EFFECT OF TRYPSIN-DIGESTED BOVINE GROWTH HORMONE ON WHOLE-BODY PROTEIN SYNTHESIS *IN VITRO* IN CHICKEN EMBRYOS

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Summary

The effect of bovine growth hormone digested with trypsin on whole-body protein synthesis *in vitro* of chicken embryos was investigated by using a whole-embryo culture system. Bovine growth hormone at 5.3 and 530 ng/ml was digested partially and completely with trypsin for 4 min and 18 h, respectively. After culturing chicken embryos with a synthetic medium containing L-[4-^{3}H] phenylalanine, whole-embryo protein synthesis was determined from the ratio of specific radioactivities of free and protein-bound phenylalanine. Whole-embryo protein synthesis of the control group cultured with no bovine growth hormone was 49.5 ± 2.2 %/d. There was no significant interaction between digestion time and the concentration of trypsin-digested bovine growth hormone. Tryptic digestion of bovine growth hormone increased fractional synthesis rates of whole-body protein compared to the 0-min groups, and there was no significant difference between the 4-min and 18-h groups. The higher concentration (530 ng/ml) of trypsin-digested bovine growth hormone was more effective in enhancing whole-embryo protein synthesis than the lower concentration (5.3 ng/ml).
(**Key Words**: Protein Synthesis, Bovine Growth Hormone, Trypsin Digestion, Embryo, Chicken)

Introduction

Growth hormone has an important role in enhancing growth of young animals, and the effects of exogenous bovine growth hormone on growth and protein metabolism have well been investigated in mammalian and avian species. Injection of bovine growth hormone improved milk production, and increased the concentration of milk protein, milk fat and milk lactose in dairy cows (Machlin, 1973; Bauman et al., 1985; Peel et al., 1985; Heap et al., 1988). Body weight and feed conversion rate of female lambs were increased by the injection of bovine growth hormone (Johnsson et al., 1985). Pell and Bates (1987) reported that the administration of bovine growth hormone caused a significant increase in muscle growth, which was accounted for by increased rate of muscle protein synthesis. In contrast to mammalian species, however, little effect of the administration of bovine growth hormone on growth and protein metabolism was found in young chickens (Carter et al., 1955; Glick, 1960).

The biological activity of bovine growth hormone is not completely lost by hydrolysis with trypsin (Sonenberg et al., 1965) and chymotrypsin (Sonenberg et al., 1969), and it was reported that in young broilers feed conversion rate and carcass protein content were improved by administrating bovine growth hormone digested partially with trypsin (Myers and Peterson, 1974). However, there was no available information with respect to the effect of trypsin-digested bovine growth hormone on protein synthesis in the chicken. In the present study, the effect of bovine growth hormone digested with trypsin on whole-body protein synthesis of chicken embryos was investigated by using *in vitro* whole-embryo culture system, a quick and convenient method for screening growth-promoting potential of a variety of physiologically active compounds in the chicken (Muramatsu et al., 1992).

Materials and Methods

Tryptic digestion of bovine growth hormone

Bovine growth hormone (USDA-bGH-B-1)

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which was kindly gifted by Dr. S. Raiti (National Hormone and Pituitary Program, University of Maryland School of Medicine, Baltimore, MD 21201-3472, USA), trypsin (sequencing grade; Sigma Chemical Company, St. Louis, MO 6 3178-9916, USA) and soybean trypsin inhibitor (Sigma Chemical Company, St. Louis, MO 631 78-9916, USA) were dissolved in a Tris-HCl buffer (.1M, pH 8.5). Tryptic digestion was performed by mixing 120 μl of the bovine growth hormone solution (2.3 mg/ml) and 30 μl of trypsin solution (250 μg/ml) and incubating at 25℃. At 4 min or 18 h after the incubation, the digestion reaction was terminated by adding soybean trypsin inhibitor solution (2.5 mg/ml). For those digested as 0 min, soybean trypsin inhibitor solution was added to the bovine growth hormone solution before the incubation.

