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Kinetic Analyses of Enzyme Reaction Curves with New Integrated Rate Equations and Applications

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1. Introduction

A reaction system of Michaelis-Menten enzyme on single substrate can be characterized by the initial substrate concentration before enzyme action (S_0), the maximal reaction rate (V_m) and Michaelis-Menten constant (K_m), besides some other required parameters. The estimations of S_0, V_m and K_m can be used to measure enzyme substrates, enzyme activities, epitope or hapten (enzyme-immunoassay), irreversible inhibitors and so on. During enzyme reaction, the changes of substrate or product concentrations can be monitored; continuous monitor of such changes provides a reaction curve while discontinuous monitor of such changes provides signals just for the starting point and the terminating point of enzyme reaction. It is an end-point method when only signals for the starting point and the terminating point are analyzed. It is a kinetic method when a range of data from a reaction curve are analyzed, and can be classifieid into the initial rate method and kinetic analysis of reaction curve. The initial rate method only analyzes data for initial rate reaction whose instantaneous rates are constants; kinetic analysis of reaction curve analyzes data whose instantaneous rates show obvious deviations from the initial rate (Bergmeyer, 1983; Guilbault, 1976; Marangoni, 2003). To estimate those parameters of an enzyme reaction system, kinetic analysis of reaction curve is favoured because the analysis of one reaction curve can concomitantly provide V_m, S_0 and K_m. Hence, methods for kinetic analysis of reaction curve to estimate parameters of enzyme reaction systems are widely studied.

An enzyme reaction curve is a function of dependent variables, which are proportional to concentrations of a substrate or product, with respect to reaction time as the predictor variable. In general, there are two types of enzyme reaction curves. The first type involves the action of just one enzyme, and employs either a selective substrate to detect the activity of one enzyme of interest or a specific enzyme to act on a unique substrate of interest. The second type involves the actions of at least two enzymes, and requires at least one auxiliary enzyme as a tool to continuously monitor a reaction curve. The second type is an enzyme-coupled reaction system. For kinetic analysis of reaction curve, there are many reports on

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one enzyme reaction system, but are just a few reports on enzyme-coupled reaction system (Atkins & Nimmo, 1973; Liao, et al., 2005; Duggleby, 1983, 1985, 1994; Walsh, 2010).

In theory, enzyme reactions may tolerate reversibility, the activation/inhibition by substrates/products, and even thermo-inactivation of enzyme. From a mathematic view, it is still feasible to estimate parameters of an enzyme reaction system by kinetic analysis of reaction curve if the roles of all those factors mentioned above are included in a kinetic model (Baywenton, 1986; Duggleby, 1983, 1994; Moruno-Davila, et al., 2001; Varon, et al., 1998). However, enzyme kinetics is usually so complex due to the effects of those mentioned factors that there are always some technical challenges for kinetic analysis of reaction curve. Hence, most methods for kinetic analysis of reaction curve are reported for enzymes whose actions suffer alterations by those mentioned factors as few as possible.

In practice, kinetic analysis of reaction curve usually employs nonlinear-least-square-fitting (NLSF) of the differential or integrated rate equation(s) to either the reaction curve *per se* or data set(s) transformed from the reaction curve (Cornish-Bowden, 1995; Duggleby, 1983, 1994; Orsi & Tipton, 1979). The use of NLSF rather than matrix inversion is due to the existence of multiple minima of the sum of residual squares with respect to some nonlinear parameters (Liao, et al., 2003a, 2007a). When a differential rate equation is used, numerical differentiation of data from the reaction curve has to be employed to derive instantsneous reaction rates. In this case, there must be intervals as short as possible to monitor reaction curves (Burden & Faires, 2001; Dagys, 1990; Hasinoff, 1985; Koerber & Fink, 1987). However, the instantaneous reaction rates from reaction curves inherenetly exhibit narrow distribution ranges and large errors; the strategy by numerical differentiation of data in a reaction curve is unfavourable for estimating V_m and S_0 because of their low reliaiblity and unsatisfactory working ranges. On the other hand, when an integrated rate equation of an enzyme reaction is used for kinetic analysis of reaction curve, there is no prerequisites of short intervals to record reaction curves so that automated analyses in parallel can be realized for enhanced performance with a large number of samples. As a result, integrated rate equations of enzymes are widely studied for kinetic analysis of reaction curve to estimate parameters of enzyme reaction systems (Duggleby, 1994;Liao, et al, 2003a, 2005a; Orsi & Tipton, 1979).

Due possibly to the limitation on computation resources, integrated rate equations of enzymes in such methods are usually rearranged into special forms to facilitate NLSF after data transformation (Atkins & Nimmo, 1973; Orsi & Tipton, 1979). In appearance, the uses of different forms of the same integrated rate equation for NLSF to data sets transformed from the same reaction curve can give the same parameters. However, kinetic analysis of reaction curve with rearranged forms of an integrated rate equation always gives parameters with uncertainty too large to have practical roles (Newman, et al, 1974). Therefore, proper forms of an integrated rate equation should be selected carefully for estimating parameters by kinetic analysis of reaction curve.

In the past ten years, our group studied chemometrics for kinetic analysis of reaction curve to estimate parameters of enzyme reaction systems; the following results were found. (a) In terms of reliability and performance for estimating parameters, the use of the integrated rate equations with the predictor variable of reaction time is superior to the use of the integrated rate equations with predictor variables other than reaction time (Liao, et al., 2005a); (b) the integration of kinetic analysis of reaction curve with other methods to quantify initial rates

and substrates has more absorbing advantages (Liu, et al., 2009; Yang, et al., 2010); such integration strategies can be applied to enzyme-coupled reaction systems and enzymes sufferring inhibition by substrates/products. Herein, we discuss chemometrics for both kinetic analysis of reaction curve and its integration with other methods, and demonstrate their applications to quantify enzyme initial rates and substrates with some typical enzymes.

2. Kinetic analysis of enzyme reaction curve: chemometrics and application

To estimate parameters by kinetic analysis of reaction curve, the desired parameters are included in a set of parameters for the best fitting. Regardless of the number of enzymes involved in a reaction curve, there are the following two approaches for kinetic analysis of reaction curve based on different ways to realize NLSF and their data transformation.

In the first approach, with a differential or integrated rate equation, a series of dependent variables are derived from data in a reaction curve with each set of preset parameters. Such dependent variables should follow a predetermined response to predictor variables that are either reaction time or data transformed from those in the reaction curve. The goodness of the predetermined response is the criterion for the best fitting. In this approach, NLSF is realized with a model for data transformed from a reaction curve (Burguillo, 1983; Cornish-Bowden, 1995; Liao, 2005; Liao, et al., 2003a, 2003b, 2005a, 2005b; Orsi & Tipton, 1979).

In the second approach, reaction curves are calculated with sets of preset parameters by iterative numerical integration from a preset staring point. Such calculated reaction curves are fit to a reaction curve of interest; the least sum of residual squares indicates the best fitting (Duggleby, 1983, 1994; Moruno-Davila, et al., 2001; Varon, et al., 1998; Yang, et al., 2010). In this approach, calculated reaction curves still utilize reaction time as the predictor variable and become discrete at the same intervals as the reaction curve of interest. Clearly, there is no transformation of data from a reaction curve in this approach.

With any enzyme, iterative numerical integration of the differential rate equation(s) from a starting point with sets of preset parameters can be universally applicable regardless of the complexity of the kinetics. Thus, the second approach exhibits better universality and there are few technical challenges to kinetic analysis of reaction curve *via* NLSF. In fact, however, the second approach is occasionally utilized while the first approach is widely practiced.

In the following subsections, the differential rate equation of simple Michaelis-Menten kinetics on single substrate is integrated; the prerequisites for kinetic analysis of reaction curve with integrated rate equations, kinetic analysis of enzyme-coupled reaction curve, the integrations of kinetic analysis of reaction curve with other methods, and the applications of such integration strategies to some typical enzymes are discussed.

2.1 Integrated rate equation for one enzyme on single substrate

Assigning instantaneous substrate concentration to S, instantaneous reaction time to t, steady-state kinetics of Michaelis-Menten enzyme on single substrate follows Equ.(1).

$$-dS/dt = (V_{\mathrm{m}} \times S)/(K_{\mathrm{m}} + S) \tag{1}$$

Assigning the substrate concentration at the first point for analysis to S_1, Equ.(1) is integrated into Equ.(2) when the enzyme is stable, the substrate and product do not alter the intrinsic activity of the enzyme and the reaction is irreversible (Atkins & Nimmo, 1973; Marangoni, 2003; Orsi & Tipton, 1979; Zou & Zhu, 1997). In Equ.(2), t_{lag} accounts for the lag time of steady-state reaction. After transformation of data in a reaction curve according to Equ.(3), there should be a linear response of the left part in Equ.(2) to reaction time, as in Equ.(4). The goodness of this linear response is judged based on regression analysis. However, to estimate parameters by kinetic analysis of reaction curve *via* NLSF, there are the following general prerequisites for Equ.(2) or any of its equivalency.

$$(S_1 - S)/K_m + \ln(S_1/S) = (V_m/K_m) \times (t - t_{lag}) \tag{2}$$

$$y = (S_1 - S)/K_m + \ln(S_1/S) \tag{3}$$

$$y = a + b \times t \tag{4}$$

The first prerequisite is that enzyme reaction should apparently follow kinetics on single substrate. For enzyme reactions with multiple substrates whose concentrations are all changing during reactions, kinetic analysis of reaction curve always give parameters of too low reliability to have practical roles no matter what methods are used for NLSF (data unpublished). From our experiences to estimate parameters by kinetic analyses of reaction curves, any substrate at levels below 10% of its K_m can be considered negligible; the use of one substrate at levels below 10% of those of other substrates can make enzyme reactions follow single substrate kinetics (Liao, et al, 2001; Liao, et al, 2003a, 2003b; Li et al., 2011; Zhao et al., 2006). For any enzyme on multiple substrates, therefore, there are two approaches to make it apparently follow kinetics on single substrate. The first is the use of one substrate of interest at levels below 10% of those of the other substrates; this approach has universal applicability to common enzymes such as hydrolases in aqueous buffers and oxidases in air-saturated buffers. The second is the utilization of special reaction systems to regenerate the substrate of the enzyme of interest by actions of some auxiliary enzymes, and indeed this approach usually yields enzyme-coupled reaction curves of complicated kinetics.

