Abstract: The opening of protein globules and corresponding exposure of their internal peptide bonds, the so-called demasking effect, is required for successful hydrolysis of peptide bonds by proteases. Under the proteolytic action of trypsin on β-lactoglobulin (β-LG), the evolution of tryptophan fluorescence spectra showed that the demasking process consists of two stages with different demasking rate constants for each stage. It was found that the ratio of these constants depends on the concentration of trypsin and changes are approximately threefold when the concentration of trypsin changes in the range of 0.3–15 mg/L. Simulation of hydrolysis taking into account the demasking effect demonstrated how the apparent first-order rate constants obtained experimentally are related to the true hydrolysis rate constants and demasking parameters. The lag phase in the kinetic curves corresponding to the hydrolysis of various peptide bonds in β-LG was also analyzed. The increased lag times indicated sites that are hydrolyzed by a two-stage demasking mechanism.

Keywords: enzyme kinetics; proteolysis; trypsin; tryptophan fluorescence; demasking; peptide bond hydrolysis

1. Introduction

Modeling of enzymatic hydrolysis of proteins, known as proteolysis, has always been given considerable attention due to the importance of proteolysis processes in biology, biotechnology and food science [1,2]. Among the many modern biotechnological tasks that require models of proteolysis, we note first the release of bioactive peptides as a result of proteolysis for their collection at due time to prevent further hydrolysis. Adequate models of proteolysis are also needed to obtain protein hydrolysates with desired properties and to improve proteomic analysis when peptide fragments of tryptic protein hydrolysates are used to identify their amino acid sequences by mass spectrometry.

Proteolysis is a complex multicomponent process that takes place over time. It includes a number of processes that also occur in time in accordance with their own laws. Of these, the main one is the hydrolysis of peptide bonds, first specific for a given enzyme, and then less specific. As the protein globule breaks down, intrinsic peptide bonds open up for the enzyme, so that they can only be hydrolyzed from some time of proteolysis [3,4]. This process of opening peptide bonds, called demasking, often limits the hydrolysis of peptide bonds and is an important component of proteolysis [5]. For the quantitative description of demasking, it is necessary to determine the degree of protein degradation by an appropriate spectroscopic method as a function of time or degree of hydrolysis [6]. Inhibition of the enzyme by proteolysis products and even its irreversible inactivation also determines the overall kinetics of proteolysis.

For the quantitative analysis of proteolysis, it is advisable to use methods of chemical kinetics, which allow describing the processes that make up proteolysis using differential equations. Their
solution and, thus, prediction of the course of proteolysis is possible if the rate constants included in

differential equations are known. The sets of rate constants for individual stages were determined,

for example, for casein proteolysis by chymotrypsin [7] and β-lactoglobulin (β-LG) proteolysis by

trypsin [8]. Recently, a technique has been developed to determine the relative hydrolysis rate constants

(selectivity values) for all specific bonds in the substrate, based on HPLC-MS analysis of peptide

fragments formed at different degrees of hydrolysis [9]. It is important that the kinetic parameters

listed correspond to processes that have a certain physic-chemical meaning. In this, they differed

from the empirical coefficients used in empirical models of proteolysis for the best approximation of

experimental kinetic curves [10].

Research into proteolysis has identified a number of processes that proceed according to the

first-order kinetics. For example, it was shown that a decrease in the concentration of intact protein

under the action of chymotrypsin obeys an exponential law [11]. The corresponding rate constants

quantitatively described the initial degradation of the proteins from milk [11]. A decrease in the

concentrations of most specific peptide bonds in β-LG during proteolysis by Bacillus licheniformis

protease was also exponential [9], although more complex dependences were observed for some

peptide bonds [12]. The degree of unfolding of the polypeptide chain, which quantitatively describes

the change in the availability of peptide bonds for the enzyme, was found to be an exponential

function [13]. For these processes, the rate constants were determined under the assumption that the

processes correspond to the first-order kinetics. However, the exponential courses of dependencies

may be apparent, and the processes may consist of a number of elementary stages. Although, the rate

constants may be effective, they are, nevertheless, very useful for modeling of whole proteolysis, as a

composite process.

The mechanism of action of proteolytic enzymes and their specificity are being intensively studied

for small synthetic substrates with ester or amide bonds and for relatively short peptides with specific

cleavable sites [14]. Trypsin (EC 3.4.21.4) is a serine protease first found in the mammalian digestive

system, where it breaks down proteins into peptides [15,16]. The maximum rate of the action of trypsin

on protein substrates is pH 7.8 and temperature 37 °C [17]. Trypsin has a catalytic triad within its

active site that involves Ser195, His57, and Asp102 residues. Positively charged side chains of Arg and

Lys residues in protein substrates participate in electrostatic interactions with Asp102 at the bottom

of the trypsin active site [18]. Consequently, trypsin cleaves peptide bonds on the carboxyl side of

which there are lysine or arginine residues (Arg-X, Lys-X bonds), if these residues are not followed by

proline [16–18]. In addition, the rate of hydrolysis of these bonds depends also on other neighboring

amino acid residues providing so-called secondary specificity. This means that tryptic hydrolysis of

demasked peptide bond in the polypeptide R1-COONH-R2 + H2O → R1COOH + NH2R2 (R1 and R2

are the N- and C-terminal fragments of the polypeptide) is dependent on the amino acid sequences

of the polypeptide fragments R1 and R2. Small peptide substrates with one hydrolysable bond are

hydrolyzed by trypsin according to Michaelis–Menten kinetics.

Proteolysis models require the definition of key variables and corresponding kinetic schemes.

