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TECHNICAL INFORMATION

Catalog Number: 100501, 100502, 150704, 150705, 151459, 195109, 195110
Collagenase

Description
Collagenases degrade native helical collagen fibrils. For an extensive review and bibliography see Harris and Krane (1974), Seifter and Harper (1970 and 1971) and Harper (1980).
The substrate, collagen, is the major fibrous component of animal extracellular connective tissue: skin, tendon, blood vessels, bone, etc. Its structural intricacies must be appreciated for an understanding of collagenolysis. (See review by Seifter and Gallop, 1966. See also Robertson and Miller (1972) on cartilagenous collagen.) In brief, collagen consists of fibrils composed of laterally aggregated, polarized tropocollagen molecules (MW 300,000). Each rod-like tropocollagen unit consists of three helically wound polypeptide a-chains around a single axis. The strands have repetitive glycine residues at every third position and an abundance of proline and hydroxyproline. The amino acid sequence is characteristic of the tissue of origin.
Tropocollagen units combine uniformly in a lateral arrangement reflecting charged and uncharged amino acids along the molecule thus creating an axially repeating periodicity (See also Ninmi 1968). Cross linkages continue to develop and collagen becomes progressively more insoluble and resistant to lysis on aging (Hamlin et al. 1975; Harris and Farrell 1972). See also Piez and Torchia (1975), Bruns and Gross (1973) and Tanzer (1973).
Gelatin results when soluble tropocollagen is denatured, for example on mild heating, and the polypeptide chains become randomly dispersed. In this state the strands are vulnerable to cleavage by a wide variety of proteases.
True collagenase may cleave simultaneously across all three chains or attack a single strand (Seifter and Harper 1970).The enzyme has an important role in connective tissue metabolism (Dresden 1971) and is produced by specific cells involved in repairs and remodelling processes (Wahl et al. 1975; Robertson et al. 1972; Sakamoto et al. 1975a). Table I in the review by Seifter and Harper (1971) lists known collagenases. Under normal conditions the mammalian enzyme is bound to a 2-macroglobulin or other serum antiproteases (Eisen et al. 1971). Where the enzyme is functional as in the postpartum uterus, it may be obtained directly from the tissue homogenate (Ryan and Woessner 1971). Mammalian collagenases split collagen in its native triple-helical conformation at a specific site yielding fragments, TCA and TCB representing 3/4 and 1/4 lengths of the tropocollagen molecule. (Woolley et al. 1975b; Gross et al. 1974). After fragmentation the pieces tend to uncoil into random coil polypeptides (gelatin).
The first mammalian enzyme to be studied was that from a culture of tadpole tail tissue. (Nagai et al. 1966; Skai and Gross 1967; Bauer et al. 1971; Harper et al. 1971; Harper and Gross 1972). Collagenases from human and other mammalian sources have been reported on by Bauer et al. (1975), Brady (1975), Huang and Abramson (1975), Sakamoto et al. (1975a and b), Wahl et al. (1985), Wooley et al. (1975), Fujiwara et al.(1974), Gross et al. (1974), Werb and Burleigh (1974), Sakamoto et al. (1973), Woessner and Ryan (1973), Tokoro et al. (1972), Robertson and Miller (1972), Vaes (1972), Bauer et al. (1971), Donoff et al. (1971), Eisen et al. (1971, 1970, and 1968), Ryan and Woessner (1971), Lazarus et al. (1968). Chesney et al. (1974) report on human platelet collagenase. Eisen et al. (1973) report finding a collagenase in the fiddler crab hepato-pancreas and Phillips and Dresden (1973) in a land planarium. There also exist bacterial collagenases, usually from host invasive strains. These enzymes differ from mammalian collagenases in that they attack many sites along the helix (Seifter and Harper 1971). Collagenases from *Clostridium histolyticum*, first prepared by Mandl et al. (1953), have been most thoroughly studied. Welton and Woods (1975 and 1973) and Keil et al. (1975) report on a collagenase from *Achromobacter iophagus*, Carrick and Berk (1975) and Schoellmann and Fisher (1966) on one from *Pseudomonas aeruginosa* and Merkel et al. (1975) on collagenase from a marine bacterium.
Of particular interest is the relationship between active collagenase and the pathology of rheumatoid arthritis. It has been suggested that the normally inhibited collagenase in articular structures may be activated thereby causing the characteristic tissue destruction in joints. (Abe and Nagai 1973; Kruze and Wojtecka 1972; Bauer et al. 1971; Evanson et al. 1968). Rheumatoid synovial fluid contains an activating material not found in joint fluid from patients with osteoarthritis (Harris and Krane 1974). Harris et al. (1969) reported that the collagenase inhibiting capacity of rheumatoid arthritic patients is reduced by one half. They also reported on two synovial collagenases. See also Woolley et al. (1975a and b), Harris and McCroskery (1974) and Leibovich and Weiss (1971).
Collagenases have also been associated with invasive tumors in rabbits. (Harris et al. 1972; McCroskery et al. 1975 and 1973). Collagenase has found widespread application in the isolation of specific cell types from attendant connective tissue (Kono 1969; Peck 1964). For example, there is much interest in the experimental transplantation of pancreatic islet cells to alleviate diabetic symptoms (Barker 1975). See also Ewart et al. (1975), Shibato et al. (1975), Gates et al. (1972), Ashcroft et al. (1971) and Lacy and Kostianovsky (1967). Intact parenchymal cells from rat liver tissue have also been isolated. (Seglen 1973 and 1972; Berry and Friend 1969; Howard et al. 1967). Kitabchi et al. (1971) isolated rat adrenal cells and Czech and Fain (1971) fat cells. DeOca and Malinin (1975) report on primary cell cultures from human kidney and Trusler (1975) on a G-banding technique for human chromosomes. See also Benya et al. (1973) for many additional references.
Particular enzymatic activities of crude collagenases have correlated with the tissues from which the cells were obtained (or with the uses to which the cells are put), and as a result of the correlations several formal types have been established.

