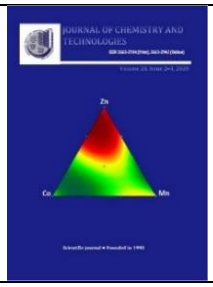
Journal of Chemistry and Technologies, 2020, *28*(3), 269-277

Journal of Chemistry and Technologies

pISSN 2663-2934 (Print), ISSN 2663-2942 (Online)

journal homepage: http://chemistry.dnu.dp.ua



UDC 633.31:66.097.8

ISOLATION OF ALFALFA SEED TRYPSIN INHIBITOR USING AFFINITY CHROMATOGRAPHY

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Received 13 June 2020; accepted 26 November 2020; available online 9 January 2021

Abstract

A protein inhibitor of trypsin of the cultivar Eva alfalfa seeds was isolated and characterized. It has significant antiproteolytic activity and is promising for the creation of compositions that are designed to correct nutrition in various conditions, accompanied by increased activation of proteolytic enzymes. The stages of purification of the trypsin inhibitor from alfalfa seeds included: extraction of 0.05 M borate buffer (pH 7.6), fractionation of the protein component of the extract with ammonium sulfate, followed by dialysis of the fraction between 75% and 100% saturation and affinity chromatography on a biospecific sorbent trypsin - sepharose 4B. Calculations showed that from 100 g of alfalfa seeds of the cultivar Eva, 67.14 mg of trypsin inhibitor was obtained, the inhibitory activity of which is 27.6 IU / mg protein. Experimental data show that the obtained inhibitor reduces trypsin activity by 63.05 % with a weight ratio of inhibitor: enzyme of 1: 1. It is known from literature that a soybean trypsin inhibitor reduces trypsin activity by 30 %. The isolated trypsin inhibitor has significant inhibitory activity, the value of which is higher than the inhibitory activity of the plant trypsin inhibitor from soybeans. Affinity chromatography allowed us to obtain a pure, homogeneous trypsin inhibitor, which has significant antiproteolytic activity, which can be compared with the antiproteolytic activity of known plant trypsin inhibitors. From the obtained experimental data, it follows that both, the obtained inhibitor and the inhibitor, which is part of a commercial preparation, effectively reduce trypsin activity, slightly inhibit chymotrypsin activity, and do not affect α-amylase and protease activity. The above data indicate that the trypsin inhibitor of alfalfa seeds of the cultivar Eva is not a bifunctional inhibitor, since it does not affect the activity of amilolytic enzymes. The isolated inhibitor belongs to proteins with a high degree of hydrophobicity. The composition of amino acids, the side chains of which can take part in the formation of hydrogen bonds, is 25 % of the total number of amino acid residues of the alfalfa seed trypsin inhibitor. For STI, this value is 23%.

Keywords: alfalfa seeds, extraction, affinity chromatography, trypsin inhibitor, amino acid composition, molecular weight.

ВИДІЛЕННЯ ІНГІБІТОРА ТРИПСИНУ НАСІННЯ ЛЮЦЕРНИ З ДОПОМОГОЮ АФФІННОЇ ХРОМАТОГРАФІЇ

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Анотація

Виділено та охарактеризовано інгібітор білка трипсину сорту насіння люцерни Eva. Він має значну антипротеолітичну активність і є перспективним для створення композицій, призначених для корекції харчування в різних умовах, що супроводжується посиленою активацією протеолітичних ферментів. Етапи очищення інгібітора трипсину з насіння люцерни включали: екстракцію 0.05 М боратного буфера (pH 7.6), фракціонування білкової складової екстракту сульфатом амонію з подальшим діалізом фракції від 75 % до 100% насичення та афінна хроматографія на біоспецифічному сорбенті трипсин – сефароза 4В. Розрахунки показали, що із 100 г насіння люцерни сорту Ева отримано 67.14 мг інгібітора трипсину, інгібуюча активність якого становить 27.6 МО / мг білка. Експериментальні дані показують, що отриманий інгібітор знижує активність трипсину на 63, 05% при ваговому співвідношенні інгібітор : фермент 1 : 1. З отриманих експериментальних даних випливає, що як отриманий інгібітор, так і інгібітор, який входить до складу комерційного препарату, ефективно знижують активність трипсину, дещо пригнічують активність хімотрипсину та не впливають на активність α-амілази та протеази.

