A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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THE PROTEINS SECRETED BY WASHED PIG PLATELETS *

The aim of this work was to study the amounts and composition of proteins released from washed pig platelets after treatment of platelets with different aggregating agents. Released protein material was separated on Sepharose 4B, Sepharose 6B, Sephadex G-200 and by means of SDS-polyacrylamide gel electrophoresis. Among the released proteins albumin, fibrinogen and specific platelet protein - β-thromboglobulin constitute the main components. As a result of release reaction caused by thrombin the presence of high molecular protein - thrombospondin was observed.

Introduction

Blood platelets respond to stimuli in various ways. When exposed to thrombin, collagen or other substances they secrete selectively the contents of their granules. It is well established that secreted compounds contain also different proteins which participate in haemostasis, inflammation and cell growth [6, 7]. Classification of released platelet proteins is based on characteristics of the proteins, biosynthetic origin, localization and function [6].

Secreted proteins have been classified into four groups:

- proteins identical or similar to plasma proteins,
- lysosomal enzymes,

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- cationic proteins,
- platelet specific proteins.

The purpose of this paper is to determine amounts and characterize the proteins released from washed pig platelets upon the different stimuli.

Material and methods

Blood was taken from pigs into 1% EDTA, 0.14 M NaCl, pH 7.4 (1 volume of anticoagulant to 9 volumes of blood). Blood was centrifuged at 120 x g for 20 min. Platelet rich plasma was removed and centrifuged at 1200 x g for 20 min. The platelet poor plasma was discarded and the platelets were resuspended in the first washing solution that consisted of 0.11 M NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.4 mM NaH_2PO_4, 5.5 mM glucose, pH 6.5 [1]. Any red cells at the bottom of the tube were not resuspended with the platelets and this resulted in some loss of platelets. The platelet suspension was then centrifuged at 1200 x g for 20 min and the platelets resuspended in the second washing solution consisting of 0.14 M NaCl, 15 mM Tris-HCl, 5.5 mM glucose, pH 7.5 [1].

The procedure of washing with second buffer was repeated twice. The platelet suspension was then centrifuged at 1200 x g for 20 min. The final suspension medium was 0.109 M NaCl, 4.3 mM K_2HPO_4, 16 mM Na_2HPO_4, 8.3 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4. The platelet suspension was divided into four equal parts and platelet aggregation was induced by the addition of different inducers:

- bovine thrombin (Biomed Serum and Vaccine Manufacturers in Lublin) at final concentrations: 1, 3, 5, 7.5 or 10 units per mg of platelet protein, without or with calcium ions ($CaCl_2$) at final concentration 10 mM,
- collagen from chicken tendon prepared according to S t i l l e r et al. [8] and used at concentration 5 or 10 µg per mg of platelet protein,
- ADP (Sigma London Chemical Co. Ltd) at the concentration 10^{-3} M or 10^{-4} M,

- one part of platelet suspension was incubated with buffer (control).

After the incubation time (0.5-5 min, 37°C) the aggregated platelets were centrifuged at 1200 x g for 20 min at 4°C. The supernatant containing the soluble platelet release products was carefuly separated.

In the control supernatant and supernatant after aggregation as well as in suspensions of platelets, protein was determined by microbiuret method [2] using bovine serum albumin as a standard.

In some experiments loss of lactic dehydrogenase (E.C.1.1.1.2) from platelets was measured by the optical test with pyruvate [3].

Chromatographic analysis: platelet suspensions (700-1500 mg of platelet protein) were incubated for 2 min with aggregating agents: thrombin (10 u. per mg of platelet protein with 10 mM $CaCl_2$) or collagen (10 µg per mg of platelet protein) or ADP (10^{-5} M). After centrifuging the supernatant was stored overnight at 4°C and formed precipitates were removed by centrifugation. This precipitated material particularly abundant after thrombin action, was fibrin. The clear concentrated solution containing about 40 mg of protein was applied on Sepharose 4B column. The column (1.8 x 60 cm) was equilibrated and eluated with 3.8 mM borate, 25 mM Tris, 1 mM EDTA, 0.15 M NaCl, pH 8.8 at 30 ml/h. Fractions of 3 ml were collected and absorbance at 280 nm was estimated.

The thrombin-released proteins derived from 2-3 separated preparations were analysed for presence of β-thromboglobulin using rechromatography of fraction 3 obtained from Sepharose 4B [9]. Rechromatography was carried out on Sephadex G-200 using the same elution buffer as by Sepharose 4B [5].

