

## Purification of enzymes related to host penetration and pathogenesis from entomopathogenic fungi

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Entomopathogenic fungi are widely distributed throughout the fungal kingdom. Some insect-pathogenic fungi have restricted host ranges, while others have a wide host range, with individual isolates being more specific. Several species of fungi are potent biocontrol agents of plant pathogenic fungi and arthropods. Entomopathogenic fungi display different strategies in their attachment to insect cuticle which forms the first formidable barrier to pathogens and fungal spores have to pass through certain discrete stages before breaching the insect cuticle. Conidial contact with an arthropod surface is the first step of the fungal penetration and infection process. Through the combined action of hydrolytic enzymes such as chitinase, protease and lipase, the fungal mycelia are able to penetrate through these barriers (Bidochka and Khachatourians 1987; St. Leger et al. 1986). Entomopathogenic fungi produce many enzymes that also are toxic components found in bacteria and animal venoms and thus may be considered as potential virulence factors. The aim of this work is to explain the strategies and processes which can be used to extract, purify and characterize different enzymes related to penetration, toxic effects, and pathogenic process in entomopathogenic fungi. Such studies can increase our knowledge on enzyme production in entomopathogenic fungi opening new avenues for the study of the role of these enzymes in virulence against different insect pests during the infection process.

**Keywords** Host infection; pathogenesis; chitinase; lipase; protease; Hyphomycetes; Biological control; Environmental microbiology.

### 1. Background

Entomopathogenic fungi are widely distributed throughout the fungal kingdom. Some insect-pathogenic fungi have restricted host ranges, while others have a wide host range, with individual isolates being more specific [1]. Several species of fungi are potent biocontrol agents of plant pathogenic fungi and arthropods. Isolates of *Zoophthora radicans* [2], *Metarhizium anisopliae*, *Fusarium* sp., and *Beauveria bassiana* can infect different insect species in screen house or field conditions [3, 4, 5]. The insect integument is composed of enzymes and chitin with associated lipids and phenolic compounds which serve as a barrier against invading microorganisms [6]. Through the combined action of hydrolytic enzymes such as chitinase, protease and lipase, the fungal mycelia are able to penetrate through these barriers [7,8]. The importance of any one of these enzymes is dependant upon the cuticular characteristics of insects and invasion mechanism of the fungus. Proteolytic enzymes are important factors in virulence of entomopathogenic fungi [9]. Thus, particular attention has been focused on the role of proteases in the penetration process [10]. Entomopathogenic fungi produce distinct extracellular serine proteases, such as subtilisin-like proteases, trypsin-like proteases, metalloproteases, as well as several families of exo-acting peptidases that are believed to be important for host cuticle degradation [11]. The subtilisin protease family Pr1 is the main enzyme produced by entomopathogenic fungi during the infection process, although the trypsin-like protease Pr2 is the first to appear during in vitro growth on the cuticle [12]. Antiserum against Pr1 protease interferes with penetration of the host cuticle and reduces infection, indicating that the level of active Pr1 may determine the capacity of the fungus to cause disease [13].

Chitinases catalyze the hydrolysis of chitin, which is a b-(1,4)-linked polymer of N-acetyl-D- glucosamine and one of the important structural components of insect cuticle [14]. Chitinases are produced by a large number of organisms including plants, fungi, and bacteria, and play an important role in the defense mechanism of plants against pathogens and in the mycoparasitic process of fungi. They also play an important role in nutrition, development, and morphogenesis of fungi. However, the role of the chitinases in the host infection process is not yet fully understood.

The participation of proteases and chitinases in the infection process of entomopathogenic fungi has been demonstrated, and it has been suggested that lipases can also be involved in the process by hydrolyzing the ester bonds of lipoenzymes, fats and wax layers of the insect integument [15]. Without the action of lipases, some of these materials would be a barrier to fungal entry. Lipolytic enzymes have been described as toxic activity components in venoms and as virulence factors in several microbial pathogens with different roles in the infection process. One of such roles could be the enhancement of the adhesion to the host cuticle, which is experimentally supported by the presence of lipase activity in *M. anisopliae* fungal spores. Lipolytic activity increases hydrophobic interactions by releasing free fatty acid, favoring adhesion to the host. Moreover, lipids in epicuticle are the first barrier against arthropod pathogenic microorganisms, which reinforces the importance of these enzymes in penetration and initial infection stages, such as the contact of conidia with host surface. Enzymes involved in protection against reactive oxygen species (ROS) formed by solar ultraviolet radiation (UV-A and UV-B) and heat are fundamental for conidia protection in the environment.

