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Articles

SigrafW: An Easy-to-use Program for Fitting Enzyme Kinetic Data

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SigrafW is Windows-compatible software developed using the Microsoft® Visual Basic Studio program that uses the simplified Hill equation for fitting kinetic data from allosteric and Michaelian enzymes. SigrafW uses a modified Fibonacci search to calculate maximal velocity (*V*), the Hill coefficient (*n*), and the enzyme-substrate apparent dissociation constant (*K*). The estimation of *V*, *K*, and the sum of the squares of residuals is performed using a Wilkinson nonlinear regression at any Hill coefficient (*n*). In contrast to many currently available kinetic analysis programs, SigrafW shows several advantages for the determination of kinetic parameters of both hyperbolic and nonhyperbolic saturation curves. No initial estimates of the kinetic parameters are required, a measure of the goodness-of-the-fit for each calculation performed is provided, the nonlinear regression used for calculations eliminates the statistical bias inherent in linear transformations, and the software can be used for enzyme kinetic simulations either for educational or research purposes.

Keywords: Enzyme kinetics, allosteric enzyme, Michaelian enzyme, nonlinear fitting.

The accurate estimation of kinetic parameters is of fundamental importance for biochemical studies. The use of partially purified enzyme preparations and the apparently complex relationship between velocity and substrate concentration are perhaps the main reasons that encourage enzyme characterization to be carried out in a simplified manner. In addition, enzyme kinetics analyses are often difficult to comprehend and apply because of confusing theoretical explanations and excessive use of mathematical extrapolation. Furthermore, enzyme kinetics teaching requires the association of theoretical lectures with time consuming experiments and calculations. In addition, it is frequently difficult and expensive to obtain enzymes with a specific and known mechanism of action. As a consequence, most aspects of enzymatic kinetics are often superficially exploited for teaching purposes [1, 2].

According to their kinetic behavior enzymes are classified as Michaelian [3] or allosteric [4]. For allosteric enzymes, fitting and plotting of data are usually performed according the simplified Hill equation [5]:

$$v = \frac{V \times [S]^n}{K + [S]^n} \qquad \text{(Eq. 1)}$$

where v is the reaction rate for the substrate concentration [*S*], V is the maximal rate, and K is the enzyme-substrate complex dissociation constant.

The plot of log ($v/(V - v)$) *versus* log [*S*] results in a straight line that allows the determination of the kinetic parameters after linear regression data treatment. However, fitting experimental data for allosteric enzyme kinetics using linear regression of the Hill plot can produce unreliable results due to the uncertainty of the estimates of V for the reaction. The Hill treatment has been successfully applied to steady-state kinetics by Monod and colleagues in their classical work on allosteric enzymes [6]. However, it is well known that there are limitations in the interpretation of Hill coefficients determined from steady-state kinetics as compared with direct binding experiments. The first is that the Hill equation is empirical and so K and n do not have any physiological significance. The second limitation is that substrate concentration values are restricted to those that give initial velocities in the range of 10–90% of V. As a consequence, n is not constant over the whole range of substrate concentrations.

The advantages of nonlinear regression data treatment for the determination of the kinetic parameters of enzyme-catalyzed reactions are well established [7–9]. In contrast, there are few available computer programs [10–12] that perform the calculation of kinetic parameters from raw data by nonlinear regression, and those specifically developed for this purpose often only can fit the Michaelian model [11].

