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OBTAINING AND CHARACTERISTICS OF A PAPAIN AND MAIZE ARABINOXYLAN COMPLEX

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Abstract. The Ukrainian people's diet lacks a number of biologically active substances. But their addition to the food is not effective enough as aggressive bodily fluids influence their activity and substantially reduce it. There are undesirable changes in the properties of biologically active substances during their storage, too. That is why it is so urgent a task to increase the effectiveness of biologically active substances by compounding them with polysaccharides in order to protect them from the unfriendly environment. It has been considered how practical it is to form a complex of papain and maize germs arabinoxylan to modify the properties of the enzyme in the intended direction. It has been proved that the complex formation taking place when biopolymer solutions are combined results in the enzyme activity increase. A number of factors (the concentrations of biopolymers solutions, their volumetric ratios, the duration of the contact, pH medium) influencing the enzyme activity in the complex have been studied. The rational conditions have been determined under which a complex can be obtained far more active than the original papain. These conditions are: a 0.25 % polysaccharide solution mixed with a 4.0 % enzyme solution, with the volumetric ratio being 1:1 and pH 6, at room temperature, for 20 minutes. Introducing papain into the complex makes it more resistant to pH and temperature changes. For the physiological pH values ranging 2 to 8, the enzyme activity in the complex composition is higher than free papain. Complex formation increases the enzyme resistance to higher temperatures, especially in the first 90 minutes. Obtaining of the complex is proved by thermogravimetric analysis.

Key words: arabinoxylan, papain, complex, activity, maize.

ОТРИМАННЯ ТА ХАРАКТЕРИСТИКА КОМПЛЕКСУ ПАПАЇНУ З АРАБІНОКСИЛАНОМ КУКУРУДЗИ

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Анотація. Раціон харчування населення України є дефіцитним за вмістом низки біологічно активних речовин. Але безпосереднє додавання їх до складу їжі є малоефективним внаслідок суттєвого зниження їхньої активності під дією агресивних фізіологічних рідин. Небажані зміни властивостей біологічно активних речовин відбуваються і під час їхнього зберігання. Тому, актуальним є підвищення ефективності використання біологічно активних речовин шляхом сполучення з полісахаридами, як засобу захисту від несприятливих умов навколишнього середовища. Розглянуто доцільність утворення комплексу папаїну з арабіноксиланом кукурудзяних зародків як засобу спрямованої модифікації властивостей ферменту. Доведено, що результатом коплексоутворення, яке відбувається за умов суміщення розчинів біополімерів, є підвищення активності ферменту. Досліджено вплив низки факторів на активність ферменту у складі комплексу, а саме: концентрацій розчинів біополімерів та їхніх об'ємних співвідношень, тривалості контакту, pH середовища. Визначено раціональні умови отримання комплексу, який за активністю значно перевершує вихідний папаїн: змішування розчинів полісахариду з концентрацією 0.25% та ферменту з концентрацією 4.0% в об'ємному співвідношенні 1:1 при pH 6 при кімнатній температурі протягом 20 хв. Включення папаїну до складу комплексу дозволяє підвищити його стійкість до зміни pH та температури. В інтервалі фізіологічних значень pH від 2 до 8 активність ферменту у складі комплексу вища, ніж вільного папаїну. Комплексоутворення підвищує стійкість ферменту при підвищених температурах, особливо протягом перших 90 хвилин. Отримання комплексу доведено методом термогравіметрії.

Ключові слова: арабіноксилан, папаїн, комплекс, активність, кукурудза.

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Introduction. Formulation of the problem

To improve the Ukrainian people's health, it is practical to use dietary supplements and foods that are intended to prevent the most common human diseases now. They are supposed to enrich the diet with necessary biologically active substances that help in making its

content of essential ingredients more balanced and in enhancing immunity.

But the addition of biologically active substances directly to food does not allow using their potential fully, because they are labile in aggressive physiological environments, as well as during storage [1]. Biologically active substances can be made more effective by compounding them with polysaccharides that protect them from adverse environment, prolong storage time, increase their activity [2].

The advantages of polysaccharides as components of dietary supplements are their biocompatibility, biodegradability, no allergenic capacity [3]. Besides, most polysaccharides are biologically active substances. Over the past decades, it has been shown that high molecular weight carbohydrates are responsible for biological effects, either directly manifesting them or causing them through complex reaction cascades [4].

Cereals are an important source of polysaccharides, in particular, non-starch ones.

