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The Isolation of Invertase from Baker's Yeast – An Introduction to Protein Purification Strategies

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1. Introduction

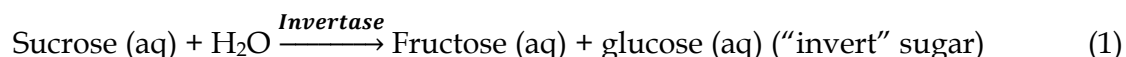
The vast number of roles that proteins serve in cell structure and function is unrivaled by any other type of compound found in nature and, in this sense, it can be argued that proteins are the most functionally and structurally diverse class of all known substances. Despite this vast diversity, each one of the hundreds to thousands of different proteins expressed in a cell possesses the same general composition: they are all un-branched polymers constructed from a common set of twenty amino acid building blocks. Because of this remarkable similarity in composition, the task of selectively isolating a functionally active protein from its biological source in the amounts required to study its function and shape might appear at first consideration to be a hopeless, if not an impossible, separation to achieve at the lab bench. However, by carefully taking advantage of variations in many physical properties between different types of proteins (including their mass, length, solubility, charge, and ability to selectively bind to specific ligands) the technology and art of protein purification has evolved into both a routine and rewarding exercise.

The purpose of this chapter is to provide a broad overview of some common concerns and corresponding strategies used to purify a protein from a natural source by way of a case study entitled, "The Isolation of Invertase from Baker's Yeast" which is a series of three-hour laboratory exercises designed to introduce methods of protein purification to a large group of undergraduate students. The reader should note that the specific, procedural details of each part of this project can be found in a methods paper recently published in the Journal of Chemical Education (Timerman et al., 2009) and, for the most part, will not be repeated here. Instead, this chapter focuses on the rationale and development of the sequence of steps used in this project as well as a brief description of each method used to measure, extract, isolate and characterize the enzyme. Other chapters of this book will provide more complete information and details regarding many of these and other selected topics in protein purification.

2. Function and properties of invertase

Invertase is the common name of the enzyme that catalyzes the hydrolysis of table sugar (i.e. sucrose) into a much sweeter, equimolar mixture of glucose and fructose called "invert"

sugar (equation 1 and figure 1). Because invert sugar is a key ingredient in a number of sweets and confectionary products, the bakery industry provides one of the most important commercial applications of this enzyme reaction. For this reason, the enzyme has been extensively characterized and commercial sources of pure invertase are readily available.



While aqueous solutions of either pure sucrose or glucose display weakly dextrorotatory behavior, meaning they cause a slight right-handed rotation of plane polarized light, solutions of pure fructose are strongly levorotatory and cause a much greater left-handed rotation of the light. The enzyme reaction, therefore, catalyzes the inversion of the right-handed rotation of polarized light observed through sucrose solutions to the left-handed rotation observed for solutions of "invert" sugar, hence the enzyme's common name of "invertase". For similar reasons, the common monosaccharides glucose and fructose are also known as dextrose and levulose, respectively.

Because enzymes are systematically named and classified by the substrate and subclass of reaction that they catalyze, the systematic name of invertase is "sucrose glycosidase" implying that it is a member of the subclass of enzymes that hydrolyze glycosidic (or acetal) linkages with a substrate specificity for sucrose. The yeast form of the enzyme has been assigned the unique four digit enzyme classification code (EC) number of 3.2.1.26 and it is also commonly called β -fructofuranosidase or sucrase. The intestinal enzyme lactose glycosidase (or lactase, EC 3.2.1.108), which hydrolyzes milk sugar into an equimolar mixture of galactose and glucose, is a related member of this enzyme subclass that may be more familiar because a deficiency of this enzyme is associated with symptoms related to lactose intolerance.

In yeast cells, invertase is classified as an extra-cellular, glycoprotein which is localized to the thin volume of space that exists between the yeast's plasma membrane and its outer cell wall (this peripheral volume is often called the periplasmic space). The enzyme serves the important biological function of cleaving sucrose on the outside of the cell into monosaccharides that can be transported (and subsequently metabolized) in the cytoplasm. That is, in the absence of invertase, yeast would have a difficult time utilizing table sugar as an energy source. Kinetic studies indicate that this extracellular form of invertase has a pH and temperature optima of about 4.8 and 40° C, respectively, and the K_m for its substrate is about 5 mM sucrose. The enzyme's native mass of about 270 kiloDaltons is constructed from two identical and heavily glycosylated subunits with a molecular weight of about 135 kiloDaltons (Neumann & Lampen, 1967). Because extracellular proteins are typically conjugated with oligosaccharide chains (i.e. glycosides) by post-translational modification before they are exported from eukaryotic cells, it is not surprising that the periplasmic form of yeast invertase is indeed a glycoprotein. However, invertase is unusual in that the numerous oligosaccharide chains attached to the two identical subunits account for nearly 50% of enzyme's native mass (Lampen, 1971).

These cellular and structural features of yeast invertase offer several advantages in this purification project: (i) first, the enzyme can be gently and selectively extracted from yeast cells by using conditions that disrupt the cell wall while leaving the plasma membrane intact; (ii) the high oligosaccharide content increases the stability of the extracted enzyme

(either by preventing protein aggregation or reducing its susceptibility to attack by proteases and other undesirable reactions) (Schulke & Schmid, 1988); and (iii) variations in the sugar content of each subunit causes them to migrate as a smeared band that is easy to detect during SDS-PAGE analysis (Moreno et al., 1980). On the other hand, this unusually high sugar content also reduces the ability of the protein to bind to Coomassie brilliant blue, the key component of the Bradford dye-binding protein assay. For this reason, solutions of pure commercial invertase prepared by dissolving a weighed mass of the solid enzyme to a final concentration of 1 mg per mL are observed to have a relative or equivalent concentration of only 0.10 mg per mL when compared to bovine serum albumin as the standard, reference protein in the Bradford assay.

3. Measurement of invertase activity

In order to selectively purify a specific protein from a mixture containing many other different proteins, it is essential to be able to selectively identify and measure the amount of the target protein without interference from others in the sample. For this reason, the first and perhaps most crucial step of any protein purification project is the **detection assay** used to monitor and compare the amount of a specific target protein contained in different mixtures.

Because enzymes catalyze very specific cellular reactions, the relative amount of an enzyme in a sample is traditionally defined by the amount it increases the reaction rate under a strictly defined set of conditions. By convention, relative amounts of enzyme are expressed in terms of “**International Enzyme Units**” where “**1-enzyme unit**” is defined as the amount of enzyme required to either: (i) consume 1.00 μmole of reactant per minute; or (ii) produce 1.00 μmole of product per minute. Furthermore, because the units used to describe enzyme activity rates ($\mu\text{mole} \cdot \text{min}^{-1}$) are different than the traditional units used to describe chemical reaction rates ($\mu\text{moles} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$), enzyme units are conveniently calculated by simply multiplying the concentration change observed in a solution by its volume (mL).

In the case of invertase, 1-unit of activity is therefore defined as the mass of enzyme required to either: (i) hydrolyze 1- μmole of sucrose per minute, or (ii) produce 1- μmole of invert sugar per minute under a precisely defined set of reaction conditions. According to this convention, a sample with 100 total units of invertase activity contains twice the mass (and thus twice the number of moles) of invertase as a sample with only 50 units of activity. Because enzyme reaction rates can change significantly with very small changes in temperature, pH, or substrate concentration (that is, enzyme activity units are a conditional property of matter) the precise value of each of these three conditions must be unambiguously described for the detection assay. For invertase, the enzyme detection assay is typically performed near its optimal pH (4.8) and temperature (40° C) at initial sucrose concentrations in excess of enzyme's substrate K_m of 5 mM sucrose.

3.1 Spectroscopic measurement of enzyme activity units

Recall that Beer's law states that the absorbance (**ABS**) of a solution is proportional to the product of (**a**•**b**•**c**) where (**a**) represents the millimolar absorptivity constant (or extinction coefficient) of the solute (expressed in units of $\text{mM}^{-1} \cdot \text{cm}^{-1}$) at a specified wavelength of light; while (**b**) defines the distance of the light-path through the cuvette (expressed in cm units);

and **(c)** is the solute concentration (expressed in milli-molar, mM units). Therefore, if the **a** and **b** terms of the equation are held at a known and constant value, then rearrangement of Beer's law indicates that any rate of change observed in the absorbance of the solution ($\Delta\text{ABS}/\text{min}$) must correspond to a proportional rate of change in the millimolar concentration ($\Delta\text{C}/\text{min}$) of the colored solute, as outlined in equation (2):

$$\Delta\text{C}/\text{min} = \frac{\Delta\text{ABS}/\text{min}}{a \cdot b} = \frac{1/\text{min}}{(\text{mM} \cdot \text{cm})^{-1}(\text{cm})} = \frac{\text{mM}}{\text{min}} = \frac{\text{mmole}}{\text{L} \cdot \text{min}} = \frac{\mu\text{moles solute}}{\text{mL} \cdot \text{min}} \quad (2)$$

And, the corresponding number of enzyme units (μmoles of solute per minute) contained in any reaction that produces (or consumes) of a colored solute is simply calculated from the rate of the absorbance change ($\Delta \text{Abs}/\text{min}$) and the fixed volume (**VmL**) of the colored solution in the cuvette with a fixed light path, as summarized in equation 3.

$$\text{Enzyme units} = \frac{\Delta\text{ABS}/\text{min}}{a \cdot b} \cdot \text{VmL} = \frac{\mu\text{moles solute}}{\text{mL} \cdot \text{min}} \cdot \text{mL} = \frac{\mu\text{moles solute}}{\text{min}} \quad (3)$$

3.2 The standard 5-minute “stop-assay” of invertase activity

Because both the reactants and products of the invertase reaction are colorless, the reaction is followed spectrophotometrically by using the monosaccharides produced from the reaction to subsequently reduce brightly yellow-colored solutions of 3, 5-dinitrosalicylate (DNS) to dark orange-colored solutions of 3-amino-5-nitrosalicylate which can be detected with an inexpensive spectrophotometer at a wavelength of 540 nm (Melius, 1971 and Sumner & Sisler, 1944). In this manner, the standard 5-minute stop assay of invertase activity used in this project can be summarized in four-steps.

