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Empirical kinetic models for tryptic whey-protein hydrolysis

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Abstract

Enzymic hydrolyses of naturally occurring protein sources represent complex reactions at a molecular level because of the specificity pattern of proteases and the composition of the substrates involved. In addition, rather global criteria are in practical use for describing the extent of protein hydrolysis. Therefore, mechanistically based kinetic modelling may not be applicable under these circumstances. For practical purposes it is often sufficient to use a descriptive mathematical model, which leads to a reliable interpolation of the experimental data. Empirical models are proposed for the kinetics of the limited tryptic whey-protein hydrolysis. These models are identified by fitting profiles of the fraction of soluble protein (non-protein nitrogen, NPN) as a function of operating time of discontinously operated stirred-tank reactors. Models with at least four parameters are required in order to describe profiles obtained at temperatures at which strong enzyme inactivation is observed. A simple model of only two parameters based on a kinetics with exponential decrease of the activity with increasing fraction of soluble protein is sufficient to simulate the tryptic digestion of whey-protein in batch reactors under conditions of moderate enzyme inactivation applicable for temperatures lower than 60°C. Copyright © 1997 Elsevier Science Ltd

Keywords: trypsin, whey-protein, protein hydrolysis, kinetics, modelling, infant food.

Nomenclature

a_i	Parameter i
E_0	Enzyme concentration (kg kg^{-1})
k_2	Rate constant (kg kg^{-1} min^{-1})
k_2'	Apparent rate constant, lumped parameter (kg kg^{-1} min^{-1})
K_{ic}	Inhibition constant for a competitive product inhibition (kg kg^{-1})
K_m	Michaelis–Menten constant (kg kg^{-1})
K_m'	Apparent Michaelis–Menten constant, lumped parameter (kg kg^{-1})
S_0	Initial substrate (protein) concentration (kg kg^{-1})
t	Time of batch-reactor operation (min)
T_c	Temperature (°C)
X	Fraction of soluble protein, SN-TCA; soluble nitrogen content; NPN, yield of soluble protein (–)

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Introduction

Porcine pancreatic trypsin (E.C. 3.4.21.4) is a protease readily available in high purity as well as in technical grade and quantities. It is a serine endoprotease which preferentially cleaves only Arg-X and Lys-X peptide bonds of denatured proteins. Like many other proteases, trypsin is not absolutely specific and its activity depends very much on the environment of the particular peptide bond susceptible for tryptic hydrolysis.[1,2] With these characteristics it is well suited for limited protein hydrolysis.

The hydrolysis of short-chain peptides may follow simple enzyme kinetics, but the proteolysis of proteins or even naturally occurring protein mixtures would lead to rather complex models if they were based on mechanistic reasoning. As a consequence, empirical models are commonly applied for modelling protein hydrolysis.[3] Thus, for the tryptic digest of myosin and fibrinogen Mihalyi *et al.*[4,5] found that the reaction could be described by two first-order reactions taking place in parallel. The extent of reaction was given by

these authors in terms of base consumption obtained by means of the pH-stat method. Similar findings were reported by Archer *et al.*[6] for a tryptic hydrolysis of partially soluble fish proteins. The amount of strong base consumed in pH-controlled reactors is not a direct measure of the extent of hydrolysis, but it may be related to other more commonly used criteria, like the fraction of peptide bonds cleaved[3] or some kind of solubility index[7] by calibration. The SN-TCA index which is the fraction of nitrogen soluble in aqueous trichloroacetic acid under defined conditions is the most significant criterion for characterizing the extent of hydrolysis, if the aim is a product of instant solubility. This index is also often called non-protein nitrogen (NPN).