The second prerequisite is that enzyme reaction should be irreversible. In theory, the estimation of parameters by kinetic analysis of reaction curve is still feasible when reaction reversibility is considered, but the estimated parameters possess too low reliability to have practical roles (data unpublished). Generally, a preparation of a substance with contaminants less than 1% in mass content can be taken as a pure substance. Namely, a reagent leftover in a reaction accounting for less than 1% of that before reaction can be negligible. For convenience, therefore, an enzyme reaction is considered irreversible when the leftover level of a substrate of interest in equilibrium is much less than 1%of its initial one. To promote the consumption of the substrate of interest, the concentrations of other substrates should be preset at levels much over 10 times the initial level of the substrate of interest. In this case, the enzyme reaction is apparently irreversible and follows kinetics on single substrate. Or else, the use of scavenging reactions to remove products can drive the reaction forward. The concurrent uses of both approaches are usually better.

The third prerequisite is that there should be steady-state data for analysis (Atkins & Nimmo, 1973; Dixon & Webb, 1979; Liao, et al, 2005a; Marangoni, 2003; Orsi & Tipton, 1979).

For this prerequisite, the first and the last points of data in a reaction curve for analysis should be carefully selected. The first point should exclude data within the lag time of steady-state reaction. The last point should ensure data for analyses to have substrate concentrations high enough for steady-state reaction. Namely, substrate concentrations should be much higher than the concentration of the active site of the enzyme (Dixon & Webb, 1979). The use of special weighting functions for NLSF can mitigate the contributions of residual squares at low substrate levels that potentially obviate steady-state reaction.

The forth prerequisite is that the enzyme should be stable to validate Equ.(2), or else the inactivation kinetics of the enzyme should be included in the kinetic model. Enzyme stability should be checked before kinetic analysis of reaction curve. When the inactivation kinetics of an enzyme is included in a kinetic model for kinetic analysis of reaction curve, the integrated rate equation is usually quite complex or even inaccessible if the inactivation kinetics is too complex. For kinetic analysis of reaction curve of complicated kinetics, numerical integration to produce calculated reaction curves for NLSF to a reaction curve of interest, instead of NLSF with Equ.(4), can be used to estimate parameters (Duggleby, 1983, 1994; Moruno-Davila, et al., 2001; Varon, et al., 1998; Yang, et al., 2010).

The fifth prerequisite is that there should be negligible inhibition/activation of activity of an enzyme by products/substrates, or else such inhibition/activation on the activity of the enzyme by its substrate/product should be included in an integrated rate equation for kinetic analysis of reaction curve (Zhao, L.N., et al., 2006). For validating Equ.(2), any substrate that alters enzyme activity should be preset at levels low enough to cause negligible alterations; any product that alters enzyme activity can be scavenged by proper reactions. When such alterations are complex, numerical integration of differential rate equations for NLSF to a reaction curve of interest can be used (Duggleby, 1983, 1994; Moruno-Davila, et al., 2001; Varon, et al., 1998).

Obviously, the first three prerequisites are mandatory for the inherent reliability of parameters estimated by kinetic analysis of reaction curve; the later two prerequisites are required for the validity of Equ.(2) or its equivalency for kinetic analysis of reaction curve.

2.2 Realization of NLSF and limitation on parameter estimation

To estimate parameters by kinetic analysis of reaction curve based on NLSF, the main concerns are the satisfaction to the prerequisites for the quality of data under analysis, the procedure to realize NLSF, and the reliability of parameters estimated thereby.

For the estimation of parameters by kinetic analysis of reaction curve, there are two general prerequisites for the quality of data under analysis: (a) there should be a minimum number of the effective data whose changes in signals are over three times the random error; (b) there should be a minimum consumption percentage of the substrate in such effective data for analysis. In general, at least two parameters like V_m and S_0 should be estimated; the minimum number of the effective data should be no less than 7 (Atkins & Nimmo, 1973; Baywenton, 1986; Miller, J. C. & Miller, J. N., 1993). The minimum consumption percentage of the substrate can be about 40% if only V_m and S_0 are estimated while other parameters are fixed as constants. In general, the estimation of more parameters requires higher consumption percentages of the substrate in the effective data for analysis.

With a valid Equ.(2), data in a reaction curve can be transformed according to Equ.(3) to realize NLSF with Equ.(4). The use of Equ.(4) for NLSF needs no special treatment of the unknown t_{lag}. For any method to continuously monitor reaction curve, there may be an unknown but constant background in signals (Newman, et al., 1974; Liao, et al., 2003a, 2005a; Yang, et al., 2010). Thus, the background in the signal for S_1 in Equ.(2) is better to be treated as a nonlinear parameter to realize NLSF; this procedure gives the term of NLSF but causes the burden of computation; as a result, a rearranged form of Equ.(2) is suggested for kinetic analysis of reaction curve (Atkins & Nimmo, 1973; Liao, et al., 2005a).

In theory, Equ.(2) can be rearranged into Equ.(5) as a linear function of V_m and K_m. In Equ.(5), the instantaneous reaction time at the moment for S_1 is preset as zero so that there is no treatment of t_{lag}. When the signal for S_1 is not treated as a nonlinear parameter, kinetic analysis of reaction curve by fitting with Equ.(5) can be finished within 1 s with a pocket calculator. However, parameters estimated with Equ.(5) always have so large errors that Equ.(5) is scarcely practiced in biomedical analyses. Hence, the proper form of an integrated rate equation after validating should be selected carefully.

$$(S_1 - S)/(t - t_{lag}) = V_m - K_m \times (\ln(S_1/S)/(t - t_{lag})) \quad (5)$$

In principle, to reliably estimate parameters based on NLSF, the distribution ranges of both the dependent variables and the predictor variables in any kinetic model should be as wide as possible while their random errors should be as small as possible (Baywenton, 1986; del Rio, et al., 2001; Draper & Smith, 1998; Miller, J. C. & Miller, J. N., 1993). By serial studies with common enzymes, we found the use of Equ.(4) or similar forms of integrated rate equations with the predictor variables of reaction time for kinetic analysis of reaction curve could give reliable V_m and S_0, when K_m was fixed at a constant after optimization (Liao, 2005; Liao, et al, 2001, 2003a, 2003b, 2005a, 2005b, 2006, 2007b; Zhao, Y.S., et al., 2006, 2009). Reaction time as the predictor variable has the widest distribution and the smallest random errors, in comparison to the predictor variable in Equ.(5). The left part in Equ.(4) also possess a wider distribution range. Such differences in predictor variables and dependent variables should account for different reliability of parameters estimated with Equ.(2) and Equ.(5), and thus an integrated rate equation with the predictor variable of reaction time may be the proper form for kinetic analysis of reaction curve. However, when NLSF with Equ.(4) is realized with S_1 as a nonlinear parameter, there is nearly 10 s for computation with Celeron 300A CPU on a personal computer. Currently, computation resource is no longer a problem and thus Equ.(4) or its equivalent equations should always be adopted.

