DOI: https://doi.org/10.21323/2414-438X-2024-9-3-236-248
Received 08.08.2024
Accepted in revised 21.08.2024
Accepted for publication 26.08.2024
Available online at https://www.meatjournal.ru/jour
Original scientific article
Open Access

BIOTECHNOLOGICAL METHODS FOR SEPARATION OF PIGS PANCREAS GLANDS PROTEIN SUBSTANCES WITH MEMBRANE TECHNOLOGIES

Maria E. Spirina, Ekaterina K. Polishchuk*, Liliya V. Fedulova, Ekaterina R. Vasilevskaya
V. M. Gorbatov Federal Research Center for Food Systems, Moscow, Russia

Keywords: *pancreas gland, electrophoresis, diafiltration, ultrafiltration, dialysis, trehalose, glycine, proline*

Abstract
The pancreas gland (PG) is a secondary product of livestock processing; it contains a wide range of biologically active compounds. The purpose of this article is to analyze the efficiency of technological approaches for pancreas gland extraction with the help of trehalose and a glycine-proline mixture aimed for recovery and separation of the gland's protein-peptide compounds. The extraction was conducted with 0.9% NaCl, 0.9% NaCl, with addition of 0.5 M trehalose (0.9% NaCl-0.5 M trehalose) and 0.9% NaCl with addition of 1% glycine and 0.1 M L-proline (0.9% NaCl-1% Gly-0.1M Pro), the ratio of pancreas gland to extractant was equal to 1:5. The concentration of the protein in the supernatants after their extraction was measured by the biuret reaction in a semi-automatic biochemical analyzer Biochem SA. The proteomic composition of the extracts and the native pancreas gland was assessed by one-dimensional Laemmli electrophoresis in a 12.5% polyacrylamide gel and by two-dimensional O'Farrell electrophoresis. When determining the intensity of the protein fractions, it was noted that the methodology of separation of protein-peptide mixtures extracted from the pigs pancreas gland with the extractant 0.9% NaCl-1% Gly-0.1M Pro, ensured the higher extraction of the proteins in comparison with the method of 0.9% NaCl-0.5 M trehalose. Notwithstanding the fact that application of amino acids (glycine and proline) mixture provided for a greater yield of proteins from the extract into the diafiltrate, the experiments in vitro showed that the diafiltrate obtained though trehalose featured higher activity. This may be explained by the fact that after dialysis removal of trehalose from the protein fraction with a molecular weight of less than 50 kDa, its residual quantities were still sufficient to prevent proteins aggregation and, as a consequence, the biological activity of the extracted proteins was preserved, while in the diafiltrate obtained through amino acids mixture where numerous protein aggregates were detected by 2-DE. This study allowed testing the biotechnological methodics on pig pancreatic tissues aimed to intensifying the extraction and separation of protein compounds. The results of the study are important for development of methodological approaches to obtaining the targeted substances for their further utilizing for various purposes.

For citation: Spirina, M.E, Polishchuk, E.K., Fedulova, L.V., Vasilevskaya, E.R. (2024). Biotechnological methods for separation of pigs pancreas glands protein substances with membrane technologies. *Theory and Practice of Meat Processing*, 9(3), 236–248. https://doi.org/10.21323/2414-438X-2024-9-3-236-248

Funding:
The article was published as part of the research topic No. FGUS-2024-0003 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS.

Introduction

The raw materials of animal origin, as well as secondary products of their processing, contain a wide assortment of biologically active compounds with various purposefulness and mechanisms of action. To prevent metabolic disorders related to age and diet, which are predictors of diabetes mellitus, obesity, dyslipidemia, and cardiovascular diseases [1], food products and dietary supplements are widely used [2]. A promising source of bioactive substances of animal origin is the pancreas gland (PG), which produces a huge amount of endocrine and exocrine compounds and can serve as a source of hormones, enzymes and their precursors, structural, regulatory, secretory and receptor substances [3]. In particular, the medications like insulin, glucagon, trypsin, chymotrypsin, and pancreatin are produced from the pancreas gland [4,5].

The extraction of some individual valuable protein substances from the pancreas gland is pretty challenging, so new technological approaches are currently being sought. One of the promising directions of researches is membrane technologies, which is an effective option for the separation, fractionation and purification of biologically active compounds obtained from various animal tissues [6]. Depending on the purpose, various types of filtration processes, various materials and polymers are used for producing the membranes.

Ultrafiltration is a widely used method for proteins separation, fractionation and purification. However, the issues of adsorption, aggregation of protein molecules and their denaturation are the main challenges that can be faced during the ultrafiltration of proteins in native form [7,8]. The simplest ways to prevent proteins aggregation and adsorp-

tion during their ultrafiltration are dilution followed by diafiltration to maintain a constant pH and ionic strength [9,10] of the mixture exposed to separation. The selection of membrane material, optimal pore size and the rate of penetration of the protein solution through it also affect the optimization of the ultrafiltration process [11]. In addition, sugars and polyhydric alcohols, including glucose, sucrose, trehalose, lactose, glycerol, sorbitol, mannitol, xylitol, and inositol, are also widely used to prevent protein aggregation. It has been suggested that trehalose may stabilize protein structure and may also prevent proteins from interacting with each other [12].

Also, the use of amino acids as anti-aggregating (anti-clogging) agents is in demand in the food industry and the manufacture of dietary supplements. According to the researchers, the pharmacological activity of a substance increases after it is combined with an amino acid, and its solubility in water also improves and cytotoxicity decreases [13]. Glycine, for example, stabilizes aggregated conformations of hydrophobic elastin-like polypeptides through the classical preferential depletion mechanism (traditional depletion mechanism) [14]. Glycine and proline have shown some ability to stabilize hemoglobin [15]. Experimental data demonstrate that proline inhibits protein aggregation by binding to a folding intermediate product and converting it to an enzymatically inactive, "aggregation-insensitive" state [16].

The purpose of the work was to study the efficiency of technological approaches for the separation of protein-peptide substances of pig pancreas glands extracted using extractants containing trehalose and a glycine-proline mixture.

Objects and methods

The object of the study was the pancreas glands of pigs, obtained from Pushkinsky Myasnoy Dvor LLC, Moscow region, Pushkino town. Animal raw materials were trimmed of connective tissue, frozen at minus 18 °C, then crushed and frozen down to minus 40 °C until further extraction. The crushed pancreas gland (PG) was thawed at a temperature of 4 °C and mixed with the extractant in a ratio of 1:5. Extraction was run in a laboratory dispersing unit LDU-3 MPR (Labotex, Russia) with a speed of steering at 400 rpm; extraction lasted 150 min at 4 °C.

To study the efficiency of technological approaches for the separation of protein-peptide compounds extracted from pancreas glands, a series of experiments were conducted. In total of 3 extractions were carried out with the following extractants:

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

Upon extraction process completion, the supernatant was separated by centrifugation for 5 minutes at a speed of 3500 rpm on a centrifuge CM-6M (ELMI, Latvia), after that it was filtered through a cotton-gauze filter to clear from suspended matter. Part of the extract was frozen at minus 40 °C in a freezer IW-401–262 Deep Freezer (Haier, China) followed by its freeze-drying on a laboratory freeze-dryer LS-1000 (Prointeh-bio, Russia). Part of it was saved for further measuring of the protein concentration in the extract, for ultrafiltration and diafiltration. In each sample, the protein concentration was measured by a biuret reaction on a semi-automatic biochemical analyzer Biochem SA (HTI, USA) with the help of the commercial Total Protein reagent (HTI, USA). The measurements were run in triplicate.

To determine the most effective approach to the separation of biologically active protein-peptide mixtures from pigs pancreatic tissues, the ultrafiltration and diafiltration of pancreatic extracts obtained using extractants 0.9% NaCl-0,5 M trehalose and 0.9% NaCl-1% Gly-0.1M Pro were conducted. Press-type ultrafiltration was run in the centrifugal ultrafilters Amicon Ultra-4 50 kDa (Millipore, Germany), the regenerated cellulose was used as the material of the membrane. To run the diafiltration, the device Vivaflow 200 (Sartorius, Germany) was used, polyether sulfone was used as the membrane material, the pass-through capacity amounted to 50 kDa. The resulting ultrafiltrates and diafiltrates were frozen at minus 40 °C in a freezer IW-401–262 Deep Freezer (Haier, China) followed by freeze drying LS-1000 (Prointeh-bio, Russia).

To purify the extracts, ultrafiltrates (UF) and diafiltrates (DF) obtained after extraction with 0.9% NaCl-0.5 M trehalose and 0.9% NaCl-1% Gly-0.1 M Pro, a dialysis method was used, in which method the gradient of concentration promoted the release of low molecular weight substances through the semi-permeable membrane. SnakeSkin Dialysis Tubing regenerated cellulose dialysis bags were used for dialysis (Thermo Fisher Scientific, USA) with a pass-through capacity of 3.5 kDa and a diameter of 16 mm. The samples after dialysis were frozen at minus 40 °C in a freezer IW-401–262 Deep Freezer (Haier, China) followed by freeze drying in the device LS-1000 (Prointech-bio, Russia).

