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Kinetics of peptide bond demasking in enzymatic hydrolysis of casein substrates

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ABSTRACT

Proteolysis of casein substrates includes demasking stage, the transition of masked bonds to the demasked stage, where peptide bonds become accessible to the enzyme attack. Therefore, proteolysis was regarded as a two-stage process with consequent demasking and hydrolysis stages. When demasking process is kinetically significant, the peptide bonds are hydrolysed with some lag. It was shown both by theoretical simulations and experimentally that the increase of amino nitrogen can be a non-monotonous function of the hydrolysis degree or proteolysis time. The non-monotonously dependence was found for chymotryptic proteolysis of β-casein, while for α-casein the monotonous dependence was obtained. This was treated as an indication of the prevalence of the hydrophobically induced masking effect for β-casein. For the proteolysis of β-casein by wild-type and engineered trypsins, the kinetic analysis allowed us to conclude that demasking stage was initiated by the splitting of the main peptide chain, which compact conformation was initially stabilized by the interaction of hydrophobic regions of peptide chain.

1. Introduction

Proteolysis, the enzymatic hydrolysis of a protein, plays an important role in various fields of bioscience and biotechnology, where proteolysis is studied on the qualitative and quantitative levels. Since Linderstrom-Lang's proteolysis model [1], several quantitative studies of the proteolysis kinetics were made mostly on the example of food proteins [2]. Practically important task is the prediction of optimal proteolysis conditions, which ensure hydrolysis of the undesired peptide fragments (bitter peptides, for example), or provide accumulation of the desired peptides (bioactive peptides, for example) [3,4]. Meanwhile, the problem of the proteolysis modeling for the arbitrary enzyme-substrate pairs is still far from being solved.

Among proteins, caseins represent an important case of the conformationally flexible polypeptide chains, possessing at the same time predominant spatial structures [5]. Such loosely packed structures were determined by the molecular modeling computations in conjunction with the data on casein secondary structure [6]. Caseins, especially β-casein as most hydrophobic among κ-, α_{s1}-, and α_{s2}-casein, may form micelles and submicelles [7]. In comparison with globular proteins, caseins are known to be easy hydrolysed by proteases because of their elevated conformational flexibility and the abundance of the enzyme-accessible peptide bonds. Meanwhile, clusters of hydrophobic amino acid residues in the polypeptide chain of casein substrates cause hydrophobic interaction to be responsible for the steric obstacles shielding peptide bonds against enzymatic attack [8].

Proteolysis as a polysubstrate process involves the hydrolysis of the multiplicity of peptide bonds of different specificities. The hydrolysis is complicated by the non-equal and often limited accessibility of peptide bonds for the enzyme action, since different sites are differently exposed to the surrounding solvent and/or possess different conformational flexibility [9]. This effect known as masking of peptide bonds was pointed to be the main limiting factor of proteolysis [2,10]. Additionally, enzyme inhibition and inactivation in proteolysis are complex processes, which include interaction of the enzyme with the set of various proteolysis products. That is why one needs in considerable simplifications to propose reasonable proteolysis model.

Proteolysis was regarded in our previous works as a two-stage process [10,11]. In the first stage, the initially enzyme-inaccessible peptide bonds X_m are converted to the demasked bonds Y_d, which become accessible for the action of enzyme. On the second stage, these bonds are hydrolysed as [10,11]

$$X_m \xrightarrow{k_d} Y_d \xrightarrow{k_h} N, \qquad \text{(Scheme I)}$$

where k_d is the rate constant of demasking, k_h is the rate constant of hydrolysis of the demasked bonds, and N is the amino nitrogen, the chemical outcome of the reaction.

Proteolysis to the limited extent (limited proteolysis) is an example of the considerable simplification of proteolysis analysis. In this particular case, the analysis can be reduced to the identification of the sites of initial splitting [9,12]. Contrary to Scheme 1, the probability of peptide bond demasking at limited proteolysis was assumed to be unchanged during proteolysis, depending

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on the initial state of three-dimensional structure of the protein substrate.

Presentation of the proteolysis kinetic with the averaged values of the rate constants was proposed in our previous works as a way for the considerable simplification of the analysis of the total proteolysis kinetics [13,14]. At low concentration of the protein substrate ($S \ll K_M$), fraction of free enzyme, $1/(1+S/K_M)$, is close to 1. Changes in K_M during proteolysis cannot influence significantly the fraction of free enzyme and thereby the rate of hydrolysis. In this case, total rate of the peptide bond hydrolysis can be presented in the following form:

$$V = E_0 Y_d \sum_j k^j \frac{Y_d^j}{Y_d} \tag{1}$$

where E_0 is the concentration of enzyme, Y_d is the total concentration of demasked peptide bonds, k^j is the second order kinetic constant for the j-th bond, which corresponds to the hydrolysis of the demasked peptide bonds with concentration Y_d^j. $Y_d^j/Y_d = P^j$ is the probability of peptide bond demasking, which is regarded as a function of the hydrolysis degree d, the portion of the hydrolysed peptide bonds $N/(N+Y_d+X_m)$. The averaged rate constant $k(d)$ as a function of d can be introduced as

$$k(d) = \sum_j k^j \frac{Y_d^j}{S_0} \tag{2}$$

where S_0 is the substrate concentration (concentration of all amino acid residues).