The selection of a weighting factor for kinetic analysis of reaction curve is also a concern. Based on error propagation and the principle for weighted NLSF with y defined in Equ.(3), squares of instantaneous rates can be the weighting factors (W_f) with Equ.(4) for NLSF to get the weighted sum of residual squares (Q), as described in Equ.(6), Equ.(7) and Equ.(8) (Baywenton, 1986; Draper & Smith, 1998; Gutierrez & Danielson, 2006; Miller, J. C. & Miller, J. N., 1993). The use of a weighting function like Equ.(7) can mitigate the effects of errors in substrate or product concentrations near the completion of reaction. The resistance of an estimated parameter (the variation within 3% in our studies) to reasonable changes in data ranges for analysis can be a criterion to judge the reliability of parameter estimated.

$$\partial y/\partial S = -(K_m + S)/(K_m \times S) \quad (6)$$

$$Wf = \partial S/\partial y = -K\mathrm{m} \times S/(K\mathrm{m} + S) \tag{7}$$

$$Q = \sum Wf^2 \times (y_{\mathrm{predicted}} - y_{\mathrm{calculated}})^2 \tag{8}$$

It is also concerned which parameter is suitable for estimation by kinetic analysis of reaction curve. In theory, all parameters of an enzyme reaction system can be simultaneously estimated by kinetic analysis of reaction curve. However, there is unknown covariance among some parameters to devalue their reliability; there is the limited accuracy of original data for analyses and the estimation of some parameters with narrow working ranges will have negligible practical roles. V_m is independent of all other parameters and so is S_0, and the assay of V_m and S_0 are already routinely practiced in biomedical analyses. Therefore, V_m and S_0 may be the parameters suitable for estimation by kinetic analysis of reaction curve. Additionally, K_m is used for screening enzyme mutants and enzyme inhibitors; but K_m estimated by kinetic analysis of reaction curve usually exhibits lower reliability and is preferred to be fixed for estimating V_m and S_0. If K_m is estimated as well, S_1 should be at least 1.5-fold K_m and there should be more than 85% consumption of the substrate in the data selected for analysis (Atkins & Nimmo, 1973; Liao, et al., 2005a; Newman, et al., 1974; Orsi & Tipton, 1979). To estimate K_m, the initial datum (S_1) and its corresponding ending datum from a reaction curve for analysis should be tried sequentially till the requirements for data range are met concurrently. In this case, the estimation of S_1 has no practical roles. In general, the resistance of V_m and S_0 to reasonable changes in ranges of data for analyses can be a criterion to select the optimized set of parameters that are fixed as constants.

In comparison to the low reliability to estimate K_m independently for screening enzyme inhibitors and enzyme mutants, the ratio of V_m to K_m as an index of enzyme activity can be estimated robustly by kinetic analysis of reaction curve. Reversible inhibitors of Michaelis-Menten enzyme include competitive, noncompetitive, uncompetitive and mixed ones (Bergmeyer, 1983; Dixon & Webb, 1979; Marangoni, 2003). The ratios of V_m to K_m will respond to concentrations of common inhibitors except uncompetitive ones that are very rare in nature. Thus, the ratio of V_m to K_m can be used for screening common inhibitors. More importantly, the ratio of V_m to K_m is an index of the intrinsic activity of an enzyme and the estimation of the ratios of V_m to K_m can also be a promising strategy to screen enzyme mutants of powerful catalytic capacity (Fresht, 1985; Liao, et al., 2001; Northrop, 1983).

For robust estimation of the ratio of V_m to K_m of an enzyme, S_0 can be preset at a value below 10% of K_m to simplify Equ.(2) into Equ.(9). Steady-state data from a reaction curve can be analyzed after data transformation according to the left part in Equ.(9). For validating Equ.(9), it is proposed that S_0 should be below 1% of K_m (Mey1er-Almes & Auer, 2000). The use of extremely low S_0 requires special methods to monitor enzyme reaction curves and steady-state reaction can not always be achieved with enzymes of low intrinsic catalytic activities. On the other hand, the use of S_0 below 10% of K_m is reasonable to estimate the ratio of V_m to K_m (Liao, et al., 2001). To estimate the ratio of V_m to K_m, the use of Equ.(9) to analyze data is robust and resistant to variations of S_0 if Equ.(9) is valid; this property makes the estimation of the ratio of V_m to K_m for screening reversible inhibitors superior to the estimation of the half-inhibition concentrations (Cheng & Prusoff, 1973).

$$\ln(S1/S) = a + (V\mathrm{m}/K\mathrm{m}) \times t \tag{9}$$

Kinetic analysis of reaction curve requires more considerations when activities of enzymes are altered by their substrates/products. In this case, more parameters can be included in kinetic models similar to Equ.(2) for kinetic analysis of reaction curve, but there must be complicated process to optimize reaction conditions and preset parameters. Based on the principle for kinetic analysis of reaction curve described above, we developed some new integration strategies to successfully quantify enzyme initial rates and substrate with absorbing performance even when the activities of enzymes of interest are altered significantly by substrates/products (Li, et al., 2011; Liao, 2007a; Zhao, L.N., et al., 2006).

2.3 Kinetic analysis of enzyme-coupled reaction curve

When neither substrate nor product is suitable for continuous monitor of reaction curve, a tool enzyme can be used to regenerate a substrate or consume a product of the enzyme of interest; the action of the tool enzyme should consume/generate a substrate/product as an indicator suitable for continuous monitor of reaction curve. Namely, the reaction of the tool enzyme is coupled to the reaction of an enzyme of interest for continuous monitor of reaction curve (Bergmeyer, 1983; Guilbault, 1976; Dixon & Webb, 1979). When such enzyme-coupled assays are used to measure initial rates of an enzyme, there are always unsatisfactory linear range because the activities of the tool enzyme is always limited and the concentration of the substrate of the tool enzyme is also limited (Bergmeyer, 1983; Dixon & Webb, 1979). It is expected that kinetic analysis of enzyme-coupled reaction curve may effectively enhance the upper limit of linear response. However, kinetics of enzyme-coupled reaction systems is described with a set of differential rate equations, which cause difficulty in accessing an integrated rate equation with the predictor variable of reaction time.

In this case, iterative numerical integration to obtain calculated reaction curves for NLSF to a reaction curve of interest can be used (Duggleby, 1983, 1994; Moruno-Davila, et al., 2001; Varon, et al., 1998; Yang, et al., 2010). Lactic dehydrogenase (LDH) is widely used as a tool enzyme for enzyme-coupled assay. The assay of activity of alanineaminotransferase (ALT) in sera has important biomedical roles and usually employs LDH-coupled assay. For LDH-coupled ALT assay, iterative numerical integration of the set of differential rate equations with each set of preset parameters from a preset starting point can produce a calculated reaction curve; such a calculated reaction curve can be made discrete at the same intervals as the reaction curve of interest and then be used for NLSF to the reaction curve of interest.

The process of iterative numerical integration for LDH-coupled ALT assay is given below (Yang, et al., 2010). In an LDH-coupled ALT reaction system, assigning instantaneous concentration of NADH to $C_{n,i}$, instantaneous concentration of pyruvate to $C_{p,i}$, instantaneous absorbance at 340 n for NADH to A_i, the molar absorptivity of NADH to ε, the initial rate of ALT under steady-state reaction to V_{1k}, the maximal activity of LDH to V_m, the integration step to Δt, there are Equ.(10), Equ.(11) and Equ.(12) to describe the iterative integration of the set of differential rate equations. Calculated reaction curves according to Equ. (12) using different sets of preset parameters become discrete and are fit to the reaction curve of interest, and background absorbance at 340 nm is treated as a parameter as well.

$$C_{n,i} = (A_i - A_b)/\varepsilon \tag{10}$$

$$C_{p,i+1} = C_{p,i} + V_{1k} \times \Delta t - V_m \times \Delta t / (1 + K_a / C_{n,i} + K_b / C_{p,i} + K_{ab} / (C_{n,i} \times C_{p,i})) \quad (11)$$

$$A_{i+1} = A_i - \varepsilon \times V_m \times \Delta t / (1 + K_a / C_{n,i} + K_b / C_{p,i} + K_{ab} / C_{n,i} \times C_{p,i}) \quad (12)$$

By simulation with such a new approach for kinetic analysis of enzyme-coupled reaction curve recorded at 1-s intervals, the upper limit of linear response for measuring ALT initial rates is increased to about five times that by the classical initial rate method. This new approach is resistant to reasonable variations in data range for analysis. By experimentation using the sampling intervals of 10 s, the upper limit is about three times that by the classical initial rate method. Therefore, this new approach for kinetic analysis of enzyme-coupled reaction curve is advantageous, and can potentially be a universal approach for kinetic analysis of reaction curve of any system of much complicated kinetics.

The computation time for numerical integration is inversely proportional to the integration step, Δt; the use of shorter Δt is always better but Δt of 0.20 s at low cost on computation is sufficient for a desirable upper limit of linear response. This new approach with Celeron 300A CPU on a personal computer needs about 10 min for just 30 data in a LDH-coupled reaction curve, but it consumes just about 5 s with Lenovo Notebook S10e. The advancement of personal computers surely can promote the practice of this approach.

2.4 Integration of kinetic analysis of reaction curve with other methods

Any analytical method should have favourable analysis efficiency, wide linear range, low cost and strong robustness. Kinetic analysis of reaction curve for V_m and S_0 assay can have much better upper limit of linear response, but inevitably tolerates low analysis efficiency when wide linear range is required. Based on kinetic analysis of reaction curve, however, our group developed two integration strategies for enzyme initial rate and substrate assay, respectively, with both favourable analysis efficiency and ideal linear ranges.

