4. METHODS

4.1 Preparation of crude enzymes

About 50 g of the digestive gland of green mussel was homogenized in 100 ml of 0.1 M sodium acetate buffer [4.2.3.2] for 15 minutes. The contents were centrifuged at 6000 g for 15 minutes in a refrigerated centrifuge. The supernatant was used as crude for isolation of endoglucanase.

4.2 Assay of endoglucanase activity

Endoglucanase activity was routinely measured according to the method of Wood and Bhat (1998) after slight modification in the procedure described under 4.2.4.

4.2.1 Principle

The enzyme cleaves carboxymethylcellulose releasing glycosylic moieties which gets oxidized in an alkaline milieu by forming orange-yellow compound with 2-hydroxy-3,5-dinitrobenzoic acid (DNSA). These orange-yellow compounds were measured at a wavelength of 530nm and the released reducing ends were quantified with glucose standard curve.

4.2.2 Reaction conditions and expression of enzyme activity.

The reaction mixture was assayed in 0.1M sodium acetate buffer (pH 5) with 1% CMC by incubating at 40°C for 30 minutes.
After assay the enzyme activity is expressed in terms of units (μmoles of glucose released/ml/min). One unit of endoglucanase activity is thus defined as the amount of enzyme that released 1μmole of reducing moieties from CMC under the above reaction conditions.

Specific activity is defined as the number of μmoles of glucose released/ min /mg of protein.

4.2.3 Reagents

4.2.3.1 Carboxymethylcellulose (1%)

Carboxymethylcellulose was used as substrate for enzyme assay. The substrate was prepared by dissolving 1g of CMC in 50 ml acetate buffer pH 5.0 by heating in a water bath for 10 min under constant stirring. After cooling to room temperature, the solution was made upto 100ml with pH 5.0 acetate buffer. The substrate could be stored at room temperature for maximum life of 4 weeks.

4.2.3.2 Acetate buffer 0.1M, pH 5.0 with 0.04% Tween 20.

0.1M acetate buffer was prepared by dissolving 8.2039 of anhydrous sodium acetate and 2.94g (200mM) of CaCl₂·2H₂O in approximately 900ml of deionised water and the pH of the buffer was adjusted to 5.00 ± 0.02 with 25% hydrochloric acid. 4ml of 10% Tween 20 (4.2.3.7) was added and the solution made upto 1000ml with deionised water. The buffer is stable and can be stored at room temperature for two weeks.
4.2.3.3 Sodium/ Potassium hydroxide

16g of sodium hydroxide and 22.4g of potassium hydroxide was dissolved in deionised water and made upto 100ml with the same. This could be stored in a plastic bottle for indefinite time.

4.2.3.4 DNSA reagent

About 2.5 g of DNSA was dissolved in 150ml of deionised water. To this 25ml Sodium/ potassium hydroxide (4.2.3.3) was added drop-wise. Then 75g of potassium sodium tartarate tetrahydrate (Rochelle’s salt) was added and the solution cooled to room temperature and filled upto 500ml with deionised water. The reagent was stored at room temperature in dark. Maximum storage life is one month.

4.2.3.5 100mM Glucose stock standard solution

1.8016 g of D glucose was dissolved and made upto 100ml with acetate buffer (4.1.3.2). Molecular weight of glucose: 180.16.

4.2.3.6 Working standard solution:

Glucose standard stock solution (4.2.3.5) was diluted to give concentrations of 5, 10, 15, 20 and 30 micromole per ml of the solution.

4.2.3.7 10% Tween 20:

10 g of Tween 20 was made upto 100ml with deionised water. Stored at room temperature. Maximum storage life is one week.
4.2.4 Procedure

50 μl of the enzyme solution was added to 0.5ml of 1% CMC dissolved in 0.1 M sodium acetate buffer, pH 5.5. After incubation at 40°C for 30 min, 1ml of DNSA reagent was added, kept for 5 minutes in a boiling water bath and cooled on ice for 5 minutes. The contents were centrifuged at 3000 rpm for 10 minutes to remove unreacted carboxymethylcellulose and diluted with 1.5ml of deioised water. The degree of enzymatic hydrolysis of the CMC was determined spectrophotometrically by measuring the absorbance at 530nm. A reaction blank was prepared similarly but the reaction mixture without incubation was heated in a boiling water bath for 5 min to denature the endoglucanase.

