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ON ENZYMIC CLOTTING PROCESSES V. RATE EQUATIONS FOR THE CASE OF ARBITRARY RATE OF PRODUCTION OF THE CLOTTING SPECIES

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The rate theory for enzyme-triggered coagulation reactions, such as the clotting of fibrin or casein, is extended to the case of an arbitrary rate of production of the clotting species. It is shown that the general expression for the growth of the weight-average molecular weight of the clotting product, $\bar{M}_w$, is given by $\bar{M}_w = M_1 \{1 + k_s \{\int_0^t P(t)^2 \, dt\}/P(t)\}$, where M_1 is the "monomer" molecular weight, k_s the smoluchowskian flocculation rate constant and $P(t)$ the total number of monomers produced by the enzyme in t. In the purely smoluchowskian case $P(t)$ stands for the total number of monomers at the beginning of the clotting process. Numerical examples in which the rate of enzymic production is governed by complete Michaelis–Menten kinetics, are compared to cases in which this rate equals V_{max}. It is shown that after exhaustion of the substrate the system continues to coagulate in a purely smoluchowskian way. Turbidimetric experiments on the clotting of micelles of whole and κ-casein are presented which suggest inactivation of the enzyme by non-productive binding in the flocs formed.

1. Introduction

Enzyme-triggered coagulation reactions usually are set going by proteinases, which by the limited proteolysis of their substrate, yield an unstable product that starts to clot. Well-known examples are the clotting of fibrin and casein brought about by thrombin (E.C. 3.4.21.5) and chymosin (E.C. 3.4.23.4) respectively [1,2]. The formation of plakalbumin from ovalbumin by subtilisin (E.C. 3.4.21.14) is another example [3]. Also the precipitation of pectic acid by pectin esterase (E.C. 3.1.1.11) in the presence of calcium ions, which was studied extensively by Krop and Pilnik [4], falls in the same category of coagulation reactions.

Most strikingly these reactions are characterized by the occurrence of a lag in the coagulation, the length of which is widely used to assess the activity of the clotting enzyme. In a number of previous publications [5–8] we have analyzed the kinetics of such clotting processes and shown that the time necessary for the clotting to occur, t_c, to a fair extent is given by

$$t_c \approx (2/k_s V_{max})^{1/2} ,$$

where k_s is the flocculation rate constant of the clotting species and V_{max} the maximum rate of proteolysis of the substrate.

The above relation is obtained by accounting for the enzymic production of the "monomer" species in von Smoluchowski's rate theory for the coagulation of unstable colloids [9,10] and the lag phase was shown to be due to the second order of the coagulation reac-

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tion per se.

A drawback of the above theory is that it has been set up for a constant rate of production of the clotting species, i.e. at V_{max}. In the present study this restriction is removed and the theory extended to the case of arbitrary V. It has been worked out for complete Michaelis–Menten kinetics and numerical examples pertaining to the clotting of micelles of whole and κ-casein by milk-clotting chymosin are given.

The previous theory predicts that the turbidity of the total clotting system is a cubic function of the reaction time [7]. The experiments, especially those at relatively high enzyme activity, show, however, that the turbidity often ends up to increase linearly with time (e.g. fig. 2 of ref. [8] and figs. 2 and 3 of ref. [11]). We shall demonstrate that such linear behaviour sometimes can be explained by substrate exhaustion and continued smoluchowskian flocculation of the enzyme product. Deviations from the theory are discussed in terms of non-productive binding of the enzyme in the flocs formed.

2. Theory

2.1. The time-dependence of the weight-average molecular weight of the clotting enzymic product

Since the clotting of fibrin or casein usually is monitored by the absorbance or light scattering of the system, we are first of all interested in the rate of change of the weight-average molecular weight, $\bar{M}_w$, of the clotting species [12]. By definition we have

$$\bar{M}_w = M_1 \sum_j j^2 P_j \Big/ \sum_j j P_j \,, \tag{2}$$

where M_1 is the "monomer" molecular weight of the clotting product and P_j the number concentration of particles of degree of aggregation j. Obviously in eq. (2) the denominator

$$\sum_j j P_j \equiv P(t) \tag{3}$$

represents the total number of monomers produced by the enzyme in t.

According to von Smoluchowski [9] we have for the rate of change of the number concentration of particles of degree of aggregation i:

$$dP_i/dt = -k_s P_i \sum_j F_j + \tfrac{1}{2} k_s \sum_{j=1}^{i-1} P_j P_{i-j} \,,$$

$$i = 2, 3, \ldots, \tag{4}$$

where k_s is the so-called flocculation rate constant. The solution to eq. (4), satisfying the boundary condition $P_i = 0$ at $t = 0$, reads

$$P_i = \tfrac{1}{2} k_s X(t) \int_0^t \frac{\sum_{j=1}^{i-1} P_j P_{i-j}}{X(t)} \, dt \,, \quad i = 2, 3, \ldots, \tag{5}$$

where $X(t)$ is defined as

$$X(t) = \exp\{-k_s \int_0^t \sum_j P_j(t) \, dt\} \,. \tag{6}$$

Consider the generating function $\Psi(z, t)$, defined as

$$\Psi(z, t) = \sum_{i=1}^{\infty} z^i P_i(t) \,, \tag{7}$$

which, after inserting eq. (5) and some manipulations, (cf. ref. [6], Appendix II), can be rewritten as

$$\Psi(z, t) = z P_1(t) + \tfrac{1}{2} k_s X(t) \int_0^t \frac{\Psi^2(z, t)}{X(t)} \, dt \,. \tag{8}$$