Gel electrophoresis of proteins:

- liberated from platelets during washing procedure,
- released from platelets after thrombin action,
- total proteins of plasma.

Solution after second washing of platelets was concentrated, dialysed against 10 mM Tris-HCl, pH 7.4 and analysed by means of SDS-polyacrylamide gel electrophoresis. The proteins released

by thrombin and total proteins of platelet poor plasma (200 μg of protein) were analysed in the same way.

SDS-polyacrylamide gel electrophoresis was carried out in 0.01 M phosphate buffer essentially described by W e b e r and O s b o r n [10]. Molecular weight determination was made by using standard proteins: reduced and non-reduced fibrinogen, serum albumin, myoglobin and cytochrom c.

Results

Washed pig platelets upon the addition of the high concentration of aggregating agents release a great number of proteins from their granules. It is a specific process since no liberation of lactic dehydrogenase activity was simultanously observed (Tab. 1).

T a b l e 1

The effects of thrombin, collagen and ADP on loss of lactate dehydrogenase (LDH) from washed pig platelets

Wpływ trombiny, kolagenu i ADP na spadek dehedrogenazy mleczanowej (LDH) z przemywanych wieprzowych płytek

Der Effekt von Thrombin, Kollagen und ADP auf den Verlust von LDH aus gewaschenen Schweineblutplättchen

Control	Thrombin	Collagen	ADP
1.9	1.8	2.1	1.9
6.6	6.2	6.9	7.1
11.4	12.1	11.9	11.3

N o t e: Pig platelet suspension (15-20 mg of platelet protein in ml) was divided into 4 parts. Each part was incubated with thrombin (10 units per mg), collagen (10 μg per mg) or ADP (10^{-3} M) for 2 min at 37°C and then centrifuged. The activity of lactate dehydrogenase was determined in total supernatant after platelets centrifugation and expressed in Wróblewski units. Controll incubation was performed in the same conditions as the absence of aggregating agents.

The effects of tested aggregating agents on amounts of released proteins from washed pig platelets are presented in Tab. 2.

T a b l e 2

The protein released from washed pig platelets after action of aggregating agents. Values expressed as the percentage of total platelet protein

Białka z przemywanych wieprzowych płytek po reakcji agregacji. Wartości podano w procentach białka całkowitego płytek

Die Proteine freigesetzten aus Schweineblutplättchen nach der Wirkung von Aggregationsfaktoren. Die Werte sind in % von Gesamtplättchenproteine gegeben

% protein released from pig platelets by								
Thrombin units NIH/mg of platelet protein								
Ca	-	+	-	+	-	+	-	+
	1 u.		3 u.		5 u.		10 u.	
X	5.9	7.8	9.7	12.9	11.6	16.0	14.9	23.0
SD	0.5	0.4	0.2	0.6	0.7	0.4	0.6	1.4

% protein released from pig platelets by				
Control (without aggregating agents)	ADP 10^{-4} M	ADP 10^{-3} M	collagen 5 µg/mg of platelet protein	
X	2.5	5.8	9.3	10.0
SD	0.3	0.6	0.5	1.2

N o t e: Washed pig platelets (30-50 mg of platelet protein) were incubated with thrombin at different concentration (with and without 10 mM $CaCl_2$), with ADP or collagen in a medium consisting of 0.109 M NaCl, 4.3 mM KH_2PO_4, 16 mM Na_2HPO_4, 8.3 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4. Control incubation without any aggregating agent was carried out in the same ways. Protein content was measured by microbiuret method [2] in supernatant after platelet centrifugation.

As we can see thrombin at the highest concentration (10 u. NIH per mg of platelet protein) causes the release of 14.9 ∓0.6 and 23.0 ∓1.4% of total platelet proteins in the absence and in the presence of calcium ions, respectively. Collagen and ADP in used

concentrations released considerably less proteins. It is also indicated that platelets both during the washing procedure and suspending in buffer lose some proteins.

