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BIOTECHNOLOGICAL TECHNIQUES FOR INTENSIFICATION OF PROTEIN EXTRACTION FROM THE PORCINE PANCREAS

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Abstract
Processing of secondary products after slaughter of farm animals is in demand. The pancreas is a rich source of bioactive protein substances, effective extraction of which is a serious problem today due to their aggregation. The aim of the work was to assess the extractivity of protein substances of the porcine pancreas using sodium chloride, trehalose, arginine, and combination of glycine and proline. The protein concentration was determined in the obtained extracts by the biuret reaction and their protein composition was assessed by densitometry of two-dimensional electropherograms using software ImageMaster™ 2D Platinum powered by Melanie 8.0. The results showed a positive effect of anti-aggregation agents on the release of protein substances into a solution. The highest protein concentration (33.36±0.64 g/l) was observed when adding 1M L-arginine; however, it was conditioned mainly by an increase in the content of three major protein fractions rather than by diversity of the protein composition. In general, the use of 0.9% NaCl as an extractive agent was quite effective, but selectivity to certain protein groups was observed for anti-aggregation agents such as sodium chloride, trehalose, arginine, glycine and proline, as well as their combination. The obtained results are important for intensifying extraction of protein substances including target ones with the subsequent application in different fields.

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Introduction

Many secondary products are generated during processing of agricultural products. The questions of their effective and rational use often remain to be unsolved. In animal slaughter, the yield of secondary products is quite high and accounts for about 30% of live weight [1,2], including by-products and low-value non-edible raw materials. Certainly, there are traditional approaches to the utilization and processing of such non-edible raw materials [3,4], however, the use and implementation of alternative technologies to intensify the rational environmental management are particularly in demand within the framework of the concept of circular economy [5].

One of such by-products is the porcine pancreas, which was used earlier in high quantities to produce insulin for people suffering from diabetes mellitus [6]. Nowadays, insulin is produced mainly using the technology of recombinant DNA [7], as a result, a high demand for the processing of the pancreas is absent. Nevertheless, bioinformatic analysis with the use of the UniProt database [8] shows that this type of raw materials contains quite a large quantity of biologically active substances of protein nature such as pancreatic alpha-amylase, triacylglycerol lipase, phospholipase A2, proglucagon, pancreatic elastase and so on. This allows regarding the pancreas as a potential object for extracting target protein compounds with the following use in the pharmaceutical and/or food industries as well as in the modern laboratory practice [9].

Today, effective extraction and purification of target protein substances from animal raw materials is a serious problem for researchers due to tendency of protein molecules to aggregation [10]. A degree of aggregation depends on many factors, which in a broad sense can be classified as internal (primary, secondary, tertiary or quaternary structure of proteins) and external (type of solution for extraction, conditions and type of isolation process) [11]. Protein aggregation can lead to a decrease in the biological activity of a molecule or its complete loss, an increase in the potential immunogenicity, sedimentation of protein aggregates, as well as other side undesirable effects [11,12]. There are many additives to solutions that stabilize the protein structure preventing thereby their aggregation and enhancing their extractivity from the initial raw materials. Such stabilizers (anti-aggregation agents) include several

amino acids, sugars, polyhydric alcohols, osmolytes and cosmotropic salts [13], which are characterized by safety and can be used to intensify extraction of protein substances, including target ones, with the following use with various purposes. The aim was to study an effect of anti-aggregation agents, such as sodium chloride, trehalose, several amino acids and their combination, on extractivity of protein substances from the porcine pancreas.

Objects and methods

The porcine pancreas was taken in LLC "Pushkinsky myasnoy dvor", Moscow region, Pushkino. Animal raw materials were cleaned of connective tissues, frozen at minus 18 °C, then minced in the frozen state and stored until the subsequent extraction.

The minced pancreas was thawed at a temperature of 4 °C and mixed with an extracting agent in a ratio of 1:5. Extraction was carried out on a laboratory dispersing equipment (LDU, Labotex, Russia) with a mixing speed of 400 rpm; extraction time was 150 min.