For preparing standard curve, 50 μl of glucose standard solution (5, 10, 15, 20 and 30 μmol/ml) was mixed with 0.5ml of 1% CMC and incubated similarly followed by addition of 1ml DNSA reagent, boiling, centrifuging and estimation of the color at 530nm. A blank was prepared with 50μl acetate buffer instead of glucose standards. The amount of sugar produced by enzyme reaction was calculated from its optical density using standard curve obtained from glucose standards.

4.3 Protein estimation

4.3.1 Lowry method

The protein content was estimated by the method of Lowry et al., 1951 as given below.
4.3.1.1 Reagents:

1. Reagent A: Reagent A was prepared by dissolving 0.5g of copper sulphate (CuSO₄·5H₂O) and 1g of sodium citrate in 100ml of deionised water. This solution can be kept indefinitely.

2. Reagent B: 20g of sodium carbonate (Na₂CO₃) and 4 g of sodium hydroxide (NaOH) were dissolved in 1 litre of deionised water to obtain reagent B.

3. Reagent C: Reagent C was freshly prepared just before use by adding 50 ml reagent B to 1ml of reagent A.

4. Reagent D: It was prepared by adding Folin Ciocalteau reagent and deionised water in 1:1 ratio just before use.

5. BSA standard: 5mg of BSA was dissolved in deionised water and made upto 5ml.

4.3.1.2 Procedure:

Various concentrations of the BSA standards (0.1 – 0.5 ml), and samples (0.2ml after appropriate dilution) were made upto 0.5ml with deionised water. To each of the tubes were added 2.5 ml of reagent C, mixed and allowed to stand for 5-10 minutes. Then 0.25 ml of reagent D was added, mixed and allowed to stand for 20 to 30 minutes. The absorbance was read at 700 nm against reagent blank. Standard curve was plotted with concentration of BSA on x-axis and optical density on y-axis. The protein concentrations of the samples were estimated.
with the help of standard curve. The protein concentration in the solution was calculated and expressed in mg protein per ml of extract.

4.3.2 Spectrophotometric method

The eluent fractions of Gel filtration chromatography were monitored, by measuring the absorbance at 280nm against buffer solution. The absorbance is due to the aromatic amino acid residues of tyrosine and tryptophan.

4.4 Extraction procedure

4.4.1 Desalting of the extract:

About 6 g of Sephadex G-25 (30 x 3.1cm) was suspended in water and allowed to swell overnight. The swollen Sephadex was deaerated and the fine particles removed by decantation. The gel suspension was brought to 4°C and poured into a column, which was previously half filled with sodium acetate buffer (0.1M) and maintained at 4°C. The partially purified endoglucanase obtained from fractionation with 60% ammonium sulphate was applied to the column and eluted with 0.1M sodium acetate buffer. The eluents were collected in 10ml fractions and monitored at 280nm. The protein fractions were collected and pooled. The activity and protein content of the desalted endoglucanase were determined.

4.4.2 Sephadex G-100 chromatography

Gel filtration was performed on Sephadex G-100. About 3 g of gel was suspended in 100ml of water and allowed to swell for 48 hours. The swollen
Sephadex was deaerated and the gel poured into a glass column (45cm x 1.7cm), which was previously half filled with sodium acetate buffer (0.1M). As the gel was poured, the excess buffer was allowed to percolate gradually through the bed. The gel height was measured. The column was equilibrated with the same buffer, the flow rate and void volume noted and this column was used for further purification of the enzyme preparation.

4.4.3 Enzyme homogeneity tests:

The homogeneity of endoglucanase was tested by electrophoresis on polyacrylamide gel and chromatography on sephadex G100 column as described below.

