

Assay and Purification of Enzymes-Oxalate Decarboxylase*

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5.1 Overview

5.1.1 Enzyme Assay and Kinetics

Enzymes are proteins that catalyze various chemical reactions which occur in living organisms. The various classes of enzymes are presented in Table 5.1. In an enzyme-catalyzed reaction, enzyme (E) and substrate (S) form a complex, i.e., $E + s \rightarrow ES \rightarrow E + P$. Figure 5.1 depicts the time-dependent changes in the concentrations of various components of the equation. Enzyme activity is influenced by pH, temperature, enzyme amount, substrate specificity, inhibitors, modulators, and

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TABLE 5.1
Summary of the International Classification of Enzymes

Classification	Reaction catalyzed
Oxidoreductases:	$A^- + B = A + B^-$ Electron transfer
Transferases:	$A-B + C = A + B-C$ Group transfer reactions
Hydrolases:	$A-B + H_2O = A-H + B-OH$ Hydrolysis reactions
Lyases:	$\begin{array}{c} X \ Y \\ \ \\ A-B = A=B + X-Y \end{array}$ Group addition removal to/from double bond
Isomerases:	$\begin{array}{c} X \ Y \ Y \ X \\ \ \ \ \\ A-B = A-B \end{array}$ Transfer of groups in molecules resulting in isomeric forms
Ligase (synthesis):	$A-B = A-B$ Generation of C-C, S-S, G-O, and C-N bonds condensation and ATP cleavage

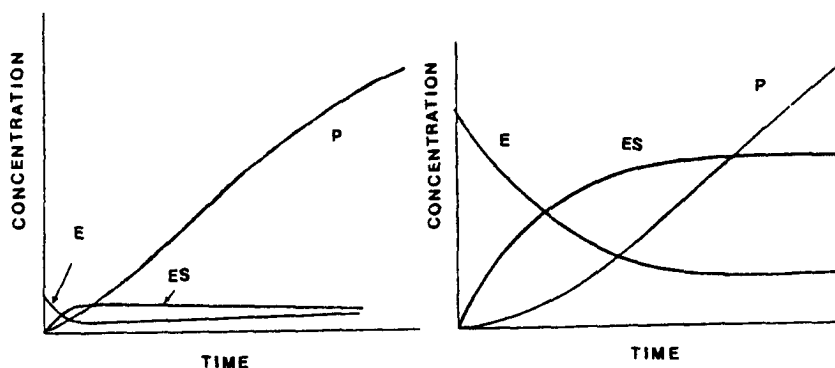


Figure 5.1

Time-dependent changes in the concentration of various components of $E + S \rightarrow ES \rightarrow E + P$. E = enzyme. S = substrate. P = product.

product amounts. Figures 5.2 and 5.3 show how enzymes possess pH and temperature optima for maximum activity. While these optima are often quite narrow, Figure 5.2 indicates that the pH optima for the activity of some enzymes can be quite broad. Enzyme amount and substrate concentration also effect enzyme activity. Increasing the amount of enzyme within a reaction mixture results in an initial linear rise in enzyme activity which then levels off. In contrast, enhancing the substrate concentration yields a hyperbolic curve (Figure 5.4).¹⁻⁹ This curve reflects Michaelis-Menten kinetics in which velocity (Y-axis) is plotted against the substrate concentration

(X-axis). The Michaelis-Menten constant $K_m \left(V_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \right)$ equals the substrate concentration

at half maximum velocity ($1/2 V_{max}$). The value of K_m is that it indicates how tightly a particular substrate binds to the active site of an enzyme. For a multistep reaction. Zubay¹⁰ points out that the relationship between the K_m and rate constants is not so simple. The K_m for certain enzymes are presented in Table 5.2. It is possible to graph the Michaelis-Menten plot (Figure 5.4) as a double reciprocal plot, i.e., $1/v$ vs. $1/[S]$, thereby yielding a Lineweaver-Burk plot (Figure 5.5)

based upon the equation $1/V_0 = \frac{K_m}{V_{max}} 1/[S] + \frac{1}{V_{max}}$. The negative reciprocal of K_m , $-1/K_m$, is

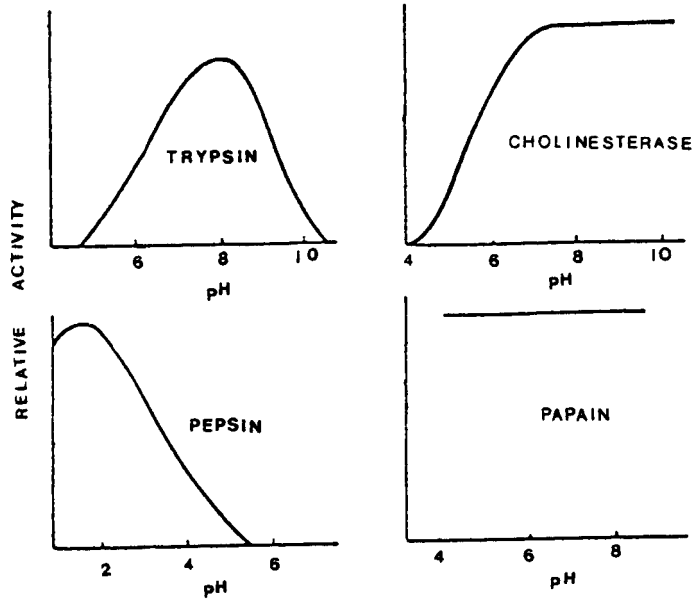


Figure 5.2
pH optima of various enzymes.
Note: cholinesterase is a mammalian enzyme.

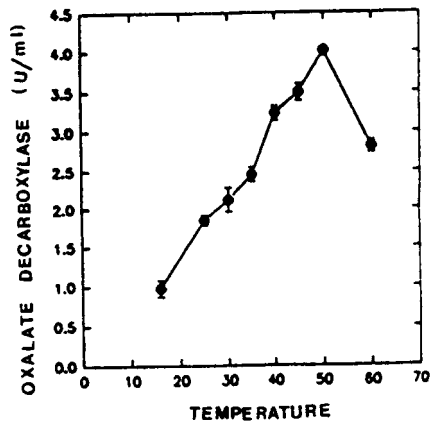


Figure 5.3
Temperature optimum of oxalate decarboxylase.

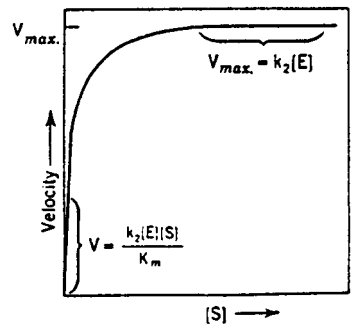


Figure 5.4
Effect of increasing substrate concentration on enzyme activity; Michaelis-Menten plot. V_{max} = maximum velocity. K_1 = rate constant. K_m = Michaelis-Menten constant.

TABLE 5.2
Summary of K_m for Certain Enzymes

Enzyme	K_m (mM)	Substrate
Aspartate aminotransferase	0.9	Aspartate
	0.1	2-Ketoglutarate
Carbonic anhydrase	9	HCO_3^-
Catalase	25	H_2O_2
Chymotrypsin	108	Glycyltyrosinylglycine
Fumarase	0.005	Fumarate
	0.025	Malate
β -Galactosidase	4	D-Lactose
Hexokinase	0.4	ATP
	0.05	D-Glucose
	1.5	D-Fructose
Threonine dehydratase	5.0	L-Threonine

Figure 5.5
Lineweaver-Burk plot for an enzyme-catalyzed reaction in the absence of an inhibitor.