Type 1

Containing average amounts of assayed activities (collagenase, caseinase, clostripain, and tryptic activities). It is generally recommended for fat, adrenal, and liver cells.

Type 2

Containing greater clostripain activity. It is generally used for heart, bone, muscle, thyroid, cartilage, and liver cells.

Type 3

Containing low proteolytic activity. It is usually used for mammary and fetal cells.

Type 4

Containing low tryptic activity. It is commonly used for islets and other applications where receptor integrity is crucial.

The wide spectrum of activity of crude collagenase makes it especially useful as a debriding agent. Such clinical applications were highlighted at a symposium reported by Mandl (1972). See also Howes et al. (1959) and Hamit and Upjohn (1960). Sussman (1968) has indicated its possible value for the treatment of herniated discs. He found that on injection into the intervertebral space a selective dissolution of the nucleus pulposus and fibrocartilaginous tissue is effected.

Characteristics of Collagenase from *Cl. histolyticum*:

Crude preparations contain not only several collagenases but also a sulhydryl protease, clostripain (Mitchell 1968), a trypsin-like enzyme (Peterkofsky and Dregelmann 1971; Sparrow and McQuade 1973) and an aminopeptidase (Kessler and Yaron 1973). Sugasawara and Harper (1984) and Bond and Van Wart (1984) report on purification of the collagenases of *Cl. histolyticum*.

Molecular weight: Harper r, et al. (1965), isolated two fractions, A and B. Their molecular weights are 105,000 and 57,400 respectively.

Composition: Mandl et al. (1964) reported two distinct collagenases: one very active on native collagen and synthetic substrates but with negligible activity on gelatin and azocoll, and a second active on gelatin but less active on synthetic substrates. Schaub and Strauch (1968) also purified two collagenases: Clostridiopeptidase A and Collagenase 2, as did Yoshida and Noda (1965); Collagenases I and II. Harper and Kang (1970) and Mitchell (1968b) also report two collagenases. Kono (1968) describes three fractions: Aa and Ba and Bb. It has been hypothesized that the enzymes are composed of subunits: a tetramer of approximately 105,000 M.W. and a dimer of 57,000. The presence of zinc or cobalt in the molecule is also indicated. (Harper et al. 1965; Takahashi and Seifter 1970). Amino acid compositions have been reported by Mandl et al. (1964), Harper et al. (1965) and Yoshida and Noda (1965). The enzymes contain neither cysteine nor methionine (Takahashi and Seifter 1970).

Optinum pH: 7-9 for the A-a enzyme (Kono o, 1968).

Activators: Ca^{2+} is required. (Takahashi and Seifter, 1970).

Inhibitors: Metal chelating agents such as cysteine, EDTA or o-phenanthroline but not DFP. It is also inhibited by a 2-macroglobulin - a large plasma glycoprotein (Werb et al. 1974). Nagase et al. (1983) and Stricklin and Welgus (1983) report on natural collagenase inhibitors. The human skin enzyme is inhibited by human serum (Eisen et al. 1970) but granulocyte collagenase is not (Lazarus et al. 1968). Human serum contains a2-macroglobulin and a1-antitrypsin that may inhibit certain collagenases as well as a third inhibitor reported by Woolley et al. (1975). It has also been shown that collagenase A is photo-inactivated in the presence of methylene blue (Takahashi and Seifter, 1970) See also Karakiulakis et al., 1991.

