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IDENTIFICATION, PURIFICATION AND CHARACTERISTICS OF GLYCOSIDASES OF HUMAN BLOOD PLATELETS

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SUMMARY

Human blood platelets were found to contain active glycosidases, acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31). The enzymes were assayed using *p*-nitrophenyl derivatives and 0.1% Triton X-100 extracts of human blood platelets. The activities fell into three categories: those with high activity (greater than 700 nmoles/h per mg protein), *N*-acetyl glucosaminidase (chitobiose acetamidodeoxyglucohydrolase, EC 3.2.1.29) and acid phosphatase; those with moderate activity (less than 700 but more than 100 nmoles/h per mg protein), β-*N*-acetyl galactosaminidase and β-mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25); and those with low activity (less than 100 nmoles/h per mg protein), α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22), α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24), β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), β-glycyronidase, β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37), β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21), β-fucosidase and α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20); α-fucosidase was absent. The enzymes were purified 16- to 292-fold by centrifugation and gel chromatography: several of the enzymes gave multiple peaks on gel crhomatography. The enzymes were severely inhibited by Pb^{2+} and Hg^{2+} but were not dependent on divalent cations for activity. The purified enzymes were shown to be able to release carbohydrate from intact platelet plasma membranes.

INTRODUCTION

The glycosidases are enzymes thought to be responsible for the hydrolysis of bonds between sugars (hexoses, pentoses and hexosamines, primarily) and amino acids (primarily asparagine, but also serine, threonine, and hydroxylysine), between sugars and lipid moieties and between adjacent monosaccharides primarily in glycoproteins, glycolipids, oligo- and polysaccharides and glycosaminoglycans[1]. Glycosidase activity has been studied in a variety ot tissues and cell types[2–11] but little work has been done on glycosidase activity in platelets.

The glycosidase activity of platelets seemed to be of importance for the following three reasons: (1) platelets contain glycoproteins[12,13] and hence platelet glycosidases would be primary sources for degradation of the platelet glycoproteins; (2) another formed element of blood, namely erythrocytes have recently[14] been shown to contain glycosidases in their plasma membranes; and, most importantly, (3) human platelets have been demonstrated to contain glycoprotein:glycosyl transferases[15,16] and these transferases have been postulated[16] not to be responsible in the mature platelet for glycoprotein synthesis at all, but rather that they may function for intracellular adhesion and the primary step in hemostasis, the adhesion of collagen to platelets. Thus glycosidases would be of prime importance to modify platelet glycoproteins or the carbohydrate portions of extra-platelet glycoproteins such as collagen.

The present report describes the identification, purification and properties of platelet glycosidases; in addition, acid phosphatase and β-glucuronidase were also studied.

The glycosidases studied in this report were β-*N*-acetylglucosaminidase (chitobiose acetamidodeoxyglucohydrolase, EC 3.2.1.29), α-D-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21), α-D-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22), β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), α-L-fucosidase (EC 3.2.1.–), β-L-fucosidase (EC 3.2.1.–), β-D-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37), α-D-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24), β-D-mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25), and β-*N*-acetyl-D-galactosaminidase (EC 3.2.1.–). In addition the platelet localization of acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) was determined.

MATERIALS AND METHODS

Blood

Units of acid–citrate–dextrose blood from young healthy male donors were used. Blood was used immediately after drawing.

Platelet isolation

Platelets were isolated by the method of ZUCKER AND LUNDBERG[17].

Fractions tested for enzyme activity

Platelets were extracted for 30 strokes with a Ten Broeck homogenizer in 0.1% Triton X-100, and a 20 000 × *g* supernatant of the 0.1% Triton X-100 extract and a Sephadex G-150 and Sephadex G-200 column eluate were tested for enzyme activity.

