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STUDIES ON SOME COMPONENTS IN SARCOPLASM PROMOTING THE COLOR FORMATION OF PROCESSED MEAT PRODUCTS

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Contents

Abbreviations

The abbreviations used in this study are defined as follows:

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPDA	Adenosine monophosphate deaminase
ATP	Adenosine triphosphate
CCMC	Cooked cured meat color
CFA	Color formation ability
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)

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FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GSH	Glutathione, reduced form
GSSG	Glutathione, oxidized form
Hb	Hemoglobin
IDP	Inosine diphosphate
IMP	Inosine monophosphate
ITP	Inosine triphosphate
LDH	Lactate dehydrogenase
Mb	Myoglobin
MetMb	Metmyoglobin
$MetMbNO_2$	Metmyoglobin nitrite
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NEM	N-Ethylmaleimide
NO	Nitric oxide
NOMb	Nitric oxide myoglobin
NOMetMb	Nitric oxide metmyoglobin
P	Phosphorus
Pi	Inorganic phosphorus
PK	Pyruvate kinase
RA	Reducing ability
SDS	Sodium dodecyl sulfate
SH groups	Sulfhydryl groups
SS groups	Disulfide groups
TCA cycle	Tricarboxylic acid cycle

CHAPTER I

INTRODUCTION

Curing of meat is a very important process in the meat technology. Historically, curing meant the addition of sodium chloride to the meat for the purpose of preservation. In recent years, with the development of refrigeration and freezing and their application to the preservation of meat, the main purpose of meat curing changed from preservation to the development of unique color, flavor, texture and palatability properties of meat products. Today, the formation of CCMC is equally as important as flavor and texture changes, even though preservation is the original purpose of meat curing.

Nitrite and/or nitrate have been conventionally used as color developing agents (color fixatives). When nitrite *per se* was first used in curing meat is unknown, but some of the classical studies[1,2] have demonstrated that the typical color of cured meats was due to nitrite but not to nitrate. The effect of nitrite in forming the CCMC in meat products was discovered probably through the accident of impurities in natural salt.[3] When nitrate is used to cured meats, it must be converted to nitrite through nitrate reducing microorganisms before reaction occurs with meat pigment to give the characteristic color of cured meat products.[4-6] Actually, the nitrite itself is not the active nitrosating agent, but it reacts more rapidly and effectively at a less amount than nitrate and, therefore, the direct use of nitrite as a curing ingredient gradually came into practice rather than relying on a unpredictable bacteriological conversion of nitrate to nitrite. Since nitrate has no direct influence on either color or flavor except as a reservoir for nitrite, nitrate is now being removed from use in meat curing. Nitrite is, however, a toxic substance and regulations about its use were enacted to protect the consumers' health.

It is well known that nitrite performs the following four favorable functions in cured meat products: (1) It is responsible for the typical color formation of meat products. (2) The growth of *Clostridium botulinum* and other spoilage and food poisoning organisms is inhibited by nitrite. (3) Nitrite contributes to the development of the characteristic flavor of cured meats. (4) Nitrite contributes to retard the development of rancidity in processed meat products.

Recently, there has been increasing concern about the formation of nitrosamines in cured meat products,[7-11] since a class of these compounds can be formed by the reaction of nitrite with amines or some other similar compounds.[12-14] The carcinogenic activity of many nitrosamines

in some experimental animals has been confirmed.[15-17] Further studies have demonstrated that nitrosamine formation may occur in cured meat products and at conditions similar to those in the human stomach.[18] This demonstration of a possible human health hazard due to nitrite has made it necessary to reevaluate the above mentioned merits of nitrite in cured meats and to determine the nitrite levels required to process quality meat products and minimize (or prevent) potential nitrosamine formation. Some effort[19-22] has also been done in order to find out a nitrite substitute in meat curing, but an additive equal or superior to nitrite for the color formation and antibotulinal action has not yet been found. Therefore, nitrite is widely used at present in meat curing. Other additives such as sodium chloride, sugar, ascorbate, *etc.*, besides sodium nitrite are usually present in a curing formula. The modern curing technology employs a number of additives for a variety of purposes. When the curing mixture or solution is added, nitrite in the cure reacts with the heme pigments in meat.

The heme pigments in meat consist mainly of two proteins; the muscle pigment, Mb and the blood pigment, Hb. Although other lesser pigments such as the cytochromes, catalase and the flavins are also present, their contribution to meat color is very slight. In a well-bled piece of meat, Mb comprises 80 to 90% of the total pigments present and is much more abundant than Hb. The two major pigments are similar in structure and function, except that the Mb molecule is one-fourth as large as the Hb molecule. Both Hb and Mb are complex proteins, which means that in addition to the protein moiety, the globin, there is another moiety called the heme. The heme portion of the pigment is of special interest because the color of meat is partially dependent on the chemical state of the iron within the heme ring. Thus, the condition of the iron plays an important role in the curing process. If the iron is in the ferrous state, the meat color is desirable red, but if the iron is in ferric or oxidized state, brown-gray color results. The role of Mb and Hb in living tissue is quite different; Hb acts as an oxygen carrier in the blood stream and Mb is essentially a storage mechanism for oxygen in the cells. Although certain reactions such as autoxidation and denaturation also occur at different rate, it should be pointed out that the two pigments undergo identical reactions of concern to meat and meat products, therefore discussion of the chemistry of these pigments may be limited to Mb.

In meat curing, when nitrite is added to meat, Mb is first oxidized to a brown state, MetMb, by a strong heme pigment oxidant of nitrite. Then, the MetMb must be converted to the desired pink-red cured meat pigment, NOMb. The chemical reactions involved in the transformation of Mb to NOMb after the addition of nitrite to meat are so complicated that the exact mechanism has not been elucidated in complete detail in spite of a lot of work on the color formation of meat products. The main proposed pathways which form the cured meat pigment NOMb in the literature are summarized as follows:

Pathway-(1)[23]; MetMb is reduced to Mb by endogenous active components or added reducing agents. Nitrite is also simultaneously reduced to NO. The reduced Mb and NO react directly to form NOMb.

Pathway-(2)[24,25]; NO formed by the reduction of nitrite reacts with MetMb to form NOMet-Mb, which is subsequently reduced to NOMb by the same reduction systems as those mentioned in pathway-(1).

Pathway-(3)[26-28]; $MetMbNO_2$ is formed by the reaction of MetMb with nitrite, and the ionic complex $MetMbNO_2$ is reduced to NOMb by endogenous active components or added reductants.

Pathway-(4)[29]; MetMb is reduced to Mb by the action of NADH or NADPH and by the reducing system containing FMN, FAD or riboflavin. Reduced Mb reduces nitrite to NO. NOMb is then formed by the direct reaction of the reduced Mb and NO.

Pathway-(5)[30-32]; Nitrite reacts with SH groups of muscle proteins to form a nitrosothiol, "protein-S-NO". The formed unstable intermediate is split by heavy metal ions in muscle and the NO group is transferred from the nitrosothiol to Mb to give NOMb.

Pathway-(6)[33,34]; NO-ferricytochrome C is formed by the reaction of nitrite with ferrocyto-

chrome C in the presence of cytochrome oxidase under anaerobic conditions. It is reduced by the NADH dehydrogenase system of muscle, and the NO is transferred to MetMb. The NOMet-Mb thus formed is then reduced to NOMb by the enzyme systems of mitochondria.

In the usual processing of meat products, it is not clear which of these pathways described above would be responsible for the color formation of meat products. However, it is thought that the pathway is probably determined by several other factors in addition to those previously mentioned, that is, the property of meat, added food additives, processing conditions in curing meat, *etc.*

Although the mechanism involved in the color formation of meat products has not been amply demonstrated, it has been established that NOMb, which is the principal pigment of cured meat, is converted to heat-stable NO-hemochrome, the pink so-called CCMC, by the heat of the cooking process. The CCMC is pink and more stable than the color of cured meat pigment (NOMb). The CCMC is the principal pigment of cooked cured meat products.

The formation of NO-hemochrome involves denaturation of the protein moiety of Mb, but leaves the heme structure intact with the NO attached. TARLADGIS[35] has postulated that the cooked cured meat pigment is dinitrosyl-hemochrome. This means that the cooked cured pigment presents two binding sites for NO compared to only one site in the unheated pigment, *i. e.*, two coordination positions of the protoheme are occupied by NO. MÖHLER[36] described in his review that the solubility of NO-hemochrome in acetone is probably caused by the exchange of the imidazole-N-bond of the 5th coordination position with acetone. The TARLADGIS' hypothesis was recently supported by the findings of LEE and CASSENS.[37] With ^{15}N-labeled nitrite the heated preparation of NOMb contained twice the amount of ^{15}N as unheated preparations. There has still been some question as to the identity of the final pigment.[38] RANKEN[39] noted that when a piece of raw cured meat was slowly heated, the color did not change directly from raw red (NOMb) to cooked pink (CCMC) but passed through an intermediate gray stage. This suggests the intermediate formation of an uncharacterized gray pigment during cooking.[40]

In order to obtain the full color development of meat products, the meat must be held as strongly as possible under reducing conditions as evidenced by the proposed pathways described above.

WALTERS *et al.*[41,42] investigated the participation of endogenous enzymes and their reduction mechanism in postmortem muscle in color formation. These authors recognized that the anaerobic incubation of nitrite with skeletal muscle minces at pH 6.0 led to the formation of NO, and suggested that nitrite was reduced to NO by the residual enzyme systems of mitochondria. Furthermore, from the experiment with a model system consisting of muscle mitochondria which contained enzymes responsible for TCA cycle (cyclophorase system), they found[43] that NO-ferricytochrome C was reduced by the NADH dehydrogenase system, and proposed[33,34] a mechanism for the formation of NOMb [see pathway-(6) in page 371]. They also assumed[44] that the overnight incubation of bacon homogenate (pH 6.0) under anaerobic condition led to the formation of ferrocytochrome C and reduced Mb. Recently, they confirmed the presence of mitochondria in a commercial vacuum packed bacon by using electron microscopy. The mitochondria contained fewer cristae than those observed in fresh meat. CHEAH[45] suggested that NOMb formation involved MetMb reduction by NADH formed from NAD^+ and lactate by lactate dehydrogenase present in cured bacon.

ROSE and PETERSON[46] noted a reducing system in fresh pork which caused a progressive decomposition of nitrite, independent of bacterial contamination. The relation of endogenous components of muscle having reducing activity to cured meat color was investigated by WATTS *et al.*[47] They heated a model mixture containing egg white, Hb and sodium nitrite in phosphate buffer (pH 5.8), and they suggested that the reduction of MetMb and nitrite necessary for the color formation may be brought about by free SH groups released from denatured muscle proteins by heat treatment. Recently, HOFMANN and HAMM[48] pointed out that the reactivity of the

SH groups in egg white is very different from the reactivity of the SH groups in meat protein: Meat proteins always contain free SH groups in the native state as well as in the denatured state, whereas there are no SH groups detectable in native egg albumin.[49-53] KUBBERØD *et al.*[54] recognized by investigating the reactivities of myosin with nitrite that the reaction rates between SH groups in myosin and nitrite were fairly low under conditions similar to the routine processing of meat products. MIRNA and HOFMANN[30] reported the phenomenon by which SH groups in muscle tissue reacted approximately stoichiometrically with the added nitrite. These findings were supported by the observation of OLSMAN and KROL.[55] MIRNA and HOFMANN[30] postulated the formation of a nitrosothiol (protein-S-NO) by the reactions of the SH groups of muscle proteins with nitrite in the curing process, and they concluded that the nitrosothiol was probably involved in the color formation [see pathway-(5) in page 371]. Thus, many studies related to the effect of muscle proteins on the color formation have been mainly carried out from the aspect of the reactivities of SH groups in the proteins with nitrite.

The investigations on the relation of cysteine to cured meat color were carried out by SIEDLER and SCHWEIGERT,[56] FOX and THOMSON,[25] REITH and SZAKÁLY[57] and WALTERS *et al.*,[44] and cysteine was found to possess the ability to promote the color formation. FOX and ACKERMAN[58] suggested that cysteine is probably involved in the direct reduction of MetMb.

It was shown that NADH in muscle could reduce not only NOMetMb to NOMb but also NO-ferricytochrome C to ferrocytochrome C [see pathway-(6) in page 371], and that the NADH was concerned with the reduction of $MetMbNO_2$ as described in pathway-(3) (see page 371). Furthermore, KOIZUMI and BROWN[29] found that NADH or NADPH in the presence of FMN, FAD or riboflavin affected the formation of NOMb [see pathway-(4) in page 371]. It was recognized by WALTERS *et al.*[44] and FOX and ACKERMAN[58] that GSH and hydroquinone exhibited a promoting effect on the color formation. OLSMAN[59] suggested that the small amount of endogenous ascorbic acid in meat plays a key role in the mechanism of nitrite depletion by acting as an electron carrier in the reduction of nitrite by the SH groups of meat products.

Modern meat curing technology has provided efficient methods using several reductants (compounds capable of donating electrons) in meat curing mixtures in order to accelerate the color formation. The most commonly used reductant is a sodium salt of ascorbic acid or of an isomer, isoascorbic acid.

The first investigations on the relation of ascorbic acid to cured meat color were carried out by GIBSON.[60] He confirmed that ascorbic acid accelerates the reduction of MetHb. SIEDLER and SCHWEIGERT[56] also showed that ascorbate is a very efficient reductant for the desired color formation. FOX and THOMSON[25] suggested from their kinetic studies that ascorbic acid forms an intermediate complex by the reaction with nitrite, and the complex decomposes slowly to form NO[see pathway-(2) in page 371]. FOX and NICHOLAS[61] tested various compounds in meat slurries for their effects on nitrite loss and found that the reductants, ascorbate and cysteine, and an amino acid, histidine, caused a nitrite loss. Loss of nitrite was related to the reductive reaction which produces NO, and they suggested that the production of NO accounts for a large part of total nitrite loss and the lost nitrite (not accounted for) was involved in the formation of NO-reductant intermediates or products. REITH and SZAKÁLY[57] studied the rate and extent of the formation of NOMb by the use of aqueous model solutions (pH 6.0) containing 1-1.5 mg/ml Mb and various amounts of nitrite and ascorbate. They demonstrated that ascorbate accelerated the formation of NOMb and the conversion of Mb to NOMb was optimum when 200 moles ascorbate was present with 1 mole nitrite per mole Mb. Furthermore, WALTERS *et al.*[43] reported that ascorbate accomplished the transnitrosation from NO-ferricytochrome C to MetMb directly without the action of the muscle enzymes [see pathway-(4) in page 371]. It has been reported[62] that the addition of nicotinamide with sodium ascorbate and nitrite could further promote the color formation than that of only sodium ascorbate and nitrite. NAGATA and ANDO[63] found that the addition of nicotinamide significantly promoted not only the decomposition of nitrite but also

the formation of NO from nitrite in the presence of sodium ascorbate, and they presumed that this finding could explain the promoting effect of nicotinamide on the color formation.

In the processing of meat products, various food additives in addition to reductants are used. Some of these food additives have been found to promote the color formation in addition to their substantial effects. NAGATA and ANDO[63,64] recognized from the experiments using an aqueous model system consisting of nitrite, ascorbate and various food additives that most of the food additives tested were found to have a tendency to promote the decomposition of nitrite in the presence of sodium ascorbate after cooking. Of all the food additives sodium chloride is incorporated at the highest amount into processed meat products. There are some reports concerning the promoting effect of this salt on the color formation. FUJIMAKI *et al.*[65] as well as WATTS and LEHMANN[23] showed that sodium chloride enhanced the color formation in a model system; whereas REITH and SZAKÁLY[57] reported that sodium chloride had an adverse effect on the color formation in model systems consisting of Mb, nitrite, ascorbate, and sodium chloride. OLSMAN and KROL[55] found that sodium chloride did not have an effect on the rate of nitrite depletion during storage of meat. LEE and CASSENS,[66] using both model systems and bacon products, demonstrated that increased levels of sodium chloride decreased analyzable or residual nitrite. ANDO *et al.*[67] observed that the addition of sodium chloride to meat increased the content of free Mg^{2+}, Ca^{2+} and Zn^{2+}, and they assumed that the increased content of free bivalent metal ions may conceivably have enhanced the color formation in the presence of the reductants in meat products.[68]

It has been known that metal ions in meat are responsible for the formation of cured meat color. GIBSON[60] recognized that Fe^{2+} and Cu^{2+} catalyzed the reduction of MetHb to Hb. WEISS *et al.*[69] reported that four metal ions, Cu^{2+}, Fe^{2+}, Fe^{3+} and Zn^{2+}, accelerated not only the reduction of MetHb by ascorbic acid but also the color formation. REITH and SZAKÁLY[57] as well as SIEDLER and SCHWEIGERT[56] also observed that Fe^{2+} improved both the rate and degree of conversion to NOMb. SIEDLER and SCHWEIGERT[56] explained the reason for this from the nitrite sparing effect by ferrous ion. This assumption was supported by OLSMAN and KROL[55] and NAGATA and ANDO.[63] The latter observed that Fe^{2+} promoted the decomposition of nitrite by heat treatment at pH 6.0 as well as at pH 5.0, and Fe^{2+} and Fe^{3+} fairly enhanced the decomposition of nitrite in the presence of sodium ascorbate. The Fe^{3+} was active only after it was reduced to Fe^{2+} by ascorbate. Mg^{2+}, Ca^{2+} and Zn^{2+} did not promote the decomposition of nitrite, whereas the reaction was accelerated slightly by heating with ascorbate.

