Human Pituitary Growth Hormone

XI. Rate of Hydrolysis by Trypsin, Chymotrypsin, and Pepsin; Effect of Trypsin on the Biological Activity

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SUMMARY

Human pituitary growth hormone (HGH), both native and oxidized, has been subjected to hydrolysis with chymotrypsin, trypsin, and pepsin. With chymotrypsin and trypsin, the course of enzymic digestion was followed in the pH stat by recording the alkali uptake during digestion, and in addition, quantitative information about the dinitrophenylation of the digestion products was obtained. Hydrolysis of HGH with chymotrypsin was completed in 12 hr at 32° and with trypsin in 20 hr at 37°. With pepsin, a different procedure was followed; the rates of peptic digestion for varying lengths of time were studied, and the number of NH_2-terminal residues released was determined by quantitative dinitrophenylation in the pH-stat.

The NH_2-terminal amino acids released by digestion with the three enzymes were identified by quantitative paper chromatography of hydrolyzates of the dinitrophenylated digestion products. The effect of trypsin on the biological potency of HGH has also been investigated. It was found that partial hydrolysis of the hormone with trypsin does not diminish the growth-promoting and lactogenic activities.

INTRODUCTION

In structural studies on proteins, digestion with proteolytic enzymes to obtain smaller peptide fragments is a standard procedure. In a previous report (1), it was shown that information about the completeness of enzymic digestion of ovine prolactin can be obtained by recording the alkali uptake during digestion and during dinitrophenylation of the digestion products. This communication is a report of the results of digestion of native and oxidized HGH with chymotrypsin, trypsin, and pepsin, with the purpose of finding the proper conditions for complete digestion by these three enzymes, investigating the release of new amino groups in the digestion, and recording the rate of hydrolysis by these enzymes.

Human pituitary growth hormone has been shown to retain its biological activity after partial chymotryptic (2) and peptic (3) hydrolysis. This paper also records the results with trypsin; it will be noted that partial hydrolysis of HGH by this enzyme confirms the observation that the whole protein molecule is not necessary for its biological potency.

MATERIALS AND METHODS

HGH was prepared from fresh glands by procedures previously described (2–5). Protein content was computed from nitrogen determinations, all calculations being based on the fact that the nitrogen content for the ash-free anhydrous HGH is 16.20% (3). Oxidized HGH was prepared by treatment

with performic acid as described previously (6).

Trypsin was a commercial preparation obtained from Worthington Biochemical Corporation, Freehold, New Jersey (lot no. 6122, salt free, 2 × cryst.). Chymotrypsin was obtained from Armour Laboratories (lot no. 381092). Pepsin was also an Armour product (lot no. 323). Solutions of the enzymes were prepared in water in appropriate concentrations immediately before use. Other chemicals used were of reagent grade and were used without further purification.

The pH stat used was the Combititrator 3D from Methrom Ltd., equipped with a combined glass-calomel microelectrode EA 125 with shock-resistant membrane. The titrations were performed as described in (1). The growth-promoting activity was assayed in hypophysectomized rats by the tibia test (7), and the pigeon crop sac-stimulating activity by the method of Lyons (8).

Digestions with chymotrypsin and trypsin were performed as described previously (1). The determination of protein content of the digested samples, dinitrophenylation, and identification and determination of NH_2-terminal amino acids were also performed according to (1).

Since the optimum pH for pepsin is 2, the rate of the digestion could not be followed with the pH stat. The following procedure was therefore used with this enzyme: In the reaction vessel of the pH stat HGH, 10 mg, was dissolved in 1.0 ml of 0.01 N HCl, and the pH was adjusted to 2.0 by the addition of 1 N HCl (≃0.05 ml). A solution of pepsin (1.0 mg in 7.5 ml 0.01 N HCl), in the amount of 0.5 ml, was then added and the pH was adjusted to 2.0 with 1 N HCl (≃0.04 ml). The enzymic reaction was allowed to proceed at room temperature (ca 25°) for various periods of time. The reaction was terminated at the appropriate moment by the addition of 1 N NaOH (≃0.1 ml); the pH was adjusted to a final value of 8.0 by the addition of 0.05 N NaOH from the burette of the pH stat. The temperature of the solution was raised to 40°, the reaction mixture was dinitrophenylated by the addition of 0.1 ml of fluorodinitrobenzene, and the uptake of 0.05 N NaOH was recorded as described previously (1). The number of peptide bonds broken was calculated from the difference in alkali uptake during dinitrophenylation of intact protein and of enzymic digests. The ether-soluble NH_2-terminal dinitrophenylated (DNP-) amino acids in the peptic digests were identified as described in (1).

