Isolation and Properties of Two Biologically Active Fragments from Limited Tryptic Hydrolysis of Bovine and Ovine Pituitary Growth Hormones†

László Gráf‡ and Choh Hao Li*

ABSTRACT: Limited tryptic hydrolysis of bovine and ovine pituitary growth hormones has been investigated. From both hormones, a peptide fragment consisting of 38 amino acid residues corresponding to residue positions 96–133 in the structure has been isolated and characterized. It possesses measurable activity in the tibia test for growth-promoting activity. Another peptide fragment derived from the COOH-terminal portion (residues 151–191) of bovine growth hormone has also been isolated and characterized. It too gives measurable response in the tibia test.

Bovine pituitary growth hormone was first isolated and partially characterized by Li *et al.* (Li and Evans, 1944; Li *et al.*, 1945). It is a protein with a dimeric form of mol wt 45,000 in neutral solutions (Li *et al.*, 1945) and becomes a monomer in acid solutions (Dellacha *et al.*, 1968; Bewley and Li, 1972). The primary structure of the BG[1] molecule (191 amino acids) has recently been elucidated by three groups of investigators (Santomé *et al.*, 1973; Wallis, 1973; Gráf and Li, 1974b; see Figure 1). Earlier studies indicated that limited digestion of BGH with chymotrypsin or trypsin (Li, 1956) does not lead to inactivation. Subsequently, Sonenberg and coworkers (Yamasaki *et al.*, 1970; Sonenberg *et al.*, 1972; Levine *et al.*, 1973) have reported that a peptide fragment of 37 amino acid residues obtained from limited tryptic digests of BGH has significant biological activity in experimental animals and human subjects. Sequence analysis of this peptide (Yamasaki *et al.*, 1972) indicated it originated from residues 96–133 of the BGH molecule (Santomé *et al.*, 1973 Wallis, 1973; Gráf and Li, 1974b).

This paper details our investigations on limited tryptic hydrolysis of BGH as well as SGH. It will be seen that we were able to isolate the 38 amino acid peptide (instead of 37) in highly purified form from these digests. In addition, a peptide fragment corresponding to the last 41 amino acids in the COOH terminus of BGH was isolated and found to possess measurable activity in the tibia assay.

Experimental Section

Materials. BGH and SGH were prepared by methods previously published (Li, 1954; Papkoff and Li, 1958). They were further purified by gel filtration on Sephadex G-100 in 0.01 M NH_4HCO_3 buffer (pH 8.4) to obtain the dimeric form of BGH and SGH (Bewley and Li, 1972).

Trypsin and urokinase were obtained from Calbiochem, and lima bean trypsin inhibitor, α-chymotrypsin, leucine-aminopeptidase, and carboxypeptidase A were products of Worthington Biochemical Corporation. Aminopeptidase M was obtained from Röhn-Haas. Partially purified human plasminogen was obtained from the Institute for Serobacteriological Production and Research (Hungary). Recrystallized α-iodoacetamide and dithiothreitol were obtained from Calbiochem.

Limited Tryptic Digestion of Growth Hormones. In a typical experiment, 160 mg of growth hormone was dissolved in 25 ml of 0.1 M KCl solution and the digestion was initiated by adding 0.8 mg of trypsin. The digestion was carried out at pH 9.6 in the pH-Stat (Radiometer Titrator type TTT 1 lb, pH meter type 26C) at 25° under a nitrogen atmosphere with continuous stirring. The pH was maintained automatically by the addition of standard 0.100 M NaOH. After 0.24 ml of the NaOH solution had been consumed (this corresponds to the cleavage of three peptide bonds per mole of growth hormone), the proteolysis was terminated by adding 1.6 mg of lima bean trypsin inhibitor. The tryptic digest was dialyzed and recovered by lyophilization.

Isolation of the Fragments. Gel filtration was carried out on Sephadex G-75 columns in 50% acetic acid. The fractions were pooled, diluted to 10% acetic acid, and lyophilized. For further purification of some fragments, partition chromatography was applied on a Sephadex G-25 column (Yamashiro, 1964). A system of 2-butanol–pyridine–0.1% aqueous acetic acid (40:24:96, v/v) was used. Fractions obtained from the partition column were evaporated at 40°, redissolved in water, and lyophilized. Some smaller fragments were separated by chromatography on Sephadex G-25 in 0.5 M acetic acid and/or preparative high voltage paper electrophoresis at pH 6.4 for 2 hr at 2000 V. Guide strips of the electrophoretograms were developed by ninhydrin.

Reduction and Alkylation. A fraction of fragments containing disulfide bridges was reduced with a 20-fold molar excess of dithiothreitol over cystine content in the presence of 8 M urea at pH 8.4 under nitrogen for 1 hr. Alkylation was carried out under the same conditions by adding iodoacetamide in ten-fold molar amounts to dithiothreitol. The excess reagents were removed by gel filtration on a Sephadex G-15 column in 0.02 M NH_4HCO_3 buffer of pH 8.5.

† From the Hormone Research Laboratory, University of California, San Francisco, California 94143. *Received July 24, 1974.* This work was supported in part by the American Cancer Society, the Allen Foundation, and the Geffen Foundation.

‡ Permanent address: Research Institute for Pharmaceutical Chemistry, Szabadsagharcosok U. 47-49, Budapest 4, Hungary.

[1] Abbreviations used are: BGH, bovine pituitary growth hormone; SGH, sheep pituitary growth hormone; HGH, human pituitary growth hormone; V_e/V_0, elution volume/void volume.