The amounts of secreted proteins depend on nature, dose of aggregating factor as well as on temperature and presence of

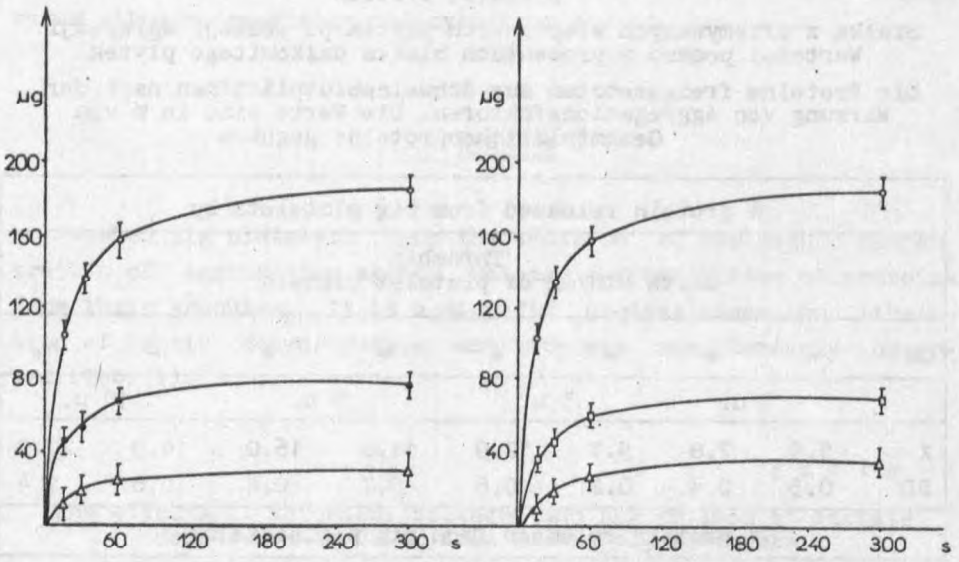

Fig. 1. Different amounts of protein released from washed pig platelets after treatment with different aggregation agents. Platelet suspension (2.5 mg of platelet protein in ml) was incubated with thrombin (7.5 u. NIH/mg of platelet protein) at 37°C -o- at 37°C with 1 mM EDTA -●- and at 4°C -Δ-. On the right side the amount of released proteins at 37°C after treatment with thrombin (7.5 u. NIH/mg of platelet protein) -o-, collagen -□- at concentration 25 µg/ml and ADP -Δ- (10^{-4} M) is presented

Ilości białka uwolnionego z przemytych płytek wieprza w wyniku działania różnych czynników agregujących. Zawiesinę płytek (2,5 mg białka płytkowego/ml) inkubowano z trombiną (7.5 u. NIH/mg białka płytek) w 37°C -o-, w 37°C z 1 mM EDTA -●- i w 4°C -Δ-. Z prawej strony ilość białka uwolnionego w 37°C w yniku działania trombiny (7,5 u. NIH/mg białka płytkowego) -o-, kolagenu -□- w stężeniu 25 µg/ml i ADP -Δ- (10^{-4} M)

Die Menge der freigesetzten Proteine aus gewaschenen Schweineblutplättchen nach der Wirkung von verschiedenen Aggregationsfaktoren. Blutplättchensuspensionen (2.5 mg von Plättchenproteine in ml) wurden mit Thrombin (7.5 µ/mg von Proteine - 37°C) -o-, mit Thrombin zusammen mit 1 mM EDTA -●- und 4°C -Δ- inkubiert. Es wird die Menge der freigesetzten Proteine (rechts) nach Wirkung (37°C) der Thrombin -o-, -□- Kollagen (25 µg pro ml und ADP -Δ (10^{-4} M gezeigt)

EDTA (Fig. 1). All the values presented are corrected for spontaneous liberation of protein and added protein. The means of 5-8 experiments with SD have been given.

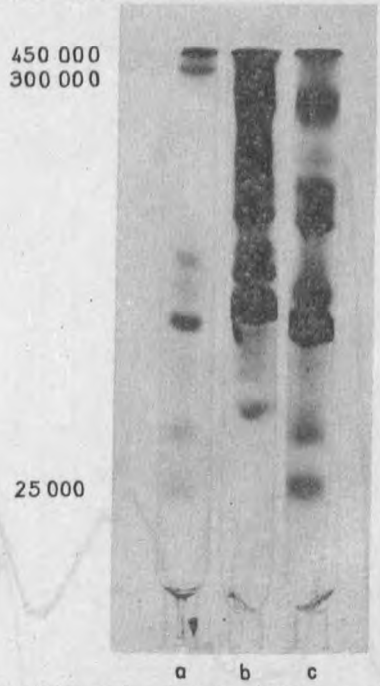

Fig. 2. SDS-polyacrylamide gel electrophoresis (5% gel) of proteins released from platelets after thrombin action (a), plasma proteins (5 µl of plasma) (b) and proteins liberated from platelets to the buffer during washing procedure (c)

