COLOUR-MONITORED SOLID-PHASE MULTIPLE PEPTIDE SYNTHESIS UNDER LOW-PRESSURE CONTINUOUS-FLOW CONDITIONS. SYNTHESIS OF MEDIUM-SIZE PEPTIDES: THE PROPART OF HUMAN PROCATHEPSIN D AND THE GROWTH-HORMONE RELEASING FACTOR

Josef VÁGNER[a], Viktor KRCHŇÁK[a], Josef PÍCHA[a], Drahomíra PÍCHOVÁ[a] and Martin FUSEK[b]

[a] *Research Institute for Feed Supplements and Veterinary Drugs, 254 49 Jílové near Prague*

[b] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6*

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The synthesis of two medium-size peptide amides, the propart of human procathepsin D (1-45) and the bovine growth-hormone releasing factor (1-44) has been accomplished by the solid-phase multiple peptide synthesis under low-pressure continuous-flow conditions on standard methylbenzhydrylamine polystyrene-based resin in concatenated flow reactors with adjustable volume. The completeness of acylation of resin-bound free amino groups was monitored by the acid-base indicator bromophenol blue. The efficiency of the coupling reaction was increased by exposure to elevated temperature in an ultrasonic bath. Crude peptides were purified on a mass-overloaded Vydac C18 reversed-phase semipreparative HPLC column. The synthetic propart of human procathepsin D (1-45) exhibited inhibition activity to bovine cathepsin D and to four other aspartic proteases. Significantly increased plasma concentration in gilts was determined after application of synthetic bovine growth-hormone releasing factor (1-44).

We have recently described a procedure for continuous-flow solid-phase multiple peptide synthesis[1,2] that requires very simple instrumentation and provides the capacity for synthesizing 10 peptides simultaneously with good results both in respect of the yield and purity of crude peptides. The method was routinely used for syntheses of small-size peptides (i.e. up to 25 amino acid residues). To exemplify the utility of this configuration of multiple peptide synthesis for the synthesis of medium-size peptides we prepared a fragment of the propart of human procathepsin D (1-45) (ref.[3]) and bovine growth-hormone releasing factor (1-44) (for amino acids sequences see Table I). The course of the acylation reaction was monitored by an acid-base indicator, bromophenol blue[4]. The favourable effect of elevated temperature and ultrasonic bath[5,6] is demonstrated on difficult couplings. The peptides were purified on mass-overloaded analytical reversed-phase column[1].

EXPERIMENTAL

General Methodology

Solvents (dichloromethane, technical grade (Lachema, Brno)), all others analytical grade (Fluka, Buchs, Switzerland)) were redistilled before use and stored over type 4A molecular sieves (Fluka, Buchs, Switzerland). Trifluoracetic acid (TFA, Riedel de Haen, Seelze, Germany) was redistilled and used without drying. Other chemicals were used as supplied: N,N-dimethylacetamide (DMA), N-methylpyrrolidone, piperidine, N-hydroxybenzotriazole (HOBt), N,N'-diisopropylcarbodiimide (DIC), bromophenol blue, hydrogen fluoride (HF), *p*-cresol, *p*-thiocresol, (all Fluka, Buchs, Switzerland), *p*-methylbenzhydrylamine copoly(styrene–1% divinylbenzene) resin (MeBHA resin, 0.4 meq/g, Chemical Dynamics Co., South Plainfield, New Jersey). Side chain functional groups for Fmoc/t-Bu strategy were protected as follows: Asp, Glu, Ser, and Thr by the tert-butyl group; Arg, by the 4-methoxy-2,3,6-trimethylbenzenesulfonyl; His, by the trityl group; and Lys, by the Boc group (Bachem, Bubendorf, Switzerland). Pre-coated Silikagel 60 F_{254} plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography, the detection being performed by chlorine–toluidine.

