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Synthesis of the segment (11–23) located in the first tandem repeat of plasma kallikrein: comparative binding studies of this and another segment (328–343) to high-molecular-mass kininogen☆

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ABSTRACT

The synthesis of porcine plasma kallikrein (pPK) segment (11–23), of sequence Phe-Phe-Arg-Gly-Gly-Asp-Val-Ser-Ala-Met-Tyr-Thr-Pro, present in the first tandem repeat sequence of the regulatory chain of PK, has been accomplished following the peptide fragments (5 + 4 + 4) condensation strategy in solution, as well as by fluorenylmethoxycarbonyl solid-phase chemistry. This and another synthetic PK segment of residues (328–343) present in the fourth tandem repeat sequence [Cys(ACM)-Ser-Leu-Arg-Leu-Ser-Thr-Asp-Gly-Ser-Pro-Thr-Arg-Ile-Thr-Tyr] and synthesized by a solid-phase method, were fully characterized by ^{1}H nuclear magnetic resonance, fast atom bombardment mass spectrometry, amino acid composition and reversed-phase high-performance liquid chromatography. Proteolysis of these peptides by either rat PK (rPK) or trypsin resulted in cleavages between Arg↓Gly for pPK (11–23) and between Arg↓Leu and Arg↓Ile for rPK (328–343). Kinetic studies revealed that for peptide pPK (11–23), the catalytic efficiency (k_{cat}/K_m) of rPK is ≃9-fold higher than that of trypsin, but for the other peptide, rPK (328–343), k_{cat}/K_m of trypsin is ≃49-fold higher than that of rPK. The facile cleavage of pPK (11–23) by rPK confirms the Arg^{13}↓Gly^{14} position as the site of autolytic degradation of PK and also explains its special preference for Phe-Phe-Arg sequence.

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Using a synthetic 31-amino acid segment of high-molecular-mass kininogen (HMK-31), representing the most effective PK binding region, we were able to demonstrate that of the two peptides rPK (328–343) and pPK (11–23) only the latter exhibited any significant affinity towards HMK (≃30%). This and other data suggested that although none of the above two peptide segments appeared to represent the true physiological structural domain necessary for optimum binding to HMK, the segment pPK (11–23) partially possesses the structural features for the protein recognition.

INTRODUCTION

The role of plasma kallikrein (PK) along with other proteins, like factor XI, factor XII and high-molecular-mass kininogen (HMK) (a non-enzymatic cofactor) in surface-dependent activation of blood coagulation, fibrinolysis, activation of plasminogen to plasmin, kinin generation and inflammation are well documented in the literature [1–6]. It is known that both PK and factor XI circulate in the blood as a complex with HMK. The binding site of HMK (a 31-amino acid segment, representing residues 194–224) for PK has recently been located [7–9] but a similar domain in PK for HMK has not yet been fully characterized although like factor XI [10], the heavy chain of PK has been implicated in such binding [11,12]. Furthermore, PK is known to undergo autolytic degradation producing a number of lower-molecular-mass bands on sodium dodecyl sulphate polyacrylamide gel electrophoresis [1,2,10,13–15] and this is again believed to occur in the heavy chain. One autolytic site was presumed to be between $Arg^{13}\downarrow Gly^{14}$ since an N-terminally truncated form of PK beginning with the sequence Gly^{14}-Gly^{15}-Asp^{16}- has recently been isolated [15]. Based on this consideration, a segment, pPK(11–23), present in the first tandem repeat sequence and containing the proposed autolytic site, was chosen to further test the evidence of the autolytic cleavage of PK and at the same time to perform comparative binding studies towards HMK with another PK segment peptide (residues 328–343), located in the fourth tandem repeat sequence [11]. Since there is extensive homology in sequence among the PKs isolated from rat (rPK), porcine (pPK) and human (hPK) [1,11,16,17], segments were selected irrespective of their sources of origin. Thus in our current studies, one of the segments selected belongs to pPK and the other to rPK sequence. The chemical syntheses, characterization and the results pertaining to the above studies are presented in the current paper.

EXPERIMENTAL

Materials

All the amino acid derivatives (Institute Armand Frappier, Laval, Canada, and Chemical Dynamics, NJ, USA) are of the L form. Thin-layer (TLC) analyses were performed on precoated silica gel G plates (250 μm, E. Merck), and the peptides were revealed with ammonium molybdate–sulfuric acid, ninhydrin or Sakaguchi color reaction [18]. The following solvent systems were used for ascending TLC (all v/v): chloroform–methanol (2:1) (A), (4:1) (B), (8:1) (C), (12:1) (D), (20:1) (E), *n*-butanol–acetic acid–water (4:1:1) (F), (4:1:5, upper phase) (G), *n*-butanol–acetic acid–water–ethyl acetate (1:1:1:1) (H). Optical rotations were measured with a Perkin-Elmer 141 polarimeter in a 1-cm cell at 25°C. Melting points were uncorrected and recorded in digital electrothermal model. The solid-phase peptide synthesis was carried out using the Pharmacia Biolynx 312 model.

The products were purified by chromatography on silica gel (230–400 mesh, 60 Å, Aldrich) or by semi-preparative high-performance liquid chromatography (HPLC). The semi-preparative runs were performed on Gilson equipment (Model 302) consisting of pumps (6000A and M45), a programmer (720), and a semi-preparative Vydac C_{18} reversed-phase column (30 cm × 1.0 cm I.D.), with a linear gradient of acetonitrile–trifluoroacetic acid (TFA) (0.025%), a flow-rate of 3.0 ml/min, and UV detection at 225 nm. For analytical runs a flow-rate of 1.0 ml/min and a Vydac C_{18} analytical column (30 cm × 0.5 cm I.D.) were employed and even a microgram quantity of a peptide could be easily detected at 0.05 a.u.f.s.

The amino acid analyses were carried out on a

Beckman 120C automatic analyzer following hydrolysis in 6 *M* HCl–phenol for 20 h at 110°C in sealed evacuated tubes.

^{1}H- Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Assoc. Bruker 400-MHz spectrometer in [^{2}H]dimethylsulphoxide unless otherwise stated. Mass spectrometry (MS) was performed on MS-50 HMTCTA in the fast atom bombardment (FAB) mode. The enzymatic activity is expressed in nmol/h of D-Phe-L-Phe-Arg-MCA (MCA is 4-methylcoumaryl-7-amido) hydrolyzing activity measured on a Perkin-Elmer MPF-31 spectrofluorimeter using initial rates. The protein contents were determined by Bio-Rad assay [19].

