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Quasi-steady state kinetics of simple sequential multienzyme reactions with single substrates

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Applications of computer algebra technique to kinetic analysis of multienzyme reaction including two and three distinct enzymes were described in the literature, which mainly depend on Gröbner Basis theory. In the present study, we have applied the same methodology to a hypothetical system with four enzymes. After deriving a composite rate law for a reaction system with four distinct enzymes using the computer algebra system MAPLE, the composite rate law was fitted to simulated data for time versus product concentration to yield estimates of the kinetic parameters.

KEY WORDS: kinetics of multienzyme reactions, quasi-steady state assumption, mathematics, computer algebra, mixed symbolic and numerical computations

1. Introduction

To date, analyses of properties of enzymes have usually been conducted on isolated enzymes in *vitro* by performing initial rate experiments. Since the biochemical properties of both individual enzymes and a system in which they act may depend on interactions with other enzymes and metabolites in *vivo*, an approach in which systems of enzymes could be studied simultaneously provides advantages.

It is, however, sometimes essential to couple a reaction with a second reaction. For instance, when it is not possible or convenient to follow a reaction directly in a spectrophotometer, it may nonetheless be possible to follow it indirectly by coupling it with another reaction. Furthermore, even if the reaction of interest can be assayed directly, it is sometimes essential to couple it with a second reaction. For example, if one of the products of the first reaction is a powerful inhibitor, or if a reversible reaction is being studied in the less favored direction, so that equilibrium is reached after only a small percentage of substrate has reacted, it may be difficult to measure the initial velocity accurately [1].

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The rate behavior of a multienzyme reaction consisting of several individual reactions linked by freely diffusible intermediates depends on the rate behavior of the individual reactions. Hence, a logical approach to the problem of determining the kinetics of a multienzyme reaction of this type would be to determine the kinetics of the individual reactions and use the resulting rate equations to obtain a rate equation for the multienzyme reaction. The kinetics of multienzyme reactions with one substrate containing three enzymes has been studied elsewhere [2–4].

We could not find any study related to kinetic analysis of a multienzyme reaction with four distinct enzymes, although it was reported that Gröbner basis theory can be employed to derive a composite rate law for any multienzyme system under a quasi-steady state assumption [4]. In this study, we examined the applicability of a combination of symbolic and numerical computation techniques to a hypothetical multienzyme reaction including four distinct enzymes.

2. Methods

Our approach includes a combination of both symbolic (solution of simultaneous equations, substitution of variables, determination of higher derivatives and so on) and numerical (solution of differential equations, finding roots of a univariate polynomials, fitting experimental data and so on) computation methods.

Gröbner Basis theory is a systematic approach to solving polynomial equations. Details on Gröbner Basis theory can be found in [5,6].

However finding the exact solutions of polynomial equation is not the only goal. Often it is enough to answer questions such as:

- Is the system of polynomial equations solvable?
- Does the system of polynomial equations have a finite number of solutions? If so, how many solutions exist?
- Are there equivalent systems of equations that give more insight to their solutions?

Answering these questions in the kinetic analysis of a multienzyme reaction under quasi-steady state assumption provide extra advantageous that a more classical approach could not.

In 1965, Buchberger [5] presented an algorithm in order to compute the Gröbner Basis of any given ideal. Many computer algebra systems implement a version of Buchberger's algorithm. These systems usually compute a reduced Gröbner Basis. MAPLE's Gröbner Basis package includes a sub-package *gbasis* that computes a *reduced Gröbner Basis*. The syntax of *gbasis* is "*gbasis*(F,X,*termorder*)". Here, F is a list of polynomials, X is a list of intermediates and termorder is either *tdeg* or *plex* that are the names of term ordering which will be used. *plex* represents lexicographical ordering while *tdeg* means total degree ordering which is out of this study's scope. With this syntax, *gbasis* computes the reduced Gröbner Basis of the ideal $\langle F \rangle$ with respect to the intermediates X and given term ordering.