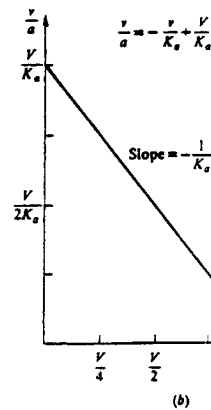
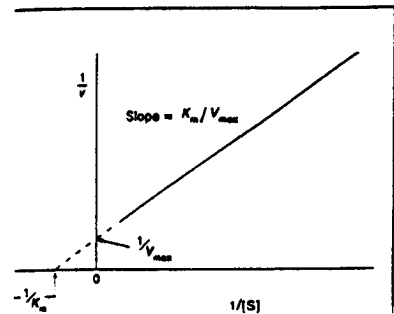


Figure 5.6
Eadie-Hofstee plot.

TABLE 5.3
Summary of the Effects of Inhibitors on
Lineweaver-Burk Plots $1/V$ vs. $1/[S]$

	Slope	Intercept on ordinate
No inhibitor	$\frac{K_m}{V_{max}}$	$\frac{1}{V_{max}}$
Competitive	$\frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i} \right)$	$\frac{1}{V_{max}}$
Uncompetitive	$\frac{K_m}{V_{max}}$	$\frac{1}{V_{max}} \left(1 + \frac{[I]}{K_i} \right)$
Noncompetitive	$\frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i} \right)$	$\frac{1}{V_{max}} \left(1 + \frac{[I]}{K_i} \right)$

obtained. Rearrangement of the Michaelis-Menten equation to a linear form $V_o = -K_m \frac{V_o}{[S]} + V_{max}$

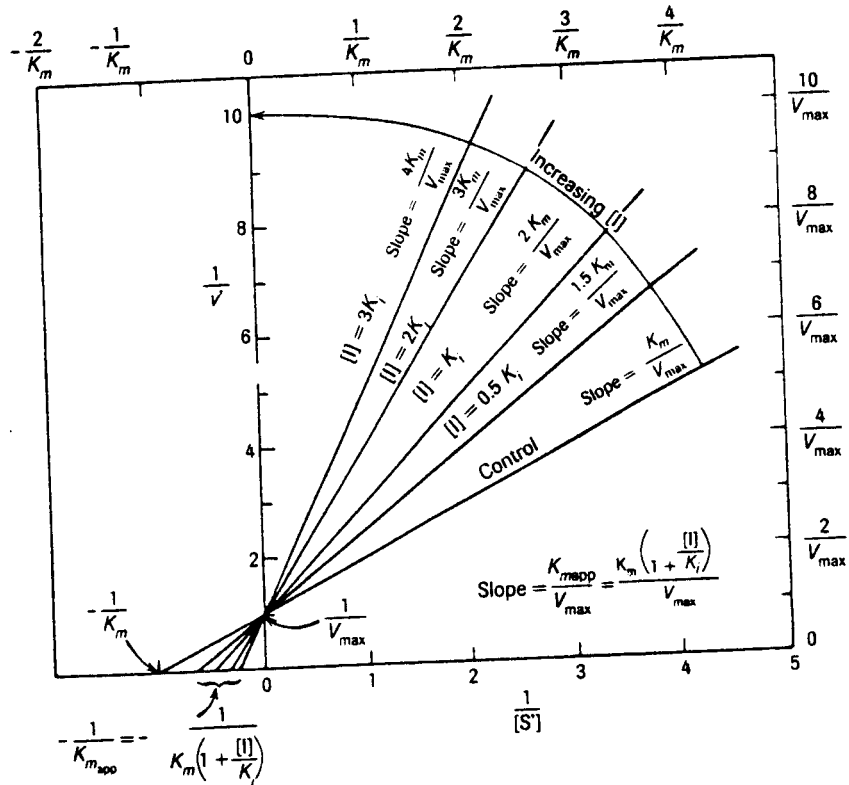
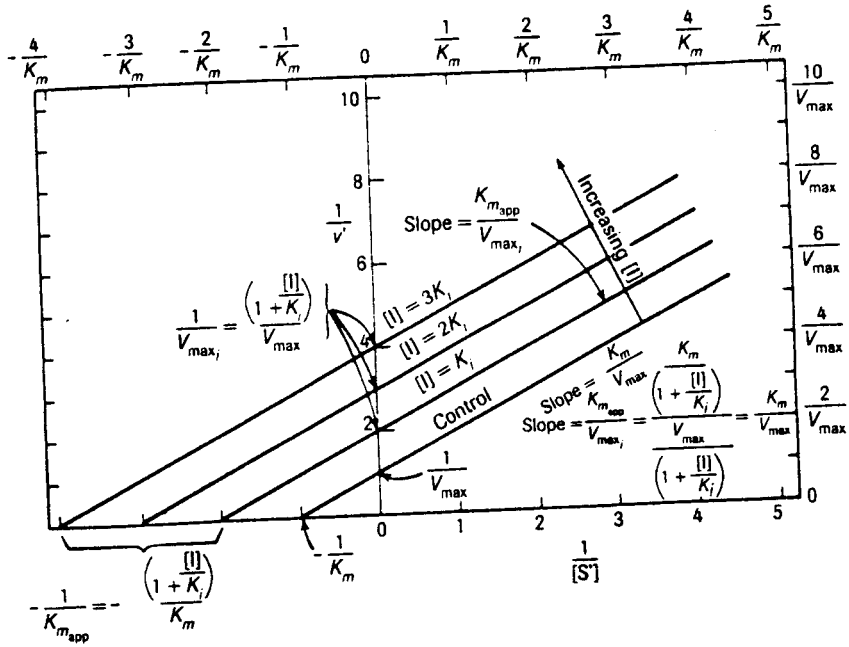
provides an Eadie-Hofstee plot (Figure 5.6), another linear relationship between substrate concentration and initial velocity. The utility of employing Lineweaver-Burk plots is in investigating the effects of inhibitors on enzyme-catalyzed reactions. Competitive, noncompetitive, and uncompetitive inhibitors represent the three major types of enzyme inhibitors (Table 5.3). Figures 5.7 and 5.8 present Lineweaver-Burk plots for uncompetitive and competitive inhibitors, respectively. Such plots enable investigators to distinguish the type of enzyme inhibitor that they are working with.

5.1.2 Protein Purification

The protocols yielding proteins purified to homogeneity involve various biochemical techniques.¹¹ The protocols are protein dependent, but usually begin with dialysis and ammonium sulfate fractionation followed by chromatographies. The latter may include: affinity,¹² gel filtration,¹³ high-pressure liquid (HPLC),¹⁴ hydroxyapatite, hydrophobic interaction,¹⁵ and ion exchange¹⁶ chromatographies. The extent of purification is monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and/or isoelectric focusing (SDS-PAGE/IEF).¹⁷

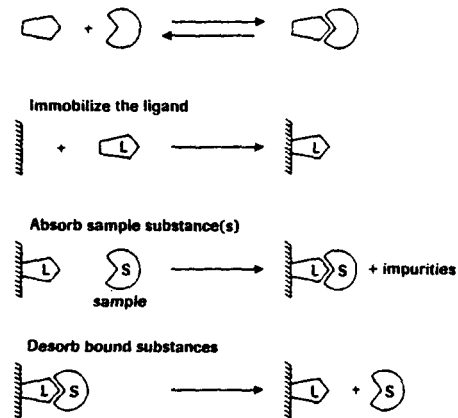
5.1.2.1 Purification Techniques

Dialysis is an early purification step by which a protein preparation, e.g., cell/tissue homogenates, can be partially purified using dialysis tubing that possesses various molecular weight "cut offs". This step can eliminate low-molecular weight peptides and free aromatic amino acids that absorb at 280 nm. This is critical as UV spectroscopy at 280 nm is often employed to quantify protein, since this test is nondestructive, allowing further utilization of the sample. After dialysis, ammonium sulfate fractionation (salting out) is often used to remove proteins other than the one being purified. This technique is based upon the fact that proteins can be precipitated by high concentrations of certain salts. The technique involves adding increasing concentrations of ammonium sulfate to the protein solution and centrifuging out the precipitated material in certain concentration ranges or "cuts" (e.g., 0 to 30, 30 to 60, and 60 to 90% ammonium sulfate), thereby "salting out" proteins at various saturation levels. The amount of ammonium sulfate added to reach a particular saturation level is best obtained from published tables and is temperature dependent.¹⁸ The ammonium sulfate should be added to the solution gradually and with gentle stirring. Frothing of the solution should be avoided, since it can promote oxidation and denaturation of proteins.