Analysis of molecular weight distribution of the protein fractions in the freeze-dried samples of native pancreas glands, extracts before and after dialysis, UF and DF after dialysis was run by one-dimensional electrophoresis according to Laemmli [17]. For preparation of the sample of freeze-dried native pancreas gland, 2000 mcl of lysis solution was used (9 M urea (PanReac, Germany), 5% β- mercaptoethanol (PanReac, Germany), 2% Triton X-100 (Helicon, Russia), 2% ampholines pH 3–10 (Serva, Germany)). Freeze-dried samples of pancreatic extracts before and after dialysis, as well as UF- and DF-s after dialysis, were diluted in a minimum volume of distilled water, after which 100 mcl of the sample was mixed with 100 mcl of protein buffer (1 ml sodium do-

decyl sulfate (SDS, Panreac, Spain) 10%, 250 µl concentrated β- mercaptoethanol (PanReac, Germany), 625 µl Tris-HCl 0.5 M (PanReac, Germany), 1.5 g urea (PanReac, Germany), bromophenol blue (Helicon, Russia) and heated in a boiling water bath in the device Assistant 26026–1 (Karl Hecht, Germany) for 5 minutes. Next, the resulting homogenate solution was centrifuged at 14,000 rpm for 20 minutes on an Eppendorf 5427 R centrifuge (Eppendorf, Germany). To run one-dimensional electrophoresis, the "VE-10" chamber (Helicon, Russia) was used; it was filled with 12.5% polyacrylamide gel.

O'Farrell electrophoresis was performed using the Bio-Rad camera (Bio-Rad (USA)) with isoelectric focusing (IEF) in glass tubes in the first direction and SDS-PAGE in the second direction, as described by Matsumoto N. [18] with slight modifications [19]: IEF in the first direction was performed in 2.4 mm × 160 mm tubular gels until reaching 3,650 volt-hours. To run the two-dimensional electrophoresis, 100 mg of pancreatic diafiltrate after dialysis was taken and mixed with 1000 mcl of distilled water. The mixture was stirred for 10 minutes at rate of 600 rpm using a MPS-1 vortex (BioSan, Latvia). The resulting homogenate was used in isoelectric focusing. Sedimentation of the samples was also conducted in order to concentrate the proteins and purify them from the various contaminants. For this the samples of diafiltrates were mixed with 10% TCA (PanReac, Spain) in a ratio of 1 to 1 and incubated for 15 hours at a temperature of +4–6 °C. After that the centrifugation was run at rate of 10,000 rpm for 10 minutes at 4 °C on an centrifuge Eppendorf 5427 R (Eppendorf, Germany). The supernatant was poured off, then the resulting precipitate was dissolved with lysis buffer (9 M urea (PanReac, Germany), 5% β-mercaptoethanol (PanReac, Germany), 2% Triton X-100 (Helicon, Russia), 2% ampholines with pH 3–10 (Serva, Germany)). The obtained protein extracts were used for isoelectric focusing.

Marker composed of preparations –standards of molecular weights — was used as a standard solution. (Thermo Scientific, USA). Protein staining was conducted in the solution of the following composition: 10% acetic acid (Component-Reaktiv; Russia), 25% isopropanol (PanReac, Germany), 0.05% coomassie G-250 (Helicon, Russia). To remove the unbound dye, 10% acetic acid (Component-Reaktiv; Russia) was used. To increase resolution capacity, the additional staining was run with silver nitrate (PanReac, Germany) according to Blum's method [20].

In order to run the computer densitometry, wet electropherograms were used. Their full digital images were obtained via the scanner Bio-5000 Plus (Serva, Germany) in 600 ppi mode for one-dimensional electropherograms, and 300 ppi for two-dimensional electropherograms, 2D-RGB and 1D-Gray. The resulting 2D electropherogram images were analyzed using the software ImageMaster ™ 2D Platinum based on Melanie 8.0 (GE Healthcare and Genebio, Switzerland).

To study the biological properties obtained by DF during the *in vitro* experiments, pancreas gland fragments were obtained from male mice (n = 2) of the line C57Bl/6J (8–9 weeks). Animals were euthanized in a euthanasia chamber (VetTech, UK) filled with carbon dioxide, then the abdominal cavity was opened in sterile conditions, the pancreas gland was taken out, placed in a Petri dish and poured with a cold solution (4 °C) of phosphate-buffered saline (Servicebio, China). Pancreatic tissues were washed from erythrocytes with phosphate-buffered saline, then thoroughly chopped with scissors and enzymatic disaggregation was run in a 0.1% solution of type I collagenase (Gibco, USA) at 37 °C, 20 min with constant gentle stirring (250 rpm) on a multifunctional device ImmunoChem-2200 with a function of thermal shaker (HTI, USA). The resulting pancreatic cells were filtered through a sterile nylon filter with a pore diameter of 200–250 µm, then centrifuged at 150 g, 3 minutes (Eppendorf, Germany). The supernatant was removed, and the precipitated cells were flushed 3 times with phosphate-buffered saline. After 3 flushes with phosphate-buffered saline, the isolated cells were incubated in Petri dishes (Thermo Scientific, USA) in DMEM/F12 culture medium (Servicebio, China) with the addition of L-glutamine (PanEco, Russia), 10% fetal bovine serum (HyClone, USA), 1% penicillin-streptomycin (PanReac Applichem, Germany). Petri dishes were put in a CO2-incubator BC — J 160 (Boxun, China), at 37 °C, with 5% CO_2.

After 72 hours, the culture medium was replaced with DMEM/F12 that contained the studied DF samples 0.9% NaCl-0.5 M trehalose and 0.9% NaCl-1% Gly-0.1 M Pro at the concentrations of 100 ng /ml, incubated at 37 °C, with 5% CO_2 for 6 days long. As a control reference, a cell culture was used which was cultivated in a nutrient medium without the addition of the studied samples.

On day 9, cells were removed with 0.05% trypsin-EDTA (Sigma-Aldrich, Germany). To record the number and viability of cells, an automatic counter LUNA-FL (Logos Biosystems, South Korea) was used. A 2 mcl cell suspension was mixed with 18 mcl of acridine orange/ propidium iodide dye solution, and 10 mcl of this solution was transferred to the chamber of a PhotonSlide counting plate (Logos Biosystems, Korea). During the measurement process, the following data were obtained: the total number of cells in 1 ml, the number of unstained (living) cells, the share of viable cells, histogram of cell size distribution within the selected range and a photograph of the plate contents.

The morphology of mice pancreatic cells was visually assessed using an inverted phase-contrast biological microscope MIB-R, a digital camera MC-5 and MS-View software (LOMO, Russia).

Statistical processing was conducted using the Statistica 10.0 software package. The results were presented as mean values and standard deviations (Mean ± SD). Statistical significance was calculated using nonparametric Mann–Whitney U tests. A probability of 0.05 was selected as the significant level.

Results and discussion

The results of determining the protein concentration in pancreatic extracts obtained using various extractants are presented in Table 1.

Table 1. Results of determining protein concentration in extracts using various anti-aggregating agents

Extragent	0.9% NaCl,	0.9% NaCl-0.5 M trehalose	0.9% NaCl-1% Gly-0,1 M Pro
Protein concentration, g/l	24.80 ± 0.89^{a}	$33.33 \pm 0.98^{b, c}$	$28.79 \pm 0.13^{b, d}$

$^{a-b, c-d}$ — statistically significant difference (Mann-Whitney *U*, $p < 0.05$).

It was shown that the addition of 0.5 M trehalose, 1% Gly-0.1MPro to 0.9% NaCl contributed to the yield of protein substances into the extractant — this protein content exceeded the protein content in the extract obtained with 0.9% NaCl by 34.4% ($p < 0.05$) and 16.1% ($p < 0.05$), respectively.

Freeze-dried samples obtained using various extractants are presented below in the Figure 1, which clearly demonstrates not only the color variation during the processes of extraction and purification of protein substances, but also the white color of DF after dialysis in case of using 0.9% NaCl-0.5 M trehalose as an extractant, while DF after dialysis in case of using 0.9% NaCl-1% Gly-0.1 MPro as an extractant featured yellowish tint which indicates a higher presence of protein substances.

The results of the analysis of the protein fractions distribution among the freeze-dried samples of native pancreas glands, the extracts before and after dialysis, UF and DF after dialysis, obtained with the various extractants, are presented below in one-dimensional (Figure 2) and two-dimensional (Figures 3 and 4) electropherograms.