The proteolysis of natural protein sources by non-tryptic proteases has also been studied. Marquez Moreno and Fernandez Cuadrado[8] found a simple kinetic equation for the proteolysis of vegetable proteins by Alcalase (from *Bacillus licheniformis*, subtilisin Carlsberg). The kinetic model contained an exponential decay of activity with increasing degree of hydrolysis, which was interpreted by assuming strong substrate inhibition together with the autodigestion of the enzyme. Their kinetic model contained but two lumped parameters. Autodigestion is commonly modelled by means of Michaelis–Menten kinetics containing the concentration of the active enzyme species as the substrate concentration. Constantinides and Adu-Amankwa[9] have analysed the proteolysis of vegetable proteins by a protease from *Penicillium duponti*. Their model combined a reversible reaction with a first-order enzyme deactivation and required five parameters. For the proteolysis of lean meat protein by Alcalase, O'Meara and Munro[10] arrived at a model similar to that of Constantinides and Adu-Amankwa. They postulated product inhibition in parallel to a first-order irreversible activity decay of the enzyme. Their basic model contained four parameters and did not improve significantly by adding an adsorptive interaction of the protease with the heterogeneous substrate. Non-tryptic proteases like Alcalase are rather non-specific enzymes leading to higher degrees of hydrolysis than trypsin, which is particularly suited for limited hydrolysis.

The main characteristics of the overall kinetics of proteases seem to be inhibition by the peptides formed as well as inactivation of the enzymic activity by autohydrolysis and thermal unfolding. We were interested in limited tryptic digestion of whey-protein concentrates. Easy to perform measurements in pH-controlled, discontinously operated reactors should serve as a basis for reactor design. Therefore, a formal balance equation or kinetics was required to fit the experimental data in order to be able to convert the results into reliable rate data. This approach did not necessarily require models to reflect the underlying reaction mechanism. Some models are proposed which are able to yield an almost perfect description of the data under diverse reaction conditions.

Materials and Methods

Partially demineralized whey-protein concentrate (WPC) with an average protein content of 22% (w/w) and porcine pancreatic trypsin, PTN 6·0 S (Novo Nordisk, Denmark) were used. The trypsin preparation contained 10·2 μmol g^{-1} of active enzyme with respect to active-site titrations according to the method of Chase and Shaw.[11]

All experiments were performed at an initial substrate concentration of WPC of 20% (w/w) ($S_0 = 0{\cdot}2$) and a pH of 7·3. The WPC contained an initial fraction of (apparently) soluble protein of 4·8% ($X_0 = 0{\cdot}048$) consisting of soluble peptides as well as ammonia. Prior to starting the reaction, trypsin was solubilized in 1 mM HCl containing 5 mM $CaCl_2$. Hydrolysis experiments were carried out in a discontinuously operated, stirred-tank reactor under pH control. The protein solution was treated for 5 min at 90°C prior to starting the reaction by addition of the enzyme solution. The consumption of a strong base (4 M KOH solution) was monitored as a primary criterion of the extent of reaction. The fraction of protein soluble in 13·6% trichloroacetic acid (SN-TCA index) expressed as non-protein nitrogen (NPN) or fraction of soluble protein (X) was calculated from correlations with base consumption. Details about the reactor operation, the analytical procedures and the correlation of the SN-TCA index (NPN) with base consumption may be found in a previous paper.[7] A parameter estimation procedure based on a modified Gauß–Newton algorithm was applied to the profiles of the fraction of soluble protein as a function of reactor operating time, taking the sum of the squares of the deviations in terms of the fraction of soluble protein (NPN or X) as the objective function to be minimized.

Results and Discussion

The conversion of heterogeneous substrates like proteins requires soluble proteases to be applied. Immobilized enzymes would not work because of severe mass-transfer problems of the substrate in porous carriers. Since these soluble enzymes are commonly not expected to be recovered their activity has to be used as economically as possible. In addition to the operating pH the main variable for optimizing the utilization of enzymes for once-only use is the operating temperature, since both the rate of reaction and the rate of irreversible inactivation increase with increasing temperature. The optimum temperature is normally found at the edge of strong enzyme deactivation. Modelling of the temperature dependence of the batch-reactor performance of enzymic reactions may be quite demanding and is only feasable in the case of

