FORMATION OF NITROSOTHIOL GROUPS IN MYOSIN

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The reaction of sodium nitrite with sulfhydryl groups in meat proteins was studied, using a model system. Isolated rabbit myosin was incubated with sodium nitrite in buffered solutions at pH 3.0 and 5.0. The temperature was 25°C and 100°C. Nitrite concentration was 10 times that of sulfhydryls from myosin.

The results clearly demonstrated the formation of nitrosothiol groups from the sulfhydryl groups. The increase in nitrosothiols was accompanied by a simultaneous decrease in sulfhydryl groups. The highest nitrosothiol content was reached within 10 minutes of reaction. At pH 3.0 and 100°C the nitrosothiols detected corresponded to a nearly quantitative reaction of the sulfhydryl groups.

Prolonged incubation reduced the amount of nitrosothiols. At pH 3.0 the stability was higher at 25°C than at 100°C. The breakdown of nitrosothiols at 100°C was approximately the same at pH 3.0 and 5.0.

FORMATION DE GROUPES DE NITROSOTHIOLE DANS LE MYOSIN

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La réaction entre le nitrite de sodium et les groupements sulphydriques dans les protéines de viande était étudiée en utilisant un système de modèle. Le myosin isolé de lapin a été incubé avec du nitrite sodium dans les solutions dampon de pH 3.0 et 5.0. La température était 25°C et 100°C. La concentration de nitrite était 10 fois celle de sulphydrique de myosin.

Les resultats ont nettement montré la formation des groupes de nitrosothiole par les groupes sulphydriques. l'augmentation de nitrosothiole a été suivi par une réduction dans les groupes de sulphydrique. Le contenu le plus haut de nitrosothiole était obtenu avant 10 minutes de réaction. A pH 3.0 et a 100°C le nitrosothiole découvert correspond à une réaction à peu près quantitative des groupes de sulphydrique.

l'incubation prolongée a diminué la quantité de nitrosothiole. À pH 3.0 la stabilité était plus haute à 25°C qu'à 100°C. La démolition de nitrosothiole 100°C était à peu près la même à pH 3.0 et à 5.0.

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DIE BILDUNG VON NITROSOTHIOLGRUPPEN IN MYOSIN

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Die Reaktion des Natriumnitrits mit Sulfhydrylgruppen in den Fleischproteinen ist, unter Verwendung eines Modelsystems, studiert worden. Isoliertes Kaninchenmyosin wurde mit Natriumnitrit in Pufferlösungen mit pH 3.0 und 5.0 inkubiert. Die Temperatur war 25°C und 100°C. Nitritkonzentration war die Zehnfache des Sulfhydryls vom Myosin.

Die Ergebnisse haben deutlich die Bildung der Nitrosothiolgruppen von Sulfhydrylgruppen gezeigt. Die Zunahme der Nitrosothiole war mit einer gleichzeitigen Abnahme der Sulfhydrylgruppen geknüpft. Der höchste Gehalt an Nitrosothiole wurde in den ersten zehn Minuten der Reaktion erreicht. Bei pH 3.0 und 100°C entsprach die erhaltene Menge der Nitrosothiole fast den quantitativen Reaktion der Sulhydrylgruppen.

Verlängerte Inkubation reduzierte die Menge der Nitrosothiole. Mit pH 3.0 war die Stabilität besser bei 25°C als bei 100°C. Der Abbau der Nitrosothiole bei 100°C war ungefähr der gleiche bei pH 3.0 und 5.0.

ОБРАЗОВАНИЕ НИТРОЗОТИОЛЬНЫХ ГРУПП В МИОЗИНЕ

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Исследовано, при употреблении модельной системы, реакцию нитрита натрия с сульфгидрильгруппами в мясных протеинах. Изолированный кроличий миозин инкубирован с нитритом натрия в буферных растворах при pH 3,0 и 5,0, при температуре 25°C и 100°C. Концентрат нитрита был десятикратным по сравнении с сульфгидрилом миозита.

Результаты явно показали образование нитрозотиольных групп из сульфгидрильных групп. Рост нитрозотиолов был связан с одновременным уменьшением сульгидрильных групп. Наибольшее содержание нитрозотиолов было получено в течении первых десяти минут реакции. Полученная доза нитрозотиолов при pH 3,0 и 100°C почти соответствовала количественной реакции сульфгидрильных групп.