From the definition of $\Psi(z, t)$ we further have

$$\sum_{j=1}^{\infty} j^2 P_j(t) = \sum_{j=1}^{\infty} i P_j(t) + \left.\frac{\partial^2 \Psi}{\partial z^2}\right|_{z=1}$$

$$= P(t) + \left.\frac{\partial^2 \Psi}{\partial z^2}\right|_{z=1} \,. \tag{9}$$

The second term on the right-hand side of this relation can be calculated as follows. By successive differentiation of eq. (8) we find for the last term of eq. (9):

$$\left.\frac{\partial^2 \Psi}{\partial z^2}\right|_{z=1} = \tfrac{1}{2} k_s X(t) \int_0^t \frac{1}{X(t)} \left\{ 2 \left(\left.\frac{\partial \Psi}{\partial z}\right|_{z=1} \right)^2 + 2\Psi \Big|_{z=1} \cdot \left.\frac{\partial^2 \Psi}{\partial z^2}\right|_{z=1} \right\} dt$$

$$= k_s X(t) \int_0^t \frac{1}{X(t)} \left\{ P(t)^2 + \sum_j P_j(t) \cdot \left.\frac{\partial^2 \Psi}{\partial z^2}\right|_{z=1} \right\} dt \,. \tag{10}$$

Defining the function $h(t)$ as

$$h(t) = \frac{1}{X(t)} \left.\frac{\partial^2 \Psi}{\partial z^2}\right|_{z=1} \,, \tag{11}$$

one finds, after substituting (10), that

$$dh(t)/dt = k_s P(t)^2/X(t) + k_s \sum_j P_j(t) \cdot h(t) . \qquad (12)$$

It can readily be verified that the solution to this equation reads

$$h(t) = \exp\{k_s \int_0^t \sum_j P_j(t)\,dt\} \cdot k_s \int_0^t P(t)^2\,dt ,$$

which by the definition of $X(t)$ given above ([6]), becomes

$$h(t) = k_s \left\{ \int_0^t P(t)^2\,dt \right\} \Big/ X(t) . \qquad (13)$$

Combining (11) and (13) we finally obtain ‡

$$\left.\frac{\partial^2 \Psi}{\partial z^2}\right|_{z=1} = k_s \int_0^t P(t)^2 dt . \qquad (14)$$

For the reduced weight-average molecular weight, $\bar{M}_w/M_1$, we thus find from eqs. (3), (9) and (14):

$$\bar{M}_w/M_1 = \sum_j j^2 P_j \Big/ \sum_j j P_j = 1 + k_s \left\{ \int_0^t P(t)^2 dt \right\} \Big/ P(t) . \qquad (15)$$

In the purely smoluchowskian case the function $P(t) = \Sigma_j j P_j$, eq. (3), is constant, say S_0. Inserting this constant value for $P(t)$ in (15) yields

$$\bar{M}_w/M_1 = 1 + k_s S_0 t . \qquad (16)$$

This indeed is the smoluchowskian weight-average molecular weight as can be verified from refs. [9] and [10].

2.2. *The time-dependence of the weight-average molecular weight of the total solute*

Since by light scattering one can only observe the weight-average molecular weight of the whole solute, the above result has to be completed with the contributions of the residual substrate (if any) and the peptide(s) split off by the enzyme to this average. This correction can easily be made, because there exists a stoichiometric relationship between the substrate, the clotting product and the peptide(s) split off by the enzyme.

Let the molecular weights of substrate, product monomer and peptide be M_0, M_1 and M_2 respectively and the original substrate concentration c_0 (g/ml).

The masses of product and peptide per ml, which are produced by the enzyme then are given by $M_1 \Sigma_j j P_j$ and $M_2 \Sigma_j j P_j$ respectively (P_j now in moles/ml). The residual substrate concentration is ($c_0 - M_0 \Sigma_j j P_j$) and the weight-average molecular weight of the total solute thus becomes

$$(\bar{M}_w)_{total} = \left\{ \left(c_0 - M_0 \sum_j j P_j \right) M_0 + M_1^2 \sum_j j^2 P_j + M_2^2 \sum_j j P_j \right\} \Big/ c_0 . \qquad (17)$$

Defining the ratio of the molecular weights of the peptide(s) split off to that of the original substrate as

$$f = M_2/M_0 , \qquad (18)$$

and substituting for $\Sigma_j j P_j$ and $\Sigma_j j^2 P_j$ from eqs. (3) and (15), eq. (17) reduces to

$$(\bar{M}_w/M_0)_{total} = 1 - M_0(1-f)\,P(t) \times \left\{ 2f - (1-f) k_s \left\{ \int_0^t P(t)^2 dt \right\} \Big/ P(t) \right\} \Big/ c_0 . \qquad (19)$$

