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A Judgment on Postmortem Aging in *Longissimus Dorsi* Based on a Peptide Substrate Library

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We attempted to develop a method to determine easily and effectively the degree of postmortem aging of pork *longissimus dorsi* (LD) by measuring the activity of proteases in the LD using fluorogenic peptide substrates. LD was used to measure the change with time in the protease activity detected with these substrates. Determining the variations within the LD muscles, strong positive correlations were found between changes in hardness and fluorescence intensities against Ac-Ala-MCA, Ac-Met-MCA, Ac-Ser-MCA, Ac-Thr-MCA, and Ac-Ala-Phe-MCA ($P < 0.005$), and strong negative correlations were found between changes in total amounts of free amino acids and Ac-Ala-MCA, Ac-Met-MCA, Ac-Ser-MCA, Ac-Thr-MCA, and Ac-Ala-Phe-MCA ($P < 0.001$). Negative correlations were also observed between changes in the amounts of free Ala, Arg, Lys, Leu, Met, Phe, and Tyr and the fluorescence intensities against Ala, Arg, Lys, Leu, Met, Phe, and Tyr-MCA respectively ($P < 0.001$).

Key words: 4-methyl-7-coumarylamide (MCA) substrate; peptide substrate library; pork *longissimus dorsi*; postmortem aging; protease

Meat is aged by storing it at a low temperature for a certain period of time to increase its palatability. Various biochemical changes occur during postmortem aging. Nishimura *et al.*[1] reported that postmortem aging of bovine rounds at 4 °C for 12 d, pork *longissimus dorsi* (LD) muscle at 4 °C for 6 d, and chicken *pectoralis superficialis* muscle at 4 °C for 2 d significantly increased the amounts of free amino acids and free peptides. Bowers[2] reported that storage of pork LD muscle at 2 °C for 8 d increased the levels of several amino acids, including glutamic acid. Moreover, Feidy *et al.*[3] reported that storage of beef *longissimus dorsi*, *triceps brachii caput longum*, and *rectus femoris* at 4 °C for 14 d increased levels of several free amino acids, and Okumura *et al.*[4–6] reported that storage of vacuum-packed LD muscle at 4 °C for at least 20 d increased the tenderness of the meat and the amounts of free amino acids and peptides, as well as enhancing the taste and flavor. As mentioned above, meat with an optimum degree of postmortem aging is the most palatable. However, the degree of postmortem aging is not actually appreciated at meat counters. Because the expiry date is set on the basis of microbial safety, people tend to eat meat when it is fresh and not at the stage when it is most palatable. This is partly because consumers do not realize that postmortem aging increases the taste, and partly because a complicated procedure is required to evaluate the postmortem aging process. Although common conventional physical and chemical analyses, such as the measurement of hardness using a Tensipresser (Taketomo Electric, Tokyo) and the amounts of free amino acids can be used to determine the degree of postmortem aging, these methods are not practicable because of the long times and high costs involved in measurement. An easier method that takes only a short time is desirable.

It is known that endogenous protease is involved in postmortem aging.[7,8] Nishimura[7] reported that calpains and cathepsins in meat after slaughter degraded meat proteins, such as Z lines, and thereby increased the

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Abbreviations: LD, *longissimus dorsi*; Ac, acetyl; MCA, 4-methyl-7-coumarylamide; Boc, *t*-butoxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; DCC, *N*,*N*′-dicyclohexyl carbodiimide; TFA, trifluoroacetic acid; DMF, *N*,*N*-dimethylformamide; HPLC, high performance liquid chromatography; FAB-HRMS, fast atom bombardment high-resolution mass spectra; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate; HOBt, 1-hydroxy-1*H*-benzotriazole; DIEA, diisopropyl-ethylamine; pmc-,2,2,5,7,8-pentamethylchroman-6-sulfonyl-; MES, 2-morpholinoethanesulfonic acid

tenderness of the meat, and that aminopeptidases caused increases in the amounts of free amino acids, and hence an improvement in taste. This suggests that the degree of postmortem aging can be evaluated by quantifying endogenous proteases over time.

Of the proteases involved in postmortem aging, calpains,[9] cathepsins,[10,11] and aminopeptidases[12,13] have been purified, but the purification of proteases requires considerable effort and cost. Furthermore, because postmortem aging of meat is caused by the combined effects of proteases, it is not practical to determine the degree of postmortem aging using only one isolated protease.

We attempted to develop a new and simple method to estimate the degree of postmortem aging of pork LD by adopting the following steps: (i) preparing a library of fluorogenic peptide substrates consisting of a number of candidate substrates that can react with proteases, (ii) reacting these substrates with LD muscle extracts to measure some protease activities simultaneously, and (iii) investigating the correlation between the physical and chemical properties of LD and its degree of postmortem aging.