Arabinoxylans dominate in the non-starch polysaccharides complex of cereals. There are also beta-glucans [5], and traces of arabinogalactans [6] and xyloglucans [7]. It seems promising to obtain non-starch polysaccharides from grain processing by-products mostly used as fodder. Wheat bran and various middlings are mainly used as dietary fibres. Usually they are added in their native state into bakery products, or are substrates when manufacturing fermented products [8].

In Ukraine, there are no technologies for extracting non-starch biopolymers from these products, nor are there any data on whether they can be used in food nanobiotechnology. Thus, this direction of studies (in particular, the prospects for obtaining supramolecular complexes of non-starch polysaccharides with biopolymers of protein nature as potential physiologically functional ingredients) is important.

Analysis of recent research and publications

Arabinoxylans of cereal crops are similar in their structure. Their backbone chain is composed of β-1.4 linked D-xylopyranosyl residues. Monomeric α-L-arabinofuranoside can be present at the C (O)-3 and/or the C (O)-2 positions of xylose moieties [9]. A comparison of the molecular structures of arabinoxylans from whole cereal grains and cereal by-products [10] indicates that the arabinoxylans from brans of rice, sorghum, finger millet, and maize have more complex side chains (including xylopyranose, galactopyranose, and α-$_D$-glucuronic acid or 4-O- methyl-α-$_D$-glucuronic residues) than those from wheat, rye, and barley. Some terminal arabinoxylan residues can be cross-linked to ferulic acid at the C (O)-5 positions via an ester linkage [11].

The molecules with higher branching are more resistant to fermentative hydrolysis. It is believed to be due to steric complications [12].

The presence of ferulic acid in the composition of arabinoxylans allows them to manifest their antioxidant properties. Phenols that can absorb free radicals are chelators for catalyst metals or singlet oxygen quenchers [13]. However, in the human body, there are no enzymes able to cleave an ester bond between arabinose and ferulic acid. As a result, the latter has but limited bioaccessibility [14].

Nowadays, active studies are being carried out, in which arabinoxylans are viewed as substrates for oxidative gelation. Under the influence of peroxidase and laccase, due to bridging between ferulic acid, cross-linking of molecules takes place, and gels are formed. This makes their use in food industry promising. However, at the same time, their antioxidant activity decreases significantly. Besides, cross bonds decrease the rate of arabinoxylan fermentation in the caecum [15].

It is believed that in the future, ferulized gels based on maize bran arabinoxylans can be used as microencapsuling systems with antioxidant properties. These gels are resistant to changes in the temperature, pH, ionic strength. It allows their passage through the gastrointestinal tract and their following fermentation by the caecum microflora [16]. It has been shown that arabinoxylan gels can be used for the controlled release of model proteins, metylxanthin [17], lycopene [18]. So, in the future, they will be candidates to be used for the development of new systems of delivering biologically active substances. Due to their functional and technological properties, arabinoxylans can be used as thickening agents and stabilizers of food systems [19].

Cereals differ, first of all, in the way of arabinose residue substitution in the xylan backbone chain, in the relative proportions and sequence of various bonds between the two carbohydrates (xylose and arabinose), and in the presence of other substituents [20].

The arabinose to xylose ratio in arabinoxylans from wheat endosperm can range 0.50 to 0.71 [21], but it is usually lower than that found in wheat bran (1.02–1.07) [22]. Rye arabinoxylan endosperm is less substituted (0.48–0.55) than the equivalent wheat material. In contrast, the arabinose to xylose ratio of maize bran is usually within a high range of 0.75 to 1.82 [23].

Besides, extraction and modification methods are to be developed in order to obtain specific arabinoxylans having each of the numerous specific molecular features. They can be used to make foods and drinks healthier.

Arabinoxylans are non-starch polysaccharides present in cereal grains, mainly in rye, and in smaller amounts, in wheat. Along with starch and gluten, arabinoxylans influence the quality of dough in bread making processes, improve its water binding capacity and mechanical properties, stabilize its gassing ability, which results in a higher bread volume and excellent crumb structure. In rye bread, where there is no gluten matrix, it is mainly a high content of arabinoxylans that is responsible for the dough quality. For their positive role, they are often added to wheat flour when making dough.

Arabinoxylans are known for many health benefits. A daily intake of as much as 2–10 g of arabinoxylans reduces cholesterol and glucose in blood. Quite recently,

arabinoxylans have been reported to have various biological effects. They can reduce cholesterol in blood serum, modify the sugar level in blood, have an antioxidant effect, reduce the postoperative glycaemic response, improve immunity, and reduce the risk of ischemic heart disease [24].

