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MEMBRANE FRACTIONATION OF BOVINE PANCREAS ENZYMES

The paper describes some laboratory attempts to select suitable membranes for fractionation of bovine pancreas enzymes. Investigation of enzyme stability allows us to suggest that its separation should be performed in the solution of pH = 6.3 and at 5–10 °C and completed within 24 hours after preparing a pancreas enzyme extract. It is shown that use of collagen membranes as a pre-filter is profitable for two reasons: reduction of membrane fouling and lipase freeing from its micelle structures. The use of YM 100 followed by YM 10 membranes (both from Amicon) results in obtaining preparation enriched with lipase and/or amylase. The lipase purification factor reaches the value of 30 that may be interesting when its industrial applications are taken into account. Protease purification factor, approaching the value of 3, allows us to adjust the amylase:protease ratio as 1:1 (against the initial ratio of 5:1). The enzyme preparations obtained permit us to obtain with ease the enzyme composition required.

1. INTRODUCTION

In the last two decades, a rapid development in biotechnology goes on. One of the areas most intensively investigated is separation of very delicate and sensitive compounds. Such methods as membrane and chromatography separations are widely used for bioactive molecules' purification and isolation tasks. Of membrane methods the following processes have found their industrial application: micro-, ultra- and nanofiltrations, electrodialysis, reverse osmosis and pervaporation [1], [2].

Despite maintaining the activity of separated molecules, the replacement of extraction-based and thus harmful technologies by more environment-friendly processes seems to play the important part in development of membrane-based technologies.

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Generally, it is stated that multi-step methods of protein isolation and purification are too expensive to prepare enzymes on a large scale. One of the possible ways to cut the production cost is the use of some membrane processes. Filtration processes seem to be most useful for enzyme fractionation and concentration. In these processes, shape and diameter of enzyme in solution are the crucial process parameters. Each filtration membrane is well characterized by its cut-off factor, the number showing molecular weight of standards (usually dextrans) rejected by the membrane in at least 90% [3]. It is assumed that effective separation appears when molecular weight of the molecule processed is 10-folds higher than membrane cut-off. However, this condition is not commonly required for protein isolation [1]. The shape of molecule, molecule hydration (controlled by pH-value and ionic strength of solution) and interaction forces between proteins and membrane surfaces are also involved in separation.

Membrane materials and particularly its surface govern nonspecific interactions of the membrane with filtered molecules (electrostatic, hydrophobic or hydrophilic interactions). In consequence, proteins may deposit on the membrane and pore surfaces and form resistant filtration cake [1], [3]. The decrease of permeate flux results from such a deposition. That phenomenon is known as the membrane fouling. Application of high-flux membranes (for example, asymmetric membranes) allows us to observe a marginal extent of fouling unless pores are not plugged. Unfortunately, in most cases filtration membranes are made from hydrophobic polymers (polypropylene, polytetrafluoroetylene, polysulfone) or moderately hydrophobic materials (polyacylonitrile). These materials have remarkable ability to adsorb proteins [1], [3]. In order to change the sorbability of membranes, some of them are surface modified by anchoring ionogenic groups, or whole membrane is prepared from hydrophilic materials (collagen, cellulose, chitosane, ceramic or glass) [4]–[11]. Moreover, when deposited species reach their own specific gel concentration a dynamic membrane is formed on the surface. Properties of the whole filtration system alter dramatically. In order to minimize this drawback some efforts are made. Cross-filtration or diafiltration, simple methods in engineering activity appear to be one of the best solutions. In the latter case, molecules smaller than pore diameter are gradually removed from the solution. They do not block intensively the pores by bridging mechanism (diafiltration is conducted without raising retentate concentration and viscosity) [1].

Usually, the membrane separation is followed by pre-filtration. Such a pretreatment allows the removal of coarse contaminants such as cells or their fragments and other forms of macromolecule aggregates. In the following step, i.e. diafiltration, the enzymes are separated according to their molecule sizes and membrane cut-off factors. Finally, both solutions of enzymes (retentate and permeate) can be concentrated by means of dead-end ultrafiltration or fractionated again. The goal of our studies was to check whether the method described above can be used for the separation of enzymes. The work was focused on the protein mixture obtained from bovine pancreas. Pancreatin, the trade name of this mixture, is produced in Pharmaceutical Plant JELFA by extraction of fatty and dye components from disintegrated pancreas tissue [12], [13]. This extraction is followed by pancreatin polishing in

which the residual amounts of fatty bodies are removed. Unfortunately, also in this step organic extraction is applied. The method has at least two drawbacks: it requires some environmentally harmful technologies (some amounts of organics are vaporized) and causes inactivation of some parts of enzyme being in contact with organic solvent. Hence, there is a need to implement new, hopefully membrane technology in use. There is another goal of the research on enzyme separation. Pancreatin is the mixture of α-amylase, lipase, DNase, RNase, proteolytic enzymes and their preenzymatic forms, as well as ballast proteins. The use of such a mixture for various malfunctionings of digestion system is not the best solution. Some patients should be treated with the preparation enriched with lipase, others need proteolytic treatment. In order to adjust a preparation for a particular request, the enzymes should be separated to their pure/enriched forms, and then mixed together to obtain a formula prescribed. So far the separation has been done by means of chromatography.