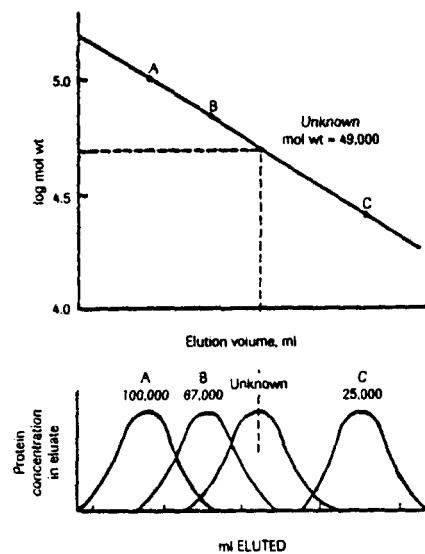


Figures 5.7,8

Lineweaver-Burk plot for enzyme-catalyzed reactions in the presence of an uncompetitive (7-top) and competitive (8-bottom) inhibitor. (From Segel, T. H., Enzyme Kinetics. John Wiley & Sons, New York, 1975. With permission.)

**Figure 5.9**

Mechanism of affinity chromatography. (From Pharmacia Laboratory, Separation Division. With permission.)

**Figure 5.10**

Theory of gel filtration. (From Zeidan, H. and Dashek, W, V., *Experimental Approaches in Biochemistry and Molecular Biology*, Wm. C. Brown Communications, Dubuque, IA, 1993. With permission.)

Subsequent to dialysis and ammonium sulfate fractionation, proteins can be further purified by the sequential application of chromatographies. Affinity chromatography centers about the binding of a stereospecific ligand to a support matrix (Figure 5.9). The ligand possesses significant specificity for the compound being purified, i.e., an antibody for its antigen, an enzyme for its substrate, etc. Nonbound compounds are eluted with appropriate reagents following binding of the compound to its ligand. Lastly, the bound species is selectively desorbed, often by changing the buffer composition.

Gel filtration is another chromatographic procedure which is often employed in an evolving protein purification protocol. This chromatography separates proteins and other compounds via their molecular weights (Figure 5.10). Even viruses, ribosomes, and cell nuclei can be separated. A mixture of proteins dissolved in a suitable buffer is layered onto a column packed with beads consisting of an inert, highly hydrated polymeric material (such as Sephadex G[®] or Bio-Gel P[®]). These materials, which must be washed and equilibrated with buffer before use, are commercially available with different degrees of porosities (Tables 5.4 and 5.5). The Sephadex G[®] and Bio-Gel[®] gels possess upper and lower molecular weight fractionation ranges, or "exclusions". The upper range (V_0 or void volume) can be determined via Pharmacia's blue dextran 2000, a carbohydrate polymer of 2,000,000 mol wt. If the V_0 of a Sephadex[®] G-150 column is determined, any protein

Summary of Pharmacia Sephadex® Resins

Sephadex	Fractionation range (mol wt)		Dry bead diameter (µm)	Approx. bed volume (ml/g dry Sephadex)
	peptides/globular proteins	Dextrans		
G-10	-700	-700	40-120	2-3
G-15	-1,500	-1,500	40-120	25-35
G-25	1,000-5,000	100-5,000	100-300	4-6
	Coarse			
	Medium		50-150	
	Fine		20-80	
	Superfine		20-50	
G-50	1,500-30,000	500-10,000	100-300	9-11
	Coarse			
	Medium		50-150	
	Fine		20-80	
	Superfine		20-50	
G-75	3,000-80,000	1,000-50,000	40-120	12-15
	Superfine	3,000-70,000	20-50	
G-100	4,000-150,000	1,000-100,000	40-120	15-20
	Superfine	4,000-100,000	20-50	
G-150	5,000-300,000	1,000-150,000	40-120	20-30
	Superfine	5,000-150,000	20-50	18-22
G-200	5,000-600,000	1,000-200,000	40-120	30-40
	Superfine	5,000-250,000	20-50	20-25

From Pharmacia Laboratory, Separation Division. With permission.

of 150,000 mol. wt or more would elute within the void volume coincident with the blue dextran. In contrast, proteins of molecular weight less than 150,000 would be retarded, with the lowest molecular weight protein being retarded the greatest.

Ion exchange chromatography is often very useful in protein purification. This chromatographic procedure uses the net charges of the molecules to achieve their separation. Pharmacia (Table 5.6) and Bio-Rad (Table 5.7) companies manufacture various strong and weak ion exchange resins. Two commonly employed ion exchange resins are the cationic and anionic resins, carboxymethyl (CM) and diethylaminoethyl (DEAE) cellulose. Their mechanisms of action illustrate the theory of ion exchange chromatography. Each Sephadex® molecule is comprised of a backbone derivatized with a charged functional group with an appropriately attached charged counterion (Table 5.7). When a mixture of neutral, positively charged, and negatively charged molecules is layered onto Sephadex® C-50, the negatively charged and neutral molecules will wash through (Figure 5.11). The positively charged molecules will exchange with the counterions to varying degrees. The exchanged molecules can be desorbed by application of either salt or pH gradients. Resorption via salt gradients is dependent upon an order of ion affinities selective for the functional group (Table 5.7). Tightly bound molecules can be desorbed by increasing the ionic strength of the eluting salt.

Hydroxyapatite and hydrophobic interaction chromatographies are two other useful separation techniques. The latter chromatography separates proteins via their relative hydrophobicities (Figure 5.12) and employs insoluble supports such as agarose or polyacrylamide. Hydrophobic alkyl and aryl groups are covalently attached to the supports. Proteins which have been bound to the resin through hydrophobic interactions are eluted according to the strength of this interaction.

TABLE 5.5
Summary of Bio-Rad Bio-Gels

Product polyacrylamide gels	Applications		U.S. standard wet mesh designation (hydrated)	Diameter of hydrated beads (microns)	Fractionation range (daltons)	Hydrated bed volume ml/g dry gel
Bio-Gel P-6DG	Desalting Gel	Desalting, buffer exchange	80-170	90-180	1,000-6,000	7
Bio-Gel P-2	Fine	Analytical fractionation	200-400	40-80	100-1,800	3.5
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-4	Medium	Group separations	100-200	80-150	800-4,000	5
	Fine	Analytical fractionation	200-400	40-80		
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-6	Coarse	Preparative industrial use	50-100	150-300	1,000-6,000	7
	Medium	Group separations	100-200	80-150		
	Fine	Analytical fractionation	200-400	40-80		
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-10	Coarse	Preparative industrial use	50-100	150-300	1,500-20,000	9
	Medium	Group separations	100-200	80-150		
	Fine	Analytical fractionation	200-400	40-80		
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-30	Coarse	Group separations	50-100	150-300	2,500-40,000	11
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Medium pressure fractionations	-400	<80		
Bio-Gel P-60	Coarse	Group separations	50-100	150-300	3,000-60,000	14
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Medium pressure fractionations	-400	<80		
Bio-Gel P-100	Coarse	Group separations	50-100	150-300	5,000-100,000	15
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Special applications	-400	<80		
Bio-Gel P-150	Coarse	Group separations	50-100	150-300	15,000-150,000	18
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Special applications	-400	<80		
Bio-Gel P-200	Coarse	Group separations	50-100	150-300	30,000-200,000	25
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Special applications	-400	<80		

