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# Pumpkin Seed Inhibitor of Human Factor $XII_a$ (Activated Hageman Factor) and Bovine Trypsin†

Yoshio Hojima,‡ Jack V. Pierce, and John J. Pisano*

ABSTRACT: A strong inhibitor of human Hageman factor fragment ($HF_f$, β-factor $XII_a$) and bovine trypsin was isolated from pumpkin (*Cucurbita maxima*) seed extracts by acetone fractionation, by chromatography on columns of diethylaminoethylcellulose and carboxylmethyl-Sephadex C-25, and by Sephadex G-50 gel filtration. Pumpkin seed Hageman factor inhibitor (PHFI) is unusual in its lack of inhibition of several other serine proteinases tested—human plasma, human urinary, and porcine pancreatic kallikreins, human α-thrombin, and bovine α-chymotrypsin. Human plasmin and bovine factor $X_a$ are only weakly inhibited. PHFI also inhibits the $HF_f$-dependent activation of plasma prekallikrein and clotting of plasma. Other properties of PHFI are a p*I* of 8.3, 29 amino acid residues, amino-terminal arginine, carboxyl-terminal glycine, 3 cystine residues, undetectable sulfhydryl groups and carbohydrate, and arginine at the reactive site. The minimum molecular weight of PHFI is 3268 by amino acid analysis. PHFI may be the smallest protein inhibitor of trypsin known.

In the course of screening plant materials for serine proteinase inhibitors, especially for inhibitors of Hageman factor fragment ($HF_f$, β-factor $XII_a$)[1] and the kallikreins, we found that corn extracts contain a highly specific $HF_f$ inhibitor (Hojima et al., 1980a). Certain other seeds and especially flower bulbs are extraordinarily rich sources of inhibitors with a variety of specificities (Hojima et al., 1980b). Of these, pumpkin seed extracts are noteworthy because they contain an unusually low molecular weight trypsin inhibitor that strongly inhibits $HF_f$ but does not inhibit plasma kallikrein. We now report the

† From the Section on Physiological Chemistry, Laboratory of Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received June 22, 1981; revised manuscript received October 6, 1981.*

‡ Present address: Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

[1] Abbreviations: $HF_f$, Hageman factor fragment (β-factor $XII_a$); PHFI, pumpkin $HF_f$ inhibitor; PCPI, potato carboxypeptidase inhibitor; PTI, basic pancreatic trypsin inhibitor (Kunitz); BSA, bovine serum albumin; *p*NA, *p*-nitroanilide; Bz, benzoyl; Pip, L-pipecolyl; Tos, tosyl; OMe, methyl ester; OEt, ethyl ester; Z, carbobenzoxy; SBzl, thiobenzyl ester; IU, inhibitor unit; $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl; DEAE, diethylaminoethyl. Common abbreviations for amino acid residues are used; all are of the L configuration unless otherwise indicated.

isolation and characterization of this interesting inhibitor.

## Materials and Methods

*Materials.* The following were obtained commercially: D-Pro-Phe-Arg-*p*NA (S-2302), Bz-Ile-Glu-Gly-Arg-*p*NA (S-2222), D-Val-Leu-Arg-*p*NA (S-2266), D-Phe-Pip-Arg-*p*NA (S-2238), and D-Val-Leu-Lys-*p*NA (S-2251) (Kabi Group Inc.); bovine TPCK-trypsin (232 Tos-Arg-OMe units/mg), $\alpha$-chymotrypsin (47 Bz-Tyr-OEt units/mg), and basic PTI (Worthington Biochemical Corp.); BSA (crystal, Reheis Chemical Co.); human plasminogen (Abbott Laboratories); bovine factor $X_a$, iodoacetic acid, dithiothreitol, and PMSF-treated carboxypeptidase A (Sigma Chemical Co.); hog pancreatic kallikrein (KZC 183, Bayer Pharmaceutical Co.); insulin B chain (oxidized) and carboxypeptidase Y (Boehringer Mannheim); bradykinin (Schwarz/Mann); Platelin Plus Activator (General Diagnostics); Sephadex G-25 and G-50 and CM-Sephadex C-25 (Pharmacia Fine Chemicals); DEAE-cellulose (DE-52, Whatman Inc.); ampholines, pH 3.5–10 and pH 7–9 (LKB Instruments, Inc.). Other reagents were the best available commercially. Active trypsin in solutions of the commercial preparation was determined by active site titration with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967).