SigrafW is useful for the calculation of kinetic parameters for allosteric enzymes having more than one species

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of ligand sites. In this case, Equation 1 is expanded to a sum of two Hill equations (Equation 2), and the representation of *v/V versus* log [*S*] provides a satisfactory way of plotting data over a large range of substrate concentrations.

$$v = \frac{V_1 \times [S]^{n_1}}{K_1 + [S]^{n_1}} + \frac{V_2 \times [S]^{n_2}}{K_2 + [S]^{n_2}} \quad \text{(Eq. 2)}$$

In particular, in the case where both sites are identical and independent [13], or the enzyme is Michaelian (the enzyme has only one active site), Equation 2 reduces to the Michaelis-Menten equation:

$$v = \frac{V \times [S]}{K + [S]} \quad \text{(Eq. 3)}$$

For Michaelian enzymes, the plot of *v versus* [*S*] generates a rectangular hyperbola, which is an unsatisfactory representation because it is very difficult to accurately draw a rectangular hyperbola and to estimate asymptotic values. As a consequence, most workers have preferred to use linear representations derived from the Michaelis-Menten equation. Except for the direct linear plot [14], all available representations for the evaluation of kinetic parameters of enzyme-catalyzed reactions require calculations of varying complexity [15–18]. In addition, the statistical bias inherent to all linear transformations of the Michaelis-Menten equation argues against all linear plots [19]. SigrafW overcomes such problems by providing a satisfactory way of presenting and plotting data over a wide range of substrate concentrations for Michaelian enzymes showing rate curves characterized by a Hill coefficient of 1 [20].

In this article, we present SigrafW, an accurate and easy-to-use software package that can be used to estimate the kinetic parameters of either allosteric or Michaelian enzymes for both educational and research purposes.[1]

SOFTWARE DESCRIPTION AND RATIONALE

SigrafW uses the semi-logarithmic form of the Hill equation (*v/V versus* log [*S*]) to plot kinetic data, providing a satisfactory way of plotting data over a wide range of substrate concentrations for both Michaelian and allosteric enzymes.

SigrafW was developed according to the procedure of Atkins [21] by using the sum of two Hill equations. Briefly, the routine used to calculate *V*, *n*, and *K* is a modified Fibonacci search [22] in which values of *n* are chosen within a restricted but valid range. The modified Fibonacci search is used to find the minimum in the curve and the corresponding *n* value. At each *n* value, a nonlinear Wilkinson regression is performed [19]. Each regression estimates *V*, *K*, and the sum of squares of the residuals. The program does not calculate standard deviations for estimated parameters because these quantities are of doubtful value in a nonlinear regression. The curve is fitted to the experimental data by minimizing the sum of squares of the residuals, and a correlation coefficient is calculated as a measure of the goodness-of-the-fit. SigrafW does not requires initial estimates for the kinetic parameters and avoids the possibility to fit the experimental results to the wrong model because is not necessary to know whether the enzyme is Michaelian or allosteric.

TABLE I
Hydrolysis of PNPP by alkaline phosphatase in 50 mM 2-amino-2-hydroxymethylpropane 1–3-diol buffer, pH 9.4, containing 2 mM $MgCl_2$

[PNPP] (M)	*v* (nmol/min/mg)
5×10^{-5}	1.6
1×10^{-4}	3.2
2×10^{-4}	5.5
3×10^{-4}	8.4
5×10^{-4}	13.5
7×10^{-4}	16.6
1×10^{-3}	19
2×10^{-3}	29
3×10^{-3}	36
5×10^{-3}	43
7×10^{-3}	43.5
1×10^{-2}	45.7
1.5×10^{-2}	47
2×10^{-2}	48

SigrafW runs well on any Intel©-based computer running Microsoft Windows 98 and is distributed as a self-extracting and installing package free of charge. The online help included at each step provides detailed information on how to perform the necessary calculations.

WORKED EXAMPLES

Tutorial Example 1—Determination of kinetic parameters for *p*-nitrophenylphosphate (PNPP)[2] hydrolysis by alkaline phosphatase.

In this example, data for the hydrolysis of PNPP by alkaline phosphatase will be used for the calculations of the kinetic parameters *V*, *n*, and *K*. Using data from Table I, SigrafW will display the following results:

Parameters calculated for curve 1:
V = 5.132E+01 nmol/min/mg
n = 1.081E+00
K = 1.427E–03 M
V/K = 3.597E+04 (nmol/min/mg)/(M)
Correlation = 9.986E–01
Total activity = 5.132E+01 nmol/min/mg
Baseline used for this curve = 0.000E+00 nmol/min/mg

Note that total activity (51.32 nmol/min/mg) represents the sum of *V* (51.32 nmol/min/mg) and the baseline value (0 nmol/min/mg). Fig. 1 shows the plot of *v/V versus* log [*PNPP*] for the hydrolysis of PNPP by the enzyme.

