Human Protein Z

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Abstract. Protein Z was purified from human plasma by a four-step procedure which included barium citrate adsorption, ammonium sulfate fractionation, DEAE-Sepharose chromatography, and blue agarose chromatography with a yield of 20%. It is a 62,000 mol wt protein with an extinction coefficient of 12.0. The concentration of Protein Z in pooled, citrated plasma is 2.2 μg/ml and its half-life in patients starting warfarin anticoagulation therapy is estimated to be <2.5 d. The NH_2-terminal sequence is Ala-Gly-Ser-Tyr-Leu-Leu-(Gla)-(Gla)-Leu-Phe-(Gla)-Gly-Asn-Leu. Neither Protein Z nor its cleavage products, which were obtained by treatment of Protein Z with thrombin or plasmin, incorporated [^{3}H]diisopropyl fluorophosphate. The physiological function of Protein Z remains unknown.

Introduction

Vitamin K is required for the posttranslational formation of a unique amino acid, gamma carboxyglutamic acid, which is present in a number of plasma proteins, including Factors II, VII, IX, X, Protein C, and Protein S, all of which play a role in hemostasis (1, 2). The gamma carboxyglutamate residues appear to be essential to the functional properties of these proteins, mediating their binding to phospholipid surfaces via calcium bridges (3). Recently Prowse and Esnouf described another vitamin K-dependent protein in bovine plasma and they named it Protein Z (4). Initially this protein was thought to represent a form of Factor X (single chain), but it was later identified as a discrete gamma carboxyglutamic acid-containing protein (5, 6). The physiological function of bovine Protein Z remains unknown. Here we describe the purification and characterization of a human plasma protein that appears to represent the human counterpart to bovine Protein Z.

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Materials

DEAE Sepharose and cyanogen bromide-activated 4B Sepharose were purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ), and blue agarose and low molecular weight standards for polyacrylamide gel electrophoresis from Bio-Rad Laboratories (Richmond, CA). Amberlite CG-50 was obtained from Mallinckrodt, Inc. (Science Products Div., St. Louis, MO). Soybean trypsin inhibitor, bovine serum albumin, Trizma base, acrylamide and bis-acrylamide, and benzamidine were bought from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Diisopropyl fluorophosphate (DFP)[1] was purchased from Amersham Corp. (Arlington Heights, IL). All other chemicals were of reagent grade or better and came from Fisher Scientific Co. (Pittsburgh, PA) or from Sigma Chemical Co.

Human plasmas. Plasmas from patients with Factors V/VIII deficiency were gifts from Dr. Walter Kisiel (University of Washington, Seattle, WA—four patients) and Dr. Roger Edson (University of Minnesota, Minneapolis, MN—one patient).

Plasmas deficient in Factors II, V, VII, VIII, IX, X, XI, XII, and von Willebrands factor, and Passavoy trait plasma were obtained from George King Biomedical (Overland Park, KS), as was normal control plasma pooled from 20 donors.

Individual normal control plasmas were obtained from laboratory personnel. Samples from eight patients starting warfarin therapy were taken from specimens sent to the Barnes Hospital hemostasis laboratory (St. Louis, MO).

Methods

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed on 10% gels (ratio acrylamide/bis, 37.5:1) by the method of Laemmli (4% stacking gel) (7) or by the method of Weber and Osborn (8). Gels were stained for protein with Coomassie Brilliant Blue R. Molecular weights were estimated on Weber-Osborn gels by interpolation from linear semilogarithmetic plots of apparent molecular weights vs. migration distance using the following protein standards: phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 20,000; and lysozyme, 14,300.

Amino acid composition and NH_2-terminal sequence

A sample of purified Protein Z was dialyzed against 0.1 M acetic acid for 48 h. Portions that contained 1.5–2.0 nmol of protein were lyophilized and then hydrolyzed in 6 N HCl at 105°C in sealed, evacuated tubes for 24 h. Amino acid compositions were determined in duplicate using a Beckman 119C amino acid analyzer (Beckman Instruments, Inc., Berkeley, CA) with a modified program (9). The results were not corrected

1. *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; Z_p, plasmin-cleaved Protein Z; Z_t, thrombin-cleaved Protein Z.

for losses due to hydrolysis. Half-cystine was determined as cysteic acid by the method of Hirs (10).