Nishino *et al.*[14,15] proposed that complex characteristics of protease mixtures can be elucidated from a limited number of measurements performed using a series of synthetic protease substrates arrayed on a plate. By applying this concept, the activities of some proteases contained in the extract should be measurable immediately and simultaneously. Moreover, it is known that the use of 4-methyl-7-coumarylamide (MCA)-labeled peptide substrates, which fluoresce (ex. 380 nm, em. 460 nm) when cleaved, allows sensitive measurements to be made of protease activities.[16–18]

In this study, to establish a simple system to analyze the degree of postmortem aging, (i) a fluorogenic peptide substrate library (endopeptidase substrates: Ac-X-MCA, Ac-X-Ala-MCA, Ac-X-Val-MCA, Ac-X-Phe-MCA, Ac-X-Lys-MCA, and Ac-X-Arg-MCA; aminopeptidase substrates: X-MCA and Leu-X-MCA; X, standard amino acids omitted cysteine) was prepared and used to measure protease activities in pork LD, and (ii) the relationships between the protease activities and physical and chemical properties, which constitute indicators of postmortem aging, were investigated. The results confirmed that the physical and chemical properties correlated with protease activities. Further, on the basis of this correlation, several substrates were identified that might be useful in estimating the postmortem aging of pork LD.

Materials and Methods

Synthesis of MCA substrates.

Synthesis of Ac-X-MCA. *t*-Butoxycarbonyl-(Boc-) or 9-fluorenylmethyloxycarbonyl-(Fmoc-) amino acids were coupled with 7-amino-4-methylcoumarin by the symmetrical anhydride method using *N*,*N*′-dicyclohexyl carbodiimide (DCC) in dichloromethane, as in the methods of Zimmerman[16] and Kanaoka[18] to give Boc- or Fmoc-X-MCA. The Boc- group was removed with trifluoroacetic acid (TFA) at 0 °C for 30 min to give X-MCA•TFA, while the Fmoc- group was removed with 20% piperidine in *N*,*N*-dimethylformamide (DMF) for 2 h to give H_2N-X-MCA, which were successively reacted with acetic anhydride and triethylamine in DMF. The products were purified by silica gel chromatography to give Ac-X-MCA. The protecting groups of Ac-X-MCA, if any, were finally removed by TFA. In the case X was Lys or Arg, Ac-X-MCAs given as TFA salt. The 19 Ac-X-MCAs thus synthesized were analyzed by HPLC and FAB-HRMS (data not shown).

Synthesis of Ac-X-Ala-MCA. Fmoc-Ala-MCA was deprotected with 20% piperidine in DMF, as described above, and coupled with Fmoc-amino acids to give Fmoc-X-Ala-MCA using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-1*H*-benzotriazole (HOBt), and diisopropylethylamine (DIEA) as coupling agents. Furthermore, the Fmoc- group of Fmoc-X-Ala-MCA was deprotected, acetylated as described above, and purified by silica gel chromatography to give Ac-X-Ala-MCA. Final side-chain deprotection of X was done by TFA. The 19 Ac-X-Ala-MCAs were analyzed by HPLC and FAB-HRMS (data not shown).

Synthesis of Ac-X-Val-MCA, Ac-X-Phe-MCA, Ac-X-Lys-MCA, and Ac-X-Arg-MCA. Ac-X-Val-MCA, Ac-X-Phe-MCA, Ac-X-Lys-MCA, and Ac-X-Arg-MCA were synthesized using Fmoc-Val-MCA, Fmoc-Phe-MCA, Fmoc-Lys(Boc)-MCA, and Fmoc-Arg(pmc)-MCA (pmc-,2,2,5,7,8-pentamethylchroman-6-sulfonyl-) respectively by the same procedure as described for the synthesis of Ac-X-Ala-MCA. All of the dipeptide-MCAs were analyzed by HPLC and FAB-HRMS (data not shown).

Synthesis of X-MCA•TFA. Boc-X-MCA was deprotected by TFA treatment to give X-MCA•TFA. In the case of X having side-chain protection, Fmoc-X-MCA was used, and side-chain deprotection using TFA was performed, followed by Fmoc-deprotection.

Synthesis of Leu-X-MCA•TFA. H_2N-X-MCA or X-MCA•TFA as synthesized above were coupled with Boc-Leu hydrate by the HBTU/HOBt method to give Boc-Leu-X-MCA, and were purified by silica gel chromatography. Finally, they were treated with TFA to remove Boc- and side-chain protection to give Leu-X-MCA•TFA. The 19 Leu-X-MCAs were analyzed by HPLC and FAB-HRMS (data not shown).

