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Influence of NaCl and pH on Hydrolysis of Chicken Myofibrillar Proteins by Leukocyte Lysosomal Proteinases

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Abstract

The influence of NaCl and pH on degradation of chicken breast muscle myofibrillar proteins by porcine leukocyte lysosomal proteinases was investigated. The degradation reactions were carried out at 38 °C for 24 hours under different conditions. The degradation of myofibrillar proteins by leukocyte lysosomal enzymes at various pH values was limited to partial hydrolysis. Reactions at higher pH values resulted in lower molecular weight degradation products while reactions at lower pH resulted in higher molecular weight degradation products. When NaCl was added into the reaction mixture, enzyme activities of degradation were increased at all pH values studied, as evidenced by NPN-analysis and SDS-PAGE. More severe degradation was observed with higher salt concentration. The concentration of 0.5 M NaCl in the reaction mixture gave more degradation of myosin heavy chain by enzyme than that of 0.1M NaCl.

Key words: lysosomal proteinases, leukocytes, myofibrillar proteins, SDS-PAGE, degradation

Introduction

The blood consists of several species of cells including polymorphonuclear leukocytes (PMNS), lymphocyte and red cells. Among these, PMNS have been shown to contain predominant proteolytic activity[1] as well as other typical lysosomal hydrolases[2].

Since leukocytes contain very active typical lysosomal hydrolytic enzymes including proteinases active at neutral to alkaline pH, these enzymes are possibly involved in protein turnover *in vivo* and may be involved in protein degradation postmoterms. These enzymes may be of particular importance for protein degradation of muscle in the animal carcass due to its high content of residual blood and possibly influence meat tenderness during meat aging.

The degradation of myofibrillar protein has special interest and importance in food science, since the texture of meat is one of the most important quality attributes of this food. The proteins of myofibrils constitute about 60% of the intracellular protein of muscle and so more than 25% of total body proteins.

Therefore, this research was intended to demonstrate that lukocyte lysosomal enzymes can hydrolyze myofibrillar proteins *in vitro* and to determine the effect of NaCl and pH on the degradation of myofibrillar proteins.

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Materials and Methods

Preparation of myofibrillar proteins

Chicken breast muscle myofibrillar proteins were prepared at 1.5 hours (hr) post mortem. The method used was that described by Briskey and Fukawaza[3].

Preparation of enzyme

Porcine leukocytes were isolated from porcine blood by the method of Kim[4]. Porcine blood was collected from healthy hogs during execution. The resulting white leukocyte pellet was well suspended in 0.25M sucrose, 1 mM EDTA, and 5 mM sodium azide solution (pH 7.0). The suspension of leukocyte was then sonicated at 4 °C using an ice bath for 3 minutes (min). The lysed leukocytes were then subjected to homogenation to disperse the lysosomal particles sticking to nuclear and cell wall debris. The sonicated suspension was centrifuged at 3,500 × g for 20 min to remove cellular debris and intact cells. The supernatant obtained after centrifugation at 3,500 × g was centrifuged again at 20,000 × g for 30 min to obtain a final lysosomal

fraction of leukocytes.

The pellet obtained from 20,000 × g centrifugation was resuspended with a minimum volume of 0.25M sucrose solution and dialyzed against 5 mM phosphate buffer (pH 7.0) for 2 days in 4 °C. The whole dialysate mixture was used as lysosomal fraction of leukocytes containing proteinases. The protein concentration of the prepared lysosomal enzyme was adjusted to 25 mg/m*l* with the same 5 mM phosphate buffer, after protein concentration was determined.

Determination of enzyme activity

Two methods were used to measure the proteolytic activity of enzymes. Folin-Lowry method was used to determine the non-protein nitrogen (NPN) production and 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weights (M.W.) of myofibrillar proteins and their degradation products.

NPN analysis

For measuring protein degradation, the myofibrillar proteins were reacted with leukocyte lysosomal enzymes at 38 °C in a ratio of 10:1 in a water bath. Samples were removed and added to equal volumes of cold 15% trichloroacetic acid (TCA). The samples were then incubated at 4 °C for overnight and centrifuged at 10,000 × g for 10 min. The resulting supernatant was assayed for NPN production by the method of Barrett[5]. The color was developed at room temperature for 30 min and the absorbance of blue color was measured at wavelength 595nm. Two blanks were made for proper calculation of enzyme activity, substrate and enzyme blanks. The relative enzyme activity was calculated from the differences by substracting absorbance of both substrate and enzyme blanks from that of reaction mixture containing both myofibrillar proteins as substrate and leukocyte lysosomal enzymes.

