Yeast Cell Envelopes: Biochemistry, Biophysics, and Ultrastructure

Volume II

Editor

Wilfred Niels Arnold, Ph.D.

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PREFACE

Yeasts hold important associations with man in at least three arenas. As industrial agents their positions in the fermentative and baking industries are household knowledge. A few yeast or yeast-like species are pathogenic. And, as experimental eukaryotic cells, yeasts continue their long history as useful subjects for studies on metabolism, genetics, and molecular biology.

A comprehensive review of the yeast cell envelope has not appeared previously and we trust that this attempt will be timely. The title of this volume was chosen to reflect the three major areas of contribution to our current understanding of the cell envelope, but we have not attempted to group chapters into subdivisions. That would be somewhat arbitrary at best. In fact, the contributing authors were recruited for their interdisciplinary work as well as their special expertise.

The approach is to describe phenomena, to review the literature, and to illuminate outstanding problems. We have also attempted to generate working hypotheses which may stimulate further studies. That some of these ideas be of germinal value is of more concern to us than that all of the hypotheses should stand the test of further experimentation.

Brenda Johnson has given special assistance in the assemblage of this volume. I also wish to acknowledge my former teachers and mentors Drs. G. Langdon, J. Middleton, J. Bald, and J. Thompson, as well as two colleagues, E. Juni and I. Goldstein, for encouragement during my formative years in biological chemistry.

W. N. Arnold
Westwood Hills, Kansas
July 1980

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Chapter 1

ENZYMES

W. N. Arnold

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I. BACKGROUND

The yeast cell envelope contains an important complement of enzymes. Self-evident as that statement is now, it was rather the mechanical role of the cell wall that dominated earlier biological considerations. Consequently the envelope was looked upon as a relatively inert complex of integuments rather than an "outer metabolic region of the cell."[1]

Studies on the enzymology of the yeast cell envelope have their beginning in turn of the century work on isolation and partial purification of β-fructofuranosidase. By 1921 Willstätter's laboratory[2] had incidentally documented the peripheral location of β-fructofuranosidase, and other enzymes were subsequently associated with the cell envelope. These enzymes fall into two classes: first, those which cleave substrates that do not permeate the plasma membrane, and second, those enzymes involved with the turnover of envelope polymers during growth. The first group includes nutritionally important enzymes, and some members are well documented; while the second group includes both synthetic and degradative members that have been sparingly studied and, indeed, their existence in some cases is only inferred from experiments with inhibitors.

The methodology for studying enzymes of the cell envelope is generally common to that used with cell-free extracts. However, certain operational advantages accrue because of facile addition and later substraction of substrates or effectors. This is achieved by washing cells (or derivative structures) on the centrifuge or on filters. Conversely, macromolecular probes (enzymes for coupled-assay, labeled antibodies, etc.) are debarred from the periplasmic space by dint of the impermeability of the cell wall, and this may present certain operational disadvantages and problems in interpretation.

This review follows a division according to apparent location of enzymes within the cell envelope. Operational definitions for the component regions of the envelope have been given in Volume I, Chapter 1. It will become evident that the depth of characterization among the individual enzymes varies greatly.

II. PROJECTED BEHAVIOR OF CELL ENVELOPE ENZYMES

Certain criteria can be assembled as hallmarks of this group of enzymes. Other tests help to distinguish fine locations within the envelope, and meeting all or some of these criteria will more or less strengthen the assignment of a particular enzyme to a specific locale. The practical results which speak to these parameters are subject to interpretations that are summarized in succeeding sections. Table 1 is a summary of this type of analysis for the cell wall, periplasmic space, and plasma membrane. Capsulated yeasts may have some enzymes associated with the slime layer, but there is no

Table 1
PROJECTED BEHAVIOR OF CELL ENVELOPE ENZYMES

Criterion	Cell wall	Periplasmic space	Plasma membrane
1. Accessibility to substrate without need of prior perturbation of membranes	+	+	+
2. Modification by reagents excluded by plasma membrane	+	+	+
3. Identity of pH profiles of cell and cell-free extract	+	+	+
4. Release of nonsedimentable activity by protoplasting	+[a]	+	−
5. Release of nonsedimentable activity by cracking.	−	+	−
6. Secretion by protoplasts that are capable of *de novo* enzyme synthesis	+	+	−
7. Cytochemical localization	Yes	Yes	Yes
8. Artificial cross-linking to plasma membrane	−	+	N.A.