NH_2-Ala-Phe-Pro-Ala-Met-Ser-Leu-Ser-Gly-Leu-Phe-Ala-Asn-Ala-
1 10
Val-Leu-Arg-Ala-Gln-His-Leu-His-Gln-Leu-Ala-Ala-Asp-Thr-
20
Phe-Lys-Glu-Phe-Glu-Arg-Thr-Tyr-Ile-Pro-Glu-Gly-Gln-Arg-
30 40
Tyr-Ser-Ile-Gln-Asn-Thr-Gln-Val-Ala-Phe-Cys-Phe-Ser-Glu-Thr-
50
Ile-Pro-Ala-Pro-Thr-Gly-Lys-Asn-Glu-Ala-Gln-Gln-Lys-Ser-
60 70
Asp-Leu-Glu-Leu-Leu-Arg-Ile-Ser-Leu-Leu-Leu-Ile-Gln-Ser-Trp-
80
Leu-Gly-Pro-Leu-Gln-Phe-Leu-Ser-Arg-Val-Phe-Thr-Asn-Ser-
90 100
Leu-Val-Phe-Gly-Thr-Ser-Asp-Arg-Val-Tyr-Glu-Lys-Leu-Lys-
110
Asp-Leu-Glu-Glu-Gly-Ile-Leu-Ala-Leu-Met-Arg-Glu-Val/Leu-Glu-
120
Asp-Gly-Thr-Pro-Arg-Ala-Gly-Gln-Ile-Leu-Lys-Gln-Thr-Tyr-
130 140
Asp-Lys-Phe-Asp-Thr-Asn-Met-Arg-Ser-Asp-Asp-Ala-Leu-Leu-
150
Lys-Asn-Tyr-Gly-Leu-Leu-Ser-Cys-Phe-Arg-Lys-Asp-Leu-His-
160 170
Lys-Thr-Glu-Thr-Tyr-Leu-Arg-Val-Met-Lys-Cys-Arg-Arg-Phe-
180
Gly-Glu-Ala-Ser-Cys-Ala-Phe-OH
190

FIGURE 1: The amino acid sequence of bovine pituitary growth hormone.

The protein peak excluded from the column was recovered by lyophilization.

Peptide Analytical Procedures. Tryptic and chymotryptic digestions were carried out in 0.02 M NH_4HCO_3 of pH 8.2 with a 1:20 (w/w) enzyme:peptide ratio at 37° for 4 and 2 hr, respectively. Leucineaminopeptidase hydrolysis was done in 0.1 M Tris buffer of pH 8.5 with an enzyme:peptide ratio of 1:20 (w/w) for 15 hr at 37°. Aminopeptidase M digestion was carried out with a 1:1 (w/w) ratio of enzyme to peptide under the same conditions as the leucineaminopeptidase hydrolysis. Digestion with carboxypeptidase A was performed in 0.1 M $NaHCO_3$ with a 1:20 (w/w) enzyme: peptide ratio for 10 hr at 37°. Digestion with plasmin was carried out in 0.02 M NH_4HCO_3 of pH 8.2 with a 20:1:0.1 (w/w) peptide:plasminogen:urokinase ratio for 20 hr at 37°.

Tryptic digests (2 mg) were mapped by paper chromatography [butanol–acetic acid–water, 4:1:5 (v/v)] and subsequent low voltage paper electrophoresis at pH 3.7 (40 ml of acetic acid, 4 ml of pyridine, 1150 ml of water) for 4 hr at 400 V. Maps were sprayed by 0.1% ninhydrin solution in acetone and the spots were cut out as they appeared, eluted with 0.5 M NH_4OH solution, and subjected to acid hydrolysis for amino acid analysis.

Fractionation of the tryptic, chymotryptic, and plasmic fragments was accomplished by high voltage paper electrophoresis at pH 2.1 (218 ml of 90% formic acid and 63 ml of glacial acetic acid per l.) for 1.5 hr at 2000 V and at pH 6.4 (8.9 ml of collidine and 3.1 ml of acetic acid per l.) for 2 hr at 2000 V. In one case, low voltage paper electrophoresis was applied at pH 3.5 (50 ml of acetic acid, 4 ml of pyridine, 1150 ml of water) for 4 hr at 400 V. Peptide bands were excised from the paper, eluted with 0.5 M acetic acid, and lyophilized.

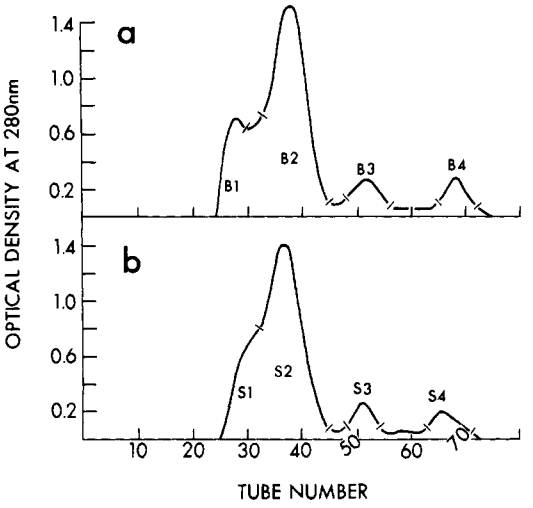

FIGURE 2: Exclusion chromatography of tryptic digests (160 mg each) of (a) BGH and (b) SGH on Sephadex G-75 (77 cm × 2.9 cm; void volume 135 ml) in 50% acetic acid; tube volume, 5 ml.

TABLE I: Yields and NH_2-Terminal Residues of the Main Fractions of Limited Tryptic Digests of BGH and SGH Obtained from Exclusion Chromatography.