Elektroforeza w żelu poliakryloamidowym (5%) w obecności SDS białek uwolnionych z płytek w wyniku działania trombiny (a), białek plazmy (5 µl plazmy) (b) i białek uwolnionych z płytek podczas przemywania (c)

SDS-Polyakrylamidegelelektrophorese von: a - freigesetzten Gesamtproteine, b - Plasmaproteine, c - Proteine aus der Flüssigkeit nach den zweiten Washen der Plättchen

After washing the platelets and removing them, the supernatant contains not only plasma proteins but the proteins derived from platelets too (Fig. 2). Among the platelet proteins liberated to the washing medium there is a low molecular protein fraction (about 25 000 daltons). This protein is absent in the plasma but present in the material released by thrombin (Fig. 2).

During our experiments we have noticed that protein material

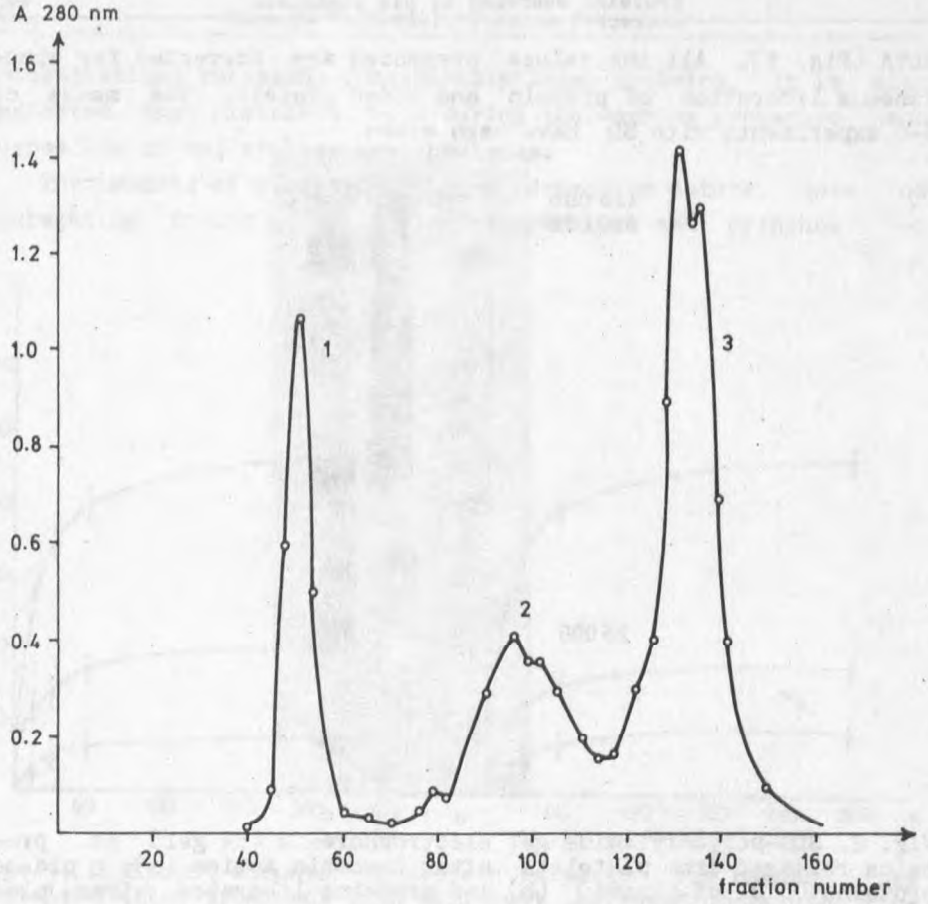

Fig. 3. Sepharose 4B profile of the released proteins (deprived of fibrinogen) from the supernatant of thrombin-aggregated washed pig platelets (10 u. NIH per mg of platelet protein). The concentrated supernatant (6 ml) containing 40 mg of protein was applied to a Sepharose column (60 x 2.5 cm) and gel filtration was performed at a flow rate of 25 ml/h using 25 mM Tris-HCl, 1 mM EDTA, 3.8 mM borate, 0.15 M NaCl, pH 8.8, 3 ml fractions were collected