It was shown previously that functional dependence (2) is quite different for various specificity parameters [13]. We believe that the comparative study of this function could be useful for the selection of various mechanisms of demasking and hydrolysis.

The aim of the present work was to study peculiarities of the proteolysis of casein substrates as a two-stage process of peptide bond demasking and subsequent hydrolysis.

2. Materials and methods

2.1. Materials

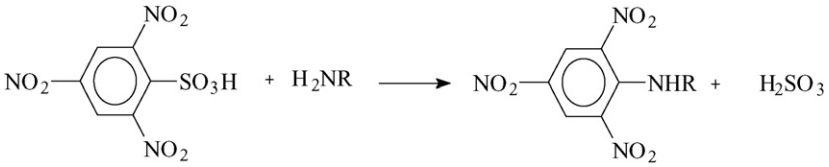
β-Casein, α-casein, and α-chymotrypsin were products of Sigma.

Summary submicellar casein was obtained from defatted milk. Casein proteins were precipitated three times by HCl (pH 4.6, temperature 6–8 °C), filtered, washed and solved by adding NaOH (pH 7.0). After the addition of acetic acid (pH 4.0), the precipitate was carefully washed, solved by adding NaOH, purified by charcoal, centrifuged and dried in vacuum.

2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from Biokhimreactive, Russia.

2.2. Proteolysis as registered by TNBS method

Proteolysis of the casein substrates (α-casein, β-casein, or summary casein) by α-chymotrypsin was carried out in 0.01 M phosphate buffer (pH 7.5) at 25 °C with stirring by a magnetic stirrer. The enzyme concentration was constant (2.5 mg/L). For termination of proteolysis and for monitoring of the reaction by TNBS method [11,14], after a required time 1 mL samples from proteolysis reactor were placed in the test-tubes with 1 mL 1% (w/v) SDS solution at 80 °C and held at this temperature for 10 min. Then 2 mL of trinitrobenzenesulfonic acid solution (TNBS, 0.2 g/L) in 0.1 M borate buffer (pH 8.2) was added to each of the tubes. Trinitrophenylation of the amino groups of the proteolysis products H_2NR



was performed at 40 °C for 2 h [11,14]. The reaction was stopped by the adding 1 ml 1 N HCl to each tube. Then the absorption was measured immediately at 340 nm against a blank sample. The latter was prepared in the same way except for the absence of the enzyme and substrate. In result, the time courses of amino nitrogen $N(t)$ during proteolysis of various casein substrates by chymotrypsin were determined.

2.3. Simulation of the kinetics of demasking and hydrolysis

Hydrolysis and demasking kinetics for both the masked peptide bonds of the j-th type X^j and the demasked bonds Y^j was described by the following equation:

$$\frac{dY^j}{dX^j} = \frac{-k^j Y^j + k_d X^j}{-k_d X^j} \tag{3}$$

where k^j is the hydrolysis rate constant for the bonds Y^j, k_d is the rate constant of the demasking, which is proposed to be the same for any type of peptide bonds j. The solution of Eq. (3) is

$$Y^j = \frac{X^j}{a^j}\left[1 + b^j\left(\frac{X^j}{X_0^j}\right)^{a^j}\right] \tag{4}$$

where the coefficients a^j and b^j were defined as

$$a^j = \frac{k^j}{k_d} - 1 \tag{5}$$

$$b^j = a^j \frac{Y_0^j}{X_0^j} - 1 \tag{6}$$

where X_0^j is the initial concentration of the masked peptide bonds of the j-th type, Y_0^j is the initial concentration of the demasked peptide bonds of the j-th type.

The mass balance equations were presented in the following form:

$$X^1 + X^1\frac{X_0^2}{X_0^1} + X^1\frac{X_0^3}{X_0^1} + \cdots = \sum_j X^j, \tag{7}$$

$$X_0^1 + X_0^1\frac{X_0^2}{X_0^1} + X_0^1\frac{X_0^3}{X_0^1} + \cdots = X_0 = S_0 - N_0 - Y_0 \tag{8}$$

where S_0 = const is the sum of the total concentration of all peptide bonds $\left(\sum_j X^j + \sum_j Y^j\right)$ and amino nitrogen N, N_0 is the initial concentration of amino nitrogen, Y_0 is the sum of all initially demasked peptide bonds $\sum_j Y_0^j$, X_0 is the sum of all initially masked peptide bonds $\sum_j X_0^j$.

