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THE INVESTIGATION OF THE POTENTIONAL COMPLEX FROM *PLANTAGO MAJOR* TO COAGULATE MILK PROTEINS

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Abstract: *The possibility of coagulating milk proteins by wild plants was confirmed. The prospects of this direction in the technology of milk – protein concentrates obtaining was provided. The potential ability of Plantago major leaves to coagulate milk proteins is considered in the article. The mass fraction of dry matters in the juice was within (4.5±0.23). It was found, that the addition of plant coagulant in the amount of 7...9 % from the general amount of milk allows obtaining a clot with such indicators: active acidity – 6.3…6.4 units, moisture mass fraction – 66.00…69.00 %, water – retaining capacity – 60.61...64.26 %. Organoleptic indicators of samples are suitable for consumption. The yield of the protein clot was 184.0...232.7 g from 1000 ml of normalized milk mixture. The state of moisture in herbal – protein clots was established by the method of infrared spectroscopy. The intensive zone of C=O – groups valence oscillations in the area of 1638 cm^{-1} was observed. This fact indicates the presence of tightly applied moisture. Microstructural study of clot samples, which were obtained by the use of Plantago major juice showed more developed spatial configuration of gel frame as compared with the control sample. High structural and mechanical characteristics and therefore water-retaining capacity of the clot were studied. The obtained results are the base for snack cheese products, semi-finished and processed cheeses technology decisions.*

Keywords: *Plantago major leaves' juice, coagulation of milk proteins, protein –herbal clot, clot's quality indicators.*

1. Introduction

The inventions of milk – protein products of general purpose with use of local plant raw materials are actual. These raw materials serve as an enrichers and technological ingredients. The production of different types of cheeses is accompanied by the milk proteins coagulation.

Destabilization of the colloid state of the casein micelle occurs under the action of proteolytic enzymes and the change of the pH value. Milk Gel is a structure that consist of a gel frame filled with serum. A characteristic feature of the gel is the synthesis – reducing of the water-retaining ability under the influence of temperature, pH and mechanical action [1-3].

The process of milk coagulation occurs as follows: χ-casein binds free calcium, what causes casein deposition. As a result, the milk coagulates and is divided into two fractions – protein clot (casein in insoluble form) and whey (containing soluble proteins) [4].

The different methods of coagulation are used for the cheese making: acid, acid-rennet (the most widespread), thermo-acid and thermocalcium. Enzyme milk coagulation – is modification of casein micelles with a help of imited hydrolysis

of casein by the enzyme. This process is accompanied with micelles' aggregating [5]. Various factors have contributed to the search for alternative sources of milk collection, which include higher cheese prices, aspects of restrictions on use related to religion, diet (vegetarianism), or the prohibition of the use of veal sticks in some countries (France, Germany, and the Netherlands) [6]. A series of studies, that were aimed at milk-clotting enzyme determination for provision of the classical rennet enzyme replacement were conducted [7]. Milk coagulation can be carried out by proteases capable of providing χ-casein' hydrolytic decomposition.

The conditions of such coagulants' industrial introduction were formulated. Also the specific physico-chemical and technological properties which should provide the appropriate characteristics of protein concentrates were determined. It is necessary that the proteolytic activity of the enzyme was not very high, otherwise, the splitting of excessive amounts of peptide bonds and the formation of a significant amount of soluble proteins may occur, and as a result, formation of weak clot [8]. As a result, the loss of dry matters and the acceleration of the serum secretion from the clot will occur.

Microbial enzymes (proteases) are used as a rennet enzymes substitutes. They synthesize proteinases that are localized in cells (endo-enzymes), or are extracted into the culture medium (exoenzymes). There are proteinases of bacteria, mold, fungi and yeasts. Inside these groups they are divided into acid, neutral and alkaline. Proteinases of mold fungi are used for a long time in the food industry for cooking food and seasonings, soy sauces and in the technology of certain types of soft cheeses [9]. Herbal milk-clotting enzymes are the subject of increasing scientific and practical interest of specialists in the field of dairy – protein production. The interest in using increases due to the availability of sources and the relative simplicity of their obtaining. According to the literature [8, 10] almost all types of plant tissues have proteolytic enzymes. They have the ability to coagulate milk proteins in the appropriate conditions [8]. Enzymes that are used as the above-mentioned coagulants belong to the aspartic proteases. Cysteine and serine proteases are also fit for milk coagulation.