Pumpkin seeds (*Cucurbita maxima*, commercial variety Big Max), produced at Yuba City, CA, in 1978, were purchased from Northrup King Co. $HF_f$ (23 units/mg with 0.1 mM D-Pro-Phe-Arg-*p*NA) (Hojima et al., 1980c), human plasma prekallikrein, p*I* 9.1 (96 units/mg with 0.5 mM D-Pro-Phe-Arg-*p*NA) after activation with $HF_f$, plasma kallikrein (86 units/mg with D-Pro-Phe-Arg-*p*NA), human urinary kallikrein (40 units/mg with 20 mM Tos-Arg-OMe and 30 °C), and human B4$\gamma$ high molecular weight kininogen (Pierce & Guimaraes, 1976) were prepared in our laboratory. One unit of enzyme hydrolyzes 1 $\mu$mol of substrate per minute at 25 °C and pH 8.0. Anhydrotrypsin immobilized on Affi-Gel 10 (Bio-Rad) and trypsin immobilized on Bio-Gel A-50m (Bio-Rad) were described previously (Hojima et al., 1980a). Fresh, normal ACD plasma was obtained from the Blood Bank of the National Institutes of Health. Plasma protein fraction reference no. 1 (8% protein concentration) (Alving et al., 1978), human $\alpha$-thrombin (2080 NIH thrombin units/mg), Z-Tyr-SBzl, and PCPI (Ryan et al., 1974) were kindly provided by Dr. J. S. Finlayson, the Bureau of Biologics, Dr. J. W. Fenton, II, New York State Department of Health, Drs. C. Kettner and E. Shaw, Brookhaven National Laboratory, and Dr. C. A. Ryan, Washington State University, respectively.

*Assay of Fractions for Trypsin Inhibition.* For determination of inhibition, one part of 0.2 M Tris-HCl/0.02% BSA, pH 8.0, two parts of sample, and one part of trypsin, 40 $\mu$g/mL in 1 mM HCl, were preincubated for 5 min at 25 °C. The mixture (20 $\mu$L) was then added to 0.98 mL of 0.05 M Tris-HCl/0.01 M $CaCl_2$, pH 8.0, containing 0.1 mM Bz-Ile-Glu-Gly-Arg-*p*NA. Samples were incubated for 0.5 min at 25 °C, and the corrected $\Delta A_{405nm}$ was noted.

*Assay of Fractions for $HF_f$ Inhibition.* (*A*) *Direct Assay.* Equal volumes of $HF_f$, 20 $\mu$g/mL (weight determined by the Lowry method with BSA as standard) in the Tris/BSA buffer, and sample were mixed and preincubated for 10 min at 25 °C. The mixture (40 $\mu$L) was added to 0.96 mL of 0.05 M Tris-HCl, pH 8.0, containing 0.1 mM D-Pro-Phe-Arg-*p*NA (Hojima et al., 1980c). Samples were incubated for 1 min at 25 °C and the $\Delta A_{405nm}$ was noted.

(*B*) *Indirect Assay.* This method is based on the $HF_f$-dependent activation of plasma prekallikrein. Equal volumes of $HF_f$, 0.1 $\mu$g/mL in 0.2 M Tris-HCl/0.02% BSA, pH 8.0, or plasma protein fraction reference no. 1 (adjusted to pH 8.0) and PHFI, 0.0125–1.0 $\mu$g/mL, were preincubated for 10 min, and then an equal volume of 2.3 $\mu$M prekallikrein (0.2 mg/mL in 0.02 M $NH_4OAc$, pH 5.0) was added. At 2 and 4 min, 20-$\mu$L portions were assayed for kallikrein activity with 0.5 mM D-Pro-Phe-Arg-*p*NA in 0.05 M Tris-HCl, pH 8.0, for 1 min.

*Kinetics of the Inhibition of Trypsin and $HF_f$ by Purified PHFI Using Synthetic Substrates.* (*A*) *Trypsin.* To 0.96 mL of 0.104–1.04 mM Bz-Ile-Glu-Gly-Arg-*p*NA in 0.05 M Tris-HCl/0.01 M $CaCl_2$, pH 8.0, were added 20 $\mu$L of a 2.5 or 5.0 $\mu$g/mL solution of PHFI and 20 $\mu$L of a 10 $\mu$g/mL solution of trypsin. Amidase activity was measured immediately for 0.5 min at 25 °C.

(*B*) *$HF_f$.* To 0.96 mL of 0.104–1.04 mM D-Pro-Phe-Arg-*p*NA in 0.05 M Tris-HCl, pH 8.0, were added 20 $\mu$L of a 20 or 40 $\mu$g/mL solution of PHFI and 20 $\mu$L of a 20 $\mu$g/mL solution of $HF_f$. Amidase activity was measure immediately for 1 min.

