日本ウズラにおける骨格筋組織のcalpain活性と筋肉タンパク質分解速度との関係

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Correlation between Skeletal Muscle Calpain Activity and Fractional Rate of Muscle Degradation of Japanese Quail *Coturnix coturnix japonica*

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The present study was conducted to clarify the relationship between calpain (EC 3.4.22.17) and muscle protein degradation system using Japanese quail. The fractional rates of muscle protein degradation (Kd) were determined by the N^{τ}-methylhistidine methods, and both of calpain and calpastatin were separated by DEAE-Sephacel and Phenyl-Sepharose column chromatography, and measured their activities with alkali-denatured casein as a substrate.

The correlation coefficients between the Kd and the calpain or calpastatin activities were 0.728 and −0.453, respectively. Multiple regression equation of muscle protein degradation rate (Y) to calpain activity (X_1) and calpastatin activity (X_2) was calculated as $Y=5.892+14.033X_1-88.128X_2$ ($R^2=0.706$). The standardized partial regression coefficients of calpain and calpastatin to Kd were 0.605 and −0.453, respectively.

These values suggest that 1) activities of calpain and calpastatin affect the muscle protein degradation rate, and 2) individual information on calpain or calpastatin activities in muscle may be available as a selection characteristic for the protein turnover and growth.

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Key words : calpain, calpastatin, muscle protein turnover rate, Japanese quail

Introduction

The accumulation rate of muscle protein is specified by the balance between two antagonistic processes, myofibrillar protein synthesis and degradation. In the systems of myofibrillar protein degradation, it is well known that endogenous proteolytic enzyme activities are associated with the degradation (GOLL *et al.*, 1989).

Calpain (EC 3.4.22.17) is one of the typical endopeptidase specifically cleaves the cytoskeletal structure or myofibrillar proteins. The Z-line in the myofibril is very susceptible to digestion by this protease (BUSCH *et al.*, 1972 ; GOLL *et al.*, 1989). The activity of calpain is absolutely dependent on the presence of Ca^{2+} (SUZUKI *et al.*, 1987). An endogenous inhibitor known as calpastatin was also identified (ISHIURA *et al.*, 1980). However, the regulation of the activity of calpain which is controlled by calpain/calpastatin interaction *in vivo* have not been identified (GOLL *et al.*, 1989).

Many researchers have reported the relationship between calpain and animal growth. MAEDA *et al.* (1991) demonstrated that the Japanese quail line selected for small body weight had a higher degradation rate of muscle protein and calpain activity

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when compared to the line selected for large body weight. In the chicken, the native breeds showed a higher degradation rate and calpain activity than those of improved chicken groups, and egg breed showed higher degradation rates and calpain activities than meat breeds (MAEDA *et al.*, 1984, 1990 ; JOHARI *et al.*, 1994). Based upon these observations, the following study was designed to extend these findings to test the hypothesis that a direct relationship exists between the calpastatin activity in the skeletal muscle, the calpain activity and the fractional rate of protein degradation in Japanese quail. Determination of this relationship would confirm the knowledge that the specific alterations in proteinase and the inhibitor affect the protein turnover and enable finding of selection trait for the protein turnover and growth.

Materials and Methods

Birds and Management

Fifteen male birds of the random bred control population maintained in Faculty of Agriculture, Kagoshima University were used. All birds were reared in an electric brooder from hatch to 14 days of age, and then placed in wire-floored individual cages with 14 hr of artificial light per day and maintained at 22℃ from 15 to 23 days of age. Feed contained 19% crude protein and 13.1 MJ/kg metabolizable energy was fed *ad libitum* by using an individual feeder cup for each bird. Body weight gain and feed intake were recorded everyday during intervals from 21-23 days of age, and fractional growth rate (%/day) and feed conversion were calculated.