Four extractions were carried out with the following extracting agents:

1) 0.9% sodium chloride solution (LLC Gematek, Russia), (0.9% NaCl);
2) 0.9% sodium chloride solution (LLC Gematek, Russia) with addition of 1 M L-arginine (PanReac, Germany) (0.9% NaCl, 1M L-Arg);
3) 0.9% sodium chloride solution (LLC Gematek, Russia) with addition of 0.5 M trehalose (Narodnaya zdrava, Russia) (0.9% NaCl, 0.5 M trehalose);
4) 0.9% sodium chloride solution (LLC Gematek, Russia) with addition of 1% glycine (PanReac AppliChem, Germany), 0.1 M L- proline (Sigma-Aldrich, USA) (0.9% NaCl, 1% Gly, 0.1M Pro).

After the end of the extraction process, supernatant was separated by centrifugation at a speed of 3500 rpm on CM-6M centrifuge for 5 min (ELMI, Latvia). The protein concentration was measured in each sample by the biuret reaction on a semi-auto biochemistry analyzer BioChem SA (HTI, USA) using the standard total protein reagent (HTI, USA). The measurements were carried out in triplicate. The results were calculated with the use of the software STATISTICA 10.0 and presented as "mean ± SD". Significant differences were tested by non-parametric statistical Mann–Whitney *U*-tests for independent variables. Differences with P-values of <0.1 were considered statistically significant.

The proteomic composition of extracts and the pancreas was assessed by two-dimensional gel electrophoresis (2-DE). A sample (100 mg) was taken and a lysing solution (2000 µl) was added. The lysing solution consisted of 9 M urea (PanReac, Germany), 5% β-mercaptoethanol (PanReac, Germany), 2% triton X-100 (Helicon, Russia), 2% ampholines pH 3–10 (Serva, Germany). The obtained homogenate was purified by centrifugation (Centrifuge 5427 R, Eppendorf, Germany) at 14,000 rpm for 20 minutes. At the first stage, isoelectric focusing (IEF) was carried out in tube gels (2.4 mm x 160 mm) in a chamber (Bio-Rad, USA) up to reaching 3,650 volt-hours; an aliquot of the introduced samples contained 140 µg of protein. As an anode buffer and a cathode buffer, 0.01 M orthophosphoric acid (Component-Reactive, Russia) and 0.02 M sodium hydroxide (Panreac, Spain), respectively, were used. After IEF, gels were incubated during 10 min in 2.5 ml of equilibration buffer I (6 M urea (Panreac, Germany), 20% glycerol (Panreac, Germany), 2% SDS (Panreac, Spain) and 1% DTT (Panreac, Spain) in 50 mM Tris-HCl buffer, pH 8.8 (Panreac, Germany)). Then, incubation was carried out in equilibration buffer II (6 M urea (Panreac, Germany), 20% glycerol (Panreac, Germany), 2% SDS (Panreac, Spain) and 4% iodoacetamide (SIGMA, USA) in 375 mM Tris-HCl buffer, pH 8.8 (Panreac, Germany)) [14]. After that, electrophoresis was carried out in 12.5% polyacrylamide gel (170 mm × 180 mm × 1.5 mm) in a chamber VE-20 (Helicon, Russia) using buffer containing 25 mM Tris-HCl (Panreac, Germany), 192 mM glycine (Panreac, Germany) and 0.1% SDS (Panreac, Spain). The process was performed at amperage of 30 mA/gel until the dye front reached the gel edge. Two-dimensional electropherograms were obtained in triplicate for each sample.

Computer densitometry of two-dimensional electropherograms in a wet state was performed using a Bio-5000 plus scanner (Serva, Germany) at a resolution of 300 ppi 1D-Gray. The obtained images were analyzed using one-way ANOVA (between gels of different samples) and ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland). Protein fractions were compared by volume, and the fold-value, an excess of which by more than two units is generally considered statistically significant difference, was calculated. All results are presented as mean ± SD from three independent experiments.

Results and discussion

The results of the determination of the protein concentration in the extracts obtained with the use of 0.9% sodium chloride or with addition of anti-aggregation agents are presented in Table 1.