2. EXPERIMENTAL PART

2.1. MATERIAL

The following chemicals used in the study were supplied by Sigma (the USA): tri(hydroxymethyloamino)methane (TRIS), 3,5-dinitrosalicylic acid (DNS), water-soluble starch, protein assay kit by Lowry method and lipase activity assay kit. The rest of chemicals (analytical grade) were bought in POCh (Poland).

Trypsin preparation was obtained according to procedure described by WILIMOWSKA [14].

Pancreatin was kindly gifted by Pharmaceutical Plant JELFA, Jelenia Góra, Poland.

The evaluated membranes are donated by the representatives of manufactures and some of the research teams. Their properties established are summarized in table 1.

2.2. METHODS

Preparation of pancreatin solution. Pancreatin solution was prepared according to the procedure recommended by the manufacturer: 0.3 g of dusty preparation was mixed with 100 cm^3 of cold (4 °C) 0.1 M phosphate buffer, pH = 6.3, for 15 min. Then the suspension was centrifuged (4 °C, 8000 rpm, 20 min) and supernatant was collected for future studies.

Lipase activity. Lipolytic activity of samples was determined according to Sigma test. The amount of enzyme that catalyzes the formation of fatty acids

(under the test conditions: 37 °C, 6 h, pH = 6.3 and 6 h) and can be neutralized by 280 cm^3 of 0.05 NaOH is assumed to be one activity unit (U/L).

Table 1

Properties of the membrane examined

Membrane symbol	Membrane material	Manufacturer	Water flux [l/(m^2h)]	Pressure [MPa]	Other properties
045FSMO	politetra-flouroethylene	Dow Denmark A/S	648	0.02	
	politetra-flouroethylene	Milipore	1311	0.05	pore diammeter 0.45 μm
	modified olysulfone	Inst. Org. Polym. Tech., Tech. Univ, Wrocław	640	0.05	NH_2 concentration 1.5 mmol/g
V21	collagen	Inst. Leather Industry, Łódź	2276	0.02	
V23	collagen	Inst. Leather Industry, Łódź	2165	0.02	
YM100	cellulose	Amicon	3050	0.15	cut-off 100 kDa
YM10	cellulose	Amicon	102	0.10	cut-off 10 kD
XM300	polysulfone	Amicon	835	0.05	cut-off 300 kDa

Amylase activity. Amylolytic activity assay of samples was carried out according to Bernfeld test [15]. The amount of enzyme that catalyzes the formation of 1 mg of equivalent of glucose (under the test conditions: 37 °C, 3 min, pH = 6.9, 0.5% soluble starch as a substrate) is assumed to be one activity unit (U).

Protease activity. Proteolytic activity assay was conducted by the modified KUNITZ method [16]. The method is based on measuring the absorbance (280 nm) of digested products which are still soluble in trichloroacetic acid. One unit of proteolytic activity (U) is defined as the amount of enzyme that gives an absorbency rise of 0.1 compared to blank. The test is carried out under the following conditions: 37 °C, 10 min, 0.5% soluble casein as a substrate.

Stability. Enzyme stability was evaluated in a one-month test. Preparation was kept at 5 and 25 °C, and activity was measured during this period. Degree of activity loss was compared to activity at the beginning of the process. Thermo- and pH-stability as well as temperature and pH-profiles were measured according to procedures described elsewhere [17].

Specific activity. Specific activity of enzyme was expressed as the number of activity units per 1 mg of protein. Amount of protein was determined by means of modified Lowry test according to Sigma procedure.

Shear inactivation. Effect of mixing on enzyme activity was evaluated in a thermostated reactor (4 °C) equipped with a magnetic stirrer. Enzyme was tested at the stirrer speed of 200–220 rpm.

2.3. MEMBRANE EVALUATION

All measurements were conducted in the Amicon 8200 ultrafiltration cell. The cell was placed in a vessel filled with ice. Before measurements each membrane was rinsed successively with water, 70% ethanol, water solutions of 0.5 M NaCl, 0.1 M HCl and 0.1 M NaOH, and finally with deionized (DI) water. The flux of DI water was measured for each membrane.

Diafiltration was conducted according to the following procedure. The Amicon cell was filled with 100 cm^3 of cooled pancreatin solution and subjected to various pressures (0.01–0.15 MPa) to obtain a constant permeate flux. Any reduction of the flux value was compensated by increasing pressure. Retentate was mixed with magnetic stirring bar (200–220 rpm) placed on the membrane surface. The obtained fractions of permeate were collected in 100 cm^3 beakers in which an average protein concentration and the enzyme activity were determined. The loss of retentate volume was compensated by the addition of phosphate buffer (0.1 M, pH = 6.3). Diafiltration was finished after 4-fold replacement of retentate volume. The obtained retentate was concentrated 2-fold for determination of protein amount and enzyme activity. Just after diafiltration, membranes were washed with 50 cm^3 of buffer, buffer with 0.5 M NaCl, and DI water. Protein concentration and enzyme activity were determined in each washing mixture (wash-out component). In the case of membranes tested in prefiltration process, their cleaning was performed by means of surfactant (Sil) solution.

In some cases the above procedure was modified:

The possibility of applying a membrane to lipase isolation was examined using the retentate obtained after 3-fold replacement of pancreatin solution volume.