This also includes simplifying the reaction pathway to be able to reduce the number of variables and

differential equations. For example, the two-step proteolysis model includes only one differential

equation to describe the masking effect, which is responsible for the limited availability of peptide bonds

for the enzyme and another differential equation to describe the hydrolysis of peptide bonds [5,7,13].

For this model, calculations were performed for hydrolysis of β-casein with trypsin, total casein with

chymotrypsin, and β-LG by trypsin. However, in these works the demasking process was not analyzed

in detail, but was considered as an effective process characterized by one rate constant of demasking.

β-LG is a globular protein that is the main protein of milk whey [19]. In vivo, it has the ability
to bind and transport small biomolecules, such as fatty acids and vitamin A. Bovine β-LG (18.3 kDa)
consists of 162 amino acid residues, and its compact globule is stabilized by two disulfide bonds [20,21].
It contains 15 lysine and three arginine residues, which are specific for trypsin, and two tryptophan
residues Trp19 and Trp61, which provide intrinsic tryptophan fluorescence. β-LG forms dimers at
physiological environment conditions and exists in monomeric form at high temperatures and pH values higher than 8 [22].

The aim of this study was to determine the demasking parameters using fluorescence spectroscopy for the proteolysis at constant substrate concentration and different enzyme concentrations, as well as modeling of the hydrolysis of peptide bonds taking into account a more complex demasking mechanism than in the two-step proteolysis model [6]. In the present work, proteolysis of β-LG by trypsin was studied in a wide range of enzyme concentrations from 0.3 to 15 mg/L. We suggested that demasking manifests itself differently at the initial stage of hydrolysis, when the protein globule is destroyed, and at the intermediate stage of proteolysis. Two processes were considered that make up demasking, the first associated with the unfolding of the globule and the second associated with the consequent destruction of a hard-hydrolysable core of the remaining polypeptide chains of β-LG. Hydrolysis of peptide bonds was simulated for these two demasking mechanisms. The obtained simulation results for peptide bond hydrolysis were compared with the available experimental data on the proteolysis of β-LG by trypsin.

2. Results

2.1. Time-Dependent Fluorescence during β-Lactoglobulin (β-LG) Proteolysis by Trypsin

Proteolytically-induced changes in conformation of β-LG and its peptide fragments were studied by analyzing the intrinsic fluorescence spectra of tryptophan residues Trp19 and Trp61 (Figure 1). The spectra were obtained with a temporal resolution of 1–2 min for the proteolysis reaction carried out at the enzyme concentration from 0.3 up to 15 mg/L. A shift in fluorescence spectra to higher wavelengths (red shift) was observed for the proteolysis reaction studied (Figure 1).

![Figure 1](image-url). Fluorescence spectra of intact β-lactoglobulin (β-LG) at 0.25 g/L (black) and its peptide fragments at various times of hydrolysis by trypsin at 15 mg/L. Hydrolysis times are 1 min (blue), 3 min (light blue), 6 min (green), 10 min (light green), 15 min (yellow), 30 min (orange), 65 min (red). The arrow indicates the direction of the red shift of tryptophan fluorescence in the course of hydrolysis and numbers are the hydrolysis times.

It is known that the position of the fluorescence emission maximum of tryptophan residues is sensitive to their nearest environment [23,24]. Therefore, the red shift of fluorescence in the course of proteolysis was attributed to an increase in the polarity of the medium into which tryptophan residues
are located when the polypeptide chain of β-LG unfolds. Because trypsin remains intact in the course of proteolysis, its tryptophan fluorescence spectrum does not shift.

The position of the emission fluorescence maximum, determined by the quadratic approximation as described in the Materials and Methods section, was monotonically shifted in the process of proteolysis. We used the fluorescence maximum wavelength \( \lambda_{\text{max}} \) as a quantitative characteristic of protein demasking and studied the increasing dependences \( \lambda_{\text{max}}(t) \) of hydrolysis time \( t \) (Figure 2a). The rate and range of this shift were dependent on the enzyme concentration, being in the maximum range from 340 nm to 355 nm for the highest enzyme concentration used, while at the lowest concentration these characteristics were minimal.

![Figure 2](image)

**Figure 2.** Determination of demasking kinetics by monitoring the maximum fluorescence wavelength \( \lambda_{\text{max}} \) in course of proteolysis β-LG by trypsin: (a) dependences of experimental values of \( \lambda_{\text{max}} \) on hydrolysis time at trypsin concentration of 15 mg/L (●), 4.5 mg/L (■), and 0.9 mg/L (○). The fitting using Equation (10) gave curves in blue; (b) linearization of kinetic curves in the semilogarithmic coordinates \( \ln(\lambda^* - \lambda_{\text{max}}(t)) \) vs. \( t \), for proteolysis reactions at trypsin concentration of 15 mg/L (●), 4.5 mg/L (■), and 0.9 mg/L (○).

### 2.2. Quantification of the Demasking Rate Constants

The fluorescence data were presented in semilogarithmic coordinates \( \ln(\lambda^* - \lambda_{\text{max}}(t)) \) vs. \( t \), where \( \lambda^* \) corresponds to the maximum spectrum shift at the end of proteolysis (\( \lambda^* = 355 \text{ nm} \)), and \( \lambda_{\text{max}}(t) \) is the current value \( \lambda_{\text{max}} \) at time \( t \) [11]. In the first approximation, a straight line was obtained, which formally corresponded to the kinetic law of first order. This is how this process was considered in the first study on the proteolysis of β-LG by trypsin using fluorescence spectroscopy [11]. However, a more detailed examination of the dependence showed that line was broken, consisting of two straight lines as is shown for concentrations of 15 and 4.5 mg/L in Figure 2b. Based on the slopes of these lines, it could be concluded that at the beginning of proteolysis, the rate at which Trp residues pass into the polar medium is higher compared to the remaining part of the process. An analysis of the sequence of release of peptide fragments during proteolysis of β-LG by trypsin showed that, first, peptides containing Trp19 are released quickly, and then products containing Trp61 are slowly released [8,25–28]. Therefore, the fast and slow processes observed with the help of fluorescence spectroscopy were associated with Trp19 and Trp61, respectively.

