KINETICS OF CASEIN HYDROLYSIS BY PEPTIDASE
FROM *Bacillus thuringiensis var. israelensis*

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The kinetics of enzyme reaction is generally studied using the Michaelis-Menten equation and various methods of its linearization. Each method has its advantages and drawbacks, so their comparison for determining the kinetics of new enzymes action is topical. The aim of this work was to study the kinetics of casein hydrolysis catalyzed by new peptidase from Bacillus thuringiensis var. israelensis IMB B-7465 using several methods of enzyme activity assessment and Michaelis-Menten equation linearization. The satisfactory agreement between kinetic constants values obtained by the methods of Lineweaver-Burk, Hanes, Eadie-Hofstee, Cornish-Bowden-Eisenthal was established. The Lineweaver-Burk method was shown to be optimal for determining K_m and V_{max} of casein hydrolysis. Estimation of caseinolytic activity with the use of ortho-phthalic dialdehyde allowed more accurate V_{max} determination compared to the use of Anson and Kunitz methods.

K e y w o r d s: casein hydrolysis, kinetics, linearization methods, activity determination, peptidase, Bacillus thuringiensis var. israelensis.

Studying new enzymes suggests the investigation of important enzyme characteristics such as catalytic property, affinity for substrates and mechanism of action. The main factors that influence the enzyme reaction rate are substrate concentration and chemical nature, medium pH, temperature and the presence of inhibitors. Therefore, the study of the kinetics of enzymatic reactions is important.

The steady-state enzyme kinetics has been studied with the use of the Michaelis-Menten equation and various forms of its linearization, such as Lineweaver-Burk, Hanes, Eadie-Hofstee, Cornish-Bowden-Eisenthal methods [1-5]. Various results were obtained regarding the best linearization method in kinetics studies of protease-catalyzed casein hydrolysis. Devi S.G. et al. in their research of alkaline protease from activated sludge of a tannery, considered the Eadie-Hofstee method to be optimal, while the Lineweaver-Burk equation resulted in significant standard errors [3]. Yoshii et al., studying the kinetics of two proteases, obtained similar values for the kinetic constants, determined by the Lineweaver-Burk and Hanes methods. However, the standard errors were slightly lower in the later [4]. Verma M.K. et al. showed, that the kinetic parameters, determined by the Hanes and Lineweaver-Burk methods had closer values and were 2-fold lower than determined by Eadie-Hofstee one [5]. Thus, comparing various linearization methods for the study of the kinetics of new proteases is important for obtaining most accurate kinetic constants.

Methods for the assessment of the proteolytic enzyme activity can be divided into two main groups: with low or high molecular mass substrates [6]. The method to be chosen depends on the objective of a study and the protease specificity; besides, various methods differ in accuracy, measurement units and duration of research.

This work aimed to determine the kinetic parameters of casein hydrolysis, catalyzed by new peptidase from *B. thuringiensis var. israelensis* IMB B-7465, using different linearization of the Michaelis-Menten equation and three approaches for the enzyme activity assessment.

It was previously shown [7], that the studied peptidase is metal-dependent serine peptidase with the optimum pH 10.0 and temperature 50 °C. It hydrolyzes native protein substrates such as elastin, collagen, fibrin, fibrinogen, casein, gelatin, as well as chromogenic substrates: S2238 (H-D-Phe-Pip-Arg-pNA), S2251 (D-Val-Leu-Lys-pNA), S2302 (HD-Pro-Phe-Arg-pNA), S1040 (Glp-Ala-Ala-Leu-pNA).

Materials and Methods

In our work we studied the *Bacillus thuringiensis var. israelensis* strain IMB B-7465 obtained from the water area of Snake Island (Black Sea) and kindly provided by the staff of the Department of Microbiology, Virology and Biotechnology of Odessa I.I. Mechnikov National University. The strain is registered in the Culture Depository of D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine, No. IMB-7465 as a collagenolytic peptidase producing bacteria [8].

The strain was cultivated in an incubation medium of the following composition (g/l): KH_2PO_4 – 1.6; $MgSO_4 \cdot 7H_2O$ – 0.75; $ZnSO_4 \cdot 7H_2O$ – 0.25; $(NH_4)_2SO_4$ – 0.5; maltose – 1.0; gelatin – 10.0; yeast autolysate – 0.15; pH 6.5-6.7 on a shaking platform at 250 rpm for 24 h, at 28 °C [9]. Erlenmeyer flasks (750 ml) containing 200 ml of the incubation medium were seeded with inoculum in the middle of the exponential phase growth on the same medium.

