Int. J. Peptide Protein Res. **8**, 1976, 323–329

Published by Munksgaard, Copenhagen, Denmark

FIBRINOPEPTIDES

IV. Synthesis of Human Fibrinopeptide A

K. CHANNABASAVAIAH AND K. M. SIVANANDAIAH

Department of Chemistry, Central College, Bangalore University, Bangalore, India

Received 24 July 1975

Human fibrinopeptide A, a hexadecapeptide, released by the action of thrombin on fibrinogen during clotting of blood, has been synthesized by conventional methods. The synthetic peptide as well as some of the intermediates in the synthesis have been examined for anticoagulant activity. Though all of them were found to be active, the terminal carboxyl protected peptides are more potent inhibitors of clotting than the carboxyl free peptides.

The plasma protein fibrinogen, composed of three pairs of nonidentical chains α(A), β(B) and γ, undergoes limited proteolysis on treatment with thrombin due to splitting of the first Arg-Gly* bonds (from the N-terminal end) leading to the formation of fibrinopeptides A and B along with fibrin monomer, and the latter polymerizes to form a network structure or clot (1). Fibrinopeptides A from different species have different amino acid sequences and human fibrinopeptide A has the sequence,

Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-
1 2 3 4 5 6 7 8
Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg
9 10 11 12 13 14 15 16

(2). A brief report on the solid phase synthesis of this hexadecapeptide by Johnson & May has appeared (3). The present paper details the synthesis of this peptide by conventional methods. As part of this study we have reported earlier the synthesis of the three fragments, Z-Ala-Asp(OBzl)-Ser-Gly (4), Boc-Glu(OBzl)-Gly-Asp(OBzl)-Phe-Leu-Ala-OMe (5) and Boc-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OMe (6) spanning the entire sequence of fibrinopeptide A. However, for the present synthesis we have used the four fragments, Z-Ala-Asp(OBzl)-Ser(Bzl)-Gly, Boc-Glu(OBzl)-Gly-Asp(OBzl), Boc-Phe-Leu-Ala and Boc-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl, and of these, the first three were obtained by the stepwise method using exclusively hydroxysuccinimide esters. Fig. 1 summarizes the assembling of the required fragments and their subsequent use in the synthesis of the final product. Since the DCC-HOBt procedure of Konig & Gieger (7) is reported to be almost racemization free, this method was employed for the condensation of fragments.

There are reports that smaller fragments related to the C-terminal part of fibrinopeptide A exhibit anticoagulant activity (8, 9). We have therefore studied the activities of our synthetic fibrinopeptide A as well as some of the intermediates. The clotting studies were carried out following essentially the method of Magnusson (10) and the results in Table 1 refer to the concentration of the peptide at which the clotting time is estimated

* Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1972) in *Biochemistry*, **11**, 1726–1732. Other abbreviations used: DMF, N,N'-dimethylformamide; DCC, N,N'-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; NMM, N-methylmorpholine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