It was shown that addition of anti-aggregation agents to 0.9% NaCl facilitated release of protein substances into the extracting agent. The highest protein content was observed upon addition of 1 M L-Arg and was 33.36 ± 0.64 g/l,

Table 1. Results of the determination of the total protein concentration in the extracts

Extracting agent	0.9% NaCl	0.9% NaCl, 1 M L-Arg	0,9% NaCl, 0.5 M trehalose	0.9% NaCl, 1% Gly, 0.1 M L-Pro
Protein concentration, g/l	24.84 ± 1.08	33.36 ± 0.64*	29.47 ± 1.58*	28.22 ± 1.36*

* statistically significant difference from extraction with 0.9% NaCl ($p < 0.1$)

which exceeded the value of the total protein content in the extract obtained using 0.9% NaCl by 34.3% ($p < 0.1$). Addition of 0.5 M trehalose and a mixture of 1% Gly with 0.1 M L-Pro facilitated an increase in the protein content in the extract by 18.6% ($p < 0.1$) and 13.6% ($p < 0.1$), respectively, compared to its content in the extract when using 0.9% NaCl.

Then, the protein composition of the obtained extracts was assessed by densitometry of two-dimensional electropherograms with the same protein load. The two-dimensional electropherograms of the extracts and pancreas tissue are presented in Figure 1. The two-dimensional electropherogram of the pancreas tissue shows the presence of protein fractions P1 (47 kDa), P2 (48 kDa), P3 (27 kDa) and P4 (36 kDa), marked in Figure 1, which are not extracted by the types of used extracting agents. In addition, many protein fractions with a molecular weight of less than 15 kDa were observed in the pancreas tissue; the relative volume change of these fractions was significantly lower in the obtained extracts. It is interesting that the highest content of total protein in the extract with addition of 1 M L-Arg was conditioned mainly by an increase in the content of three major protein fractions rather than by diversity of the protein composition. Also, two-dimensional electropherograms show protein fractions D1 (16.279 kDa), D2 (28.92 kDa), D3 (29 kDa), D4 (30 kDa), D5 (26 kDa), which were not detected on other gels.

On the two-dimensional electropherograms, fraction F4 (13 kDa) located in the alkaline side was additionally detected in the extracts obtained using 0.9% NaCl with 1 M L-Arg, while the extracts obtained using 0.9% NaCl, 0.9% NaCl with 0.5 M trehalose or 1% Gly, 0.1 M L-Pro contained protein fractions F1 (16.56 kDa), F2 (14.581 kDa), F3 (17 kDa) in the alkaline area. Furthermore, upon extraction with the use of 0.9% NaCl, 0.9% NaCl with 0.5 M trehalose or 1% Gly, 0.1 M L-Pro, two protein groups were observed in the alkaline area in a range of 24–32 kDa and 37–40 kDa marked in Figure 1 with the violet color; when 0.9% NaCl was used as an extracting agent, protein fraction L1 (34 kDa) was found. These fractions were not detected in the initial pancreas tissue but were found in the extracts.

The statistically significant relative volume change of protein fractions is depicted in Figures 2 and 3.

Extraction with the use of 0.9% NaCl facilitated enrichment of the extract practically with all protein fractions, excluding S20 (28.699 kDa), S27 (35 kDa) and S29 (13.969 kDa), the relative volume change of which was 1.5, 4 and 1.7 times lower, respectively, than in pancreas tissue. The relative volume change of fractions S24 (27.419 kDa) and