TABLE 5.6
Composition of Pharmacia Ion Exchange Resins

Ion exchange resin	Functional group	Counterion
Anion exchangers DEAE (diethylaminoethyl)	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \diagup \\ \text{O}-\text{CH}_2\text{CH}_2\text{N}^+-\text{H} \\ \diagdown \\ \text{C}_2\text{H}_5 \end{array}$	Cl ⁻
QAE (quaternaryaminoethyl)	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \diagup \\ \text{OCH}_2\text{CH}_2\text{N}^+-\text{CH}_2\text{CHCH}_3 \\ \diagdown \\ \text{C}_2\text{H}_5 \end{array}$	Cl ⁻
Q (quaternary amine) Cation exchangers	CH ₂ -N(CH ₂) ₂	SO ₄ ²⁻
CM (carboxymethyl)	OCH ₂ COO	Na ⁺
SP (sulfopropyl)	OCH ₂ CH ₂ CH ₂ SO ₂	Na ⁺
S (sulfonate)	CH ₂ -SO ₂	Na ⁺

From Pharmacia Laboratory, Separation Division. With permission.

Elution is promoted by alterations in hydrophobicity, ionic strength, pH, or temperature of the eluting buffer. In contrast, hydroxyapatite involves the use of calcium phosphate gels.

5.1.2.2 Protein Quantification

The amount of protein in the sample must be quantified in order for specific activities to be calculated. Many different assays are available for this. This topic has been reviewed recently.¹⁹ Ultraviolet (UV) spectroscopy, in which the absorbance of the solution is measured at specific wavelengths, is a rapid and nondestructive technique of estimating the amount of protein in a sample (Protocol 5.1). The most commonly used wavelengths are 280 and 205 nm. Care must be taken, however, since nucleic acids, some detergents, and other compounds can also absorb UV at these wavelengths. The presence of contaminating nucleic acids can be corrected by zeroing the cuvette at 260 nm and then reading the absorbance of the solution at 260 and 280 nm. The protein concentration can be obtained with the formula:²⁰

$$\text{Protein concentration (mg/mL)} = 1.55 A_{280\text{nm}} - 0.76 A_{260\text{nm}}$$

Many other assays are calorimetric. These include the classical Lowry technique²¹ (Protocol 5.2), the Bradford technique^{22,23} (Protocol 5.3A, B), and the BCA protein reagent²⁴ (Protocol 5.2). The advantages and disadvantages of these techniques are discussed by Stoscheck.¹⁹ Other contemporary assays that detect extremely low levels of protein are presented in Table 5.8.²⁴⁻³⁰ In all cases, a standard curve should be created each time the assay is used. This can be done by making serial dilutions of 140 μg 100 μL⁻¹ bovine serum albumin (BSA). An optimum standard curve (Figure 5.13A,B) can be generated with a linear regression program using the method of least squares.

TABLE 5.7
Bio-Rad Ion Exchange Resins and Order of Ion Affinities

Resin type	Active group*†	Order of selectivity for monovalent ions for multivalent ions	Stability		
			Thermal	Solvent (alcohols, hydrocarbons, etc.) ¹ oxidation	Reduction
AG 1 Bio-Rex MSZ 1 Aminex anion resins strongly basic anion exchangers	R-CH ₂ N ⁺ (CH ₃) ₃	1>phenolate>HSO ₄ >ClO ₄ > NO ₃ >Br>CN>HSO ₃ >NO ₂ > Cl>HCO ₃ >IO ₃ >H ₂ COO>Ac> OH>F	OH form-fair to 50°C Cl and other forms good to 150°C	¹ Very good ² Slow solution in hot 15% HNO ₃ or conc. H ₂ O ₂	Good ¹
AG 2 Strongly basic anion exchanger	R-CH ₂ N ⁺ (CH ₃) ₂ C ₂ H ₄ OH	phenolate>i>HSO ₄ >ClO ₄ >NO ₃ > Br>CN>HSO ₃ >NO ₂ >Cl> OH>IO ₃ >H ₂ COO>Ac>F Ag>Rb>Cs>K>NH ₄ >Na> H>Li	OH form fair to 30°C Cl-form Good to 150°C Good to 150°C	¹ Very good ² Slow solution in hot 15% HNO ₃ or conc. H ₂ O ₂ ¹ Very good ² Slow solution in hot 15% HNO ₃	Good ¹
AG 50W Bio-Rex MSZ 50 Aminex cation resins strongly acidic cation exchangers	R-SO ₃	Zn>Cu>Ni>Co	Good to 150°C	¹ Very good ² Slow solution in hot 15% HNO ₃	Very good
Bio-Rex 5 intermediate base anion exchanger	R-N(CH ₂) ₂ HCl & R-N ⁺ (CH ₃) ₂ C ₂ H ₄ OH	—	Good to 60°C	¹ Good ² Good	Excellent
Bio-Rex 70 Weakly acidic carboxylic cation exchanger	R-COO	H>Ag>K>Na>Li H>Fe>Ba>Sr>Ca>Mg	Good to 100°C	¹ Good ² Good	Good
AG 4-X4 AG 3-X4 weakly basic anion exchnagers	R-CH ₂ N ⁺ (CH ₃) ₂	SO ₄ >H>HCi>CrO ₄ >HCO ₃ tartaric>oxilic>H ₃ PO ₄ > H ₃ AsO ₄ >HNO ₃ >HI>HBr> HCl>HF>HCOO>HAc>H ₂ CO ₃ ⁺	Good to 60°C	¹ Good ² Good	Unknown

From Bio-Rad Laboratories, Richmond, CA. With permission.

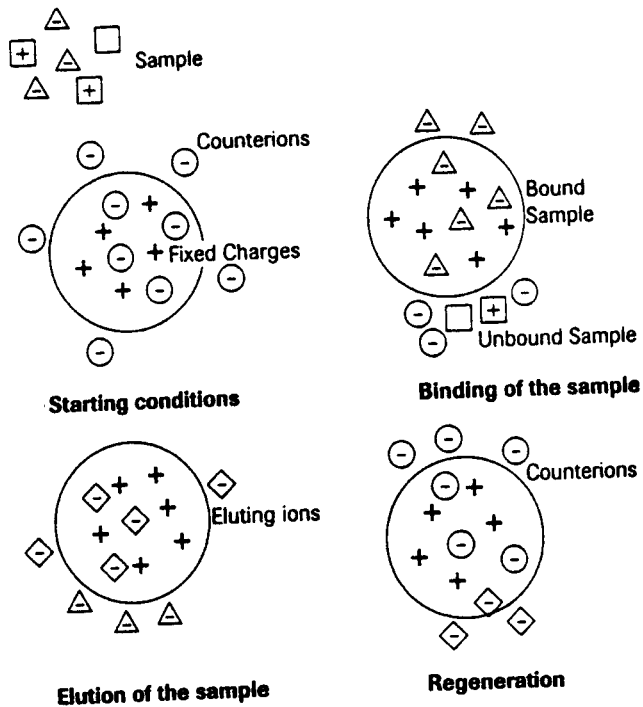


Figure 5.11
Theory of ion exchange chromatography. (From Bio-Rad Laboratories, Richmond, CA. With permission.)