Peptidase of *B. thuringiensis var. israelensis* IMB B-7465 was isolated from the supernatant after centrifugation of the culture medium (5000 g, 30 min) and by ammonium sulfate (60% saturation) precipitation. The precipitate was collected by centrifugation (5000 g, 30 min), then dissolved in 0.01 M Tris-HCl buffer (pH 7.5) and applied on a column (2.5×40 cm) with a TSK DEAE 650 anion exchanger (M) (Toyosoda, Japan). The proteins were eluted with the same buffer in the NaCl gradient from 0 to 1 M (flow rate 0.5 ml/min). Protein fractions with caseinolytic activity were combined and applied to a column (2.5×40 cm) with a neutral TSK-gel, Toyopearl HW-55 (F-grade) (Toyosoda, Japan). Elution was performed with the same buffer (flow rate 0.85 ml/min).

The molecular mass of the purified native enzyme was determined by size-exclusion chromatography on a column (1.5×25 cm) with Sepharose 6B (Pharmacia, Sweden), equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). Enzyme solution (1 ml) was applied on the column. Elution was performed with the same buffer (flow rate 0.3 ml/min). To determine the molecular mass, a calibration curve was plotted using marker proteins (Pharmacia, Sweden): bovine serum albumin (67.0 kDa), proteinase K (28.9 kDa), trypsin (24.0 kDa), lysozyme (14.5 kDa).

Denaturing electrophoresis (SDS-PAGE) was performed by the Laemmli method [10]. The studied enzyme preparation was dissolved in a sample buffer (0.5 M Tris-HCl with 2-mercaptoethanol, pH 8.8) containing 10% sodium dodecyl sulfate, 20% glycerol and 0.001% bromophenol blue), then boiled for 1 min, and applied to a gel (50-100 µg per well). Electrophoresis was performed in a 5% stacking and 12% separating acrylamide gels with constant power supply 30 mA. After electrophoresis, the gel was stained with Coomassee G-250. The following protein markers were used (Pharmacia, Sweden): bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), a soya bean trypsin inhibitor from soya (20.0 kDa), α-lactalbumin (14.4 kDa).

During purification, the total peptidase activity was measured by the modified Anson assay [11], based on the quantitative determination of tyrosine formed during casein hydrolysis under the peptidase activity. The culture medium supernatant (0.5 ml) and a 1% casein solution (0.5 ml) were added to the test tube. The control tube contained the culture medium supernatant (0.5 ml) and a 4% solution of trichloroacetic acid (TCA) (2 cml). The samples were incubated on a water bath at 37 °C for 30 min, and then 2 ml of 4% trichloroacetic acid (TCA) solution was added to the test tube. The tubes were kept at room temperature for 20 min and then centrifuged (10,000 g, 5 min). 2.5 ml of 0.5 M Na_2CO_3 solution and 0.5 ml of diluted Folin's reagent (1:3) were added to 0.5 ml of the supernatant, and the mixture was kept at room temperature for 20 min. The cleavage products were determined at 670 nm on spectrophotometer SF-26. The ability of the enzyme to convert casein into non-precipitating TCA-form in an amount, corresponding to 1 µmol of tyrosine for 1 min at 37 °C, was taken as an activity unit.

The total protein level was determined by the modified Lowry assay (Hartree-Lowry method) [12]. Caseinolytic activity was determined by the spectro-

photometric Kunitz method at 280 nm [13], by the modified Anson method with the Folin-Ciocalteu's reagent [11], and by determining the amino groups released during proteolysis, using *o*-phthaldialdehyde (OPA) [14]. The latter method was modified by increasing the reagent concentration (4-fold) in 1.0 M H_3BO_3/NaOH buffer (pH 9.2). This modification enabled us to significantly improve the sensitivity of the method.

The kinetics of casein hydrolysis in the presence of isolated peptidase was studied at 37 °C in 0.017 M Na-phosphate buffer, pH 7.5, by measuring the initial rates of the substrate hydrolysis. The amount of enzyme was 12.5 µg of protein. K_M and V_{max} values were determined within the ascending part of the curve (5 points) relating the initial reaction rate and the substrate concentration. The data obtained were linearized by Hanes, Lineweaver-Burk, Eadie-Hofstee, Cornish-Bowden-Eisenthal methods [15]. The chosen conditions were for a substrate conversion of no more than 5% when using the lowest enzyme concentration.

Casein concentration range was 0.31-10.0 g/l. Statistical data analysis was performed using Statistica software with the implementation of Student's *t*-test. The results were considered significant at $P < 0.05$. To calculate the standard errors of the kinetic constants, first we calculated the value and standard errors of the linear regression coefficients of the ascending part of the kinetic curve, then the K_M and V_{max} values and their standard errors were calculated [16].