We have used MAPLE 4.0 as a computer algebra system for symbolic computations [7]. Both symbolic and numerical computations were performed in a PC with 64 MB RAM and Pentium III processor running under Windows 98 operating system.

Enzyme kinetic theory and application of Gröbner Basis theory to derive a quasi-steady state rate law via MAPLE

Mathematical model of the kinetics of enzyme reactions is described by a set of differential or differential–algebraic equations. With the underlying first order mechanism, a single enzyme reaction can be represented as in scheme 1.

$$S+E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P+E$$

Scheme 1. A simple sequential enzyme catalyzed reaction.

In scheme 1, $[S]$ represents substrate concentration and $[P]$ stands for product concentration. Under quasi-steady state assumptions, the kinetics of this reaction can be mathematically described as a system of polynomial equations

$$\begin{aligned} f1 &= v - k_2[ES] + k_{-2}[P][E] = 0, \\ f2 &= \frac{\mathrm{d}[ES]}{\mathrm{d}t} = k_1[E][S] + k_{-2}[E][P] - (k_{-1}+k_2)[ES] = 0, \\ f3 &= [E_0] - [E] - [ES] = 0, \end{aligned} \tag{1}$$

where $v = \mathrm{d}[P]/\mathrm{d}t$. The following MAPLE syntax produces rate law for reaction depicted in scheme 1:

```
with(grobner);
Out1:=gbasis([f1,f2,f3],[E,ES,v],plex);
v=solve(Out1[3],v);
```

After appropriate arrangements, the rate of conversion of S to P becomes

$$v = \frac{(V_{\mathrm{Max}}/K_{\mathrm{M,F}})([S] - [P]/K_{\mathrm{EQ}})}{1 + [S]/K_{\mathrm{M,F}} + [P]/K_{\mathrm{M,R}}}, \tag{2}$$

where K_{EQ} is the overall equilibrium constant, V_{Max} is the maximum velocity and $K_{\mathrm{M,F}}$ and $K_{\mathrm{M,R}}$ are forward and reverse Michaelis Menten constants, respectively. Definitions of kinetic parameters in terms of individual rate constants are given by

$$K_{\mathrm{EQ}} = \frac{k_1 k_2}{k_{-1}k_{-2}}, \qquad K_{\mathrm{M,F}} = \frac{k_{-1}+k_2}{k_1}, \qquad K_{\mathrm{M,R}} = \frac{k_{-1}+k_2}{k_{-2}}, \qquad V_{\mathrm{Max}} = k_2[E_0]. \tag{3}$$

When a sequence of such reactions form a simple sequential multienzyme reaction, because of the nonlinearities of the mathematical model and a great number of parameters, the kinetic analyses becomes harder. However, that composite rate law using Gröbner

Basis theory for multienzyme reactions can be derived with the aid of powerful computers and enables us to analyze the kinetics of such reactions.

3. Mathematical model of a simple sequential multienzyme reaction

To demonstrate the applicability of the methods in kinetic analysis of multienzyme reactions, we have chosen a hypothetical linear system including four distinct enzymes in which a substrate S is converted into a product P via feely diffusible intermediates X_1, X_2 and X_3 which link four individual reaction catalyzed by the enzymes E_1, E_2, E_3 and E_4 as shown in scheme 2.

$$S \underset{E_1}{\overset{v_1}{\rightleftharpoons}} X_1 \underset{E_2}{\overset{v_2}{\rightleftharpoons}} X_2 \underset{E_3}{\overset{v_3}{\rightleftharpoons}} X_3 \underset{E_4}{\overset{v_4}{\rightleftharpoons}} P$$

Scheme 2. A simple sequential multienzyme reaction including four distinct enzymes.