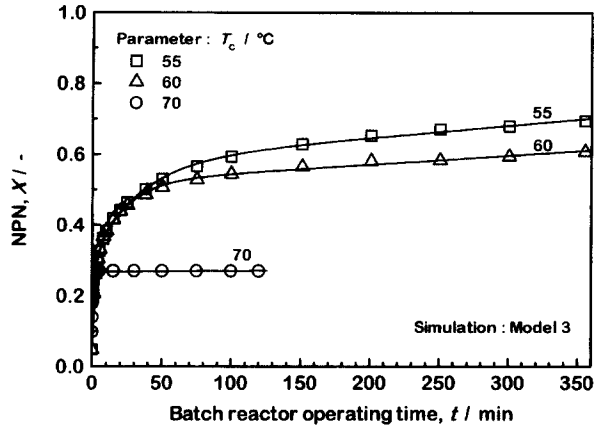

Fig. 1. Tryptic whey-protein hydrolysis at different temperatures. Simulations according to model 3 (S_0=0·2; E_0/S_0=0·005; pH=7·3).

relatively simple reaction systems.[12] Therefore, process-temperature optimization will normally be carried out purely by empirical procedures.

The optimum temperature for the tryptic digestion of whey-proteins in batch-reactors was found to be at about 55°C.[13] Typical batch-reactor experiments for different operating temperatures are shown in Fig. 1. The lower performance at 60°C compared to that at 55°C shows that the overall kinetics are already influenced by strong irreversible enzyme deactivation. Another characteristic feature of the profiles is the steep increase in the fraction of soluble protein at the beginning of the reaction followed by a rapid decrease of the rate of reaction at moderate degrees of hydrolysis, visible from the abrupt flattening of the profile of the degree of hydrolysis as a function of operating time. The fraction of soluble protein may become constant when the enzyme is completely deactivated during the time of reactor operation, as can be seen from the profile at 70°C as an extreme example. For convenience, the deactivation of trypsin during actual reactor experiments is shown in Fig. 2 for different operating temperatures. The activity has been assessed by means of an assay system based on DL-BAPNA (*N*-α-benzoyl-D,L-arginine-*p*-nitroanilide).[14] Obviously, the deactivation of trypsin follows a rather complex pattern, which may be explained by a series mechanism.[14]

If an overall kinetic model cannot be derived, the problem persists that numerical values have to be extracted from experimental data in order to be able to do some basic calculations, e.g. for reactor design. Commonly, only a few data are available from batch-reactor experiments, which would have to be interpolated or from which the first derivative would have to be estimated as a function of, for example, substrate conversion. Such a task requires a smooth model function which should fit the data more or less exactly.

This task has been undertaken for the tryptic digestion of whey proteins. Some typical data are shown in Figs 3 and 4. Figure 3 shows batch-reactor experiments carried out at the operating temperature of 55°C and

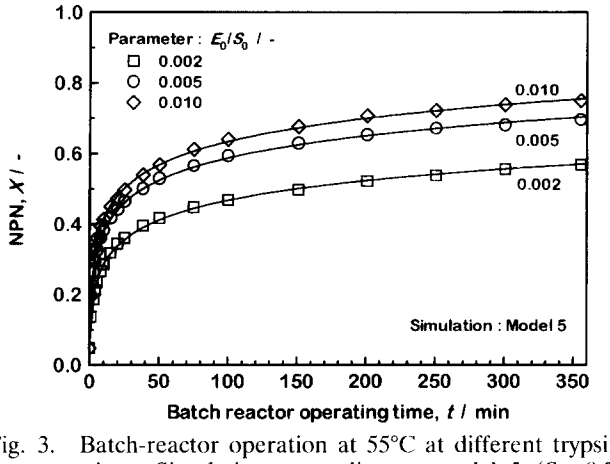

Fig. 3. Batch-reactor operation at 55°C at different trypsin concentrations. Simulations according to model 5 (S_0=0·2; T_c=55°C; pH=7·3).

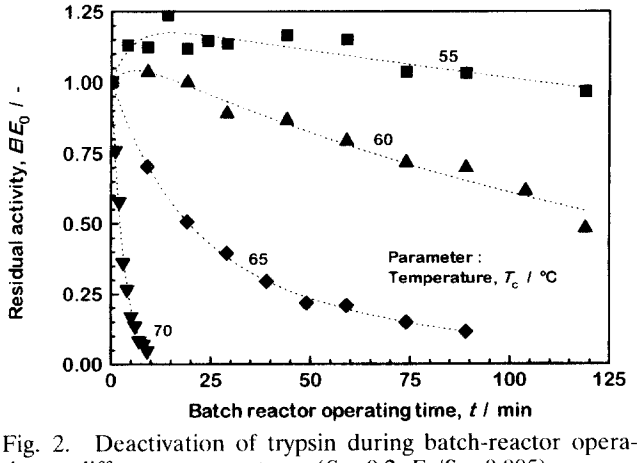

Fig. 2. Deactivation of trypsin during batch-reactor operation at different temperatures (S_0=0·2; E_0/S_0=0·005).