*Inhibition Assay for Other Enzymes.* All enzymes were preincubated for 10 min at pH 8.0 with PHFI. A plasma kallikrein/inhibitor mixture (40 $\mu$L), containing 10 $\mu$g of kallikrein/mL, was added to 0.96 mL of 0.05 M Tris-HCl, pH 8.0, containing 0.5 mM D-Pro-Phe-Arg-*p*NA. Another 40 $\mu$L of mixture was added to 40 $\mu$L of 10 $\mu$M kininogen in water, and kinin generation was determined with the guinea pig ileum bioassay. Other enzymes were similarly preincubated and 40-$\mu$L aliquots containing 2–25 $\mu$g of enzyme/mL were added to 0.96 mL of substrate in the Tris buffer. Plasmin, 25 $\mu$g/mL (converted from plasminogen with streptokinase), factor $X_a$, 2 $\mu$g/mL (amount determined by titration with soybean trypsin inhibitor), urinary or pancreatic kallikren, 25 $\mu$g/mL, $\alpha$-thrombin, 5 $\mu$g/mL, and $\alpha$-chymotrypsin, 5 $\mu$g/mL, were determined with 0.5 mM D-Val-Leu-Lys-*p*NA, 0.5 mM Bz-Ile-Glu-Gly-Arg-*p*NA, 0.5 mM D-Val-Leu-Arg-*p*NA, 0.5 mM D-Phe-Pip-Arg-*p*NA, and 0.1 mM Z-Tyr-Sbzl, respectively. Hydrolysis of Z-Tyr-SBzl was determined by the method described for Bz-Tyr-SBzl (Farmer & Hageman, 1975).

*Inhibitor Unit (IU).* One IU is defined as the amount of inhibitor that inhibits 1 mg of enzyme. Trypsin and $HF_f$ inhibition in the direct assays were proportional to the amount of inhibitor up to 95% and 85% inhibition, respectively.

*Measurement of Activated Partial Thromboplastin Time.* To polyethylene cups in a fibrinometer at 37 °C were added 0.05 mL of normal human plasma and 0.05 mL of 0.05 M Tris-HCl/0.15 M NaCl, pH 7.4, or PHFI solution, and 0.1 mL of Platelin Plus Activator. After 5 min, clotting was initiated by the addition of 0.1 mL of 0.025 M $CaCl_2$.

*Disc Polyacrylamide Gel Electrophoresis.* Samples in 20% sucrose were layered on top of 0.5 × 4.9 cm gels [7.5% acrylamide and 0.20 bis(acrylamide)] and electrophoresed for 2 h at 2 mA/gel and pH 8.9 (Davis, 1964) or for 1 h and 10 min at 4 mA/gel and pH 4.3 (Reisfeld et al., 1962). The gels were stained with 0.1% Coomassie brilliant blue R-250 dissolved in 10% HOAc/50% methanol/40% water and destained with 7.5% HOAc/5% methanol/87.5% water.

*Isoelectric Focusing.* Samples were focuses in a 110-mL LKB column with pH 7–9 ampholines for 39-41 h at 500 V and 25 °C.

*Estimation of Molecular Weight.* (*A*) *NaDodSO$_4$–Polyacrylamide Gel Electrophoresis.* Samples and reference peptides were dissolved in 0.01 M $H_3PO_4$/1% $NaDodSO_4$/20% glycerol adjusted to pH 6.8 with solid Tris–OH and in the

same buffer containing 5% β-mercaptoethanol. The latter set of samples was heated at 95 °C for 5 min. All the samples were layered on 0.5 × 9.0-cm gels [16.7% acrylamide and 0.33% bis(acrylamide)] in 0.1 M $H_3PO_4$-Tris/0.1% NaDod-$SO_4$, pH 6.8, and electrophoresed for 10 h at 2 mA/gel. The gels were stained as described above. The reference standards were PTI ($M_r$ 6500), PCPI ($M_r$ 4200) (Ryan et al., 1974; Hass et al., 1975), and insulin B chain ($M_r$ 3400).

*(B) Gel Filtration.* PHFI and molecular weight standards PTI, PCPI, insulin B chain, and bradykinin ($M_r$ 1060) were filtered through a Sephadex G-25 column (1.5 × 87 cm) equilibrated with 0.1 M Tris-HCl/045 M NaCl, pH 8.0, at a flow rate of 10 mL/h and 4 °C. Elution volumes were determined by appropriate inhibition assays, absorbance at 280 nm, or bioassay (bradykinin).

*Stability of Purified PHFI on Anhydrotrypsin–Agarose and Trypsin–Agarose Columns.* Samples of PHFI (0.5 mg) in 0.05 M Tris-HCl/0.02 M $CaCl_2$, pH 8.0, were adsorbed to 0.7 × 5.7 cm columns and eluted with 0.5 M HOAc. Eluates were freeze-dried as previously described for corn $HF_f$ inhibitor (Hojima et al., 1980a).