Note: (+) = Enzyme in this locale should meet the stated criterion; (−) = should not meet the criterion; and N.A. = not applicable.

[a] May be (−) if enzyme is associated with bud scar residues. See Volume I, Chapter 5, Figures 6 and 7.

information to this effect and in this chapter we can only mention certain salient points about the slime layer in passing.

Criteria 1, 2, and 3 arise from the fact that the cell wall is freely permeated by most low molecular weight substrates, hydrogen ions, and several potential inhibitors or inactivators, that normally do not cross the plasma membrane. (This subject is discussed further in Volume I, Chapter 3.) As well as demonstrating that a candidate enzyme is directly assayable in the intact cell, and that it feels the bathing medium, it becomes equally important to demonstrate that the same conditions do not support assay or modification of cytoplasmic enzymes. If the activity per cell is increased by pretreatments that perturb membranes, then an additional location may be indicated. However, such an increase may be deceptive because the perturbant may also function by inhibiting a secondary event (metabolic or transport) that would otherwise deplete the assayable product in question. This is discussed in more detail under kinetic restraints.

Useful inactivators under criterion 2 include H^+ and heavy metals. The proper functioning of the plasma membrane (see also Volume I, Chapter 4) enables the cytoplasm to be maintained at a fairly constant pH in spite of external changes. Likewise, salts are excluded, especially if the bathing medium is free of metabolizable substrates. However, at extremes of pH the plasma membrane is perturbed and the differential between internal and external pH is abolished. That the integrity of the plasma membrane has not been compromised needs to be demonstrated by the proper controls if criteria 1 to 3 are to be interpreted meaningfully.

Criterion 4 follows from the fact that dissolution of the cell wall by exogenous glucanases will remove the network of the wall, and those enzymes associated with the periplasmic space and the cell wall will now freely diffuse into the bathing medium. Accordingly, the released enzyme will resist sedimentation by high-speed centrifuga-

tion. Important controls under this heading include documentation of incidental enzymes in the protoplasting mixture (e.g., snail digestive juice is endowed with many additional enzymes as well as the vital β-glucanases needed for yeast cell wall dissolution) and, in the case of a negative finding, checking the candidate enzyme for susceptibility of inactivation during the protoplasting treatment. Further discussion on protoplast formation is given in Volume II, Chapter 5, but it is worth mentioning here that osmotic vulnerability alone is not sufficient evidence for protoplast formation. That protoplasts devoid of cell wall remnants have indeed been produced needs to be confirmed by electron microscopy.

Release of the enzyme in question during protoplasting does not distinguish between the periplasmic space and the cell wall because the latter is dissolved by the treatment. Criterion 5 provides distinctive evidence in this context because mechanical disruption (See Volume I, Chapter 3 for specific methods) cracks the cell wall in a limited number of locations and large pieces of cell wall are discernible under the light microscope. If the candidate enzyme is associated with the cell wall it will be sedimented by centrifugation, whereas if the enzyme is located in the periplasmic space then but one crack in the wall will be a sufficient opening through which the enzyme can diffuse into the medium. In the latter case there is no need to completely homogenize the wall to generate a nonsedimentable enzyme, and indeed there should be a good correlation between the percentage of cells broken by the treatment and that fraction of the population of candidate enzyme released.

The secretion of enzymes by protoplasts, criterion 6, also follows from the removal of the cell wall, which would restrain those enzymes that normally lodge in the periplasmic space. Incidentally, retention of periplasmic enzymes by regenerating protoplasts should be a good sign for achievement of at least a semblance of functional wall structure (see Volume II, Chapter 6)

At first glance criterion 7, cytochemical localization, would seem to be straightforward. It can be, although several artifactual problems which are general to cytochemical methods are also in play here. As an example one may consider the demonstration of phosphatases by capture of the nascent phosphate as an insoluble and electron-opaque precipitate. Disparate diffusion rates for substrate and trapping agent, or an unfavorable rate of precipitation relative to phosphate ion generation, may lead to artifactual precipitation of phosphate salt some distance from the site of its generation. Additional problems with periplasmic space enzymes relate to their inaccessibility to external enzymes that might otherwise be invoked for a coupled assay (e.g., glucose oxidase and formazan formation as a means to demonstrate sites of glucose generation). This problem of cell wall porosity is not circumvented by working with partly disrupted material because the periplasmic space enzymes will then diffuse into the medium. A study of thin sections embedded in plastic may be feasible for identification of specific compounds, but is unlikely to be worth trying in the case of enzymatic activity. The utility of labeled antibodies in conjunction with thin sections and electron microscopy is feasible but relatively unexplored in yeast.

