

New Kinetic Approach to the Enzymatic Hydrolysis of Proteins – A Case of Thermolysin-Catalyzed Albumin

Anna Trusek-Holownia, Andrzej Noworyta

Abstract—Using an enzyme of known specificity the hydrolysis of protein was carried out in a controlled manner. The aim was to obtain oligopeptides being the so-called active peptides or their direct precursors. An original way of expression of the protein hydrolysis kinetics was introduced. Peptide bonds contained in the protein were recognized as a diverse-quality substrate for hydrolysis by the applied protease. This assumption was positively verified taking as an example the hydrolysis of albumin by thermolysin. Peptide linkages for this system should be divided into at least four groups. One of them is a group of bonds non-hydrolyzable by this enzyme. These that are broken are hydrolyzed at a rate that differs even by tens of thousands of times. Designated kinetic constants were $k'_F = 10991.4$ L/g h, $k'_M = 14.83$ L/g h, k'_S about 10^{-1} L/g h for fast, medium and slow bonds, respectively. Moreover, a procedure for unfolding of the protein, conducive to the improved susceptibility to enzymatic hydrolysis (approximately three-fold increase in the rate) was proposed.

Keywords—Peptide bond hydrolysis, kinetics, enzyme specificity, biologically active peptides.

I. INTRODUCTION

BOVINE SERUM ALBUMIN (BSA) is an easily accessed protein occurring profusely in whey which is often treated as a waste product. BSA is a rich source of potentially bioactive peptides. On the basis of the information available in the BIOPEP database [1] it is possible to determine the profile of a potential biological activity of this protein. According to it, BSA may be decomposed to more than 85 biologically active peptides of which 42 are responsible for generating bitter taste, 17 possess antihypertensive properties and 15 of them exhibit the activity of a dipeptidyl-peptidase IV inhibitor. In turn, the remaining peptides reveal many other activities, including opioid, immunomodulatory, antithrombotic and antioxidative ones [2]. These are mainly dipeptides, some tripeptides, and one residue consisting of 9 amino acids.

Protein can be hydrolyzed using both chemical and enzymatic methods. Nevertheless, the latter appear more useful because they enable control of peptides formation process due to the possibility of obtaining expected final products, using the range of enzymes of known substrate specificity in different configurations. Furthermore, in most cases, enzymatic digestion is performed under mild conditions

(for example quite low temperature), it does not require the use of harmful chemicals and finally, products obtained in these reactions are generally recognized as safe (GRAS) in accordance with the definition specified by American Food and Drug Administration (FDA).

In the case of enzymatic hydrolysis it is not easy, however, to describe the process kinetics necessary to design continuous processes often integrated with membrane separation. Using a nanofiltration membrane the products of low molecular weight can be easily separated from the fraction of the unreacted substrate and enzyme [3], [4]. This is a multisubstrate consecutive-parallel reaction, where a given protein is decomposed into a number of oligomers being a substrate for a subsequent polymerization process. Resulting products may have significant mass distribution starting from a single unit (amino acid) up to a polymer with only one unit cut-off.

This work is focused on basic hydrolysis of bovine serum albumin to potential bioactive peptides carried out using thermolysin as a biocatalyst. A preliminary approach was taken to create a kinetic model of protein hydrolysis. The essential innovation in relation to the standard form of kinetic equations is the use of an amount (concentration) of bonds in substrate molecules. Each protease is characterized by particular substrate specificity. Hence, each bond is attacked at different rates (or not hydrolyzed at all) by the use of protease. This approach to kinetics is a complete novelty. So far, the hydrolysis of protein was determined only by the total degree of hydrolysis (DH) [5], [6].

II. MATERIALS AND METHODS

A. Materials

Thermolysin from *Bacillus thermoproteolyticus rokko* (T7902), albumin from bovine serum (BSA) as a substrate (Cat.No.05470), α -lactalbumin as a HPLC marker, gel filtration markers kit for protein molecular weights 6,500 – 66,000 Da (MWGF70), vitamin B₁₂ as a HPLC marker, serine as an OPA assay marker, phthaldialdehyde and DL-dithiothreitol to OPA assay were purchased from Sigma-Aldrich (Germany). The other chemicals, all of analytical or HPLC grade, were obtained from POCh (Poland).