The following buffer systems were employed in the current study: buffer A: 100 m*M* 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (BES) plus 1 m*M* EDTA, pH 8.0; buffer B: 25 m*M* $NaH_2PO_4 \cdot H_2O$ plus 1 m*M* EDTA, pH 6.0; buffer C: 10 m*M* BES plus 1 m*M* EDTA, pH 8.0.

Syntheses of peptides

A liquid-phase method [18] (shown schematically in Fig. 1) was adopted for the synthesis of pPK(11–23) of sequence Phe-Phe-Arg-Gly-Gly-Asp-Val-Ser-Ala-Met-Tyr-Thr-Pro-OH. The following protecting groups were used during the synthesis: Boc (butyloxycarbonyl) for amino terminal, NO_2 for Arg, OBz (β-O-benzyl) for Tyr, Ser, Thr hydroxyl and Asp carboxyl groups.

N-tert.-Butyloxycarbonyl-phenylalanyl-phenylalanine (I)

Coupling of Boc-Phe-OH (2.65 g, 1.0 mmol) with Phe-OH (1.65 g, 1 mmol) in tetrahydrofuran (THF)–water–$KHCO_3$ with dicyclohexylcarbodiimide (DCC)–N-hydroxysuccinimide (NHSu) yielded crystalline I (ethyl acetate–hexane, 3.41 g, 83%), m.p. 143–144°C, R_{FB} 0.42, $[\alpha]_D$ −1.8° (c6.8, methanol).

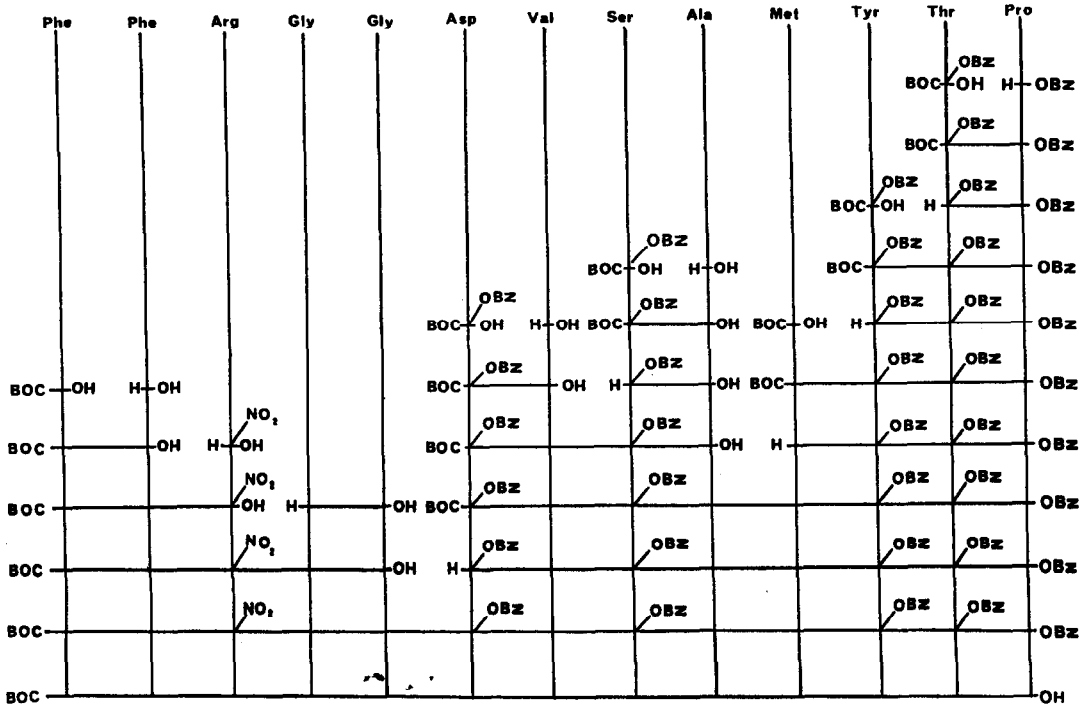

Fig. 1. Synthetic scheme for pPK(11–23)

N-tert.-Butyloxycarbonyl-phenylalanyl-phenylalanyl-ω-nitroarginyl-glycylglycine (III)

Boc-Phe-Phe-OH (I, 2.06 g, 5 mmol) on further coupling with H-Arg(NO_2)-OH (1.1 g, 5 mmol) produced N-*tert.*-butyloxycarbonyl-phenylalanyl-phenylalanyl-ω-nitroarginine (II) as crystal (ethyl acetate–diethyl ether, 2.25 g, 73%), m.p. 148–150°C, R_{FB} 0.21. This (2 g, 3.26 mmol) was again coupled with H-Gly-Gly-OH (0.43 g, 3.26 mmol) to yield upon silica gel column chromatography [26 cm × 2.5 cm I.D., eluted with chloroform–methanol(1:1)] crystalline III (ethanol–diethyl ether, 0.8 g, 42%,), m.p. 147–149°C, $[\alpha]_D$ −9.11° (c6.2, methanol), R_{FA} 0.21; ^{1}H NMR consistent with its structure; FAB-MS, *m/z* 684 ($M^+ + H - CO_2$).

N-tert.-Butyloxycarbonyl-β-O-benzyl-aspartyl-valyl-β-benzyl seryl-alanine (VI)

Two dipeptides, N-*tert.*-butyloxycarbonyl-β-O-benzyl-aspartyl-valine (IV) and N-*tert.*-butyloxycarbonyl-β-O-benzyl-seryl-alanine (V), were first prepared separately from Boc-Asp (OBz)-OH + TFA · H-Val-OH and Boc-Ser (OBz)-OH + alanine, respectively, following carbodiimide-mediated reaction. V (4.75 g, 9.4 mmol) was deprotected with TFA–dichloromethane (DCM) (1:1) and coupled to IV (3.3 g, 7.82 mmol) to furnish N-*tert.*-butyloxycarbonyl-β-O-benzyl-aspartyl-valyl-β-benzyl-seryl-alanine (VI) as crystalline solid (ethyl acetate–hexane, 3.5 g, 67%), m.p. 155-160°C, $[\alpha]_D$ −20.5° (c8.45, methanol), R_{FC} 0.39; ^{1}H NMR, δ 8.36 (d, *J* = 7.7 Hz, CON*H*), 8.20 (br m, 1H, COO*H*), 7.68 (m, 2H, CON*H*), 7.41 (s, 5H, arom), 7.38 (s, 5H, arom), 7.12 (d, 1H, *J* = 4 Hz, BocN*H*), 5.12 (s, 2H, Asp COOCH_2Ph), 4.52 (s, *J* = 6.2 Hz, 2H, Ser OCH_2Ph), 4.32 (br m, 2H, Asp and Val αH), 3.92 (m, 2H, Ser and Ala αH), 2.62 (d, 1H, *J* = 8.8 Hz, Asp βH), 2.05 (octet, 1H, Val βH), 1.37 (s, 9H, Boc), 1.18 (d, 3H, *J* = 6.9 Hz, Ala Me), 0.83 (d, 3H, *J* = 6.5 Hz, Val Me), 0.79 (d, 3H, *J* = 6.5 Hz, Val Me); FAB-MS, *m/z* 671 ($M^+ + H$).