Ключові слова: насіння люцерни; екстракція; афінна хроматографія; інгібітор трипсину; амінокислотний склад; молекулярна маса.

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doi: 10.15421/082029

Journal of Chemistry and Technologies, 2020, *28*(3), 269-277

ВЫДЕЛЕНИЕ ИНГИБИТОРА ТРИПСИНА ИЗ СЕМЯН ЛЮЦЕРНЫ С ПОМОЩЬЮ АФФИННОЙ ХРОМАТОГРАФИИ

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Аннотация

Выделен и охарактеризован белковый ингибитор трипсина из семян люцерны сорта Eva. Он обладает значительной антипротеолитической активностью и перспективен для создания композиций, предназначенных для коррекции питания в различных условиях, сопровождающихся повышенной активацией протеолитических ферментов. Этапы очистки ингибитора трипсина из семян люцерны включали: экстракцию 0.05 М боратного буфера (pH 7.6), фракционирование белкового компонента экстракта сульфатом аммония с последующим диализом фракции от 75 % до 100 % насыщения и аффинная хроматография на биоспецифическом сорбенте трипсин-сефароза 4В. Расчеты показали, что из 100 г семян люцерны сорта Ева было получено 67.14 мг ингибитора трипсина, ингибирующая активность которого составляет 27.6 МЕ / мг белка. Экспериментальные данные показывают, что полученный ингибитор снижает активность трипсина на 63.05 % при массовом соотношении ингибитор : фермент 1 : 1. Из полученных экспериментальных данных следует, что как полученный ингибитор, так и ингибитор, входящий в состав коммерческого препарата, эффективно снижают активность трипсина, незначительно подавляют активность химотрипсина и не влияют на активность α-амилазы и протеазы.

Ключевые слова: семена люцерны; экстракция; аффинная хроматография; ингибитор трипсина; аминокислотный состав; молекулярная масса.

Introduction

One of the topical and promising areas of biotechnology and pharmacy is the development of drugs that directly affect the enzymatic processes in the body.

Protein inhibitors of proteolytic enzymes play an important role in maintaining homeostasis of a living organism. Due to the low level of stress resistance and reduced immune activity, attacks by viruses of unknown origin are becoming more and more dangerous and lead to the development of gastroepithelial disorders, diabetes, and oncological diseases. Today, the results of numerous laboratory and clinical studies prove that inhibitors of proteolytic enzymes are factors that neutralize disturbances in the processes of blood coagulation, angiogenesis, apoptosis, activation of the complement system [1–3]. In this regard, the question of the search for protease inhibitors is becoming global for many countries of the world. For example, new drugs are being developed, but these developments are only able to influence damaged tissues, reduce the manifestation of symptoms or carry out prevention, but, unfortunately, cannot solve the problem of a genetic nature or congenital pathologies that entail a violation of enzymolysis.

It is known that the secretion of pancreatic juice is regulated by the digestive process. The digestibility of food depends on the level of trypsin and chymotrypsin in the intestine. When the level of these enzymes falls below a critical value, the pancreas begins to produce more enzymes. Under conditions of binding of trypsin to an inhibitor, digestion can be slowed down and the process of absorption of nutrients normalizes [4; 5].

The effect of trypsin inhibitor on the human body is not limited, however, to digestion functions. Some pathological conditions, such as rheumatoid arthritis, bacterial pneumonia, peritonitis, are characterized by excessive activation of proteolysis. The introduction of additional amounts of inhibitors into the body is one of the methods of treatment of these diseases [1].