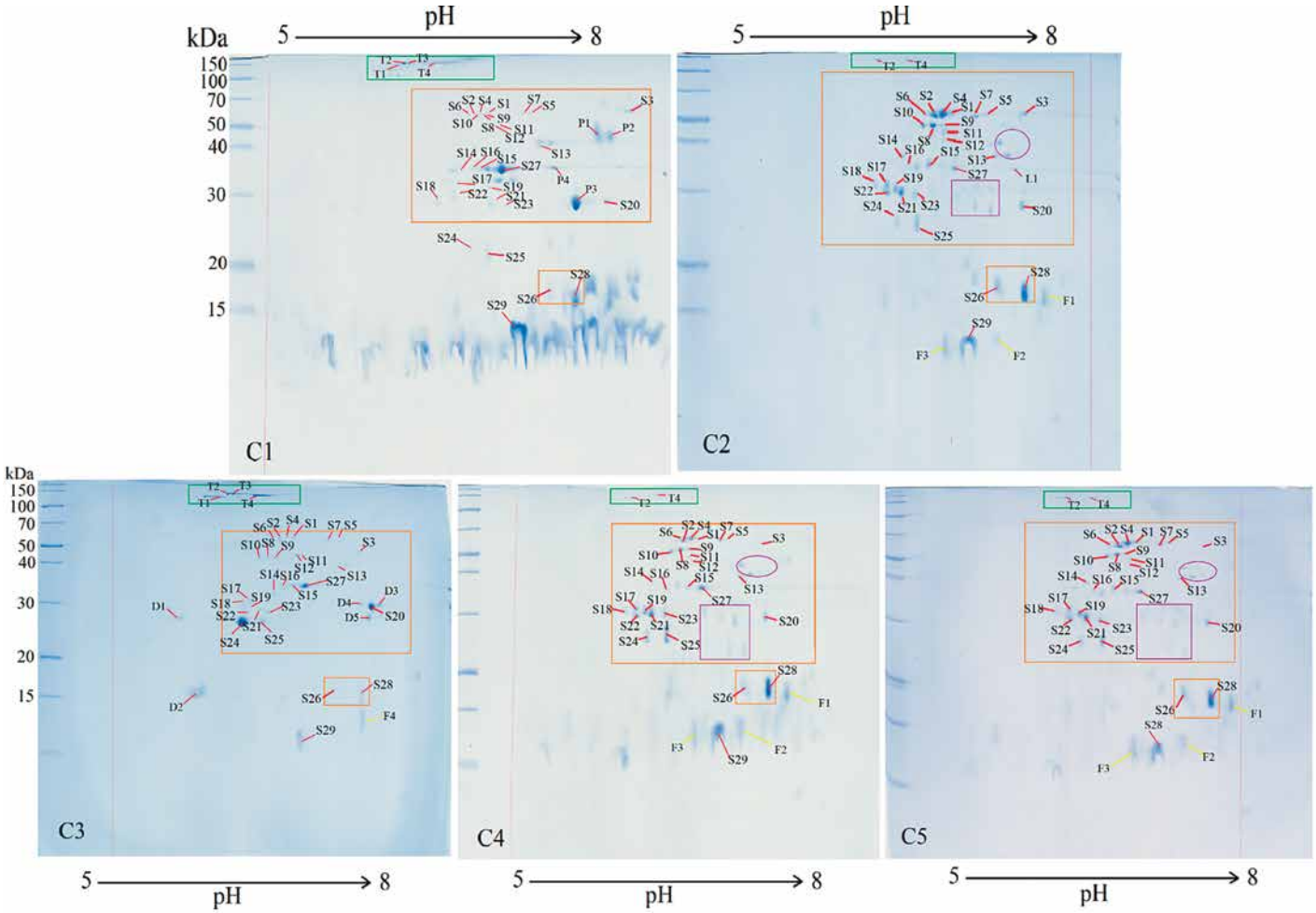

Figure 1. Two-dimensional electropherograms of extracts and pancreas tissue
C1 — pancreas; C2–0.9% NaCl; C3–0.9% NaCl, 1 M L-Arg; C4–0.9% NaCl, 0.5 M trehalose; C5–0.9% NaCl, 1% Gly, 0.1 M L-Pro.
Protein fractions, the relative volume change of which was significantly different, are marked with red arrows