The one-stage transition of Trp19 residue from the masked to demasked state can be represented by the following scheme: \( S^1_m \xrightarrow{k_f} S^1_d \), where \( S^1_m \) stands for the masked state and \( S^1_d \) stands for the demasked
state, where tryptophan is hydrated and surrounding peptide bonds can be attacked by the enzyme, and $k_{d}^{f}$ is the rate constant of this transition.

The two-stage transition of Trp61 residue from the masked to demasked state can be represented by the following scheme: $S_{m}^{2} \rightarrow S_{d}^{2} \rightarrow S_{dd}^{2}$, where $S_{m}^{2}$ stands for the masked state, $S_{d}^{2}$ stands for the state, where tryptophan is partially demasked but surrounding peptide bonds still are unhydrolysable, $S_{dd}^{2}$ stands for the fully hydrated tryptophan with hydrolysable surrounding bonds, and $k_{d}$ is the rate constant for the second stage of the transition. The superscripts 1 and 2 denote the one-stage or two-stage demasking.

The demasking rate constant for the first part of the demasking process ($k_{d}^{f}$) was not previously determined by us within the framework of the two-step proteolysis model; only the $k_{d}$ constant was determined [3,5,11]. In the terms of the two-step proteolysis model, the first part of the demasking proceeds very quickly yielding “initially demasked peptide bonds” that could be freely hydrolysed from the beginning of proteolysis. In the present study, this fast process was taken into account and the corresponding rate constant $k_{d}^{f}$ was introduced.

The theoretical course of the dependence $\lambda_{max}(t)$ was based on the concentrations of $S_{m}^{1}, S_{m}^{2}, S_{d}^{1}, S_{d}^{2}$, and $S_{dd}^{2}$ (Equations (4)–(8)). These concentrations were derived from the proposed schemes [Equations (1) and (2)], while $\lambda_{max}$ was calculated as a linear combination of the wavelengths $\lambda_{1}, \lambda_{2},$ and $\lambda_{3}$, which correspond to the positions of the emission fluorescence maxima of the masked, partially demasked, and fully demasked tryptophan residues, respectively (Equations (9) and (10)). It was found (Equation (11)) that the initial rate of demasking $V_{0}$ ($d\lambda_{max}/dt$ at $t = 0$) is equal to $k_{d}^{f} (\lambda_{2} - \lambda_{1})$ that was used for the determination of $k_{d}^{f}$ as follows: $k_{d}^{f} = V_{0}/(\lambda_{2} - \lambda_{1})$. In further calculations, this proportionality (Figure 3a) was used to determine $k_{d}^{f}$ at various enzyme concentrations (Table 1). The proportionality of $k_{d}^{f}$ to $E_{0}$ is consistent with reference [29], where the proteolysis of maltose binding protein by trypsin was investigated even at a higher concentration of trypsin up to 0.4 g/L, and a proportional relationship was established.

![Figure 3](image-url)

**Figure 3.** Determination of demasking rate constants at various trypsin concentrations: (a) determination of $k_{d}^{f}$ using linear dependence of $V_{0}/(\lambda_{2} - \lambda_{1})$ on trypsin concentration; (b) dependence of the ratio of demasking rate constants $k_{d}^{f}/k_{d}$ on trypsin concentration.
The rate constants $k_d$ were obtained by fitting with Equation (10) of the experimental points $\lambda_{\text{max}}(t)$ presented in Figure 2a. It was found that $k_d$ decreased higher than $k_d^f$ with decreasing $E_0$ and, therefore, the ratio $k_d^f/k_d$ increases with decreasing $E_0$ (Table 1 and Figure 3b). The ratio of these constants was in the range of 3–9.5 with varying of enzyme concentration from 15 to 0.3 mg/L. This ratio is of interest for modeling the hydrolysis of peptide bonds. In fact, at low enzyme concentrations, it was not possible to achieve large shift in fluorescence, as it was at $E_0 = 15$ mg/L (Figure 2). Similarly, when evaluating the results of proteolysis by the degree of hydrolysis (DH), the final DH values were noticeably lower at low $E_0$ than at high.

Table 1. Determination of the rate constants of demasking.

| $E_0$ mg/L | $k_d^f$ min$^{-1}$ | $k_d$ min$^{-1}$ | $k_d^f/k_d$ $^1$ |
|------------|-------------------|-----------------|-----------------|
| 15         | 0.309 ± 0.006     | 0.103 ± 0.003   | 3.0±0.2         |
| 10         | 0.206 ± 0.005     | 0.0644 ± 0.002  | 3.2±0.3         |
| 5          | 0.103 ± 0.002     | 0.0278 ± 0.0015 | 3.7±0.3         |
| 4.5        | 0.0824 ± 0.0010   | 0.0201 ± 0.0015 | 4.1±0.4         |
| 0.9        | 0.0185 ± 0.0025   | 0.00303 ± 0.0005| 6.1±0.7         |
| 0.3        | 0.0062 ± 0.0029   | 0.00065 ± 0.00014| 9.5±2.0        |

$^1$ Calculations were performed with Equations (9) and (10) at $\lambda_1 = 340$ nm, $\lambda_2 = 344.5$ nm, $\lambda_3 = 360$ nm, and $S_{0d}^f/S_{0d}^c = 1$.