If the rate of enzymic production is constant and equal to V_{max} we have

$$k_s \left\{ \int_0^t P(t)^2 dt \right\} \Big/ P(t) = \tfrac{1}{3} k_s V_{max} t^2 \qquad (20)$$

and, after defining the enzymic clotting time, τ, as

$$\tau = (2/k_s V_{max})^{1/2} , \qquad (21)$$

we find on substituting (20) and (21) in eqs. (15) and (19) that

$$\bar{M}_w/M_1 = 1 + \tfrac{2}{3}(t/\tau)^2 \qquad (22)$$

and

$$(\bar{M}_w/M_0)_{total} = 1 - M_0(1-f)(8V_{max}/k_s)^{1/2} \times \{f(t/\tau) - (1-f)(t/\tau)^3/3\}/c_0 , \qquad (23)$$

which are identical to the expressions found previously for the case of constant V [6,7].

‡ The combined equations (9) and (14), as one of the referees points out to us, can also be obtained directly from eq. (4) by the calculation of $d/dt\ (\Sigma_i i(i-1)P_i)$.

2.3. Numerical examples; influence of substrate exhaustion on the rate of clotting

The influence of substrate exhaustion on the kinetics of the clotting process is certainly the most interesting case to be investigated by the above theory. In the following numerical examples we shall restrict ourselves to the clotting of micelles of whole and κ-casein by chymosin [2,5,7,13]. Theoretical and experimental work on the thrombin/fibrin system is in progress and will be dealt with in a future publication.

According to Henri and Michaelis-Menten [14,15] we now have for the rate of production of the clotting species

$$dP(t)/dt = V_{max}\{S_0 - P(t)\}/\{K_m + S_0 - P(t)\}\,, \quad (24)$$

where S_0 is the initial substrate concentration and K_m the Michaelis constant (both in mM).

In the application of eq. (24) we are faced with the fact that the Michaelis-Menten parameters for the splitting of the chymosin-sensitive phe-met bond between residues 105 and 106 of κ-casein are only poorly known. In table 1 we have collected some recent estimates of K_m and k_{cat} found with intact κ-casein and κ-casein analogs. The actual computations have been carried out using the data of refs. [16] and [17]. The data for the synthetic peptides have not been considered, because they were obtained at an unusually low pH and they probably, as a consequence of the shortness of these peptides, do not reflect the electrostatic enzyme/substrate interaction found with the intact κ-casein [11].

To simulate the milk clotting reaction it was accepted that the total casein concentration in milk is about 30 g/l, 12 per cent of which is κ-casein [20]. Therefore, if all κ-casein were accessible to the enzyme, we have for the initial substrate concentration $c_0 = 3.6 \times 10^{-3}$ g/ml. Further, from the sequence studies of Mercier et al. [21] and the molecular weight determinations by Vreeman et al. [22] it is readily calculated that $S_0 = 0.19$ mM and $f = 0.35$–0.38. The previous measurements of the clotting time suggest that the flocculation rate constant of whole and κ-casein is about 10^5–10^6 ml mol^{-1} s^{-1} [7,8,11], which completes the set of parameters needed to simulate the course of the clotting process.

The number of monomers produced by the enzyme in t can conveniently be computed by the recurrent difference equation

$$P(t + \Delta t) = P(t) + V_{max}\{S_0 - P(t)\} \times \Delta t/\{K_m + S_0 - P(t)\}\,, \quad P(0) = 0\,, \quad (25)$$

whereas $\int_0^t P(t)^2 dt$ was obtained by trapezoidal integration with a time-interval of 6.25 s.

Slopes were calculated by Newton's interpolation formula. All calculations were carried out on the Hewlett-Packard 9830 A/9862 A calculator/plotter system.

The results of the computations, comparing the growth of the reduced weight-average molecular weight of the product and the total solute with and without substrate exhaustion taking place, are collected in figs. 1–4 and tables 2–5. They give rise to the following conclusions.

1) Exhaustion of the substrate can lead to a drastic decrease in the rate of clotting. As is obvious from eq. (24) this decrease is more pronounced the larger the ratio K_m/S_0.