Results and Discussion

Peptidase from *B. thuringiensis var. israelensis* IMB B-7465 was isolated by multistep purification using classic methods of protein chemistry: fractionation of the culture supernatant with ammonium sulfate (60% saturation), ion-exchange chromatography and exclusion chromatography. It was shown (Fig. 1), that when separating the complex enzyme preparation using ion-exchange chromatography with a NaCl gradient from 0 to 1 M on a column with an anion exchanger TSK Toyopearl DEAE 650 (M), there was one major peak of peptidase with caseinolytic activity (No. 1) at 0.1 M NaCl. This allowed us to remove some protein contaminants from the enzyme preparation.

Isolated fraction 1 was analyzed using exclusion chromatography on TSK Toyopearl HW-55 (F-grade). The studied peptidase was detected in fraction 2 when 0.01 M Tris-HCl buffer (pH 7.5) was used (Fig. 2).

The homogeneity of the enzyme from *B. thuringiensis var. israelensis* IMB B-7465 and its subunit structure were confirmed by SDS-PAGE (Fig. 3). To
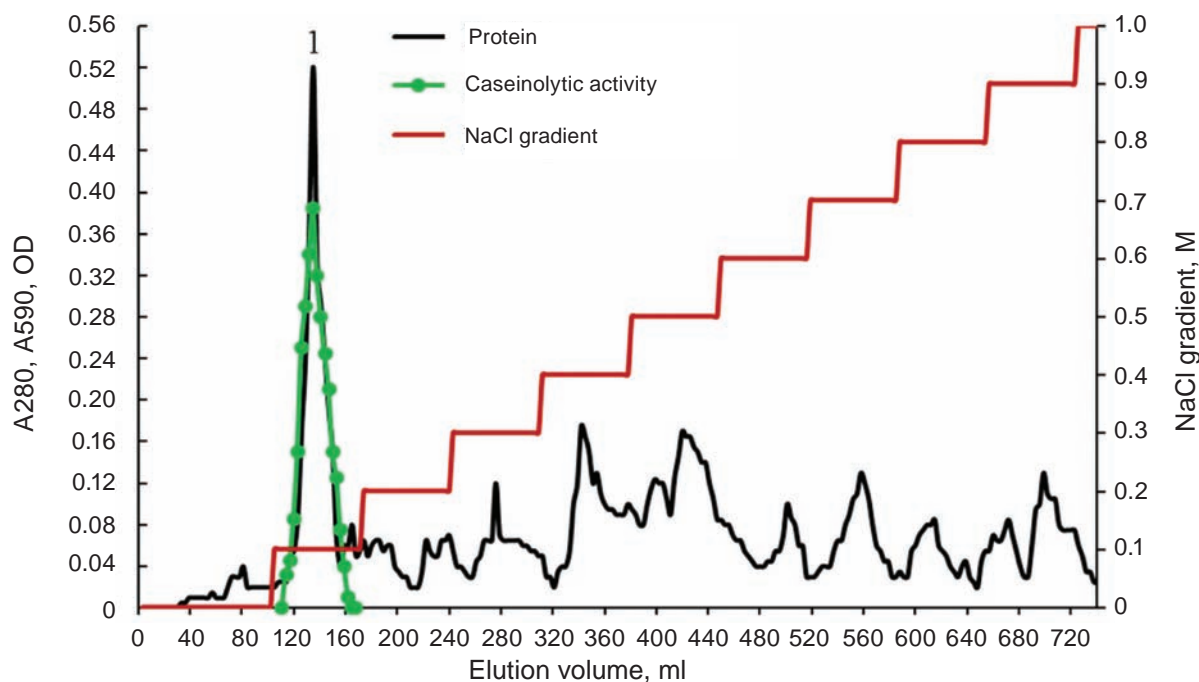