4.4.3.1 Polyacrylamide gel electrophoresis

4.4.3.1.1 Principle: Vertical gel electrophoresis was conducted by the method of Laemmli (1970) with some change in sample preparation. It is based on the principles that, in the presence of 10% SDS and 2-mercaptoethanol, proteins dissociate into their sub units and bind large quantities of the detergent which mask the charge of the proteins and giving a constant charge to mass ratio so that the proteins move according to their molecular weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are made up in the electrode buffer, Tris-glycine. During electrophoresis, the leading ion is chloride while the trailing ion is glycine.
4.4.3.1.2: Preparation of reagents and gel:

1) Tris-HCl: 0.5M, pH 6.8: 6 g of Tris(hydroxymethyl)aminomethane was dissolved in deionised water and made up to 100ml with after adjusting the pH to 6.8.

2) Tris-HCl: 1.5M, pH 8.8: 27.23g of Tris(hydroxymethyl)aminomethane was dissolved in deionised water and made up to 150ml with after adjusting the pH to 8.8.

3) SDS 10%: 10 g of SDS was dissolved in 100 ml of deionised water.

4) APS 10%: Freshly prepared by dissolving 100 mg of APS in 1ml of deionised water to get 10% APS.

5) 1% bromophenol blue: 1 g of bromophenol blue was dissolved in 100 ml of water.

6) Acrylogel 30%: 30 g of acrylogel was dissolved in 100 ml of deionised water.

7) Coomassie blue R250 stain (1% in 10% acetic acid and 40% methanol): Prepared by dissolving 0.1g of Coomassie blue R250 in 50ml acetic acid, then added 200ml of methanol and 250ml of deionised water.

8) Preparation of sample buffer:

   The sample buffer was prepared by adding 1ml of Tris-HCl 0.5M, 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4ml of 2-mercaptoethanol and 0.4ml of bromophenol blue to 3.8ml of deionised water.

9) Preparation of sample:

   The crude extract was diluted thrice to get low concentration of protein. Then 0.1ml of this diluted sample was mixed with 0.3ml of sample buffer. Purified
extract was as such diluted by adding 0.2ml sample buffer to 0.1ml of extract in microfuge tube. Microfuge tubes containing crude and purified extracts separately were heated in boiling water bath for 4 minutes, cooled and kept frozen until used for investigation.

10) Preparation of Electrode buffer:

The electrode buffer was prepared by adding 9g of Tris base, 43.2g of glycine and 3g of SDS to 600ml of deionised water.

Working solution: For each electrophoretic run working solution was prepared by diluting 100ml of the electrode buffer to 500ml with deionised water.

11) Preparation of separating gel: 10%

Deionised water: 4.05ml
1.5M Tris-HCl: 2.5ml
10%SDS: 100μl
Acrylogel: 3.3ml
10% APS: 50μl
TEMED: 20μl

Running gel (10ml) of 10% was prepared by mixing 4.05ml of deionised water, 2.5ml of 1.5M Tris – HCl pH 8.8, 100μl of 10% SDS and 3.3ml of 30% acrylogel. Then it was degassed for more than 15 minutes at room temperature. After degassing, 20μl of TEMED was added and shaken well. To this 50μl of 10% APS was added and mixed well.
12) Preparation of stacking gel 4%

Deionised water: 6.1ml
1.5M Tris-HCl: 2.5ml
10% SDS: 100µl
Acrylogel: 1.33ml
10% APS: 50µl
TEMED: 20µl

Stacking gel (10ml) of 4% acrylogel was prepared by mixing 6.1ml deionised water, 2.5ml of 0.5M Tris – HCl, pH 6.8, 100µl of 10% SDS and 1.33ml of 30% acrylogel. Then it was degassed till no bubble was observed (15-20 minutes). To this 20µl of TEMED was added followed by 50 µl of 10% APS and mixed well.

4.4.3.1.3. Casting of discontinuous (Laemmli) Polyacrylamide Gel:

Comb was inserted in between the assembled gel sandwich of “Biorad” mini protein II electrophoresis plates. Mark was made on glass plate 1cm below the teeth of comb, this was the level to which the separating gel was poured and comb was removed. Prepared separating gel was poured immediately after adding TEMED and APS and overlaid with water slowly with steady even rate of delivery to prevent mixing. The gel was allowed to polymerize for 45 minutes. Over layer of water was removed and comb was placed in a tilted position. After adding TEMED and APS to the stacking gel, it was poured till the gel had covered the gap between the teeth of the comb. The comb was properly placed.
Gel was allowed to polymerize for 45 minutes. Comb was removed and gels were assembled on the inner cooling case.