Apparatus

An analytical chromatograph was assembled from three pumps Waters 510, autosampler WISP 712, diode array detector Waters 991, and PowerMate personal computer (Waters, Milford, Massachusetts). A preparative chromatograph was assembled from two pumps Waters 510, Rheodyne 9125 valve with 2 ml sample loop, tunable UV detector Waters 484, fraction collector Isco Foxy, and PowerMate personal computer (Waters, Milford, Massachusetts). Analytical (4 × 250 mm) and semi-preparative (8 × 250 mm) columns were packed with Vydac C18 RP 10 μm sorbent (Vydac, Hesperia, California).

Analytical runs were carried out on a 4 × 250-mm column, flow-rate 1.0 ml/min, detection by UV absorption at 200 – 300 nm, sample injection 40 μl, concentration of sample 1 mg/ml, gradient slope 1% methanol or acetonitrile/min. Gradient was formed from solvent A containing 0.1% aqueous TFA, and solvent B containing 80% MeOH or acetonitrile in water and 0.07% TFA. The composition of the mobile phase is expressed in vol.%.

Samples for amino acid analysis were hydrolyzed in 6M-HCl at 110 °C for 24 h in the presence of phenol in evacuated sealed tubes and analyzed on an amino acid analyzer type AAA 881 (Mikrotechna, Praha). An ultrasonic compact cleaner type UC 005 AJ1 (TESLA, Praha) was used to accelerate the coupling reaction. Fast-atom-bombardment mass spectrometry (FAB MS) was carried out on a ZAB EQ spectrometer (VG Analytical, Manchester, U.K.), the bombarding gas being xenon at 8 kV. Automatic Edman degradation was carried out in Model 470A Gas-phase Protein Sequencer (Applied Biosystem, Foster City, California). The phenylthiohydantoins were identified by HPLC (Beckman Liquid Chromatography System 345, Beckman Instruments, San Ramon, California) on an Ultrasphere ODS column.

TABLE I
Amino acid sequence of peptide

Peptide	Amino acid sequence
bGRF	H-YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAKVRL-NH_2
hPD	H-LVRIPLHKFYSIRRTMSEVGGSVEDLIAKGPVSKYSQAVPAVTEG-NH_2

Peptide Synthesis

The continuous-flow multiple peptide synthesis was performed on manually operated synthesizer in flow reactors with adjustable volume[1,13]. The scheme of the synthesizer and the synthetic protocol were published in our recent paper[1]. Two medium-size peptide amides were synthesized simultaneously together with eight other sequence-unrelated twenty amino-acid-residue peptides in ten concatenated flow reactors. Fmoc/t-Bu protection strategy on MeBHA resin was employed. The completeness of acylation of resin-bound amino groups was monitored by the acid-base indicator bromophenol blue[4]. The 0.01M solution of bromophenol blue in DMA was added to the solution of activated amino acid and the coupling was carried out till the blue colour disappeared. Flow reactors with activated amino acid were immersed into ultrasonic bath to accelerate the reaction rate[5,6]. The deprotection of acid-labile side chain was accomplished by TFA containing 3% thioanisole and 3% 1,2-ethanedithiol, followed by peptide splitting with anhydrous hydrogen fluoride[7]. The crude peptides were passed through a Sephadex G15 2.5 × 100-cm column in 1M aqueous acetic acid, flow rate 20 ml/h. Fractions were checked by TLC in system 1-butanol–pyridine–acetic acid–water 30 : 20 : 6 : 17 and those containing the main product were pooled and lyophilized. The yield and purity are summarized in Table II.

The peptides were purified on mass-overloaded semi-preparative column (8 × 250-mm), flow-rate 2.0 ml/min, UV detection at 230 nm, sample loop 2 ml, concentration of sample 30 – 50 mg/ml, sample load 110 mg of bGRF, 85 mg of hPD. A sample was dissolved in 0.1% aqueous TFA, filtered through 0.2 μm Minisart NML filter (Sartorius, Göttingen, Germany) and injected on column equilibrated in solvent A. The gradient reached 40% or 50% of MeOH in 30 min for purification of bGRF or hPD, respectively. Shallow gradient of 0.2% MeOH/min was applied during the elution of product. Fractions were collected at 1 min intervals and examined for purity on analytical isocratic HPLC at 57% MeOH (bGRF) and 68% MeOH (hPD). Yield 15 mg of bGRF, 26.9 mg of hPD.