Considering the net rates according to Michaelis Menten kinetics as given by equation (2), we can write four differential equations of the form

$$\begin{aligned}
v_1 &= \frac{V_{1,\mathrm{Max}}}{K_{1,\mathrm{M,F}}} \frac{[S] - [X_1]/K_{1,\mathrm{EQ}}}{1 + [S]/K_{1,\mathrm{M,F}} + [X_1]/K_{1,\mathrm{M,R}}}, \\
v_2 &= \frac{V_{2,\mathrm{Max}}}{K_{2,\mathrm{M,F}}} \frac{[X_1] - [X_2]/K_{2,\mathrm{EQ}}}{1 + [X_1]/K_{2,\mathrm{M,F}} + [X_2]/K_{2,\mathrm{M,R}}}, \\
v_3 &= \frac{V_{3,\mathrm{Max}}}{K_{3,\mathrm{M,F}}} \frac{[X_2] - [X_3]/K_{3,\mathrm{EQ}}}{1 + [X_2]/K_{3,\mathrm{M,F}} + [X_3]/K_{3,\mathrm{M,R}}}, \\
v_4 &= \frac{V_{4,\mathrm{Max}}}{K_{4,\mathrm{M,F}}} \frac{[X_3] - [P]/K_{3,\mathrm{EQ}}}{1 + [X_3]/K_{4,\mathrm{M,F}} + [P]/K_{4,\mathrm{M,R}}},
\end{aligned} \tag{4}$$

for kinetics of the system even though there are eight individual reactions in scheme 2. Here the rates v_i $(i = 1, 2, 3, 4)$ are

$$v_1 = \frac{\mathrm{d}[X_1]}{\mathrm{d}t}, \qquad v_2 = \frac{\mathrm{d}[X_2]}{\mathrm{d}t}, \qquad v_3 = \frac{\mathrm{d}[X_3]}{\mathrm{d}t}, \quad v_4 = \frac{\mathrm{d}[P]}{\mathrm{d}t}. \tag{5}$$

The conservation law yields

$$[S] + [X_1] + [X_2] + [X_3] + [P] = [S]_0, \tag{6}$$

where the subscript 0 indicates initial concentrations of the metabolite. When the reaction depicted in scheme 2 is in a quasi-steady state, the flux v of the system is the same for all of individual reactions. So, we have

$$v = v_1 = v_2 = v_3 = v_4. \tag{7}$$

This assumption reduces the system of equations given in equation (4) to a system of multivariate polynomial equations.

Computation of a Gröbner Basis of the ideal generated by a set of polynomials formed by equations (6) and (7) yields another set of polynomials whose solution set is the same as that of the former. Therefore, the mathematical model for the kinetics of the reaction and the computation of Gröbner Basis for this system in MAPLE's syntax can be obtained as follows:

```
Eq1:=numer(v-v1);
Eq2:=numer(v-v2);
Eq3:=numer(v-v3);
Eq4:=numer(v-v4);
Eq5:=[S]+[X1]+[X2]+[X3]+[X4]-[S]0;
Out2:=gbasis([Eq1,Eq2,Eq3,Eq4,Eq5],[S,X1,X2,X3,v],plex);
```

We need to take numerators of $v - v_i$ $(i = 1, 2, 3, 4)$ to get the polynomials for computation of Gröbner Basis. The denominators of v_i $(i = 1, 2, 3, 4)$ are nonzero because of the fact that the rate constants and also concentration values cannot be negative. It turns out that the last polynomial in *Out2* is a fourth order polynomial for v with coefficients in terms of concentration P and some kinetic parameters. This polynomial is given in appendix A. Since the other four polynomials in *Out2* are linear in concentrations of X_1, X_2, X_3 and S, we could then call MAPLE's *solve* procedure to obtain expressions for their concentrations in terms of concentration of P and various kinetic parameters analytically, although the size of these expressions would be extremely large.

