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ACTIVE ENZYME GEL CHROMATOGRAPHY: II. Computer simulations*

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The behavior of an enzyme undergoing reaction while on a gel chromatography column has been studied by computer simulation using the steady state assumption for a system with a single enzyme–substrate complex. The profiles of the enzyme–substrate complex, product, and substrate were examined varying the parameters of k_{cat}, flow rate, partition coefficient, dispersion coefficient, and time. These investigations confirm that much information about both the active enzyme and the product may be obtained by examining the product profile alone, varying the power of applying scanning gel chromatography to active enzyme systems.

1. Introduction

Hydrodynamic studies of enzymes using conventional techniques require enzyme concentrations on the order of mg/ml and require very pure preparations. The feasibility of performing physical studies using impure enzyme preparations on the μg/ml level has been demonstrated using active enzyme ultracentrifugation [1]. This technique involves layering a small amount of enzyme on a substrate solution and observing spectrophotometrically either the appearance of a product or the disappearance of a substrate. From the resulting substrate or product profile the sedimentation coefficient may be determined [1].

Recently, the capability of applying active enzyme studies to scanning gel chromatography has been demonstrated [2]. Using the latter technique, 100 μℓ samples of L-glutamate dehydrogenase and homoserine dehydrogenase ranging in concentration from 1.3 to 66 μg/ml were applied to the top of a column with substrate-containing buffer. A small volume (25 μℓ) of buffer without substrate was then allowed to enter the column and subsequent elution carried out with the substrate-containing buffer. The product profile of the L-glutamate dehydrogenase reaction was recorded by direct optical scanning of the gel column. In the homoserine dehydrogenase experiments both "forward" and "reverse" reactions were observed, generating both substrate depletion and product formation profiles. From these experiments the enzymic activities could be determined by measuring the amount of product produced between scans and the partition cross sections determined from the rate of movement of the leading boundaries of the profiles [2,3]. The technique was found to provide reliable estimates of the enzyme transport parameters.

Computer simulations have been successfully used to describe macromolecular behavior on a gel column [4–9]. Such simulations provide a means of predicting the behavior of interacting systems, based upon given assumptions about the nature of the interactions. Comparison of the predicted effects with experimentally observed behavior then enables one to test and modify such assumptions until good agreement is achieved. It is the purpose of this paper to extend the simulation approach to active enzyme gel chromatography using the steady state approximation for the system.

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P\,, \qquad (1)$$

where k_f and k_r are the forward and reverse rate constants respectively so that $k_f/k_r = K_{eq}$. The parameter k_{cat} is the catalytic constant. These simulations show

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that by measuring the product profile* alone one may determine the partition coefficient of both enzyme and product, obtain information about the dispersion coefficient of each, and in addition obtain the same kinetic data as is available from classical kinetic experiments.

2. Methods of analysis

2.1. Transport equations

The computer simulations generate the profiles of all species as they would be observed by direct measurement of each species on the gel column. The profile of each species is determined by the transport equation for species j undergoing reaction and is given below [4]:

$$J_j' = FC_j'/\xi_j - L_j\,\mathrm{d}C_j'/\mathrm{d}X + r_j \qquad (2)$$

In this equation J_j' is the solute flux per unit cross sectional area and ξ_j is the partition cross section for solute j, representing the fraction of the cross-sectional area occupied by solute j. In the present simulations a constant cross-sectional area A is assumed and uniform packing so that $\xi_j A$ is constant for each solute species. C_j' is the column concentration in mass units, F is the flow rate of eluent, L_j is the dispersion coefficient and r_j is the rate of production (positive) or consumption (negative) of species j by chemical reaction. The parameter ξ_j is related to the partition coefficient, σ_j, by $\xi_j = \alpha + \beta\sigma_j$ [10] where α and β are dimensionless parameters related to the fraction of the volume occupied by the void volume and internal volume respectively. The partition coefficient represents the fraction of the internal volume occupied by the solute and is of interest becasue of its relation to the molecular radius, a_j, through the expression [12].

$$\sigma_j = \mathrm{erfc}[(a_j - a_0)/b_0]\ . \qquad (3)$$

In eq. (3), a_0 and b_0 are calibration constants related to the porosity of the gel**. The column concentration, C_j', is defined in terms of the bulk solution concentration, C_j, by $C_j' = \xi_j C_j$ [11].

* The substrate depletion profile was also measured and could as easily have been used for calculations as the product profile.