B. Enzymatic Hydrolysis

Operating conditions (pH, T, enzyme concentration) were chosen experimentally. The degree of hydrolysis (DH) was determined on the basis of the OPA assay known in the literature [7]. The standard curve for this assay was based on

Anna Trusek-Holownia and Andrzej Noworyta are with the Wrocław University of Technology, Department of Chemistry, Wrocław, Poland (phone: 4871-320-2653; fax: 4871-328-1318; e-mail: anna.trusek-holownia@pwr.wroc.pl, andrzej.noworyta@pwr.wroc.pl).

serine to give the equation of standard curve $A(340\text{nm}) = 1.202 \cdot 10^{-21} [\text{number of NH}_3\text{group/L}]$. Before undergoing the enzymatic hydrolysis of protein, it has been unfolded by maintaining albumin solution (pH7.0) at 60°C for 30min. (time chosen experimentally).

The process of hydrolysis was carried out for 2 days in a thermostated (60°C) stirred (250 rpm) reactor. Bovine serum albumin (BSA) was dissolved in 0.1 M phosphate buffer at pH 7.0. Final concentration of the substrate was 5 g/L, enzyme 0.0087 and 0.0156 g/L. During this process samples were withdrawn from the reactor in certain time intervals, cooled rapidly in an ice water bath and then analyzed by SE-HPLC.

C. Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

Molecular weight distribution of albumin hydrolyzates was analyzed by size exclusion chromatography under isocratic conditions using two columns connected in series: a 300 × 7.8mm BioSep-SEC-s2000 column (Phenomenex, USA) and a 300 × 7.8mm Yarra-SEC-2000 column (Phenomenex, USA) on an HPLC system (Shimadzu, USA).

Samples, filtered through a 0.22µm syringe filter, were eluted with 0.1 M phosphate buffer at pH 6.8 and 25°C for 60 min. The flow rate was 0.036 L/h and peak absorbance was monitored at 214nm. Bovine serum albumin (66.0 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), α-lactalbumin (14.2 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa) and vitamin B₁₂ (1.3 kDa) were run as standards, see Fig. 1. The percentage abundance (area under the peak) of the determined molecular weight fractions was derived from the HPLC software (LabSolutions LC/GC version 5.51, Shimadzu Corporation, Kyoto, Japan).

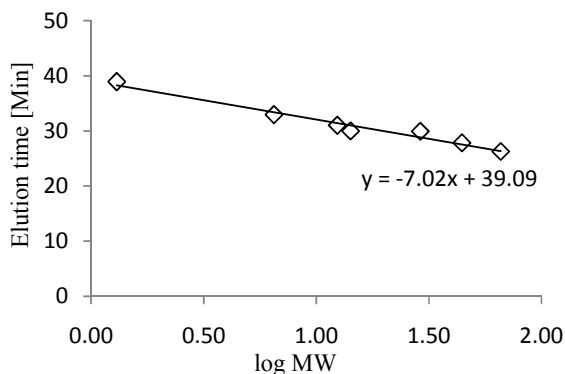


Fig. 1 Standard curve on proteins and peptides HPLC analyses

III. RESULTS AND DISCUSSION

The analysis of thermolysin substrate specificity for albumin (based on BIOPEP) indicates that the enzyme can very deeply cut this substrate. The final product should contain peptides (including active ones) which consist of less than twenty amino acids, fractions of oligopeptides (2-10 amino acids) and individual amino acids. This corresponds to a distribution of the molecular masses in the range of 0.1-2.3 kDa. The dominant fractions are short oligopeptides with

molecular weight ranging from 0.2 to 0.8 kDa and amino acids, cf. Fig. 2.

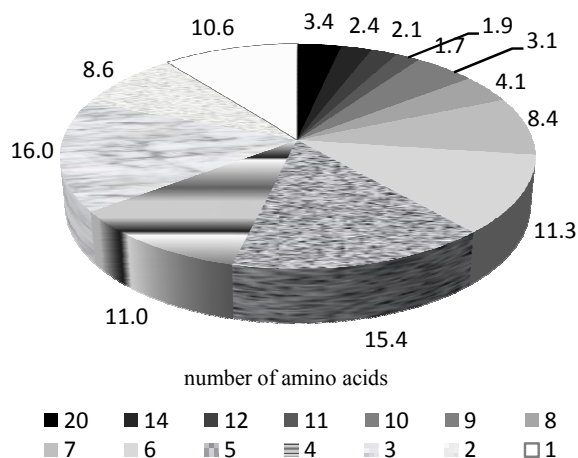


Fig. 2 The percentage of different fractions of peptides after complete BSA hydrolysis using thermolysin as a catalyst