Measurement of the fractional rate of muscle protein degradation

The fractional rates of muscle protein degradation of Japanese quail were measured by the methods previously described (HAYASHI *et al.*, 1985 ; MAEDA *et al.*, 1986 ; MAEDA *et al.*, 1991). Complete 24 hr excreta collection was individually obtained during intervals from 21-23 days of age and stored at −40℃ until analyzed for N^{τ}-methylhistidine content. Following the homogenization of each excreta sample, an aliquot was hydrolyzed in 50 m*l* of 6 N HCl in a sealed Erlenmeyer's flask at 115℃ for 24 hr. After filtration (Whatman No. 1), the filtrate was reduced to dryness and redissolved in 10 m*l* 0.2 M pyridine and applied to a Dowex AG50W×8 (Dow chemical Co.) column (pyridine form). After the acidic and neutral amino acids were eluted with 200 m*l* of 0.2 M pyridine, N^{τ}-methylhistidine was eluted with 100 m*l* of 1 M pyridine. An assay of N^{τ}-methylhistidine was conducted by the method previously described (WARD, 1978).

For estimation of the muscle protein degradation rate, the pool size of N^{τ}-methylhistidine in the whole body skeletal muscle was calculated by 0.233 (μ mmol/g body weight)×body weight (MAEDA *et al.*,1986). Since approximately 90% of the N^{τ}-methylhistidine in the excreta originated from the degradation of skeletal muscle, the daily amount of N^{τ}-methylhistidine excretion released by degradation of skeletal muscle protein can be estimated by the following equation (MAEDA *et al.*, 1986) :

Daily amount of excretion of N^{τ}-methylhistidine released
by degradation of skeletal muscle
$=0.9\times(X_i-F_i\times C)$

where 0.9 is the amount of N^{τ}-methylhistidine in skeletal muscle relative to the total N^{τ}-methylhistidine in the body, X_i is the daily apparent N^{τ}-methylhistidine excretion of the i_{th} bird, C is a constant (μmol/g) of N^{τ}-methylhistidine in the feed, and F_i is the feed intake of the i_{th} bird. The fractional rates of degradation of muscle protein was calculated as described by FUNABIKI *et al.* (1976).

Measurement of calpain and calpastatin activities in skeletal muscle

Quail were killed by cervical dislocation at 23 days of age. The breast muscle was quickly excised, weighed and frozen in liquid nitrogen. Samples were stored at −80℃ until they were used. Unless otherwise indicated, all procedures of enzyme extraction and enzyme assay were carried out at 4℃. The calpain and calpastatin from skeletal muscle were isolated by the method described by INOMATA *et al.* (1983). Briefly, the breast muscles were dissected and minced, and homogenized with 3 volumes of ice cold buffer A containing 20 mM Tris-HCl, pH 7.5 ; 5 mM EDTA ; 10 mM 2-mercaptoethanol, in an ULTRA-TURRAX TP 10 N (Janke & Kunkel Gmbh & Co. KG) tissue grinder. The homogenate was centrifuged at 10,000×g for 15 min and the supernatant fraction was filtered through glass filter to remove fat materials. The resultant supernatant was immediately applied to a column to separate the calpain and calpastatin. The crude extract was poured onto a DEAE-Sephacel (Pharmacia LKB) column (21×200 mm equilibrated with buffer A) and the column was eluted with a linear gradient of NaCl from 0 to 0.6 M NaCl in buffer A. Extractable muscle protein concentration was measured by the method of LOWRY *et al* (1951).

The fraction containing calpastatin was applied onto a phenyl-Sepharose CL-4 B (Pharmacia LKB) column (15×33 mm equilibrated with 0.3 M NaCl-buffer A) and eluted with 0.3 M NaCl-buffer A.

Calpain activity was measured using casein (nach Hammerstein ; Merck) as a substrate. The reaction mixture contained a final concentration of 0.24% alkali-denatured casein, 28 mM 2-mercaptoethanol, 6 mM $CaCl_2$, and 0.1 M Tris-HCl (pH 7.5). The reaction was started by the adding the enzyme solution and stopped by adding 1 m*l* of 10% trichloroacetic acid (TCA) after incubation at 30℃ for 20 min. After centrifugation at 1,300×g for 10 min, the absorbance of the supernatant at 280 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which catalyzed an increase of 1.0 absorbance unit at 280 nm at 30℃ in 30 min under the standard assay conditions.