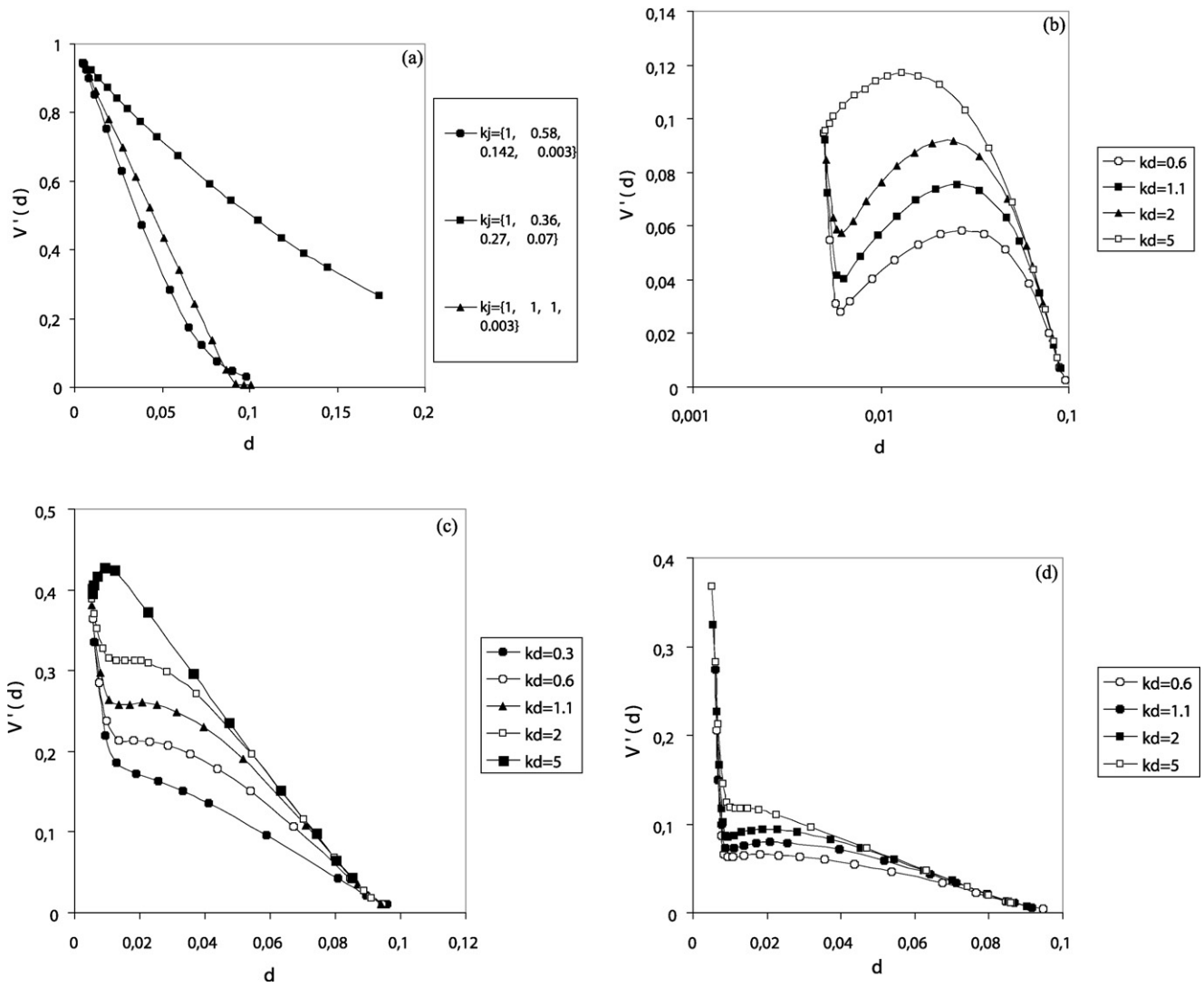


Fig. 1. Simulations of the demasking and hydrolysis stages, in accordance with Scheme 1. (a) Unchanged parameters were $m = 0.05$, and $k_d = 1.1$. Variables were $k^{Trp} = 1$ (●), 1 (■), 1 (▲); $k^{Tyr} = 0.58$ (●), 0.36 (■), 1 (▲); $k^{Phe} = 0.142$ (●), 0.27 (■), 1 (▲); $k^{Leu+Met+Asn+Gln} = 0.003$ (●), 0.07 (■), 0.003 (▲). (b) Unchanged parameters were $m = 0.9$, $k^{Trp} = 50$, $k^{Tyr} = 0.5$, $k^{Phe} = 0.5$, $k^{Leu+Met+Asn+Gln} = 0.003$. Variables were $k_d = 0.6$ (○), $k_d = 1.1$ (■), $k_d = 2$ (▲), $k_d = 5$ (□). The X-axis is plotted in logarithmic scale. (c) Unchanged parameters were $m = 0.6$, $k^{Trp} = 10$, $k^{Tyr} = 0.5$, $k^{Phe} = 0.5$, $k^{Leu+Met+Asn+Gln} = 0.003$. Variables were $k_d = 0.3$ (●), $k_d = 0.6$ (○), $k_d = 1.1$ (▲), $k_d = 2$ (□), $k_d = 5$ (■). (d) Unchanged parameters were $m = 0.6$, $k^{Trp} = 50$, $k^{Tyr} = 0.5$, $k^{Phe} = 0.5$, $k^{Leu+Met+Asn+Gln} = 0.003$. Variables were $k_d = 0.6$ (○), $k_d = 1.1$ (●), $k_d = 2$ (■), $k_d = 5$ (□).