The initial conditions for our hypothetical system are given by

$$\begin{aligned} &[S]_0 = 250.0 \text{ mM}, \qquad [X_1]_0 = 0.0 \text{ mM}, \qquad [X_2]_0 = 0.0 \text{ mM}, \\ &[X_3]_0 = 0.0 \text{ mM}, \qquad [P]_0 = 0.0 \text{ mM}. \end{aligned} \tag{8}$$

Hypothetical values of all parameters for our system are given in table 1. After sensitivity analysis of the dependence of these parameters on the flux v, five parameters ($K_{2,\mathrm{M,F}}$, $K_{3,\mathrm{M,F}}$, $K_{4,\mathrm{M,F}}$, $K_{2,\mathrm{M,R}}$ and $K_{3,\mathrm{M,R}}$) were chosen to be estimated. Using the parameters given in table 1, we first obtain simulated data for concentration P against time and then we get a set of artificial data which will be used for estimation of these five parameters after superimposing pseudorandom error on the simulated data.

Table 1
Hypothetical values of all kinetic parameters for the system depicted in scheme 2.

Maximum velocities (mM/sec)	Forward Michaelis Menten parameters (mM)	Reverse Michaelis Menten parameters (mM)	Equilibrium constants
$V_{1,\mathrm{Max}} = 99.0 \times 10^0$	$K_{1,\mathrm{M,F}} = 12.0 \times 10^{-2}$	$K_{1,\mathrm{M,R}} = 12.0 \times 10^{-3}$	$K_{1,\mathrm{EQ}} = 13.6 \times 10^0$
$V_{2,\mathrm{Max}} = 17.0 \times 10^0$	$K_{2,\mathrm{M,F}} = 13.0 \times 10^{-2}$	$K_{2,\mathrm{M,R}} = 13.0 \times 10^{-3}$	$K_{2,\mathrm{EQ}} = 14.0 \times 10^0$
$V_{3,\mathrm{Max}} = 19.0 \times 10^0$	$\mathrm{K}_{3,\mathrm{M,F}} = 15.0 \times 10^{-2}$	$K_{3,\mathrm{M,R}} = 15.0 \times 10^{-3}$	$K_{3,\mathrm{EQ}} = 11.0 \times 10^0$
$V_{4,\mathrm{Max}} = 15.0 \times 10^0$	$K_{4,\mathrm{M,F}} = 16.0 \times 10^{-2}$	$K_{4,\mathrm{M,R}} = 16.0 \times 10^{-3}$	$K_{4,\mathrm{EQ}} = 14.6 \times 10^0$

4. Estimation of kinetic parameters by fitting the rate law to simulated data

For estimation of the parameters, we preferred FORTRAN rather than MAPLE to implement numerical computations due to the limitations of numerical computation packages in MAPLE. Furthermore, the time required to end up any computation in MAPLE is often longer than the time needed for FORTRAN.

The simulated data consist of ordered pairs of concentration P against time. The rate law we are fitting relates $v = \mathrm{d}[P]/\mathrm{d}t$ to $[P]$. We need to integrate the rate equation in order to obtain theoretical values for concentrations P to fit to the simulated data.

Estimation of the selected parameters can be determined by nonlinear least square regression. This procedure has three stages. These are numerical solution of a fourth order univariete polynomial, numerical solution of an initial value problem, and a multivariate optimization.

As mentioned before, the last polynomial in the *Out2* is a fourth order univariate polynomial, and it must be solved numerically first. The NAG [8] routine of C02EAF was used for this purpose. C02EAF computes all roots of a univariate polynomial with real coefficients (including complex roots). The error tolerance chosen for C02EAF is 10^{-8}. Since v is $\mathrm{d}[P]/\mathrm{d}t$, after determining a meaningful root which makes all metabolite concentrations non-negative, v must be integrated to construct an objective function expressing deviations of theoretical values of $[P]$ from simulated data for multivariate optimization. For this purpose, we have employed NAG routine of D02AEF, that integrates a stiff system of first-order ordinary differential equations over a range with suitable initial conditions using a variable-step method implementing the backward differentiation formulae, is used as an integration routine. The error tolerance for this routine is chosen as 10^{-3}. The objective function F obtained in this manner is of the form

$$F = \sum_i \left([P]_{\mathrm{Simulated}_i} - [P]_{\mathrm{Theoretical}_i}\right)^2. \tag{9}$$

F is a function of $K_{2,\mathrm{M,F}}$, $K_{3,\mathrm{M,F}}$, $K_{4,\mathrm{M,F}}$, $K_{2,\mathrm{M,R}}$ and $K_{3,\mathrm{M,R}}$. PRAXIS [9] is used for multivariate optimization and 10^{-6} is chosen as an error tolerance for PRAXIS.