Catalase, Cu–Zn SOD, and peroxidase activities, which are important to spore protection in the environment, were found in SSP. High tolerance to UV radiation is very important to the successful use of entomopathogenic fungi as commercial biocontrol agent in crop protection strategies. Also, catalase activity protects against cytotoxic effects of host-derived H<sub>2</sub>O<sub>2</sub>, as reported for *Claviceps purpurea* in rye.

Enzyme purification is generally a multi-step process exploiting a wide range of biochemical and biophysical characteristics of the target enzyme, such as its source, relative concentration, solubility, charge, and hydrophobicity. The ideal purification strives to obtain the maximum recovery of the desired enzyme, with minimal loss of activity, combined with the maximum removal of other contaminating enzymes. There is no set procedure for isolating enzymes. Purification schemes should be tailored to take advantage of the biochemical properties of the target enzyme, as well as the cellular properties of the tissue that provides the most abundant source of material. Whenever possible try to reduce the complexity of the sample. For example, isolate specific enzyme subsets or subcellular organelles thereby enriching for the low abundance target molecule.

When designing a purification protocol one should aim for the following: (1) high recovery; (2) highly purified end product; (3) reproducibility, within the lab, in other labs and also when either scaled up or down; (4) economical use of reagents; and (5) convenience with regard to time. This wide enzymatic arsenal comprises activities related to several physiologic and ecologic functions that are vital for fungal survival and adaptation in a broad array of habitats, such as saprophyte or pathogen. Activities related with conidia protection and germination, adhesion to host, fungal nutrition, cell wall, and membrane cell modification and disruption, which are obviously important to pathogenesis and host infection were identified, characterized, and discussed in this work. The aim of this work is to explain the strategies and processes which can be used to extract, purify and characterize different enzymes related to penetration, toxic effects, and pathogenic process in entomopathogenic fungi. Such studies can increase our knowledge on enzyme production in entomopathogenic fungi opening new avenues for the study of the role of these enzymes in virulence against different insect pests during the infection process.

## 2. Different strategies used for enzyme purification

In general, the most successful isolation procedures involve only a few steps, chosen to give the highest yields. Rarely will a single technique fulfill the requirements of any specific separation. More frequently, two, three, or more steps are needed for the purification of the desired enzyme. When undertaking any purification, always begin with enough starting material so that a workable amount of final product can be isolated. Delays during a multistep protocol should be avoided. The stability of the target enzyme will determine the time necessary for the protocol. For example, in cases where stability is limited due to the inherent liability of the enzyme or co purifying proteolytic activity, the speed of operation can be much more important than the enzyme purity. Therefore, there will be instances when it will be beneficial to rush one step after another, sacrificing purification, rather than carrying out each step to perfection and taking a long time in the process. Whenever possible, fractionation steps should be arranged to follow one from the other without extensive manipulation between steps. Some typical protocols that do not involve intermediate treatments are listed below:

Salt precipitation → Gel filtration → Ion exchange chromatography

Salt precipitation → hydrophobic interaction chromatography → Ion exchange chromatography → Reversed phase HPLC

Ion exchange chromatography → hydrophobic interaction chromatography → Affinity chromatography → Gel filtration chromatography

Organic solvent extraction → Affinity chromatography

Affinity chromatography → Salt precipitation → Gel filtration chromatography

The positions of the steps in the above strategies are predicated on the volume and ionic strength of the sample. In a typical purification protocol, the first step after extracting the target enzyme in soluble form from the starting material could be ion exchange chromatography, which has excellent resolving power that can concentrate the target enzyme from a dilute starting solution. The target enzyme is usually eluted with high concentrations of salt. An interim desalting step can be avoided if the next step is hydrophobic interaction chromatography in which the enzyme is loaded onto the column in a high salt containing solution. The eluate from this step, now free of lipids and other potential problem-causing contaminants, can be put onto an affinity column, if one is available. Using an immobilized ligand, the target enzyme is specifically bound, and the contaminants are washed away. The enzyme of interest is then eluted. Often, this step is the most powerful in the scheme, being good enough to stand alone when a large quantity of semi-purified enzyme is desirable. The final step in a purification protocol is frequently size exclusion chromatography, which, in addition to contributing to the purity of the final product, also accomplishes two other important goals. Size exclusion chromatography will yield the Stokes radius of the specific enzyme which can be used as a close approximation of the enzyme molecular weight. Secondly, the final step can be used to adjust or change the buffer, transferring the enzyme into a solution that is compatible with the intended usage.