** $\mathrm{erfc}(X) = (2/\pi)\int_X^\infty \mathrm{e}^{-t^2}\,\mathrm{d}t = 1 - (2/\pi)\int_0^X \mathrm{e}^{-t^2}\,\mathrm{d}t$.

The dispersion coefficient for a species is given by the expression [12]:

$$L_j = FL_p + \xi_j D_j + qd^2F^2/\xi_j^3 A^2 D_j\ . \qquad (4)$$

In eq. (4) L_p is the contribution to dispersion resulting from flow perturbation around gel particles, D_j is the free diffusion coefficient, A is the cross-sectional area of the column, q is a gel particle packing factor, and d is the gel particle diameter. Both F and ξ have been defined earlier. The parameters L_j were evaluated from eq. (4) using column parameters measured by Halvorson and Ackers [18]. The values of D_j were evaluated from the expression

$$D_j = D_{\mathrm{rel}}(M_{\mathrm{ref}}/M_j)^{1/3}\ , \qquad (5)$$

obtained by assuming that the radius is proportional to the cube root of the molecular weight, where M_j and M_{ref} are the molecular weights of species j and the reference species respectively. The values of the reference species are those given by Zimmerman et al. [7].

2.2. The chemical systems

The program simulates the chemical reaction of eq. (1) occurring concurrently with the transport processes on a uniformly packed column of unit cross-sectional area saturated with substrate. The column is mathematically divided into a series of small boxes. At time zero a microgram quantity of enzyme is added to one box and at various times during the course of transport the profile of all four species is determined by monitoring the contents of each box.

The kinetic reactions within each box are evaluated using the integrated form of the Michaelis–Menton eq. [17], making the additional assumption that the equilibrium between E, S, and ES is rapid and consequantly the Michaelis constant, K_m reduces to

$$K_m = k_r/k_f \qquad (6)$$

$$= 1/K_{eq}\ . \qquad (7)$$

Results using this method were compared to those obtained by a finite difference approximation to the differential equations describing the reaction of each species (e.g., $\mathrm{d}(E) = [(k_r + k_{cat})(\mathrm{ES}) - k_f(\mathrm{E})(\mathrm{S})]\,\mathrm{d}(t)$). The results were identical for cases where $k_r \gg k_{cat}$. Since the integrated form required considerably less computer time, this form was used throughout.

Table 1
Species parameters for the reference system

Species	Partition coefficient, σ	Dispersion coefficient L(cm^{-2}/min)	Partition cross section, ξ
Enzyme	0.300	1.95×10^{-3}	0.496
Enzyme–substrate	0.300	1.95×10^{-3}	0.496
Substrate	1.000	5.82×10^{-4}	0.965
Product	1.000 (cm^2/min)	5.82×10^{-4}	0.965

Table 2
Constants used in the reference system

Constant	Value
Equilibrium constant, K_{eq} (ml/mg)	1.000×10^{4}
Michaelis constant, K_m (mg/ml)	1.000×10^{-4}
Catalytic constant, k_{cat} (min^{-1})	2.000×10^{-1}
Flow rate, F(ml/hr)	2.000
Time, t (min)	192

2.3. Physical constants

The parameters used in these simulations unless otherwise indicated are given in tables 1 and 2 and apply to a column of Sephadex G-200R. All reactions involve an enzyme of molecular weight 160 000, a substrate of molecular weight 2 000, an enzyme–substrate complex of molecular weight 162 000 and a product whose physical parameters are identical to those of the substrate.

2.4. The computer program

The program used was originally derived from that of Cox [13–15] and is a direct modification of programs previously described by Zimmerman and Ackers [4–6], Zimmerman et al. [7] and Zimmerman [8, 9]. Computations were performed on an IBM 370/158 digital computer.

A finite difference approximation to eq. (2) is used to determine the profile of each species on the column. The profile of each species is calculated by alternating rounds of translation and dispersion. Internal to each dispersion round are several kinetic rounds and after each round of translation, dispersion or kinetics there is a reequilibration step for the enzyme, substrate, and enzyme–substrate complex.

3. Results and discussion

The profiles of enzyme, enzyme–substrate complex, substrate and product using the standard parameters are given in fig. 1. The product profile generated is similar in appearance to the profile generated by a single species in large-zone experiments. The leading edge is more disperse than the trailing edge as a result of the greater dispersion of the enzyme–substrate complex compared to substrate alone. The centroid of the leading edge corresponds to the position of the enzyme–substrate peak confirming that the centroid and the ξ value of the active enzyme–substrate complex may be calculated by measuring the centroid of the leading eadge of the product peak.

