PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 297–299 (1981)

Inhibition of Ellagic Acid-Activated Hageman Factor (Factor XII) and Hageman Factor Fragments by Popcorn Inhibitor[1] (41063)

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Abstract. An inhibitor derived from popcorn prolonged the partial thromboplastin time of normal human plasma. The effect of this agent was localized to its inhibition of activated Hageman factor (factor XII), as demonstrated in amidolytic assays employing ellagic acid-activated Hageman factor and the carboxy-terminal fragment of Hageman factor (HF_f).

Swartz *et al.* (1) have described an agent derived from opaque-2-corn that inhibits trypsin. An immunologically identical inhibitor, isolated from fresh sweet corn, also inhibits the enzymatically active carboxy-terminal fragment of Hageman factor (HF, factor XII), but neither kallikrein nor α-thrombin (2, 3). The present study demonstrates additionally that this or a related agent derived from popcorn inhibits ellagic acid-activated HF, but does not appear to interfere either with the activation of HF by glass nor other steps of the intrinsic or extrinsic pathways of clotting.

Materials and Methods. Popcorn inhibitor, the gift of Dr. Y. Hojima, National Institutes of Health, was prepared by essentially the same method that was used to isolate the inhibitor derived from fresh sweet corn (3). In essence, an aqueous extract of ground popcorns was subjected to diethylaminoethyl-cellulose chromatography. The active fraction was then precipitated with acetone, rechromatographed on the same gel, filtered through a column of Sephadex G-50, and lyophilized. The preparation was about 50% pure and appeared to have multiple inhibitory components. The inhibitor was dissolved at a concentration of 1 mg/ml buffer and stored at −20° until used.

Human plasma of normal subjects and of a patient deficient in HF was separated from venous blood to which 1/50th volume of 0.5 *M* sodium citrate buffer (pH 5.0) had been added (4). Citrated bovine PTA-deficient plasma was the gift of Dr. Gary Kociba, Columbus, Ohio. A pool of 24 normal adult male plasmas was prepared and stored as reported earlier (4).

Ellagic acid-activated HF (HF_{ea}) (5) and the carboxy-terminal fragment (HF_f) separated by tryptic digestion (6) were prepared from purified human HF (46–113 u/mg) (6, 7).

Activated plasma thromboplastin antecedent (PTA, factor XI), prepared by tryptic activation, was derived from purified human PTA (200 u/mg protein) (8).

One unit of HF or PTA was that amount found in 1 ml of pooled normal human plasma.

Russell's viper venom (Sigma Chemical Co., St. Louis, Mo.) was dissolved in buffer at a concentration of 0.1 mg/ml. Hexadimethrine bromide (Aldrich Chem. Co., Milwaukee, Wisc.) was dissolved in buffer at a concentration of 1 mg/ml of buffer. Protamine sulfate (10 mg/ml of 0.9% sodium chloride, Eli Lilly and Co., Ind.) was diluted to 1 mg/ml with buffer. Bovine serum albumin (Pentex, crystallized, Miles Laboratories, Inc., Elkhart, Ind.) was dissolved at a concentration of 1.0% buffer.

Unless otherwise noted, buffer was 0.025 *M* sodium barbital in 0.125 *M* sodium chloride (pH 7.5).

[1] This study was supported in part by Grant HL 01661 from the National Heart, Lung, and Blood Institute, the National Institutes of Health, U.S. Public Health Service, and in part by grants from the American Heart Association and its Northeast Ohio affiliate.

[2] Career Investigator of the American Heart Association.

0037-9727/81/020297-03$01.00/0

The prothrombin time (9), thrombin time (10), and partial thromboplastin time (10), the last modified by replacing crude "cephalin" with a suspension of 10 mg kaolin (acid-washed, American standard, Fisher Scientific Co., Fair Lawn, N.J.) per milliliter of 0.1% crude soybean phosphatides (Centrolex "P," the gift of Central Soya Co., Fort Wayne, Inc.) in 0.15 *M* sodium chloride, were measured as described earlier in 10 × 75 mm disposable glass tubes. The Russell's viper venom time was measured by substituting 0.1 ml of venom (0.1 mg/ml) for tissue thromboplastin in assays of the prothrombin time. In each case, the effect of popcorn inhibitor was tested by incubating 0.1 ml normal human plasma with 0.1 ml popcorn inhibitor (1 mg/ml) or buffer for 1 min at 37°, after which the appropriate agents were added.

The effect of popcorn inhibitor upon activated PTA was determined by incubating 0.01 ml activated PTA and 0.09 ml popcorn inhibitor (1 mg/ml buffer) or buffer for 10 min at 37° in 10 × 75-mm disposable glass tubes. Thereafter, 0.1 ml PTA-deficient plasma and 0.1 ml Centrolex P (0.1% in 0.15 *M* sodium chloride) were added and incubation was continued for 1 min. Prewarmed 0.025 *M* calcium chloride, 0.1 ml, was then added and the partial thromboplastin time measured by continual testing.

The effect of hexadimethrine bromide (1 mg/ml), protamine sulfate (1 mg/ml), and popcorn inhibitor (1 mg/ml) on surface activation of HF was tested by swirling 0.1 ml of each agent onto the surface of 10 × 75-mm disposable glass tubes. The tubes were then rinsed 20× with 1 ml buffer and 5× with distilled water, and allowed to dry at room temperature. The partial thromboplastin time of normal plasma was then measured in these tubes in comparison to that obtained in tubes similarly prepared with the omission of the three agents tested.