The antioxidant effect of chymotrypsin inhibitors from soy, beans and potatoes is proved, which depends on both the dose and the type of inhibitor. It is generally accepted that inhibitors protect cells of the gastrointestinal tract from oxidation, first of all [5]. The mechanism of this protection is not studied enough, but it is believed that the sulfur atoms of the inhibitor molecules are able to bind radicals, prevent their oxidation and the formation of hydrogen peroxide.

Inhibitors also exhibit antiviral, antimicrobial activity, have anti-inflammatory, anticoagulant effects [1].

The last example explains the importance of searching not only for natural substances, especially of domestic origin, but also for new approaches to obtaining protease inhibitors due to the fact that today the prompt solution to the problem of maintaining homeostasis, which is

associated with the life and health of the population, is a fundamental factor in the development of food technologies, biomedicine, biotechnology and pharmacy.

Recently, more and more attention of researchers is attracted by plant seeds, in particular buckwheat, wheat, lupine, rye, pepper, mustard, potato, as promising technological plant sources of protease inhibitors [5]. However, despite this, the search for new, alternative sources has been and remains relevant.

The daily rate of protease inhibitors, for example, in Russia is 0.5 % [6]. This indicates the need to find useful edible sources.

Researchers [7] studied the issue of trypsin immobilization in a chitosan matrix, however, the use of chitosan in many cases is limited due to its high cost. The authors [8-16] proposed alginic and pectic acid and xanthanum as matrices for the immobilization of protease inhibitors, but the developments can only be used for pharmaceutical or food compositions. These developments do not solve congenital pathologies or acquired disorders of the enzyme system.

The authors [17] investigated a new thermostable trypsin inhibitor obtained from chanyar seeds *(Geoffroea decorticans)*, which can be used as a natural and hyperstable antidiabetic drug with antithrombotic and hypoglycemic effects.

In the research [18], the effect of a trypsin inhibitor (CgTI) obtained from cassia (Cassia grandis) was studied. Previous research on CgTI has shown it to be a promising biotechnology and biomedical drug.

Legumes, in particular alfalfa (Medicago sativa), contain a significant amount of biologically active compounds, especially low molecular weight proteins, which are natural inhibitors and have a wide spectrum of biological activity. From the literature review, it is clearly seen that the raw materials for inhibitors are mainly vegetable. The monitoring of raw materials was carried out, according to which the seeds of alfalfa of the Eva species were selected as the most economically profitable, efficient, rational [19].

The choice of raw materials was carried out according to the criterion of inhibitory activity.

The aim of the work is the development of methods for isolation and description of the action of a protein trypsin inhibitor, derived from alfalfa seeds of the cultivar Eva, zoned in Ukraine, which has high antiproteolytic activity [20] and is promising as a component of food compositions intended for nutrition correction in various conditions, accompanied by increased activation of proteolytic enzymes.

Objects of research were alfalfa seeds of the cultivar Eva. Trypsin of the human pancreas (Sigma, T6424) Sepharose 4B was used as the sorbent. Its activation was carried out using benzoquinone [20], which was synthesized according to the procedure [21] from previously purified hydroquinone [22].

Experimental

1. Covalent binding of the activated carrier to trypsin was carried out by the following method: to 3 ml of activated Sepharose 4B gel 3 ml of 0.1 M phosphate buffer (pH 7.6) was added. The binding reaction was carried out at 4 ° C for 24 hours. The resulting trypsin-sepharose 4B gel was washed with distilled water on a glass filter, and then in a column (1x15 cm), sequentially treated with 1 M KCl in 0.1 M Na-acetate buffer, pH 4 for 24 hours, 1 M KCl in 0.1 M phosphate buffer, pH 8.0 for 24 hours and washed with distilled water until no absorption at 280 nm.

2. Degreasing of alfalfa seeds was performed in a Soxhlet apparatus using petroleum ether. Extraction of trypsin inhibitor from alfalfa seeds was performed with 0.05 M borate buffer (pH 7.6), containing 0.5 M NaCl (hydromodule 100) at temperature of 18–25 ° C. The extract was heated at 70 °C for 10 minutes to inactivate proteases that could be extracted from alfalfa seeds. The precipitate was separated from the supernatant by centrifugation at 8000 rpm for 20 minutes.