N-tert.-Butyloxycarbonyl-methionyl-β-O-benzyl-tyrosyl-β-O-benzyl-threonyl-proline benzyl ester (IX)

Boc-Thr(OBz)-OH (3.09 g, 10 mmol) on coupling with H-Pro-OBz · HCl (2.42 g, 10 mmol) using DCC–1-hydroxybenzotriazole (HOBT) yielded N-*tert.*-butyloxycarbonyl-β-O-benzyl-threonyl-proline benzyl ester (VII). This was then deprotected with TFA–DCM and coupled to Boc-Tyr(OBz)-OH. The tripeptide thus obtained was similarly deprotected and joined to Boc-Met-OH to furnish N-*tert.*-butyloxycarbonyl-methionyl-β-O-benzyl-tyrosyl-β-O-benzyl-threonyl-proline benzyl ester (IX) as crystal (ethyl acetate–hexane, 1.1 g, 64%), m.p. 121–122°C, $[\alpha]_D$ −35.4° (c4.0, methanol), R_{FE} 0.41; ^{1}H NMR ($CDCl_3$), δ 7.7 (m, 2H, 2 CON*H*), 7.37 (s, 15H, arom), 7.16 (d, 2H, *J* = 8.1 Hz, $Tyr_{3,5}$ H), 6.87 (d, 2H, *J* = 8.2 Hz, $Tyr_{2,6}$ H), 6.5 (br m, 1H, BocN*H*), 5.19 (s, 2H, Pro COOCH_2Ph), 5.02 (s, 2H, Tyr OCH_2Ph), 4.55 (finely splitted d, 2H, Thr OCH_2Ph), 4.5–4.8 (m, 4H, Met, Tyr, Thr, Pro αH), 4.3 (br m, 1H, Thr βH), 3.77 (m, 2H, Pro δH), 2.95 (br d, 2H, *J* = 6 Hz, Tyr βH), 2.44 (t, 2H, *J* = 6.5 Hz, Met γH), 2.09 (s, 3H, Met SCH_3), 1.8–2.1 (m, 2H, Met βH), 1.79 (br s, 4H, Pro βγH), 1.48 (s, 9H, Boc), 1.19 (d, *J* = 6.5 Hz, 3H, Thr Me); FAB-MS, *m/z* 881 (M^+).

N-tert.-Butyloxycarbonyl-β-O-benzyl-aspartyl-valyl-β-O-benzyl-seryl-alanyl-methionyl-β-O-benzyl-tyrosyl-β-O-benzyl-threonyl-proline benzyl ester (X)

Boc-Met-Tyr(OBz)-Thr(OBz)-Pro-OBz (0.3 g, 0.3 mmol) was deprotected with TFA–DCM and coupled to Boc-Asp(OBz)-Val-Ser(OBz)-Ala-OH (VI, 0.206 g, 0.30 mmol), with DCC–HOBT to furnish upon silica gel chromatography (31 cm × 2 cm I.D., eluted with 2% methanol–chloroform) crystalline X (methanol–chloroform, 0.202 g, 46%), R_{FD} 0.54, m.p. 230–235°C, $[\alpha]_D$ −2.60° (c3.15, water); ^{1}H NMR consistent with its structure; amino acid analysis: Asp 0.98 (1), Val 0.97 (1), Ser 1.01 (1), Ala 1.0 (1), Met 0.80 (1), Tyr 0.90 (1), Thr 1.10 (1), Pro 0.96 (1); FAB-MS, *m/z* 1435 ($M^+ + H$).

N-tert.-Butyloxycarbonyl-phenylalanyl-phenylalanyl-ω-nitroarginyl-glycyl-glycyl-β-O-benzyl-aspartyl-valyl-β-O-benzyl-seryl-alanyl-methionyl-β-O-benzyl-tyrosyl-β-O-benzyl-threonyl-proline benzyl ester (XI)

Octapeptide (X, 0.1 g, 0.069 mmol) on deprotection with TFA–DCM and coupling to Boc-Phe-Phe-Arg(NO_2)-Gly-Gly-OH (III, 50 mg, 0.069 mmol) furnished upon silica gel column chromatography (26 cm × 1.8 cm, I.D., eluted with 20% methanol–chloroform) XI, (0.72 g, 48%), R_{FC} 0.38, m.p. 228–230°C. ^{1}H NMR spectrum is fully consistent with its structure.

Deprotection and purification of the final peptide

The protected tridecapeptide (XI, 17 mg) on hydrogenolysis [H_2/Pd-black (50 mg) for 16 h] in N,N-dimethylformamide (DMF)–methanol–acetic acid (1:1:0.1) and reversed-phase HPLC yielded XII [retention time (t_R) 46.7 min, 6 mg, 52%], R_{FG} 0.61, $[\alpha]_D$ −16.8° (c3.15, water); ^{1}H NMR, δ 8.5–7.8 (br m, 11 CON*H*), 7.35–7.15 (m, 10H, arom), 6.98 (m, 2H, $Tyr_{3,5}$ H), 6.9 (s, 1H, BocN*H*), 6.59 (br s, 2H, $Tyr_{2,6}$ H), 4.7–4.1 (m, 10αH), 3.75 (br s, 4H, 2Gly αH), 3.60–3.57 (m, 3H, Ser and Thr βH), 3.15 (br s, 2H, Arg δH), 3.07 (m, 2H, Tyr βH), 2.82* (m, 4H, 2Phe βH), 2.66* (m, 2H, Pro δH), 2.60* (m, 2H, Asp βH), 2.13–2.0 (m, 3H, Met βH + Val βH), 2.09 (m, 2H, Met γH), 1.90 (s, 3H, Met SC*H*$_3$), 1.85 (m, 4H, Pro βγH), 1.70–1.45 (m, 4H, Arg βγH), 1.27 (s, 9H, Boc), 1.19 (br s, 3H, Thr Me), 1.10 (s, 3H, Ala Me), 0.81 (near q, 6H, Val 2Me), (*interchangable); FAB-MS, *m/z* 1547 (M^+ + H).