Lipase was concentrated by decreasing the retentate volume from 100 to 20 cm^3. Concentration and activity of lipase were checked in 10 cm^3 fraction of permeate, in feed and in retentate.

Membrane YM 10 was tested by means of trypsin solution of 112 $\mu g/cm^3$.

Effectiveness of membrane separation was evaluated by means of *SR* parameter

$$SR = 1 - \frac{C_p}{C_r} \tag{1}$$

where C_p and C_r are concentrations of enzymes (in mass or activity units) in permeate and retentate, respectively.

2.4. ENZYME ISOLATION

Process of enzyme isolation was carried out in the Amicon ultrafiltration cell. At first, the pancreatin solution was prefiltered through V23 membrane. Then, 100 cm^3 of permeate was diafiltered on YM 100 membrane. Retentate was concentrated 5-fold on the same membrane. Finally, 100 cm^3 of permeate was concentrated on YM 10 membrane until 5-fold volume reduction was reached. The whole procedure was triplicated. DI water flux through washed membrane was a measure of membrane fouling extent.

3. RESULTS AND DISCUSSION

The research on the separation of enzymes should be followed by their thermo- and pH-stability determinations. Well-established conditions of enzyme stability allow us to carry out the separation without significant damage of enzyme activity and can be considered as essential prerequisite of a search for optimal parameters of membrane process. Lipase stability was tested in thermostated bath at 5 and 25 °C during 30 days of storage. The data presented in figure 1 show that lipase lost a half of its activity when stored for one day at 25 °C. When temperature fell to 5 °C, lipase kept its activity during the first 10 days. Studies of the storage stability of other enzymes showed 50% reduction of amylolytic activity after 10 days at 5 °C and 20% rise of proteolytic activity after 18 days at the same temperature. An unexpected increase in proteolytic activity might be connected with autocatalytic activation of trypsinogen caused by trypsin as well as activation of chymotrypsinogen and pre-elastase. The studies presented above resulted in establishing the separation conditions: pancreatin solution should be processed within 24 hours after dissolving its powder, and the process should proceed at 5 °C.

Lipase is the dominant enzyme in the pancreatin preparation. Hence, the effect of pH (figure 2) and temperature (figure 3) on lipase stability and activity may additionally limit the process conditions. Careful examination of both figures allows observation of a wide range of the enzyme pH-stability (from 4.0 to 7.0). In this range, lipase is also most active – 90% of its maximal activity covers the region from 5.3 to 6.6 pH units. Hence, this range offers the best pH for the experiments on lipase. Temperature is the second process parameter to be studied. The data in figure 3 prove that more careful temperature adjustment is needed. When temperature is over 10 °C lipase is inactivated to a large extent. Over 60 °C, the inactivation is completed. The maximal lipase activity is observed in the wide range, i.e. from 27 to 45 °C. The last observations allowed us to modify the test conditions. In this paper, lipase activity was determined for pH = 6.3 and at 37 °C.

Similar experiments were performed for the other enzymes. The obtained data are juxtaposed in table 2. On the basis of presented data, it was decided to select the following parameters: pH = 6.3 and temperature below 10 °C.

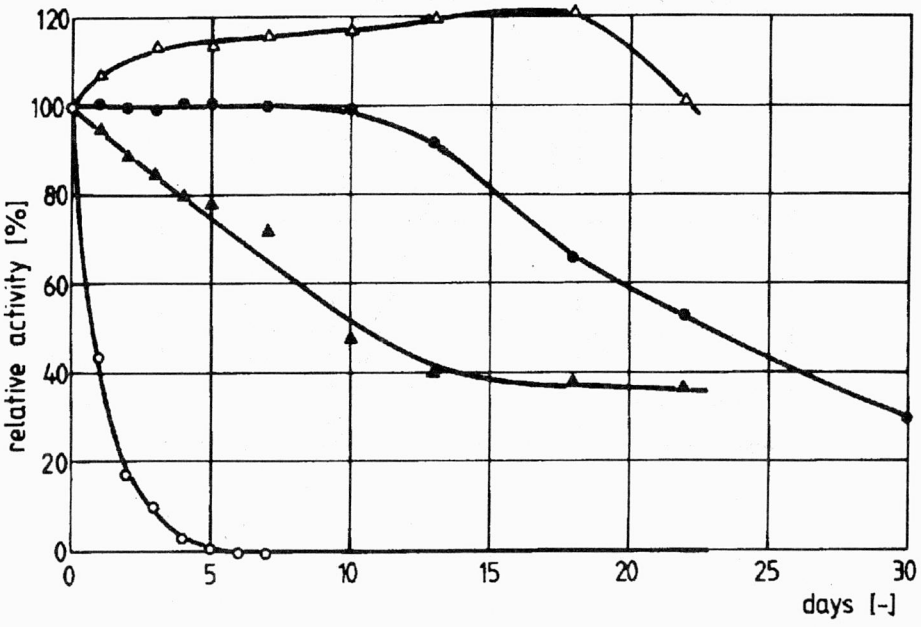

Fig. 1. Storage stability of lipase at 25 °C (open circle) or 5 °C (filled circle), amylase at 5 °C (open triangle) and proteases at 5 °C (filled triangle)