The calpastatin activity was determined as follows. Appropriate amounts of calpastatin were added to fixed amounts of calpain with standard assay mixture. The reaction mixture was incubated for 30 min at 30℃. After addition of TCA solution to stop the reaction, the mixture was centrifuged and the absorbance of the supernatant at 280 nm was determined. One unit of inhibitory activity was defined as the amount of calpastatin that inhibited 50% of 2 units of calpain (BALLARD *et al.*, 1988).

Statistical Analysis

For choosing the best model to specify the strategy for selecting variables, data were analyzed by the multiple regression analysis described by KLEINBAUM *et al.* (1987) with seven equation models as follows :

1) $X_1=\beta_0+\beta_2X_2+e$
2) $Y=\beta_0+\beta_1X_1+e$
3) $Y=\beta_0+\beta_2X_2+e$
4) $Y=\beta_0+\beta_1X_1+\beta_2X_2+e$
5) $Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_1^2+\beta_4X_2^2+e$
6) $Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_1^2+e$
7) $Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_1^2+\beta_4X_2^2+\beta_5X_1X_2+e$

The fractional rate of muscle protein is established to response variable (Y) and, the independent variables were set up in terms of calpain activity X_1, and calpastatin activity X_2, except on equation 1 which the calpain and calpastatin activities were defined as a response variable (X_1) and independent variable (X_2).

Results

Body weight, daily gain (D.G.), fractional growth rate (F.G.R.), feed efficiency (F.E.), fractional rate of muscle protein degradation (Kd), and calpain and calpastatin activities of the Japanese quail at 3 weeks of age are given in Table 1. The body weight ranged from 61.1 to 95.45 g ; D.G. ranged from 3.2 to 11.4 g/day ; F.G.R. ranged from 1.25 to 5.66%/day ; Kd ranged from 0.96 to 12.25%/day ; calpain activity ranged from 0.045 to 0.881 units/mg extractable muscle protein ; calpastatin activity ranged from 0.019 to 0.074.

Figure 1. shows three dimensional projection of 15 quail onto calpain activity (X_1), calpastatin activity (X_2), and Kd (Y). The Kd exhibited upward trends with calpain activity. On the contrary, the Kd exhibited downward trends with calpastatin activity.

Table 2 shows correlation coefficients between Kd and the activities of calpain and calpastatin. The positive correlation coefficient between calpain activity and Kd was significant (0.728, $p<0.01$). The negative correlation coefficient between calpastatin activity and Kd was also significant (-0.453, $p<0.01$). The negative correlation coefficient between calpain and calpastatin activities was also estimated (-0.397, $p<0.01$).

The equation of the fractional rate of muscle protein degradation by multiple regression was estimated as follows :

Table 1. Body weight, growth rate, feed efficiency, fractional rate of muscle protein degradation, calpain activity, and calpastatin activities of Japanese quail at 3 weeks of age

	Mean±S.E. (n=15)
Body weight (g)	77.137±2.371
Daily gain (g/day)	2.068±0.651
Fractional growth rate (%)	3.523±0.166
Feed efficiency	0.195±0.023
Fractional rate of muscle protein degradation (%/day)	4.768±0.823
Calpain activity (units/mg extractable muscle protein)	0.536±0.043
Calpastatin activity (units/mg extractable muscle protein)	0.047±0.004