A. Kinetics of Polymer Hydrolysis

In general, depolymerization (polymer hydrolysis) is a very specific reaction defined as a serial-parallel reaction. Starting from a given polymer, a number of oligomers are formed, constituting substrates for further depolymerization. For typical biopolymers like proteins, polysugars containing hundreds of units, the reaction system is very complex. In the simplest case, when the initial macromolecule consists of N identical units, the reaction system contains $N-1$ substrates complicating the description of the reaction kinetics. In the case of enzymatic hydrolysis of proteins it is even much more complex, since particular bonds in the polymer are characterized by different susceptibility to hydrolysis arising from the substrate specificity of the enzyme applied.

In the paper a model of depolymerization kinetics of protein was proposed. It was assumed that the reaction substrate was a mer-mer bond. The activity of each bond determined the reaction rate. Furthermore in the model it was assumed that:

- (1). Each bond was characterized by its activity dependent on the amino acids in P1 and P1' position [8];
- (2). Each mer (amino acid) was of the same molar mass;
- (3). All reactions were of the first order.

Assumption (3) was introduced to provide a further modeling of the process. Assumed average weight of the mer (an amino acid) was equal to 113.2 Da, which resulted from dividing the molecular weight of albumin (66 kDa) by the number of mers contained in it (583 amino acids).

The number of bonds in a given polymer/oligomer (W_i) is about one less than the number of monomer units:

$$W_i = c_i \cdot \frac{1}{M-i} \cdot (i-1) \cdot N_A \quad (1)$$

Thus, the total number of bonds is described as

$$\sum_{i=1}^{i-1} W_i = \frac{1}{M} \cdot N_A \sum_{i=1}^{i-1} c_i \cdot \left(1 - \frac{1}{i}\right) \quad (2)$$

and is the function of time.

In order to determine final products of albumin hydrolysis we analyzed its structure and enzyme specificity. It was found that, with good approximation, among 582 bonds 44 called type *F* bonds were highly reactive, 70 (binding type *M*) were medium reactive, 118 are slowly reactive (binding type *S*) and 310 of bonds *N* were not susceptible to the enzyme attack. According to the substrate specificity of thermolysin, *F* bonds were assigned as binding, wherein in both P1 and P1' amino acid the residues were leucine or valine or phenylalanine or isoleucine or alanine or serine and at position P2' there was no proline. *M* and *S* bonds referred to the above-mentioned amino acids, but requirement of their presence applied only to P1' (+ no Pro at position P2').

Hence, one may write:

$$\frac{d(W_F+W_M+W_S+W_N)}{dt} = k_F \cdot W_F + k_M \cdot W_M + k_S \cdot W_S + k_N \cdot W_N \quad (3)$$

where $k_N = 0$ and whole values of kinetic constants include enzyme concentration, e.g.

$$k_F = k'_F \cdot c_E \quad (4)$$

$$k_M = k'_M \cdot c_E \quad (5)$$

$$k_S = k'_S \cdot c_E \quad (6)$$

Assuming that the reaction of bonds *F* is very fast and it occurs only in the first few minutes, one can write:

$$\frac{d(W_F+W_S+W_N)}{dt} \approx \frac{d(W_F)}{dt} = k_F \cdot W_F \quad (7)$$

hence

$$W_F = W_F(t=0)e^{-k_F t} \quad (8)$$

while the reaction of *M* bonds occurs after the *F* bonds have become depleted and the total rate of hydrolysis is equal to the rate of *M* bonds hydrolysis:

$$\frac{d(W_F+W_M+W_S+W_N)}{dt} \approx \frac{d(W_M)}{dt} = k_M \cdot W_M \quad (9)$$

hence

$$W_M = W_M(t=0)e^{-k_M t} \quad (10)$$

and like *M* bonds, *S* bonds hydrolysis was calculated.

B. BSA Hydrolysis with Thermolysin

The conditions of biocatalysis were selected as a preliminary point of experiments. Enzyme concentration selected on the basis of the OPA assay (albumin concentration of 5 g/L, pH 7.0, 48°C) is presented in percentage in Fig. 3. The range of linear relationship is observed for the enzyme concentration until 0.016g/L.