Sephadex G-150 and G-200 chromatography

Routinely, platelets were homogenized in 5 vol. of 0.1% Triton X-100 for 30 strokes with a Ten Broeck homogenizer at 4° and then centrifuged at 20 000 × *g* for 20 min and the supernatant was used as the starting material for purification of glycosidases by Sephadex G-150 and Sephadex G-200 column chromatography. Sephadex G-150 was prepared in 0.1% Trition X-100 as recommended by the manufacturers. The 20 000 × *g* supernatant of the 0.1% Triton X-100 extract was placed

on the gel column. The column (83.0 cm × 3.4 cm) was eluted at 4° with 0.1% Triton X-100 with a flow rate of 10 ml/h; fractions of 6 ml were collected. Fractions were kept at 4° during collection and assayed immediately. Sephadex G-200 was prepared in a similar manner and Fractions 4 to 18 from the Sephadex G-100 chromatography were placed on a column (83.0 cm × 3.4 cm) and eluted at 4° with 0.1% Triton X-100 with a flow rate of 4 ml/h; fractions of 4 ml were collected.

Protein

Total protein in any of the various enzyme preparations was determined by the method of LOWRY *et al.*[18]. Crystalline bovine serum albumin was used as a standard. In each instance of protein determination, the sample for analysis was precipitated with 30% trichloroacetic acid, washed twice with 10% trichloroacetic acid and once with ethanol–diethyl ether (2:1, v/v) and the resultant insoluble material dissolved in NaOH for analysis. Protein in column eluents was determined by measuring the absorbance at 280 nm against a 0.1% Triton X-100 blank.

Enzyme assay

The amount of a given glycosidase, acid phosphatase, or β-glucuronidase activity at pH 4.3 in any of the various enzyme preparations was determined using the *p*-nitrophenyl derivative in the following manner for the optimal conditions[11,14]: 50 μl (in some instances of high activity, 10 μl) of the 0.1% Triton X-100 enzyme extract of the fraction to be tested were incubated with 6.0 μmoles of the *p*-nitrophenyl derivative (the final volume was made up to 1.050 ml with 0.05 M in citrate, adjusted to pH 4.3) for 1 h at 37°. The amount of 0.1% Triton X-100 was constant in all assays at 50 μl. The reaction was terminated by the addition of 2 ml of 0.4 M glycine–NaOH buffer, pH 10.5. The reaction mixtures were then centrifuged at 5000 × *g* for 10 min, and the absorbance of the released *p*-nitrophenol in the supernatant measured at 420 nm. From this measurement and a standard *p*-nitrophenol curve run simultaneously the nmoles hydrolyzed per h were calculated. In each instance reactions were terminated at 10-min intervals to insure the linearity of the reactions up to 1–6 h. The substrates used were *p*-nitrophenyl phosphate, *p*-nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-α-L-fucopyranoside, *p*-nitrophenyl-β-L-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-α-D-galactopyranoside. *p*-Nitrophenol was used as a standard. Each of the above compounds was purchased from Pierce Biochemicals, Rockford, Ill. In each experiment, assays and controls were run in duplicate or triplicate. Controls consisted of assays in which glass distilled water was substituted for either the *p*-nitrophenyl derivative substrate or the enzyme in the reaction mixture. These control values from triplicate assays were averaged, added together, and subtracted from the appropriate assays. Enzyme blank values were always less than 1% of the assay and substrate blank values were never greater than 10% of the total assay value; these blank values were always subtracted from the assay values run simultaneously. Thus, light scattering contributions from the enzyme preparations or absorbance contributions from non-specific hydrolysis of the *p*-nitrophenyl derivative were subtracted from the results

presented herein. Each experiment was performed at least 4 times. All solutions, suspensions, and buffers were made in distilled water that was deionized by an ion exchange column and then distilled in a glass still.

Carbohydrate

Total carbohydrate released from the blood platelets by the purified Sephadex G-150 enzymes of human blood platelets was measured by the anthrone procedure[19].

RESULTS

Glycosidase activity of human blood platelets

The data in Table I indicate that except for α-fucosidase, each of the glycosidases was present in the crude 0.1% Triton X-100 extract of human blood platelets, as were acid phosphatase and β-glucuronidase. The activities fell into three categories: those with high activities (greater than 700 nmoles per h per mg protein of activity), β-*N*-acetylglucosaminidase and acid phosphatase; those with moderate activity (less than 700 but more than 100 nmoles per h per mg protein of activity), β-*N*-acetylgalactosaminidase and β-mannosidase; and those with low activity (less than 100 nmoles per h per mg protein of activity), β-galactosidase, α-mannosidase, α-galactosidase, β-glucuronidase, β-xylosidase, β-glucosidase, β-fucosidase, and α-glucosidase.

