Mean values of fibrinolytic components in human seminal plasma (percent of the content of a normal standard consisting of pooled serum from 20 healthy persons)

	No. of samples	Mean ± SD
Fibrinolytic activity on unheated fibrin plates (mm^2)	51	1016 ± 765
Fibrinolytic activity on heated fibrin plates (mm^2)	51	0
Plasminogen (%)	20	traces
Urokinase inhibitors (%)	42	25 ± 10
α_2-macroglobulin (%)	30	15 ± 8
Antiplasmin (%)	20	traces
α_1-antitrypsin (%)	18	1.3 ± 0.8
Total antitrypsin activity (%)	38	8 ± 13
Fibrin/fibrinogen degradation products (µg/ml)	51	0

ent in only low concentrations. The fibrinolytic activity on unheated fibrin plates was high, and no activity was found on heated plates, in agreement with earlier observations[3,4,25]. Hence, the high fibrinolytic activity of human seminal plasma seems to be due to the presence of large amounts of fibrinolytic activators in association with only a low concentration of fibrinolytic inhibitors.

Zusammenfassung. Die menschliche Samenflüssigkeit wurde auf ihre fibrinolytische Aktivität und auf ihre Konzentration von Inhibitoren der Plasminogenaktivierung (Urokinaseinhibitoren), Antiplasmin, α_2-Macroglobulin, α_1-Antitrypsin, totale Antitrypsinaktivität sowie Plasminogen und Antithrombin-III untersucht. Dabei wurden niedrige Konzentrationen der Inhibitoren, die auf die hohe fibrinolytische Aktivität des Seminalplasmas nur eine geringe Hemmwirkung ausüben können, gefunden.

P. LIEDHOLM and B. ÅSTEDT[26]

Coagulation Laboratory and Department of Obstetrics and Gynecology, University of Lund, Allmänna Sjukhuset, S-21401 Malmö (Sweden), 6 June 1974.

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A Carbohydrate Inhibitor of Cathepsin B Activity Associated with Haptoglobin

Human plasma contains at least 6 different well characterized proteinase inhibitors, recently reviewed by HEIMBURGER, HAUPT and SCHWICK[1]. They called attention to the high content of carbohydrate residues of these inhibitors, mainly hexoses, acetylhexosamines and sialic acid, and they further stressed the unknown role of these components as far as enzymic inhibition was concerned. Turning to the lysosomal cathepsin B1 we reported in 1967 that haptoglobin purified from Cohn's fraction IV-4 showed a marked inhibition of cathepsin B activity[2]. The question was already at that time left open if this was due to haptoglobin as such or an associated component. Data presented below suggest that haptoglobin preparations seem to carry a glucosamine-containing saccharide which has all the proteinase inhibitory activity found in haptoglobin.

Haptoglobin was prepared from clear yellow ascites fluid sampled from mice carrying the Ehrlich-Landschütz tumor (15–20 days after i.p. inoculation of 2×10^6 cells) by means of an acetone precipitation method followed by column chromatography on Sephadex G-200, equilibrated with 0.05 *M* phosphate buffer pH 7.0 and with 0.1 *M* NaCl added. The absorbance at 280 nm was measured on the eluate as well as the inhibitory effect on samples of pure cathepsin B1. The inhibition assays were performed according to the same system as used for activity measurements of the enzyme[3]. The substrate was benzoylarginine-2-naphthylamide (BANA)/ 0.4 mg/ml / in a 0.05 *M* phosphate buffer pH 6.0 containg 5 m*M* EDTA + 4 m*M* cysteine HCl for activation. Incubations were performed for 30 min at 40 °C. The reaction was stopped by added TCA and the released naphtylamine was diazotized and assayed at E_{560} as usual.

The dissociation of the cathepsin B inhibitor from the haptoglobin preparation was shown in the following way. Starting with a purified haptoglobin solution, the total inhibiting effect contained in the solution was not changed following the addition of an excess of haemoglobin. However, when this solution was analyzed on a Sephadex G-200 column at pH 6.0 the well-defined haptoglobin peak had disappeared. A new component appeared near the elution front (Figure, tube No. 16–22) representing the haemoglobin-haptoglobin complex, and this was lacking all cathepsin inhibitor activity. All the inhibitor activity previously associated with the original haptoglobin moiety, appeared instead later at the end of the elution (Figure, tube No. 30–40); This new component

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had no absorbance at 280 nm and was completely free from haptoglobin.

