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Use of the Foster-Niemann Equation in Study of the Enzymic Processes

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Dedicated to the memory of Professor Vera Johanides

Summary

The Foster-Niemann equation enables the analysis of kinetic data from long-term enzymic reactions in which products of the reaction inhibit a process. In this way the inevitable errors in estimation of initial rates, needed for reaction constants calculation, can be avoided. Initially, Foster-Niemann equation was used in reevaluation of the kinetic constants of previously studied specific substrates of α-chymotrypsin. Enzymic hydrolysis of biopolymers are very complex, and reaction products generally inhibit the reaction. Therefore, the equation was very useful both in the preliminary studies of unknown processes and in the elucidation of the reaction mechanisms. In the processes in which soluble enzyme reacts with insoluble substrate the basic Foster-Niemann equation was modified for the reactions in heterogeneous systems. Thus, the appropriate forms of the Foster-Niemann equation were employed in the study of starch, cellulose, cellobiose, barley 1,3 and 1,4-β-D-glucan and protein hydrolysis. By means of these equations the rection mechanisms were elucidated, the kinetic constants evaluated and kinetic and mathematical models of the reaction systems developed.

Key words: Foster-Nieman equation, enzyme kinetics, proteinase, cellulase, β-glucosidase, amylase

Introduction

A number of cases, in which reaction products inhibit the enzymic processes, has been described. Thus, the overall reaction can be satisfactorily described kinetically as

$$S+E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E+P \qquad /1/$$

$$P+E \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EP \qquad /2/$$

The products of the reaction are generally competitive inhibitors. The velocity of the reaction for these cases is given by the equation

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$$v = -\frac{ds}{dt} = \frac{k_{+2}ES}{K_m\left(1+\frac{P}{K_i}\right)+S} \qquad /3/$$

The kinetic constants can be estimated from measurements of initial reaction velocities. However, since the evaluation of the initial rates includes extrapolation to $t = 0$, it is possible that the values of initial rates have an inevitable error due to the inhibition by reaction products. Integrated rate equation /3/ rearranged to the slope-intercept form is

$$\frac{1}{t}\ln\frac{S_0}{S_t} = \frac{K_m - K_i}{K_m(K_i + S_0)}\frac{P}{t} + \frac{k_{+2}EK_i}{K_m(K_i + S_0)} \qquad /4/$$

Accordingly, plot of $1/t\ ln\ (S_0 / S_t)$ *vs.* P/t will give a family of lines of slope $(K_m - K_i)/(K_m(K_i + S_0))$ and ordinate intercept $k_{+2}\,E\,K_i/(K_m(K_i + S_0))$ for various values of E. For different enzyme concentrations and constant S_0 the straight lines will be parallel. It can be seen from the relation /4/ that when $K_i > K_m$ the slope is negative, when $K_i = K_m$ the lines are parallel with the abscissa and when $K_i < K_m$ the slope is positive. It has also been shown that reaction constants can be estimated using Foster-Niemann coordinates without evaluating the initial velocities separately (*1–3*). K_i may be simultaneously evaluated under the conditions which do not require the separate addition of the reaction products. It should be emphasised that Foster-Niemann plot employs the same coordinates as the plot of Walker and Schmidt (*4*) when no inhibitory reactions are taking place. However, the terms for the slopes and intercepts in Foster-Niemann plot are extended with K_i making it superior to the plot of Walker and Schmidt. Integrated rate equation /4/ was for the first time used in reevaluation of the kinetic constants of sixteen specific substrates of α-chymotrypsin (*5*). The features of integrated rate equations have been comprehensively presented by Laidler (*6*).