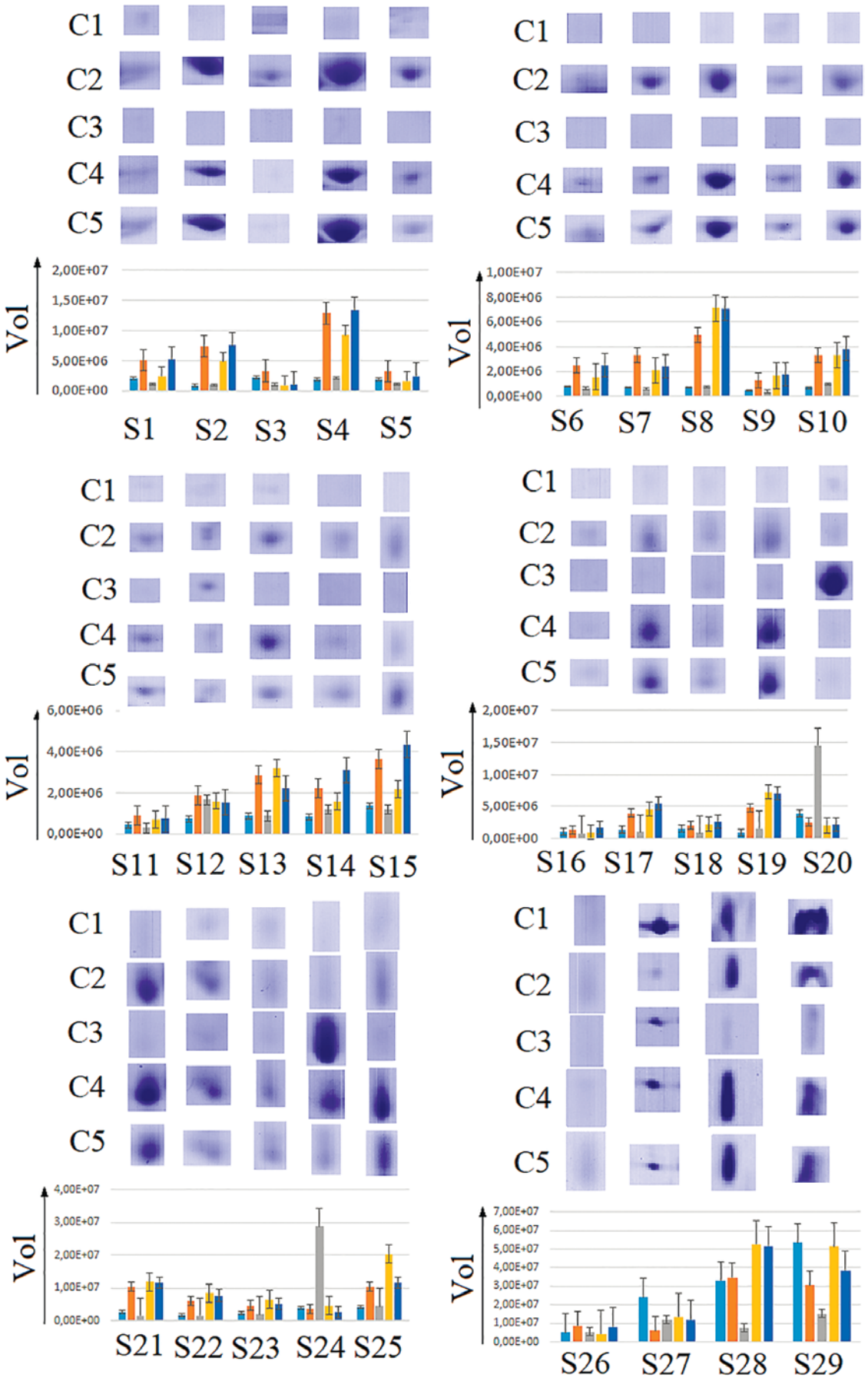

Figure 2. Relative volume change of protein fractions.
Light blue– C1, pancreas; orange– C2, 0.9% NaCl; grey– C3, 0.9% NaCl, 1 M L-Arg; yellow– C4, 0.9% NaCl, 0.5 M trehalose; dark blue– C5, 0.9% NaCl, 1% Gly, 0.1 M L-Pro; S1-S29 — protein fractions, the relative volume change of which was significantly different

Note: the spot intensity was normalized by the total valid spot intensity and the mean value for duplicate analytical gels from triplicates. The data presented are mean ± SD for three independent experiments.