### 3. Deciding on your objectives

Purity, yielding and cost are the major considerations, and the relative importance of these factors will depend on the purpose of the purification. For studies on the biological activity of an enzyme (e.g. an enzyme), only  $\mu\text{g}$  amounts may be required and, as long as there are no interfering substances present, 100% purity may not be necessary. For structural studies, mg amounts may be needed and the enzyme must be pure. So we should aim to use the smallest numbers of purification steps that will not only give us the yield and purity required for our application, but also reduce inevitable loss of interesting at each stage of the purification procedure.

#### 3.1 Preliminary considerations

Rather than approaching every purification steps on a 'trial and error' basis, try to find out as much as we can: about the physical and biological properties of the desired enzyme before you begin your practical work. Even if the enzyme to be purified is novel, it is likely that similar enzymes will be described in the literature. Knowledge of various influencing factors can allow you to take steps to preserve the tertiary structure and biological activity of the desired enzyme during the purification process. For most enzymes, the initial procedures should be carried out at  $4^{\circ}\text{C}$ , to minimize the risk of proteolysis.

#### 3.2 Devising a strategy for purification

Any successful enzyme purification scheme will exploit the unique properties of the desired enzyme in terms of its size, net charge, hydrophobic nature, biological activity, etc. However, the order in which the various purification steps are carried out needs some thought, and each stage needs to be considered in relation to the following factors, including capability, resolution, and yield. Commonly, the purification is carried out in two stages so as to obtain highly purified enzyme, crude separation and refined separation.

## 4. Protein purification

### 4.1 Mass production of enzyme culture

#### 4.1.1 Production of extracellular cuticle degrading enzymes

The extracellular enzymes like chitinase, protease and lipase can be produced in the basal medium (pH 7.2) consisting of glucose 0.2% (w/v), peptone 0.5% (w/v),  $\text{MgSO}_4$  0.01% (w/v),  $\text{K}_2\text{HPO}_4$  0.1% (w/v) and SDS 0.25 % (w/v). Add Chitin (1% w/v) as a carbon source to previously sterilized basal medium ( $121^{\circ}\text{C}$ , 15 min) while the basal medium without chitin will serve as control. The flasks will be inoculated with one ml of  $1 \times 10^7$  spores/ml and incubated at 180 rpm and  $30^{\circ}\text{C}$  for 5 days. The crude culture filtrate of enzyme will be obtained by filtration through Whatman no. 1.

#### 4.1.2 Production of antioxidizing enzymes

The composition of the basal medium for the production of antioxidizing enzymes (SOD, CAT and peroxidase is glucose 2% (w/v), yeast extract 0.3% (w/v),  $\text{K}_2\text{HPO}_4$  0.1% (w/v),  $\text{MgSO}_4$  0.04% (w/v), glycine 1% (w/v),  $\text{CuSO}_4$  0.1% (w/v),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.1% (w/v) and  $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$  0.1% (w/v). Inoculate the flasks with one ml of  $1 \times 10^7$  spores/ml and incubated at 180 rpm and  $30^{\circ}\text{C}$  for 5 days.

The cell-free extract was by the following procedure. Briefly, mycelium biomass will be harvested by filtration, washed in distilled  $\text{H}_2\text{O}$ , and then in cold 50 mM potassium buffer (pH 7.8), and resuspended in the same buffer. Disrupt the cell suspension was by homogenization. The temperature during treatment should be maintained at  $4-6^{\circ}\text{C}$  by chilling in an ice-salt bath and by filtration through filter paper. Cell-free extracts will be clarified at 8,000 g for 15 min at  $4^{\circ}\text{C}$ .

### 4.2 Concentrating enzymes from crude filtrates

Often the volume and protein concentration of the initial soluble extract will be quite high. Application of a differential solubility technique at this stage results in the precipitation of selected proteins. These can be removed by filtration or centrifugation, washed, and then resuspended in an appropriate buffer. This will reduce the sample volume and may give a small degree of purification, making the sample more suitable for subsequent chromatographic steps.

