CHAPTER - 5

DETERMINATION OF MOLECULAR WEIGHT OF *A. blitum* L.
INORGANIC PYROPHOSPHATASE BY GEL FILTRATION THROUGH
SEPHADEX G-100
Introduction

When a mixture of molecules is passed through a column of porous gel granules, the molecules may appear in the effluent in order of decreasing size. Fractionation is believed to occur when diffusion of the molecules into the gel pores is restricted but not prevented because of their size and because they pass through the column at rates that are related inversely to the fluid volume accessible to them within the column. So gel filtration is a liquid column chromatography to separate solute molecules according to differences in molecular size. Lathe and Ruthren (108) suggested that the dimensions of protein and polysaccharide molecules might be estimated by this technique, now widely known as gel filtration. Although molecular weight is unlikely to be a good approximation for size in comprising such dissimilar molecules as proteins and polysaccharides, the correlation between molecular weights and gel filtration behaviour of dextrans (109) indicates that for a homogeneous series of macromolecules size and molecular weights are closely related.
Andrews (110) obtained evidence from experiments with sephadex gel columns that gel filtration can be used as a comparative method to give useful estimates of molecular weights of proteins.

The present chapter in this connection describes the experiment for the estimation of the molecular weight of inorganic pyrophosphatase from the leaf of *A. blitum* L. by gel filtration through sephadex G-100 column.

**Experimental**

**Materials and Methods**

**Chemicals**

**Sephadex G-100**

This dextran is manufactured by Pharmacia, Upsala, Sweden.

**Marker proteins**

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome-C</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Trypsin soyabean inhibitor</td>
<td>Sigma</td>
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</tbody>
</table>
Each protein solution of concentration 3 mg/ml was prepared in 50 mM tris acetate buffer (pH 7.5).

Source of enzyme inorganic pyrophosphatase

The purified enzyme preparation from A. blitum L. (mentioned in Chapter 2) was used for the determination of the molecular weight of inorganic pyrophosphatase.

Preparation of column

Preparation of sephadex G-100 gel column was described in Chapter 2. The bed height was adjusted to 30 cm and equilibrated with 50 mM tris acetate buffer (pH 7.5) for 24 hours.

Procedure for column run

The experiment was performed with column equilibrated with 50 mM tris acetate buffer, pH 7.5 and a flow rate through the column of approximately 10 ml/hr. was maintained by adjusting the hydrostatic pressure over the surface of the gel bed. All the

*/ Specifications and preparation of tetrasodium pyrophosphate, MgCl₂ and tris buffers were mentioned in Chapter 1.
column runs were performed at 8-10°C. Proteins (listed in Table 9) were dissolved in the equilibration buffer at a concentration of 3 mg/ml. 0.3 ml of each protein solution was used for gel filtration. In case of inorganic pyrophosphatase 0.3 ml of the purified enzyme solution of activity 4.1 units was used. The rate of effluent collection was maintained at 10 ml/hr. 0.5 ml of the eluate fraction was collected in a series of test tubes. Each run was continued for two and half hours.

**Examination of the column effluent**

**Determination of proteins:**

Proteins were scanned spectrophotometrically at 280 nm by Hilger Uvispek spectrophotometer by using whole of each effluent fraction diluted with 3 ml of distilled water in a cuvette with a 1 cm light path. The reference cuvette contains a sample of column effluent collected before the emergence of the protein.
Determination of the inorganic pyrophosphatase activity in the column effluent:

Inorganic pyrophosphatase activity in the column effluent is estimated by incubating 0.1 ml of each of the eluate fraction with 100 mM tris acetate buffer (pH 9.0), 10 mM MgCl$_2$ and 1.0 mM tetrasodium pyrophosphate in a total volume of 1 ml at 30°C for 5 minutes, the liberated phosphate was determined as usual.

Results and Discussion

The proteins listed in Table 9 were used to determine their elution volumes from sephadex G-100 column. Table 9 also gives their molecular weights from corresponding references. Figure 10 shows the elution profile of the listed proteins (cytochrome-c, ovalbumin, trypsin soyabean inhibitor) and the enzyme inorganic pyrophosphatase. The elution volumes of cytochrome-c, ovalbumin and trypsin soyabean inhibitor are 19.5 ml, 13.5 ml and 17.0 ml respectively. A standard curve (Figure 11) was drawn by plotting the elution volume against
logarithm of the molecular weights obtained from Table 9. A straight line curve was obtained. From Figure 10 the elution volume of inorganic pyrophosphatase was found to be 15.0 ml. This value of the elution volume gave the corresponding molecular weight of inorganic pyrophosphatase 32,860 from Figure 11.

According to previous reports, the molecular weights of inorganic pyrophosphatases obtained from E. coli, yeast, the mold Aspergillus oryzae and rabbit skeletal muscle were found to be 121,000 \( \pm \) 2,000; 63,000; 60,800; and 67,000 respectively (61, 65, 84, 112) which are very high in comparison to A. blitum L. inorganic pyrophosphatase. Each of the above four enzymes were reported to possess multiple subunits. But the molecular weight of A. blitum L. inorganic pyrophosphatase is approximately similar to maize leaf inorganic pyrophosphatase which was reported to be 32,000 \( \pm \) 4,000 (76) as determined by gel filtration through sephadex G-150. It is notable
that *A. blitum* L. inorganic pyrophosphatase when passed through sephadex G-100 was not resolved into two peaks in contrast to its behaviour on DEAE-cellulose column in which case this enzyme was found to be resolved into two clearly separated peaks with inorganic pyrophosphatase activity (described in Section 1 of Chapter 6). Electrophoretic study (described in Section 2 of Chapter 6) also proves that at least two isoenzymes are present in the leaf of *A. blitum* L. This signifies that the two forms of inorganic pyrophosphatase are charged isomers not being different in molecular size.
Table 9

Proteins used as molecular weight-markers in sephadex G-100 gel chromatography.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Molecular weight</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome-c (from horse heart)</td>
<td>12,400</td>
<td>(113)</td>
</tr>
<tr>
<td>Trypsin inhibitor (from soyabean)</td>
<td>21,500</td>
<td>(114)</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
<td>(115)</td>
</tr>
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