.12	0.0095	43	3.4	6.0	0.47	59	4.6
γ_2	0.021	0.0017	0.19	0.015	42	3.3	5.2	0.41	47	3.7
δ	0.0069	0.00069	0.048	0.0048	100	10	48	4.8	590	59
ϵ	0.0054	0.00060	0.026	0.0029	140	15	71	7.8	450	50
ζ	0.0059	0.00073	0.027	0.0034	77	9.7	37	4.6	530	66

[a] Activities for both collagen and gelatin were calculated from a substrate M_r of 300 000; see text for explanation. All protein concentrations were determined by the method of Whitaker & Granum (1980). [b] *p*-Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg.

somewhat from those given in Table II of the preceding paper (Bond & Van Wart, 1984a), which were calcualted on the basis of the Bradford method (1976). The molar activities toward collagen vary from 0.00060 to 0.0019 nkats/nm. These rates are extremely high compared with those of proteinases such as thermolysin, trypsin, or chymotrypsin, which are at least 1000 times less active with this same assay. Interestingly, all of the collagenases are approximaately 5–10 times more active against gelatin than collagen. For simplicity, the gelatinase activities were calculated by assuming the same molecular weight for gelatin as for native collagen (300 000), since the same total amount of substrate and hydrolyzable bonds are present in both the collagenase and gelatinase assays.

All of the collagenases are highly active against the synthetic peptides FALGPP and FALGPA. Activities toward PZ-Pro-Leu-Gly-Pro-D-Arg have also been measured, since this substrate has been used in many earlier studies. The relative activities of all six collagenases toward these three substrates are very similar. The assays carried out with FALGPA and FALGPP as substrates follow first-order kinetics since the substrate concentration (50 μM) is below K_M. The molar activities listed in Table II are easily converted into the more useful values of k_{cat}/K_M in units of M^{-1} min^{-1} by multiplying by 1.2×10^6. Thus, the value of k_{cat}/K_M for the hydrolysis of FALGPA by α_1-collagenase is 6.0×10^5 M^{-1} min^{-1}.

As pointed out in the preceding paper (Bond & Van Wart, 1984a), the relative activities of these enzymes toward collagen vs. the synthetic peptides used here enables them to be separated into two classes. The class I collagenases (α, β, and γ) have higher activities toward collagen and gelatin and lower activities toward FALGPP, FALGPA, and the PZ peptide. The class II collagenases (δ, ϵ, and ζ) have about one-third of the activity toward collagen and gelatin but have significantly higher activities toward the synthetic peptides. The subspecies (α_1 and α_2; γ_1 and γ_2) have the same activities as each other within experimental error toward all substrates examined. Within each class, the molar activities of each enzyme toward each substrte are extremely similar.

The effect of incubation at room temperature in 10 mM Tris and 5 mM $CaCl_2$, pH 7.5, and of freeze–thawing on the activity of these collagenases toward FALGPA has been examined (Figure 3). Data are shown only for β- and ϵ-collagenases; the results for other collagenases in the same class are very similar. Incubation at room temperature causes an almost parallel, progressive decrease in the activities of all six collagenases (Figure 3A). In 24 h, almost half of the starting activity is lost. This inactivation is not affected by addition of 1 M sucrose. Freeze–thawing has a more deleterious effect on the activities of the enzymes (Figure 3B). The class II collagenases are particularly sensitive and lose approximately half their activity after four cycles. These enzymes also lose activity faster than the class I enzymes as a function of the time that they are stored frozen (data not shown). The loss of activity of both classes of enzymes on freeze-thawing is much less serious when 1 M sucrose is present. In view of the instability of highly purified samples of the class II and, to a much lesser extent, the class I collagenases, the specific activities listed in Table II should be considered to be lower limits. During the last stages of purification of the class II collagenases in particular, the enzymes may have lost some activity when frozen for storage.

