豚筋肉中に存在する蛋白分解酵素について(畜産学科)

Studies of the Proteolytic Enzymes in Porcine Muscle

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I INTRODUCTION

Though the proteolytic action to be caused by protease of a cathepsin type existing in animal muscles is lower in comparison with those existing in other tissues, the study thereof is considered to be important from the standpoint of meat storage and use as well as from the viewpoint of proteometabolism *in vivo*. In particular, meat decomposition products to be generated by muscular tissue protease-cathepsin are considered to become a constituent of meat essence as the so-called "flavoring component" in the stage of aging and in this sense the activity of proteolytic enzymes existing in muscle is deemed to exercise an important effect on meat aging, tenderization and flavoring.

The past reports on cathepsin had been made with internal organ tissues such as liver, kidney and pancreas, etc. as the objects of study and subsequently a large number of reports have been published on rabbit, beef, chicken and fish meat.

For instance, Hata et al. (4) detected protease activity on the neutral and alkaline sides besides pH 4 in rabbit muscle, while Bandack-Yuri et al. (1) fractionated chicken protease and reported activity at pH 4 and 7. Frederrick et al. (2) fractionated porcine muscle cathepsin and found the strongest activity at pH 4 and 8 and further, they (3) reported similarly activity at pH 4 and 9 in respect of beef, too. Recently, it has been reported that there are three types of muscular cathepsin with optimum pH of 5, 8-9 and 10 respectively, while Lutalo-Bosa et al. (6) measured activity of cathepsins B, C and D obtained from porcine muscle and reported that among them, B had the strongest activity.

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In this study, to bring to light the chemical characteristics of proteolytic enzymes existing in muscle from the standpoint of meat storage, processing or flavoring, a preliminary experiment was conducted to measure principally the activity of muscular enzyme solution existing in fresh pork and its fundamental properties were examined first.

II EXPERIMENTAL MATERIAL AND METHOD

1. Experimental material

All the porcine muscles used in the present experiment were taken from the carcasses of Randrace hybrid pigs slaughtered at the slaughterhouse of Koami Animal Industry Co., Ltd. in Ami-machi, Inashiki-gum, Ibaraki pref. and in particular, head meat (M. Masseter) was collected within an hour or two after slaughter (and before cold storage) and similarly, ham (M. biceps femoris) after an overnight cold storage, as 1 to 2kg meat lumps.

2. Preparation of enzyme solution

Within the experimental room utilizing sterilization lamps, the abovementioned muscles were immediately minced by means of a sterilized mincing machine after removal of adipose tissue, connective tissue and surface muscles. By way of the processes of extraction using principally 2% potassium chloride solution twice in volume the weight of a fixed amount of such meat, adjustment to a required pH by means of acid and alkali and 10-minute incubation at 40°C, the precipitates were removed and the supernatant liquid was taken as crude enzyme source.

3. Measurement of enzyme activity

As the substrate, to 1 *ml* of 2% casein were added 2 *ml* of enzyme solution adjusted to a required pH and 2 *ml* of buffer solution. The solution thus prepared was digested for a certain period of time while shaking in a thermostatic water bath at 40°C. After digestion, 5 *ml* of 20% trichloroacetic acid were added for filtration and 1 *ml* of the filtrate was colored under the Folin method (5) and colorimetrically determined at 660mμ. The measured value was expressed in terms of absorbancy or mM of tyrosine in the filtrate.

III EXPERIMENTAL RESULT AND DISCUSSION

1. Reaction time

Using M. Masseter thawed after about a month of freeze storage at

−20°C and Sörensen buffer solution under the condition of pH 6.0, in changing the reaction time from 0.5 to 1, 2, 4, 8 and 16 hours reaction was caused to take place at 40°C and the amount of free tyrosine generated during that time was measured. The result was shown in Fig. 1.