According to the data [6] there is production of cheeses with a use of artichoke Cynara spp. as a herbal coagulant in Spain and Portugal. Cheeses from the sheep's milk: Serra and Serpa [11], Los Pedroches, La Serena [12], Torta del Casar. Los Ibores cheese – from the goat's milk. Flor de Guía from the mix of sheep's and cow milk [13-15]. Extracts of Cynara spp. were used for the production of both Spanish and Portuguese cheeses. An extract of Calotropis procera is used for cheese production in Nigeria and Benin Republic [6]. Except of the mentioned above, there are a lot of other plants that can be used as the source for milk coagulation: nettle (Urticadioica), fig (Ficuscarica), burdock (Arctium minus), black currant (Centureaspp.), Ambrosia artemisiifolia, and others. Nevertheless, the excessive proteolytic activity of most of plant coagulants limited their use in the milk-protein products manufacturing. As a result – law cheese yield and flavor and texture defects [16].

Hemlock (Coniummaculatum) and Ricinus seeds (Ricinuscommunis) cause the combined coagulation of milk with a help of acids and enzymes. Mainly they are used in the production of cheeses with soft dough. Therefore, the search for new potential enzymes from traditional herbal plants is relevant.

Proteases are divided into groups on the ground of the catalytic mechanism, which

is used during the hydrolytic process. The main catalytic types are aspartate, serine and cysteine [17]. Types and sources of milk – clotting proteases are presented in Table 1.

Table 1.

The characteristic of milk-clotting plant proteases [8]

Type of protease	**The name of the protease**	**Source**	**Link**
Aspartic acid (Aspartic)	Cardosins and cyprosins	Cynara cardunculus (Spanish Artichoke)	[12, 15, 18-26]
	Protein extract	Silybum marianum (Saint-Mary-thistle)	[27-28]
	Oryzasin	Oryza sativa (rice)	[29]
	Protein extract	Centaurea calcitrapa (maize thistle)	[30-32]
	Procirsin	Cirsium vulgare (sow thistle)	[33]
	Protein extract	Solanum elaeagnifolium (silver-leaved nightshade)	[34]
Cystein	Protein extract	Helianthus annuus (common sunflower)	[35]
Serin	Neriifolin, Neriifolin S	Euphorbia neriifolia (euphorbia)	[36-37]
	Dubiumin	Solanum dubium Fresen (Solanum)	[38-40]
	Lettucine	Lactuca sativa (cutting lettuce)	[41]

Serine and cysteine proteases catalytically differ from asparagine and metalloproteases. Inasmuch as the nucleophile of the catalytic node is a part of the amino acid, while in the other two groups, it is a molecule with the activated moisture [42]. The wild plantain (Plantago major) – is the source of alternative kinds of plant milk-clotting enzymes. It belongs to the genus of single- and perennial grasses, less often half shrubs of the Plantain family (Plantaginaceae). Plantago media is widespread on Ukrainian territory.

The coagulating ability of milk proteins depends not only from the presence of proteases, but also from organic acids.Thus, the acid complex of plantain leaves is represented by the following components: fumaric, oxalic (31...103 mg%) [43], wine (1.60...1.87 %), citric (1.22...1.53 %), apple (0.20...0.51 %), malonic (0.11...0.35 %) and succinic (0.25...0.55 %) acids [44]. The total content of organic acids is 10...12%, of which up to 60 % are bound acids. The dominant components of the complex are wine and citric acid.

The plananthosine enzyme from the Plantago maior' leaf extract was identified by affinity chromatography on bacitracin sepharose and ion exchange chromatography on Mono Q in FPLC at 70 °C and pH 11 [45].

Plant milk-clotting enzymes have a number of technological and economic benefits: low cost, ease of allocation, a wide range of producer plants, etc. This allows to consider them as promising technological components for the cheeses production. It is necessary to take into account the special characteristics of plant milk coagulants. The conditions of their use for the production of certain types of cheeses need additional research. The specific chemical composition makes possible to use Plantago major juice for the milk proteins' coagulation in the technology of soft cheese.