Selection of substrates that react with pork LD extract. Each of the synthetic MCA substrates (0.05 mmol) was dissolved in dimethyl sulfoxide (500 ml) to give a 100 μM solution. Each well of a 96-well plate was charged with 90 μl of 100 mM MES (2-morpholinoethanesulfonic acid)–NaOH buffer (pH 6.0), and then the various substrate solutions (10 μl each) were added to

make a detection plate with a final substrate concentration of 10 μM in each well. Minced LD muscle (15 g) was homogenized with 3 volumes of 0.1 mM MES–NaOH buffer (pH 6.0) in a juicer (National MX-X-60, Matsushita Electric.) for 3 min. The homogenate was centrifuged at 10,000 × *g* for 15 min. After an extract was pre-incubated at 37 °C for 5 min, 100 μl of the solution was poured into each of the wells containing the substrates. After 30 min, the fluorescence intensity (ex. 380 nm, em. 460 nm) was measured using a microplate reader (ALVO™ MX 1420 multilabel counter, Perkin Elmer, Wellesley, MA.). To check for the presence of proteases that require metal ions for activation, EDTA (5 mM) was added to an extract buffer solution and the reactions were investigated.

Preparation of pork loins. Nine loins of ordinary hybrid pork line Landrace × Large white × Duroc were purchased from the local slaughterhouse. A portion of pork loins was cut into six equal pieces on the day of slaughter and the surfaces were sterilized with a burner. The burned surfaces were later removed on a clean bench and the LD muscle samples were vacuum packed into sterile bags in a sterile environment and stored in a refrigerator at 4 °C.

The samples were stored for up to 30 d. On the 2nd, 5th, 10th, 20th, and 30th days of storage, the protease activity, the amounts of free amino acids, and physical hardness were measured for each piece of LD muscle.

Measurement of the response of substrates to changes in pork LD properties with time. Of 152 synthetic MCA substrates that were examined in the first analysis in the samples stored for 2 d, 58 that showed a measured fluorescence intensity of 1,000 or more following reaction with the extract were selected (Table 1). These 58 selected substrates were used to measure changes in LD properties with time. The procedures for preparing substrate plates and measuring the reaction of substrates were as described above. On the basis of the results of the substrate-selection test, the times for fluorescence intensity measurement were set at 30 min for endopeptidase substrates (the same as those described above) and 1 min for aminopeptidase substrates, which give faster reactions.

Pork LD hardness. Pork LD hardness was tested by the method of Okumura *et al.*[4] The LD muscles were sliced to 20 mm-thick meat and vacuum sealed. They were heated in a water bath at 70 °C for 20 min and cooled in an ice bath for 20 min. After removal of excessive surface water, hardness values were determined with a Tensipresser™ (TTP-50BXII, Taketomo Electric, Tokyo), using a cylindric plunger with an outer diameter of 5.5 mm.

Free amino acid analysis. A free amino acid analysis in LD muscle was performed by the method of Okumura *et al.*[4] Five g of LD muscle was homogenized with 22 ml of 0.1% 2-mercaptoethanol for 1 min, and then 3 ml of 50% (w/v) trichloroacetic acid was added. The mixture was homogenized again for 1 min, and then left on ice for 3 h. The homogenate was centrifuged at 10,000 × *g* for 20 min and the supernatants were filtered. After the pH of the filtrates were adjusted to pH 2.3, free amino acids were analyzed with an amino acid analyzer (JLC-500/V, JEOL).

Statistical analysis. The T test was used to identify significant associations of fluorescence intensity, hardness, and amount of free amino acids within the LD muscles during 2–30 d of storage. Multiple regression was used to determine variations between fluorescence intensity against endopeptidase-substrates and hardness or total amounts of free amino acids. We used fluorescence intensity as the outcome variable, and hardness or total amounts of amino acids of LD muscle as predictor variables. LD muscle was treated as a categorical factor using a dummy variable with 8 degrees of freedom. The P value from the t test for the regression slope of hardness or total amounts of amino acids was used to determine the probability of the analysis. The magnitude of the correlation coefficient between fluorescence intensity and hardness or total amounts of free amino acids within the LD muscle was calculated as the square root of (sum of squares for hardness, or total amounts of free amino acids)/(sum of squares for hardness, or total amounts of free amino acids + residual sum of squares). The sign of the correlation coefficient was given by that of the regression coefficient for hardness or total amounts of free amino acids.[19] This method was similarly used to determine the variations between fluorescence intensity against aminopeptidase-substrates and free amino acids.