The track 3 in the Figure 2A featured a solid colored band and the absence of clearly outlined protein fractions, which proved the destruction of proteins during dialysis of the pancreas gland extract with 0.9% NaCl-0.5 M trehalose, which phenomenon was not observed when 0.9% NaCl-1% Gly-0,1M Pro was used as an extractant. Thus, on the track 4B, the well-expressed bands of protein frac-

Figure 1. Freeze-dried samples

[I — pancreas gland (PG), II — pancreas gland extract with 0.9% NaCl-0.5 M trehalose before dialysis, III — pancreas gland diafiltrate with 0.9% NaCl-0.5 M trehalose after dialysis, IV — pancreas gland extract with 0.9% NaCl-1% Gly-0.1M Pro before dialysis, V — pancreas gland extract with 0.9% NaCl-1% Gly-0.1M Pro after dialysis, VI — pancreas gland diafiltrate with 0.9% NaCl-1% Gly-0.1M Pro after dialysis]

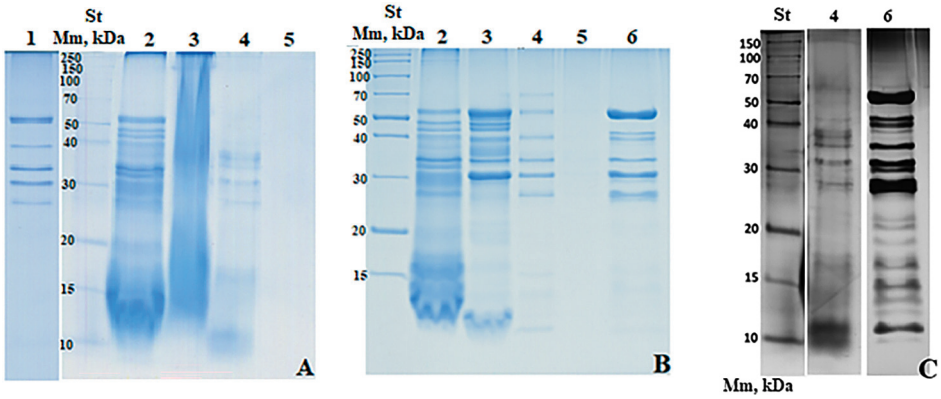

Figure 2. One-dimensional electropherograms of the samples prepared using 0.9% NaCl-0.5 M trehalose (A) and 0.9% NaCl-1% Gly-0.1 M Pro (B); fragments of DF electropherograms stained with silver nitrate (C).

Legend keys: St — molecular weight standard, kDa, 1 — pancreas gland extract (PG) with 0.9% NaCl, 2A — native pancreas gland, 3A — pancreas gland extract after dialysis, 4A — DF after dialysis, 5A — UF after dialysis, 2B — native pancreas gland, 3 B — pancreas gland extract before dialysis, 4 B — pancreas gland extract after dialysis, 5 B — UF after dialysis, 6B — DF after dialysis, 4C — DF using 0.9% NaCl-0.5 M trehalose after dialysis, stained with silver nitrate, 6C — DF using 0.9% NaCl-1% Gly-0.1M Pro after dialysis, stained with silver nitrate

tions were clearly visible, however, when compared with the track 3B, it was clear that some protein fractions were lacking, which also proved the partial destruction of proteins after dialysis. The inefficiency of press-type ultrafiltration with subsequent dialysis was determined, since there were no protein fractions on the tracks 5A and 5B. When comparing tracks 4A and 6B, it became obvious that the methodology for separating protein-peptide mixtures obtained from pancreas gland with the help of 0.9% NaCl-1% Gly-0,1M Pro as an extractant had higher efficiency than in case of using 0.9% NaCl-0.5 M trehalose. The mechanism of trehalose stabilization of proteins may partially depend on the environment as well as the type of molecule being stabilized. The mobility of biomolecules may decrease upon water binding with trehalose or vitrification, which can sometimes result to minor protein denaturation [21,22]. In its turn, proline promotes the solubility of sparingly soluble proteins and also behaves as a chaperone during protein folding [23]. In track 6C a diversity of protein fractions was observed within the range from 50 to 10 kDa, with the most expressed fractions within the band of 50, 40, 39, 35, 31, 30, 27 and 11 kDa (Figure 2 C). It can be assumed that these protein bands may correspond to pancreatic triacylglycerol lipase (50.0 kDa) [24], protein associated with pancreatic lipase (53.2 kDa) [25], phospholipase 2A (39–40 kDa) [26], elastase (27–28 kDa) [27], chymotrypsin-like elastase (28.8 kDa) [28], trypsin (24.4 kDa) [29], secretin (14.6 kDa) [30], gastrin (11.5 kDa) [31], insulin (11.6 kDa) [32] and colipase (12.1 kDa) [33]. The presented protein fractions are mainly proteolytic enzymes that implement the specific functions. Lipases are responsible for the hydrolysis of ester bonds in triacylglycerols, leading to the formation of glycerol, free fatty acids, diacylglycerols and monoacylglycerols [34]. Phospholipase A2 belongs to the phospholipid-hydrolyzing enzymes family and participates in the metabolism of phospholipids in the cell membranes, including the synthesis of prostaglandins, into the transmission of cell signals and in the serum lipoproteins metabolism [35]. Trypsin is the serine endopeptidase, which catalyzes the peptide bonds hydrolysis at the carboxyl terminus (C-terminus) of the amino acids lysine and arginine, thus releasing polypeptides. Elastase is responsible for the hydrolysis of peptide bonds on the C-terminal side of the amino acids valine, alanine and glycine with releasing of the polypeptides [36]. Secretin is a key gastrointestinal hormone involved in the regulation of pH of duodenal content [37]. Gastrin stimulates the gastric mucosa to produce hydrochloric acid and the pancreas gland to produce digestive enzymes, also stimulates the contraction of smooth muscles, enhances blood circulation and water secretion in the stomach and intestines [38]. Insulin reduces the concentration of glucose in the blood, increases permeability of cells to monosaccharides, amino acids and fatty acids, it accelerates glycolysis, the pentose phosphate cycle and glycogen synthesis in the liver [39]. Colipase is a cofactor of pancreatic lipase, which allows the lipase to get fixated at the interface of lipid and water [40].

For the convenience of two-dimensional electropherograms analyzing, the groups of proteins on them located in the same ranges of molecular weights (MW) and isoelectric points (pI), were divided into the blocks (Figures 3 and 4, Tables 2 and 3). Comparing two-dimensional electropherograms of A, B and C in Figure 3 using 0.9% NaCl-0.5 M trehalose as an extractant, it is necessary to note that the electropherogram of the extract (A) was characterized by the largest quantity of protein fractions; and the electropherogram of DF without sedimentation (C) showed the greater diversity of protein fractions in comparison with the DF electropherogram with sedimentation (B), thus proving a greater efficiency of the sample preparation without sedimentation. Within the area of molecular weights (MW) from 30 to 50 kDa and isoelectric points from 6.3 to 7.3 (block 1), 9 protein fractions were found that were present in all studied samples. However, a slight shift was observed in their molecular weights and isoelectric points (pI) towards a lesser extent when comparing the different methods of the sample preparation, which differed significantly from the extract. Thus, the isoelectric points of the proteins No. 1–9 shifted to the alkaline area in both DFs in reference to the extract, which is especially noticeable for the proteins No. 1–4. The molecular weights of the protein fractions were slightly shifted and on average were within the range of 35–38 kDa. Molecular weights and pI can shift into one direction or another due to post-translational modifications (phosphorylation, glycosylation, etc.), with the cleavage of signal sequences or the other sequences, with the formation of protein complexes, various protein isoforms, as well as due to nonspecific proteolytic cleavage and the degradation of the protein [41,42].

The fraction No. 6was the most intensely expressed in 1 block of proteins, and in the DF electropherogram without sedimentation (C) the volume of this protein fraction accounted for 25,627,460 c. u., which was 1.3 times greater than in the electropherogram A, and also 7 times greater than in electropherogram B. The intensity of the remaining protein spots was also higher in the DF electropherogram without sedimentation (C), compared to the electropherograms of the extract (A) and DF with sedimentation (B).

Within this range of molecular weights and pI, the following proteins can be found: phospholipase [26], annexin A1 [43,44], annexin A4 [45], annexin A2 [46], cathepsin B [47], cathepsin D [48], procathepsin L [49] and procathepsin H [50,51]. Presumably, the most expressed protein in block 1, which is marked by number 6, may be annexin A2 (38.8 kDa, pI 6.92) [52].