3. It has previously been stated that the trypsin inhibitor of alfalfa seed, which belongs to the Kunitz family of inhibitors, is an albumin protein. In this regard, its fractionations were performed with ammonium sulfate with a degree of salt saturation between 75 and 100 % (F75–100). The protein solution was stirred and centrifuged at 8.000 rpm for 20 minutes after each salt injection. Fractions with ammonium sulfate were performed at 4 °C for 40 minutes [23]. The resulting precipitate was dissolved in distilled water, the protein suspension was transferred to a dialysis chamber with a porous membrane and dialyzed against distilled water for 3 days. The precipitate was separated from the liquid phase by centrifugation at 5.000 rpm for 30 minutes. The resulting supernatant was added to a column with an affinity sorbent.

4. The inhibitor solution was passed through a column (1x15 cm) with trypsin-sepharose 4B sorbent at a rate of 15 ml / min. After saturation of the sorbent, which was controlled by the

appearance of inhibitor activity in the eluate with respect to trypsin, the gel was washed with 0.05 M Tris / HCl buffer, pH 8.0. The gel in the column was washed with 1 M NaCl solution, 8 M urea 0.05 M Tris / HCl buffer (pH 8). The desorption of the inhibitor was carried out with a 10.0 M HCl solution. The active fraction was neutralized to pH 8.0 with 1 M NaOH solution and freeze-dried.

5. The purified inhibitor was investigated by gel electrophoresis in Tris-glycine buffer pH 8.3 using 15 % polyacrylamide gel (PAGE) on a 2219 Multitemp II Thermostatic Circulator. As markers were used: phosphorylase B (92500 Da), BSA (37000 Da), egg albumin (15000 Da), carbohydrase (29000 Da), soy trypsin inhibitor (21000 Da), cytochrome C (12000 Da), inhibitor from cattle lungs (6000 Da). Samples were treated at 4 ° C for 3 hours with a current of 20 mA. Protein was fixed with a 60% aqueous trichloroacetic acid solution for 3 hours at room temperature. The gel was stained with Kumashi-250 paint for 4 hours at temperature of 18–25 ^{0}C. The gel was washed with a 7 % solution of acetic acid in a 10 % solution of isopropyl alcohol [24].

6. Inhibitory activity (IA) was determined by the degree of inhibition of the enzymatic activity of trypsin and expressed in inhibitory units (IU). Trypsin activity was expressed by the amount of casein, which was processed with 1 g of protein prepared at 37 °C for 1 minute. Casein acc. to Hammarsten was used as a substrate.

7. Protein content was determined by the Lowry-Hartree method [25].

After degreasing and extraction of protein substances, the protein component of alfalfa seeds was fractionated. The most common and classic is the method of fractionation of extracted proteins with salts to obtain solutions of varying degrees of saturation. The analysis of resource [26] indicates that inhibitors of proteolytic enzymes are in the albumin fraction. This explains the study of inhibitory activity in the fraction of protein substances obtained by the degree of saturation of saline solutions from 75 % to 100 %.

Results and discussions

The stages of purification of the trypsin inhibitor from alfalfa seeds included: extraction of 0.05 M borate buffer (pH 7.6), fractionation of the protein component of the extract with ammonium sulfate, followed by dialysis of the fraction between 75 % and 100 % saturation $(NH_4)_2SO_4$ and affinity chromatography on a biospecific sorbent trypsin – sepharose 4B (table 1).