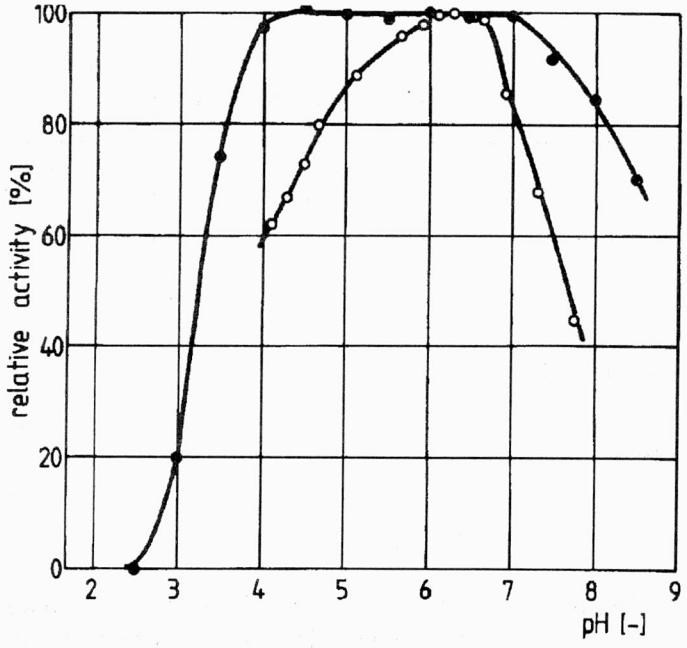

Fig. 2. Effect of pH on lipase activity (open circle) and stability (filled circle)

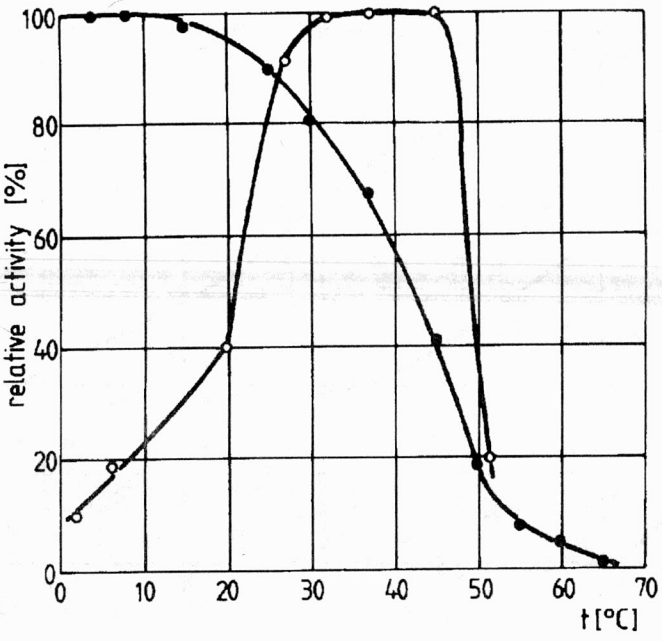

Fig. 3. Effect of temperature on lipase activity (open circle) and stability (filled circle)

Table 2

Properties of pancreatic enzymes

Enzyme	Lipase	Proteases*	α-amylase**
Optimal pH	6.1–6.6	7.6	6.8–7.0
pH-stability	4.0–7.0	2.0–9.0	
Optimal temperature [°C]	27–45	37	37
Thermostability [°C]	up to 10	up to 40	up to 50

*Data from Ref. [18].
**Data from Ref. [19].

3.1. MEMBRANE EVALUATION

Membrane fouling is one of the negative processes accompanying each filtration. Solute molecules deposited on membrane block pores intensively and irreversibly. Hence, the permeate flux is reduced and filtration process becomes less profitable. In

order to control this phenomenon filtration processes are performed with mixing in the retentate phase. In order to overcome the problem of fouling, two approaches are usually applied: tangential flow filtration (cross-filtration) and stirring the solution nearby a membrane surface. In both cases, shear forces may significantly inactivate enzyme by disrupting sensitive structure of protein coils. According to available literature [20]–[23] stirring the retentate at the rate of 250 rpm is still safe for lipase and other enzymes. Experimental verification of this statement (4 hours of permanent mixing at 200–220 rpm at 6 °C and pH = 6.3) has shown marginal changes of the enzyme activity (lipolytic activity decreased by 4%, proteolytic activity rose by 5% and amylolytic activity was constant).

As we have mentioned in the introduction, crude pancreatin preparation needs to be degreased before its subsequent use in membrane processes. Removal of fine fatty bodies by means of extraction has adverse effect on enzyme activity and may act against legislative regulation for environment protection. Hence, the use of pre-filtration procedure seems to be the best choice. Pre-filtration was carried out on several kinds of microfiltration membranes: Dow Denmark and Milipore Teflon's membranes, modified polysulfone membrane and on both collagen membranes. The studies showed that the use of diafiltration procedure allowed only 2-fold replacement of retentate volume when three mentioned membranes were evaluated. After this time, the fatty bodies present in retentate fouled membrane so extensively that the permeate flux was reduced by 0.2–3% of its initial value. Additionally, decrease in the enzyme concentration was observed in permeate which was substantially connected with the formation of a less permeable dynamic layer on the top of the membrane. Such enzymes as lipase, amylase or proteases were rejected by this layer. The calculated *SR* parameters, after 2-fold replacement of retentate volume, are shown in table 3.