Tutorial Example 2—Determination of kinetic parameters for ATP hydrolysis by alkaline phosphatase.

Data from Table II will be used for calculation of *V*, *n*, and *K*. The user will observe that ATP hydrolysis by alkaline phosphatase resulted in a *v/V versus* log [*ATP*] biphasic curve. The curve segment in the range of 10^{-8} to 10^{-5} M ATP represents substrate binding to high affinity sites, and the higher segment curve, in the range of 10^{-5} to 3×10^{-3} M ATP, the binding to low affinity sites. During calculation

[1] Persons interested in receiving a free copy of the software should contact Dr. F. A. Leone.

[2] The abbreviation used is: PNPP, *p*-nitrophenylphosphate.

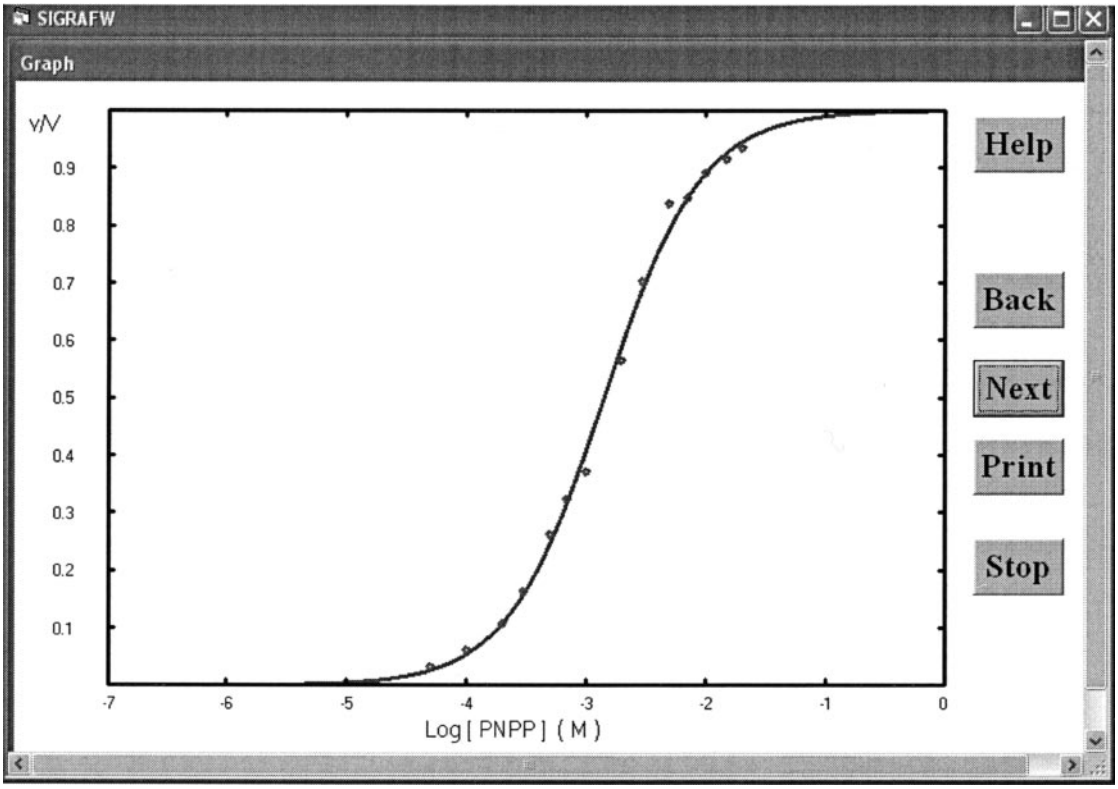

FIG. 1. Plot of *v/V versus* log [*PNPP*] for the hydrolysis of PNPP by an alkaline phosphatase.