Block 2 in the electropherograms of all samples featured the availability of groups of the proteins with molecular weights from 26 to 31 kDa and pI within the range of 6.8–7.5. There were 5 protein spots found on the electropherograms A, B and C (protein fractions No. 10–14), and protein No. 13, with a MW ~of 30 kDa and pI ~7.35, was most expressed on the DF electropherogram without

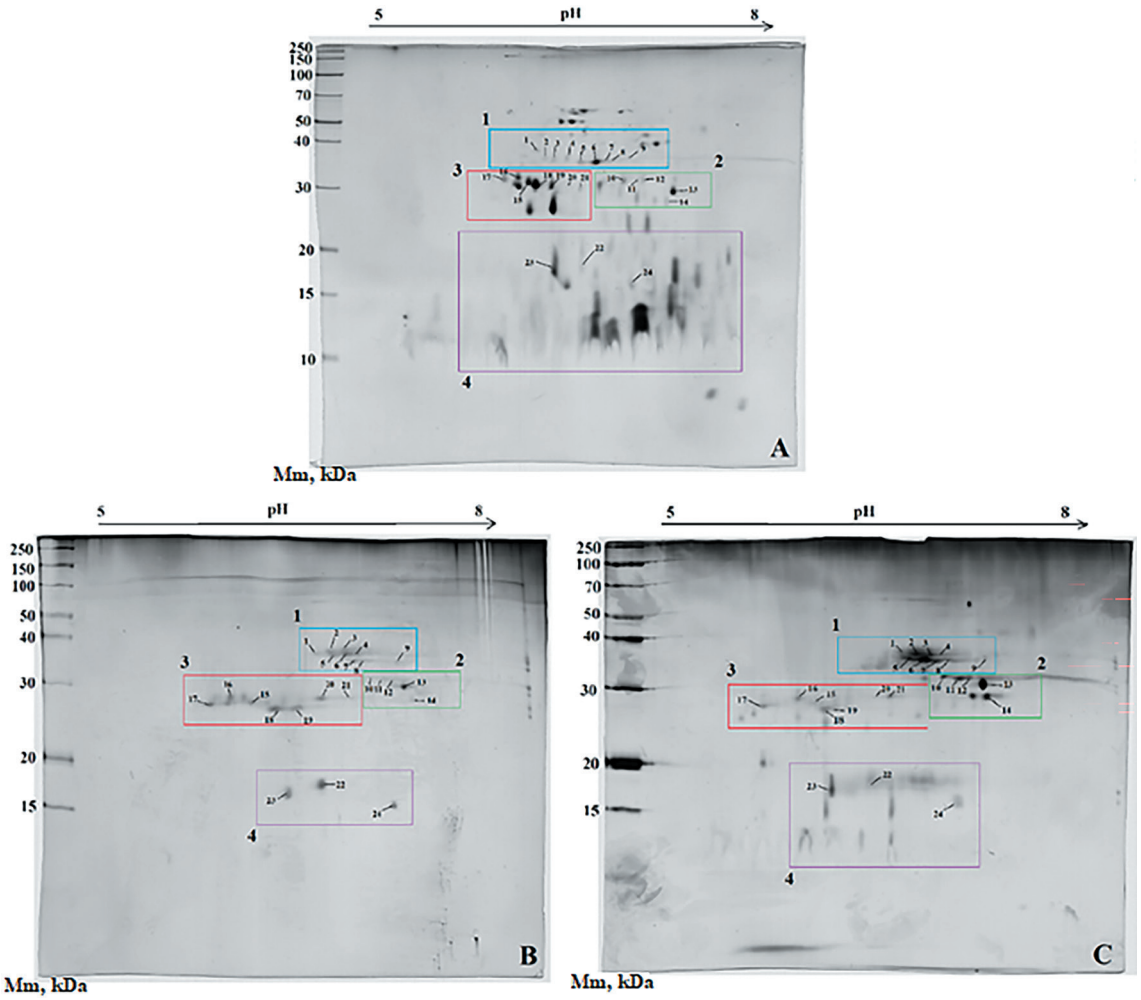

Figure 3. Two-dimensional electropherograms of PG extract (A), precipitated diafiltrate (B), and non-precipitated diafiltrate (C) obtained using 0.9% NaCl-0.5 M trehalose as extractant. Legend keys: Block 1 — marked blue; Block 2 — marked green; Block 3 — marked red; Block 4 — indicated mauve

sedimentation and exceeded the intensity values on electropherograms of the extract and DF with sedimentation by 1.46 times and 1.22 times, respectively. It is interesting to note that the proteins No. 10–12, as well as No. 14, barely noticeable in electropherograms A and B, were clearly visible in electropherogram C. The protein fraction with a MW of 29 kDa and pI 7.2 is clearly visible in the DF electropherogram without sedimentation (C) and had a coloring intensity of 17,042,702 c. u., while in the electropherogram of the extract (A) its intensity was 2,623,714, which was 6.5 times lower than its intensity in DF without sedimentation (C). In general, the electropherogram of extract (A) featured the greatest diversity of protein fractions in the studied area, and the electropherogram of DF with sedimentation featured the smallest diversity.

The following proteins may be found within this molecular weights range: chymotrypsin C [53], chymotrypsinogen B2 [54,55], member of the chymotrypsin-like family elastase 1 [28], and elastase [56]. The most expressed protein in this block, marked with number 13, presumably may correspond to chymotrypsinogen B2 (29.1 kDa, pI 7.43) [57].

In block 3 the groups of protein fractions were found that belonged to the MW range of 25–31 kDa and pI from 5.6 to 7.0. Seven protein spots were found in the electropherograms of all studied samples (fractions No. 15–21), and for the proteins 16 and 17 pI shift towards the area of 5.7–5.8 was observed in the electropherograms of the diafiltrates (B and C), compared with the electropherogram of the extract (A), where the pI values of proteins 16 and 17 were equal to 6.2 and 6.1, respectively. It is also necessary to note the shift in the molecular weights of protein fractions 15–21 in the diafiltrates by an average of 2–3 kDa to the lesser values, in comparison with the extract. In general, the intensity of staining of protein spots 15–21 was higher in the electropherogram of the extract (A) and it reached 60,599,552 c. u. (fraction 18), while in the DF electropherograms with and without sedimentation this value reached 27,059,680 c. u. (fraction 18) and 12,459,113 c. u. (fraction 17) respectively. However, it is worth paying attention to the fraction number 20, which intensity in the 2-DE extract was 770,700 c. u., which was 17.8 times less than in the 2-DE diafiltrate with sedimentation, and 3.4 times less than in the 2-DE diafiltrate without sedimentation. Comparing electropherograms B and C, one can note that block 3 in electropherogram C featured a wide variety of protein spots, on which basis it can be concluded that the sedimentation technology in the preparation of diafiltrate sample for two-dimensional electrophoresis leads to "adhesion" of some protein fractions.

Table 2. Major protein fractions observed in 2D-electropherograms

		Extract (A)		DF with sedimentation (B)		DF without sedimentation (C)	
	Fraction number	MW, kDa / pI	Volume of the spot, c. u.	MW, kDa / pI	Volume of the spot, c. u.	MW, kDa / pI	Volume of the spot, c. u.
1 block	1	39/6.4	3250480	37/6.8–6.9	2462218	37/6.6	8209223
	2	37/6.3	2078607	38/6.8–6.9	18222116	37/6.7–6.8	22283304
	3	37/6.5	6814863	38/6.9–7.0	11433116	37/6.9	18283564
	4	37/6.6	3902051	37/7.0	7775438	37/6.9–7.0	9530793
	5	35/6.7	2578116	35/6.9	9815580	36/6.7	14523944
	6	35/6.9	19726360	35/7.0	3651586	36/6.7–6.8	25627460
	7	35/6.9	2620893	34/7.1	6899868	34/6.9	11582044
	8	35/7.0	1129037	34/7.2	5317755	34/7.0	6214498
	9	35/7.1	2019942	34/7.3	1770069	36/7.3	2407177
2 block	10	31/7.2	3844677	31/7.3	7252735	31/7.2	13198519
	11	31/7.1	1728022	31/7.2	9658015	31/7.1–7.2	22687422
	12	31/7.0	5705523	31/7.1	3543859	31/7.0	12672727
	13	29/7.3–7.4	41674312	29–30/7.4	50013524	30–31/7.3	60929312
	14	28/7.4	4011557	28/7.4	18876574	29/7.3	27470042
3 block	15	31/6.3–6.4	31991576	27/6.3	10863911	28/6.1	5363564
	16	32/6.2	21427866	28/6.0	10201778	29/5.9	8694539
	17	31/6.1	10504652	27/5.8	18248104	28/5.7	12459113
	18	30–31/6.4	60599552	26/6.4–6.5	27059680	27/6.3	11679986
	19	30–31/6.5	27205454	26/6.6	25008948	27/6.3–6.4	3282697
	20	30/6.6	770700	28/6.7–6.8	13699304	29/6.5	2657022
	21	30/6.7	3634289	28/7.0	5857993	29/6.6	9958788
4 block	22	18/6.7	5988978	17/6.7–6.8	44363600	18/6.4–6.5	13464702
	23	17–18/6.5	91543784	16/6.5	4798642	17–18/6.2	28625686
	24	16/7.0	5304107	15/7.3	36110256	16/7.1	6687567

Block 3 may contain proteins such as trypsin [29] and trypsinogen [58,59]. Fraction number 20, which was most expressed in DF extracts, presumably may correspond to trypsinogen (28.2 kDa, pI 6.85).

The protein spots marked in the block 4 of the 2-DE extract (A), had molecular weight range from ~22 kDa to 10 kDa and isoelectric points from 5.4 to 7.8. In case of diafiltrate, in electropherograms B and C the molecular weights of the proteins in the block 4 ranged from 20 kDa to 10 kDa, and the isoelectric point ranged from 6.2 to 7.4. Block 4 on the electropherogram of the extract featured a wide variety of protein spots, as well as the availability of proteolytic changes in them in the area below 15 kDa. In block 4 on the electropherogram C (DF without sedimentation), protein fractions were noted within the area of MW ~19 kDa and pI 6.9–7.3, which were not observed in the electropherogram of DF with sedimentation (B). In general, in the block 4, 3 the protein fractions were found that were present in the electropherograms of all studied samples (fractions No. 22–24). The highest intensity of staining among the total protein fractions was characterized by fraction 23 on electropherogram A, having a MW of 17 kDa and pI of 6.5; the volume of this protein spot was 91,543,784 c. u., which was 20.5 times greater than in the electropherogram B and 3.2 times greater than in the electropherogram C.