Step 1: Invertase catalyzed hydrolysis of sucrose: At 30 second intervals, small aliquots (0.10 mL or less) of each enzyme-containing fraction (or diluted samples of each fraction) are quantitatively transferred and gently mixed into 1.0 mL of substrate solution (composed of 20 mM sucrose in 40 mM sodium acetate buffer at pH 4.80 and an ambient temperature of 20-24° C) contained in separate 13 x 100 mm disposable test-tubes where the invertase reaction (see Figure 1) is allowed to proceed for a precise time of 5.0 minutes.

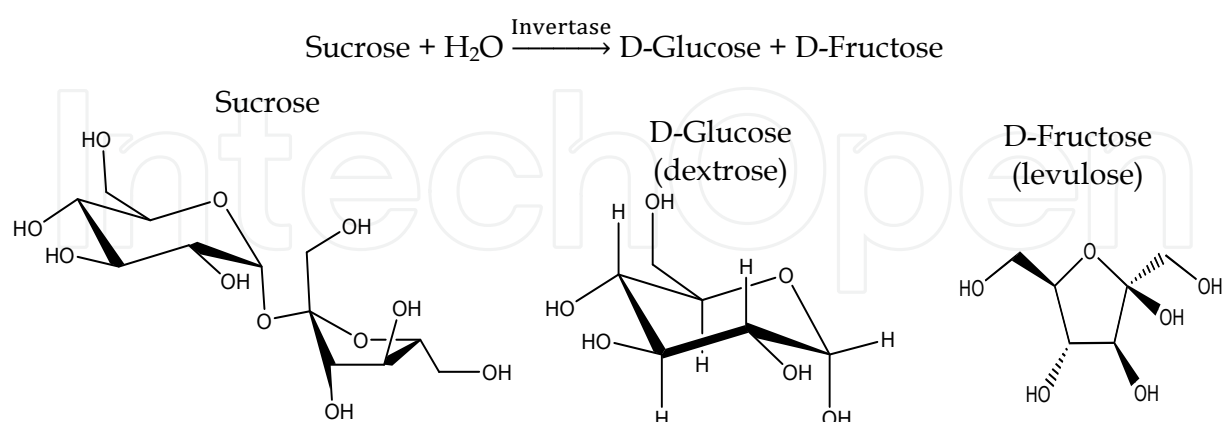


Fig. 1. Structures of sucrose and the cyclic (anomeric) conformations of each monosaccharide produced in step 1 of the invertase detection assay. The reaction proceeds for precisely 5.0 minutes in 20 mM sucrose at pH 4.8 and ambient temperatures of 20-24° C before it is abruptly stopped by denaturation of the enzyme with the rapid addition of an alkaline solution of 3,5 dinitrosalicylate (DNS).

Step 2: Denaturation of invertase and the reduction of DNS: At the end of each 5.0 minute reaction period, the invertase activity in each tube is instantaneously stopped at 30 second intervals by denaturation with the rapid addition of 2.0 mL of alkaline DNS solution (composed of 0.20 M NaOH, 23 mM 3, 5-dinitrosalicylate, and 0.53 M sodium potassium tartrate). After each reaction is “stopped”, the entire set of assay tubes is transferred to a boiling water bath for 7-8 minutes. Under these conditions, the D-Fructose produced in step 1 rapidly isomerizes to D-glucose (see figure 2) which, along with the other mole of glucose produced by the enzyme reaction, reduces the brightly yellow-colored 3,5-dinitrosalicylate to dark orange-colored solutions of 3-amino-5-nitrosalicylate (see figure 3). The entire set of assay tubes is then cooled in a beaker of tap water before each solution is diluted to a final volume of 6.1 mL with the addition of 3.0 mL of 50 mM sodium acetate buffer at pH 4.8.

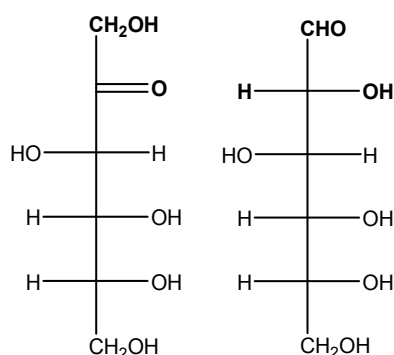
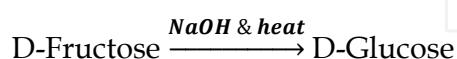


Fig. 2. Fisher projections of the open-chain conformations of the reactants and products of the base-catalyzed isomerization of D-fructose (a non-reducing keto-hexose) to D-glucose (a reducing aldo-hexose) which proceeds in a boiling water bath for about 7-8 minutes during step 2 of the invertase detection assay.

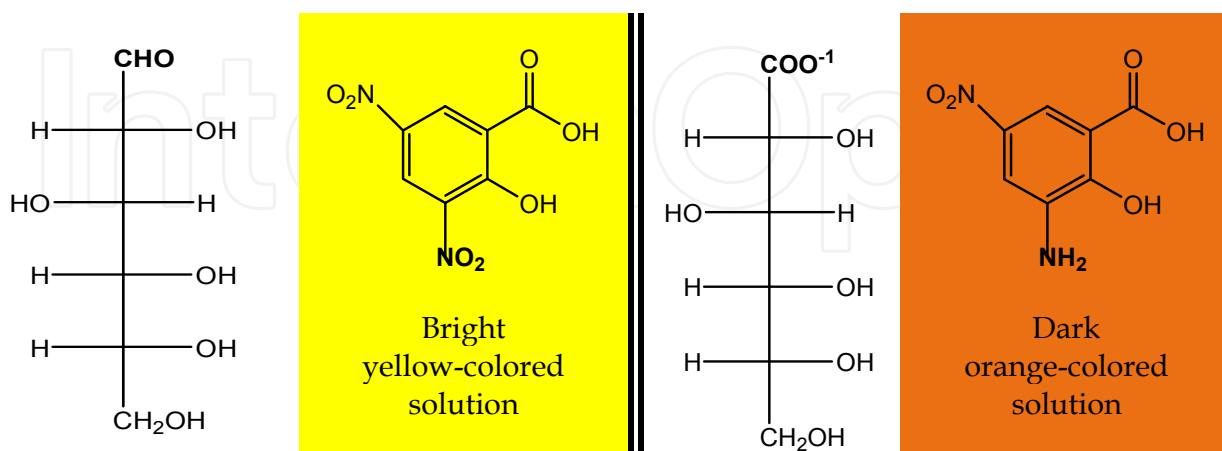
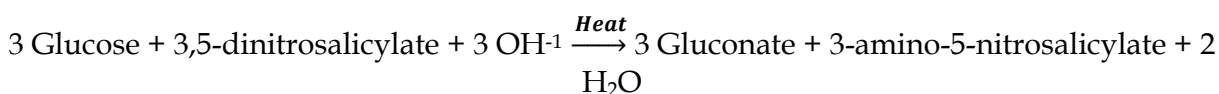


Fig. 3. The structures of the reactants and products for the base catalyzed reduction of brightly yellow-colored solutions of 3,5-dinitrosalicylate into dark orange-colored solutions of 3-amino-5-nitrosalicylate by glucose which is oxidized to gluconate. The reaction proceeds for about 7-8 minutes in a boiling water bath during step 2 of the invertase detection assay.

Step 3: Calculation of invertase activity in each assay tube: A spectrophotometer is used to read the absorbance of the solution in each disposable test tube at 540 nm against a reagent blank prepared exactly as one of the assay tubes in steps 1 and 2 except for the volume of enzyme added to the substrate solution in step 1 is replaced with water (or simply just omitted). Equation 4 is then used to calculate the number of units of invertase activity contained in each tube based upon the absorbance change over the reagent blank (ΔABS) that resulted from the 5.0 minute reaction period and measured in a tube with a 1.0 cm light path and a final volume of 6.1 mL:

$$\text{Units of invertase activity in each assay tube} = \frac{\Delta\text{ABS}/5.0 \text{ min}}{a \cdot 1.0 \text{ cm}} \cdot 6.1 \text{ mL} \quad (4)$$

The value of the milli-molar absorptivity constant (**a**) in equation (4) is determined from the absorbance change (ΔABS) of a calibration tube prepared exactly as one of the assay tubes in steps 1 and 2, except that the volume of enzyme added to the substrate solution in step 1 is replaced with 0.10 mL of a 20 mM stock solution of invert sugar (i.e. a mixture that contains both 20 mM glucose and 20 mM fructose). The dilution of 0.10 mL of 20 mM invert sugar to a final volume of 6.1 mL yields a final concentration of 0.33 mM invert sugar and the absorptivity constant (**a**) is calculated by equation (5):

$$a = \frac{\Delta\text{ABS of calibration tube}}{0.33 \text{ mM invert sugar} \cdot 1 \text{ cm}} = \text{mM}^{-1} \cdot \text{cm}^{-1} = \frac{\text{mL}}{\mu\text{mole invert sugar} \cdot \text{cm}} \quad (5)$$

The calibration tubes prepared in this manner typically have absorbance values of about 0.66 (above the reagent blank) which yields millimolar absorptivity constant values (**a**) of about $2.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ which, for the purpose of calculating enzyme units, is more conveniently expressed as $2.0 \text{ mL} \cdot \mu\text{mole}^{-1} \cdot \text{cm}^{-1}$.