TABLE II

Hydrolysis of ATP by alkaline phosphatase in 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM $MgCl_2$

[*ATP*] (M)	*v* (nmol/min/mg)	[*ATP*] (M)	*v* (nmol/min/mg)
1×10^{-8}	3.0	3×10^{-5}	27.9
2.5×10^{-8}	3.2	5×10^{-5}	32.2
5×10^{-8}	4.0	6×10^{-5}	33.7
7.5×10^{-8}	4.5	7.5×10^{-4}	36.4
1×10^{-7}	6.0	1×10^{-4}	38.9
2.5×10^{-7}	11.0	2×10^{-4}	50.7
5×10^{-7}	14.0	3×10^{-4}	61.5
7.5×10^{-7}	16.0	4×10^{-4}	64.7
1×10^{-6}	21.7	5×10^{-4}	69.5
2×10^{-6}	22.3	6×10^{-4}	77.5
3×10^{-6}	24.0	7.5×10^{-4}	86.5
5×10^{-6}	23.0	1.5×10^{-3}	91.3
7.5×10^{-6}	23.5	2×10^{-3}	102.6
1×10^{-5}	25.3	3×10^{-3}	102.9
2×10^{-5}	25.8		

of the kinetic parameters, SigrafW deconvolutes this biphasic saturation curve, and each segment curve is treated separately as a single saturation curve. Finally, both curve segments are fitted to the experimental data to produce the final biphasic curve as shown in Fig. 2.

Parameters calculated for curve 1:

V_1 = 2.202E+01 nmol/min/mg

n_1 = 1.315E+00

K_1 = 4.247E−07 M

V_1/K_1 = 5.186E+07 (nmol/min/mg)/(M)

Correlation = 9.936E−01

Total activity = 2.482E+01 nmol/min/mg

Baseline used for this curve = 2.800E+00 nmol/min/mg

Note that total activity (24.82 nmol/min/mg) represents the sum of V_1 (22.02 nmol/min/mg) and the baseline value (2.8 nmol/min/mg). The baseline value always represents 95% of the lowest experimental rate. This value must be introduced in the calculations to obtain the best fit to the experimental results.

Parameters calculated for curve 2:

V_2 = 8.872E+01 nmol/min/mg

n_2 = 1.170E+00

K_2 = 4.278E−04 M

V_2/K_2 = 2.074E+05 (nmol/min/mg)/(M)

Correlation = 9.976E−01

Total activity = 1.135E+02 nmol/min/mg

Baseline used for this curve = 2.482E+01 nmol/min/mg

Note that total activity (1.135E+02 nmol/min/mg) represents the sum of V_2 (88.72 nmol/min/mg), V_1 (22.02 nmol/min/mg), and the baseline value (2.8 nmol/min/mg). Fig. 2 shows the plot of *v/V versus* log [*ATP*] for the hydrolysis of ATP by the enzyme. Independent of the fact that $n < 1.0$ (for curve 1) or $n > 1.0$ (for curve 2), SigrafW gave the best fit to the experimental points with a very good correlation.

CONCLUSIONS

The most common way of calculating kinetic parameters for enzymes showing cooperative effects is to use the Hill equation [5, 23]. However, unreliable evaluations may result due to the uncertainty of estimating the *V* value. Furthermore, although several programs have been developed to fit enzyme kinetic data for Michaelian enzymes, they have been written for the DOS operating system and do not provide good quality graphs [10, 11, 24].