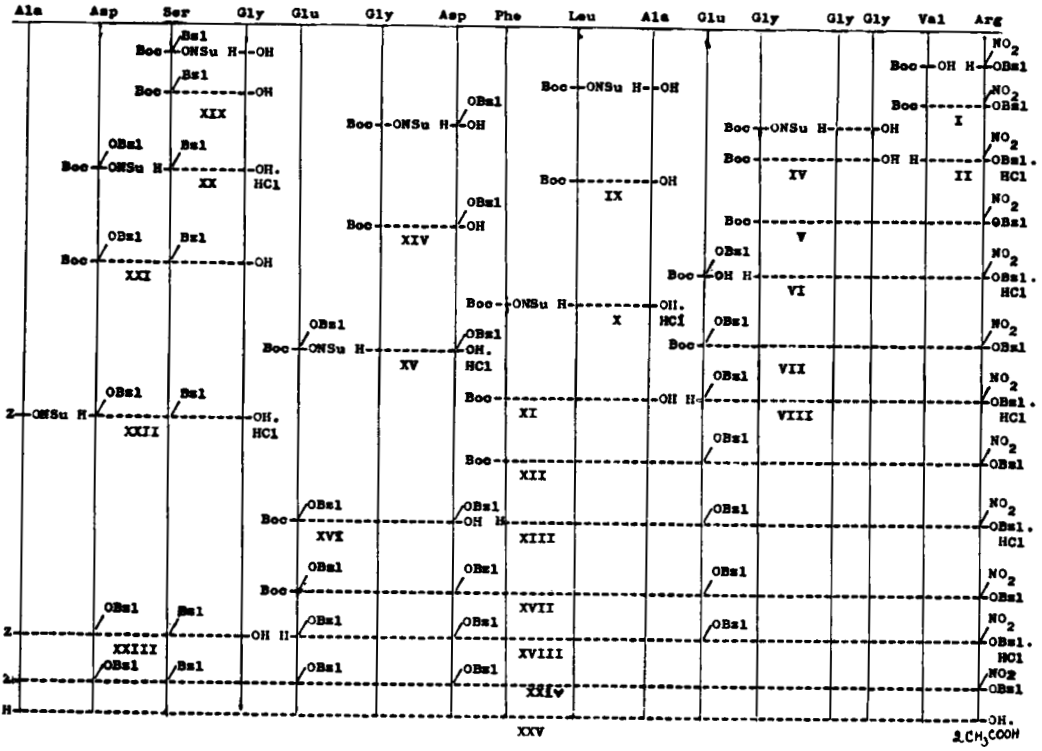

FIGURE 1

Schematic representation of the synthesis of human fibrinopeptide A

TABLE 1

Inhibition of clotting of fibrinogen by synthetic peptides

	Peptide	c[a] (μmol/lit)
II	Val-Arg(NO_2)-OBzl HCl	1.00 ± 0.05
VI	Gly-Gly-Gly-Val-Arg(NO_2)-OBzl HCl	0.60 ± 0.05
VIII	Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl HCl	0.35 ± 0.05
XIII	Phe-Leu-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl HCl	0.25 ± 0.05
XVIII	Glu(OBzl)-Gly-Asp(OBzl)-Phe-Leu-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl HCl	0.15 ± 0.05
XXVI	Gly-Gly-Gly-Val-Arg(NO_2). TFA	11.00 ± 0.10
XXVII	Gly-Gly-Gly-Val-Arg. 2TFA	3.00 ± 0.05
XXVIII	Glu-Gly-Gly-Gly-Val-Arg. 2TFA	1.70 ± 0.05
XXIX	Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg. 2TFA	1.00 ± 0.05
XXX	Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg. 2TFA	0.70 ± 0.05
XXV	Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg. 2TFA	0.45 ± 0.05

[a] c is the concentration of the peptide at which the clotting time was twice the clotting time without the peptide.

to have doubled (cf Liem, Andreatta & Scheraga (11)). A perusal of these results reveals that the protection of the terminal carboxyl group of the peptide greatly enhances its ability to inhibit clotting and this is in conformity with the observations of Blomback and co-workers (9). As the chain length of the peptide increases, its potency also increases and this is true of both protected as well as deprotected peptides. We have found that the pentapeptide (XXVI), having the nitro group in the arginine moiety, is less potent than the one without it (XXVII). One can therefore presume that peptides with a free guanido group but having a protected terminal carboxyl group will have greater ability to inhibit clotting than those containing the nitro group.

EXPERIMENTAL PROCEDURES

All the amino acids referred to (except glycine) are of L-configuration. The melting points were taken on a Lietz-Wetzlar unit and are uncorrected. Evaporations of solvents during working up were routinely carried out below 40° under reduced pressure. Thin layer chromatography (t.l.c.) on silica gel G (E. Merck, Darmstadt, West Germany) plates was carried out using the solvent systems ethyl acetate, chloroform-methanol-acetic acid (45:5:2), *n*-butanol-acetic acid-water (4:1:1) and *n*-butanol-acetic acid-pyridine-water (15:3:12:10) and the R_f values are expressed as R_fI, R_fII, R_f III and R_f IV, respectively. Paper chromatography was carried out on Whatman No. 1 filter paper strips by the ascending method and R_f V, R_f VI and R_f VII refer to the solvent systems *n*-butanol-acetic acid-water (4:1:5), *n*-butanol-acetic acid-water (2:2:1) and *n*-butanol-acetic acid-pyridine-water (15:3:12:10) respectively. Petroleum ether used for the crystallization of the peptides was of b.p. 60–80°C. Aminopeptidase M (AP-M) digestion of the peptide was carried out according to Hofmann et al. (12) and the amino acid composition of the digest was determined (13) on a Hitachi Model KLA 3B amino acid analyser. Elementary analyses were performed by the Regional Research Laboratory, Hyderabad, India. Bovine fibrinogen fraction 1, thrombin and AP-M were purchased from Sigma Chemical Company, St. Louis, U.S.A.