The NH_2-terminal sequence analysis of Protein Z was performed on a gas-phase sequenator (Applied Biosystems, Foster City, CA); phenylthiohydantoin derivatives were identified by high-performance liquid chromatography (11, 12). Two separate analyses were performed using 1 nmol of Protein Z and gave identical results.

Extinction coefficient of Protein Z

Protein Z was dialyzed extensively into 0.1 M NaCl, 0.05 Tris, pH 7.5, and its extinction coefficient was determined by the method of Babul and Stellwagen (13). No correction was made for light scattering.

Proteins

Human prothrombin, Factor X, Factor IX, and Factor VII were isolated as previously described (14, 15). Protein C was first purified from fresh-frozen plasma by barium citrate adsorption, $(NH_4)_2SO_4$ elution, and affinity chromatography using a murine monoclonal antibody, and then converted to activated Protein C with thrombin (16, 17). Protein S was isolated by a procedure similar to that of Dahlbach, with the exception that DEAE Sepharose rather than DEAE Sephacel ion-exchange chromatography was used (18). Thrombin with a specific activity of 2.7 NIH units/μg was prepared from prothrombin (19).

Human plasminogen was purified from fresh-frozen plasma by affinity chromatography on lysine-Sepharose; plasmin was prepared with urokinase linked to Sepharose 4B (20, 21). To produce plasmin-Sepharose, 1 ml of 0.1 M $NaHCO_3$, pH 8.3, containing 2 mg plasmin was added to an equal volume of CNBr-activated 4B Sepharose and allowed to react overnight at 4°C. Unreacted sites were then blocked with glycine ethyl ester, and the gel was washed extensively with 0.1 M NaCl, 0.05 M Tris, pH 7.5. The plasmin-Sepharose contained 1 mg protein/ml hydrated gel and it hydrolyzed 2.8 μmol of S2251/min per ml of gel at room temperature.

Functional assays

Prothrombin, Factor VII, Factor IX, and Factor X were assayed as previously described (14, 15).

Purification of Protein Z

Starting material. Human citrated plasma was kindly provided by the Missouri-Illinois Regional Red Cross Blood Program. 35 liters were thawed at 37°C, 137 g of benzamidine (25 mM) and 350 mg of soybean trypsin inhibitor (10 mg/liter) were added, and the plasma was transferred to the cold room. All subsequent steps were performed at 4°C in plastic containers.

Barium citrate adsorption and ammonium sulfate fractionation. 1.75 liters of 1.0 M $BaCl_2$ was added dropwise over 45 min and the mixture was stirred an additional 15 min. The barium citrate precipitate was collected by centrifugation at 3,000 *g* for 15 min and the supernatant plasma was decanted. The precipitate was washed once with 16 liters, and then twice with 8 liters, of 0.15 M NaCl, 0.01 M $BaCl_2$, 0.01 M benzamidine, pH 7.0, and finally resuspended in 3.5 liters of 35% saturated $(NH_4)_2SO_4$, 0.01 M benzamidine, 0.02 M Tris-HCl, pH 8.1 This was stirred for 30 min, the precipitate was removed by centrifugation at 3,000 *g* for 20 min, and enough dry $(NH_4)_2SO_4$ was added to bring the supernatant to 65% saturation. The mixture was stirred for 30 min, and the protein precipitate was collected by centrifugation at 10,000 *g* for 20 min and resuspended in 350 ml of 0.02 M sodium citrate, 0.001 M benzamidine, pH 6.0. The solution was dialyzed overnight against two changes of 40 liters each of 0.02 M sodium citrate, 0.001 M benzamidine, pH 6.0.

DEAE Sepharose chromatography. The sample was then applied to a 5 × 95 cm column of DEAE Sepharose equilibrated in 0.02 M sodium citrate, 0.001 M benzamidine, pH 6.0, at a flow rate of 100 ml/h. The column was washed with 200 ml of the same buffer and then eluted with a linear gradient to 0.60 M NaCl, 0.02 M sodium citrate, 0.001 M benzamidine, pH 6.0, over 9 liters. The Protein Z was pooled as indicated in Fig. 1, concentrated to 20 ml (PM-10 membrane, Amicon Corp., Scientific Sys. Div., Danvers, MA) and dialyzed against 4 liters of 0.15 M NaCl, 0.02 M sodium citrate, 0.001 M benzamidine, pH 7.0.