Step 4: Calculation of total invertase activity in each fraction: The final step of the detection assay is to calculate the total number of enzyme units contained in each fraction collected during the purification procedure. This is essentially a straight-forward application of the factor-label method from general chemistry where the number of enzyme units per assay tube (calculated in equation 4) must be converted to the total number of units in an entire fraction by accounting for: (i) the volume of diluted enzyme solution added to the substrate solution in step 1; (ii) the dilution factor of the enzyme solution; and finally, (iii) the total volume of the stock fraction. To keep life simple, all volumes are expressed in mL units:

$$\text{Total units} = \frac{\# \text{ of units}}{6.1 \text{ mL assay}} \cdot \frac{6.1 \text{ mL assay}}{\text{mL of dilute enzyme added}} \cdot \frac{\text{total mL of dilute enzyme}}{\text{mL of stock added}} \cdot \frac{\text{mL of stock fraction}}{1} \quad (6)$$

3.3 Sample calculation

Using the information provided below, calculate the total number of units of invertase activity contained in a 25.0 mL sample of yeast extract.

Data: The 25.0 mL of yeast extract is first diluted by mixing a 0.250 mL sample with 4.75 mL of buffer (for a total dilution volume of 5.00 mL). The dilute extract is then analyzed for invertase activity using the standard 5-minute stop assay described above. Briefly, 0.100 mL of the dilution is mixed into 1.0 mL of substrate solution at 22° C. The reaction is stopped at precisely 5.0 minutes with the addition of 2.0 mL of DNS solution, heated in a boiling water

bath for about 7 minutes and diluted with 3.0 mL of acetate buffer to a final volume of 6.1 mL. The solution in the assay tube has an absorbance value at 540 nm of 0.744 (above a reagent blank) compared to an absorbance value of 0.620 for a calibration tube containing a final concentration of 0.330 mM invert sugar.

Solution:

Step 1, use equation 5 to solve for the value of the millimolar absorptivity constant ($a = 1.86 \text{ mL} \cdot \mu\text{mole}^{-1} \cdot \text{cm}^{-1}$).

Step 2, use equation 4 to calculate the number of units of invertase activity in the assay tube (0.488 units).

Step 3, finally, use equation 6 to calculate the total number of units in 25.0 mL of extract (2440 units).

4. Extraction of Invertase from yeast

After the method for measuring the amount of a specific protein in different samples is established, the second order of business is to optimize a procedure for extracting the functionally active protein from the initial raw material into a defined aqueous solution (the extraction medium). The objectives of this process are straight-forward: First, the tissue must be homogenized or disrupted adequately enough for the protein to be released as a soluble form into the extraction medium. Second, the homogenate must be filtered and centrifuged sufficiently enough to remove any solid, cellular debris from the soluble extract.

The apparent simplicity of this procedure may suggest that tissue extractions are the least complicated task of the entire project; however, in reality, the success of this step, and therefore the outcome of the entire purification, often depends upon the precise control of a surprisingly large number of variables. Some of the more common concerns include: (1) proteins can be denatured by the shearing forces (or chemicals) used to disrupt the initial raw material; (2) proteins can be degraded by digestive enzymes co-extracted from the tissue; (3) proteins can become insoluble or inactivated due to differences between the composition of the cellular fluid and extraction medium (including pH, ionic strength, and the concentration of reducing agents or other specific solutes). In many cases, it is not an exaggeration to state that the success of an entire purification procedure requires a very specific set of extraction conditions including the amount of force, length of time or temperature in which the tissue is homogenized or the precise pH, ionic strength, or concentration of specific supplements (such as protease inhibitors) included in the extraction medium.

While specific details of each extraction must be optimized empirically, there are several rules of thumb that apply in most cases (Scopes, 1982). First, the initial raw material should be the simplest biological system that contains the greatest concentration of the target protein in order to both (i) maximize the final yield and (ii) reduce the amount of other proteins extracted during the homogenization step, especially digestive enzymes or related, but unwanted, isoforms of the target protein. This first point is simply stating the obvious fact that it is always easier to work with as pure and as large of an amount of a protein as possible. For example, in order to isolate an enzyme compartmentalized to bovine heart mitochondria, it would be advantageous to use isolated beef heart mitochondria as the initial raw material rather than an entire beef heart (or an entire cow for that matter).

Second, the initial raw material should be disrupted as gently as possible in order to reduce the risk of protein denaturation. For this reason, protein purification labs are typically equipped with a host of instruments designed to provide a wide range of shearing forces required to physically disrupt different types of tissues (including glass-teflon or glass-glass hand-held homogenizers, electric blenders, freeze clamps, sonicators, and French presses). In addition to these physical methods of disruption, a wide variety of the three general classes of detergents (non-ionic, ionic, or zwitterionic) are available to solubilize (i.e. chemically extract) specific proteins from different tissues and isolated organelles. Third, all of the materials, solutions and equipment should be kept as cold as possible (ideally between 0-4° C) in order to decrease the rate of undesirable proteolytic and other unwanted side reactions. For this reason, preparative (i.e. large scale) operations are often performed in a dedicated cold room while scaled-down procedures can be carried out on buckets of ice. Regardless of the scale of the operation, refrigerated centrifuges (ranging from low to ultra speed devices) are almost always used for separating the soluble extract from the insoluble fraction of the homogenate. Finally, the pH, ionic strength, and other components of the extraction medium are often adjusted to match that of the original cellular conditions in order to maximize both the solubility and stability of the extracted protein.

Since jars of dried baker's yeast can be stored on grocery store shelves for several months at room temperature, it is not surprising that many proteins extracted from dried yeast are also quite stable at ambient temperatures for prolonged periods of time. The isolation of invertase in this project provides the additional benefit that extracellular proteins, in general, tend to be much more stable than intracellular proteins. This observation has been attributed, in part, to the role of protein glycosylation and, since oligosaccharides account for nearly 50% of the composition of invertase, may explain the observation that very little, if any, loss of invertase activity is detected in yeast extracts stored for up to five weeks at 0-4° C. Furthermore, because the yeast cell wall is selectively lysed in dilute solutions of sodium bicarbonate, the extracellular form of invertase is gently extracted from the periplasmic space by simply mixing the contents of a 113 gram (4 oz.) jar of dried yeast from the grocery store into 400 mL of 0.10 M NaHCO₃ and incubating the suspension in a tightly sealed reagent bottle for about 15 hours at 35° C. The insoluble debris (which accounts for roughly one-half of the volume of the lysate) is then separated from the soluble fraction by centrifugation for 30 minutes at 15,000 x g and 4° C. The invertase enriched extract is then simply poured off of the solid pellet, diluted with extraction medium to a final volume of 250 mL and stored at 0-4° C.

Two common types of yeast currently stocked in many grocery stores include: (i) traditional "active-dry" strains which have been used in baking for many generations; and (ii) "bread-machine" strains that have been recently selected or engineered for the purpose of reducing the length of time required for dough to rise in electronic bread makers. Because the dough rising reaction is fueled primarily by table sugar (sucrose), it seems plausible that the new "bread-machine" strains might be characterized by higher concentrations of invertase in order to increase the rate of sucrose digestion and, therefore, allow the dough to rise more rapidly. For this reason, the invertase content of extracts prepared from different commercial brands of "active-dry" and "bread-machine" yeast strains was analyzed to determine the best choice of raw material to use in this project. Not surprisingly, this survey (summarized in table 1) demonstrated that extracts prepared from the bread-machine strains from two different companies contained significantly higher concentrations of

invertase activity (90 to 300 units per mL) compared to the extracts prepared from their corresponding active-dry strains (16 to 82 units per mL). Furthermore, the Red-Star brand of bread-machine yeast was clearly the top choice for the raw material in this project because it consistently produced extracts that contained 2-3 times higher concentrations of invertase activity (190 to 300 units per mL) compared to extracts prepared from Fleischmann's bread-machine yeast (90 to 140 units per mL).

In summary, the isolation of yeast invertase is an especially attractive target for a protein purification project designed for a large group of undergraduate students working in a laboratory at ambient temperatures not only because the starting material is readily available and relatively inexpensive but also because the exceptionally uncomplicated extraction procedure produces a large volume of a solution that is enriched with a remarkably stable enzyme!

Commercial Brand of Yeast	Concentration of Invertase activity in extracts prepared from Active-Dry Yeast (units per mL)	Concentration of invertase activity in extracts prepared from Bread-Machine Yeast (units per mL)
Fleischmann's	44-82 (n = 4)	90-140 (n = 4)
Red-Star	16-20 (n = 3)	190-300 (n = 3)

Table 1. Comparison of the concentration of invertase activity contained in extracts prepared from different commercial sources of dried baker's yeast.

Each extract was prepared by incubating the contents of a 113 g jar of yeast in 400 mL of a 0.10 M aqueous solution of sodium bicarbonate for about 15 hours at 35° C. Each lysate was cooled in an ice-bath and centrifuged for 30 minutes at 15,000 x g and 4° C before the soluble extract was poured off of the pellet, diluted with extraction medium to a final volume of 250 mL and stored at 4° C. One-unit of invertase activity is defined as the hydrolysis of 1-μmole of sucrose per minute at ambient temperatures of 20-22° C in a 40 mM solution of sodium acetate at pH 4.8 containing an initial substrate concentration of 20 mM sucrose. The results in the table represent the range of values measured from extracts prepared from (n) number of different lots of each yeast strain.