Thermolysin is known to act at high temperature [9], [10]. However, at 70°C, the substrate used (BSA) underwent coagulation and in this form it was not susceptible to hydrolysis. Therefore, the enzyme activity and stability was examined at lower temperatures (24-60°C) in 0.1 M phosphate buffer at pH 7.0. In order to determine the stability, the enzyme solution was incubated at a given temperature for 15h, after which the standard test for proteolytic activity was conducted. To determine the degree of hydrolysis (DH), the OPA assay was used. The results are shown in percentage in Fig. 4.

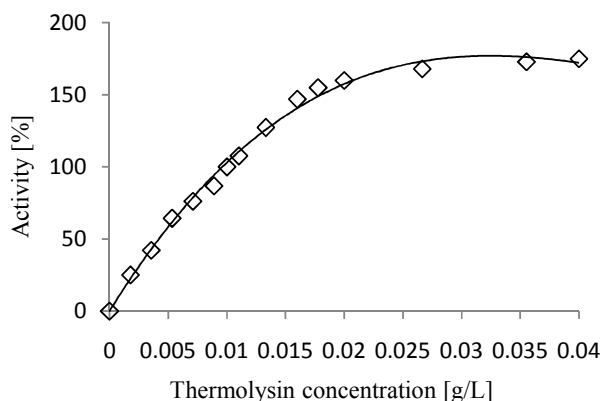


Fig. 3 Proteolytic activity at different enzyme concentrations in percentage according to the activity at $c_E=0.010$ g/L

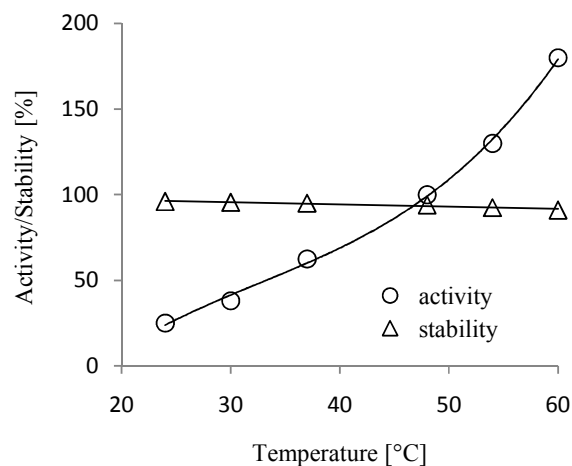


Fig. 4 Activity of thermolysin as a function of temperature in percentage according to the activity at 48°C and stability after 15h incubation

Thermolysin has very good stability in the whole investigated temperature range. Since the activity increased strongly with increasing temperature, the highest possible (due to the substrate) –60°C was selected for further studies.

The study of pH effect showed that the enzyme was relatively active and stable in a wide Ph range, from 7 to 10. For further studies a set pH of 7.0 was taken.

In selected conditions (60°C, pH=7.0) stability of the enzyme was determined. Inactivation constant is equal to 0.006071/h. Thus, the half-life of activity of 114h indicates that the enzyme used is quite stable under the reaction conditions.

Albumin is a globular protein, folded, forming a compact unit, cf. Fig. 5. In such form, the enzyme availability to some bonds is difficult or even impossible. Therefore, the conditions for unfolding the structure were developed. The thermal method was applied. Albumin solution was incubated at 60°C for a period of 10-60min. Then a test was performed on the proteolytic activity. It was observed that the incubation for 30min. was sufficient (no impact of the long incubation on DH). With the unfolding structure the reaction rate increased about three times, cf. Fig. 6.

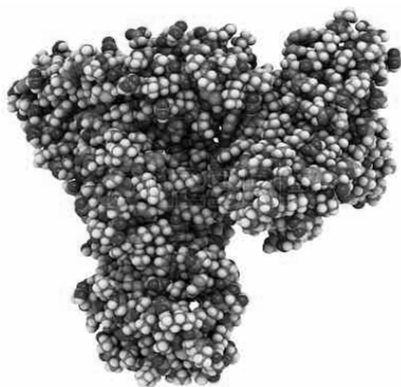


Fig. 5 Structure of albumin [11]