4.4.3.1.4. Sample application and run

Electrode buffer (2 - 4°C) was poured into the chamber and about 10μl of the samples were applied per well of stacking gel. The cathode and anode of the gel assembly were then connected to the power supply. A constant voltage of 200 volts was then applied and kept constant throughout the run. When the dye front reached the bottom of the gel, power supply was switched off. The slabs were dismantled from the unit and allowed to cool.

4.4.3.1.5. Staining of gels

The gels from the glass plates were then removed carefully and stained with 0.1% coomassie blue R-250 dissolved in 10% acetic acid and 40% methanol for 30 minutes. Destaining was done with 7% acetic acid, with several changes to remove background dye.

4.4.3.2. Sephadex G100 chromatography:

To test the homogeneity of the purified sample, gel filtration chromatography was performed on Sephadex G100. The gel suspension was deaerated and poured into a glass column (1.7 x 45 cm) already half filled with deionised water. The gel was poured until the bed volume reached a height of 30 cm. The column was equilibrated with 0.1M sodium acetate buffer and the flow rate noted. The
sample was applied and eluted with 0.1M sodium acetate buffer. The eluent fractions were collected and tested for endoglucanase activity was under (4.2) and protein content by absorbance measurement at 280nm.

4.4.3.3 Absorption spectrum: Endoglucanase diluted in 0.1M sodium acetate buffer was measured for absorbance at various wavelengths from 200 to 400 nm using Spectronic Genesys 5. The UV absorption spectrum of green mussel endoglucanase was obtained by plotting optical density values on y-axis and absorbance on x-axis.

4.5 Characteristics of green mussel endoglucanase

4.5.1 Molecular weight determination

The molecular weight of pure endoglucanase was determined by SDS PAGE method [4.4.3.1]. Both high and low molecular weight markers were used for comparison as described below.

Procedure:

Electrode buffer (2 - 4°C) was poured into the chamber after assembling the gel in the chamber. To consecutive wells of the stacking gel were added 10 μl each of high molecular weight protein marker (Myosin, rabbit muscle: 205000, β-galactosidase, E. coli: 116000, Phosphorylase b, rabbit muscle: 97000, Fructose-6-phosphate kinase, rabbit muscle: 84000, Albumin, bovine serum: 66000, Glutamic dehydrogenase, bovine liver: 55000, ovalbumin, chicken egg, 45000, Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle: 36000),
crude sample and purified sample and low molecular weight markers (Albumin, bovine serum: 66000, Ovalbumin, chicken egg: 45000, Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle: 36000, Carbonic anhydrase, bovine erythrocytes: 29000, trypsinogen, bovine pancreas: 24000, trypsin inhibitor, soybean: 20000, α-Lactalbumin, bovine milk: 14200, aproptinin, bovine lung: 6500). The electrodes were connected to the power supply unit and a constant voltage of 200 volts was then applied. After electrophoretic run the gel was stained (4.4.3.1.5) and bands identified.

From the electrophorograms obtained, the relative mobility of each protein applied was calculated as per the equation

\[
\text{Relative mobility} = \frac{\text{Distance of protein migration} \times \text{Gel length before staining}}{\text{Gel length after destaining} \times \text{Dist. of bromophenol blue migration}}
\]

4.5.2 Effect of substrate concentration on endoglucanase activity

To study the effect of substrate concentration on endoglucanase activity assay was performed as in 4.2 with 30μl of endoglucanase and various concentrations of CMC (0.1-0.8 mg/ml) in a series of test tubes. Line Weaver Burk plot was drawn with reciprocal of substrate concentration (CMC) (1/S) on x-axis and reciprocal of velocity of reaction (1/v) on y-axis. From the graph y - intercept and slope were measured and Vmax and Km were calculated using Michaelis Menten equation, \( V = \frac{V_{\text{max}}[S]}{[S] + K_m} \), where \( V \) = reaction rate, \([S]\) = substrate concentration, \( K_m \) = Michaelis Menten constant (Measures enzyme substrate
affinity), \( K_m \) is the substrate concentration at half the maximal velocity, \( V_{\text{max}} = \) maximum rate