Results

Selection of substrates for determination of postmortem aging

First we selected substrates that react intensely with LD muscle extracts. Using 152 synthesized MCA substrates of eight combinations such as Ac-X-MCA, Ac-X-Ala-MCA, Ac-X-Val-MCA, Ac-X-Phe-MCA, Ac-X-Lys-MCA, Ac-X-Arg-MCA X-MCA, and Leu-X-MCA (X, standard amino acids omitted cysteine), protease activities were measured for an extract prepared from the LD muscle 2 d after slaughter. Among these substrates, fluorescence intensities against 58 of six combinations such as Ac-X_1-MCA (X_1 = Ala, Met, Ser, Thr), Ac-Ala-Phe-MCA, Ac-X_2-Lys-MCA (X_2 = Val, Leu, Ile, Met, His, Phe, Tyr, Trp), Ac-X_3-Arg-MCA (X_3 = Val, Leu, Ile, Met, Ser, Thr, His, Phe, Tyr, Trp), X_4-MCA ($X_4 \neq$ Asp), and Leu-X_5-MCA ($X_5 \neq$ Asp, Thr) were as high as 1,000 or more (Table 1). Although the fluorescence intensities against Ac-X_1-MCA, Ac-Ala-Phe-MCA, X_4-MCA, and Leu-X_5-MCA

Table 1. Selection of the Substrates by Fluorescence Intensity (F. I.)
Substrates were reacted with extract from LD muscle (A) or extract containing 5 mM EDTA (B). — Not detected.

Substrate	F. I.		Substrate	F. I.		Substrate	F. I.	
	(A)	(B)		(A)	(B)		(A)	(B)
Ac-Gly-MCA	170	16	Ac-Gly-Ala-MCA	—	—	Ac-Gly-Val-MCA	—	—
Ac-Ala-MCA	10,236	2,531	Ac-Ala-Ala-MCA	924	381	Ac-Ala-Val-MCA	345	—
Ac-Val-MCA	391	—	Ac-Val-Ala-MCA	144	163	Ac-Val-Val-MCA	—	—
Ac-Leu-MCA	345	256	Ac-Leu-Ala-MCA	139	100	Ac-Leu-Val-MCA	—	—
Ac-Ile-MCA	—	—	Ac-Ile-Ala-MCA	219	266	Ac-Ile-Val-MCA	—	—
Ac-Met-MCA	2,388	705	Ac-Met-Ala-MCA	933	224	Ac-Met-Val-MCA	20	—
Ac-Asp-MCA	—	—	Ac-Asp-Ala-MCA	—	—	Ac-Asp-Val-MCA	—	—
Ac-Glu-MCA	—	—	Ac-Glu-Ala-MCA	—	—	Ac-Glu-Val-MCA	—	—
Ac-Ser-MCA	2,883	569	Ac-Ser-Ala-MCA	—	—	Ac-Ser-Val-MCA	—	—
Ac-Thr-MCA	3,561	727	Ac-Thr-Ala-MCA	55	—	Ac-Thr-Val-MCA	157	53
Ac-Asn-MCA	154	—	Ac-Asn-Ala-MCA	—	—	Ac-Asn-Val-MCA	29	—
Ac-Gln-MCA	77	—	Ac-Gln-Ala-MCA	247	1	Ac-Gln-Val-MCA	88	—
Ac-His-MCA	15	—	Ac-His-Ala-MCA	8	—	Ac-His-Val-MCA	—	—
Ac-Lys-MCA	—	—	Ac-Lys-Ala-MCA	100	108	Ac-Lys-Val-MCA	—	—
Ac-Arg-MCA	—	—	Ac-Arg-Ala-MCA	119	—	Ac-Arg-Val-MCA	—	—
Ac-Pro-MCA	—	—	Ac-Pro-Ala-MCA	915	850	Ac-Pro-Val-MCA	—	—
Ac-Phe-MCA	945	225	Ac-Phe-Ala-MCA	367	380	Ac-Phe-Val-MCA	—	—
Ac-Tyr-MCA	180	103	Ac-Tyr-Ala-MCA	674	595	Ac-Tyr-Val-MCA	158	20
Ac-Trp-MCA	143	108	Ac-Trp-Ala-MCA	407	441	Ac-Trp-Val-MCA	159	219