Boc-Val-Arg(NO_2)-OBzl (I)

A solution of Boc-Val (14) (1.52 g, 7.0 mmol), Arg(NO_2)-OBzl (15) (1.8 g, 6.0 mmol) and DCC (16) (1.45 g, 7.0 mmol) in dichloromethane (35 ml) was stirred for 5 h at 0° and for 12 h at room temperature. The precipitated N,N′-dicyclohexylurea was filtered and washed with dichloromethane. The combined filtrate and washings were evaporated. The residue was triturated with hot ethyl acetate, when the product separated out as white granules. This was collected and washed with ethyl acetate (2.3 g, 78%); m. p. 168–170°; $[\alpha]_D^{24}+0.3°$ (c, 0.5, DMF); R_f I 0.7; R_f II 0.6.

Anal. Calcd. for $C_{26}H_{36}N_6O_7$ (508.5): C, 54.31; H, 7.13; N, 16.51. Found: C, 54.45; H, 7.23; N, 16.21.

Val-Arg(NO_2)-OBzl (III)

The protected dipeptide I (1.27 g, 2.5 mmol) was treated with 3N HCl-THF (12 ml) for 40 min and then concentrated *in vacuo* to an oil. This was then dissolved in water (20 ml) and extracted with ethyl acetate (2 × 15 ml). The aqueous phase was neutralized at 0° with 50% K_2CO_3 and the liberated free ester was extracted into chloroform (3 × 30 ml). The combined organic phase was washed with water, dried (Na_2SO_4) and evaporated. The product was crystallized from chloroform-petroleum ether (0.65 g, 65%); m. p. 117–118°; $[\alpha]_D^{24}+0.2°$ (c, 0.45, DMF); R_f III 0.67.

Anal. Calcd. for $C_{18}H_{28}N_6O_5$ (408.45): C, 52.93; H, 6.91; N, 20.57. Found: C, 53.20; H, 6.82; N, 20.87.

Boc-Gly-Gly-Gly (IV)

A mixture of Boc-Gly-ONSu (17) (1.1 g, 4.0 mmol), Gly-Gly (0.66g, 5.0 mmol) and $KHCO_3$ 1.0 g, 10.0 mmol) in dioxane-water (1:1, 20 ml) was stirred for 24 h at room temperature, diluted with water (20 ml) and extracted with ether (2 × 30 ml). The aqueous phase was concentrated to about 10 ml, acidified with solid citric acid and extracted with *n*-butanol (2 × 30 ml). The organic phase was washed with saturated NaCl solution and evaporated. The residue was crystallized from THF (0.8 g, 60%); m. p. 156–157°; R_f III 0.45.

Anal. Calcd. for $C_{11}H_{19}N_3O_6$ (289.29): C, 45.66; H, 6.62; N, 14.52. Found: C, 45.35; H, 6.82; N, 14.29.

Boc-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl (V)

Boc-Gly-Gly-Gly (0.55 g, 1.9 mmol) and Val-Arg(NO_2)-OBzl (0.65 g, 1.6 mmol) were dissolved in a mixture of DMF (3 ml) and dichloromethane (30 ml) and cooled to $-10°$. DCC (0.39 g, 1.9 mmol) was added and the reaction mixture was stirred for 5 h at 0° and for 12 h at room temperature. Dichloromethane was evaporated and the residue was treated with N H_2SO_4 (50 ml). The resulting solid was collected, washed with water and recrystallized from hot acetone (1.05 g, 95%); m. p. 116–117°; $[\alpha]_D^{24}$ +0.33° (c, 0.45, DMF); R_f II 0.48; R_f III 0.6; R_f IV 0.75.

Anal. Calcd. for $C_{29}H_{45}N_9O_{10}$ (679.7): C, 51.23; H, 6.70; N, 18.54. Found: C, 50.95; H, 7.10; N, 18.91.