Criterion 8 is postulated on the rationale of possible cross-linking of candidate enzyme molecules to the nearest neighboring structure. In this context a periplasmic space enzyme might be anchored to the inner aspect of the cell wall as well, although this would depend upon the reactivity of the polymers involved and the choice of cross-linking agent. This approach has been demonstrated recently with periplasmic enzymes from bacteria. Preincubation with diimidoesters makes periplasmic proteins partly inextractable and subsequent separation of envelope components enables some discrimination to be made between the inner and outer membrane as nearest neighbor for a particular enzyme.[3] No published work on yeast cell envelopes along these lines

has appeared, but this approach is feasible and will depend on the ingenuity with which potential cross-linking agents are synthesized and applied.

III. ENZYMES OF THE PERIPLASMIC SPACE

Criteria 1 through 6 are met by the β-fructofuranosidase of *Saccharomyces cerevisiae*, while a lesser number of these criteria have been explored for other enzymes that are apparently located in the periplasmic space of various species. Quantitation is very important, i.e., it is essential that bookkeeping be performed on any candidate enzyme through any fractionation scheme. For example, a claim that isolated cell walls are endowed with "high" activity for a particular enzyme is a meaningless statement of localization if the fraction that is still sedimentable is only a few percent of the original total activity. More likely explanations in that particular case are that some adventitious whole cells have escaped the preliminary disruptive treatment or, alternatively, that a small fraction of the monitored enzyme has been artifactually trapped within sedimentable debris. In other cases more than one location within the intact cell may be definitely indicated. Likewise, a similar catalytic activity may be exhibited by more than one protein in a given cell so that some confusion about specific location may result until the two or more proteins are separated and characterized. Documentation along these lines obviously has important implications for precursor to product relationships and for mechanisms of translocation of enzymes destined for the periplasmic space.

A. Detection

The presence of an enzyme in the periplasmic space may be missed for a variety of reasons. Furthermore, quantitative assessments must be made with an awareness of potential problems. The summary that follows is based on reported interferences and means for their rectification and includes also situations that might reasonably be anticipated.

1. Kinetic Restraints

Figure 1 is a diagram of sequential events in the assimilation of a substrate A via intermediates B and C. The regions of the cell envelope are indicated (without regard to scale), and velocities of the individual steps are assigned values of v_1, v_2, ... v_n. Enzyme 1 is assigned to the periplasmic space and carrier-mediated transport of B across the plasma membrane is proposed although that particular mechanism is not essential to the general argument. (A complete discussion of transport processes is to be found in Volume I, Chapter 4.)

If a suspension of cells is incubated with A then compound B may accumulate in the medium according to the scheme in Figure 1, provided that v_2 is larger than v_3. In practice the amount of enzyme 1 is estimated from the maxium velocity for the reaction $A \rightarrow B$. The velocity is measured from the rate of disappearance of A or, more commonly, the appearance of B. If v_2 is much greater than v_3 then the rate of appearance of B in the medium will indeed be close to the absolute rate of its generation. But the greater the magnitude of v_3 relative to v_2, then the lower the activity of enzyme 1 will seem by routine monitoring of B in the medium. The latter problem can be rectified by including a specific transport inhibitor in the assay mixture, i.e., v_3 is reduced to zero in the ideal.

Another approach is to add an excess of a coupling-enzyme that will convert B into a nontransported derivative. The coupling-enzyme is unlikely to be able to diffuse through the cell wall, so that its access to B (generated in the periplasmic space) will still