Fraction[a]	Yield[b] (mg)	NH_2-Terminal Residues[c]
B1	30	Phe, Ala (Val)
B2	84	Phe, Ala (Ser, Val)
B3	25	Val (Phe)
B4	8	Ala (Glx)
S1	26	Phe, Ala (Val, Ser)
S2	80	Phe, Ala (Ser, Val)
S3	23	Val (Phe)
S4	6	Ala (Glx)

[a] See Figure 2. [b] From 160 mg of BGH or SGH. [c] NH_2-terminal residues which appeared in visually smaller amounts than the others are indicated in parentheses.

Amino acid analysis of the acid (6 M HCl, 110°, 24 hr, in evacuated tubes), leucineaminopeptidase, and carboxypeptidase hydrolysates were carried out according to Spackman *et al.* (1958) in an automatic amino acid analyzer (Model 120, Beckman Instruments). The amino acid sequence of the peptides was determined by the dansyl-Edman procedure (Gray, 1967; Woods and Wang, 1967) as previously described (Li *et al.*, 1970).

Bioassay. The growth promoting activity was measured by the tibia test (Geschwind and Li, 1955) in hypophysectomized rats.

Results

Isolation and Characterization of Fractions B3-1 and S3-1. The chromatographic profiles of limited tryptic digests of BGH and SGH on a Sephadex G-75 column in 50% acetic acid are shown in Figure 2. The yields and NH_2-terminal residue analyses of the fractions are summarized in Table I. It is evident that the hydrolytic products of BGH and SGH are identical and that certain peptide bonds are preferentially cleaved by trypsin in these two proteins.

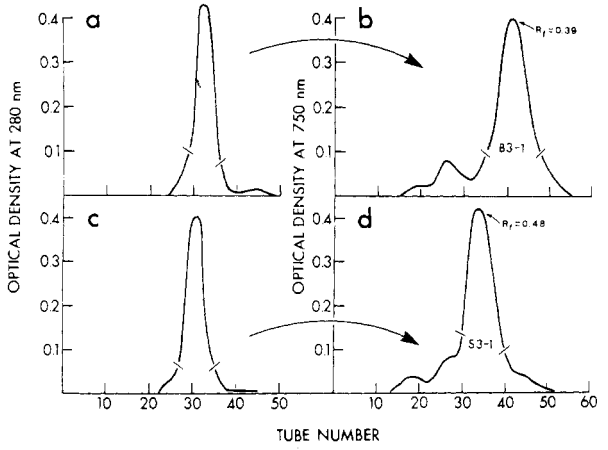

FIGURE 3: Exclusion chromatography on B3 (a) and S3 (c) (17 mg each) on Sephadex G-75 column (86 cm × 1.5 cm) in 50% acetic acid; tube volume, 3 ml. The pooled and lyophilized fractions were subjected to partition chromatography (b and d, respectively) on Sephadex G-25 column (64 cm × 2.0 cm); tube volume, 3 ml; 0.2-ml aliquots of the tube were used for peptide content determination by Folin-Lowry reagent. Peaks B3-1 (b) and S3-1 (d) were evaporated at 40° and lyophilized separately.

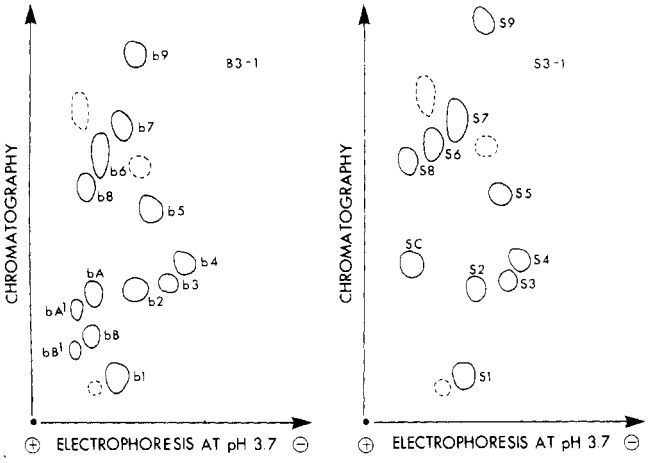

FIGURE 4: Peptide maps of tryptic digests of B3-1 and S3-1. Peptide spots indicated by solid line were cut out and analyzed for amino acid composition (Table III).

Fractions B3 and S3 (17 mg each, Figure 2) were rechromatographed on Sephadex G-75 in 50% acetic acid as shown in Figure 3. The pooled and lyophilized fractions were subjected to partition chromatography on Sepahdex G-25 in 2-butanol–pyridine–0.1% aqueous acetic acid solvent system (Figure 3) and two homogeneous peptide fragments (B3-1, R_F 0.39, 9 mg; S3-1, R_F 0.48, 11 mg) were obtained. Both fragments were shown to be single components as evidenced by NH_2-terminal residue analyses (sole valine end group), paper electrophoresis at pH 3.7, paper chromatography, and amino acid analysis (Table II).[2]

The peptide maps obtained from the tryptic hydrolysates of B3-1 and S3-1 are shown in Figure 4. The peptide spots were cut from the maps and analyzed for amino acid content. The results of these analyses are summarized in Table III. From these data, together with those in Table II, the fragments must have been derived from amino acid residues 96–133 in the sequences of BGH (Figure 1) and SGH (Li *et al.*, 1973), respectively.

The single amino acid difference between B3-1 and S3-1 (Table II) gave rise to the occurrence of bA–bB (bA′–bB′) and sC in the corresponding peptide maps (Figure 4). In ad-

[2] It is worth mentioning that the partition chromatography step in the purification of fragments B3 and S3 (Figure 3) was applied with advantage to exclude some disulfide containing contaminant(s) from the preparations. This was shown by oxidation and subsequent amino acid analysis of the fragments before and after partition chromatography.