2.4.1 New integration strategy for enzyme initial rate assay

The classical initial rate method to measure enzyme initial rates requires S_0 much higher than K_m to have desirable linear ranges (Bergmeyer, 1983; Dixon & Webb, 1979; Guilbault, 1976; Marangoni, 2003). Due to substrate inhibition, limited solubility and other causes, practical substrate levels are always relatively low and thus the linear ranges by the classical initial rate method are always unsatisfactory (Li, et al., 2011; Morishita, et al., 2000; Stromme & Theodorsen, 1976). As described above, kinetic analysis of reaction curve can measure enzyme V_m, and many approaches based on kinetic analysis of reaction curve are already proposed (Cheng, et al., 2008; Claro, 2000; Cornish-Bowden 1975, 1995; Dagys, et al., 1986, 1990; Duggleby, 1983, 1985, 1994; Hasinoff, 1985; Koerber, & Fink, 1987; Liao, et al., 2001; Lu & Fei, 2003; Marangoni, 2003; Walsh, et al. 2010). Such approaches all require substrate consumption percentage over 40% with K_m preset as a constant. As a result, there should be intolerably long reaction duration to monitor reaction curves for samples of low enzyme activities, or else the lower limits of linear response are unfavourable.

The integration of kinetic analysis of reaction curve using proper integrated rate equations with the classical initial rate method gives an integration strategy to measure enzyme initial

rates with expanded linear ranges and practical analysis efficiency. This integration strategy is effective at substrate concentrations from one-eighth of K_m to three-fold of K_m (Li, et al., 2011; Liao, et al., 2009; Liu, et al., 2009; Yang, et al., 2011). The integration strategy for enzyme initial rate assay uses a special method to convert V_m into initial rates so that the indexes of enzyme activities by both methods become the same; it is applicable to enzymes suffering strong inhibition by substrates/products (Li, et al., 2011). Walsh et al. proposed an integration strategy to measure enzyme initial rate but they employed Equ.(9) that requires substrate levels below 10% of K_m (Walsh, et al. 2010). Our integration strategy is valid at any substrate level to satisfy Equ.(2) and hence can be a universal approach to common enzymes of different K_m. The principles and applications of the integration strategy to one enzyme reaction systems and enzyme-coupled reaction systems are discussed below.

As for one enzyme reaction systems, kinetic analysis of reaction curve can be realized with an integrated rate equation after data transformation; the integration strategy for enzyme initial rate assay requires enzyme kinetics on single substrate and an integrated rate equation with the predictor variable of reaction time (Liao, et al., 2003a, 2005a, Zhao, L.N., et al., 2006). Moreover, the integration strategy should solve the following challenges: (a) there should be an overlapped range of enzyme activities measurable by both methods with consistent results; (b) there should be consistent slopes of linear response for enzyme activities to enzyme quantities by both methods (Figure 1). After these two challenges are solved, the linear segment of response by the classical initial rate method is an extended line of the linear segment of response by kinetic analysis of reaction curve (Liu, et al., 2009).

To solve the first challenge, a practical S_0 and reasonable duration to monitor reaction curve for favourable analysis efficiency are required as optimized experimental conditions. By mathematic derivation and simulation analyses to solve the first challenge, it is demonstrated that a ratio of S_0 to K_m from 0.5 to 2.5, the duration of 5.0 min to monitor reaction curves at intervals no longer than 10 s can solve the first challenge for most enzymes, any ratio of S_0 to K_m smaller than 0.5 or larger than 2.5 requires longer duration to monitor reaction curves. The use of S_0 about one-eighth of K_m requires no shorter than 8.0 min to monitor reaction curves at 10-s intervals to solve the first challenge (Li, et al., 2011; Liu, et al., 2009). When S_0 is too much larger than three times K_m, reaction time to record reaction curves for analysis to solve the first challenge should be much longer than 5 min. Clearly, the first challenge can be solved with practical S_0 for favourable analysis efficiency.

To solve the second challenge, K_m and other parameters should be optimized and fixed as constants to estimate V_m by kinetic analysis of reaction curve, and a preset substrate concentration (PSC) should be optimized to covert V_m into initial rates according to the differential rate equation. In theory, a reliable V_m should be independent of ranges of data when they are reasonably restricted, and CVs for estimating parameters by enzymatic analysis are usually about 5%. Hence, the estimation of V_m with variations below 3% for the changes of substrate consumption percentages from 60% to 90% can be a criterion to select the optimized set of preset parameters. For converting V_m into initial rates, the optimized PSC is usually about 93% of S_0 and can be refined for different enzymes (Li, et al., 2011; Liao, et al., 2009; Liu, et al., 2009; Yang, et al., 2011). Optimized K_m and PSC to solve the second challenge are parameters for data processing while optimized S_0 and reaction duration to solve the first challenge are experimental conditions. The concomitant solution of the two challenges provides feasibility and potential reliability to the integration strategy.

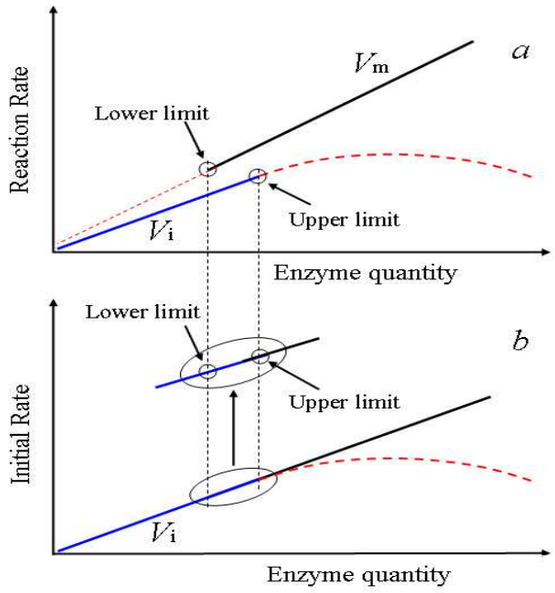

Fig. 1. The integration strategy for enzyme initial rate assay (Modified from Liu et al. (2009)).

After the integration strategy for enzyme initial rate assay is validated, a switch point should be determined for changing from the classical initial rate method to kinetic analysis of reaction curve. The estimation of V_m by kinetic analysis of reaction curve usually prefers substrate consumption percentages reasonably high. Therefore, the substrate consumption percentage that gives an enzyme activity from 90% to 100% of the upper limit of linear response by the classical initial rate method can be used as the switch point.

It should be noted that the lower limit of linear response is difficult to be defined for enzyme initial rate assay by an integration strategy. For most methods, their lower limits of linear response are usually defined as three times the standard errors of estimate (Miller, J. C. & Miller, J. N., 1993). Usually, enzyme initial rate assay utilizes just one method for data processing and the difference between the lower limit and the upper limit of linear response is seldom over 30-fold. By the integration strategy, the measurable ranges of enzyme quantities cover two magnitudes and the detection limit is reduced to that by the classical initial rate method. By manual operation, different dilution ratios of a stock solution of the enzyme have to be used and any dilution error will increase the standard error of estimate for regression analysis. The measurement of higher enzyme activities will inevitably have larger standard deviation. Thus, regression analysis of the response of all measurable enzyme initial rates by the integration strategy to quantities of the enzyme will give higher standard error of estimate and thus an unfavourable lower limit of linear response. By this new integration strategy, we arbitrarily use twice the lower limit of linear response by the classical initial rate method as the lower limit if the overall standard error of estimate is more than twice that by the classical initial rate method alone; or else, the lower limit of linear response is still three times the overall standard error of estimate.

Taken together, for measuring initial rates of enzyme acting on single substrate by the integration strategy based on NLSF and data transformation, there are the following basic steps different from those by the classical initial rate method. The first is to work out the

integrated rate equation with the predictor variable of reaction time. The second is to optimize individually their parameters fixed as constants for kinetic analysis of reaction curve. The third is to optimize a ratio of S_0 to K_m and duration to monitor reaction curves; usually a ratio of S_0 to K_m from 0.5 to 2.5, the duration of 5.0 min and intervals of 10 s are effective. The forth is to refine PSC around 93% of S_0 to convert V_m into initial rates.

As for enzyme-coupled reaction system, initial rate itself is estimated by kinetic analysis of reaction curve based on numerical integration and NLSF of calculated reaction curves to a reaction curve of interest. Consequently, neither the conversion of indexes nor the optimization of parameters for such conversion is required and the integration strategy can be realized easily. By kinetic analysis of enzyme-coupled reaction curve, there still should be a minimum number of the effective data and a minimum substrate consumption percentage in the effective data for analysis; these prerequisites lead to unsatisfactory lower limits of linear response for favourable analysis efficiency (the use of reaction duration within 5.0 min). The classical initial rate method is effective to enzyme-coupled reaction systems when activities of the enzyme of interest are not too high. Therefore, this new approach for kinetic analysis of enzyme-coupled reaction curve can be integrated with the classical initial rate method to quantify enzyme initial rates potentially for wider linear ranges.

With enzyme-coupled reaction systems, only the first challenge should be solved to practice the integration strategy. Namely, reaction duration and sampling intervals to record reaction curve should be optimized so that there is an overlapped region of enzyme initial rates measurable by both methods with consistent results. The upper limit of the classical initial rate method should be high enough so that data after reaction of about 5.0 min for enzyme activity at such an upper limit are suitable for kinetic analysis of reaction curve. The integration strategy gives an approximated linear range from the lower limit of linear response by the classical initial rate method to the upper limit of linear response by kinetic analysis of LDH-coupled ALT reaction curve (Yang, et al., 2010).