TABLE I

GLYCOSIDASE, ACID PHOSPHATASE AND β-GLUCURONIDASE ACTIVITY OF HUMAN PLATELETS

Preparation of the various fractions are as given in MATERIALS AND METHODS. 0 indicates no measurable activity was found.

Enzyme	*Enzyme activity (nmoles/h per mg protein)*					
	0.1% Triton X-100 extracts of human blood platelets			*20 000 × g Supernatant*	*Column chromatography fractions*	
	Complete	*0°**	*Boiled extract***		*Sephadex G-150*	*Sephadex G-200*
α-Glucosidase	20.6	0	1	41.3	140	811 (39)***
β-Glucosidase	35.0	1	0	70.2	630	2 914 (83)
α-Galactosidase	72.1	0	0	148.2	1610	7 118 (99)
β-Galactosidase	84.4	0	0	167.2	420	1 622 (19)
α-Fucosidase	0	0	0	0	0	0 (—)
β-Fucosidase	24.7	0	0	49.1	1610	7 222 (292)
β-*N*-Acetylgalactosaminidase	168.8	4	0	339.8	1190	5 433 (32)
β-*N*-Acetylglucosaminidase	905.9	12	4	1911.4	700	31 920 (34)
α-Mannosidase	80.3	0	0	158.1	490	1 247 (16)
β-Mannosidase	175.0	11	0	347.3	1470	5 711 (33)
β-Xylosidase	35.0	1	0	71.0	2800	11 092 (32)
Acid phosphatase	763.8	12	4	1581.3	3924	17 425 (23)
β-Glucuronidase	35.0	0	0	71.2	910	5 187 (148)

* Incubations performed at this temperature.
** Extracts were boiled 10 min before assay.
*** Number in parentheses is amount of purification achieved.

The data in Table I indicate the enzymes were dependent on temperature for activity and could be heat denatured by boiling for 10 min. The purification scheme outlined in MATERIALS AND METHODS yielded purifications of the human blood platelet glycosidase ranging from 16- to 292-fold. It is interesting to note that relatively high purifications were achieved with β-glucosidase (as opposed to α-glucosidase), α-galactosidase (as opposed to β-galactosidase), β-fucosidase (α-fucosidase absent), and β-glucuronidase, indicating that the glucosidases and galactosidases of human blood platelets are