TABLE II: Amino Acid Composition[a] of Fragments[b] B3-1 and S3-1.

Amino Acid	B3-1		S3-1	
	Expt	(96–133)[c]	Expt	(96–133)[d]
Lys	1.9 (2)	2	1.9 (2)	2
Arg	2.8 (3)	3	2.7 (3)	3
Asp	4.2 (4)	4	4.0 (4)	4
Thr	2.7 (3)	3	2.9 (3)	3
Ser	1.9 (2)	2	1.9 (2)	2
Glu	5.0 (5)	5	5.0 (5)	5
Pro	1.1 (1)	1	0.9 (1)	1
Gly	3.2 (3)	3	2.2 (2)	2
Ala	1.1 (1)	1	1.1 (1)	1
Val	3.1 (3)	3.3[e]	4.1 (4)	4
Met	0.8 (1)	1	0.7 (1)	1
Ile	1.1 (1)	1	0.9 (1)	1
Leu	5.8 (6)	5.7[e]	6.3 (6)	6
Tyr	0.8 (1)	1	0.9 (1)	1
Phe	2.1 (2)	2	2.0 (2)	2

[a] Composition in molar ratio; Thr and Ser values are uncorrected. [b] See Figure 3. [c] Residue positions in the BGH structure (Figure 1). [d] Residue positions in the SGH structure (Li *et al.*, 1973). [e] Fraction values are due to the microheterogeneity at sequence position 127.

TABLE III: Amino Acid Composition of Tryptic Peptides from Fragments B3-1 and S3-1.

Peptides[a]	Amino Acid Composition[b]	Location in the Sequence[c]
b1, s1	Asp_1, Thr_1, Ser_1, Gly_1, Arg_1	104–108
b2, s2	Glu_1, Val_1, Tyr_1, Lys_1	109–112
b3, s3	Met_1, Arg_1	124–125
b4, s4	Leu_1, Lys_1	113–114
b5, s5	Ala_1, Met_1, Leu_1, Arg_1	122–125
b6, s6	Asp_1, Glu_2, Gly_1, Ile_1, Leu_3, Lys_1	113–121
b7, s7	Asp_1, Thr_1, Ser_1, Val_1, Leu_1, Phe_1	98–103
b8, s8	Asp_1, Glu_2, Gly_1, Ile_1, Leu_2	115–121
b9, s9	Val_1, Phe_1	96–97
bA, bA′	Asp_1, Thr_1, Glu_2, Pro_1, Gly_1, Leu_1, Arg_1	126–133
bB, bB′	Asp_1, Thr_1, Glu_2, Pro_1, Gly_1, Val_1, Arg_1	126–133
sC	Asp_1, Thr_1, Glu_2, Pro_1, Val_1, Leu_1, Arg_1	126–133

[a] See Figure 4. [b] Molar ratio. In view of the small size and the high purity of the peptides, the nearest whole residue numbers are given. [c] Residue numbers in the BGH and SGH structures (Figure 1; Li *et al.*, 1973).

TABLE IV: Amino Acid Composition[a] of Purified Fragments[b] from Plasmic and Chymotryptic Digests of Fragment B3-1.

Amino Acid	P1	P2	K51	K52	K53	K6
Lys	0.9 (1)	0.9 (1)	2.1 (2)			
Arg	1.0 (1)	1.9 (2)		2.0 (2)	0.9 (1)	1.9 (2)
Asp	2.2 (2)	1.9 (2)	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Thr	1.8 (2)	0.8 (1)		0.9 (1)	0.9 (1)	1.0 (1)
Ser	1.9 (2)				0.9 (1)	
Glu	1.2 (1)	3.9 (4)	3.1 (3)	2.0 (2)		2.2 (2)
Pro		0.9 (1)		1.0 (1)		0.9 (1)
Gly	1.1 (1)	2.0 (2)	0.9 (1)	1.0 (1)	0.9 (1)	1.0 (1)
Ala		1.1 (1)		0.9 (1)		
Val	2.9 (3)	0.2		0.3	1.1 (1)	0.3
Met		0.7 (1)		0.8 (1)		0.7 (1)
Ile		0.9 (1)	0.9 (1)			
Leu	1.2 (1)	5.2 (5)	3.0 (3)	1.8 (2)		0.7 (1)
Tyr	0.8 (1)				1.0 (1)	
Phe	2.0 (2)					

[a] Molar ratio; Thr and Ser values are uncorrected; values for residues less than 0.1 are omitted. [b] See Figure 5.

TABLE V: Structural Investigations on Peptides Derived from Fragment B3-1.

Peptides[a]	Structure[b]
B3-1	96 100 105 Val-Phe-Thr-Asn-Ser-Leu-Val-Phe-Gly-Thr-( )
P1	96 99 Val-Phe-Thr-Asn-( )
K53	104 110 Gly-Thr-Ser-Asp-Arg-Val-Tyr
K51	111 115 Glu-Lys-Leu-Lys-Asp-(Leu,Glu,Glu,Gly,Ile,Leu)
P2	113 115 120 124 Leu-Lys-Asp-Leu-Glu-Glu-Gly-Ile-Leu-Ala-Leu-Met-( )
K52	122 125 130 133 Ala-Leu-Met-Arg-Glu-Val/Leu-Glu-Asp-Gly-Thr-Pro-Arg
K6[c]	124 125 130 133 Met-Arg-Glu-(Val/Leu,Glu,Asp,Gly,Thr,Pro)-Arg

[a] Peptides are listed in the order of their appearance in fragment B3-1. [b] →, dansyl-Edman procedure; —, phenylthiohydantoin amino acid derivative identified; ⇀, leucineaminopeptidase digestion. [c] Asp-Gly peptide bond at positions 129–130 was resistant to aminopeptidase digestion. The assignment of aspartic acid residue for position 129 was based on the electrophoretic mobility of peptides K6 and K52 at pH 6.4 (Offord, 1966).