Table 3

Rejection of enzymes on membranes used in pre-filtration step

Membrane	Supplier	*SR* (amylase)	*SR* (protease)	*SR* (lipase)
045FSMO	Dow Denmark	0.52	0.59	0.53
	Milipore	0.35	0.51	0.47
Modified poly-sulfone	IOPT, Tech. Univ. Wrocław	0.14	0.11	0.53

The results obtained prove that the performances of the hydrophobic membranes are not very effective in the process of fine degreasing. Diafiltration effectiveness depends mostly on the membrane material. Much more effective process was observed for hydrophilic membranes.

Collagen membranes (V21 and V23) lost no more than 35% of the initial flux when used in diafiltration (5-fold volume replacement) and then in retentate concen-

tration (2-fold decrease of volume). They did not disturb nearly all enzymes transported through them (marginal values of *SR* parameter for amylase, proteases and lipase). The application of a membrane cleaning procedure with surface active agent (Sil) results in the flux recovery on the level of 70–75%. Performance of the collagen membranes is presented in table 4.

Table 4

Diafiltration of pancreatin solution through collagen membrane V23

	Amylase		Proteases		Lipase		
	Activity [U]	Specific activity [U/mg]	Activity [U]	Specific activity [U/mg]	Activity [U/L]	Specific activity [U/L mg]	Protein amount [mg]
Feed	8868	61.4	10602	73.4	3564	24.7	144.4
Permeate	9983	66.9	10615	71.1	3681	24.6	149.3
Retentate	95	70.4	57	42.0	45	33.3	1.4
Wash-out*	26	145	37	185	0	0	0.2
Together [%]	114.0		101.0		104.5		104.5
Yield [%]	112.6		100.1		103.3		103.4

*Enzymes wash-out from membrane under gentle conditions.

The membrane investigated accomplishes satisfactorily the requirements of the pre-filter: most proteins passed freely through it and large particles and fatty bodies were stopped. To have a better insight into a chance of using the V 23 membrane one should observe the change of the membrane performances in the course of the diafiltration. The results of such studies are shown in figure 4.

For the reader's sake, the enzyme activity at the beginning of the diafiltration process is assumed to be 100%. The careful examination of the data allows us to state that enzymes as well as total protein are weakly rejected by the membrane. They pass easily enough to permeate. However, lipase shows extraordinary behaviour. Its specific activity (as a matter of fact the enzyme purity) increases dramatically with the progress of diafiltration. Any traces of activity were not detected in wash-out protein. After passing the critical point (the first replacement of retentate volume) lipase is preferentially transported across the membrane. There are two possibilities to explain such a behaviour. Some qualitative changes in building-up a polarization layer (like gelation of proteins and reconstruction of deposited layer) may appear in progress of diafiltration. There is another explanation for lipase transport – the enzyme is freed from micelles. After removing most of polypeptides (about 92% of protein are taken

out during the first two volume replacements) the micelles are not stabilized by surface active agent – the solution is suspected to be below its Critical Micelle Concentration (CMC). Taking the latter explanation as more plausible one easily finds at least two profits coming from the pre-filtration step. The first profit comes from removing the harmful fatty bodies from the mixture, and the second one from the disruption of micelles and freeing a lipase (the pre-filtration action is equivalent to molecular milling). Figure 4a shows that after the first replacement of retentate volume more than 75% of proteases and amylase are transferred to permeate, while the transfer of lipase approaches 50%. The second half of the enzyme is removed from retentate during the subsequent retentate volume replacements.

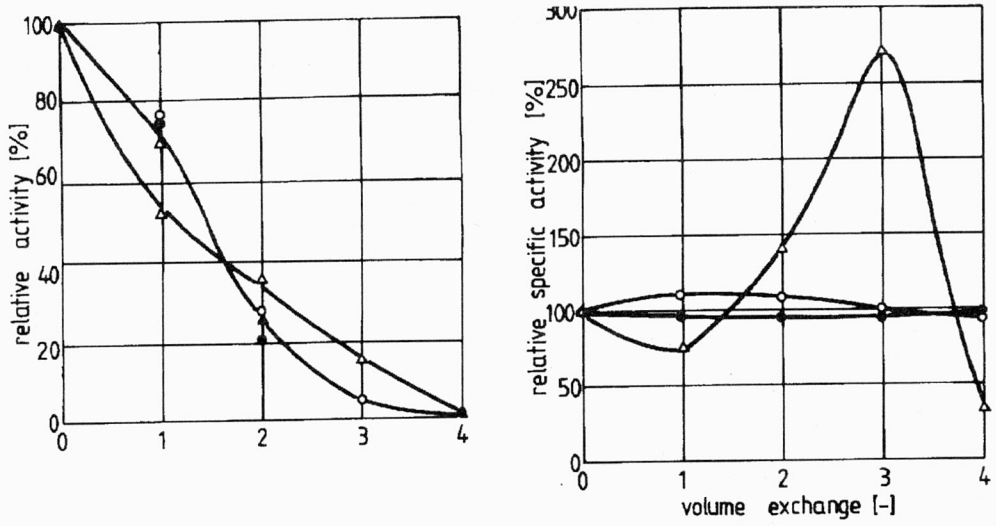

Fig. 4. Effect of diafiltration volume replacement on enzyme activity (a) and its specific activity (b) in permeate. The case of V23 membrane. α-amylase – open circle, proteases – filled circle, lipase – open triangle, total protein – filled triangle