The following protein fractions may be found within this molecular weight range: colipase [33], insulin [32], proglucagon [60], secretin [30], trefoil factor-2 [61], gastrin [31], cholecystokinin [62], phospholipase major isoenzyme [63].

Comparing the electropherograms in Figure 3 of the extract (A), DF with sedimentation (B) and DF without sedimentation (C) using 0.9% NaCl-1% Gly-0.1M Pro as an extractant, it was found that protein profile of the DF radically differed from the extract, and therefore it was difficult and uninformative to compare the DF electropherograms (B and C) with the electropherogram of the extract. However, it was found that both the DF electropherogram without sedimentation and the DF electropherogram with sedimentation showed fractions of the protein aggregates with MW above 50 kDa, although the membrane with a 50 kDa cutoff was used for diafiltration. The extensive protein aggregates were also observed in the alkaline area. The availability of protein aggregates gave the ground to suggest that the presence of amino acids in the extract allowed the proteins to acquire/remain in a conformation that prevented them from adhesion to each other, and after their removal during dialysis, the proteins prone to aggregation formed this area at 2-DE. During the preparation of the samples with sedimentation, it was noted that the intensity of protein fractions increased.

Comparing the protein groups in the block 1 in the electropherograms B and C, one can note a shift in the pI of the protein fractions from the range of 6.6–7.1 (electropherogram C) to the range of 7.0–7.6 (electropherogram B). The change in the molecular weights of the proteins from 49–65 kDa was also detected (electropherogram C) to 49–69 kDa (electro-

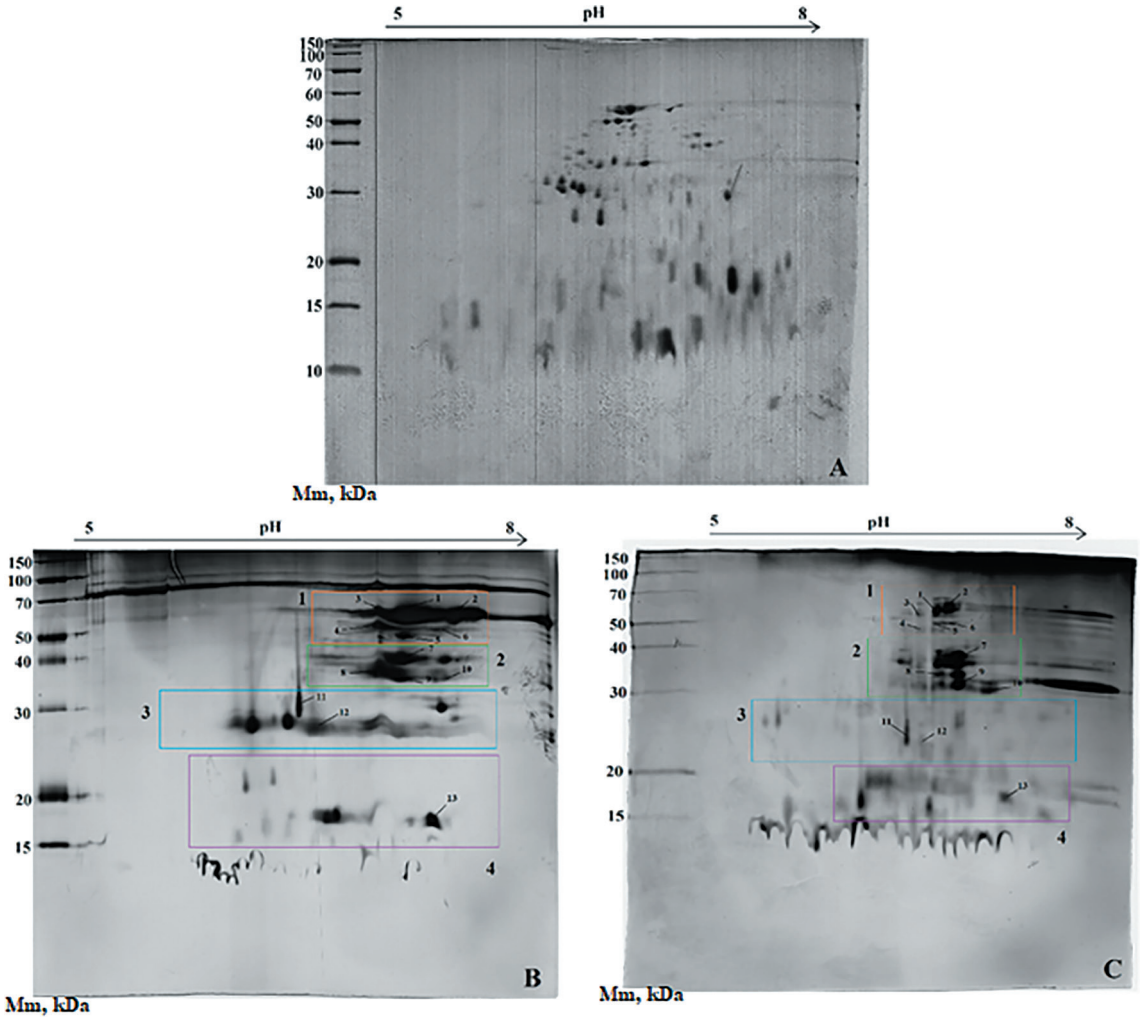

Figure 4. Two-dimensional electropherograms (2- DE) of pancreas gland extract (A), sedimented diafiltrate (B) and diafiltrate without sedimentation (C), obtained using 0.9% NaCl-1% Gly-0.1M Pro as extractant. Legend: Block 1 — marked orange; Block 2 — marked green; Block 3 — marked blue; Block 4 — indicated mauve

pherogram B). Despite the difficulty in interpreting the results due to presence of protein aggregates, 6 protein fractions were detected that were present in both electropherograms (fractions 1–6, Table 3). Fraction 1 was characterized by the highest intensity of staining on the DF electropherogram with sedimentation (B); the volume of the spot made up 151,776,770 c. u. (MW 55–69 kDa; pI 7.1–7.4), which was 19.2 times more than in the DF electropherogram without sedimentation (C). The least intensely expressed fraction in this block of proteins was the fraction No. 4 on the DF electropherogram without sedimentation (C), its volume was 799,690 c. u. (MW 49 kDa; pI 6.7), while the volume of this fraction in the DF electropherogram with sedimentation (B) comprised 17,187,413 c. u. (MW 53–60 kDa; pI 6.9–7.1), which was 21.5 times more. In this range of protein weights, the most expressed fractions may presumably be triacylglycerol lipase [64], pancreatic triacylglycerol lipase [24] and pancreatic lipase-associated protein [25].

Block 2 in the electropherograms was represented by groups of the proteins with pI 6.6–7.3 and MW 30–39 kDa (electropherogram C) and with pI 6.9–7.5 and MW 35–41 kDa (electropherogram B). Four protein fractions were found that matched both electropherograms. Block 2 on the electropherograms was represented by groups of proteins with pI 6.6–7.3 and MW 30–39 kDa (electropherogram C) and with pI 6.9–7.5 and MW 35–41 kDa (electropherogram B). Four protein fractions were found that coincided in both electropherograms (fractions No. 7–10, Table 3). Fraction No. 8 on 2-DE diafiltrate with sedimentation (B) featured the highest intensity of staining, its volume was 65,789,330 c. u., while its volume on the 2-DE diafiltrate without sedimentation (C) was 8.9 times less, and its molecular weight on the electropherogram C shifted into the range of 33–34 kDa, in comparison with electropherogram B, where its MW ranged within the area of 38–40 kDa. The fraction No. 10 was the least expressed (among the total protein fractions) on 2-DE diafiltrate with sedimentation, with MW of 37 kDa and pI 7.4–7.5, its intensity made up 26,984,492 c. u., which is 2.4 times less than for 2-DE diafiltrate without sedimentation. Presumably, the most pronounced fraction in the block 2 corresponded to phospholipase 2A (39–40 kDa) [26].