RESULTS AND DISCUSSION

Digestion with Chymotrypsin

The alkali uptake as a function of time during digestion of HGH with chymotrypsin is shown in Fig. 1. Digestion of oxidized HGH proceeded at a higher rate at the beginning of the reaction (11.5 bonds broken in 32 min, 14.9 bonds broken in 90 min), but the number of bonds broken in 12 hr was the same as in the native material (Table 1). Table 2 shows the results of dinitrophenylation of the digests; the estimated number of NH_2-terminal groups released is in good agreement with the number calculated from the alkali uptake recorded during digestion. Since chymotrypsin is known to hydrolyze specifically peptide bonds containing tyrosine and tryptophan, it can be calculated that complete chymotryptic hydrolysis of

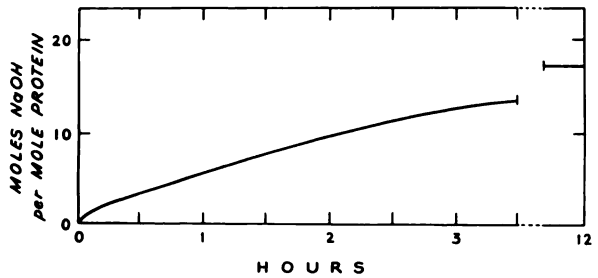

FIG. 1. *Alkali uptake during digestion of HGH with chymotrypsin as recorded by pH stat* Enzyme:hormone ratio = 1:100 (w/w). Temp. 32°, pH 8.5, 1% solution of HGH.

TABLE 1
Alkali uptake and estimated number of bonds broken during digestion of native and oxidized HGH with chymotrypsin and trypsin

Preparation[a]	Digestion with chymotrypsin		Digestion with trypsin	
	Alkali uptake (mole per mole of protein)	Estimated number of bonds split per mole of protein	Alkali uptake (mole per mole of protein)	Estimated number of bonds split per mole of protein
HGH	18.0	19.4	21.2	22.8
O-HGH	17.1	18.4	22.8	24.5

[a] O-HGH, performic acid oxidized HGH.

TABLE 2
Alkali uptake during dinitrophenylation of HGH and oxidized HGH before and after enzymic digestion

Preparation[a]	Enzyme[b]	Alkali uptake (mole per mole of protein)	Difference in alkali uptake in dinitrophenylation of digested and intact hormones (mole per mole of protein)	Estimated number of released NH_2-terminal groups
HGH	None	23.0	0	0
O-HGH	None	22.0	0	0
HGH	Chymotrypsin	43.4	20.4	18.6
O-HGH	Chymotrypsin	42.4	20.4	18.6
HGH	Trypsin	47.4	24.4	22.2
O-HGH	Trypsin	47.5	25.5	23.2
HGH	Pepsin, 1/2 hr	33.0	10.0	9.1
HGH	Pepsin, 2 hr	39.1	16.1	14.7
HGH	Pepsin, 24 hr	51.2	28.2	25.6
O-HGH	Pepsin, 24 hr	47.4	25.4	23.1

[a] O-HGH, performic acid-oxidized HGH.
[b] Conditions for enzymic digestions, see text.

HGH results in the rupture of 20 peptide bonds, a number to be expected from the content of aromatic amino acids (9, 10) in the hormone molecule.

In Table 3a are shown the NH_2-terminal groups in the enzymic digests. The content of DNP-amino acids in the chymotryptic digests of HGH and oxidized HGH suggests the presence of Phe-Phe, x-Asp, x-Glu, x-Ser, x-Ala, x-Leu bonds where x is either tyrosine or tryptophan. It may be recalled that hydrolysis of HGH with chymotrypsin to the extent of 10% did not cause inactivation, but longer digestion did diminish growth-promoting activity (2).