The protecting Boc group was finally removed to furnish pPK(11–23) (XIII), identical by HPLC, ^{1}H NMR and FAB-MS with that prepared by solid-phase method using fluorenylmethoxycarbonyl (Fmoc) chemistry where the protecting groups employed were 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) for Arg and *tert.*-butyl for Asp, Ser, Thr and Tyr. Reversed-phase HPLC: t_R = 26.3 min. Amino acid analysis: Phe 2.03 (2), Arg 1.08 (1), Gly 2.03 (2), Asp 1.13 (1), Val 1.08 (1), Ser 0.97 (1), Ala 1.09 (1), Met 1.23 (1), Tyr 0.82 (1), Thr 0.98 (1), Pro 0.85 (1).

Synthesis of rPK(328–343)

This peptide, H-Cys(ACM)-Ser-Leu-Arg-Leu-Ser-Thr-Asp-Gly-Ser-Pro-Thr-Arg-Ile-Thr-Tyr-OH, was synthesized by a solid-phase method, purified and characterized by reversed-phase HPLC (t_R = 34.1 min); FAB-MS, *m/z* 1841 (M^+ + H); amino acid analysis: Asp 1.0 (1), Thr 2.92 (3), Ser 2.84 (3), Pro 1.02 (1), Gly 1.12 (1), Cys 0.6 (1), Ile 0.97 (1), Leu 1.98 (2), Tyr 0.86 (1) and Arg 2.11 (2).

Synthesis of HMK-31

This peptide of sequence Ser-Asp-Asp-Asp-Trp-Ile-Pro-Asp-Ile-Gln-Thr-Asp-Pro-Asn-Gly-Leu-Ser-Phe-Asn-Pro-Ile-Ser-Asp-Phe-Pro-Asp-Thr-Thr-Ser-Pro-Lys (molecular mass = 3567) was also prepared by solid-phase chemistry and characterized by amino acid analysis and MS.

Immobilization of HMK-31 on affi-Gel 15

HMK-31 (11 mg, 3.08 μmol) was immobilized at 4°C on affi-Gel 15 (5 ml packed gel, active ester content 9 μmol/ml) in 0.1 *M* $KHCO_3$ (5 ml) according to the manufacturer's procedure. The coupling was followed by reversed-phase HPLC and was found to be complete in 2 h. This yielded a gel containing ≃0.60 μmol HMK-31 per ml.

Affinity of HMK-31 immobilized gel towards rPK activity

Partially purified crude preparations of rPK [15] (enzymatic activity = ≃4500 nmol/h) was shaken with HMK-31 immobilized gel (1 ml, concentration of ligand 0.6 μmol/ml) at 4°C for 2h. The binding was monitored by following the enzymatic activity in the supernatant at each 15-min interval.

Determination of kinetic parameters K_m, V_{max} and k_{cat} for the enzymatic digestions of the synthetic peptides

Rate assays for the determination of kinetic constants for the digestions of the synthetic peptides were started by the addition of 20 μl of solution containing ~75 ng of active trypsin or ~250 ng to 3 μg of active rPK to 480 μl of buffer A or buffer B containing various amounts of the syn-

TABLE I

FAB MS DATA ON THE PEPTIDES

No.	Peptide	Molecular mass	Peaks at m/z
1	Boc-Phe-Phe-Arg-Gly-Gly-Asp-Val- -Ser-Ala-Met-Tyr-Thr-Pro-OH; pPK(11–23); XII	1546	1547 (M^+ + H), 1532 (M^+ + H − Me), 1515 (M^+ − CH_2OH), 1485 (M^+ + H − Me − SCH_3), 1447 (M^+ + H − Boc), 738 (X^a − C ≡ O − Gn), 723 (X^a − Gn − CH_3COOH), 638 (738 − Boc)
2	Boc-Asp(OBz)-Val-Ser(OBz)-Ala- -Met-Tyr(OBz)-Thr(OBz)-Pro-OBz; X	1434	1435 (M^+ + H), 1417 (M^+ + H − H_2O), 1344 (M^+ − Bz), 1335 (M^+ + H − Boc)
3	Boc-Asp(OBz)-Val-Ser(OBz)-Ala-OH; VI	670	709 (M^+ + K), 671 (M^+ + H), 655 (M^+ − Me), 627 (M^+ + H − $C0_2$), 609 (M^+ + K − Boc), 571 (M^+ + H − Boc)
4	Boc-Met-Tyr(OBz)-Thr(OBz)-Pro-OBz; IX	880	881 (M^+ + H), 676 (M^+ +H − Pro OBz), 576 (M^+ + H − Pro OBz − Boc) 397 [H_3N^+ − Thr (OBz) − Pro OBz], 306 (397 − Bz), 206 (H_3N^+ − Pro OBz)
5	Boc-Phe-Phe-Arg(NO_2)-Gly-Gly-OH; III	727	684 (M^+ + H − CO_2), 653 (M^+ − Gly), 640 [M^+ + H − Gn(NO_2)], 584 (M^+ + H − CO_2 − Boc)
6	Cys(ACM)-Ser-Leu-Arg-Leu-Ser- -Thr-Asp-Gly-Ser-Pro-Thr-Arg- -Ile-Thr-Tyr-OH; rPK(328–343)	1840	1841 (M^+ + H)

[a] X = Boc-Phe-Phe-Arg-Gly-Gly-Asp-C ≡ O^+.

thetic peptides ranging from 2 to 320 μg. The amount of reactions, terminated after 3 h of digestion at 25°C by adding 100 μl of 50% TFA–buffer, was determined by HPLC and K_m and V_{max} were estimated by Michealis–Menten kinetics following the computer-assisted algorithm as described [20]. k_{cat} was calculated by dividing V_{max} with the molar concentration of active enzyme. Active site titration [21] of trypsin indicated it to be about 80% active by mass. For rPK, the amount of active enzyme was estimated from our earlier work [15,17].

Radioiodination of rPK(328-343) and Boc-pPK-(11–23)

Both the synthetic peptides, rPK(328–343) and pPK(11–23), were radioiodinated [12,13] and purified by reversed-phase HPLC. The t_R for cold and hot Boc-pPK(11–23) were 46.7 min (52% acetonitrile) and 47.2 min (52% acetonitrile) respectively, whereas for cold and hot rPK(328–343) they were 34.1 min (39% acetonitrile) and 36 min (41% acetonitrile), respectively.