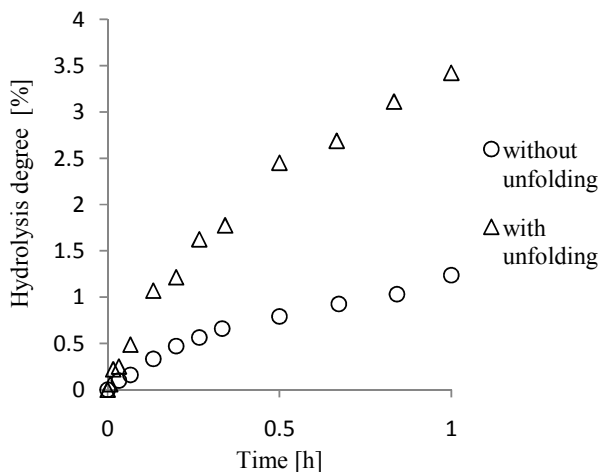


Fig. 6 Effect of unfolding albumin (60°C, 30min) on its susceptibility to hydrolysis Results of the OPA test after hydrolysis (albumin 5 g/L, thermolysin 0.016g/L, 60°C, pH 7.0)

A qualitative and quantitative analysis of the reaction mixture was based on the chromatograms for each sample taken during the process. Due to similar properties of the individual oligomers the experimental data can only provide information on a particular mass of the fraction of oligomers –

it is not possible to obtain analytical values of the concentrations of individual oligomers. An example is shown in Fig. 7.

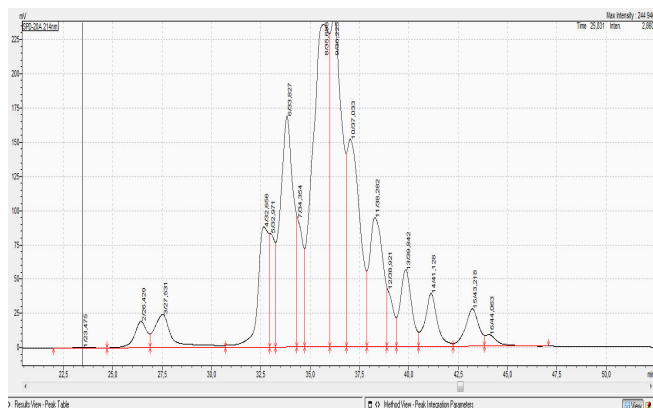


Fig. 7 An example of a chromatogram showing the hydrolysis of protein

The hydrolysis reaction was conducted at an initial concentration of albumin 5 g/L which corresponds to $2.74 \cdot 10^{22}$ bonds per liter of the reaction mixture. The enzyme concentration was 0.0087g/L and 0.0156g/L. Fig. 8 shows a degree of hydrolysis, calculated on the basis of chromatograms, obtained in the reaction at the enzyme concentration of 0.0087g/L.

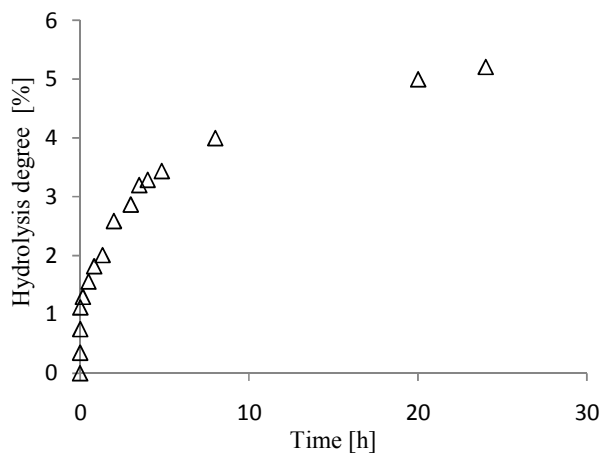


Fig. 8 The hydrolysis of albumin ($c_{s,0} = 5 \text{ g/L}$) in a reaction catalyzed by thermolysin at a concentration of 0.0087g/L (60°C, pH 7.0)

The analysis of samples taken in the first minute of the reaction (Fig. 9) allowed us to designate constant $k_F = 95.625 \text{ 1/h}$ (at $c_E = 0.0087 \text{ g/L}$ from here $k'_F = 10991.4 \text{ L/g h}$), where the number of bonds rapidly available corresponds to $W_F = 2.83 \cdot 10^{20} \text{ 1/L}$ (and not, as assumed in the analysis of substrate specificity of the number $2.07 \cdot 10^{21}$).