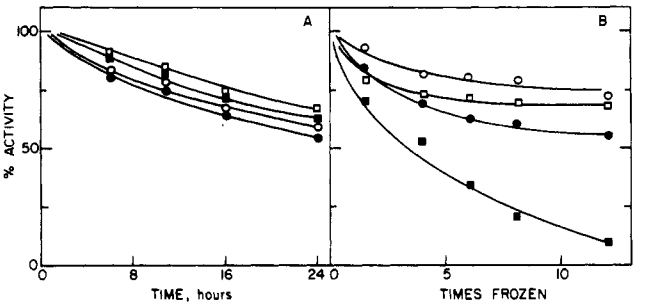

FIGURE 3: (A) Effect of incubation at 25 °C in 10 mM Tris and 5 mM $CaCl_2$, pH 7.5, on the activities of (●) β-collagenase and (■) ϵ-collagenase. (○) and (□) Data for β- and ϵ-collagenases, respectively, in the same buffer containing 1 M sucrose. (B) Effect of freeze–thawing on the activities of (●) β-collagenase and (■) ϵ-collagenase in 10 mM Tris and 5 mM $CaCl_2$, pH 7.5. (○ and □) Data for β- and ϵ-collagenases, respectively, freeze-thawed in the same buffer containing 1 M sucrose.

Table III: Amino Acid Composition of *Clostridium histolyticum* Collagenases[a]

amino acid	α_1	α_2	β	γ_1	γ_2	δ	ϵ	ζ
Asp + Asn	90	91	156	98	101	131	144	160
Thr	42	42	80	57	57	60	68	73
Ser	37	37	67	46	47	59	69	72
Glu + Gln	59	60	98	69	70	95	104	115
Gly	56	55	92	60	60	61	73	99
Ala	38	38	66	47	47	43	46	57
Val	31	32	52	32	32	38	42	62
Met	3.7	3.7	8.0	4.9	4.9	9.6	12	15
Ile	32	31	50	31	30	38	38	56
Leu	51	51	72	51	50	68	69	87
Tyr	43	42	70	52	51	68	75	78
Phe	30	27	51	37	37	36	39	34
His	6.1	8.5	17	13	13	19	21	22
Lys	51	51	91	62	61	83	94	101
Arg	20	20	30	23	23	31	33	36
Pro	16	16	27	18	18	32	38	47
Cys[b]	2.2	2.2	3.5	2.4	2.5	4.1	4.5	4.5

[a] Expressed as residues per molecule of enzyme. Trp was not determined or included in the calculations, and the values cited were uncorrected for losses during hydrolysis. [b] Determined as cysteic acid.

Table IV: Carbohydrate Content of *Clostridium histolyticum* Collagenases

sample	neutral sugars (% w/w)[a]	hexosamines (% w/w)[b]
Sephacyl S-200 fraction A	1.6	0.52
β-collagenase	<0.45	<1.0
γ-collagenase	<0.20	<1.0
ε-collagenase	<1.0	<1.0
β-glucuronidase	4.8[c]	

[a] Determined by the method of Dubois et al. (1956). [b] Determined by the method of Gatt & Berman (1966). [c] Literature values vary from 3.6 to 6.6% w/w (Himeno et al., 1974).

The amino acid compositions of all the collagenases are listed in Table III in the form of residues per molecule of enzyme. The analyses for α_1 and α_2 are extremely similar to each other, as are those for the γ_1- and γ_2-collagenases. Thus, it is likely that these subspecies differ by only a small peptide. When calculated in terms of residues per thousand (not shown), it is clearly seen that the compositions of the class I and class II enzymes are internally very similar. However, there are marked differences in the amounts of Glx, Ala, Met, Phe, His, and Pro. Comparison of these analyses with those of five collagenases reported in the literature shows that collagenase I (Yoshida & Noda, 1965) has a composition that is in satisfactory agreement with that for β-collagenase, the same relationship suggested in the preceding paper (Bond & Van Wart, 1984a). Moreover, collagenase II has a composition that is intermediate between those of the two classes of enzymes, suggesting that it could have been a mixture. The analyses for collagenases A and B and the enzyme isolated by Emod et al. (1981), although similar in many ways, do not agree well with any of the collagenases isolated here.

To provide a measure of carbohydrate content of the collagenases, analysis for neutral sugars and hexosamines has been carried out (Table IV). Glycoproteins contain mainly galactose, mannose, glucose, L-fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acids (Wagh & Bahl, 1981), and these analyses will detect all of these species except sialic acid. Sephacryl S-200 fraction A, which contains greater than 90% by weight collagenases, was found to contain 1.6 and 0.52% w/w neutral sugars and hexosamines, respectively. Hence, there is very little carbohydrate in these enzymes. The sugar contents of the β-, γ-, and ε-collagenases were measured (Table IV) and confirm that they contain less than 1% w/w of either of these classes of sugars.