5. Purification of invertase from yeast extract

The next goal of the project is to selectively isolate a single, specific protein from the tissue extract while removing as many of the other polypeptides as possible, a task that is traditionally accomplished with a series of sequential **isolation steps** that take advantage of differences in two or more physical properties between individual proteins, such as variations in their size, charge, and solubility. A minimum of two “back-to-back” isolation steps is usually required because, while it may be common for many proteins to have a similar size or a similar charge, it is far less likely for two different proteins to possess both the same size and charge (or some other combination of physical properties). In summary, the overall objective of this stage of the purification is to obtain as pure or homogenous of a

sample as possible in the fewest number of isolation steps. In this project, invertase is purified from a 25 mL sample of fresh yeast extract by (i) differential precipitation with ethanol; (ii) gel filtration; and (iii) ion-exchange chromatography.

5.1 Precipitation of invertase with ethanol

The selective precipitation of a protein from an aqueous solution is one of the oldest, most effective, and technically simple isolation steps used in protein purification. Furthermore, the solid protein precipitated from a large volume of extract can also be concentrated by dissolving it back into a much smaller volume of solvent that is more convenient to apply to a variety of chromatography columns used in subsequent isolation steps. For this reason, it is not uncommon for protein precipitation to be used as the initial isolation step of many purification procedures.

In order for a large protein molecule to become “solvated” or dissolved in an aqueous solution, the majority of its surface must be able to form complexes with an enormous number of water molecules producing a large “hydration shell” that is energetically stabilized by ion-dipole, hydrogen bonds, and dipole-dipole attractive forces between the water molecules and side chains of polar amino acids exposed on the protein’s surface. Because these interactions with the water molecules (i.e. the hydration shell energy) required to keep a protein solvated are sensitive to the pH, ionic strength, and polarity of the solvent; AND, because each protein has a unique hydration shell network, it is possible to selectively entice the surfaces of specific proteins in a mixture to become less and less hydrated, such that their newly exposed surfaces are forced to stick or clump together into an insoluble aggregate or precipitate by simply adding a high enough concentration of an acid (to lower the pH of the solvent), or a salt (to increase the ionic strength of the solvent), or an organic liquid (to decrease the polarity of the solvent). It should be emphasized that protein precipitation is very different from protein denaturation in the vital sense that a protein contained in the solid aggregate retains its native, three-dimensional shape so that it is possible to fully restore its biological function by simply dissolving it back into solution.

In summary, it is possible to partially purify a protein from a mixture by adding a precipitating agent which selectively perturbs the complex set of interactions between its surfaces with the large excess of water molecules from the solvent. The two most common types of precipitating agents used for this purpose are sulfate salts (especially ammonium sulfate) and miscible organic liquids (such as acetone or ethanol). Because salts and organic liquids have a different affect on protein solubility, salts are better precipitating agents for some proteins while organic solvents are better for others (Scopes, 1982). In theory, salts are expected to be more efficient for precipitating proteins which contain larger areas of hydrophobic patches on their surfaces while organic solvents are better for those with surfaces that are almost exclusively dominated by polar amino acid side chains and other hydrophilic groups (such as the carbohydrate chains of a glycoprotein like invertase). Despite these differences in their physical behavior, the same straight-forward and simple set of steps is used by each type of precipitating agent in the procedure: (i) A precipitating agent is added to the extract above the “threshold-concentration” required for the desired protein to become insoluble; (ii) The mixture is incubated for a short period of time to allow the precipitation reaction to go to completion; (iii) The mixture is centrifuged into both a soluble fraction (the decantate) that contains other proteins but is devoid of the target

protein and an insoluble fraction (the solid pellet) which is enriched in the desired protein; and, finally, (iv) The decantate is carefully poured off the pellet which is then dissolved into a much smaller volume of a desired solvent.

In this project, invertase is enriched and concentrated from the initial extract using a 2-step (or differential) method of precipitation with ethanol. In the first precipitation reaction, 10.0 mL of ethanol is added to 25.0 mL of fresh yeast extract, for a final ethanol concentration of 29% (by volume) which is below the threshold concentration required to precipitate invertase activity. After centrifugation, the first decantate (which contains nearly all of the initial invertase activity) is poured off and saved while the first pellet (of contaminating proteins, lipid complexes and other cellular debris) is discarded. In the second precipitation reaction, another 7.0 mL of ethanol is added to the first decantate, for a final volume of about 42 mL and ethanol concentration of about 40% (by volume) which now exceeds the required threshold concentration. Following a short incubation period, the mixture is centrifuged so that the second decantate can be poured off of the invertase enriched pellet which is dissolved in 2.0 mL of gel filtration column buffer composed of 5 mM each NaH_2PO_4 , Na_2HPO_4 , and NaN_3 (included as an anti-bacterial agent) at pH 7.0. In summary, this first isolation step both partially purifies the invertase activity contained in a 25.0 mL sample of yeast extract (fraction 1) and concentrates it about 10-fold in gel filtration column buffer to a final volume of 2-2.5 mL (fraction 2).

5.2 Separation of Invertase by gel filtration

Gel filtration (or size-exclusion) chromatography is a powerful method commonly used to separate proteins based upon their differences in size (McLoughlin, 1992 and Melius, 1971)). More specifically, gel filtration separates particles according to the length of their “**Stokes radius**” which defines the “rotational volume” occupied by the particle as it spins freely in solution. In this sense, separations by gel filtration are affected by particle shape because an elongated, rod-shaped protein will have a much longer Stokes radius (and corresponding rotational volume) than a spherically shaped protein with the same molecular weight. However, because a large percentage of soluble proteins extracted from tissues are characterized as ‘spherically-shaped’ globular proteins, a reasonable correlation often exists between their Stoke’s radius and molecular weight.

In the first step of this procedure, a small volume of a concentrated protein mixture is carefully loaded on to the top of a long column that is packed with a size-exclusion resin composed of tiny, porous beads with a defined mesh-size or cut-off limit. After the sample is loaded, additional buffer is pumped through the resin which drives each particle through the length of the column which acts as a filter that forces larger particles to migrate much more rapidly through the column than smaller proteins. In this manner, it is possible to collect proteins of different sizes into separate fractionation tubes as they elute from the end of the column in the order of larger proteins that come off first in a lower elution volume followed by smaller proteins that are collected in higher elution volumes. Figures 4A and 4B provide a display of the gel filtration apparatus used by students in this project as well as the separation observed shortly after a 1.0 mL solution of blue dextran (a large polysaccharide with a molecular weight of 2,000 kiloDaltons) and hemoglobin (a red colored protein with a molecular weight of 65-70 kilodaltons) is loaded on to the top of the column.

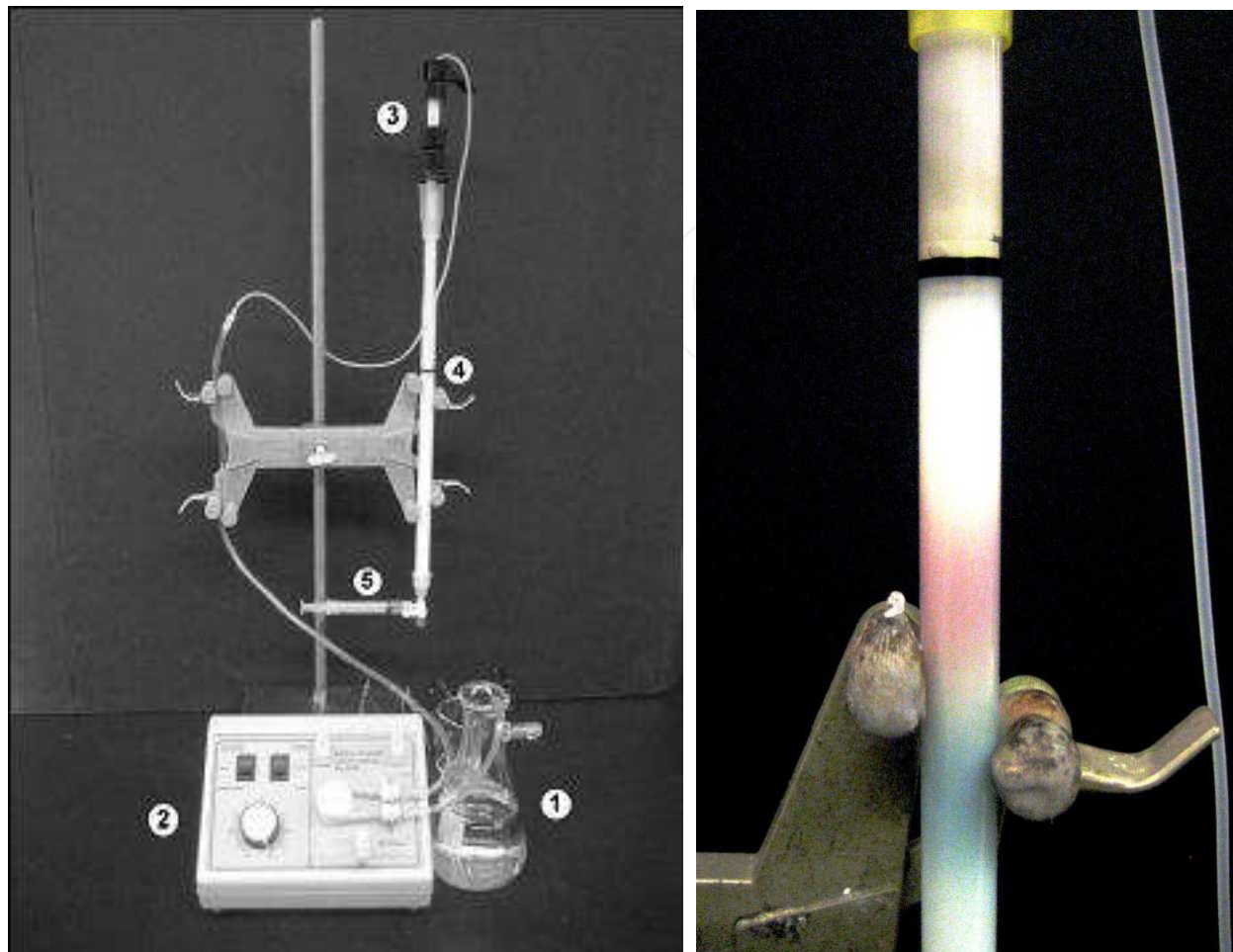


Fig. 4A. (left). The apparatus for purification of yeast invertase by gel filtration chromatography consists of: (1) a reservoir of column buffer contained in the Erlenmeyer flask; (2) a peristaltic pump; (3) An adjustable column flow adapter; (4) a 1 x 42 cm packed column of Sephacryl HR-300 (a shorter column is presented in the photo); and (5) a syringe connected to a three way Luer lock positioned at the bottom of the column.