Characteristics of Biopolymers and Complex Substrates Hydrolysis

The Foster-Niemann equation has a marked impact on the study of biopolymers and complex substrates hydrolysis. Indeed, enzymic hydrolyses of biopolymers are very complex. They cannot be regarded as the reaction of one substrate, but as consecutive reactions in which intermediate products compete with the original substrate for the reaction with the enzyme. In heterogeneous systems, the situation is even more complex. When solubilised enzyme reacts with concentrated, highly nonideal solid polymeric substrate, thermodynamic variables should be taken into account in the quantitative study. The whole complexity of insoluble substrate hydrolysis can be imagined on the basis of the degradation of yeast cell wall (*7*) with the β-glucanase from *Polyporus* sp. (*8*) (see Fig.1). In addition, one should keep in mind that physico-chemical properties of cell wall depend on the composition of nutrition medium and growth conditions (*9*).

Furthermore, cereals contain inhibitors which strongly affect the enzyme activity in mashing. Correspondingly, the reactions like these cannot be described by classical Michaelis-Menten concept that was developed for enzymic reactions in true solutions without any kind of inhibition. Though most of these reaction systems obey the initial rate concept and reaction rate is commonly proportional to the enzyme concentration. For relevant industrial conditions it is possible to obtain hydrolysis curves that can be very useful in the process of optimisation (*10*). However, the hydrolysis curves have not been very convenient for the comparison of the hydrolysis of differently pretreated substrates, taking into account the efficiency of enzymes of different origin *etc*. In such circumstances the need to develop systematic kinetic models was obvious. Taking into account all characteristics of complex substrate hydrolysis it was evident that more useful information can be obtained by procedures that are based upon a rate equation which describes the reaction throughout its entire course.

Hydrolysis of Carbohydrates

Among carbohydrates, starch and cellulose are the most important polysaccharides in industrial processing and life maintenance. The products of starch degradation inhibit the enzymes catalysing starch hydrolysis. The coordinates $1/t\ ln\ (S_0/S_t)$ *vs.* P/t were used in the analysis of kinetic data owing to the fact that the first-order rate constant declined as the process proceeded. Soluble potato starch in the concentration range of 0.23–5.0 % was hydrolysed with the culture filtrate of *Aspergillus awamori* and *A. batatae*. For low E/S ratios high linearity was obtained, however, with E/S ratio increasing, marked deviations from linearity appeared. It was pointed out that the procedure could be employed in estimation of the characteristics of enzymic preparations in starch hydrolysis (*11*).

The Foster-Niemann equation was even of greater importance in the research of cellulose hydrolysis. The products of cellulose hydrolysis, glucose, cellobiose and lower oligomers, inhibit action of the cellulase. The inhibition was shown during the initial stages of hydrolysis in the presence and absence of glucose and cellobiose. Strong product inhibition was also proved by plotting kinetic data of long term reaction for three initial cellulose concentrations in the Foster-Niemann coordinates (*12,13*). In further studies the kinetic parameters were estimated also from long term reactions for cellulose concentrations up to 15 g/L and at hydrolysis extents up to 15 %. The simple noncompetitive model gave better results in predicting the reaction course for up to 10 h than the competitive model, particularly at higher substrate concentrations (*13*). The results shown in this work are presented as another step towards the proposal for generalized kinetic description of cellulose hydrolysis using basic enzymological principles.

The kinetics of β-glucosidase, which is a part of the cellulase complex, was also studied. The action of β-glucosidase is inhibited by glucose. The reliable estimation of initial rate data is experimentally difficult, and therefore inhibition mechanism was studied by applying the Foster-Niemann equation. For various initial cellobiose concentrations a set of linear correlations with different slopes but with a single intercept was obtained. Thus, it