To check the result obtained, we need to have an estimate of the error behavior of our system. A simple approach to this is a statistical technique known as bootstrap [10]. At the minimum point of the objective function F, we have small residual errors in each point when using our estimated values for selected kinetic parameters. For each simulated pair of concentration P against time, we select one of the residual errors randomly, and then adding it to the simulated data in turn, we get a set of *new* values for P concentrations. Then, we use these values as though they were simulated data in a second round of minimization to calculate new estimates for selected parameters. After repeating this many times, we may compute standard deviations for each parameter. It may be suggested that standard deviations are similar, so we get an idea about the accuracy of our initial estimates.

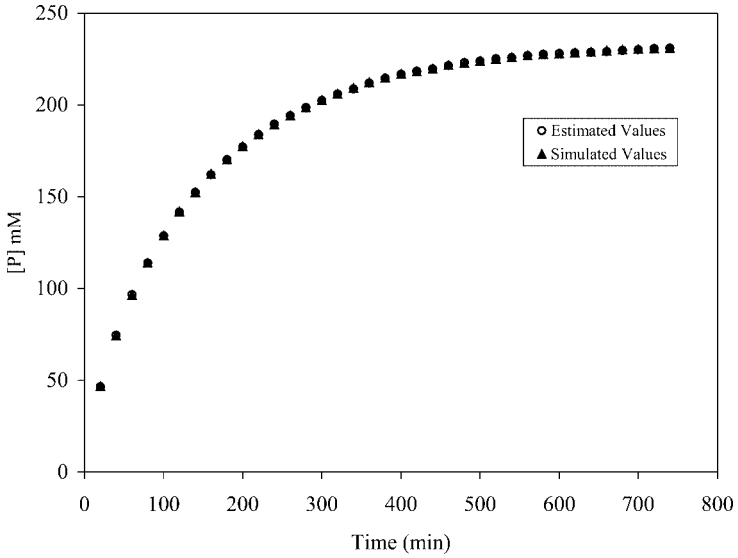

Figure 1. Graphical form of simulated curve and the curve drawn using the estimated values of selected against time.

5. Results and discussions

Changes in concentrations P over time were fitted to the rate equation given in appendix A using a FORTRAN program. Before starting the estimation procedure, initial values for each of the parameters must be specified. The choice of initial values is more important if a large number of parameters are being varied.

Graphical results of least square fit are shown in figure 1. The simulated data and the theoretical fitting model are in a very good agreement. Estimated values of the parameters and the result of confidence interval obtained by bootstrap method are summarized in table 2. Convergence of the estimation procedure is tested for several sets for initial starting values of the selected 5-parameters. The estimated values of three parameters ($K_{2,M,F}$, $K_{3,M,F}$ and $K_{4,M,F}$) deviate from their original values by less than 5%. However, $K_{2,M,R}$ and $K_{3,M,R}$ deviate from their original values by less than 12% (table 1). We can conclude that forward Michaelis Menten parameters may have lower standard deviations than reverse Michaelis Menten parameters. The graphical representation for distribution residuals against time (figure 2) exhibits a random behavior indicating that our parameters fit generally very well.

We have presented the applicability of mixed symbolic and numerical computation techniques to the kinetic analyses of a simple sequential multienzyme reaction including four distinct enzymes. It has been demonstrated that it is possible to obtain some kinetic parameters for this system.

Implementation of symbolic computations in MAPLE also provides advantages because of the availability of the program. There are some studies on use of MAPLE in biochemical kinetic theory as a computer algebra system in the literature [11–13].