Enzymatic digestion of synthetic pPK(11–23) and rPK(328–343) by rPK and trypsin in the absence or presence of HMK-31

Synthetic Boc-pPK(11–23) or rPK(328–343) (7.8 nmol) was incubated at 25°C for 3 h with either trypsin or rPK (enzymatic activity = 600 nmol/h) in buffer A (for PK) or buffer B (for trypsin) (500 μl). The digestion of rPK(328–343) by trypsin was followed after every hour. In the rest of the cases, the digestions were monitored after each successive addition of enzymes (activity = 600 nmol/h) at intervals of 3, 6, 9 and 18 h. For evaluation of the reaction, 100 μl, equivalent to ~5 μg of the peptide, were injected into the HPLC column.

The extent of above digestions were also investigated by reversed phase HPLC after 3-h prior incubations of the peptides (7.8 nmol) with HMK-31 (7.8 nmol).

Affinity of pPK(11–23), rPK(328–343) and the corresponding ^{125}I-labeled materials towards HMK-31-bound affi-Gel

Boc-pPK(11–23), rPK(328–343) (≃0.6 μmol) or their ^{125}I-labeled materials (≃1 · 10^6 cpm of radioactive counts representing ≃16 pmol) were shaken separately overnight in either buffers A or C, pH 6.0 or 8.0 (1.0 ml) with HMK-31 immobilized on affi-Gel 15 (1.0 ml packed gel, concentration = ≃0.60 μmol of ligand per ml of gel) in a rotary shaker at 4°C. The percentage of peptide bound was measured either by reversed-phase HPLC (for cold material) or by radioactive counts (for ^{125}I-labeled material) of an aliquot of the supernatant.

Effects of synthetic pPK(11–23) and rPK(328–343) on the binding affinity of rPK to affi-Gel 15–HMK-31 column

rPK (5 μl, ~0.28 μg, ~4510 nmol/h enzymatic activity) in buffer A plus 50% glycerine (500 μl) was shaken with HMK-31-bound affi-Gel 15 (1 ml packed gel) at 25°C for 3 h, centrifuged, and an aliquot of 100 μl was withdrawn from the supernatant for enzymatic assay. The above experiment was repeated with 3 h prior incubation of the gel with either synthetic pPK(11–23) or rPK (328–343) (0.6 μmol), and the progress of binding was followed by both HPLC and enzymatic assay. A control experiment was also performed using ethanolamine-bound affi-Gel 15 column.

RESULTS AND DISCUSSION

FAB-MS data

Boc-pPK(11–23) (XII) exhibited a weak (M^+ + H) peak at *m/z* 1547, commensurate with its molecular structure (Table I). The weak intensity is explained by its relatively high hydrophobicity value (−139.0, based on Bull and Breese index model [22]). XII also displayed important peaks at *m/z* 1532 (M^+ + H − Me), 1515 (M^+ − CH_2OH), 1485 (M^+ + H − Me − SCH_3) and 1447 (M^+ + H − Boc). Two intense peaks at *m/z* 738 and 722 are believed to be originated from the cleavage of Asp↓Val bond following the loss of HCHO (of serine side-chain) from one fragment and guanidine (of arginine side-chain) from the other. Similar fragmentation was also noticed for the free peptide (XIII) (M^+ + H =

TABLE II

ENZYMATIC DIGESTION OF SYNTHETIC pPK(11–23) AND rPK(328–343) IN THE ABSENCE OR PRESENCE OF HMK-31

Enzyme used	Enzymatic acitvity[a] (nmol/h)	Enzymatic activity/peptide (nmol/h)/nmol	Percentage of peptide cleaved[b]				Percent of peptide cleavage affected by HMK-31	
			pPK(11–23)		rPK(328–343)			
			No HMK-31	With HMK-31[c]	No HMK-31	With HMK-31[c]	Inhibition for pPK(11–23)	Activation for rPK(328–343)
Trypsin	611	78	4.2		98			
	1222	156	40		ND[d]			
	1833	234	59		ND[d]			
rPK	611	78	59	41	9	11.0	30.5	22
	1222	156	62		22			
	1833	234	63		39			
	2444	312	–		90			

[a] The enzymatic activity was assayed by monitoring the fluorescence of released AMC from D-Phe-L-Phe-Arg-MCA.

[b] The cleavage of the peptide was estimated by HPLC after an incubation period of 3 h at 25°C each time. A fresh solution of enzyme of 611 nmol/h activity was added after each incubation. A buffer system consisting of 100 m*M* BES plus 1 m*M* EDTA, pH 8.0 was used for the digestion by rPK and 0.025 *M* NaH_2PO_4 plus 1 m*M* EDTA, pH 6.0 for the same by trypsin.

[c] Synthetic HMK-31 of 1:1 molar ratio with respect to the peptide and a 3-h period of incubation of the peptide were used.

[d] Not determined.

1447). The FAB-MS of synthetic rPK(328–343) ($M^+ + H = 1841$) in chemical ionization mode exhibited peaks corresponding to the cleavage -CH↓CO-NH- of each peptide bond, the intensity decreasing gradually with the number of amino acids in the fragment ion.

^1H NMR data

^1H NMR spectra of the two synthetic peptides (see Experimental) are fully consistent with their structures. The removal of benzyl ether/ester-protecting groups during solution phase preparation of pPK(11–23) could be easily monitored by following the disappearence of signals for benzylic protons in the ^1H NMR spectrum (δ 5.07 for Asp, 5.11 for Pro, 5.0 for Tyr, 4.55 for Thr and 4.52 for Ser).

Enzymatic digestions of synthetic Boc-pPK(11–23) and rPK(328–343) with trypsin and rPK

Digestion of Boc-pPK(11–23) (XII) with rPK [16,19] yielded Boc-Phe-Phe-Arg-OH and H-Gly-Gly-Asp-Val-Ser-Ala-Met-Tyr-Thr-Pro-OH as established by HPLC (Fig. 2), amino acid composition and sequencing suggesting a cleavage between the Arg↓Gly residues. The identity of one of them, Boc-Phe-Phe-Arg-OH, was further confirmed by chemical synthesis and coelution on HPLC. To simplify the purification we have kept the Boc functionality on the N-terminal since, when this group is removed, the resulting free peptide elutes much earlier due to a decreased hydrophobicity (Fig. 2). There was 59% cleavage when the ratio of enzymatic activity (nmol/h)/peptide (nmol) was ~78:1. Addition of further enzyme or extension of incubation time did not have any significant effect on the percentage cleaved (Table II). A comparative Michealis–Menten kinetic analysis (as shown in Fig. 3 for the digestion of pPK(11–23) by rPK) of the cleavage of Boc-pPK(11–23) shows that rPK is about 9-fold more potent than trypsin in cleaving this peptide following the Phe-Phe-Arg↓ sequence (Table III).