Relative Enzyme Activity = Abs. Es–(Abs. E + Abs. S)

ES : Enzyme reacted with myofibrillar proteins,
E : Enzyme alone,
S : Myofibrillar proteins alone,
Abs.: Absorbance at 595 nm

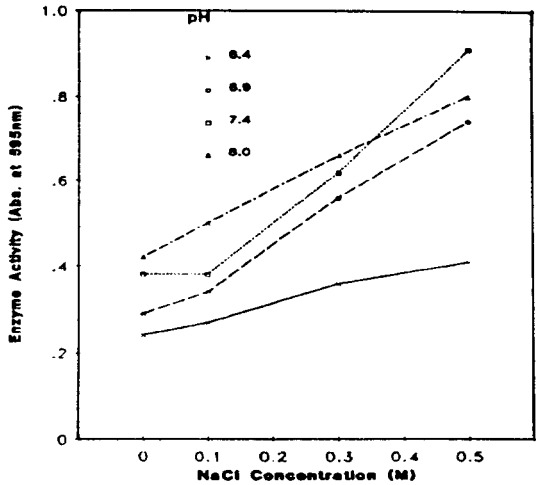

Fig. 1. The effect of NaCl and pH on procine leukocyte lysosomal enzyme activity assayed with chicken myofibrillar proteins

SDS-PAGE analysis of activity

To measure proteolytic activity of enzymes for the degradation of myofibrillar proteins, SDS-PAGE was performed. The method used was the procedure described by Weber and Osborn[6]. Seven % acrylamide gel was used for electrophoresis.

Determination of molecular weight

The molecular weights of polypeptide chains obtained by proteolysis of myofibrillar proteins and separated by SDS-PAGE were calculated from their electrophoretic mobilities using the standard curve. The standard protein contained myosin (M.W. 200,000), beta-galactosidase (M.W. 92,500), bovine serum albumin (M.W. 66,200), ovalbumin (M.W. 45,000), carbonic anhydride (M.W. 31,000), soybean trypsin inhibitor (M.W. 21,500) and lysozyme (M.W. 14,000). These proteins were used to draw standard curve.

Results and Discussion

In order to observe the effect of NaCl at different pH values, a mixture containing myofibrillar proteins as substrate and leukocyte lysosomal enzyme in a ratio of 10:1 (w/w) was reacted with four different concentrations (0, 0.1, 0.3, 0.5M) of added NaCl. The pH of the reaction mixture were then adjusted to 6.45, 6.9, 7.4 and 8.0 with Tris-maleate

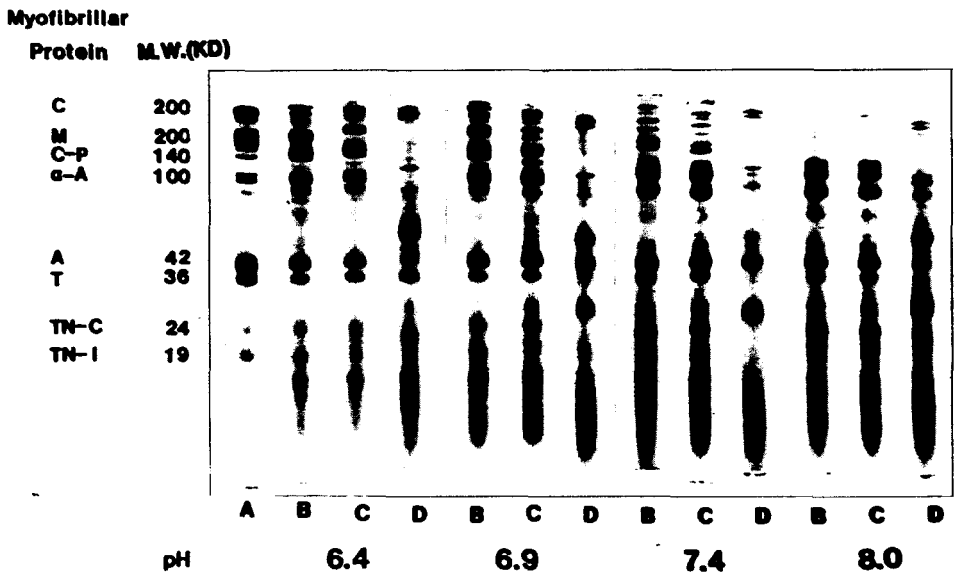

Fig. 2. SDS-PAG Electrophoregrams of chicken myofibrillar proteins reacted with porcine leukocyte lysosomal enzymes at different conc. of NaCl and pH

Each gel contained 50 μg of myofibrillar proteins.

