Die Bildung von Nitrosohäm und Häm in Dauerwurst bei Verwendung von katalasepositiven Bakterienstämmen als Startkulturen

ERIK SLINDE und JOHN NORDAL
Norwegisches Institut für Nahrungsmittelforschung, Postboks 50, N-1432 AAS-NLH/Norwegen

Die Farbe in Dauerwurst wird gewöhnlich Nitrosomyoglobin und Nitrosohämoglobin zugeschrieben. Bei Anwendung von Startkulturen fällt gewöhnlich der pH-Wert in der Dauerwurst von ca. 6.5 auf 4.8. Das Studium von den Dissosiationskurven des Myoglobins und des Hämoglobins in Zitronensäurepuffern zeigt, dass die Häm-Gruppe dissossiert von den Proteinen bei pH-Werten vergleichbar mit denen, die gewöhnlich in Dauerwurst während der Gärung vorkommen. Nitrosohäm und Häm sind weit mehr hydrofobe als Myoglobin oder Hämoglobin. Aus ungereifter Rohwurst konnte durch Extraktion mit Fosfatpuffer das rote Pigment entfernt werden, welches sich aus Dauerwurst mit pH-Werten zwischen 4.5 - 5.0 nicht extrahieren liess. Diese Versuche deuten darauf hin, dass der Ursprung der Farbe der Dauerwurst hauptsächlich Nitrosohäm und Häm ist. *Lactobacillen* sind gewöhnlich katalasenegative, und Peroxide, die während des Fermentierungs-Prozesses produziert werden, können deshalb im Einzelfall die Pigmente durch Oxidation zerstören, als auch das Wachstum der Startkulturen verzögern. Eine Lösung dieses Problems ist jedoch durch Zusatz von katalasepositiven *Micrococcen* möglich. Es zeigt sich aber, dass acht Milchsäure-Bakterienstämme, die bei der Dauerwurstherstellung üblich sind, alle Katalase produzieren konnten, wenn sie in der Anwesenheit von Häm, entweder als Hämoglobin oder Myoglobin, gezüchtet wurden.

Formation of nitrosoheme and heme in dry sausages using catalase positive strains of lactic acid bacteria as starter cultures.

ERIK SLINDE and JOHN NORDAL
Norwegian Food Research Institute, P.B. 50, N-1432 Aas-NLH, Norway

The colour of dry sausages is generally attributed to nitrosomyoglobin and nitrosohemoglobin. By the use of starter cultures, the pH of dry sausages usually decreases from about 6.5 to 4.8. Studies of the dissociation curves for myoglobin and hemoglobin in citric acid buffers show that the heme group dissociates from the proteins at pH values comparable to those normally found in dry sausages during fermentation. Nitrosoheme and heme are far more hydrophobic than myoglobin or hemoglobin. Extraction of fresh sausages with phosphate buffer removed the red pigment, which could not be extracted from the dry sausage at a pH of about 4.5 - 5.0. These experiments indicate that the colour of dry sausages is mainly due to nitrosoheme and heme. *Lactobacilli* are generally catalase negative, and peroxide produced during the fermentation process may therefore in some cases destroy the pigments by oxidation as well as decrease the growth of the starter culture. This can be overcome by the addition of the catalase positive *Micrococci*. However, of eight strains of lactic acid bacteria used in dry sausage production, all possess the property of producing catalase when grown in the presence of heme, added either as hemoglobin or myoglobin.

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La formation de nitrosohème et d´hème dans des saucisses sèches en utilisant comme cultures initiales des souches de bactéries acide lactique qui sont catalase positives.