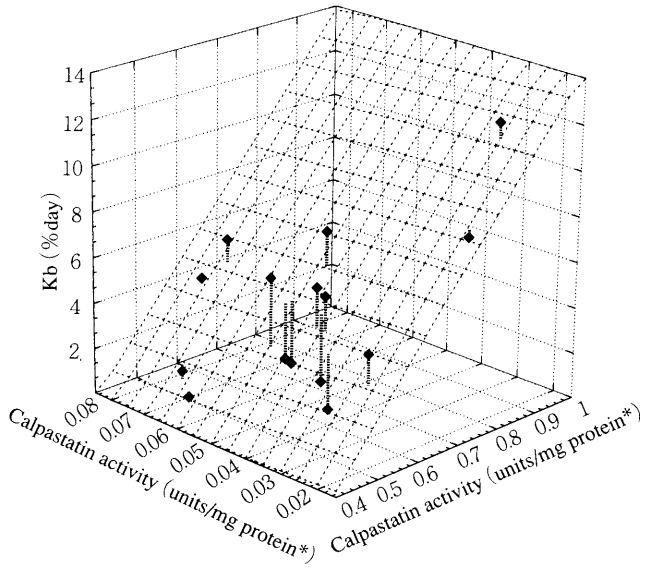

Fig. 1. Three dimensional projection of 15 quails onto fractional rate of muscle protein degradation (Kd), calpain and calpastatin activity with equation 1.
*protein : extractable muscle protein ; ▬▬ : distance from plane for multiple regression equation.

Table 2. Correlation coefficients among fractional rate of muscle protein degradation (Kd), calpain activity, calpastatin activity of Japanese quail at 3 weeks of age

	Calpain activity	Calpastatin activity
Kd	0.728**	−0.453**
Calpain activity		−0.397**

**: $P<0.01$

1) $X_1=0.367-2.058X_2$
2) $Y=-0.175+22.921X_1$
3) $Y=8.873=87.806X_2$
4) $Y=5.892+14.033X_1-88.128X_2$
5) $Y=7.592+1.573X_1-112.941X_2+25.048X_1^2+258.594X_2^2$
6) $Y=-12.099+61.933X_1+247.894X_2-893.200X_1X_2$
7) $Y=-20.455+64.860X_1+521.484X_2+22.979X_1^2-1982.349X_2^2-1101.165X_1X_2$

Table 3. shows summary of results of these regression models. Consider the overall F statistic, the equation 4 shows much higher than the values for other models. On the contrary, the R-square value show higher in the order of equations 7, 6, 5, 4, 2, 3, and 1. The response surface for variable Y of three equations is given in Table 4. For a given variable in given model, the significant associated partial F statistic were assessed the contribution made by the variable of equation 2, 3, and 4.

The standardized partial regression coefficients of calpain and calpastatin ac-

Table 3. Summary of results of regression procedure

Equation	MSE	R-square	F-value
1	0.017	0.060	0.397
2	4.657	0.513	12.649**
3	6.098	0.362	6.820*
4	3.064	0.706	13.688**
5	3.518	0.724	5.905*
6	2.362	0.794	12.860**
7	2.622	0.817	7.153**

*: $P<0.05$, **: $P<0.01$

Table 4. Response surface for variable Y (Kd) and X_1 (calpain activity) of seven equations

Variable	Df	Parameter estimate	Standard error	Partial F statistic	Standardized partial regression coefficient
Equation 1					
Intercept	1	0.367	0.121	0.001	
X_2	1	− 2.058	2.345	0.158	−0.246
Equation 2					
Intercept	1	− 0.175	1.366	0.394	
X_1	1	22.921	4.672	12.652**	0.716
Equation 3					
Intercept	1	8.873	2.306	22.915**	
X_2	1	− 87.806	44.807	6.823*	−0.602
Equation 4					
Intercept	1	5.892	2.174	7.344*	
X_1	1	14.033	3.910	12.881**	0.605
X_2	1	− 88.128	32.128	7.236*	−0.453
Equation 5					
Intercept	1	7.592	5.675	1.790	
X_1	1	1.573	18.192	0.001	0.068
X_2	1	− 112.941	215.722	0.275	−0.581
X_1^2	1	25.048	38.706	0.419	0.536
X_2^2	1	258.594	2762.266	0.014	0.130
Equation 6					
Intercept	1	− 12.099	8.916	1.841	
X_1	1	61.933	23.440	6.980*	2.670
X_2	1	247.894	165.188	2.253	1.275
$X_1 X_2$	1	− 893.200	432.386	4.268	−2.364
Equation 7					
Intercept	1	− 20.455	14.732	1.927	
X_1	1	64.860	35.065	3.423	2.796
X_2	1	521.484	365.312	2.196	2.683
X_1^2	1	22.979	33.432	0.472	0.492
X_2^2	1	−1982.349	2771.871	0.465	−0.995
$X_1 X_2$	1	−1101.165	545.487	4.448	−2.914

*: $P<0.05$, **: $P<0.01$

tivities to Kd in equation 4 were 0.605 and −0.453, respectively.