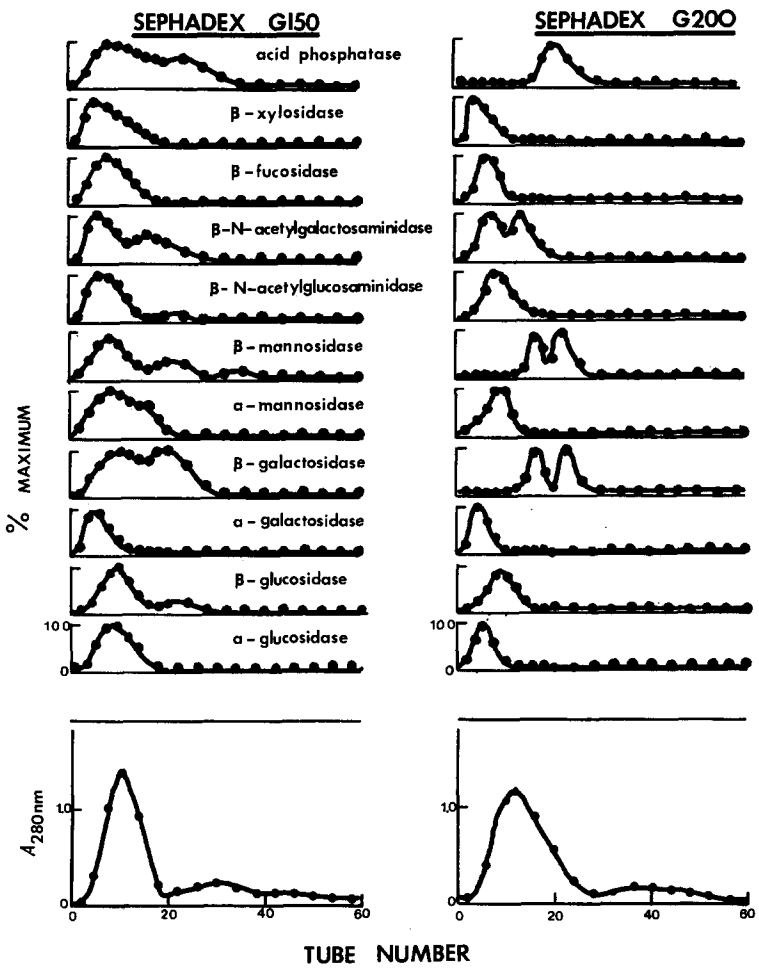

Fig. 1. Gel chromatography of platelet glycosidases. A sample of the 20 000 × g supernatant of the 0.1% Triton X-100 extract of human blood platelets was applied to a Sephadex G-150 column (83.0 cm × 3.4 cm) previously equilibrated with 0.1% Triton X-100 (left). A maximum hydrostatic head of 20 cm was maintained during elution of the column with 0.1% Triton X-100. Fractions of 6 ml were collected. Assays were performed with p-nitrophenyl glycosides or p-nitrophenyl phosphate, as described in MATERIALS AND METHODS. Fractions 4–18 of the Sephadex G-150 column were pooled, dialyzed and lyophilized to a volume of 6 ml. This material was applied to a Sephadex G-200 column (83.0 cm × 3.4 cm) previously equilibrated with 0.1% Triton X-100 (right). Fractions of 4 ml were collected. The lowest panel in each instance represents the protein elution profile.

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TABLE II

EFFECT OF IONS ON HUMAN BLOOD PLATELET GLYCOSIDASES

EDTA had a final concentration of 1 mM. The incubation mixture contained 6.0 μmoles of substrate in 0.2 M citric acid–sodium citrate buffer (pH 4.3), 25 μl of the purified G-200 glycosidase of human blood platelets *plus* the EDTA and cation tested to a final volume of 1.675 ml. The incubation mixture was incubated at 37° for 1 h and the activity determined. Activities for the EDTA assays are expressed as percentages of controls assayed with H_2O substituted for the EDTA; activities for the cations are expressed as percentages of the EDTA assays with deionized water substituted for the cations. In each experiment, both controls and tests were assayed in duplicate. The experiment was repeated 4 times. Means ± 1 S.E. All divalent cations were added as the chloride and were present at a concentration of 2 mM.

Enzyme	*EDTA*	Mg^{2+}	Ca^{2+}	Ba^{2+}	Cu^{2+}	Cd^{2+}	Hg^{2+}	Co^{2+}	Pb^{2+}	Mn^{2+}
α-D-Galactosidase	111 ± 6	76 ± 2	67 ± 5	76 ± 3	81 ± 1	72 ± 1	0	92 ± 2	21 ± 2	101 ± 3
β-D-Galactosidase	102 ± 4	84 ± 4	82 ± 2	72 ± 2	83 ± 2	71 ± 2	2 ± 0.1	94 ± 3	18 ± 2	103 ± 4
α-D-Glucosidase	99 ± 3	86 ± 4	64 ± 1	81 ± 1	86 ± 3	68 ± 2	3 ± 0.1	93 ± 2	27 ± 2	88 ± 2
β-D-Glucosidase	116 ± 4	82 ± 3	79 ± 1	82 ± 1	88 ± 2	79 ± 1	2 ± 0.1	98 ± 3	40 ± 3	92 ± 3
α-L-Fucosidase	—	—	—	—	—	—	—	—	—	—
β-L-Fucosidase	121 ± 4	72 ± 2	81 ± 1	83 ± 4	81 ± 1	81 ± 1	7 ± 0.1	101 ± 3	28 ± 1	96 ± 4
α-D-Mannosidase	109 ± 3	76 ± 1	87 ± 3	82 ± 3	82 ± 4	83 ± 2	3 ± 0.1	92 ± 3	36 ± 1	95 ± 1
β-D-Mannosidase	117 ± 2	92 ± 3	82 ± 2	87 ± 2	81 ± 1	72 ± 3	3 ± 0.1	86 ± 2	37 ± 1	100 ± 2
β-D-Xylosidase	118 ± 2	90 ± 2	81 ± 1	84 ± 1	80 ± 2	76 ± 4	0	97 ± 4	24 ± 1	96 ± 3
N-Acetyl-β-D-glucosaminidase	99 ± 7	87 ± 1	84 ± 4	83 ± 4	86 ± 3	87 ± 4	7 ± 0.1	92 ± 1	32 ± 2	102 ± 3
N-acetyl-β-D-galactosaminidase	106 ± 4	85 ± 4	71 ± 3	82 ± 1	84 ± 2	68 ± 2		94 ± 5	19 ± 1	101 ± 3