2) The lag phase proper, occurring at the earliest stages of the clotting process, is not seriously affected by substrate exhaustion (cf. fig. 2). If, however, as is

Table 1
Summarizing recent estimates of Michaelis-Menten parameters for the limited proteolysis of κ-casein and κ-casein analogs by chymosin.

substrate	residues	K_m (mM)	k_{cat} (s^{-1})	exptl. conditions			ref.
				pH	I	°C	
κ-casein	1–169	0.004	10	6.7	0.08	37	[16]
tryptic peptide	98–112-OH	0.025	48	6.6	0.05	30	[17]
synthetic peptide	103–112-OH	0.40	25	4.7	0.05	30	[18]
synthetic peptide	103–108 OMe	0.85	18	4.7	0.05	30	[19]

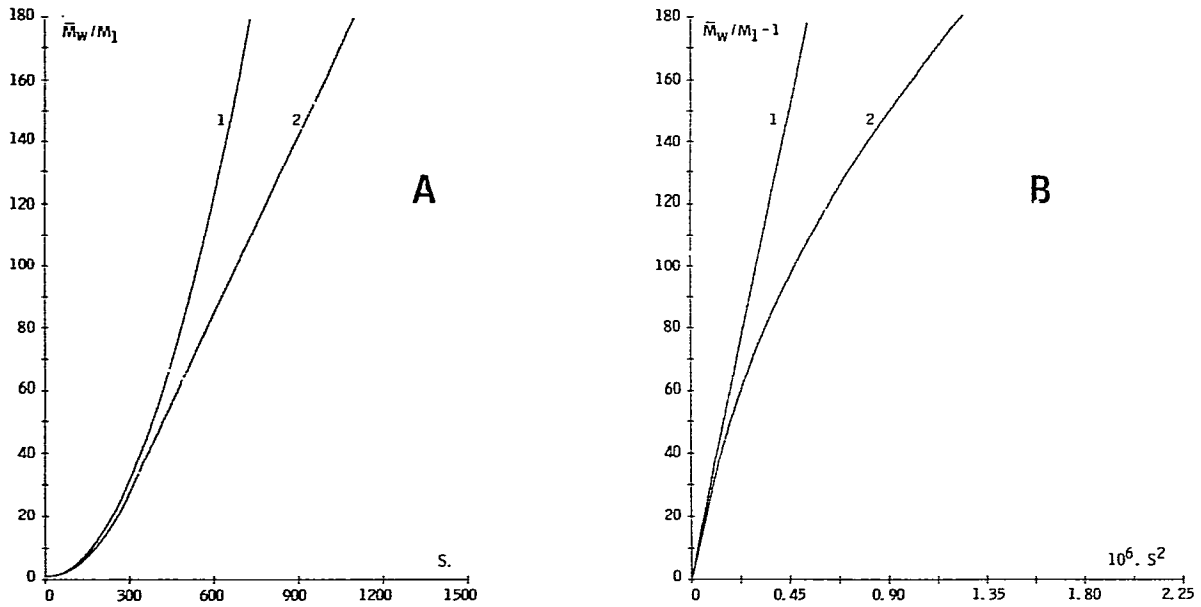

Fig. 1. Comparing the product clotting of (κ-)casein brought about by chymosin without (curve 1; eq. (22)) and with (curve 2; eq. (15)) exhaustion of the substrate. Computational parameters: $V_{max} = 10^{-9}$ (mol/ml s), $K_m = 0.025$ (mM) and the flocculation rate constant $k_s = 10^6$ (ml/mol s). A: $\bar{M}_w$/M.W. (product) versus t. B: $\bar{M}_w$/M.W. (product) − 1 versus t^2.

Table 2
Kinetics of clottable product.

t(s)	V/V_{max}	$P(t)/S_0$	$M(W)/M(1)$	slope
0	1	0	1	0
100	0.81	0.45	3.91	0.06
200	0.57	0.83	12.51	0.12
300	0.06	0.99	27.37	0.18
400	0.00	1.00	46.09	0.19
500	0.00	1.00	65.09	0.19
600	0.00	1.00	84.09	0.19
700	0.00	1.00	103.09	0.19
800	0.00	1.00	122.09	0.19
900	0.00	1.00	141.09	0.19
1000	0.00	1.00	160.09	0.19
1100	0.00	1.00	179.09	0.19
1200	0.00	1.00	198.09	0.19
1300	0.00	1.00	217.09	0.19
1400	0.00	1.00	236.09	0.19

Mol. weight of substrate = 19000 and substrate conc. = 0.19 mMol. The Michaelis-Menten parameters are: $V_{max} = 10^{-9}$ mol/ml s and $K_m = 0.025$ mMol. The flocculation rate constant $K_s = 1000\,000$ ml/mol s.

Table 3
Kinetics of total solute.

t(s)	V/V_{max}	$P(t)/S_0$	$M(W)/M(0)$
0	1	0	1
100	0.81	0.45	1.29
200	0.57	0.83	4.27
300	0.06	0.99	10.59
400	0.00	1.00	17.86
500	0.00	1.00	25.16
600	0.00	1.00	32.47
700	0.00	1.00	39.77
800	0.00	1.00	47.07
900	0.00	1.00	54.38
1000	0.00	1.00	61.68
1100	0.00	1.00	68.99
1200	0.00	1.00	76.29
1300	0.00	1.00	83.59
1400	0.00	1.00	90.90

Mol. weight of substrate = 19000 and substrate conc. = 0.19 mMol. f = M.W. (peptide)/M.W. (substrate) = 0.38. The Michaelis-Menten parameters are: $V_{max} = 10^{-9}$ mol/ml s and $K_m = 0.025$ mMol. The flocculation rate constant $K_s = 1000\,000$ ml/mol. s.