Fig. 1. Elution profile of the complex enzyme preparation from B. thuringiensis var. israelensis IMB B-7465 on TSK Toyopearl DEAE 650 (M) with step NaCl gradient
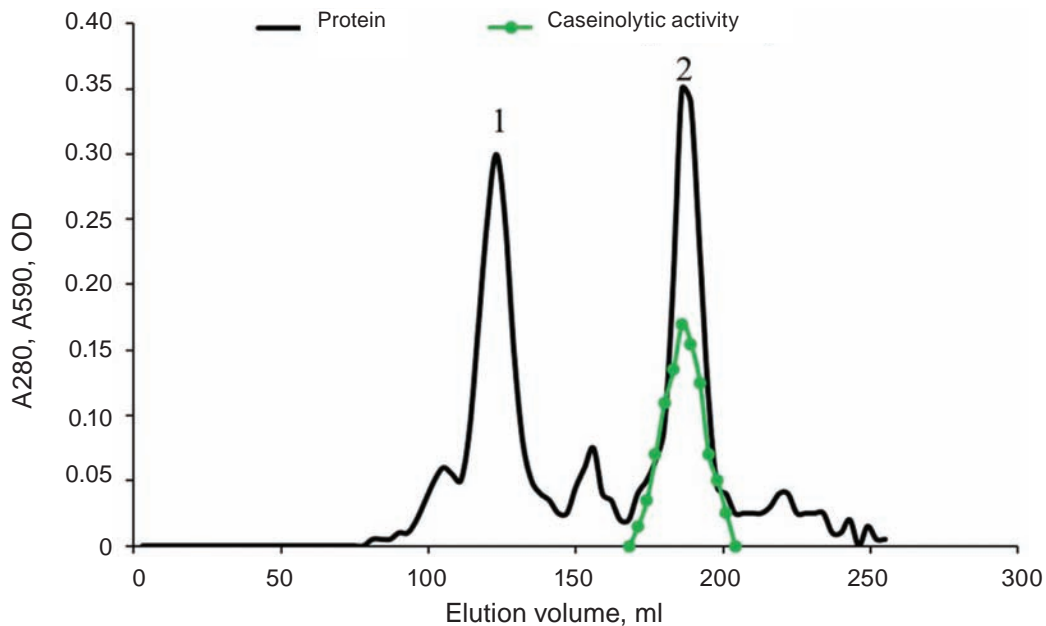
Fig. 2. Elution profile of fraction 1 of the complex enzyme preparation from B. thuringiensis var. israelensis IMB B-7465 on TSK Toyopearl HW-55
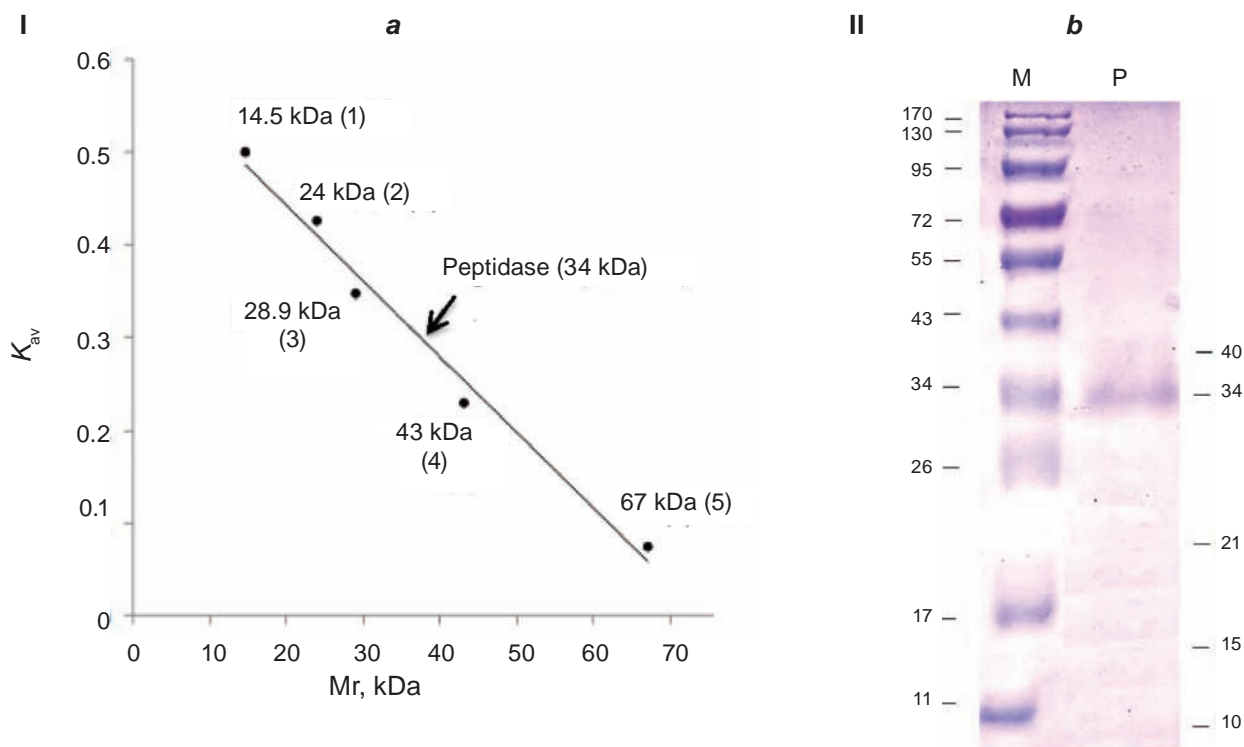

*Fig. 3. Determination of the molecular mass of peptidase from B. thuringiensis var. israelensis IMB B-7465 in native (**I**) and denaturing (SDS-PAGE) (**II**) conditions: **a** – size exclusion chromatography on Sepharose 6B (calibration plot); K_{av} – distribution coefficient; protein markers: lysozyme (1), trypsin (2), proteinase K (3), peroxidase (4), bovine serum albumin (5); **b** – SDS-PAGE M - protein markers; P - peptidase from B. thuringiensis var. israelensis IMB B-7465*

determine the molecular mass, we used native and denaturing conditions, in particular, analytical exclusion chromatography on Sepharose 6B and SDS-PAGE. The column was calibrated with the protein molecular mass markers. The partition coefficient (K_{av}) was calculated as described in [17].