Boc-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl (VII)

The pentapeptide V (0.82 g, 1.2 mmol) was dissolved in 3N HCl-THF (8 ml) and left at room temperature for 40 min. The solid, precipitated by the addition of ether, was recrystallized from methanol-ether to yield the hydrochloride VI. This was then dissolved in DMF (4 ml), cooled to $-10°$ and treated with NMM (0.16 ml, 1.5 mmol). After 15 min a solution of Boc-Glu(OBzl) (18) (0.5 g, 1.5 mmol) was added followed by DCC (0.31 g, 1.5 mmol). The mixture was stirred for 4 h at 0° and for 12 h at room temperature and diluted with ethyl acetate. The white solid that separated was collected, washed with water and dissolved in hot acetonitrile. The insoluble N, N′-dicyclohexylurea was filtered and the filtrate was cooled when the product separated out. This was filtered and washed with ethyl acetate (0.75 g, 73%); m.p. 126–129°; $[\alpha]_D^{24}$ −5.4° (c, 0.37, DMF); R_f II 0.5; R_f III 0.72; R_f IV 0.85.

Anal. Calcd. for $C_{41}H_{58}N_{10}O_{13}$ (898.94): C, 54.79; H, 6.50; N, 15.58. Found: C, 54.56; H, 6.76; N, 15.71.

Boc-Leu-Ala (IX)

A solution of Boc-Leu-ONSu (17) (3.0 g, 9.0 mmol), Ala (0.89 g, 10.0 mmol) and $KHCO_3$ (2.0 g, 20.0 mmol) in dioxane-water (1:1, 40 ml) was stirred for 24 h at room temperature. The reaction mixture was diluted with water (40 ml) and washed with ether (2 × 30 ml). The aqueous phase was neutralized at 0° with solid citric acid and extracted with ethyl acetate (3 × 30 ml). The organic phase was washed with water, dried (Na_2SO_4) and evaporated. The residue was reprecipitated from ethyl acetate-petroleum ether and dried *in vacuum* to yield the dipeptide as a hygroscopic foam (2.5 g, 95%); R_f II 0.55; R_f III 0.7.

Boc-Phe-Leu-Ala (XI)

The dipeptide IX (1.2 g, 4.0 mmol) was treated with 3N HCl-THF (10 ml) for 40 min at room temperature. The solvent was evaporated and the residue was washed with dichloromethane. This was then dissolved in DMF (15 ml), cooled to 0° and neutralized with NMM (0.8 ml, 8.0 mmol). The mixture was stirred for 16 h at room temperature after adding Boc-Phe-ONSu (17) (1.5 g, 4.0 mmol) and partitioned between N H_2SO_4 (50 ml) and ethyl acetate (50 ml). The organic phase was extracted with 5% $KHCO_3$ (3 × 20 ml). The combined aqueous phase was acidified with solid citric acid and the liberated tripeptide acid was extracted with ethyl acetate (2 × 30 ml). The organic phase was washed with water, dried (Na_2SO_4) and evaporated. The product was recrystallized from ethyl acetate-petroleum ether (1.2 g, 66%); m. p. 110–113°; $[\alpha]_D^{24}$ − 18.2° (c, 0.27, DMF); R_f II 0.6; R_f III 0.8.

Anal. Calcd. for $C_{23}H_{35}N_3O_6$ (449.58): C, 61.45; H, 7.85; N, 9.35. Found: C, 61.52; H, 8.11; N, 9.18.

Boc-Phe-Leu-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl (XII)

The hexapeptide VII (0.35 g, 0.4 mmol) was deprotected following the procedure described for the preparation of VI, the resulting hydrochloride was dissolved in DMF (4 ml) and cooled to $-10°$. NMM (0.05 ml, 0.5 mmol) was added followed by Boc-Phe-Leu-Ala (0.22 g, 0.5 mmol), DCC (0.1 g, 0.5 mmol) and HOBt (0.06 g, 0.5 mmol). The mixture was stirred for 2 h at 0° and for 20 h at room temperature. The solid that separated out by the addition of ethyl acetate to the reaction mixture was dissolved in DMF (3 ml), cooled to 0° and filtered to remove N, N′-dicyclohexylurea. Addition of ether to the filtrate precipitated the product (0.42 g, 85%); m. p. 220–222°; $[\alpha]_D^{24}$ − 12.5° (c, 0.2, DMF); R_f II 0.5; R_f III 0.85; R_f IV 0.92.

Anal. Calcd. for $C_{59}H_{83}N_{13}O_{16}$ (1230.35):

C, 57.60; H, 6.80; N, 14.80. Found: C, 57.48; H, 6,53; N, 14.93.