Modern meat curing technology has provided efficient methods to accelerate the meat curing process because of the automation and rationalization of meat processing. Thus, the newest curing method of brine pumping, "multiple injection", with the combined use of tumbler or massage processes has been used widely in the technology of meat products such as hams instead of the conventional method. The utilization of this method could greatly shorten the curing period. Similary, in the processing of many kinds of sausage products, nitrite as well as many other ingredients is simultaneously added to meat at the cutting process. The sausage emulsion is stuffed into casings after cutting and immediately subjected to the thermal process (smoking and/or cooking). This method is referred to as a rapid emulsion curing process and is currently available in processing emulsion-type sausage. Since the rapid emulsion curing process has a much shorter period of meat curing than the conventional manner, the color formation of sausage products through this method probably proceeds mainly during the thermal process by non-enzymatic reactions. The cooking just after curing eliminates the possibility of enzyme coupled reaction in the color formation. This suggests that numerous substances in muscle tissue, in addition to the food additives such as reductants, are involved in the color formation of sausage products.

Lately, ANDO and NAGATA[70] have observed that the sarcoplasm fraction exhibited a distinct promoting effect on the decomposition of nitrite and the color formation in the rapid emulsion curing process under anaerobic condition.

Sarcoplasm is the cytoplasm of muscle fibers, and contains the intracellular colloidal substances in which all the organelles are suspended. The sarcoplasm is readily extractable in water or low ionic strength buffers (0.15 or less). About 75% of the volume of muscle fibers is taken up to by the myofibrils, from which it can be calculated that the probably *in situ* concentration of sarcoplasmic proteins is from 25 to 30%.[71] The sarcoplasmic proteins include the enzymes associated with glycolysis, Mb, Hb, F-protein,[72] *etc.* SCOPES[72,73] identified many enzymes of the glycolytic sequence in the starch-gel electrophoretic patterns of porcine muscle sarcoplasmic proteins. He reported[71] that the glycolytic enzymes normally constitute about at least 80% of the sarcoplasmic proteins.

The sarcoplasmic proteins are known to act as emulsifying agents in sausage emulsions, even though they are not so effective as the myofibrillar proteins. However, the contribution of these protein fractions to the color formation has not been investigated.

On the other hand, the sarcoplasm includes numerous non-protein nitrogenous compounds, carbohydrates, non-nitrogenous compounds and inorganic constituents dissolved in water. Much attention has been devoted to the chemical constituents of the low-molecular weight meat fraction, since the precursors of the characteristic flavor [and odor compounds in meat are largely water soluble.[74-77] Several investigators have reported on the separation of the low-molecular weight components in meat by dialysis techniques[78-81] and by the use of paper chromatography.[82-85] ZAIKA *et al.*[86] separated many aroma precursors from the water-soluble low-molecular weight fraction of bovine muscle using column chromatography on Bio-Gel P-2, Amberlite XAD-2 and DEAE-Sephadex A-25 and also thin-layer chromatography. Many individual components were found to be present in the low-molecular weight fraction of meat. However, attempts to identify the components involved directly in the development of meat aroma from the fraction have been unsuccessful.

TINBERGEN[87] investigated the reaction of nitrite with a water-soluble low-molecular weight fraction extracted from bovine skeletal muscle and he suggested that the component related to the nitrite reduction and NOMb formation was either an amino acid or a lower peptide, probably with a SH group, having a molecular weight below 500.

Since nitrite can react with amines or amides to form nitrosamines which are carcinogenic to several species of animals, [88] considerable attention has recently been forcused on the fate of added nitrite in meat.

SEBRANEK *et al.*[89,90] have extensively studied the fate of nitrite in a comminuted, cured, canned luncheon meat by applying ^{15}N as a tracer. They found that nitrite added to the meat product was rapidly changed to other various compounds during and after processing, and that the rate of change slowed until a constant low level of residual nitrite was reached. When residual nitrite decreased, the amount of ^{15}N in the pigment fraction was 9 to 12% of the level, which did not vary greatly due to amount of heat processing, and this was relatively constant during storage. They also demonstrated that an increase of ^{15}N occurred in two fractions, a protein bound fraction and a non-nitrite, water-soluble fraction of the meat, and they detected 25 to 35% of the formulated nitrite as non-nitrite compounds in the water-soluble low-molecular weight fraction of cured meat. Furthermore, they[91] reported that this fraction was separated into one large and one small peak of ^{15}N content by Sephadex G-10 gel chromatography, and the ^{15}N peak fractions did not include ninhydrin-positive or ultraviolet absorbing compounds, and were also negative for SH groups. FUJIMAKI *et al.*[65] as well as EMI-MIWA *et al.*[92] have quantitatively analyzed for ^{15}N in model and meat systems using ^{15}N-labeled nitrite. In the meat systems, the recovery of the added nitrite was from 66 to 90%, while it was almost 100% in the model systems in the presence of ascorbate. They[93] attempted to separate reaction products of nitrite from low-salt-soluble, low-molecular weight fraction of meat which had been treated with ^{15}N-labeled nitrite, and found that the one ^{15}N-containing peak which was obtained from the reaction compounds did not contain nucleotides, amino acids or peptides, but it included an acidic

compound.

It is worth noting that the highest amount of unidentified non-nitrite ^{15}N compounds has been found in the water-soluble or low-salt-soluble, low-molecular weight meat fraction. Studies on the fate of nitrite might provide some valuable information to characterize the endogenous components promoting the color formation.

The purpose of this study is to clarify the mechanism of the color formation in sausages by the current rapid emulsion curing process. The present work was undertaken to identify the endogenous components promoting the color formation in porcine skeletal muscle, especially in the sarcoplasm fraction and to characterize the endogenous active components.

The rationale is also to develop information about the possibility of regulating the color formation of cooked cured meat products. These studies could provide meaningful information about meat processing techniques which could always give a uniform and desired color to meat products. The studies may also contribute to lessening the potential health hazards by the reasonable usage of nitrite and possibly result in a nitrite substitute for meat curing.

CHAPTER II

PROMOTING EFFECT OF PORCINE MUSCLE SARCOPLASM ON THE COLOR FORMATION

INTRODUCTION

The formation of NO-hemochrome in cooked cured meat products is of great commercial importance to the meat processing industry, since this compound has been shown to be the principal red pigment in cooked cured meat products. The color of meat products is indispensable to consumer acceptance. Formation of the pigment basically involves the reaction of Mb with nitrite employed in the curing formula, but the precise sequence of events whereby the pink color is formed is not fully understood. Considerable research has been carried out on the mechanism of the reaction and the proposed pathways were reviewed in Chapter I. There is a number of methods of meat curing. In the usual processing of meat products, curing agents are added to meat, and curing is carried out for a given period of time. It has been found[36] that the reducing systems endogenous to meat, which include not only non-enzymatic system but also enzymatic one, play an important role in the color formation during the curing process. However, recent trends have been toward decreased time of curing in the meat industry. In preparing modern emulsion-type sausages such as frankfurters or bologna, nitrite as well as many other ingredients is simultaneously added to meat, and after comminution the sausage emulsion is immediately subjected to the thermal process (smoking and/or cooking). Since this processing method has a very short curing period compared with the conventional manner, the color formation of cooked sausage through this rapid emulsion curing process appears to proceed mainly by non-enzymatic reactions during cooking. Recently, KOIZUMI and BROWN[29] have reported that NADH or NADPH in the presence of FMN, FAD or riboflavin was responsible for the color formation in non-enzymatic systems. It has been shown that some components which are present in muscle tissues such as SH groups released by heat denaturation,[47] ascorbic acid,[25,44,56,57,59-61] cysteine,[25,44,56-58] GSH,[44,58] hydroquinone[44,58] and metal ions[55-57,60,63,67-69] are also involved in the color formation of meat products. In the rapid emulsion curing process, it is conceivable that unknown components in muscle tissues other than these active compounds also could be responsible for the color formation. However, the identification of these endogenous components and the mechanisms of the color formation are not clear at the

present stage.

Lately, ANDO and NAGATA[70] have observed that of all the four fractions obtained from the porcine skeletal muscle, *viz.*, sarcoplasm, myofibrils, mitochondria and microsomes fraction, sarcoplasm fraction exhibited the most favorable effects on the color formation and the decomposition of nitrite in the rapid emulsion curing process under anaerobic condition.

The objective of this study was to make clear the possible endogenous components in sarcoplasm which promote the color formation. The work reported herein was to separate sarcoplasm prepared from porcine skeletal muscle into low- and high-molecular weight fractions and to examine the promoting effect on the color formation of these two fractions. Furthermore, an experiment was designed to fractionate the low-molecular weight fraction by gel filtration techniques.

MATERIALS & METHODS

1. Preparation and fractionation of sarcoplasm

Sarcoplasm fraction was prepared from porcine skeletal muscle (*M. adductor*, 3 hr postmortem) by the method of GREASER *et al.*[94] with a slight modification. After external fat and connective tissues were removed as far as possible, 100 g of the porcine muscle was ground with a hand chopper having a plate with 3.2 mm diameter holes. It was then homogenized with 400 ml of ice-cold 0.1 M NaCl-5 mM histidine buffer (pH 7.6) for 90 sec in a Waring Blendor. The homogenate was centrifuged at 1,000 × g for 20 min and the supernatant was further centrifuged at 60,000×g for 60 min. The entire procedure was carried out at 4°C. The obtained supernatant was concentrated to 80 ml in a rotary vacuum evaporator (Shibata Model A) kept at 10°C. The resulting concentrated supernatant was used as sarcoplasm. Forty ml of the sarcoplasm (corresponding to 50 g of the porcine muscle) was dialyzed against distilled water overnight at 4°C with two changes of water of 500 ml each using a Visking tubing (size: 27/32). The combined outside solution (ca. 1,500 ml) of Visking tubing was concentrated to 40 ml with a rotary evaporator kept at 10°C. The concentrated outside solution and the inside solution were adjusted to pH 5.5 with 1 N HCl and made up to 50 ml each with M/35 veronal buffer[95] of pH 5.5. The resulting outside and inside preparations were used as low- and high-molecular weight fractions, respectively.

2. Sephadex G-50 gel chromatography of the low-molecular weight sarcoplasm fraction

The low-molecular weight sarcoplasm fraction was further concentrated to 15 ml with the same method as described before and all of the concentrated preparation was applied to a 2.5 × 40 cm column of Sephadex G-50 (Pharmacia) and eluted with 0.1 M NaCl-5 mM histidine buffer (pH 7.6) at a flow rate of 0.5 ml/min and at 4°C. Five ml fractions were collected each. Since the low-molecular weight fraction has been found to have an absorption maximum at 248 nm,[80] the absorbance of the eluted fractions was measured at this wavelength. All spectrophotometric data were obtained using a Hitachi Model 124 Spectrophotometer equipped with a Hitachi Model QPD_{73} Recorder. Each fraction was adjusted to pH 5.5 with 0.1 N HCl or NaOH solution and was made up to 10 ml with M/35 veronal buffer, pH 5.5. Each fraction was analyzed for RA, SH groups, ninhydrin-positive substances and carbohydrates. CFA and nitrite decomposition were also measured by the following methods.

3. Determination of CFA and nitrite

(1) Preparation of cooked reaction mixture

To 2 ml of each fraction adjusted to pH 5.5, 0.1 ml of 0.055% $NaNO_2$ and 0.1 ml of 2.75% porcine heart Mb (purified by SNYDER and AYRES' s method[96]) were mixed in a Thunberg tube (final concentrations of $NaNO_2$ and Mb were 25 ppm and 0.125%, respectively). The reaction mixture was then cooked at 75°C for 1 hr at a vacuum of 5 mmHg, and cooled with running tap water for 10 min.

(2) Determination of CFA

CFA was determined by the method of OKAYAMA and NAGATA[97] which modified the method of HORNSEY.[98] Two ml of each cooked reaction test mixture was extracted with 75% acetone in water at 0°C for 5 min with intermittent stirring and filtered through a Toyo No. 5A filter paper. In order to stabilize the NO-heme pigments extracted, 5 ml of the filtrate was adjusted to pH

5.5 by adding 0.1 ml of 5.5 N acetic acid aqueous solution and then the absorbance was measured at 395 nm.

(3) Determination of nitrite

Determination of nitrite was carried out by NAGATA and ANDO's method[99] which modified the method of FOLLETT and RATCLIFF.[100] This method includes the following steps to avoid interference by reducing substances in the colorimetric determination of nitrite. Each test sample was diluted with distilled water, adjusted to pH 9.0 with sodium diethylbarbiturate, and heated at 80 °C for 90 min. After cooling, potassium ferricyanide was added to the filtrate and adjusted to pH 7.0 with 1 N HCI, and lead acetate was added in order to precipitate ferrocyanide formed by the reaction of ferricyanide with reducing substances. After filtering off the precipitate, the amount of nitrite in the filtrate was estimated colorimetrically with Orange I reagent by the method of FOLLETT and RATCLIFF.[100]

4. Determination of RA

RA was measured by the modified ferricyanide-reducing method of ANDO and NAGATA,[70] which has initially been developed by KAJITA.[101] The procedures are as follows: To 2 ml of each fraction, 5 ml of 1 mM potassium ferricyanide (dissolved in M/35 veronal buffer of pH 7.0) was added and incubated for 60 min at 0°C. Then, 0.1 ml of 10% ammonium sulfamate and 0.4 ml of 0.25 M lead acetate were added, and allowed to stand for 10 min at room temperature. After adding 2.5 ml of 20% TCA and filtering, the absorbance of this filtrate was measured at 420 nm. The difference in absorbance between a control solution (used distilled water instead of the fraction sample) and the sample solution was expressed as RA.

5. Determination of SH groups

Determination of SH groups was carried out by HAMM and HOFMANN' s method[102] and expressed as μmoles SH reacted with NEM per ml each fraction.

6. Determination of ninhydrin-positive substances

Ninhydrin-positive substances were determined by YEMM and COCKING's method[103] and expressed as mg leucine per ml each fraction.

7. Determination of carbohydrates

Determination of carbohydrates was carried out by SCOTT and MELVIN's method using an anthrone reagent[104] and expressed as mg glucose per ml each fraction.

RESULTS & DISCUSSION

1. Comparison of the low- and high-molecular weight fraction of sarcoplasm

Table 1 shows the ability to promote the color formation present in both the low- and high-molecular weight fractions. CFA was found in both fractions and was stronger in the low-molecular one, which was also more effective in decomposing nitrite than the high-molecular one. About 90% of RA exhibited by the whole sarcoplasm fraction was detected in the high-molecular one (data not shown).

It is well known that the sarcoplasm fraction contains a great many glycolytic enzymes.[71] Therefore, it seems reasonable to assume that RA of the high-molecular weight fraction is mostly attributed to SH groups released from these glycolytic enzymes by heat treatment[47] and RA based on the SH groups was greatly responsible for both the color formation and the decomposition of nitrite in the high-molecular weight fraction. On the other hand, the low-molecular weight fraction was found to be stronger in CFA and in nitrite decomposing ability in spite of its lower RA than that of the high-molecular one. This suggests that the mechanism promoting the color formation of the low-molecular weight fraction differs from that of the high-molecular one.

2. Investigation of the low-molecular weight fraction by gel chromatography

Since the low-molecular sarcoplasm fraction exhibited stronger CFA, the low-molecular one was fractionated by gel chromatography using Sephadex G-50 column. The absorbance of each fraction obtained by gel filtration was measured at 248 nm. As can be seen in Fig. 1, the fractions having an absorbance at 248 nm were found

in the region from tubes 35 up to 50, and tube 40 had the highest absorption peak. CFA was present to a varying extent in Region A (tubes 35 to 42, Kd=0.8–1.1), and the highest CFA peak was tube 38. It was also recognized that the fractions having higher CFA were not merely more effective in decomposing nitrite but also higher in levels of RA and SH groups. Fig. 2 shows the distribution patterns of ninhydrin-positive substances and carbohydrates in the low-molecular one as separated on Sephadex G-50, which corresponded on the whole to that of CFA curve.