Specificity: Clostridial collagenase I or clostridiopeptidase A degrades the helical regions in native collagen preferentially at the Y-Gly bond in the sequence -Pro-Y-Gly-Pro- where Y is most frequently a neutral amino acid. This bond in synthetic peptide substrates may also be split. See also Soberano and Schoellmann (1972a) and Harris and Farrell (1972). The specificities of the three Kono enzymes have been studied (Kono o, 1968).

Applications

For collagen structural and biosynthetic studies researchers generally use more highly purified collagenase preparations free of other proteolytic activities.

Clostridiopeptidase A (MP catalog # 194124), also called collagenase 1, is a highly purified enzyme essentially free of collagenase 2, clostripain, and other proteases.

For tissue dissociation most researchers employ either crude collagenase preparations or chromatographically purified collagenase combined with other enzymes such as elastase, trypsin, and/or papain.

Typical Assay

Assay methods utilizing labelled collagen have been reported by Gisslow and McBride (1975), Robertson et al. (1972) and Sakamoto et al. (1972). Since true collagenase attacks the helical region of the molecule, change in optical rotary dispersion reflects collagen degradation (Keil et al. 1975).

Method: A modification of the procedure of Mandl et al. (1953). Collagenase is incubated for 5 hours with native collagen. The extent of collagen breakdown is determined using the Moore and Stein (1948) colorimetric ninhydrin method. Amino acids liberated are expressed as micromoles leucine per milligram collagenase. One unit equals one micromole of L-leucine equivalents from collagen in 5 hours at 37°C and pH 7.5 under the specified conditions.

Reagents

– 0.05 M TES [tris(hydroxymethyl)-methyl-2-aminoethane sulfonate] buffer with 0.36 mM calcium chloride, pH 7.5

– 4% Ninhydrin in methyl cellosolve

– 0.2 M Sodium citrate with 0.71 mM stannous chloride, pH 5.0

– Ninhydrin-citric acid mixture: Prepare by mixing 50 ml of the 4% ninhydrin in methyl cellusolve with 50 ml of 0.2 M citrate with 0.71 mM stannous chloride, pH 5.0. Allow mixture to stir for 5 minutes.

– 50% n-Propanol

– Substrate: bovine achilles tendon Collagen and vitamin free casein

– 50% (w/v) Trichloroacetic acid

Enzyme

Dissolve enzyme at a concentration of 1 mg/ml in 0.05 M TES with 0.36 mM calcium chloride, pH 7.5. Dilutions run are 1/10 and 1/20 in the above buffer.
Procedure
Weigh 25 mg of bovine collagen into each of four test tubes. Include at least two tubes to serve as blanks which will contain no enzyme. Add 5.0 ml of 0.05 M TES buffer to the tubes and incubate at 37°C for 15 minutes. Start the reaction by adding 0.1 ml of enzyme dilution to appropriate tubes.
After 5 hours, stop the collagenase reaction by transferring 0.2 ml of solution (leaving behind the collagen) to test tubes containing 1.0 ml of ninhydrin-citric acid mixture. Include an enzyme blank (collagen incubated with 0.1 ml TES buffer in place of enzyme). Heat for 20 minutes in a boiling water bath. After cooling, dilute with 5 ml of 50% n-propanol. Let stand for 15 minutes and read absorbance at 600 nm. From an L-leucine standard curve determine micromoles amino acid equivalent to leucine liberated.
Non-specific protease activity (i.e. caseinase activity) is determined using the above assay and substituting 25 milligrams vitamin free casein for collagen. The reaction is stopped after 5 hours by the addition of 0.5 ml of 50% trichloroacetic acid. After centrifugation, 0.2 ml of the supernatant is transferred to 1.0 ml of ninhydrin and treated as above. Caseinase activity is calculated as collagenase activity.
Calculation
Units/mg = (micromoles L-leucine equivalents liberated) / (mg enzyme in digestion mixture)

Assay for Clostridiopeptidase A
Collagenase I - *Cl. histolyticum*
Method: The assay is based on the enzyme-specific splitting of a Pz-Pro-Leu-Gly-Pro-Arg substrate between leucine and glycine (Wünch and Heidrich, 1963). This results in a colored lipophilic fragment and a non-colored tripeptide. The change in absorbance of the fragment at 320 nm per unit time is a measure of enzyme activity.
Reagents

– 0.1 M Tris*HCl, pH 7.1

– 0.1 M Calcium chloride

– 25 mM Citric acid, pH 3.5

– Ethyl acetate

– Anhydrous sodium sulfate

– Substrate: Pz-Pro-Leu-Gly-Pro-Arg (molecular weight 812.93). Dissolve to 5 mg/ml in methanol. Dilute to 1 mg/ml with Tris buffer.