Fig. 4B. (right). Separation of blue dextran from hemoglobin by gel filtration chromatography shortly after a 1.0 mL solution of 0.75 mg/mL of blue dextran and 1.0 mg/mL hemoglobin was loaded onto a 1 x 42 cm column of Sephacryl HR-300 and separated at a flow rate of about 1.2 mL per minute.

The mesh-size or cut-off limit of the size-exclusion resin is an important parameter of a gel filtration column because it defines the size of the largest, spherically shaped particle that can penetrate the surface of the porous beads and equilibrate into the “internal volume” (V_i) of the column (Scopes, 1982). Since this equilibration is responsible for the slower migration rate of particles that are small enough to be retained by the filtering action of the size-exclusion resin, all particles that are larger than this cut-off limit cannot penetrate into the beads and rapidly migrate through the entire length of the column in the interstitial spaces between the beads called the excluded or “void volume” (V_o). Meanwhile, any protein on the other extreme side of this size-spectrum that is small enough to freely equilibrate between the internal and void volumes must migrate through the column’s total volume

(V_t) which can be calculated from its bed-height (H) and inner diameter (d) [$V_t = H \pi (\frac{1}{2} d)^2$] and is also equivalent to the sum of the void and internal column volumes ($V_t = V_o + V_i$). Due to the restrictions summarized above, the effective range of sizes that can be actually separated on a gel filtration column is limited to moderately sized particles that elute in the internal volume of the column ($V_i = V_t - V_o$) because they are too small to elute in the void volume (V_o) and yet too large to elute in the total volume (V_t). For globular proteins within this limited size range, a linear relationship is observed between the log of their molecular weight ($\log MW$) and the peak of their elution volume (V_e). This relationship has a practical application in that it allows for the native mass of unknown proteins to be estimated by comparing their elution volumes from a size-column that has been calibrated against the elution volumes of standard proteins with known molecular weights (as the one demonstrated in figure 5).

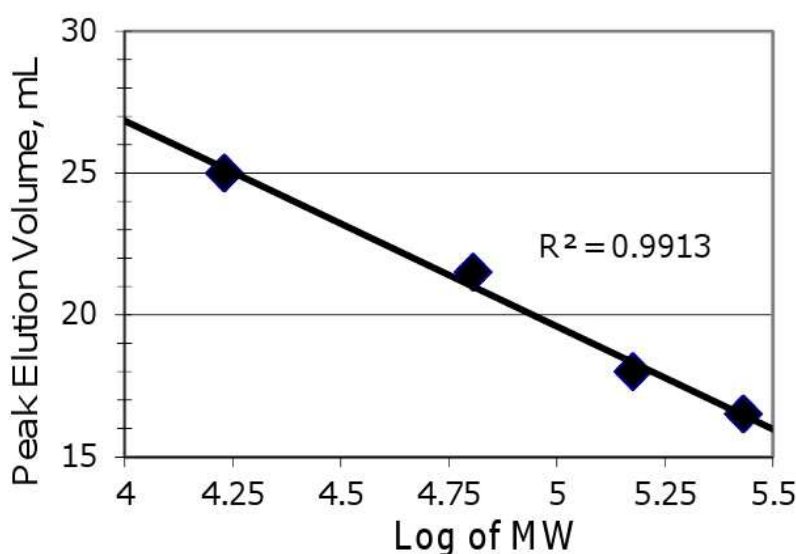


Fig. 5. Sephacryl HR-300 calibration curve. Correlation of the log of the molecular weight and peak elution volumes (V_e) observed for four different proteins collected from a 1 x 42 cm column of Sephacryl HR-300 at a flow rate of about 1.2 mL per minute. The four proteins analyzed (from left to right) are (i) myoglobin, MW 17 kiloDaltons; (ii) hemoglobin, MW 64 kiloDaltons; (iii) alcohol dehydrogenase, MW 150 kiloDaltons; and (iv) yeast invertase, MW 270 kilodaltons.

In order to prepare a gel filtration column that provides an adequate level of resolution for a specific separation, a large number of size-exclusion resins with a wide range of mesh sizes and mechanical flow properties are commercially available. These resins are typically composed of very tiny beads constructed from specific polymers (such as dextrans used to synthesize Sephadex resins and polyacrylamide contained in Sephacryl resins) that are chemically cross-linked to a defined mesh-size. In order to pack the column, a small sample of the dry beads is first hydrated and equilibrated in column buffer which causes the volume of the beads to swell by a factor of ten or more. The mixture is shaken to allow the slurry of resin to be poured into a column where the swollen beads eventually settle by gravity to a desired packed height or column length. Since the actual separation of particles by gel filtration (as mentioned above) is confined to the columns internal volume (V_i), the resolution or degree of separation achieved between two proteins can be improved by

increasing its internal volume which, for a column with a fixed diameter, is proportional to the length of the column. In short, resolution tends to increase as the length of the gel filtration column increases (Scopes, 1982). On the other hand, increasing the length of the column also generates a number of procedural complications (including higher column pressures, lower flow rates, and sample dilution due to zone-broadening) that must be considered in determining the most practical column length to use for the separation.

In this project, the yeast invertase from fraction 2 is further purified by gel filtration chromatography on a (1 x 42 cm) packed column of Sephacryl HR-300, a resin composed of poly-acrylamide beads with a mesh-size designed to exclude globular proteins with molar masses in excess of 300 kilodaltons and engineered to withstand the column pressures encountered at the relatively high rates, HR, of flow of 1-mL per minute. Because invertase is a colorless solute with a native mass of about 250 kiloDaltons, a 1.0 mL sample from fraction 2 (approximately one-half of the 2.0 mL fraction obtained by ethanol precipitation) is first spiked with three visual column markers before it is loaded onto the size-column: (i) blue dextran, the blue colored polymer with a molecular weight of over 2,000 kiloDaltons

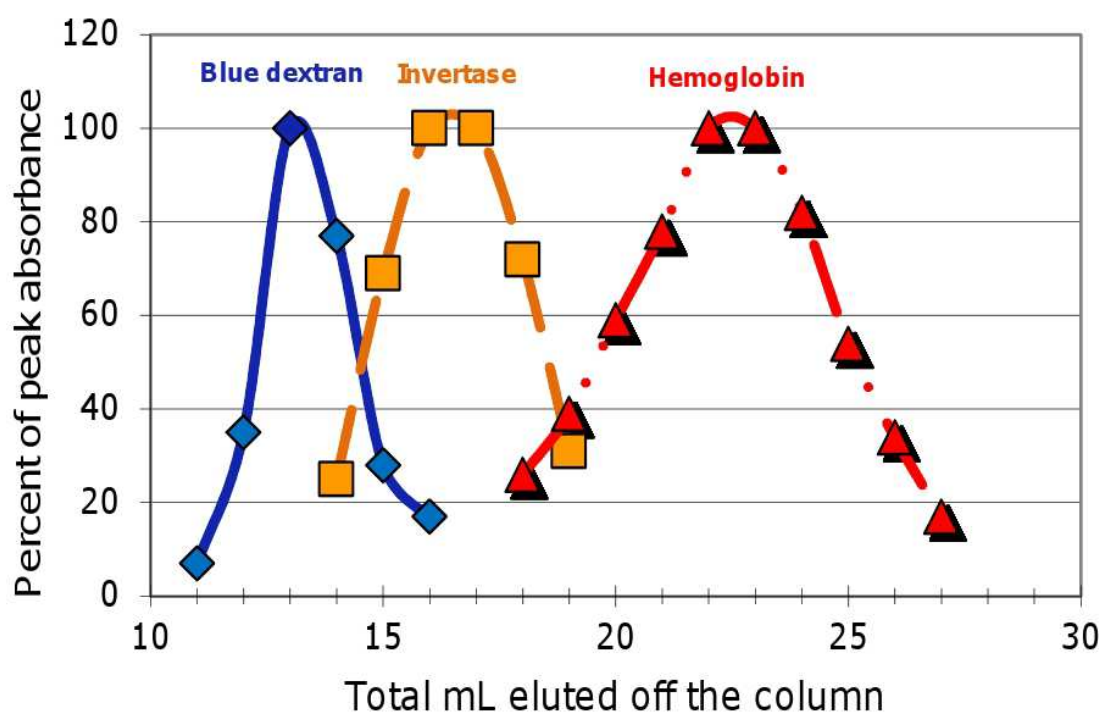


Fig. 6. Elution profiles for the separation of blue dextran, invertase activity, and hemoglobin by gel filtration chromatography. A 1.0 mL sample of F2 (29-40% ethanol cut) was spiked with 0.050 mL each of 15 mg/mL blue dextran and 20 mg/mL hemoglobin. The mixture was then separated on a 1 x 42 cm column of Sephacryl HR-300 at a flow rate of 1.2 mL per minute. The blue dextran in fractions 11-16 was detected on a Novaspec spectrophotometer at 620 nm with a peak absorbance value of 0.295 in fraction 13. The red tinted hemoglobin fractions (18-27) were detected at 420 nm with a peak absorbance value of 0.800 in fractions 22 and 23. Invertase activity in 10.0 μ L samples of fractions 14-19 was monitored by the reduction of DNS by glucose to produce the orange-colored product (3-nitro-5-amino salicylate) which is detected at 540 nm.