Table 1. Effects of whole and fractionated sarcoplasm fractions of porcine skeletal muscle on the color formation and the decomposition of nitrite in the rapid emulsion curing process.

Sample	CFA		Nitrite decomposition after cooking[a]	
	Found	Index number[b]	ppm	Index number[c]
Whole sarcoplasm fraction	0.360	100	9.0	100
High-molecular weight fraction	0.143	40	4.8	53
Low-molecular weight fraction	0.223	62	7.6	84

a) Cooked for one hour at 75°C immediately after 25 ppm of $NaNO_2$ had been added to each fraction.
b) Figures for index number were calculated on the basis of the absorbance value for whole sarcoplasm fraction as 100.
c) Figures for index number were calculated on the basis of the amount of nitrite decomposed in whole sarcoplasm fraction as 100.
Mean values from three experiments are denoted.

From the results obtained by Sephadex G-50 gel chromatography, it was clear that the fraction exhibiting greater effect on the color formation in the low-molecular one had not only the more effective ability in decomposing nitrite, but also a large amount of reducing substances, ninhydrin-positive substances and carbohydrates.

3. Ultraviolet absorption spectrum of tube 40

Since tube 40 obtained by Sephadex G-50 gel chromatography of the low-molecular weight sarcoplasm fraction was found to have high CFA as well as the highest absorbance at 248 nm (Fig. 1), the ultraviolet spectral characteristics of tube 40 were investigated in order to identify the substances having strong ultraviolet absorption in connection with CFA. At first, the ultraviolet absorption spectrum of this fraction was meas-

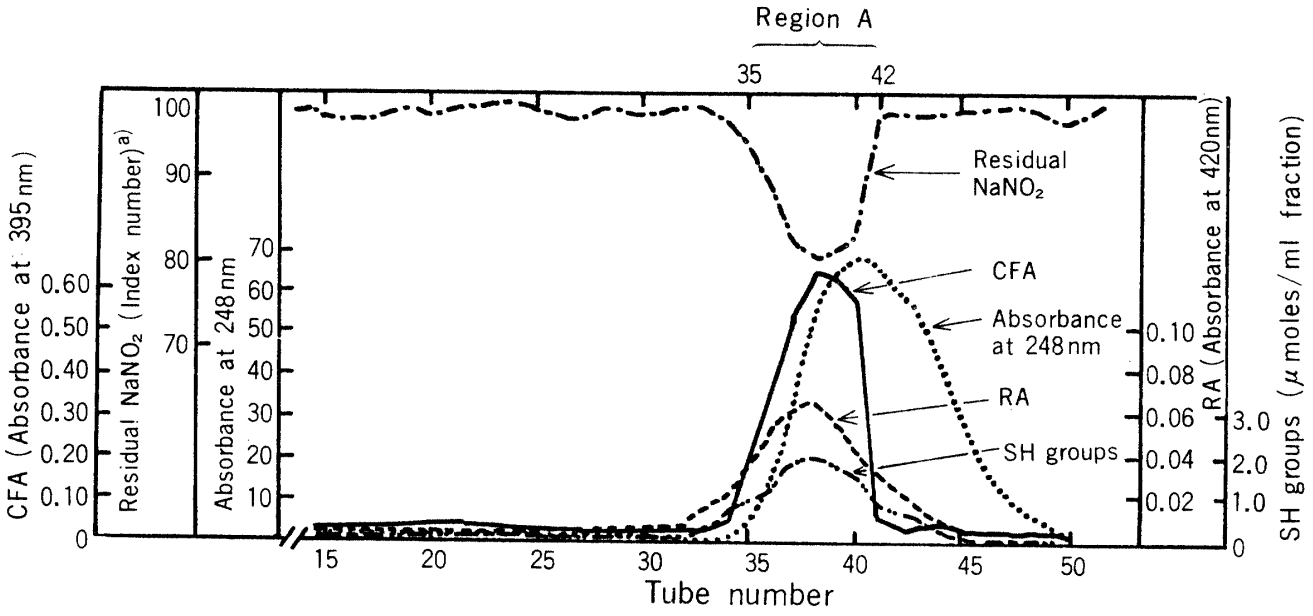

Fig. 1. Distribution patterns of absorbance at 248 nm, CFA, residual $NaNO_2$, RA and SH groups in the low-molecular weight fraction of sarcoplasm from porcine skeletal muscle on Sephadex G-50.
a) Figures for index number were calculated on the basis of 25 ppm of $NaNO_2$ added as 100.

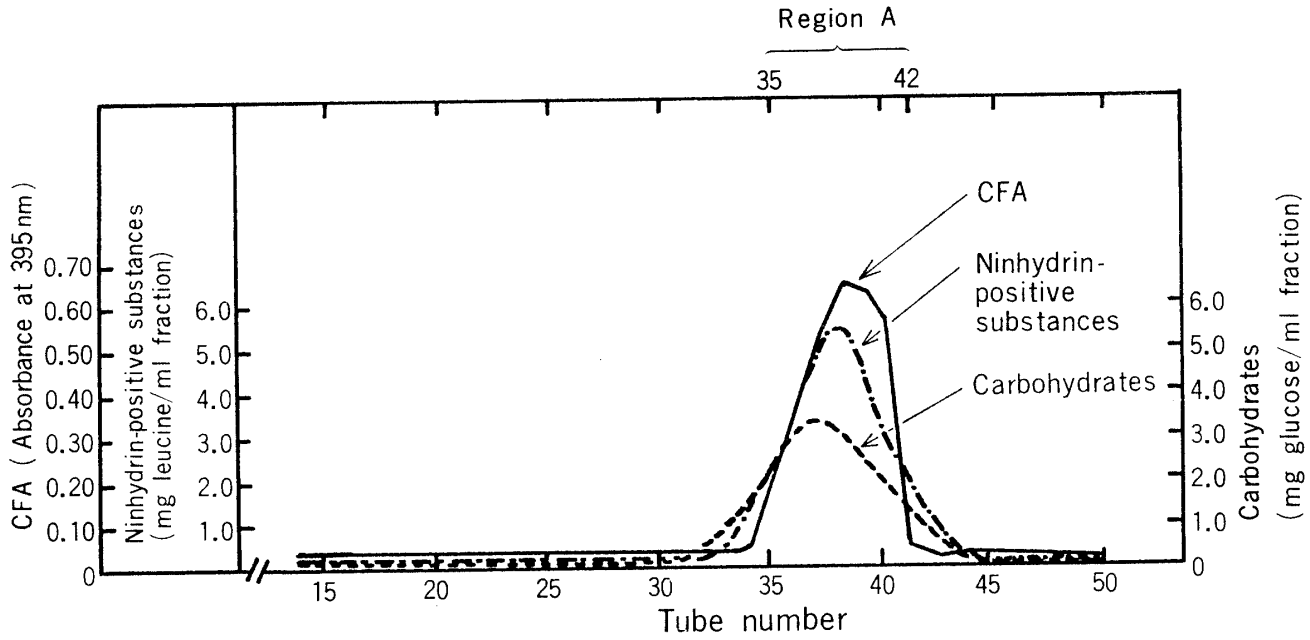

Fig. 2. Distribution patterns of CFA, ninhydrin-positive substances and carbohydrates in the low-molecular weight fraction of sarcoplasm from porcine skeletal muscle on Sephadex G-50.

ured after being diluted 100-fold with 0.1 M NaCl-5 mM histidine buffer of pH 7.6. Tube 40 was indicated to have an absorption maximum at 248 nm. This suggested the existence of such components as inosine and some nucleotides, *i.e.*, IMP, IDP and ITP which had an absorption maximum at 248 nm.[105] The ultraviolet absorption spectrum of 0.05 mM inosine preparation (Ishizu) in the same NaCl-histidine buffer was examined next and the spectrum was recognized to be in complete agreement with that of tube 40. The absorption maximum and the absorption ratio (E 280/E260) of tube 40 were also compared with those of inosine in pH 2.0, 7.0 and 12.0 solutions. Tube 40 showed approximately the same values as the inosine preparation.[105,106] However, E280/E260 ratio of tube 40 was slightly lower at pH 2.0 and 7.0 than those of inosine solution. These spectral differences were assumed likely to be caused by the presence of other components in tube 40.

Wasserman and Gray[80] and Zaika *et al.*[86] have proved the presence of inosine and inosinic acid in the water-soluble fraction of bovine muscle from their studies on the meat aroma precursors.

The present study indicates that a region can be separated from the low-molecular weight sarcoplasm fraction by Sephadex G-50 and the main peak fraction of Region A contains not only reducing substances, ninhydrin-positive substances and carbohydrates, but also probably a considerable amount of inosine and/or its derivatives from its spectral characteristics. However, it should be

Table 2. Ultraviolet absorption spectral data of main peak tube obtained by Sephadex G-50 gel chromatography of the low-molecular weight fraction from sarcoplasm of porcine skeletal muscle and inosine preparation.

Sample	λmax (nm)			E280/E260		
	pH 2.0	pH 7.0	pH 12.0	pH 2.0	pH 7.0	pH 12.0
Main peak tube (tube 40)	248	248	253	0.21	0.22	0.17
Inosine	248	248	253	0.26	0.25	0.17

taken into consideration that many other substances are also contained in the region and any other substances may be involved in the color formation.

The effects of inosine and its derivatives on the CFA must be investigated. Further works are also necessary to fractionate Region A more in detail using effective techniques and to identify the components active in promoting the color formation.

SUMMARY

Sarcoplasm prepared from porcine skeletal muscle was first separated into two fractions, low- and high-molecular weight fractions, by dialysis using Visking tubing. The low-molecular weight fraction was evidently found to be more efficacious in promoting the color formation and the decomposition of nitrite than the high-molecular weight fraction. The low-molecular weight fraction was further separated into fifty tubes of 5 ml each by Sephadex G-50 gel chromatography, and a main peak exhibiting the most favorable effect on the color formation was obtained in Region A (tubes 35 to 42, Kd=0.8-1.1). Region A was also found to be effective in decomposing nitrite and was rich in reducing substances, ninhydrin-positive substances and carbohydrates. From the ultraviolet absorption spectral characteristics, it was shown that the main peak tube 40 (Kd=1.0) having a maximum absorbance value at 248 nm contained a considerable amount of inosine and/or its derivatives.

CHAPTER III

LOW-MOLECULAR WEIGHT COMPONENTS IN SARCOPLASM PROMOTING THE COLOR FORMATION

INTRODUCTION

It is well known that the low-molecular weight sarcoplasm fraction contains numerous non-protein nitrogenous compounds, carbohydrates, non-nitrogenous compounds and inorganic constituents dissolved in water. Much attention has been devoted to the chemical constituents of this fraction since the precursors of the characteristic flavor and odor compounds in meat are largely water soluble.[74-77] These works were reviewed in some detail in Chapter I. So many individual components are present in the low-molecular weight, water-soluble fraction, consequently, it has not been possible to identify the compounds most directly associated with aroma development. Moreover, there is a general lack of agreement on the exact constituents present in this fraction.

It has been found that some of the endogenous components such as coenzymes, amino acids, SH compounds, ascorbic acid and metal ions are involved in the color formation of cured meat products. These findings were also discussed in some detail in Chapter I.

In Chapter II, the sarcoplasm from the porcine skeletal muscle was separated into the two fractions, low- and high-molecular weight fractions, by a dialysis procedure and it was observed that the low-molecular one possessed higher abilities in promoting the color formation of cooked cured meat products and in decomposing nitrite than those of the high-molecular one. It was also demonstrated that the Region A separated from the low-molecular one by Sephadex G-50 gel chromatography apparently exhibited the promoting effect for the color formation and the decomposition of nitrite. It was also found that Region A contained a large amount of reducing substances, ninhydrin-positive substances and carbohydrates.

The objective of this study was to make clear the possible endogenous components in low-

molecular weight sarcoplasm fraction which promote the color formation. Region A was further separated by gel chromatography. Furthermore, thin-layer chromatography was designated to identify the components which promote the color formation. The identified components were tested for their promoting effect on the color formation with both aqueous model and meat systems.

MATERIALS & METHODS

1. Preparation and fractionation of the low-molecular weight sarcoplasm fraction

The low-molecular weight fraction of sarcoplasm was prepared from porcine skeletal muscle (*M. adductor*, 3 hr postmortem) and was separated by Sephadex G-50 gel chromatography according to the method given in Chapter II (see page 377). The tubes from 35 up to 42 (Kd=0.8-1.1) were combined together into one fraction and used as Region A. Since our preliminary experiment has indicated that considerably good separation of Region A could be achieved by Sephadex G-15 chromatography, Region A was further fractionated with Sephadex G-15 (Pharmacia) in the following way : Region A (total volume: 55 ml) was concentrated to 15 ml in a rotary evaporator under the same conditions as in the sarcoplasm preparation (see page 377), and the concentrated preparation (15 ml) was applied to a 2.5 ×40 cm column of Sephadex G-15 and eluted with 0.1 M NaCl-5 mM histidine buffer (pH 7.6) at a flow rate of 0.5 ml/min and at 4°C. Five ml fractions were collected. The ultraviolet absorbance of the fractions was measured at 248 nm. Each fraction was analyzed for RA, SH groups, ninhydrin-positive substances, carbohydrates and Fe^{2+}. CFA and nitrite decomposition were also measured by the following methods.

2. Determination of CFA and nitrite

Each fraction was adjusted to pH 5.5 with 0.1 N HCl or NaOH solution and was made up to 10 ml with M/35 veronal buffer, pH 5.5. To each buffered fraction, porcine heart Mb and sodium nitrite were added to give levels of 0.125% Mb and 25 ppm $NaNO_2$. The reaction mixture was then cooked at 75°C for 1 hr in a Thunberg tube at a vacuum of 5 mmHg. The formed cooked cured meat pigments and the residual nitrite were determined according to the procedures given in Chapter II (see page 377-378). Absorbance of the 75% acetone extract containing NO-hemochrome was measured at 395 nm and the absorbance value was expressed as CFA.

3. Determination of RA, SH groups, ninhydrin-positive substances, carbohydrates and Fe^{2+}

RA, SH groups, ninhydrin-positive substances and carbohydrates in the fractions were analyzed according to the procedures described in Chapter II (see page 378). Fe^{2+} was determined by the *o*-phenanthroline method[107] using the NH_2OH reagent for reduction.

4. Thin-layer chromatography

Identification of fraction components was carried out by thin-layer chromatography. Ninhydrin-positive substances were examined by MOFFAT and LYTLE's method[108] using Silica Gel G (Merck) plates, A ; ethylalcohol : water (3 : 1 v/v) and B ; n-butanol : acetic acid : water (3 : 1 : 1 v/v) as developing solvents and ninhydrin-$Cu(NO_3)_2$ reagent for detection. Carbohydrates were chromatographed by STAHL and KALTENBACH's method[109] using Silica Gel G (Merck) plates, ethylacetate : isopropanol (65%) (65 : 35 v/v) as the developing solvent, and anisaldehyde sulfuric acid reagent for detection. Nucleotides and nucleosides were separated according to POTTHAST and HAMM's method[110] on Silica Gel HF_{254} (Merck) plates with ethyleneglycol-monoethylether : ethyleneglycol-monobutylether : dioxane : ammonia : water (15 : 50 : 70 : 35 : 80 v/v) as the developing solvent and were detected as dark, absorbing spots under ultraviolet light at 254 nm.

5. Ultraviolet absorption spectrum

All spectral data were obtained using a Hitachi Model 124 Spectrophotometer equipped with a Hitachi Model QPD_{73} Recorder.

6. Aqueous model system

Each aqueous model system initially contained Mb and $NaNO_2$ in M/35 veronal buffer of pH 5.5. The reaction mixture was then cooked at 75°C for 1 hr in a Thunberg tube at a vacuum of 5 mmHg. Further details appear in the Table

legends.

7. Meat system

Pork sausage was used as a meat system. Each sample initially contained 2% NaCl, 10 ppm $NaNO_2$ and the designated amount of each test compound (see Table 4). The pH was adjusted to 5.5 with 1N HCl or NaOH. Water was added to each sample at a rate of 10% by weight of meat. Just after curing, samples were cooked at 75°C for 1 hr. Further details appear in the Table legends.