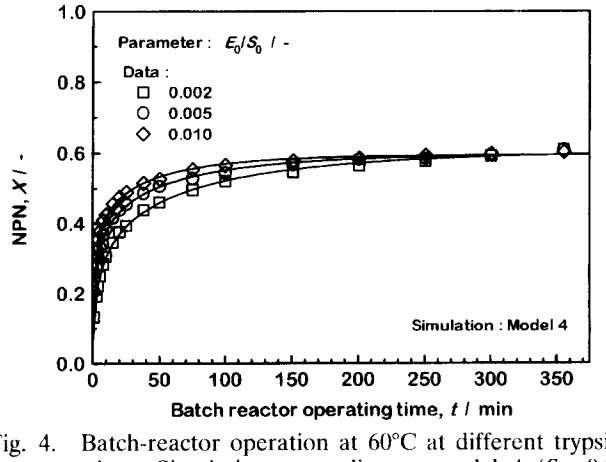

Fig. 4. Batch-reactor operation at 60°C at different trypsin concentrations. Simulations according to model 4 (S_0=0·2; T_c=60°C; pH=7·3).

three different enzyme:substrate ratios. Figure 4 shows the equivalent profiles for an operating temperature of 60°C. These data have been applied for screening of models, which would fulfill the requirements discussed above. Most of the models applied did show systematic deviations. The most promising models are presented in Table 1; the summary of the results is shown in Table 2. The mean errors were calculated as the square root of the quotient of the sum of squares and the number of data decremented by one.

Model 1 has been taken into consideration, because a hyperbolic function often fits conversion-time profiles of batch-reactor experiments with enzymes rather well, subject to product inhibition.[15] Such a model can conveniently be applied for estimating initial rates.[16] The linear term in time was added because of the slow rise of the profile at 55°C. It describes the profiles quite well at moderate temperatures, but is less useful at high temperatures, which are characterized by fast enzyme deactivation.

Model 2 describes a double-exponential decay of two fractions of the whey-protein being digested with different rate constants. Models based on double-exponential kinetics have been proposed by Mihalyi *et al.*,[4,5] for example. This model simulates the profile fairly well at both temperatures. However, it has the same drawback as model 1, because both lead to rate laws in terms of operating time instead of the fraction of soluble protein.

Model 3 goes back to a proposition of Hugo and Pham,[17] who applied a similar model to conversion-time data in order to extract reaction rates. The original model had to be altered by splitting one parameter in the arguments of the exponents into two. The pure fitting capacity of this model is outstanding. The simulations shown in Fig. 1 have been carried out

Table 1. Models used for fitting batch-reactor experiments

Model no.	No. of parameters	Batch reactor balance equation	Corresponding kinetic model
1	3	$X=X_0+\frac{a_1 t}{a_2+t}+a_3 t$	$\frac{dX}{dt}=\frac{a_1 a_2}{(a_2+t)^2}+a_3$
2	4	$X=X_0+a_1[1-\exp(-a_2 t)]+a_3[1-\exp(-a_4 t)]$	$\frac{dX}{dt}=a_1 a_2\exp(-a_2 t)+a_3 a_4\exp(-a_4 t)$
3	5	$X=X_0+a_1\left[1+a_2 t-\frac{\exp(-a_3 t)}{1+a_4[1-\exp(-a_5 t)]}\right]$	$\frac{dX}{dt}=a_1\left\{a_2+\frac{[a_3+a_3a_4+(a_4a_5-a_3a_4)\exp(-a_5 t)]\exp(-a_3 t)}{[1+a_4-a_4\exp(-a_5 t)]^2}\right\}$
4	4	$t=\frac{S_0}{a_1E_0}(X-X_0)+\frac{a_2}{a_1E_0}\ln\left(\frac{a_4-X_0}{a_4-X}\right)+\frac{a_3}{a_1E_0}S_0^2(X-X_0)^2 \quad a_4=X_{eq}$	$\frac{S_0}{E_0}\frac{dX}{dt}=\frac{a_1S_0(a_4-X)}{a_2+S_0(a_4-X)+2a_3S_0^2(X-X_0)(a_4-X)}$ $a_4=X_{eq}$
4a	3	$a_4=1$	$a_4=1$
5	2	$X=\frac{1}{a_2}\ln\left\{\exp(a_2X_0)+\frac{a_1a_2}{S_0^2}E_0 t\right\}$	$\frac{dX}{dt}=a_1\frac{E_0}{S_0^2}\exp(-a_2X)$