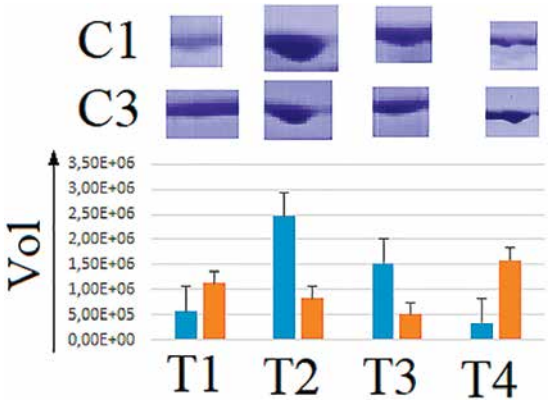

Figure 3. Relative volume change of protein fractions
Light blue– C1, pancreas; orange– C3, 0.9% NaCl, 1 M L-Arg; T1–T4 — protein fractions, the relative volume change of which was significantly different.

Note: the spot intensity was normalized by the total valid spot intensity and the mean value for duplicate analytical gels from triplicates. The data presented are mean ± SD for three independent experiments.

S28 (17.08 kDa) almost did not differ from the pancreas. On the contrary, addition of 1M L-Arg to 0.9% NaCl facilitated enrichment of the extract with fractions S20 (28.699 kDa) and S24 (27.419 kDa), the relative volume change of which was 3.7 and 7.1 times higher, respectively, than in pancreas tissue. Also, an increase by 1.4–2.3 times, on average, in the content of fractions S10 (48.25 kDa), S12 (40 kDa), S14 (36.11 kDa) and S19 (30.68 kDa), respectively, was observed. The relative volume change of other protein fractions was either significantly lower than the level of pancreas tissue or did not differ from it. Addition of 0.5 M trehalose to 0.9% NaCl led to enrichment of the extract practically with all protein fractions excluding S3 (52.168 kDa), S20 (28.699 kDa) and S27 (35 kDa), the relative volume change of which was 2.6, 2.0 and 1.8 times lower, respectively, then in pancreas tissue. The relative volume change of fractions S5 (52.306 kDa), S16 (34.908 kDa), S26 (19.203 kDa) and S29 (13.969 kDa) almost did not differ from pancreas tissue. The use of 0.9% NaCl with 1% Gly, 0.1 M L-Pro as an extracting agent also facilitated enrichment of the extract with all protein fractions excluding fractions S3 (52.168 kDa) S20 (28.699 kDa), S24 (27.419 kDa), S27 (35 kDa) and S29 (13.969 kDa), the relative volume change of which was 2.2, 1.8, 1.5, 2, and 1.3 times lower, respectively, then in pancreas tissue.

In general, the use of 0.9% NaCl as an extracting agent was quite effective. Addition of 1 M L-Arg to 0.9% NaCl significantly reduced extractivity of practically all protein fractions excluding S12 (40 kDa), which did not differ from the extract obtained using 0.9% NaCl. Also, fractions S20 (28.699 kDa) and S24 (27.419 kDa), turned to be an exclusion; their content was the highest among all types of extracting agents. Addition of 0.5 M trehalose to 0.9% NaCl facilitated enrichment of the extract with protein fraction S25 (27.172 kDa), the relative volume change of which was 2.0 times higher than in 0.9% NaCl; the content of fraction S23 (28.821 kDa) also increased by 1.4 times. Addition to the 0.9% NaCl solution of anti-aggregation agents such as 0.5 M trehalose and the mixture 1% Gly and 0.1 M L-Pro led to an increase by 1.3–1.6 times, on average, in the content of fractions S8 (49.328 kDa), S9 (48.27 kDa), S19 (30.68 kDa), S22 (30.594 kDa), S28 (17.08 kDa) and S29 (13.969 kDa). Addition of the mixture of 1% Gly and 0.1 M L-Pro facilitated enrichment of the extract with protein fractions S14 (36.11 kDa), S15 (35.29 kDa), S16 (34.91 kDa), S17 (31.63 kDa), S18 (32.74 kDa), the relative volume change of which was 1.2–1.4 times higher on average than in 0.9% NaCl.