2.4.2 New integration strategy for enzyme substrate assay

Analysis of a biochemical as the substrate of a typical tool enzyme, *i.e.*, enzymatic analysis of substrate in biological samples, is important in biomedicine (Bergmeyer, 1983; Dilena, 1986; Guilbault, 1976; Moss, 1980). In general, there are the kinetic method and the end-point method for enzyme substrate assay; the end-point method is called the equilibrium method, and it determines the difference between the initial signal for a reaction system before enzyme action and the last signal after the completion of enzyme reaction; such differences proportional to S_0 can serve as an index of substrate concentration (Dilena, et al., 1986; Guilbault, 1976; Moss, 1980; Zhao, et al., 2009). For better analysis efficiency and lower cost on tool enzymes, kinetic methods for enzyme substrate assay are preferred. Among available kinetic methods, the initial rate method based on the response of initial rates of an enzyme at a fixed quantity to substrate concentrations is conventional; however, it tolerates sensitivity to any factor affecting enzyme activities, requires tool enzymes of high K_m, and has narrow linear ranges. Kinetic analysis of reaction curve with a differential rate equation to estimate S_0 is proposed with favourable resistance to variations in enzyme activities and has upper limit of linear response over K_m, but it suffers from high sensitivity to background and has unfavourable lower limit of linear response (Dilena, et al., 1986; Hamilton & Pardue, 1982; Moss, 1980). Hence, new kinetic methods for enzyme substrate assay are still desired.

For enzymatic analysis of substrate, the equilibrium method can still be preferable as long as it has desirable analysis efficiency and favourable cost on tool enzyme. In theory, the last signal for the stable product or the background in the equilibrium method can be estimated by kinetic analysis of reaction curve with data far before the completion of reaction. This process can be a new kinetic method for enzyme substrate assay and is distinguished from the equilibrium method and other kinetic methods by its prediction of the last signal after the completion of enzyme reaction (Liao, 2005; Liao, et al., 2003, 2005a, 2006; Zhao, L.N., et al., 2006; Zhao, Y.S., et al., 2006, 2009). This new kinetic method should have resistance to factors affecting enzyme activities and upper limit of linear response higher than K_m besides all advantages of common kinetic methods.

An enzyme reaction curve can be monitored by absorbance of a stable product or the substrate itself (Figure 2). The initial absorbance before enzyme action (A_0) thus is the background (A_b) when absorbance for a stable product is quantified, or is the absorbance of the substrate plus background when absorbance of the substrate is quantified. The last absorbance after the completion of enzyme reaction, which is predicted by kinetic analysis of reaction curve, is the maximum absorbance of the stable product plus the background (A_m) or A_b itself. There is strong covariance between the initial signal and the last signal for the same reaction system; this assertion enhances precision of this kinetic method for substrate assay (Baywenton, 1986; Liao, et al, 2005b; Zhao, Y.S., et al., 2009).

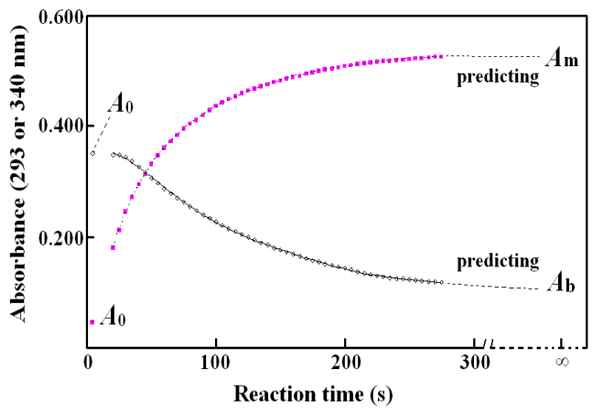

Fig. 2. Demonstration of reaction curves of uricase (293 nm) and glutathione-S-tranferase (340 nm), and the prediction of the last absorbance after infinite reaction time

However, this new kinetic method for substrate assay can by no means concomitantly have wider linear ranges and desirable analysis efficiency. Due to the prerequisites of the quality of data for kinetic analysis of reaction curve, the activity of a tool enzyme for enzymatic analysis of substrate should be reasonably high for higher upper limit of linear response, but it should be reasonably low for favourable lower limit of linear response. On the other hand, the duration to monitor reaction curves should be long enough to have higher upper limit at reasonable cost on a tool enzyme, but should be as short as possible for favourable analysis efficiency. Thus, this new kinetic method alone requires tough optimizations of conditions. Moreover, there are the inevitable random noises from any instrument to record an enzyme reaction curve; when there is much small difference between the initial signal before enzyme action and the last signal recorded after the preset reaction duration, this kinetic method for

substrate assay always has unsatisfactory precision. Therefore, this new kinetic method itself is still much beyond satisfaction for substrate assay.

To concomitantly have wider linear ranges, desirable analysis efficiency and favourable precision for enzyme substrate assay, the integration of kinetic analysis of reaction curve with the equilibrium method can be used. The indexes of substrate quantities by the two methods have exactly the same physical meanings, and thus the integration strategy can be easily realized for enzyme substrate assay. By this integration strategy, there should still be an overlapped range of concentrations of the substrate measurable consistently by both methods, besides a switch threshold within such an overlapped region to change from the equilibrium method to kinetic analysis of reaction curve. Additionally, this overlapped region of substrate concentration measurable by both methods with consistent results should localize in a range of substrate concentration high enough for reasonable precision of substrate assay based on kinetic analysis of enzyme reaction curve. These requirements can be met as described below. (a) The upper limit of linear response by the equilibrium method should be optimized to be high enough, so that the difference between the initial signal before enzyme action and the last recorded signal for about 80% of this upper limit is 50 times higher than the random noise of an instrument to record enzyme reaction curves; such a difference can be used as the switch threshold. (b) The activity of a tool enzyme and the duration to monitor reaction curve as experimental conditions should be optimized; kinetic parameters except V_m for kinetic analysis of reaction curve are optimized as well. The resistance of the predicted last signal to reasonable variations in data ranges for analysis can be a criterion to judge the optimized set of preset parameters. For favourable analysis efficiency in clinical laboratories, reaction duration can be about 5.0 min. This reaction duration results in a minimum activity of the tool enzyme for the integration strategy so that the upper limit of linear response by the equilibrium method can be high enough to switch to kinetic analysis of reaction curve. This integration strategy after optimizations can simultaneously have wider linear ranges, higher analysis efficiency and lower cost, better precision and stronger resistance to factors affecting enzyme activities.

Similarly, with the integration strategy for enzyme substrate assay, we also use twice the lower limit of the equilibrium method as the lower limit by the integration strategy if the standard error of estimate is much larger; or else, three times the standard error of estimate by the integration strategy is taken as the lower limit of linear response.

In general, the following steps are required to realize this integration strategy for enzyme substrate assay: (a) to work out the integrated rate equation with the predictor variable of reaction time; (b) to optimize individually the (kinetic) parameters preset as constants for kinetic analysis of reaction curve; (c) to optimize the activity of the tool enzyme so that data for the upper limit of linear response by the equilibrium method within about 5.0-min reaction are suitable for kinetic analysis of reaction curve. As demonstrated later, this integration strategy is applicable to enzymes suffering from strong product inhibition.

2.5 Applications of new methods to some typical enzymes

We investigated kinetic analysis of reaction curve with arylesterase (Liao, et al., 2001, 2003a, 2007b), alcohol dehydrogenase (ADH) (Liao, et al., 2007a), gama-glutamyltransfease (Li, et al., 2011), uricase (Liao, 2005; Liao, et al., 2005a, 2005b, 2006; Liu, et al., 2009; Zhao, Y.S., et

al., 2006, 2009), glutathione-S-tranferase (GST) (Liao, et al., 2003b; Zhao, L.N., et al., 2006), butylcholineasterase (Liao, et al., 2009; Yang, et al., 2011), LDH (Cheng, et al., 2008) and LDH-coupled ALT reaction systems (Yang, et al., 2010). Uricase of simple kinetics is a good example to study new methods for kinetic analysis of reaction curve; reactions of GGT and ADH suffer product inhibition and kinetic analyses of their reaction curves are complicated because they require unreported parameters. Hence, our new methods for kinetic analysis of reaction curve and the integration strategies for quantifying enzyme substrates and initial rates are demonstrated with uricase, GST and ADH as examples.

2.5.1 Uricase reaction

Uricase follows simple Michaelis-Menten kinetics on single substrate in air-saturated buffers, and suffers neither reversible reaction nor product inhibition (Liao, 2005; Liao, et al., 2005a, 2005b; Zhao, Y.S., et al., 2006). Uricase reaction curve can be monitored by absorbance at 293 nm. The potential interference from the intermediate 5-hydroxylisourate with uric acid absorbance at 293 nm can be alleviated by analyzing data of steady-state reaction in borate buffer at high reaction pH (Kahn & Tipton, 1998; Priest & Pitts, 1972). The integrated rate equation for uricase reaction with the predictor variable of reaction time is Equ.(4). Uricases from different sources have different K_m (Liao, et al., 2005a, 2006; Zhang, et al., 2010; Zhao, Y.S., et al., 2006). Using Equ.(4), K_m of *Candidate* utilis is estimated with reasonable reliability (Liao, et al., 2005a). Using Equ.(9) to estimate the ratio of V_m to K_m, uricase mutants of better catalytic capacity and their sensitivity to xanthine are routinely characterized (data unpublished). Thus, we used uricases of different K_m as models to test the two integration strategies for enzyme substrate assay and initial rate assay, respectively.