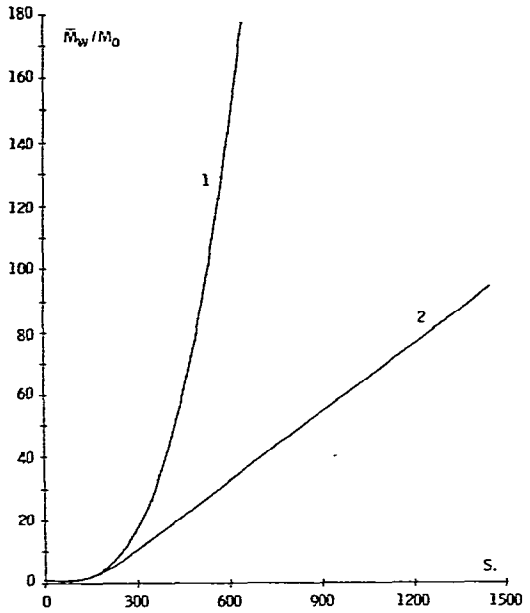

Fig. 2. Comparing the clotting of the total solute (κ-)casein brought about by chymosin without (curve 1; eq. (23)) and with (curve 2; eq. (19)) exhaustion of the substrate. Computational parameters: substrate conc. = 0.19 (mM); substrate M.W. = 19 000; f = M.W. (peptide)/M.W. (substrate) = 0.38. Rate constants as with fig. 1.

Table 4
Kinetics of clottable product.

t(s)	V/V_{max}	$P(t)/S_0$	$M(W)/M(1)$	slope
0	1	0	1	0
100	0.98	0.09	1.55	0.01
200	0.98	0.17	3.18	0.02
300	0.97	0.26	5.90	0.03
400	0.97	0.34	9.71	0.04
500	0.96	0.43	14.60	0.05
600	0.96	0.51	20.57	0.07
700	0.95	0.60	27.63	0.08
800	0.94	0.68	35.77	0.09
900	0.92	0.76	45.00	0.10
1000	0.88	0.84	55.36	0.11
1100	0.80	0.92	66.94	0.12
1200	0.52	0.98	80.27	0.15
1300	0.03	1.00	97.22	0.19
1400	0.00	1.00	116.15	0.19

Mol. weight of substrate = 19000 and substrate conc. = 0.19 mMol. The Michaelis-Menten parameters are: V_{max} = 1.67 × 10^{-10} mol/ml s and K_m = 0.004 mMol. The flocculation rate constant K_s = 1000 000 ml/mol s.

Table 5 Kinetics of total solute.

t(s)	V/V_{max}	$P(t)/S_0$	$M(W)/M(0)$
0	1	0	1
100	0.98	0.09	0.98
200	0.98	0.17	1.06
300	0.97	0.26	1.36
400	0.97	0.34	1.99
500	0.96	0.43	3.03
600	0.96	0.51	4.61
700	0.95	0.60	6.82
800	0.94	0.68	9.76
900	0.92	0.76	13.51
1000	0.88	0.84	18.17
1100	0.80	0.92	23.77
1200	0.52	0.98	30.31
1300	0.03	1.00	37.49
1400	0.00	1.00	44.79

Mol. weight of substrate = 19000 and substrate conc. = 0.19 mMol. f = M.W. (peptide)/M.W. (substrate) = 0.38. The Michaelis-Menten parameters are: V_{max} = 1.67 × 10^{-10} mol/ml s and K_m = 0.004 mMol. The flocculation rate constant K_s = 1000 000 ml/mol s.

customary practice [7,23], its length is determined by extrapolation of the steepest part of the turbidity plot to the time of zero turbidity increase, it could be seriously in error.

3) After practically complete exhaustion of the substrate, the clotting product continues to grow linearly with a slope equal to the product $k_s S_0$ (cf. tables 2 and 4 and eq. (16)). This is exactly what was predicted by Troelstra [24] and Oster [25, see also refs. 9 and 10] for the behaviour of a purely smoluchowskian system. Furthermore we see from fig. 3 that for a small ratio K_m/S_0 the curves which do and do not allow for substrate exhaustion are almost coincident on the time interval under consideration. This could be expected from eq. (24). The "exhaustion theory", however, predicts an eventual smoluchowskian behaviour (see table 4), whereas in a "constant production theory" the slope of $\bar{M}_w/M_1$ retains a constant variation (see eq. (22)).

4) The final slope of the growth curve of the total solute after exhaustion of the substrate is considerably lower than that of the growing product (cf. figs. 1 and 2), because of the contribution of the non-aggregating peptide to the total weight-average molecular weight.