Boc-Gly-Asp(OBzl) (XIV)
Boc-Gly-ONSu (0.44 g, 1.6 mmol) was reacted with Asp(OBzl) (19) (0.45 g, 2.0 mmol) in presence of $KHCO_3$ (0.4 g, 4.0 mmol) in dioxane-water (1:1, 10 ml) following the procedure described for the preparation of IX. The product was obtained as an oil (0.35 g, 60%); R_f II 0.45; R_f III 0.7.

Boc-Glu(OBzl)-Gly-Asp(OBzl) (XVI)
The Boc group in the dipeptide XIV (0.35 g, 0.9 mmol) was cleaved in the usual manner and the resulting hydrochloride was dissolved in water (10 ml) and treated with NMM (0.1 ml, 1.0 mmol). To this was added a solution of Boc-Glu(OBzl)-ONSu (20) (0.32 g, 1.0 mmol) in dioxane (10 ml) and the mixture was stirred for 16 h at room temperature. The product was isolated following the procedure described for the preparation of IX to yield an oil (0.3 g, 55%); R_f II 0.3; R_f III 0.5.

A small quantity of this acid was dissolved in ether, and treated with dicyclohexylamine. The crystalline dicyclohexylamine salt obtained was recrystallized from methanol-ether; m. p. 142–145°; $[\alpha]_D^{24}$ −28.2° (c, 0.14, DMF); R_f III 0.45.

Anal. Calcd. for $C_{42}H_{60}N_4O_{10}$ (780.93): C, 64.70; H, 7.74; N, 7.18. Found: C, 64.86; H, 7.86; N, 7.60.

Boc-Glu(OBzl)-Gly-Asp(OBzl)-Phe-Leu-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl (XVII)
The nonapeptide XII (0.25 g, 0.2 mmol) was deprotected with 3N HCl-THF (6 ml). The hydrochloride obtained was dissolved in DMF (2 ml), neutralized at 0° with NMM (0.02 ml, 0.2 mmol) and stirred with the tripeptide XVI (0.12 g, 0.2 mmol), DCC (0.04 g, 0.2 mmol) and HOBt (0.03 g, 0.2 mmol) for 1 h at 0° and for 16 h at room temperature. The product was isolated following the procedure described for the preparation of XII and recrystallized from DMF-ether (0.21 g, 62%); m. p. 225–226°; $[\alpha]_D^{24}$ −19.1° (c, 0.21, DMF); R_f III 0.8; R_f IV 0.85.

Anal. Calcd. for $C_{84}H_{110}N_{16}O_{23}$ (1711.83): C, 58.90; H, 6.48; N, 13.09. Found: C, 58.48; H, 6.55; N, 13.48.

Boc-Ser(Bzl)-Gly (XIX)
This dipeptide was obtained as an oil by reacting Boc-Ser(Bzl)-ONSu (21) (0.8 g, 2.0 mmol) with Gly (0.15 g, 2.0 mmol) following the procedure described for the preparation of IX (0.72 g, 80%); R_f II 0.6; R_f III 0.75.

Boc-Asp(OBzl)-Ser(Bzl)-Gly (XXI)
The dipeptide XIX (0.72 g, 1.6 mmol) was deprotected and reacted with Boc-Asp(OBzl)-ONSu (21) (0.65 g, 1.6 mmol) as described for the preparation of XVI. The product was obtained as a sticky solid after two precipitations from ethyl acetate-petroleum ether (0.53 g, 50%); $[\alpha]_D^{24}$ −7.4° (c, 0.54, DMF); R_f II 0.55, R_f III 0.65.

Anal. Calcd. for $C_{28}H_{35}N_3O_9$ (557.58): C, 60.30; H, 6.33; N, 7.53. Found: C, 59.82; H, 5.93; N, 7.64.

Z-Ala-Asp(OBzl)-Ser(Bzl)-Gly (XXIII)
The tripeptide XXI (0.28 g, 0.5 mmol) was deprotected and converted into the tetrapeptide XXIII, by reacting with Z-Ala-ONSu (17) (0.16 g, 0.5 mmol) as described for the preparation of XVI. The product was crystallized from ethyl acetate (0.18 g, 55%); m. p. 132–135°; $[\alpha]_D^{24}$ −10.8° (c, 0.32, DMF); R_f II 0.4; R_f III 0.6.