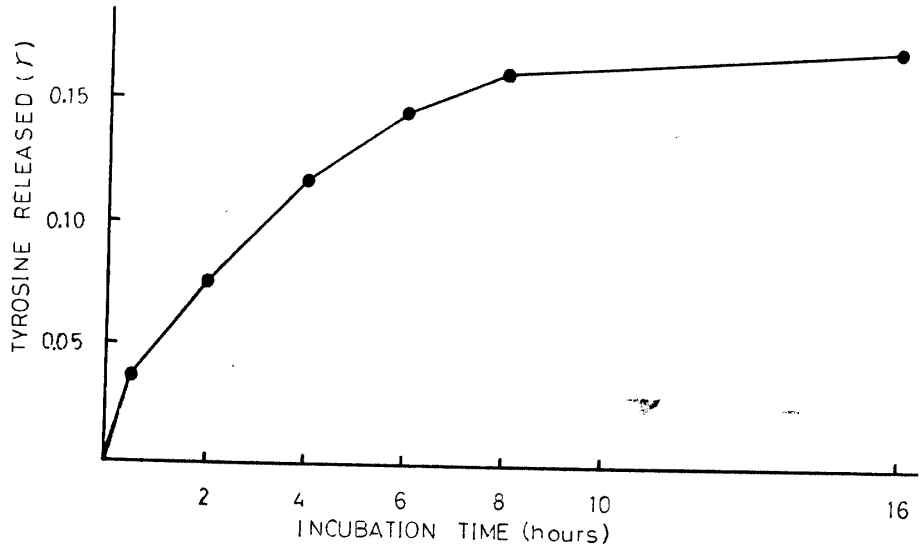

Fig. 1. Relationship of proteolytic activity vs. incubation time at pH 6.0 and 40°C for porcine muscle

The amount of generated free tyrosine increased with the lapse of reaction time ; up to 8 hours of reaction it showed a sharp increase and thereafter, a somewhat slow increase. Accordingly, the author decided to employ a reaction time of 16 hours in subsequent experiments to obtain a maximum hydrolytic value.

2. Reaction temperature

Using M. Masseter thawed after a week of freeze storage at −20°C and in setting the reaction temperatures to 5, 20, 40 and 60°C, incubation was carried out for 16 hours at pH 6.0 and the amount of free tyrosine generated at each temperature was measured. The result is shown in Fig. 2.

As the buffer solution, Söresen buffer was employed. In changing the reaction temperature as above, the highest activity was observed at 40°C. Activity to be observed still at 60°C is assumed, according to Lutalo-Bosa et al. (6), to represent enzyme having properties similar to cathepsin C.

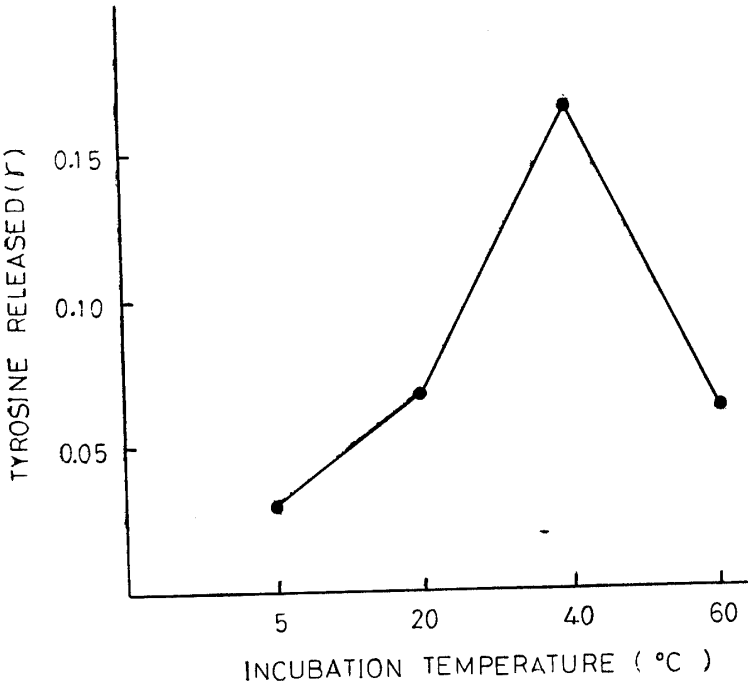

Fig. 2. Effect of reaction temperature on proteolytic activity at pH 6.0 and 16 hours for porcine muscle

3. pH-effect curve

The relation between the activity of enzyme solution extracted from M. Masseter immediately after slaughter and M. biceps femoris about 5 hours after slaughter and pH is shown in Fig. 3 and 4.