It is convenient to operate with the degree of peptide bond masking, being introduced as

$$x = \frac{X^1}{X_0^1} = \frac{\sum_j X^j}{S_0 - N_0 - Y_0} \tag{9}$$

The solution (4) can be transformed regarding to x and the degree of peptide bond hydrolysis d in the following form:

$$mx\left(\sum_j \frac{1 + x^{a^j} b^j}{a^j} \frac{X_0^j}{\sum_i X_0^i}\right) = 1 - d - mx \tag{10}$$

where m is the parameter, which is equal to the initial degree of masking, i.e. $m = X_0/S_0$.

Averaged rate constant can be calculated as

$$k(d) = mx \sum_j \frac{k^j(1 + x^{a^j} b^j)}{a^j} \frac{X_0^j}{\sum_i X_0^i} \tag{11}$$

To calculate $k(d)$ from Eq. (11) one needs to determine firstly x as a function of d from the transcendent Eq. (10).

One can evaluate the relative rate of the peptide bond hydrolysis V' as

$$V'(d) = \frac{V(d)}{V_{max}} = \frac{k(d)}{k_{max}} \tag{12}$$

where V_{max} and k_{max} are the constant values. k_{max} was calculated by the formula:

$$k_{max} = \sum_i k^i \frac{X_0^i + Y_0^i}{S_0 - N_0} \tag{13}$$

3. Results and discussion

3.1. Simulation of the demasking and hydrolysis stages

In our theoretical consideration, the type of the peptide bond j was determined by the type of amino acid residue, forming C=O group of hydrolysed bond. This approach is reasonable for trypsin and chymotrypsin because for these proteases the reaction ability is