Profil rozdziału na Sepharozie 4B białek (pozbawionych fibrynogenu) uwolnionych podczas agregacji przemytych płytek wieprza spowodowanej trombiną (10 u. NIH/mg białka płytkowego). Zatężony supernatant (6 ml) zawierający 40 mg białek nanoszono na kolumnę (60 x 2,5 cm) wypełnioną Sepharozą i prowadzono sączenie na żelu stosując szybkość elucji 25 ml/h i 25 mM Tris-HCl, 1 mM EDTA, 3,8 mM boran, 0,15 M NaCl, pH 8,8 jako eluent. Zbierano frakcje o objętości 3 ml

Ein typisches Elutionprofil (Sepharose 4B) der freigesetzten Proteine (ohne Fibrinogen) aus dem Supernatant der Thrombinaggregierten Blutplättchen. Das konzertrierte Supernatant (6 ml) mit 40 mg von Proteine wurde auf Sepharose 4B aufgegeben und mit Geschwindigkeit von 25 ml/h fraktioniert. 3 ml Fraktionen wurden gesammelt

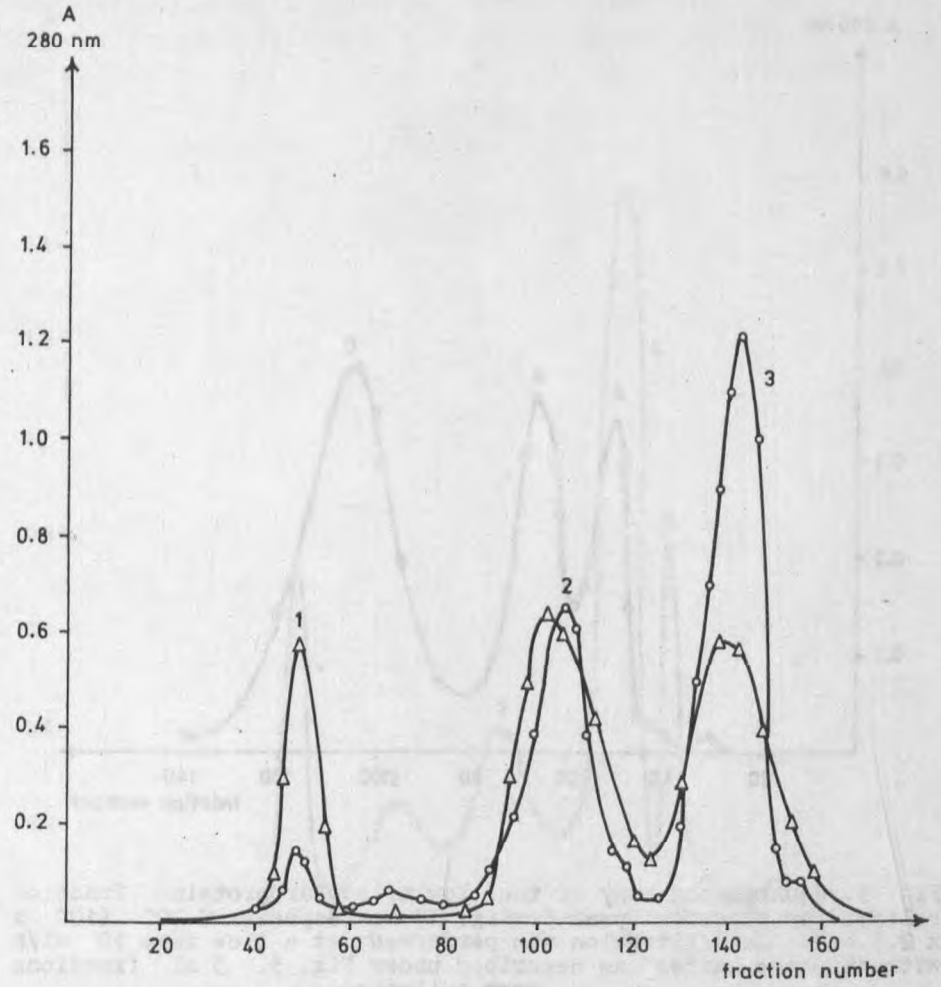

Fig. 4. Sepharose 4B profiles of the proteins released from washed pig platelets by collagen -Δ- (25 μg per ml) and by ADP -o- (10^{-4} M). Concentrated supernatant was chromatographed as described in Fig. 3

Profile rozdziału chromatograficznego na Sepharozie 4B białek uwolnionych z przemytych płytek w wyniku działania kolagenu -Δ- (25 μg/ml) i ADP -o- (10^{-4} M). Warunki rozdziału zatężonego supernatantu jak w opisie rys. 3