Uricase from *Bacillus* fastidiosus A.T.C.C. 29604 has high K_m to facilitate predicting A_b (Zhang, et al., 2010; Zhao, Y.S., et al., 2006, 2009). Reaction curves at low levels of uric acid with this uricase at 40 U/L are demonstrated in Fig. 3. Steady-state reaction is not reached within 30 s since reaction initiation; it is difficult to get more than 5 data with absorbance changes over 0.003 for kinetic analysis of reaction curve at uric acid levels below 3.0 μmol/L. At 40 U/L of this uricase, the absorbance after reaction for 5.0 min has negligible difference from that after reaction for 30 min for uric acid below 5.0 μmol/L. To quantify the difference between A_0 and A_b after reaction for 5.0 min, the equilibrium method has an upper limit of about 5.0 μmol/L, while kinetic analysis of reaction curve with K_m as a constant is feasible for S_0 of about 5.0 μmol/L. Thus, the change of absorbance over 0.050 between A_0 and the absorbance after reaction for 5.0 min can be the switch threshold to change from the equilibrium method to kinetic analysis of reaction curve.

This integration strategy for enzyme substrate assay gives the linear response from about 1.5 μmol/L up to 60 μmol/L uric acid at 40 U/L uricase (Fig.4, unpublished), and shows resistance to the action of xanthine at 30 μmol/L in reaction solutions (this level of xanthine always caused negative interference with all available kits commercialized for serum uric acid assay). Therefore, the integration strategy for uric acid assay is clearly superior to any other uricase method reported.

Uricases from *Candida* sp. with K_m of 6.6 μmol/L (Sigma U0880) and *Bacillus* fastidious uricase from A.T.C.C. 29604 with K_m of 0.22 mmol/L are used to test the integration strategy for initial rate assay. The use of uric acid at S_0 of 25 μmol/L to monitor reaction curves

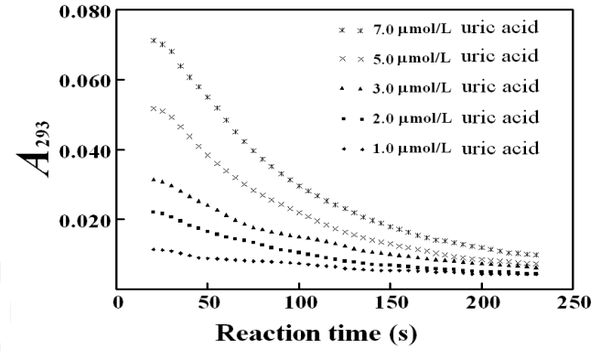

Fig. 3. Reaction curves (absorbance at 293 nm) at low levels of uric acid and 40 U/L uricase (recombinant uricase in *E*. Coli BL21 was as reported before (Zhang, et al., 2010)).

within 8.0 min or at S_0 of 75 μmol/L to monitor reaction curves within 5.0 min, the integration strategy to measure initial rates of both uricases is feasible; the use of PSC of 93% S_0 to convert V_m into initial rates gives the linear range of about two magnitudes (Liu, et al., 2009). Therefore, the integration strategy for enzyme initial rate assay is also advantageous.

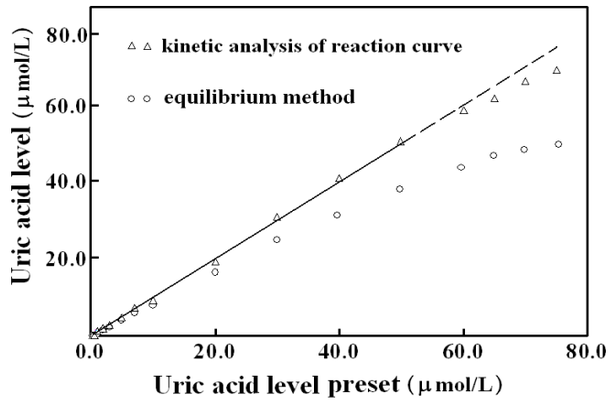

Fig. 4. Response of absorbance change at 293 nm to preset uric acid levels at 40 U/L uricase.

2.5.2 Glutathione-S-transferase reaction

Using purified alkaline GST isozyme from porcine liver as model on glutathione (GSH) and 2,4,-dinitrochlorobenzene (CDNB) as substrates, GST reaction curves are monitored by absorbance at 340 nm (Kunze, 1997; Pabst, et al, 1974; Zhao, L.N., et al., 2006). To promote reaction on single substrate, CDNB is fixed at 1.0 mmol/L while GSH concentrations are kept below 0.10 mmol/L (Zhao, L.N., et al., 2006). Because the concentration of product is calculated from absorbance at 340 nm, the background absorbance before GST reaction is adjusted to zero so that there is no need to treat A_b as a parameter. This treatment of background absorbance eliminate the estimation of A_b and thus confronts with no problem of covariance between A_b and A_m for NLSF. However, GST reaction is more complicated than uricase because it suffers strong product inhibition with an unreported inhibition constant (Kunze, 1997; Pabst, et al, 1974). Thus, the effectiveness of the two integration strategies is tested for measuring initial rate and GSH levels after the inhibition constant of the product is optimized for kinetic analysis of GST reaction curve.

The following symbols are assigned: C to instantaneous concentration of CDNB, B to instantaneous concentration of GSH, Q to instantaneous concentration of the product, K_{ma} to K_m of GST for CSNB, K_{mb} to K_m of GST for GSH, K_{ia} to the dissociation constant of GSH, K_{iq} to the dissociation constant of the product, A for instantaneous absorbance, A_m for the maximal absorbance of the product, ε to difference in absorptivity of product and CDNB, V_m for the maximal reaction rate of GST. The differential rate equation for GST reaction is Equ.(13). After the definition of M_1, M_2 and M_3, the integrated rate equation with the predictor variable of reaction time is Equ.(19) if GST reaction is irreversible and a process similar to that for Equ.(4) is employed (Zhao, L.N., et al., 2006).

$$\frac{1}{V} = \left(K_{mb}/V_m\right) \times \left[1 + K_{ib} \times K_{ma} \times Q/\left(K_{iq} \times K_{mb} \times C\right)\right]/B + \left[1 + K_{ma} \times \left(1 + Q/K_{iq}\right)/C\right]/V_m \tag{13}$$

$$M_1 = K_{ma}/(\varepsilon \times K_{iq}) \tag{14}$$

$$M_2 = K_{ma} - K_{ib} \times K_{ma}/K_{iq} - A_m \times K_{ma}/(\varepsilon \times K_{iq}) + C - A_0 \times K_{ma}/(\varepsilon \times K_{iq}) \tag{15}$$

$$M_3 = K_{ma} \times A_m + \varepsilon \times K_{mb} \times C + C \times A_m - K_{ib} \times K_{mb} \times A_0/K_{iq} - K_{ma} \times A_m \times A_0/\left(K_{iq} \times \varepsilon\right) \tag{16}$$

$$\frac{M_1 \times A^2 + M_2 \times A - M_3}{A - A_m} \times dA = C \times \varepsilon \times V_m \times dt \tag{17}$$

$$Y = M_1 \times \left(A - A_m\right)^2/2 + \left(2 \times M_1 \times A_m + M_2\right) \times \left(A - A_m\right) + \left(M_1 \times {A_m}^2 + M_2 \times A_m - M_3\right) \times Ln\left|A - A_m\right| \tag{18}$$

$$Y = C \times \varepsilon \times V_m \times \left(t - T_{lag}\right) = a + b \times t \tag{19}$$

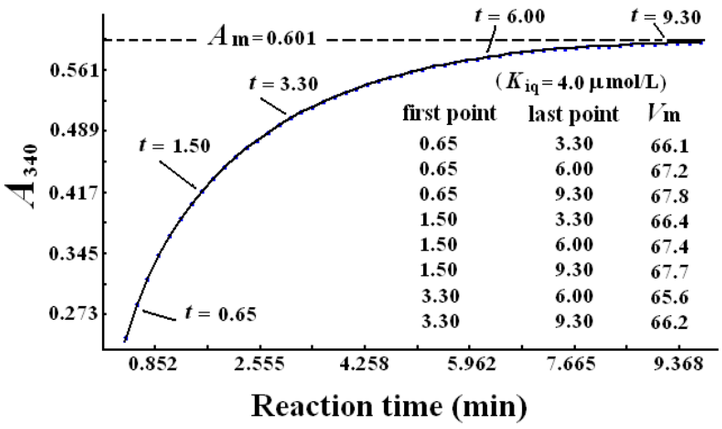

Fig. 5. Estimated V_m to changes in data ranges for analyses with 60 μmol/L GSH.