Having obtained the fatty-bodies-free permeate, which is still the dilute mixture of enzymes and ballast protein, we are able to select such a membrane that separates selectively lipase. The quick comparison of molecular weights of the enzymes investigated (proteases in the range of 24–35 kDa, amylase of 56 kDa, and lipase in the range of 300–500 kDa; lipase consists of 50 kDa subunits), allows us to limit the number of the membranes being evaluated. The membranes worth considering should have the cut-off factor of several hundreds of kDa. Two membranes, i.e. XM 300 (Amicon) and YM 100 (Amicon), matched this requirement. Their performances were evaluated in the enzyme separation process. Hydrophobic polysulfone membrane XM 300 was almost totally fouled just after two replacements of retentate volume. Permeate flux dropped down to 6% of its initial value. It was evident that the hydrophobic molecules of lipase were adsorbed on polymer surface and formed a dynamic layer. In consequence, the separation of amylase and proteases was substantially retarded ap-

proaching 31.0% and 34.5%, respectively. Due to the high concentration of lipase on the membrane surface, *SR* parameter for this enzyme (*SR* = 69.8) did not satisfy separation requirements. Much better separation efficiency was obtained for membranes made from cellulose (YM 100). Though the YM 100 membrane had smaller nominal cut-off factor than the XM 300 type (100 kDa versus 300 kDa, respectively), it separated enzymes more effectively (see table 5 and figure 5). Additionally, proteins did not foul dangerously the YM 100 membrane – the permeate flux during the fourth replacement of volume was only by 10% smaller than the initial one.

Table 5

Diafiltration through YM 100 membrane

	Amylase		Proteases		Lipase		
	Activity [U]	Specific activity [U/mg]	Activity [U]	Specific activity [U/mg]	Activity [U/L]	Specific activity [U/L mg]	Protein amount [mg]
Feed	1779	31.0	3600	95.0	900	24	38.0
Permeate	1399	39.7	2959	84.1	120	9	35.2
Retentate	102	85.0	545	454.1	837	697	1.2
Wash-out*	155	163.1	79	83.	0	–	0.95
Together [%]	93.1		99.5		106.3		98.3
Yield [%]	78.6**		81.6**		93.0***		–

*Enzyme wash-out from membrane under gentle conditions.
** Yield related to activity in retentate.
*** Yield related to activity in permeate.

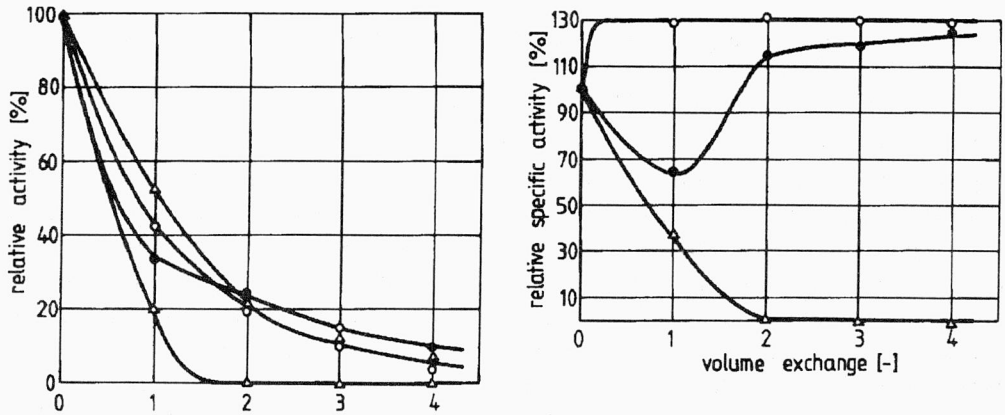

Fig. 5. Effect of diafiltration volume replacement on enzyme activity (a) and its specific activity (b) in permeate. The case of YM100 membrane. α-amylase – open circle, proteases – filled circle, lipase – open triangle, total protein – filled triangle

The data obtained show similar efficiencies of membrane in rejection of amylase (*SR* = 21%) and protease (*SR* = 18%). Sorption of both enzymes on membrane surface varied significantly. Sorption of amylase reached 5.7% (amount of enzyme washed-out), while that of proteases – 2.2%. Hence, filtration cake consisted mostly of amylase and formed a dynamic layer for separation of smaller molecules of protease.

Lipase balance (figure 5) shows clearly that lipase in permeate is present only at the beginning of diafiltration (the first volume replacement). During the entire process, the lipase rejection was equal to 97% and the enzyme did not deposit on the membrane. The hypothesis of the protein dynamic layer formation seemed to explain this behaviour. The layer, less permeable than the neat membrane, rejected larger amount of protein and was not so prone to deposition of hydrophobic lipase. Its formation resulted in alteration of the membrane permeability for proteases (the increase of proteolytic specific activity in permeate after first volume replacement). Moreover, the layer enrichment with amylase facilitated the transport of this enzyme. It seems that strong affinity of amylase for cellulose structure is of crucial importance in these interactions.