*Analytical Studies.* Samples (40 μg) of PHFI were hydrolyzed in 20 μL of distilled 6 N HCl at 110 °C in sealed, evacuated tubes for 24, 48, and 72 h. Amino acid analyses of the hydrolysates were preformed with a Beckman Model 121-M amino acid analyzer (Spackman et al., 1958). Tryptophan was determined by the method of Edelhoch (1967). PHFI was reduced and carboxymethylated by the method of Crestfield et al. (1963). The dansyl method (procedure B) of Tamura et al. (1973) was used to determine the amino-terminal residue. Dansyl amino acids were identified by the method of Woods & Wang (1967) and confirmed by a high-performance liquid chromatographic method developed in our laboratory (unpublished). The carboxyl-terminal residue was determined by incubation with carboxypeptidases A (Ambler, 1972) and Y (Hayashi, 1977). The released amino acid was identified with a Toyo Soda Model HLC-805 high-speed liquid chromatograph (Benson & Hare, 1975; Yoshida & Nakajima, 1980). The phenol–sulfuric acid method (Dubois et al., 1956) was used to test for carbohydrates, and 5,5′-dithiobis(2-nitrobenzoate) (in 0.05 M Tris-HCl, pH 7.5, containing 0.2% $NaDodSO_4$ or 6 M guanidine hydrochloride) was used to measure sulfhydryl groups (Ellman, 1959). Modification of arginine residues was performed with 1,2-cyclohexanedione (Liu et al., 1968) and modification of lysine with 2,4,6-trinitrobenzenesulfonic acid (Haynes et al., 1967).

## Results

*Purification. (Step 1) Extraction.* Pumpkin seeds, 390 g, were homogenized in a Waring Blendor with 2 L of 0.1 M Tris-HCl/0.15 M NaCl/0.1% $NaN_3$, pH 8.0, for 2 min at room temperature, stirred overnight in a cold room at 4 °C, and then centrifuged at 16000*g* for 20 min.

*(Step 2) Acetone Fractionation.* The extract (1.2 L) was fractionated with acetone at room temperature. The precipitate, which formed between 40 and 70% (v/v) acetone, was suspended in 200 mL of 0.02 M Tris-HCl, pH 7.5, stirred for 30 min, and centrifuged. The supernatant solution (205 mL) was again fractionated with acetone, and the precipitate, which formed between 50 and 80% acetone, was suspended in 30 mL of buffer, dialyzed for 5 h against 4 L of buffer in the cold room, and centrifuged.

*(Step 3) DEAE-cellulose Chromatography.* The supernatant solution from step 2 (dialyzed 80% acetone precipitate) was subjected to chromatography on a DEAE-cellulose column equilibrated at 4 °C with 0.02 M Tris-HCl, pH 7.5. The main

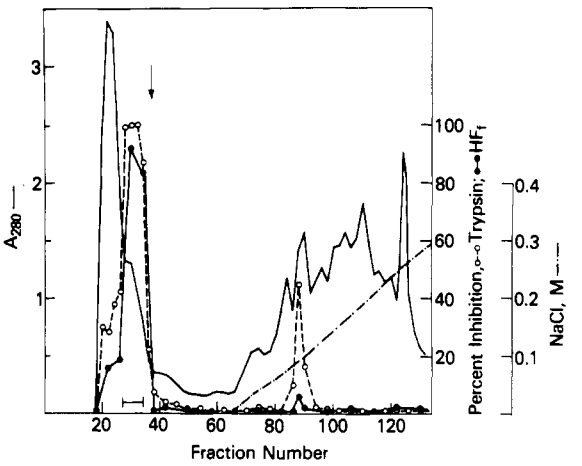


FIGURE 1: DEAE-cellulose chromatography of PHFI. Column, 2.6 × 64 cm; sample, 50 mL; eluents, 260 mL of 0.02 M Tris-HCl, pH 7.5; 1000-mL linear gradient of 0–0.5 M NaCl in buffer started at the arrow; flow rate, 50 mL/h; fraction volume, 8.4 mL; assay, fractions diluted 1:50.

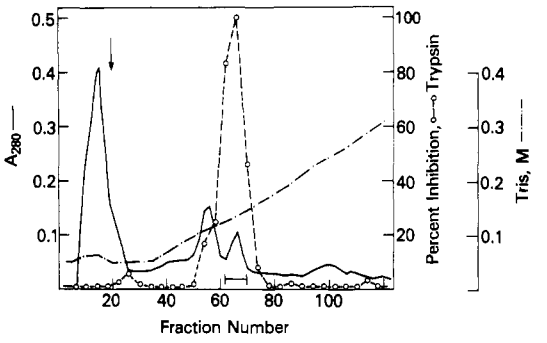


FIGURE 2: CM-Sephadex C-25 chromatography of PHFI. Column, 2.6 × 34 cm; sample, 65 mL; eluents, 120 mL of 0.05 M Tris-HCl, pH 7.5; 1000-mL linear gradient of 0.05–0.4 M Tris-HCl, pH 7.5, started at the arrow; flow rate, 50 mL/h; fraction volume, 9.0 mL; Assay, fractions diluted 1:20.