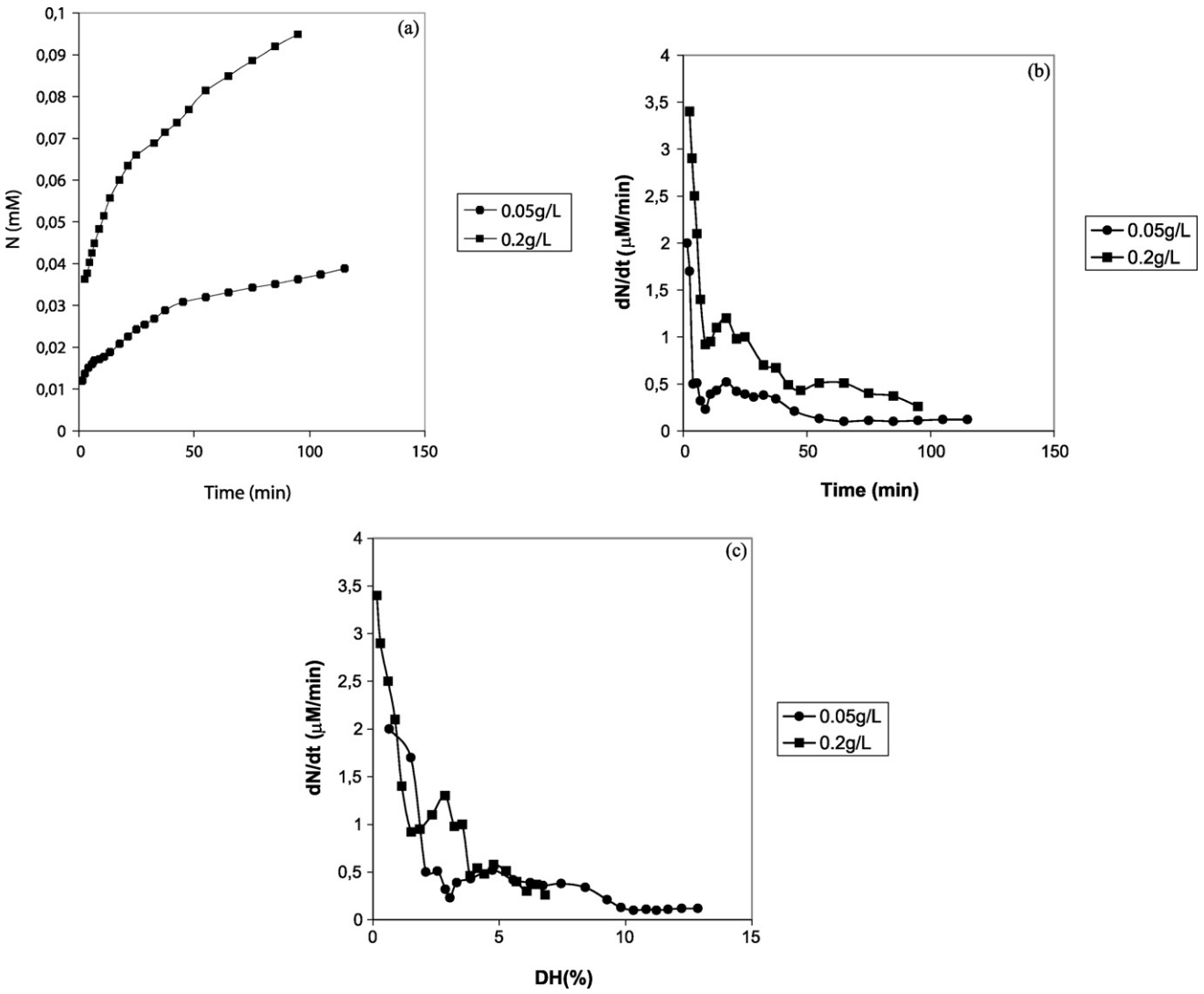


Fig. 2. Kinetic data for the proteolysis of summary casein by chymotrypsin. Proteolysis by α-chymotrypsin (2.5 mg/L) was carried out in 0.01 M phosphate buffer (pH 7.5) at 25 °C. Substrate concentration was 0.05 g/L (●) and 0.2 g/L (■). (a) Typical profiles of the amino nitrogen vs. proteolysis time. (b) Typical profiles of the hydrolysis rate vs. proteolysis time. (c) Typical profiles of the hydrolysis rate vs. hydrolysis degree.

determined mostly by the primary specificity and only partly by the interaction of an enzyme with other amino acid residues (secondary specificity) [13,15]. For chymotrypsin, the most specific amino acid residue is Trp in the comparison with less specific Tyr and Phe. For the amino acid residues Leu, Met, Asn and Gln, the values of k^j are not equal to 0, since these amino acid residues were found at the C-end of the peptides entering in the chymotryptic hydrolisates of proteins [13]. For total casein, the occurrence of the peptide bonds was calculated taking into consideration the relations between individual caseins (α_{s1}-, β- and κ-casein) as 4:3:1. The following occurrence of the peptide bonds was used in the simulations: 0.51% (Trp), 3.85% (Tyr), 3.98% (Phe), 23.5% (Leu + Met + Asn + Gln), and 68.16% for other natural amino acid residues, for which $k^j = 0$.

To elucidate kinetic regularities of the proteolysis with a demasking stage (Scheme 1) we calculated the $V'(d)$ dependences for the following characteristic sets of the parameters:

A. Most of the peptide bonds are initially demasked, $m \ll 1$.
B. Most of the peptide bonds are initially masked, $m \approx 1$.
C. Quantities of the initially masked and demasked bonds are comparable ($m \sim 1/2$) at low excess in specificity of the most specific bond ($k^{Trp} = 10$).
D. Quantities of the initially masked and demasked bonds are comparable ($m \sim 1/2$) at high excess in specificity of the most specific bond ($k^{Trp} = 50$).

Dependently on the mentioned above kinetic conditions A, B, C and D, the $V'(d)$ curves possess the following characteristics:

A. For $m = 0.05$, monotonously decreasing $V'(d)$ dependences (Fig. 1a).
B. For $m = 0.9$, non-monotonous dependences, which include region with noticeable maximum of $V'(d)$ (Fig. 1b).
C. For $m = 0.6$ and $k^{Trp} = 10$, non-monotonous dependences with poorly recognized broad maximum of $V'(d)$. $V'(d)$ in the beginning of proteolysis is comparable with that at the maximum (Fig. 1c).
D. For $m = 0.6$ and $k^{Trp} = 50$, non-monotonous dependences with poorly recognized broad maximum. $V'(d)$ in the beginning of proteolysis is significantly higher than that at the maximum (Fig. 1d).

