

## Production, Purification and Application of Collagenase

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A bacterial strain of *Staphylococcus aureus* producing collagenase was isolated from scales of fish and characterized. Optimum growth requirements of the organism for production and activity of the enzyme were determined. The collagenase was purified by ammonium sulphate precipitation method and dialysis. The molecular weight of the partially purified enzyme was estimated to be 62 kDa by NATIVE-PAGE. The enzyme was also immobilized by Sodium alginate gel entrapment method and the enzyme was used in tenderization of meat.

**Key words:** Collagenase, *Staphylococcus aureus*.

Collagenases are enzymes that are capable of hydrolyzing native collagen or collagen substrates at or near physiological pH. For the extraction of collagenase enzymes, marine sources (fish) are selected. As the organism producing collagenase enzyme are bound to the surface of the scales of fish as a source of collagen as substrate. *Staphylococcus aureus* produces the enzyme collagenase growing on the scales of fishes. Skin, tendon, blood vessels and bone are used by the organism to produce the enzyme collagenase (Daatselaar & Harder, 1974).

Collagenase is used in treatment of wounds, removal of dead and scar tissue as they

are non - toxic and eco- friendly biocatalysts. Dyeing is an important process in the Leather industry, which employs many synthetic colorants. Many good dyes suffer from incomplete exhaustion and this causes concern, as the biotreatability of the exhausted dyes in effluent is normally difficult. The removal of dyes using bacterial collagenase enzymes as biocatalyst has proved to be useful. The strength is not significantly altered and the bulk properties like softness of leather have been found to be improved by the use of collagenase (Lapiere, 1962).

### MATERIAL AND METHODS

#### Isolation and characterization of the organism

Microorganisms were isolated from scales of fish and then streaked it directly on the nutrient agar plates. They were characterized by performing various staining techniques (simple staining, gram staining) and biochemical tests (catalase test, indole production test, methyl red test, Voges-Proskauer test, nitrate reduction test, coagulase test).

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**Media for production of collagenase**

Gelatin	-	1.5 g
Yeast extract	-	1g
KHPO <sub>4</sub>	-	0.4g
Distilled water	-	100ml
pH	-	7.0

The medium was autoclaved. A loopful of the isolated *Staphylococcus aureus* culture was inoculated and incubated at 37°C overnight. The medium was filtered and the filtrate was assayed.

**Collagenase assay**

The chemistry of ninhydrin reaction has been considered in respect to qualitative test and they can be modified to give qualitative assay methods. Not all the amino acids give same intensity of color. The imino acids (Proline and hydroxyproline) give a yellow color while the rest give a bluish purple color. Collagenase is incubated for 5 hours with native collagen. The extent of collagen break down is determined using

Moore and Stein colorimetric method. Aminoacids 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 specified conditions (Emod and Tong, 1981).

**Table 1.** Biochemical tests

S.No.	Biochemical test	Test results
1.	Catalase	Positive
2.	Indole production	Negative
3.	Methyl red	Positive
4.	Voges Proskauer	Positive
5.	Fermentation of simple Carbohydrates	Positive
6.	Nitrate reduction	Postive
7.	Coagulase	Positive

**Table 2.** Estimation of aminoacids - ninhydrin method

S.No.	Rreagents	B	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>4</sub>	S <sub>5</sub>	U <sub>1</sub>	U <sub>2</sub>
1.	Volume of amino acids (ml)	-	0.2	0.4	0.6	0.8	1.0	-	-
2.	Concentration of amino acids(µg)	-	20	40	60	80	100	-	-
3.	Volume of unknown (ml)	-	-	-	-	-	-	0.5	1.0
4.	Volume of ninhydrin (ml )	2	2	2	2	2	2	2	2
5.	Volume of water (ml)	2.0	1.8	1.6	1.4	1.2	1.0	1.5	1.0
Heat in a boiling water bath for 15 mins and after cooling add 5ml of isopropanol and read at 570nm									
6.	Optical density	0.00	0.20	0.42	0.64	0.81	0.99	0.80	0.99

**Table 3.** Estimation of protein by lowry's method

S.No.	Rreagents	B	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>4</sub>	S <sub>5</sub>	U <sub>1</sub>	U <sub>2</sub>
1.	Volume of protein (ml)	-	0.5	1.0	1.5	2.0	2.5	-	-
2.	Concentration of protein(µg)	-	25	50	75	100	125	-	-
3.	Volume of unknown (ml)	-	-	-	-	-	-	0.5	1.0
4.	Volume of water(ml)	2.5	2.0	1.5	1.0	0.5	-	2.0	1.5
5.	Volume of alkaline copper tartarate (ml)	5	5	5	5	5	5	5	5
Incubate for 10 minute at room temperature.									
6.	Volume of Folin's reagent  (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubate for 20 minutes at room temperature. Read at 620 nm.									
7.	Optical density at 620nm	0.00	0.37	0.60	0.70	0.76	0.94	0.11	0.20

### Procedure

0.2- 1 ml of standard amino acids were taken into test tubes and the solution was made up to 2 ml with water. Appropriate blank was set up with 2 ml with water. 0.1 and 0.2 ml of filtrate was taken as unknown. 25 mg of bone substrate was added to unknown tubes and incubated for 5 hours. After 5 hours, 0.2 ml Ninhydrin – Citric acid mixture was added. The tubes were heated for 20 minutes, then cooled, diluted with 5 ml of 50% n- propanol, incubated at room temperature for 15 minutes and read at 600nm (Mandl, 1961).