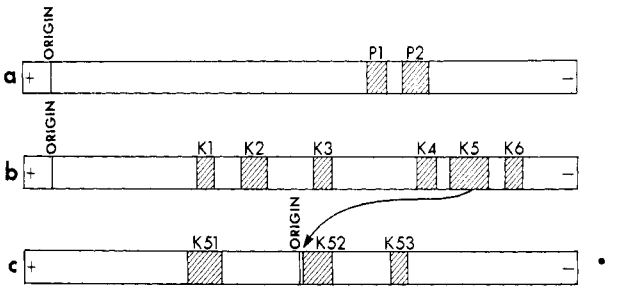

FIGURE 5: Paper electrophoretic separation of (a) the plasmic digest and (b) the chymotryptic digest of fragment B3-1 at pH 2.1. (c) The material obtained from band K5 of b was submitted to electrophoresis at pH 6.4.

dition, this particular tryptic fragment of B3-1 [residues 126–133 in the BGH structure (Figure 1)] appeared in four homologous forms on the tryptic map (Figure 4: bA, bA′; bB, bB′) as was already shown by Seavey *et al.* (1971).

Sequence Analysis of Fragment B3-1. The NH_2-terminal sequence of fragment B3-1 was found to be Val-Phe-Thr-Asn-Ser-Leu-Val-Phe-Gly-Thr- as revealed by the dansyl-Edman procedure.

Further elucidation of the primary structure of B3-1 was derived from data obtained with plasmic and chymotryptic fragments. The plasmic and chymotryptic digests of B3-1 were subjected to high voltage paper electrophoresis at pH 2.1 as shown in Figure 5. The two large plasmic fragments, P1 and P2, required an additional purification by high voltage paper electrophoresis at pH 2.1, whereas band K5 of chymotryptic digest was further fractionated by paper electrophoresis at pH 6.4 as shown in Figure 5C.

Table IV presents the amino acid composition of the purified plasmic and chymotryptic fragments which were submitted to complete or partial sequence determination. These structural investigations are summarized in Table V.

From these data the complete primary structure of fragment B3-1 can be formulated as shown in Figure 6. The amino acid composition of the tyrosine-containing tryptic peptide, b2 (Table III), provided evidence for the assignment of peptides K53 and K51.

Since the Asx-Gly bond of peptides K52 and K6 was completely resistant to leucineaminopeptidase and also aminopeptidase M cleavage, the assignment of aspartic acid for Asx-129 (Table V, Figure 6) was derived from the following observations. When K6 was submitted to paper elec-

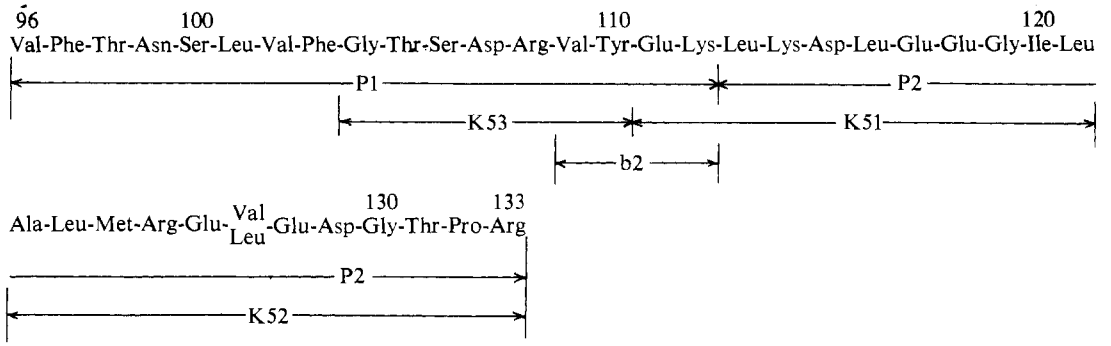

FIGURE 6: Amino acid sequence of fragment B3-1.

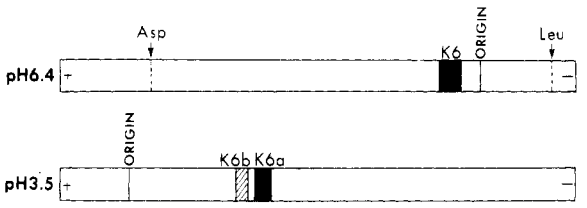

FIGURE 7: Paper electrophoresis of fragment K6 (Figure 5, Table IV) at pH 6.4 and 3.5. The two components, K6a and K6b, were eluted and further characterized (Table VI).

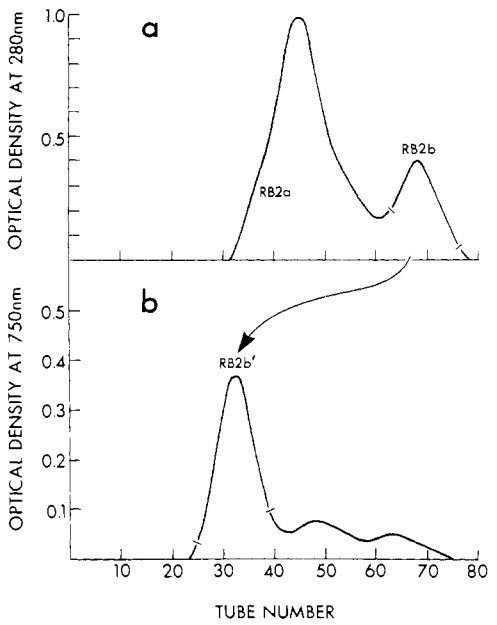

FIGURE 8: (a) Chromatography of 100 mg of reduced and alkylated B2 (Figure 2) on Sephadex G-75 (75 cm × 3.3 cm) in 20% acetic acid; tube volume, 5 ml. The yield of fraction RB2b was 25 mg. (b) RB2b (13 mg) was further purified by partition chromatography on Sephadex G-25 (64 cm × 2.0 cm); tube volume, 3 ml. Aliquots (0.2 ml) were applied for protein content determination by the Folin-Lowry method. Peak RB2b′ was eluted with an R_F value of 0.5 and yielded 8 mg.