Reversed phase HPLC

Separation of various peptide fragments of the trypsin-digested bovine growth hormone was performed on a C-8 reversed phase column (particle size 5 μm, 250 mm × 4.6 mm I.D.; CAPCELL PAK C8 SG300, Shiseido Co. Ltd., Tokyo 104, Japan) with a HPLC (LC-6A system, Shimadzu Co. Ltd., Kyoto 604, Japan). After 50 μl of sample solution was injected into the chromatographic system, a linear gradient of the eluate was applied from 15% (v/v) acetonitrile - 85% (v/v) water to 60% (v/v) acetonitrile - water 40% (v/v) water at a flow rate of 1 ml/min. A washing step was done for 5 min with 15% (v/v) acetonitrile - 85% (v/v) water. The column temperature was kept at 40℃. The photometric intensity of derived peptides was measured at a wavelength of 215 nm.

Whole-embryo culture *in vitro*

Fertilized eggs from Single Comb White Leghorn hens maintained in our laboratory were incubated at 38℃ and about 70% relative humidity for 7 d. On day 7 of incubation, 48 eggs were randomly distributed into 6 groups of 8 eggs. A part of egg shell from the blunt end was carefully removed, and an embryo was removed and placed onto a plastic net. The embryo was then washed with physiological saline (0.85 NaCl) pre-warmed at 38℃, transferred individually into a glass bottle with 9.2 ml of culture medium, and cultured as described previously (Muramatsu et al., 1992). The culture medium (9.2 ml) was prepared by mixing with 5.4 ml of Ham's F-10 with L-glutamine, 1.8 ml of MEM Eagle's essential amino acids solution without L-glutamine, 1.8 ml of non-essential amino acids solution (Whittaker Bioproducts, Walkersville, MD 21793, USA), and 0.2 ml of trypsin-digested bovine growth hormone solution. Final concentrations of trypsin-digested bovine growth hormone were set at 5.3 and 530 ng/ml. Labeled L-[4-^{3}H] phenylalanine (37.0 MBq/ml, 1.07 TBq/mmol; Amersham Japan Co. Ltd., Tokyo 112, Japan) was added to the culture medium to give specific radioactivity of phenylalanine at approximately 800 dpm/nmol. Prior to the culture, pH of the medium was adjusted to 7.0 with 3N NaOH. The culture medium was then bubbled with $O_2 : CO_2$ (19 : 1) at 38℃ for 60 min. The chicken embryo was injected intraperitoneally with 0.5 ml of the culture medium, and then the glass bottle containing the embryo and culture medium was set in a rotatory whole-embryo culture system (Type 10-0310, Ikemoto Scientific Technology Co. Ltd., Tokyo 113, Japan). Embryos were incubated under the $O_2 : CO_2$ (19 : 1) gas phase at 38℃ at a rotation speed of 45 rpm for 10 or 60 min. The gas flow rate was set at 17 ml/min. After 10 and 60 min of culture period, three and five embryos respectively were removed from glass bottles, washed with physiological saline, blotted gently, frozen by plunging into liquid nitrogen, and were stored at −20℃ until analysis.

Fractional synthesis rate (FSR, %/d) of whole-body protein, expressed as percentage of protein synthesized per day to the total protein mass, was calculated according to the equation of Garlick et al. (1980). Two way analysis of variance was done on all data, and significance of differences between means was assessed by Duncan's multiple range test using the GLM procedure of SAS (1985).

Results

Chromatograms of the trypsin-digested bovine growth hormone with a reversed-phase HPLC are shown in figure 1. Intact bovine growth hormone was detected at 17.1 min of the retention time. At 4 min of digestion, the peak of intact bovine growth hormone was lower than that of the 0-min group, and 76% of bovine growth

hormone was estimated to be hydrolyzed during 4 min of tryptic digestion. Partial digestion of bovine growth hormone with trypsin generated new peaks corresponding to fragmented peptides which were detected at 4.6, 5.7, 7.4, 10.5, 12.1, 15.3 and 17.7 min of the retention time. After complete digestion for 18 h, there were more new peptide peaks at 4.3, 5.0, 6.1, 7.1, 9.5, 10.4, 10.8, 11.6 and 15.0 min of the retention time.

