საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 16, №2, 2022
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 16, no. 2, 2022

Biochemistry

Colorimetric Assay to Determine Total Proteolytic Activity

Tamriko Khobelia[*], Kristine Museliani[*], Tea Ninua[*], Edisher Kvesitadze[*]

[*] *Faculty of Chemical Technology and Metallurgy; Educational Center "Biomed", Georgian Technical University, Tbilisi, Georgia*

(Presented by Academy Member George Kvesitadze)

The present paper presents a new method for determining proteolytic activity, using Egg White Modified Protein as a substrate. The given protein is an almost insoluble complex in a buffer solution. At the same time, it is subject to the enzymatic influence. Due to its properties, it has become possible to determine the exact total proteolytic activity compared to other alternative methods. The developed method can be used to determine the total proteolytic activity. *© 2022 Bull. Georg. Natl. Acad. Sci.*

proteolytic activity, EWMP substrate, protease, tricloracetic acid

Determination of protease activity is associated with certain problems. First of all, it is related to the substrate structure where casein is traditionally used. Casein is a polymer and can be exposed to both endo and exo-type enzymes. Depending on the specificity of the protease action, the product may have high molecular weight, low molecular weight, peptide, and/or amino acids [1]. In view of all the above, the problem is to identify the total product obtained by the enzymatic reaction. There are various methods for determining protease activity [2-10], but, due to its availability and simplicity, the most common method for measuring protease activity is considered to be the McDonald's and Chen method [11], where the authors note that TCA (which precipitates a certain fraction of proteins) is added to the product obtained by the enzymatic reaction, and the fragments obtained by hydrolysis, which are soluble in TCA, are measured by the Folin & Ciocalteu assay. The question arises (if casein will be taken as a substrate) will the enzymatic reaction produce an insoluble product in TCA (which is precipitate)? How important is this insoluble product for measuring enzymatic activity? As a result of some experiments and based on the data available in the literature, it was substantiated that casein has an insoluble and soluble protein conglomerate called α, β, and k parts [12]. It should be noted that most proteases act on both soluble and insoluble fragments. Exposure

to proteases results in the formation of soluble fragments from the insoluble fraction, part of which is precipitated with TCA. These precipitated fragments are excluded from the TCA solution since they are precipitated and removed by centrifugation before the measurement. This action causes a false negative result. Solution requires a substrate that does not have a soluble part and is exposed to enzyme hydrolysis. The screening was performed to find out such type of substrate and a practically insoluble substrate was selected to determine this enzymatic activity. The experiment with the selected substrate does not require the addition of TCA, which ignores the above flaws and provides a full manifestation of proteolytic activity.

Materials and Methods

Materials. For the assay, casein was purchased from Sigma Aldrich. Salts for buffer solution Na2HPO4, KH2PO4 were purchased from Alfa Chemical (India). Folin & Ciocalteu's phenol reagent and EWMP substrate were purchased from L.T.D. Biologica. Enzymes with different proteolytic activity were prepared by Educational Center "Biomed", Georgian Technical University. Chymoral produced by Gelenika a.d. was used as a commercial protease

Enzyme samples. For assay different types of biological liquids were used. As a plant source, Galium Verum extract was used. To 10 g of the dried plant was added 100 mL of 50 mM Potassium Phosphate Buffer, pH 7.4, and were homogenized, after which it was centrifuged at 1500 rpm for 15 minutes. For the reaction, 10 µl of the extract was taken. As the fungal origin, a 10 µl liquid culture of Aspergillus Oryzae was used. Concerning a commercial protease 1 tablet of Chymoral which is equal to 121000 USPj was crushed finely and dissolved in 10 ml of 50 mM Potassium Phosphate Buffer, pH 7.4 after which was centrifuged at 1500 rpm for 15 minutes, for the reaction was used 10 µl of supernatant.