2.3. Verification of the Proposed Demasking Schemes

Another method of the processing fluorescence data used here is based on the comparison of fluorescence spectra over the entire emission fluorescence range. It can be assumed that the demasking of both Trp$^{19}$ and Trp$^{61}$ are one-step processes, one fast and the other slow. This method of processing spectra made it possible to eliminate this alternative to Equations (1) and (2).

The fluorescence intensity $I(\lambda, t)$ at any proteolysis time $t$ can be represented as a linear combination of the intensities of the following reference spectra: initial $I_0(\lambda)$, semifinal $I_1(\lambda)$ and final spectra $I_2(\lambda)$. The initial spectrum is the spectrum of non-hydrolysed β-LG, in which both Trp residues are masked, since they are hidden in the protein globule. The semifinal spectrum is a spectrum in which one tryptophan residue (Trp$^{10}$) is hydrated and another one is still masked. This spectrum was obtained in the final part of proteolysis at the lowest enzyme concentration used (0.3 mg/L). The final spectrum is a spectrum of the completely hydrolysed β-LG, which was obtained at the highest enzyme concentration of 15 mg/L. The initial, semifinal and final spectra with the wavelengths at the maximum of 340, 344.5, and 356 nm, respectively, are independent of time (Figure 4a).

The linear combination was composed with weights corresponding to the fractions of masked, partially demasked and completely demasked tryptophan:

$$I(\lambda, t) = [1 - c_1(t) - c_2(t)] I_0(\lambda) + c_1(t) I_1(\lambda) + c_2(t) I_2(\lambda)$$

(1)

where $c_1$ is the fraction of the fragments of β-LG molecule, where Trp$^{19}$ is demasked, but Trp$^{61}$ is masked; the $c_2$ fraction of the fragments of another β-LG molecule in which both tryptophans are demasked. Fraction $c_1$ includes $S_{0d}^f$ and $S_{0d}^c$, while $c_2$ includes only $S_{0d}^c$. Both parameters $c_1$ and $c_2$ are independent of $\lambda$ and are only functions of the hydrolysis time. These parameters were determined by the fitting of experimental data $[I(\lambda, t), I_0(\lambda), I_1(\lambda)$ and $I_2(\lambda)]$ using Equation (3). An example of the original and fitted spectrum is shown in Figure 4a.
Figure 4. Comparison of fluorescence spectra in course of proteolysis β-LG by trypsin: (a) reference spectra includes initial $I_0(\lambda)$ (green), semifinal $I_1(\lambda)$ (blue), and final spectrum $I_2(\lambda)$ (red). Original fluorescence spectrum $I(\lambda, 2.5)$ at 2.5 min of hydrolysis (●), and its fitted variant obtained with Equation (3) (dotted line); (b) symbols indicate values obtained by Equation (3). One Trp residue is demasked in fraction $c_1$ (●), both Trp residues are demasked in fraction $c_2$ (■), and in fraction $1 - c_1 - c_2$ (□) both Trp residues are masked. Lines indicate course of fractions $c_1$ (blue), $c_2$ (red), and $1 - c_1 - c_2$ (green) in accordance with Equations (1), (2) and (4)–(8).

The time-dependences of the fractions are shown in Figure 4b for proteolysis at $E_0 = 15 \text{ mg/L}$. The fraction of intact protein $(1 - c_1 - c_2)$ decreases with time and the fraction of the completely demasked tryptophans $(c_2)$ increases. The semifinal fraction $(c_1)$ first increases and then decreases. If both tryptophans were demasked in one step with different rates, the dependence $c_1$ on time would increase monotonically. Intermediate maximum for $c_1$ corresponds to $S^2_{0}$ since $S^1_{0}$ only monotonically increases; hence, this confirms the transition of Trp$_{61}$ through an intermediate state in accordance to the two-stage scheme. This is also confirmed by the presence of a lag phase for the $c_2$ fraction (Figure 4b), since the transition in one step does not give lagged kinetic curves.

In the approach used in this subsection, the course of fractions was calculated using Equation (3), while kinetic schemes were not used (symbols in Figure 4b). To compare this approach with that used in the Section 2.2, we calculated the concentrations of fractions using Equations (4)–(8) with the values of $k^f_d = 0.3 \text{ min}^{-1}$ and $k_d = 0.1 \text{ min}^{-1}$, obtained in the Section 2.2 at $S^1_{0}/S^2_{0} = 1$ (lines in Figure 4b). Comparison shows the qualitative agreement of these approaches, although some values obtained by Equation (3) for $1 - c_1 - c_2$ were negative and underestimated. This is apparently due to the simplicity of Equation (3), although, there is no doubt that it can be used to adopt or reject possible demasking schemes.

2.4. Hydrolysis of Peptide Bonds Taking into Account Demasking Mechanisms

If we assume that peptide bonds are initially masked in the protein globule, and can be attacked by enzyme after their demasking, then their hydrolysis can proceed according to one of the following schemes. For the bonds hydrolyzed immediately after unfolding of the protein globule, the hydrolysis process corresponds to the scheme: $B^j_m \xrightarrow{k_{fj}} B^j_d \xrightarrow{k_d} N^j$, where $B^j_m$ is the jth peptide bond in the intact β-LG, $B^j_d$ is the jth peptide bond in the partially demasked state, $N^j$ is the product of hydrolysis of the jth bond, and $k^j$ is the true constant of the hydrolysis rate.
For the bonds hydrolyzed after demasking of some core resistant to proteolysis, the hydrolysis process corresponds to the scheme: $B_m^j \xrightleftharpoons{\kappa_d^j} B_d^j \xrightarrow{k_d^j} N_d^j \rightarrow B_{dd}^j \xrightarrow{k_{dd}^j} N_{dd}^j$, where $B_m^j$ is the jth peptide bond in the completely demasked state, and other designations are the same as in the previous scheme. The time dependences derived for the concentrations $N^j(t)$ are collected in the Materials and Methods section [Equations (12) and (13)]. It was proposed that the ratio of the rate constants $k_d^j$ and $k_d$ are the same as in the demasking schemes, since the removal of masking environment of tryptophans and the opening of peptide bonds in the globule and in resistant to hydrolysis core are interrelated processes.

