Improved Extraction and Determination of Some Glycolytic Enzymes in the Human Erythrocyte

In the human erythrocyte, determination of maximal enzyme activity in vitro, although only loosely connected with in vivo metabolic fluxes, may be useful for many practical purposes (screening for enzyme defects, clinical tests, etc.). Widely divergent results in the literature[1-5] prompted us to reconsider hemolysis techniques and assay methods for crucial glycolytic enzymes. Short sonication and modifications of the assay mixtures were followed by significantly higher activities.

Materials and methods. Red cells were obtained from venous blood freshly drawn from healthy adults of both sexes. Plasma and leucocytes were removed by suction; the red cells were washed 3 times with an isotonic buffer (*Tris*(hydroxymethyl)aminomethane (*Tris*) 17 m*M*, NaCl 153 m*M*, pH 7.5). The leucocyte contamination was less than 1000/µl.

Hemolysis. 3 different techniques were employed: 1. Sonication: the packed red cells, resuspended at 20% in isotonic *Tris*-buffer (see above) were sonicated for 5 sec with a Branson sonifier. 2. Freezing and thawing and 3. hypotonic shock were performed according to MARKS[6].

Assay of enzyme activity. The standard assay procedure by BÜCHER et al.[7] was modified in the following points (concentrations are given as µmoles/ml test solution): hexokinase:ATP 4.95 instead of 1.65; phosphofructokinase:ATP 0.66 instead of 1.65; aldolase:fructose-1,6-P_2 12 instead of 4; glyceraldehyde-3-P dehydrogenase: ATP 8.3 instead of 1.65; 3-P-glycerate kinase:ATP 8.3 instead of 1.65; pyruvate kinase:phosphoenolpyruvate 1.6 instead of 0.8. The pH of the test mixture was 8.2 for hexokinase and phosphofructokinase and 7.6 for the other enzymes.

Results and discussion. The activity of the 7 glycolytic enzymes tested at 25° and 37 °C in the 3 different hemolysates are given in Table I. The best yields were obtained by short sonication. The hypotonic shock procedure gives unsatisfactory yields at both temperatures. Freezing-

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Table I. Influence of hemolysis procedure and temperature on the activity of some glycolytic enzymes in the human erythrocyte

	25 °C Sonication	Freezing	Hypotonic shock	37 °C Sonication	Freezing	Hypotonic shock
Hexokinase	8.5 ± 3.5	6.2 ± 1.9	7.3 ± 1.6[a]	20.0 ± 2.9	15.8 ± 3.8	14.8 ± 5.9[a]
Phosphofructokinase	274 ± 49	239 ± 49[a]	145 ± 40	469 ± 40	437 ± 76[a]	265 ± 44
Aldolase	51 ± 7	45 ± 12[a]	=	117 ± 11	117 ± 13[a]	76 ± 16
Glyceraldehyde-3-P dehydrogenase	1701 ± 180	1747 ± 435[a]	1308 ± 275	3023 ± 648	3209 ± 309[a]	1993 ± 444
3-P-glycerate kinase	2736 ± 497	2355 ± 554[a]	2329 ± 415[a]	4171 ± 678	3675 ± 335[a]	3975 ± 914[a]
Pyruvate kinase	158 ± 22	146 ± 48[a]	95 ± 22	334 ± 29	309 ± 53[a]	243 ± 32
Lactate dehydrogenase	1567 ± 127	1143 ± 189	1313 ± 256[a]	2531 ± 108	2332 ± 659[a]	2261 ± 434[a]

Mean values of 6 experiments ± SD. The activities are given as µmoles substrate transformed/h/ml packed red cells. [a] Differences with respect to the sonicated cells are not significant ($P > 0.5$).

Table II. Comparison between sonicated red cell glycolytic activities (Table I) and the highest literature values

	25 °C Glycolytic activity	Results by LÖHR AND WALLER[2,3]	37 °C Glycolytic activity	Results by RAPOPORT[4]
Hexokinase	8.5 ± 3.5	21 ± 2.9	20.0 ± 2.9	14
Phosphofructokinase	274 ± 49	97 ± 15	469 ± 40	205
Aldolase	51 ± 7	56 ± 4.8[a]	117 ± 11	80
Glyceraldehyde-3-P dehydrogenase	1701 ± 180	1083 ± 160	3023 ± 648	1800
3-P-glycerate kinase	2736 ± 497	1580 ± 247	4171 ± 678	2250
Pyruvate kinase	158 ± 22	179 ± 32[a]	334 ± 29	360
Lactate dehydrogenase	1567 ± 127	932 ± 104	2531 ± 108	2050

Mean values ± SD. The activities are given as µmoles substrate transformed/h/ml packed red cells. [a] Differences are not significant ($P > 0.5$).