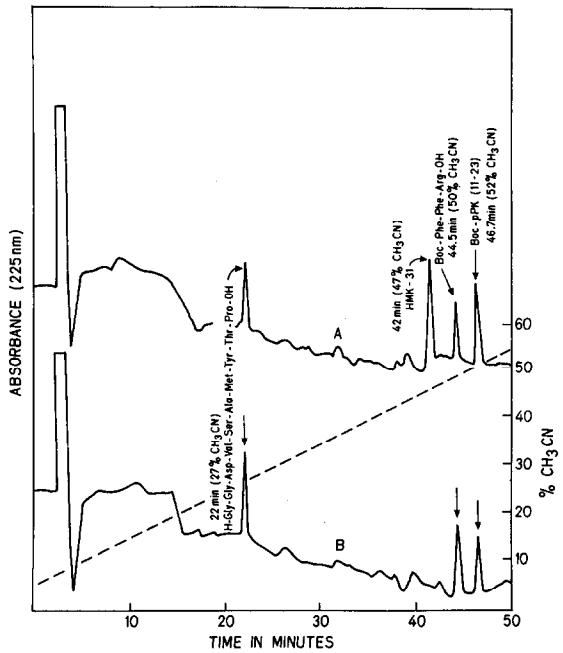

Fig. 2. Reversed-phase HPLC chromatograms of enzymatic digestions of Boc-pPK(11–23) by rPK on a Vydac C_{18} analytical column in the presence (A) or absence (B) of HMK-31 under the gradient profile as depicted (see Experimental). Incubation time for each digestion: 3 h at 25°C.

Digestion of synthetic rPK(328–343) by either trypsin or rPK yielded three cleavage products [H-Cys(ACM)-Ser-Leu-Arg-OH, rPK(328–331); H-Leu-Ser-Thr-Asp-Gly-Ser-Pro-Thr-Arg-Ile-Thr-Tyr-OH, rPK(332–343); and H-Cys(ACM)-Ser-Leu-Arg-Leu-Ser-Thr-Asp-Gly-Ser-Pro-Thr-Arg-OH, rPK(328–340); ACM = acetomethyl] as determined by amino acid composition and sequencing indicating a marked preference for the cleavage between Arg↓Leu (major product) as compared to Arg↓Ile (minor product) (Fig. 4). Furthermore, rPK is 33-fold more potent in cleaving pPK(11–23) than rPK(328–343), in contrast to trypsin which exhibits a 13-fold higher K_{cat}/K_m for the cleavage of rPK(328–343) as compared to pPK(11–23) (Table III). For cleavage of rPK(328–343), trypsin is 49-fold more potent than rPK. The facile cleavage of pPK(11–23) by rPK supports our previous hypothesis [15] that the isolation of a partially cleaved form of rPK beginning with the sequence Gly^{14}-Gly-Asp... is due to autolysis at the Arg^{13}↓Gly^{14} site in rPK, and it emphasizes the special preference of rPK for the cleavage of Phe-Phe-Arg↓ sequences.

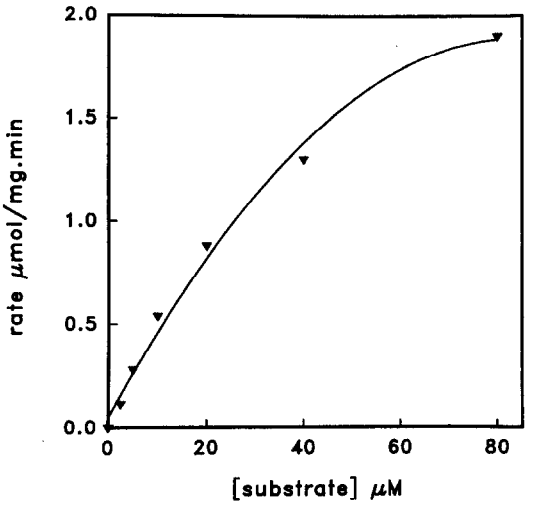

Fig. 3. Kinetic studies on the digestion of synthetic pPK(11–23) peptide by rPK.

Comparative studies on the affinity of synthetic pPK(11–23) and rPK(328–343) towards HMK-31

The binding affinity of HMK-31 towards rPK was displayed when it was observed that HMK-31-immobilized gel was able to bind the enzymatic activity of rPK almost quantitatively within 2 h of shaking at 4°C. The majority of this activity (>60%) could be recovered by elution with a mixture consisting of 1 *M* guanidine · HCl, 1 *M* NaCl, 100 m*M* 2-(N-morpholino)ethanesulfonic acid (MES), 1 m*M* EDTA, pH 8.0. The above column was also able to bind 25–30% of pPK(11–23) in either buffer A or C, pH 6.0 or 8.0 at 25°C, as observed with both cold and ^{125}I-labeled material compared to only <1% for rPK (328–343) (see Experimental). For binding studies with the cold peptides, an equimolar ratio of pPK(11–23) or rPK(328–343) with HMK-31 immobilized on the gel (in this case 0.60 µmol) was employed since PK is known to form a 1:1 complex with HMK. A control ethanolamine-immobilized affi-Gel 15 column did not bind either of these peptides, cold or ^{125}I-labeled (<0.2%). This observation suggested some degree of affinity between pPK(11–23) and HMK-31. When the same experiment was repeated in buffers containing 1 *M* Gn · HCl, 1 *M* NaCl, 1 m*M* EDTA, pH 8.0 or 6.0, no significant binding was observed as expected, since, as already shown above, such high-ionic-strength buffers containing chaotropic reagents may disrupt the bond between the peptide and HMK-31.

The above notion is also supported by the fact that the enzymatic digestion of Boc-pPK(11–23) by rPK can be partially retarded (30%) by prior incubation of the peptide with HMK-31 (Table II and Fig. 2). In contrast, under identical condition, prior incubation of synthetic rPK(328–343) with HMK-31 led to 22% more cleavage by rPK (Table II and Fig. 4). This can be explained by our observation that enzymatic activity of rPK as measured by the cleavage of D-Phe-Phe-Arg-MCA, a potent fluorogenic substrate of rPK, increased linearly upon incubation with HMK-31 alone (4.9% per nmol of HMK-31 added) (Fig. 5). Such an effect was previously noted during the binding of rPK with HMK [13]. Because of the fact that a concentration of 7.8 nmol of HMK-31

TABLE III

KINETIC PARAMETERS FOR THE PROTEOLYTIC DIGESTIONS OF SYNTHETIC rPK(328–343) AND pPK(11–23) WITH TRYPSIN AND PLASMA KALLIKREIN