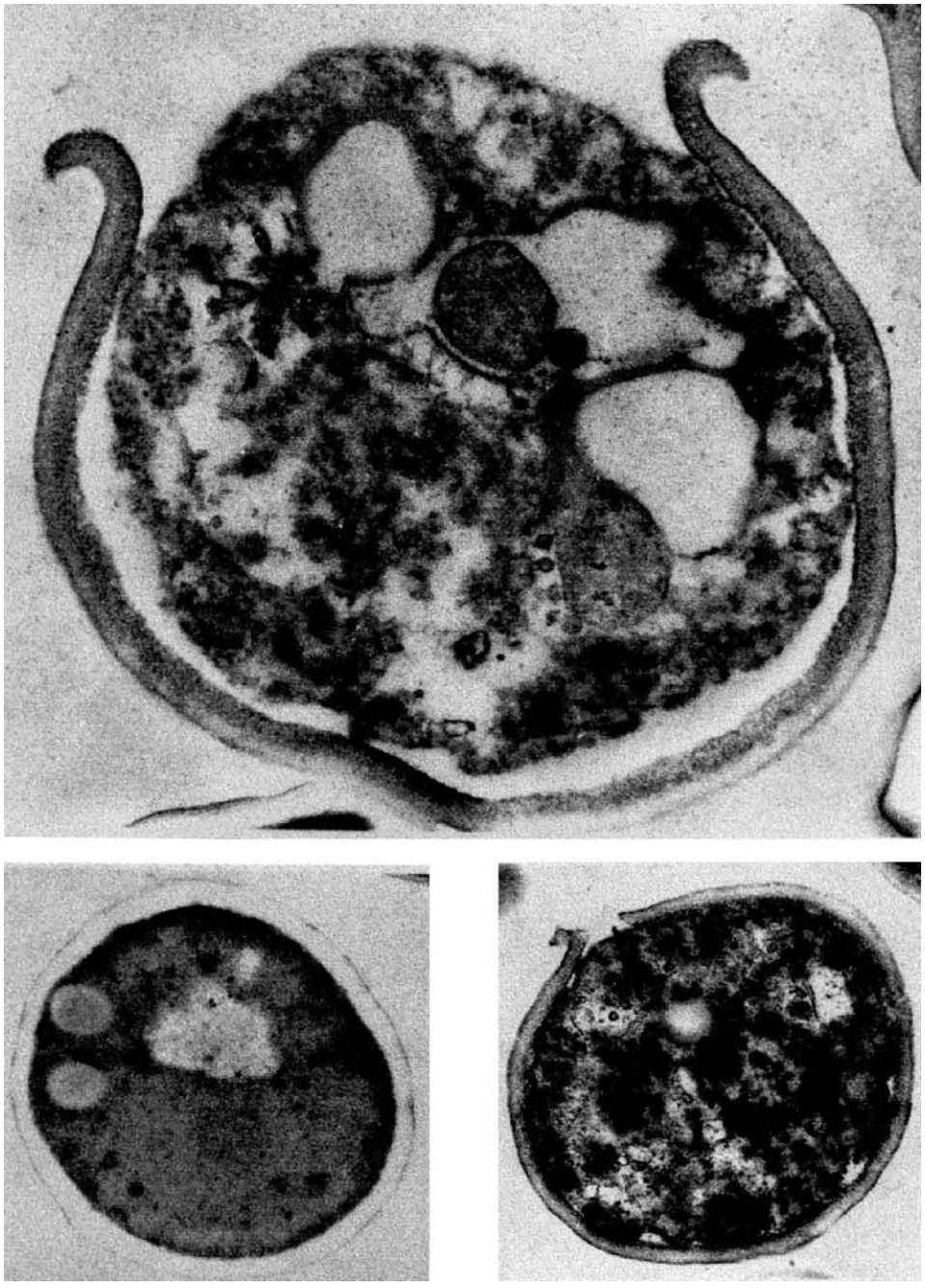

Fig. 1. Degradation of the cell wall in yeast *Saccharomyces cerevisiae* with β-glucanase from *Polyporus* sp. (*7*). Magnification 20 000 x and 6 000 x (photo: N. Ljubešić)

was concluded that the action of β-glucosidase is inhibited by glucose through a competitive mechanism. The inhibition constant K_i evaluated from the mean value of the intercept was found to be 1.32 g/L (*14*).