RESULTS AND DISCUSSION

Continuous-Flow Solid-Phase Multiple Peptide Synthesis

Continuous-flow solid-phase multiple peptide synthesis (CF SP MPS)[1,2] is routinely applied in our laboratory for the synthesis of small-size peptides (up to 25 amino acid residues). To document the compatibility of CF SP MPS with the synthesis of medium-

TABLE II
Summarized data on synthetic peptides

Peptide	Resin		Cleavage yield[a]	Gel chromatography	R_F[b]
	yield	cleavage			
bGRF	980	300	144.0	115.4	0.35
hPD	990	440	208.7	153.8	0.27

[a] All amounts are given in mg, initial amount of resin 300 mg; [b] TLC system 1-butanol–pyridine–acetic acid–water 30 : 20 : 6 : 17.

size peptides, we prepared the bovine releasing factor of growth hormone 1-44 (bGRF) and the propart of human procathepsin D 1-45 (hPD). Both peptides were synthesized on MeBHA resin using Fmoc/t-Bu protection strategy. Couplings were performed by in situ prepared HOBt esters, DIC having been used for activation. To drive the reaction to completeness, we increased the coupling efficiency by a combination of exposure to elevated temperature (40 – 50 °C) and ultrasonic field. The completeness of acylation was monitored by bromophenol blue.

The synthesis was carried out in standard synthetic run together with eight sequence-unrelated twenty amino acid-residue peptides. After assembling the first twenty amino acids, the synthesis of two medium-sized peptides continued with another set of eight twenty amino acid-residue peptides.

After finishing the synthesis the side chain protection groups were removed by 1 h treatment with TFA containing 3% thioanisole and 3% 1,2-ethanedithiol. Both peptides were split from the resin in liquid HF (ref.[7]) and crude peptides were gel-filtrated on Sephadex G15 column to remove non-peptidic impurities. This procedure is routinely applied to all crude synthetic peptides since impurities and also the possible excess of strong acid (HF) can deteriorate the purity of crude synthetic peptides[8]. The purity of gel-filtrated peptides was evaluated by analytical gradient HPLC (Figs 1 and 2) and TLC.

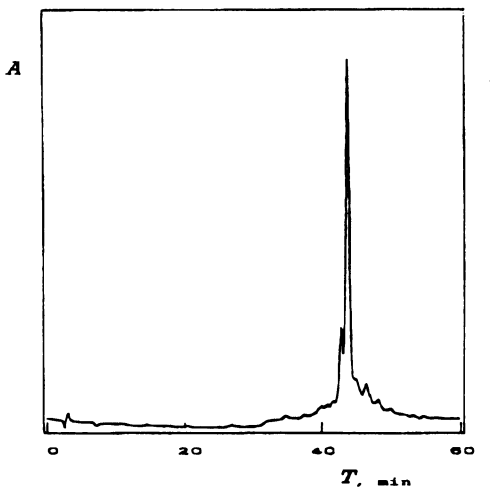

FIG. 1
Gradient HPLC profile of crude bGRF(1-44). Mobile phase A: 0.1% TFA in water; B: 0.07% TFA in methanol–water (80 : 20). Gradient 20 – 80% of methanol in 60 min, flow rate 1 ml/min, UV absorption at 210 nm

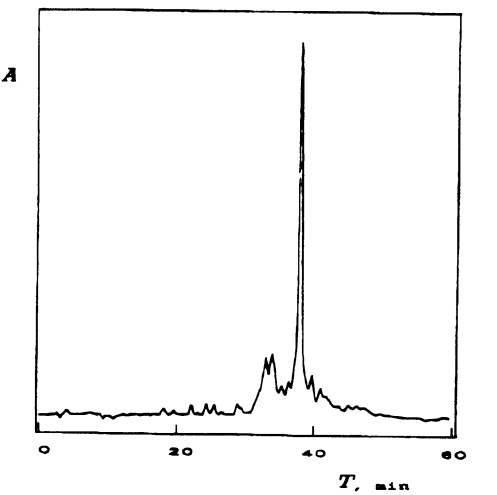

FIG. 2
Gradient HPLC profile of the crude propart of hCD(1-45). Mobile phase A: 0.1% TFA in water; B: 0.07% TFA in methanol–water (80 : 20). Gradient 20 – 80% of methanol in 60 min, flow rate 1 ml/min, UV absorption at 210 nm