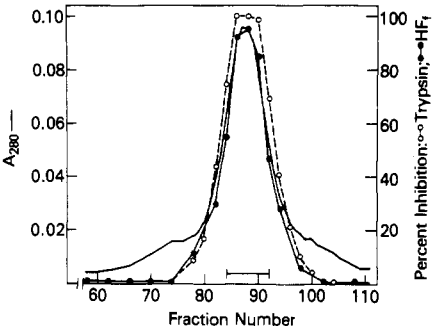


FIGURE 3: Sephadex G-50 gel filtration of PHFI. Column, 2.6 × 93 cm; sample, 6 mL; eluent, 0.02 M $NH_4OAc$, pH 8.5; flow rate, 20 mL/h; fraction volume, 4.3 mL; assay, fractions diluted 1:40.

peak of activity was in fractions 27–34 and accounted for 50% of the applied activity (Figure 1).[2]

*(Step 4) CM-Sephadex C-25 Chromatography.* Fractions 27–34 from step 3 were combined (63 mL), mixed with 2 mL of 1 M Tris-HCl, pH 7.5, and added to a CM-Sephadex column equilibrated at 4 °C with 0.05 M Tris-HCl, pH 7.5.

[2] Fractions 86–90 (Figure 1) had 10% of the activity against trypsin of fractions 27–34 but only 2% of the activity against $HF_f$. The $M_r$ of this minor inhibitor estimated by Sephadex G-25 gel filtration was 4500. Electrofocusing (with pH 3.5–10 ampholines) revealed two peaks at pH 5.2 and 5.7.

Table I: Purification of PHFI from 390 g of Seeds

| step | total protein ($A_{280}$ units) | total activity[a] (IU) | specific activity (IU/$A_{280}$) | yield (%) | purification (x-fold) |
|---|---|---|---|---|---|
| 1. extract[b] | 30600 | 234 | 0.0076 | 100 | 1 |
| 2. acetone fractionation | 1150 | 172 | 0.15 | 74 | 20 |
| 3. DEAE-cellulose chromatography | 72 | 82 | 1.1 | 35 | 150 |
| 4. CM-Sephadex C-25 chromatography | 5.9 | 49 | 8.3 | 21 | 1090 |
| 5. Sephadex G-50 | 2.6[c] | 35 | 13.6 | 15 | 1790 |

[a] Determined with trypsin. [b] All of the trypsin inhibitory activity is in the albumen, but whole pumpkin seeds were extracted for convenience. [c] Freeze-drying of this fraction gave 5.8 mg dry weight.

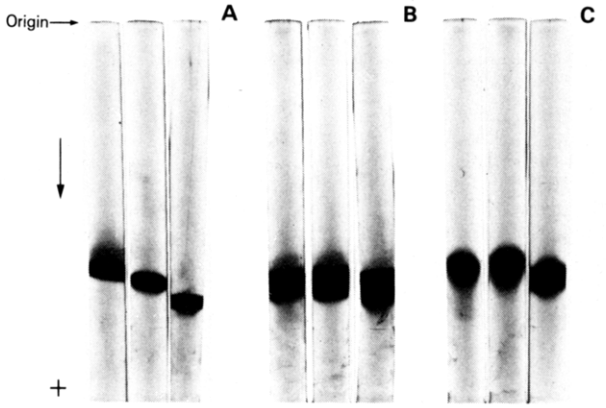


FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of PHFI after anhydrotrypsin–agarose and trypsin–agarose affinity chromatography. (A) Reduced; 10 μg each of the molecular weight standards PTI, PCPI, and insulin B chain, from left to right. (B) Reduced; 20 μg of PHFI, 25 μg of PHFI after the anhydrotrypsin–agarose column, and 30 μg of PHFI after the trypsin–agarose column, from left to right. (C) Nonreduced; same as (B).

Fractions 62–70 (0.12–0.16 M Tris-HCl) were combined (Figure 2) in 79 mL and concentrated in an Amicon apparatus (UM 2 membrane, 202 cell) to 6 mL.

(*Step 5*) *Sephadex G-50 Gel Filtration.* The concentrate from step 4 was filtered at 4 °C through a Sephadex G-50 column equilibrated with 0.02 M $NH_4OAc$, pH 8.5 (Figure 3). Fractions 84–92 were pooled and freeze-dried (5.8 mg).

PHFI was purified 1800-fold in 15% yield (Table I) when assayed against trypsin and in 16% yield when assayed against $HF_f$. A single diffuse band was observed by NaDodSO$_4$–polyacrylamide gel electrophoresis (lane 1 of B and C in Figure 4) and disc polyacrylamide gel electrophoresis. The specific activity of PHFI is 6.0 IU/mg with trypsin and 4.0 IU/mg with $HF_f$. Disc polyacrylamide gel electrophoresis at pH 8.9 revealed PHFI–$HF_f$ complexes, which appear as sharp bands with distinctly lower mobilities than $HF_f$ alone, while at pH 4.3 no differences were observed (not shown).