Table 2. Results of fitting models to experimental data

Model no.	No. of parameters	Mean deviation at 55°C	Mean deviation at 60°C	Global mean deviation	Relative error	Comment
1	3	0·01576	0·01963	0·0177	3·35	
2	4	0·01443	0·01273	0·0136	2·58	
3	5	0·00772	0·00604	0·00688	1·30	
4	4	0·00481	0·00575	0·00528	1·00	$a_4=X_{eq}$
4a	3	0·02163	0·03973	0·0307	5·81	$a_4=1$
5	2	0·00532	0·01356	0·00944	1·79	

Table 3. Summary of the evaluation of model 4

T_c/°C	E_0/S_0	a_1/min^{-1}	a_2/–	a_3/–	a_4/–	Mean deviation
55	0·002	−1·304	−0·1101	5·118	0·5837	0·00595
55	0·005	−0·7378	−0·1364	4·674	0·7108	0·00511
55	0·010	−0·3492	−0·1497	4·419	0·7778	0·00337
60	0·002	−2·142	−0·1165	4·571	0·6049	0·00579
60	0·005	−1·239	−0·1158	6·470	0·5962	0·00663
60	0·010	−0·8917	−0·1200	8·107	0·5943	0·00483

by applying this model. Unfortunately, it contains five parameters and the reactor-balance equation translates into clumsy kinetics in terms of operating time.

Model 4 represents a general solution of the batch-reactor balance for enzymic equilibrium reactions subject to competitive and non-competitive product inhibition as well as substrate inhibition.[16] The actual parameters may be identified with the following lumped parameters as:

$$a_1=k'_2 \quad a_2=K'_m \quad a_3=K' \quad a_4=X_{eq}$$

where X_{eq} is the equilibrium conversion. This model shows by far the best fit of the reaction profiles at both temperatures. The curves shown in Fig. 4 have been simulated by means of this model. In addition, the reactor balance is based on extended Michaelis–Menten kinetics. Therefore, the parameters may be interpreted accordingly. A summary of the results with respect to the parameters obtained is given in Table 3. The parameters a_1 and a_2 are both negative under all conditions tested. This is due to the fact that the reaction is subject to severe product inhibition. If the affinity of the enzyme is higher for the product than the substrate, e.g. the inhibition constant (K_{ic}) less than the Michaelis–Menten constant (K_m), both parameters should be negative. This may be deduced from the definition of the lumped parameters for Michaelis–Menten kinetics accompanied by a competitive product inhibition:[16]

$$k'_2=k_2\frac{K_{ic}}{K_{ic}-K_m} \quad K'_m=K_m\frac{K_{ic}+S_0}{K_{ic}-K_m}$$

The parameter a_4 gives an estimation of the maximum product yield in terms of the fraction of soluble protein. If the same model is taken, but the equilibrium of reaction neglected, the outcome would be model 4a. This model is only given for comparison with model 4 in order to show the effect of the equilibrium assumption. Model 4a is not able to fit the profiles with sufficient precision.