– Standard: Pz-Pro-Leu (molecular weight 466.54). Dissolve to 5 mg/ml in methanol. Dilute to 1 mg/ml with Tris buffer.

Enzyme
Stock solution: Dissolve at 1 mg/ml in reagent grade water. Dilute stock 1:20 and 1:50 for assay.
Procedure

	Micrograms of Standard						
Standard	**0**	**50**	**100**	**200**	**400**	**600**	**800**
Tris buffer	0 ml	0.05 ml	0.1 ml	0.2 ml	0.4 ml	0.6 ml	0.8 ml

1.1 ml

1.05 ml

1.0 ml 0.9 ml 0.7 ml 0.5 ml 0.3 ml

Calcium chloride	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml

Mix well and transfer 0.5 ml to labeled tubes containing 1 ml citric acid and 5 ml ethyl acetate. Vortex for 15 seconds. All the phases separate at room temperature. Transfer the organic phase to tubes containing 350 mg sodium sulfate. Shake gently to allow ethyl acetate to dry. Read the A_{320} of the dried ethyl acetate vs. air.
Determine the net A_{320} (test - blank) and plot A_{320} nm/ug standard from the slope of the curve. Determine the calibration factor as follows:

Factor = (1) / (A_{320} nm/ug X 466 ug/umole)

Enzyme Assay: Pipette 0.2 ml calcium chloride and 1.0 ml substrate into a series of numbered tubes. Incubate in a water bath at 25ºC to achieve temperature equilibration. At timed intervals, add 0.1 ml enzyme dilution to the respective tubes. Include 2 tubes with 0.1 ml water as blanks. Incubate exactly 15 minutes at 25ºC and at timed intervals withdraw 0.5 ml and transfer to tubes containing 1 ml citric acid and 5 ml ethyl acetate. Proceed as with standard curve, reading A_{320} of dried ethyl acetate vs. air. Determine net A_{320}.
Calculation

Units/mg = (Net A_{320} X dilution X Factor) / (0.1 X 15 X mg/ml in stock)

Availability:

Catalog Number	Description	Sizes Available
194124	Clostridiopeptidase A from Clostridium histolyticum. Activity approximately 5 units/dry weight. Supplied as a lyophilized powder tested for BAEE activities and casein digestion.	5 U 10 U
362001	Collagenase from Clostridium histolyticum. Activity approximately 300 Mandl units/mg. This mixture is suited for dissolving connective tissue and exposing embedded cells without destroying the cell membranes and other structures, since the content of unspecific proteases is very low. The high purity and complete absence of pathogenic spores makes this enzyme best suited for cell research and cell culture applications.	300 KU
100501	Collagenase from Clostridium histolyticum. Sterile, salt-free, lyophilized. Activity approximately 125-250 units/mg dry weight.	50 mg
150705	Collagenase from Clostridium histolyticum. Cell Culture Reagent.	1 gm 5 x 1 gm
151459	Collagenase from Clostridium histolyticum, highly purified, free of detectable non-specific proteases. Activity 2,000 - 3,000 units per ml when reconstituted with one ml buffer.	1 vial
150704	Collagenase from Clostridium histolyticum. Type 3, Activity approximately 3500 u/gm; clostripain, trypsin and caseinase $\geq$ 100 u/gm (by casein-folin method, pH 7.0)	1 gm 5 x 1 gm
195109	Collagenase from Clostridium histolyticum, Grade I, salt-free, lyophilized. Activity approximately 125-220 units per mg solid.	25 mg 100 mg 500 mg 1 gm
100502	Collagenase from Clostridium histolyticum, Grade II, lyophilized powder. Activity approximately 125 - 250 units/mg dry weight	25 mg 100 mg 500 mg 1 gm
195110	Collagenase from Clostridium histolyticum, Type 4, salt-free, lyophilized. Activity approximately 125 units per mg solid. Hepatocyte Isolation Grade, contains clostripain, neutral protease and trypsin activities.	25 mg 100 mg 500 mg

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