In addition to starch, cellulose and cellobiose degradation, the Foster-Niemann equation was used in analysis of the kinetics of barley 1,3 and 1,4-β-D-glucan hydrolysis. High correlation (r = 0.999) was found in β-glucan hydrolysis with fungal (*Polyporus* sp.) and bacterial (*Bacillus subtilis*) β-glucanase. These two enzymes, when mixed in definite ratio, showed synergistic effect in β-glucan hydrolysis. The enzyme ratio did not affect the slope of the straight lines. However, with the alteration of the enzyme ratio the ordinate intercept changed. The highest ordinate intercept appeared with enzyme ratio which showed the strongest synergistic effect (*15*). Thus, the Foster-Niemann equation could be also successfully used in studies of cooperative and synergistic effects between enzymes involved in degradation of biopolymers.

Hydrolysis of Proteins

The inhibition of proteolytic enzymes by reaction products was first described in soluble synthetic substrate hydrolysis (*16,17*). As already mentioned, the Fos-

ter-Niemann equation was also initially used in soluble substrates studies (*5*). The functional and nutritional properties may be improved by the use of specific proteases to partially hydrolyse a protein. However, in various processes, the native protein is the insoluble component, thus the enzyme reaction system is heterogeneous. The kinetic models for proteolytic enzyme reactions in heterogeneous systems have been developed much later. Integrated rate equation with product inhibition was used for evaluation of the model parameters for soybean protein solubilisation. The substrate concentration was in the range of 10–75 g/L and the parameters K_m, $k_{+2}E$ and K_i were evaluated from regression line with a correlation coefficient r = 0.978. The model fitted satisfyingly to the experimental data (*18*).

Approximately at the same time the barley protein degradation with bacterial proteinase was studied. Barley syrup production, brewing with barley and animal feeding with barley represent an important use of hydrolytic enzymes. Process of barley protein degradation was studied very often independently of amylolysis. The time course of barley protein degradation was examined during mashing raw barley and spent grains. The analysis of kinetic data for protein degradation during mashing of barley (30 % dry matter) and spent grains (21–22 % dry matter) has shown that the first-order coefficients are falling as the reaction proceeds (*19*). The gel-chromatography of supernatants also indicated product inhibition during the hydrolysis. Since in this case the substrate is in solid phase and the product of reaction in liquid phase, the Foster-Niemann coordinates were adapted to this system. For that purpose the concentration of total soluble and α-amino nitrogen was expressed on the mash volume taking into acount the extract concentration, dry matter of mash and specific density. The estimation of specific density was facilitated owing to the fact that in this branch of industrial analysis there are very precise tables for relationship between extract concentration and specific density. By plotting the kinetic data for protein solubilisation during mashing of barley with neutral proteinase (*B. subtilis*) in Foster-Niemann coordinates, straight lines with rather unexpectedly high correlation (r = 0.9999) have been obtained. Similar results were achieved for protein solubilisation during mashing of spent grains from barley syrup preparation (*19*). So high kinetic regularity was not expected due to the heterogeneity of the system and very high concentration of mash (30 % dry matter). Still more surprising was the positive slope of straight lines, indicating that the solubilisation process was strongly inhibited by solubilised proteins. At first the author doubted the correctnes of the calculations. The results also reminded of »*negative values*« for K_m appeared in carboxypeptidase study, when it was concluded that more extensive measurements were required to elucidate the reaction mechanism (*20*). However, comparing different enzyme concentrations, the straight lines were, in accordance with equation /4/, closely parallel. Furthermore, the ordinate intercept was proportional to the enzyme concentration. The relationships of kinetic parameters obtained for native and denaturated protein degradation were in accordance with known characteristics of the protein hydrolysis. It has been noted that presented kinetic regularities and experimental and analytical techniques could be used for the analysis of protein degradation with different enzymes in cereals which have been subjected to physical or chemical changes (*21,22*). Therefore, it could be useful to mention that the zero time concentrations of total and α-amino nitrogen had crucial place in evaluation of kinetic parameters. For this reasons, in experiments particular attention should be paid to their estimation. Furthermore, inherent proteolytic enzymes of barley and, already in the phase of the mash preparing, hydrolyse proteins and change the values of S_0 and P. To avoid the effect of this uncontrolled process on later kinetics with external enzymes added, the mashes should be prepared in as identical conditions as possible and during a definite time.