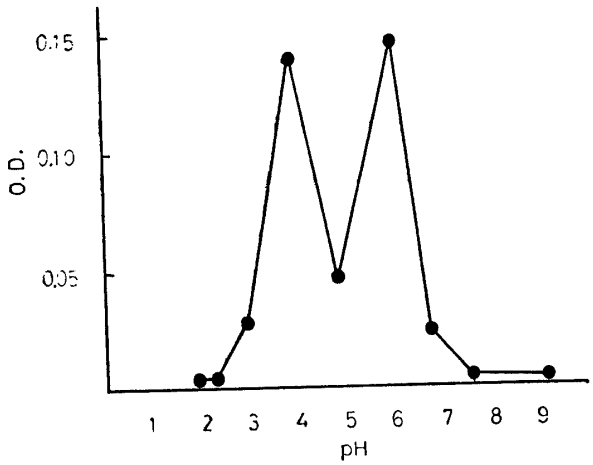

Fig. 3. Effect of pH on proteolytic activity at 40°C and 16 hours for porcine muscle (M. masseter)

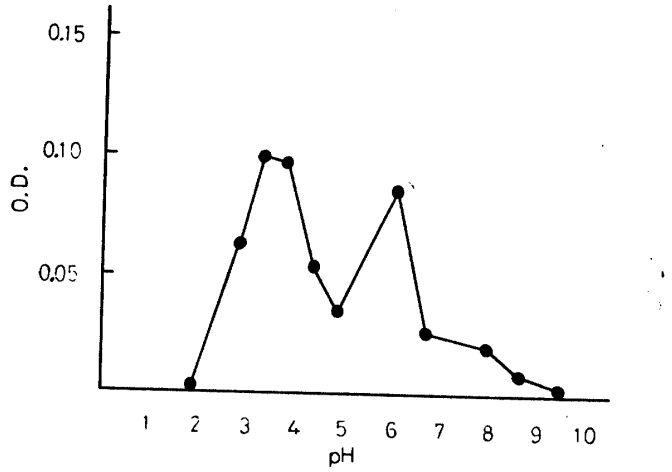

Fig. 4. Effect of pH on proteolytic activity at 40°C and 16 hours for porcine muscle (M. biceps femoris)

As a result, in both cases of M. Masseter and M. biceps femoris, a high activity was observed at about pH 3.5 and 6.0. Though the activity of proteolytic enzymes was observed in two pH-regions in the extracted solutions of the two kinds of muscle used as above, it took some time in that case to prepare the enzyme solutions and it was difficult to obtain a fixed enzyme activity in each experiment and therefore, using similar M. biceps femoris the author then studied the effect on activity in the case of cold acetone treatment and freeze drying.

The extraction of enzyme solution was carried out in the same manner as before except that the volume of 2% potassium chloride was made corresponding to that of fresh meat in consideration of moisture content due to drying. That is, as 18*g* of dry matter were obtained by acetone-drying of 75*g* of fresh meat, 200 *ml* of extraction solution were applied. Further, as 19.7*g* of freeze-dried meat were obtained, 190 *ml* of extraction liquid were employed to extract the enzyme solution, trying to make meat dry matter and water volume nearly equal in any cases.

The result obtained by using acetone-dried meat is as shown in Fig. 5. In this case the concentration of potassium chloride in the extraction solution is somewhat different and an accurate comparison cannot be made, but activity slightly higher in comparison with fresh meat is indicated. However, the optimum pH-region lies, as before, in the vicinity of pH 3.5 and pH 6.0.

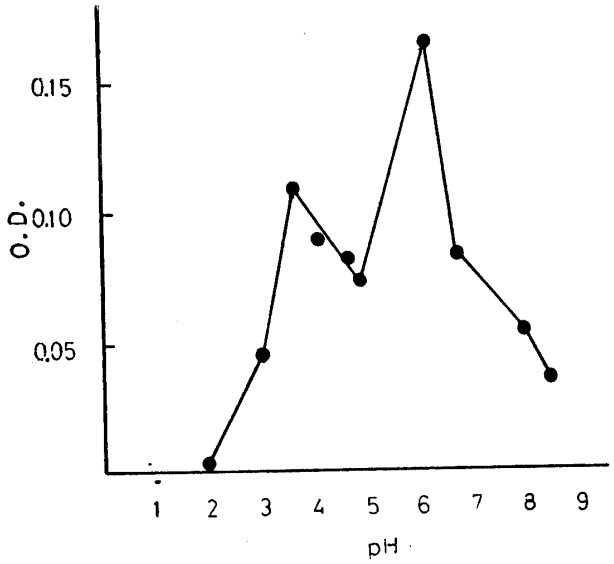

Fig. 5. Effect of pH on proteolytic activity at 40° C and 16 hours for acetone-drid porcine muscle

Next, the result in the case of freeze drying is shown in Fig. 6. Though pH changes in this case show a trend similar to the previous one, activity shows a relatively higher value in comparison with fresh meat. This is deemed to be the result of the destruction of lysosome within tissue by the freezing or thawing process and an increased frequency owing to exposure of internal cathepsin.