In another series of experiments, the collagenases were treated with β-glucosidase, β-galactosidase, β-glucuronidase, and neuraminidase to assess whether removal of different sugars had any effect on either the activities of the enzymes or their behavior in either electrophoresis or electrofocusing experiments. The enzymes were also treated with alkaline phosphatase to determine whether the removal of phosphate had an impact on these properties. In no case did any of the treatments mentioned have any discernible effect on any of the properties of the enzymes studied. In addition, treatment of each of the collagenases with a series of lectins specific for a wide range of sugars had no influence on their activities. Thus, we have found no evidence for the presence or involvement of carbohydrate in the activities of these enzymes, nor can its presence explain the different physicochemical properties observed.

The metal contents of the collagenases were measured at various stages of the purification. A representative sampling of the results, expressed as micromoles of metal per gram of protein, is listed in Table V. Analyses for Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} were carried out by atomic absorption spectrometry. The crude starting enzyme contains large quantities of Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+}. Sephacryl S-200 fraction A from the purification is enriched only in Ca^{2+} and Zn^{2+} but still contains substantial quantities of Cu^{2+}, Fe^{2+}, and Mg^{2+}. At the completion of the purification, only Ca^{2+} and Zn^{2+} were present in any of the collagenases in stoichiometrically significant (>0.02 mol/mol) quantities. Thus, only Zn^{2+} and Ca^{2+} can be considered catalytically essential for these enzymes.

Table V: Metal Content of *Clostridium histolyticum* Collagenases at Various Stages of Purification[a]

	μmol of M^{2+}/g of protein[b]			
	crude enzyme	hydroxylapatite fraction C	Sephacryl S-200 fraction A	β
Ca^{2+}	25	55	61	57
Cd^{2+}	0.063	0.088	0.056	<0.02
Co^{2+}	0.20	0.29	0.18	<0.10
Cu^{2+}	1.3	3.9	1.3	<0.15
Fe^{2+}	17	4.5	2.1	<0.08
Mg^{2+}	24	13	3.0	<0.10
Mn^{2+}	0.23	0.43	0.20	<0.0047
Ni^{2+}	0.69	1.1	0.62	<0.028
Zn^{2+}	4.0	4.3	8.0	8.7

[a] The samples listed are identified in Figure 1 of Bond & Van Wart (1984a). [b] Protein concentration determined by method of Whitaker & Granum (1980).

Table VI: Metal Content of *Clostridium histolyticum* Collagenases Calculated from Different Methods of Protein Determination[a]

	Zn^{2+} (mol/mol of protein)			Ca^{2+} (mol/mol of protein)		
	method 1	method 2	method 3	method 1	method 2	method 3
α		0.80	0.97		1.9	2.4
β	1.00	1.03	1.37	6.6	6.8	9.0
γ_1		0.81	1.03		2.8	3.5
γ_2		0.85	1.08		2.4	3.0
δ		1.10	1.45		2.6	3.4
ε		0.91	1.40		3.5	5.4
ζ		0.88	1.37		5.1	7.9
carbonic anhydrase[b]	1.06					

[a] Protein concentration was determined gravimetrically (method 1), by the method of Whitaker & Granum (1980) (method 2), and by the method of Bradford (1976) (method 3). [b] Protein concentration determined spectrophotometrically with $\epsilon_{280} = 5.70 \times 10^4$.

On the basis of the analyses for Ca^{2+} and Zn^{2+} in the purified collagenases, the stoichiometries of binding of these metals have been calculated (Table VI). The calculations have been carried out by using ϵ_{280} values determined by the three methods discussed earlier. With use of the most accurate value of ϵ_{280} for β-collagenase determined gravimetrically, a stoichiometry of 1.00 mol of Zn^{2+} and 6.6 mol of Ca^{2+}/mol is obtained. Carbonic anhydrase, used as a protein standard, was found to have 1.06 mol of Zn^{2+}/mol. With values from the other two methods of calculation, the collagenases are all found to have either slightly less or slightly more than 1 mol/mol of Zn^{2+}. In contrast, the enzymes contain variable amounts of Ca^{2+}, ranging from a low of 1.9 for α-collagenase to a high of 6.8 mol/mol for β-collagenase. In general, within each class the Ca^{2+} content correlates with the molecular

weight of the collagenase. The Ca^{2+} contents of the enzymes depended to some extent on the length of dialysis of the samples prior to analysis.

