Active, Soluble Product Obtained by Enzymatic Hydrolysis of Plasminogen.* (28538)

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Plasminogen, when purified by methods which involve exposure to acid, becomes insoluble within the pH range 3.5 to 8.5. Treatment of such plasminogen with a commercial carboxypeptidase (CP) preparation under conditions described in this paper has resulted in partial hydrolysis of the molecule. Partially hydrolyzed plasminogen (PHP) has been isolated as a non-dialyzable protein, soluble at pH 7.4 to at least 0.5% concentration, possessing caseinolytic, fibrinolytic and esterolytic activity comparable to the untreated plasminogen.

Not all CP preparations were effective, and it was found that plasminogen was hydrolyzed and solubilized by an effective CP preparation after acid destruction of all detectable CP activity. The contaminant responsible for the partial hydrolysis of plasminogen may be α-chymotrypsin, since this enzyme produced a soluble product identical with that isolated after CP treatment as measured by nitrogen analyses and ultracentrifugal studies.

Materials and methods. Plasminogen was purified from Fraction III according to our published procedure(1). The preparations varied from 90 to 135 Remmert & Cohen caseinolytic units(2) per mg N. The Fraction III was released for use by Dr. James Pert of American National Red Cross and supplied through the courtesy of E. R. Squibb & Sons. Fibrinogen (Bovine Fraction I) was purchased from Armour & Co., Chicago, Ill. Carboxypeptidase 5 × recrystallized, α-chymotrypsin 3 × recrystallized, and lysine methyl ester monohydrochloride were obtained from Mann Research Laboratories, New York. Caseinolytic, lysine esterase and fibrinolytic assays were performed as previously described(3).

Procedure. The plasminogen solutions contained about 25 caseinolytic units per ml suspended in 0.075 M sodium phosphate, pH 7.4. A minimum of 6.4 μg of CP per casein unit of plasminogen was required (Table I). Additional CP did not increase the yield. The required volume of CP suspension was added to the plasminogen solution, and the reaction mixture was kept at 4° for 17 hours. Stirring during this time did not alter the results. The suspension was then centrifuged for 10 minutes. The clear supernatant solution was decanted and adjusted to pH 3.0 with N HCl. After 10 minutes at room temperature to destroy CP activity, the solution was readjusted to pH 7.0 with N NaOH, and a small precipitate was removed by recentrifugation. The water-clear solution obtained was kept frozen or was freeze-dried after dialysis against water.

The procedure used to prepare PHP by treatment of plasminogen with α-chymotrypsin was the same as that outlined above except that 1.0 μg of chymotrypsin per casein unit of plasminogen was required (Table I). After overnight incubation at 4° and centrifugation, the solution was adjusted to pH 2.0 and solid NaCl was added to make the solution 1.5 M. Soluble plasminogen precipitated

TABLE I. Partial Hydrolysis and Solubilization of Plasminogen.

Pl'gn treated (cas.u/ml)	CP (γ/cas.u)	CT (γ/cas.u)	% yield of PHP
24	1.10		10
24	2.12		18
24	4.25		22
24	6.40		34
24	8.50		34
24	11.00		34
25		.06	24
25		.12	17
25		.36	18
25		.50	28
25		1.00	32
25		2.00	31
25		4.00	26

Abbreviations: pl'gn, plasminogen; CP, carboxypeptidase; CT, α-chymotrypsin; PHP, partially hydrolyzed plasminogen.

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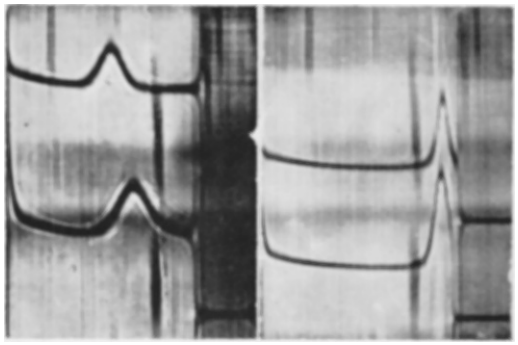

FIG. 1. Sedimentation patterns of insoluble plasminogen (upper) and soluble plasminogen (lower). Concentration of protein, 0.3% insoluble and 0.4% soluble plasminogen; buffer-0.10 M acetate, pH 3.0. Photographs were taken 8 and 72 minutes after rotor reached speed of 59.780 r.p.m. in a Spinco model E ultracentrifuge. Direction of sedimentation is to the left. This run was kindly carried out by Dr. K. Munkres, Dept. of Biochemistry.

quantitatively, free of detectable CP activity.

Results. The yields obtained in more than 25 experiments constituted from 32 to 50% of the original activity. Approximately ¼ to ⅓ of the starting activity remained in the insoluble residue even with longer treatment and higher concentrations of hydrolyzing enzyme, and a similar amount disappeared during the experiments. The PHP isolated approached and sometimes equaled the starting material in specific activity and was free of CP activity as measured by casein hydrolysis in the absence of streptokinase.