C: Connectin, M: Myosin heavy chain, C-P: C-protein, α-A: α-actinin, A: actin, T: Tropomyosin and Troponin-T, TN-C: Troponin-C and Myosin alkali light chain-1, TN-I: Troponin-I and Myosin alkali light chain-2

Reactions were carried out for 24 hr at 38 °C.

A: Substrate blank (No enzyme added)

B: Reaction mixture contained myofibrillar proteins and enzymes in the absence of added salt.

C: Reaction mixture contained myofibrillar proteins and enzymes in the presence of 0.1M NaCl.

D: Reaction mixture contained myofibrillar proteins and enzymes in the presence of 0.5M NaCl.

buffer. The final concentration of Tris-maleate buffer was 50 mM in the reaction mixture. The reactions were carried at 38 °C for 24 hr. NPN analysis (Fig. 1) and SDS-PAGE (Fig. 2) were used to measure enzyme activities.

When NPN-analysis was used to measure the effect of NaCl at different pH value, it was shown that the effect of NaCl varied with changes in pH of reaction medium (Fig. 1). The reaction mixture incubated at higher pH values in the absence of NaCl had higher activity measured by NPN. At concentrations of 0.1M NaCl, it showed slight increase in enzyme activities. However, in the presence of 0.5M NaCl in the reaction mixture, the highest enzyme activity was observed at pH 7.4 and next at pH 8.0. At pH 6.4 and 6.9, the enzyme activity was stimulated to 200% while pH 7.4 and 8.0 stimulated to 270% of original activities when the activities were measured at different pH values in the presence of 0.5M NaCl.

Electrophoretic patterns of myofibrillar proteins reacted with enzyme for 24 hr and different pH values at different salt concentrations are presented in Fig. 2.

At pH 6.4 (Fig. 2), the major reaction products observed without salt were 200,000, 165,000, 100,000, 42,000, and 36,000 daltons with other minor degradation products as well as other low molecular weight native proteins. The addition of NaCl to give 0.1M to the reaction mixture resulted in greater degradation of myosin heavy chain as evidenced by its intensity. Electrophoretic pattern of the degradative system in the presence of 0.1M NaCl was very similar to that of the reaction mixture incubated without added NaCl. The addition of NaCl to give 0.5M NaCl, however, resulted in more extensive degradation of all myofibrillar proteins except tropomyosin. The major products produced under this condition were 63,000, 56,000, and 36,000 daltons with other minor products. The band corresponding to actin molecule was very thin, indicating that this protein degraded under

this condition. The myosin heavy chain and other products appeared in the presence of low NaCl concentrations were almost completely degraded in the presence of 0.5M NaCl, leaving proteins with M.W. of 63,000, 56,000 and 36,000 daltons as major reaction products.

Results obtained with reaction at pH 6.9 (Fig. 2) showed the increased enzyme activity both in the absence and presence of NaCl. The addition of salt to the reaction mixture brought more extensive degradation of whole myofibrillar proteins. The major products obtained in the presence of 0.5M NaCl were 56,000, 42,000 and 27,000 daltons with other minor bands.

At pH 7.4 (Fig. 2), the major degradation products had M.W. of 100,000 and 83,000 daltons in the absence of salt. The addition of NaCl to give 0.1M did not alter the degradation pattern of the myofibrillar proteins. As in other pH conditions, the addition of salt to give 0.5M to the reaction mixture resulted in extensive degradation of myofibrillar proteins. The major products remaining after reacting with 0.5M NaCl were 42,000 and 27,000 dalton. The product with M.W. of 56,000 dalton was present only in trace under this condition. Complete degradation of tropomyosin was also observed.