Blue agarose chromatography. The sample was then applied to a 2 × 20 cm column of blue agarose equilibrated in 0.15 M NaCl, 0.02 M sodium citrate, 0.001 M benzamidine, pH 7.0, at a flow rate of 10 ml/h and eluted with the same buffer. The protein that flowed through the column under these conditions was pooled, concentrated (PM-10 membrane) to 1–2 mg/ml, and stored at −70°C.

Preparation of antisera

Purified Protein Z, 1 mg in 1 ml 0.1 M NaCl, 0.05 M Tris, pH 7.5 was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously at multiple sites into the back of a 2-kg male New Zealand white rabbit. A booster injection that contained the same dose of Protein Z in incomplete Freund's adjuvant was given at 4 wk, and the rabbit was bled at 2-wk intervals. The blood was allowed to clot and the antisera were collected after centrifugation. When analyzed by the Ouchterlony immunodiffusion technique (22), these antisera gave a precipitant band against Protein Z but failed to recognize human Factors II, VII, IX, X, and Proteins C and S.

Rocket immunoelectrophoresis

The concentration of Protein Z in plasmas was determined by the rocket immunoelectrophoresis method of Laurell (23). 1% agarose gels in 0.024 M Tris, 0.075 M barbital, 2 mM EDTA, pH 8.7 and containing rabbit anti-Z antisera at a 1:90 dilution were cast on GelBond film (FMC Corp., Marine Colloids Div., Springfield, NJ) (18 ml agarose solution for each 12.5 × 10 cm slab). 15-μl samples were placed in 4-mm wells and run overnight in a horizontal slab electrophoresis apparatus with a cold water (4°C) recirculator at 2 V/cm. The gels were then washed in 0.145 M NaCl, 0.05 sodium phosphate, pH 7.2, dried at 65°C for 1 h, and stained with Coomassie Brilliant Blue R.

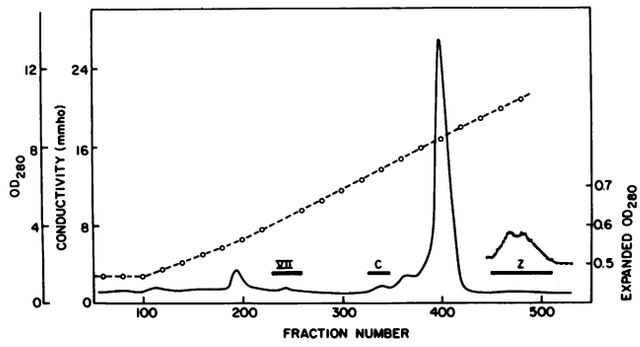

Figure 1. Elution of Protein Z from DEAE-Sepharose. Protein was eluted from the column (5 × 95 cm) with a linear gradient from 0 to 0.6 M NaCl in 0.02 M sodium citrate, 0.001 M benzamidine, pH 6.0, over 9 liters. Fractions (14 ml) were collected at a flow rate of 180 ml/h. ——, OD_{280}; - - - o - - -, conductivity in mmho; — • —, expanded OD_{280}. The persistent baseline OD_{280} (~0.5) is due to the presence of benzamidine.

Enzymatic cleavage of Protein Z

Thrombin-cleaved Protein Z (Z_t) was produced by incubating 1 mg/ml Protein Z with 200 U/ml thrombin in 0.1 M NaCl, 0.05 M Tris, pH 8.0 for 3 h at 37°C. To remove thrombin, the mixture was passed through a small column of CG-50 in the same buffer.

Plasmin-cleaved Protein Z (Z_p) was produced by incubating 1–2 mg/ml Protein Z with an equal volume of hydrated plasmin-Sepharose in 0.1 M NaCl, 0.05 M Tris, pH 8.0 for 20 h at room temperature. The plasmin-Sepharose was then filtered to isolate the Z_p.

