Basic concept of chromatography

= a group of separation techniques, which are characterized by a distribution of the molecules to be separated between two phases, one stationary and the other mobile phase

molecules with a high tendency to stay in the stationary phase will move through the system at a lower velocity than will those which favor the mobile phase.

the shape, rigidity and particle size distribution profile of the gel matrix are important parameters

Ion-Exchange Chromatography

Example:
Cation-exchange chromatography

Fig .5. Ion exchange chromatography.
Fig. 6. Gel filtration chromatography.

** Basic concept of gel filtration (different amount of time different solute within the liquid phase that is entrapped by the matrix)**

Protein Separation and Purification

Purification can be monitored at each stage by measuring the enzyme activity, in units per ml, where 1 unit conventionally means the activity required to convert 1mmole of substrate per minute.
**TABLE 3-5** A Purification Table for a Hypothetical Enzyme

<table>
<thead>
<tr>
<th>Procedure or step</th>
<th>Fraction volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activity (units)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude cellular extract</td>
<td>1,400</td>
<td>10,000</td>
<td>100,000</td>
<td>10</td>
</tr>
<tr>
<td>2. Precipitate with ammonium sulfate</td>
<td>280</td>
<td>3,000</td>
<td>96,000</td>
<td>32</td>
</tr>
<tr>
<td>3. Ion-exchange chromatography</td>
<td>90</td>
<td>400</td>
<td>80,000</td>
<td>200</td>
</tr>
<tr>
<td>4. Size-exclusion chromatography</td>
<td>60</td>
<td>100</td>
<td>60,000</td>
<td>600</td>
</tr>
<tr>
<td>5. Affinity chromatography</td>
<td>6</td>
<td>3</td>
<td>45,000</td>
<td>15,000</td>
</tr>
</tbody>
</table>

Note: All data represent the status of the sample after the designated procedure has been carried out. Activity and specific activity are determined on page 91.

A measure of enzyme purity: it increases during purification of an enzyme and becomes maximal and constant when the enzyme is pure.

1.0 unit of enzyme activity = amount of enzyme causing the transformation of 1.0 µmol of substrate per minute at 25°C under optimal conditions of measurement.

Protein concentration can also be measured using *colorimetric assays, in mg protein per ml. The ratio of enzyme activity to protein concentration (i.e. units per mg of protein) is known as the specific activity of the enzyme, and is a measure of the **purity of the enzyme. As the enzyme is purified the specific activity of the enzyme should increase until the protein is homogeneous and pure, which can be demonstrated using (for diagnosis of portions) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Fig. 8).

**Electrophoresis**

Cross-linked polymer polyacrylamide acts as a molecular sieve, slowing the migration of proteins approximately in proportion to their charge-to-mass ratio.

SDS-polyacrylamide gel stained with a protein-specific dye (e.g. coomassie blue) can be used to separate proteins based on their molecular weight. SDS disrupts the native state of proteins, allowing them to migrate according to their size. 

**Fig. 8. Electrophoresis**
The purification of the enzyme can be seen from the increase in specific activity at each stage of the purification. An SDS-polyacrylamide gel containing samples of protein at each stage of the purification is shown in Figure 9. You can see that in the crude extract there are hundreds of protein bands, but that as the purification proceeds for example, the 28-kDa protein becomes more and more predominant in the gel.

![Fig. 9 samples of protein at each stage of the purification](image)

In order to test the activity of the enzyme we must first of all have an assay: a quantitative method for measuring the conversion of substrate into product by two methods direct and indirect (Fig. 10). In some cases conversion of substrate to product can be monitored directly by ultraviolet (UV) spectroscopy, *if the substrate or product has a distinctive UV absorbance.* Failing this, *a chromatographic method can be used to separate substrate from product* and hence monitor conversion. In order to quantify a chromatographic assay a *radioactive label (radiochemical) is usually required in the substrate,* so that after separation from substrate the amount of product can be quantitated by scintillation counting. Such an assay is *highly specific and highly sensitive, but unfortunately is rather tedious for kinetic work.* A more convenient assay for *kinetic purposes* is to monitor consumption of a *stoichiometric* cofactor or cosubstrate, for example the cofactor *nicotinamide adenine dinucleotide* (NADH) by UV absorption at 340 nm, or consumption of oxygen by an *oxygenase enzyme* using an oxygen electrode. In other cases a coupled assay is used, in which the product of the reaction is immediately consumed by a second enzyme (or set of enzymes) which can be conveniently monitored.
Why do we need pure enzyme? If we can see enzyme activity in the original extract, why not use that? The problem with using unpurified enzyme for kinetic or mechanistic studies is that there may be interference from other enzymes in the extract that use the same substrate or cofactor. There may also be enzymes that give rise to UV absorbance changes which might interfere with a UV-based assay.

What are GMO’s?
GMO is the acronym for “genetically modified organism”. It is used to describe organisms (plant, animal or microbial) that have been altered through use of modern biotechnology.

Why are GM microorganisms used to produce enzymes?
Modern biotechnology has improved enzyme production and enzyme quality in several ways: 1) Increased efficiency of enzyme production resulting in higher yields; 2) Increased enzyme purity through reduction or elimination of side activities; 3) Enhancing the function of specific enzyme proteins, e.g., by increasing the temperature range over which an enzyme is active. The results are better products, produced more efficiently, often at lower cost and with less environmental impact.

Enzymes have five distinct areas of application: (1) as scientific research tools; (2) in cosmetics; (3) for diagnostic purposes; (4) in therapeutic treatment; and (5) for use in industry. The first important product produced with the aid of enzyme technology is high fructose corn from maize.

Figure 4.1 Types of enzyme assays. $A_{340}$, ultraviolet (UV) absorbance at 340 nm; $A_{394}$, UV absorbance at 394 nm; ENZ, enzyme; NADH, nicotinamide adenine dinucleotide; P, product; Q, product of coupling enzyme; S, substrate.