*Properties.* The minimum $M_r$ of PHFI is 3268 by amino acid analysis (Table II) whereas the $M_r$ values estimated by NaDodSO$_4$–polyacrylamide gel electrophoresis and Sephadex G-25 gel filtration are 4200 and 4000, respectively. The 29-residue inhibitor contains 3 cystine residues and no detectable sulfhydryl or neutral carbohydrate groups. Amino sugars, phenylalanine, and threonine were not observed in the amino acid analyzer, nor was tryptophan detected by the method of Edelhoch (1967). The amino terminus is arginine, as upon dansylation the only dansyl amino acids observed in hydrolysates were α-dansylarginine, *O*-dansyltyrosine, and ε-dansyllysine. The carboxyl-terminal residue is glycine, since it was recovered in 75% yield from digestion of reduced and alkylated PHFI with carboxypeptidase Y. Timed hydrolyses indicated that the carboxyl-terminal sequence of PHFI is -Tyr-Cys-Gly. Treatment with carboxypeptidase A gave only glycine. The $E^{1\%}_{280nm}$ is 4.4 in 0.05 M Tris-HCl, pH 8.0. Isoelectric focusing revealed one peak of PHFI at pH 8.3.

Table II: Amino Acid Composition of PHFI

| amino acid | residues/mol[a] 24 h | 48 h | 72 h | integral |
|---|---|---|---|---|
| Asx | 2.19 | 2.10 | 2.16 | 2 |
| Ser | 0.96 | 0.90 | 0.98 | 1 |
| Glx | 2.18 | 2.12 | 2.14 | 2 |
| Pro | 1.09 | 1.01 | 1.01 | 1 |
| Gly | 2.11 | 2.11 | 2.11 | 2 |
| Ala | 1.07 | 1.06 | 1.19 | 1 |
| $^1/_2$-Cys | 6.18 | 5.72 | 4.92 | 6 |
| Val | 2.00 | 2.00 | 2.00 | 2 |
| Met | 0.94 | 0.86 | 0.56 | 1 |
| Ile | 1.20 | 1.15 | 1.07 | 1 |
| Leu | 3.25 | 3.12 | 3.16 | 3 |
| Tyr | 0.95 | 0.86 | 0.49 | 1 |
| Lys | 3.01 | 2.94 | 2.98 | 3 |
| His | 1.01 | 1.00 | 0.97 | 1 |
| Arg | 2.06 | 1.98 | 1.92 | 2 |
| | | | | 29[b] |

[a] Residues/mol assuming 2 mol of Val/mol. Less than 0.1 residue/mol of Thr and Phe was found. Also, no tryptophan was found (Edelhoch, 1967). [b] Total.

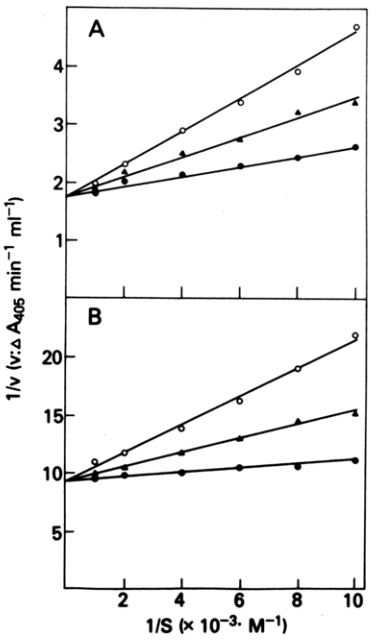


FIGURE 5: Lineweaver–Burk plots showing competitive inhibition by PHFI of the hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*NA by trypsin and D-Pro-Phe-Arg-*p*NA by $HF_f$. (A) trypsin: (●) no PHFI; (▲) 0.05 μg; (○) 0.1 μg. (B) $HF_f$: (●) no PHFI; (▲) 0.4 μg; (○) 0.8 μg.

*Trypsin and $HF_f$ Inhibition.* Competitive inhibition was observed with both trypsin and $HF_f$ (Figure 5). Using $M_r$ = 3268 for PHFI, we found that $K_i = 1.3 \times 10^{-8}$ M with trypsin and Bz-Ile-Glu-Gly-Arg-*p*NA ($K_m = 4.6 \times 10^{-5}$ M;

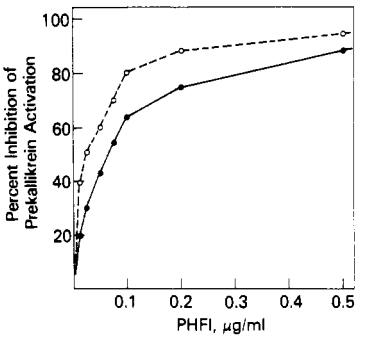


FIGURE 6: PHFI inhibition of human plasma prekallikrein activation by $HF_f$ and plasma protein fraction. (●) $HF_f$; (○) plasma protein fraction.