Table 2
Hypothetical and estimated values with confidence intervals for selected kinetic parameters for the system depicted in scheme 2.

Kinetic parameters	Original values (mM)	Estimated values (mM) (Mean ± SD)
$K_{2,M,F}$	13.0×10^{-2}	$12.33 \times 10^{-2} \pm 3.60 \times 10^{-3}$
$K_{3,M,F}$	15.0×10^{-2}	$14.39 \times 10^{-2} \pm 3.95 \times 10^{-3}$
$K_{4,M,F}$	16.0×10^{-2}	$16.16 \times 10^{-2} \pm 1.29 \times 10^{-3}$
$K_{2,M,R}$	13.0×10^{-3}	$11.89 \times 10^{-3} \pm 5.80 \times 10^{-4}$
$K_{3,M,R}$	15.0×10^{-3}	$15.99 \times 10^{-3} \pm 4.91 \times 10^{-4}$

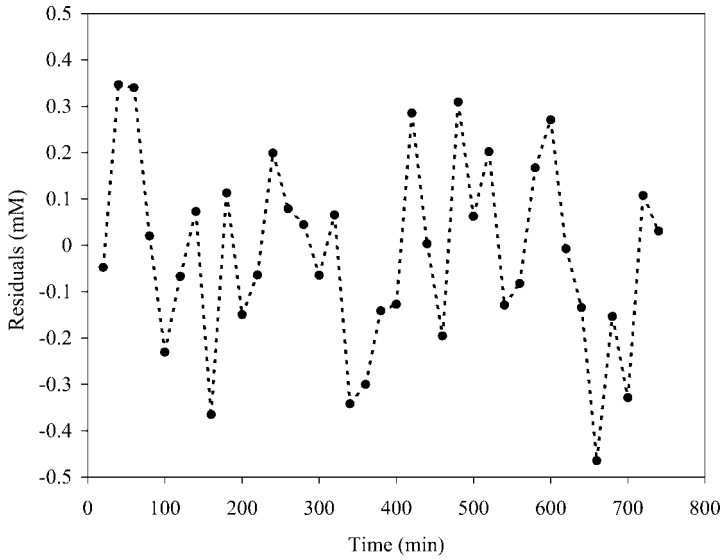

Figure 2. Graphical form of the distribution of the residuals against time. This graph shows that our parameters fit very well.

The method presented here is general and there is practically no upper limit for the number of parameters that can be estimated. It mainly depends on the number of data file, the quality of the data and the initial starting values for the parameters.

However, there are also difficulties before the techniques can become generally useful. One of the major problems is the time required for calculation of a Gröbner basis for system of polynomial equations if the multienzyme reaction becomes more complicated. Secondly, a more insidious problem is the selection of a minimization method, some of which need the evaluation of the first and second derivatives of the objective function. When the expression for the rate law is a massive algebraic expression, converting such expression into FORTRAN format and making numerical computations there is prone to round off error [4] due to the huge numbers produced by MAPLE even if one select double precision option.

The method introduced in this paper can fit progress-curve data to widely different enzyme mechanisms including more than one substrate and product and provide us to test the resultant fits by statistical methods.

As a result, although there are some limitations, the method described in this study to analyze multienzyme reaction is mathematically accurate, fast and easy to use. Furthermore after making some modifications, this method can also be employed for analysis of larger systems with the help of powerful computers.