We may define the degree of enzyme purification as the ratio of specific enzyme activity before each separation step to this activity after each separation step. In the case of discussed diafiltration, lipase was purified in 30-fold extent (see table 5). Retentate was also enriched with amylase (3-fold purification) and proteases (5-fold purification). However, the relative ratios of enzymes altered significantly. The pancreatin solution was characterized by amylase:protease:lipase ratio of 2.49:3.97:1.00, while in the retentate after YM 100 diafiltration this ratio was 0.12:0.65:1.00. The mixture obtained matches the requirements for enzyme preparation that is being searched for.

Having a lipase-free permeate, we can try to split amylase and proteases. Cellulose membrane developed in the previous step of fractionation proved to have the desired properties; thus the same material (membrane YM 10, Amicon) was applied to the further enzyme purification. The membrane used has been designed by its supplier to reject molecules with molecular weight higher than 10 kDa. Pure trypsin (25 kDa), applied as a proteases marker, should entirely be rejected by the membrane. The data presented in table 6 prove once more that the cut-off criterion for membrane selection is offered only by the first approach. The *SR* parameter, based on proteolytic activity comparison, reached the value of 72%. It seemed to be too small to satisfy the potential customer. Additionally, the increase of trypsin specific activity in permeate clearly shows that most proteins are rejected by membrane and do not appear in permeate (the total protein rejection coefficient reached the values ranging from 86 to 94% in progress of concentration). In consequence, the 5-fold decrease of feed volume resulted in 3.8-fold increase of protein concentration and 2.5-fold increase of trypsin concentration in retentate. We may suspect that multi-cycle enzyme separation on the YM 10 membrane results in formation of amylase-rich, dense and dynamic layer. That layer might act as a specific separator and make trypsin to be rejected to a larger extent.

Table 6

Dead-end filtration of trypsin on YM 10 membrane

	Activity [U]	Specific activity [U/mg]	Protein amount [mg]
Feed	1120	100	11.20
Permeate	432	258	1.67
Retentate	563	63.1	8.50
Wash-out	190	283.6	0.67
Balance [%]	105.6		96.8
Yield [%]	50.3		75.9

3.2. IDEA OF ENZYME ISOLATION FROM BOVINE PANCREASES

The separation system consisting of three units (pre-filtration on collagen V 23 membrane, diafiltration on YM 100 membrane and concentration on YM 10 membranes) was used for triplicated separation of the pancreatin enzymes. After each run, the membranes were cleaned under gentle conditions and V 23 membrane was washed in a surfactant solution (Sil). The scheme of the separation procedure investigated is presented in figure 6.

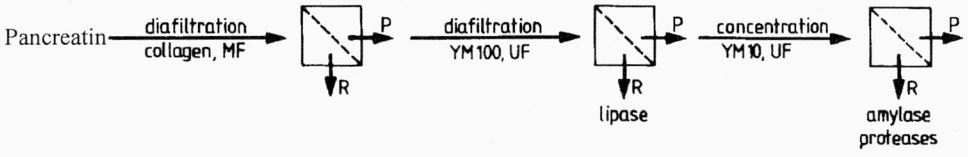

Fig. 6. Scheme of membrane system used for purification of bovine pancreas enzyme: P – permeate, R – retentate

The properties of the preparations obtained, just after each run, are juxtaposed in table 7. When lipase has not been found in permeate obtained after higher number of repeated separation cycle, both other enzymes have changed their ratios in concentrated retentate. The amylase to protease ratios altered from 1:1.12, through 1:1.36, to 1:1.56 for first, second and third separation runs, respectively. Comparison of the amounts of washed-out enzymes (about 10% of proteases in each cycle, and 25, 31 and 32% of amylase during each cycle) allows us to conclude that preferential sorption of amylase occurs on the YM 10 membrane. Taking account of the discussion presented for the YM 100 membrane, the phenomenon seems to be clear: both membranes made from cellulose and amylase show strong affinity to them. Hence, the

Table 7

Yield and separation efficiency in separation of pancreatic enzymes repeated three times

	Run	Amylase	Protease	Lipase	Protein
Yield of pre-filtration [%]	1	96.1	104.4	100.0	101.3
	2	99.5	99.5	102.1	99.4
	3	96.3	99.4	104.2	94.4
Yield of diafiltration and concentration for lipase separation [%]	P1	97.2	87.0	9.8	102.4
	P2	94.0	82.6	0.0	96.8
	P3	90.8	79.5	0.0	88.5
	R1	1.7	5.9	47.7	3.5
	R2	3.1	3.9	48.5	4.9
	R3	3.5	9.5	53.6	7.7
	R1+W1	6.3	6.9	90.7	4.9
	R2+W2	8.4	7.0	90.8	7.4
	R3+W3	12.6	11.6	92.1	10.1
Yield of concentration for separation of amylase and protease [%]	R1	83.3	88.2		47.5
	R2	67.6	86.3		56.2
	R3	57.3	84.6		56.9
	R1+W1	108.2	99.7		54.4
	R2+W2	98.6	97.3		62.9
	R3+W3	88.1	95.0		65.9
Purification factor	R1	1.71	1.81	11.85	
	R2	1.20	1.54	9.06	
	R3	1.01	1.49	7.00	
	R1+W1	1.93	1.82	16.10	
	R2+W2	1.57	1.55	11.85	
	R3+W3	1.34	1.44	9.08	