RESULTS & DISCUSSION

1. Distribution patterns of CFA and absorbance at 248 nm in Region A rechromatographed on Sephadex G-15

Region A was rechromatographed by Sephadex G-15 gel filtration. As shown in the distribution patterns of the absorbance at 248 nm, Region A was separated into five peaks (Fig. 3). The fractions from 15 up to 39 were found to be active in promoting the color formation and two peaks in CFA were observed at the tubes 23 and 28. Tube 23 was the highest CFA peak and was followed by tube 28. Consequently, the tubes 15 to 39 were divided into two regions, Region B (tubes 15 to 26, Kd=0.04-0.48) and Region C (tubes 27 to 39, Kd=0.52-0.99). Region B exhibited higher CFA in spite of its lower ultraviolet absorption than that of Region C.

2. Distribution patterns of residual nitrite, SH groups and RA in Region A rechromatographed on Sephadex G-15

It was recognized that the fractions having higher CFA were not merely more effective in decomposing nitrite but also higher in levels of RA and SH groups. This tendency was especially clear in Region B (Fig. 4). From these results, it was shown that Regions B and C contained a considerably larger amount of reducing substances and SH groups. TINBERGEN[87] observed that a component which caused strongly the reduction of nitrite to NO was contained in the water-soluble low-molecular weight fraction extracted from bovine skeletal muscle. From the experimental results due to the ion-exchange chromatography and ultrafiltration techniques, he proposed that the component involved in NOMb formation was either an amino acid or a lower peptide, probably with a SH group, having a molecular weight below 500. This suggests that reducing substances and SH groups detected in Regions B and C contributed to promoting the color formation in the two regions.

3. Distribution patterns of ninhydrin-positive substances, carbohydrates and Fe^{2+} in Region A rechromatographed on Sephadex G-15

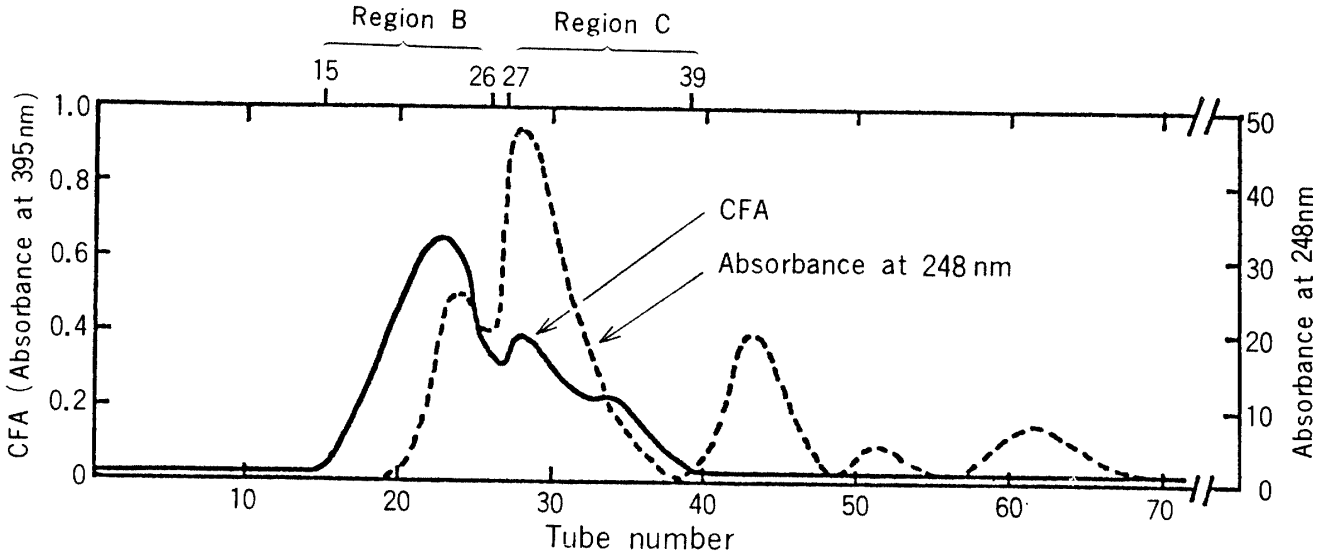

Fig. 3. Distribution patterns of CFA and absorbance at 248 nm in Region A rechromatographed on Sephadex G-15.

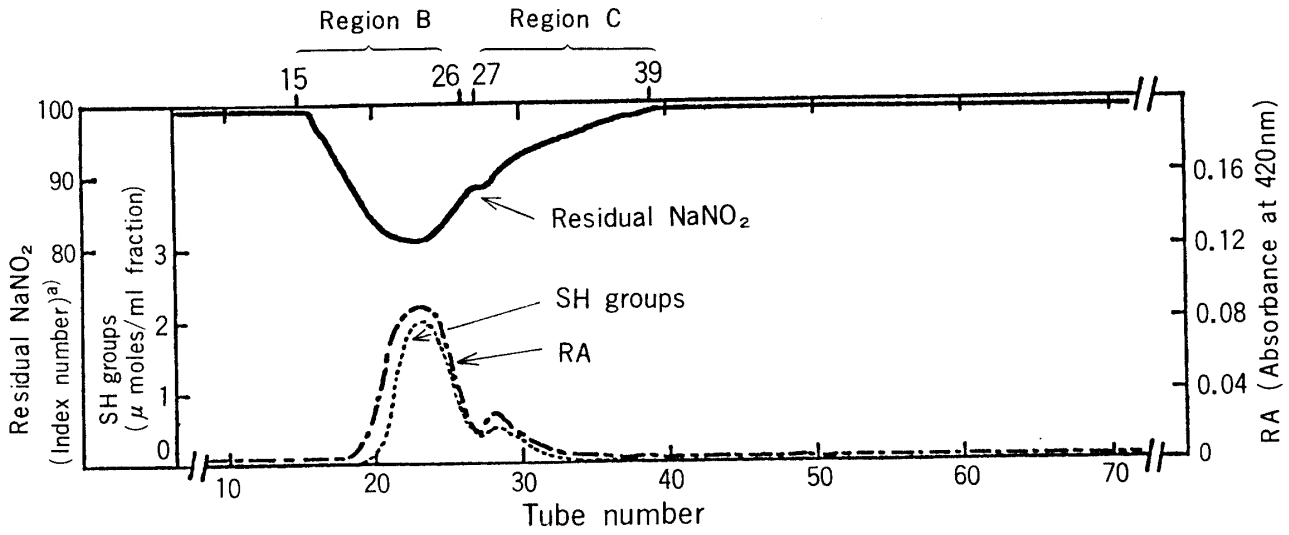

Fig. 4. Distribution patterns of residual $NaNO_2$, SH groups and RA in Region A rechromatographed on Sephadex G-15.
a) Figures for index number were calculated on the basis of 25 ppm of $NaNO_2$ added as 100.

Ninhydrin-positive substances and carbohydrates were contained in both Regions B and C. Region C included more carbohydrates than Region B (Fig. 5). Tube 28, the CFA peak of Region C, contained the largest amount of carbodydrates, and thus it was assumed that some of these carbohydrates may have responsibility for the color formation in Region C. Fe^{2+} was detected only in Region B (Fig. 5). Fe^{2+} is known to promote not only MetMb reduction[60] but also the nitrite decomposition[63] and the color formation.[56,57,69] Hamm and Bünnig [111-113] found that a fairly large amount of nonheme-iron was present in porcine and bovine muscles and suggested that the nonheme-iron may be bound to other components in muscle tissue. Olsman and Krol[55] also showed that endogenous irons exist in some coordination complexes. From the above observations, it was suggested that a component seemed to be bound to Fe^{2+} is present in Region B and this component plays a positive role in the color formation.

Tinbergen[87] confirmed that the addition of water-soluble low-molecular weight fraction from bovine muscle to a meat product caused a significant increase in the reaction rate of the

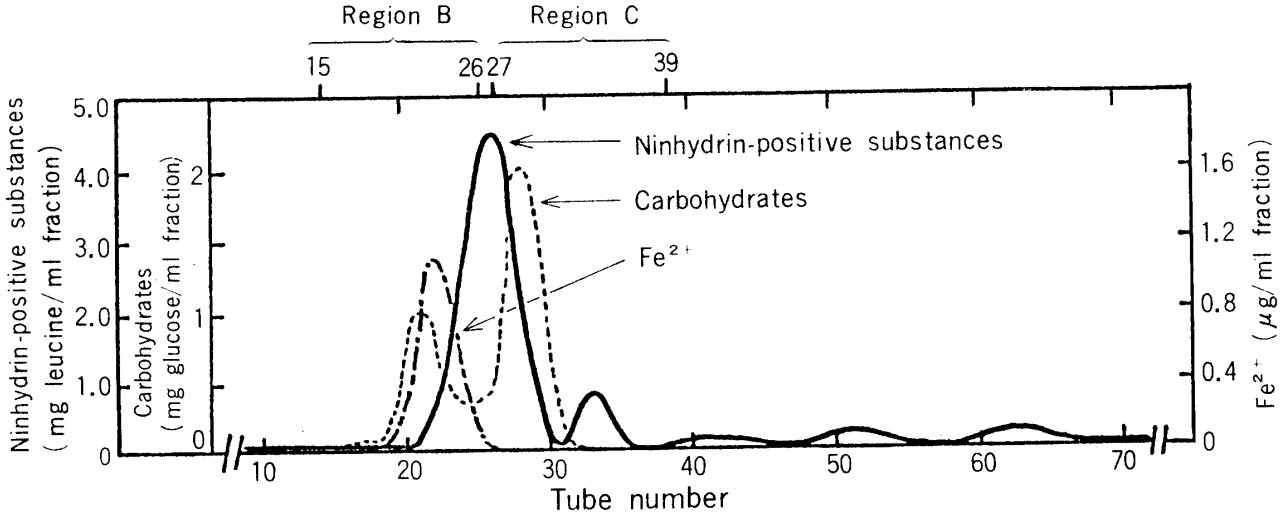

Fig. 5. Distribution patterns of ninhydrin-positive substances, carbohydrates and Fe^{2+} in Region A rechromatographed on Sephadex G-15.

nitrite depletion. We also investigated the additional effects of Regions B and C on the color formation with a cooked cured sausage. Each of the applied regions was prepared from an equal amount of porcine skeletal muscle used for the experimental meat product, which initially contained 2% NaCl and 10 ppm $NaNO_2$ with and without addition of Regions B or C. It was confirmed that the addition of Regions B and C to meat product resulted in a significant increase both in CFA and in the nitrite decomposition. The increase in CFA was 25% and 20% for Regions B and C, respectively, and the nitrite decomposition was the same increase of 10% in the both regions.

From the results, it was recognized that active components promoting the color formation and the decomposition of nitrite were present in Regions B and C.

4. Thin-layer chromatography

Ninhydrin-positive substances in the tubes 23 and 28, which were in the CFA peak of Regions B and C, were subjected to thin-layer chromatography on plates of Silica Gel G. Results are shown in Fig. 6. Authentic amino acids (Wako) and GSH (Sigma) were used as the standards. Tube 23 was found to contain GSH, histidine, glutamic acid, alanine and a substance which appeared to be GSSG, while tube 28 contained histidine, glutamic acid, alanine, glycine, valine and tyrosine. Several workers[114,115] reported that basic amino acids, lysine and arginine, promoted the color formation of cooked cured meat products, although the mechanism remained uncertain. In the present experiment, spots corresponding to the basic amino acids were not observed for Regions B and C. Cysteine[25,44,56-58] and GSH[44,58] are well known to promote the color formation. As shown in Fig. 6, cysteine was not detected in either region. The presence of cysteine in these fractions also could not be confirmed by the reaction with 2,6-dichloro-p-benzoquinone which is a specific colorimetric reagent for cysteine. GSH was detected only in Region B.

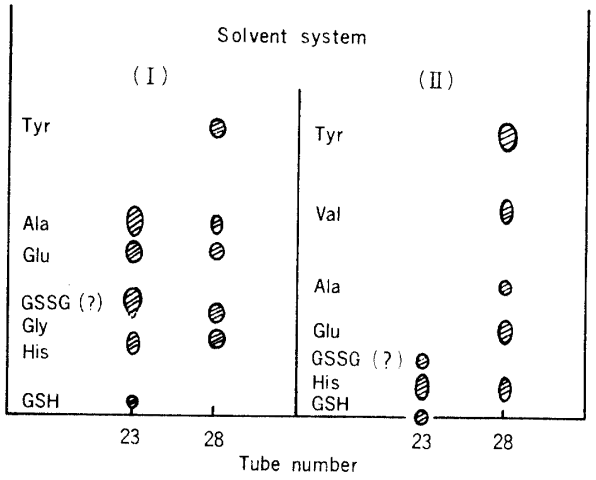

Fig. 6. Thin-layer chromatograms of ninhydrin-positive substances in the CFA peak tubes 23 and 28 of Regions B and C.
Thin-layer : Silica Gel G (Merck)
Solvent system :
(I) Ethylalcohol : water (3 : 1 v/v)
(II) n-Butanol : acetic acid : water (3 : 1 : 1 v/v)
Development distance : (I) 17 cm in 7 hr
(II) 17 cm in 8 hr
Detection : Ninhydrin-$Cu(NO_3)_2$ reagent

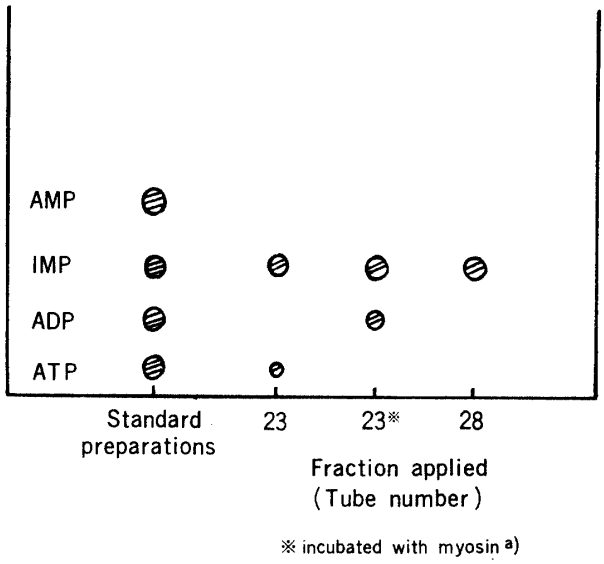

Fig. 7. Thin-layer chromatograms of nucleotides and nucleosides in the CFA peak tubes 23 and 28 of Regions B and C.
Thin-layer : Silica Gel HF_{254} (Merck)
Solvent : Ethyleneglycol-monoethylether : ethyleneglycol-monobutylether : dioxane : ammonia : water (15 : 50 : 70 : 35 : 80 v/v)
Development distance : 17 cm
Detection : Ultraviolet lamp
a) Incubation conditions : 1 : 1 diluted tube 23, 1.245 mg/ml myosin (prepared from porcine skeletal muscle), 5mM $CaCl_2$, 20 mM Tris-acetate, pH 7.0 ; 25°C, 60 min.

Carbohydrates in tubes 23 and 28 were separated by thin-layer chromatography and identified. Ribose was detected in tube 28 but not in tube 23 (data not shown).

Nucleotides and nucleosides in both fractions were separated by thin-layer chromatography. The results are shown in Fig. 7. In tube 23 are contained ATP and IMP, and in tube 28 only IMP. In order to ascertain the presence of ATP, tube 23 was incubated with myosin prepared from fresh porcine skeletal muscle under the conditions similar to those used in measurement of Ca^{2+}-activated ATPase activity.[144] As can be seen in Fig. 8, the amount of P*i* liberated in the reaction mixture increased with incubation time although the absolute quantity of P*i* detected was relatively small. This might be due to inhibition of the enzyme reaction caused by a considerably large amount of P present in the tube. After incubating this fraction with myosin at 25°C for 1 hr, aliquots of the reaction mixture was again applied onto a thin-layer plate. The result showed that the ATP spot disappeared and a new spot of corresponding to ADP appeared instead (Fig. 7). Thus, ATP was confirmed to be present in tube 23 of Region B.