Substrate	F. I.		Substrate	F. I.		Substrate	F. I.	
	(A)	(B)		(A)	(B)		(A)	(B)
Ac-Gly-Phe-MCA	113	135	Ac-Gly-Lys-MCA	—	—	Ac-Gly-Arg-MCA	—	—
Ac-Ala-Phe-MCA	1,999	—	Ac-Ala-Lys-MCA	354	364	Ac-Ala-Arg-MCA	633	611
Ac-Val-Phe-MCA	189	75	Ac-Val-Lys-MCA	1,852	2,131	Ac-Val-Arg-MCA	2,267	3,065
Ac-Leu-Phe-MCA	184	93	Ac-Leu-Lys-MCA	2,163	2,628	Ac-Leu-Arg-MCA	3,441	4,538
Ac-Ile-Phe-MCA	332	96	Ac-Ile-Lys-MCA	1,360	1,494	Ac-Ile-Arg-MCA	1,767	2,371
Ac-Met-Phe-MCA	746	247	Ac-Met-Lys-MCA	1,017	1,348	Ac-Met-Arg-MCA	2,219	2,749
Ac-Asp-Phe-MCA	128	45	Ac-Asp-Lys-MCA	101	213	Ac-Asp-Arg-MCA	510	712
Ac-Glu-Phe-MCA	144	—	Ac-Glu-Lys-MCA	257	466	Ac-Glu-Arg-MCA	794	482
Ac-Ser-Phe-MCA	354	—	Ac-Ser-Lys-MCA	418	503	Ac-Ser-Arg-MCA	2,546	1,836
Ac-Thr-Phe-MCA	787	—	Ac-Thr-Lys-MCA	231	324	Ac-Thr-Arg-MCA	1,096	636
Ac-Asn-Phe-MCA	—	—	Ac-Asn-Lys-MCA	120	365	Ac-Asn-Arg-MCA	395	379
Ac-Gln-Phe-MCA	—	—	Ac-Gln-Lys-MCA	385	511	Ac-Gln-Arg-MCA	620	627
Ac-His-Phe-MCA	—	—	Ac-His-Lys-MCA	2,588	3,111	Ac-His-Arg-MCA	3,048	3,705
Ac-Lys-Phe-MCA	—	—	Ac-Lys-Lys-MCA	859	1,050	Ac-Lys-Arg-MCA	993	1,156
Ac-Arg-Phe-MCA	47	61	Ac-Arg-Lys-MCA	479	620	Ac-Arg-Arg-MCA	567	535
Ac-Pro-Phe-MCA	—	—	Ac-Pro-Lys-MCA	105	—	Ac-Pro-Arg-MCA	72	173
Ac-Phe-Phe-MCA	225	44	Ac-Phe-Lys-MCA	3,954	4,768	Ac-Phe-Arg-MCA	4,194	5,720
Ac-Tyr-Phe-MCA	497	321	Ac-Tyr-Lys-MCA	4,592	5,941	Ac-Tyr-Arg-MCA	4,032	5,153
Ac-Trp-Phe-MCA	882	767	Ac-Trp-Lys-MCA	2,846	3,706	Ac-Trp-Arg-MCA	2,034	2,635

Substrate	F. I.		Substrate	F. I.	
	(A)	(B)		(A)	(B)
Gly-MCA	1,855	754	Leu-Gly-MCA	4,213	860
Ala-MCA	24,106	2,723	Leu-Ala-MCA	28,442	2,453
Val-MCA	10,462	1,641	Leu-Val-MCA	10,294	462
Leu-MCA	28,307	20,474	Leu-Leu-MCA	27,268	12,906
Ile-MCA	24,611	18,622	Leu-Ile-MCA	17,715	6,535
Met-MCA	23,909	13,368	Leu-Met-MCA	27,216	12,760
Asp-MCA	335	—	Leu-Asp-MCA	—	—
Glu-MCA	5,525	2,516	Leu-Glu-MCA	3,430	897
Ser-MCA	11,671	3,543	Leu-Ser-MCA	10,381	2,384
Thr-MCA	9,849	5,967	Leu-Thr-MCA	503	343
Asn-MCA	20,424	7,828	Leu-Asn-MCA	10,408	2,127
Gln-MCA	19,008	14,956	Leu-Gln-MCA	17,608	8,974
His-MCA	9,716	5,985	Leu-His-MCA	11,380	4,745
Lys-MCA	26,973	7,152	Leu-Lys-MCA	31,373	8,127
Arg-MCA	32,884	17,249	Leu-Arg-MCA	32,522	13,582
Pro-MCA	3,545	56	Leu-Pro-MCA	3,375	3,216
Phe-MCA	28,915	4,003	Leu-Phe-MCA	35,080	4,876
Tyr-MCA	33,660	3,910	Leu-Tyr-MCA	38,961	5,500
Trp-MCA	36,968	4,578	Leu-Trp-MCA	40,199	6,275