Digestion with Trypsin

The rate of tryptic digestion is shown in Fig. 2. The reaction is rapid at the beginning, resulting in the cleavage of about 60% of the total expected number of peptide bonds within 1 hr. The digestion of oxidized HGH proceeds at a still higher rate (14.0 bonds cleaved in 12 min, 17.5 bonds in 60 min). The results of the digestion are shown in Table 1, and of the subsequent dinitrophenylation of the digestion products, in Table 2. The number of bonds broken during 20 hr of digestion with trypsin are about the same for the oxidized and for the native protein hormone.

Because trypsin is highly specific for the rupture of peptide bonds containing lysine and arginine, it can be calculated from the content of these two basic amino acids in the HGH molecule (9, 10) that complete tryptic hydrolysis results in the hydrolysis of 19 peptide bonds, yielding 19 new NH_2-

TABLE 3a

NH_2-terminal amino acid residues in chymotryptic and tryptic digests of HGH and oxidized HGH

DNP-amino acid[a]	Chymotryptic digest						Tryptic digest					
	HGH			O-HGH[b]			HGH			O-HGH		
	Precipitated	Ether soluble	Sum	Precipitated	Ether soluble	Sum	Precipitated	Ether soluble	Sum	Precipitated	Ether soluble	Sum
Aspartic acid	1.5	0.4	1.9	1.5	0.1	1.6	1.8	0.3	2.1	1.9	0.5	2.4
Glutamic acid	0.6	0.2	0.8	0.8	0.1	0.9	1.4	0.5	1.9	0.9	0.6	1.5
Serine	0.7	0.4	1.1	1.1	0.2	1.3	1.5	0.5	2.0	1.1	0.4	1.5
Threonine	0.1	0.1	0.2	0.2	0.1	0.3	0.8	0.2	1.0	0.9	0.1	1.0
Glycine	0.2	0.2	0.4	0.6	0.2	0.8	0.2	0.1	0.3	0.3	0.1	0.4
Alanine	0.8	0.2	1.0	1.1	0.1	1.2	0.5	0.3	0.8	0.6	0.1	0.7
Leucine	1.7	0.3	2.0	1.8	0.2	2.0	2.3	0.4	2.7	2.3	0.5	2.8
Phenylalanine	0.3	0.3	0.6	1.2	0.3	1.5	1.1	0.2	1.3	1.1	0.3	1.4

[a] Residues per mole of protein. Values not corrected for losses during hydrolysis and chromatography.
[b] O-HGH, performic acid-oxidized HGH.

TABLE 3b

NH_2-terminal amino acid residues in peptic digests of HGH and oxidized HGH

DNP-amino acid[a]	HGH									O-HGH[b]		
	1/2-hr digestion			2-hr digestion			24-hr digestion					
	Precipitated	Ether soluble	Sum	Precipitated	Ether soluble	Sum	Precipitated	Ether soluble	Sum	Precipitated	Ether soluble	Sum
Aspartic acid	0.5	0.2	0.7	0.5	0.2	0.7	0.7	0.2	0.9	0.8	0.1	0.9
Glutamic acid	0.2	0.1	0.3	0.3	0.3	0.6	0.4	0.7	1.1	0.4	0.4	0.8
Serine	—	0.2	0.2	0.2	0.2	0.4	0.1	0.2	0.3	0.2	—	0.2
Alanine	0.4	0.1	0.5	0.8	0.1	0.9	0.7	0.2	0.9	0.8	0.1	0.9
Leucine	1.9	0.2	2.1	3.4	0.3	3.7	3.1	0.9	4.0	3.6	0.4	4.0
Phenylalanine	1.0	0.5	1.5	1.0	0.6	1.6	0.8	0.9	1.7	1.5	0.4	1.9
Dilysine	0.2	—	0.2	0.7	0.1	0.8	0.8	0.3	1.1	0.9	0.2	1.1
Dityrosine	0.4	0.1	0.5	1.0	0.3	1.3	0.7	1.0	1.7	1.1	0.5	1.6

[a] Residues per mole of protein. Values not corrected for losses during hydrolysis and chromatography.
[b] O-HGH, performic acid oxidized HGH; only 24-hr digest was used.