Previous studies have demonstrated that the bioactivity of arabinoxylans can be related to their specific molecular characteristics by means of modifying them. Modified wheat bran arabinoxylans with low Mw (6.6×10^4 Da) have potential prebiotic properties *in vitro*, and those of modified rice bran with Mw (30–50 kDa) exhibit immunomodulatory activity *in vitro* and *in vivo*. In contrast, high molecular weight arabinoxylans have proved able to reduce the postoperative glycaemic response *in vivo* [25].

Digestive enzymes of plant origin are in demand because, unlike those of animal and microbial origin, they do not inhibit the production of the body's own enzymes, and are toxic and allergic but to a small extent. However, quite few proteases of plant origin (one of which is papain) are manufactured on an industrial scale.

Papain is a polypeptide consisting of 212 amino acid residues. The N-final amino acid of its molecule is isoleucine, and the S-final amino acid is asparagine. It is well soluble in water and water-salt solutions. The enzyme has the following physiological properties: anti-inflammatory, antioxidative, antibacterial, antihelminthic, immunostimulatory, wound-healing, antipyretic, cardiotonic, hypotensive, cholagogic, diuretic. It also improves digestion, normalizes metabolism, has a rejuvenating effect on the skin. Papain has a local effect on the gastrointestinal tract and is absorbed in minimum quantities, which prevents negative side effects on the inner organs, especially the liver [26]. However, when this enzyme is used, the aggressive medium of the digestive canal has a negative effect on it and reduces its activity. That is why it is practical to make it part of a complex, thus preventing the negative influence of the environment.

Maize is one of the six most important cereals grown all over the world. In Ukraine, it ranks third in overall production. A by-product of maize grain processing is a germ which is separated while processing grain into flour and grits. It constitutes about 10% of maize grain mass and contains up to 32–37% of fat. Maize oil is made from germs. A by-product of its production is oilcakes – now they are only used as fodder. Unlike non-starch polysaccharides of wheat and rye flour, hemicelluloses of maize germs have not been paid enough attention to.

The **aim of this research** was obtaining and characterizing the of a maize germ arabinoxylan complex with the papain enzyme.

Tasks of the research:

1. Obtaining arabinoxylan and its characterization;
2. Obtaining a papain-arabinoxylan complex;
3. Characterization of papain in the composition of the complex.

Research materials and methods

Obtaining arabinoxylan and its characterization. The raw material used was oilcake of maize germs by the LLC *Kama* (Poltava). It was heated for 90 min at 130 ºC to inactivate its own enzymes, and then milled. To determine the content of easy hydrolysed polysaccharides, the raw material was hydrolysed by heating it with a 2% HCl solution for 4 hours [27]. The monosaccharides from the hydrolysates were converted into their corresponding alditol acetates and identified by gas-liquid chromatography (Hewlett-Packard 5890 A chromatograph; Hewlett-Packard, St. Louis, MO, USA) with a flame ionization detector and integrator 3393 A with an Ultra-1 capillary column (25 m×0.2 mm) at temperatures 175 to 270°C and a rate of 10°C/min in the stream of nitrogen [28].

Arabinoxylane was obtained by the methods [29] with some modifications made. Alkali extraction of arabinoxylane was carried out with a 0.17 M NaOH solution at 65–70°C for 1.5 h. The suspension obtained was centrifuged. Then, the pH of the supernatant was acidified to pH=5.0. The precipitate was separated by means of centrifugation. The supernatant was incubated with papain at 37°C for 4 h. The enzyme was inactivated by boiling for 15 min. Then, it was incubated with α-amylase at 37°C for 4 h. The enzyme was inactivated. The suspension was centrifuged, the precipitate separated. The arabinoxylane was precipitated by adding three times as much ethanol. After the centrifugation, the precipitate was dried at 50 °C.

The content of carbohydrates in the resulting product was determined by the anthrone method [30] using glucose (Sigma-Aldrich, USA) as a standard, that of protein by the Lowry method [31]. The hydrolysis of the polysaccharide was determined and monosaccharides identified as described above.

Sephadex G-100 was used for gel chromatography of arabinoxylane, with column dimensions H= 38 cm; D=3.1 cm; V=121 cm^3. The constant elution rate was set with a pump. The column with sephadex was calibrated with markers the molecular weights of which were already known.