4.5.3 Effect of temperature on endoglucanase activity

To study the effect of temperature on endoglucanase activity, assay was performed as in 4.2 with 30\( \mu \)l of endoglucanase and 0.5ml of 1% CMC in a series of test tubes and incubated at various temperatures (-20°C, 0°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 100°C) for 30 minutes. The endoglucanase activity values (in terms of percentage of maximum) obtained at various temperatures was plotted on y-axis and the temperatures of incubation on x-axis. The optimum temperature was determined from the graph.

4.5.4 Effect of pH on endoglucanase activity

The effect of pH on endoglucanase activity was studied with McIlvaine and glycine - sodium hydroxide buffers as described below. The buffers were prepared as given by Dawson et al., (1986).

a) McIlvaine buffers (pH 2.6-7.6):

Citric acid monohydrate (0.1M): 21.01 g of citric acid monohydrate was dissolved in 1 litre of deionised water.

Disodium hydrogen phosphate (0.2M): 35.61 g of \( \text{Na}_2\text{HPO}_4 \) was dissolved in 1 litre of deionised water.
Mcllvaine buffers (pH 2.6-7.6) were prepared by adding 0.1M Citric acid and 0.2M disodium hydrogen phosphate (Na₂HPO₄) as described in the Table 4.1.

Table 4.1: Mcllavaine buffers

<table>
<thead>
<tr>
<th>pH</th>
<th>0.1M Citric acid</th>
<th>0.2M Na₂HPO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6</td>
<td>89.10</td>
<td>10.90</td>
</tr>
<tr>
<td>3.0</td>
<td>79.45</td>
<td>20.55</td>
</tr>
<tr>
<td>4.0</td>
<td>61.45</td>
<td>38.55</td>
</tr>
<tr>
<td>5.0</td>
<td>48.50</td>
<td>51.50</td>
</tr>
<tr>
<td>6.0</td>
<td>36.85</td>
<td>63.15</td>
</tr>
<tr>
<td>7.0</td>
<td>17.65</td>
<td>82.35</td>
</tr>
<tr>
<td>7.6</td>
<td>6.35</td>
<td>93.65</td>
</tr>
</tbody>
</table>

(pH 2.6-7.6)

b) Glycine – NaOH buffer (pH 8.6-10.0):

Glycine (0.2M): 4.03 g of glycine was dissolved in 300ml of deionised water.

Table 4.2: Glycine – NaOH buffers

<table>
<thead>
<tr>
<th>pH</th>
<th>Volume of 0.2N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.6</td>
<td>2.0</td>
</tr>
<tr>
<td>9.0</td>
<td>4.4</td>
</tr>
<tr>
<td>10.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

(pH 8.6-10.0)
Glycine – NaOH buffer was prepared by adding different volumes of 0.2 N NaOH (Table 4.2) to 25 ml 0.2M Glycine solution and then making up the mixture to 100ml with deionised water.

The substrate was prepared in 1% concentration of various buffers (2.6-10.0). To study the effect of pH on the activity of endoglucanase, assay was performed as in 4.2 with 30μl of purified endoglucanase and 0.5ml of the substrate prepared with various pH solutions taken in a series of test tubes. The degree of enzymatic hydrolysis of the CMC at different pH was determined spectrophotometrically by measuring the absorbance at 530nm. Graph was plotted with pH values on x-axis and activity (in terms of percentage of maximum) on y-axis. The optimum pH was determined from the graph.

4.5.5 Determination of isoelectric point

The isoelectric point of endoglucanase was determined as detailed below according to the procedure given by Stroev and Makarova, 1989.