distinct enzymes for the α- and β-anomeric configuration. The data in Fig. 1 indicate that several of the enzymes separated into multiple peaks on gel chromatography; this was most pronounced for β-*N*-acetylgalactosaminidase, β-mannosidase, and β-galactosidase.

Ion effects on human blood platelet glycosidases

The data of Table II demonstrate the effects of EDTA and various divalent cations on the activities of the platelet glycosidases. EDTA had no deleterious effect on the glycosidases; indeed, in many instances activity was enhanced by the EDTA probably due to chelation of an inhibitory ion(s) in the incubation mixture. The response of each of the glycosidases to divalent cations in the assay was qualitatively similar (Table II). Of the ions tested Mn^{2+} and Co^{2+} had little deleterious effect, Mg^{2+}, Ca^{2+}, Ba^{2+}, Cu^{2+} and Cd^{2+} caused moderate inhibition of the human blood platelet

TABLE III

OPTIMUM pH, TEMPERATURE, K_m AND v_{max} OF THE GLYCOSIDASES OF HUMAN BLOOD PLATELETS

In the instances of pH and temperature, if the condition was optimum for a range of values the mean value of this range is given. The enzyme source for these determinations was the purified Sephadex G-200 blood platelet enzymes.

Enzyme	*Optimum pH*	*Optimum temp. (°)*	$K_{m\ (apparent)}$ *(mM)*	v_{max} *(nmoles/h)*
α-D-Glucosidase	4.2	37	36	27
β-D-Glucosidase	4.3	38	21	24
α-D-Galactosidase	4.3	37	41	18
β-D-Galactosidase	3.8	40	50	21
α-L-Fucosidase	—	—	—	—
β-L-Fucosidase	4.2	37	27	9
α-D-Mannosidase	4.2	37	23	21
β-D-Mannosidase	4.3	37	4	9
β-D-Xylosidase	4.3	38	31	18
N-Acetyl-β-D-galactosaminidase	4.0	37	1	14
N-Acetyl-β-D-glucosaminidase	4.3	40	3	19

glycosidases, and Pb^{2+} and especially Hg^{2+} were severely inhibitory to the activity of the human blood platelet glycosidases (Table II).

Optimum pH, temperature, K_m and v_{max} for the human blood platelet glycosidases

The data in Table III demonstrate that the optimum pH of reaction for the glycosidases ranged from 3.8 to 4.3 and the optimum temperature from 37° to 40°; there was virtually no activity above 60° or below 10°. Linear Lineweaver–Burk reciprocal plots of $1/[S]$ *vs.* $1/v$ yielded the $K_{m\ (apparent)}$ and v_{max} values given in Table III for the human blood platelet glycosidases. The $K_{m\ (apparent)}$ values ranged from 1 to 50 mM with v_{max} values ranging from 9 to 27 nmoles/h.