Determination of Enzyme-substrate dependence. To determine the Enzyme kinetic parameters were used EWMP substrate concentrations in the range of 2.5-50 mg/ml (1.5-29.6 micromol), for the reaction was used 50 mM Potassium Phosphate Buffer, pH 7.4. The substrates (several concentrations) were mixed with 0.01 ml of enzyme and incubated for 30 min at 25°C. After incubation, the reaction was stopped by placing samples in an ice bath. The reaction mixtures were centrifuged at 1500 rpm using an Eppendorf centrifuge. Then, the supernatants were taken, and Na_2CO_3 and Folin & Ciocalteu reagent (proportion of 2:5:1) were added, and were incubated at 37°C for 30 minutes [13]. Observations were carried out by measuring the OD at 660 nm using a UV–VIS spectrophotometer (Perkin Elmer, USA) against a reagent blank. In our case, for each sample, blank value was taken 30 minute incubated substrate samples without enzyme. After cooling samples we added Enzyme and immediately measure with Folin & Ciocalteu reagent. This type of blanks was taken to exclude enzyme and substrate influence on absorption.

Determination of Enzyme activity in various biological extracts. After the substrate concentration was determined, the protease activity was set. For reaction, 30 mg EWMP substrate and proteases from different biological objects (fungal, plant, and commercial origin) were taken. The reaction was performed analogous to the enzyme-substrate dependence mentioned above. To calculate the activity of the enzyme in units, units per/mL and units per/mg were used in the following formulas:

$$A(U) = \frac{[PR]_{\mu mol} * [V_t]_{ml}}{[R_t]_{min} * [V_m]_{ml}}$$

$$A(U/ml) = \frac{[PR]_{\mu mol} * [V_t]_{ml}}{[R_t]_{min} * [V_m]_{ml} * [V_E]_{ml}} = \frac{A(U)}{[V_E]_{ml}}$$

$$A(U/mg) = \frac{A(U/ml)}{E(mg/ml)}.$$

$[PR]_{\mu mol}$ – Product in micromoles

$[V_t]_{ml}$ – Reaction total volume in milliliters

$[R_t]_{min}$ – Reaction time in minutes

$[V_m]_{ml}$ – Measurement volume of the cuvette in milliliters

$[V_E]_{ml}$ – Volume of Enzyme in mililiters

E (mg/ml) – Enzyme (mg/ml)

Results

Measurement of soluble fragments obtained from the insoluble parts of Casein by Enzymatic reaction. In order to confirm the loss information of casein soluble part gated by enzymatic reaction, we conducted an experiment on the given substrate using the Folin & Ciocalteu assay [13], for which 0,6% casein and a commercial enzyme (Chymoral) 10 µl was taken. The test result obtained is shown in Table 1 according to Scheme 1, where the amount of soluble casein's protein equal of tyrosine per minute was 0.018 micromol, and the results obtained by adding the enzyme to casein was 0.081 micromol tyrosine. The amount of soluble fragments obtained from the insoluble parts of casein by the enzymatic reaction is the ratio of solution 2 minus the rate of solution 1, which corresponds to 0.063 micromols of tyrosine.

Table 1. The amount of Tyrosine measured in the supernatants of solution 1 and solution 2 from Scheme 1 by the Folin&Ciocalteu's method

Name	Tyrosine (micromole) 0 min	Tyrosine (micromole) 30 min	Tyrosine (micromole) Δ
Casein (solution 1)	0,071	0,089	0,018
Casein+Enzyme (solution 2)	0,075	0,156	0,081

Characterization of EWMP substrate. The EWMP was used as a substrate and its insolubility rate was measured. Fig. 1 shows the solubility of a substrate without enzyme exposure over different time ranges. 30 minutes was selected as the incubation time for proteolytic activity.