used to mark the void volume (V_0) and is expected to elute ahead of the smaller, colorless molecules of invertase; (ii) hemoglobin, a red colored protein with a native mass that is about one-fourth the size of invertase that is expected to elute from the column after invertase. In this manner, the colorless molecules of invertase are expected to elute from the column in between the peak elution volumes observed for blue dextran and hemoglobin; and finally, (iii) dinitrophenol aspartic acid (DNP-Asp) a yellow-colored derivative of a small amino acid which marks the total volume of the column (V_t). After loading, the sample is separated on the column at a flow rate of 1.2-1.3 mL per minute while hand collecting 1-mL fractions from the end of the column in small disposable test-tubes. During the run, the three visual markers are observed to separate (as in figure 4B) and then eventually elute from the column in peak volumes of 13 mL for blue dextran, followed by 22-23 mL for hemoglobin, and finally over 30 mL for DNP-Asp. Small samples (10 μ L) from each of the 1-mL fractions collected between the peak tubes of blue dextran and hemoglobin are analyzed for invertase activity (using the standard 5-minute stop assay) so that the four fractionation tubes containing the most invertase activity (typically tubes 15-18) can be pooled together to obtain a new 4-mL fraction of purified invertase (fraction 3). Figure 6 shows the results of a typical elution profile for blue dextran, invertase and hemoglobin from the gel filtration column.

5.3 Isolation of Invertase by ion exchange (Adsorption) chromatography

While ion exchange chromatography is commonly used to separate proteins on the basis of their charge differences, it is a specific example of a more general separation process called adsorption chromatography that also includes both hydrophobic-interaction chromatography and affinity (or ligand-binding) chromatography. In each case, the protein of interest selectively binds or adsorbs to a solid resin packed in a column while other proteins simply flow through or are washed off before the target protein is selectively dissociated from the column (Scopes, 1982). In addition to being a powerful purification tool, adsorption chromatography also provides a practical method of concentrating dilute protein solutions. For example, a protein contained in 100 mL of solution can be concentrated 50-fold by adsorbing it to a column with a 1.0 mL bed volume and eluting it off in a final volume of 2.0 mL. The general procedure for each type of adsorption chromatography method can be divided into four common steps:

Packing: the resin is first mixed with a small volume of equilibration solution (which allows the protein of interest to bind the column) to form a slurry that can be poured into a column and allowed to settle by gravity to a desired bed height or column volume.

Loading: the sample (ideally contained in the same solution used to pack the column) is then pumped through the resin at a flow rate that is slow enough to permit the specific adsorption reaction to come to completion. To insure maximal binding, the flow through is often collected and re-applied to the column (or continuously pumped through the column for a defined period of time). Batch loading refers to an alternative method of packing and loading a column in which the packing slurry is prepared by mixing the resin directly into the sample solution prior to packing the column.

Washing: after loading the sample, the column is washed by pumping a minimum of two to three bed volumes of solution to remove both (i) non-adsorbed proteins trapped in the

interstitial spaces of the resin, and (ii) other proteins adsorbed to the column less tightly than the target protein.

Dissociating: the protein of interest is finally eluted from the column using 2-3 bed volumes of solution and a slow enough flow rate to maximize its yield off the column.

Separations by ion exchange chromatography are sub-classified into anion vs. cation exchange chromatography based upon the charge of the particles that bind to the resin. In the case of anion exchange chromatography used in this project, negatively charged proteins in the sample are first absorbed via salt bridges (i.e. electrostatic attractive forces) to a positively charged anion-exchange resin commercially produced by conjugating an insoluble matrix (such as cellulose) with a quaternary amine or other positively charged functional group. In contrast, cation-exchange resins are prepared by the incorporation of a negatively charged functional group (such as a carboxylate or sulfate) to the matrix. The loaded anion-exchange column is then washed with increasing concentrations of salt, typically NaCl, until the chloride ion reaches a high enough concentration to displace each protein from the column by replacing the salt bridge formed between the resin and protein (hence the term anion exchange chromatography). In this manner, proteins are sequentially eluted from the ion exchange column in order of the weakest to strongest binding affinities to the resin which, in turn, is a function of the charge density contained on localized regions of the protein's surface.

Therefore, the purpose of the third and final isolation step of this procedure is to use an anion-exchange column to further purify and concentrate the dilute solution of invertase contained in fraction 3. For this purpose, 3.5 mL of the blue-tinted, 4.0 mL sample pooled from the gel filtration column is slowly loaded onto a 0.5 mL packed bed volume of DEAE cellulose (i.e. an anion exchange resin composed of cellulose derivitized with diethyl-amino-ethane). After loading, the non-adsorbed particles are washed from the one-half milliliter column with 2.0 mL (i.e. four bed volumes) of gel filtration column buffer before the final fraction of invertase activity (fraction 4) is obtained by dissociating it from the column with 1.0 mL (or 2 bed volumes) of column buffer supplemented with 50 mM NaCl. For demonstrative purposes, proteins that bind to the DEAE-cellulose column more tightly than invertase are then dissociated with 1.0 mL of 250 mM NaCl and also saved for analysis by SDS-PAGE).

6. Assessment of invertase purity

In the last stage of this project, the relative purity of the initial yeast extract (fraction 1) and each fraction obtained from the three sequential isolation steps (fractions 2-4) is compared to that of a commercial source of invertase (Sigma Product Information Bulletin I4504) using both a visual, qualitative method (SDS-PAGE) in addition to the more precise quantitative evaluation of specific activity measurements.

6.1 SDS-PAGE analysis

SDS-PAGE is an acronym for sodium dodecyl sulfate polyacrylamide gel electrophoresis which is a common technique used to separate, visualize, and therefore compare the relative amount of individual polypeptide chains contained in different fractions (Jisnusun &

Bhinyo, 1977 and Roberts et al., 1977). In practice, the procedure takes place in three general steps:

Denaturation: a small sample from each fraction is first mixed with an excess of SDS (sodium dodecyl sulfate, an ionic detergent), β ME (β -mercaptoethanol, a thiol reducing agent), and bromophenol blue (an intensely colored dye with a negative charge and low molecular weight that is used to track the progress of each sample through the gel during electrophoresis) (Laemmli, 1970). Each mixture is then placed in a boiling water bath for several minutes where the β ME reduces all disulfide bridges to sulfhydryl groups (disrupting tertiary protein structure) and the dodecyl sulfate anions bind to the backbone of each polypeptide at a ratio of about 1 anion for every 2 residues (disturbing secondary, tertiary and quaternary levels of protein structure). In the end, each polypeptide chain is unfolded into a negatively-charged, rod-shaped complex with a relatively constant charge to mass ratio (that is, the native charge of the polypeptide in the complex is essentially masked by the huge excess of detergent anions).

Electrophoresis: a small aliquot from each denatured sample is transferred to separate wells of a solid, rectangular gel of polyacrylamide that is crosslinked to a desired mesh or size-exclusion limit. An electric field is applied across the loaded gel with the positive pole positioned on the opposite side of the samples in order to drive the negatively-charged complexes through the mesh of polyacrylamide which effectively filters them according to the length of their Stokes radius as they wiggle through the porous matrix to the opposite side of the gel. Since small particles move through the gel more rapidly than larger ones, the electrophoresis is visually followed by the movement of the small bromophenol blue tracking dye to insure that the power supply is shut off before any protein in the sample reaches the bottom of the gel.

Staining and destaining: following electrophoresis, the entire gel is soaked in a stain solution containing a dye that tightly binds to the backbone of each denatured polypeptide chain (usually Coomassie brilliant blue). Excess stain is then washed from the gel by soaking it in a destain solution long enough for the blue-stained bands of each polypeptide to be visualized against the clear, colorless background of the gel.

Comparison of separations by gel filtration vs. SDS-PAGE: Because the separations achieved by SDS-PAGE and gel filtration chromatography are based upon the same physical property, i.e. differences in the Stokes radius of each particle, it is not surprising that a linear relationship is observed between the log of the protein's molecular weight and its relative mobility through a size column or polyacrylamide gel. Despite this similarity, there are two crucially important differences between the separations obtained by these two methods. First, larger proteins migrate down gel filtration columns more rapidly than smaller ones because proteins with a large Stokes radius are less likely to enter the internal volume of the resin and are restricted to the interstitial spaces between the beads. In SDS-PAGE, however, this relationship is exactly reversed as smaller proteins move through a gel more rapidly than larger ones because they are being forced to migrate through a single, continuous barrier instead of a column of packed beads. Since there is no exit route around the barrier in SDS-PAGE (i.e. there is no equivalent of the void volume), the larger proteins are simply forced to stack up on the top of the gel while exceptionally small proteins migrate towards the bottom of the gel very closely to the small tracking dye.

The second important difference is that while the separation of native proteins by gel filtration is affected by the shape of the particle (elongated proteins have a larger Stokes radius and move through the column more rapidly and appear to be larger than spherically shaped proteins of the same mass), shape is not a factor in SDS-PAGE because the proteins have been completely denatured into complexes with similar shapes and charge densities prior to electrophoresis. For this reason, protein separations by SDS-PAGE are based solely upon the length (i.e. the number of amino acids) of each polypeptide chain, which is, in turn, essentially proportional to its molecular weight. In fact, under ideal conditions, SDS-PAGE can effectively separate two polypeptide chains that differ in length by just a few amino acids, a degree of separation that is simply impossible to achieve by gel filtration chromatography.