The experimentally observed curves $N^j(t)$ are usually considered as kinetic curves of the first order with apparent rate constants [9,30]. The apparent rate constants $k_{app}^j$ were determined from these curves for the specific peptide bonds in several protein substrates of trypsin [30]. By contrast with this approach, here we propose that the hydrolysis of peptide bonds consists of several stages and depends both on the parameters of demasking and on the true hydrolysis rate constants $k_d^j$.

We analyzed the dependences $N^j(t)$ theoretically using Equations (12) and (13) at $k_d^j = 1$ and at the ratio $k_d^j / k_d = 3$, which we determined for high concentrations of trypsin, at which practically important studies of tryptic proteolysis are carried out. In our simulations, eight possible combinations were for two demasking options (one-stage or two-stage demasking) and four categories of true hydrolysis rate constants ($k_d = 50, 10, 1$, or $0.2$).

Increasing dependences were obtained for all these cases with demasking (Figure 5b), as well as for the proteolysis model without demasking in accordance with Equation (14) (Figure 5a). The specific course of the dependences for the one-stage demasking was determined by the values of demasking parameters and true hydrolysis rate constants (Figure 5b). For the one-stage demasking and $k_d^j$ value 50 and 10 in Equation (12), the dependences were almost exponential with the apparent rate constants close to $k_d = 1$ (Figure 5b).

Figure 5. Comparison of kinetic curves obtained for proteolysis model without and with masking/demasking effect: (a) simple exponential kinetic curves without demasking at $k_d^j = 50$ (black), 10 (green), 1 (blue) and 0.2 (red), calculated with Equation (14) at $N_0 = 1$; (b) kinetic curves with one-stage demasking ($k_d^j = 1$) at $k_d^j = 50$ (black), 10 (green), 1 (blue) and 0.2 (red), calculated with Equation (12) at $N_0 = 1$.

For one-stage and two-stage demasking, and $k_d^j = 1$ or 0.2, the dependences were sigmoidal with noticeable lag phase (Figure 6). For other parameters of demasking and hydrolysis, the $N^j(t)$
dependences had a shorter length of the lag time \( t_{\text{lag}} \). The value of \( t_{\text{lag}} \) was determined as shown in Figure 6. It is equal to the segment on the time-axis, which is cut off by the tangent to the curve \( N_j(t) \) at the point of its inflection.

Figure 6. Comparison of initial parts of kinetic curves obtained for one-stage (Equation (12)) and two-stage (Equation (13)) demasking models. (a) One-stage demasking \( (k^f_j = 1) \) at \( k^f = 1 \) (blue) and 0.2 (red). (b) Two-stage demasking \( (k^f_d = 1, k^f_j / k_d = 3) \) at \( k^f = 1 \) (blue) and 0.2 (red).

The dependences \( N_j(t) \) were approximated by the exponential function \( 1 - \exp(-k^j_{\text{app}}t) \) to quantify the \( k^j_{\text{app}} \) values. The values \( k^j_{\text{app}} \) and \( t_{\text{lag}} \) calculated for eight options are shown in Table 2. The same characteristics can be obtained by processing real experimental curves. We would like to use the values of \( k^j_{\text{app}} \) and \( t_{\text{lag}} \) obtained by processing the experimental curves of bond hydrolysis in order to assign peptide bonds to one or another demasking mechanism.

Table 2. Theoretical analysis of the kinetic curves obtained at various values of true hydrolysis rate constants and different types of demasking.

| Type of Demasking | True Hydrolysis Constant \( k^f \) | Apparent Hydrolysis Constant \( k^j_{\text{app}} \) | Time Lag \( t_{\text{lag}} \) |
|-------------------|----------------------------------|-----------------------------------|------------------|
| 1                 | 50                               | 0.972 ± 0.005                     | 0.017            |
| 1                 | 10                               | 0.866 ± 0.020                     | 0.064            |
| 1                 | 1                                | 0.402 ± 0.028                     | 0.28             |
| 1                 | 0.2                              | 0.139 ± 0.007                     | 0.53             |
| Mean              | 15.3                             | 0.595                             |                  |
| Standard deviation | 23.6                             | 0.392                             |                  |
| Coefficient of variation (cv) | 1.54                     | 0.66                              |                  |
| 2                 | 50                               | 0.202 ± 0.013                     | 0.47             |
| 2                 | 10                               | 0.194 ± 0.014                     | 0.55             |
| 2                 | 1                                | 0.144 ± 0.015                     | 1.12             |
| 2                 | 0.2                              | 0.080 ± 0.007                     | 1.92             |
| Mean              | 15.3                             | 0.155                             |                  |
| Standard deviation | 23.6                             | 0.0562                            |                  |
| Coefficient of variation (cv) | 1.54                     | 0.36                              |                  |

1 stands for one-stage demasking, 2 stands for two-stage demasking, \( k^f_j = 1 \) was in the calculations for one-stage demasking, \( k^f_d = 1 \) and \( k^f_j / k_d = 3 \) were in the calculations for two-stage demasking.
At the same values of $k'$, one-stage demasking gave higher $k'_{\text{app}}$ and lower values of $t_{\text{lag}}$ than two-stage demasking. Two-stage demasking caused a longer lag phase in comparison with the one-stage scheme; therefore, the values of $t_{\text{lag}}$ were in the intervals of 0.47–1.92 and 0.017–0.53, respectively (Table 2). As one can see, these intervals overlap only in the case of large $k'$ (50) for two-stage demasking and small $k'$ (0.2) for one-stage demasking, that is, when the difference in $k'$ values is a hundredfold. Only for this combination of $k'$, the assignment of peptide bonds to one of the two types of demasking can be misleading.