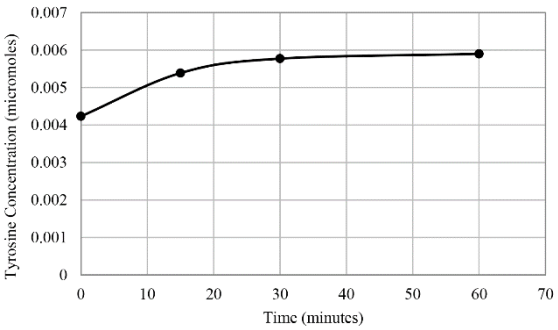


Fig. 1. EWMP Substrate insolubility rate over time.

Enzyme-substrate dependence. The enzyme-substrate dependence on the EWMP substrate was studied, for which the Michaelis-Menten graph was constructed (see Fig. 2), which shows the effect of substrate concentration on the reaction rate [14]. We used commercial enzymes Chymoral 10 µl as the source of the Enzymes.

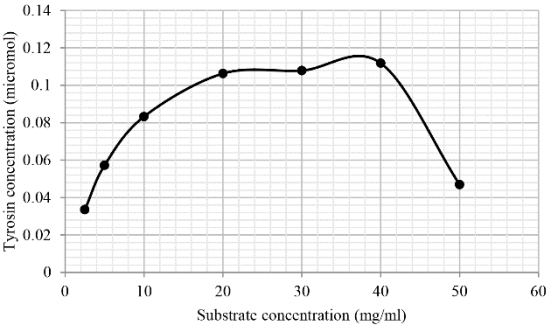


Fig. 2. Dependence of reaction rate and substrate concentration.

Determination of proteolytic activity from different sources of the enzyme. In our experiments, we used the company L.T.D Biologica's product EWMP as substrate, which is characterized by exceptional stability; namely, its solubility is negligible. According to our data for 30 minutes, which is quite sufficient to measure the proteolytic activity, the amount of liberated tyrosine was measured with Folin & Ciocalteu method at 660 nm

was 0,0057 micromol. This indicator is very low, so it may be considered as a baseline or zero point when measuring protease activity. To test the universality of the substrate, we tested protease activities obtained from various biological objects (see Table 2).

Table 2. Activity of Proteases obtained from various biological objects

№	Name	A (U/ml) 0 min	A (U/ml) 30 min	A (U/ml) Δ
1	Commercial protease (Chymoral)	0,228	0,428	0,200
2	Protease of plant origin (Galium verum)	0,240	0,356	0,092
3	Protease of fungal origin (Aspergillus Oryzae)	0,920	1,076	0,152