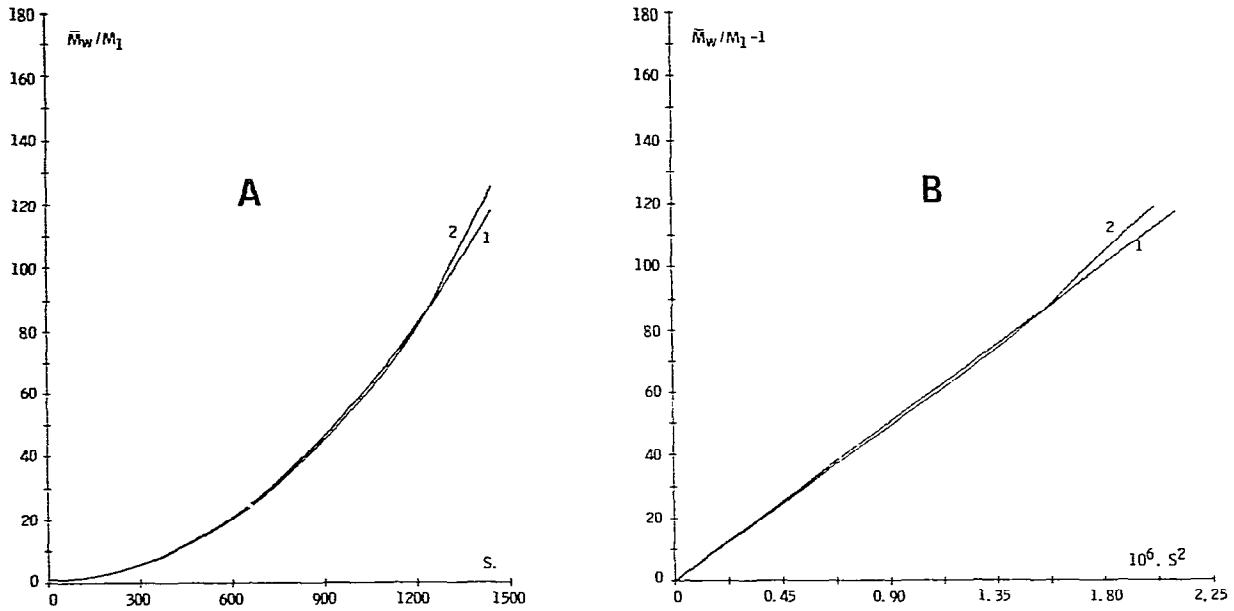

Fig. 3. Comparing the product clotting of (κ-)casein brought about by chymosin without (curve 1; eq. (22)) and with (curve 2; eq. (15)) exhaustion of the substrate. Computational parameters: $V_{max} = 1.67 \times 10^{-10}$ (mol/ml s), $K_m = 0.004$ (mM) and the flocculation rate constant $k_s = 10^6$ (ml/mol s). A: $\bar{M}_w$/M.W. (product) versus t. B: $\bar{M}_w$/M.W. (product) − 1 versus t^2.

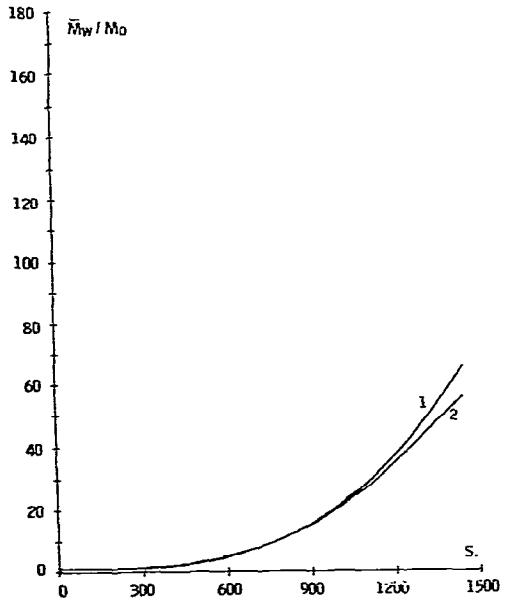

Fig. 4. Comparing the clotting of the total solute (κ-)casein brought about by chymosin without (curve 1; eq. (23)) and with (curve 2; eq. (19)) exhaustion of the substrate. Computational parameters: substrate conc. = 0.19 (mM); substrate M.W. = 19000 f = M.W. (peptide)/M.W. (substrate) = 0.38.

3. Discussion; comparison with experiment

The present theory generalizes the previous description of enzymic clotting processes [6–8] to cases in which the rate of production of the clotting species is arbitrary. As its predecessor it makes clear that the lag phase in the clotting is due to the second order of the coagulation reaction proper (see eq. (4)). In the beginning of the clotting process the concentrations of the clotting species are simply too low for appreciable aggregation to occur. Moreover, the flocculation rate constants involved, without exception, are very low [8,11].

At this point it should be mentioned that von Smoluchowski in his original theory used the assumption that the number of coagulating particles is practically infinite, whereas in clotting processes we start with a zero concentration. The application of von Smoluchowski's theory becomes justified, however, when it is reliazed that under the usual enzymic conditions, the enzyme produces already a very large number of such particles in a very short time.