On the basis of kinetic characteristics of barley protein degradation with microbial proteinase (*22*) a sequential Michaelis-Menten kinetic model with product inhibition and enzyme activity decay for protein solubilisation was proposed (*23*). Following the interpretation of insoluble (gelatin) protein hydrolysis kinetics of Schurr and Mc Laren (*24*), basic Foster-Niemann equation was modified by introducing appropriate constants for protein hydrolysis in heterogeneous systems:

$$\frac{1}{t}\ln\frac{N_0^{is}}{N_t^{is}} = \frac{K_B^{is} - K_y}{K_B^{is}(K_y + N_0^{is})}\frac{N_t^s}{t} + \frac{k_A^{is}E_T K_y}{K_B^{is}(K_y + N_0^{is})} \qquad /5/$$

To provide further experimental validity of the proofs for the approach presented, the effect of barley concentration on protein degradation kinetics has been studied. By plotting the kinetic data in Foster-Niemann coordinates for barley concentration range of 10–30 % linear relationships ($r \geq 0.999$) were obtained. It was also observed that the slopes of straight lines were very close to corresponding reciprocal initial insoluble nitrogen concentrations ($1/N_0^{is}$) in case of $K_y << 1$, providing an additional argument for the product inhibition concept.

The effect of proteinase inhibitors on protein solubilisation in spent grains mashing with alkaline proteinase (*B. subtilis*) was also studied. The addition of proteolytic inhibitors affected the ordinate intercept. The value of the ordinate intercept decreased in agreement with the equation /5/.

In order to verify suitability of equation /5/, a mathematical model describing protein degradation during barley mashing was developed. To avoid difficulties and possible mistakes in the individual estimation of constants K_B^{is}, K_Y and k_A^{is} the equation /5/ was transformed in model equation by taking derivative with respect to time. Here protein solubilisation rate was expressed by lumped constants *i.e.* by related slope and ordinate intercept in Foster-Niemann plot. The validity of the model was tested by computer simulation. The results of computer simulation were in good agreement with experimental data (*23*). The modified Foster-Niemann equation was also successfully applied in the study of the effect of barley peptidase extract on protein solubilisation during spent grains mashing (*25*). By detailed analysis of the relationships between kinetic parameters it was presumed that a decrease in protein solubilisation rate with peptidase extract was due to the endopeptidase inhibitors. The assumption based upon kinetic reasoning was

proved by isolation of microbial proteinase inhibitor from the peptidase extract (*25*).

The presented interpretation of enzymic proteolysis kinetics in heterogeneous systems facilitate the comparison of different enzymes or proteins from different sources. Since the solubilisation rate is proportional to the intercept (h), the usage of this parameter for comparison between different enzyme preparations was proposed. Also it has been distinguished that the ratio of the intercept (h) and the slope (K), $h/K = E_T\, k_A^{is}\, K_Y/(K_B^{is}–K_Y)$, consists of parameters related to the solubilisation rate. In this way it was found that h/K ratio in spent grains hydrolysis with the peptidase extract was over seven times lower than in hydrolysis with proteinase only. Thus, due to its easy determination, this parameter could be a convenient criterion for the comparison of different enzymes and processes carried out under various conditions.