Block 3 shows groups of protein fractions within the range of MW 20–29 kDa and pI 5.5–7.8. Despite the wide variety of protein fractions available in this block in both electropherograms, there were only 2 common protein fractions (fractions No. 11 and 12). Fraction No. 11 featured

Table 3. Major protein fractions observed in 2 D electropherograms

		DF with sedimentation (B)		DF without sedimentation (C)	
	Faction number	MW, kDa / pI	Volume of the spot, c. u.	MW, kDa / pI	Volume of the spot, c. u.
1 block	1	55–69/7.1–7.4	151776770	55–65/6.8–6.9	7901400
	2	55–69/7.4–7.6	141606173	55–65/6.9–7.0	9312176
	3	60–69/6.9–7.0	24085491	55–60/6.7	3969649
	4	53–60/6.9–7.1	17187413	49/6.7	799690
	5	50/7.1–7.2	17276026	50/6.8–6.9	1935011
	6	53/7.4	4868449	50/6.9–7.0	1373070
2 block	7	39–41/7.0–7.4	56484832	35–39/6.8–6.9	47240403
	8	38–40/6.9–7.0	65789330	33–34/6.8–6.9	7359654
	9	37–40/7.1–7.2	40374312	30–31/6.9–7.1	12979550
	10	37/7.4–7.5	26984492	29–30/7.2–7.3	64556730
3 block	11	29–33/6.5–6.6	77202204	23–27/6.7	18918796
	12	27–28/6.6–6.7	55503144	21–23/6.8	2752734
4 block	13	16–17/7.3–7.4	98002590	16–17/7.4	25712488

a higher intensity of staining both in the DF electropherogram with sedimentation (B) and in the DF electropherogram without sedimentation (C). On 2-DE diafiltrate with sedimentation its molecular weight and volume made up 29–33 kDa and 77,202,204 c. u., while for 2-DE diafiltrate without sedimentation this value was 23–27 kDa and 18,918,796 c. u. The isoelectric point of this fraction was also shifted: in the case of sedimentation of the diafiltrate, the pI value accounted for 6.5, and without sedimentation it was 6.7. The lowest intensity among the total protein fractions in this block was observed in the fraction No. 12 at 2-DE diafiltrate without sedimentation (C) and amounted to 2,752,734 c. u., which was 20.2 times less than in 2-DE diafiltrate with sedimentation. Elastase is present in this range of protein weights [27], chymotrypsin-like elastase [28] and chymotrypsin [65].

Protein fractions isolated in the block 4 had a molecular weight within the range of 15–22 kDa and isoelectric points from 6.3 to 7.4. As in the case of the block 3, despite the rather large number of protein fractions in the block 4, there was only one common fraction for electropherograms B and C (fraction no. 13). Its molecular weight was the same in both electropherograms and amounted to 16–17 kDa, the isoelectric point was within the area of 7.3–7.4. The intensity of fraction No. 13 was greater at 2-DE diafiltrate with sedimentation (B) and amounted to 98,002,590 c. u., while for 2-DE diafiltrate without sedimentation (C) it amounted to 25,712,488 c. u. The most pronounced protein in this block, presumably, may be a phospholipase (16.4 kDa).

When studying the biological properties of the obtained DFs in experiments in vitro, it was found that after 6 days the pancreatic cells spread over the surface of the Petri dish the culture was represented by islet of alpha-, delta-, acinar, ductal and stellate myofibroblast-like cells (capable of switching from tranquil phenotype to an activated phenotype and back), the latter were more numerous in the control Petri dish (Figure 4). After 9 days, in the control dish the predominantly contaminating substances were observed, it was fibroblast-like cells and a small quantity of spindle-shaped cells.

It was noted that when the studied samples were added into the medium, on the 6th day the islet cells showed good adhesion and spreading over the surface, forming a denser monolayer of cells.

It is interesting to note that when the samples of DF 0.9% NaCl-0.5 M trehalose and DF 0.9% NaCl-1% Gly-0.1 M Pro were added into the medium, the cells retained their rounded shape. It was particularly expressed when added to the medium DF 0.9% NaCl-0.5 M trehalose, despite the fact that, in accordance with [66], primary isolated pancreatic cells grown on 2D substrates usually possess flatter and elongated morphology. In experimental dishes the cells formed rosette-shaped micro-communities, in dishes with a nutrient medium with DF 0.9% NaCl-0.5 M trehalose added the beta cells were detected taking on the typical morphology of polarized polyhedron.

In result of studying the biological properties obtained by DF in experiments *in vitro* on the primary isolated mice pancreatic cells, it was found that when the studied samples were added to the medium, the islet cells showed good adhesion and spread over the surface on the 6th and 9th days, forming a denser monolayer of cells (Figure 4). After 9 days, in the control dish the predominantly contaminating substances were observed, it was fibroblast-like cells and a small quantity of spindle-shaped cells.

In experimental dishes cells formed rosette-shaped micro-communities, in dishes with a nutrient medium with DF 0.9% NaCl-0.5 M trehalos e added, the beta cells were detected taking on the typical morphology of a polarized polyhedron.

The results of recording the viability and number of the cells on the 9th day are presented below in the Table 4.

Table 4. Impact of DF 0.9% NaCl-0.5 M trehalose and DF 0.9% NaCl-1% Gly-0.1 M Pro at a concentration of 100 ng /ml on the number and viability of the pancreatic cells

	Concentration of living cells (cells /ml)	Survival rate (%)	Average cell size (µm)
Control	3.68×10^5	47.4	16.6
Gly	3.17×10^5	85.5	15.5
Treg	2.88×10^5	73.9	16.8

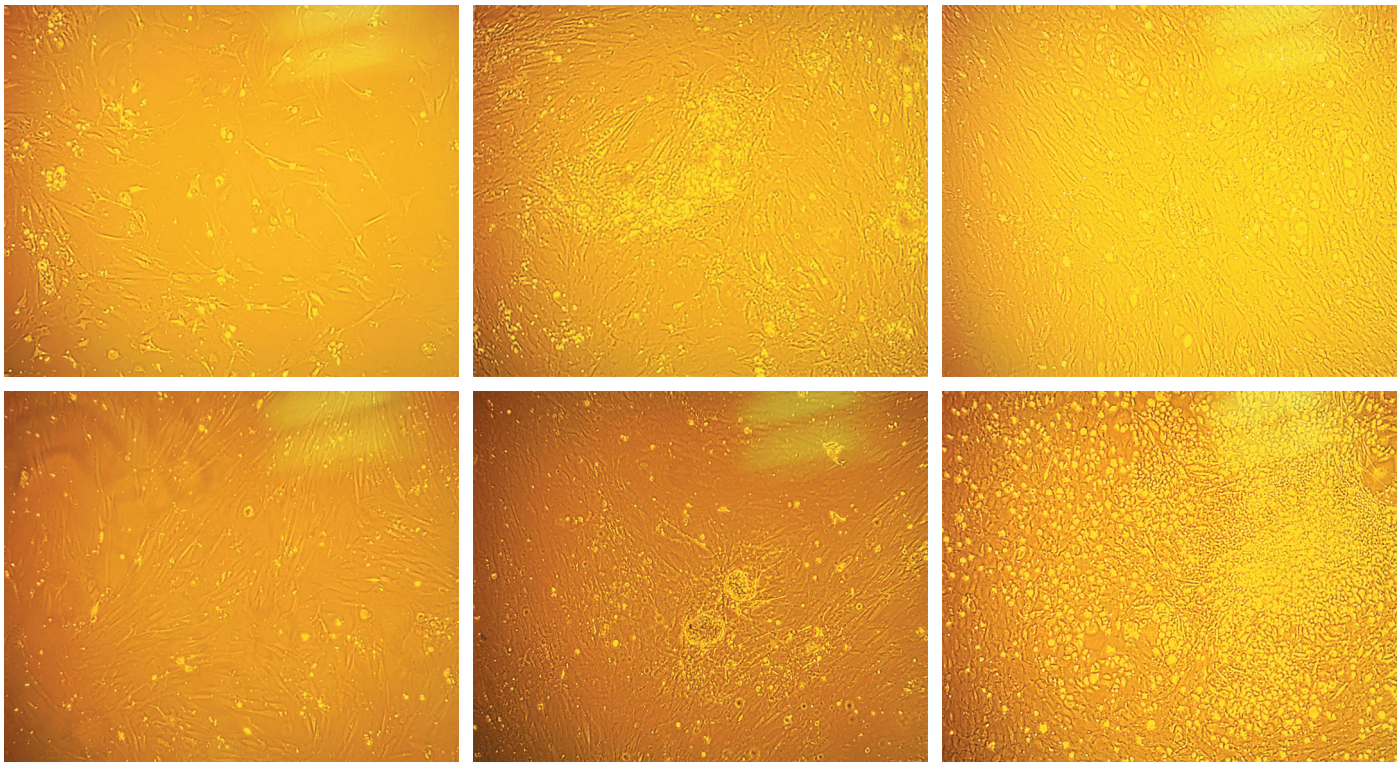

Figure 4. Culture of pancreatic cells on a Petri dish: from above — after 6 days, from below — after 9 days. Inverted microscope, magnification ×100

The high concentration of living cells of a larger volume found in the control sample, as well as their high survival rate, is related to the predominance of fibroblast-like cells in the culture, while there is a smaller number and lower survival rate of cells grown in a medium with DF 0.9% NaCl-0.5 M trehalose added is related to the predominance of islet cells, possibly beta cells, in the medium. This is quite important due to the fact that beta cells are particularly get affected by hypoxia, showing reduced viability and loss of insulin secretory capacity soon after isolation and cultivation on a flat 2D substrate [66].