The aim of study was to investigate the potential capacity of the Plantago major juice from the aerial part of plant to coagulate milk proteins.

2. Matherials and methods

2.1. Obtaining of Plantago major juice

The *Plantago major* leaves were collected on the territory of the Kyiv-Svyatoshinsky district of the Kyiv region. The juice of herbaceous plant was obtained according to the scheme shown in Figure 1.

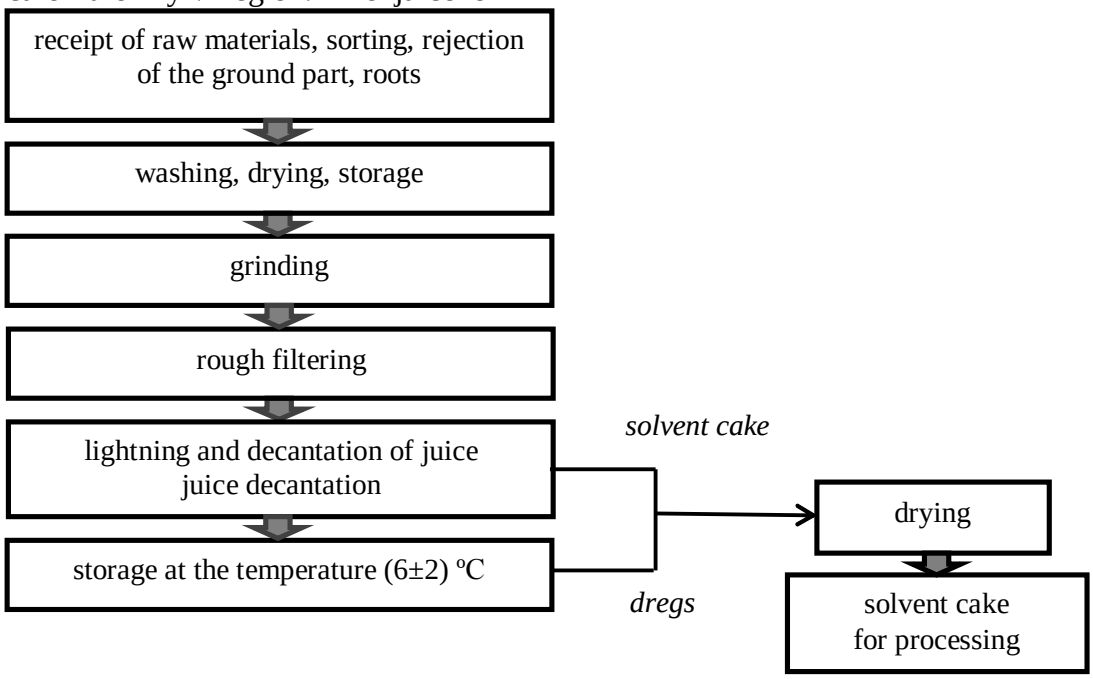


Fig. 1. Technological scheme of herbaceous plant' juice obtaining

In the Plantago major juice, the amount of dry matter was (4.5±0.23) %, the index of active acidity at the level (5.85±0.18).

2.2. The obtaining and study of herbal – protein clots

To prepare model samples was used normalized milk with the following indicators: dry matters mass fraction – (12.3±0.62) %, fat – (2.6±0.13) %, protein– (2.8±0.14) %, with active acidity – (6.9±0.35) pH units, density– 1027 kg/m^3.

The preliminary preparation of normalized milk according to classical technology was carried out for the milk proteins' coagulation. The Plantago major juice was introduced into the milk heated to 96…98 °C. The mixture was slightly stirred and held for 1 to 2 minutes till the clott was formed. The mass of the wet protein clot for each sample was determined. It was fixed by weighing after self-pressing for 15 minutes. The yield of herbal – protein mass was 184.0…232.7 g from 1000 ml of normalized mixture.

The samples, obtained by the coagulation by plant coagulant in the amount 5…12 % from the normalized milk volume were prepared for the experiment. The indicators of active acidity were determined by the potentiometric method (Sartorius pH meter). The moisture mass fraction was determined by drying the sample to a constant mass. The moisture-retaining capacity was determined by Grau-Hamm method in modification of A.A. Alekseyev. This technique consists in determination of water mass that allocates from the product in mild compression and is absorbed by the filter paper [46].