Analysis under native and denaturing conditions showed, that the molecular mass of the peptidase from *B. thuringiensis var. israelensis* IMB B-7465 is ~ 34 kDa (Fig. 3).

A purified preparation of new peptidase (34 kDa) with a total proteolytic activity of 0.21 U/ml was isolated from *B. thuringiensis var. israelensis* IMB B-7465 [7].

In our work, we studied the kinetics of casein hydrolysis catalyzed by peptidase from *B. thuringiensis var. israelensis* IMB B-7465 (Fig. 4).

At relatively low substrate concentrations, the reaction rate increased proportionally. When substrate concentration increased, the rate value approached its limit, and then began to decrease. That is, in the certain casein concentration range, the enzyme is inhibited by the substrate.

The kinetic constants were measured within the ascending part of the curve relating the initial reaction rate and the substrate concentration. The obtained data were then linearized.

A comparative analysis of the linearization methods of the Michaelis-Menten equation revealed that K_m parameters, obtained for the selected methods of the enzyme activity assay (Kunitz method, modified Anson method, NH_2-groups determination with OPA reagent) using Hanes plot (Fig. 5) have similar values (Table 1).

The obtained K_m values are quite low compared to most of microbial proteases (0.3-19.2 g/l) [1, 3, 18, 19].

The V_{max} value, determined with the OPA method, was 1.7-fold greater than that with modified Anson method (Table 1). The data obtained indicate that not all the substrate fragments, generated during hydrolysis, contain tyrosine or other aromatic amino acids, which react with the Folin-Ciocalteu's reagent (when using Anson assay). However, almost all amino acids or peptides react with OPA. In the Kunitz method, V_{max} value is measured in relative proteolytic units. Therefore, it cannot be compared with the other two methods for the caseinolytic activity assay.

For the Hanes plot, high degree approximation coefficients were obtained ($R^2 = 0.999$); however, the standard errors of K_m values were fairly high (Table 1).

A satisfactory agreement between the K_m and V_{max} parameters (Fig. 6, Table 2) was observed, when using the Lineweaver-Burk method, compared to the Hanes method. The V_{max} values measured using the OPA method for enzyme assay also found to be greater than determined by the Anson method.

The Lineweaver-Burk method demonstrated slightly lower approximation coefficients (0.977-0.984) compared to the Hanes method; but with significantly lower standard errors of K_m, (for the OPA
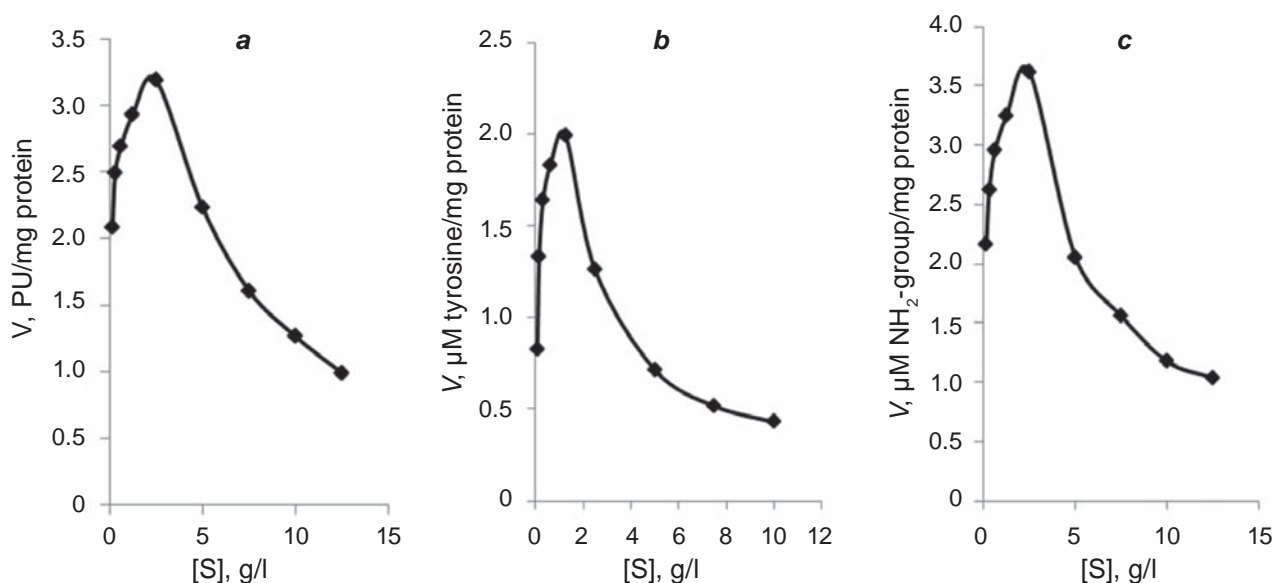