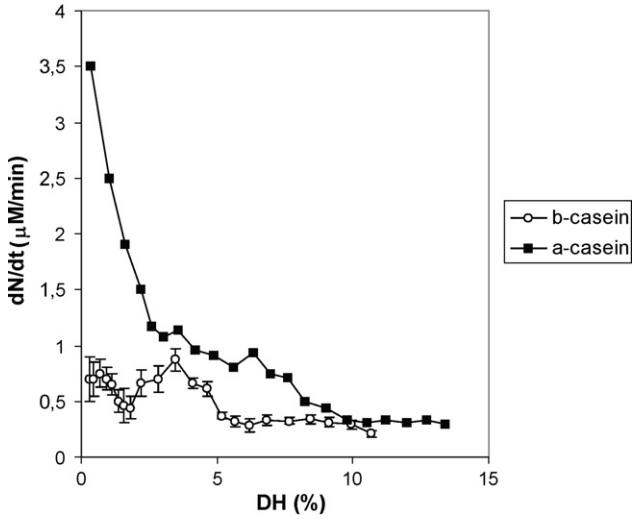


Fig. 3. Typical profiles of the hydrolysis rate vs. hydrolysis degree for the proteolysis of β-casein (○) and α-casein (■). Proteolysis of individual caseins by α-chymotrypsin (2.5 mg/L) was carried out in 0.01 M phosphate buffer (pH 7.5) at 25 °C.

3.2. Total kinetics for the chymotryptic proteolysis of casein substrates

According to Scheme 1, the hydrolysis rate is able to be maximal in two situations. Firstly, at the beginning of proteolysis when initially demasked peptide bonds start to hydrolyze. Secondly, in the middle part of proteolysis when the maximum of B_d is achieved after the demasking of the majority of masked bonds (maximum points of the broad peaks in Fig. 1b–d).

To find subtle features of the proteolysis kinetics, we used frequent sampling of the probes from the reaction mixture. Trinitrophenyl derivatives of the proteolysis products in the aliquots were engaged for the determination of amino nitrogen. For the proteolysis of summary casein, kinetic curves showed local retardation with the consequent acceleration of the hydrolysis (Fig. 2a). These non-monotonous trends in the $N(t)$ curves corresponded to the local extremes of the $V(t) = dN/dt$ (Fig. 2b). To be able to compare theoretical and experimental curves we plotted hydrolysis rate vs. hydrolysis degree $DH = d \cdot 100\%$, the percentage of the hydrolysed peptide bonds (Fig. 2c). For the chymotrypsin hydrolysis of the individual caseins (Fig. 3), one can see quite different dependences for α- and β-casein. Monotonously decreasing dependence was obtained for the proteolysis of α-casein, while for proteolysis of β-casein the $V(DH)$ dependence had maximum. The reproducibility of the hydrolysis rate as measured by TNBS method was studied systematically for β-casein hydrolysis (error bars in Fig. 3). In the average over all points on the curve, the relative error was 16%.

The comparison of the theoretical curves (Fig. 1) with experiment (Figs. 2 and 3) allowed us to conclude that proteolysis of α-casein is close to A case (most of peptide bonds are demasked), while proteolysis of β-casein is close to B case (most of peptide bonds are masked). Proteolysis of summary casein has a feature of C and D types when initially a part of peptide bonds is masked and another part of peptide bonds is free for the enzyme attack. Certainly, our comparative study was rather qualitative than quantitative, but at least it allowed us to conclude that the masking effect for β-casein was the highest among the studied substrates.

Thus, the analysis of the demasking kinetics is required for the correct depiction of the proteolysis for casein substrates. The well-known enhanced amphiphatic properties of β-casein could be responsible for the considerable masking of peptide bonds in β-casein.

In the common case of proteolysis, the hydrolysis rate is [13,14]:

$$V = \frac{k(DH)E_0S_0}{1 + S_0/K_M(DH)} \quad (14)$$

Functional dependences of the second order rate constant $k(DH)$ and effective Michaels constant $K_M(DH)$ (inhibition and productive binding) are necessary to be determined for the complete kinetic depiction of proteolysis. In present article, the measurements were performed at low concentration of substrate (0.05–0.2 g/L). In this case, the term $1/(1 + S_0/K_M)$ is close to 1, since the values of $1/K_M$ were estimated to change in the interval of 0.48–0.15 L/g for the hydrolysis of casein substrate by chymotrypsin [14].

Inactivation of enzyme as monotonous slow decrease of active enzyme during proteolysis can proceed [13,14]. But this process cannot be responsible for the non-monotonous dependencies, especially at the beginning of proteolysis.

We studied demasking effect for the proteolysis of β-casein by chymotrypsin. However, one can assume the masking properties mostly to be a characteristic of the substrate structure. In this case, the used protease would not be crucial. For the support of this hypothesis, it is useful to demonstrate significant role of demasking stage for the tryptic proteolysis as well.

3.3. Demasking and hydrolysis of the hydrophobic C-end of β-casein

Above, the total hydrolysis of all peptide bonds entering in the peptide chain of protein substrate was studied. To make easier the analysis of the total kinetics we tested averaged rate constants as the functions of *DH*. This subsection was devoted to the analysis of the hydrolysis of some selected bonds in β-casein. For this purpose, the individual constants of the hydrolysis of these bonds were analyzed.