2.3. Infrared (IR) spectroscopy

To determine and compare the moisture condition, IR spectra of milk – protein and herbal–protein clots were shooted by the method of a drop, inflated between the RRS-5 windows on the IR-Fourier Nexus spectrometer of Thermo Nicolet company, USA. Nujol, which has no additional strips in the field of water absorption, was used as an internal standard. The conditions of spectrum shooting: scan range – 400...4000 cm^{-1}, number of scans per second – 7, scan interval – 1 cm^{-1}, resolution – 1 cm^{-1}.

2.4. Microphotographs of herbal – protein clots

Microphotographs of herbal – protein clots were obtained with a help of a luminescent and phase-contrast microscope XSP-139A-TP with a digital camera Canon-66 (NNGOA Compani LTD). Organoleptic indicators were determined according to the generally accepted methods for soft cheeses.

3. Results and discussion

The amount of *Plantago major* juice that was used in the milk proteins coagulation and its influence on the active acidity and the moisture mass fraction of the protein – herbal clots were studied in the first stage of research. The corresponding results are presented in Figure 2.

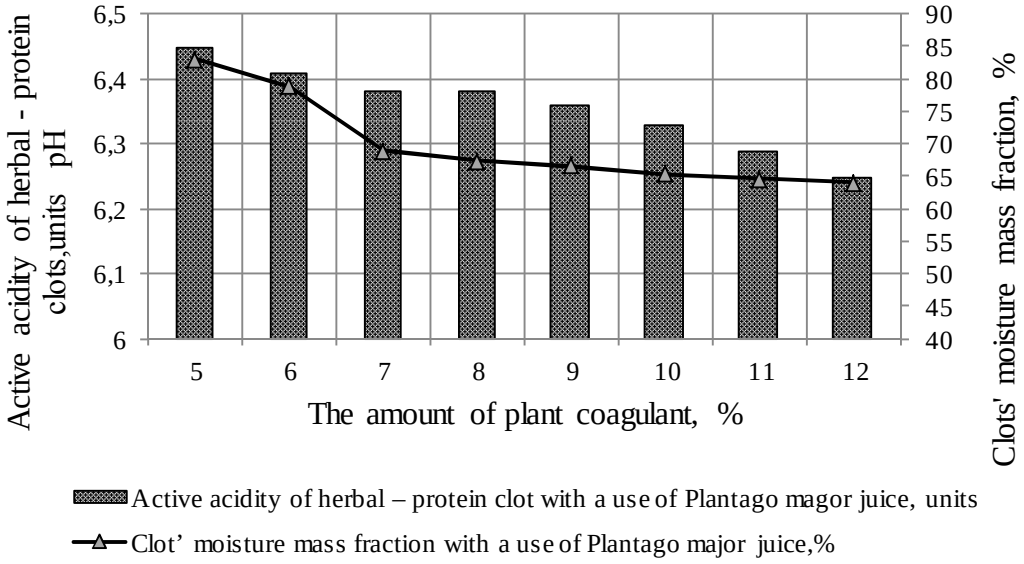


Fig. 2. The dependence of active acidity and moisture mass fraction of herbal –protein clot from the amount of Plantago juice

Experimental studies (fig. 2) showed a tendency to decrease the index of active decrease when the amount of plant coagulant increases. The addition of 12 % *Plantago major* juice to milk promotes the setting of active acidity at the level 6.25 units. With decreasing of coagulant amount, the level of clot's active acidity, which was 6.45 pH units at the adding of 5 % of juice, increases. Perhaps it is connected with a presence of organic acids, namely oxalic, citric, apple, wine, and others in the *Plantago major* juice. They affect the process of milk proteins' coagulation.

The obtained results of experimental samples correspond to the pH of the Adyghey soft cheese – (5.9±0.3). It is produced by thermo-acid coagulation with a help of milk whey with increased acidity. The indicator of moisture mass fraction of the clots, obtained by the adding of plant

coagulant from 7 to 9 %, changed significantly and varied from 66.00 to 69.00 %.

The moisture-retaining ability of the herbal – protein clots depending from the plant coagulant amount was investigated. This rate affects on such soft cheese indicators as structure and quality of the finished product (figure 3).