Barley protein hydrolysis can also be considered as a potential model system for other somewhat similar processes. Such example can be found in hydrolytic enzyme use for animal nutrition. The enzymes mixed into the feedstuffs improve animal digestive capacity, the degradation of specific polysaccharides, plant cell membranes, and proteins. The knowledge of enzyme characteristics and kinetic parameters of protein degradation achieved by using modified Foster-Niemann equation /5/ significantly facilitated the enzyme choice, determination of optimal formulation and dose in animal fattening (*26*). In addition, several methods are provided in enzyme engineering for the improvements of enzymes for the conditions in industrial processes. Using chemical enzyme modification, mutagenesis and *in vitro* recombination it is possible to alter the structure of an enzyme providing more suitable catalyst (*27*). Due to easy determination of kinetic parameters (*e.g.* h/K) the basic Foster-Niemann equation and its extended form for the reactions in heterogeneous systems /5/ could also be an efficient tool in the enzyme characterisation in this fruitful field of research.

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Symbols

E enzyme concentration (mol/L)

E_T total enzyme concentration (mol/L)

EP enzyme-product complex (mol/L)

ES enzyme-substrate complex (mol/L)

h ordinate intercept in Foster-Niemann coordinates (min^{-1})

k_A^{is} average reaction rate constant for break down of complexes of enzyme with different peptide bonds of insoluble protein (min^{-1})

k_{+i}, k_{-i} reaction rate constants for forward (subscript $+i$) and reverse (subscript $-i$) reactions (min^{-1})

$K_B = \overline{K}_m$ average Michaelis-Menten coefficient for binding of enzyme with different peptide bonds of soluble protein (mg/L)

K_B^{is} average Michaelis-Menten coefficient for binding enzyme with different peptide bonds of insoluble protein (mg/L)

$K_B^{is} = \overline{K}_m^{is} \cdot K_F^{-1}$

K_F equilibrium constant for partition of free enzyme between solution and solid phase (dimensionless)

K_i product inhibition coefficient (mol/L)

K_m Michaelis-Menten coefficient (mol/L)

$\overline{K}_m^{is}$ average Michaelis-Menten coefficient for binding of enzyme with different peptide bonds of insoluble protein (mg/L)

K_Y average product inhibition coefficient for binding of enzyme with peptide bonds in solubilised protein (mg/L)

N_0^{is},N_t^{is} insoluble nitrogen concentrations at beginning of reaction and after time t (mg/g dry matter or mg/L)

N_t^s total soluble nitrogen liberated in process after time t (mg/g dry matter or mg/L)

P product (mol/L)

S_0,S_t substrate concentration at the beginning of the reaction and after time t (mol/L)

t reaction time (min)

v reaction velocity (mol/L min)

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Primjena Foster-Niemannove jednadžbe u proučavanju enzimskih procesa

Sažetak

Foster-Niemannova jednadžba omogućuje analizu kinetičkih podataka dugotrajnih enzimskih reakcija u kojima produkti reakcije inhibiraju proces. Njezinom primjenom moguće je izbjeći pogreške pri procjenjivanju početnih brzina potrebnih za izračunavanje konstanti reakcije. Najprije je Foster-Niemannova jednadžba primjenjivana pri novoj procjeni kinetičkih konstantâ prethodno proučavanih supstrata α-kimotripsina. Budući da su enzimske hidrolize biopolimera vrlo kompleksne a produkti općenito inhibiraju reakciju, Foster-Niemannova jednadžba može se uspješno primjenjivati i u preliminarnim istraživanjima nepoznatih procesa i za temeljito objašnjenje reakcijskih mehanizama. U procesima u kojima topljivi enzim reagira s netopljivim supstratom, osnovna je Foster-Niemannova jednadžba modificirana za reakcije u heterogenim sustavima. Odgovarajući oblici Foster-Niemannove jednadžbe primijenjeni su u istraživanju hidrolize škroba, celuloze, celobioze, 1,3 i 1,4-β-D-glukana ječma i proteina. S pomoću jednadžba objašnjeni su reakcijski mehanizmi, procijenjene kinetičke konstante i razvijeni kinetički i matematički modeli reakcijskih sustava.