

Collagenases for Tissue Dissociation

Cell aggregates or distinctive cell types can be isolated from different human or animal tissues. Collagenase mediated tissue dissociation is a crucial step in cell isolation procedures influencing yield, viability and function of the cells.

Viability and function of the cells is essential for their use in:

- research studies
- pharmacological test systems applied to development of new drugs
- transplantation e.g. in diabetes therapy
- tissue engineering and transplantation eg. cartilage transplants

SERVA Collagenases are designed for different fields of applications. Superb quality and performance are assured by:

- stringent quality control
- optimized purification steps
- accurate analysis of proteolytic enzyme activities
- manufacturing according to GMP guidelines incl process control

Collagen and connective tissue

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, teeth and extracellular matrices of skin and blood vessels.

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. The primary structure of the collagen molecule mainly consists of the repetitive sequence -Gly-Xaa-Yaa-, with Xaa often being Pro and Yaa often being Hyp, and builds a triple helix (tropocollagen). The collagen fibrils are complex structures which are assembled from tropocollagen sub-units, 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. One group of large proteoglycans interacts specifically with hyaluronic acid to form aggregates. In the presence of water, these aggregates form a matrix which connects and maintains the network of collagen fibrils. Small proteoglycans do not form aggregates and are regularly arrayed at the surface of the collagen fibrils in soft connective tissue. Furthermore, type I collagen fibrils have been shown to have specific binding sites for these proteoglycans.

Tissue dissociation is best achieved by means of the tissue-dissociating collagenases, assisted by other proteolytic enzymes.

Collagenases - Structure and mode of action

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⁽¹⁻⁴⁾.

Collagenase from *Clostridium histolyticum*

SERVA Collagenase NB qualities 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 SERVA Collagenase NB preparations for dissociating tissues is partly due to the presence of balanced amounts of these enzymes.

Moreover, SERVA Collagenase NB qualities are characterised by a low endotoxin content which guarantees a high viability of the isolated cells.

All Collagenase NB qualities are manufactured at the pharmaceutical plant of Nordmark Arzneimittel GmbH & Co. KG in Uetersen, Germany, by means of a patented process in a series of chromatographical steps in order to obtain purified enzymes. Our supplier Nordmark is not only the world's largest manufacturer of collagenase but also is experienced in collagenase production according to GMP-guidelines since several years. Therefore, the production guarantees batch to batch consistency and high quality products.

Properties

M _r :	70 - 120,000
Optimum pH:	7.5 - 8.0
Isoelectric point:	5.35 - 6.2
Optimum temperature:	37 °C

Cofactors

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

Inhibitors

Inhibitors of collagenase include cysteine, EDTA, o-phenanthroline, 8-hydroxyquinoline-5-sulfonate, bipyridyl and 2,3-dimercaptopropanol. It is also inhibited by tris buffer above pH 7.5⁽⁶⁾. Collagenase is not inhibited by diisopropylphosphorofluoridate.

Stability and Storage

Collagenase NB qualities are soluble in water and diluted salt solutions. The enzyme is reversibly inactivated at high pH values, and irreversibly inactivated at low pH values. Collagenase NB and Neutral Protease NB products are delivered as lyophilized powders and remain stable without loss of activity for at least one year when stored at 2-8°C. 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

<p>"Wünsch" units:</p>	<p>Collagenase cleaves the substrate PZ-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine producing a yellow fragment PZ-L-prolyl-L-leucine which is determined spectrophotometrically after extraction into ethyl acetate ⁽⁷⁾.</p> <p>Unit definition: 1 U catalyzes the hydrolysis of 1 µmole 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine per minute at 25 °C, pH 7.1.</p>
<p>"FALGPA" units:</p>	<p>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 <i>Clostridium histolyticum</i> ⁽⁶⁾</p> <p>Unit definition: 1 U is defined as the hydrolysis of 1 µmole of N-(3-[2-furyl]-acryloyl)-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) per minute at 25 °C, pH 7.5.</p> <p>Conversion to "Wünsch" U/mg: 1 U/mg "Wünsch" ≈ 3.9 U/mg "FALGPA"</p>
<p>"Mandl" unit or CDU (Collagenase Degrading Units):</p>	<p>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.</p> <p>Unit definition: 1 U liberates 1 µmole amino acid (expressed as L-leucine equivalents) from collagen per 5 hours at 37 °C, pH 7.5.</p> <p>Conversion to "Wünsch" U/mg: 1 U/mg "Wünsch" ≈ 1000 U/mg "Mandl" or "CDU"</p> <p><i>Please note:</i> These two activity determinations cannot be directly converted into each other: The activity determined according to "Wünsch" is based on hydrolysis of a synthetic peptide thereby reflecting mainly the activity of collagenase class II, whereas CDU determination reflects the activity of both collagenase class I and collagenase class II !</p>
<p>Clostridiopeptidase A, "HP Units"</p>	<p>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 ⁽⁹⁾.</p> <p>Unit definition: 1 U catalyzes the hydrolysis of 1 µmole N-carbobenzyloxy-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine per minute at 37 °C</p> <p>Conversion to "Wünsch" U/mg: 1 U/mg "Wünsch" ≈ 10 HPU/mg</p>

SERVA Collagenase preparations are assayed according to Wünsch in our quality control department.

