NITROSYLMYOGLOBIN FORMATION BY THE ACTION OF *LACTOBACILLUS FERMENTUM*

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Objectives

Cured meat colour is important to consumers for judging meat product quality. Cured meat pigment, nitrosylmyoglobin (NOMb), is formed by reaction of myoglobin in meat with nitric oxide (NO) generated from nitrite. There is concern, however, that carcinogenic nitrosamines may possibly be produced from nitrite in the meat curing process[1]. Thus, substitutes for nitrite and nitrate are eagerly being sought.

Lactobacillus fermentum, a lactic acid bacteria, converts metmyoglobin (MMb) to NOMb in media with no requirement for nitrite[2]. NO is synthesized in mammals, with nitric oxide synthase (NOS) catalyzing the stepwise oxidation of L-arginine to NO and L-citrulline. However, this enzyme has not been found in any bacterial cell.

In this study, ten strains of *L. fermentum* were screened for their capacity to generate NOMb from MMb. The mechanism of MMb conversion to NOMb was studied and assessment was made of NOS activity in bacterial cells.

Experimental Methods

Bacterial strains : *L. fermentum* strains JCM 1560, JCM 2761, IFO 3071, IFO 3956, IFO 3959, NRIC 1047, NRIC 1598, NRIC 1736, NRIC 1952 and NRIC 1955 were used in this study.

MMb conversion capacity to red myoglobin derivatives : The strains were examined for the ability to generate red myoglobin derivatives from MMb in MRS broth (Oxoid) as previously described[3]. MRS broth supplemented with 2.0 mg MMb (Sigma) per ml was termed, MRS-Mb broth.

Red myoglobin derivative detection : To detect MMb, oxymyoglobin (O_2Mb) and NOMb, spectral scans were made from 450 to 650 nm after removing the bacterial cells by centrifugation, and absorption spectra of 75% acetone extracts from MRS-Mb cultures were measured from 350 to 450 nm by the method of Okayama and Nagata[4].

Electron spin resonance (ESR) techniques : Each sample (400μl) was transferred to a ESR tube. ESR spectra were recorded on an ESR spectrometer (JES-TE2X, JEOL Co, Ltd.). Measurement conditions: microwave power, 4mW; modulation frequency and widths, 100kHz and 0.5 or 1.0mT; temperature, 77K; measurement time, 4min.

MMb reduction and NO production by L. fermentum : The experimental systems is depicted in Fig.1. Nitrocellulose tubing of three different molecule permeabilities (Mol. wt.$<$ 3,500, 8,000 and 12,000) was used. In Experiment 1, A_1 (MRS-Mb broth + IFO 3956) surrounded the outside of the tubing in a flask, while B_1 (MRS-Mb broth) was present inside the tubing. In experiment 2, A_2 (MRS-Mb broth + IFO 3956) was outside and B_2 (MRS-Mb broth) inside the tubing. The bacterial strains were inoculated in the outside medium. Myoglobin derivatives were detected after incubation at 37℃ for 20hr.

Measurement of NOS activity : NOS activity was determined by measuring production of nitrite alone or nitrite plus nitrate (stable oxidation products of NO) by the method of Baek *et al.*[5]

Principal Results

Ten strains were found capable of converting MMb to a red myoglobin derivative in MRS-Mb broth. The acetone extract from each red MRS-Mb culture was detected at 395, 476, 535 and 563 nm, this being characteristics of NOMb. Conversion capacity was greatest for *L. fermentum* IFO 3956 and thus it was used in subsequent experiments. ESR of MRS-Mb broth showed a signal of MMb (Fig. 2-A). ESR signals of MRS-Mb culture demonstrated the presence of NOMb and traces of MMb (Fig. 2-B).

A $NOMb^-$ mutant was isolated spontaneously and found incapable of generating NOMb but able to convert MMb to O_2Mb. The formation of O_2Mb is caused by oxygenation of reduced myoglobin. The $NOMb^-$ mutant may possibly be able to reduce MMb but not produce NO.