Principle: Proteins are amphoteric in nature that is they carry both positive and negative charges. The charge on a protein molecule determines the agglomeration of protein particles and their precipitation. The net charge is affected by pH of the medium. Each protein has a characteristic pH value at which the sum of positive and negative charges on the protein is zero or the molecule is electrically neutral. This is its isoelectric point i.e., at this pH proteins loose biological activity and deposit as precipitate especially in the presence of dehydrating agents like ethanol, acetone, and others.
Reagents:

(1) McIlvaine phosphate and glycine - sodium hydroxide buffers were prepared as given under [4.5.4]

(2) 96% Ethanol

Procedure: To 3 ml of buffer solutions (pH 3 to 9) taken in a series of test tubes were added 20 μl of purified enzyme and shaken well. The tubes were observed for cloudiness. Then 2 ml of ethanol (96%) was added to each of the tubes and cloudiness visually examined and compared. The turbidity was measured using a turbidometer. The results were expressed in terms of NTU.

4.5.6 Estimation of amino acids other than tryptophan

Amino acids were estimated according to the procedure of Ishida et al. (1981) with little modification.

4.5.6.1 Preparation of reagents:

1. Mobile phase buffers: The mobile phase buffers were prepared by mixing the reagents and adjusting to required pH have been given in Table 4.3.

2. OPA buffer: OPA buffer is prepared by dissolving the reagents (Table 4.4) in water and making up to 3 liters.

3. OPA reagent: OPA reagent was prepared by dissolving the reagents listed as per Table 4.5 in 500ml OPA buffer.
Table 4.3: Composition of mobile phase buffers for amino acid analysis

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Buffer A</th>
<th>Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tri sodium citrate</td>
<td>58.80</td>
<td>58.80</td>
</tr>
<tr>
<td>Boric acid (ml)</td>
<td>-</td>
<td>12.40</td>
</tr>
<tr>
<td>Ethanol (99.5%)ml</td>
<td>210.00</td>
<td>-</td>
</tr>
<tr>
<td>Perchloric acid (60%) ml</td>
<td>50.00</td>
<td>-</td>
</tr>
<tr>
<td>4N NaOH (ml)</td>
<td>-</td>
<td>45.00</td>
</tr>
<tr>
<td>Final volume (l)</td>
<td>3.00</td>
<td>1.00</td>
</tr>
<tr>
<td>pH</td>
<td>3.59</td>
<td>9.21</td>
</tr>
</tbody>
</table>

Table 4.4: Composition of OPA buffer

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium carbonate</td>
<td>122.10</td>
</tr>
<tr>
<td>Boric acid</td>
<td>40.75</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>56.40</td>
</tr>
</tbody>
</table>

Table 4.5: Preparation of OPA reagent

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA</td>
<td>400 mg</td>
</tr>
<tr>
<td>99% Ethanol</td>
<td>7 ml</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>1 ml</td>
</tr>
<tr>
<td>30% Brij-35 solution</td>
<td>0.75 ml</td>
</tr>
</tbody>
</table>
4. Sodium hypochlorite solution: 0.5ml of sodium hypochlorite solution was added to 100ml of OPA buffer.

5. Preparation of standard: 0.1ml of the aminoacid standard was made upto 1ml with 0.05M HCl.

6. Preparation of Sample: 1ml of purified enzyme extract containing (3mg protein) was pipetted into a heat sealable test tube into which 5ml of 6N HCl was added. Air was removed from the test tube by passing nitrogen, before it was sealed. Hydrolysis was carried out in hot air oven at 110°C for 24 hours. After the hydrolysis was over the test tube was broken open. The contents were removed quantitatively and filtered into a round bottom flask through Whatman Filter Paper No.42 the filter paper was washed 2-3 times with deionised water. The contents of the flask was flash evaporated at 50 - 60°C to remove traces of HCl, the process was repeated 2-3 times after adding deionised water. The residue was made upto 2 ml with 0.05M HCl. The sample thus prepared was filtered again through a 0.45µm membrane filter.