The computations demonstrate that exhaustion of the substrate ultimately leads to purely smoluchowskian flocculation of the clotting product. Since the course of the clotting had to be computed numerically, the nature of the clotting time is not immediately apparent from the present calculations. If, however, we introduce a time-average rate of enzymic production:

$$\langle V \rangle = \left\{ \int_0^{t_e} \mathrm{d}P(t) \right\} / t_e \qquad (26)$$

where t_e is the time at which the substrate is practically exhausted, then it is readily seen that the analytical expressions (22) and (23) can be maintained with the time-average of τ defined as

$$\langle \tau \rangle = (2/k_s \langle V \rangle)^{1/2} . \qquad (27)$$

Substituting $\langle \tau \rangle$ for τ in eq. (23) shows that an explosive increase of the weight-average particle weight is only to be expected for reaction times exceeding $\langle \tau \rangle$. The clotting time, t_c, is therefore given by

$$t_c \approx \langle \tau \rangle . \qquad (28)$$

As an example: from the figures given in table 2 it is readily calculated that at practically complete exhaustion $\langle V \rangle \approx 5 \times 10^{-10}$ (mol ml^{-1} s^{-1}) to be compared with $V_{max} = 10^{-9}$ (mol ml^{-1} s^{-1}). The clotting times estimated by eqs. (21) and (27) therefore differ by less than a factor of 1.5. The calculation demonstrates that still fairly reliable estimates of the clotting time can be made using the simple theory based on V_{max} (cf. eq. (23)). It also justifies the former conclusions based on that theory with regard to the flocculation rate constants of fibrin and casein [7,8,11].

It has been mentioned above that the turbidity plots of the clotting of fibrin and casein often end up with a linear slope instead of following the cubic equation (23). The present calculations show that such behaviour could be explained by the exhaustion of the substrate and continued smoluchowskian flocculation of the product formed. The following examples may serve, however, to illustrate that in practice substrate exhaustion may be difficult to diagnose.

In fig. 5 the clotting of micellar whole casein at ionic strengths of 0.04 and 0.08 M and under otherwise identical conditions are compared. It has been shown before [11] that the increase in t_c at $I = 0.08$ should be ascribed to a reduction of the rate of proteolysis by the electrolyte. By comparing figs. 5a and b it is seen that the plot at the highest enzyme velocity indeed ends up with a perfect linear slope, whereas at $I = 0.08$ it remains curved upto the highest absorbance measured. This suggests indeed that the enzymic reaction has come to an end at $I = 0.04$ after about 200 s.

Consider now the clotting experiments with micelles of κ-casein at different enzyme concentrations shown in fig. 6. They all end up with a more or less linear slope of the turbidity plot, which at first sight

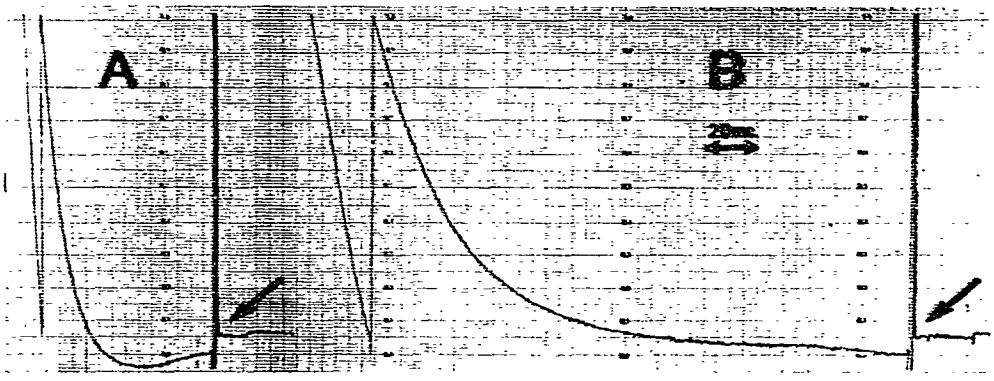

Fig. 5. Clotting of casein micelles by chymosin. Absorbance measurements with the Cary 14 spectrophotometer at 500 nm in 0.5 cm cuvettes. Total scale expansion: 0–0.2 abs., switching at 0.1 abs. Skim milk powder diluted 15 times with 0.01 M $CaCl_2$; ionic strength adjusted with NaCl; 35°C. A: $I = 0.04$ M. B: $I = 0.08$ M. Note the minimum in the absorbance at $I = 0.04$ in agreement with eqs. (19) and (23). Enzyme addition indicated by arrow; time flows to the left.