#### 4.2.1 Ammonium sulphate precipitation ('salting out')

This is the most widely used solubility technique, having the advantage that most precipitated enzymes are not permanently denatured, can be redissolved with restoration of activity. The basis of fractionation in this method is that,

as the salt concentration of the extract is increased, proteins with larger or more abundant hydrophobic patches will precipitate before those with smaller or fewer patches. Although the phenomenon of salting out is seen with several salts,  $(\text{NH}_4)_2\text{SO}_4$  is the most widely used due to its high solubility and low cost. The  $(\text{NH}_4)_2\text{SO}_4$  salt should be added slowly while stirring, and the mixture left at  $4^\circ\text{C}$  for several hours before centrifuging to obtain precipitation.

#### 4.2.2 Precipitation by changing pH

Proteins are least soluble at their isoelectric points because at that pH there is no longer the repulsion that occurs between positively or negatively charged protein molecules at pH values. If the precipitated proteins are required for further purification, it is essential that the protein of interest is not irreversibly denatured.

#### 4.2.2 Heat denaturation

Exposure of most proteins to high temperatures disrupts their conformation through effects on non-covalent interactions such as hydrogen bonds and Van Der Waals forces. However, different proteins are denatured and hence precipitated, at different temperatures, and this can provide a basis for the separation of some heat stable proteins.

#### 4.2.3 Solvent and polymer precipitation methods

Organic solvents (e.g. acetone, ethanol) and polymers (e.g. PEG) cause precipitation of proteins by lowering the dielectric constant of the solution. Performing the precipitation at  $0^\circ\text{C}$  minimizes permanent denaturation. Stepwise, concentration (%v/v) increments are used, giving 'cut' of precipitated proteins, as with ammonium sulphate precipitation.

#### 4.2.4 Concentration by ultrafiltration

This involves forcing water and small molecules through a semi-permeable membrane using high pressure or centrifugation. Ultrafiltration not only concentrates the sample, but also may give a degree of purification. It also can be used to change the buffer composition by diafiltration.

#### Protocol 1: Ammonium sulfate precipitation

This is the most widely used solubility technique, having the advantage that most precipitated enzymes are not permanently denatured, can be redissolved with restoration of activity. The basis of fractionation in this method is that, as the salt concentration of the extract is increased, proteins with larger or more abundant hydrophobic patches will precipitate before those with smaller or fewer patches. Although the phenomenon of salting out is seen with several salts,  $(\text{NH}_4)_2\text{SO}_4$  is the most widely used due to its high solubility and low cost. The  $(\text{NH}_4)_2\text{SO}_4$  salt should be added slowly while stirring, and the mixture left at  $4^\circ\text{C}$  for several hours before centrifuging to obtain precipitation. It is best to operate at a neutral pH between 6–7.5.

After deciding to perform an ammonium sulfate fractionation the next decision is what percentage saturation to try. The percentage saturation for different enzymes are Chitinase 80%, Protease 75%, Lipase 70% and SOD 55%. According to the volume of the protein solution, perform a quick calculation and add solid ammonium sulfate to reach the final desired concentration by consulting Kaplan [16]. Material precipitating prior to 25%  $(\text{NH}_4)_2\text{SO}_4$  saturation is generally particulate, preaggregated or very high molecular weight protein. The best conditions for enriching a specific protein from a complex mixture are reached empirically. As a first approximation the following percentages should be helpful: 0–25%, 25–40%, 40–60%, 60–80%, and the 80% supernatant.

#### 4.3 Removing salts and changing the buffer

It is necessary to remove salts or to change the buffer before the next step will work effectively (e.g. when carrying out IEC, the ionic strength or the pH of the sample may need to be changed before the target protein binding to the column). Several methods are available, including dialysis, diafiltration and gel permeation chromatography.

##### 4.3.1 Removal of salts through dialysis

Dialysis is a separation process that takes advantage of osmotic forces between two liquids or a liquid and a solid. Dialysis is used for removing excess low molecular weight solutes and simultaneously equilibrating the sample in a new buffer, and as a means of concentrating a dilute solution. Dialysis tubing is a semi-permeable membrane, usually made from cellulose acetate, available in a wide range of dimensions and nominal molecular weight cut-offs (NMWC) allowing molecules below a certain molecular weight to freely equilibrate on both sides of the membrane. Practical removal of a dialyzable component from within the bag cannot be accomplished without changing the dialysate solution at least once.