Obtaining a papain-arabinoxylan complex. In the experiments, papain from Carica papaya (Merck) was used. The complex was obtained by combining water solutions of papain and arabinoxylan, varying the concentrations of the polysaccharide (0.1–0.75 %) and enzyme (0.4–4.0%) in the solutions, their volume ratios (2:1, 1:1, 1:2), the pH of the solution (5–8), the duration of the process (10–40 minutes). The samples were lyophilized. The proteolytic activity of free papain and of that in the complexes was determined by the method [32].

To determine the dependence of proteolytic activity of the samples on the pH value of the medium, they were incubated in the solutions with pH 2.0–10.0. After that, the solutions were brought to pH 6.0, and the proteolytic activity (PA) was determined.

The dependence of the samples activity on the temperature was evaluated by incubating them at +24ºC, +37ºC, +50ºC, +65ºC and +80ºC, with pH 6.0. After that,

the temperature was brought to +37ºC and proteolytic activity was determined again.

Results of the research and their discussion

Obtaining arabinoxylan and its characterization. The maize germ meal contained 12 % of non-starch polysaccharides. In the hydrolysate, arabinose, xylose, glucose, galactose, and traces of uronic acids were identified. The arabinose to xylose mass ratio was approximately one. Such a mass ratio of arabinose to xylose is typical of arabinoxylans with a branched molecule structure. It is known that if arabinoxylans with this structure are extracted with alkaline solutions, they become water soluble. That is why such conditions for extracting arabinoxylan were chosen.

The obtained sample contained 90.2% of carbohydrates and 6.5% of proteins. In the hydrolysate, arabinose, xylose, glucose, galactose and uronic acids were identified, their mass ratios being 12:5:42:38:3, respectively. Residues of galactose and uronic acids are also typical for arabinoxylans extracted from maize, rice, and sorghum bran. It is known that arabinoxylans from rice, sorghum, finger millet, and maize have complex side chains including xylose, galactose, and glucuronic acid residues. The starch content was checked by iodine staining in the sample, with a negative result. As reported, the alkali arabinoxylans extracted from wheat and rye contained more proteins, starch oligomers, β-glucan, galactan. In our opinion, the presence of glucose in the sample obtained can be due to the presence of not only starch, but of β-glucan and xyloglucan as well.

As shown in Fig.1, the carbohydrate and protein elution profiles overlap. This indicates a close relationship between them. Gel chromatography of the product on a Sephadex G-100 column has shown its inhomogeneity. The molecular weights of the vast majority of carbohydrates range 30–100 kDa. Carbohydrates with a molecular weight of about 10 kDa are also present.

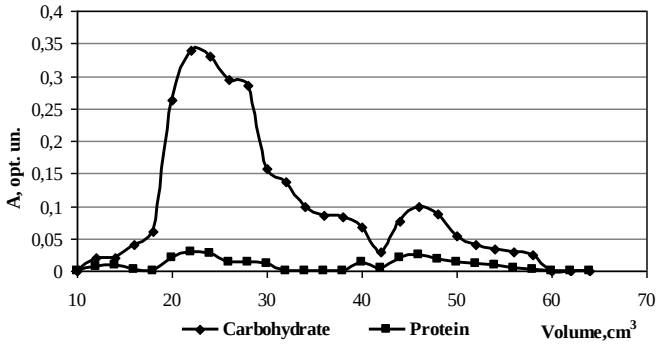

Fig. 1. Elution curves of arabinoxylan

It should be noted that there is a co-elution of carbohydrate and protein components. This indicates a connection between them and matches the results [24].

Obtaining a papain-arabinoxylan complex. The complex was obtained by mixing arabinoxylane and papain solutions. Fig. 2 presents the research data of how the pH of the reaction medium and the length of the biopolymer contact influence the immobilized papain activity.

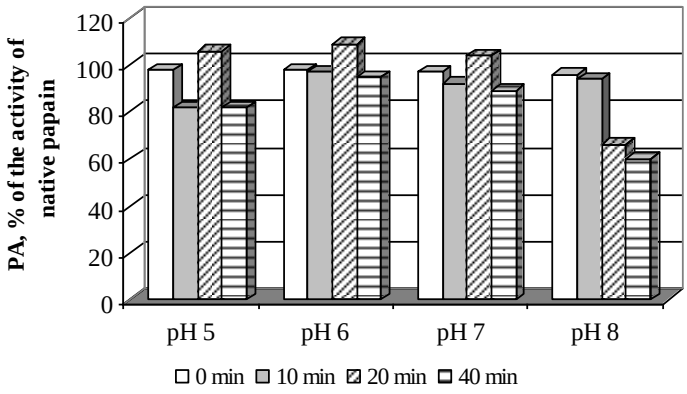

Fig. 2. Dependence of the enzyme activity in the complex on the pH and on the duration of the contact of the solutions

The best result was obtained when the medium was slightly acidic. In an alkaline medium, the result was significantly worse. The complex with the highest activity was obtained at pH 6 and incubation time 20 min. With a longer contact, no increase in papain activity was observed. That is why, these conditions were applied in the further experiments.