For the purposes of this study, Z_t and Z_p were assumed to have the same extinction coefficient as native Protein Z.

Incorporation of [^{3}H]DFP into Protein Z, Z_t, and Z_p

10 μl of 1.14 mM [^{3}H]DFP (1 mCi/ml) in propylene glycol was added to 10 μl of Protein Z (1.0 mg/ml), Z_t (1.0 mg/ml), Z_p (1.0 mg/ml), activated Protein C (1.0 mg/ml), or thrombin (0.07 mg/ml), in 0.1 M Tris, pH 8.1. The samples were incubated for 20 h at 4°C, reduced with 5% 2-mercaptoethanol, and, after SDS-polyacrylamide gel electrophoresis, the gel was prepared for fluorography as previously described (24). Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) was exposed for 1–8 wk at −70°C in a cassette containing Cronex intensifying screens (DuPont Instruments, Wilmington, DE). Under these conditions [^{3}H]DFP incorporation into the heavy chain of thrombin and both the α- and β-heavy chains of activated Protein C could be easily detected.

Results

Purification of Protein Z. Based upon its concentration in plasma as determined by immunoelectrophoresis (see below), we estimate that Protein Z was purified 35,000-fold by a four-step process with a yield of 20% (16.8 mg Protein Z isolated from 35 liters citrated plasma). In this procedure, the vitamin K-dependent proteins are removed from plasma by adsorption to barium citrate, and the Protein Z is separated from the other adsorbed proteins by chromatography on DEAE Sepharose (Fig. 1). We found, as noted by others for bovine Protein Z (4, 6), that the use of an agarose- (as opposed to a dextran-) based ion-exchange support was required for optimal separation of the Protein Z. The bars in Fig. 1 represent the fractions that were pooled for Factor VII, Protein C, and Protein Z, respectively (left to right in figure). Factor X and Protein S eluted just before the large protein peak which contained Factor IX and prothrombin. As shown, the Protein Z occasionally eluted in at least two partially separated peaks. The Protein Z pool following DEAE Sepharose chromatography was usually >95% pure as judged by SDS-polyacrylamide gel electrophoresis, but subsequent blue agarose chromatography was at times required to remove trace quantities of contaminants. Functional assay revealed no detectable prothrombin, Factor VII, Factor IX, or Factor X in the isolated Protein Z.

Characterization. The final preparation of Protein Z appeared to be homogeneous as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2). In the Laemmli discontinuous system (7), Protein Z frequently appeared as multiple, tightly spaced bands, and it migrated with an apparent molecular weight greater than that found when the continuous system of Weber and Osborne (8) was employed. When the latter method was used, Protein Z migrated as a single band with an apparent molecular weight of 62,000, both before and after reduction. The extinction coefficient of Protein Z was estimated to be 12.0 using the method of Babul and Stellwagen (13).

The amino acid composition of human Protein Z is shown in Table I, as is that previously published for bovine Protein Z (6). The NH_2-terminal amino acid sequence is shown in Table II; for comparison, those of human Factors II, VII, IX, X, Protein C, Protein S, and bovine Protein Z (25–31) are also shown. No amino acids were detected in cycles 7, 8, and 11, and these residues were assumed to represent gamma carboxyglutamic acids.

Plasma levels of Protein Z. Heterologous rabbit anti-Protein Z antibodies were used to determine plasma levels of Protein Z by the rocket immunoelectrophoresis technique of Laurell. Normal citrated plasma pooled from 20 donors, when tested on 12 occasions, gave a Protein Z level of 2.2±0.14 μg/ml (mean±standard deviation). The mean level of Protein Z in 10 individual normal donors was 2.2±0.4 with a range of 1.6 to 2.95 μg/ml.

Protein Z immunoelectrophoresis rockets could not be visualized (<0.2 μg/ml) by the third to fifth day after initiation of warfarin therapy in eight patients undergoing anticoagulant therapy for thromboembolic disease. Plasmas from five patients with Factor V/VIII deficiency and from one patient with Passavoy trait had normal levels of Protein Z, as did plasmas from patients with deficiencies of Factor II, V, VII, VIII, IX, X, XI, XII, or von Willebrands factor.