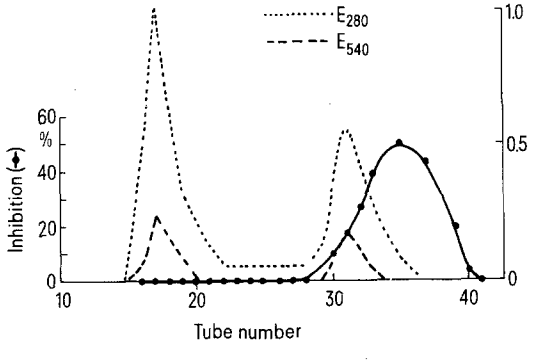
In a second set of experiments, it was more convenient to add haemoglobin dissolved at pH 6.0 to an equal amount of dissolved haptoglobin and then precipitate the complex with an equal volume of acetone. The liberated inhibitor left in the supernatant could then be further isolated on a Dowex column using graded ethanol concentrations. The inhibitor appeared in the solution between 50–60% ethanol. The yield was about 90%, meaning that from a haptoglobin solution containing 100 arbitrary inhibitor units about 90 units were recovered. The inhibitor solution could be further purified by running it through a Biogel P2 column; all the inhibitor activity emerged at a zone corresponding to a molecular weight of near 900.

The inhibitor could also be liberated from the haptoglobin moiety by treatment at a low pH of about 1.5 (0.2 *M* H_2SO_4 added).

Inhibition tests using a standard amount (0.250 mg/ml) of cathepsin B1 and increasing amounts of added carbohydrate inhibitor

Amount of carbohydrate inhibitor (mg)	Inhibition of enzyme activity (%)
0.023	15
0.040	28
0.065	45
0.083	55
0.100	60
0.140	65

Conditions: 0.05 *M* phosphate buffer pH 6.0, BANA used as a substrate, incubations at 40 °C and 1 h. Cysteine and EDTA added for activation.



Gel filtration of a mixture of haptoglobin and haemoglobin on a Sephadex G-200 column equilibrated with 0.1 *M* NaCl and 0.05 *M* phosphate buffer pH 6.0. Protein absorption measured at 280 nm and haemoglobin at 540 nm. The 1st peak (left) corresponds to the haptoglobin-haemoglobin complex. The 2nd peak represents excess of haemoglobin. The black squares denote the distribution of inhibitory activity of cathepsin B1 measured against BANA as a substrate (cf. text). It should be noticed that the haptoglobin-haemoglobin complex was lacking inhibitory activity while all this activity appeared in a liberated form (3rd peak) in tubes No. 30 to 40.

The inhibitor material was hydrolyzed in sealed ampoules with 2 *M* HCl for 12 h at 105°C. After evaporation twice, the hydrolysis products were dissolved in water and subjected to thin-layer chromatography on cellulose powder MN 300 plates according to LAMKIN et al.[4]. Chromatography according to GAL[5] was also performed, using Silica gel G plates. Following suitable development, only 3 components could be resolved namely fucose, galactose and glucosamine. Plates sprayed with ninhydrine showed only glucosamine but no amino acids. Mannose was absent.

Using this purified non-hydrolyzed carbohydrate compound it was shown that the inhibition of cathepsin B1 activity was pH dependent; 50% inhibition was reached at pH 6.3 and 100% at pH 7.0 (cf. curve for haptoglobin inhibition in ref.[6]). The quantitative degree of inhibition with increasing amounts of inhibitor is shown in the Table. The biological effect was not limited to cathepsin B1 only. Papain and trypsin were also partially inhibited to about 50% suggesting a broad specificity of the inhibitor acting both on a cysteine and a serine proteinase. The specificity of these interactions need further study. Impurities were almost certainly ruled out since very small amounts of inhibitor were effective (Table). Only a metal contamination could be considered, but in the tests involved EDTA was present.

Thus, the important point is that the proteinase inhibition ascribed to haptoglobin as such[2] seems to be due to an associated carbohydrate moiety of low molecular weight, which is relatively firmly attached to haptoglobin and perhaps also to other serum proteins, like the α_2-macroglobulin recently claimed by STARKEY and BARRETT[7] to be the only cathepsin B inhibitor in blood plasma. This carbohydrate compound could be attached to and carried by different proteins without constituting an essential structural part of such proteins. The results appear to open up new lines of study as to the biological interaction and regulation of proteinase activity.

Zusammenfassung. Die Natur von Proteinase-Inhibitoren, die mit gewissen Plasmaproteinen assoziiert sind, wurde untersucht und gezeigt, dass die hemmende Wirkung des Haptoglobins, isoliert aus Tumor Ascitesflüssigkeit, auf Cathepsin B1 auf einer Kohlehydrat-Komponente des Haptoglobins beruht.

O. SNELLMAN and B. SYLVÉN[8]

The Cancer Research Division of Radiumhemmet, Karolinska Institute, Stockholm 60 (Sweden), 11 April 1974

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