P – permeate, R – retentate, W – protein wash-out the membrane under gentle conditions.
Subscripts 1, 2 and 3 denote the separation run.

sorption of amylase on the membrane surface is most responsible for the alteration of the membrane performance and run-to-run changes of retentate composition. If the process stability is taken into account, it seems convenient to use more effective procedures of membrane cleaning and/or undertake some efforts allowing us to adjust flow properties and to reduce the effect of protein deposition. How a deposited layer may cause malfunctioning of particular membrane can be seen in table 8. The collagen membrane loses about 30% of its permeability in the first run and then the flux becomes stable. It means the membrane is prone to solute deposition only in its neat form. In the next separation runs, the membrane is stable. Two other membranes (YM 100 and YM 10) are slowly fouled by solutes in each separation step. Flux recovery is an index of the cleaning degree.

Table 8

Changes of water flux passing through washed-out membranes.
The course of separation runs. Pressure of 0.05 MPa

	V23 membrane	YM100 membrane	YM10 membrane
Neat membrane [L/(m^{2}h)]	5722	1061	58
After 1-st run [L/(m^{2}h)]	3838	1072	56
After 2-nd run [L/(m^{2}h)]	3993	975	53
After 3-rd run [L/(m^{2}h]	3923	897	53
Flux recovery [%]	68.6	84.5	91.4

4. CONCLUSIONS

It seems that the main goal of this paper is accomplished: the membrane systems investigated can be used for purification of enzyme mixture prepared from bovine pancreas. The separation scheme offers some possibilities to create environmentally friendly technology which reduces the use of harmful and volatile solvent and guarantees better adjustment of pharmacological formula to the particular customer's requirement.

The use of collagen membranes in pre-filtration step allows us to remove almost all fatty bodies from solution. Additionally, these membranes do not alter their separation properties in the case of multi-cycle procedure of separation. Hydrophobic membranes (like Teflon or polypropylene microfilters) are not effective because they foul very quickly and it is impossible to reconstitute their preliminary flux by conventional cleaning procedures.

The kind of material used for a membrane preparation influences strongly the separation and concentration of enzyme fractions. The hydrophobic XM 300 membrane proved to be worse than its hydrophilic YM 100 alternative. The membrane cut-off parameter, even that determined by the same manufactures (thus by means of the same procedure), shows that the former membrane is more effective in separation of lipase (molecular weight approaches 500 kDa). When selection of a membrane is based only on this factor, it may lead to unenviable choice. The kind of material (polymer) used in a membrane production should also be considered in the process of optimization.

The membrane system presented allows us to separate lipase and the mixture of amylase and protease from a bovine pancreas extract. Simultaneous diafiltration and enzyme concentration resulted in obtaining the preparation enriched with lipase (retentate after YM 100 membrane treatment) and consisting of amylase:protease:lipase in the ratio of 0.12:0.65:1.00. It means the initial composition of enzyme system (2.49:3.97:1.00) was modified by 30-fold increase of lipase concentration. The second effluent, retentate after YM 10 membrane treatment, contains mixture of amylolytic and proteolytic enzymes with the ratio ranging from 1.00:1.12 to 1.00:1.56, while the initial preparation is the mixture of 1.0:5.4 ratio.

Further progress in designing the membrane separation system allowing enzyme isolation depends mostly on investigations undertaken in order to select more effective cleaning procedures and adjust the process parameters (pressure, kind of module, flow rate for cross-flow filtration, scaling-up, etc.). These problems must be solved by end-manufacturer of R&D stuff.

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ROZDZIAŁ ENZYMÓW Z TRZUSTKI WOŁOWEJ ZA POMOCĄ UKŁADÓW MEMBRANOWYCH

Omówiono kryteria wstępnego doboru membran przeznaczonych do frakcjonowania enzymów z trzustki wołowej. Stwierdzono, że proces separacji powinien być prowadzony w roztworze o pH = 6,3 i w temperaturze 5–10 °C. Począwszy od chwili przygotowania ekstraktu białkowego, cały proces należy zakończyć w ciągu 24 godzin. Wykazano, że zastosowanie membrany kolagenowej jako filtra wstępnego jest opłacalne z dwóch powodów: maleje tendencja do zatruwania membran w kolejnych stopniach separacji oraz uwalnia się lipaza zawarta w strukturach micelarnych. Zastosowanie membran YM100, a następnie YM10 (obie produkowane przez Amicon) umożliwia otrzymanie preparatów enzymatycznych wzbogaconych w lipazę czy proteazy. Współczynnik wzbogacenia lipaz, osiągający wartość 30 w jednym stopniu separacji, jest zbliżony do wartości otrzymanej podczas frakcjonowania kolumnowego. Współczynnik wzbogacenia proteaz, o wartości około 3, umożliwia otrzymanie mieszaniny amylaz i proteaz w stosunku 1:1 (w surowcu wynosi 5:1). Posługując się otrzymanymi preparatami, można łatwo przygotować mieszaniny o składzie pożądanym dla określonej terapii.