trophoresis at pH 6.4, it behaved as a homogenous acidic peptide (Figure 7) indicating that the three dibasic amino acids (Glu-126, Glu-128, and Asp-129) are present as acids according to Offord (1966). However, paper electrophoresis of K6 at pH 3.5 resolved into two components (K6b and K6a, Figure 7), K6a being predominant. The amino acid compositions of the acid and leucineaminopeptidase hydrolysates of both components are shown in Table VI, indicating the common structural origin of these components. The possible reasons for this microheterogeneity are discussed in the Discussion section.

TABLE VI: Amino Acid Content[a] of the Acid and Leucine-aminopeptidase (LAP) Hydrolysates or Peptides[b] K6a and K6b.

Amino Acid	K6a		K6b	
	Acid Hydrolysate	LAP Hydrolysate	Acid Hydrolysate	LAP Hydrolysate
Arg	2.0	1.0	1.9	1.1
Asp	1.1	0	0.9	0
Thr	0.9	0	0.9	0
Glu	2.2	2.2	2.1	2.1
Pro	0.9	0	1.1	0
Gly	1.0	0	1.1	0
Val	0.3	0.4	0.3	0.3
Met	0.9	1.1	1.0	1.0
Leu	0.8	0.7	0.7	0.7

[a] Molar ratio; values for residues less than 0.05 are omitted. [b] See Figure 7.

Studies on the Resistance of Fragments B3-1 and S3-1 to Enzymic Hydrolysis. During the structural investigations of fragments B3-1 and S3-1 it became apparent that these peptides were highly resistant to enzymic hydrolyses. Because of this, unusually high enzyme to peptide ratios were used for the tryptic and chymotryptic digests (Experimental Section). These fragments are especially resistant to leucineaminopeptidase hydrolysis as revealed by the amino acid analyses of the whole digests. In contrast with the restricted leucineaminopeptidase digestion of B3-1, the NH_2-terminal 17 amino acid fragment derived from plasmic digest (P1, Figure 5, Table IV) was easily hydrolyzed by leucineaminopeptidase. The amount of leucine in the hydrolysates of B3-1 and P-1 [Leu-101 is the single leucine residue of peptide P-1] served as reference for the comparison.

Purification and Chemical Characterization of Fractions B2 and B4.[3] For further characterization of fraction B2 (Figure 2) it was completely reduced and carbamidomethylated. This derivative of B2 (RB2) was resolved into two main fractions by exclusion chromatography as shown in Figure 8. The more retarded peak of this chromatography (RB2b) was further purified by partition chromatography (Figure 8b). The material obtained from peak RB2b′ was found to be homogeneous by NH_2-terminal residue. Amino

[3] B1 was found to contain aggregated material and was not investigated further.

TABLE VII: Amino Acid Composition[a] of Fragments[b] RB2b′, B41a, B41n, and B41b.

Amino Acid	RB2b′		B41a		B41n		B41b	
	Expt	(151–191)[c]	Expt	(140–150)[c]	Expt	(140–150)[c]	Expt	(134–139)[c]
Lys	4.2	4	1.0	1	1.0	1	1.0	1
His	1.0	1						
Arg	3.8	4	1.0	1	1.1	1		
Cys[d]	2.9	3						
Asp	4.0	4	3.1	3	3.2	3		
Thr	2.1	2	2.0	2	2.1	2		
Ser	2.8	3						
Glu	2.0	2	1.1	1	1.2	1	1.0	1
Gly	2.2	2					1.0	1
Ala	2.8	3					0.9	1
Val	0.8	1						
Met	0.9	1	0.9	1	0.8	1		
Ile							1.0	1
Leu	6.2	6					1.0	1
Tyr	1.8	2	1.0	1	1.0	1		
Phe	2.9	3	1.0	1	1.0	1		

[a] Molar ratio. [b] See Figures 8 and 9. [c] Residue numbers in the BGH sequence (Figure 1). [d] Cys determined as carboxymethylcysteine.

TABLE VIII: Growth-Promoting Activity of Peptide Fragments Obtained by Limited Tryptic Digests of BGH and SGH.

Preparation[a]	Total Dose (μg)	Response[b]
B3-1	0	183 ± 6 (4)
	200	208 ± 16 (5)
	600	223 ± 19 (5)
S3-1	0	176 ± 10 (6)
	200	210 ± 8 (4)
	600	228 ± 9 (4)
RB2b′	0	173 ± 3 (6)
	200	119 ± 12 (5)
	600	233 ± 11 (4)
BGH	0	168 ± 2 (5)
	20	211 ± 4 (4)
	60	269 ± 5 (5)

[a] For fragments B3-1, S3-1, and RB2b′, see Figures 3 and 8. [b] Tibia width in micra; mean ± standard error (number of rats).