Ein typisches Elutionprofil (Sepharose 4B) der freigesetzten Proteine nach der Inkubation der gewaschenen Schweineblutplättchen mit Kollagen -Δ- 25 μg/ml und mit ADP (10^{-4} M) -o-. Das konzentrierte Supernatant wurde wie früher (Fig. 3) chromatographiert

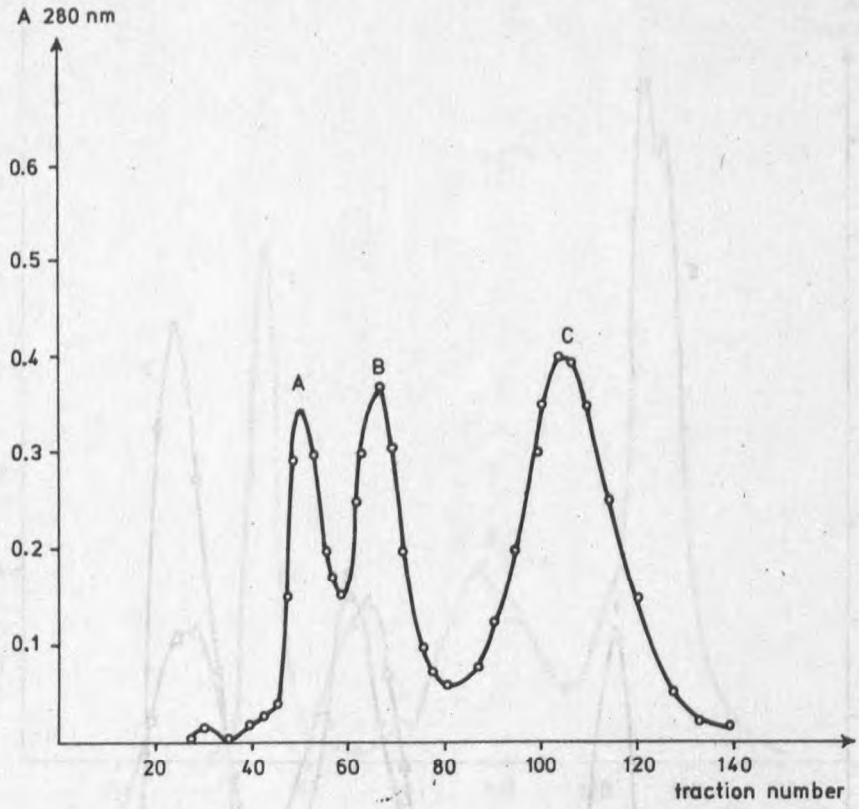

Fig. 5. Rechromatography of the low molecular protein fraction released by thrombin (peak 3, Fig. 3) on Sephadex G-200 (100 x x 2.5 cm). Gel filtration was performed at a flow rate 10 ml/h with the same buffer as described under Fig. 3. 3 ml fractions were collected

Rechromatografia frakcji białek niskocząsteczkowych uwolnionych w wyniku działania trombiny (rys. 3, szczyt 3) na Sephadeksie G--200 (100 x 2,5 cm). Sączenie na żelu prowadzono przy szybkości elucji 10 ml/h stosując ten sam bufor (rys. 3). Zbierano frakcje o objętości 3 ml

Die Auftrennung auf Sephadex G-200 (100 x 2.5 cm) der niedermolekulären Proteine nach Thrombininduzierter Freisetzung. Gelfiltration wurde mit Geschwindigkeit von 10 ml/h, Puffer wie früher (Fig. 3) verwendet. 3 ml Fraktionen wurden gesammelt

released by thrombin contains fibrinogen which constitutes about one tenth of total protein secreted by platelets. The fibrinogen has been clotted overnight at 4°C.