Продленная инкубация уменьшила количество нитрозотиолов. Стабильность при pH 3,0 была выше при 25°C чем при 100°C. Разложение нитрозотиолов при 100°C было приблизительно таким же при pH 3,0 и 5,0.

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Introduction

The reaction between nitrite and sulfhydryl groups, resulting in the formation of nitrosothiols, is regarded as being responsible for some nitrite loss in cured meat products (Mirna, 1970; Olsman, 1973). The formation of S-nitrosocystein is known to be quantitative when cystein is incubated with nitrite under strong acidic conditions (Saville, 1958). With increasing pH (pH 2.3 to 5.5) Mirna and Hofmann (1969) obtained decreasing formation of nitrosothiol from glutathione. The nitrosothiols from cysteine and glutathione were unstable compounds, having half-lives of 2 and 3 hours, respectively, at pH 5.5.

Kubberød et al. (1974) demonstrated a similar pH dependence when nitrite was reacted with sulfhydryl groups of isolated myosin. Using myosin and nitrite in an anaerobic model system, they found a nearly equimolar decrease in sulfhydryl groups and nitrite at pH 5.0. Only 20% of the total nitrite loss was detectable as nitrosothiols, however.

The work presented here was undertaken to investigate further the products from the reaction between sulfhydryl groups of myosin and nitrite (Kubberød et al., 1974). The formation and stability of nitrosothiols at different pH and temperatures, and the possible formation of disulfides from the breakdown of nitrosothiols were investigated.

Materials and Methods

Rabbit muscle myosin was isolated according to Nauss et al. (1969) as described elsewhere (Kubberød et al., 1974). All solutions used, were made with double-destilled water and were treated with chelating resin, Chelex-100 (Bio-Rad Laboratories) to remove traces of heavy metals (Buttkus, 1971). The myosin was stored as ammonium sulfate precipitates at -80°C under N_2-atmosphere. Before use, aliquots were dialyzed three times versus 0.5 M KCl, 5 mM TES (N-tris(hydroxymethyl)methyl-2-aminoethane), 6mM EDTA at pH 7.0 and were then centrifuged at 100.000 x g for 60 minutes. In all analyses, protein solutions were weighed into reaction vessels. Samples prepared this way contained 15 - 20 mg protein/g solution, determined by the Kjeldahl method (AOAC, 1970) and the conversion factor 6.25. For routine work, protein was determined by the biuret method (Colowick and Kaplan, 1957), using bovine serum albumin as standard.

In the experiments, aliquots of 0.15 - 0.20 g of myosin solution were treated with 1.0 ml buffered $NaNO_2$-solution at different conditions. In all experiments the nitrite concentration was 10 times the initial sulfhydryl group concentration of the myosin in solution. Buffers used for incubation were mixtures of 0.1 M citric acid and 0.2 M dipotassium phosphate (Colowick and Kaplan, 1955).

Nitrosothiol groups of myosin were determined using the method given by Saville (1958) except that 6 M guanidine-hydrochloride was used to make up the volum of the sample after incubation. Cystein was used as standard.

For sulfhydryl group determination, a modification of the Ellman (1959) procedure (Butterworth et al., 1967) was used. After incubation, the sample was mixed with 5.0 ml 6 M guanidine-hydrochloride (pH 7.5) and stirred with a glass rod until the solution was homogeneous. 2.0 ml 0.01 M DTNB (5.5'-dithiobis(2-nitrobenzoic acid)) were added. After 2 minutes of reaction, 8.5 ml 50% saturated ammonium sulfate were added. The sample was centrifuged (30.000 x g) for 25 minutes and the precipitate washed 4 times using 20 ml 50% saturated ammonium sulfate solution each time. The precipitate was dissolved in 3.0 ml 6 M guanidine-hydrochloride and the pH adjusted to 7.0. After addition of 0.06 ml 0.4 M DTE (1,4-dithioerythritol), the volume was made up to 20 ml with a 0.1 M potassium-phosphate buffer containing 0.5 M KCl and 6 mM EDTA at pH 7.5. The optical density was read at 412 nm against a blank. Glutathione was used as standard. The same procedure was used when determining the sulfhydryl groups in myosin without nitrite incubation.