Fig. 4. Kinetics of casein hydrolysis catalyzed by isolated peptidase (proteolytic activity by Kunitz method (**a**), modified Anson method (**b**), OPA method (**c**))
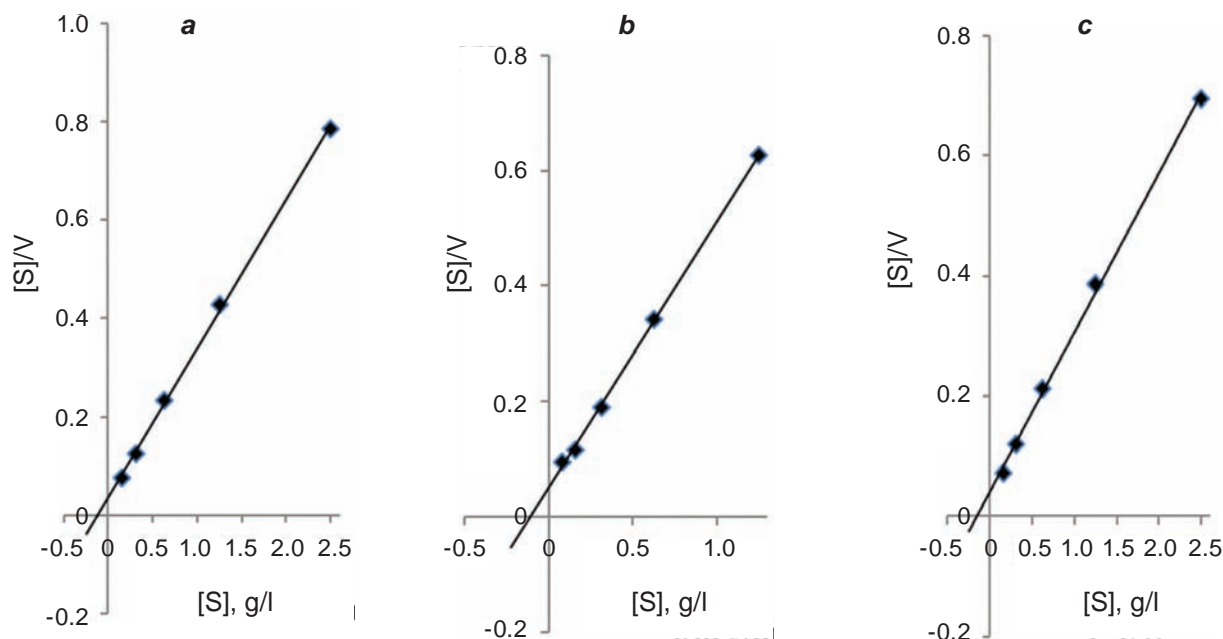
*Fig. 5. Determination of the kinetic parameters of casein hydrolysis catalyzed by peptidase by Hanes plot (proteolytic activity by Kunitz method (**a**), modified Anson method (**b**), OPA method (**c**))*

T a b l e 1. *Kinetic parameters of casein hydrolysis, catalyzed by peptidase from Bacillus thuringiensis, determined by the Hanes method*

Caseinolytic activity assay	K_m, g/l	V_{max}	R^2
Kunitz method	0.119 ± 0.027	3.31 ± 0.06, PU/mg protein	0.999
Modified Anson method	0.111 ± 0.012	2.17 ± 0.03, µmol tyrosine/mg protein per min	0.999
OPA method	0.151 ± 0.033	3.77 ± 0.09, µmol NH_2-group/mg protein per min	0.999

and Kunitz methods) and relatively low errors of V_{max} values.

The kinetic constants K_m and V_{max} obtained by Eadie-Hofstee method also were close to the values, determined by other linearization methods (Fig. 7, Table 3).

However, when using the Eadie-Hofstee method, we obtained low approximation coefficients (0.950-0.956) and comparable values of standard errors for kinetic parameters, obtained by the Lineweaver-Burk method.

The obtained kinetic data were analyzed by the Cornish-Bowden-Eisenthal method. The results were checked for belonging to one experimental sample using the "V" criterion and for normal distribution. The calculated ratios of the asymmetry and excess to their standard errors were below 3, that is, the data are normally distributed. Therefore, they could be processed by Student's test. The K_m values, obtained

for three methods of caseinolytic activity assay, were found to have close values (Table 4). However, the standard errors of K_m constants, when using the OPA and Kunitz methods for the enzyme activity assays, were greater, compared to those by Lineweaver-Burk.