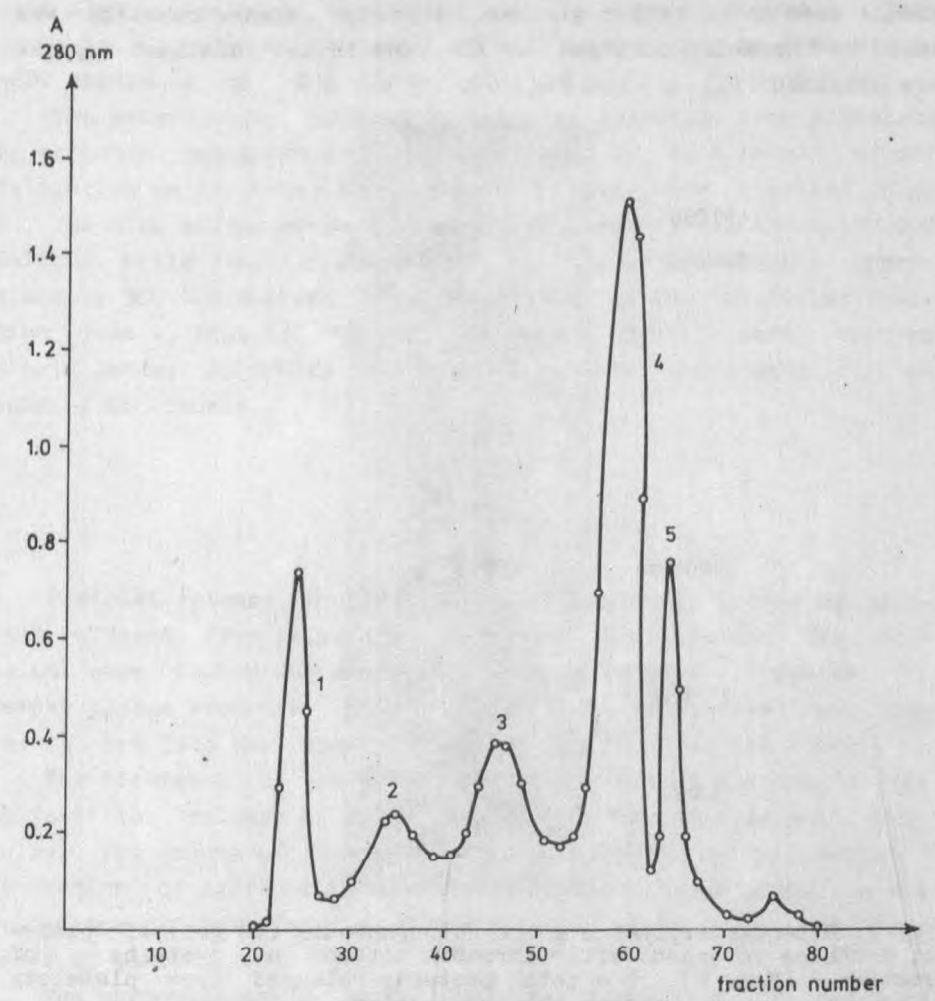

Fig. 6. Sepharose 6B profile of the proteins released by thrombin (10 u. NIH per mg of platelet protein). Gel filtration was performed in the same way as on Sepharose 4B (see Fig. 3)

Profil rozdziału na Sepharozie 6B białek uwolnionych w wyniku działania trombiny (10 u. NIH/mg białka płytkowego). Warunki sączenia na żelu takie same, jak w przypadku Sepharozy 4B (rys. 3)

Ein typisches Elutionprofil auf Sepharose 6B Proteine nach Thrombininduzierter Freisetzung (10 u. pro mg). Gelfiltration wurde wie fürher mittels Sepharose 4B durchgeführt

Chromatohraphic analysis revealed that Sepharose 4B elution profiles of protein released from pig platelets by different

stimuli seem to be rather similar. Whenever release reaction was caused by thrombin, collagen or ADP, the three distinct peaks were obtained (Fig. 3 and Fig. 4).

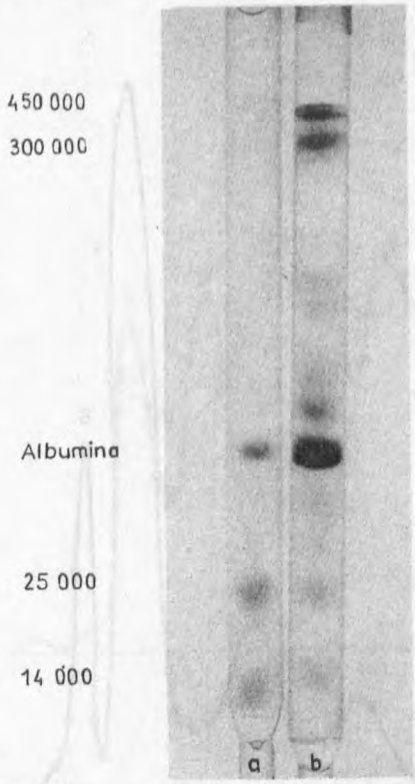

Fig. 7. SDS-polyacrylamide gel electrophoresis (5% gel) of platelet proteins released after thrombin action: a - proteins of fraction 4 (Fig. 6), b - total proteins released from platelets after thrombin action