For these reasons, gel filtration and SDS-PAGE provide different, but related, information about the mass of purified multimeric proteins that are composed of two or more polypeptide chains (Scopes, 1982). That is, gel filtration data is used to estimate the mass of the entire set of subunits that comprise the quaternary structure of the functional molecule while SDS-PAGE yields the mass of each individual subunit derived from the denatured complex. In the end, the mass of the native structure estimated by gel filtration must be equal to the sum of its individual polypeptide chains determined by SDS-PAGE. Hemoglobin, a hetero-tetrameric oxygen transport protein composed of two alpha and two beta subunits (Perutz, et al, 1960), provides a classic example of the mass information obtained by these two methods. Gel filtration experiments indicate the functional hemoglobin molecule has a native mass of about 65 kiloDaltons while SDS-PAGE analysis reveals the pure protein is composed of two different polypeptide chains with masses of about 16-17 kiloDaltons. Because the color intensity of the two bands observed in the gel is very similar, the results further suggest that the native molecule contains a similar mass of each subunit such that the quaternary structure must contain two copies of each polypeptide in order to account for a native mass of 65-70 kiloDaltons.

In summary, SDS-PAGE analysis has developed into a vital assessment tool in protein purification because it provides a rapid and visual comparison of both the relative purity and the amount of a specific protein contained in different fractions collected during the isolation procedure. The degree of purity between different fractions, and therefore the effectiveness of each isolation step, is evaluated by simply comparing the total number of blue stained bands observed in each fraction. That is, with each isolation step in the procedure, one expects each new fraction to yield fewer and fewer bands on the gel until a homogeneous fraction (composed of just one protein) is obtained. For the simplest case of proteins composed of just one single type of polypeptide chain, the point of homogeneity in the purification is defined by the observance of just a single band in the gel. Furthermore, since the color intensity or darkness of each band is proportional to protein mass, the relative amount of a specific protein loaded onto the gel from each different fraction is estimated by simply comparing the darkness of each band. Finally, the molecular weight of each polypeptide in the gel can be estimated by simply comparing its distance of migration or relative mobility (R_f) with that of a set of proteins of known molecular weight (i.e. molecular weight markers) loaded onto a separate well of the gel.

The results of SDS-PAGE analysis on a typical set of fractions collected by students during the isolation of invertase from baker's yeast is presented below in figure 7. The first lane on

the left (labeled M) contains a set of molecular weight markers used to estimate the mass of proteins contained in samples prepared from the following fractions (from left to right): F1 (the initial 25 mL of fresh yeast extract); F2 (the 2.0 mL fraction obtained by precipitation in 29-40% ethanol); F3 (the 4 mL peak of invertase activity collected off the gel filtration column); FT (the flow through of proteins that did not absorb to the DEAE resin); F4 (the 1 mL invertase enriched fraction eluted from the DEAE resin with 50 mM NaCl); HSW (the 1 mL high salt wash of proteins eluted from the DEAE column with 250 mM NaCl); and, finally, Σ (a solution of commercial invertase purchased from Sigma Chemical Company prepared by dissolving a weighed mass of the solid protein in column buffer to a final concentration of 1 mg per mL).

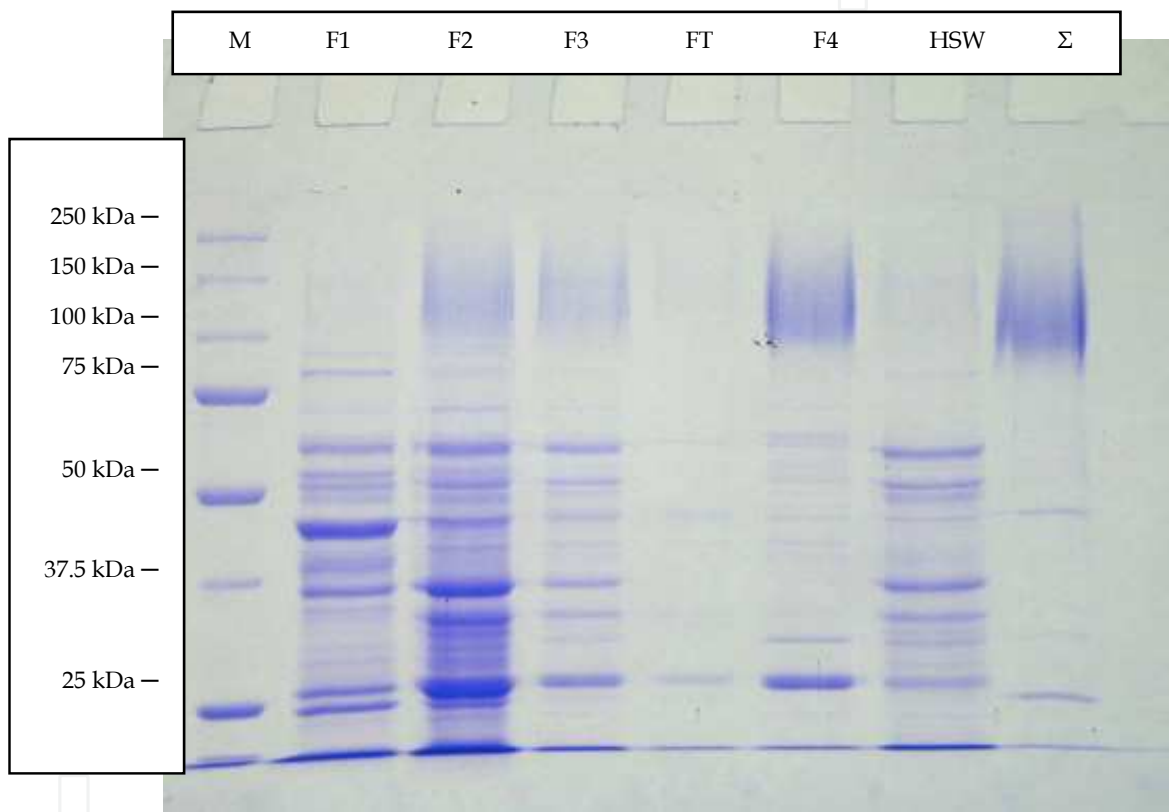


Fig. 7. SDS-PAGE analysis of fractions obtained in the isolation of invertase from baker's yeast. All samples were denatured (Laemmli, 1970) and reduced by mixing with an excess of SDS, β -mercaptoethanol and heated in a boiling water bath prior to separation by electrophoresis at 2 W of constant power on a discontinuous gel (composed of an 8% acrylamide resolving gel and a 4.5% stacking gel each with an acrylamide to bis-acrylamide ratio of 1:29).

M = Biorad Precision Plus Molecular Weight Markers with masses listed for the seven visible bands (as shown from the top to bottom) equal to 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37.5 kDa and 25 kDa.

Other lane labels are described in the text.

The lane containing the commercial sample of invertase (Σ) on the far right is clearly dominated by the presence of a large band that is both intensely stained and smeared as it migrates (compared to all other bands) over an unusually wide range of molecular weights

extending from 100 to 150 kDa. This observation is consistent with previous work characterizing yeast invertase as a large protein with a native mass of 270 kDa composed of two identical and heavily glycosylated subunits with masses of about 135 kDa. For an undergraduate exercise, this unusually smudged appearance of the invertase band (which is due to the extensive but variable amount of sugars attached to the subunits) is a benefit that allows an inexperienced observer to readily identify the position of invertase in each sample and visually assess the relative degree of purity in each fraction by comparing the darkness of the invertase band vs. that of the other bands in each lane.

The SDS-PAGE analysis unambiguously demonstrates that an impressive and significant degree of purification is achieved between the initial yeast extract (F1) and final fraction (F4) which is dominated by the presence of the same large, smeared band observed in the commercial sample which was barely visible in the initial extract (F1). Furthermore, this analysis also indicates that this final fraction (F4) is significantly less pure than the commercial source of the enzyme (Σ) as evidenced by the difference in the number of contaminating bands with masses between 25-75 kDa in the two fractions. On the other hand, it is difficult to assess the sequential increase in purity anticipated with each step of the procedure (from F1-F4) by SDS-PAGE analysis because, in addition to being purified, the proteins precipitated in the first isolation step were also concentrated 10-fold in F2 and then subsequently diluted 4-fold in the second isolation step by gel filtration chromatography in F3. In spite of this complication with large increases and decreases in concentrations, the expected trend in which the invertase band represents a larger and larger percentage of the total protein in each fraction seems to be apparent on the gel.

6.2 Specific activity measurements

Specific activity is defined as the ratio of the total number of units of enzyme activity vs. the total mass of protein contained in a sample and is most commonly expressed as “enzyme units per mg of protein” (or more simply as units/mg). Because the overall goal of the purification is to obviously maximize the yield of the total number of enzyme units from the initial tissue extract while removing as much other protein as possible, the specific activity of each fraction is expected to increase with each isolation step until it plateaus to a maximal value that is a characteristic, intrinsic property (independent of the concentration or amount) of the purified enzyme. That is, it is impossible for the specific activity to increase beyond the homogeneity point of the purification procedure because any additional isolation step will result in a proportional loss of both total protein (which is now 100% enzyme) and enzyme activity to produce a constant specific activity ratio.