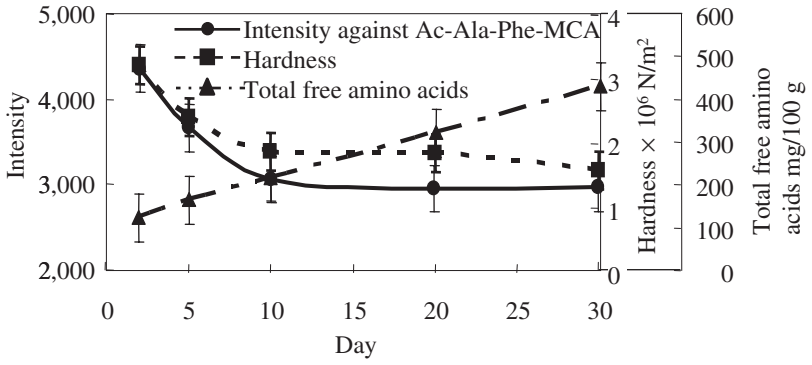

Fig. 1. Changes in Fluorescence Intensity against Ac-Ala-Phe-MCA, Hardness and Total Volume of Free Amino Acids of LD Muscle Stored at 4 °C.
The bar indicates the standard error.

were as high as 1,000 or more when treated with an EDTA-free extract, they showed reduced fluorescence intensity when treated with an EDTA-containing extract. The fluorescence intensities against Ac-X-Ala-MCA, Ac-X-Val-MCA, and Ac-X-Phe-MCA (except for Ac-Ala-Phe-MCA) were as low as 1,000 or less.

The 58 substrates were used to measure changes in LD muscle properties.

Correlation between measurements on the substrate library and the physical and chemical properties of pork LD

Figure 1 shows changes with time in fluorescence intensity against Ac-Ala-Phe-MCA, hardness, and total amounts of free amino acids measured for LD muscle samples stored for 2, 5, 10, 20, and 30 d. The fluorescence intensity against Ac-Ala-Phe-MCA decreased during storage. This tendency was similar for other Ac-X_1-MCA substrates (data not shown), but no significant changes were observed in fluorescence intensity against Ac-X_2-Lys-MCA or Ac-X_3-Arg-MCA. From the 2 d through the 10 d, the hardness of LD muscle decreased by about $1.6 \times 10^6\,N/m^2$, although this decrease was not statistically significant. Determining variations within the LD muscles, strong positive correlations between changes in hardness and fluorescence activities against Ac-Ala-MCA, Ac-Met-MCA, Ac-Ser-MCA, Ac-Thr-MCA, and Ac-Ala-Phe-MCA were observed correlation coefficients of 0.779, 0.746, 0.778, and 0.775 respectively ($P < 0.005$, Table 2). The total amounts of free amino acids, however, increased significantly. As Table 2 indicates, the change in total amounts of free amino acids shows a negative correlation with changes in the intensities against Ac-Ala-MCA, Ac-Met-MCA, Ac-Ser-MCA, Ac-Thr-MCA, and Ac-Ala-Phe-MCA; the corresponding correlation coefficients were 0.779, 0.756, 0.782, 0.681, and 0.821 ($P < 0.001$).

Figure 2 shows changes with time in fluorescence intensity against Leu-MCA and the amount of free leucine. As the amount of free leucine increased, fluorescence intensity against Leu-MCA decreased ($P < 0.01$). A significant decrease in fluorescence intensity against X_4-MCA substrates for X_4 = Ala, Arg, Lys, Met, Phe, Tyr or Leu, and significant increases in the amounts of free amino acids except for Gln, were observed. As Table 3 indicates, a negative correlation was observed between the amount of free Ala, Arg, Lys, Leu, Met, Phe, and Tyr and fluorescence intensity against Ala, Arg, Lys, Leu, Met, Phe, and Tyr-MCA ($P < 0.001$); the corresponding correlation coefficients were 0.780, 0.782, 0.831, 0.892, 0.891, 0.852, and 0.821 respectively ($P < 0.001$).

Discussion

Nishimura,[7] Koohmaraie,[8] and other researchers have shown that endogenous proteases in muscle contribute markedly to the mechanism of meat tenderness and an improvement in meat flavor. In this study, we focused on the change with time in the activity of proteases in meat as an indicator that permits measurements of the degree of postmortem aging of meat to be made more easily. The use of only a few substrates to monitor changes in the activities of the proteases that are involved in postmortem aging may not cover the entire range of changes that can occur during the postmortem aging process. In this study, a large number of fluorescently labeled peptide substrates were synthesized to produce a substrate library, and this was used to examine whether postmortem aging of LD can be evaluated in one experiment by simultaneously measuring the activities of a number of proteases in LD muscle during postmortem aging.