During the SP synthesis of GRF(1-32)OH the couplings of amino acids Ala^{19}, Ser^{18}, Leu^{17}, Gln^{16}, Ala^{15}, and Leu^{14} were reportedly incomplete after second coupling and the incorporation of Gln^{16} was incomplete even after three cycles[9]. This phenomenon was explained in terms of conformational predisposition to form an antiparallel β-sheet structure with β-turn forming sequence Ala^{19}-Arg^{20}. To find out any possible correlation between the capacity of peptidyl resin to swell and the position of difficult sequences we measured the swollen volume in each step after coupling in DMF. Figure 3 shows the dependence of peptidyl resin volume on the length of peptide. In the above mentioned region from Ala^{19} to Leu^{14} we observed a continuous drop in the swollen volume. The reported aggregation of peptide chain[9] is thus closely related to the swelling capacity of peptidyl resin and its decreased swollen volume also correlated with slower rate of coupling[10]. Nevertheless, the application of ultrasonic bath for the acceleration of coupling appeared to be very useful, since even in the case of Gln^{16} the coupling was complete. The effect may be attributed to enhanced solvation of resin-bound peptide chains by disruption of aggregates.

In fact, there is a second sudden drop in swollen volume, namely from the Glu^{33} to Gly^{32}. However, this sequence is not included in the GRF(1-32)OH. It should be noted that the coupling difficulties described by Deber at al.[9] were observed on the synthesis of shorter peptide GRF(1-32) synthesized by Boc/Bzl strategy. This illustrates that the difficult couplings are an inherent feature of sequences and that they are not dependent on the length of peptide or on the synthetic strategy.

HPLC gradient profile of gel-filtrated crude bGRF (Fig. 1) revealed the presence of impurity with shorter retention time and its content was not constant in different batches of HF treatment using the same synthetic batch of peptidyl resin (the amount of impurity varied from 15 to 56%). We isolated this side product. Amino acid analysis after hydrolysis in 6M-HCl showed identical amino acid composition with bGRF and

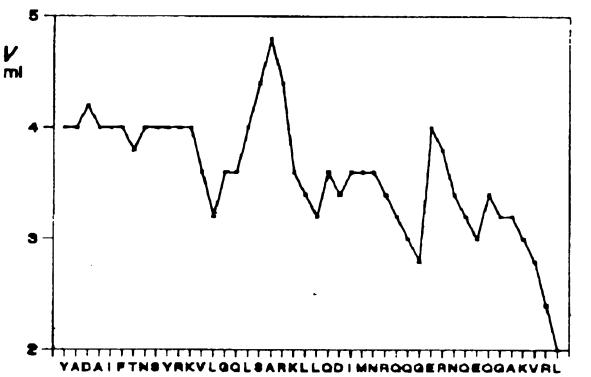

FIG. 3
Volume (V) of swollen peptidyl resin in DMF in dependence on amino acid sequence during the synthesis of bGRF

the molecular peak in FAB-MS was greater by about 16 when compared with bGRF. These two facts together with the known tendency of Met to oxidation indicate that the impurity is [Met(O)^{27}]bGRF(1-44). To prove that the impurity is [Met(O)^{27}]bGRF(1-44), the crude product was treated with 100 equivalents of 2-mercaptoethanol in 6M urea under nitrogen. After 36 h at room temperature the amount of [Met(O)^{27}]bGRF decreased from 56% to 17%.