Table I. Amino Acid Composition of Protein Z (Normalized to 35 Leucine Residues)

	Human	Bovine
Aspartic acid	28.2*	29.7
Threonine	25.9	21.1
Serine	24.7	21.5
Glutamine	49.5	45.0
Proline	15.8	29.7
Glycine	34.5	37.5
Alanine	17.0	31.4
Half-cystine	18.8	20.8
Valine	31.3	28.5
Methionine	4.0	3.9
Isoleucine	17.5	8.2
Leucine	35.0	35.0
Tyrosine	9.7	9.4
Phenylalanine	11.2	11.8
Histidine	13.9	10.4
Lysine	14.5	11.0
Arginine	20.3	28.5
Tryptophan	ND	6.9

* Number of residues
ND, not determined

Table II. Comparison of the NH_2-Terminal Sequences of Human Prothrombin, Factor VII, Factor IX, Factor X, Protein C, Protein S, Protein Z, and Bovine Protein Z

	Sequence Position														
Factor	1				5					10					15
Human prothrombin	Ala	Asn	Thr	—	Phe	Leu	Gla	Gla	—	Val	Arg	Lys	Gly	Asn	Leu
Human Factor VII	Ala	Asn	—	Ala	Phe	Leu	(Gla)	(Gla)	Leu	—	Arg	Pro			
Human Factor IX	Tyr	Asn	Ser	Gly	Lys	Leu	(Gla)	(Gla)	Phe	Val	—	Gln	Gly	Asn	Leu
Human Factor X	Ala	Asn	Ser	—	Phe	Leu	Gla	Gla	—	Met	Lys	Lys	Gly	His	Leu
Human Protein C	Ala	Asn	Ser	—	Phe	Leu	Gla	Gla	Leu	—	Arg	His	Ser	Ser	Leu
Human Protein S	Ala	Asn	Ser	—	Leu	Leu	(Gla)	(Gla)	?	—	Lys	Gln	Gly	Asn	Leu
Human Protein Z	Ala	Gly	Ser	Tyr	Leu	Leu	(Gla)	(Gla)	Leu	Phe	—	(Gla)	Gly	Asn	Leu
Bovine Protein Z	Ala	Gly	Ser	Tyr	Leu	Leu	Gla	Gla	Leu	Phe	—	Gla	Gly	His	Leu

Gla refers to gamma carboxyglutamic acid. Parentheses surround Gla residues that are suspected but not yet proven. Dashes refer to spaces which have been inserted to bring the sequences into alignment for better homology.

Cleavage of Protein Z by thrombin and plasmin. Treatment of Protein Z with high levels of thrombin or plasmin produced cleavage products (see Methods) that we have called Z_t and Z_p, respectively (Fig. 2). Z_t was a single chain protein both reduced and unreduced on SDS polyacrylamide gel electrophoresis with an apparent molecular weight of 56,000. Z_p migrated as a single chain unreduced of 60,000 D, and following reduction was composed of a heavy chain of 44,000 D and a light chain (not seen well in Fig. 2) of 20,000 D. Neither Protein Z, Z_t, nor Z_p incorporated [^{3}H]DFP under conditions in which [^{3}H]DFP in-