By turning the kinetic data around for detecting simple correlations, it eventually came about that straight lines were observed, when the fraction of soluble protein was plotted against the logarithm of the operating time. This is shown in Fig. 5. The only problem was that an initial fraction of soluble protein had to be included in the model. This can be done as in model 5, which has been derived from the rate law given by Marquez Moreno and Fernandez Cuadrado.[8] The most remarkable characteristic of this model is that it only contains two parameters. Model 5 describes the experimental data obtained at 55°C accurately, but shows systematic deviations at 60°C. The interesting behaviour of model 5 may be deduced from Fig. 3, for which the simulations have been carried out by applying this model. In addition, an evaluation of the model is given in Table 4. The rate law is astonishingly simple by postulating an exponential decay of the rate of reaction with the fraction of soluble protein. This model is in principle equivalent to the well known Elovich equation commonly used for describing chemisorption kinetics.[18]

However, these models should be applied with care and only for the purposes for which they are designed. The rates of reaction extracted from two models, model 4 and 5, for experiments at 55°C are given in Fig. 6. Although the overall fit is almost the same, the outcome is quite different in both cases.

Finally, comparing the results given in Fig. 2 and those in Fig. 6 shows clearly that the steep decrease of the rate of reaction with time or with protein conver-

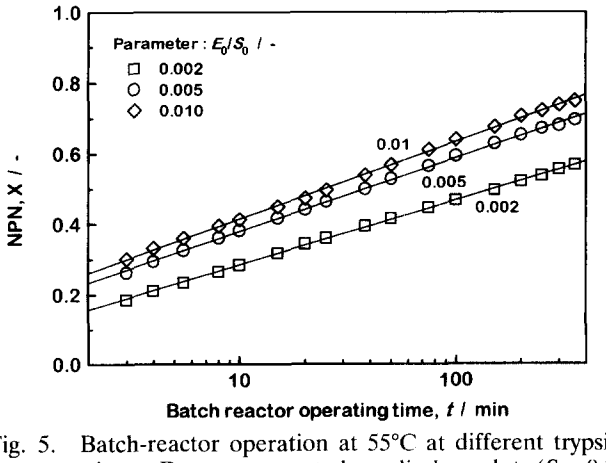

Fig. 5. Batch-reactor operation at 55°C at different trypsin concentrations. Data represented as lin-log plot (S_0=0·2; T_c=55°C; pH=7·3).

Table 4. Summary of the evaluation of model 5

T_c/°C	E_0/S_0	a_1/min^{-1}	a_2/–	Mean deviation
5	0·002	25·527	12·321	0·00451
55	0·005	22·735	10·959	0·00515
55	0·010	14·819	10·506	0·00631
60	0·002	26·111	11·211	0·01090
60	0·005	62·557	14·412	0·01338
60	0·010	136·587	16·922	0·01641

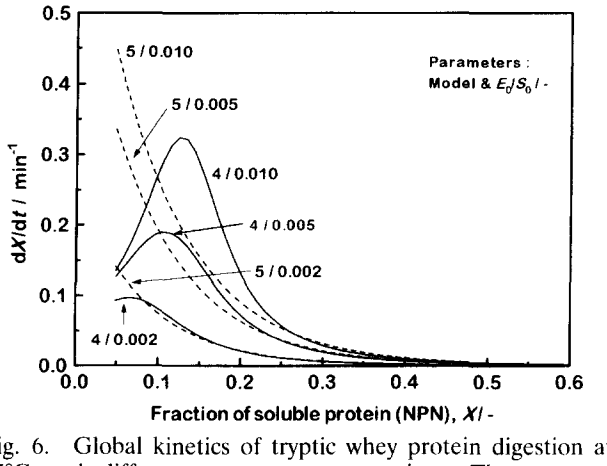

Fig. 6. Global kinetics of tryptic whey protein digestion at 55°C and different enzyme concentrations. The rates of increase of the fraction of soluble enzymes were simulated by the models indicated (S_0=0·2; T_c=55°C; pH=7·3).

sion even at 55°C is not caused by deactivation, but by strong product inhibition.

Conclusion

Some empirical models of a purely mathematical nature as well as others with a strong background in enzyme-reaction mechanisms are presented, and can advantageously be used for fitting data from batch-reactor experiments of protein hydrolysis. Two models are particularly useful. One of these (model 5), identifiable as the Elovich equation, contains only two parameters and should be well adapted for describing reaction systems with strong product inhibition and low rates of catalyst deactivation. Since this is a rather stiff model it may be adequate for estimating initial rates from batch-reactor experiments, which is another significant problem in data evaluation.[16]

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