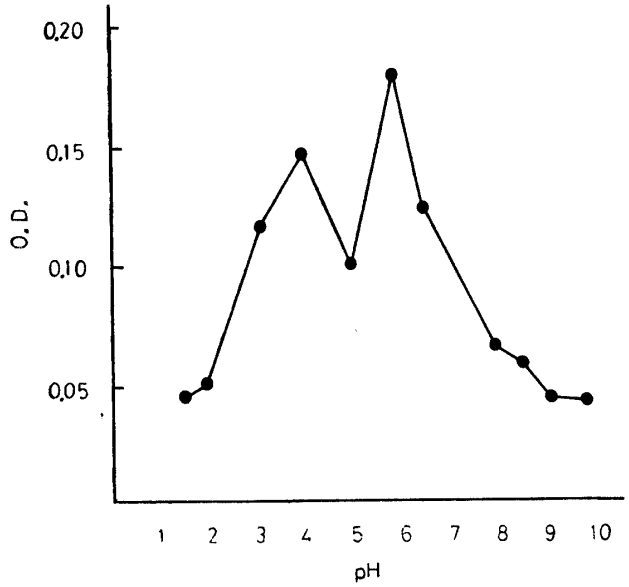

Fig. 6. Effect of pH on proteolytic activity at 40°C and 16 hours for freeze-dried porcine muscle

Next, similar meats were freeze-stored at −20°C for 10 days and then thawed : the pH-effect curve in this case is as shown in Fig. 7. In this case, too, the optimum pH showed a trend similar to the previous one.

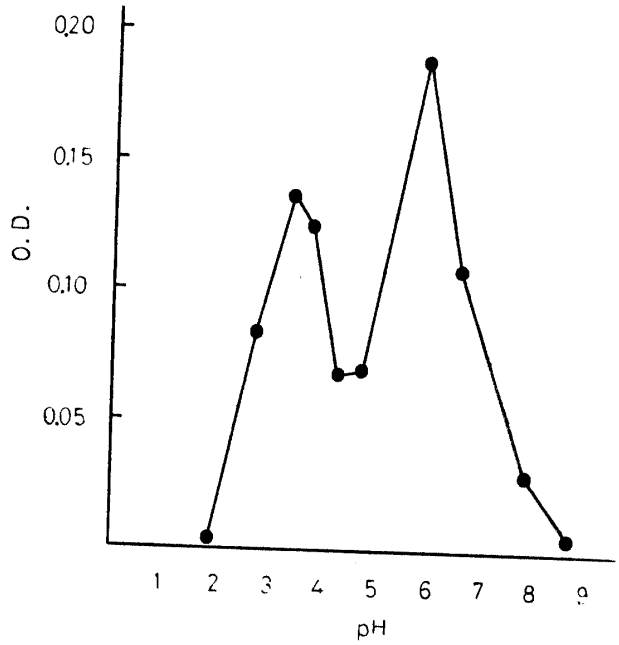

Fig. 7. Effect of pH on proteolytic activity at 40° C and 16 hours for frozen-stored porcine muscle

As a result of a comparative study of enzyme activiy of the extraction solutions obtained from the abovementioned treated muscles, a trend was observed, in which fresh meat, frozen meat, acetone-drieed meat and freeze-dried meat showed lower values in this order.