At pH 8.0 (Fig. 2), the major degradation products of myofibrillar proteins in the absence of salt were products with M.W. of 120,000, 110,000, 100,000 and 83,000 daltons. Although extensive degradation of myofibrillar proteins occurred at pH 8.0 by 0.5M NaCl, the degradation caused by 0.5M NaCl was less compared to that at other pH. The major degradation products were 56,000 and 27,000 dalton polypeptides in the presence of 0.5M NaCl. The degradation of actin, troponin and tropomyosin molecules was also noticed.

The above results indicated that the effect of NaCl varied with pH of the reaction mixture as well as concentration of salt. The degradation products obtained in the absence of added NaCl at different pH values had different M.W. depending on pH of reaction medium. The addition of salt to reaction mixtures at different pH values caused more extensive degradation of all the myofibrillar proteins than reactions without added salt.

Since salt was shown to enhance the protein degradation by lysosomal proteinase isolated from

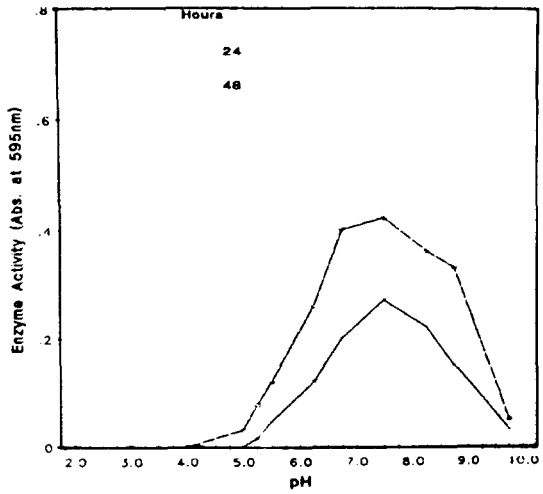

Fig. 3. The effect of 0.5M NaCl on porcine leukocyte lysosomal enzyme activity assayed with chicken myofibrillar proteins at different pH values

leukocytes, optimum pH conditions were studied in the presence of 0.5M NaCl. Optimum pH for the activities of leukocyte lysosomal enzymes in the presence of 0.5M NaCl were determined using myofibrillar proteins as substrate at different pH values from pH 4.0 to pH 9.0. The final pH was measured after the enzyme reaction. Fifty mM Tris-maleate buffer was used to give the same concentration of salt at all pH values to control the salt concentration of the reaction mixture, since salt was shown to increase the proteolytic activity of leukocyte lysosomal enzymes. The reactions were carried out for 24 hr at 38 °C in substrate to enzyme ratio of 10:1 (w/w). Both NPN analysis (Fig. 3) and SDS-PAGE (Fig. 4) were used to measure the enzyme activities. SDS-PAGE was performed with samples reacted at pH 5.3, 7.5 and 8.6 in the absence of NaCl as control and in the presence of 0.5M NaCl.

SDS-PAGE was performed at pH 5.3, 7.5 and 8.6 to understand better the role of NaCl at different pH values. The results obtained presented in Fig. 4. Results indicated that presence of 0.5M NaCl stimulated enzyme activities at all reactions, but stimulation of enzyme activity by NaCl was highly dependent on pH of the incubation mixture. Most extensive degradation of myofibrillar proteins in the presence of 0.5M occurred at neutral pH (pH 7.5),

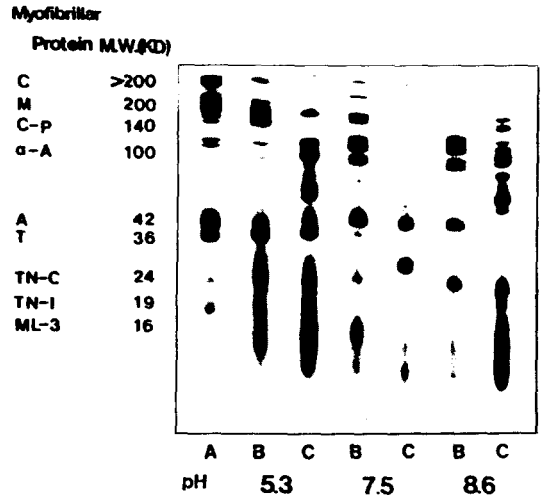

Fig. 4. SDS-PAG Electrophoregrams of chicken myofibrillar proteins reacted with porcine leukocyte lysosomal enzymes in the absence and in the presence of 0.5M NaCl at different pH values

Each gel contained 50 μg of myofibrillar proteins reacted.