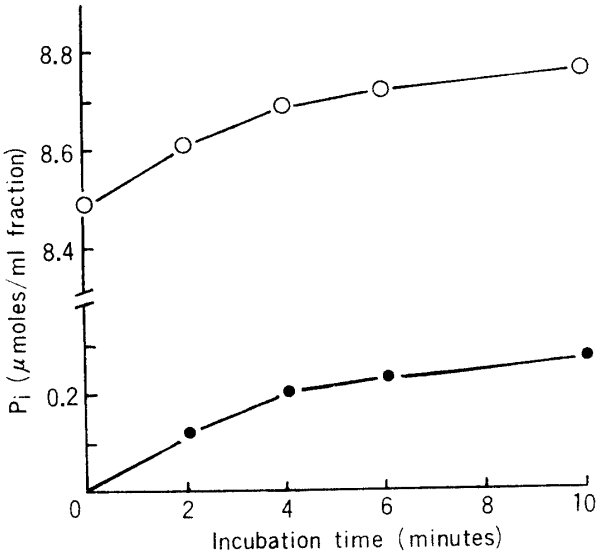

Fig. 8. Increase of P*i* liberated from the CFA peak tube 23 of Region B during incubation with myosin.
Myosin : Prepared from porcine skeletal muscle
Conditions : 1 : 4 diluted tube 23, 0.62 mg/ml myosin, 5 mM $CaCl_2$, 20 mM Tris-acetate buffer, pH 7.0 ; 25°C.
○——○ : P*i* found in the reaction mixture,
●——● : P*i* liberated during incubation. (net increase)

5. Ultraviolet absorption spectral characterization

In order to ascertain the endogenous components in tube 23, which was in the CFA peak of Region B, detected by thin-layer chromatography, ultraviolet absorption spectral characteristics were investigated. Fig. 9 shows the ultraviolet absorption spectrum of tube 23. A large absorption peak at 232 nm and small one at 252 nm were observed as shown in Fig. 9. It seemed reasonable to assume that the one component having an absorption maximum at 252 nm is IMP [pH 6.0-λmax 245.8 nm[105]] on the basis of the thin-layer chromatogram of Region B (Fig. 7).

GSH is known to have an absorption maximum at 232 nm.[116] Therefore, an absorption maximum observed at 232 nm may be attributable to GSH in the tube. To ascertain the existence of GSH in tube 23, ultraviolet absorption spectra of this fraction and GSH (Sigma) were examined in the pH range from 5.0 to 7.0. A GSH solution in the presence or absence of IMP was first subjected to the experiment. Fig. 10 shows the ultraviolet absorption spectrum of GSH solution. The wavelength of the great absorption maximum of GSH (232 nm) was not shifted by the pH, but the

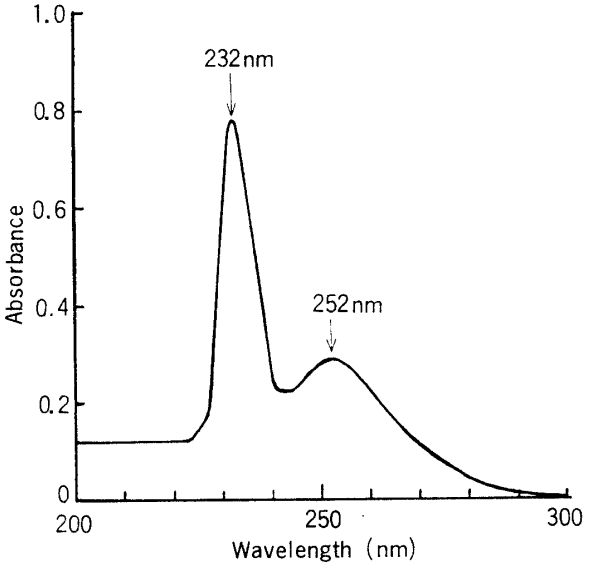

Fig. 9. Ultraviolet absorption spectrum of the CFA peak tube 23 of Region B.
(This fraction was diluted 50-fold with 0.1 M NaCl-5 mM histidine buffer of pH 7.6.)

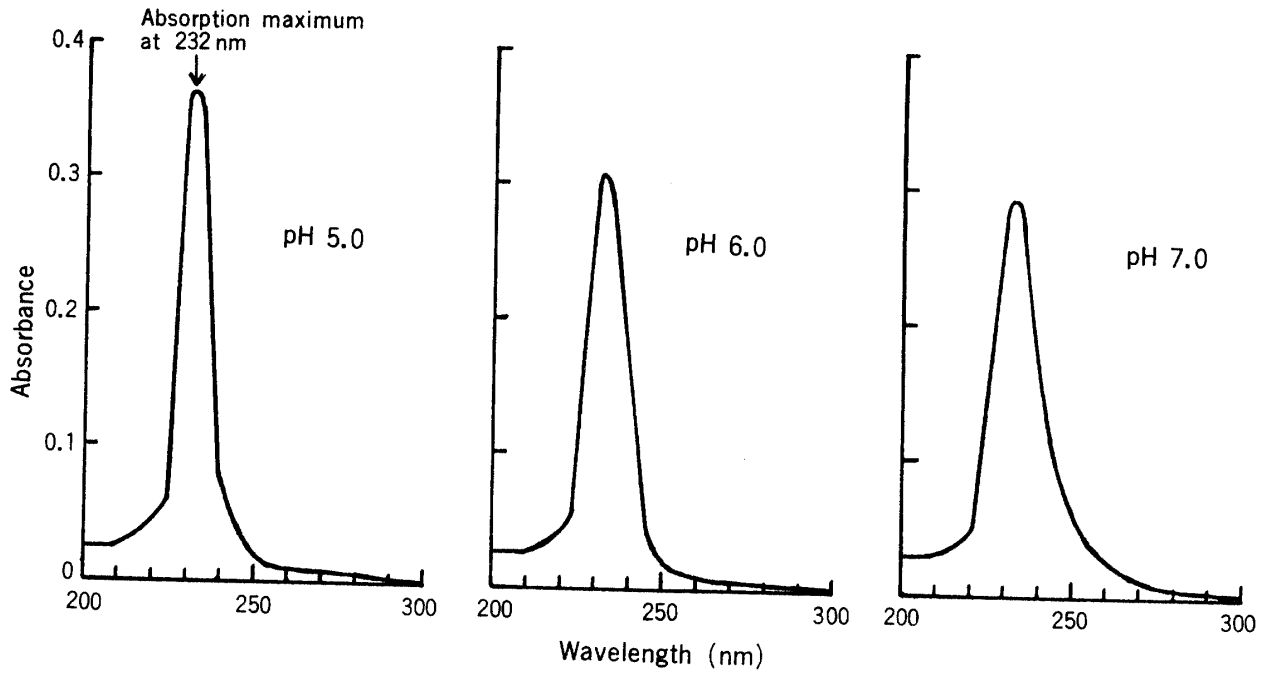

Fig. 10. Effect of pH on the ultraviolet absorption spectrum of GSH.
(At the concentration of 1 mM in 0.1 M NaCl-5 mM histidine buffer of the designated pH)

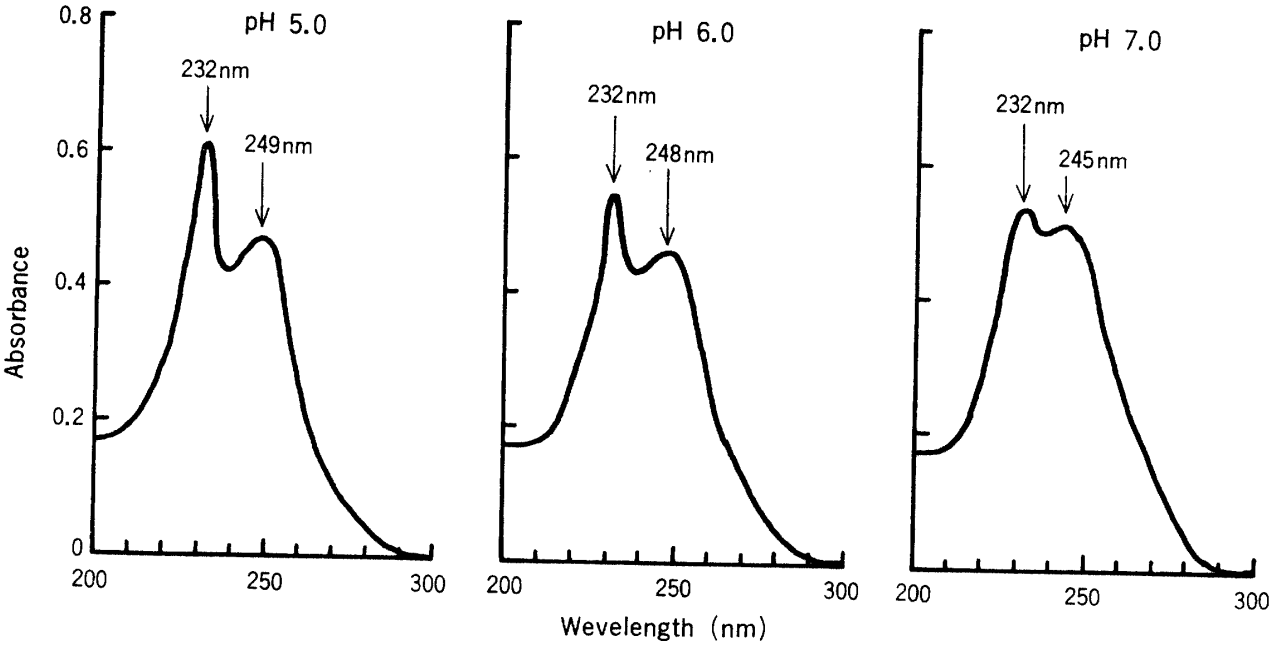

Fig. 11. Effect of pH on the ultraviolet absorption spectrum of GSH plus IMP.
(Each sample solution contained 0.2 mM GSH and 0.05 mM IMP in 0.1 M NaCl-5 mM histidine buffer of the designated pH.)

absorbance value was higher at pH 5.0 than at pH 6.0 and 7.0. The ultraviolet absorption spectra of GSH coexisted with IMP are shown in Fig. 11. The ultraviolet absorption spectra obtained were found to be similar to those of tube 23 in Region B (Fig. 9). Effect of pH on the ultraviolet absorption spectrum of tube 23 was examined and the results are shown in Fig. 12. The absorbance at 232 nm of this fraction increased with a lowering of the pH. This tendency essen-

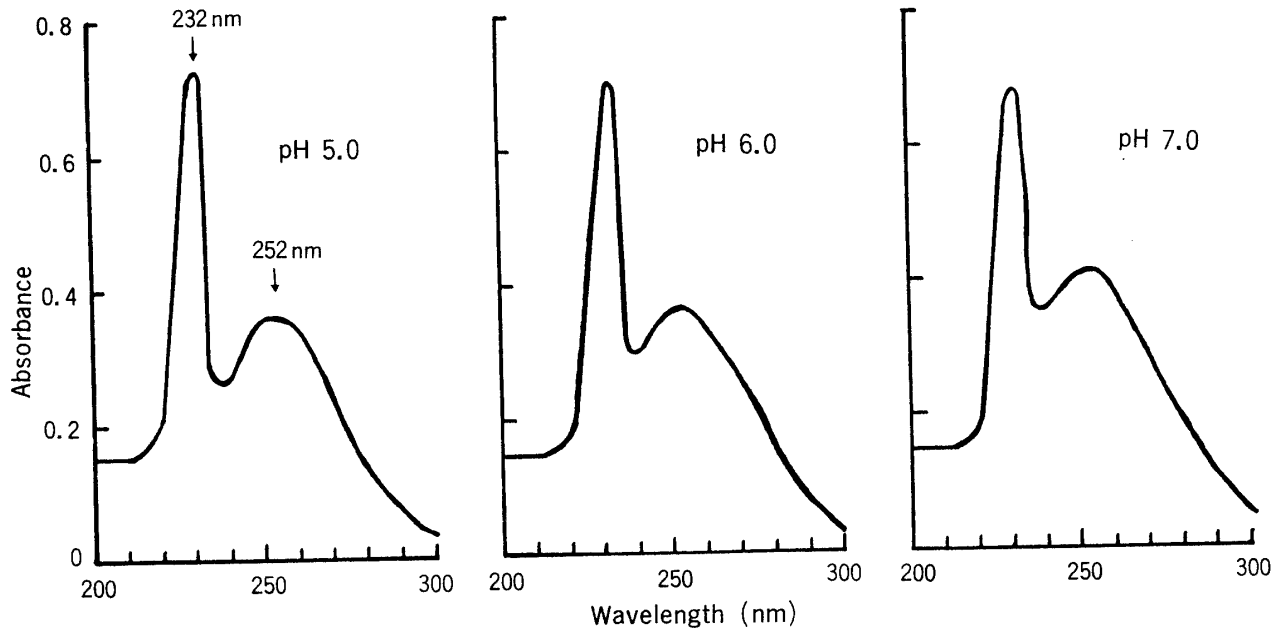

Fig. 12. Effect of pH on the ultraviolet absorption spectrum of the CFA peak tube 23 of Region B. (The fraction was diluted 100-fold with 0.1 M NaCl-5 mM histidine buffer of the designated pH.)

tially agrees with the concept of GSH coexisting with IMP (Fig. 11). Tube 23 was suggested to contain GSH and IMP by the thin-layer chromatography (Figs. 6 and 7). These results support the view that both GSH and IMP are contained in tube 23.

From the above investigations it was found that Region B contained GSH, IMP, ATP, alanine, glutamic acid, histidine and Fe^{2+}, while Region C contained IMP, alanine, glutamic acid, glycine, histidine, tyrosine, valine and ribose.

6. Contribution of the endogenous components to the color formation of meat products

Effects of the endogenous components detected in Regions B and C on the color formation and the decomposition of nitrite were examined with the following two systems. Of the identified endogenous components, GSH, ATP, IMP and ribose were selected for the analysis. The amount of each component tested was based on the data reported in the literature.[117-119]

1) Aqueous model system

Table 3 shows the effect of addition of GSH (Sigma), ATP (Sigma), IMP (Sigma) and ribose (Wako) in an aqueous model system. GSH greatly enhanced the color formation and the decomposition of nitrite. Ribose and ATP were fairly active in promoting the color formation and in

Table 3. Effects of GSH, ATP, IMP and ribose on the color formation and the decomposition of nitrite.
—Aqueous system[a] —

Compound added[b]	CFA	Nitrite decomposition[c] (%)
Control (Mb+$NaNO_2$)	0.031	1.2
+GSH	0.578	44.8
+ATP	0.043	5.2
+ATP+GSH	0.645	44.8
+IMP	0.026	2.4
+IMP+GSH	0.602	26.8
+Ribose	0.161	6.8
+Ribose+GSH	0.708	46.0

a) Each model system initially contained 0.125% Mb, 25 ppm $NaNO_2$ and the designated amount of each compound in M/35 veronal buffer, pH 5.5. The pH was corrected to 5.5 with 1 N HCl or NaOH. Just after preparation, each sample was cooked at 75°C for 1 hr.

b) Final concentrations added : 5 mM GSH ; 1 mM ATP ; 10 mM IMP ; 5 mM ribose.

c) "Nitrite decomposition" refers to the percentage of the decomposed nitrite to the added nitrite. Mean values from three experiments are denoted.

decomposing nitrite, but the corresponding values for IMP were almost the same as those of the control in this experimental conditions. In the presence of GSH, ATP and ribose enhanced the color formation significantly, but not the decomposition of nitrite. IMP did not exhibit any promoting effect but rather lowered the rate of nitrite decomposition in the presence of GSH. Quite recently, MIWA *et al.*[120] reported that IMP reacted with nitrite and ascorbate to produce notable amounts of an unidentified nitrogen compound. Contrary to their findings, in the aqueous model system IMP was found to inhibit greatly the decomposition of nitrite in the presence of GSH. The reason for this apparent contradiction is not clear.

2) Meat system

Table 4 shows the additional effects of GSH, ATP, IMP and ribose obtained with a meat system. Pork sausage was used as the meat system. In order to detect the effects more clearly, each compound used in this experiment was examined at the concentration ten times higher than that used in the aqueous model system. In spite of the addition of larger amounts of GSH, the promoting effect of this compound on the color formation and the decomposition of nitrite was not greater in the meat system than in the aqueous model system.

MÖHLER noted in his review[36] reducing agents such as thioglycollic acid and thioacetamide enhance the formation of cured pigment when added to meat products ; whereas GSH or cysteine exhibit no effect at the natural pH (6.0) in meat though the reason for this discrepancy is unknown. However, the present study could recognize the positive effect of GSH. IMP differed from the result obtained with the aqueous model system, that is, IMP enhanced CFA appreciably in the meat system. Each additive tended to enhance both the effects. Above all, ribose promoted the color formation remarkably.

Table 4. Effects of GSH, ATP, IMP and ribose on the color formation and the decomposition of nitrite.