The results of Experiments 1 and 2 are listed in Table 1. In Experiment 1, IFO 3956 converted MMb on the outside to NOMb (A), while MMb on the inside was converted to O_2Mb (B) and consequently, the passage of solutes less than 8,000 and 12,000 daltons became possible. MMb inside the tubing (Mol. wt. $<$ 3,500) remained unchanged. In Experiment 2, MMb was converted to NOMb inside the tubing (Mol. wt. $<$ 8,000 and 12,000). In Experiment 1, NO may have had a strong affinity for the outside putative reduced myoglobin and thus would have been consumed in the formation of NOMb. When MRS-Mb broth supplemented with L-ascorbate (0.3%) was introduced into the tubing (Mol. wt. $<$ 3,500), the MMb was converted to NOMb during incubation. Without inoculation of IFO 3956, MMb in the tubing was converted to O_2Mb.

IFO 3956 and the NOMb$^-$ mutant showed NOS activity, which was not defective but relatively low in the latter. To our knowledge, this demonstrates for the first time the presence of NO activity in bacterial cells. NO may be generated enzymatically from L-arginine by IFO 3956. Bacterial NOS should be characterized. A manufacturing process of meat product is suggested in Fig. 3.

Conclusions

Lactobacillus fermentum IFO 3956 converted metmyoglobin to nitrosylmyoglobin, showing this strain to reduce metmyoglobin and the bacterial cells to synthesize nitric oxide enzymatically. Constituents from 3,500 to 8,000 daltons in MRS culture may possibly contribute to the reduction of metmyoglobin during incubation.

References

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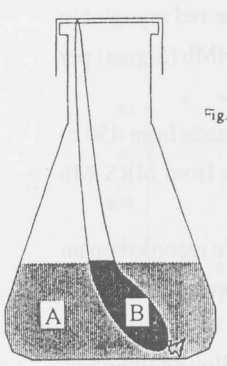

Fig. 1. Experiments using nitrocellulose tubing (Mol. wt. $<$ 3,500, 8,000 or 12,000).
Experiment 1 A_1: MRS-Mb broth + IFO 3956
B_1: MRS-Mb broth
Experiment 2 A_2: MRS broth + IFO 3956
B_2: MRS-Mb broth

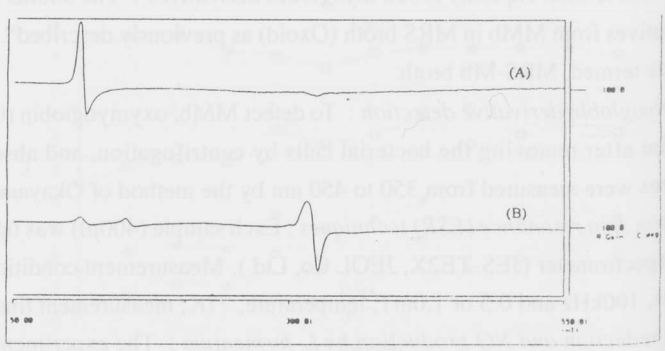

Fig. 2. ESR signals of MRS-Mb broth (A) and MRS-Mb culture of *L. fermentum* IFO 3956 (B).

Table 1. MMb reduction and NO production by *L. fermentum* IFO 3956

IFO 3956	Mol. wt. (Nitrocellulose tubing)	MMb addition	
		Inside	Inside and outside
NOMb$^+$ strain	$<$ 12,000	NOMb	O_2Mb
	$<$ 8,000	NOMb	O_2Mb
	$<$ 3,500	MMb	MMb
NOMb$^-$ mutant	$<$ 12,000	O_2Mb	O_2Mb
	$<$ 8,000	O_2Mb	O_2Mb
	$<$ 3,500	MMb	MMb

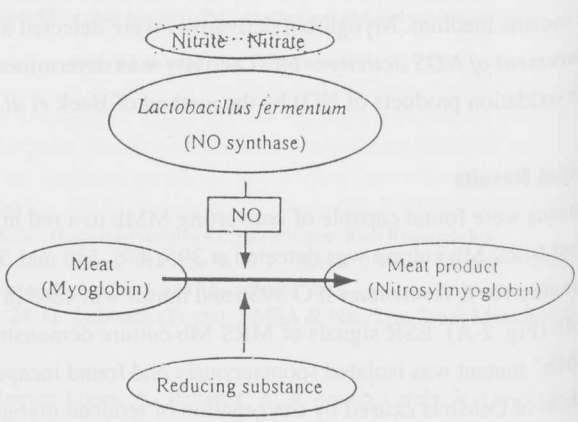

Fig. 3. A conception of advancement in meat technology.