Among the 152 synthesized substrates, fluorescence intensities against 58 substrates were stronger for crude LD muscle extract. These substrates were considered to be easily decomposed by some of the proteases present in LD. The remaining substrates were considered to have a low specificity, although some fluorescence intensities against them were detected (Table 1). The addition of EDTA decreased the fluorescence intensity

Table 2. Correlation Coefficients between Hardness or Total Volume of Free Amino Acids and Activity against Endpeptidase Substrates

	Tenderness			Total free amino acids		
	Correlation coefficient	Sign	Difference	Correlation coefficient	Sign	Difference
Ac-Ala-MCA	0.779	+	**	0.779	−	***
Ac-Met-MCA	0.701	+	*	0.756	−	***
Ac-Ser-MCA	0.746	+	**	0.782	−	***
Ac-Thr-MCA	0.778	+	**	0.681	−	***
Ac-Ala-Phe-MCA	0.775	+	**	0.821	−	***
Ac-Val-Lys-MCA	0.244	−	NS	0.344	−	NS
Ac-Leu-Lys-MCA	0.249	−	NS	0.406	−	NS
Ac-Ile-Lys-MCA	0.268	−	NS	0.396	−	NS
Ac-Met-Lys-MCA	0.304	−	NS	0.362	−	NS
Ac-His-Lys-MCA	0.258	−	NS	0.455	−	**
Ac-Phe-Lys-MCA	0.216	−	NS	0.431	−	*
Ac-Tyr-Lys-MCA	0.200	−	NS	0.428	−	*
Ac-Trp-Lys-MCA	0.261	−	NS	0.413	−	NS
Ac-Val-Arg-MCA	0.282	−	NS	0.446	−	*
Ac-Leu-Arg-MCA	0.237	−	NS	0.448	−	*
Ac-Ile-Arg-MCA	0.226	−	NS	0.438	−	*
Ac-Met-Arg-MCA	0.263	−	NS	0.429	−	*
Ac-Ser-Arg-MCA	0.634	−	NS	0.001	+	NS
Ac-Thr-Arg-MCA	0.446	−	NS	0.349	−	NS
Ac-His-Arg-MCA	0.236	−	NS	0.501	−	**
Ac-Phe-Arg-MCA	0.240	−	NS	0.437	−	*
Ac-Tyr-Arg-MCA	0.236	−	NS	0.498	−	**
Ac-Trp-Arg-MCA	0.297	−	NS	0.475	−	**

Significant differences are indicated by asterisks ***P < 0.001, **P < 0.005, *P < 0.01. NS, not significant.

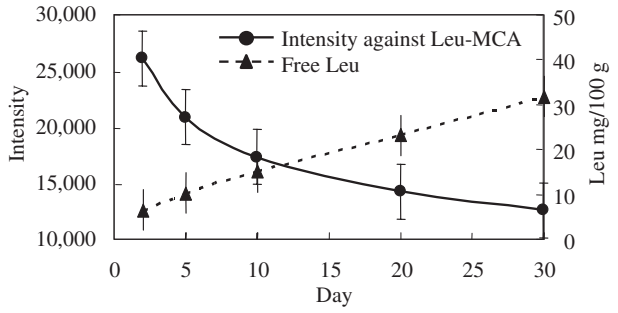

Fig. 2. Changes in Fluorescence Intensity against Leu-MCA and in the Amounts of Free Leu of LD Muscle Stored at 4 °C.
The bar indicates the standard error.

against Ac-X_1-MCA and Ac-Ala-Phe-MCA. On the basis on Davey's report,[20] which suggests that EDTA inhibits the degradation of Z lines by chelating calcium, it was hypothesized that the reaction of these substrates might involve calpains, a calcium-dependent protease. It is known that cathepsin B reacts specifically with the substrate Z-Arg-Arg-MCA or Z-Phe-Arg-MCA,[21] and that cathepsin L reacts specifically with the substrate Z-Phe-Arg-MCA.[21] Based on this information, it is hypothesized that a reaction with a substrate that has a basic amino acid in the P1 part, such as Ac-X_2-Lys-MCA and Ac-X_3-Arg-MCA, might involve cathepsin B or L.