4. pH-effect curve per type of buffer solution

Though, in par. 3, boric acid-potassium chloride buffer solution was employed as buffer on the alkali side, Hata et al. (4) used Tris-buffer solution and observed activity in the vicinity of pH 7 and pH 9 in the measurement of muscular protease effect of rabbits. In the present experiment, too, Tris-buffer solution was employed and the existence of a high activity was observd in the vicinity of pH 8. Accordingly, the author conducted a digestion test in using a few kinds of buffer solution ; the result is as shown in Table 1, 2 and 3. In the meantime, though measurements were made in applying the buffer solutions in the range from pH 3.5 to 9.0, activity varies with even a slight change in pH and therefore actual pH's measured are shown. As a result of comparison made in consideration of such differences, the difference in activity due to the kinds of buffer solution seems to be small in the acid region and pH involving the highest activity is con-

sidered to be the same regardless of the kinds of buffer solution. However, in the case of pH 7.5, 8.0 and 9.0, obviously the use of Tris-buffer solution and Michaelis buffer solution resulted in higher values than in other cases, with an especially high activity being recognizable in the vicinity of pH 8.0.

Table 1. Effect of various buffer solutions on proteolytic activity at 40°C and 16 hours for porcine muscle (M. biceps femoris)

pH	3.5				3.7				4.0			
Buffer	S[a]	Mi[b]	Mc[c]	K[d]	S	Mi	Mc	K	S	Mi	Mc	K
Proteolytic activity(O. D.)[e]	0.123	0.128	0.104	0.142	0.133	0.131	0.131	0.172	0.079	0.078	0.077	0.083
Blank (O.D.)[f]	0.029	0.052	0.035	0.051	0.053	0.082	0.071	0.086	0.085	0.046	0.047	0.042
Measured pH	3.3	3.4	3.3	3.5	3.7	3.9	3.8	3.8	4.1	4.3	4.2	4.2

a: S rensen citrate buffer b: Michaelis veronal buffer c: Mcllvaine phosphate and citrate buffer d: Kolthoff succinic and borate buffer e & f: expressed as optical density at 660 mμ (O. D.)

Table 2. Effect of various buffer solutions on proteolytic activity at 40°C and 16 hours for porcine muscle (M. biceps femoris)

pH	5.0			5.9		
Buffer	S[a]	Mi[b]	K[c]	S	Mi	K
Proteolytic activity(O. D.)[d]	0.041	0.061	0.039	0.103	0.060	0.063
Blank (O. D.)[e]	0.015	0.020	0.017	0.006	—	0.005
Measured pH	5.0	4.7	4.9	5.8	5.4	5.5

a: Sörensen citrate buffer b: Michaelis veronal buffer c: Kolthoff succinic and borate buffer d & e: expressed as optical density at 660mμ (O. D.)

Table 3. Effect of various buffer solutions on proteolytic activity at 40°C and 16 hours for porcine muscle (M. biceps femoris)

pH	7.5				8.0				9.0			
Buffer	B[a]	T[b]	S[c]	Mi[d]	B	T	S	Mi	B	T	S	Mi
Proteolytic activty (O. D.)[e]	0.049	0.715	0.054	—	0.016	0.808	0.040	0.399	0.016	0.086	0.018	0.235
Balnk (O. D.)[f]	0.014	0.008	0.006	0.004	0.006	0.004	0.005	—	—	0.006	0.006	0.017
Measured pH	7.6	7.5	7.2	7.2	8.2	8.1	7.7	7.2	9.0	8.6	8.9	8.6

a: 0.2 M Boric acid, gallium chloride plus sodium carbonate buffer b: Tris buffer c: Sörenesen sodium borate-sodium hydroxide buffer d: Michaelis ammonium chloride-ammonia buffer e & f: expressed as optical density at 660 mμ (O. D.)

5 Influence of preservatives on pH-effect curve

As described above, Hata et al. (4) used Tris-buffer solution and found a high activity in the vicinity of pH 9, while in the present experiment, too, using Tris- or Michaelis buffer solution a peak was observed at near pH 8. In that case, in consideration of a possible putrefaction after digestion, the use of preservatives was tried.

Table 4. Effect of preservative addition on proteolytic activity at 40°C and 16 hours for porcine muscle (M. biceps femoris)

Preservative[a]	added						nonadded					
pH	5.9	6.7	7.0	7.5	8.0	8.9	5.9	6.7	7.0	7.5	8.0	8.9
Proteolytic activity (O. D.)[b]	0.159	0.035	0.042	0.045	0.029	0.010	0.218	0.045	0.668	0.209	0.159	0.010
Blank (O. D.)[c]	—	0.006	0.002	—	0.042	0.013	—	—	0.022	0.022	0.017	—
Measured pH	6.2	6.6	7.4	7.8	8.3	9.5	6.5	6.8	7.1	7.6	8.1	9.5

a: fluoro toluene-ethylene chloride-butyl chloride (1: 1: 3) b & c: expressed as optical density at 660 mμ (O. D.)