The propensity of Met in bGRF towards oxidation was tested on a sample of HPLC purified bGRF. The solution of pure bGRF in elution phase (50% aqueous MeOH containing 0.1% TFA) was left respite at 30 °C for two days. Figure 4 shows overlaid analytical isocratic profiles of pure bGRF and HPLC-isolated Met(O)^{27}bGRF, Fig. 5 documents on overlaid chromatograms the course of oxidation after 6 h (estimated amount of Met(O)^{27} bGRF 8 %) and after 36 h (22 %).

Crude bGRF was purified on semi-preparative column under mass-overloaded conditions[11] that take advantage of the high resolving capacity of shallow gradient. Since the retention time of peptides under mass-overloaded conditions decreases, it is necessary to estimate the probable composition of mobile phase when the product starts to elute

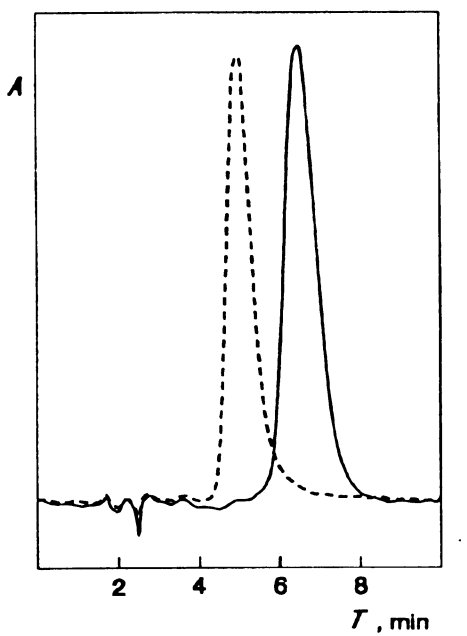

FIG. 4

Isocratic HPLC profiles of bGRF (——), R_t 6.46 min, and Met(O)^{27}bGRF (- - - -), R_t 4.95 min. Mobile phase 60% MeOH containing 0.1% TFA, flow rate 1 ml/min, UV absorption at 210 nm

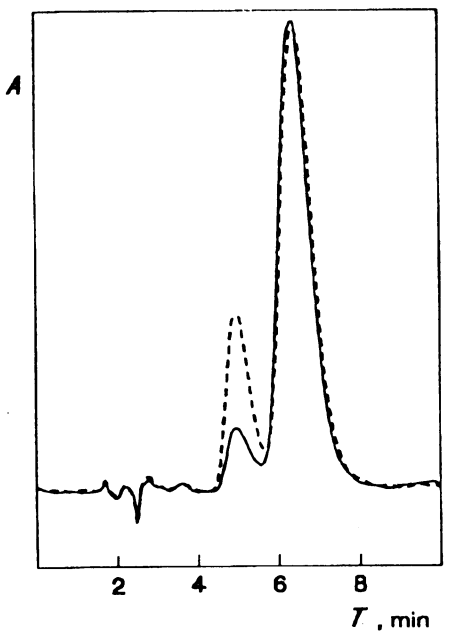

FIG. 5

The course of oxidation of Met in bGRF followed by HPLC. Sample after 6 h (——), 8% of Met(O)^{27}bGRF, and after 36 h (- - - -), 22% of Met(O)^{27}bGRF. Conditions as described in Fig. 4

(elution phase). Using a set of experimental data we constructed a graph exhibiting the dependence of elution phase on analytical retention time[1]. The calculated composition of elution phase for the purification of bGRF was 47% MeOH. The purification started from neat solvent A with a sharp 20 min gradient to 40% MeOH (7% less than expected elution phase) and followed with a shallow gradient of 0.2% MeOH/min during the elution of product. The actual elution phase contained 46.5% MeOH showing a good agreement with the calculated value. The preparative HPLC profile is depicted in Fig. 6 and it shows a typical behaviour of mass-overloaded column. Analytical isocratic HPLC was used to evaluate the purity of individual collected fractions (1 tube/min). Only the first two tubes and the last two ones containing the product were contaminated by impurities (see Fig. 6).