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Appendix A

The fourth order polynomial satisfied by v can be written as

$$Av^4 + Bv^3 + Cv^2 + Dv + E = 0,$$

where the coefficients are

$$\begin{aligned}
A = &-1737183448.0K_{3,\mathrm{M,F}}K_{2,\mathrm{M,R}} + 6945400.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P] \\
&+ 62508600.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}}K_{2,\mathrm{M,R}} - 62508600.0K_{2,\mathrm{M,F}}K_{3,\mathrm{M,F}}K_{3,\mathrm{M,R}} \\
&+ 62508600.0K_{2,\mathrm{M,F}}K_{3,\mathrm{M,F}}K_{4,\mathrm{M,F}} + 3906787500.0K_{2,\mathrm{M,F}}K_{3,\mathrm{M,F}}[P]K_{4,\mathrm{M,F}} \\
&- 6945400.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}K_{3,\mathrm{M,R}} + 6945400.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}K_{4,\mathrm{M,F}} \\
&+ 434087500.D0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}[P]K_{4,\mathrm{M,F}} - 6945400.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}K_{4,\mathrm{M,F}} \\
&- 434087500.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P]K_{4,\mathrm{M,F}}, \\
B = &-45610193750.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}[P]K_{4,\mathrm{M,F}} - 1048913250.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P] \\
&+ 59351600000.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P]K_{4,\mathrm{M,F}} + 949625600.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}K_{4,\mathrm{M,F}} \\
&+ 63929250.0[P]K_{3,\mathrm{M,F}}K_{2,\mathrm{M,F}} + 260495005848.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}} \\
&- 1387386000.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}}K_{2,\mathrm{M,R}} + 46119150000.0K_{2,\mathrm{M,F}}K_{3,\mathrm{M,F}}[P]K_{4,\mathrm{M,F}} \\
&+ 737906400.0K_{2,\mathrm{M,F}}K_{3,\mathrm{M,F}}K_{4,\mathrm{M,F}} + 199722600.0K_{2,\mathrm{M,F}}K_{3,\mathrm{M,F}}K_{3,\mathrm{M,R}} \\
&+ 7103250.0[P]K_{3,\mathrm{M,F}}K_{2,\mathrm{M,R}} + 833944100.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}K_{3,\mathrm{M,R}} \\
&- 729763100.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}K_{4,\mathrm{M,F}} + 107969400.0K_{4,\mathrm{M,F}}K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}} \\
&+ 6748087500.0[P]K_{4,\mathrm{M,F}}K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}}, \\
C = &\,786571087500.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}[P]K_{4,\mathrm{M,F}} + 42032412800.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P] \\
&- 1766079356250.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P]K_{4,\mathrm{M,F}} \\
&- 28257269700.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}K_{4,\mathrm{M,F}}
\end{aligned}$$

$$
\begin{aligned}
&+ 754677000.0[P]K_{3,\mathrm{M,F}}K_{2,\mathrm{M,F}} - 10266020465624.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}\\
&- 7273866600.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}}K_{2,\mathrm{M,R}} + 11068596000.0K_{2,\mathrm{M,F}}K_{3,\mathrm{M,F}}K_{3,\mathrm{M,R}}\\
&- 746348625.0[P]K_{3,\mathrm{M,F}}K_{2,\mathrm{M,R}} - 23531583900.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}K_{3,\mathrm{M,R}}\\
&+ 12585137400.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}K_{4,\mathrm{M,F}} + 110423250.0[P]K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}}\\
&+ 1274565600.0K_{4,\mathrm{M,F}}K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}} + 79660350000.0[P]K_{4,\mathrm{M,F}}K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}},\\
D = &-655944083175.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P] + 15239438775000.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P]K_{4,\mathrm{M,F}}\\
&+ 243831020400.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}K_{4,\mathrm{M,F}} + 156765188755560.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}\\
&+ 210303324000.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}}K_{2,\mathrm{M,R}} + 12871163250.0[P]K_{3,\mathrm{M,F}}K_{2,\mathrm{M,R}}\\
&+ 188777061000.0K_{2,\mathrm{M,R}}K_{3,\mathrm{M,F}}K_{3,\mathrm{M,R}} + 1303533000.0[P]K_{3,\mathrm{M,R}}K_{2,\mathrm{M,F}},\\
E = &\,3580768471500.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}[P] - 832848959250000.0K_{3,\mathrm{M,R}}K_{2,\mathrm{M,R}}.
\end{aligned}
$$

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