Table 5. Effect of preservatives on proteolytic activity at 40°C and 16 hours for porcine muscle (M.biceps femoris)

	expressed as optical density			
Toluene	0.057	0.047	0.031	0.031
Three combined solution *	0.049	0.028	0.021	0.026
pH	5.8	6.3	7.0	7.5

*: fluoro toluene-ethylenene chloride-butyl chloride (1: 1: 3)

The digestion test was conducted in applying a few kinds of preservatives and the results are shown in Tables 4 and 5. As can be seen from Table 4, when the mixed solution of fluoro-toluene, ethylene chloride and butyl chloride was employed, little difference in activity was seen in pH 5.9 and 6.7 regions, while a difference was observed in the vicinity of pH 8, with a similar trend being indicated in Table 5 showing the result of use of toluene.

Table 6. Effect of preservative additon on proteolytic activity at 40°C, 4 and 16 hours for porcine muscle (M. biceps femoris)

Kind of buffer	Borate				Michaeris				Tris			
Preservative	added		nonadded		added		nonadded		added		nonadded	
Reaction time	4	16	4	16	4	16	4	16	4	16	4	16
Proteolytic activity*	0.023	0.067	0.023	0.075	0.035	0.090	0.049	0.516	0.030	0.073	0.044	0.703
Blank*	—	0.014	0.009	0.002	0.004	0.025	0.012	0.027	0.007	0.018	—	0.012
pH	8.3	8.3	8.3	8.4	7.7	7.5	7.5	7.5	8.1	8.1	8.1	8.1

*: expressed as optical density at 660 mμ

Next, a comparison was made in applying the borate buffer solution showing no peak in the vicinity of pH 8 and Tris- and Michaelis buffer solutions showing peaks there, the result of which is shown in Table 6. As can be seen from this table, regardless of application and no-application of toluene, only a slight difference was observed when the borate buffer solution was used and a 16-hour digestion test conducted, whereas the use of Michaelis- and Tris- buffer solutions with no addition of toluene resulted in quite a high absorbancy, being especially marked in the case of the 16-hour digestion test. In the meantime, in the acid region, no change in optimum pH was observed regardless of whether preservatives were applied or not.

Using M. biceps femoris as in par. 3, Tris-buffer solution and the aforesaid mixed solution, a 14-hour digestion test was conducted; the pH-effect curves as compared with the case of no-application are shown in Fig. 8.

From this figure a difference in value can be noticed at and around pH 6.0, which is due to the difference in pH between the both experiments, and it is considered that nearly the same degree of activity will show up even when applying preservatives if caused to act near pH 6.

From the above results, with respect to casein, one can assume the existence of such proteolytic enzyme as works best in the vicinity of pH 3.5 and 6.0. The author found a high activity at pH 3.5 when hemoglobin was applied as the substrate, but not clearly in the vicinity of pH 6 and therefore, want to study the influence per type of substrate separately.

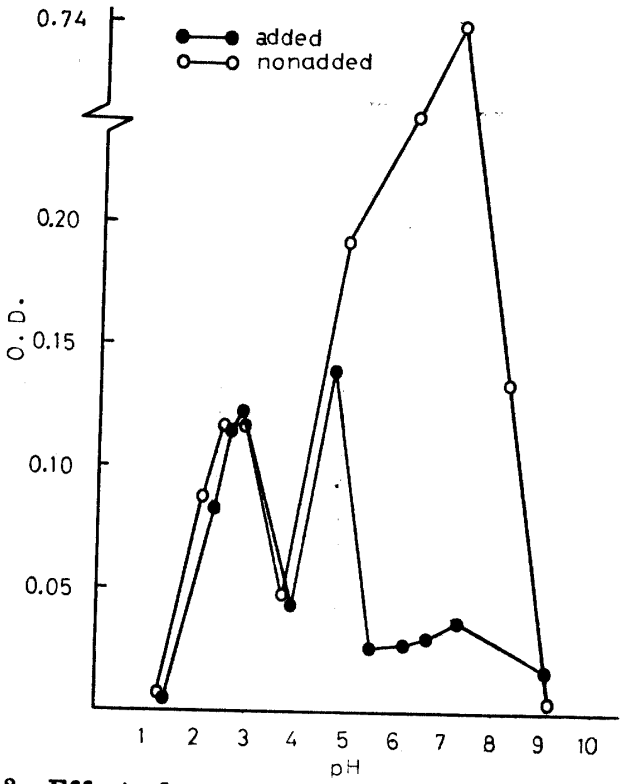

Fig. 8. Effect of preservative addition on proteolytic activity at 40°C and 16 hours for porcine muscle (M. biceps femoris) used Tris buffer