Since the hPD(1-45) has not been synthesized before, there are no synthetic data available for comparison. All coupling steps during the synthesis proceeded without difficulties, and only two couplings, Ser^{36} and Arg^{13}, were repeated. However, we observed that during the condensation of Gly^{20} and in the subsequent synthetic cycles bromophenol blue remained adsorbed on the peptidyl resin and intensive blue colour, which appears after treatment with base (deprotection by piperidine), was not washed out even by piperidine. Interestingly, when tested with ninhydrin no colour reaction was observed even with the Fmoc-deprotected resin.

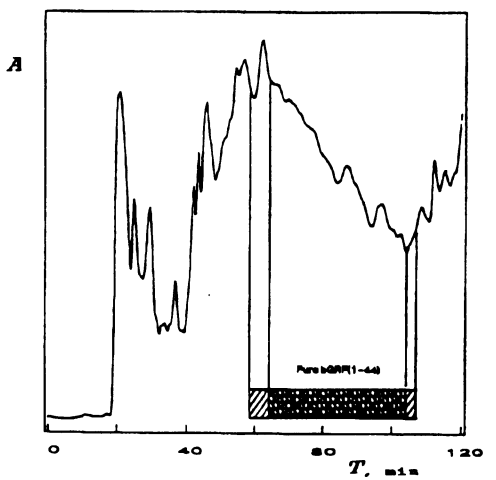

FIG. 6
Preparative gradient profile of bGRF(1-44) purification. For experimental details see Experimetal

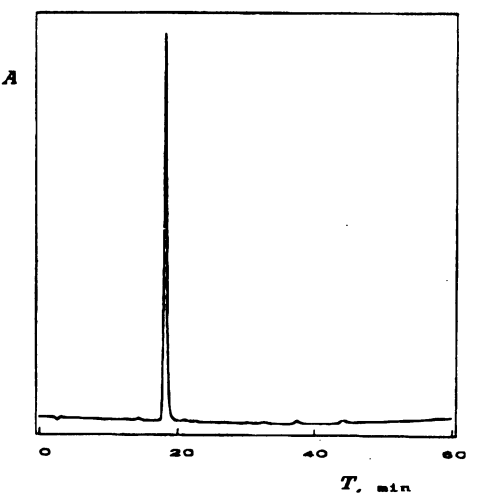

FIG. 7
Gradient HPLC profile of the pure propart of hCD(1-45). Mobile phase A: 0.1% TFA in water; B: 0.07% TFA in acetonitrile–water (80 : 20). Gradient 20 – 80% of acetonitrile in 60 min, flow rate 1 ml/min, UV absorption at 210 nm

The gradient HPLC elution profiles of crude and HPLC-purified propart of procathepsin D (R_t 37.9, area 58.9%) are shown in Figs 2 and 7. Amino acid analysis and Edman degradation of the propart of hPD were in accordance with the target sequence.

Biological Activities

The biological activity of hPD(1-45) was tested by the inhibition of bovine cathepsin D. The residual activity of cathepsin was measured by cleaving the synthetic substrate KPAEFF(p-NO_2)AL. Previous results showed that the inhibition constant K_i ranged from $1 . 10^{-6}$ to $1 . 10^{-8}$ mol l^{-1} depending on the pH of the kinetical assay (Fig. 8). Also other aspartic proteases from different sources were tested (pig pepsin, chicken pepsin, endothiaparasitica pepsin, and penicillopepsin). The K_i values also ranged from $1 . 10^{-6}$ to $1 . 10^{-8}$ mol l^{-1}.