Figs. 2 and 3, respectively, show that profiles of the

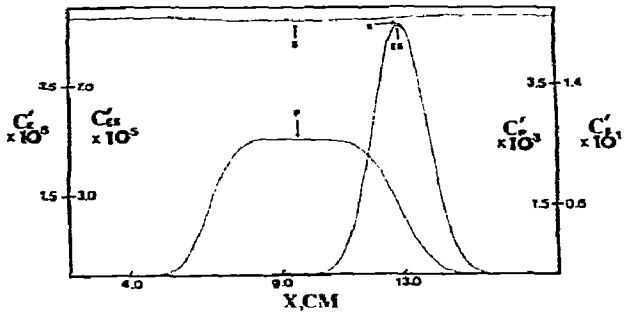

Fig. 1. Profiles of enzyme, enzyme–substrate complex, product and substrate using parameters in tables 1 and 2.

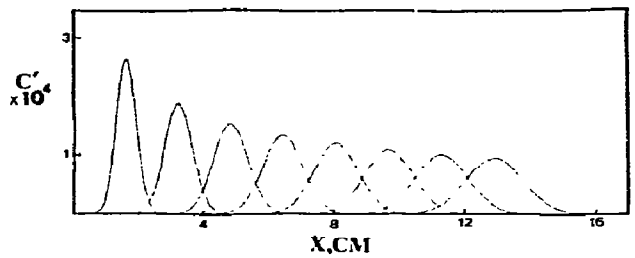

Fig. 2. Enzyme–substrate profiles from 24 minutes to 192 minutes in 24 minute intervals for the system shown in fig. 1.

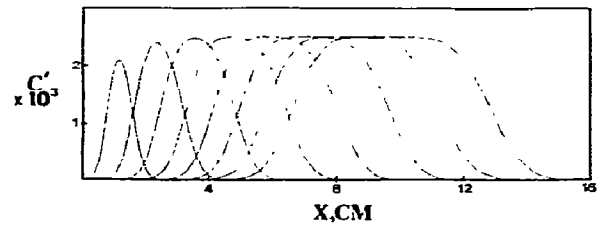

Fig. 3. Product profiles from 24 minutes to 192 minutes in 24 minute intervals for the system shown in fig. 1.

ES complex and the product for eight different times, 24 minutes apart. The ES complex profile follows the expected pattern for a macromolecule applied as a small zone. The profile of free enzyme is almost identical to that of the ES complex except for magnitude and thus is not shown.

3.1. Calculation of partition cross section

The product profile begins as a peak but gradually widens until the formation of a plateau is apparent. It has been shown previously [2], that the relation,

$$d\bar{X}/dt = F/\xi A \,, \qquad (8)$$

may be used to calculate the parameter ξ for the active ES complex, where $d\bar{X}/dt$ is the rate of movement of the centroid of the leading edge of the product peak. When the ξ value of the ES complex is calculated from eq. (8) using the leading edge of the product peak (table 3), the time intervals before the plateau appears (times 1–4 in this case) give only a rough estimate of the correct ξ value; however, for longer time intervals after which the plateau has been formed (times 5–8), eq. (8) provides excellent agreement with the correct value given in table 1.

Eq. (8) was also applied to the trailing edge of the product profile to determine how well the ξ value of the product can be measured from the rate of movement of the trailing centroid. It was found (table 4), similar to the above case, that the time intervals before a plateau is established provide a rough estimate of the ξ value of the product while time intervals after the plateau is established provide an excellent estimate of the input value.

3.2. Calculation of kinetic parameters

The increase in the area under the product curve, or equivalently the increase in mass of product, with time may be used to calculate the rate of conversion of substrate to product [1,2]. Table 5 compares the catalytic constant calculated from the observed velocity from the final 24 minute time interval with the input

Table 3
Calculation of partition cross section for ES complex

Time interval	Elapsed time (min)	Centroid position of product[a] (cm)	Distance centroid moved during time interval (cm)	Calculated ξ_{ES}
1	24	1.656	1.656	0.483
2	48	3.227	1.569	0.509
3	72	4.817	1.590	0.503
4	96	6.419	1.602	0.499
5	120	8.027	1.608	0.497
6	144	9.638	1.611	0.497
7	168	11.250	1.613	0.496
8	192	12.862	1.613	0.496

[a] Calculated from leading edge of product profile.