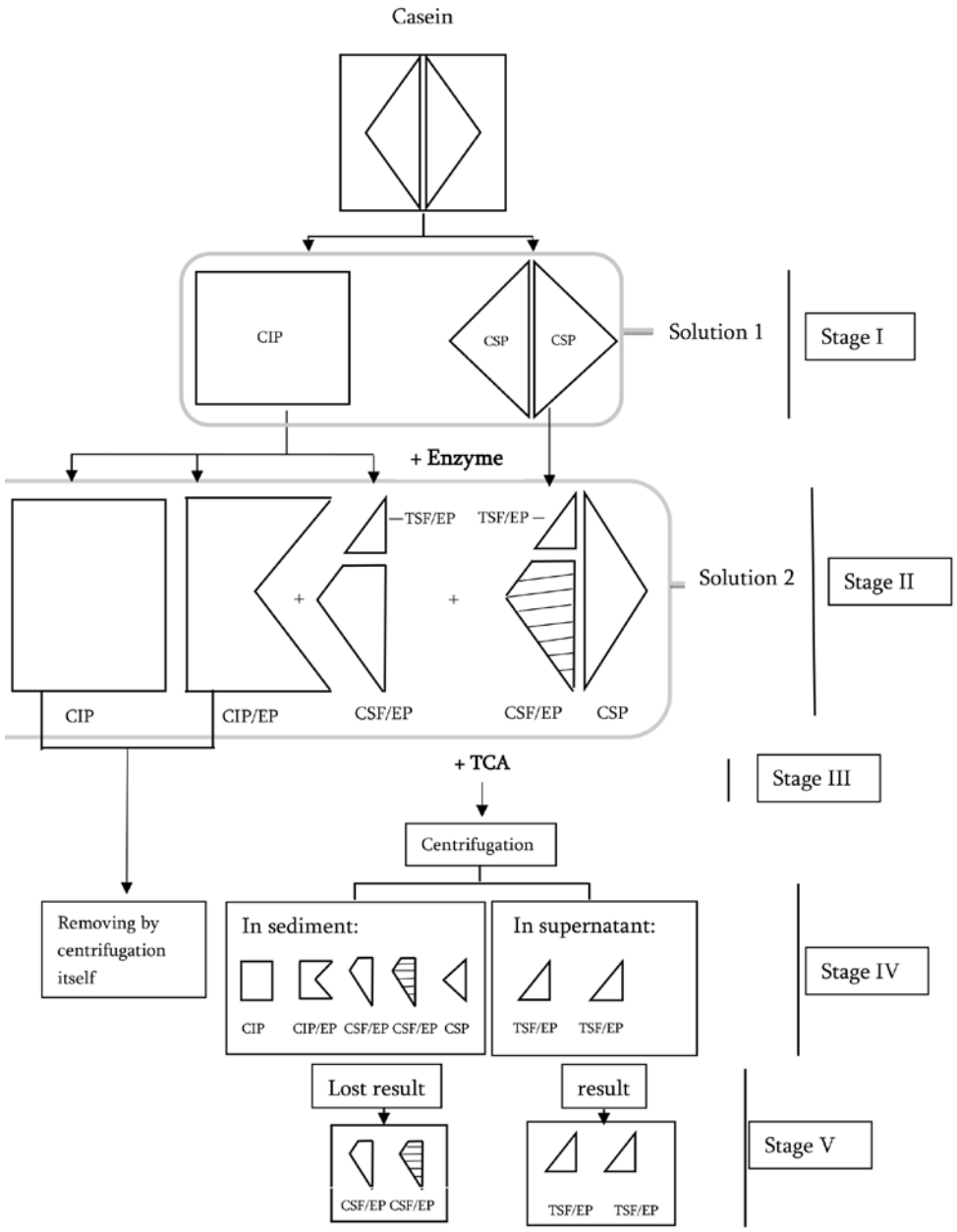
Discussion

The universal scheme 1 shows the degradation of proteins, on the example of casein, as a result of exposure to proteolytic enzymes, consider the case when the substrate is hemoglobin or any other soluble protein. This process in the scheme begins from stage-I, where the soluble protein analog of CSP acts as a substrate, and CIP acts as an insoluble protein, and the path of its degradation is not considered in this case. As shown in Stage II as a result of enzymatic exposure to CSP, TSF/EP, CSF/EP, and CSP are obtained in solution, which by the addition of TCA (Stage III) and centrifugation (Stage IV) are divided into 2 parts. The first part is the CSP and CSF/EP sediment, which are precipitated and are not subjected to measurement, and the second part is the supernatant, TSF/EP, which is a TCA soluble peptide or amino acid, by which the enzymatic activity is determined. However, the enzymatic activity must also be assessed by the fraction shown in the (V stages) lost result – CSF/EP, which is also the product of the enzymatic activity. Therefore, the addition of TCA loses information about the product obtained by enzymatic exposure, which gives a false negative response.

In the presence of an insoluble substrate such as casein, similar enzymatic activity information is also lost. Casein, because of its properties, is most commonly used as substrate to determine protease activity using the Folin & Ciocalteu method. The I-stage in this scheme is the dissociation of casein into two parts, the soluble part – CSP and the insoluble part – CIP (solution 1).