Discussion

Our extensive characterization of the six *Clostridial* collagenases serves to confirm many of the ideas presently held about these enzymes (Seifter & Harper, 1970, 1971). In addition, the data contained herein contradict other widely held views. Over the years, many different molecular weights have been reported for *Clostridial* collagenases. Without specifying the designation given to the collagenase or the method used to determine the molecular weight, values that have been reported include 57 400 (Harper et al., 1965), 66 000 (Keil, 1979), 70 000 (Emod et al., 1981), 72 000 (Lwebuga-Mukasa et al., 1976), 79 000 (Yoshida & Noda, 1965), 81 000 (Lwebuga et al., 1976), 95 000 (Yoshida & Noda, 1965), 105 000 (Harper et al., 1965), 109 000 (Seifter et al., 1959; Strauch & Grassman, 1966), 112 000 (Mandl et al., 1964), and 147 000 (Soru & Zaharia, 1972). There is little doubt that some of the quoted values differ due to the fact that investigators have simply isolated different individual or subsets of the individual collagenases. The results reported here firmly establish that there are at least six distinct enzymes with molecular weights in the 68 000–125 000 range. This explains, at least in part, the unusually large spread in the published values.

A second factor that may well have influenced the determination of the molecular weights of these collagenases is their susceptibility to proteolysis. This has a specially serious impact on the behavior of the enzymes on sodium dodecyl sulfate electrophoresis gels. Trace quantities of contaminating protease activity present in the samples on heating degrade the high molecular weight collagenases and produce species with molecular weights close to some of those cited in the literature. All of our observations concerning this proteolysis are consistent with clostripain being the source. Mitchell & Harrington (1968) have previously noted that clostripain is heat stable in the presence of calcium ions and also that it is inhibited by EDTA. Thus, proteolysis of collagenase samples will occur during heating to a degree determined by the amounts of clostripain and calcium ions present. It is not clear whether this problem may have been related to the claim that *Clostridial* collagenases run anomalously on sodium dodecyl sulfate–polyacrylamide gels (Lwebuga-Mukasa et al., 1976). In any event, when the samples are prepared as described, the sodium dodecyl sulfate gel electrophoresis technique is as applicable to the molecular weight determination of *Clostridial* collagenases as any other protein. The carbohydrate analyses confirm that these enzymes contain almost no sugar and therefore would not be expected to have the anomalous electrophoretic mobilities observed for true glycoproteins.

It is possible that the proteolysis described here is responsible for the early claim that collagenase consists of subunits of M_r 25 000 (Levdikova et al., 1963) or the later claim that collagenase A is a dimer of collagenase B (Harper et al., 1965). With regard to subunits, all of the collagenases appear to be single polypeptide chains. The molecular weights obtained for the individual collagenases do not change when 2-mercaptoethanol is present during electrophoresis or when they are reduced and alkylated prior to application to the gel. Since the metal analyses establish that each of the enzymes has only 1 mol of zinc/mol of enzyme, each of the collagenases appears to be a single chain with a single active site.

There has been a certain reluctance to believe that the molecular weights of *Clostridial* collagenases could be as high as 100 000–125 000 (Keil, 1979). For example, the collagenase isolated by Emod et al. (1981) is clearly shown from their own sodium dodecyl sulfate electrophoresis data to have a molecular weight greater than 100 000. Yet, the authors favor the molecular weight of 70 000 obtained from molecular seive chromatography, a method known to give inherently unreliable values when applied to undenatured proteins. Indeed, *Clostridial* collagenases do have abnormally high molecular weights compared to those of bacterial neutral metallohydrolases and other bacterial collagenases (Keil, 1979), which are closer to 35 000. Nevertheless, these high values are real and are thought to be the result of the evolution of these collagenases by gene duplication (Bond & Van Wart, 1984b).

The isoelectric points of the collagenases all lie in the 5.35–6.20 range, and many of the values for the individual collagenases are almost identical. This explains the difficulty encountered in separating the individual collagenases by ion-exchange chromatography alone. Few attempts have been made to measure the isoelectric points of these collagenases, but these values are generally in good agreement with those cited by Lwebuga-Mukasa et al. (1976). The value of 8.6 cited by Nordwig (1962) is not consistent with our results.