Table 4
Calculation of the partition cross section for product

Time interval	Elapsed time (min)	Product centroid position [a] (cm)	Distance centroid moved during time interval (cm)	Calculated ξ_p
1	24	0.725	0.725	1.103
2	48	1.585	0.861	0.928
3	72	2.431	0.846	0.945
4	96	3.269	0.838	0.954
5	120	4.102	0.833	0.960
6	144	4.932	0.831	0.962
7	168	5.762	0.830	0.964
8	196	6.591	0.829	0.965

a) Calculated from trailing edge of product profile.

Table 5
Calculation of velocity and k_{cat} from product peak [a]

Time interval (min)	Mass of product produced during time interval [b]	Velocity (mg/min)	Mass of ES complex (mg)	k_{cat} (min^{-1})
24	1.959×10^{-3}	8.163×10^{-5}	4.081×10^{-4}	2.000×10^{-1}

a) Results were the same for each of the eight time intervals.
b) The mass of product is proportional to the area under the product curve.

value. They are the same, indicating that the program gives the correct velocity. Thus, if one knows k_{cat}, one may calculate K_m or vice versa.

3.3. Variation of flow rate

Fig. 4 shows the product profile for five different flow rates. These profiles were generated using the standard parameters given in tables 1 and 2 except that the times and flow rates were varied to maintain the centroid of the ES complex at a constant position. The primary effect on the product profiles when the flow rate is increased is a gradual increase in the dispersion of the leading edge to the increase in the dispersion coefficient of the ES complex. The effect on the free enzyme and ES complex profiles is not shown but is a small increase in the dispersion of the profiles with increased flow rate as predicted by eq. (4).

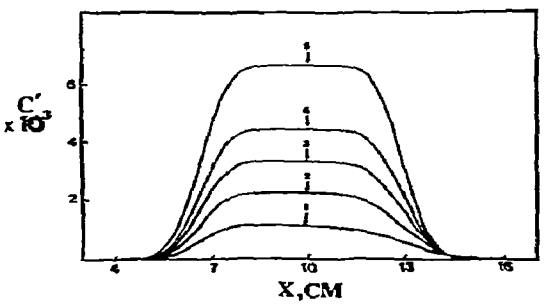

Fig. 4. Product profiles for the following flow rates: (1) 6.0, (2) 3.0, (3) 2.0, (4) 1.5, and (5) 1.0 ml/hr. The times have been varied to maintain a constant centroid position. Other parameters are as in fig. 1.

3.4. Variation of enzyme partition coefficient

Fig. 5 shows the product profiles after 192 minutes using the standard parameters except for the indicated variations in the partition coefficient, σ, of both the free enzyme and the ES complex. The effect of increasing σ is to decrease the distance traveled and to decrease the dispersion coefficient as predicted by eq. (4). The product profiles exhibit a sharp peak at high enzyme partition coefficients with the formation of plateaus at lower enzyme partition coefficients. The

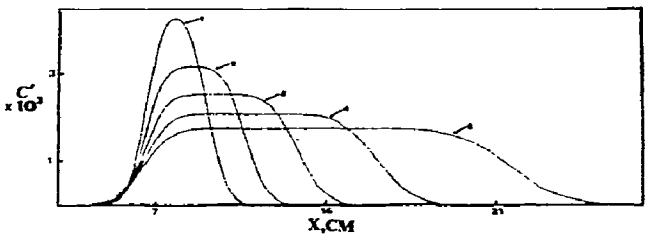

Fig. 5. Product profiles for the following enzyme and ES complex partition coefficients: (1) 0.600, (2) 0.450, (3) 0.300, (4) 0.150, (5) 0.000. These partition coefficients result in dispersion coefficients of: (1) 9.74×10^{-4}, (2) 1.30×10^{-3}, (3) 1.95×10^{-3}, (4) 3.43×10^{-3} and (5) 7.69×10^{-3} cm²/min. Other parameters are as in fig. 1.

plateaus become lower as the distance traveled becomes greater, as they must, since the time on the column and thus the amount of product produced is the same for all five systems. Of special interest is the fact the leading edge becomes more disperse as σ is decreased, while the trailing edge is affected only slightly.

