Advances in **Protein Purification**

Caroline Gardner

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Edited by **Caroline Gardner**

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Preface

This book provides an in-depth knowledge of protein purification. The book is designed and written to expedite speedy access to important information regarding protein purification and its various methodologies. It intends to present a summary on the latest methods for the purification, examination and quantification of proteins in composite samples utilizing various enrichment techniques.

This book has been the outcome of endless efforts put in by authors and researchers on various issues and topics within the field. The book is a comprehensive collection of significant researches that are addressed in a variety of chapters. It will surely enhance the knowledge of the field among readers across the globe.

It is indeed an immense pleasure to thank our researchers and authors for their efforts to submit their piece of writing before the deadlines. Finally in the end, I would like to thank my family and colleagues who have been a great source of inspiration and support.

Editor

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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.

$$\text{Sucrose (aq)} + H_2O \xrightarrow{Invertase} \text{Fructose (aq)} + \text{glucose (aq) ("invert" sugar)} \quad (1)$$

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 Km 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 (µmole • min^{-1}) are different than the traditional units used to describe chemical reaction rates (µmoles • mL^{-1} • 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 Km 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 mM^{-1}•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 **(ΔABS/min)** must correspond to a proportional rate of change in the millimolar concentration **(ΔC/min)** of the colored solute, as outlined in equation (2):

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

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 **(Δ Abs/min)** and the fixed volume **(VmL)** of the colored solution in the cuvette with a fixed light path, as summarized in equation 3.

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

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.

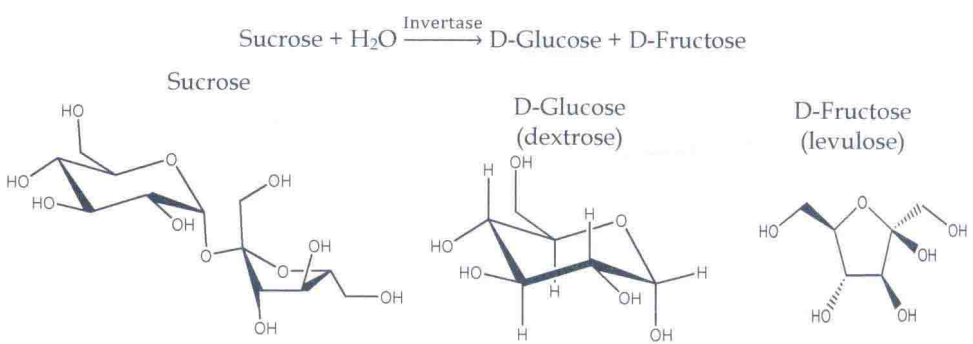

Fig. 1. Structures of sucrose and the cyclic (anomeric) conformations of each monosaccharide produced in step 1 of the invertase detection assay. The reaction proceeds for precisely 5.0 minutes in 20 mM sucrose at pH 4.8 and ambient temperatures of 20-24° C before it is abruptly stopped by denaturation 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.

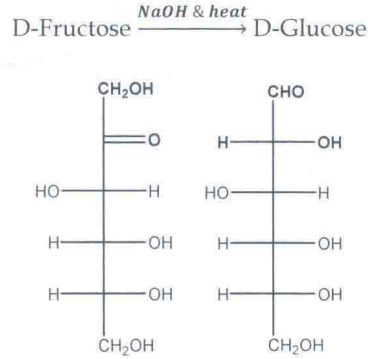

Fig. 2. Fisher projections of the open-chain conformations of the reactants and products of the base-catalyzed isomerization of D-fructose (a non-reducing keto-hexose) to D-glucose (a reducing aldo-hexose) which proceeds in a boiling water bath for about 7-8 minutes during step 2 of the invertase detection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

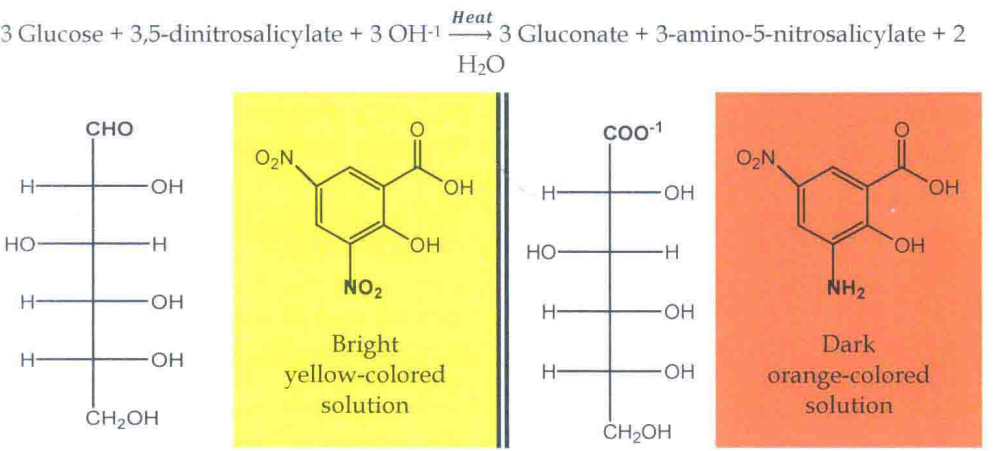

Fig. 3. The structures of the reactants and products for the base catalyzed reduction of brightly yellow-colored solutions of 3,5-dinitrosalicylate into dark orange-colored solutions of 3-amino-5-nitrosalicylate by glucose which is oxidized to gluconate. The reaction proceeds for about 7-8 minutes in 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:

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

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):

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

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 $\text{mM}^{-1} \bullet \text{cm}^{-1}$ which, for the purpose of calculating enzyme units, is more conveniently expressed as 2.0 $\text{mL} \bullet \mu\text{mole}^{-1} \bullet \text{cm}^{-1}$.

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:

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

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 $mL \cdot \mu mole^{-1} \cdot cm^{-1}$).

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 $NaHCO_3$ 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.