As demonstrated in the definition of M_1, M_2 and M_3, kinetic parameters preset as constants for kinetic analysis of GST reaction curve should have strong covariance. Except K_{iq} as an unknown kinetic parameter for optimization, other kinetic parameters are those reported (Kunze, 1997; Pabst, et al, 1974). To optimize K_{iq}, two criteria are used. The first is the consistency of predicted A_m at a series of GSH concentrations using data of 6.0-min reaction with that by the equilibrium method after 40 min reaction (GST activity is optimized to complete the reaction within 40 min). The second is the resistance of V_m to reasonable changes in data ranges for analyses. After stepwise optimization, K_{iq} is fixed at 4.0 μmol/L; A_m predicted for GSH from 5.0 μmol/L to 50 μmol/L is consistent with that by the equilibrium method (Zhao, L.N., et al. 2006); the estimation of V_m is resistant to changes of data ranges (Fig. 5). Therefore, K_{iq} is optimized and fixed as a constant at 4.0 μmol/L.

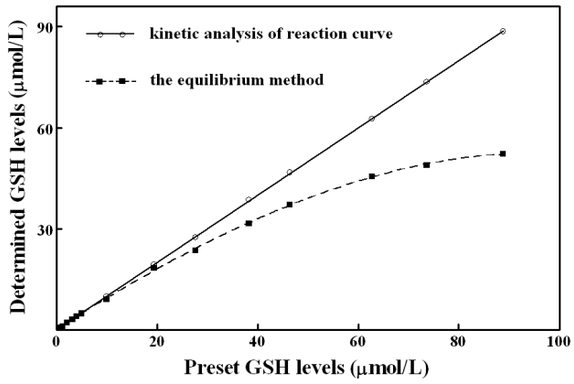

Fig. 6. Response of GSH concentration determined to preset GSH concentrations (the equilibrium method uses data with 6.0 min reaction).

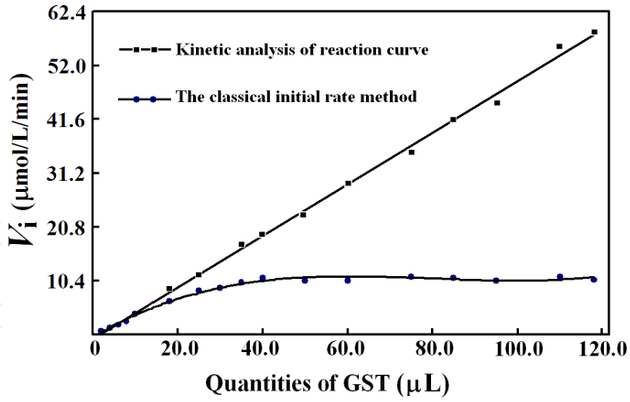

Fig. 7. Response of initial rates to quantities of purified porcine alkaline GST.

Kinetic analysis of GST reaction curve can predict A_m for GSH over 4.0 μmol/L, but there are no sufficient data for analyses at GSH below 3.0 μmol/L; after optimization of GST activity for complete conversion of GSH at 5.0 μmol/L within 6.0 min, reaction curve within 5.0 min for GSH at 5.0 μmol/L can be used for kinetic analysis of reaction curve to predict A_m. With the optimized GST activity for reaction within 5.0 min, the linear range for GSH assay is from 1.5 μmol/L to over 90.0 μmol/L by the integration strategy while it is from 4.0

μmol/L to over 90.0 μmol/L by kinetic analysis of reaction curve alone (Fig. 6, unpublished). By the equilibrium method alone for reaction within 5.0 min, the assay of 80.0 μmol/L GSH requires GST activity that is 50 folds higher due to the inhibition of GST by the accumulated product. Therefore, the integration strategy for GSH assay is obviously advantageous.

The integration strategy for measuring GST initial rates is tested. For convenience, S_0 of the final GSH is fixed at 50 μmol/L and the duration to monitor reaction curve is optimized. After the analyses of reaction curves recorded within 10 min, it is found that reaction for 6.0 min is sufficient to provide the required overlapped region of GST activities measurable by both methods. By using K_{iq} fixed at 4.0 μmol/L as a constant, the reaction duration of 6.0 min and PSC at 48 μmol/L to convert V_m to initial rates, the integration strategy gives a linear range from 2.0 U/L to 60 U/L; kinetic analysis of reaction curve alone gives the linear range from 5.0 U/L to 60 U/L while the classical initial rate method alone gives a linear range from 1.0 U/L to 5.0 U/L (Fig. 7, unpublished). Clearly, with enzyme suffering strong product inhibition, the integration strategy for enzyme initial rate assay is advantageous.

2.5.3 Alcohol dehydrogenase reaction

ADH is widely used for serum ethanol assay. ADH kinetics is sophisticated due to the reversibility of reaction and the inhibition by both acetaldehyde and NADH as products. To simplify ADH kinetics, some special approaches are employed to make ADH reaction apparently irreversible on single substrate (alcohol). Thus, reaction pH is optimized to 9.2 to scavenge hydrogen ion; semicabarzide at final 75 mmol/L is used to remove acetaldehyde as completely as possible; final nicotinamide adenine dinucleotide (NAD^+) is 3.0 mmol/L; final ADH is about 50 U/L (Liao, et al., 2007a). By assigning the maximal absorbance at 340 nm for reduced nicotinamide adenine dinucleotide (NADH) by the equilibrium method to A_{me} and that by kinetic analysis of reaction curve to A_{mk}, kinetic analysis of ADH reaction curve should predict A_{mk} consistent with A_{me}, but requires some special efforts.

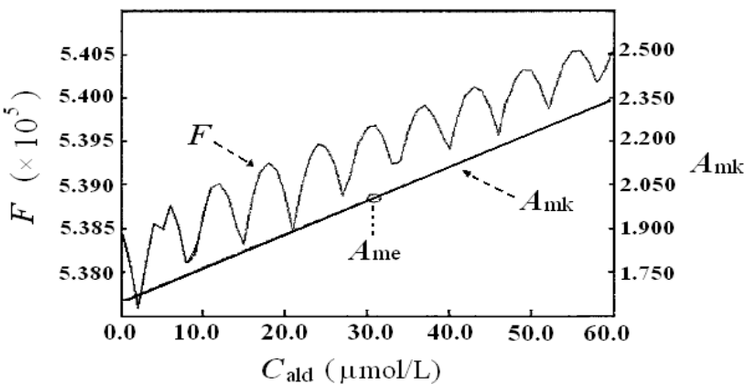

Fig. 8. Response of F values to preset C_{ald} for kinetic analysis of reaction curve for 0.31 mmol/L ethanol (reproduced with permission from Liao, et al, 2007a).

The use of semicabarzide reduces concentrations of acetaldehyde (C_{ald}) to unknown levels, and thus complicates the treatment of acetaldehyde inhibition on ADH. The integration rate equation with the predictor variable of reaction time can be worked out for ADH (Liao, et

al., 2007a). All kinetic parameters and NAD^+ concentrations are preset as those used or reported (Ganzhorn, et al. 1987). However, there are multiple maxima of the goodness of fit with the continuous increase in steady-state C_{ald} for kinetic analysis of reaction curve (Fig. 8). Thus, C_{ald} can not be concomitantly estimated by kinetic analysis of reaction curve, and a special approach is used to approximate steady-state C_{ald} for predicting A_{mk}.

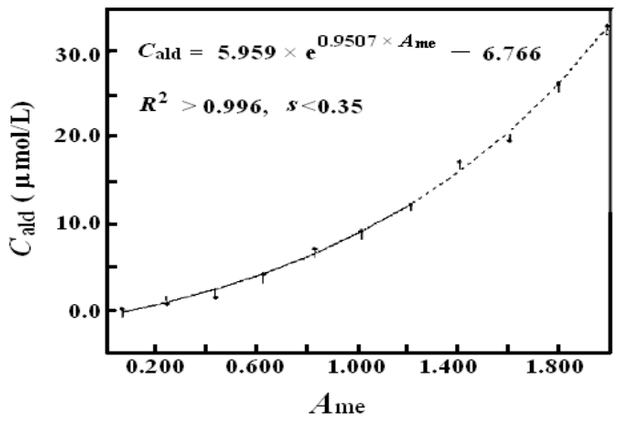

Fig. 9. Correlation function of the best steady-state C_{ald} with A_{me} (reproduced with permission from Liao, et al, 2007a).

Under the same reaction conditions, the equilibrium method can determine A_{me} for ethanol below 0.20 mmol/L after reaction for 50 min. For kinetic analyses of such reaction curves, the lag time for steady-state reaction is estimated to be over 40 s and is used to select data of steady-state reaction for analysis. Using the equilibrium method as the reference method, the best steady-state C_{ald} for data of 6.0-min reaction is obtained for consistency of A_{mk} with A_{me} at each tested ethanol level from 10 μmol/L to 0.17 mmol/L. After dilution and determination by the equilibrium method, A_{me} for each tested ethanol level from 0.17 mmol/L to 0.30 mmol/L is also available. Consequently, an exponential additive function is obtained to approximate the correlation of the best C_{ald} for predicting A_{mk} consistent with A_{me} (Fig. 9). This special correlation function for C_{ald} and A_{mk} is used as a restriction function to iteratively adjust C_{ald} for predicting A_{mk}; namely, iterative kinetic analysis of reaction curve with C_{ald} predicted from the restriction function using previous A_{mk} finally gives the desired A_{mk}. Such an artificial intelligence approach to the steady-state C_{ald} for kinetic analysis of reaction curve can hardly be found in publications.