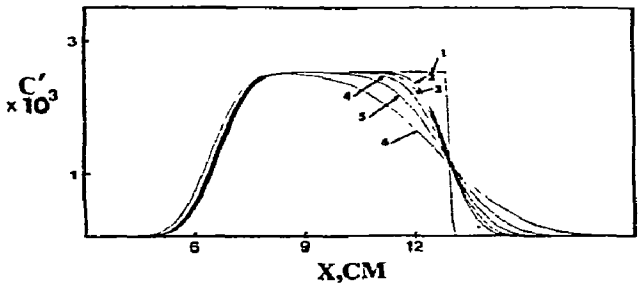

Fig. 6. Product profiles for the following enzyme and ES complex dispersion coefficients: (1) 0.00, (2) 9.74×10^{-4}, (3) 1.30×10^{-3} (4) 1.95×10^{-3}, (5) 3.43×10^{-3} and (6) 7.69×10^{-3} cm²/min. Other parameters are as in fig. 1.

Table 6
Calculation of dispersion coefficient of ES complex from product leading edge

Case number	Input dispersion coefficient L (cm²/min)	Calculated dispersion coefficient L (cm²/min)
1	9.74×10^{-4}	9.75×10^{-4}
2	1.30×10^{-3}	1.30×10^{-3}
3	1.95×10^{-3}	1.95×10^{-3}
4	3.43×10^{-3}	3.45×10^{-3}
5	7.69×10^{-3}	7.69×10^{-3}

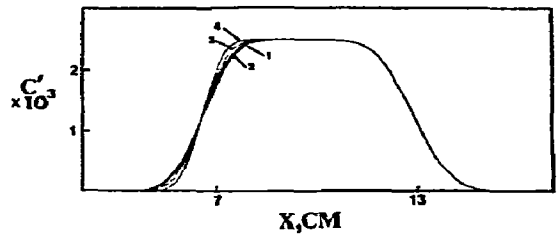

Fig. 7. Product profiles for the following substrate and product dispersion coefficients: (1) 0.00, (2) 3.00×10^{-4}, (3) 5.80×10^{-4}, and (4) 8.00×10^{-4} cm²/min. Other parameters remain as in fig. 1.

3.5. Variation of dispersion coefficient

The dispersion coefficients calculated by the computer program for the flow rate study previously given were used to study the variation of product profiles resulting from changes in the dispersion coefficient of the free enzyme and ES complex. Fig. 6 shows more clearly the dependence of the leading edge on the protein species with relatively little effect on the trailing edge. The dispersion coefficient of the leading edge can be calculated from the slope of the line given by the relation [16],

$$\operatorname{erfc}^{-1}(2C/C_0) = (\xi AE - Ft)\,(4L\xi^2A^2t)^{-1/2}\,, \qquad (10)$$

where C_0 is the plateau concentration and all other parameters have been previously defined. The results agree excellently (table 6) with the dispersion coefficient of the ES complex.

The effect of artificially varying the dispersion coefficient of both the substrate and product on the product profile is shown in fig. 7. Notice that the leading edge is not affected while the trailing edge becomes more disperse with increasing product dispersion coefficient.

The results of figs. 5, 6 and 7 indicate that the leading edge of the product profile provides information predominantly about the dispersion coefficient of the active ES complex while the trailing edge gives information concerning the dispersion coefficient of the product.

3.6. Variation of kinetic parameters

The effect of increasing k_{cat} in the ranges tested is only to increase or decrease the product profile by the same numerical factor as the rate constant is changed.

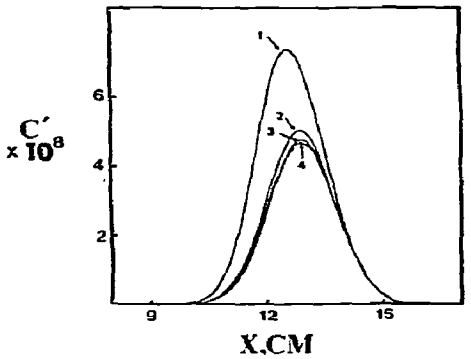

Fig. 8. Enzyme profiles for varying values of k_{cat}: (1) 10.0, (2) 2.0, (3) 0.2, and (4) 0.02 min^{-1}. Other parameters remain as in fig. 1.

However, as can be seen in fig. 8, as the rate constant is increased, the free enzyme peak becomes slightly shifted to the left as substrate is depleated. This is necessary in order for equilibrium to be maintained between enzyme, ES complex and substrate. The peak of the ES complex is too massive (relative to the free enzyme) to be affected significantly. Preliminary work using other conditions indicates that k_{cat} may play a more important role than has been observed in this study in the determination of the profile shapes.

4. Conclusions

Active enzyme gel chromatography has great potential as a tool for providing both kinetic and physical information about an enzyme which is actively undergoing reaction at physiological concentrations. The extension of these simulations to monomer–*n*-mer systems is now underway.

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