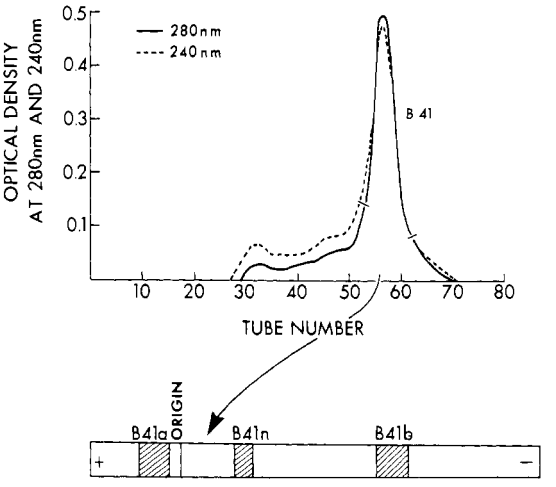

FIGURE 9: Fractionation of B4 (Figure 2). Fraction B4 (8 mg) was chromatographed on Sephadex G-25 (95 cm × 1.5 cm) in 0.5 M acetic acid; tube volume, 2 ml. The main peak (B41) was subjected to paper electrophoresis at pH 6.4.

acid analyses (Table VII) also indicated that RB2b′ is a homogeneous peptide. The dansyl-Edman method revealed its NH_2-terminal sequence to be: Ser-Asx-Asx-. Analysis of carboxypeptidase A digests of RB2b′ gave: -Ala-Phe-OH. From these data it is apparent that fragment RB2b′ was derived from the COOH-terminal portion of the sequence between residues 151 and 191 (Figure 1).

Fraction B4 (Figure 2) was subjected to gel filtration on Sephadex G-25 and subsequently to paper electrophoresis at pH 6.4 as shown in Figure 9. The peptide eluted from band B41n was further purified by paper electrophoresis at pH 2.1. Amino acid compositions of all these purified peptides are summarized in Table VII. The NH_2-terminal sequences of peptides B41n and B41b were found to be Glx-Thr-Tyr- and Ala-Gly-Glx-, respectively. These data clearly indicate that they originate from residues 140–150 and 134–139, respectively, of the BGH molecule (Figure 1). The NH_2-terminal residue of peptide B41a having the same amino acid composition as B41n (Table VII) was undetectable by the dansyl method. These characteristics and the difference between the electrophoretic mobilities of peptides B41a and B41n suggest that they have the same structure except that the NH_2-terminal glutamine of B41a has been cyclized to pyrrolidonecarboxylyl residue.

Growth Promoting Activity of Fragments B3-1, S3-1, and RB2b′. Bioassay results of these fragments are summarized in Table VIII. It may be noted that these fragments give similar response in the tibia test. A total dose of 200 or 600 μg caused an increment of tibial width of 25–50 over the control value.

Discussion

It is evident from Figures 2-4 and Tables I-III that the behavior of BGH and SGH toward limited tryptic digestion is identical. For further characterization of peptide fragments obtained from these digests, only those derived from the bovine hormone were investigated. It was shown that fraction B3 (Figure 2) consisted of a fragment (B3-1, Figure 3) originating in residues 96–133 of the BGH structure (Figure 6). Fragments B41b, 41a, and 41n (Figure 9) isolated from fraction B4 (Figure 2) were located between positions 134–139 and 140–150 of the sequence, respectively (Table VII). The identity of fragment RB2b′ with the COOH-terminal portion, residues 151–191, of the BGH (Figure 8, Table VII) together with some preliminary data on the composition of fraction RB2a (Figure 8) indicate that fraction B2 (Figure 2) is mainly composed of two large fragments (residues 1–95 and 151–191) of BGH, connected by the disulfide bridge at positions 53 and 164 (see Figure 1).

Some observations on structure investigations of fragment B3-1 (Tables IV–VI) may be emphasized. Human plasmin cleaved specifically the lysylleucine bond[4] of B3-1 at position 112–113 of the BGH structure, resulting in the formation of two fragments, P1 and P2 (Figure 5, Table IV). One of these plasmic fragments, P2, was useful in connecting peptides K51 with K52 (Figure 6).

Both fragments B3-1 and S3-1 are resistant to digestion with leucineaminopeptidase, whereas the two plasmin fragments of B3-1 are easily hydrolyzed by the enzyme. However, the plasmic digest of B3-1 before fractionation is not attacked by leucineaminopeptidase. These data suggest that the conformational integrity of fragment B3-1 was not destroyed by this single cleavage at residue position 112–113. It requires further physicochemical studies to define what specific intramolecular interactions[5] are responsible for the resistance of B3-1 and S3-1 to enzymic attacks.

Analyzing the sequence of peptide K52 by the dansyl-Edman method, both leucine and valine were found at residue position 127 (Table V). From the amino acid composition of K52 and K6, the Leu and Val forms of BGH are estimated to have a molar ratio of 7:3 (Tables IV and VI). The resolution of the corresponding tryptic fragment of B3-1 into forms A and B by paper chromatography–gel electrophoresis (Figure 4, Table III) is evidently due to the same partial Leu/Val substitution at sequence position 127. All these data are in fair agreement with those reported by Seavey *et al.* (1971) in regard to the occurrence of allelic BGH forms.

As to the presence of peptides bA′ and bB′ on the tryptic peptide map (Figure 4), Seavey *et al.* (1971) proposed that they were probably deamidated forms of peptides bA and bB, respectively. This explanation seemed rather reasonable in view of the sequence of this portion of BGH (Table V, Figure 6) and the ability of asparagine in the asparaginylglycine sequence for deamidation (Gráf *et al.*, 1970, 1971). However, the paper electrophoresis of peptide K6 at pH 6.4 (Figure 7) gave evidence that Asp-129 was not admidated at all. The electrophoretic heterogeneity of peptide K6 appeared only when run at lower pH value, *i.e.*, 3.5 (Figure 7). Since this value is near to the dissociation constant of the β-carboxyl group of aspartic acid, it is very probable that peptide K6b, the more acidic component, contains a β-aspartylglycine bond at positions 129–130, whereas K6a represents the α peptide.