The influence of biopolymer concentrations in the solutions and of their volume ratios on the enzyme activity in the complex obtained is shown in Table 1.

The best results were obtained when the volume ratio of the biopolymer solutions was 1:1. With a 0.25% arabinoxylan solution used, there was an increase in immobilized papain activity for all concentrations of the enzyme being part of the complex (0.4% to 4.0%), as compared to free papain. So, as a result of interaction with the polysaccharide, the enzyme is activated. The best result was obtained when a 4.0% papain solution was used. But the use of polysaccharide solutions of other concentrations reduced the activity of the enzyme in the complexes.

Table 1 – Influence of biopolymer concentrations in the solutions and of their volume ratios on the enzyme activity in the complex, n=3; p≤0.95

Polysaccharide concentration in the solution, %	Papain concentration in the solution, %	Volumetric ratios of the components of the solutions		
		2:1	1:1	1:2
0.1	0.4	19.0	23.2	20.0
	1.0	47.0	64.2	50.0
	2.0	55.0	77.8	60.0
	3.0	58.0	77.0	60.0
	4.0	66.0	81.8	64.0
0.25	0.4	81.0	119.7	88.0
	1.0	79.0	111.5	82.0
	2.0	75.0	109.6	78.0
	3.0	70.0	107.5	75.0
	4.0	68.0	106.9	75.0
0.5	0.4	34.0	66.0	50.0
	1.0	45.0	41.1	50.0
	2.0	50.0	42.2	52.0
	3.0	50.0	58.4	56.0
	4.0	53.0	70.1	58.0
0.75	0.4	52.0	80.1	55.0
	1.0	67.0	97.9	68.0
	2.0	65.0	93.7	68.0
	3.0	55.0	84.2	60.0
	4.0	52.0	80.1	57.0

Based on the above, the rational conditions for obtaining a complex are: mixing of a 0.25% arabinoxylane solution and a 4.0% papain solution at pH 6 for 20 min at room temperature. In these conditions, a papain-arabinoxylane complex was obtained, in which the enzyme activity was maximum.

The formation of the complex is confirmed by the results of thermogravimetric analysis. They are shown in Fig. 3.

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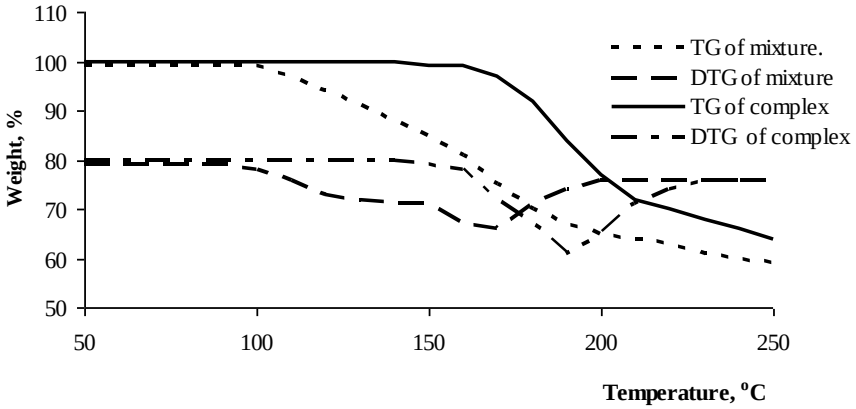

Fig 3. Thermograms DSC

The mechanical mixture of papain with arabinoxylan was stable at a temperature of 100 °C. In the temperature range 104–201 °C, two processes occur that partially overlap. The maximum rate of the first one was at a temperature of 144 °C, of the second – 176 °C. Similar processes took place with the papain-arabinoxylan complex, but at higher temperatures. So, at a temperature of 162–226 °C, there are two processes that partially overlap. The first ends at a temperature of 171 °C. The maximum splitting rate of the second one is at a temperature of 194 °C. A weak endothermic reaction is observed when analysing the complex at 152 °C.