—Meat system[a] —

Compound added[b]	CFA		Nitrite decomposition[d] (%)
	Found	Index number[c]	
Control (NaCl+$NaNO_2$)	0.249	100	50.3
+GSH	0.309	124	54.9
+ATP	0.273	110	51.3
+ATP+GSH	0.331	133	57.7
+IMP	0.310	124	51.8
+IMP+GSH	0.343	138	57.7
+Ribose	0.325	131	53.8
+Ribose+GSH	0.346	139	57.7
+GSH+ATP+IMP+ Ribose	0.371	149	59.3

a) Each sample initially contained 2% NaCl, 10 ppm $NaNO_2$ and the designated amount of each compound. The pH was adjusted to 5.5 with 1 N HCl or NaOH. Water was added to each sample at a rate of 10% by weight of meat. Just after curing, samples were cooked at 75 °C for 1 hr.

b) Final concentrations added : 50 mM GSH ; 10 mM ATP ; 100 mM IMP ; 50 mM ribose.

c) Figures for index number were calculated on the basis of the CFA for control sample as 100.

d) "Nitrite decomposition" refers to the percentage of the decomposed nitrite to the added nitrite.
Mean values from three experiments are denoted.

Sugar is one of the common additives employed in the curing mixture and their main functions are flavoring and reduction of the harshness of the salt. Extensive experimentation by LEWIS and VOSE[121] indicated that the sugars, particulary glucose, produced a desired initial color in hams. The effect of sugar as an aid to the full color formation has been explained on the fact of lowering the pH of meat by the organic acids formed from the sugar by its bacterial fermentation in meat, and besides from its antioxidative action and reducing ability to the heme pigments.[99] NAGATA and ANDO[64] also found that sucrose and glucose promoted the decomposition of nitrite in the presence of ascorbate. On the other hand, TEN CATE[122] observed that in repeatedly reused brines an augmentation of dissolved sugars in meat took place, but he noted their role as a hydrogen donator during the denitrification was quite doubtful. MACY *et al.*[117] carried out the qualitative analysis of water-soluble carbohydrates in bovine and porcine muscle. He found

that ribose was the carbohydrate most labile to heating and discussed the importance of this component in the browning of meat as a flavor precursor. In contrast to other studies, TARR[123] indicated that only ribose was involved in Maillard reactions of fish muscle. Whether the promoting effect of ribose on the color formation is caused by its own ability or through the action of an "intermediate" such as reductones which might be formed by the reaction of ribose with amino acids in meats remains to be shown.

In the presence of GSH, all of the components used in this experiment clearly promoted the color formation and the decomposition of nitrite. Therefore, the color formation in the rapid emulsion curing process must be enhanced at least by the combined action of these components. Amino acids detected in Regions B and C, and Fe^{2+} found in Region B also may be correlated to the color formation.

More detailed investigations are necessary to clarify the exact mechanism of the promoting effects of the endogenous low-molecular components in sarcoplasm on the color formation of cooked cured meat products in the rapid emulsion curing process.

SUMMARY

Region A separated from the low-molecular weight sarcoplasm fraction was further fractionated by Sephadex G-15 gel chromatography. Region B (tubes 15 to 26, Kd=0.04-0.48) and Region C (tubes 27 to 39, Kd=0.52-0.99) were found to be active in promoting the color formation and the decomposition of nitrite. Region B exhibited CFA to high extent in spite of its lower ultraviolet absorption than that of Region C. Regions B and C contained a considerably large amount of reducing substances, SH groups, ninhydrin-positive substances and carbohydrates. Fe^{2+} was detected only in Region B. Thin-layer chromatography indicated that Region B contained GSH, ATP, IMP, alanine, glutamic acid and histidine, while Region C contained IMP, alanine, glutamic acid, glycine, histidine, tyrosine, valine and ribose. The presence of GSH and IMP in Region B was confirmed by the ultraviolet absorption spectra. In an aqueous model system, GSH, ATP and ribose enhanced the CFA and the decomposition of nitrite. In a meat system, GSH, ATP, IMP and ribose enhanced the color formation and the decomposition of nitrite. It was ascertained that the combined action of the identified endogenous components, *i. e.*, GSH, ATP, IMP and ribose contribute to promote the color formation of meat products in the rapid emulsion curing process.

CHAPTER IV

HIGH-MOLECULAR WEIGHT COMPONENTS IN SARCOPLASM PROMOTING THE COLOR FORMATION

INTRODUCTION

The sarcoplasm of skeletal muscle contains numerous water-soluble proteins and a number of low-molecular weight constituents in addition to water. The water-soluble, high-molecular weight fraction of sarcoplasm consists of sarcoplasmic proteins such as the enzymes associated with glycolysis, Mb and F-protein.[72] The glycolytic enzymes normally constitute about 80% of the sarcoplasmic proteins.[71] Hb is also contained, because even with ideal slaughtering techniques a small amount of blood remains in the muscle.

The first investigation on the relation of muscle proteins to cured meat color has been carried out by WATTS *et al.*[47] They heated a model mixture containing egg white, Hb and sodium nitrite in phosphate buffer (pH 5.8), and for comparison, they added ascorbic acid to the mixture and iodoacetic acid to another sample of the mix-

ture before heating. The color formation was most intense in the mixture containing ascorbic acid and was least intense in the mixture with the SH reagent. They concluded that the reduction of MetMb and nitrite necessary for the color formation of cured meat products may be brought about chemically by SH groups released by heat denaturation of muscle protein rather than by reducing enzyme systems. Recently, HOFMANN and HAMM[48] pointed out that the reactivity of the SH groups in egg white is very different from the reactivity of the SH groups in meat protein. KUBBERØD *et al.*[54] investigated the reactivities of myosin with nitrite and suggested that the rate of the reaction was fairly low under usual cured meat processing conditions. MIRNA and HOFMANN[30] found that the addition of nitrite to minced bovine and porcine muscle in amounts nearly equimolar to the SH content caused a decrease of about 20 to 30% in both components during storage for 1-2 weeks at 2°C and pH 5.6-5.8. Since it has been proved that in meat to which no nitrite was added the SH groups were stable during 12 days of storage at 2-3°C, [53] the decrease of SH groups in the presence of nitrite might be due to a reaction of the SH groups with nitrite. This explanation was supported by the observation of OLSMAN and KROL[55] that a smaller loss of nitrite occurred when the SH groups in meat had been previously blocked by a SH reagent. MIRNA and HOFMANN[30] proposed a new NO-transfer mechanism for the color formation of meat products: They suggested that the role of meat proteins in the formation of the cured meat color perhaps consists of the production of a nitrosothiol from SH groups of meat proteins with added nitrite and of a transfer of the NO group from the nitrosothiol to Mb [see pathway-(5) in page 371]. Thus, the effect of muscle proteins on the reactivity of nitrite with Mb has been investigated from an aspect of the SH groups by many workers. These reactions are so complicated that the responsibility of muscle proteins for promoting the color formation has not yet been elucidated in complete detail.

It was shown in Chapter II, the high-molecular weight fraction separated from porcine muscle sarcoplasm by dialysis procedures possessed a fairly great ability in promoting the color formation, although its ability was somewhat lower than that of the low-molecular one.

The present study was carried out to identify an endogenous component promoting the color formation in the high-molecular weight fraction and to obtain some informations about the contribution of this component to the color formation in the rapid emulsion curing process.

MATERIALS & METHODS

1. High-molecular weight fraction of sarcoplasm

The high-molecular weight fraction of sarcoplasm was prepared from the porcine skeletal muscle (*M. adductor*, 3 hr postmortem) according to the procedures given in Chapter II (see page 377).

2. Sephadex G-100 gel chromatography

A 10 ml aliquot of the high-molecular weight fraction was applied to a 2.5 × 40 cm column of Sephadex G-100 (Superfine, Pharmacia), which had indicated good separation in preliminary experiments, and eluted with 0.1 M NaCl-5 mM histidine buffer (pH 7.6) at a flow rate of 10 ml/hr and at 4°C. Five ml fractions were collected. The ultraviolet absorbance of each fraction was measured at 280 nm. Each fraction (5 ml) was adjusted to pH 5.5 with 0.1 N HCl or NaOH solution and was made up to 10 ml with M/35 veronal buffer of pH 5.5. Each fraction was analyzed for RA, SH and SS groups. CFA and nitrite decomposition were also determined.

3. Sepharose 6B gel chromatography

The fraction of tube 16 (5 ml) was further fractionated by Sepharose 6B gel chromatography. For the gel chromatography, Sepharose 6B (Pharmacia) was found to be more suitable than Sephadex G-200 or Sepharose 4B. Except using a 2.6 × 35 cm column, the conditions of Sepharose 6B gel chromatography were the same as those of Sephadex G-100. The absorbance of each fraction was measured at 280 nm, and then analyzed for protein concentration by the biuret method[124] using the bovine serum albumin (Sigma) as the standard. CFA and enzyme activity were measured by the following methods.

4. Determination of CFA, nitrite, RA and free SH groups

CFA and nitrite were determined according to the procedures given in Chapter II (see page 377). When heme pigments were contained in the fraction, the content was determined by HORNSEY's method.[98] Thus, the corrected amount of Mb was added to the fraction by subtracting the heme pigment content prior to the determination of CFA. RA and free SH groups were measured according to the procedures given in Chapter II (see page 378) both before and after cooking.

5. Determination of total SH and SS groups

The total amount of SH and SS groups was determined by the amperometric titration method of HAMM and HOFMANN[125] and expressed as μmoles SH or SS groups per ml fraction.

6. Measurement of enzyme activity

Enzyme activity of the fractions was assayed as follows: Phosphofructokinase activity was measured according to the method of LING *et al.*[126] AMPDA activity was measured by LEE's method.[127] PK activity was measured by a method given by BÜCHER and PFLEIDERER.[128] The activity of phosphorylase b and glyceraldehyde phosphate dehydrogenase were assayed by the method of CORI *et al.*[129] and VELICK,[130] respectively. In addition, the measurement of LDH activity was performed by DENNIS and KAPLAN's method.[131] Activity of these enzymes was represented as each unit per ml fraction.

7. Preparation of AMPDA

AMPDA was prepared from porcine skeletal muscle (*M. adductor*, 3 hr postmortem) by the method of SMILEY *et al.*[132]

8. Enzyme preparation

AMPDA (Sigma), PK (Boehringer) and LDH (Boehringer), all of which were prepared from rabbit muscle, were used as enzyme preparations.

9. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of WEBER and OSBORN.[133] Molecular weight marker(BDH) for gel electrophoresis was used as the standard for molecular weight estimation.

10. SH reagent solution

As a SH reagent solution, 0.3 M iodoacetic acid (Wako) aqueous solution was prepared by adjusting the pH to 5.5 with 1 N NaOH.

11. Aqueous model system

Each aqueous model system was prepared according to the procedures given in Chapter III (see page 382). Further details appear in the Figure and Table legends.

12. Meat system

Pork sausage was used as a meat system. Each meat system was prepared by the method described in Chapter III (see page 383). AMPDA was added at 0.2% by reference to the AMPDA content of sarcoplasmic protein reported in the literature.[71] For comparison, PK and LDH preparations were also added separately instead of AMPDA. Iodoacetic acid as a SH reagent was added at varying concentrations with AMPDA when necessary. Further details appear in the Table legends.

RESULTS & DISCUSSION

1. Sephadex G-100 gel chromatography of the high-molecular weight fraction

The high-molecular weight fraction of sarcoplasm was chromatographed on Sephadex G-100 column. Fig. 13 shows the distribution patterns of CFA and residual nitrite. Region I (tubes 14 to 18, Kd=0-0.2), Region II (tubes 22 to 33, Kd =0.3-0.8) and Region III (tubes 39 to 43, Kd= 1.0-1.2) in Fig. 13 were found to be active in promoting the color formation. These regions were effective in decomposing nitrite. The fraction having higher CFA was also more active in nitrite depletion. This tendency was the same as that observed in the low-molecular weight fraction (Chapter III). However, the effect of these fractions was not always directly proportional to the protein concentration of each fraction. Tube 25 of Region II showing the highest CFA was considerably shifted from that of a absorption peak measured at 280 nm.

Fig. 14 shows the distribution patterns ob-

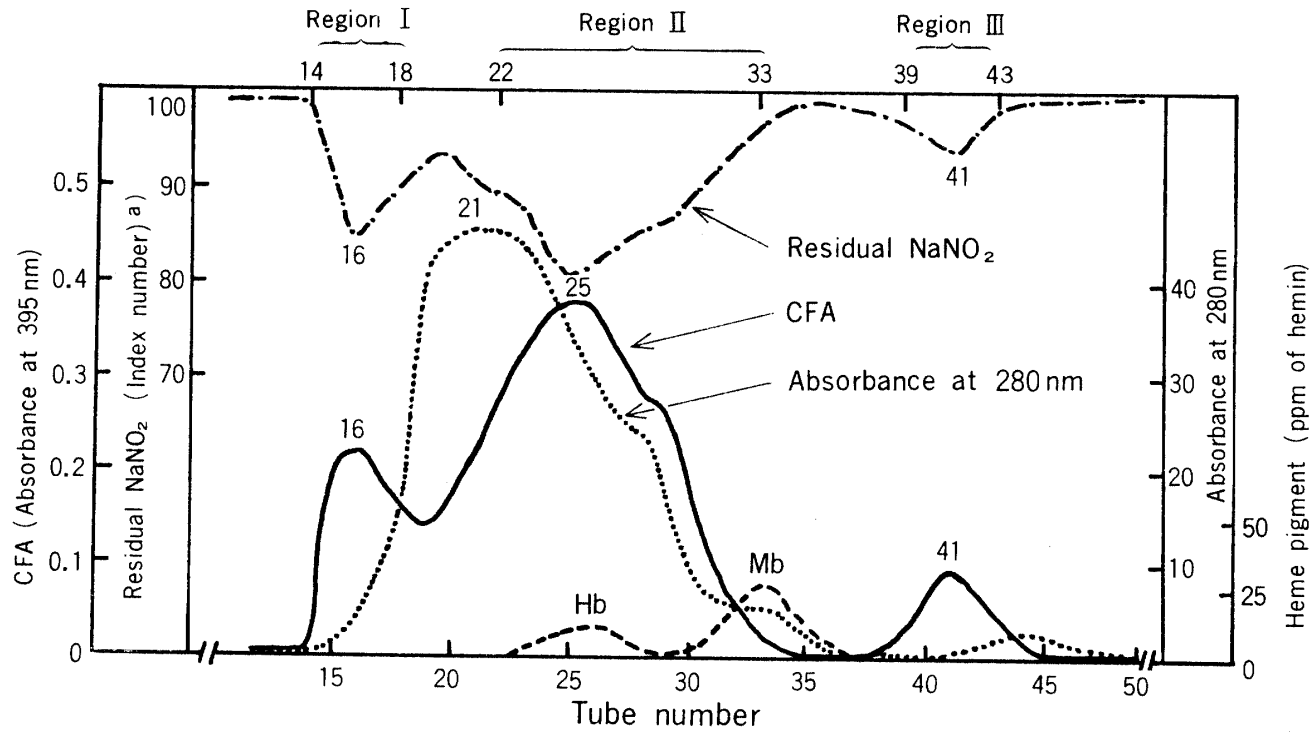

Fig. 13. Distribution patterns of CFA, the amounts of residual $NaNO_2$, absorbance value at 280 nm and heme pigments in the high-molecular weight fraction of sarcoplasm from porcine skeletal muscle on Sephadex G-100.

a) Figures for index number were calculated on the basis of 25 ppm of $NaNO_2$ added as 100.