Substrate	Enzyme	Enzyme concentration (µmol/l)	V_{max} (µmol/mg · s)	k_{cat} (s^{-1})	K_m (*M*)	k_{cat}/K_m (M^{-1} s^{-1})
rPK(328–343)	Trypsin	0.007	1.118	159.7	$5.42 \cdot 10^{-4}$	$2.95 \cdot 10^{5}$
rPK(328–343)	rPK	0.07	0.133	1.9	$3.14 \cdot 10^{-4}$	$6.05 \cdot 10^{3}$
pPK(11–23)	Trypsin	0.007	0.012	1.7	$7.47 \cdot 10^{-5}$	$2.29 \cdot 10^{4}$
pPK(11–23)	rPK	0.005	0.0515	10.3	$5.16 \cdot 10^{-5}$	$2.0 \cdot 10^{5}$

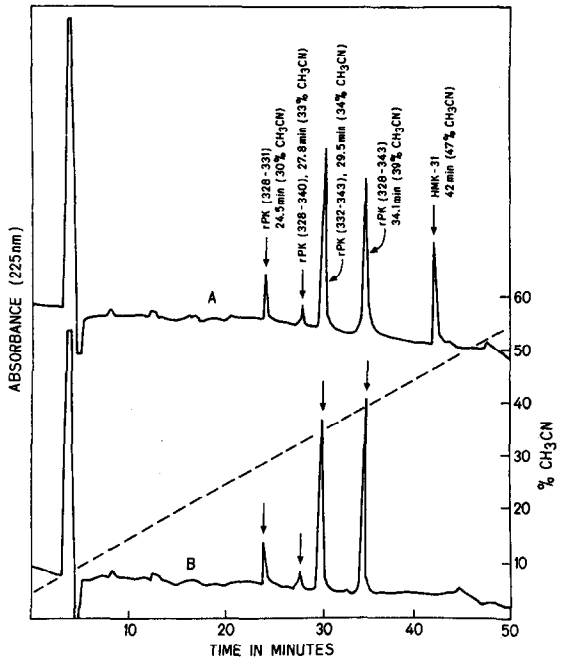

Fig. 4. Reversed-phase HPLC profile of enzymatic digestion of synthetic rPK(328–343) by rPK on a Vydac C_{18} column in the presence (A) or absence (B) of HMK-31, all other conditions being same as before.

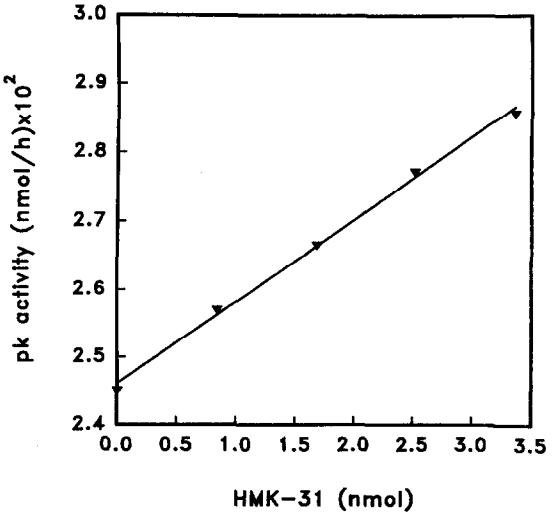

Fig. 5. Effect of synthetic HMK-31 on the enzymatic activity of rPK, as followed by the cleavage of fluorogenic substrate, D-Phe-Phe-Arg-MCA.

was used in the above experiment, one would expect an increase of about 38% (4.9 × 7.8) of cleavage of the peptide if there is no association between the peptide and the enzyme. The observed 22% increase in cleavage may be interpreted as indicating the near absence of a significant association between rPK(328–343) and rPK, whereas the 30% increase in cleavage for pPK(11–23) implies some degree of affinity between rPK and pPK(11–23). It was further noted that the binding of rPK to immobilized HMK-31 is affected only by prior incubation with synthetic pPK(11–23) (≃26% inhibition) and not by rPK (328–343) (Table IV) also suggesting some affinity (26%) between pPK(11–23) and HMK-31. It is important to note that as expected from its amino acid sequence, HMK-31 is not a substrate but only a ligand whereas the two synthetic peptides are substrates and not ligands for rPK.

It is interesting to note that the HMK binding domain of factor XI [2], a protein of very closely related structure and function to PK [1,11], resides in the segment 55–86 located in the first tandem repeat of factor XI [10]. As shown in Fig. 6, the presence of a 54% similarity (8% identity) with hPK(59–71), may explain why pPK(11–23) only partially binds to HMK. Furthermore, as estimated from Kyte and Doolittle [23] hydropathy calculations, both peptides possess a common feature of high hydrophilicity in the central region of the sequence (data not shown). The data presented in this work suggest that of the two synthetic peptides, only pPK(11–23) possesses some elements common to the PK binding domain. Two domains of PK were implicated previously [12] for such a binding, namely one which includes the segment 55–86 and the second one 141–371. Our data demonstrated that the rPK (328–343) peptide did not show any binding to HMK. Therefore, the fact that this peptide is included in the hPK(141–371) segment which was suggested to contain two distinct binding sites, one for HMK and the other for Factor-XII, reveals that sequences other than those found in the segment 328–343 are involved in the binding of PK to HMK.

TABLE IV

EFFECT OF PRIOR INCUBATION WITH SYNTHETIC pPK(11–23) AND rPK(328–343) ON THE AFFINITY OF rPK TOWARDS HMK-31-BOUND GEL

Volume of affi-Gel 15-bound HMK-31 used (ml)	Concentration of HMK-31 in the gel (μmol/ml)	Peptide used	Amount taken (μmol)	Total enzymatic activity loaded (nmol/h)	Enzymatic activity in the flow through plus buffer wash (nmol/h)	Percentage of enzymatic activity bound on the column	Percentage of binding inhibited by the peptide
1.0	0.6	None		4510	1014	78	NA[a]
1.0	0.6	rPK(328–343)	0.6	4510	983	79	0
1.0	0.6	pPK(11–23)	0.6	4510	2165	52	26

[a] Not applicable.