Therefore, the concept of M and S bonds, was introduced. These further bonds are hydrolyzed slowly or much more slowly. Their number was calculated on $W_M = 1.79 \cdot 10^{21} \text{ 1/L}$ and $W_S = 2.81 \cdot 10^{21} \text{ 1/L}$. The value of $k_M = 0.1291 \text{ /h}$ ($k'_M = 14.83 \text{ L/g h}$)

was obtained on the basis of data presented in Fig. 10 and k'_S is about 10^{-1} L/g/h (very difficult to calculation because of very long-time reactions).

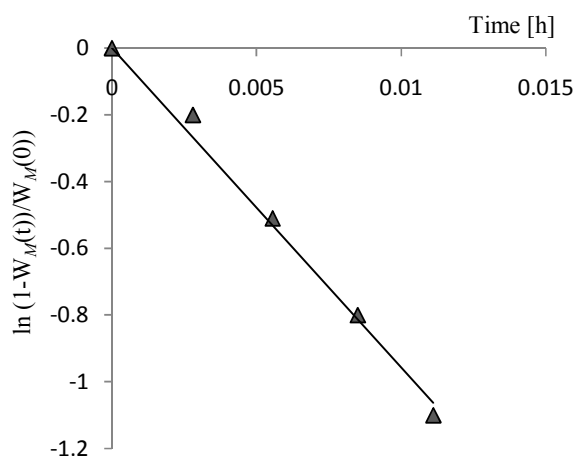


Fig. 9 Fast bonds kinetic constant estimation- k_F (albumin Concentration of 5 g/L, thermolysin of 0.0087g/L, 60°C, pH 7.0)

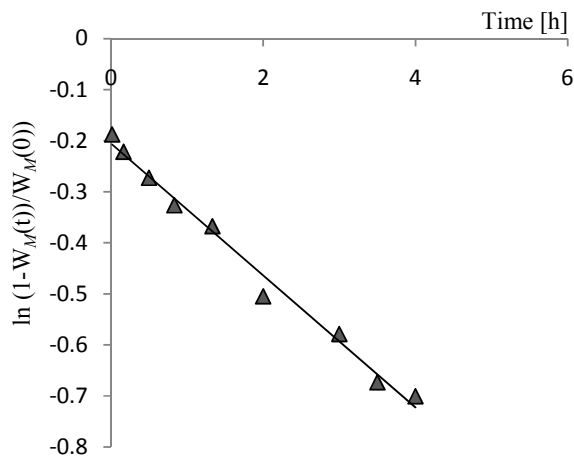


Fig. 10 Kinetic constant estimation of medium bonds - k_M (albumin concentration of 5 g/L, thermolysin of 0.0087g/L, 60°C, pH 7.0)

When all bonds F and M are hydrolyzed DH reaches the value of 7.9%. When the reactions were carried out by one or two days, this value of DH was exceeded indicating that hydrolysis bonds S started, cf. Table I. The fact that at $DH=9.7$ or 11.1% a quantity of albumin and non-hydrolyzed high molecular fraction $>26kDa$ remains in the solution indicates that the hydrolysis of S -bonds begins during the hydrolysis of M bonds. Hence, the calculation of constant k_S requires a significantly great number of measuring points and this task will be continued in the near future. The order of this constant can only be estimated as $10^{-1}L/g/h$.

TABLE I
THE DEGREE OF HYDROLYSIS AND DISTRIBUTION OF MASS FRACTION CORRESPONDING TO THE RELEASE OF PARTICULAR GROUP OF BONDS AND THE STATE AFTER REACTION TIME OF 24 AND 51 H

MW [kDa]	F and M bonds cut [%]	F , M and S bonds cut [%]	$c_E=0.0087g/L$ 24h	$c_E=0.0156g/L$ 24h	$c_E=0.0156g/L$ 51h
66	-	-	1.4	0.9	0.5
26-65.9	-	-	2.5	1.9	1.7
16-25.9	-	-	-	-	-
9-15.9	-	-	5.8	-	-
6.5-8.9	12.5	-	12.7	3.7	2.1
4-6.4	25.7	-	16.9	21.5	19.6
3-3.9	16.5	-	23.3	29.9	30.8
2-2.9	12.3	3.4	18.9	18.7	17.2
1.3-1.9	18.2	4.5	7.6	6.2	6.0
0.7-1.2	5.7	30.5	5.4	10.0	13.4
0.4-0.6	7.0	26.4	3.5	5.2	6.4
<0.4	2.1	35.2	2.0	2.0	2.3
DH	7.6	19.6	5.2	9.7	11.1

IV. CONCLUSIONS

The study confirms validity of the assumption that the rate of hydrolysis of each bond is different and depends mainly on substrate specificity of the enzyme used. However, probably because of the dimensional structure of protein molecules and the tendency of proteins to interact with each other and

creating a floccules, some bonds specifically meant for the hydrolysis are not cut.