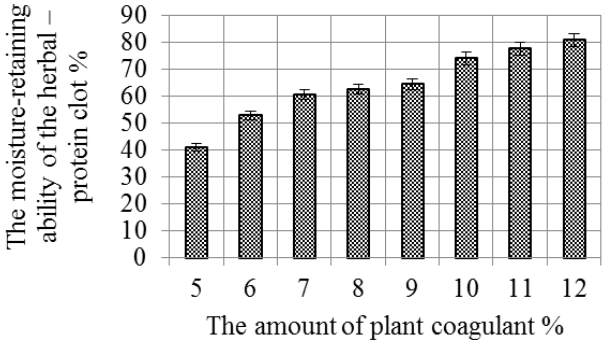


Fig. 3. The dependence of moisture – retaining capacity of herbal – protein clot from the amount of plant coagulant

The results of research (fig. 3) indicate a strengthening of the clot with the increasing of Plantago major juice amount. Moisture -retaining capacity of clots' model samples increased with a compaction of their structure. Thus, with the maximum amount of Plantago major juice, the moisture-retaining capacity index is 80.68 %, which indicates about active removal of the surface charge from the micelle, that is a factor of the milk protein phase' colloid destabilization. The organoleptic evaluation of herbal – protein clots' experimental samples is presented in Table 2.

Table 2.

The organoleptic evaluation of herbal – protein clots

Indicator	The amount of *Plantago major* juice introduction into the milk		
	5±0.25	8±0.4	11±0.55
			
Taste and smell	Dairy with a weakly herbal flavor	Dairy with herbal flavor	Excessive herbal taste and smell
Color	White with a light green tint	Light green	Saturated green
Consistence	The clot is mild, weak, and there is a slight whey selection	The clot is soft, smeared moderately tight	The clot is strong, too tight

Due to the change of color and its intensity, it is necessary to take into account the colority of the samples. A similar situation occurs with taste and smell. The samples, obtained by proteins coagulation with a help of 10…12 % of *Plantago major* juice have the excessive taste, color and smell. It is necessary for

them to apply organoleptic assessment as a limiting factor for wide use in the form of the basis for milk-protein products.

As a result of the investigation of the moisture' state by the IR spectroscopy, in both samples – control and herbal – protein clot , the assignment of the bandwidths of the IR spectra of the experimental samples and the identification of the functional groups were obtained. The examples of experimental clots samples graphic image are shown in Fig. 4 (a-b).