Conclusion

The methodology for separation of the protein-peptide mixtures, obtained from pigs' pancreas glands using 0.9% NaCl-1% Gly-0.1M Pro as an extractant, allowed extracting more proteins than when using 0.9% NaCl-0.5 M trehalose. Press-type ultrafiltration followed by dialysis was proved inefficient in both approaches. However, when analyzing two-dimensional electropherograms, it was found that during the diafiltration technology using 0.9% NaCl-0.5 M trehalose as an extractant, the proteomic distribution of diafiltrates on 2-DE in the area of less than 50 kDa (protein cutoff threshold for diafiltration) corresponded to the proteomic distribution in the extract. The application of 0.9% NaCl-1% Gly-0.1M Pro contributed to significantly higher yield of the proteins into the diafiltrate, however on 2D-EF there were many protein aggregates noted, including those with MW above 50 kDa, though during diafiltration a membrane with a cutoff of 50 kDa was used. The availability of the protein aggregates suggests that the presence of the amino acids in the extract and diafiltrate allowed the proteins to acquire/remain in a conformation that prevented them from sticking to each other and after their removal during dialysis, the proteins aggregated to each other again. When using 0.9% NaCl-0.5 M trehalose as an extractant, the protein fractions of annexin A2, chymotrypsinogen B2 and trypsinogen were most intensely expressed, and the use of 0.9% NaCl-1% Gly-0.1M Pro contributed to the highest yield of the following fractions: triacylglycerol lipase, pancreatic triacylglycerol lipase — the protein associated with pancreatic lipase and phospholipase.

The results of experiments *in vitro* showed that diafiltrates of pigs' pancreas glands obtained with addition of 0.9% NaCl-0.5 M trehalose and 0.9% NaCl-1% Gly-0.1 M Pro into the nutrient medium, contribute to the preservation of the function and rate of survival of islet cells of mice pancreas gland. Diafiltrate obtained with trehalose promoted the forming of rosette-shaped microcommunities, preservation of the round shape of islet cells and beta cells, which may be explained by the fact that after removal of trehalose by dialysis from a protein fraction with a molecular weight of less than 50 kDa, its residual quantities were still sufficient to prevent the proteins aggregation and, as a consequence, the extracted proteins preserve their biological activity.

REFERENCES

1. Moszak, M., Szulińska, M., Bogdański, P. (2020). You are what you eat–the relationship between diet, microbiota, and metabolic disorders — a review. *Nutrients,* 12(4), Article 1096. https://doi.org/10.3390/nu12041096
2. Teodoro, A. J. (2019). Bioactive compounds of food: Their role in the prevention and treatment of diseases. *Oxidative Medicine and Cellular Longevity,* 2019, Article 3765986. https://doi.org/10.1155/2019/3765986
3. Karpińska, M., Czauderna, M. (2022). Pancreas gland–its functions, disorders, and physiological impact on the mammals' organism. *Frontiers in Physiology,* 13, Article 807632. https://doi.org/10.3389/fphys.2022.807632
4. Silano, V., Barat Baviera, J.M., Bolognesi, C., Cocconcelli, P.S., Crebelli, R., Gott, D.M. et al. (2021). Safety evaluation of a food enzyme containing trypsin, chymotrypsin, elastase and carboxypeptidase from porcine pancreas gland. *EFSA Journal,* 19(1), Article e06368. https://doi.org/10.2903/j.efsa.2021.6368
5. Salhi, A., Amara, S., Mansuelle, P., Puppo, R., Lebrun, R., Gontero, B. et al. (2020). Characterization of all the lipolytic activities in pancreatin and comparison with porcine and human pancreatic juices. *Biochimie,* 169, 106–120. https://doi.org/10.1016/j.biochi.2019.07.004
6. Castro-Munoz, R., Garcia-Depraect, O., Leon-Becerril, E., Cassano, A., Conidi, C., Fila, V. (2021). Recovery of protein-based compounds from meat by-products by membrane-assisted separations: A review. *Journal of Chemical Technology and Biotechnology,* 96(11), 3025–3042. https://doi.org/10.1002/jctb.6824
7. Miron, S. M., Dutournié, P., Thabet, K., Ponche, A. (2019). Filtration of protein-based solutions with ceramic ultrafiltration membrane. Study of selectivity, adsorption, and protein denaturation. *Comptes Rendus. Chimie,* 22(2–3), 198–205. https://doi.org/10.1016/j.crci.2018.09.011
8. Arakawa, T., Ejima, D., Akuta, T. (2017). Protein aggregation under high concentration/density state during chromatographic and ultrafiltration processes. *International Journal of Biological Macromolecules,* 95, 1153–1158. https://doi.org/10.1016/j.ijbiomac.2016.11.005
9. Ratnaningsih, E., Reynard, R., Khoiruddin, K., Wenten, I.G., Boopathy, R. (2021). Recent advancements of UF-based separation for selective enrichment of proteins and bioactive peptides — a review. *Applied Sciences,* 11(3), Article 1078. https://doi.org/10.3390/app11031078
10. Fernandez-Cerezo, L., Rayat, A. C. M. E., Chatel, A., Pollard, J. M., Lye, G. J., Hoare, M. (2020). The prediction of the operating conditions on the permeate flux and on protein aggregation during membrane processing of monoclonal antibodies. *Journal of Membrane Science,* 596, Article 117606. https://doi.org/10.1016/j.memsci.2019.117606
11. Wang, W., Roberts, C. J. (2018). Protein aggregation — Mechanisms, detection, and control. *International Journal of Pharmaceutics,* 550(1–2), 251–268. https://doi.org/10.1016/j.ijpharm.2018.08.043
12. Maruf, A., Milewska, M., Varga, M., Wandzik, I. (2023). Trehalose-bearing carriers to target impaired autophagy and protein aggregation diseases. *Journal of Medicinal Chemistry,* 66(23), 15613–15628. https://doi.org/10.1021/acs.jmedchem.3c01442
13. Xu, Q., Deng, H., Li, X., Quan, Z.-S. (2021). Application of amino acids in the structural modification of natural products: A review. *Frontiers in Chemistry,* 9, Article 650569. https://doi.org/10.3389/fchem.2021.650569
14. Liao, Y.-T., Manson, A. C., DeLyser, M. R., Noid, W. G., Cremer, P. S. (2017). Trimethylamine N-oxide stabilizes proteins via a distinct mechanism compared with betaine and glycine. *Proceedings of the National Academy of Sciences,* 114(10), 2479–2484. https://doi.org/10.1073/pnas.1614609114
15. Dou, M., Lu, C., Sun, Z., Rao, W. (2019). Natural cryoprotectants combinations of l-proline and trehalose for red blood cells cryopreservation. *Cryobiology,* 91, 23–29. https://doi.org/10.1016/j.cryobiol.2019.11.002
16. Patriarca, E. J., Cermola, F., D'Aniello, C., Fico, A., Guardiola, O., De Cesare, D. et al. (2021). The multifaceted roles of proline in cell behavior. *Frontiers in Cell and Developmental Biology,* 9, Article 728576. https://doi.org/10.3389/fcell.2021.728576
17. Laemmli, UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227 (5259), 680–685. https://doi.org/10.1038/227680a0
18. Matsumoto, H., Haniu, H., Kurien, B. T., Komori, N. (2019). Two-dimensional gel electrophoresis by glass tube-based IEF and SDS-PAGE. Chapter in a book: Electrophoretic Separation of Proteins (Vol. 1855). Humana Press, New York, NY, 2019. https://doi.org/10.1007/978-1-4939-8793-1_11
19. Ahremko, A. G. (2019). Application of two-dimensional electrophoresis method for assessing meat products composition on model stuffing example. *Vsyo o Myase,* 3, 46–48. https://doi.org/10.21323/2071-2499-2019-3-46-48 (In Russian)
20. Blum, H., Beier, H., Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis,* 8(2), 93–99. https://doi.org/10.1002/elps.1150080203
21. Vinciguerra, D., Gelb, M. B., Maynard, H. D. (2022). Synthesis and application of trehalose materials. *JACS Au,* 2(7), 1561–1587. https://doi.org/10.1021/jacsau.2c00309
22. Kadoya, S., Fujii, K., Izutsu, K., Yonemochi, E., Terada, K., Yomota, C. et al. (2010). Freeze-drying of proteins with glass-forming oligosaccharide-derived sugar alcohols. *International Journal of Pharmaceutics,* 389(1–2), 107–113. https://doi.org/10.1016/j.ijpharm.2010.01.027
23. Samuel, D., Ganesh, G., Yang, P.-W., Chang, M.-M., Wang, S.-L., Hwang, K.-C. et al. (2008). Proline inhibits aggregation during protein refolding. *Protein Science,* 9(2), 344–352. https://doi.org/10.1110/ps.9.2.344
24. P00591 LIPP_PIG (Pancreatic triacylglycerol lipase). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P00591/entry#sequences Accessed August 21, 2023
25. D7EZN2 LIPR2_PIG (Pancreatic lipase-related protein 2). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/D7EZN2/entry#sequences Accessed August 21, 2023
26. van den Berg, B., Tessari, M., Boelens, R., Dijkman, R., Kaptein, R., de Haas, G. H. et al. (1995). Solution structure of porcine pancreatic phospholipase A2 complexed with micelles and a competitive inhibitor. *Journal of Biomolecular NMR,* 5(2), 110–121. https://doi.org/10.1007/BF00208802
27. Ardelt, W. (1974). Partial purification and properties of porcine pancreatic elastase II. *Biochimica et Biophysica Acta (BBA) — Enzymology,* 341(2), 318–326. https://doi.org/10.1016/0005-2744(74)90224-1
28. P00772 · CELA1_PIG (Chymotrypsin-like elastase family member 1). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P00772/entry Accessed August 21, 2023.
29. P00761 · TRYP_PIG (Trypsin). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P00761/entry#function Accessed August 21, 2023
30. P63298 SECR_PIG (Secretin). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P63298/entry Accessed July 30, 2024