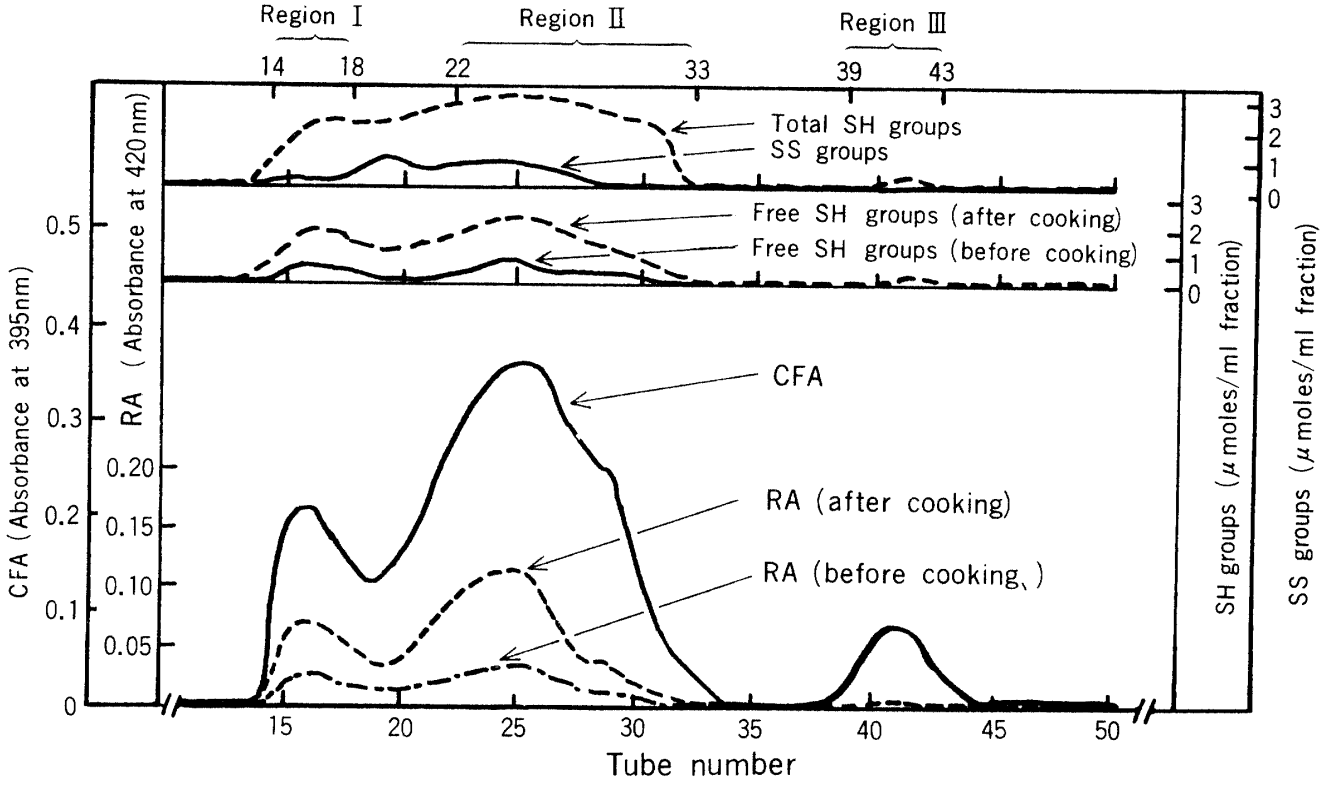

Fig. 14. Distribution patterns of CFA, RA and the amounts of free SH, total SH and SS groups in the high-molecular weight fraction of sarcoplasm from porcine skeletal muscle on Sephadex G-100.

tained by measuring RA and free SH groups. Both RA and free SH groups in Regions I and II increased considerably by the cooking treatment. The higher RA a cooked fraction possessed, the stronger CFA the fraction exerted. This suggested that the RA due to free SH groups released from sarcoplasmic proteins in the fraction during cooking, contributed to promoting the color formation. The total amounts of SH and SS groups in the fractions did not change by the cooking treatment (data not shown). Tubes 19 to 21 contained a considerable amount of SS groups which could not be involved in the color formation. Low CFA of the fraction may be accounted for by the presence of small quantities of free SH groups, in other words, by the low RA. A small amount of Region III was eluted finally, which seemed to contain relatively low-molecular weight constituents. Region III, which was distinct from Regions I and II, showed a higher absorbance at 260 nm rather than at 280 nm and fairly low RA. Tube 41 of this region had a absorption maximum at 250 nm. This suggests that Region III contains a nucleoprotein. FUJIMAKI and DEATHERAGE[134] separated a fraction having an absorption maximum at approximately 245 nm from bovine muscle sarcoplasm by cellulose phosphate column chromatography, and supposed the presence of a nucleoprotein in this fraction from its spectral characteristic.

From the calculation on the basis of CFA and absorbance at 280 nm (A_{280}) shown in Fig. 13, it was found that Region I possessed about 20% of the total CFA of all the tubes and that the ratio of CFA/A_{280} for Regions I and II was 0.024 and 0.009, respectively, that is, the CFA value per unit weight of the protein in Region I was about 2.7 times that of Region II. In addition, the cooking treatment of Region I resulted in a considerable increase of RA and SH groups and in a relatively strong decomposition of nitrite. From these observations, it was noteworthy that Region I exerted a greater CFA per unit weight of the protein.

2. Sepharose 6B gel chromatography of Region I

Tube 16 which exhibited the highest CFA in Region I was further fractionated by Sepharose 6B gel chromatography. Fig. 15 shows the distribution patterns obtained by measuring the protein content and CFA. Tube 16 was separated into two fractions, Fraction A (tubes 12 to 17, Kd=0-0.2) and Fraction B (tubes 22 to 33, Kd =0.4-0.8). Despite its lower protein contents, Fraction A showed approximately the same CFA as Fraction B. The CFA per mg protein in both fractions was calculated from the results, and found to be 0.29 in Fraction A and 0.14 in Fraction B. Thus, it was confirmed that the Fraction A possessed twice the CFA per unit weight of the protein compared with that of Fraction B. The Fraction A separated from tube 16 was shown to have the highest protein contents and the greatest CFA in Region I. These results suggested that a high-molecular component considerably active in promoting the color formation in spite of its lower protein contents was present in the Fraction A.

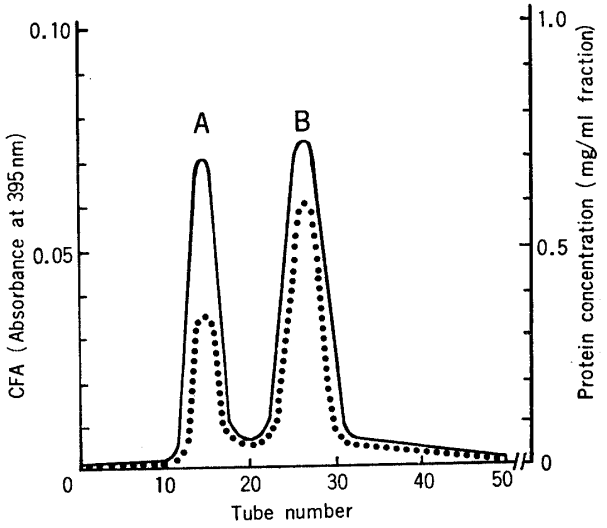

Fig. 15. Distribution patterns of proteins and CFA in tube 16 of Region I rechromatographed on Sepharose 6B.
—— : CFA, •••• : Protein concentration.

3. Identification of a component in Fraction A active in promoting the color formation

It is well known that a number of enzymes associated with glycolysis was contained in the high-molecular weight fraction of sarcoplasm.[71] If an endogenous component promoting the color formation in Fraction A was one of the glycolytic or associated enzymes, it is conceivable that the component might be identified by measuring the enzyme activity. Of the glycolytic or associ-

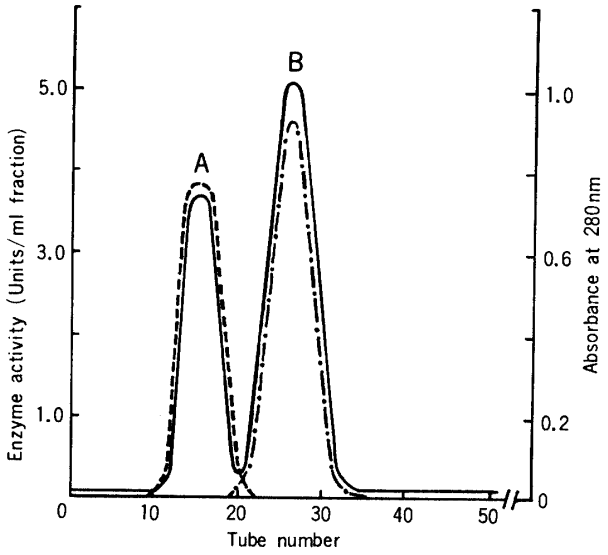

Fig. 16. Distribution patterns of AMPDA and PK activities in tube 16 of Region I rechromatographed on Sepharose 6B column.
——— : Absorbance at 280 nm,
– – – : AMPDA activity,
— • — : PK activity.

ated enzymes having relatively high-molecular weights, phosphofructokinase, AMPDA, PK, phosphorylase b, glyceraldehyde phosphate dehydrogenase and LDH were selected and these enzymatic activities were measured in each fraction eluted by Sepharose 6B gel chromatography of Region I.

Fig. 16 shows the chromatographic patterns of tube 16 of Region I obtained by measuring the AMPDA and PK activities. AMPDA activity was found in all the Fraction A separated from each fraction (tube 14–18) of Region I. Especially, Fraction A from tube 16 exhibited the highest AMPDA activity. Fraction B separated from tube 16 exhibited only PK activity, but Fraction B from fractions 17 and 18 had a considerably strong LDH activity as well as PK activity. Any of the other enzymes tested (phosphofructokinase, phosphorylase b and glyceraldehyde phosphate dehydrogenase) could not be detected in the tubes. In this experiment, the activities of six enzymes were examined as the possible known components.[71]

As the existence of AMPDA in Fraction A was shown from the measurement of its enzyme activity, the effect of AMPDA *per se* on the color formation was examined by the use of an aqueous model system containing Mb and $NaNO_2$ with varying concentrations of AMPDA prepared from porcine skeletal muscle. The conditions for determining CFA are given in Fig 17. The same experiments were also performed with PK and LDH (Boehringer's preparatoins). As shown in Fig. 17, the CFA values obtained with AMPDA, PK and LDH were all proportional to the concentration of each enzyme. Comparison of the CFA of these enzymes was indirect, because AMPDA was prepared from porcine skeletal muscle, while both PK and LDH preparations were prepared from rabbit skeletal muscle. However, AMPDA was found to exhibit the highest CFA per unit weight of the protein of these three enzymes from the slopes of these linear plots in Fig. 17.

Fig. 18 shows SDS-polyacrylamide gel (5%) electrophoretic patterns of AMPDA preparation and the Fraction A separated from tube 16. Although some faintly stained bands which seemed to be impurities appeared in the AMPDA preparation, AMPDA was observed as a single band. Based on the electrophoresis with proteins having known molecular weights, the molecular

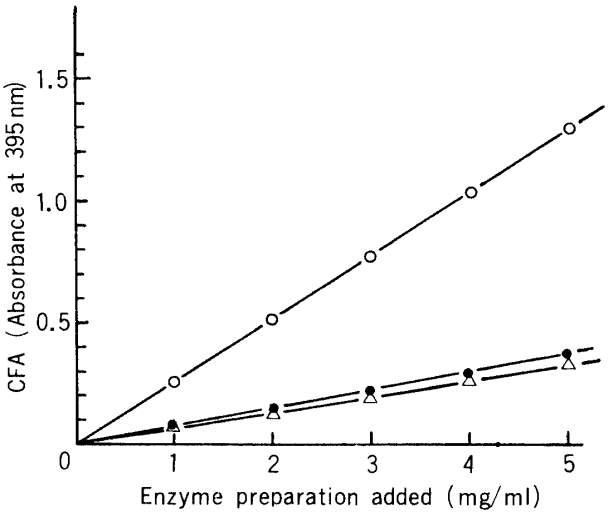

Fig. 17. Promoting effects of AMPDA, PK and LDH on the color formation in an aqueous model system.
Each sample solution contained 0.125% Mb, 25 ppm $NaNO_2$ and a designated concentration of each enzyme preparation in M/35 veronal buffer, pH 5.5. The CFA of each enzyme sample was presented after subtraction of the CFA value of the corresponding control which contained only Mb and $NaNO_2$.
Mean values from three experiments are denoted.
○——○ : AMPDA, ●——● : PK, △——△ : LDH.

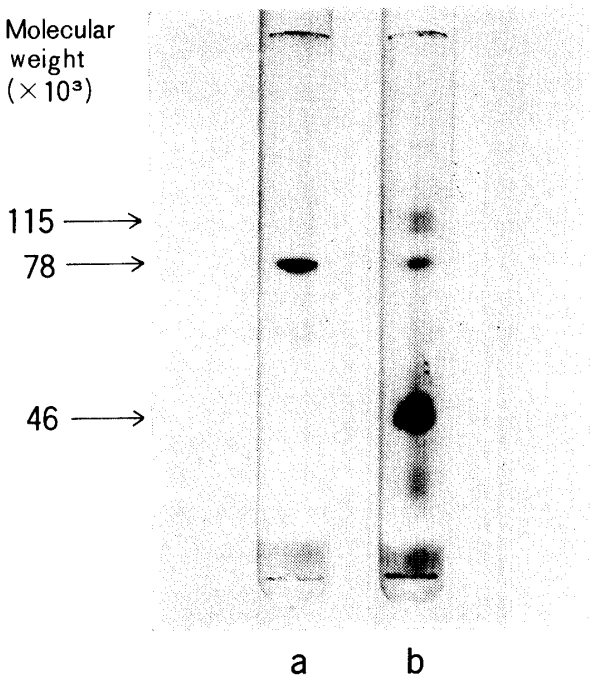

Fig. 18. SDS-polyacrylamide gel (5%) electrophoretic patterns of an AMPDA preparation and Fraction A.
a) AMPDA preparation (Sigma, 1.25 μg protein loaded).
b) Fraction A separated from tube 16 of Region I (2.5 μg).

weight of AMPDA was estimated to be 78,000 and the result approximately agreed with those described by other workers.[135-137] The position of a band with considerably high intensity in Fraction A was consistent with that of the band of AMPDA preparation. Since the AMPDA preparation and the Fraction A were prepared from the different species of animal, the co-electrophoresis of AMPDA preparation with Fraction A was performed to examine whether the migration distance of the AMPDA bands was quite the same or not. The result indicated that the AMPDA from the porcine and rabbit muscles exhibited quite the same electrophoretic behavior. By the assay of enzyme activity, AMPDA was confirmed to be present in Fraction A. Some unknown proteins having molecular weights in the range from 46,000 to 115,000 were also detected in Fraction A. The AMPDA content of Fraction A was estimated by scanning the SDS-polyacrylamide gel electrophoretic patterns obtained from four different porcine muscles with a Toyo Model DMU-33C Digital Densitorol and found to be in the range from 11 to 14%.

4. Promoting effect of AMPDA on the color formation

From the findings of SDS gel electrophoresis, it was confirmed that Fraction A contained some unknown protein components other than AMPDA. Therefore, the CFA of AMPDA and unknown protein components in Fraction A was compared by the following experiment. Fraction A was fractionated from tube 16 of Region I. The AMPDA concentration in this fraction was estimated to be 0.04 mg/ml from the area calculated by scanning of the SDS-polyacrylamide gel electrophoretic pattern. As the control, 0.04 mg/ml solution of AMPDA prepared from porcine skeletal muscle was used. Since the protein concentration of the Fraction A used in this experiment was 0.28 mg/ml, the protein content of the Fraction A was about seven times that of the control which contained only AMPDA. The experimental conditions for determining CFA are described in Table 5. As shown in Fig. 17, CFA increased in proportion to the protein concentration of an enzyme preparation. Therefore, if the unknown protein components in Fraction A have the same effect as AMPDA, the CFA of Fraction A should exhibit about seven times that of the control

Table 5. Contribution of AMPDA in Fraction A to the CFA.

Sample	Protein concentration (mg/ml)	CFA (N=5)		
		Mean	S.D.	Index number[c]
AMPDA[a]	0.04	0.011	0.003	100
Fraction A[b]	0.28	0.026	0.005	236

a) Prepared from porcine skeletal muscle by the method of SMILEY *et al.*[132]
b) Fractionated from tube 16 of Region I. The AMPDA concentration in this sample was estimated to be 0.04 mg/ml from the area calculated by scanning of the SDS-polyacrylamide gel electrophoretic pattern. One ml of each protein aqueous solution was mixed with $NaNO_2$ and Mb. The other conditions for determining CFA were the same as those in Fig. 17.
c) Figures for index number were calculated on the basis of the CFA for AMPDA sample as 100.

Table 6. Effects of the addition of AMPDA, LDH and PK on the color formation and the decomposition of nitrite in a meat system.[a]

Enzyme preparation added	CFA		Nitrite decomposition[c] (%)
	Found	Index number[b]	
None	0.250	100	50.2
AMPDA	0.303	121	64.6
LDH	0.263	105	52.2
PK	0.260	104	52.2

a) Each sample initially contained 2% NaCl, 10 ppm $NaNO_2$ and 0.2% enzyme preparation. The pH was adjusted to 5.5 with 1 N HCl. Water was added to each sample at a rate of 10% by weight of the meat. Just after curing, samples were cooked at 75°C for 1 hr.

b) Figures for index number were calculated on the basis of the CFA for control sample as 100.

c) "Nitrite decomposition" refers to the percentage of the decomposed nitrite to the added nitrite. Mean values from three experiments are denoted.