SigrafW serves not only for allosteric but also for Michaelian enzymes. It can be easily employed by users with no previous experience in the use of computers to fit kinetic data. In addition, it is not necessary to perform

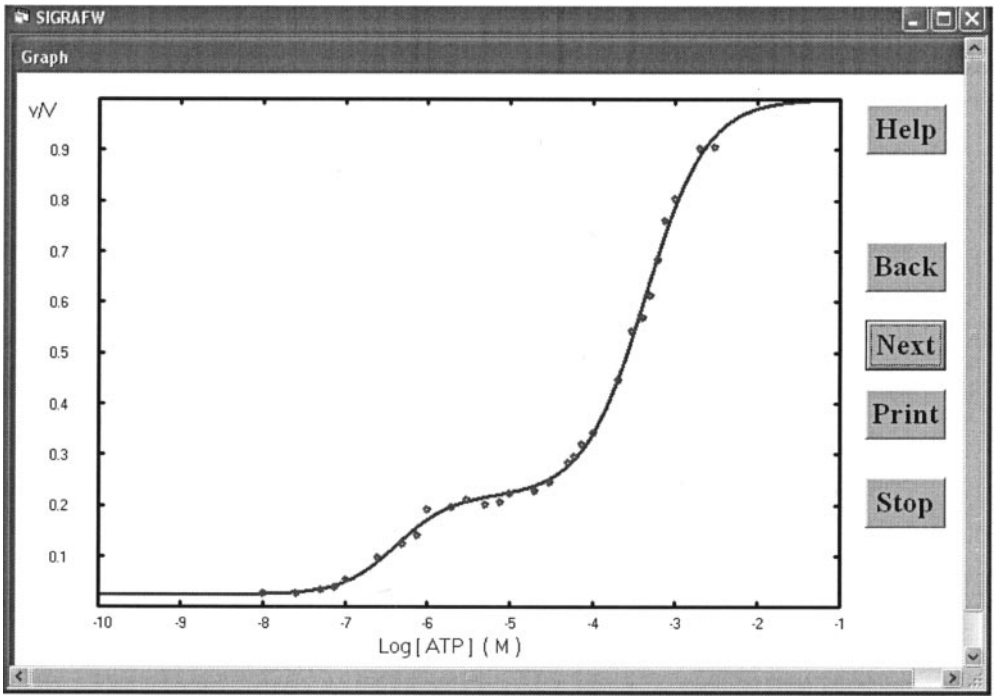

FIG. 2. Plot of *v/V versus* log [*ATP*] for the hydrolysis of ATP by an alkaline phosphatase.

initial estimates of the kinetic parameters to proceed with calculations and plotting, and the program is versatile enough for calculating the kinetic parameters for enzymes showing more than one species of ligand site. In the case of two ligand-binding sites, a sum of two simplified Hill equations is used and SigrafW decomposes the biphasic curve into two segments representing the high and low affinity sites, respectively. Each curve segment is then considered as an isolated substrate saturation curve for the determination of the kinetic parameters. This characteristic allows SigrafW to be used in enzyme kinetic simulations in which the user can verify the effect of a given substrate concentration on the estimates of *n*, *V*, and *K*. Deletion or introduction of experimental data is a feature of SigrafW that enables the user to gain insights to simulations of enzyme kinetic analysis, resulting in detailed observations on the weight of each data point to the fitting procedure. Furthermore, SigrafW also provides for the refinement of the data-fitting procedure after the acquisition of new experimental data. Finally, the use of the semilogarithmic plot of *v/V versus* log [*S*] facilitates comparative studies, giving the user a realistic view of the active site occupancy at any position on the saturation curve.