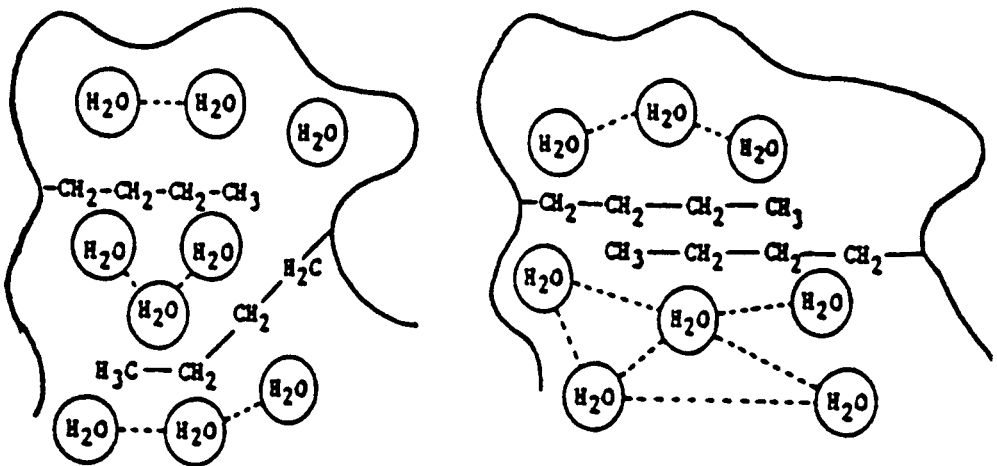
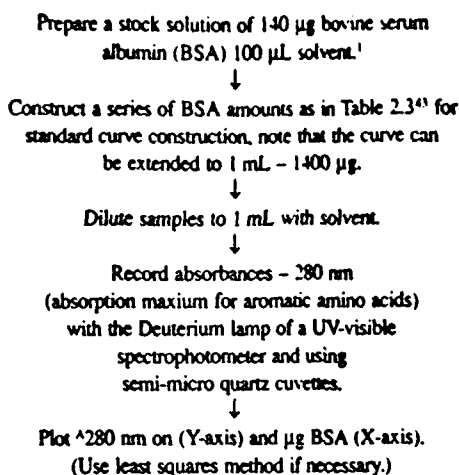


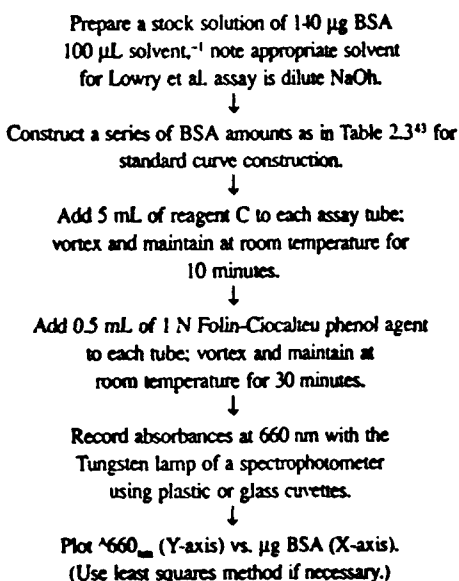
Figure 5.12
Principle by which hydrophobic interaction chromatography separates proteins. (From Bhagaran, N. V. *Biochemistry: A Comprehensive Review*, J. P. Lippincott, Philadelphia. With permission.)

5.1.2.3 Assessment of Purification — Electrophoresis

Electrophoresis is usually used to ascertain the effectiveness of the purification protocol. PAGE can be done under nondenaturing or denaturing conditions. When nondenaturing conditions are used, the enzymes usually retain their activity and can be visualized with various specific stains.³¹

**Protocol 5.1**

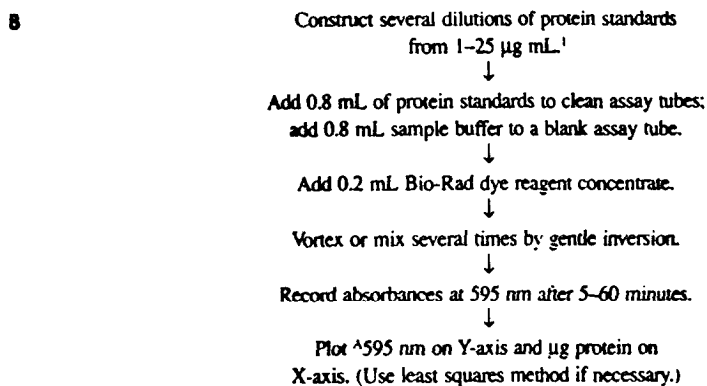
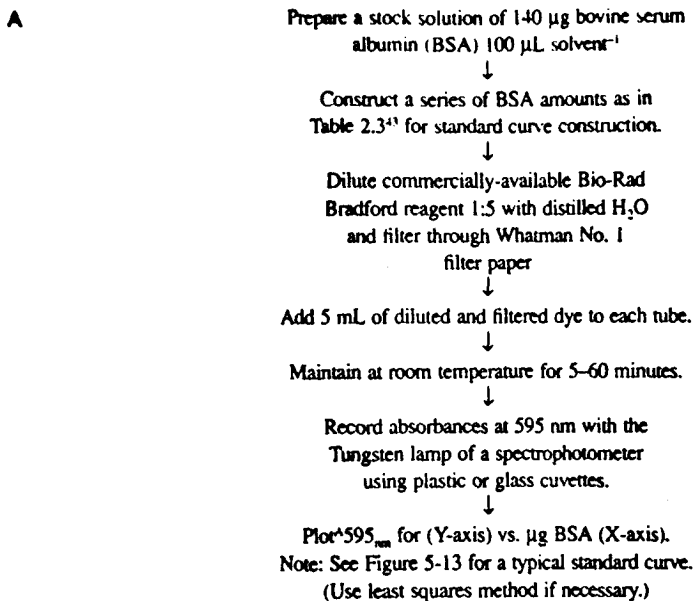
Protocol for UV spectroscopic quantification of proteins.

**Protocol 5.2**

Summary of Lowry et al. colorimetric procedure for quantitation of protein. (Adapted from Lowry, O. H. et al., *J. Biol. Chem.*, 193, 265, 1956. With permission.)

Denaturing conditions, which are usually achieved by adding the negatively charged detergent SDS to the gel, are more commonly employed for determinations of molecular weight. IEF, in which proteins are separated in a pH gradient using ampholytic salts, will give information on the isoelectric point of the protein. Two-dimensional electrophoresis, combined with the use of highly sensitive silver stains, gives the most critical assessment of the purity of an enzyme preparation. Protocols for all these techniques are presented by Hames and Rickwood³² and Deutscher.³³

At each step in a purification protocol, aliquots containing 30 μg of protein should be withheld and stored in such a way that minimizes proteolysis. These aliquots are then subjected to SDS-PAGE employing marker enzymes of known molecular weights. Bio-Rad Laboratories and other companies manufacture such markers. After electrophoresis, the proteins are visualized using a variety of staining procedures. Silver staining is the most sensitive and can reveal proteins that are



Protocol 5.3

Flowchart for the quantification of protein by the standard (A) and micro (B) Bio-Rad Bradford assay. (From Bio-Rad Laboratories, Protein Assay instruction booklet, Richmond, CA. With permission.)

present in nanogram quantities.³⁴ The molecular weight of the protein of interest can be calculated by measuring its relative mobility (R_f) and comparing that to a calibration curve created from the standards. The relative mobility is calculated by measuring the amount of movement of the protein from the origin (i.e., where the sample was applied) and dividing that by the distance of the tracking dye from the origin. A semilogarithmic plot is then constructed from the logarithms of the molecular weights of the protein standards graphed as a function of their R_f values. The molecular weight of the polypeptide subunits of the protein of interest can then be estimated from their R_f values by linear regression analysis. It is necessary to determine how many subunits make up the protein. This can be done by comparing the results of SDS-PAGE to chromatographic techniques.