ERIK SLINDE et JOHN NORDAL

Institut Norvégien de Recherche Alimentaire, P.B. 50, N-1432 Aas-NLH, Norvège

La couleur des saucissons secs est généralement attribuée à la nitrosomyoglobine et à la nitrosohémoglobine. Par l´usage de cultures initiales, le pH des saucisses sèches dècroît généralement d´environ 6.5 à 4.8. Des études portant sur des courbes de dissociation de la myoglobine et de l´hémoglobine dans des tampons citrates montrent que le groupe hème se dissocie des protéines à des valeurs de pH comparables à celles normalement trouvées dans des saucisses sèches durant la fermentation. Le nitrosohème et l´hème sont de loin beaucoup plus hydrophobes que la myoglobine ou l´hémoglobine. A l´aide d´un tampon phosphate, on peut extraire le pigment rouge des saucissons frais, alors que celui-ci ne peut être extrait de saucisses sèches à un pH d´environ 4.5-5.0. Ces expériences montrent que la couleur des saucisses sèches est principalement due au nitrosohème et à l´hème.
Les *Lactobacilli* sont généralement catalase négatifs; le peroxyde produit durant le processus de fermentation peut par conséquent détruire dans certains cas les pigments en les oxydant et réduire la croissance des cultures initiales. Ceci peut être neutralisé par l´addition de *Micrococci* catalase positifs. Toutefois, parmi les huit souches de bactéries acide lactique utilisées dans la production des saucisses sèches, toutes possèdent la propriété de produire de la catalase lorsqu´elles sont cultivées en présence d´hème, ajouté soit sous forme d´hémoglobine, soit sous forme de myoglobine.

По вопросу образования nitrosoheme и heme в готовых колбасных изделиях с помощью штамм положительного фермента бактерий молочной кислоты как инициатора.

ЭРИК СЛИНДЕ и ДЖОН НУРДАЛЬ

Норвежский научно-исследовательский институт пищевых продуктов,И-1432 ААС-ИЛН,Норвегия.

Цвет готовых колбасных изделий в основном зависит от nitrosomyoglobin и nitrosohemoglobin. При помощи инициатора степень кислотности (pH) готовых колбасных изделий обычно понижается приблизительно от 6.5 до 4.8 . Данные,полученные при сравнении кривых разложения myoglobin и hemoglobin в буфере лимонной кислоты,свидетельствуют, что группа отделяется от протеинов при таких степенях кислотности (pH),которые обычно находят в колбасных изделиях во время ферментации. Nitrosoheme и heme обладают значительно большей гидрофобностью чем myoglobin или hemoglobin . Экстракция продукции в самом начале процесса фосфорно-кислым буфером снимает красный пегмент,чего нельзя сделать с изделиями готовыми к употреблению при степени кислотности около 4.5-5.0 Эти опыты показывают,что цвет готовых изделий зависит в основном от nitrosoheme и heme. *Lactobacilli* обычно негативны к каталазу и перекись,образуемая в период процесса ферментации,может поэтому разрушить пигмент окислением,а также снижением роста инициатора. Это можно преодалеть добавлением положительного каталаза *Micrococci*. Однако из восьми штамм бактерий молочной кислоты,используемых в процессе производства, все обладают способностью выделять каталаз, при условии их выращивания в непосредственной близости с hemeи с добавлением hemoglobin или myoglobin.

Formation of nitrosoheme and heme in dry sausages using catalase positive strains of lactic acid bacteria as starter cultures.

ERIK SLINDE and JOHN NORDAL

Norwegian Food Research Institute, P.B. 50, N-1432 Aas-NLH, Norway

Introduction

A number of methods have been used to obtain the characteristic colour of dry sausages (Karmas, 1977). The methods often take advantage of both pH lowering and the formation of nitric oxide. It seems generally accepted that the colour generated is due to nitrosomyoglobin and nitrosohemoglobin (Fox, 1968; Karmas, 1977). In the production of fermented sausages carbohydrates are used as fermentable substrates in sausage mixes inoculated with starter cultures. During fermentation the production of lactic acid and acetic acid lowers the pH from approximately 6 to 5 (DeKetelaere et al., 1974, Acton et al., 1977). Acid denaturation of myoglobin and hemoglobin has been found to occur in the pH region actual for dry sausages. The denaturation includes unfolding of the globin chains as well as detachment of the heme group (Steinhardt et al., 1963).

Lactic acid bacteria are often used as starter cultures in the production of dry sausages. These are generally catalase negative (Yousten et al., 1975), and peroxide produced during the fermentation process may therefore, in some cases, change the colour as well as decrease the growth of the starter culture (Flowers et al., 1977).

The aim of the present study was to obtain information on the dissociation of nitrosoheme and heme from myoglobin (hemoglobin) in dry sausages. Furthermore, it was of interest to determine the catalase activity of some lactic acid bacteria that have been used as starter cultures in dry sausage production.

Materials and methods.

The dissociation of heme from myoglobin was measured spectrophotometrically by following the decrease in absorbance in the Soret region (Allis and Steinhardt, 1970).