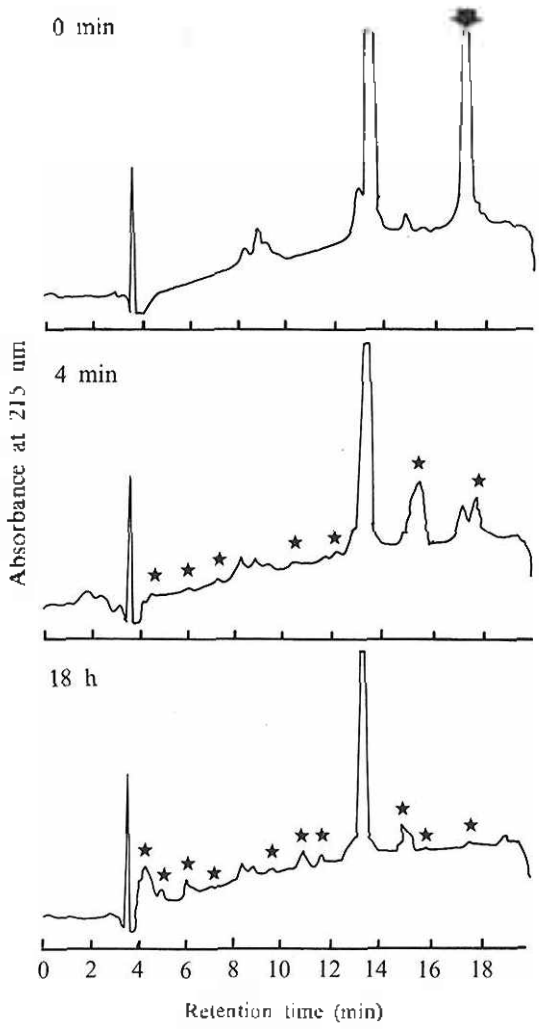

Figure 1. Chromatograms of trypsin-digested bovine growth hormone. Samples were applied to C 8 reversed phase HPLC system. A linear gradient of the eluate was applied from 15% (v/v) acetonitrile - 85% (v/v) water to 60% (v/v) acetonitrile - water 40% (v/v) water at a flow rate of 1 ml/min, and the effluent was monitored at 215 nm. The column temperature was kept at 40℃. Asterisks indicate the peaks corresponcing to newly formed peptides, and the arrow shows the peak of intact bovine growth hormone.

Table 1 represents the effect of trypsin-digested bovine growth hormone on fractional synthesis rates of whole-body protein in chicken embryos cultured *in vitro*. The value for the control group with no bovine growth hormone was 49.5 ± 2.2 %/d. There was no significant interaction between digestion time and the concentration of bovine growth hormone. Tryptic digestion of bovine growth hormone for both 4 min and 18 h increased fractional synthesis rates of whole-body protein compared to the 0-min group, and there was no significant difference between 4-min and 18-h groups. The higher concentration (530 ng/ml) of trypsin-digested bovine growth hormone was more effective in increasing whole-embryo protein synthesis than the lower concentration (5.3 ng/ml).

Discussion

Trypsin is one of proteolytic enzymes present in the lumen of small intestine and acts on the carboxyl terminal amino acid residue of lysyl and arginyl bonds of peptide chains. As bovine growth hormone is consisted of 191 amino acids containing 13 arginine and 11 lysine (Wallis, 1975), it is expected that maximally 24 peptide bonds can be digested with trypsin. As shown in figure 1, 7 newly yielded peptide peaks were detected by reversed-phase HPLC which confirmed that the tryptic digestion fragmented intact bovine growth hormone molecules. Although 10 new peptide pecks were detected by the 18-h digestion, most of retention times of these peptides were quite different from those of peptides generated by the 4-min digestion. This difference may be ascribed to the shift of peptide population with high to low molecular weights as the digestion went by from 4 min to 18 h, since the half life of proteolytic activity of trypsin is maintained for longer than 4 min (Wu et al., 1982).