Solutions may be concentrated by "dialysis" against a high molecular weight solid hydroscopic substance. The solution is placed in dialysis tubing which is then coated with an inert, high molecular weight hydroscopic substance

that “pulls” water out of the bag. A solution containing  $(\text{NH}_4)_2\text{SO}_4$  can be dialyzed without agitation. Float the dialysis tubing in a tall, graduated cylinder. The relatively dense  $(\text{NH}_4)_2\text{SO}_4$  exits the tubing and sinks to the bottom of the cylinder, leaving the solute concentration very low in the vicinity of the tubing. When removing the sample from the tubing, care should be taken to avoid spillage and sample loss. Dissolve the enzyme precipitate in small volumes of 0.05 mol/L Tris-HCl buffer (pH 7.5). Dialyze the solution overnight at 4°C against with four changes of distilled water. The dialysate can be collected by freeze-drying and used for protein measurement and enzymatic activity assay.

#### 4.4 Quantification of proteins

##### 4.4.1 Determination of total protein

Protein was monitored by measuring the absorbance at 280 nm by protein-nucleic acid detector during chromatographic separation. Protein concentration was also determined by using the Bradford method with bovine serum albumin as the standard.

Before determine the protein concentration, a standard curve is required. Firstly according to the following demands (Table 2), two parallel groups can be performed at one time. After mixed together, absorbency of the mixture can be detected at 595 nm using NO.1 as control in 5~20 min. Secondly use Origin 7.0 to draw graph, the X axis of which is used for BSA amount, while the corresponding absorbency at 595 nm can be plotted on the Y axis. And we should get the reasonable formula by curve fitting.

Thirdly, detect absorbency of the sample at 595 nm. The assay mixture consisted of 2.85 mL of Coomassie brilliant blue solution, 140 µL of PBS, 10 µL of the sample, while the control was added to a total volume of 3 ml by PBS and the equal amount of Coomassie brilliant blue solution. At last, calculate the protein amount of the sample in terms of the obtained formula.

**Table 1** Quantitative table for drawing standard curve of protein determination

No.	0	1	2	3	4	5
1 mg/mL BSA/µL	0	10	20	30	40	50
PBS/µL	150	140	130	120	110	100
Coomassie brilliant blue solution/mL	2.85	2.85	2.85	2.85	2.85	2.85
$\overline{A}_{595}$						

##### 4.4.2 Protease assay

Subtilisin- like (Pr1) activity can be assayed by using succinyl-(alanine)<sub>2</sub>-proline-phenylalanine-*p*-nitroanilide as substrate, while benzoylphenylalanine-valine-arginine-*p*-nitroanilide is used for trypsin- like (Pr2) activity. Each assay will be consistinf of 0.05 ml substrate (1 mmol L<sup>-1</sup>), 0.85 ml 15 mmol L<sup>-1</sup> Tris-HCl buffer (pH 8.5) and 0.1 ml crude enzyme. Incubated the mixture for one hour at 28°C and the reaction will be terminated by adding 0.25 ml of 30% acetic acid and left to stand for 15 min on ice, after which the samples were centrifuged at 1250 g for 5 min at 4°C. Transfer the supernatants to the wells of microtitre plate and measure the absorbance using spectrophotometer at 410 nm. One unit of activities will be expressed as nanomoles nitroanalide (NA) released per mg per hour. Protein was measured using Coomassie Brilliant Blue G-250 according to Bradford [17], with bovine serum albumin as standard.

##### 4.4.3 Lipase assay

Lipase activity can be determined as described by Pignede et al. [18]. The substrate emulsion is prepared with olive oil, 50 ml and gum Arabic, 50 ml (10% w/v). The reaction mixture will consist of one ml enzyme (sample already obtained from culture flasks), 5 ml substrate emulsion and 2ml of 50 mM phosphate buffer, pH 6.8, incubated for 1h at 37 °C with shaking. The reaction will be stopped with 4ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity will determined by titration of the fatty acid released with 50 mM sodium hydroxide. One international unit is defined as enzyme activity that produced 1µmole of fatty acid per min.