Anal. Calcd. for $C_{34}H_{38}N_4O_{10}$ (662.67): C, 61.62; H, 5.78; N, 8.46. Found: C, 61.62; H, 5.87; N, 8.90.

Z-Ala-Asp(OBzl)-Ser(Bzl)-Gly-Glu(OBzl)-Gly-Asp(OBzl)-Phe-Leu-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(NO_2)-OBzl (XXIV)
The dodecapeptide XVII (0.13 g, 0.075 mmol) was converted into the hydrochloride XVIII, and reacted with the tetrapeptide XXIII (0.07 g; 0.1 mmol) in presence of DCC (0.02 g, 0.1 mmol) and HOBt (0.013 g, 0.1 mmol). The product was isolated as described for the preparation of XII and recrystallized from DMF-ether (0.135 g, 58%); m. p. 230–232°; $[\alpha]_D^{24}$ −21.7° (c, 0.23, DMF); R_f III 0.65; R_f IV 0.7.

Anal. Calcd. for $C_{115}H_{145}N_{20}O_{32}$ (2319.45): C, 59.55; H, 6.31; N, 12.08. Found: C, 59.68; H, 6.42; N, 12.37.

Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg. 2 CH_3COOH (XXV)
The protected hexadecapeptide XXIV (0.08 g) was hydrogenated over Pd-black catalyst (0.05 g)

in acetic acid (2 ml) and water (2 ml) for 40 h. Fresh catalyst (0.05 g) was added and hydrogenation was continued for a further 20 h. The catalyst was filtered, washed with 50% acetic acid and the filtrate evaporated. The residue on t.l.c. (solvent III) showed one major spot followed by two minor spots. This was then dissolved in water (1.5 ml), applied to a column of Sephadex G 25 (30 × 2.5 cm) and eluted with water. Fractions of 3 ml each at a flow rate of 8 ml per h were collected and the desired peptide (as shown by t.l.c.) emerged first. Those fractions in which the peptide was present were pooled and evaporated when the product was obtained as a light brown powder (0.018 g); m. p. 216–219°; $[\alpha]_D^{24}$ −45.25° (c, 1.9 water); The product gave a single ninhydrin positive spot on t.l.c., R_f III 0.48; R_f IV 0.6. On paper chromatography also the peptide showed a single ninhydrin and Sakaguchi positive spot, R_f V 0.44; R_f VI 0.74; R_f VII 0.50. Amino acid analysis of the AP-M digest gave the following values: Ala 1.91, Asp 1.85, Ser 1.0, Gly 5.27, Glu 1.92, Phe 1.17, Leu 0.88, Val 0.89 and Arg 0.78.

Clotting inhibition studies

Fibrinogen solution. Bovine fibrinogen fraction 1 (0.5 g) was stirred with 0.075 M NaCl (50 ml) and 0.075 M Tris buffer (50 ml, pH 7.2) for 15 min and filtered to remove a small amount of insoluble material. From the optical density of this solution measured at 280 nm the concentration of fibrinogen was calculated to be 0.4%.

Thrombin solution. 10 NIH Units of thrombin contained in one vial were dissolved in 0.075 M NaCl (5.0 ml).

Peptide solutions. The protected peptides V, VII, XII and XVII were hydrogenated in methanol acetic acid (1:1) in presence of Pd-black catalyst and the resulting partially protected peptides were treated with TFA to furnish the free peptides, XXVII, XXVIII, XXIX and XXX respectively. The partially protected pentapeptide XXVI was prepared by the hydrolysis of V with NaOH followed by treatment with TFA. These peptides along with the others listed in Table 1 were dissolved in 0.075 M Tris buffer (pH 7.2) and used for clotting inhibition studies.

Assay. The assays were carried out at room temperature by mixing fibrinogen solution (0.2 ml) with the solution of the peptide (0.2 ml) followed by thrombin solution (0.2 ml) and noting the time required for the appearance of visible clot. Control experiments were conducted in a similar manner by adding Tris buffer (0.2 ml) instead of peptide solutions. The results in Table 1 represent the concentration of peptides at which the clotting times are estimated to have doubled.

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Address:

Dr *K. M. Sivanandaiah*
Department of Chemistry
Central College
Bangalore University
Bangalore 560001
India