These data indicate the presence of bonds between papain and arabinoxylan in the complex.

Characterization of papain in the complex composition

At all pH values in the range under study, an increase in enzyme activity is observed. Its maximum value reaches 119 % of the activity of the free enzyme. The pH optimum of free papain is about 6 units. As a result of complex formation, an increase in the pH of the optimum enzyme is observed. The optimum pH value for papain in the complex is within the range of 6–8 (Fig. 4).

In the whole interval of the temperatures investigated, the free enzyme is less active than its immobilized forms (Fig. 5). Within the temperature range 50–80 °C, the enzyme in the complex retains as much as 100 % of its activity and even more compared to the original activity of the free enzyme. It is, in particular, due to increased activity of the enzyme in the course of complex formation with the polysaccharide. The highest proteolytic activity of both samples is registered at a temperature of about 50 ºC.

The thermostability of the enzyme in the complex composition was determined at the physiological temperature value 37 °C and – considering the necessity of the further drying of the product – at 65 °C (Fig. 6, 7)

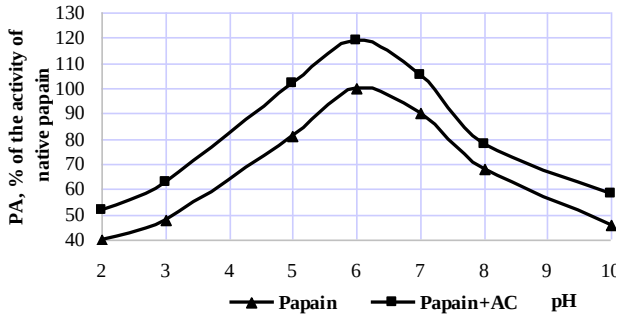

Fig. 4. Dependence of papain proteolytic activity on the pH medium

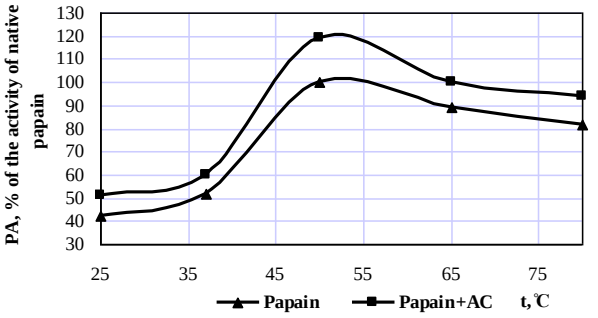

Fig. 5. Dependence of the proteolytic activity of papain on the temperature

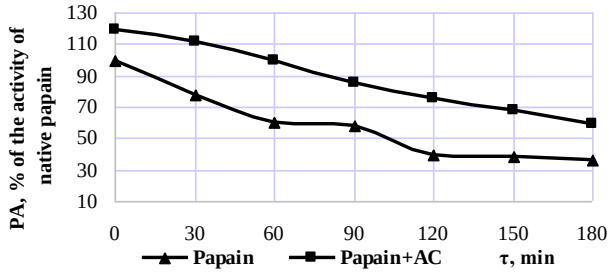

Fig.6. Thermostability of papain at 37 °C

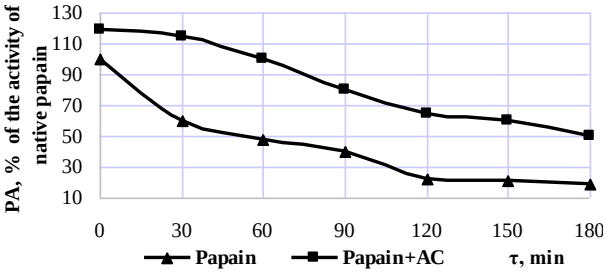

Fig.7. Thermostability of papain at 65 °C

Conclusion

The possibility has been proved and the conditions have been determined of the formation of an arabinoxylan complex with a proteolytic enzyme, papain. This was achieved by combining papain and arabinoxylan solutions, and was accompanied by the enzyme activation.

The rational conditions of the complex formation process are combining a 0.25 % arabinoxylan solution and a 4.0 % papain solution, with pH 6, for 20 minutes at room temperature.

The inclusion of the enzyme in the complex composition increases its resistance to changes of such factors of the environment, as pH and temperature. The formation of the complex is confirmed by thermogravimetric data.

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