The effect of corn inhibitor on amidolysis of H-D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide (S2238, Kabi Group, Inc., Greenwich, Conn.) was tested by a previously reported technique (12). In brief, 0.15 ml HF_{ea} or HF_f (both in 0.05% bovine albumin in buffer) was incubated at 37° for 10 min in 12 × 75-mm polystyrene tubes with 0.10 ml popcorn inhibitor (serially diluted in buffer) or buffer. Thereafter, 1.0 ml of an equal mixture of 1 m*M* S2238 in water and Tris–imidazole–saline buffer (pH 8.2), prewarmed to 37° was added. Incubation was continued for 60 min, and then halted by addition of 0.3 ml glacial acetic acid. The *p*-nitroaniline released was estimated at 405 nm against blanks of similarly treated mixtures to which glacial acetic acid had been added before addition of S2238, in comparison to solutions of *p*-nitroaniline.

TABLE I. THE EFFECT OF POPCORN INHIBITOR UPON THE COAGULANT ACTION OF ACTIVATED PTA

Agent tested	Clotting time (sec)
Buffer	>300
Activated PTA + buffer	84.4
Activated PTA + popcorn inhibitor	74.8

Note. 0.01 ml activated PTA and 0.09 ml buffer or popcorn inhibitor (1.0 mg/ml) or 0.1 ml buffer was incubated in 10 × 75-mm polystyrene tubes for 10 min at 37°. Thereafter 0.1 ml bovine PTA-deficient plasma and 0.1 ml Centrolex "P" were added. After 1 min, 0.1 ml prewarmed 0.025 *M* calcium chloride was added and the clotting time was measured.

Results. Popcorn inhibitor, at a concentration of 0.25 mg/ml in the final mixture, did not alter the thrombin time, prothrombin time, or Russell's venom time of normal human plasma. In contrast, the kaolin-activated partial thromboplastin time was increased from 60.2 sec in the presence of buffer to 137.1 sec.

The effect of popcorn inhibitor was localized to a step before the participation of activated PTA. The addition of trypsin-treated PTA shortened the partial throm-

TABLE II. THE EFFECT OF PROTAMINE SULFATE, HEXADIMETHRINE BROMIDE, AND POPCORN INHIBITOR UPON THE CLOT-PROMOTING PROPERTIES OF GLASS

Agent tested	Clotting time (sec)
Buffer	96.0
Protamine sulfate	305.9
Hexadimethrine bromide	>400
Popcorn inhibitor	94.1

Note. 0.1 ml of each agent tested (1.0 mg/ml) or buffer was swirled onto the surface of 10 × 75-mm glass tubes, which were then rinsed 20× with buffer and 5× with distilled water and air dried. The partial thromboplastin time of normal plasma was measured in duplicate in each tube, using 0.1 ml Centrolex P instead of kaolin–Centrolex.

TABLE III. THE EFFECT OF POPCORN INHIBITOR UPON AMIDOLYSIS OF S2238 BY HF_{ea} AND HF_f

Inhibitor concentration (μg/ml)	*p*-NA-Released	
	HF_{ea} (nmole/ml/hr)	HF_f (nmole/ml/hr)
1	3.3	4.1
0.5	7.0	7.5
0.25	10.4	12.1
0	18.3	17.2

Note. 0.15 ml HF_{ea} (0.04 μg *activated* HF/ml) or HF_f (0.5 μg/ml) was incubated at 37° for 10 min with 0.1 ml popcorn inhibitor in barbital–saline buffer, or buffer alone. Thereafter 1.0 ml 0.5 m*M* S2238 was added and incubation was continued for 60 min, and the amount of *p*-nitroaniline (*p*-NA) released was measured as described under Materials and Methods. The concentration of popcorn inhibitor is that in the final enzyme–substrate mixture.

boplastin time of bovine PTA-deficient plasma, an effect not inhibited by popcorn inhibitor at a concentration in the final mixture of 0.23 mg/ml (Table I).

When glass was exposed to protamine sulfate or hexadimethrine bromide solutions and then thoroughly washed, it lost its ability to induce surface-mediated activation of clotting (Table II). In contrast, glass exposed to popcorn inhibitor was unaffected by such treatment.

Amidolysis by both HF_{ea} and HF_f was strongly inhibited to the same degree by popcorn inhibitor at approximately the same concentration of each enzyme (Table III).

Discussion. The experiments described demonstrate that a protein derived from popcorn specifically inhibits two forms of activated Hageman factor (HF). Suitable experiments localized its clot-inhibitory properties to a step in the intrinsic pathway before the participation of activated PTA. Unlike protamine sulfate or hexadimethrine bromide, popcorn inhibitor did not reduce the clot-promoting properties of glass, as though it did not block the activation of HF by negatively charged surfaces. But, at a concentration of approximately 0.3–0.4 μg/ml in the final enzyme–substrate mixture, it inhibited amidolysis of H-D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide (S2238) by half.

An inhibitor of trypsin derived from corn was described by Swartz (1). A similar or identical agent derived from sweet corn does not inhibit human plasma kallikrein, urinary kallikrein, hog pancreatic kallikrein, plasmin, α-thrombin, or bovine α-chymotrypsin, but blocks the amidolytic properties of HF_f for H-D-prolyl-L-phenylalanyl-L-arginine *p*-nitroanilide (S2302) (2).

HF_{ea} and HF_f differ grossly in their V_{max}, as tested upon S2238 (13), but when preparations of approximately the same enzymatic activity were tested, inhibition of amidolytic activity by popcorn inhibitor was approximately equal. The significance of the current studies resides in the apparently specific behavior of popcorn inhibitor towards activated forms of HF, a potentially most useful tool.

This study would not have been possible without the expert help of Ms. Marilyn Vavrek and Ms. Barbara Everson.

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Received July 18, 1980. P.S.E.B.M. 1981, Vol. 166.