Table 1

Stages of purification of a trypsin inhibitor from alfalfa seeds of the cultivar Eva (0.7 g of homogenized seeds)

Stages of purification	Stage 1 (extraction)	Stage 2 (fractionation and dialysis)	Stage 3 (affinity chromatography)
Volume, cm^3	70	130	67
IA, IU / cm^3	0.27	0.14	0.19
Protein, mg / cm^3	0.940	0.020	0.007
Total protein, mg	65.80	2.60	0.47
Total IA, units	18.90	18.14	13.00
Specific IA, IO / mg	0.29	7.00	27.60
Degree of purification	1.0	25.0	95.3
Output, %	100.0	96.0	68.7

As given in table. 1 data show that the extract with a protein content of 0.940 mg / cm^3 and inhibitory activity of 0.27 IA / cm^3 was purified to a protein content in the active fraction of the eluate after affinity chromatography of 0.007 mg / cm^3, which has an inhibitory activity of 0.19 IU / cm^3. The degree of purification of the inhibitor is 95.3. Thus, calculations show that from 100 g of alfalfa seeds of the cultivar Eva, 67.14 mg of trypsin inhibitor was obtained, the inhibitory activity of which is 27.6 IU / mg protein. Experimental data show that the obtained inhibitor reduces trypsin activity by 63, 05% with a weight ratio of inhibitor: enzyme of 1: 1. It is known from literature that a soybean trypsin inhibitor reduces trypsin activity by 30%. Thus, the isolated trypsin inhibitor has significant inhibitory activity, the value of which is higher than the inhibitory activity of the plant trypsin inhibitor from soybeans.

From the table it can be concluded that the alfalfa seed inhibitor can be isolated using affinity chromatography on immobilized trypsin in almost one stage with a yield of 68.7 % and a high degree of purification. The calculation shows that 100 g of alfalfa seeds contains about 67 mg of trypsin inhibitor.

The substance is a white hygroscopic powder, highly soluble in water.

The trypsin inhibitor protein was purified using trypsin-sepharose 4B affinity chromatography. The results of the determination of IA and protein concentration in the collected (Vl) eluate fractions are shown in Fig. 1.

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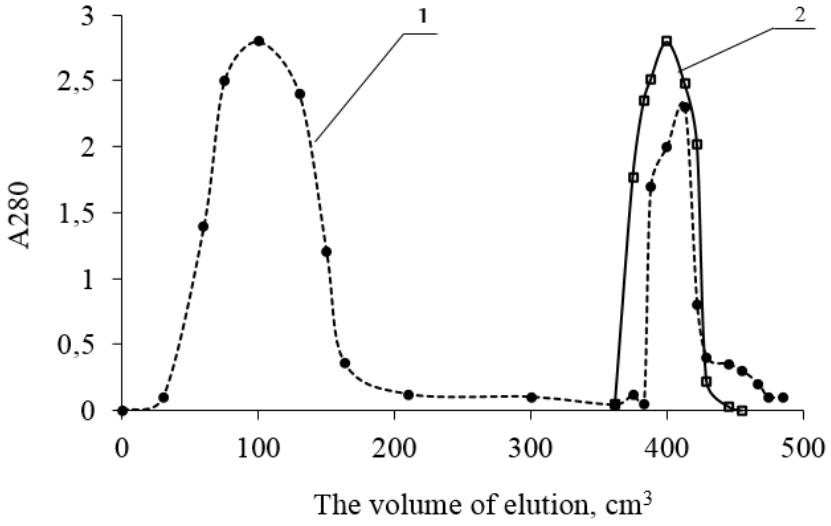

Fig. 1. Affinity Chromatography on Trypsin Sepharose: Protein – 1, Trypsin inhibitor – 2

The molecular weight (Mr) of the obtained protein was determined by electrophoresis in 15 % SDS (Fig. 2).

It was shown that using the described method of purification of the trypsin inhibitor, a protein with a molecular weight of 20.24 kDa was obtained, which was calculated using a calibration curve (Fig. 3).