It has been noted that the deamidation of an asparaginylglycine bond, accompanied by transpeptidation, results in the formation of a much larger amount of the β form than the α form of the peptide (Gráf *et al.*, 1971; unpublished data). In the case of peptide K6, the two electrophoretic forms appeared in a reverse ratio, which suggests that originally aspartic acid (at least partly, if not exclusively) was present at position 129 of the BGH structure, rather than asparagine. As to the occurrence of a small amount of the slightly more acidic form of the aspartylglycine peptides (bA′, bB′, and K6b in Figures 4 and 7), two explanations may be raised: (a) the aspartylglycine bond in residue positions 129–130 underwent a partial transpeptidation during the isolation procedure; (b) aspartic acid and asparagine together were present at position 129, similar to the microheterogeneity of genetic nature at position 127, and the asparagine fraction has undergone deamidation according to the proposed mechanism (Gráf *et al.*, 1971).

The sequence of fragment B3-1 shown in Figure 6 differs from that given by Yamasaki *et al.* (1972) in that it contains one more leucine residue at position 121 of the BGH structure. From the amino acid composition (Table II) and the tryptic peptide maps (Figure 4, Table III) it is clear that fragments B3-1 and S3-1 occupy the same positions (residues 96–133) of the BGH (Figure 1) and SGH (Li *et al.*, 1973) molecule.

It must be pointed out that the responses obtained with fragment B3-1 on the tibia assay are much less than those reported by Sonenberg and coworkers (Sonenberg *et al.*, 1972; Yamasaki *et al.*, 1970) for fragment A-II. As shown in Table VIII, the dose–response relationships for B3-1 and S3-1 are different from the native hormone. The data for these fragments give much smaller slopes in comparison with that of BGH, and it is thus impossible to statistically estimate their potencies in terms of native BGH. Nevertheless, they exhibit similar measurable responses in the tibia test. In this connection, we have recently synthesized a dotarakonta-peptide (homologous to fragment B3-1) corresponding to residues 95–136 of the HGH structure (Li, 1972) and found that the synthetic peptide had similar measurable responses in the tibia test (Blake and Li, 1973). It should also be noted in Table VIII that fragment of RB2b′, which is shown to be derived from the COOH-terminal portion (residues 151–191) of BGH, also gave practically the same tibia response as fragments B3-1 and S3-1. It is of interest to note (Li and Gráf, 1974) that a fragment which was isolated from plasmic digests of HGH and shown to be derived from the COOH-terminal residues 141–191 had similar potency as exhibited by RB2b′ described herein.

Acknowledgment

The authors thank Jean Knorr, Daniel Gordon, and J. D. Nelson for their skilled technical assistance.

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[4] The sensitivity of this particular peptide bond to plasmin in oxidized BGH has recently been reported (Gráf and Li, 1974a).

[5] Comparative gel filtration experiments carried out with fragment B3-1 on Sephadex G-50 in 0.05 M NH_4HCO_3 buffer of pH 8.5 and in 50% acetic acid (V_e/V_0 value is constantly 1.4–1.5) indicated that the fragment was in monomeric form under the conditions of leucineaminopeptidase hydrolysis.

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Cyclic Adenosine 3′,5′-Monophosphate Responses to Concanavalin A in Human Lymphocytes. Evidence that the Response Involves Specific Carbohydrate Receptors on the Cell Surface†

Leon R. Lyle‡ and Charles W. Parker*

ABSTRACT: Human peripheral blood lymphocytes, purified by isopycnic centrifugation, have been shown to undergo early increases in cAMP in response to concanavalin A (Con A). Changes in cAMP were demonstrable within 1 min suggesting that the response is initiated at the cell surface. This was confirmed by demonstrating that stimulation is also obtainable with Con A substituted polylysine–agarose beads, which cannot enter the cell. Control experiments with beads containing Con A indicated that the lectin remains attached to the beads under conditions in which stimulation of lymphocytes occurs. The cAMP response to Con A was blocked by methyl α-mannoside and methyl α-glucoside, which have affinity for the specific carbohydrate receptors on Con A, but not by other simple sugars. Taken together these observations indicate that Con A stimulates cAMP accumulation through its ability to interact selectively with carbohydrate receptors on the cell surface.

Stimulation of lymphocyte transformation by *Phaseolus vulgaris* phytohemagglutinin (PHA)[1] and other mitogenic lectins has been a widely studied model for induction of cell growth but the intriguing question of how the stimulation takes place has been difficult to elucidate. It was recently shown that PHA that is covalently bound to Sepharose can induce mitogenesis in mouse lymphocytes under conditions in which solubilization of mitogen is not demonstrable, indicating that a surface interaction is involved (Greaves and Bauminger, 1972). This creates a presumptive requirement for a secondary messenger inside the cell (Parker *et al.*, 1974).

Recent observations in human lymphocytes indicate that PHA produces changes in lymphocyte adenylate cyclase activity resulting either in a rise or a fall in cAMP (Smith *et*

† From the Washington University School of Medicine, Department of Medicine, St. Louis, Missouri 63110. *Received July 1, 1974.* Supported by a U. S. Public Health Service Training Grant AI00219.

‡ Present address: Mallinckrodt/Nuclear, Maryland Heights, Missouri 63145.

[1] Abbreviations used are: PHA, phytohemagglutinin; Con A, concanavalin A.