It is impossible to compare the collagenase activities reported here with those in the literature because there is, unfortunately, no standard assay for collagenase activity and few of those in use are directly comparable. However, many workers have measured the activities of their collagenases against PZ-Pro-Leu-Gly-Pro-D-Arg. The activities of δ-, ϵ-, and ζ-collagenases are significantly higher than those ever reported for any *Clostridial* collagenase. Our data indicate that when the activities toward collagen and gelatin are compared in assays in which the same mass of substrate is present, gelatin is hydrolyzed 5–10 times faster than native collagens. This is in good agreement with the data of Seifter et al. (1959). This difference in rates could be ascribed, in part, to the lower molarity of substrate (triple-helical rods) for native collagen compared to gelatin (mixture of component chains). However, the majority of the increase is undoubtedly due to the ease with which the collagenases can hydrolyze the dissociated chains compared to the triple helix, regardless of the substrate concentration. Thus, while collagenases are unique in their capacity to attack triple-helical collagen, these *Clostridial* collagenases more easily hydrolyze the unordered component gelatin chains. This is consistent with the high proteolytic activity of the collagenases toward synthetic substrates with sequences found in collagen (Streinbrink, 1981).

Another property of *Clostridial* collagenases about which there is poor agreement is their amino acid composition. Again, it is likely that some of the discrepancies are due to the fact that different workers have simply isolated different collagenases or mixtures of collagenases. Our data confirm the findings of Keil (1979) and Emod et al. (1984) that these collagenases do contain Met and Cys residues, in contrast to earlier claims (Seifter & Harper, 1971) that these residues are not present.

Over the last 30 years, a considerable body of circumstantial evidence has accumulated to indicate that collagenases are zinc metalloenzymes that require calcium ions for activity (Seifter & Harper, 1971). In order to draw convincing conclusions about the role of any metal in the action of an enzyme, it is essential that special precautions be taken during the purification to prevent the accumulation of adventitious metals and the loss of catalytic metals (Vallee, 1960). We have strictly adhered to the requisite procedures and report here direct analytical measurements that both confirm the assumption that zinc and calcium are catalytically essential metals and establish

their binding stoichiometry, Moreover, these are the only metals for which a functional role can be defined. The presence of zinc as a catalytic metal is consistent with the fact that the collagenases are inhibited by 1,10-phenanthroline and other agents that chelate transition-metal ions (Seifter & Harper, 1971) and that this inhibition is reversed by addition of zinc (Van Wart & Steinbrink, 1981). It is also consistent with the observation that when ^{65}Zn is included in the culture medium of the bacterium, the ^{65}Zn copurifies with the collagenases harvested (Harper & Seifter, 1974).

Variable quantities of calcium ions also copurity with the collagenases. These ions are also essential for activity, since the enzymes are inhibited by EDTA and this inhibition is reversed by addition of excess calcium but not reversed by addition of excess zinc (Van Wart & Steinbrink, 1981). The amount of calcium bound to each enzyme increases with the molecular weight of the enzyme, varying from about 2 atoms/molecule for α-collagenase to about 7 atoms/molecule for β-collagenase. The stoichiometries of calcium binding are somewhat less defined than that for zinc because the results depend slightly on the length of dialysis of the enzymes, indicating that some of the calcium ions may be less tightly bound than others. The presence of zinc and calcium in these collagenases is reminiscent of other bacterial metallohydrolases such as thermolysin.

Taken together, all of the physiochemical and functional properties of the six collagenases examined indicate the validity of their division into two distinct classes (Bond & Van Wart, 1984a). In particular, the relative activities toward collagen, gelatin, and synthetic substrates, the effects of freeze–thawing on activity, and the amino acid compositions all serve to distinguish between the class I (α, β, and γ) and class II (δ, ϵ, and ζ) collagenases. Moreover, a detailed examination of the substrate specificities of these collagenases toward a large number of synthetic peptides demonstrates further the similarities of the enzymes in each class, as well as the differences between those in the two classes (D. R. Steinbrink and H. E. Van Wart, unpublished data). In addition, the analysis of tryptic digests of these collagenases presented in the following paper (Bond & Van Wart, 1984b) substantiates this view unequivocally. This, it is now clear that *C. histolyticum* does indeed produce at least two distinct collagenases coded for by different genes. Thus, the hypothesis that the bacterium produces a single collagenase and that all of the others are derived from it by proteolysis (Emod et al., 1981) is incorrect. The question of the relationship between the individual enzymes in each class is examined in detail in the following paper (Bond & Van Wart, 1984b).

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Registry No. FALGPA, 78832-65-2; FALGPP, 89847-93-8; PZ-Pro-Leu-Gly-Pro-D-Arg, 17011-78-8; collagenase, 9001-12-1; zinc, 7440-66-6; calcium, 7440-70-2.

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