(containing only AMPDA). However, as can be seen in Table 5, the CFA of Fraction A was only 236 on the basis of CFA for the control as 100, that is, the CFA of Fraction A was only about 2.4 times that of the control. This suggested that the CFA per unit weight of the unknown protein components in Fraction A was considerably less than that of AMPDA. It might be considered that an unknown minor protein component, other than AMPDA, exhibiting high CFA per unit weight was present in Fraction A. However, these results support the view that the endogenous components having relatively high CFA in Fraction A is AMPDA.

Then, the contribution of AMPDA to the color formation was evaluated using a pork sausage as a meat system. The same experiment was also performed with PK and LDH preparations for comparison. As shown in Table 6, CFA and nitrite decomposition by the addition of AMPDA increased about 20% and 30% compared with that of the control (without addition of AMPDA), respectively, and the addition of AMPDA caused a greater increase in CFA and nitrite decomposition than that of PK or LDH. These results showed approximately the same tendency for CFA as in the aqueous model system (Fig. 17). Thus a definite promoting effect of AMPDA on the color formation could be confirmed with meat systems as well as with aqueous model systems.

5. Effect of SH reagent on the promoting effect of AMPDA

In order to clarify the promoting mechanism of AMPDA for the color formation, the effect of the addition of SH reagent on the CFA of AMPDA was investigated. As a SH reagent, iodoacetic acid was used. This reagent was the same alkylating agent as that used by WATTS *et al.*[47] The experiments were carried out with a meat system as well as an aqueous model system. The AMPDA used in this experiment was prepared from porcine skeletal muscle.

The aqueous model system containing constant levels of Mb and $NaNO_2$, and varying amounts of AMPDA (0–5 mg/ml) was prepared with or without addition of iodoacetic acid (3 mM). Other experimental conditions were given in Fig. 17. The results are shown in Fig. 19. The pro-

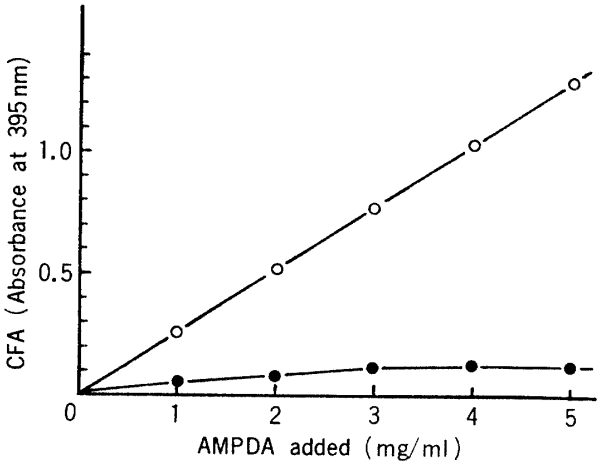

Fig. 19. Effect of the addition of SH reagent on the CFA of AMPDA in an aqueous model system.

As a SH reagent, iodoacetic acid was added to each of the test samples to give a final concentration of 3 mM. Other conditions were the same as in Fig. 17. CFA of each protein sample was presented after subtraction of the CFA value of the corresponding control which contained only Mb and $NaNO_2$.

Mean values from three experiments are denoted.
○——○ : AMPDA,
●——● : AMPDA+SH reagent.

moting effect of AMPDA on the color formation was remarkably inhibited by the addition of iodoacetic acid. SAMMONS *et al.*[138] found that 26 SH groups per mole of AMPDA prepared from chicken muscle were titrated with DTNB. RAGGI *et al.*[139] reported that in the urea-denatured AMPDA from rat muscle 30 SH groups per mole were titratable with DTNB and 32 moles as cysteic acid per mole were determined by the amino acid analysis. O'DRISCOLL and ROSS[140] also reported that 31 moles SH groups per mole of rabbit AMPDA reacted with DTNB in 6 M urea or 0.5% SDS solution. In the case of 5 mg/ml AMPDA sample used to the aqueous model system, the amount of added iodoacetic acid theoretically corresponded to about six times the SH group content of AMPDA on a molar basis.

Table 7 shows the effect of added SH reagent on CFA and nitrite decomposition in a meat system. The addition of 0.2% AMPDA to the cooked sausage caused a significant increase in CFA and nitrite decomposition as observed in Table 6. In the sample containing iodoacetic acid, the promoting effect of AMPDA was suppressed. Both the CFA and nitrite decomposition were inhibited in proportion to the iodoacetic acid level added. Although the SH groups content of meat given in literature varies considerably depending on the method of assay or the species of material investigated, HOFMANN and HAMM[48] concluded in their recent review that the average SH contents of porcine and bovine muscle are 10.2 and 10.5 moles SH/10^5g protein, respectively. HOFMANN *et al.*[141] also found that the most part of SH groups in meats (96–97%) was bound in muscle proteins ("protein-SH"). Calculation based on these data indicated that iodoacetic acid of molar concentrations from 1.5 to 15 times that of SH groups in meat was initially added to the pork sausage (Table 7). Despite a large excess of the added iodoacetic acid, the suppression of CFA in the meat system was not so remarkable as compared with that found in the aqueous model system (Fig. 19).

In generally, iodoacetic acid as well as the other alkylating agents has a relatively high specificity for SH groups. On the other hand, the reactivity of this SH reagent with protein-SH groups has been shown to be influenced by an electrostatic interaction between the charged side-chains of protein and the carboxyl anion group of iodoacetic acid, the rate of which is dependent on the pH.[142] It has also been reported that iodoacetic acid reacts with ε-NH_2, OH and S-CH_3 groups of proteins in a region below pH 8–9.[143] Since, at the present experiment, the meat samples were adjusted to pH 5.5 and cooked at 75°C for 1 hr, the SH reagent

Table 7. Effect of the addition of a SH reagent on the color formation and the decomposition of nitrite in a meat system.[a]

AMPDA[b] added (%)	SH reagent[c] added (mmoles/100g meat)	CFA		Nitrite decomposition[e] (%)
		Found	Index number[d]	
—	—	0.255	100	52.3
0.2	—	0.306	120	67.9
0.2	3	0.250	98	52.3
0.2	15	0.209	82	50.2
0.2	30	0.162	64	47.6

a) Each sample initially contained 2% NaCl, 10 ppm $NaNO_2$ and additionally 10% water by weight of the meat together with the above ingredients. The pH was adjusted to 5.5 with 1 N HCl. All the samples were cooked at 75°C for 1 hr just after curing.
b) Prepared from porcine skeletal muscle by the method of SMILEY *et al.*[132]
c) Iodoacetic acid was used. This reagent was adjusted to pH 5.5 with 1 N NaOH before use.
d) Figures for index number were calculated on the basis of the CFA for control sample as 100.
e) "Nitrite decomposition" refers to the percentage of the decomposed nitrite to the added nitrite. Mean values from three experiments are denoted.

added to the meat system was considered to react probably with these functional groups in addition to SH groups in the meat. Therefore, it seemed likely that these complicated reactions influenced on the color formation of cooked sausages in the presence of the SH reagent. WATTS *et al.*[47] investigated the role of SH groups in heat-denatured proteins using a model system and found that the addition of 0.1 M iodoacetic acid suppressed remarkably the color formation in the model system containing egg white, Hb and nitrite. They assumed that the inhibiting effect of the SH reagent was presumably exerted indirectly by eliminating the reducing activity of SH groups in egg albumin. At the present study, iodoacetic acid added to cooked cured product samples, however, did not inhibit the CFA so remarkably as the findings of WATTS *et al.*[47] OLSMAN and KROL[55] observed a still considerable decomposition of added nitrite even after blocking SH groups in meat with mercuric chloride or NEM. TINBERGEN[87] also reported on the effect of vinylpyridine or NEM to water-soluble low-molecular weight fraction of bovine muscle. He found that the addition of these SH reagents partly inhibited the NOMb formation in a reaction mixture of Mb, nitrite and the fraction, and suggested that a SH/SS redox system in the fraction was involved in the nitrite reduction.

In the present study, it was found that the SH groups of AMPDA play an important role in promoting the color formation. The exact mechanism whereby AMPDA promotes strongly the color formation remains to be investigated.

SUMMARY

High-molecular weight fraction of sarcoplasm was first separated by Sephadex G-100 (Superfine) gel chromatography. Region I (tubes 14 to 18, Kd=0-0.2), Region II (tubes 22 to 33, Kd =0.3-0.8) and Region III (tubes 39 to 43, Kd= 1.0-1.2) were found to be active in CFA and decomposing nitrite. As Regions I and II exhibited high RA, there is no doubt that the SH groups released from sarcoplasmic proteins in both regions during heat treatment participate in the reaction process of the color formation. In spite of its lower protein contents, Region I significantly enhanced the color formation. Region I could be further separated into two fractions, Fractions A (tubes 12 to 17, Kd=0-0.2) and B (tubes 22 to 33, Kd=0.4-0.8) by gel filtration on Sepharose 6B column. Fraction A was more effective in promoting the color formation on the basis of the protein concentration compared with that of Fraction B. These results demonstrated that an endogenous high-molecular weight component exhibiting the relative highly promoting effect on the color formation was present in Fraction A. From the measurement of activities of several glycolytic or associated enzymes, Fraction A was found to have an AMPDA activity. The CFA of this fraction varied depending largely upon its protein concentration and also the AMPDA activity. SDS-polyacrylamide gel electrophoresis indicated that Fraction A contained 11 to 14% AMPDA. The CFA per unit weight of the unknown protein components in Fraction A was considerably lower than that of AMPDA. These results supported the view that an endogenous component having higher promoting effect on the color formation in Fraction A is to be AMPDA. The addition of AMPDA to a pork sausage caused a significant increase in CFA. Iodoacetic acid added as a SH reagent suppressed the promoting effect of AMPDA in a meat system as well as in an aqueous model system and this finding suggested that the SH groups of AMPDA play an important role in the mechanism.

CHAPTER V

GENERAL SUMMARY

The formation of NO-hemochrome, the so-called CCMC, is very important in the technology

of meat processing, since this compound has been shown to be the principal pigment of processed meat products.

The exact mechanism of the color formation of meat products has not so far been well known, though some hypothesis have been put forward.

In recent years, a rapid emulsion curing method has widely been used in processing sausage products. It was assumed that, in the rapid emulsion curing process, many unknown endogenous components in muscle tissues as well as food additives are non-enzymatically involved in the color formation. However, the characterization of all of these endogenous components and the mechanism of the color formation are not clear at the present stage. Thus, the present work is undertaken to make clear the possible endogenous active components in porcine skeletal muscle, especially in the sarcoplasm fraction which will be able to promote the color formation in the rapid emulsion curing process.

Sarcoplasm was first separated into two fractions, low- and high-molecular weight fractions, by dialysis techniques. The low-molecular weight fraction was evidently found to be more efficacious in promoting the color formation and the decomposition of nitrite than the high-molecular one. The low-molecular weight fraction was further separated by Sephadex G-50 gel chromatography, and a main peak exhibiting the most favorable effect on the color formation was obtained in Region A (tubes 35 to 42, Kd=0.8-1.1). Region A was also found to be effective in decomposing nitrite, and rich in reducing substances, ninhydrin-positive substances and carbohydrates. From the ultraviolet absorption spectral characteristics, it seemed likely that the main peak fraction [tube 40 (Kd=1.0)] having a maximum absorbance value at 248 nm contained a considerable amount of inosine and/or its derivatives. The Region A was further chromatographed by Sephadex G-15 gel filtration. Region B (tubes 15 to 26, Kd=0.04-0.48) and Region C (tubes 27 to 39, Kd=0.52-0.99) were found to be active in CFA and decomposing nitrite. Region B exhibited CFA to a high extent in spite of its lower ultraviolet absorption compared with that of Region C. Both Regions B and C contained a considerably large amount of reducing substances, SH groups, ninhydrin-positive substances and carbohydrates. Fe^{2+} was detected only in Region B. Thin-layer chromatography indicated that Region B included GSH, ATP, IMP, glutamic acid, histidine and alanine, while Region C contained IMP, alanine, glutamic acid, histidine, glycine, tyrosine, valine and ribose. The presence of GSH and IMP in Region B was confirmed by the ultraviolet absorption spectral characteristics. In an aqueous model system, GSH, ATP and ribose enhanced the CFA and the decomposition of nitrite. In a meat system, GSH, ATP, IMP and ribose enhanced the color formation and the decomposition of nitrite. It was ascertained that the combined action of the identified endogenous components, *i. e.*, GSH, ATP, IMP and ribose contribute to promote the color formation of meat products in the rapid emulsion curing process.

The high-molecular weight fraction was separated by Sephadex G-100 (Superfine) gel chromatography. Three fractionated regions, that is, Regions I (tubes 14 to 18, Kd=0-0.2), II (tubes 22 to 33, Kd=0.3-0.8) and III (tubes 39 to 43, Kd=1.0-1.2) were found to be active in CFA and the decomposition of nitrite. As Regions I and II exhibited high RA, there is no doubt that free SH groups released from sarcoplasmic proteins in both regions during heat treatment participate in the color formation. In spite of its lower protein contents Region I significantly enhanced the color formation. Region I could be further separated into two fractions, Fractions A (tubes 12 to 17, Kd=0-0.2) and B (tubes 22 to 33, Kd=0.4-0.8) by gel filtration on Sepharose 6B column. Fraction A was more effective in promoting the color formation on the basis of the protein concentration compared with that of Fraction B. These results demonstrated that an endogenous high-molecular weight component exhibiting higher promoting effect on the color formation was present in Fraction A. From the measurement of activities of several glycolytic or associated enzymes, Fraction A was found to have an AMPDA activity. The CFA of this fraction varied depending largely upon its protein concentration and also the AMPDA activity. SDS-polyacrylamide gel electrophoresis indicated that Fraction A contained 11 to 14% AMPDA. The addition

of AMPDA to a pork sausage caused a significant increase in CFA. These results supported the view that an endogenous component having higher promoting effect on the color formation in Fraction A is to be AMPDA. Iodoacetic acid added as a SH reagent suppressed the promot ing effect of AMPDA in both the aqueous model and meat systems. This fact suggested that the SH groups of AMPDA play a key role in the mechanism.

At the present study, several endogenous components were separated from porcine muscle sarcoplasm corresponding to the water-soluble muscle fraction, and it was confirmed that most of these identified components are involved in promoting the color formation of cooked cured meat products such as pork sausages which are produced through the modern rapid emulsion curing process. However, the possibility that other endogenous factors may be present in the sarcoplasm, especially in the low-molecular weight fraction, cannot be excluded.

A more detailed investigation is necessary to characterize these endogenous components promoting the color formation and to clarify the exact mechanism. The results of such studies are awaited with great interest.

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加熱塩漬肉の発色促進に寄与する筋漿成分に関する研究

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要　約

迅速エマルジョン塩漬法における肉製品の発色機構を解明するため，筋肉構成成分のうち筋漿画分に存在する発色促進に有効な成分の検索を発色に及ぼす作用効果と共に追究した。

1．筋漿：豚内転筋より筋漿を調製し，これを透析法によって低分子量成分と高分子量成分含有の2画分に分け，前者が後者より顕著な発色促進効果と亜硝酸塩分解能を示すことを明らかにした。

2．低分子量成分：Sephadex G-50 と G-15 による2段階のゲルロ過を経て最終的に有効成分を含む2画分が得られた。すなわち，(1)還元型グルタチオン，ATP，IMP，グルタミン酸，ヒスチジン，アラニン，鉄 (2)IMP，グルタミン酸，ヒスチジン，アラニン，グリシン，チロシン，バリン，リボース。これらの中で還元型グルタチオン，ATP，IMP，リボースの各成分の単独または複数存在が発色促進効果を発揮することを明確にした。

3．高分子量成分：Sephadex G-100 と Sepharose 6B による2段階のゲルロ過を経て最終的に量的には少ないにもかかわらず強い発色促進効果を示す物質として5′-アデニル酸デアミナーゼ(AMP deaminase)を検出ならびに同定した。本物質のモデル系における発色促進効果などから高分子量成分が発揮する効果に本物質が明確に寄与するものと支持された。さらに，ヨード酢酸の同時存在が発色抑制を示すことからSH基の関与が示唆された。

以上の結果から，短時間内に遂行される加熱塩漬肉色の発現には，筋漿に含有される上述の諸成分が促進効果の役割を果たしているものと結論された。