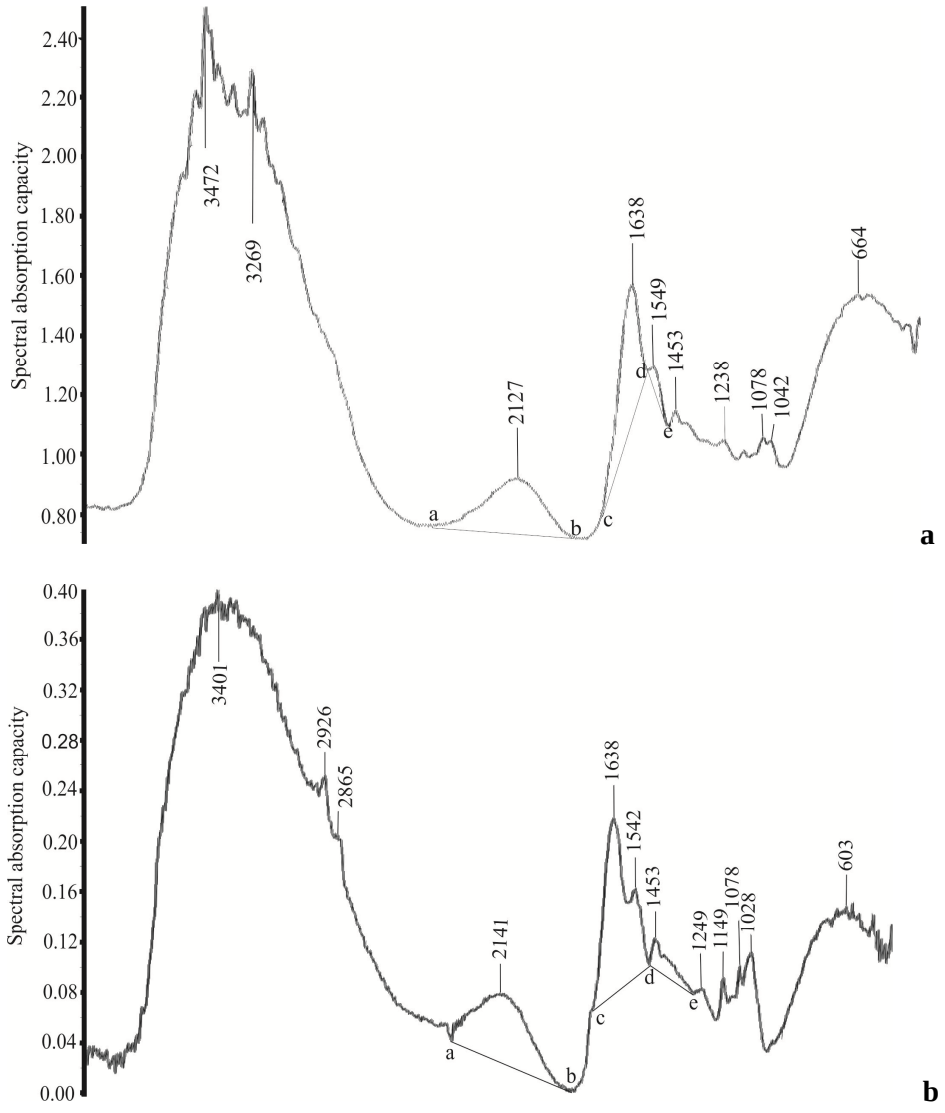


Fig. 4. Infrared transmittance spectra of samples: a) milk – protein (control); b) herbal – protein

The similarity of the main zones was observed at the comparison of the studied samples' spectra (fig. 4). The spectra of protein clot, obtained by acid – rennet coagulation differed from the herbal – protein clot in the presence of absorption zones 3269 cm^{-1}. The wide zone in the area 3400…3200 cm^{-1} was caused by valence fluctuations of H-bound OH groups.

The appearance in the infrared spectrum of an intensive zone of C=O-groups valence fluctuations at 1638 cm^{-1} showed a deformation oscillation of adsorption-bound water.

Absorption zones at 1550…1542 cm^{-1} are caused by deformation oscillations of amino acids' NH groups as the part of protein portion of the protein clot. As

shown in fig. 4 (a-b), this zone was present in all spectra. The 1549 cm^{-1} zone is characteristic for a carboxylate ion:

$$\left(-C\begin{matrix}O\\O\end{matrix}\right)^{-}.$$

The wide zone in the 2133…2127 cm^{-1} area refers to a mobile proton, which is capable to form H-ligaments of the "bridge" type. The formed in this area zone indicates on the presence of weakly bound moisture.

In the herbal – protein clot' spectra there are small changes in the area of the C-H and O-H bonds fluctuations in comparison with the spectrum of the milk-protein clot. The appearance of absorption zones of the OH –group's valence oscillations to 2962…2865 cm^{-1} indicates about the formation of carboxyl groups. The 1449, 1453 cm^{-1} zones, that indicates on deformation vibrations of methyl and methylene groups of the matrix were present in all samples. A number of zones in the 1243-1041 cm^{-1} region relates predominantly to C-O, C-C bonds of matrices.

In order to learn the mechanism of various types of H-bound moisture adsorption in the herbal–protein clot, the relative transmission intensity of the zones was calculated, where R_1 – indicator of tightly bonded moisture; R_2 – is an indicator of weakly bound moisture. The square calculation is made by the triangle method. As the baselines, the corresponding tangents are taken – ab, cd and dc. The internal standard of calculation was the 1539 cm^{-1} zone, which objectively characterized the change in the state of moisture in the experimental mixtures. For example, below is a calculation of R_1 and R_2 for milk-protein clot (control) (figure 4a):

$$R_1 = \frac{S_{1640}}{S_{1539}}; \quad R_2 = \frac{S_{2127}}{S_{1539}}.$$

S – area (or integral intensity) of the corresponding zone.

Relative intensities of various H-bound water types (X = 0.95; n = 3; S_{ch} = 10 %) of the experimental control and herbal – protein clots are presented in Table 3

Table 3.