Discussion

The objective of the present study was to investigate the relationships among the fractional rate of muscle protein degradation (Kd), skeletal muscle calpain activity, and calpastatin activity.

The correlation coefficient between Kd and calpain activity was positive. On the contrary, the correlation coefficient between Kd and calpastatin activity was negative. The correlation coefficient between calpain activity and calpastatin activity was negative. Moreover, all combination of observed correlation coefficients were statistically significant.

In the multiple regression analysis, linear effects of calpain and calpastatin activities to Kd were showed significantly. However, quadratic and crossproduct terms for both calpain and calpastatin activities had no significant partial F statistic. These results implied that the independent effect of calpain or calpastatin activities on Kd were more important than interaction effect of calpain and calpastatin activities. Therefore, we chose the linear model (equation 4). The standardized partial regression coefficient of calpain activity for Kd was shown to be higher than those of calpastatin activity. These results suggested that calpain activity in the skeletal muscle has very important effect on Kd, and calpastatin activity in the skeletal muscle also has relatively important effect on Kd. Although, calpain activity has negative correlation with calpastatin activity, it seems that calpain activity may be regulated by another factor such as Ca^{2+} concentration rather than calpastatin activity, *in vivo*. Further information on the cellular mechanisms of calpain and calpastatin relative to each other and on possible regulation of the calpain/calpastatin interaction would be important to understand how activity of the calpain is controlled *in vivo* and how this control might be modified to alter the rate of muscle protein degradation.

The present studies about calpain activity and growth have demonstrated that calpain and calpastatin activities affect the rate of muscle protein turnover and muscle growth. JOHARI *et al.* (1994) showed that negative correlation exist between growth rate and calpain activity in chickens. However, HIGGINS *et al.* (1988) reported that calpain activity and muscle growth had no direct relationships in sheep, except when protein accretion was stimulated by nutritional factor. Additionally, MAEDA *et al.* (1991) illustrated that the skeletal muscle calpain activities of the Japanese quail varied according to the lines selected for body weight. JOHARI *et al.* (1994) also demonstrated that calpain and calpastatin activities in skeletal muscle varied among breeds which have different rate of muscle production.

These findings suggest that the genetic variation of calpain activity leads the variation of muscle protein degradation. It is possible that if we can control calpain activity, we can also control muscle protein degradation, hence growth rate in livestock breeding.

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日本ウズラにおける骨格筋組織のcalpain活性と筋肉タンパク質分解速度との関係

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本研究では，タンパク質分解酵素の一種であるcalpain（カルシウム依存性中性プロテアーゼ）が筋肉タンパク質代謝回転速度にどのような影響をおよぼしているかを明らかにすることを目的として，筋肉タンパク質代謝回転速度の測定をMaeda *et al.*（1990）の方法に従ってN^{τ}-Methylhistidine法により行うとともに，calpainおよびcalpastatin活性値の測定をInomata *et al.*（1983）の方法により無作為交配集団の日本ウズラを用いて行った。

その結果，3週齢時のそれぞれの相関係数は，筋肉タンパク質分解速度（Kd）とcalpain活性値では0.728，分解速度とcalpastatin活性値では−0.453であった。Kd（Y）に対するcalpain活性値（X_1）およびcalpastatin活性値（X_2）の重回帰式を作成したところ $Y=5.892+14.033X_1-88.128X_2$ という結果が得られた。また，重回帰分析をおこなった結果より得られた偏回帰係数から，calpainおよびcalpastatin活性値が筋肉タンパク質分解速度に寄与する割合では，それぞれ0.605および−0.453と評価され，またその決定係数は70.63%を示した。

以上の結果からcalpain活性と筋肉タンパク質代謝回転速度の間には密接な関係が存在しており，またcalpastatin活性も筋肉タンパク質代謝回転速度にある程度の影響をおよぼしていることが推察された。

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キーワード：calpain，calpastatin，筋肉タンパク質代謝回転速度，日本ウズラ