Thus, in our work, we studied the kinetics of casein hydrolysis catalyzed by a new peptidase (34 kDa) from *Bacillus thuringiensis var. israelensis* IMB B-7465. A satisfactory agreement between values of the kinetic constants obtained by different linearization methods of the Michaelis-Menten equation was found. In our study, the Lineweaver-Burk method was shown to be optimal for determining the kinetic parameters, as it is characterized by the lowest standard errors of K_m and V_{max} values. The advantage of the OPA method of the enzyme activity assay over other tested methods is the interaction with almost all products of proteolysis, which enables to determine the V_{max} constants more accurately.
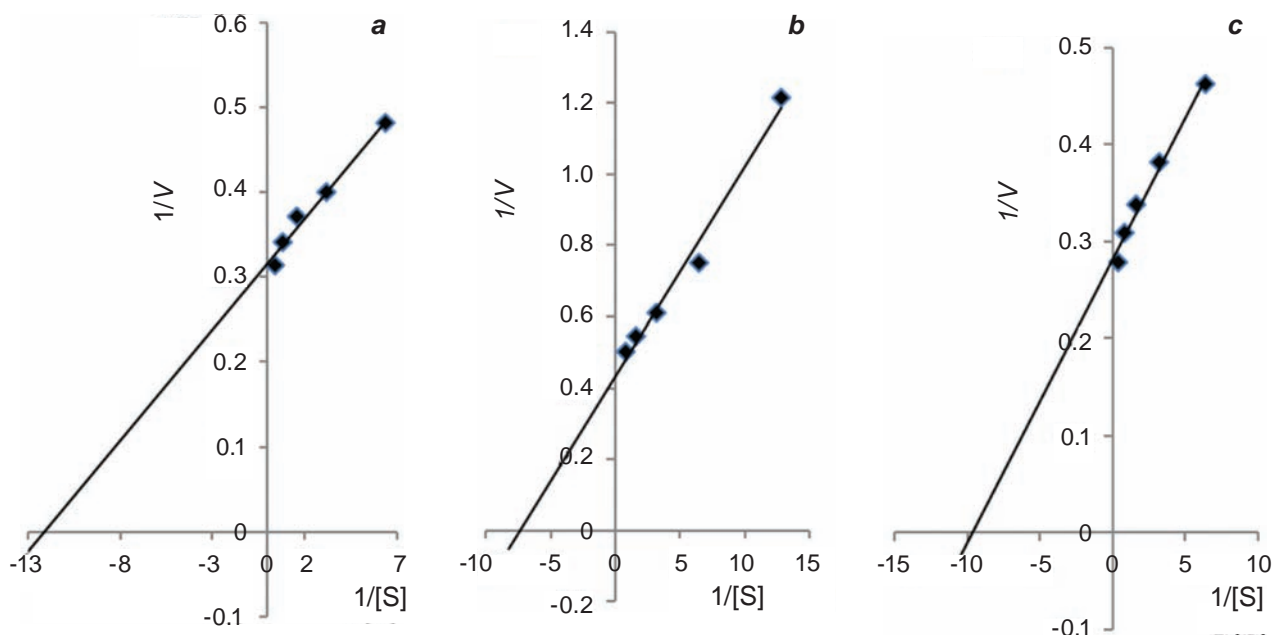
*Fig. 6. Determination of kinetic parameters of casein hydrolysis, catalysed by peptidase, by the Lineweaver-Burk plot (proteolytic activity by the Kunitz method (**a**), modified Anson method (**b**), OPA-method (**c**))*
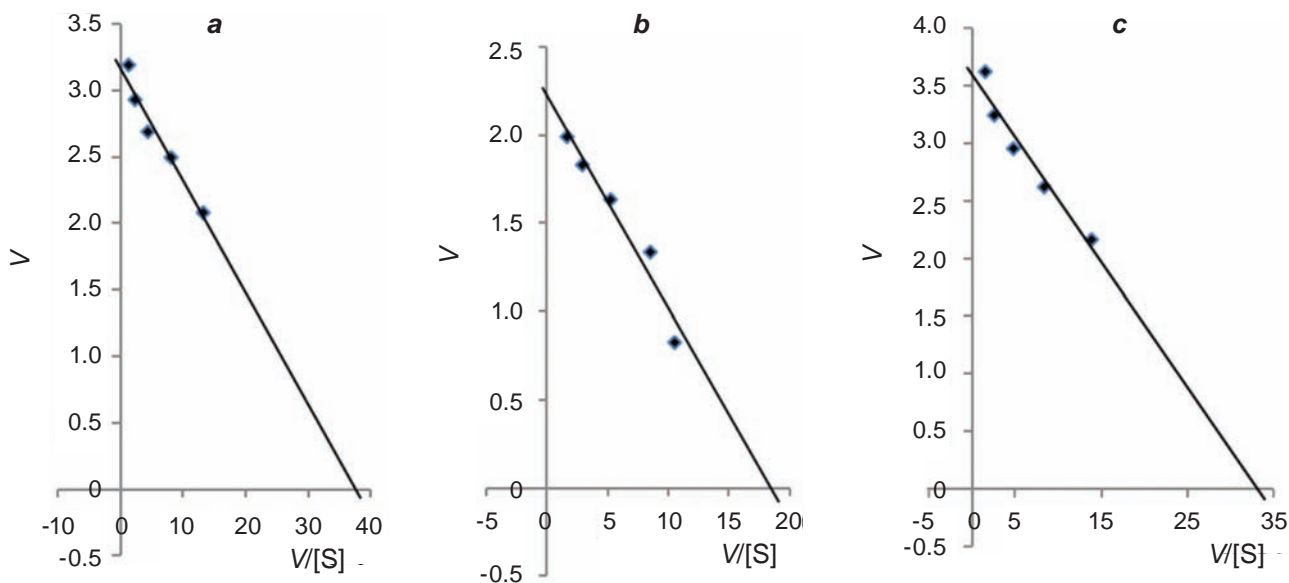