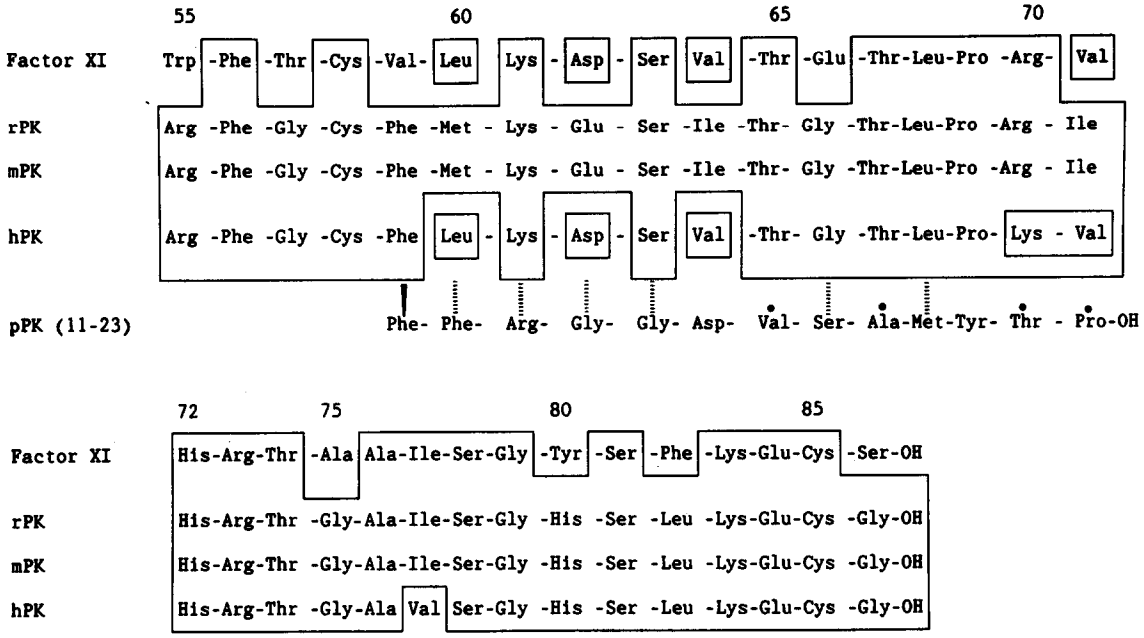

Fig. 6. Comparison of the amino acid sequences of human (h), rat (r), mouse (m) plasma kallikrein (PK) and factor XI of segments between residues 55–86. The sequences of hPK, rPK, mPK were taken from Chung *et al.* [1], Fujikawa *et al.* [2] and Seidah *et al.* [11]. Identical residues are placed within boxes. By expressing sequence homology as the percentage of identical residues *versus* analysed residues, the 55–86 segment of hPK was found to be 65.5% homologous to factor XI counterpart whereas the same belonging to mPK or rPK was 60% homologous. Synthetic pPK(11–23) peptide was aligned along the position (residues 59–71) of hPK. Some similarity could be observed between the two. The solid and broken lines indicate identical or similar groups, respectively, between the above two.

CONCLUSION

Our data reinforce the notion that the observed *in vivo* cleavage of the peptidic bond Arg^{13}–Gly^{14}- of PK is probably through an autolysis mechanism. Furthermore, these results indicate that a better recognition by PK is enhanced by the presence of two highly hydrophobic groups (Phe) at P-2 and P-3 positions (with respect to the scissile peptide bond). Based on the results so far presented, it may be concluded that, although neither of the two selected peptide segments located in the first and the fourth tandem repeat of PK represent the correct physiological domain of PK for optimum binding to HMK, the study did point out that the region 11–23 of first tandem repeat of PK contains some but not all the necessary primary structural elements of the binding domain. Future studies should clarify the exact segment within the 141–371 sequence of PK which is involved in the binding to HMK. Also the 31-residue synthetic peptide, HMK-31, may find use in future affinity purification procedure for purification of crude preparations of plasma kallikreins.

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REFERENCES

1 W. D. Chung, K. Fujikawa, B. A. McMullen and E. W. Davie, *Biochemistry*, 25 (1986) 2410.
2 K. Fujikawa, D. W. Chung, L. E. Hendriekson and E. W. Davie, *Biochemistry*, 25 (1986) 2417.
3 D. D. Revak, C. G. Cochrane, A. R. Johnson and T. E. Hugli, *J. Clin. Invest.*, 54 (1974) 619.
4 K. Fujikawa, R. L. Heinmark, K. Kurachi and E. W. Davie, *Biochemistry*, 19 (1980) 1322.
5 S. D. Revak, C. G. Cochrane and J. H. Griffin, *J. Clin. Invest.*, 59 (1977) 1167.
6 J. H. Griffin and C. G. Cochrane, *Semin. Thromb. Hemost.*, 5 (1979) 254.
7 J. F. Tait and K. Fujikawa, *J. Biol. Chem.*, 261 (1986) 15 396.
8 P. E. Bock, J. D. Shore, G. Tans and J. H. Griffin, *J. Biol. Chem.*, 60 (1985) 12 434.
9 D. M. Kerbiriou, B. N. Bouma and J. H. Griffin, *J. Biol. Chem.*, 255 (1980) 3952.
10 F. A. Baglia, B. A. Jameson and P. N. Walsh, *J. Biol. Chem.*, 265 (1990) 4149.
11 N. G. Seidah, R. Ladenheim, M. Mbikay, J. Hamelin, G. Lutfalla, F. Rougeon, C. Lazure and M. Chrétien, *DNA*, 8 (1989) 563.
12 J. D. Page and R. W. Coleman, *J. Biol. Chem.*, 266 (1991) 8143.
13 J. F. Tait and K. Fujikawa, *J. Biol. Chem.*, 262 (1987) 11 651.
14 J. A. Cromlish, N. G. Seidah and M. Chrétien, *Neuropeptides*, 5 (1985) 493.
15 J. Paquin, S. Benjannet, N. Sawyer, C. Lazure, M. Chrétien and N. G. Seidah, *Biochim. Biophys. Acta*, 999 (1989) 103.
16 J. A. Cromlish, N. G. Seidah and M. Chrétien, *J. Biol. Chem.*, 261 (1986) 10 850.
17 J. A. Cromlish, N. G. Seidah and M. Chrétien, *J. Biol. Chem.*, 261 (1986) 10 859.
18 A. Basak, Y. T. Gong, J. A. Cromlish, J. Paquin, F. Jean, N. G. Seidah, C. Lazure and M. Chrétien, *Int. J. Pept. Protein Res.*, 36 (1990) 7.
19 Bio-Rad Co., *Chromatography, Electrophoresis, Immunochemistry and HPLC Catalog*, 1986, p. 80 and also Bulletin No.1069.
20 H. U. Bergmeyer (Editor), *Methods of Enzymatic Analysis*, Vol. I, Verlag Chimie, Basel, 3rd ed., 1965, p.86.
21 H. U. Bergmeyer (Editor), *Methods in Enzymatic Analysis*, Vol. II, Verlag Chimie, Basel, 3rd ed., 1983 p.320.
22 B. H. Bull and K. Breese, *Arch. Biochem. Biophys.*, 161 (1974) 665.
23 J. Kyte and R. F. Doolittle, *J. Mol. Biol.*, 157 (1982) 105.