Myers and Peterson (1974) reported that carcass protein content was improved in young broilers receiving bovine growth hormone digested partially with trypsin. As shown in table 1, bovine growth hormone digested with trypsin increased

TABLE 1. EFFECT OF PARTIALLY DIGESTED BOVINE GROWTH HORMONE ON WHOLE-BODY PROTEIN SYNTHESIS IN CHICKEN EMBRYOS DURING WHOLE-EMBRYO CULTURE *IN VITRO*[1,2]

Time for digestion	Bovine growth hormone		Mean
	5.3 ng/ml	530 ng/ml	
	(%/d)		
0 min	48.0	66.6	57.3[a]
4 min	60.1	79.1	69.6[b]
18 hour	62.6	71.2	66.9[b]
Mean	56.9[a]	72.3[a]	

Analysis of variance			
Source of variance	df	Mean square	Significance level
Time	2	417.9	$p < .05$
Dose	1	1,768.5	$p < .01$
Time × Dose	2	87.7	$p > .05$
Residual	24	82.5	

[a,b] Means not sharing a common superscript letter are significantly different at $p < .05$.
[1] The number of embryos used was five per treatment.
[2] The value for the control group (dose 0 ng/ml medium) was 49.5 ± 2.2 %/d.

protein synthesis of whole-embryo *in vitro*. Although the amino acid length of fragmented peptides was different between the 4-min and 18-h groups as shown in figure 1, there was no significant difference in whole-embryo protein synthesis. The higher concentration (530 ng/ml) of trypsin-digested bovine growth hormone was more effective in increasing whole-embryo protein synthesis than the lower concentration (5.3 ng/ml). Muramatsu et al. (1992) reported that partially trypsin-digested bovine growth hormone (500 ng/ml) elevated phenylalanine extraction from a culture medium into whole-embryos. From these findings, it would appear that partially or completely digested bovine growth hormone with trypsin has potency to enhance protein metabolism, i.e. amino acid incorporation into cells, protein synthesis and protein accretion in the chicken.

There is discrepancy concerning to the effect of intact bovine growth hormone on protein metabolism in the chicken. Although higher concentration (530 ng/ml) of intact bovine growth hormone increased whole-embryo protein synthesis in the present study, no influence of intact bovine growth hormone on phenylalaine extraction was detected up to 1,250 ng/ml (Muramatsu et al., 1992). This may mean that phenylalanine extraction does not necessarily reflect amino acid incorporation from the medium into the body protein of chicken embryos. In this respect, direct measurement of whole-body protein synthesis as done in the present study may provide more suitable and valid result than the measurement of phenylalanine extraction.

With no bovine growth hormone, the fractional synthesis rate of whole-body protein in chicken embryos measured *in vitro* in the present study was 49.5 %/d, which was much lower than those reported *in vivo* (Muramatsu et al., 1987, 1990). Goldspink et al. (1983) have stated that protein synthesis rates measured *in vitro* depend on, and can readily be altered by, various constituents including hormones in the culture medium. In the present study the addition of trypsin-digested bovine growth hormone in the culture medium increased whole-embryo protein synthesis, suggesting the possibility that the high rate of protein synthesis observed *in vitro* might be accounted for by various hormones and growth factors present in eggs. During the development, these growth factors are also synthesized within the embryonic body. It has been well known that there are many growth promoting factors, e.g. insulin, insulin-like growth factor I and II (Girbau et al., 1987), in chicken embryos, and their effects on whole-embryo protein synthesis in the chicken have not been clarified. This issue is to be investigated in the

future using the present *in vitro* culture method.

Literature Cited

Bauman, D. E., P. J. Eppard, M. J. DeGeeter and G. M. Lanza. 1985. Responses of high-producing dairy cows to long-term treatment with pituitary somatotropin and recombinant somatotropin. J. Dairy Sci. 68:1352-1362.

Carter, R. D., R. N. Risner and H. Yacowitz. 1955. Some effects of growth hormone preparations in pullets and mature hens. Poult. Sci. 34:1407-1414.