In case of enzyme addition, in stage II we get stage I pristine, **insoluble** (CIP) and soluble parts (CSP), as well as insoluble casein's breakdown products: insoluble CIP/EP, **soluble** TSF/EP and CSF/EP, similarly casein soluble part CSP and decomposition products CSF/EP and TSF/EP. After the addition of TCA, the CSP and CSF/EP are precipitated, as well as the insoluble parts of the casein CIP and CIP/EP. Accordingly, stage IV shows the total precipitant composition **in sediment**, while in lost results (stage V) the sediment part CSF/EP, which is precipitated fragment of the protein obtained by enzymatic exposure that does not participate in or reflect the results. **In supernatants,** as a result of enzymatic action, only low molecular weight protein fragments of TSF/FP (peptides, amino acids) are also obtained, which is reflected in the determination of enzymatic activity.

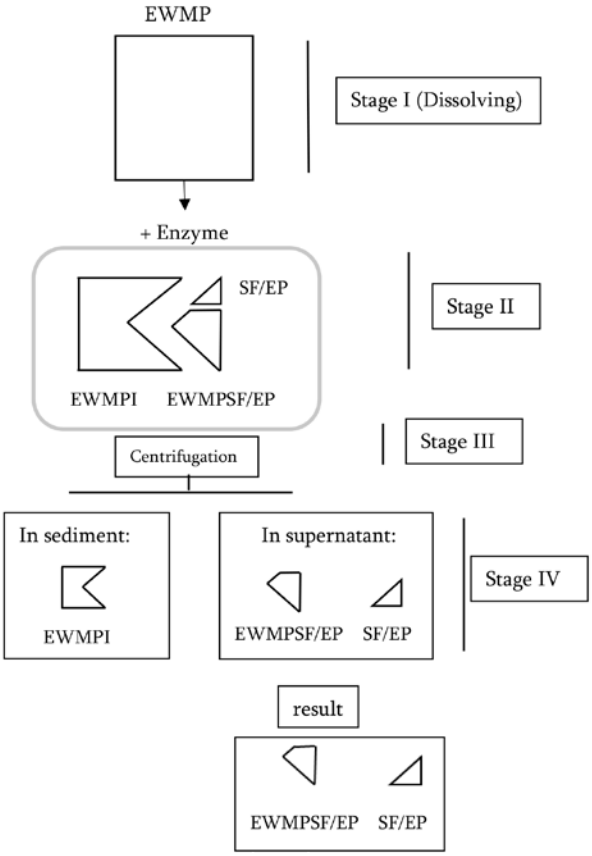
In step V, it is shown that in the lost result, 2 similar fragments from both the insoluble and the soluble substrate are precipitated, which are not reflected by spectral measurement, and consequently, this data lacks the actual activity of the enzyme. Therefore this data is lacking in actual enzyme activity.



Scheme 1. Casein hydrolysis scheme under the influence of proteolytic enzymes.

In view of the above, the problem is the need to add TCA to remove the casein-soluble part (CSP), so that it is not considered as a product of the action of the enzyme. And the addition of TCA to CSP leads to leakage of CSF/EP, which is a product of enzymatic action, and both of which are not reflected in the result.

The aim of further research was to find a substrate that would not give part of the insoluble protein in solution without the addition of enzymes, so there would no longer be a need to add TCA, It must also be subject to enzymatic degradation. To select the substrate, we conducted screening on different substrates and selected the EWMP offered by the company L.T.D. Biologica. As can be seen from Fig. 1, the substrate is stable for 30 minutes without exposure to the enzyme and does not release significant soluble protein fragments into the solution. It was also no longer necessary to add TCA to remove the insoluble part.



Scheme 2. EWMP hydrolysis scheme under the influence of Proteolytic Enzymes.

Scheme 2 shows the solubility rate of EWMP in buffer, where no insoluble part is formed in stage I. After enzyme addition in stage II, EWMP/IP is formed, also by the action of the enzyme, high and low molecular weight fragments of EWMPSF/EP and SF/EP are formed also. Stage III represents centrifugation, as a result of which the insoluble part – EWMPIP is precipitated in sediment, while in the supernatant are shown both low molecular weight peptides (EWMPSF/EP) and amino acids (SF/EP) obtained by enzymatic exposure (stage IV).