The relative intensities of various H-bound water types (X = 0.95; n = 3; S_{ch} = 10 %)

Indicators	Experimental samples	
	Milk-protein clot	Herbal-protein clot
R_1	9.8	7.4
R_2	14.8	10.7

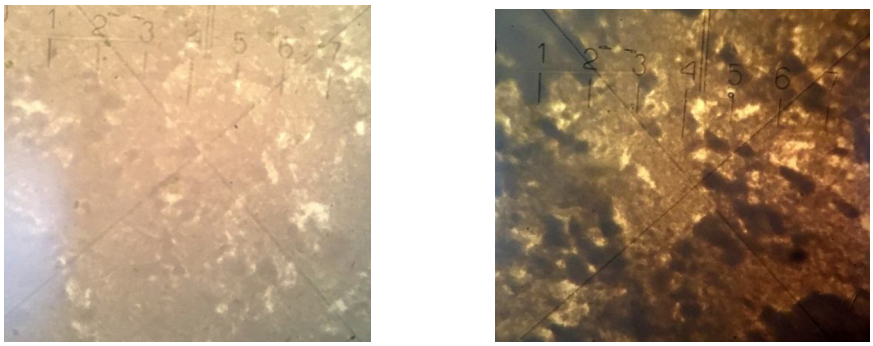
In order to determine the peculiarities of the herbal – protein clot' structure, which was obtained with a use of *Plantago major* juice, the comparative studies of the control and experimental samples microstructure were carried out (fig. 4).

The results of the microstructural analysis were compared with the data obtained at the evaluating of the qualitative parameters of soft cheese samples.

The cheese was obtained by thermo-acid coagulation with a help of milk whey with an titrated acidity index (150±2) °T.

It is clear from the presented material (figure 4), that the microstructure of the clot, formed in the presence of *Plantago major* juice had distinct features from control. Thus, in the experimental samples of clots made with plant coagulant, it is possible to see more structural units in protein clot. They have expressed homogeneous consistency and their

microstructure is more homogeneous and includes uniformly distributed micropores of minimal size. In the microphotographs of clot obtained with the *Plantago major* juice, the protein structure consists of particles that are more closely interconnected than in the control sample. Obviously, the clot structure has a more advanced spatial configuration, representing a "carcass". It is typical to connected disperse systems, which obviously prevents the free reciprocal movement of its links. More structure rigidity provides the best structural – mechanical properties and moisture-retaining ability.



a) **b)**

Fig. 4. Microphotographs of clots: a) control (protein clot); b) herbal–protein clot obtained with a use of *Plantago major* juice in the amount of 8%

4. Conclusion

The possibility of *Plantago major* juice using for milk proteins complex coagulation at the temperature of thermo-acid coagulation by a classical technology was proven.

A rational amount of *Plantago major* juice for milk proteins coagulation at the level of 7…9 % was established. The active acidity of the herbal-protein clot was 6.3...6.4 pH units, the moisture mass fraction was 66.00...69.00 %. At this case, the yield of herbal-protein clot varied from 184.0...232.7 g from the 1000 ml of normalized mixture.

The results of moisture-retaining capacity of herbal-protein clots with a meaning 60.61 to 64.26 % at addition of 7...9 % of the *Plantago major* juice, were obtained. The limiting factor for this type of clots is colority.

The efficiency of the IR spectroscopy method for determining the state of moisture in herbal–protein clots was proved. It was confirmed, that the coagulation of milk proteins by *Plantago major* proteases and organic acids promotes active synergetic occurrence. This was detected by the appearance of an intensive zone of C=O-groups valence oscillations in the infrared spectrum at 1638 cm^{-1},that indicated about deformation oscillation of adsorption-bound water.

The microstructure of the clot's samples with a use of *Plantago major* juice was observed. It was determined that clots had a more developed spatial configuration than the control. The "skeleton" of samples obtained with a maximum content of plant coagulant, results in greater rigidity of the structure. Therefore, it provides the best structural and mechanical characteristics and, accordingly, moisture-retaining ability.

The obtained results confirm the ability of the juice of the herbaceous part of *Plantago major* of coagulating milk proteins. The mechanism of the process requires an additional investigation. The obtained data are the basis for technology developing of the snack cheese pastes,

semi-finished products and processed cheese.

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