Garlick, P. J., M. A. McNurlan and V. R. Preedy. 1980. A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [^{3}H] phenylalanine. Biochem. J. 192:719-723.

Gruban, M., J. A. Gomez, M. A. Lesniak and F. de Pablo. 1987. Insulin and insulin like growth factor I both stimulate metabolism, growth, and differentiation in the postneurula chick embryo. Endocrinology 121:1477-1482.

Glick, B. 1960. The effect of bovine growth hormone, desoxycorticosterone, and cortisone on the weight of the bursa of Fabricius, adrenal glands, heart, and body weight of young chickens. Poult. Sci. 39:1527-1533.

Goldspink, D. F., P. J. Garlick and M. A. McNurlan. 1983. Protein turnover measured *in vivo* and *in vitro* in muscles undergoing compensatory growth and subsequent denervation atrophy. Biochem. J. 210:89-98.

Heap, R. B., I. R. Fleet, F. M. Fullerton, A. J. Davis, J. A. Goode, I. C. Hart, J. W. Pendleton, C. G. Prosser, L. M. Silvester and T. B. Mepham. 1988. A comparison of the mechanisms of action of bovine pituitary derived and recombinant somatotropin (ST) in inducing galactopoiesis in the cow during late lactation. Pages 73-84 in: Biotechnology in Growth Regulation. R. B. Heap, C. G. Prosser and G. E. Lamming, ed., Butterworths, London, UK.

Johnsson, I. D. and I. C. Hart. 1985. The effects of exogenous bovine growth hormone and bromocriptine on growth, body development, fleece weight and plasma concentrations of growth hormone, insulin and prolactin in female lambs. Anim. Prod. 41:207-217.

Machlin, L. J. 1973. Effect of growth hormone on milk production and feed utilization in daily cows. J. Daily Sci. 63:575-580.

Muramatsu, T., K. Kita, S. Nakagawa and J. Okumura. 1992. Whole-embryo culture as a quick and convenient method for searching growth-promoting factors in chickens: Effects of insulin, growth hormone, IGF-I and IGF-II on phenylalanine extraction. Comp. Biochem. Physiol. 104A:507-512).

Muramatsu, T., K. Hiramoto and J. Okumura. 1990. Strain differences in whole-body protein turnover in the chicken embryo. Br. Poult. Sci. 31:91-99.

Muramatsu, T., S. Muramatsu, J. Okumura and I. Tasaki. 1987. Developmental fall in whole body protein turnover of chick embryos during incubation. Br. Poult. Sci. 28:165-172.

Myers, W. R. and R. A. Peterson. 1974. Responses of six and ten-week-old broilers to a tryptic digest of bovine growth hormone. Poult. Sci. 53:508-514.

Peel, C. J., I. D. Sandles, K. J. Quelch and A. C. Herington. 1985. The effects of long-term administration of bovine growth hormone on the lactational performance of identical-twin dairy cows. Anim. Prod. 41:135-142.

Pell, J. M. and P. C. Bates. 1987. Collagen and non-collagen protein turnover in skeletal muscle of growth hormone treated lambs. J. Endocr. 115: R1-R4.

SAS Institute. 1985. SAS® User's Guide: Statistics, Version 5 Edition. SAS Institute Inc., Cary, NC.

Sonenberg, M., J. M. Dellacha, C. A. Free and A. C. Nadler. 1969. Growth hormone activity in man of chymotryptic digests of bovine growth hormone. J. Endocr. 44:255-265.

Sonenberg, M., C. A. Free, J. M. Dellacha, G. Bonadonna, A. Haymowitz and A. C. Nadler. 1965. The metabolic effects in man of bovine growth hormone digested with trypsin. Metabolism 14: 1189-1213.

Wallis, M. 1975. The molecular evolution of pituitary hormones. Biol. Rev. 50:35-98.

Wu, H. L., C. Kundrot and M. L. Bender. 1982. The denaturation of trypsin. Biochem. Biophys. Res. Comm. 107:742-745.