##### 4.4.4 Chitinase and Chitosanase assay

Colloidal chitin will be prepared by the method of Roberts and Selitrenikoff [19] with some modification. One hundred grams of chitin flakes of reagent grade chitin will be added slowly to 1.75 liter concentrated HCl and agitated gently for 3 hours on a magnetic stirrer. Filter this solution to 20 liter of pre chilled distilled water with constant mixing and allowe it to settle. Centrifuge the dense white precipitate formed at 10,000 rpm for 10 min at 4 °C. Wash The precipitate

in cold distilled water repeatedly until the pH of the wash reached near to 5.5. Discard the supernatant and keep colloidal chitin in refrigerator for future use.

Chitinase assay is based on the estimation of reducing sugars released during the hydrolysis of colloidal chitin. The reaction mixture is composed of 0.5 ml of enzyme, 0.5 ml of 0.5% colloidal chitin and 1.0 ml of citrate phosphate buffer pH 5.6. Keep the mixture in a water bath at 37 °C for 1 h. Estimate the amount of reducing sugar liberated by Miller's (1959) method. One unit (U) of activity is defined as the amount of enzyme which catalyzed the release of one µg of reducing sugar per ml per minute under the reaction conditions. Protein concentration was determined according to Bradford [16] using bovine serum albumin as a standard.

Chitosanase activity is estimated using acid swollen chitosan as the substrate. The acid swollen chitosan can be prepared following the protocol used to make acid swollen chitin. The assay mixture contains 1ml 0.7 % acid swollen chitosan, 1ml 50mM acetate buffer, pH 5.0 and 1ml enzyme that were incubated at 37°C for 1h. Estimate the reducing sugars produced using Somogyi method at 520nm. One international unit of enzyme is defined as the activity that produced 1 µmol glucosamine equivalents per min.

#### 4.4.5 SOD assay

Measure the SOD activity by the nitro-blue tetrazolium (NBT) reduction method. Add one ml solution of 0.05 M potassium phosphate buffer pH 7.8, 13mM L-methionine, 75 mM NBT, 0.1 mM EDTA and 0.025% triton X-100 to glass tubes. Start the reaction by adding sample and 10 mM riboflavin at the same time and place the tubes under fluorescent light for 15 min. After this period, measure the absorbance at 560nm. One unit of SOD activity is defined as the amount of SOD required for inhibition of the reduction of NBT by 50% ( $A_{560}$ ) and was expressed as units per mg protein, i.e. (U mg protein)<sup>-1</sup>.

#### 4.4.6 Catalase assay

Catalase assay essentially that described by Beers and Sizer [19] will be used in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. One unit decomposes one micromole of H<sub>2</sub>O<sub>2</sub> per minute at 25°C and pH 7.0 under the specified conditions. The reaction tube contains reagent grade water 1.9 ml and 0.059 M Hydrogen peroxide 1.0 ml. Incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate if any. Add 0.1 ml of diluted enzyme and record decrease in absorbance at 240 nm for 2-3 minutes. Calculate  $\Delta A_{240}/\text{min}$  from the initial (45 second) linear portion of the curve.

#### 4.4.7 Peroxidase assay

The peroxidase activity is determined by measuring the absorbance at 470 nm of tetraguaiacol produced by the reaction between 0.05 M guaiacol solution and 10.3 mM hydrogen peroxide solution in 0.1 M phosphate buffer pH 7.0 at 25°C. The increase in absorbance at 470 nm was monitored for 5 min. One activity unit is defined as the amount of enzyme which cause an increase of 0.001 in absorbance per hour.

### 4.5 Methods for refined separation – chromatography

Chromatography is used to separate individual constituents within a sample on the basis of different physical characteristics, e.g. molecular size, shape, charge, solubility and/or adsorptivity. In a chromatography system, those substances which interact strongly with the stationary phase will be retarded to the greatest extent while those which show little interaction will pass through with minimal delay, leading to differences in distances traveled or elution times. Herein separation methods of chromatography are introduced.

#### 4.5.1 Adsorption chromatography

This is a form of solid-liquid chromatography. The stationary phase is a porous, finely divided solid which adsorbs molecules of the test substance on its surface due to dipole-dipole interactions, hydrogen bonding and/ or Vander Waals interactions.