It can be concluded that a presence of lag phase is a specific feature of the proteolysis models considering the demasking effect. Moreover, the lag times can be used to determine that hydrolysis of a given peptide bond occurs with either the two-stage or one-stage demasking (Table 2). Only when $t_{\text{lag}}$ falls into the interval 0.47–0.54, it is impossible to indicate exactly what type of demasking is being implemented. In principle, the relative selectivities, as estimates of $k'_{\text{app}}$ values, can be used for the assignment procedure, but the accuracy of the determination of selectivity values is low in the case of non-exponential curves.

In the absence of masking, the relative standard deviation (coefficient of variation, cv) for true hydrolysis constants was 1.54, while this parameter was only 0.66 and 0.36 for apparent values $k'_{\text{app}}$ in one-stage and two-stage demasking mechanisms, respectively (Table 2). This is due to the fact that masking effect brings the values of the apparent hydrolysis constants closer together. The width of the distribution of apparent constants, characterized in vitro digestibility of food proteins, was previously used to judge the importance of masking effect in proteolysis of food proteins [31].

2.5. Role of Demasking Process in the Proteolysis of β-LG by Trypsin

As an example of the application of our approach to the analysis of kinetic curves for proteolysis of β-LG by trypsin, we analyzed kinetic data obtained for calculating selectivity values [30]. The relative values of the selectivity parameter [30] were used as an estimation of $k'_{\text{app}}$ (Table 3). The lag times were determined at the initial stage of proteolysis (0–8 min), where $N_i(t)$ functions were approximated by the quadratic functions of $t$. The peptide bonds were divided into bonds with high $t_{\text{lag}}$ (0.89–2.08) and low $t_{\text{lag}}$ less than 0.33 (Table 3). This made it possible to assign cleavage sites to the demasking mechanisms. The peptide bonds 8, 14, 40, 75, 138, 141, and 148 were assigned to the one-stage demasking. The peptide bonds 20, 60, 83, 91, 124, and 135 were assigned to the two-stage demasking.

The assignment of adjacent peptide bonds 83, 91, 124, and 135 to the two-stage demasking suggests that this region of the polypeptide chain contains a nucleus that may be resistant to hydrolysis. Indeed, a fragment of the polypeptide chain of β-LG with the amino acid residues 76–138 was noted as a core resistant to the action of trypsin [8,27].

Peptide bonds 8, 14, 40, 75, 141, and 148, mainly located at the N- and C-terminus of the polypeptide chain, are hydrolyzed at the highest rates. Moreover, the apparent rate constants of their hydrolysis are very close to each other (Table 3). This can be explained by the fact that the true rate constants for these bonds are almost the same. However, it is more likely that the true hydrolysis rate constants are completely different, but the apparent hydrolysis rate constants are close because they are limited by the demasking rate constant $k'_{d}$, as shown in Table 2 for $k' = 10$ and 50.
Table 3. Kinetic parameters for hydrolysis of cleavage sites in β-LG.

| Bond Index j | Cleavage Site | Selectivity 2 (%) | $k_{app}^j/k_{app}^8$ | $t_{lag}$ (min) | $t_{lag}/t_0$ | Type of Demasking |
|--------------|---------------|-------------------|----------------------|----------------|----------------|------------------|
| 8            | MK-GL         | 13.7              | 1                    | 0              | 0              | 1                |
| 14           | QK-DL         | 7.4               | 0.54                 | 0.70           | 0.33           | 1                |
| 20           | WY-SL         | 9.9               | 0.72                 | 4.45           | 2.08           | 2                |
| 60           | QK-WE         | 0.2               | 0.01                 | 4.27           | 2.00           | 2                |
| 75           | EK-TK         | 9.1               | 0.66                 | 0.69           | 0.32           | 1                |
| 83           | FK-ID         | 2.9               | 0.21                 | 2.58           | 1.21           | 2                |
| 91           | NK-VL         | 3.8               | 0.28                 | 1.90           | 0.89           | 2                |
| 124          | VR-TP         | 5.0               | 0.36                 | 2.92           | 1.36           | 2                |
| 135          | EK-FD         | 1.6               | 0.12                 | 4.16           | 1.94           | 2                |
| 138          | DK-AL         | 5.3               | 0.39                 | 0.58           | 0.27           | 1                |
| 141          | LK-AL         | 9.4               | 0.69                 | 0.38           | 0.18           | 1                |
| 148          | IR-LS         | 11.0              | 0.80                 | 0.30           | 0.14           | 1                |

1 Cleavage sites 69, 70 and 100, 101 with amino acid sequence –Lys-Lys– were not analyzed, since the influence of hydrolysis of the neighboring specific bonds on each other were not considered here. 2 Values of selectivity were from [30]. 3 Value of $k_{app}^8$ was 13.7%. 4 The characteristic time of hydrolysis $t_0$ for the most rapidly hydrolyzed bond ($j = 8$) was 2.14 min.