4.5.6.2 Procedure: 20 µl of the sample was injected into a Shimadzu HPLC-LC 10AS (This system of aminoacid analyzer consists of column packed with a strongly acidic cation exchage resin (i.e.) styrene divinyl benzene copolymer with sulfinic group. The column is Na type i.e. ISC-07/ S1504 Na with a length of 19 cm and diameter 5mm. The mobile phase of the system consists of two buffers A and B (Table 4.3). The aminoacids were eluted from the column by stepwise elution i.e. acidic aminoacids first followed by neutral and then basic aminoacids.
The aminoacid analysis was done with non-switching flow method and fluorescence detection after post column derivatization with o-phthalaldehyde. In the case of proline and hydroxyproline imino group was converted to amino group with sodium hypochlorite. Aminoacid standard was also run to calculate the concentration of sample aminoacid depending on the standard chromatogram. The amino acid content was calculated and expressed as percentage mg/100mg protein.

4.5.7 Estimation of tryptophan

Tryptophan in the enzyme was estimated by the method of Sastry and Tammuru (1985) with some modification in sample and reagent preparation. About 1ml sample was hydrolysed with 3ml of 5% NaOH at 110°C for 24 hours in a sealed tube filled with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6N HCl using phenolphthalein indicator. The volume was made up to 25ml with deionised water. This was then filtered through Whatman filter paper No.1 and the filtrate was used for estimation of tryptophan. Tryptophan content in the sample was determined as per the procedure explained in Section 4.5.7.1 below.

4.5.7.1 Preparation of standard and standard curve

Standard solution of Tryptophan containing 10μg/ml was prepared in 0.1N hydrochloric acid. 2.5% sucrose and 0.6% thioglycolic acid each at 0.1ml level were successively added to test tubes containing 4ml of 50% sulphuric acid. The test tubes were shaken and incubated at 45-50°C for 5 min and cooled.
Standard solution of tryptophan ranging from 0.05 - 0.5ml (0.5 to 5μg) was added and mixed. The volume was made upto 5ml with 0.1N HCl and was kept for 5 minutes for the development of full colour. The absorbance was measured at 500nm against a reagent blank. A standard calibration curve was prepared for deducting the amount of tryptophan. Tryptophan content was finally expressed as mg/100 mg protein.

4.5.8 Effect of certain inorganic ions and heavy metals on the activity of endoglucanase

10mM concentration of various anions F⁻, Cl⁻, I⁻ and Br⁻ and cations Fe²⁺, Fe³⁺, Cu²⁺, Zn²⁺, Sn²⁺, Mn²⁺, Mg²⁺, Co²⁺, Ba²⁺, Ca²⁺, Pb²⁺, Cd²⁺, Hg²⁺, Ag⁺ were prepared from the salts of these ions. To determine the effect of these ions on the endoglucanase activity, the assay was conducted as in 4.2 with 50μl of endoglucanase, 0.5ml of 1% CMC dissolved in 0.1M sodium acetate buffer and 1 ml of 10mM concentration of each ions taken in a series of test tubes. The degree of enzymatic hydrolysis of the CMC in presence of each ion was determined spectrophotometrically by measuring the absorbance at 530nm.

4.5.9 Immobilisation of endoglucanase

Endoglucanase was attempted immobilization with two solid phases viz. polyacrylamide gel and chitosan as described below.

4.5.9.1 Immobilisation of endoglucanase on Polyacrylamide gel

0.5ml of the enzyme solution was mixed with 3ml of 0.1M sodium acetate buffer pH 5.0, 3ml of acrylogel containing 30% acrylamide and 2.67% N, N -
methylenebisacrylamide. To this was added 50 µl of 10% APS and 20 µl TEMED and mixed well. The mixture was kept for 15 min to gel. The gel was lyophilized to get immobilized endoglucanase. The immobilized endoglucanase was stored at 0°C and activity studied.

4.5.9.2 Immobilisation of endoglucanase on chitosan

0.5g chitosan was dissolved in 50ml of 1% acetic acid of pH 5.0. To 10 ml of this solution, 0.5ml of endoglucanase was added and mixed in a vortex mixer at intervals and kept for 2 hours at 0-4°C. The solution was neutralized with 0.1N sodium hydroxide. Chitosan precipitates. The precipitate was centrifuged at 6000 g in a refrigerated centrifuge for 10 minutes. The precipitate was washed free of salt and kept at 0-4°C. The activity was studied.