In analysis of electropherograms, selective enrichment of the extracts with certain groups of protein fractions was noted. The use of 1 M L-Arg with 0.9% NaCl facilitated the highest enrichment of fractions T1–T4, which are presented in Figure 3. Furthermore, this extraction facilitated enrichment of the extract with fractions T1 (150 kDa) и T4 (153 kDa) — their relative volume change exceeded this value in pancreas tissue by 2 and 4.6 times, respectively. For fractions T2 (152 kDa) and T3 (154.7 kDa), the relative volume change was on average 3-fold lower compared to pancreas tissue. On the contrary, when using other extracting agents, trace amounts of only two fractions — T2 (152 kDa) и T4 (154.7 kDa) — were noted on the two-dimensional electropherograms.

In general, the use of 0.9% NaCl as an extracting agent was quite effective. Cosmotropic salts act as a protein stabilizer (usually small ions, low polarizability) and as creators of the polar water structure [15,16]. For weak cosmotropic salts such as NaCl and KCl, the recommended initial concentration is 300 mM and 200 mM, respectively; the recommended concentration range is 0–1 M. The 0.15 M NaCl solution was used in the experiment described above.

To prevent protein aggregation, sugars and polyhydric alcohols are also widely used [15,17]. Polyol and sugar osmolytes can disrupt protein hydrogen bonds influencing the protein function [18] and stabilizing the lattice structure of water [19]. Several papers report about stabilization of different biomolecules with trehalose [20]. It is assumed that trehalose induces the well-defined protein-protein distance, which can explain why it inhibits protein-protein interactions and protein aggregation associated with them. However, the excellent anti-aggregation effect of trehalose can also be linked with the fact that the local solvent structures are very important for explaining the mechanism of protein stabilization [21]. The recommended initial concentration of 0.5 M for trehalose and sucrose [15,17] was used in this work.

The use of amino acids as anti-aggregation agents is in demand in the food industry and production of biologically active substances. Amino acids and their derivatives increase the surface tension of water in a concentration of 20–500 mM [19]. It is assumed that hydrophobic surfaces that are present on proteins interact with the hydrophobic surface represented by the arginine clusters. Masking of the hydrophobic surface inhibits protein-protein aggregation [22]; however, scientists also describe other mechanisms, by which arginine prevents protein aggrega-

tion [22,23]. Even though arginine demonstrated the best results in prevention of aggregation among 15 tested amino acids [24], in this work its addition negatively affected diversity of extracted proteins. Glycine demonstrated two stages of stabilization. The first effect (at concentrations lower than 100 mM) is specific for protein and, possibly, is conditioned by multiple direct interactions with polar or charged side chains and partial charges on the peptide backbone of protein. The second stage (at concentrations higher than 100 mM) is similar to anions with the high charge density, where it is associated with competition for water in the unfolding process [25]. Proline has a closed circular structure in the side chain, which has the hydrophobic surface allowing it to interact with proteins by hydrophobic interactions [26]. It has been assumed that the multimeric forms of proline can be responsible for its inhibiting action on aggregation [22]. Combination of glycine (action via the polar part of protein) and proline (action via the hydrophobic part) is able to exert the complex action suppressing protein aggregation, which was demonstrated in the present work.

In general, the use of 0.9% NaCl as an extracting agent was quite effective; however, selectivity of anti-aggregation agents such as sodium chloride, trehalose, arginine, glycine and proline, and their combinations to certain protein groups was noticed.

Conclusion

Effective extraction and purification of target protein substances from animal raw materials is a serious problem for researchers due to the tendency of protein molecules to aggregation. The present work shows that the 0.9% sodium chloride solution was able to extract quite effectively a wide spectrum of protein substances from pancreas tissue, and addition of anti-aggregation agents was characterized by selectivity to certain protein groups. Although arginine demonstrated the best results in prevention of the development of aggregates in several scientific works, the highest content of total protein in the extract with the addition of 1 M L-arginine was conditioned by an increase in three major protein fractions rather than by diversity of the protein composition. Addition of 0.5 M trehalose to the 0.9% sodium chloride solution or a mixture of 1% glycine and 0.1 M L-proline led to an increase in the content of several protein fractions, including those with pI shifted to the alkaline area.

The obtained results are important for intensifying extraction of protein substances including target ones with the following application with various purposes. An effect of anti-aggregation agents on the processes of purification and separation of protein mixtures using membrane technologies will be studied in the subsequent work.

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