$k_{cat}$ = 113 $s^{-1}$), and $K_i = 4.1 \times 10^{-8}$ M with $HF_f$ and D-Pro-Phe-Arg-*p*NA ($K_m = 1.7 \times 10^{-5}$ M; $k_{cat}$ = 14 $s^{-1}$).

*Inhibition of $HF_f$ Activation of Prekallikrein.* PHFI inhibits activation of plasma prekallikrein by purified $HF_f$ or by $HF_f$ present in plasma protein fraction (Alving et al., 1978). The specific activities of PHFI were 1.0 and 0.77 IU/mg, respectively (Figure 6). [The concentration of $HF_f$ in plasma protein fraction reference no. 1 has been estimated to be 0.05 μg/mL (Hojima et al., 1980a).]

*Inhibition of Other Serine Proteinases.* Human plasma, human urinary, and porcine pancreatic kallikreins, human α-thrombin, and bovine α-chymotrypsin were not inhibited at the following enzyme–inhibitor weight ratios: 1:100, 1:25, 1:100, an 1:100, respectively. Human plasmin and bovine factor $X_a$ are very weakly inhibited with specific activities of about 0.1 IU/mg and 0.02 IU/mg, respectively.

*Prolongation of Activated Partial Thromboplastin Time.* The clotting time of normal human plasma was prolonged from 44 s ($n$ = 18) to 84, 121, 136, and 196 s ($n$ = 6) with 0.01, 0.03, 0.1, and 0.3 mg of PHFI/mL, respectively. These results are similar to those obtained with corh $HF_f$ inhibitor (Hojima et al., 1980a). The inhibitors also inhibit α-factor $XII_a$ (Griffin & Cochrane, 1979), the initial form of activated factor XII (Y. Hojima, unpublished data).

*Inactivation of PHFI.* The inhibitor is inactivated by heating in 0.1 N NaOH for 30 min at 95 °C but not by 0.1 N HCl for 30 min at 95 °C. Modification of lysine residues by incubating 0.08 mM PHFI with 10 mM 2,4,6-trinitrobenzenesulfonic acid did not alter trypsin inhibitory activity but treatment of 0.08 mM PHFI with 10 mM 1,2-cyclohexanedione for 4 h caused a 60% loss of activity, indicating that arginine probably is at the reactive site.

*Loss of Activity after Trypsin–Agarose Chromatography.* After elution from the trypsin–agarose column, PHFI was fully active against trypsin, but only 40% of the $HF_f$ inhibitory activity was recovered. On the other hand, there was no loss of activity against either enzyme when PHFI was adsorbed to and eluted from the anhydrotrypsin–agarose column. Comparison of the two preparations by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4) showed that PHFI recovered from the trypsin–agarose column had a slightly higher mobility than PHFI recovered from the anhydrotrypsin–agarose column when tested without reduction. After reduction, the migrations were similar. PHFI recovered from the anhydrotrypsin–agarose column was indistinguishable from untreated PHFI.

## Discussion

Compared with most other plant materials examined, pumpkin seed extracts are unusual in having strong inhibitory activity against $HF_f$ and trypsin but little or no activity against several other serine proteinases. DEAE-cellulose chromatography revealed two peaks of inhibitory activity (Figure 1). The major activity, p*I* 8.3, found in the unadsorbed fraction, was further purified by CM-Sephadex chromatography and Sephadex G-50 gel filtration.

In a previous study with corn $HF_f$ inhibitor (Hojima et al., 1980a), the inhibitor was modified during trypsin–agarose chromatography (converted to the two-chain form). The activity of the modified inhibitor against $HF_f$ was reduced 40% under the assay conditions employed, whereas the activity against trypsin was unchanged. No modification or reduction in activity was evident when the corn inhibitor was subjected to anhydrotrypsin–agarose chromatography. Similar results were obtained with PHFI: no change was observed on passing it through an anhydrotrypsin column but a 60% reduction in activity (assayed after 10-min preincubation of PHFI and $HF_f$) on passing it through a trypsin–agarose column. Our experience with the corn inhibitor and PHFI indicates that anhydrotrypsin–agarose (and not trypsin–agarose) should be used when only virgin inhibitor is desired. It has been noted previously that the rate of complex formation between enzyme and modified inhibitor can be much lower than the rate between the enzyme and the virgin inhibitor (Laskowski & Sealock, 1971). Rates of complex formation of $HF_f$ with virgin and modified inhibitor remain to be determined.