BCA Assay Procedure

Because the ratio of working reagent to sample volume is the same (i.e. 20 parts working reagent to 1 part sample) all of the recommended protocols are identical except for their incubation temperatures and times.

Prepare a set of protein standards of known concentration by diluting the stock 2 mg/ml BSA standard (bovine serum albumin), or other suitable protein, in the same diluent as your unknown samples. The set of protein standards should cover the range of concentrations suitable for the assay protocol you are following. For best results, prepare standards using known concentrations of the protein you are testing.

Pipet 0.1 ml of each standard or unknown protein sample into the appropriately labeled test tube. For blanks, use 0.1 ml of diluent.

Add 2.0 ml working reagent to each tube. Mix well.

Incubate all tubes at the selected temperature and time

Standard Protocol 37°C for 30 minutes

Room Temperature Protocol..... R.T. for 2 hours

Enhanced protocol 60°C for 30 minutes

After incubation, cool all tubes to room temperature.

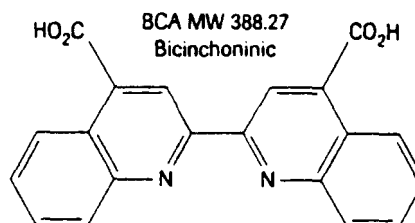
Measure the absorbance at 562 nm of each tube vs. water reference.

Subtract the absorbance of the blank from the value found for the standards of unknowns.

Note: If you have a double beam spectrophotometer, blank correction can be made automatically. Simply zero your instrument with both the sample and reference cuvettes filled with the solutions from duplicate developed blanks.

Leave the reference cell filled with blank solution and then read the absorbance of the samples/standards placed in the sample cuvette. These absorbance readings are now blank corrected.

Prepare a standard curve by plotting the net (blank corrected) absorbance at 562 nm vs. protein concentration. Using this standard curve, determine the protein concentration for each unknown protein sample.



Protocol 5.4

Reaction of protein with the BCA reagent. (From Pierce Chemical Co., BCA Protein Assay Reagent instructions booklet, Rockford, IL. With permission.)

TABLE 5.8
Summary of Procedures for the
Quantification of Low Levels of Protein
Using Colloidal Gold Technique

Sensitivity	Investigator(s)
Nanogram	25
Nanogram	26
Low nanogram	27
Low nanogram	28
Subnanogram and nanogram	29
Nanogram	30

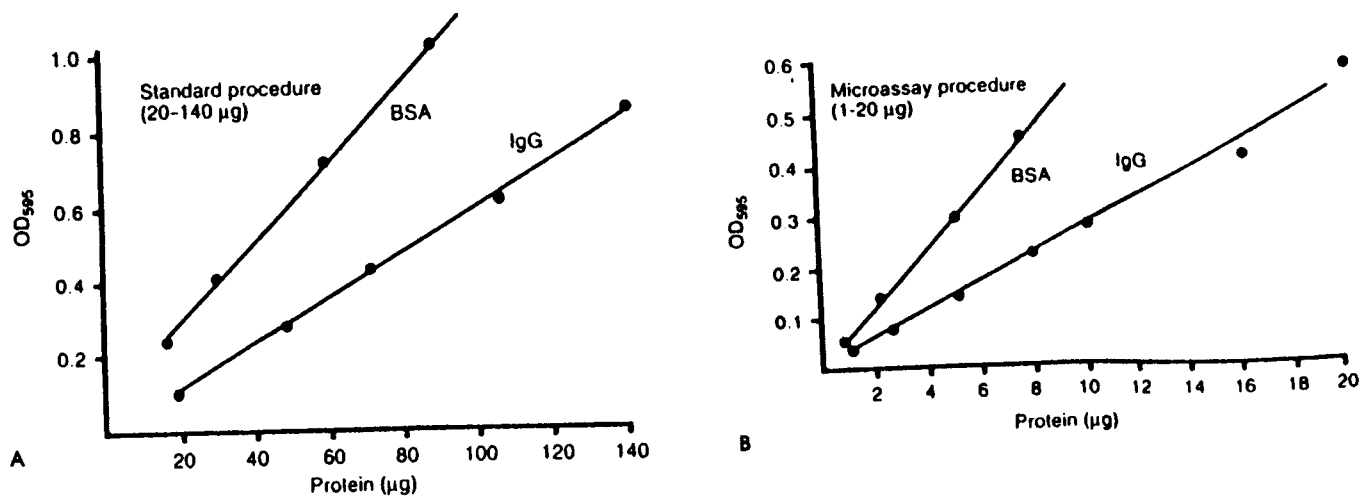


Figure 5.13 Typical standard curves for the Bio-Rad protein assays. A, Standard assay; B, microassay. (From Bio-Rad Protein Assay instruction booklet, Richmond, CA. With permission.)

5.2 Oxalate Decarboxylase as a Model Enzyme for Enzyme Purification

Oxalate decarboxylase (ODC) (EC4.1.1.2) is an enzyme which catalyzes the conversion of oxalic acid to formic acid and CO₂ (Figure 5.14). This enzyme has been isolated from various wood-rotting basidiomycetes and it is thought to regulate the accumulation (or lack thereof) of oxalic acid during decay. The enzyme has traditionally been associated with white-rot fungi,⁹ but was recently detected in mycelial extracts from the brown-rot fungus, *Postia placenta*.³⁵ Table 5.9 summarizes the occurrence and purification protocols of ODC from diverse microorganisms. Here we will concentrate on the occurrence of ODC in *P. placenta*.

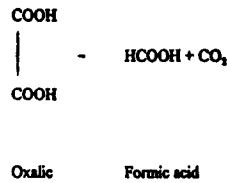


Figure 5.14
Reaction catalyzed by oxalate decarboxylase.

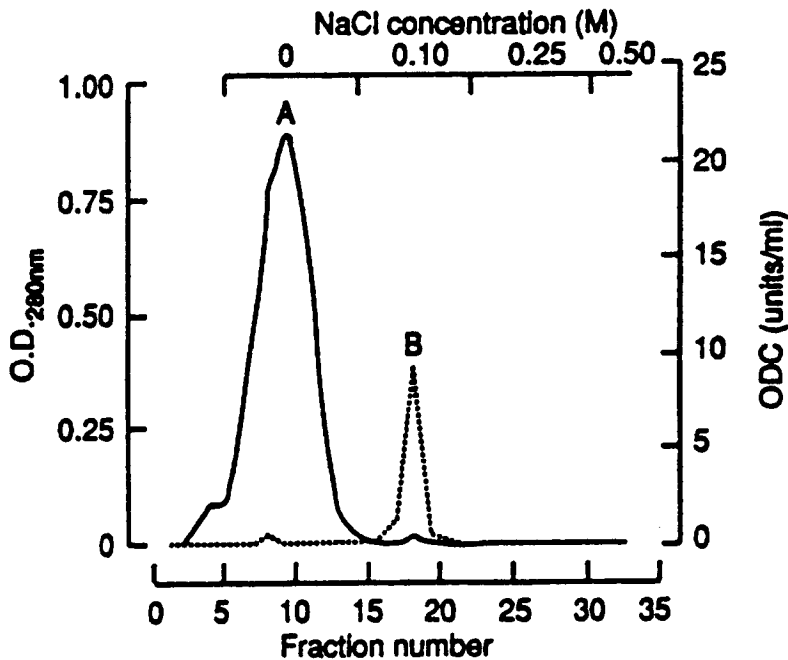


Figure 5.15
Stepwise NaCl gradient elution of *Postia placenta* ODC from CM Sepharose CL6B cation ion exchange resin. (From Micales, J. A., 1995)³⁵