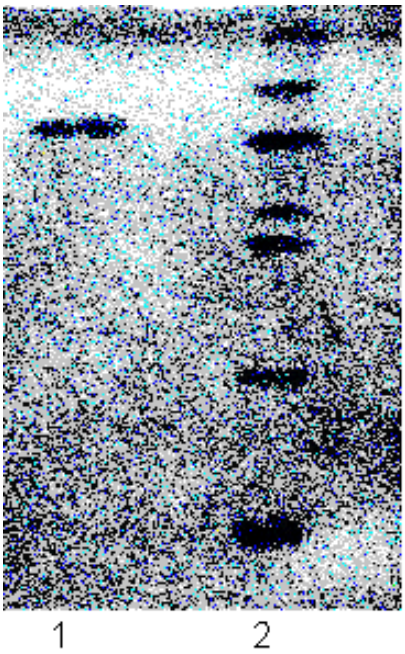

Fig. 2. Electrophoregram of the isolated trypsin inhibitor alfalfa seeds (determination of molecular weight). 1 – sample of trypsin inhibitor; 2 – markers [18]

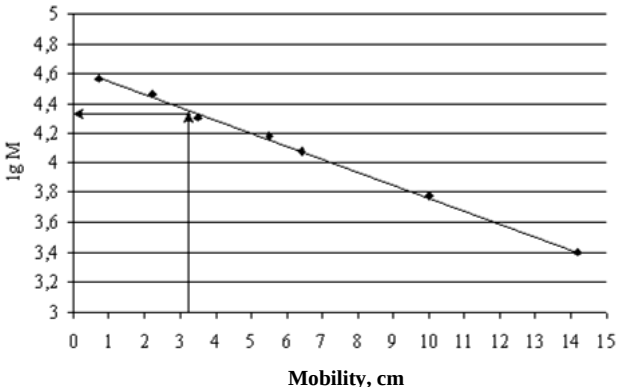

Fig. 3. Calibration curve for calculating the molecular weight of the trypsin inhibitor

Thus, affinity chromatography allowed us to obtain a pure, homogeneous trypsin inhibitor, which has significant antiproteolytic activity, which can be compared with the antiproteolytic activity of known plant trypsin inhibitors.

The amino acid composition of the obtained trypsin inhibitor was studied (Table 2).

Analysis of the data shown in table 2 indicates that the alfalfa seed inhibitor is significantly different in amino acid composition from the Bauman-Birk inhibitor. These differences are primarily expressed in a low cysteine content and a higher content of residues with hydrophobic side chains. It is important that for a smaller number of disulfide bonds, the conformation stability of an alfalfa seed inhibitor to a greater extent than that of a Bauman-Birk inhibitor depends on hydrophobic interactions.

Table 2

Amino acid composition of trypsin inhibitors of alfalfa, Kunitz (STI) and Bauman-Birk (BBI)

Amino acid	Amount of residues		
	TI-1	STI [3]	BBI [3]
Asp	16.4(16)	26	12
Tle	6.2(6)	7	2
Ser	14.8(15)	11	8
Glu	18.1(18)	18	7
Pro	15.2(15)	10	6
Gly	20.2(20)	16	0
Ala	8.0(8)	8	4
Val	1.4(14)	14	1
Cys	3.9(4)	4	14
Met	1.8(2)	2	1
Ile	13.3(13)	14	2
Leu	17.3(17)	15	2
Phe	4.8(5)	9	2
Tyr	7.3(7)	4	2
Lys	13.9(14)	10	5
His	3.1(3)	2	1
Arg	8.3(8)	9	2
Trp	0.8(1)	2	0
The total amount of residues	186	181	71
M_r	20238	20100	8000

One of the main characteristics of natural inhibitors is the specificity of their action on various enzymes. In the table. Figure 3 shows comparative data on the specificity study of a trypsin inhibitor, alfalfa seeds of the cultivar Eva and the preparation of a protease inhibitor from cattle lungs (preparation «Contrical» from AWD pharma GmbH & Co. KG).