thawing hemolysis gave the same range of values as sonication, except in the case of hexokinase at both temperatures and of lactate dehydrogenase at 25 °C. Comparison of our results with the highest activities found in the literature[1–5] shows that sonication accompanied by modification of the test composition leads to better yields in the case of many glycolytic activities. As shown in Table II, phosphofructokinase, glyceraldehyde-3-P dehydrogenase, 3-P-glycerate kinase and lactate dehydrogenase tested at 25 °C were significantly higher than reported by LÖHR and WALLER[2,3]. A statistical assessment of the activities at 37 °C was not possible, because the corresponding RAPOPORT[4] data do not contain standard deviation and number of sample values. However, our activities were higher by 40% or more in the case of hexokinase, phosphofructokinase, aldolase, glyceraldehyde-3-P dehydrogenase and 3-P-glycerate kinase[8].

Zusammenfassung. Die Aktivitäten von 7 glykolytischen Enzymen wurden in normalen menschlichen Erythrozyten gemessen. Kurze Ultraschall-Hämolyse und modifizierte Test-Bedingungen führten, verglichen mit den höchsten Werten, zu signifikant höheren Aktivitäten.

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Specificity of Potato Kallikrein Inhibitors for Kallikreins

Potato kallikrein inhibitor has been initially discovered by WERLE et al.[1] and partially purified on this material[2,3]. However, details have never been elucidated on the intrinsic material, much remaining obscure. In the present paper, inhibitors, acting specifically on human plasma kallikrein, were discovered in potatoes and studied as to their purification and properties, including their inhibitory specificity against many kinds of kallikreins or protease enzymes. Our final purpose was to clarify the pathological roles or meanings of kinin liberation in blood by means of development of such specific inhibitors.

Sixteen kinds of Japanese and 22 foreign potatoes were examined to screen their contents. Potato kallikrein inhibitors (PKI) were found to contain 60–190 kallikrein inhibitor units (KIU) per g in various kinds of potatoes. The Japanese potato '*Danshaku-Imo*' was mainly used for our study and contained about 140 KIU per 1 g of this fresh potato. Purification of PKI was performed by the combination of salting out with ammonium sulphate, dialysis against water, DEAE-cellulose treatment, chromatography on columns of CM-cellulose, CM-Sephadex and hydroxyapatite, and the 'Ampholine' electrofocusing method[4]. Two kinds of inhibitors were found in potatoes and their isoelectric points were pH 5.6 and pH 6.4. Both were isolated in homogenous form, checked by disc-electrophoresis and ultracentrifugation. Molecular weights of these inhibitors (pI 5.6 and pI 6.4) were measured as follows:

Inhibitors	Ultra-centrifugation	Gel-filtration (Sephadex G-100)	Calculation from amino acid analysis
pI 5.6	24,200	25,000	23,388
pI 6.4	28,700	26,000	22,814

Both were readily soluble in neutral saline solution and unstable on heating.

Each 2 inhibitors were pre-incubated with various proteases and the inhibitory activities at less than 50% inhibition were measured on the dog vasodilator[5], esterolytic[6,7], caseinolytic[8] and fibrinolytic[9] activities (Table) and compared with that of Trasylol (bovine lung kallikrein – trypsin – inhibitor). Purified PKI inhibited human plasma kallikrein (activated with acetone) strongly, hog pancreatic kallikrein and human plasmin (streptokinase activated euglobulin) only slightly and bovine trypsin and α-chymotrypsin also slightly, while Trasylol has broadly strong actions for hog pancreatic kallikrein, human plasmin and bovine trypsin, and slight action for human

Inhibitory effects of potato kallikrein inhibitors and trasylol

Enzymes	Method or substrate	PKI pI 5.6	PKI pI 6.4	Trasylol
Human plasma kallikrein	dog assay[5]	24,800	24,000	9,700
Hog pancreatic kallikrein	dog assay[5]	3,680	9,000	46,000
	BAEE[6]	208	313	12,025
Trypsin	casein[8]	11	11	51
	BAEE[6]	240	280	400
α-Chymotrypsin	casein[8]	25	40	47
	BTEE[7]	140	110	142
Human plasmin	fibrinolysis[9]	229[a]	246[a]	43,100[a]

Numbers are inhibited units per μmole of inhibitors, Frey units by dog assay, Kunitz's units by caseinolysis and μmoles of substrate (BAEE, BTEE) hydrolyzed per min. Inhibitory units per μmole of inhibitors are expressed as based on the molecular weights of PKI, 25,000 (assumed), and trasylol, 6500. [a] Inhibited human euglobulin equivalent to the original plasma volume (ml/μmole of inhibitors). BAEE, N^{α}-benzoyl-L-arginine ethylester; BTEE, benzoyl-L-tyrosine ethylester.

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