While the molecular weight of PHFI is ~4000 by gel filtration and ~4200 by $NaDodSO_4$–polyacrylamide gel electrophoresis, the more accurate molecular weight calculated from the amino acid analysis is 3268. PHFI contains 3 cystine residues, no detectable sulfhydryl groups, and neutral carbohydrates or amino sugars. Interaction of PHFI with trypsin occurs with 1:1 enzyme/inhibitor stoichiometry, and inactivation by 1,2-cyclohexanedione indicates that arginine is probably at the reactive site. Assignment of PHFI to one of the families of protein proteinase inhibitors described (Laskowski & Kato, 1980) must await the determination of its amino acid sequence and the localization of the disulfide bridges. Its small size and stability make it an attractive candidate for modification with group-specific reagents to probe its mechanism of action. PHFI, like the corn inhibitor, has a remarkably narrow spectrum of inhibition, and it may prove to be a very useful reagent for studying the mode of action and pathophysiologic significance of Hagement factor.

Polanowski et al. (1980) described the purification of three trypsin inhibitors from squash seeds (*Cucurbita maxima*, to which several pumpkin and squash varieties belong) and presented the amino acid analyses of all three inhibitors. Subsequently, the Polish group published the sequence and a revised amino acid analysis of isoinhibitor III, indicating that it has 28 residues, amino-terminal arginine, carboxyl-terminal tyrosine, 4 half-cystine residues, and arginine at the reactive site (Nowak et al., 1981). Recently, however, M. Wieczorek has communicated to us that (1) this isoinhibitor has 29 residues, 6 half-cystine residues, and the same amino acid composition as PHFI, (2) the published sequence of III appears to be correct for the first 13 residues at the amino terminus but that the remaining sequence is questionable, and (3) tyrosine is not the carboxyl-terminal residue. This new information suggests that PHFI and squash isoinhibitor III are identical.

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# Activation of Spin-Labeled Chicken Pepsinogen†

D. M. Glick,* M. J. Valler, C. C. Rowlands, J. C. Evans, and J. Kay

ABSTRACT: Chicken pepsinogen has been spin-labeled by the attachment of four nitroxides to ϵ-amino groups near the protein's amino terminus. Acidification results in a bond cleavage, generating a nonlabeled, enzymatically active protein. Electron spin resonance spectra of the spin-labeled zymogen, acidified in the presence or absence of pepstatin, are identical and indicate that the nitroxides are quite mobile, compared to the nonacidified zymogen. This mobilization is interpreted as the freeing of the peptide to which the spin-labels are attached, from the protein, subsequent to the acidification that causes a peptide bond cleavage. The rate at which the peptide leaves the protein is 1 order of magnitude slower than the cleavage of the peptide bond, measured by the rate of appearance of milk-clotting activity (first-order rate constants of 0.3 $min^{-1}$ vs. 6 $min^{-1}$ at pH 2, 22 °C). The inclusion of pepstatin, at molar ratios above 2 during activation, decreases the rate of peptide leaving. These observations, and those previously reported for activation of spin-labeled pig pepsinogen, are incorporated into a model of pepsinogen activation.

Conversion of pepsinogen into the active enzyme, pepsin, releases a total of about 44 amino acid residues in the activation segment (depending on the species). However, it has been shown (Dykes & Kay, 1976; Christensen et al., 1977) that these amino acids are not all released together as one peptide—instead a sequential activation mechanism is operative. Of the zymogens of this type, pig pepsinogen has been studied most extensively. By inclusion of a small molar excess of pepstatin to arrest the activation at completion of the first step, it was found that the first peptide bond to be hydrolyzed is $Leu_{16}$–$Ile_{17}$ (Figure 1). A peptide of 16 amino acid residues is released, generating an intermediate, "pseudopepsin" (Kay & Dykes, 1977). Activations of chicken pepsinogen and calf prochymosin also occur by sequential mechanisms (Kay & Dykes, 1977; Keilova et al., 1977; Pedersen et al., 1979), but in both these cases the first bond split is $Phe_{25}$–$Leu_{26}$ (Figure 1) and a larger initial peptide is released. Activation in the presence of pepstatin is restricted to cleavage of the Phe–Leu

† From the Department of Biochemistry (D.M.G.), The Medical College of Wisconsin, Milwaukee, Wisconsin 53226, and the Departments of Biochemistry (M.J.V. and J.K.) and Chemistry (C.C.R. and J.C.E.), University College, Cardiff, Wales, United Kingdom. *Received December 7, 1981.* This work was supported by U.S. Public Health Service Grant AM-09826 (to D.M.G.) and Agricultural Research Council Grant AG72/31 (to J.K.). An international collaboration was supported by an award to D.M.G. from The Burroughs Wellcome Fund.

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