The values of the kinetic constant for each group of bonds indicate a significant difference in the rate of hydrolysis. The research will be continued, inter alia, for a different initial concentration of the substrate which should explain the presence of fast (F) bonds. Due to the very slow hydrolysis S bonds, even after two days of the process a very high content

of products with MW>3kDa is present. Therefore membrane separation of reaction system is planned. The use of a membrane with a cut-off of 10-15kDa should retain the biocatalyst in the reactor and substrate molecules untouched by enzyme. Therefore, fractions of products of mass MW<10kDa are expected in the permeate. This stream will be divided by using the second membrane with a cut off of less than 1kDa. Part of oligopeptides present in the permeate will be classified as active peptides or their precursor.

ACKNOWLEDGMENT

This study was supported by the project "Characteristics of the cascade of enzymatic membrane reactors for biopolymers hydrolysis" financed by NCN 011/03/B/ST8/06029.

SYMBOLS

c concentration, g/L
 i the number of mers in the polymer/oligomer
 k kinetic constant of first order reaction, 1/h
 M molecular mass of one mer, g/mol
 N_A Avogadro constant
 t time, h
 W the number of bonds in a given oligomer, 1/L

Index:

E enzyme
 F fast reaction fraction
 M middle fraction
 N non-reactive fraction
 S slow fraction

REFERENCES

- [1] J. Dziuba, A. Iwaniak, M. Niklewicz, Database of Protein and Bioactive Peptide Sequences – BIOPEP 2003, <http://www.uwm.edu.pl/biochemia/index.php/en/biopep>.
- [2] J. Dziuba, L. Fornal, *Biologically Active Peptides and Food Proteins*, WNT, Warsaw, 2009, pp.199-203.
- [3] A. Trusek-Holownia, Production of Protein Hydrolysates in an Enzymatic Membrane Reactor, *Biochem. Eng. J.* vol.39, no.2, pp. 221-229, 2008.
- [4] C. Martin-Orue, S. Bouhallab, A. Garem, Nanofiltration of Amino Acid and Peptide Solutions: Mechanisms of Separation, *J. Membrane Sci.*, vol. 142, pp.225-233, 1998.
- [5] D. Misun, L. Curda, P. Jelen, Batch and Continuous Hydrolysis of Ovine Whey Proteins, *Small Rum. Res.* Vol.79, pp.51-56, 2008.
- [6] C. A. Prieto, A. Guadix, P. Gonzalez-Tello, E. M. Guadix, A Cyclic Batch Membrane Reactor for the Hydrolysis of Whey Protein, *J. Food Eng.*, vol.78, pp.257-265, 2007.
- [7] S. Colombini, G. A. Broderick, M. K. Clayton, Effect of Quantifying Peptide Release on Ruminant Protein Degradation Determined Using the Inhibitor *in vitro* System, *J. Dairy Sci.*, vol. 94, pp.1967-1977, 2011.
- [8] L. Stryer, *Biochemistry*, W. H. Freeman & Co., NY, 1985 pp.24-25.
- [9] A. Trusek-Holownia, Synthesis of ZAlaPheOMe, the Precursor of Bitter Dipeptide on the Two-Phase Ethyl Acetate – Water Systems Catalysed by Thermolysin, *J. Biotechnol.* vol. 102, pp. 153-163, 2003.
- [10] B. Hernandez-Ledesma, M. Ramos, I. Recio, L. Amigo, Effect of Beta-Lactoglobulin Hydrolysis with Thermolysin under Denaturing Temperatures on the Release of Bioactive Peptides, *J. Chromatogr. A*, vol. 1116, no. 1-3, pp. 31-37, May 2006.
- [11] http://pl.123rf.com/photo_16083597