*Fig. 7. Determination of the kinetic parameters of casein hydrolysis, catalyzed by peptidase, by the Eadie-Hofstee method (proteolytic activity according to the Kunitz method (**a**), modified Anson method (**b**), OPA method (**c**))*

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

T a b l e 2. *Kinetic parameters of casein hydrolysis, catalyzed by peptidase from Bacillus thuringiensis, determined by Lineweaver-Burk*

Caseinolytic activity assay	K_m, g/l	V_{max}	R^2
Kunitz method	0.082 ± 0.009	3.16 ± 0.08, PU/mg protein	0.977
Modified Anson method	0.136 ± 0.017	2.31 ± 0.15, μmol tyrosine/mg protein per min	0.979
OPA method	0.104 ± 0.012	3.55±0.10, μmol NH_2-group/mg protein per min	0.984

T a b l e 3. *Kinetic parameters of casein hydrolysis, catalyzed by peptidase from Bacillus thuringiensis, determined by the Eadie-Hofstee method*

Caseinolytic activity assay	K_m, g/l	V_{max}	R^2
Kunitz method	0.084 ± 0.011	3.17 ± 0.08, PU/mg protein	0.956
Modified Anson method	0.12 ± 0.015	2.22 ± 0.10, μmol tyrosine/mg protein per min	0.950
OPA method	0.109 ± 0.014	3.59 ± 0.11, μmol NH_2-group/mg protein per min.	0.953

T a b l e 4. *Kinetic parameters of casein hydrolysis, catalyzed by peptidase from Bacillus thuringiensis, determined by the Cornish-Bowden-Eisenthal method*

Caseinolytic activity assay	K_m, g/l	V_{max}
Kunitz method	0.128 ± 0.023	3.23 ± 0.06, PU/mg protein
Modified Anson method	0.111 ± 0.010	2.15 ± 0.02, μmol tyrosine/mg protein per min
OPA method	0.139 ± 0.024	3.59 ± 0.11, μmol NH_2-group/mg protein per min

КІНЕТИКА ГІДРОЛІЗУ КАЗЕЇНУ ЗА ДОПОМОГОЮ НОВОЇ ПЕПТИДАЗИ *Bacillus thuringiensis var. israelensis*

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Дослідження кінетики дії ензимів здійснюють, використовуючи рівняння Міхаеліса–Ментен і різні форми його лінеаризації, однак кожен з методів має свої переваги і недоліки, тому їх порівняння для визначення кінетики нових ензимів є актуальним. Метою цієї роботи було вивчення кінетичних особливостей гідролізу казеїну новою пептидазою *Bacillus thuringiensis var. israelensis* ІМВ В-7465 за допомогою різних методів лінеаризації рівняння Міхаеліса–Ментен із використанням декількох способів визначення активності ензиму. Встановлено задовільне узгодження значень кінетичних констант, одержаних методами Лайнуївера–Берка, Хейнса, Іді–Хофсті, Корніш–Боуден–Ейзенталя. Оптимальним для визначення K_m і V_{max} є метод Лайнуївера–Берка. Метод дослідження казеїнолітичної активності за допомогою *о*-фталевого діальдегіду (*о*-ФДА) порівняно з модифікованим методом Ансона і Кунітца дозволяє вірогідніше визначити V_{max} реакції.

К л ю ч о в і с л о в а: гідроліз казеїну, кінетика, методи лінеаризації, визначення активності, пептидаза, *Bacillus thuringiensis var. israelensis.*

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