If we compare schemes 1 and 2, in the second scheme it is clear how much the analysis of the products obtained by enzymatic exposure is simplified and also the measurement process includes all products gotten during the reaction. Because in case of EWMP, no self-soluble part is formed in stage I, besides, after conducting the enzymatic reaction the insoluble and soluble parts are separated from each other by centrifugation since the soluble part is the whole product obtained by enzymatic action. The precipitate is not a product of the enzymatic reaction, and the product obtained by the enzymatic action remains in the supernatant entirely. This eliminates a false negative response compared to methods that use TCA.

To determine the activity, the next step was to measure the reaction rate at different concentrations of the substrate, for which the maximum concentration of the substrate was 50 mg/ml considering on solubility. However, it must be said that the given concentration caused inhibition of the enzyme, therefore the substrate concentration is inserted in the frame where 20 mg/ml is the lower limit from where the reaction rate begins to decrease due to substrate decrease and 40 mg/ml is the maximum substrate Concentration, the increase of which has

the effect of inhibiting the enzyme. From this point of view, in order not to approach the lower and upper critical limits, we decided to take a substrate with a concentration of 30 mg/ml, which allows us to protect from both adverse effects.

As for pH, the measurement was carried out at pH 7.4, since in a neutral medium both acidic and alkaline proteases exhibit enzymatic activity. The researcher can use different buffer systems by discretion.

To verify the above and determine the universality of the substrate we conducted a determination of protease activity in crude biological liquids, and samples taken from various sourses (see Table 2). The above data allow us to formulate a method for determining total proteolytic activity in the form in which it is presented in the materials and methods we provide.

Abbreviations:

TCA – Trichloroacetic Acid

OD – Optical Density

CIP – Casein Insoluble Part

CSP – Casein Soluble Part

CSF – Casein Soluble Fraction

TSF – Trichloroacetic Acid-Soluble Fraction

TSF/EP – Trichloroacetic Acid-Soluble Fraction/Enzyme Product

CSF/EP – Casein Soluble Fraction/Enzyme Product

CIP/EP – Casein Insoluble Part/Enzyme Product

EWMP – Egg White Modified Protein

EWMPI – Egg White Modified Protein Insoluble

EWMPSF/EP – Egg White Modified Protein Soluble Fraction/Enzyme Product

SF/EP – Soluble Fraction/Enzyme Product

ბიოქიმია

კოლორიმეტრული ანალიზით საერთო პროტეოლიზური აქტივობის დადგენა

თ. ხობელია*, ქ. მუსელიანი*, თ. ნინუა*, ე. კვესიტაძე*

*საქართველოს ტექნიკური უნივერსიტეტი, ქიმიური ტექნოლოგიისა და მეტალურგიის ფაკულტეტი; საგანმანათლებლო ცენტრი ,,ბიომედი'', თბილისი, საქართველო

(წარმოდგენილია აკადემიის წევრის გ. კვესიტაძის მიერ)

მოცემულ სტატიაში წარმოდგენილია პროტეოლიზური აქტივობის განსაზღვრის ახალი მეთოდი, სუბსტრატის სახით გამოყენებულია მოდიფიცირებული კვერცხის ცილა EWMP. მოცემული ცილა წარმოადგენს ბუფერულ ხსნარში თითქმის უხსნად კომპლექსს. ამავდროულად იგი ექვემდებარება ფერმენტულ ზემოქმედებას, მისი ამ თვისების გამო შესაძლებელი გახდა ზუსტი აქტივობის დადგენა სხვა ალტერნატიულ მეთოდებთან შედარებით. შემუშავებული მეთოდი შესაძლოა გამოყენებულ იქნეს საერთო პროტეოლიზური აქტივობის განსასაზღვრავად.