The correlation between changes in the traditional indicators of degree of postmortem aging and changes in fluorescence intensity against the substrates were then

Table 3. Correlation Coefficients between Total Volume of Free Amino Acid and Activity against Aminopeptidase Substrates

	Correlation coefficient	Sign	Difference
Ala	0.780	−	**
Arg	0.782	−	**
Asn	0.735	−	**
Asp	0.218	−	NS
Gln	0.357	−	NS
Glu	0.752	−	**
Gly	0.078	+	NS
His	0.590	−	**
Ile	0.749	−	**
Leu	0.892	−	**
Lys	0.831	−	**
Met	0.891	−	**
Phe	0.852	−	**
Pro	0.065	+	NS
Ser	0.572	−	**
Tyr	0.867	−	**
Val	0.498	−	NS

Significant differences are indicated by asterisks **P < 0.001. NS, not significant.

investigated. Okumura *et al.*[4] reported that hardness decreased gradually until 20 d and that the total amounts of free amino acids increased up to 20 d, when the LD was found to have the best sensory properties. In this study, the hardness decreased until 10 d, and there was almost no change subsequently. Therefore, it may be best to eat pork LD on day 10 as for hardness. The total amounts of free amino acids in this study increased up to

20 d, which was consistent with their report.[4] In the evaluation of the relationship between changes in hardness of LD and fluorescence intensity against the substrate, a strong positive correlation was observed for Ac-Ala-MCA, Ac-Met-MCA, Ac-Ser-MCA, Ac-Thr-MCA, and Ac-Ala-Phe-MCA ($P < 0.005$, Table 2). Koohmaraie *et al.*[22] reported that after a 2 weeks of postmortem aging of beef, the remaining activities of *m*- and μ-calpain were about 90 and 20% respectively, which might be sufficient to contribute to postmortem aging. The fact that the above five substrates are cleaved by calpains indicates that these fluorescence intensities can be attributed to calpains. In the evaluation of the relationship between changes in the total amounts of free amino acids and changes in fluorescence intensity against the substrate, a strong negative correlation was observed for Ac-Ala-MCA, Ac-Met-MCA, Ac-Ser-MCA, Ac-Thr-MCA, Ac-Ala-Phe-MCA, and other endopeptidase substrates ($P < 0.001$, Table 2). It is believed that protein is degraded initially by endopeptidase and subsequently by aminopeptidase to produce free amino acids.[7] This study confirmed that the two types of protease act together to increase the total amounts of free amino acids. Nishimura *et al.*[7] reported that calpain has an important role in postmortem aging of meat. Correlations were observed between substrates suggesting the activity of calpain and hardness or total amounts of free amino acid, and hence the substrates can serve as indicators of postmortem aging.

The change in free amino acids during postmortem aging was similar to that observed by Okumura *et al.*[4] They reported that the taste of pork LD became better in sensory properties on day 20. In this study, pork LD stored for 20 d was best to eat. A negative correlation was observed between the amount of free Ala, Arg, Lys, Leu, Met, Phe, and Tyr and fluorescence intensity against Ala, Arg, Lys, Leu, Met, Phe, and Tyr-MCA ($P < 0.001$, Table 3) respectively. Thus, Ala, Arg, Lys, Leu, Met, Phe, and Tyr-MCA can serve as indicators of postmortem aging. Nishimura *et al.*[12,13] purified aminopeptidase C and H and investigated their abilities to hydrolyze amino acids. They found that aminopeptidase C tends to hydrolyze Lys, Ala, Leu, and Met, whereas aminopeptidase H tends to hydrolyze Met, Leu, and Lys. (Their study did not cover Arg, Phe, or Tyr.) The decrease in measured fluorescence intensities against Ala, Arg, Lys, Leu, Met, Phe, and Tyr-MCA in this study can be attributed to the actions of aminopeptidases C and H, but the involvement of other aminopeptidases, such as leucine aminopeptidase[23] and cathepsin H,[21] cannot be eliminated.

Our results showed that 11 of the 58 selected substrates (Ac-Ala-MCA, Ac-Ser-MCA, Ac-Thr-MCA, Ac-Ala-Phe-MCA, Ala-MCA, Arg-MCA, Lys-MCA, Leu-MCA, Met-MCA, Phe-MCA, and Tyr-MCA) responded to the degree of postmortem aging as represented by the physical and chemical properties of pork LD. It is expected that the combined use of these substrates will help to establish a system for analyzing postmortem aging that can be used easily and quickly to determine the optimum timing for eating meat. Furthermore, we can determine when meat is of the best palatability and safety to eat by combining this method with a bacterial count test. We also expect that beef, as well as pork, can easily be analyzed by applying our concept. Because beef requires longer postmortem aging, it is more important to set the timing for the consumption of beef.

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