To verify Scheme 1 on the example of tryptic hydrolysis of β-casein, we analyzed set of the rate constants, which was published previously for wild-type and engineered trypsins [8]. Different types of the engineered trypsin were produced by replacing of Lys188 by other amino acid residues [8,16]. The following mutants were used: K188H, K188F, K188Y, and K188D/D189K (in the later case the residue 189 was also replaced) [8]. Variations in the enzyme specificity give different patterns of demasking/hydrolysis kinetics, which all have to be in accordance with common Scheme 1.

β-Casein has unique amino acid sequence, since it contains a few Arg-X bonds all located at the terminus of the polypeptide chain (Arg25-Ile26 from the N-terminus, Arg183-Asp184 and Arg202-Gly203 bonds from C-end). Majority of peptide bonds susceptible to tryptic hydrolysis are of Lys-X type. They are included in the internal homogeneous fragment, Lys28-Lys176, which only includes Lys bonds, but not Arg bonds. Several studies of the proteolysis in the aqueous media and at the interface have been performed with such interesting substrate-enzyme pair as β-casein and trypsin [17–19].

Kinetic curves for the liberation of individual peptides were engaged previously to calculate the hydrolysis rate constants for the Arg-X bonds (Arg25-Ile26, Arg183-Asp184, Arg202-Gly203), and for several internal Lys-X bonds (Lys28-Lys29, Lys32-Phe33, Lys99-Glu100, Lys105-His106, Lys113-Tyr114, Lys169-Val170, Lys176-Ala177) [8]. The calculations were made at the assumption that peptide bonds were completely demasked [8]. These constants were found to depend on the type of engineered trypsin and pH [8].

Presumably, hydrophobic interaction is responsible for the incorporation of C-terminus of β-casein into hydrophobic cluster and thereby for the masking effect of corresponding peptide bonds as shown schematically in Fig. 4. Theoretical analysis revealed that for the case of considerable masking (high m), one should expect a temporal retardation of hydrolysis that may give a kinetics with a lag. In fact, several peptides in the tryptic digest of β-casein (pep-

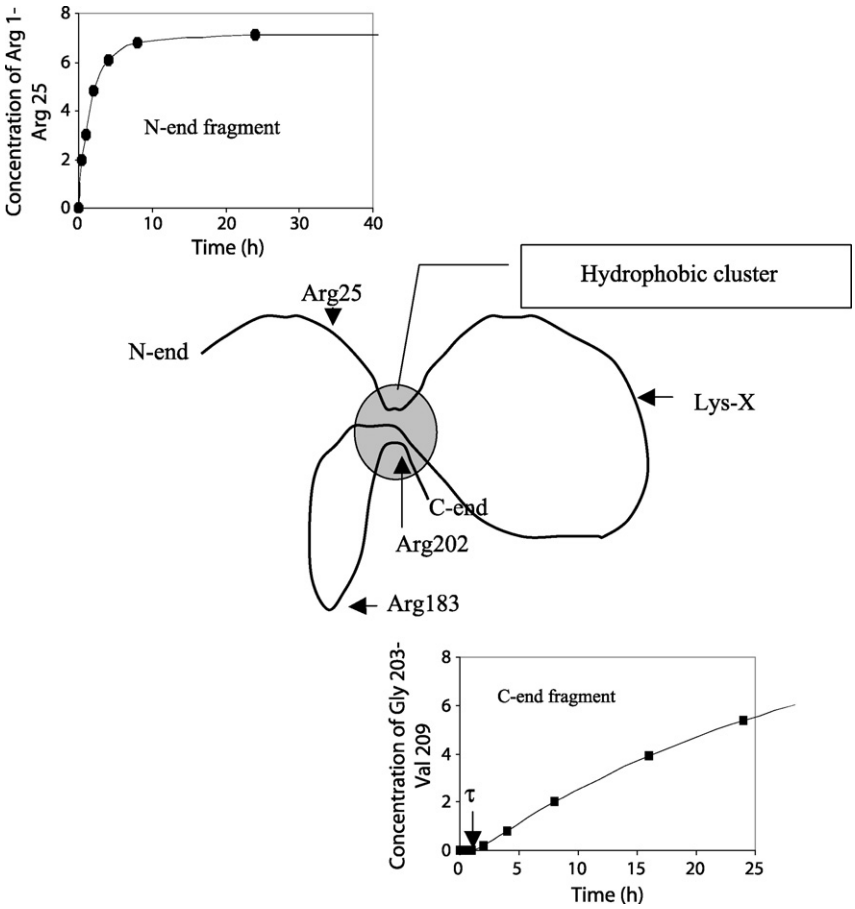


Fig. 4. Evolution of the concentrations for N- and C-terminal peptide fragments as obtained from tryptic hydrolysis of β-casein, in accordance with our previous data [8].

tide Gly203-Val209, for example) accumulated with the noticeable lag (Fig. 4).