5.2.1 Enzyme Preparations

Isolates of *P. placenta* can be grown easily in liquid culture. Strain ME20, which can be obtained from the Center of Forest Mycology, Forest Products Laboratory, One Gifford Pinchot Dr., Madison, WI, overproduces this enzyme. It also produces limited amounts of fungal glucan (or "slime"), thus making it an excellent experimental organism for enzyme purification studies. The culture

TABLE 5.9
Summary of Oxalate Decarboxylase Purification Procedures

Organism	Occurrence	Purification	Investigator
<i>Trametes hirsuta</i> and <i>Flammulina velutipes</i>		Mycelium homogenized in a mortar and pestle; extracted with dH ₂ O; centrifuged; acetone added to supernatant; precipitate dried in vacuum and redissolved in H ₂ O; centrifuged; supernatant contained enzyme	9
<i>Aspergillus niger</i>	Abundant in strains that yield greater amounts of citric acid; O ₂ is not consumed during the reaction and causes a denaturation proportional to the partial pressure. O-phenylenediamines and proteins protect and stimulate the purified enzyme in the presence of air	Homogenized frozen mycelium in a mortar and pestle in 0.1 M acetate buffer, pH 5.6, centrifuged 18,000 xg 15 min; filter through a fitted glass filter to eliminate floating lipids. fractional precipitation with lipids: MeOH	36
<i>Sclerotinia sclerotiorum</i>	Production of ODC is regulated by composition and pH of culture medium; required the presence of oxalate or succinic acid as inducers	Refrozen mycelia homogenized in a mortar and pestle on ice; when mycelia began to thaw, 5 mM citrate-phosphate buffer with 1 mM dithiothreitol added; centrifuged 30,000 xg, 20 min at 2C	37
<i>F. velutipes</i>		Two forms of ODC purified from a crude extract of frozen mycelium via precipitation with acetone and chromatofocusing and DEAE-Sephacel® CL-6B	38
<i>T. versicolor</i>	ODC detected both intra- and extracellularly in liquid cultures; both mycelial and culture filtrate ODC could be induced by oxalic acid; ODC was isolated and purified from mycelium and characterized by SDS-PAGE and Western blotting against a polyclonal antibody to ODC from <i>F. velutipes</i> ; a band of 59,000 mol wt cross reacted with the antibody	Disrupt mycelia with an Omni® mixture using glass beads in 0.2 M acetate buffer pH 3.7 and precipitation at 80% (NH ₄) ₂ SO ₄ ; centrifuge at 10,000 xg for 20 min and redissolve in 0.2 M acetate buffer pH 3.7, before dialysis; concentrated using Centrifuge -30 Amicon® concentrators (mol wt cut off 30,000); proteins separated by fast flow DEAE-Sephacel CL6B eluted with a pH gradient from 4.5 to 2.3 with an amino acid-HCl buffer	39
<i>Postia placenta</i>		ODC identified and partially purified from low and high decay cultures; ODC was partially purified by chromatography via CM Sepharose® CLB cation exchange chromatography using a stepwise NaCl gradient (see Figure 5.15)	35

medium for this fungus and the protocol for enzyme extraction are presented by Micales³⁵ and Micales and Highley.⁴⁰ Table 5.9 also lists other fungal systems that can be used as a source of ODC.

TABLE 5.10
Summary of Oxalate Decarboxylase Assays for Various Organisms

Source of ODC	Assay conditions	Investigator
<i>Myrothecium verrucaria</i>	Standard manometric methods at 30°C were used for assay of O ₂ uptake and CO ₂ production, nitrogen gas used was treated for removal of O ₂ ; standard reaction mixtures contained 0.015 M acetate buffer, 20 μmol oxalic acid, 0.8 mL of enzyme extract, pH 4.0 and either 0.2 mL of 20% KOH or 0.2 μL of H ₂ O in the Warburg flask center well; formic acid was determined by gravimetric and iodometric procedures	41
<i>Trametes hirsuta</i> + <i>Flammulina velutipes</i>	Estimated ODC by measuring in a Warburg manometer at 30°C the amount of CO ₂ produced; each manometer contained 1.3 mL of a reaction mixture consisting of 1/130 M oxalic acid	9
<i>Aspergillus niger</i> liquid surface cultures <i>Trametes versicolor</i>	Determined CO ₂ by manometric techniques; reaction carried out in an atmosphere of air, each flask contained 0.3 mL of 0.1 M oxalate solution, pH 4.7, 0.1 mL of enzyme, and 0.2 M acetate buffer up to 3.5 mL at 30°C; one unit of ODC activity was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of oxalate per minute	36
<i>Sclerotinia sclerotiorum</i>	Activity measured photometrically using a twostep method: (1) oxalate is decomposed to CO ₂ and formate at pH 5.0 (2) formate is measured at pH 7.5; reaction mixtures contained 20 μL of 40 μM oxalic acid (adjusted to pH 5.0 with KOH), 10 μL of 15 m M <i>o</i> -phenylenediamine, 50 μL of 0.1 m M BSA; aliquots of enzyme and 0.15 mL citrate-phosphate pH 5.0 in a final volume of 0.5 mL (<i>o</i> -phenylenediamine and albumin in citrate-phosphate buffer added to protect the enzyme); reaction mixtures were incubated at 25°C for 20 min and oxalate decarboxylation terminated with 0.8 mL of 0.15 M K ₂ HPO ₄ pH 9.5; then 0.25 mL of 45 m M NAD ⁺ added; reaction mixture was mixed and after 3 min, 15 μL of formate dehydrogenase were also added; estimated the amount of ODC by measuring the increase in absorbance at 340 nm before and 20 min after formate dehydrogenase addition. Enzyme activity expressed as μg oxalic acid destroyed by protein min ⁻¹	37
<i>Trametes versicolor</i>	ODC determined by measuring liberation of [¹⁴ C]CO ₂ from [¹⁴ C]-oxalic acid; one unit of activity was defined as the amount of enzyme releasing 1 μmol of ¹⁴ CO ₂ per min at 37°C	38
<i>T. versicolor</i> ; nonshaken cultures, defined liquid medium using glucose as the carbon source at 25°C: oxalic acid was added to the culture medium at 5 m M final concentration	“Breakdown” of 1.5 mL of 2 m M oxalic acid at 37°C in 0.2 M acetate buffer pH 3.7 with a final pH of 3; oxalate quantified over 60 min using reverse-phase HPLC; one unit of activity will convert 1 mol of oxalate to 1 mol of formate per min at pH 3 at 37°C	39
<i>Postia placenta</i> ME20 and 698 liquid cultures in a basal salts medium with 0.055 M glucose and 0.05 M ammonium phosphate, cultured 9-15 d	ODC determined by the rate of oxalic acid decomposition (see Protocol 5.5); one unit of ODC activity equals the amount of enzyme needed to degrade 1 mg oxalic acid per min at 40°C	35

5.2.2 Assay of Fungal Oxalate Decarboxylase

A variety of assay procedures are used for ODC detection (Table 5.10). They are generally based on the detection of the product (formic acid) or measure the decomposition of the substrate (oxalic acid). The method used in our laboratory is presented in Protocol 5.5. Recently, Labrou and Clonis⁴²

Oxalate decarboxylase can be quantified by measuring either the rate of oxalic acid disappearance or the rate of formic acid production. We have used both methods (Micales, 1995).³⁵ Measuring the rate of oxalic acid breakdown is simplified by a colorimetric assay marketed by Sigma Chemical Company (oxalate diagnostic kit, catalog number 591 -C). This kit has been adapted for use with a microtitre plate reader. This modification decreases the amount of reagent needed per test and maximizes the number of tests that can be analyzed simultaneously.