3. Discussion

Since the first proteolysis model by Linderstrom–Lang [2], several have been proposed, and therefore it is necessary to clearly indicate the difference between the approach proposed here and the already existing models of proteolysis. In our model, the apparent hydrolysis rate constants depend not only on the interaction of the polypeptide chain with the active site of trypsin, that is, on the primary and secondary specificity, but also on whether the enzyme may reach the target sites. This can be estimated from the probability of demasking for each hydrolysable bond and, according to our model, from the rate constants of demasking. Thus, the values of $k_{app}^j$ determined by extrapolating the experimental curves from a simple exponential dependence turn out to be dependent not only on $k^j$, but also on the demasking parameters $k^j_t$ and $k_d$ (Table 2).

A separate problem in proteolysis modeling is the prediction of the secondary specificity of trypsin for an arbitrary amino acid sequence of any polypeptide substrate. For this, the kinetic hydrolysis parameters for synthetic peptides [32] or statistical data on cleavage or non-cleavage of various peptide bonds in peptides identified in protein hydrolysates [33,34] were used on an experimental basis. The statistical and kinetic approaches give qualitatively similar results for trypsin, indicating, for example, the almost complete absence of charged residues at the P$_2$, P$_1'$ and P$_2'$ positions of the cleaved bonds [30]. However, it is currently difficult to obtain the quantitative estimates of $k^j$ values that are consistent for both approaches. Thus, when modeling the hydrolysis of total casein by chymotrypsin, we used two different sets of hydrolysis constants corresponding to the statistical and kinetic approaches [5].

An ambitious and practically important task is to predict the kinetics of the release of individual biopeptides as a result of proteolysis. By using the demasking parameters and taking into account the contribution of the secondary specificity, the release of the desired peptides can in principle be predicted. The easiest way to do this is to calculate the probabilities of bond hydrolysis using Equation (10) [6]. This formula is valid for uniform demasking of all peptide bonds in the substrate and is not valid when two demasking mechanisms are involved. Since it is not known a priori which peptide bond is involved in the one-step or two-step demasking, it is very difficult to find out when simulating proteolysis. We hope that the data presented here will be useful for predicting the mechanisms of demasking during proteolysis of other protein substrates.

The hydrolysis parameters for various peptide bonds in the protein substrates were previously determined for different proteolysis conditions and methods of determining these parameters [8, 30,31,35]. The sets of rate constants for different proteins hydrolyzed by the same protease can be
used for statistical analysis to investigate the impact of the folding/unfolding state of these proteins on the proteolysis kinetics. Here, we demonstrated theoretically that masking leads to a decrease in the variation between the apparent hydrolysis constants of individual bonds (Table 2). To find experimental confirmation of this, we calculated the relative standard deviations (cv) of the apparent rate constants for trypsic hydrolysis of bonds 25, 28, 32, 48, 97, 99, 105, 107, 113, 169, 176, 183, and 202 in β-casein. Using a set of rate constants of 47, 22, 3.6, 0.45, 5.1, 3.8, 12, 2.2, 1.8, 5.4, 6.1, 8.3, and 1.2 × 10⁻⁵ s⁻¹ [35], a value of 1.26 for cv was found. Using a set of selectivity values of 0.7, 8.2, 0.7, 0.02, 0.6, 15.3, 23.4, 2.7, 1.0, 32.0, 11.4, 2.8, and 0.2% [30], the close value of 1.35 was obtained for cv. Meanwhile, for hydrolysis of β-LG by trypsin, using a set of selectivity values of 13.7, 11, 10.1, 9.9, 9.4, 9.1, 7.4, 6.1, 5.3, 5.0, 3.8, 3.6, 2.9, 1.6, 1.2, 0.2, and 0.1% [30], a lower value of 0.70 was obtained. That is, for globular β-LG with masked peptide bonds, cv was less than for a well-hydrated polypeptide with largely demasked bonds, which is β-casein substrate. A similar result was obtained earlier when comparing the hydrolysis by pancreatic enzymes of the protein isolate of β-LG and total casein [31]. In this case, a set of hydrolysis rate constants was obtained using the amino acid digestibility values for proteolysis in an open reactor (digestion cell), and the width of the distribution of rate constants was estimated from the difference between rate constants for specific and non-specific amino acid residues [31].

With the help of modern HPLC-MS methods, it is possible to determine the concentration and identify most of the peptide fragments obtained during proteolysis. The degree of hydrolysis of the j bond can be calculated by adding up the concentrations of all peptide fragments with the N- and C-termini resulting from cleavage of the jth site. Thus, we can move from describing proteolysis in terms of hydrolysis and formation of peptide fragments to describing proteolysis as the process of cleavage of a set of peptide bonds. This method was used to determine the selectivity parameters [8,30], as well as to analyze product yield curves within the framework of the two-step proteolysis model [12]. For the hydrolysis of whey proteins by *Bacillus licheniformis* protease, it was shown that the yield curves had a characteristic shape, indicating a masking effect [12]. This was found for more than half of analyzed curves, which made it possible to determine the demasking parameters. The use of HPLC-MS to determine a set of kinetic parameters for all hydrolysable bonds in a protein substrate, in our opinion, has great prospects for the modeling proteolysis.

The presence of lag-phases on the hydrolysis curves was observed in publications devoted to the kinetics of peptide release during proteolysis [8,28], although the authors did not focus on this and did not analyze the reasons for the appearance of lag phases. Our study demonstrates the importance of analysis of the delay time in determining the mechanism of demasking and thus detailing the proteolysis model.

4. Materials and Methods

4.1. Materials

β-LG (L3908) from bovine milk, and trypsin from bovine pancreas (TI426) treated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma-Aldrich and used without further treatment. TPCK was used to inhibit the contaminating chymotrypsin activity without affecting the activity of trypsin. Phosphate buffer solution was prepared with doubly distilled water and stored at 4 °C before use. Trypsin solutions in phosphate buffer were freshly prepared by diluting the freeze-dried trypsin with activity of 9.8 BAEE (N-benzoyl-L-arginine ethyl ester) units per µg of trypsin. All other reagents were of analytical grade and obtained from commercial sources.