Fig. 4 shows the hydrolysis of some specific to trypsin bonds at the β-casein chain, i.e. Arg25, internal Lys-X bond (only one from these bonds is shown in Fig. 4), Arg183 and Arg202 bonds at the C-end. This figure shows schematically the masked region, in which the hydrolysis of Arg202 bond might be conditioned by the hydrolysis of other peptide bonds. C-terminal peptide Gly203-Val209 is

Table 1
Ratio of the hydrolysis rate constant to the demasking rate constant.

Enzyme type	pH	Hydrolysis rate constant[a]/demasking rate constant[b]		
		k(Arg25-Ile26)/k_d	$k_{Lys\text{-}X}/k_d$	k(Arg183-Asp184)/k_d
Wild	7	6.2	0.8	2.7
	8	7.5	1.0	2.0
	9	5.0	1.0	2.0
	10	2.7	0.7	0.7
K188H	7	1.0	–	1.9
	8	1.4	0.9	4.6
	9	1.4	1.0	2.2
	10	3.6	1.0	3.0
K188F	7	1.8	1.7	0.4
	8	4.2	3.6	0.8
	9	3.6	3.0	0.5
	10	1.8	2.6	0.4
K188Y	7	1.0	1.3	1.1
	8	8.9	0.7	2.1
	9	11.0	1.0	2.3
	10	11.6	1.2	2.6
K188D/D189K	7	9.5	0.7	3.1
	8	10.7	0.8	2.9
	9	10.1	0.9	3.6
	10	5.9	0.9	–

[a] Hydrolysis rate constants were from [8].
[b] Demasking rate constants for the bond Gly203-Val209 were calculated with Eq. (15).

accumulated after the hydrolysis of the bond Arg202-Gly203 with the lag τ [8]. Application of Scheme 1 for demasking and hydrolysis of the bond Arg202 allows us to express τ as

$$\tau = \frac{1}{k_h - k_d} \ln \frac{k_h}{k_d} - \frac{1}{k_h} \left(\frac{k_h}{k_d} \right)^{-k_h/(k_d - k_h)} + \frac{k_d + k_h}{k_d k_h} \quad (15)$$

where k_d and k_h are the demasking and hydrolysis rate constants, correspondingly. For the peptide bond Arg202, k_h was estimated as 0.02 h^{-1} and k_d was calculated from the observed τ values with the transcendental Eq. (15).

Splitting of peptide chain should cause an increase in the conformational labiality of the hydrophobic cluster, providing by this way the demasking of the peptide bonds at the C-end. For example, hydrolysis of one of the bonds shown in Fig. 4 may break peptide chain, causing an increase in its conformational flexibility.

It is interesting to know more precisely the hydrolysis of which bond might initiate the demasking process at the C-end of the chain. Hydrolysis of such bond can be regarded as a step, limiting the hydrolysis of C-terminal bonds. The hydrolysis rate constant for limiting bond should be close to k_d. By other words, the ratio of the hydrolysis rate constant for the limiting bond to the value of k_d should be close to 1.

The k_d values were compared with the hydrolysis rate constants for the Arg25 bond, for the internal Lys-X bond (mean value of apparent constants for the Lys-X bonds), and for the Arg183 bond (Table 1). The hydrolysis of Arg25-Ile26 is not appropriate for the role of the limiting step as being around one order faster than demasking. For all enzymes except K188F mutant (Table 1), $k_{\text{Lys-X}}/k_d$ is close to 1, indicating that the splitting of internal homogeneous fragment Lys28-Lys176 limits the demasking. For K188F mutant, the limiting splitting is most likely the hydrolysis of Arg183-Asp184 bond.

Previously [8], demasking parameter was assumed to depend on the averaged hydrophobicity of the several amino acid residues, which were nearest to the hydrolysed bond. For this model of masking, the predicted values of the reaction rate for the peptide bonds at the C-terminus were deviated from the experimentally observed ones [8]. On the contrary, in the present study we proposed the demasking parameter was dependent on the hydrolysis of the bond, which might be located far away from the analyzed site. To our knowledge, this is the first attempt to link together demasking and hydrolysis of the different bonds in polypeptide.

4. Conclusions

Two-step model with consequent demasking and hydrolysis stages is useful for the understanding of non-monotonous kinetic patterns, which were demonstrated for the chymotryptic and tryptic proteolysis of casein substrates. In spate of simplicity and formal character of the model, the connection of masking with amphiphatic nature of casein substrates is evident when total hydrolysis kinetics for β-casein is compared with that for less hydrophobic α-casein and when individual kinetics for the hydrolysis of the hydrophobic C-terminus of β-casein is compared with the hydrolysis of hydrophilic N-end. Thus, the analysis of peptide bond demasking is required for the correct depiction of whole proteolysis kinetics.

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