Results

The rate of formation of nitrosothiols was high when myosin was incubated with nitrite at 25°C and pH 3.0 (Figure 1). After 10 minutes of reaction, 24.7 moles/$5 \cdot 10^5$ g protein were detected. Two hours of incubation reduced the amount of nitrosothiols by approximately one third.

The detectable amount of sulfhydryl groups was reduced from 26.7 moles/$5 \cdot 10^5$ g protein to 12.3 moles/$5 \cdot 10^5$ g protein in 5 minutes of incubation. No reduction in the amount of sulfhydryl groups was obtained beyond these first minutes. Sulfhydryls were stable when incubated without nitrite at 25°C and pH 3.0 for 2 hours.

At 100°C and pH 3.0, nitrosothiols were formed at a high initial rate. After 5 minutes, 25.3 moles/$5 \cdot 10^5$ g protein was detected (Figure 2). On prolonged incubation there was a rapid decrease in the amount of nitrosothiols. After 2 hours, 3.8 moles/$5 \cdot 10^5$ g protein remained. This was nearly equal to 15% of the maximum amount detected.

The content of sulfhydryl groups was reduced from 26.7 moles/$5 \cdot 10^5$ g protein to 3.8 moles/$5 \cdot 10^5$ g protein in 5 minutes. Myosin heated without nitrite at 100°C and pH 3.0 showed a sulfhydryl loss of about 10% in 30 minutes.

After 2 hours of incubation 3.7 moles nitrosothiols/$5 \cdot 10^5$ g protein remained (Figure 3). This corresponded to 23% of the amount detected after 10 minutes of reaction.

The content of sulfhydryl groups was reduced from 25.5 moles/$5 \cdot 10^5$ g protein to 22.0 moles/$5 \cdot 10^5$ g protein in two hours when myosin was incubated without nitrite and to 3.7 moles/$5 \cdot 10^5$ g protein on incubation with nitrite. Close to 90% of this reduction occurred within the first 10 minutes of reaction.

Discussion

The reduction in detectable sulfhydryl groups when myosin was incubated with nitrite under acidic conditons, is in agreement with results obtained in previous experiments (Kubberød *et al.*, 1974). After the initial rapid fall, the amount remained nearly constant on prolonged incubation.

The results clearly demonstrated a rapid formation of nitrosothiols. When incubation was continued, the amount of nitrosothiols decreased, indicating that nitrosothiols formed in myosin are unstable. Whether the stability of these high molecular nitrosothiols was better than reported for low molecular nitrosothiols (Mirna and Hofmann, 1969) is difficult to assess from the data presented, since experimental conditions were different.

As would be expected, the stability of nitrosothiols was better at a lower temperature than at a higher. On the other hand, no effect of pH on the breakdown of nitrosothiols was observed in these experiments.

The amounts of nitrosothiols found at short time incubation might have been slightly affected by a possible additional formation of nitrosothiols due to low pH during a step in the procedure. All samples were treated identical however, so that comparisons could be made.

Nitrosothiols are split by mercuric ions, causing a reformation of nitrite, a fact which is utilized in the Saville (1958) procedure for determining nitrosothiols. However, this reformation of nitrite is not spesific for nitrosothiols, as Mirna and Coretti (1974) detected nitrite from N-nitrosamines treated with mercuric chloride. Under identical conditions, the amount of nitrite arising from nitrosamines corresponded to only 1 - 2% of that from nitrosothiols. This indicates that nitrosothiols can be analyzed by the Saville method without being considerably influenced by nitrosoamines that might be present.