### pH variation

To standardize the optimum pH at which the organism grows and produces the enzyme, the medium containing gelatin, yeast extract, Potassium hydrogen phosphate was prepared and the pH was adjusted to 5,6,7,8,9 using acid (Hydrochloric acid) or base (Sodium hydroxide pellets) and autoclaved. The substrate bone was added and the organism was inoculated and incubated at room temperature for 24 hrs. The medium was filtered and estimated for the amount of leucine liberated by Estimation of aminoacids- Ninhydrin method.

### Temperature variation

To standardize the optimum temperature for the growth of the organism and the production of the enzyme, the corn starch media was prepared and the organism was inoculated, then the flasks were incubated at various temperatures (17, 27, 37 and 47) for 3 to 5 days. The medium was filtered and assayed for the amount of Leucine liberated by Estimation of aminoacids- Ninhydrin method.

### Purification of enzymes

Purification of enzymes was carried out by ammonium sulphate precipitation method and dialysis. The optimum pH and temperature of the purified enzyme was determined.

### Estimation of protein – lowry's method

Working standard was taken into different test tubes and volume was made up to 2.5ml with water. Unknown solutions are taken within the range of the standard solution. 5ml of alkaline copper reagent was added and left it at room temperature for 10 minutes and then 0.5ml of Folin's reagent was added. After 20 minutes the intensity of the color was read at 620nm. From

the standard the amount of protein present in the unknown was calculated.

### Enzyme immobilisation

The purified enzyme was immobilized by Gel Entrapment method using Sodium Alginate.

### Application of enzyme

1gm of chicken sausage was weighed and added to 1ml of the enzyme (Test). 1gm of the chicken sausage was weighed (control) and incubated without any enzyme. After incubation for about 3hrs, it was assayed for the amount of leucine released.

## RESULTS AND DISCUSSION

### Isolation of the organism

The scales were collected from two different fishes and they were dispensed in distilled water and ground. After two hours a loopful of filtrate was streaked in nutrient media on microscopic examination, various bacterial colonies were observed. Both Gram negative rods and Gram positive cocci organisms were observed. To isolate *Staphylococcus aureus*, gram positive cocci was allowed to grow on Mannitol salt agar. After 24 hours, yellow pigmented colonies were observed (Fig. 1). These colonies were again stained and observed under the microscope. Gram positive cocci in clusters were observed. To confirm the presence of *Staphylococcus aureus*, biochemical characterization was carried out (Grant and Alburn, 1959) (Table 2). The organism isolated was confirmed to be *Staphylococcus aureus* (Mandl, *et al.*, 1953).

### Assay for aminoacids – ninhydrin method

For the production of enzyme, the organism – *Staphylococcus aureus* was inoculated in media containing Gelatin, Yeast extract, Potassium hydrogen phosphate and bone as substrate. After 24hrs of incubation, the medium filtrate was assayed for the amount of leucine liberated by Ninhydrin method (Table 2). The Concentration of amino acid in the given sample was found to be 16 mg of amino acid (Rosen, 1957).

### pH variation

To standardize the growth of the organism and the production of enzyme the pH of the medium was varied using acid or base.

The pH of the medium was found to be 7.0, Hence in order to vary the pH to 5 and 6 acid (Hydrochloric acid) was added to the medium. pH 8 and 9 was adjusted using base (Sodium Hydroxide pellets). The filtrate was assayed for the amount of amino acids by Ninhydrin method. There was considerable increase in the growth of the organism as the pH from 5- 7, but when the pH was increased further to 8-9 there was decrease in the growth of the organism. Hence, the growth of the organism was found to be optimum at 7 (Waldvogel & Swartz, 1969).

#### Temperature variation

To standardize the growth of the organism and the production of enzyme the temperature of the medium was varied. The filtrate was assayed for the amount of amino acids by Ninhydrin method. As the temperature increased, there was considerable increase in the growth of the organism and as the temperature was further increased above 37°C. There was decrease in the growth of the organism. Hence, the growth of the organism was found to be optimum at 37°C.

#### Purification of collagenase

Following the procedure of Hanada *et al*, (1971), ammonium sulphate precipitation and dialysis was carried out (Fig. 2) and the purified enzyme was obtained and was analyzed in Native Page.

#### Native page

Native page was performed using 10% acrylamide gel and the proteins were stained with Coomassie brilliant blue. The band for the sample was found to correspond with marker at 62KDa.

#### Protein determination

The amount of protein was estimated with bovine serum albumin (concentration of 50 microgram per 50 milliliters) as standard (Table 3). Within the standard value unknown was plotted and the amount of protein in the purified enzyme was 17.5 mg.

#### Effect of pH on enzyme

The optimum pH at which the enzyme is stable was determined using phosphate buffers of varying pH (6, 7, 8, 9) the optimum activity was observed at pH 7. When the graph was plotted the peak value (maximum activity) was observed

at pH 7 (Nagai & Noda, 1959) (Sugasawara & Harper, 1984) (Fig. 3).

#### Effect of temperature on enzyme

The stability of the enzyme was examined at different temperature (17°C, 27°C, 37°C & 47°C) in phosphate buffer at constant pH. When the graph was plotted the peak value (maximum activity) was observed at 37°C (Saifter & Harper, 1971) (Fig. 4).

#### Immobilisation of enzyme by gel entrapment

Enzyme was successfully immobilized by gel entrapment via Sodium Alginate. This is an efficient method and it can be used for further large scale application. The immobilized enzyme activity was assayed by estimation of Amino acids – Ninhydrin method and the activity was found to be same as that of crude enzyme (Fig. 5).

#### Application of enzyme

The Chicken sausage was treated with the enzyme and was left over night. It was found that the amount of Leucine liberated was increased when compared with control. Thus, it has been proved that the enzyme has a role in tenderization of meat (Wig, 1971).

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