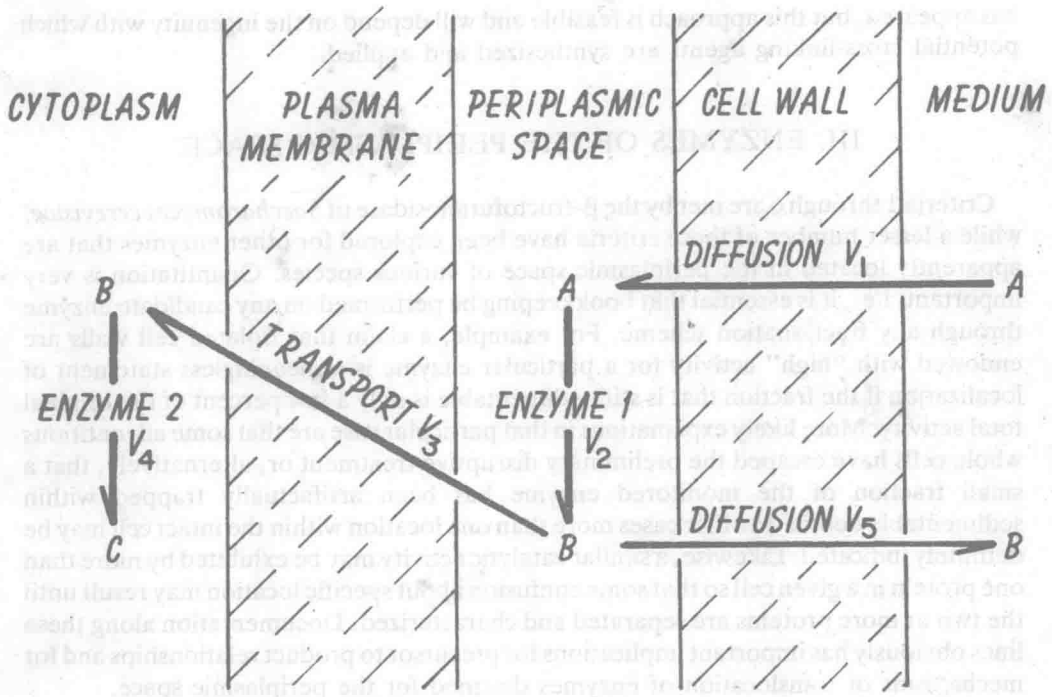

FIGURE 1. Diagrammatic representation of assimilation of a substrate (A) via intermediates (B) and (C). Enzyme 1 is located in the periplasmic space, while Enzyme 2 is in the cytoplasm. Kinetic restraints on the detection of Enzyme 1 in intact cells are discussed in the text in relation to the relative velocities (V) of the enzymatic, transport, and diffusion steps.

depend upon v_5 being substantially greater than v_3; hence this approach is inherently less satisfactory.

If the whole cell suspension is assayed for B (e.g., after stopping the incubation by pH manipulation) it is possible to include cytoplasmic B in the analysis and thus the magnitude of v_4 relative to v_2 will then determine the degree of underestimation of v_2. Addition to the assay mixture of a specific inhibitor of enzyme 2 should result in full values in this circumstance. For that matter, inhibition of some more distant enzyme (in the main pathway for the cytoplasmic metabolism of B) might at least ameliorate the situation because turnover of cosubstrates might be sufficiently compromised to inhibit the pathway. The rational approach is to explore a variety of inhibitors and to discover conditions that maximize activity estimates of the periplasmic enzyme. If other criteria suggest a unique location in the periplasmic space, then the activity of cell-free extracts should also match the values from the optimized cell-suspension assay.

More complicated situations are easily envisaged, e.g., product B might be generated in the periplasmic space and then acted upon by a second extracytoplasmic enzyme. It is not necessary to catalog all of the possibilities, but it is worth considering one specific example in this context. The trisaccharide raffinose (*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) is fermented by different *Saccharomyces* species to varying extents, as summarized in Table 2. *Sacch. inusitatus* is endowed with an α-galactosidase and a β-fructofuranosidase that are presumably located in the periplasmic space. This would be a case of two enzymes acting on one substrate (raffinose) and although the kinetics are defined for a cell-free system (see, for example, Reference 4) they would be even more complicated for intact cell assay. The actions of the two enzymes on separate molecules of raffinose result in disaccharides,

Table 2
FERMENTATION OF RAFFINOSE BY SELECTED *SACCHAROMYCES* SPECIES

Species	Fraction fermented (moieties)	Residual sugar in medium	Enzymes present, in addition to glc/fru permease		
			β-Fructosidase	α-Galactosidase	Galactozymase
unisporus	0/3	raffinose	−	−	+
cerevisiae	1/3 (fru)	melibiose	+	−	+
oleaceus	1/3 (gal)	sucrose	−	+	+
inusitatus	2/3 (fru, glc)	galactose	+	+	−
uvarum	3/3 (fru, glc, gal)	none	+	+	+

Note: glc = glucose, fru = fructose, gal = galactose. Galactozymase is a collective term for galactose permease, galactokinase, uridyl transferase, and uridine diphosphate-4-epimerase.

sucrose, and melibiose, which subsequently become substrates for the other enzyme in each case. Furthermore, two of the monosaccharides are actively absorbed by the protoplasm whereas galactose is apparently debarred due to the lack of a galactozymase system in this species. The successful assay of the two enzymes in question would obviously require individual substrates, e.g., sucrose and melibiose, in conjunction with an inhibitor of the hexose transport system.