Salami sausages were produced according to the usual formula of the manufacturers, as described by Skjelkvåle et al., (1974). Extraction of the hydrophilic colour components of the sausages was done by homogenizing 10 g sausage with 40 ml of 50 mM potassium phosphate buffer, pH 6.9. The solution was cleared by centrifugation and the supernatant beneath the lipid layer was withdrawn. The extraction was repeated once, and the concentration of the hydrophilic red colour components extracted was measured by scanning the Soret region using a Shimadzu UV-300 spectrophotometer.

The bacteria used were *Lactobacillus plantarum* ATCC 8014, two strains of *Lactobacillus* isolated from commercially available starter cultures, and five strains of lactic acid bacteria isolated from salami sausages produced at five different processing plants. The organisms were cultured aerobically in a medium composed of 1% tryptone, 0.5% yeast extract, 0.2% dextrose, 0.7% K_2HPO_4, and 0.05% myoglobin.

The cultures were harvested by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7) and disrupted by a Branson Sonifier B-12 operated at 150 W for 20 min at 4-8°C. Cell debris was removed by centrifugation.

The number of bacteria in sausages was determined by a standard plate count method on blood agar.

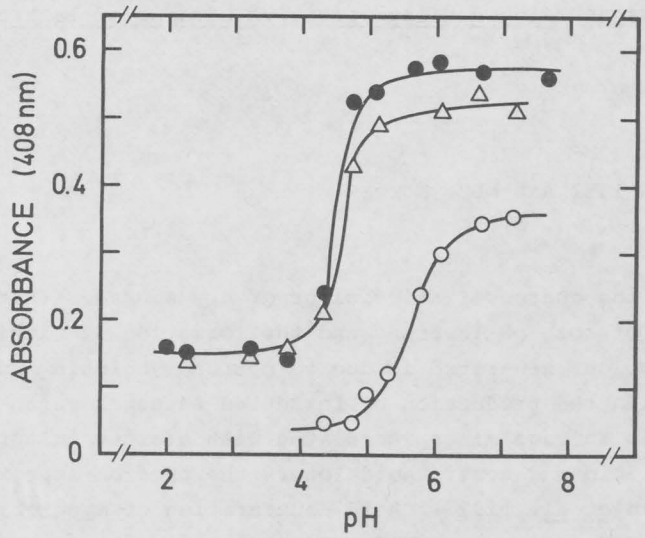


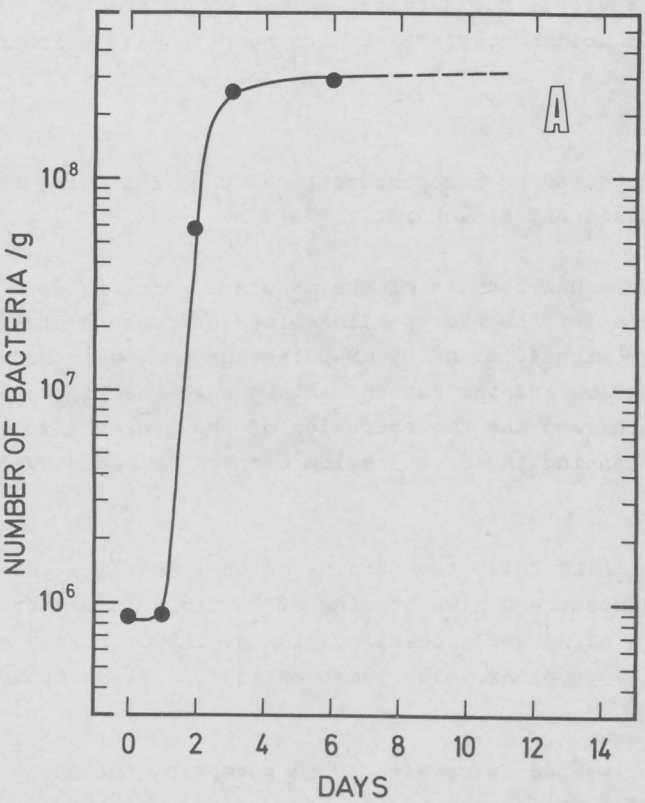
Fig. 1. Absorbance of myoglobin (408 nm) at different pH values in the presence of nitrite (●), ascorbate (Δ) and ascorbate and nitrite (o). The experiments were performed in 50 mM citrate buffers, T = 25°C

Catalase activity was measured as described by Chance and Maehly (1955).

Protein was determined by the method of Lowrey et al., (1951).