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REFERENCES

[1] A. Cornish-Bowden (1976) *Principles of Enzyme Kinetics*, Butterworths, London.
[2] F. A. Leone, L. Degreve, J. A. Baranauskas (1992) SIGRAF: A versatile computer program for fitting enzyme kinetic data, *Biochem. Educ.* **20,** 94–96.
[3] L. Michaelis, M. L. Menten (1913) Die kinetik der Invertinwirkung, *Biochem. Zeit.* **49**, 333–369.
[4] J. P. Changeux (1964) Sur les proprietés allostériques de la L-thréonine désaminase de biosynthèse. II. Cinetiques d'action de la L-thréonine désaminase de biosynthèse vis-à-vis du substrate et de l1inhibiteur naturels, *Bull. Soc. Chim. Biol.* **46**, 947–961.
[5] A. Hill (1910) The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves, *J. Physiol.* **40,** iv-vii.
[6] J. Monod, J. Wyman, J. P. Changeux (1965) On the nature of allosteric transitions: *a* plausible model, *J. Mol. Biol.* **12**, 88–118.
[7] C. H. Suelter (1985) *A Practical Guide to Enzymology*, John Wiley & Sons, New York.
[8] R. Tommasini, L. Endrenyi, P. A. Taylor, D. J. Mahuran, J. A. Lowden (1985) A statistical comparison of parameter estimation for the Michaelis-Menten kinetics of humam placental hexosaminidase, *Can. J. Biochem. Cell Biol.* **63**, 225–230.
[9] P. J. F. Henderson (1992) Statistical analysis of enzyme kinetic data, in *Enzyme Assays: A Practical Approach* (R. Eisenthal, M. J. Danson, eds.) pp. 277–316, IRL Press, Oxford, United Kingdom.
[10] P. A. Williams (1983) ENZPACK: A microcomputer program to aid in teaching of enzyme kinetic, *Biochem. Educ.* **11**, 141–143.
[11] A. Hernández, M. T. Ruiz (1998) An Excel template for calculation of enzyme kinetic parameters by non-linear regression, *Bioinformatics.* **14**, 227–228.
[12] R. J. Leatherbarrow (1988) Enzfitter: A non-linear regression data analysis program for IBM PC, *J. Am. Chem. Soc.* **110**, 4100.
[13] I. H. Segel (1975) *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, New York.
[14] R. Eisenthal, A. Cornish-Bowden (1974) The direct linear plot. A new graphic procedure for estimating enzyme kinetic parameters, *Biochem. J.* **139**, 715–720.
[15] H. Lineweaver, D. Burk (1934) The determination of enzyme dissociation constants, *J. Am. Chem. Soc.* **56**, 658–666.
[16] G. S. Eadie (1942) The inhibition of cholinesterase by physostigmine and prostigmine, *J. Biol. Chem.* **146**, 85–93.
[17] B. H. J. Hofstee (1952) Specificity of esterases. I. Identification of two pancreatic aliesterases, *J. Biol. Chem.* **199**, 357–367.
[18] C. S. Hanes (1932) CLXVII. Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley, *Biochem. J.* **26**, 1406–1421.
[19] G. N. Wilkinson (1961) Statistical estimations in enzyme kinetics, *Biochem. J.* **80**, 324–332.
[20] A. Goldbeter (1974) Kinetic negative co-operativity in the allosteric model of Monod, Wyman and Changeux, *J. Mol. Biol.* **90**, 185–190.
[21] G. L. Atkins (1973) A simple digital-computer program for estimating the parameters of the Hill equation, *Eur. J. Biochem.* **33**, 175–180.

[22] G. S. G. Beveridge, R. S. Schechter (1970) *Optimization: Theory and Practice*, McGraw-Hill, New York.
[23] H. J. Wieker, K. J. Johanes, B. Hess (1970) A computer program for the determination of kinetic parameters from sigmoidal steady-state kinetics, *FEBS Lett.* **8**, 178–185.
[24] A. Cornish-Bowden (1995) *Analysis of Enzyme Kinetic Data*, Oxford, United Kingdom.

APPENDIX 1

$$v = \frac{V \times [S]^n}{K + [S]^n}$$

$$v \times K + v \times [S]^n = V \times [S]^n$$

$$v \times K = V \times [S]^n - v \times [S]^n$$

$$v \times K = V \times [S]^n - v \times [S]^n = (V\text{–}v) \times [S]^n$$

$$\frac{v}{V - v} = \frac{[S]^n}{K}$$

$$\log\left(\frac{v}{V - v}\right) = n \times \log[S] - \log K$$