In the case of *Sacch. cerevisiae* the β-fructofuranosidase catalyzed reaction is normally about 300 times more rapid than the intracellular metabolism of the hexoses [5] so that production of reducing sugars from either sucrose or raffinose provides a reasonable assay. On the other hand, one may envisage an advantage in using raffinose for those species that have a similar enzyme complement to that of *Sacch. cerevisiae* but a lower amount of β-fructofuranosidase. This is because one product, melibiose, is not depleted by transport. Such a rationale was employed with mature cells of *Sacch. rouxii*.[6]

2. Other Causes of Quantitative Error or Nondetection

A number of conditions influence the assay of an enzyme in the periplasmic space. A rather obvious but essential requirement is that the test substrate be able to penetrate the cell wall. Other aspects such as pH optimum, substrate specificity, and buffer composition are general to all classes of enzymes, are approached by exploring a range of conditions systematically, and need not be labored here. The items that follow deserve special consideration for the present class of enzymes and assume added importance in comparisons among different species for enzyme content and localization. For these reasons the discussion that follows is couched in terms of safeguards that may help to avoid overlooking or underestimating a particular activity.

By the very nature of its location an enzyme in the periplasmic space has direct contact with the medium at all times. The bathing solutions include the growth medium, the washing solution used on harvested cells, and the medium in which cells are stored or further manipulated experimentally. All three liquids may be a source of denaturants, e.g., a transitory pH drop in the growth medium may greatly influence yield of a particular extracytoplasmic enzyme. These conditions usually have less effect on cytoplasmic enzymes. Attention to buffering capacity, pH value, ionic strength, and temperature is important at all stages. Furthermore, the washing medium may elute the enzyme in question as a result of either induced dissociation into subunits or increased porosity of the cell wall. This phenomenon can be approached by assaying all

supernatant fractions and by studying the time course of elution, or loss of enzymic activity.

The growth medium may also influence the yield of a particular enzyme through repression of its biosynthesis. Examples include repression of β-fructofuranosidase synthesis by glucose and of acid phosphatase by orthophosphate. Conversely, induction may be necessary. These subjects are discussed in more detail below. The composition of the medium and the quality of the water used in its manufacture are sometimes influential. A recent example from our laboratory involved an acid phosphatase from *Sacch. rouxii* which experienced heavy metal poisoning during a bout with inadequate-quality water that still did not noticeably affect growth.[7] Conversely, the requirement for a metal cofactor must not be overlooked—acid phosphatase from one species of yeast requires Fe^{3+} for full activity.[7] In such cases some consideration should be given to fortifying not only assay mixtures but also growth and washing media.

Recent demonstrations of enzyme inactivation by polypeptide inhibitors and activation by subsequent proteolytic digestion have opened an exciting new field of metabolic control in yeast (see, for example, Reference 8). In terms of covalent modifications the possibilities include protein phosphorylation and dephosphorylation, zymogen forms subject to limited proteolysis, and changes in sulfhydryl status. All types have cytoplasmic examples. To the best of our knowledge no systems of covalent modification have yet been discovered for extracytoplasmic enzymes in yeast.

B. β-Fructofuranosidase

The systematic designation for this enzyme is β-D-fructofuranoside fructohydrolase (EC 3.2.1.26), which is abbreviated to β-fructofuranosidase. Other names that are not recommended by the International Union of Biochemistry, but are used interchangeably in the older literature include sucrase, saccharase, β-fructosidase, and invertase. The latter appelation derives from a change in the sign of optical rotation which attends the complete hydrolysis of sucrose ($M[\alpha]_D^{20^\circ} = 22,770^\circ$) to glucose and fructose ($M[\alpha]_D^{20^\circ} = -7,200^\circ$).

Persoz reported on the inverting action of yeast in 1833, and Berthelot prepared active cell-free extracts and alcohol precipitates of the enzyme as early as 1860. Since then much literature has accumulated on yeast β-fructofuranosidase. Reviews by Neuberg and colleagues[9,10] include interesting historical background and remind us that this enzyme was the subject of landmark studies on enzyme specificity, on the concept and biological importance of pH, and on the kinetics of enzyme-catalyzed reactions. Other reviews that are either devoted to, or contain substantial summaries on yeast β-fructofuranosidase have appeared at intervals over the last 25 years.[11-17] The bulk of the literature is based on *Sacch. cerevisiae* as source material, and the descriptions below pertain to that species unless otherwise stated.