Results

The dissociation of heme and nitrosoheme from myoglobin was measured in 50 mM citric acid buffers by scanning the Soret band transition. The change in absorbance at 408 nm was used to evaluate the degree of dissociation. Fig. 1 shows that in the presence of excess nitrite (38 mM, 2500 ppm) or ascorbate (76 mM, 1.3%) the changes are the most pronounced between pH 4 and 5. When ascorbate and nitrite are added together, the dissociation shifted towards higher pH values. A decrease in absorption (colour) indicates dissociation of the heme and nitrosoheme from the myoglobin molecule. When both ascorbate and nitrite are added, a destruction of the heme chromophore is also observed (equal amounts of myoglobin are added in all cases). Due to excess of ascorbate and nitrite added destruction can be observed even at pH values where myoglobin are stable in the presence of only one of the components.



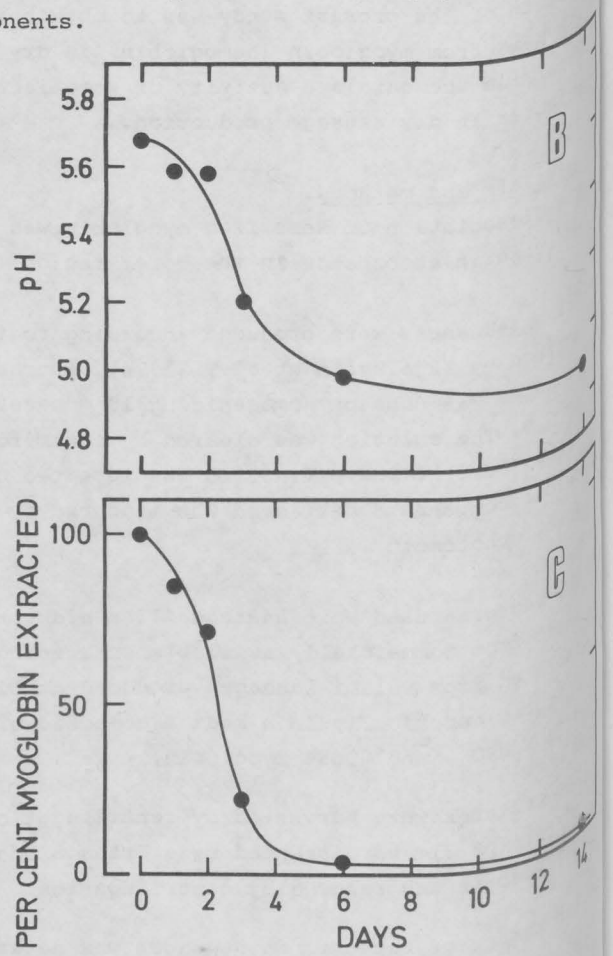


Fig. 2. (A) Growth of lactic acid bacteria in dry sausage during fermentation. (B) Decrease of pH and (C) amount of myoglobin extracted during the fermentation period.

The growth of lactic acid bacteria during fermentation of dry sausages is shown in Fig. 2A. The production of lactic acid and acetic acid lowers the pH (Fig. 2B) of the sausages. Fig. 2C shows the decrease in the amount of hydrophilic colour components extracted during the dry sausage ripening process.

Table I shows the catalase activity of eight strains of lactic acid bacteria grown in the presence of myoglobin. The catalase activity of *Lactobacillus plantarum* ATCC 8014 was used as a reference. Sausages produced with this organism as the starter culture gave acceptable sausages. The bacteria showed low or hardly any catalase activity when grown in the absence of myoglobin.

Table I. Catalase activity of lactic acid bacteria used in dry sausage production when grown in the presence of myoglobin. *Lactobacillus plantarum* ATCC 8014 was used as a reference.

ATCC 8014	100
Strain 1	156
Strain 2	92
Strain 3	121
Strain 4	119
Strain 5	96
Strain 6	76
Strain 7	35

Discussion

In dry sausage production the main precursors of the colour are myoglobin and hemoglobin. Upon addition of ascorbic acid to myoglobin or hemoglobin (Fig. 3) the oxidized forms (metmyoglobin and methemoglobin) are reduced. In the presence of both ascorbic acid and nitrite, nitrosomyoglobin and nitroshemoglobin are formed. Myoglobin, hemoglobin, nitrosohemoglobin, and nitrosomyoglobin all have hydrophilic properties and are therefore soluble in a hydrophilic medium. On the other hand, heme and nitrosoheme are hydrophobic compounds not soluble in a hydrophilic medium. Since the absorbance of heme and nitrosoheme is lower than that of myoglobin, hemoglobin, nitrosomyoglobin, and nitrosohemoglobin, the dissociation of heme or nitrosoheme from the globin chain can be studied as a function of pH as shown in Fig. 1. The acid denaturation of myoglobin shown in Fig. 1 is similar to that found for hemoglobin by Steinhardt et al. (1963) and Allis and Steinhardt (1970). Differential scanning calorimetric studies (unpublished results) used to measure the denaturation of myoglobin were found to parallel the pH profile in Fig. 1.