To start kinetic analysis of an ADH reaction curve, the highest absorbance under analysis is taken as A_{mk} to predict the best C_{ald} for the current run of kinetic analysis of reaction curve. The estimated A_{mk} is then used to predict the second C_{ald} for the second run of kinetic analysis of reaction curve (Fig. 10). Such an iterative kinetic analysis of reaction curve can predict A_{mk} consistent with A_{me} for 0.31 mmol/L ethanol when reaction duration is just 6.0 min and the convergence criterion is set for absorbance change below 0.0015 in A_{mk}. Usually convergence is achieved with 7 runs of the iterative kinetic analysis of reaction. Moreover, it is resistant to the change of ADH activities by 50% and coefficients of variation (CV) are below 5% for final ethanol levels from 20 μmol/L to 310 μmol/L in reaction solutions.

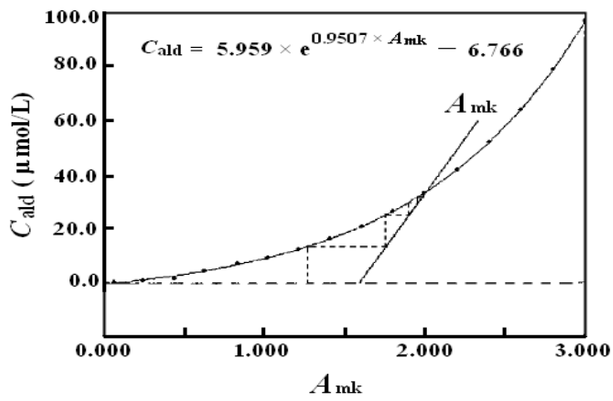

Fig. 10. Iterative adjustment of C_{ald} to predict A_{mk} for 0.31 mmol/L ethanol at 50 U/L ADH (reproduced with permission from Liao, et al, 2007a).

Obviously, by this special approach for kinetic analysis of ADH reaction curve, the upper limit of linear response is excellent, but the lower limit of linear response is over 5.0 μmol ethanol. Under the stated reaction conditions, the equilibrium method after reaction for 8.0 min is effective to quantify ethanol up to final 6.0 μmol. Thus, the equilibrium method with reaction duration of 8.0 min can be integrated with iterative kinetic analysis of reaction curve for quantifying ethanol; this integration strategy gives the linear range from about final 2.0 μmol to about 0.30 mmol/L ethanol in reaction solutions; it has CVs below 8% for ethanol below 10 μmol/L, and CVs below 5% for ethanol over 20 μmol/L (Liao, et al., 2007a). These results clearly supported the advantage of the new integration strategy for substrate assay and the importance of chemometrics in kinetic enzymatic analysis of substrate.

2.6 Programming for kinetic analysis of enzyme reaction curve

Most software package like Origin, SAS, MATLAB can perform kinetic analysis of reaction curve, but they are usually ineffective to implicit functions for kinetic analysis of reaction curve. For convenience and the use of some complicated methods for kinetic analysis of reaction curve in widow-aided mode, self-programming is still favourable.

For simplicity in programming, we used Visual Basic 6.0 to write the source code and working windows (Liu, et al., 2011). The executable program has the main window to perform kinetic analysis of reaction curve (Fig. 11). Original data for each reaction curve is stored as a text file, and keywords are used to indicate specific information related to the reaction curve including sample numbering, the enzyme used, the quantification method, some necessary kinetic parameters, and usually initial signal before enzyme action. Such information is read into memory by the software for kinetic analysis of reaction curve.

On the main window to perform kinetic analysis of reaction curve, original data are listed and plotted for eyesight-checking of data for steady-state reaction. Text boxes are used to input some common parameters like K_m, and most parameters are read from the text file for the reaction curve. Subprogram for an enzyme reaction system is called for running; results are displayed on the main window and may be saved in text file for further analysis.

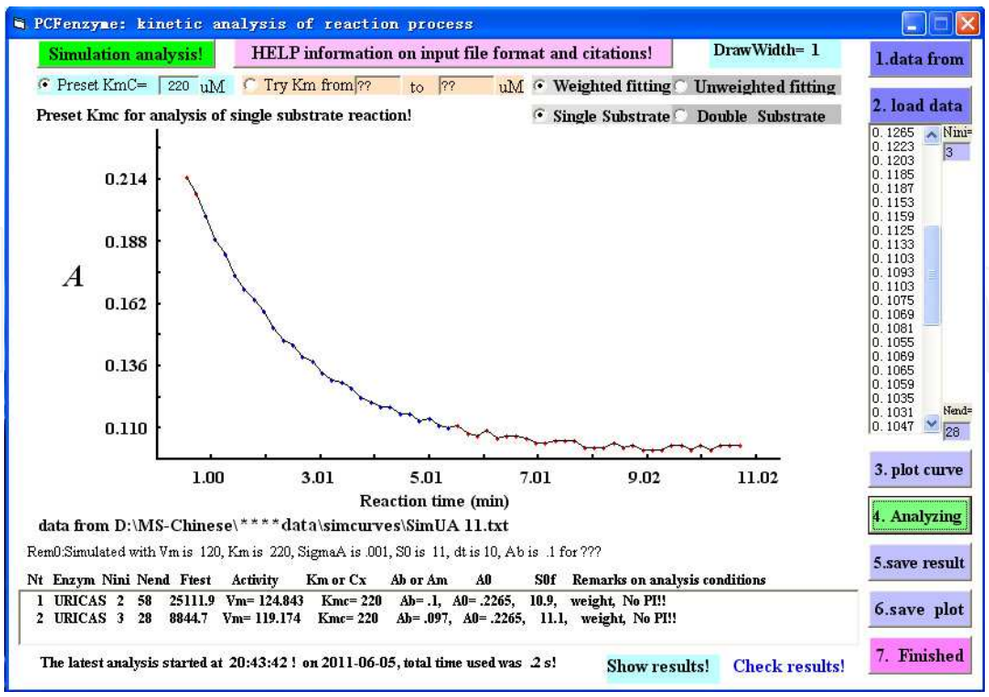

Fig. 11. Main window for the executable PCFenzyme

We called the software PCFenzyme. An old version of the executable PCFenzyme can be downloaded at http://dx.doi.org/10.1016/j.clinbiochem.2008.11.016. The latest version of the executable PCFenzyme with new methods included is available upon request by e-mail.

3. Conclusion

The following conclusions can be drawn. (a) Kinetic analysis of reaction curve can give the initial substrate concentration before enzyme action, the maximal reaction rate, Michaelis-Menten constant and other related parameters of an enzyme reaction system; for reliability, however, it is better to just estimate the initial substrate concentration before enzyme action and the maximal reaction rate with Michaelis-Menten constant and other parameters fixed as constants after optimization. (b) For an enzyme whose integrated rate equation with the predictor variable of reaction time is accessible, kinetic analysis of reaction curve can estimate parameters *via* nonlinear-least-square-fitting after transformation of data from the reaction curve under analysis. (c) For an enzyme reaction system whose kinetics is described by a set of differential rate equations or is difficult to be integrated with the predictor of reaction time, iterative numerical integration of the differential rate equation(s) with a series of preset parameters can produce serial calculated reaction curves; such calculated reaction curves can be fit to the reaction curve under analysis for estimating parameters based on nonlinear-least-square-fitting. This approach is applicable to enzyme-coupled reaction systems of sophisticated kinetics. (d) The integration of kinetic analysis of reaction curve with the equilibrium method can quantify enzyme substrates with expanded linear ranges, favourable analysis efficiency, low cost on tool enzyme, desirable resistance to factors affecting enzyme activities and enhanced precision; it can be applied to enzyme reaction suffering strong product inhibition. (e) The integration of kinetic analysis of reaction curve

with the classical initial rate method can measure enzyme initial rates with wide linear ranges, favourable analysis efficiency and practical levels of substrates; it can be applicable to enzyme-coupled reaction curve or enzyme reaction suffering product inhibition.

Taken together, kinetic analysis of enzyme reaction curves under optimized conditions can screen common reversible inhibitors and enzyme mutants; the integration strategy for measuring enzyme activities can quantify serum enzymes and enzyme labels in enzyme-immunoassays to expand the quantifiable ranges, and can be applied to quantify irreversible inhibitors as environmental pollutants; the integration strategy to quantify enzyme substrate can be the second-generation approaches and potentially find wide applications in clinical laboratory medicine. Therefore, these new methodologies for enzymatic analyses based on chemometrics can potentially find their important applications in biomedical sciences.

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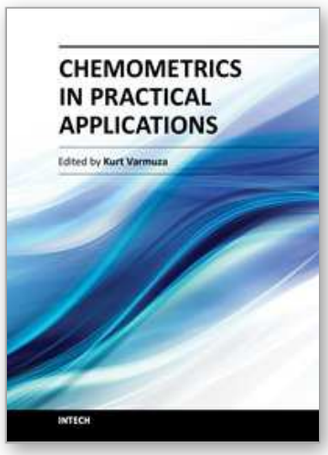

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