1. Reaction

$$\text{R-}\beta\text{-fructofuranoside} + H_2O \rightarrow \text{fructose} + \text{ROH}$$

The specificity is absolute for the unsubstituted and unchanged β-fructofuranosidic terminal. Thus α-fructofuranosides, fructopyranosides, turnaose and melezitose are not acted upon, whereas methyl-β-D-fructofuranoside, sucrose, raffinose, stachyose, and verbascose are cleaved at the fructosidic linkage[11,12] on the fructose side of the bridge oxygen.[18] Sucrose is the best substrate (lowest K_m, highest V_{max}). Gottschalk[11] postulated that sucrose associates with the active site of β-fructofuranosidase via the glycosidic

oxygen, the hydroxyl groups at C_6 and C_3 of the fructofuranosyl moiety, and possibly the hydroxyl group at C_2 of the glucopyranosyl moiety. The latter is obviously not essential because methyl-β-D-fructofuranoside is attacked, albeit less avidly. Substitution of the afructon moiety of sucrose (e.g., the raffinose series) affects kinetic parameters supposedly by dint of steric influence.

The mechanism of the reaction is visualized as an initial formation of α-D-glucopyranose and a fructosyl-enzyme intermediate which then reacts with water to liberate β-D-fructofuranose. Instead of water, any one of the three primary alcohol groups of sucrose may function as an alternative acceptor of the fructosyl group so that in the presence of excess substrate trisaccharides of the kestose type are transient products.[13,19,20] These are eventually cleaved to monosaccharides. The observations that small amounts of two difructosides (C_1 and C_6 substitutions) are also formed during incubation of purified β-fructofuranosidase with sucrose[21] or fructose alone[22] show that fructose can act as both donor and acceptor for the transfer reaction, which is in concert with the above mechanism. A trace of 6-β-fructofuranosylglucose can be isolated from incubations with fructose and glucose but no sucrose is detected.[21,22] Notwithstanding the academic interest in fructosyl-transfer products, there has been no indication that β-fructofuranosidase performs any significant synthetic function in the intact yeast cell.

2. Assay

The earlier predilection for the polarimetric method has given way to assays based upon colorimetric detection of the reducing sugar products. My preference is for the 3,5-dinitrosalicylic acid (DNS) reagent of Sumner[23] rather than the copper-reductometric, Somogyi-Nelson procedure.[12] The single-step addition of alkaline DNS reagent serves to terminate the enzymic action, and mixtures require only 5 min in a boiling water bath for color development.[24] The reddish reduction product (3-amino-5-nitrosalicylic acid) has an absorption maximum at 520 nm but we prefer to monitor at 560 nm, where the yellow reagent itself has a low absorption.

Assays of cell suspensions are handled similarly to cell-free extracts. For *Sacch. cerevisiae* the titer of β-fructofuranosidase is so high that suitably diluted suspensions contribute negligible turbidity to the final solution.[25] With less-endowed, and hence more concentrated, suspensions the mixtures need to be cleared by centrifugation after color development.[6,26] In addition, we recommend carrying companion samples through the incubation period (typically 10 to 20 min) at 30°C. These controls receive DNS reagent and substrate (in that order) at term. This enables correction of assay values for any reducing substances derived from other sources in the cells and for any color-throw.

One IU of enzyme is that amount which brings about the hydrolysis of 1 μmol substrate (here sucrose) per min at 30°C. Concentrations of enzyme are expressed in units per gram dry weight of cells or per milligram protein. Concentrations of cell suspensions are conveniently estimated by turbidity measurements in conjunction with a standard curve based on dry weight per unit volume.[27] Quantification of cell-free extracts can be based on the original cell suspension or on the protein content. The latter is most conveniently estimated by the Folin-Ciocalteu reagent but the color yield should be established on a Kjeldahl nitrogen basis. For bakers' yeast β-fructofuranosidase the color yield of the purified enzyme is 27% higher than that for bovine serum albumin (the most common standard).[28] Regrettably, corrected protein values are rarely reported.

3. Location

A variety of evidence serves to locate the β-fructofuranosidase of *Sacch. cerevisiae* in a region external to the protoplasmic membrane (see, for example, Reference 29 and