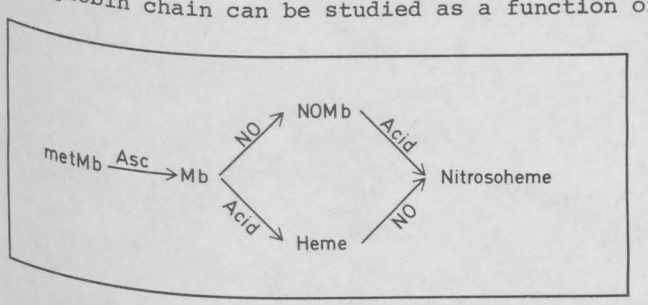


Fig. 3. Probable scheme for colour formation in dry sausage. Mb, myoblobin; Asc, ascorbate.

Since the pH values of dry sausages are comparable to those giving dissociation of heme and nitrosoheme from myoglobin (Fig. 1) studies on the extractability of the colour components in dry sausages were performed (Fig. 2). The bacterial growth (Fig. 2A), decrease in pH (Fig. 2B), and decrease in hydrophilic colour components extracted (Fig. 2C) during the dry sausage ripening process were found to parallel each other. This indicates a change from hydrophilic to hydrophobic colour components. Fig. 3 shows a probable scheme for the colour formation, and it is indicated that the main colour component formed is nitrosoheme. The reactions of importance are: 1) the generation of nitric oxide and 2) the dissociation of nitrosoheme and heme from the myoglobin (hemoglobin) molecule. These are both favoured by low pH.

It has been shown that bovine liver catalase is significantly inhibited by chloride ions in concentrations and at pH values comparable to those found in dry sausages (Litchfield, 1977). Furthermore, peroxide may be formed through the reaction; $MbO_2 + Mb^{2+} \xrightarrow{H^+} 2Mb^{3+} + H_2O_2$ (Giddings, 1977). It is therefore of importance that the lactic acid bacteria used in dry sausage production are able to degrade the peroxide formed. From Table I it is seen that the

bacteria used in dry sausage production all posess catalase activity when grown in the presence of myoglobin. A starter culture should therefore be grown in a medium giving high catalase activity. In the production of dry sausage we have found that strain 1 (Table I) with the highest relative catalase activity as a rule gave the best colour in the final product. The growth of this strain is, however, inhibited by higher concentrations of sodium chloride.

References

Acton, J.C., Dick, R.L., and Norris, E.L. (1977) J. Food Sci., 42, 174-178.

Allis, J.W., and Steinhardt, J. (1970) Biochem., 9, 2286-2293.

Chance, B. and Maehly, A.C. (1955) Methods Enzymol., 2, 764-775.

DeKetelaere, A., Demeyer, D., Vandekerkhove, P., and Vervaeke, I. (1974) J. Food Sci., 39, 297-300.

Flowers, R.S., Martin, S.E., Brewer, D.G., and Ordal, Z.J. (1977) Appl. Environ, Microbiol., 33, 1112-1117.

Fox, J.B. jr., and Ackerman, S.A. (1968) J. Food. Sci. 33, 364-370.

Giddings, G.G. (1977) CRC. Crit. Rev. Food Technol., 9, 81-114.

Karmas, E. (1977) Sausage Products Technology (Food Technol. Rev. No 39) Noyes Data Corp., Park Ridge, New Jersey, USA.

Litchfield, W.J. (1977) FEBS Lett., 83, 281-284.

Lowrey, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J.Biol. Chem., 193, 265-275.

Skjelkvåle, R., Tjaberg, T.B., and Valland, M. (1974) J. Food Sci., 39, 520-524.

Steinhardt, J., Ona-Pascual, R., Beychok, S., and Chien Ho (1963) Biochem., 2, 256-266.

Yousten, A.A., Johnson, J.L., and Salin, M. (1975) J. Bacteriol., 123, 242-247.