The biological activity of synthetic bGRF(1-44) was confirmed by measuring the release of growth hormone (GH). The concentration of GH in gilts after application of synthetic bGRF(1-44) was determined in homologous double antibody radioimmunological assay[12]. GH plasma concentration after bGRF(1-44) application is plotted in Fig. 9. The mean plasma level of GH during pulsatile secretion was 6.47 ± 0.38 ng/ml. Statistically insignificant differences in all time intervals were confirmed by Student's t-test. At the dose of 0.2 nmol/kg of body weight GH concentration significantly increased from 6.00 ± 0.59 ng/ml to 10.97 ± 0.15 ng/ml during 10 min. GH concentration was

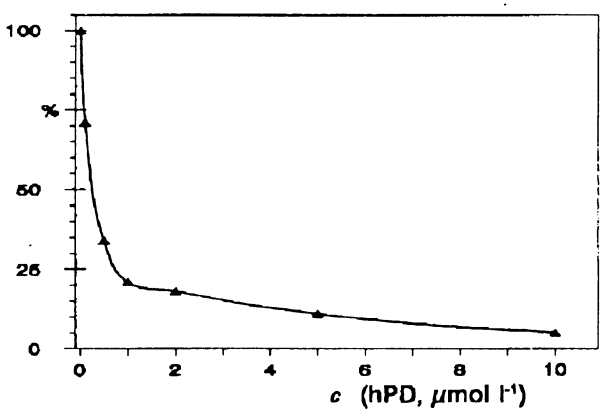

FIG. 8

Inhibition of bovine cathepsin D by hPD(1-45). The velocity of the cleavage (expressed in %, relative to controll without hPD) of synthetic substrate KPAEFF(p-NO_2)AL was monitored spectrophotometrically at 305 nm. Reaction mixture: 1 ml 0.1M acetate buffer pH 5.44, substrate concentration $2.5 . 10^{-4}$ mol l^{-1}, enzyme concentration $3 . 10^{-7}$ mol l^{-1}, the hPD concentration varied from 0.1 to 10 μmol l^{-1}. Before reaction the enzyme was preincubated with hPD for 2 h in 0.05M Tris-HCl buffer, pH 7.4

significantly increased ($P < 0.05$) between the 60th and 240th min after bGRF(1-44) application. Following administration of the dose of 0.6 nmol/kg body weight GH concentration increased during 10 min from 5.44 ± 0.63 ng/ml to 10.98 ± 2.25 ng/ml. Within 60 to 240 min after administration of bGRF(1-44) GH the concentration significantly increased ($P < 0.01$). The application of both doses of bGRF(1-44) significantly increased the mean plasma GH concentration (at the dose of 0.2 nmol/kg mean GH concentration was 9.89 ± 0.57 ng/ml; at the dose of 0.6 nmol/kg mean GH concentration was 11.66 ± 0.70 ng/ml). Details of the biological activities of the propart of human procathepsin D (1-45) and growth-hormone releasing factor (1-44) will be published elsewhere.

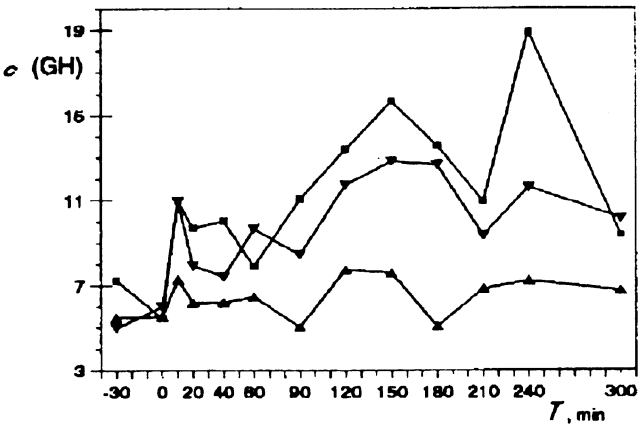

FIG. 9

Concentration of GH in gilts after application of bGRF(1-44). The biological activity (pulsatile secretion, ▲) of bGRF(1-44) was evaluated by change of GH concentration in blood plasma of three gilts at mean weight 55.3 ± 0.7 kg. bGRF(1-44) was intramuscularly injected in the doses of 0.2 (▼) and 0.6 (■) nmol/kg of body weight. Two blood samples were withdrawn at 30 min intervals before administration of the peptide. During the first hour after administration blood was sampled at 20 min intervals, during the next three hours at 30 min intervals and then at 60 min intervals

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