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Collagenase

Clostridiopeptidase A from *Clostridium histolyticum*

Description

Cell aggregates or distinct cells can be isolated from human or animal tissues by Collagenase mediated tissue dissociation. As viability and function of cells are crucial for all kinds of applications this enzymatic step is most important in cell isolation procedures influencing yield, viability and function of the cells.

Collagen is the most abundant protein of vertebrates, and occurs in virtually every tissue. Collagen proteins building collagen fibrils are the main components of the supporting tissue of connective tissue, bones, cartilage, and extracellular matrices of skin and blood vessels. It is one of the long, fibrous structural proteins whose functions are quite different from those of globular proteins such as enzymes; tough bundles of collagen called collagen fibers are a major component of the extracellular matrix that supports most tissues and gives cells structure from the outside, but collagen is also found inside certain cells. Collagen has great tensile strength, and is the main component of cartilage, ligaments, tendons, bone and teeth.

The complex composition of connective tissue has to be taken into account when tissues need to be dissociated in research or clinical situations, such as the preparation of suspensions of viable cells for metabolic studies, the isolation of pancreatic islets in diabetes research and the clinical treatment of necrotic tissue.

The structure of connective tissue has long been a subject of intensive research in biochemistry. Soft connective tissue has generally been shown to consist of collagen fibrils embedded in a gel-like matrix. A distinctive feature of collagen is the regular arrangement of amino acids in each of the three chains of these collagen subunits. The sequence often follows the pattern Gly-X-Pro or Gly-X-Hyp, where X may be any of various other amino acid residues. Gly-Pro-Hyp occurs frequently. This kind of regular repetition and high glycine content is found in only a few other fibrous proteins, such as silk fibroin. 75-80% of silk is (approximately) -Gly-Ala-Gly-Ala- with 10% serine — and elastin is rich in glycine, proline, and alanine (Ala), whose side group is a small, inert methyl. Such high glycine and regular repetitions are never found in globular proteins. The high content of Pro and Hyp rings, with their geometrically constrained carboxyl and (secondary) amino groups, accounts for the tendency of the individual polypeptide strands to form left-handed helices spontaneously, without any intrachain hydrogen bonding.

The collagen fibrils are complex structures which are assembled from tropocollagen sub-units (about 300 nm long and 1.5 nm in diameter), and which function primarily as supporting elements. These fibrils are surrounded by an extrafibrillar matrix, the macromolecular components of which are mainly proteoglycans. Proteoglycans consist of central protein cores with covalently linked heteroglycan chains radiating from them. Most connective tissues contain both large and small proteoglycans. These complex structures from proteoglycans and collagen are best dissociated by tissue-dissociating collagenases, assisted by other proteolytic enzymes.

Collagenases are enzymes that are able to cleave the peptide bonds in the triple helical collagen molecule. Besides the mammalian and amphibian tissue collagenases, the collagenases of the bacterium *Clostridium histolyticum* are of special interest and have been subject of investigations for more than 40 years. Structure and mode of action of this family of enzymes have been elucidated.

Collagenase from *Clostridium histolyticum*

Genaxxon Collagenase from *Clostridium histolyticum* also contain a number of other enzymatic activities, including clostripain, which cleaves peptides preferentially at the carboxyl side of arginine residues, a tryptic activity, which acts preferentially at the carboxyl side of arginine and lysine residues, and neutral non-specific proteases. The particular suitability of Genaxxon Collagenase I and II preparations for dissociating tissues is partly due to the presence of balanced amounts of these enzymes.

Specifications

The molecular weight of Collagenase is 70 – 120 kDa
pH-Optimum is 7.0 – 8.0
Isoelectric point is 5.4 to 6.2
Optimum temperature is 37°C

Cofactors
Collagenase requires calcium ions both for full catalytic activity and binding to the collagen molecule.

Inhibitors
Inhibitors of collagenase include Cystein, EDTA, o-phenanthroline, 8-hydroxyquinoline-5-sulfonate, bipyridyl and 2,3-dimercaptopropanol. It is also inhibited by TRIS buffer above pH7.5.
Collagenase is not inhibited by diisopropylphosphorofluoridate.

Stability and Storage
Collagenase is soluble in water and diluted salt solutions at pH above the isoelectric point (pH >7.0). The enzyme is reversibly inactivated at high pH values, and irreversibly inactivated at low pH values. All collagenases from Genaxxon are delivered as lyophilized powders that can be stored at 4 °C and remain stable without loss of activity for at least three years.
Opened vials should be protected from moisture!
We recommend to dissolve the enzymes immediately before use only, and not to store dissolved enzyme solutions because of autolysis and significant loss of activity.

Enzymatic Activities
The collagenolytic activity can be determined by different methods:

"Mandl" unit or CDU *(Collagenase Degrading Units):*	The enzyme is incubated with native collagen for 5 h. The extent of collagen breakdown is determined by quantification of released L-leucine equivalent amino acids using the colorimetric ninhydrin method [8]. This determination is partly dependent on the concentration of other proteases present in the preparation. **Unit definition:** 1 U liberates 1 µmol amino acid (expressed as L-leucine equivalents) from collagen per 5 hours at 37 °C, pH 7.5.
"Wünsch" units:	Collagenase cleaves the substrate PZ-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine producing a yellow fragment PZ-Lprolyl-L-leucine which is determined spectrophotometrically after extraction into ethyl acetate. **Unit definition:** 1 U catalyzes the hydrolysis of 1 µmol 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine per minute at 25 °C, pH 7.1. **Convertation to "Mandl" units:** 1000 U/mg "Mandl" ≈ 1 U/mg "Wünsch"
***"FALGPA" units*:**	The substrate N-(3-[2-furyl]acryloyl)-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) is a collagenase-specific substrate which is hydrolysed more rapidly than any other synthetic substrate, but is resistant to other proteases produced by *Clostridium histolyticum* **Unit definition:** 1 U is defined as the hydrolysis of 1 µmol of N-(3-[2-furyl]-acryloyl)-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) per minute at 25 °C, pH 7.5. **Convertation to "Mandl" units:** 1 U/mg "Mandl" ≈ 3900 U/mg "FALGPA"
Clostridiopeptidase A, "HP Units"	Clostridiopeptidase A cleaves the hexapeptide (HP) substrate N-carbobenzyloxy-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine producing N-CBZ-Gly-Pro-Gly and the tripeptide Gly-Pro-Ala, the latter being quantified spectrophotometrically after reaction with 2,4,6-trinitrobenzene sulfonic acid. **Unit definition:** 1 U catalyzes the hydrolysis of 1 µmol N-carbobenzyloxy-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine per minute at 37 °C. **Convertation to "Mandl" U/mg:** 1000 U/mg "Mandl" ≈ 100 HPU/mg

PLEASE NOTE:
Mandl units cannot be converted directly into the others as CDU determination reflects the activity of both collagenase class I and class II, whereus the other activity determination methods are based on synthetic peptides reflecting mainly the collagenase class II activity.

Genaxxon Collagenase preparations are assayed according to Mandl.

Collagenase Activity 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).

Genaxxon BioScience collagenase products are assayed as described below:

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 pH7.5 under the specified conditions.

Reagents:

0.05M TES [tris(hydroxymethyl)-methyl-2-aminoethane sulfonate] buffer with 0.36mM $CaCl_2$, pH7.5

4% Ninhydrin in methyl cellusolve

0.2M Sodium citrate with 0.71mM $SnCl_2$, pH5.0.

Ninhydrin-citric acid mixture: Prepare by mixing 50mL of 4% ninhydrin in methyl cellusolve with 50mL of 0.2M citrate with 0.71mM $SnCl_2$, pH5.0. Allow mixture to stir for 5 minutes.

50% n-Propanol

Substrate: Genaxxon bovine collagen and vitamin free casein

50% (w/v) Trichloroacetic acid

Enzyme:

Dissolve enzyme at a concentration of 1mg/mL in 0.05M TES with 0.36mM $CaCl_2$, pH7.5.
Dilutions run are 1/10 and 1/20 in the above buffer.

Procedure:

Weigh 25mg bovine collagen into each of four test tubes. Include at least two tubes to serve as blanks containing no enzyme. Add 5.0mL of 0.05M TES buffer to the tubes and incubate at 37°C for 15 mins.. Start the reaction by adding 0.1mL of enzyme dilution to appropriate tubes.

Reaction is stopped after 5 hours by transferring 0.2mL of solution (leaving behind the collagen) to test tubes containing 1.0mL of ninhydrin-citric acid mixture. Include an enzyme blank (collagen incubated with 0.1mL TES buffer in place of enzyme). Heat for 20 min. in a boiling water bath. After cooling, dilute with 5mL of 50% n-Propanol. Let stand for 15 min. and read absorbance at 600nm. From an L-leucine standard curve determine micromoles amino acid equivalent to leucine liberated.

Non-specific protease activity (i.e. caseine activity) is determined using the above assay, substituting 25mg vitamin free casein for collagen. The reaction is stopped after 5 hours by the addition of 0.5mL of 50% Trichloroacetic acid. After centrifugation, 0.2mL of the supernatant is transferred to 1.0mL of ninhydrin and treated as above. Cascinase activity is calculated as collagenase activity.

Calculation of Collagenase activity

Units/mg = µmoles L-leucine (equivalent liberated) / mg enzyme in digestion mixture

The 3 typical intrinsic activities of collagenase:

Clostripain	Clostripain activity is measured by the ability to hydrolyse N-benzoyl-L-arginine ethyl ester (BAEE) in the presence of the activator dithiothreitol (DTT).
	Unit definition: 1 U catalyzes the hydrolysis of 1 µmol BAEE per minute at 25 °C, pH 7.6, after activation with 1 mM calcium acetate and 2.5 mM dithiothreitol.
Trypsin-like proteases:	Trypsin activity is measured by the ability to hydrolyse N-benzoyl-L-arginine ethyl ester (BAEE).
	Unit definition: 1 U catalyzes the hydrolysis of 1 µmol BAEE per minute at 25 °C, pH 7.6.
Neutral proteases:	Neutral proteases are determined by their ability to hydrolyse dimethylcasein (DMC), liberated amino acids being determined with 2,4,6-trinitrobenzene sulfonic acid (TNBS).
	Unit definition: 1 DMC-U catalyzes the cleavage of 1µmol peptide bond from dimethylcasein per minute at 25 °C, pH 7.0, expressed in terms of newly formed terminal amino groups (determined with TNBS).

Usage of Collagenase

Tissue dissociation for cell culture

Collagenase is especially valuable when tissues are too fibrous or too sensitive to allow the use of trypsin, which is ineffective on fibrous material and damaging to sensitive material. Dissociation is usually achieved either by perfusing whole organs or by incubating smaller pieces of tissue with enzyme solution. Collagenase has been successfully used for the isolation of a broad variety of cell types.