C: Connectin, M: Myosin heavy chain, C-P: C-protein, α-A: α-actinin, A: actin, T: Tropomyosin and Troponin-T, TN-C: Troponin-C and Myosin alkali light chain-1, TN-I: Troponin-I and Myosin alkali light chain-2 ML-3: Myosin light-chain

Reactions were carried out for 24 hr at 38 °C.

A: Substrate blank (No enzyme added) heated for 24 hr at pH 7.5.

B: Chicken myofibrillar proteins reacted with enzyme for 24 hr at given pH values.

C: Chicken myofibrillar proteins reacted with enzyme for 24 hr in the presence of 0.5M NaCl at given pH values.

leaving minor protein bands above the M.W. of 56,000 daltons, in contrast to other pH values (pH 5.3 and 8.6). The degradation patterns of myofibrillar proteins was somewhat different from each other.

At pH 8.6, the stimulation of proteinase activity by NaCl was decreased. The electrophoretic profile changed in the presence of NaCl at this pH. The reaction in the absence of NaCl produced 120,000, 110,000 and 83,000 dalton proteins as major products with other minor subfragments. However, the addition of salt to give 0.5M resulted in the formation of somewhat intense bands corresponding to molecular weights higher than 42,000 daltons. The estimated molecular weight of new proteins were 180,000, 170,000, 165,000, 140,000, 125,000, 110,000, 105,000, 100,000, 83,000, 56,000 and 52,000 daltons. The bands corresponding to actin (A), tropomyosin and troponin-T (T) disappeared completely.

The above observation suggested that the effect of NaCl varied with the pH of reaction medium. The optimum pH of leukocyte lysosomal proteinases to breakdown muscle myofibrillar proteins in the presence of 0.5M NaCl occurred at near neutral pH. This agrees with the results observed by other investigators[7-9].

Starkey and Barrett[10] demonstrated the proteolytic activity of human spleen extracts at neutral pH. The fractionation procedure for separation of the proteinases demonstrated some limited solubility. They demonstrated that neutral proteolytic activity was due to two serine proteinases, an elastase and cathepsin G. The activities of these two proteinases measured against azocasin, was shown to be stimulated by high salt concentration. The stimulation of enzyme activity was greatest with crude enzyme[11,12]. Pryce-Jones *et al.*[13] found that high salt concentration was necessary for the solubilization of elastase from neutrophil leukocytes. They also found that the enzyme were activated by salt. Noguchi and Kandatsu[14] reported that autolytic activity of skeletal muscle proteins was increased by potassium chloride (KCl) up to 0.6M, and that activity was optimal at pH 7.5-9.5. Holmes *et al.*[8] reported that alkaline proteinase activity of sedimentable fraction increased in the presence of NaCl, but this effect was strongly pH dependent, being maximal near neutral pH. More recently, Reinauer and Dahlmann[15] reported on two alkaline proteinases in rat skeletal muscle which were believed to be responsible for myofibrillar protein breakdown. The enzyme that is soluble in the presence of 33% ammonium sulfate in KCl was named cytosolic proteinase while the other which was insoluble was named myofibrillar proteinase.

The leukocyte lysosomal proteinase was able to degrade native myofibrillar proteins. The degradation of myofibrillar proteins was stimulated by the addition of salt and this stimulation was strongly pH dependent. The electrophoretic patterns to degrade myofibrillar proteins were different upon the pH of the reaction medium. Moreover, the addition of salt

alters the degradation pattern more severely, suggesting different enzymes are possibly involved in this particular proteins.

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Leucocyte lysosomal proteinase에 의한 닭의 筋纖維 蛋白質 分解에 미치는 NaCl과 pH의 影響

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닭의 筋纖維 蛋白質이 돼지의 白血球에서 抽出한 lysosomal proteinase에 의해서 分解될 때 미치는 NaCl과 pH의 영향에 대해서 硏究하였다. Leucocyte lysosomal proteinase에 의한 筋纖維 蛋白質의 分解는 다른 pH를 갖는 Tris-maleate buffer에서 partial hydrolysis로 進行하였다. 분해는 높은 pH에서 더욱 深化되었으며, 여기에 NaCl이 添加되었을 때 proteinase의 activity는 pH의 高低에 關係없이 더욱 增加함을 보여주었다.