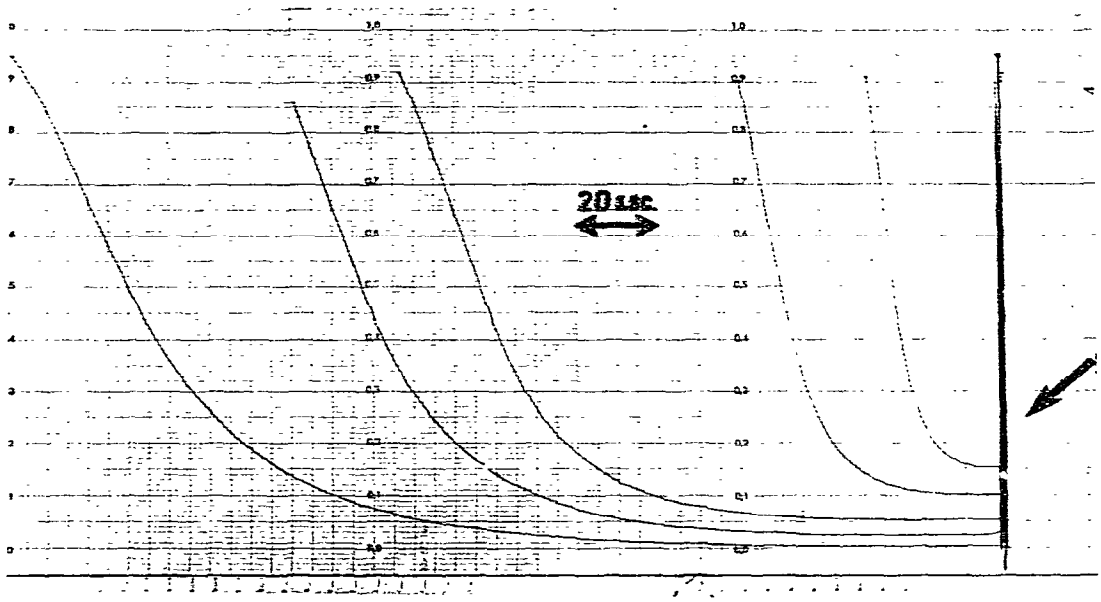

Fig. 6. Clotting of micelles of κ-casein by chymosin. Absorbance measurements with the Cary 14 spectrophotometer at 500 nm in 1 cm cuvettes. Total scale expansion 0–1 abs. 21.4°C. V_{max} (from left to right): 0.3, 0.42, 0.6, 1.2 and 2.4 × 10^{-9} (mol/ml s).

suggests exhaustion of the substrate. The theoretical expectation is, however, that after substrate exhaustion we are always left with the same system, consisting of particles completely stripped off by the enzyme and the non-aggregating peptides. Such particles would obviously possess the same flocculation rate constant and, since their concentration is the same, the turbidity would ultimately increase at the same rate. This behaviour is exemplified in fig. 7, where the graphs computed at three different enzyme concentrations are seen to end with parallel slopes. The final slopes in fig. 6 yet are found to decrease with decreasing enzyme concentration. In itself this observation is in good agreement with the earlier conclusions [6,11] about the validity of the so-called law of Segelcke and Storch: the flocculation rate constant of the micelles is roughly proportional to the concentration of the enzyme. A plausible explanation for the decreasing slopes is that they are due to non-productive binding and inclusion of the enzyme in the flocs already formed. Such binding would certainly be enhanced through electrostatic interaction between the positively charged product particles [21] and the negatively charged enzyme. It could lead to complete immobilization of the enzyme and the effect would be most pronounced at the lowest enzyme concentrations, where the micelles of the lowest flocculation rate constants would remain. The final linear slopes therefore do not only reflect the exhaustion of the substrate but rather a combination of exhaustion and the inactivation of the enzyme by non-productive binding in the flocs.

An alternative, though less likely explanation of the different final slopes is that they are related to the restricted validity of Rayleigh's scattering law and differences in the structure of the flocs formed, a point amply discussed by Overbeek [9]. Whether such abnormal scattering really contributes to the turbidity differences observed can only be decided via true light scattering measurements after correction for the dissymmetry of the scattered light. Such measurements are presently undertaken and will be dealt with in due course.

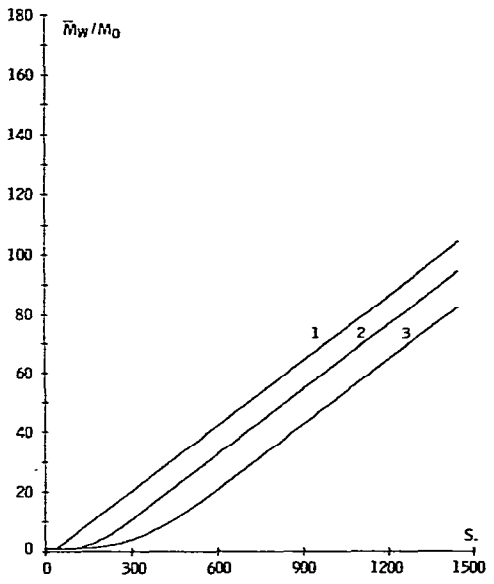

Fig. 7. Simulating the effect of substrate exhaustion on the clotting of κ-casein by chymosin. Computational parameters substrate conc. = 0.19 (mM); substrate M.W. = 19 000; f = M.W. (peptide)/M.W. (substrate) = 0.38. The Michaelis constant K_m = 0.025 (mM) and the flocculation rate constant $k_s = 10^6$ (ml/mol s). 1: $V_{max} = 5 \times 10^{-9}$ (mol/ml s); 2: $V_{max} = 10^{-9}$ (mol/ml s); 3: $V_{max} = 5 \times 10^{-10}$ (mol/ml s).

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