As the extraneous activities of these preparations are known to contribute to their performance, three further activities are assayed:

Clostripain	<p>Clostripain activity is measured by the ability to hydrolyse N-benzoyl-L-arginine ethyl ester (BAEE) in the presence of the activator dithiothreitol (DTT) ⁽¹⁰⁾.</p> <p>Unit definition: 1 U catalyzes the hydrolysis of 1 µmole BAEE per minute at 25 °C, pH 7.6, after activation with 1 mM calcium acetate and 2.5 mM dithiothreitol.</p>
Trypsin-like proteases:	<p>Trypsin activity is measured by the ability to hydrolyse N-benzoyl-L-arginine ethyl ester (BAEE) ⁽¹¹⁾.</p> <p>Unit definition: 1 U catalyzes the hydrolysis of 1 µmole BAEE per minute at 25 °C, pH 7.6.</p>
Neutral proteases:	<p>Neutral proteases are determined by their ability to hydrolyse dimethylcasein (DMC), liberated amino acids being determined with 2,4,6-trinitrobenzene sulfonic acid (TNBS) ⁽¹²⁾.</p> <p>Unit definition: 1 DMC-U catalyzes the cleavage of 1 µmole peptide bond from dimethylcasein per minute at 25 °C, pH 7.0, expressed in terms of newly formed terminal amino groups (determined with TNBS).</p>

Collagenases: Applications

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.

Isolation of pancreatic islets cells of Langerhans

An important application field is the isolation of viable pancreatic islet cells of Langerhans from different species ^(13, 14). A number of factors have limited the success of this method for the transplantation of human islets yet. These include marked variations in the activity of collagenase preparations, the presence of β-cell toxic factors, the absence of any correlation between enzyme activity and islet yield, and the influence of collagenase on the immunogenicity and chemotactic behaviour of the islets ^(16, 17). However, due to the availability of characterised collagenase preparations as well as validation and optimisation of the isolation protocols, islet cells have been successfully transplanted recently ⁽¹⁸⁾.

Isolation of cardiomyocytes

Collagenase has also been used in the isolation of cardiomyocytes suitable for the preparation of cultures. If tissue dissociation is accomplished by perfusion of the intact organ with enzyme solution, then collagenase is always the enzyme of choice, either alone or in combination with other enzymes such as hyaluronidase ⁽¹⁹⁾ or trypsin. Collagenase can also be used when dissociation is achieved by incubating small pieces of tissue in enzyme solution ⁽²⁰⁾.

Isolation of hepatocytes

Isolated hepatocytes are used in tumour promotion and initiation studies, for studying cellular control mechanisms and in drug and carcinogen assay systems ^(20, 21).

Additionally, transplantation of isolated hepatocytes from donor livers is emerging as modality of treatment for patients with acute liver failure or liver-based metabolic disorders ⁽²²⁾.

Isolation of tumour cells

All kinds of tumour cells need to be isolated from tumour tissues for use in research studies^(23, 24). Another recently emerging field is the tumour vaccination, which aims to augment anti-tumour immunity. One approach is to fuse isolated dendritic cells with isolated tumour cells to produce hybrid cell vaccines. Vaccination of patients with cancer using dendritic cells was shown to be effective for B-cell lymphoma, renal cell carcinoma, prostate cancer and malignant melanoma, advanced breast and ovarian cancer^(25, 26).

Other tissues

There is a strong interest to isolate intact cells from a broad variety of tissues for research purposes. Collagenase has been successfully used for the isolation of cells from bone⁽²⁷⁾, cartilage, thyroid glands^(28, 29), ovarian and uterine tissues⁽³⁰⁾, skin, endothelial cells⁽³¹⁾, neuronal cells⁽³²⁾ and others. SERVA collagenase NB qualities are particularly suitable for the isolation of viable cells from tissues. The strain of *Clostridium histolyticum* has been carefully selected and is characterised by production of high amounts of collagenases and balanced amounts of other proteolytic activities. Moreover, the strain does not form toxins, and accordingly the Collagenase NB qualities are non-toxic according to the requirements of the USP 23 General Safety Test. The TSE safety (transmissible spongiform encephalopathy) of raw materials from animal origin used for the production of Collagenase N is certified in a Certificate of Suitability (CoS) by the European Directorate for the Quality of Medicines (EDQM).

SERVA offers the standard grade collagenase quality, **Collagenase NB 4 Standard Grade**, for several cell isolation applications like pancreatic islet cells, tumour cells, cardiomyocytes, hepatocytes (from small animals), nerve and endothelial cells, chondrocytes. Another broad range quality, **Collagenase NB 8 Broad Range**, with a higher collagenase activity is designed for the same range of applications, but preferably for tissues from bigger animals.

Especially for pancreatic islet cell isolation SERVA offers the highly purified **Collagenase NB 1 Premium Grade** characterised by a high collagenase activity, a very low endotoxin level and a high batch to batch consistency. This highly purified collagenase is produced by means of a patented process in a series of chromatographical steps, resulting in the product mainly containing collagenase class I and II. Islet cells isolated with this collagenase quality show a very high viability and the isolations provide high yield of islet equivalents (IEQ). As Neutral Protease has been reduced to very low levels in this collagenase quality, the preparations show a high stability. Since the neutral protease activity is necessary for islet cell isolations, SERVA offers a purified **Neutral Protease NB** from *Clostridium histolyticum* which can be added individually to the enzyme preparation to achieve the best suitable solution for optimal tissue dissociation.

Several cell isolations are carried out with the objective of producing cells or cell aggregates for tissue engineering and transplantations. These isolations require production standards according to GMP-guidelines. SERVA Collagenase **NB 6** and **NB 1 GMP Grade** qualities are especially designed for these applications, and are produced and certified in accordance with international valid GMP-guidelines.

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