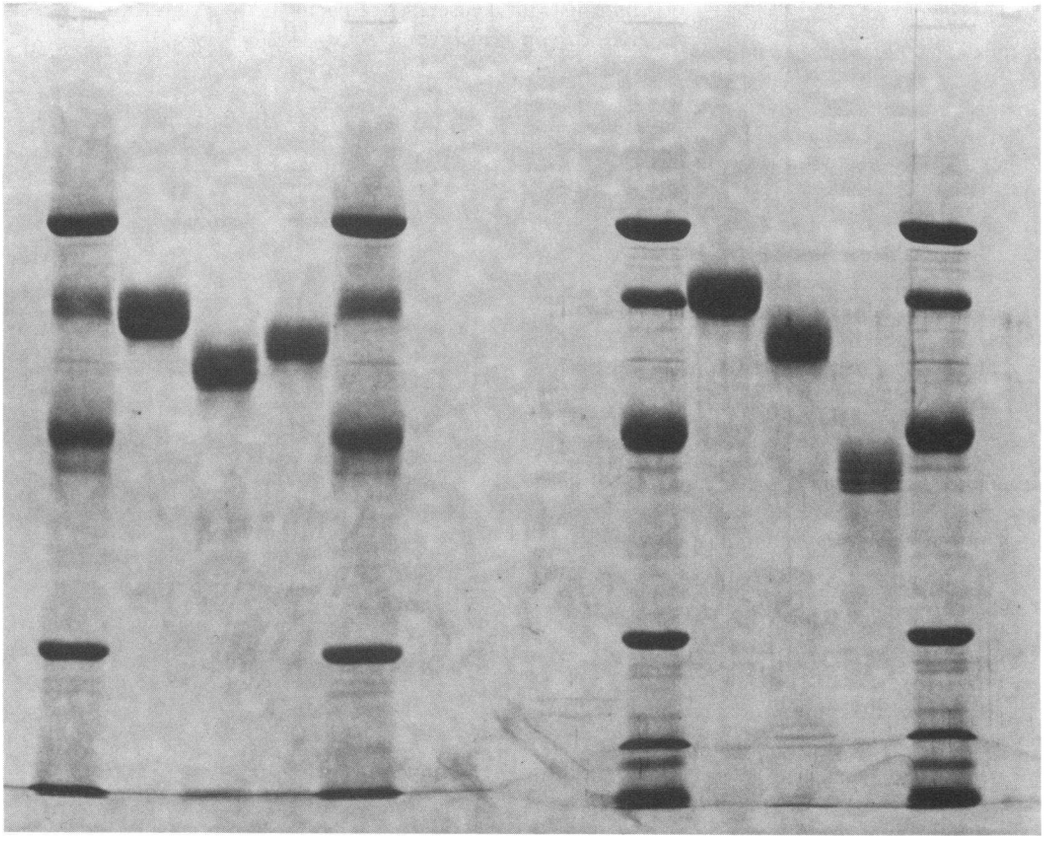

Figure 2. Sodium dodecyl sulfate polyacrylamide (10%) gel electrophoresis of Protein Z (7). On the left: protein standards; Protein Z (15 μg); thrombin-treated Protein Z (10 μg); plasmin-treated Protein Z (10 μg); and protein standards, respectively. On the right: the same samples after reduction with 5% 2-mercaptoethanol. The protein standards are phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,500; soybean trypsin inhibitor, 20,000; and lysozyme, 14,300.

corporation into thrombin and activated Protein C could be easily detected.

Discussion

The human protein isolated here has been called Protein Z because its molecular weight, amino acid composition, NH_2-terminal sequence, and chromatographic characteristics are very similar to those of the bovine protein of the same name. Protein Z does not appear to be related to the recently described Protein M, since it failed to activate Factor X in the presence of human brain thromboplastin or to affect the activation of prothrombin in the presence of Factor X_a, Factor V_a, phospholipids, and calcium. Both of these properties have been ascribed to Protein M (32, 33, and Broze, G. J., Jr., and J. P. Miletich, unpublished observations).

The level of Protein Z in human citrated plasma is ~2.2 μg/ml, and from data obtained from patients starting warfarin anticoagulation therapy, we estimate that the half-life of Protein Z is <2.5 d. Although the heterologous rabbit anti-Z antibody could no longer detect Protein Z in the plasmas of these patients treated with warfarin, it is not clear whether this is because of an actual absence of Protein Z, or because the rabbit antibody cannot detect gamma carboxyglutamate-deficient Protein Z.

The physiological function of Protein Z remains obscure. In preliminary experiments, neither the prothrombin time, partial thromboplastin time, nor euglobulin lysis time of normal plasma was affected by prior immunoadsorption of Protein Z from the plasma or by addition of Z_t or Z_p (10 μg/ml) (Broze, G. J., Jr., and J. P. Miletich, unpublished observations). Although neither Protein Z nor its cleavage products produced by treatment with thrombin or plasmin could be demonstrated to incorporate [^{3}H]DFP, it is still possible that Protein Z may be a serine protease zymogen.

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