Elektroforeza w żelu poliakryloamidowym (5%) zawierającym SDS białek płytkowych uwolnionych w wyniku działania trombiny: a - białka frakcji 4 (rys. 6), b - całkowite białko uwolnione z płytek w wyniku działania trombiny

SDS-polyakrylamidegelelektrophorese von Proteine der Schweineblutplättchen: a - Fraktion 4 (Fig. 6), b - freigesetzte Gesamtproteine

However, the proteins released by ADP contained extremely small amounts of high molecular protein (peak 1, Fig. 4). Low molecular fraction (peak 3) appeared to be the largest one of them all, particularly in case of thrombin action (Fig. 3). This frac-

tion always contained β-thromboglobulin as a component. This could be demonstrated by means of rechromatography on Sephadex G--200 according to M o o r e et al. [5] (Fig. 5, peak C).

The heterogenity of protein material secreted from platelets by thrombin was shown in Fig. 6 and Fig. 7. As a result of gel filtration on Sepharose 6B, 5 distinct peaks were obtained (Fig. 6). The high molecular weight fraction (peak 1) was about 450 000 daltons while fraction 2 seemed to have molecular weight approximately 300 000 daltons. The composition of low molecular fraction (peak 4, Fig. 6) was very changeable. Usually were observed 3 main bands: 25 000 daltons, 14 000 daltons and a band corresponding to albumin.

Discussion

Platelet release reaction was investigated by measuring protein released from cells into the extracellular phase. The platelets were washed and resuspended in an artificial medium to remove plasma proteins. However, some part of platelet proteins was removed into the washing solution during these procedures.

The treatment of pig platelets with aggregating agents results in the release of great amount of proteins from cell granules. The amount of secreted proteins depends on type and concentration of aggregating agents. Thrombin treated platelets secreted the greatest amount of protein particularly in the presence of calcium ions.

The chromatographic profiles of the separation of released compounds on Sepharose 4B are generally similar, however there are some differences. The ADP-treated platelets release only very small amounts of high molecular protein fraction. This fraction is extremely distinct in thrombin-released material and correspond to "thrombin sensitive protein" called thrombospondin - a glycoprotein described recently by L a w l e r et al. [4]. The low molecular fraction obtained by separation on Sepharose has a rather variable composition. However it can be separated on Sephadex G-200 into three distinct peaks. Peak I is possibly Ig G, peak II is serum albumin and peak III seams to be β-thromboglobulin [5, 9].

We suppose that the major component of thrombin-treated proteins is thrombospondin with its degradation products of molecular weight about 300 000 (dimer), 150 000 daltons (monomer), albumin and β-thromboglobulin.

The composition of proteins secreted from washed pig platelets resembles the composition of material released from human platelets.

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BIAŁKA UWOLNIONE Z PRZEMYTYCH KRWINEK PŁYTKOWYCH WIEPRZA

Badano ilość oraz skład białek uwalnianych z przemytych płytek wieprzowych po inkubacji z różnymi czynnikami agregującymi. Uwalniane związki białkowe rozdzielano na Sepharozie 4B, Sepharozie 6B, Sephadexie G-200 oraz w żelu poliakrylamidowym. Wśród uwalnianych białek przeważają: fibrynogen, albumina oraz specyficzne białko płytkowe β-thromboglobulina.

W wyniku reakcji uwalniania stymulowanej przez trombinę, stwierdzono obecność wysokocząsteczkowego białka - trombospondiny.

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FREIGESETZTE PROTEINE AUS GEWASCHENEN SCHWEINEBLUTPLÄTTCHEN

Es wurden die Menge und Zusammensetzung der freigesetzten Proteine aus gewaschenen Schweineblutplättchen untersucht und der Einfluss von verschiedenen Aggregationsfaktoren beobachtet. Das freigesetzte Proteinmaterial wurde mittels Gelfiltration auf Sepharose 4B, Sepharose 6B, auf Sephadex G-200 fraktioniert und mittels SDS-polyakrylamidegelelektrophorese analysiert.

Man zeigte, dass die aus Schweineblutplättchen freigesetzte Proteine als Grundkomponenten Fibrinogen, Albumin und ein spezifische Plättchenprotein - β -Thromboglobulin enthalten. Nach der Thrombin-induzierten Freisetzungreaktion ist auch das hochmolekuläres Protein - Thrombospondin beobachtet.