REFERENCES

1. Garg S. K. & Johri B. N. (1999) Proteolytic enzymes. *Thermophilic Moulds in Biotechnology*, 191–218. doi:10.1007/978-94-015-9206-2_8
2. Wilson D. D., Choudhury G. S. (2003) Development and validation of a modified pH-stat method to study arrowtooth flounder (Atheresthes stomias) protease. *J. Aquat. Food Prod. Technol*. 12: 65– 83, DOI: 10.1300/J030v12n01_05
3. Pinto M. R., Schanze K. S. (2004) Amplified fluorescence sensing of protease activity with conjugated polyelectrolytes. *Proc. Natl. Acad. Sci. U.S.A.* 101: 7505– 7510, DOI: 10.1073/pnas.0402280101
4. Peng Y. H., Chen S. Y., Ji H. W., Liu S. C. (2019) Localization of trypsin-like protease in postmortem tissue of white shrimp (Litopenaeus vannamei) and its effect in muscle softening. *Food Chem.* 290: 277– 285, DOI: 10.1016/j.foodchem.2019.03.147
5. Luo Q., Chen D., Boom R. M., Janssen A. E. M. (2018) Revisiting the enzymatic kinetics of pepsin using isothermal titration calorimetry. *Food Chem.* 268: 94– 100, DOI: 10.1016/j.foodchem.2018.06.042
6. Hu J. J., Liu F., Feng N., Ju H. X. (2016) Peptide codes for multiple protease activity assay via high-resolution mass spectrometric quantitation. *Rapid Commun. Mass Spectrom.* 30: 196– 201, DOI: 10.1002/rcm.7631
7. Quinn C. P., Semenova V. A., Elie C. M., Sandra R. S., Carolyn G., Han L., Karen S., Evelene S. C., Schmidt D. S., Elizabeth M. (2002) Specific, sensitive, and quantitative Enzyme-Linked Immunosorbent Assay for human immunoglobulin G antibodies to anthrax toxin protective antigen. *Emerging Infect. Dis*. 8: 1103–1110, DOI: 10.3201/eid0810.020380
8. Kolesnikov A. V., Kozyr A. V., Ryabko A. K., Shemyakin I. G. (2015) Ultrasensitive detection of protease activity of anthrax and botulinum toxins by a new PCR-based assay. *Pathog. Dis.* 74, ftv112 DOI: 10.1093/femspd/ftv112
9. Cheng M., Zhou J., Zhou X., Xing D. (2018) Peptide cleavage induced assembly enables highly sensitive electrochemiluminescence detection of protease activity. *Sens. Actuators, B,* 262: 516– 521, DOI: 10.1016/j.snb.2018.01.191
10. Liu J., Han B., Deng S., Sun S., Chen J. (2018) Changes in proteases and chemical compounds in the exterior and interior of Sufu, a Chinese fermented soybean food, *During Manufacture. Lwt* 87: 210–216, DOI: 10.1016/j.lwt.2017.08.047
11. McDonald C.E. and Chen L.L. (1965) The Lowry modification of the Folin reagent for determination of proteinase activity , *Anal. Biochem*. 10: 175–177
12. Mendoza D. R., Flores R.J. (2020) Casein nomenclature, structure, and association, *Encyclopedia of Dairy Scienses*. 3 **:** 870-880, DOI.org/10.1016/B978-0-12-818766-1.00277-4
13. Cupp-Enyard C. (2008) Sigma's non-specific protease activity assay - casein as a substrate. *J. Visualized Exp*. 19, e899 DOI: 10.3791/899
14. Kenneth A. J. & Roger S. G. (2011) The Original Michaelis Constant: Translation of the 1913 Michaelis–Menten Paper, *Biochemistry*. 50, 39: 8264–8269, doi.org/10.1021/bi201284u

Received April, 2022