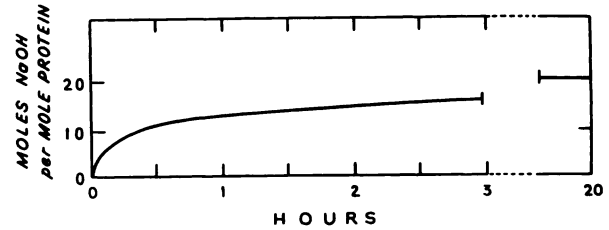

FIG. 2. *Alkali uptake during digestion of HGH with trypsin*
Enzyme:hormone ratio = 1:100 (w/w). Temp. 37°, pH 8.5, 1% solution of HGH.

terminal amino acids. The results presented in Table 1 and Table 2 indicate the cleavage of 22–23 bonds, which might reflect the presence of some chymotryptic activity in the preparation of trypsin used (cf. 1). The NH_2-terminal groups found in the enzymic digests are shown in Table 3a; the values are not corrected for losses during hydrolysis or chromatography. It is of interest to note that the number of NH_2-terminal amino acid residues in the digest of HGH is in good agreement with that in the digest of the oxidized hormone.

The effect of tryptic hydrolysis on the growth-promoting and crop sac-stimulating activities of HGH may be seen in Table 4; both activities are retained after digestion for 30 min. This indicates that 12 out of 23 bonds (see Fig. 2) can be hydrolyzed by trypsin with little loss of biological activities of the hormone.

TABLE 4a
Effect of tryptic digestion on growth-promoting activity of HGH

Time of digestion (hr)	Number of peptide bonds hydrolyzed	Number of rats	Tibia width[a] (μ)
0	0	12	232 ± 2
0.25	9	11	229 ± 1
0.5	12	12	219 ± 3
1.0	15	12	163 ± 4
20.0	23	12	154 ± 3
Controls		6	161 ± 2

[a] A total dose of 40 μg in 4 days; mean ± standard error.

Digestion with Pepsin

The peptic digestion of HGH proceeds at a rapid rate at the beginning of the reaction (Table 2). Thus, 35% of the bonds cleaved in 24 hr are broken within 30 min. It has previously been shown (3) that HGH retains its biological activity after 60 min of digestion with pepsin, but that the activity is completely lost after 2 hr of digestion.

It is of interest to note that the same number of bonds are broken during digestion for 24 hr of an oxidized sample as with the native protein. It is also interesting that pepsin digests HGH to about the same extent as does trypsin. Thus, because of the differing specificities of the two enzymes, overlapping peptide fragments which could be of great value in sequence determinations can be obtained by tryptic and peptic digestion. NH_2-terminal groups in the digests are presented in Table 3b; as with the other two enzymes, the calculations are not corrected for losses. The NH_2-terminal amino acids obtained by the 24-hr digestion are almost

TABLE 4b
Effect of tryptic digestion on pigeon crop sac-stimulating activity of HGH

Time of digestion (hr)	Number of peptide bonds hydrolyzed	Total dose[a] (μg)	Response[b]
0	0	4	1+, 2+, 3+, 1+, 2+, 2+, 2+, 2+
			1+, 2+, 1+, 1+
		2	2+, 2+, 1+, 1+
0.25	9	4	3+, 2+, 2+, 2+, 1+, 2+, 2+, 2+
			1+, 1+, 1+, 1+, 2+, 2+, 1+, 1+
		2	1+, 1+, 1+, 2+, 2+, 2+, 2+
0.5	12	4	2+, 1+, 2+, 1+, 1+, 2+, 1+, 2+
		2	1+, 2+, 1+, 1+
1.0	15	4	0, 0, 0, 0, 0, 0, 0, 0
20.0	23	10	0, 0, 0, 0, 0, 0, 0, 0

[a] A total dose in 2 days.
[b] No stimulation, −; moderate stimulation, 1+; good stimulation, 2+; marked stimulation, 3+.

identical with those of oxidized HGH, and the yields are comparable.

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