31. P01351 GAST_PIG (Gastrin). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P01351/entry Accessed July 10, 2024
32. P01315 INS_PIG (Insulin). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P01315/entry Accessed July 30, 2024
33. P02703 · COL_PIG (Colipase). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P02703/entry Accessed July 28, 2024
34. Brockman, H. L. (1981). Triglyceride lipase from porcine pancreas gland. Chapter in a book: Methods in enzymology (Vol. 71). Academic Press, 1981. https://doi.org/10.1016/0076-6879(81)71074-7
35. Neugebauer, E. A. (2019). Handbook of Mediators in Septic Shock. CRC Press, 2019. https://doi.org/10.1201/9781351071123
36. Lambré, C., Barat Baviera, J.M., Bolognesi, C., Cocconcelli, P.S., Crebelli, R., Gott, D.M. et al. (2022). Safety evaluation of the food enzyme containing trypsin, chymotrypsin, α -amylase and triacylglycerol lipase from porcine pancreas gland. *EFSA Journal,* 20(4), Artisle e07239. https://doi.org/10.2903/j.efsa.2022.7239
37. Berg, P., Svendsen, S. L., Ayasse, N., Sorensen, M. V., Leipziger, J. (2024). Secretin: A hormone for HCO3– homeostasis. *Pflugers Archiv — European Journal of Physiology,* 476(4), 545–554. https://doi.org/10.1007/s00424-024-02906-3
38. Ma, Z., Zhang, Y., Su, J., Li, X., Yang, S., Qiao, W. et al. (2018). Distribution of the pig gastrin-releasing peptide receptor and the effect of GRP on porcine Leydig cells. *Peptides,* 99, 142–152. https://doi.org/10.1016/j.peptides.2017.09.015
39. Rahman, M. S., Hossain, K. S., Das, S., Kundu, S., Adegoke, E. O., Rahman, Md. A. et al. (2021). Role of insulin in health and disease: An update. *International Journal of Molecular Sciences,* 22(12), Article 6403. https://doi.org/10.3390/ijms22126403
40. Lim, S. Y., Steiner, J. M., Cridge, H. (2022). Lipases: It's not just pancreatic lipase! *American Journal of Veterinary Research,* 83(8), 1–8. https://doi.org/10.2460/ajvr.22.03.0048
41. Szczesna, K. (2019). Why does the molecular weight of my protein differ from the theoretically expected weight? Technology Networks — Proteomics and Metabolomics. Retrieved from https://www.technologynetworks.com/proteomics/articles/why-does-the-molecular-weight-of-my-protein-differ-from-the-theoretically-expected-weight-322079 Accessed July 30, 2024
42. Zhu, K., Zhao, J., Lubman, D. M., Miller, F. R., Barder, T. J. (2005). Protein p I shifts due to posttranslational modifications in the separation and characterization of proteins. *Analytical Chemistry*, 77(9), 2745–2755. https://doi.org/10.1021/ac048494w
43. P19619 ANXA1_PIG (Annexin A1). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P19619/entry#sequences Accessed August 21, 2023
44. Sun, L., Qin, T., Liu, Y., Zhao, H., Xia, X., Lei, X. (2018). Cloning, expression, and characterization of a porcine pancreatic α -amylase in Pichia pastoris. *Animal Nutrition,* 4(2), 234–240. https://doi.org/10.1016/j.aninu.2017.11.004
45. P08132 ANXA4_PIG (Annexin A4). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P08132/entry Accessed July 30, 2024
46. P19620 ANXA2_PIG (Annexin A2). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P19620/entry Accessed August 6, 2024
47. A1E295 CATB_PIG (Cathepsin B). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/A1E295/entry Accessed August 8, 2024
48. P00795 CATD_PIG (Cathepsin D). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P00795/entry Accessed August 8, 2024
49. Q28944 CATL1_PIG (Procathepsin L). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/Q28944/entry Accessed August 8, 2024
50. O46427 · CATH_PIG (Pro-cathepsin H). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/O46427/entry Accessed August 8, 2024
51. Blanchard, P. J., Mantle, D. (1996). Comparison of proteolytic enzyme levels in chicken, pig, lamb and rabbit muscle at point of slaughter: Role in meat tenderization post mortem. *Journal of the Science of Food and Agriculture,* 71(1), 83–91. https://doi.org/10.1002/(SICI)1097–0010(199605)71:1<83::AID-JSFA552>3.0.CO;2-F
52. Peerapen, P., Boonmark, W., Thongboonkerd, V. (2023). Characterizations of annexin A1-interacting proteins in apical membrane and cytosolic compartments of renal tubular epithelial cells. *Computational and Structural Biotechnology Journal,* 21, 3796–3809. https://doi.org/10.1016/j.csbj.2023.07.037
53. G1ARD6 G1ARD6_PIG (Chymotrypsin C). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/G1ARD6/entry Accessed August 21, 2023
54. A0A480M2A7 A0A480M2A7_PIG (Chymotrypsinogen B2). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/A0A480M2A7/entry Accessed August 21, 2023
55. A0A480INX1 A0A480INX1_PIG (Chymotrypsinogen B2). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/A0A480INX1/entry Accessed August 21, 2023
56. Elastase, porcine pancreas gland, high purity, crystallized. MERCK. Retrieved from https://www.sigmaaldrich.com/RU/en/product/mm/324682 Accessed August 21, 2023
57. Hudáky, P., Kaslik, G., Venekei, I., Gráf, L. (2001). The differential specificity of chymotrypsin A and B is determined by amino acid 226. *European Journal of Biochemistry,* 259(1–2), 528–533. https://doi.org/10.1046/j.1432-1327.1999.00075.x
58. A0A4X1V2S2 · A0A4X1V2S2_PIG (Trypsinogen). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/A0A4X1V2S2/entry Accessed August 21, 2023
59. A0A8D1RDS5 A0A8D1RDS5_PIG (Trypsinogen). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/A0A8D1RDS5/entry Accessed August 8, 2024
60. P01274 GLUC_PIG (Pro-glucagon). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P01274/entry Accessed August 2, 2023
61. P01359 TFF2_PIG (Trefoil factor 2). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P01359/entry Accessed August 3, 2023
62. P01356 CCKN_PIG (Cholecystokinin). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P01356/entry Accessed August 8, 2024
63. P00592 PA21B_PIG (Phospholipase A2, major isoenzyme). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/P00592/entry Accessed August 8, 2024
64. A0A8D2CC29 · A0A8D2CC29_PIG (Triacylglycerol lipase). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/A0A8D2CC29/entry Accessed August 8, 2024
65. A0A8D1Y1K2 · A0A8D1Y1K2_PIG (chymotrypsin). UniProt. Retrieved from https://www.uniprot.org/uniprotkb/A0A8D1Y1K2/entry Accessed August 8, 2024
66. Milojević, M., Rožanc, J., Vajda, J., Činč Ćurić, L., Paradiž, E., Stožer, A. et al. (2021). In vitro disease models of the endocrine pancreas gland. *Biomedicines,* 9(10), Article 1415. https://doi.org/10.3390/biomedicines9101415

AUTHOR INFORMATION

Maria E. Spirina, Senior Laboratory Assistant, Experimental Clinic-Laboratory of Biologically Active Substances of Animal Origin, VM Gorbatov Federal Research Center for Food Systems. 26, Talalikhin str., 109316, Moscow, Russia.
Tel.: +7–495–676–95–11 ext. 207, E-mail: m.spirina@fncps.ru
ORCID: https://orcid.org/0000-0003-4544-4433

Ekaterina K. Polishchuk, Junior Researcher, Experimental Clinic-Laboratory of Biologically Active Substances of an Animal Origin, VM Gorbatov Federal Research Center for Food Systems. 26, Talalikhin str., 109316, Moscow, Russia.
Tel.: +7–495–676–95–11 ext. 129, E-mail: e.polchuk@fncps.ru
ORCID: https://orcid.org/0000-0003-2719-9649
* corresponding author

Ekaterina R. Vasilevskaya, Candidate of Technical Sciences, Researcher, Experimental Clinic-Laboratory of Biologically Active Substances of an Animal Origin, VM Gorbatov Federal Research Center for Food Systems. 26, Talalikhin str., 109316, Moscow, Russia. Tel.: +7–495–676–95–11 ext. 128, E-mail: e.vasilevskaya@fncps.ru
ORCID: https://orcid.org/0000-0002-4752-3939

Liliya V. Fedulova, Doctor of Technical Sciences, Professor, Head of the Experimental Clinic-Laboratory of Biologically Active Substances of an Animal Origin, V. M. Gorbatov Federal Research Center for Food Systems, 26 Talalikhin str., 109316, Moscow, Russia. Tel.: +7–495–676–95–11 ext. 128, E-mail: l.fedulova@fncps.ru
ORCID: http://orcid.org/0000-0003-3573-930X

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

The authors declare no conflict of interest.