6. Influences of extraction solution and dialysis

For the puropose of further enhancing the activity of proteolytic enzymes, the author also studied the case where enzyme solution is prepared by changing extraction solution in several ways and dialyzed. The ratio of extraction solution to meat was set same as before and using water, 2% potassium chloride and hydrochloric acid or sodium hydroxide, pH at the time of extraction was adjusted to 4.0, 7.0 and 8.0. In any cases, enzymic effects at or near pH 3.5 and pH 6.0 were compared. The results are shown in Tables 7 and 8. For convenience of comparison, the data obtained were expressed in terms of γ-value of tyrosine in the trichloroacetic acid filtrate after the digestion test and further, since different enzyme solutions were employed, kjeldahl nitrogen of each enzyme solution was measured and tyrosine value per mg thereof was also calculated.

Sliwinski et al.(7) report that there is a certain substance to impede enzymic action and if it is eliminated by dialysis, activity is further enhanced. According to the results shown in Table 7 and 8, there is no conspicuous difference in free tyrosine volume and, in terms of mgN, higher values were obtained by dialysis.

Table 7. Effect of dialysis on proteolytic activity in extracts obtained by using various solvents at pH 3.5 for porcine muscle (M. biceps femoris)

(40° C, 16 hr.)

Extracting solvent	Treatment	Explt. (optical density at 660 mμ)	Blanks	Difference	Tyrosine liberated (γ)	Specific activity*
pH 4.0	Dialyzed	0.030	0.028	0.023	40.7	3.93
	Undialyzed	0.044	0.023	0.021	38.1	3.58
pH 7.0	Dialyzed	0.023	0.013	0.010	18.0	3.17
	Undialyzed	0.022	0.010	0.012	21.8	2.36
pH 8.0	Dialyzed	0.030	0.015	0.015	25.2	4.48
	Undialyzed	0.020	0.008	0.012	20.8	2.28
Distilled water	Dialyzed	0.018	0.010	0.008	14.5	3.17
	Undialyzed	0.015	0.008	0.007	11.8	1.50
2% potassium chloride	Dialyzed	0.031	0.011	0.020	36.3	6.33
	Undialyzed	0.030	0.009	0.021	37.1	3.92

*: γ of tyrosine liberated per mg of extracting solvent N_2

Table 8. Effect of dialysis on proteolytic activity in extracts obtained by using various solvents at pH 6.0 for porcine muscle (M. biceps femoris)

(40° C 16 hr.)

Extracting solvent	Treatment	Explt. (optical density at 660mμ)	Blanks	Difference	Tyrosine liberated (γ)	Specific activity*
pH 4.0	Dialyzed	0.064	—	0.064	115.0	58.7
	Undialyzed	0.074	0.005	0.070	52.5	24.5
pH 7.0	Dialyzed	0.061	—	0.061	110.0	18.8
	Undialyzed	0.070	—	0.070	127.0	15.0
pH 8.0	Dialyzed	0.062	—	0.062	122.0	22.1
	Undialyzed	0.072	—	0.072	130.0	14.1
Distilled water	Dialyzed	0.052	—	0.052	93.3	20.4
	Undialyzed	0.056	—	0.056	101.0	13.2
2% potassium chloride	Dialyzed	0.049	—	0.049	87.9	15.4
	Undialyzed	0.061	—	0.061	110.0	11.9

*: γ of tyrosine liberated per mg of extracting solvent N_2

From Table 7, it is considered that enzymes acting in the vicinity of pH 3.5, if compared in terms of mgN, show a relatively high value when extracting with 2 % potassium chloride.

As in Table 8, enzymes acting at or near pH 6 showed the highest values when extracting at pH 4, showing, however, little difference in other cases.

7. Fractionation by means of ammonium sulfate

For the purpose similar to that mentioned in par. 6, fractionation was carried out by means of ammonium sulfate. That is, after 20, 40, 60, 80, and 100% saturation with ammonium sulfate, the fractions were collected and dialyzed and in using the supernatant liquids, the enzymic effect was measured at pH 3.5 and pH 6.0. The results are shown in Tables 9 and 10.