Table 3

Comparative specificity of trypsin inhibitor alfalfa seeds Eva and the preparation «Kontrikal» (weight ratio of inhibitor : enzyme – 1 : 1)

Number in sequence	Enzyme	Inhibitory activity of trypsin inhibitor alfalfa seeds, %	Inhibitory activity of the preparation «Kontrikal», %
1	Pronase E (from Streptomyces griseus)	Does not inactivate	Does not inactivate
2	Protease C	Does not inactivate	Does not inactivate
3	Pepsin	Does not inactivate	Does not inactivate
4	Pancreatin	Does not inactivate	Does not inactivate
5	Oraza	Does not inactivate	Does not inactivate
6	Chymotrypsin	23.5	37.3
7	Trypsin	92.6	97.0
8	α- Amylase	Does not inactivate	Does not inactivate
9	β- Amylase	Does not inactivate	Does not inactivate

From the obtained experimental data, it follows that the obtained inhibitor and inhibitor, which is part of a commercial preparation, effectively reduce trypsin activity, slightly inhibit chymotrypsin activity, and do not affect α-amylase and protease activity. Thus, the above data indicate that the trypsin inhibitor of alfalfa seeds of the cultivar Eva is not a bifunctional inhibitor, since it does not affect the activity of amilolytic enzymes.

In fig. 4. shows the results of studies on the inhibition of trypsin of the human pancreas by an isolated inhibitor. It effectively inhibits trypsin activity. The inhibition curve is linear until 90 % inhibition is achieved. The extrapolation of its linear portion of the curve to the point corresponding to zero enzymatic activity indicates 100% inhibition with a molar ratio of inhibitor: enzyme of 1: 1. Thus, one molecule of the trypsin inhibitor of alfalfa seeds is able to bind one molecule of trypsin. By the nature of the action on trypsin, the alfalfa seed inhibitor is similar to the Kunitz inhibitor [20].

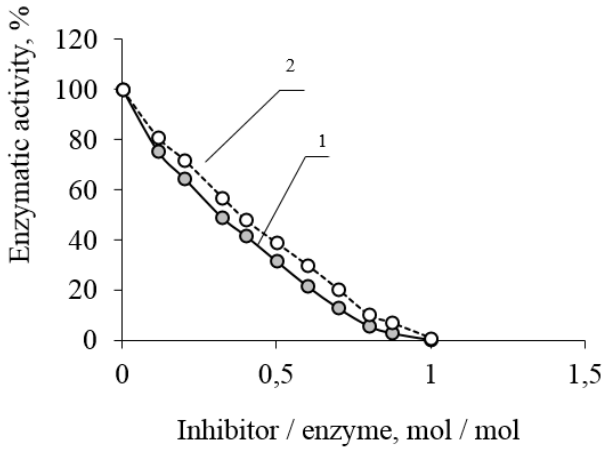

Fig. 4. Dependence of trypsin activity on the concentration of the inhibitor: TI (trypsin inhibitor) of alfalfa seeds – 1; Contrikal – 2

A study of the action of trypsin inhibitor (IT) in concentrations from 0.1 mg / cm3 to 1 mg / cm3 on the proteolytic activity of trypsin (Fig. 7) showed that the linear relationship between the amount of IT added to the incubation mixture and enzymatic activity remains almost until 90 % inhibition. Extrapolation to the value of the enzymatic activity equal to zero allowed us to conclude that when the enzyme-inhibitor complex is formed, one IT molecule of alfalfa seeds and one Kontikal molecule binds one trypsin molecule.

In general, the amino acid composition of an alfalfa seed inhibitor is characterized by properties common to STI family inhibitors. As already noted, the molecule of the isolated inhibitor contains a significant amount of acidic amino acid residues, as well as glycine and amino acids with non-polar side chains (Pro, Val, Leu, Ile). The amount of non-polar side chains (NPS) calculated by the Bigelow method [26] is 0.39 (0.38) for the alfalfa seed trypsin inhibitor. For STI, this value is 0.29. Thus, the isolated inhibitor belongs to proteins with a high degree of hydrophobicity. The composition of amino acids, the side chains of which can take part in the formation of hydrogen bonds, is 25 % of the total number of amino acid residues of the alfalfa seed trypsin inhibitor. For STI, this value is 23 %.

Conclusions

The results of the study make it possible to predict the feasibility of using alfalfa seeds of the Eva variety as a source of inhibitors of enzyme activity and to recommend them as a prescription component in food or dietary supplements, in particular, regulating the functional activity of the digestive system.

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