Dialyze enzyme preparation in 0.1 M HCl/KCl buffer, pH 1.75, or 0.2 M citrate phosphate buffer, pH 2.2 until pH has equilibrated to that of the buffer. Enzyme will not be active under more basic conditions.



Mix 50- μ L aliquots of authentic aqueous 4 m M oxalic acid with 50- μ L aliquots of enzyme preparation in wells of microtitre plates. Construct wells corresponding to 0,10,20,30,40,50,60, up to 120 min if low activity is suspected. Control wells should have oxalic acid diluted 1:1 with the appropriate buffer. At least three replicate wells should be made for each incubation time.



Incubate at 40°C in an incubator.



At appropriate times, remove 5- μ L aliquots to wells of a second microtitre plate filled with 100 μ L of oxalate reagent A (Sigma Chemical Company, St. Louis, MO). Control wells should have known concentrations of oxalic acid for the formation of a standard curve. The test kit recommends concentrations of 0,0.25,0.50, and 1.00 mM/L. These control wells should be organized in such a way that the plate reader can use them to construct a standard curve, thus converting absorbance readings into actual concentration values.



Add 10 μ L of oxalate reagent B (Sigma Chemical Company, ST. Louis, MO). Incubate for 5 min at room temperature.



Quantify color intensity at 550 nm in microtitre plate reader. The amount of enzyme activity is quantified by subtracting the amount of oxalic acid remaining in the sample wells from that present in the control wells (i.e., those that contained no enzyme solution) using the equation:

$$\text{ODC} = (\text{OA}_c - \text{OA}_s) \times 90 \times (\text{min}^{-1}),$$

where OA_c = the average amount of oxalic acid remaining in the control wells (that had no enzyme solution); OA_s = the average amount of oxalic acid remaining in the sample wells; 90 = molecular weight of oxalic acid min⁻¹ — incubation time.

One unit of oxalate decarboxylase activity is the amount of enzyme needed to degrade 1 mg of oxalic acid per minute at 40°C. Alternately, 1 U is the amount of ODC that catalyzes the conversion of 1 μ mol of oxalic acid to formic acid per minute. pH and temperature optima can be calculated as described by Micales (1995).³⁵

Protocol 5.5

Microtiter plate ODC assay procedure. (From Micales, J. A.)

Biomimetic Dye-ligand Assay⁴²

Determine the rate of formic acid formation from oxalate by coupling with formate dehydrogenase and NAD⁺ by following the increase in absorbance at 340 nm at 37°C.

Step 1— pH 5.0 (2 min)

Reaction mixture 0.6 mL containing 50 m M potassium phosphate buffer, pH 5.0, 55.7 m M oxalate, and ODC preparation (up to 0.001 U)

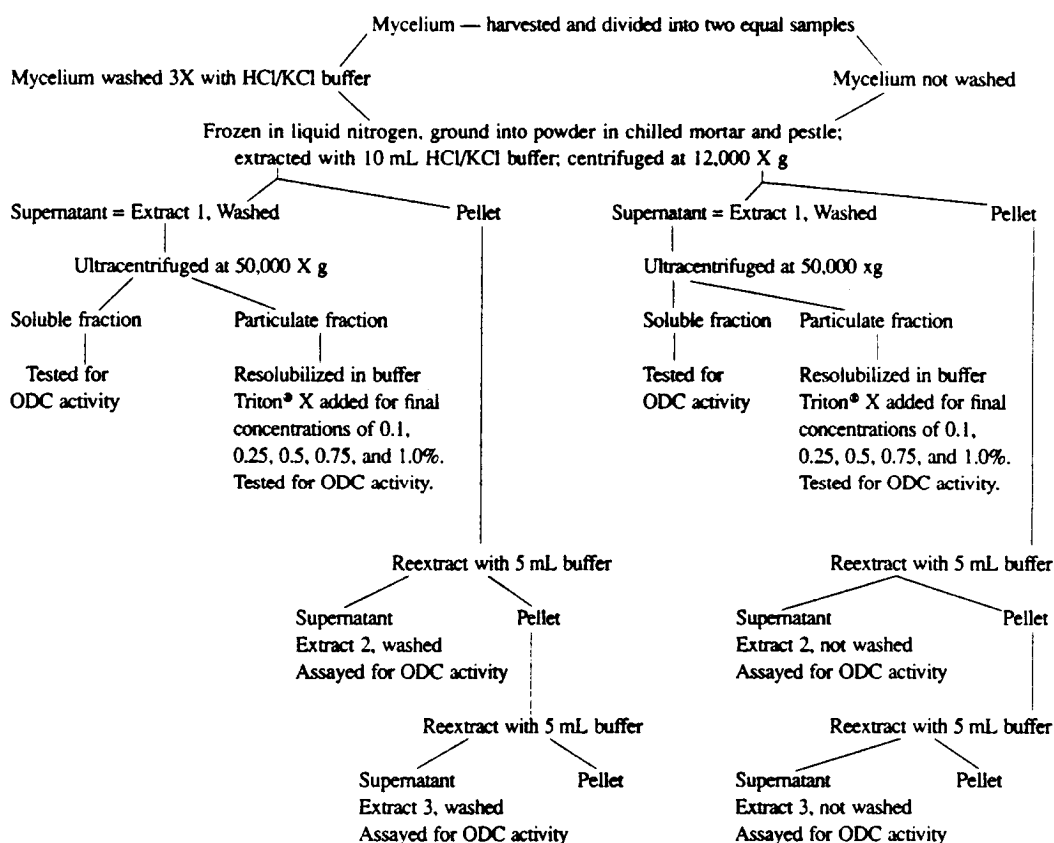
Stop reaction with 2 mL of 150 m M potassium phosphate, pH 7.5

Step 2 — Perform second step in the above mixture (final assay volume 3 mL) containing 3.8 m M NAD⁺ and 8 U formate dehydrogenase

One unit of ODC is the amount that catalyzes the conversion of 1 μ mol of oxalate to formate per minute.

Protocol 5.6

Biomimetic dye-ligand ODC assay protocol. (From Labrou, N.E. and Clonis, Y. D., J. *Biochem.*, 40, 59, 1995. With permission.)



Protocol 5.7

Protocol for the localization of ODC from mycelial extracts of *Postia placenta*. (From Micales, J. A., International Research Group on Wood Decay, Stockholm, Sweden, Doc. No IRG. WP 96-10161, 8 pp, 1996. With permission.)

reported a biomimetic dye–ligand procedure for assaying and purifying enzymes that break down dicarboxylic acids. Although this technique (Protocol 5.6) has not yet been used for ODC, it may also be applicable.

Once ODC has been purified, the K_M of the enzyme can be obtained in the presence of diverse substrates. Procedures for this have been described in Zeidan and Dashek.⁴³ Differential centrifugation has also been used to determine that the enzyme is primarily extracellular and is weakly associated with the hyphal surface.⁴⁴ A detailed flow chart describing this differential centrifugation is presented in Protocol 5.7.

5.3 Acknowledgment

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Errata

Contents

Page 49	5.2 Oxalate decarboxylase.....62 = 65
	5.2.2 Assay of Fungal Oxalate Decarboxylase.....65 = 67
	Acknowledgment.....67 = 69
Page 50	Line 4 effect = affect
Page 58	Line 15 Protocol 5.2 = Protocol 5.4
Page 60	Figure 5.12 legend Bhagaran = Bhagavan
Page 61	Line 4 under Protocol 5.1 NaOh - NaOH
Page 66	Table 5.9 Line 16 - MeoH = MeOH Line 31 $(\text{NH}_4)\text{SO}_4$ - $(\text{NH}_4)_2\text{SO}_4$
Page 71	Ref. 36 Miliari = Emiliani