Optical density readings of trichloroacetic acid filtrates at 280 mμ indicated that approximately 10% of the plasminogen originally present became TCA soluble. Since only ⅓ to ½ of the original plasminogen was solubilized, the degraded molecule may be smaller than the original by as much as 30%. Microkjeldahl and biuret analyses of lyophilized, moisture-free and ashless PHP in 3 experiments gave values of 13.7, 13.7 and 13.9% N. The insoluble plasminogen from which the PHP was made contained 15.9% N. This reduction in N content suggests that the carbohydrate portion of plasminogen was not lost during exposure to CP or chymotrypsin.

Ultracentrifuge patterns of insoluble plasminogen 3 mg/ml, and the soluble material obtained by "active" carboxypeptidase treatment of the same preparation 4 mg/ml, dissolved in the same buffer, were obtained in a single run with a wedge cell (Fig. 1). Both materials appear to be homogeneous at pH 3.0. Insoluble plasminogen (upper curve) sedimented more rapidly, $s = 4.34$, than soluble plasminogen (lower curve), $s = 2.88$. Diffusion, shown by the spreading of the curves was greater for soluble plasminogen as anticipated for a smaller molecule. PHP isolated after α-chymotrypsin treatment of plasminogen behaved identically in ultracentrifuge studies and contained the same percentage of N as did PHP prepared by "active" CP hydrolysis.

Spontaneous plasmin activity was not diminished by this procedure and may have been slightly increased. PHP hydrolyzed lysine methyl ester and had an activity on fibrin plates slightly greater than insoluble plasminogen of equal caseinolytic potency (Table II). The highly specific reaction: plasminogen (plasmin) + streptokinase → SK-activator(3) was shown to occur with PHP by fibrinolytic as well as lysine methyl esterase assays (Table II).

Discussion. Although α-chymotrypsin was effective in producing soluble plasminogen, it is possible that the contaminant in CP which also produced this partial degradation, is not identical with chymotrypsin since 1 μg of the latter and only 6.4 μg of CP per casein unit of plasminogen were required for maximal effect. This would necessitate that the 5 × recrystallized preparation of CP contained 16% chymotrypsin, an unlikely amount. Furthermore, after acid destruction of the CP, which did not diminish the ability of the preparation to hydrolyze plasminogen, the residual activity against casein was barely detectable. On the other hand, the quantity of chymotrypsin needed to hydrolyze plas-

TABLE II. Relative Activity of Insoluble and Soluble Plasminogen Against Various Substrates.

Activity	Assay	Ratio of activity	
		insol pl'gn	soluble pl'gn
Plasmin	casein	1.0	1.0
"	LME	1.0	1.6
Activator	"	1.0	1.1
"	clot	1.0	1.2
"	fibrin plate (area lysed)	1.0	1.4

minogen, attacked casein at an easily measureable rate. The fact that the hydrolysis had to be carried out at 4° (37° did not produce an active product) suggests that the intermediate chymotryptic activity described by Keller, Cohen and Neurath(4) which appeared during the conversion of procarboxypeptidase to CP at 4° may have been involved. The complexity of the activation of procarboxypeptidase and the varieties of both CP and chymotrypsin which have been described, preclude an answer at this time.

The evidence suggesting that PHP is a partially hydrolyzed product of plasminogen is (1) PHP was soluble at neutral pH. In a control experiment in which plasminogen was repeatedly extracted with phosphate buffer and the extracts pooled and lyophilized, the product obtained was only sparsely soluble at pH 7.4, (2) 10% or more of the plasminogen molecule became TCA soluble during treatment with CP or α-chymotrypsin, (3) the isolated PHP contained 13.8% N as compared with 15.9% found in the plasminogen from which it was derived, (4) in the ultracentrifuge, PHP was homogeneous at pH 3.0 and traveled slower than the equally homogeneous plasminogen from which it had been prepared. Greater diffusion during the ultracentrifuge run indicating a smaller molecule, was also noted for PHP.

Summary. Insoluble plasminogen when treated with a certain carboxypeptidase preparation after destruction of CP activity, or with α-chymotrypsin, produced a soluble, active material with all the biochemical activities of the original substance. The identity of the hydrolyzing enzyme has not been established. Evidence is presented that the product obtained is partially hydrolyzed plasminogen (PHP).

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Transport of Glycerol in Human Blood* (28539)

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The release of free fatty acids (FFA) from adipose tissue *in vitro* is accompanied by release of glycerol(1). Recent reports of increased plasma levels of glycerol as well as FFA after injection of epinephrine(2) and norepinephrine(3), which increase the rate of hydrolysis of triglycerides in adipose tissue(4), can be considered evidence that such release occurs *in vivo*. The sites of removal of glycerol from the blood have not been studied under physiological conditions. The high activity of glycerokinase in liver and kidney(5,6) suggests that these organs may be important sites of removal. We studied the concentration of glycerol in human blood in various regions. Our observations show that glycerol is released from the area drained by the saphenous vein and is taken up in the splanchnic region and kidney.

Material and methods. We studied 16 women and 14 men, aged 10 to 62 years (mean age 38) during cardiac catheterization. They were not acutely ill and had neither anemia nor polycythemia. They were catheterized in the morning after fasting overnight or between 1 and 2 P.M. after a light breakfast of toast and coffee about 8 A.M. They received no sedatives, nor was heparin injected before the blood samples were withdrawn.

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