Table 9. Effect of ammonium sulfate fractionation on proteolytic activity at pH 3.5 for porcine muscle (M. biceps femoris)

(40° C, 16 hr.)

Ammonium sulfate (%)	pH	Explt. (optical	Blanks density at	Difference 660 mμ)	Tyrosine liberated (γ)	Specific activity*
20	3.5	0.043	0.031	0.012	4.5	16.1
40	3.4	0.133	0.060	0.073	54.3	27.5
60	3.7	0.075	0.033	0.042	29.0	7.1
80	3.7	0.061	0.038	0.023	12.7	2.9
100	3.9	0.031	0.012	0.019	11.8	2.2
Residure	3.5	0.016	0.013	0.003		

*: γ of tyrosine liberated per mg of extracting solvent N_2

Table 10. Effect of ammonium sulfate fractionation on proteolytic activity at pH 6.0 for porcine muscle (M. biceps femoris)

(40° C, 16 hr.)

Ammonium sulfate (%)	pH	Explt. (optical	Blanks density at	Difference 660 mμ)	Tyrosine liberated (γ)	Specific activity*
20	5.8	0.485	0.065	0.421	380.0	1350.0
40	5.8	0.449	0.073	0.376	335.0	165.0
60	5.7	0.122	0.027	0.095	69.7	16.8
80	5.6	0.077	0.033	0.017	30.8	7.2
100	5.8	0.029	0.020	0.009	0.01	0.2
Residure	5.7	0.025	0.019	0.006		

*: γ of tyrosine liberated per mg of extracting solvent N_2

As in Table 9, enzymes acting at pH 3.5 were found more in the fractions of 40 — 60 % saturation with ammonium sulfate. This result coincides with the result obtained by Sliwinski et al. (7) reporting that in the case of bovine muscle, enzymes acting at pH 4.0 were found more in the fractions involving 50—60% of ammonium sulfate. On the other hand, it can be seen

from Table 10 that enzymes acting at or near pH 6 are comprised more in the 20% fraction, which are also found in the 40% fraction, being considered to be existing in greater part in the 20—40% fractions. Regarding them, the author intends to further purify them and study the properties.

IV SUMMARY

To know the fundamental properties of proteolytic enzymes existing in muscle, the enzymic action of the fresh porcine muscle extraction liquid was studied principally as to its activity. As a result, with respect to incubation time, a nearly linear increase was observed up to 8 hours and a gentle increase thereafter. The optimum temperature was found in the vicinity of 40°C. Concerning the relation between pH and enzymic action, as a result of experiment repeated several times, a strong activity was found in the vicinity of pH 3.5 and pH 6.0. When no preservative was applied, a strong activity was also found at and around pH 8.0 depending on the buffer solution used (Tris-buffer, for instance) . Enzymes acting at or near pH 3.5 and pH 6.0 showed a strong activity per mgN of enzyme solution when 2 % potassium chloride and extraction liquid with an initial pH of 4.0 (using hydrochloric acid) were employed, respectively. Further, enzymes acting at or near pH 3.5 were found more in the fractions of 40 — 60% saturation with ammonium sulfate and enzymes acting at or near pH 6.0, more in the 20% fraction.

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豚筋肉中に存在する蛋白分解酵素について

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要 約

筋肉中に存在する蛋白分解酵素の基本的な性質を知るために，新鮮な豚筋肉抽出液の酵素作用をその活性を中心として調査した。その結果、インキューベートした時間に対しては8時間までほぼ直線的に増加し，その後はゆるやかな増大が認められた。至適温度は，40°C附近に認められた。pHと酵素作用の関係については，数回くり返し行なったが，pH3.5附近および6附近で活性が大であった。防腐剤を入れないと，使用する緩衝液（例えばトリス緩衝液）によつては，pH 8附近にも強い活性が認められた。pH3.5附近で作用する酵素は，2%塩化カリウム、pH6附近のものは、抽出最初のpHが4（塩酸使用）の抽出液を用いた場合に酵素液のmgN当りの活性が強かった。さらに、pH 3.5で作用する酵素は、硫安40～60%胞和の区分に多く、pH6附近では，20%区分に多いことが認められた。

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