4.2. Proteolysis Reaction

The protein substrate (β-lactoglobulin) was dissolved in 20 mM phosphate buffer (pH 7.9) at 37 °C by stirring. The enzymatic hydrolysis was carried out in a 1 cm quartz cuvette for fluorescence measurements at constant concentration of substrate and various concentrations of the enzyme. This cuvette with 2ml of β-LG solution was placed on a thermostated holder with a magnetic stirrer. For
example, enzymatic hydrolysis with a substrate concentration of 0.25 g/L was initiated by adding 10 µl of trypsin solution (1 g/L) to provide trypsin concentration in the reaction mixture of 5.0 mg/L. To determine the demasking kinetics, the concentration of trypsin was 0.3, 0.9, 4.5, 5, 10, or 15 mg/L.

4.3. Fluorescence Spectroscopy and Determination of Demasking Kinetics by Fluorescence Measurements

The fluorescence emission during proteolysis was measured using a Perkin-Elmer LS 55 Luminescence Spectrometer (Waltham, MA, USA) at 90° relative to the excitation beam at an excitation wavelength of 280 nm. The spectral bandwidth of the excitation and emission light was set to 10 and 5 nm, respectively. A thermostated cuvette holder was used to keep the sample at 37 °C. The emission spectra were recorded at a scanning speed of 150 nm/min.

To determine the wavelength of fluorescence maximum, we used a parabolic function $I(\lambda) = a\lambda^2 + b\lambda + \text{const.}$ for the approximation of fluorescence spectrum, taking only a small area with a bandwidth of 30 nm. The parabolic function was used to approximate the fluorescence spectrum in a small region of 30 nm around peak maximum, which allowed us the determination of $\lambda_{\text{max}}$—the position of the parabola center at $\lambda_{\text{max}} = -b/2a$ [13].

4.4. Determination of the Demasking Rate Constants and Simulation of the Hydrolysis of Peptide Bonds

The kinetic scheme for the demasking process of Trp$_{19}$ is:

$$S^1_m \xrightarrow{k_f^1} S^1_d$$

(2)

The kinetic scheme for the demasking process of Trp$_{61}$ is:

$$S^2_m \xrightarrow{k_f^2} S^2_d \xrightarrow{k_d} S^2_{dd}$$

(3)

This scheme corresponds to the following concentration functions of time $t$:

$$S^1_m = S^1_0 e^{-k_f^1 t}$$

(4)

$$S^1_d = S^1_0(1 - e^{-k_f^1 t})$$

(5)

$$S^2_m = S^2_0 e^{-k_f^2 t}$$

(6)

$$S^2_d = \frac{S^2_0 k_d}{k_d - k_f^2} (e^{-k_f^2 t} - e^{-k_d t})$$

(7)

$$S^2_{dd} = S^2_0 (1 - \frac{k_d}{k_d - k_f^2} e^{-k_f^2 t} + \frac{k_f^2}{k_d - k_f^2} e^{-k_d t})$$

(8)

Theoretical dependence $\lambda_{\text{max}}$ on $t$ can be calculated as:

$$\lambda_{\text{max}} = \frac{(S^1_m \lambda_1 + S^1_d \lambda_2 + S^2_m \lambda_1 + S^2_d \lambda_2 - S^2_{dd} \lambda_3)}{S_0}$$

(9)

After substituting concentrations $S^1_m, S^1_d, S^2_m, S^2_d$ and $S^2_{dd}$ from Equations (4)–(8) into Equation (9), the following equation is obtained:

$$\lambda_{\text{max}}(t) = \frac{s^1_m}{s_0} \left[ \lambda_2 - (\lambda_2 - \lambda_1) e^{-k_f^1 t} \right] + \frac{s^2_m}{s_0} \left[ \lambda_1 + \frac{\lambda_2 k_f^1}{k_d - k_f^2} e^{-k_f^2 t} - \frac{\lambda_2 k_f^1}{k_d - k_f^2} e^{-k_d t} \right] + \frac{s^2_{dd}}{s_0} \left[ 1 - \frac{k_d}{k_d - k_f^2} e^{-k_f^2 t} + \frac{k_f^2}{k_d - k_f^2} e^{-k_d t} \right]$$

(10)
The initial rate of the increase of $\lambda_{\text{max}}$ at $t = 0$ is:

$$\frac{d\lambda_{\text{max}}}{dt} = k_{d}^{f}(\lambda_{2} - \lambda_{1})$$

(11)

For one-stage demasking, the concentration of the products at the hydrolysis of bond $j$ is:

$$N_{j}(t) = N_{0}\left[1 - \frac{k_{j}e^{-k_{d}t}}{(k_{j} - k_{d})} + \frac{k_{d}e^{-k_{j}t}}{(k_{j} - k_{d})}\right]$$

(12)

For two-stage demasking, the concentration of the products at the hydrolysis of bond $j$ is:

$$N_{j}(t) = N_{0}\left[1 - \frac{k_{d}k_{j}e^{-k_{d}t}}{(k_{d} - k_{j})(k_{j} - k_{d})} - \frac{k_{j}k_{d}e^{-k_{j}t}}{(k_{j} - k_{d})(k_{d} - k_{j})}\right]$$

(13)

For the proteolysis without masking/demasking processes, the concentration of the products at the hydrolysis of bond $j$ is:

$$N_{j}(t) = N_{0}(1 - e^{-k_{j}t})$$

(14)

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