Release of carbohydrate from intact platelets by the G-150 human blood platelet glycosidases

Fractions 4–18 of the Sephadex G-150 column chromatography were pooled

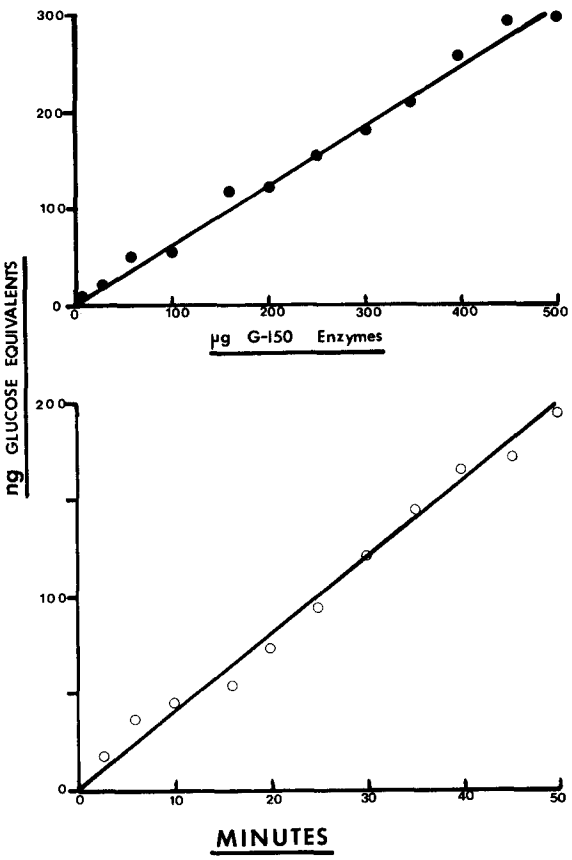

Fig. 2. (Top) Release of carbohydrate from intact platelets by Sephadex G-150 platelet glycosidase. The complete system contained 50 μl of the Sephadex G-150 platelet glycosidases containing the indicated amount of extract, and 15 mg of intact platelet protein (final volume 1.050 ml, 0.05 M in citrate pH 4.3) and was incubated at 37° for 1 h. After incubation, 2 ml of 20% trichloroacetic acid were added and the mixture was centrifuged at 2000 × g for 10 min. The decanted supernatant was analyzed for carbohydrate by the anthrone procedure. Controls consisting of the enzyme source (Sephadex G-150 enzymes) and substrate (intact platelets), incubated and precipitated as above, were subtracted from the incubated reactions. These controls were in all instances less than 4% of the test values. Experiments were performed in triplicate. If the Sephadex G-150 platelet glycosidase fraction was boiled for 10 min before incubation, no release of carbohydrate occurred. (Bottom) Experiments were performed exactly as above except that the amount of Sephadex G-150 platelet glycosidases in the assay was kept constant (400 μg) and the time of incubation was varied.

and incubated with intact platelets (15 mg as protein) for varying amounts of time and with varying amounts of the Sephadex G-150 enzyme. The data in Fig. 2 demonstrate that the glycosidases of the platelets were capable of hydrolyzing and liberating monosaccharide residues presumably from glycoproteins and glycolipids on the platelet plasma membrane.

DISCUSSION

The human blood platelets contain glycosidase activity which is not dissimilar

to glycosidase activity in other cells and tissues[2–11] with respect to divalent cation effects and optimal pH. Since glycoproteins are found in platelets[12,13] and since it was demonstrated herein that the glycosidases from human blood platelets could release carbohydrate from intact human blood platelets, it is reasonable to assume that the glycosidases could modify platelet plasma membrane glycoproteins and glycolipids. Furthermore, since platelets have been demonstrated to contain glycoprotein:glycosyl transferases[16] and since these transferases have been postulated[15,16] to participate not only in glycoprotein synthesis but also in cell adhesiveness and aggregation[15,16,20], such modifications of platelet surface glycoproteins and glycolipids by these glycosidases could greatly affect platelet–platelet interactions.

Serum contains many glycoproteins and glycolipids[21] and cellular blood group substances are glycolipid in nature[22]. Either group of glycoproteins or glycolipids could function as substrates for platelet glycosidases. Finally, collagen is a glycoprotein[23,24] and the platelet glycosidases could degrade the carbohydrate portion of collagen and perhaps affect the primary step in hemostasis, the adhesion of collagen to platelets. The results also identify glycosidases again in the formed elements of blood, the glycosidases of the erythrocyte plasma membrane having been previously described[14]. Additional purification will have to be performed before the glycosidases from these two formed elements of blood can be compared.

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