Fox and Nicholas (1974) reported that among several amines and amino acids, cystein gave the greatest contribution to the nitrite loss in buffered systems. This finding together with the results presented herein, would indicate that the predominant loss of nitrite when myosin is incubated with sodium nitrite is due to nitrosothiols. The nearly equimolar losses in sulfhydryls and nitrite found by Kubberød *et al.* (1974), indicated that no nitrite was reformed when nitrosothiols were destroyed by heat. This should be borne in mind when analyzing residual nitrite in meat products according to the AOAC (1970) procedure. The method involves heating of the samples for 2 hours before mercuric chloride is added for protein precipitation. Nicholas and Fox (1973) did not find any change in nitrite content when mercuric chloride was omitted from the analysis. The reason for this might have been that any nitrosothiols initially present, were destroyed during heat treatment. Thus, the nitrite from these nitrosothiols would not be included in the amount of residual nitrite determined. A way to solve the problem might be to split the nitrosothiols before heating the samples.

In the present investigation, sulfhydryl groups were determined according to a modification of the Ellman procedure. By including a reduction in this procedure, the method should be applicable for disulfide analysis. However, difficulties were met when reducing the sample with 1,4-dithioerytrithol in the presence of nitrite, and nitrite would possibly have to be removed before reduction. This analytical problem is presently under investigation in our laboratory.

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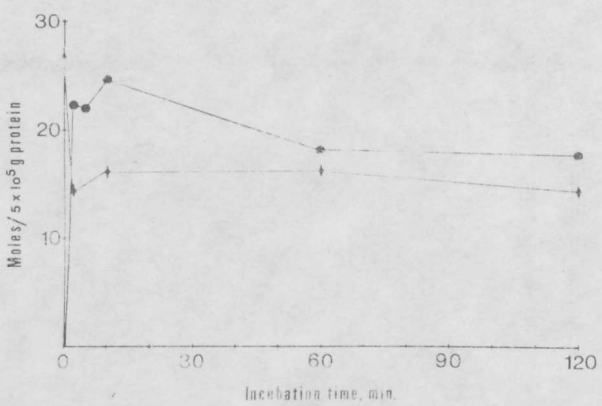

Figure 1. Nitrosothiol (●) and sulfhydryl groups (◆) of myosin treated with sodium nitrite at 25°C, pH 3.0. Sodium nitrite concentrations were 10 times sulfhydryl group concentrations in solution.

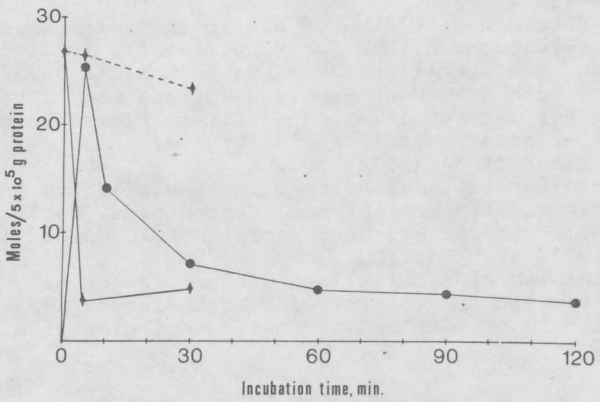

Figure 2. Nitrosothiol (●) and sulfhydryl groups (♦) of myosin treated with (——) and without (---) sodium nitrite at 100°C, pH 3.0. Sodium nitrite concentrations were 10 times sulfhydryl group concentrations in solution.

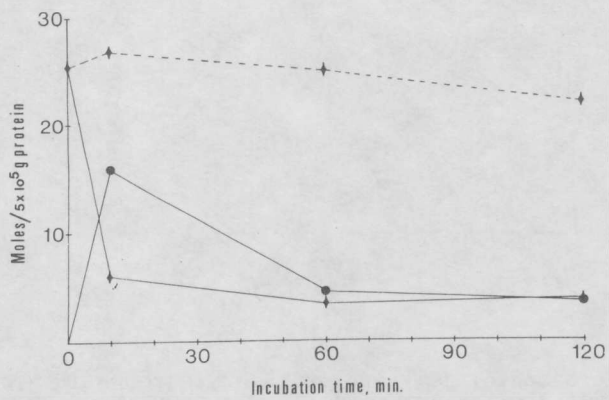

Figure 3. Nitrosothiol (●) and sulfhydryl groups (♦) of myosin treated with (——) and without (---) sodium nitrite in buffered solutions at 100°C, pH 5.0. Sodium nitrite concentrations were 10 times sulfhydryl group concentrations in solution.