#### 4.5.2 Ion-exchange chromatography

Separation is carried out using a column packed with a porous matrix which has a large number of ionized groups on its surfaces. You should select the ion-exchange resin and buffer pH so that the best substances are strongly bound by electrostatic attraction to the ion-exchange resin on passage through the system, which the other components of the sample are rapidly eluded. You can then elude the bound components by raising the salt concentration of the mobile phase, either stepwise or as a continuous gradient, so that exchange of ions of the same charge occurs at oppositely charged sites on the stationary phase. Weakly bound sample molecules will elude first, while more strongly bound molecules will elude at a higher concentration.

#### 4.5.3 Gel permeation chromatography (GPC) or gel filtration

The stationary phase is in the form of beads of a cross-linked gel containing pores of a discrete size. The size of the pores is controlled so that at the molecular level, the pores act as 'gates' that will exclude large molecules and admit smaller ones. Molecules of intermediate size partly enter the pores. So if a sample is placed at the top of such a column, the mobile phase will carry the sample components down the column, but at different rates according to their molecular size. A very large molecule will elude in the minimum possible volume, but molecules of intermediate size will elude in the mid course.

Of course, currently many other kinds of chromatography have been widely used based on biological specificity or physico-chemical properties such as covalent chromatography, affinity chromatography, and hydrophobic interaction chromatography and so on.

#### 4.6 Monitoring purification

At each step, the separated material is usually collected as a series of 'fractions'. Each fraction must be assayed for the protein of interest, and fractions containing that protein are pooled prior to the next step. The assay performed will depend on the properties of the protein of interest but specific enzyme assays or immunoassays are most commonly used. The protein concentration and the volume of the pooled fraction must be determined, and these values, together with those obtained for the amount of the protein of interest, are used to determine the purity and yield after each step. For enzymes, the biological activity is measured and used to calculate the specific activity (the enzyme activity per unit mass of protein). By determining the specific activity of the enzyme at each step, the degree of purification or purification factor (n-fold purification) can be obtained from the following relationship:

Purification factor = specific activity after a particular step/specific activity of initial sample

Increased purification usually represents a degree in total protein relative to the biological activity of the protein of interest, though in some instances it may reflect the loss of an inhibitor during a purification step. Calculation of the yield of enzyme at each step is straightforward, since:

Yield = [total enzyme activity after a particular step/total enzyme activity in initial sample] × 100%

Note that the yield equation uses the total amount of enzyme and is therefore unaffected by the volume of the solutions involved. And a record of the progress of your purification procedure at each step should be made.

##### 4.6.1 Electrophoresis assay

Electrophoresis is a separation technique based on the movement of charged molecules in an electric field. Dissimilar molecules move at different rates and the components of a mixture will be separated when an electric field is applied. It is a widely used technique, particularly for the analysis of complex mixtures or for the verification of purity (homogeneity) of isolated biomolecules. Polyacrylamide gel electrophoresis (PAGE) has a major role in protein analysis, both for one-dimensional and two-dimensional separations.

###### ( i ) SDS-PAGE

The most widely used PAGE protein separation technique uses an ionic detergent, usually sodium dodecyl sulphate (SDS), which dissociates proteins into their individual polypeptide sub-units and gives a uniform net charge along each denatured polypeptide. The technique is known as SDS-PAGE. In SDS-PAGE, the sample protein is normally heated to 100 °C for 2~5 min, in buffer containing 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol, the latter to cleave any disulphide bonds. As a result, the intrinsic net charge of each peptide is 'swamped' by the negative charge imposed by SDS, and there is a uniform negative charge per unit length of polypeptide. Since the polypeptide now have identical charge densities, when they are subject to PAGE (with SDS present) using a gel of appropriate pore size, molecular sieving will occur and they will migrate strictly according to polypeptide size. This not only gives effective separation, but the molecular mass of a given polypeptide can be determined by comparing its mobility to polypeptides of known molecular mass run under the same conditions.

###### ( ii ) Native-PAGE

In order to preserve the native protein conformation and biological activity, non-dissociating conditions are required, i.e. no SDS or other detergents and reductants are added in demanded buffers as well as no heating processing. Based on the intrinsic net charge and size of the native protein, dissimilar molecules move at different rates and the components of a mixture will be separated in an electric field. This method is useful in purity detection after a series of purification process. Valuably, the combination of SDS-PAGE and Native-PAGE can easily shows the amount of subunits of the protein.

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