Therefore, the final task of this project is to quantitatively assess the effectiveness of the isolation procedure by carefully measuring the specific activity of each isolated fraction and comparing each one against the specific activity of commercial invertase which, according to the previous analysis by SDS-PAGE analysis, represents a nearly homogenous sample of the enzyme. In the end, a reasonable agreement is expected between the analysis by SDS-PAGE and specific activity measurements. That is, with each isolation step in the procedure, the specific activity should increase as the number of contaminating bands in the gel decreases until the homogeneity point is detected by a plateau in the specific activity measurement and the appearance of just a single protein in the gel. In short, specific activity is an

important index by which the relative purity of an enzyme in different samples can be compared. That is, a fraction with a specific activity of 50 units per mg is described to be five-times more pure than a fraction with a specific activity of 10 units per mg. For this reason, the effectiveness of each isolation step in the procedure can be assessed by simply determining the extent by which it increases the specific activity of the enzyme.

In practice, the result of each isolation step used in a procedure is traditionally presented as a **purification table**, which is simply a summary of the total amount of protein, enzyme activity and specific activity contained in each isolated fraction. However, before the specific activity values of different samples are compared, it is important to recognize that specific activity represents a conditional property of an enzyme (an issue that can be very frustrating for undergraduate students accustomed to measuring non-conditional properties of matter such as length or mass). That is, because the methods used to measure both activity units and protein mass are dependent upon many variables that must be precisely controlled in each assay (including temperature, pH, and reagent concentrations which are, in turn, affected by the total assay volume), it is very plausible for two different groups to honestly report widely different specific activity values for the exact same set of samples by simply changing the assay conditions! For this reason, purification tables are required to include a detailed list of the precise set of conditions used to measure both enzyme activity and protein content (or a reference to this list). Perhaps most important in terms of laboratory procedures, this conditional nature is an important issue in order to understand and appreciate the vital role that sampling consistency plays in reducing many experimental errors that affect specific activity measurements. For this reason, whenever possible, the assays for each fraction reported in a purification table should be performed at the same time, using the same reagents, instruments, equipment, and, perhaps, even the same person.

Table 2 presents a traditional purification table summarizing the average results of specific activity measurements obtained by eight different groups of students for the same initial yeast extract (F1), the three fractions obtained from each isolation step (F2-F4), and a stock solution of commercial invertase prepared by dissolving solid protein with column buffer to a final concentration of 1.0 mg per mL (Σ). In terms of percent yields, the table clearly shows a sequential decrease in the total mass of protein in each fraction (which was measured indirectly by a modification of the Bradford dye binding technique using bovine serum albumin as the standard reference protein) starting with an average of 39 milligrams in the initial 25 mL of yeast extract to only 0.16 milligrams in the final 1-mL fraction (F4) for a combined percent yield of just 0.41% of the initial protein. Likewise, the total number of enzyme units (as measured spectrophotometrically using the 5-minute stop assay described previously) also dropped with each step from an initial value of 1070 units to 72 units in F4 which is an overall percent yield of just 6.7 % of the initial enzyme activity (but still 16 times higher than the percent yield of total protein). However, in contrast to the loses of both total protein and activity units, the table indicates a sequential and significant increase in the specific activity of each fraction starting from 28 units per mg in the initial extract to 490 units per mg in the final fraction (F4) and an overall n-fold purification of 17.5 (that is, the invertase contained in the final fraction, F4, is, on average, 17.5 times more pure than the invertase in the initial yeast extract). These results simply confirm (as previously observed by SDS-PAGE analysis) that each step of the procedure succeeded in selectively retaining a higher percentage of the invertase compared to other proteins in each sample.

Fraction	Volume (mL)	Total Protein (mg)	Total Activity (units)	Specific activity (units / mg)	n-fold purification (vs. F1)
F1	25.0	39 (± 6)	1070 (± 95)	28 (± 5)	1.0
F2	2.0	8.2 (± 2.6)	717 (± 100)	94 (± 24)	3.4
F3	4.0	0.72 (± .33)	145 (± 60)	210 (± 80)	7.5
F4	1.0	0.16 (± 0.08)	72 (± 27)	490 (± 180)	17.5
Σ	1.0	0.11 (± 6)	115 (± 15)	1100 (± 300)	39

Table 2. Purification table for the isolation of invertase from Baker’s Yeast

All data reflect the average values (± standard deviation) reported by 8 different groups of students using the same yeast extract (F1) prepared from Red-Star bread machine yeast. Protein values were measured by a modification of the Bradford dye binding method using bovine serum albumin as the reference protein (Zor & Selinger, 1996). Invertase activity was monitored at pH 4.8, ambient temperature (20-24° C), and 20 mM sucrose using the standard 5-minute stop assay to reduce 3,5 dinitrosalicylate to 3-nitro-5-amino salicylate which is detected at 540 nm . F1= Yeast extract; F2 = 29-40% ethanol cut; F3 = gel filtration peak; F4 = DEAE enriched invertase; Σ = Commercial invertase (Sigma chemical company) dissolved in gel filtration column buffer to a final concentration of 1 mg per mL.

In comparing the effectiveness of the three isolation steps used in the procedure, the precipitation with ethanol appears to have been the most efficient step by providing a 3.5-fold increase in the average specific activity of invertase compared to the additional increases of 2.2- and 2.3-fold obtained by gel filtration and anion exchange, respectively. Within this context, it should be pointed out that because the specific activity of the nearly-homogenous sample of commercial invertase (as judged by SDS-PAGE) is equal to 1100 units per mg, that the maximum n-fold purification possible from this extract (at 28 units per mg) is a 39-fold increase. Therefore, the combined 17.5-fold enrichment obtained at the end of the three steps in this project accounts for about 45% of the upper limit. Likewise, by comparing the specific activity of the commercial enzyme (1100 units per mg) with the total number of units in the initial extract (1070 units), one can estimate that invertase accounts for about 1 out of the 39 mg of total protein (or 2.5%) contained in the initial 25 mL of extract prepared from the Red-Star bread machine yeast.

Finally, the results of this project demonstrate excellent agreement between the SDS-PAGE analysis and specific activity measurements in comparing the purity of the final fraction (F4) with that of the commercial invertase (Σ). That is, the commercial enzyme appears much more pure on the gel because it contains far fewer contaminating bands than that observed in DEAE enriched fraction of invertase (F4). Likewise, the specific activity of the commercial enzyme (1100 units per mg) is about 2.2-times higher than the final fraction (490 units per mg). While it may seem disappointing that, after all of this work, the students do not obtain as pure of a sample as the commercial enzyme, it must be remembered that these steps were not optimized to maximize either the yield or purity of invertase. Instead the steps were designed to introduce general methods of protein purification to large groups of undergraduate students over the course of several three-hour laboratory periods. If a more

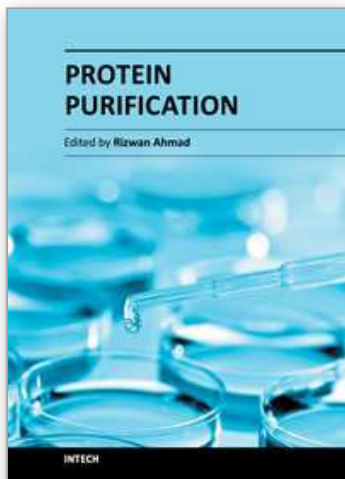
pure fraction of invertase is desired, each of the following modifications to this procedure would be worth considering: (i) first and foremost, start with a much larger volume of the initial extract (such as 250 – 1000 mL) because it is always better to work with a larger vs. smaller amount of the desired protein; (ii) use a much larger and longer gel filtration column in order to be able to both load larger samples and maximize the separation between invertase and other proteins on the column; (iii) raise the concentration of salt in the gel filtration column buffer in order to reduce the chance that other proteins might adsorb-to and co-elute with invertase off the size-column; (iv) monitor each one of the invertase enriched fractions eluted from the size-column by SDS-PAGE in order to discard the most contaminated fractions from the ones that are pooled together and applied to the anion-exchange column; (v) improve the efficiency of the ion-exchange column by using a salt gradient to gradually increase the sodium chloride concentration and dissociate the enzyme from the column more selectively; (vi) incorporate an additional or different isolation step into the procedure, such as lectin affinity chromatography (lectins, such as concanavalin A and wheat germ agglutinin, are sugar binding proteins that, when immobilized to a solid column support are useful in the purification of glycoproteins).

7. Conclusion

The exercises described in this chapter provide a practical, hands-on introduction to many general considerations and corresponding strategies encountered during the course of isolating a specific protein from its initial biological source. Furthermore, the project is especially well suited for incorporation into an undergraduate laboratory curriculum for several reasons: First, the starting material, dried baker's yeast, is relatively inexpensive and can be obtained from most local grocery stores. In addition, the procedure required to extract invertase from the periplasmic space of yeast cells is both exceptionally uncomplicated and yields a large volume of an extract that is enriched with an uncommonly stable glycoprotein that is easy to detect by SDS-PAGE analysis. Second, the materials and equipment required for the detection assay, isolation steps, and final analysis are also reasonably inexpensive and currently stocked in most undergraduate laboratories. Finally, the isolation steps are designed to infuse a large number of visual cues into the isolation of a colorless enzyme from a natural product (and therefore it represents a more typical real life example compared to isolating colored proteins, such as green fluorescent protein or myoglobin (Miller et al. 2010), that are over expressed in genetically modified sources). These visual cues include the following: (i) the 2-step precipitation in ethanol requires students to consider where their enzyme is located at all times (the decantate or pellet); (ii) the addition of visual markers to the sample prior to gel filtration lets them know where their enzyme is located on and off the column; (iii) contaminating blue dextran in the gel filtration enriched fraction of invertase is removed in the last isolation step by adsorption to the DEAE cellulose column, in this way they know the column is working; (iv) The fractions are first visually analyzed by SDS-PAGE, prior to performing the more technically challenging and less intuitive estimate of purity by specific activity measurements; and, finally (v) commercial sources of the pure enzyme to serve as a gold standard of comparison against their own results is readily available and inexpensive.

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