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N-ETHYLMALEIMIDE INFLUENCED THE EVALUATION OF DISULFIDE CROSS-LINKS IN THE OXIDIZED MYOFIBRILLAR PROTEINS USING THE NON-REDUCING SDS-PAGE

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Abstract
The present study aimed to investigate the effect of N-Ethylmaleimide (NEM) on the evaluation of disulfide formation in the oxidized myofibrillar proteins during the sample preparation of the non-reducing SDS-PAGE procedure. For this purpose, extracted myofibrillar proteins were oxidized firstly via a Fenton oxidation reaction, and non-oxidized proteins were used as a control. Before running SDS-PAGE, in the sample preparation, these oxidized and non-oxidized proteins were prepared according to the three different sample preparation methods with or without the presence of N-Ethylmaleimide or β-mercaptoethanol. Results showed that oxidized proteins treated with NEM regardless of sample preparation methods presented attenuated bands of myosin heavy chain monomer in the non-reducing SDS-PAGE gels, suggesting that the disulfide bonds formed as a result of protein oxidation could be preserved by NEM during sample preparation. Meanwhile, a possible mechanism for the effect of NEM was proposed.

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Introduction

Protein oxidation frequently occur during processing and storage of meat and meat products, and leads to impaired meat quality and recued nutritional values [1–3]. Particularly, along with microbial spoilage and lipid oxidation, protein oxidation has been proposed as one of the main reasons for quality deterioration of meat and meat products during processing and storage [4]. However, compared to the other two factors, protein oxidation is easily ignored due to its subtle effect on food flavor or appearance. Until recent decades, increasing attention from meat scientist has been focused on protein oxidation and its consequences on meat quality. Protein oxidation is manifested by modifications of amino acid side chains, peptide fragmentation, and formation of covalent protein cross-linking [5]. Furthermore, protein cross-linking is one of the most important consequences of protein oxidation, which might have important impact on meat quality, for instances, meat tenderness [6–8], water-holding capacity [9] and protein functionalities [10]. Particularly, the disulfide cross-linking has attracted lots of attention, and it has been proposed as the major form of protein cross-links when meat proteins are oxidized [11]. The possible reason could be ascribed to the fact that cysteine is the most susceptible amino acid residue and is usually one of the first to be oxidized, thus leading to the formation of disulfide bonds.

As a routine method, the non-reducing and reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has been commonly used to determine the protein covalent links [11], where protein samples were dissolved in the SDS-PAGE sample buffer with or without 5% of β-mercaptoethanol (MCE) and boiled for several minutes. The presence of MCE in the reducing SDS-PAGE serves as a disulfide bond breaking agent, leading to breakage of all the formed disulfide bonds present in the proteins. On the other hand, these disulfide bonds would remain intact in the absence of MCE. As revealed in the stained SDS-PAGE gels, for samples treated without MCE, protein cross-linking leads to the loss of myosin heavy chain (MHC) and the appearance of cross-linked products with larger molecular weights, whereas the presence of MCE recovers the lost proteins cross-linked via disulfide bonds in the identical samples treated with MCE. Based on these observations, formation of disulfide protein cross-linking as the consequences of treatment related to protein oxidation could be drawn and the role of disulfide cross-linking in affecting meat quality could be further elucidated. However, artifacts might be introduced during the boiling process in the

non-reducing SDS-PAGE since sulfhydryl group is highly susceptible to form disulfide [11]. Therefore, these artifacts formed during sample preparation would cause the overestimation of disulfide bonds, and thus the exaggerate the role of disulfide cross-linking of proteins in affecting meat quality. N-ethylmaleimide (NEM) has been reported to be a specific sulfhydryl blocking agent, and therefore, theoretically be able to prevent disulfide artifacts during sample preparation. Therefore, to solve the aforementioned problem, in the early studies [11–13], NEM was commonly used to prevent the formation of disulfide bonds during sample preparation for SDS-PAGE. However, searching throughout the published literatures, NEM treatment is often ignored in the sample preparation process in many other studies [10,14]. Moreover, it remains unknow whether NEM treatment in the sample preparation can influence the evaluation of the disulfide formation by non-reducing SDS-PAGE analysis, and the inhibition degree of NEM remains unclear.

The present study was designed to examine whether boiling of the protein samples in the non-reducing SDS-PAGE analysis could cause disulfide artifacts of protein cross-links, and whether NEM could inhibit the occurrence of these artifacts. Unexpectedly, the opposite effect of NEM was observed in this study, and the effect of NEM in the sample preparation for the non-reducing SDS-PAGE on the evaluation of disulfide formation in oxidized myofibrillar proteins was further studied.

Materials and methods

Materials

Pig loins were purchased from a local slaughter house 24 h postmortem, and the visible fat and connective tissues were removed. Afterwards, the muscles were cut into small pieces, and stored at minus 40 °C until use. All chemicals used were of reagent grade.

Extraction of myofibrillar proteins

Myofibrillar proteins were extracted according to the method as reported by Takahashi et al. [15]. In short, 5 g of minced meat were washed twice with 5 volumes of 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5), followed by the homogenization in 5 volumes of the same buffer using an Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany), and the homogenate was centrifuged at 3000 ×g for 5 min (4 °C). The obtained pellets washed three times with the above solution, and the final pellet was dissolved and regarded as myofibrillar protein (MFP) suspensions. Finally, to the MFP suspension, a solution of 3 M NaCl, 20 mM Tris-HCl (pH 7.5) was added to give a final NaCl concentration of 0.5 M NaCl. Protein concentrations of the MFP suspensions were measured and then adjusted to the same level of 4 mg/ml.

Protein oxidation

Myofibrillar proteins were oxidized in a Fenton protein oxidation system [16]. 10 mL of MFP suspension (4 mg/mL) were oxidized for 12 h at 4 °C with 10 μmol/L $FeCl_3$, 0.1 mM ascorbic acid, and 1 mmol/L H_2O_2. Oxidation was terminated by the addition of a mixture of oxidation terminators, that is, 1 mM propyl gallate, 1 mM trolox C, and 1 mM EDTA. The non-oxidized group was treated with these terminators only and incubated for 12 h at 4 °C.

Sample preparation for SDS-PAGE

Samples were prepared according to one of the following three different methods.

Traditional method: 0.5 mL of MFP suspensions were mixed thoroughly with 0.5 mL of one of the following different loading buffers: (1) NEM loading buffer: 0.1 M Tris, 4% SDS, 20% glycerol, 4 mg N-ethylmaleimide, 0.2% bromophenol blue, pH 6.8; (2) MCE loading buffer: 0.1 M Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.2% bromophenol blue, pH 6.8; (3) CON loading buffer: 0.1 M Tris, 4% SDS, 20% glycerol, 0.2% bromophenol blue, pH 6.8. Afterwards, the mixtures were incubated for 2 h at room temperature, and boiled for 5 min, followed by centrifugation at 10,000 g for 5 min.

Un-heating method: 0.5 mL of MFP suspensions were mixed thoroughly with 0.5 mL of one of the following different loading buffers: (1) NEM loading buffer: 0.5 M Tris,16 M Urea, 4% SDS, 4 mg N-ethylmaleimide, 0.4% bromophenol blue, pH 7.5; (2) MCE loading buffer: 0.5 M Tris, 16 M Urea, 4% SDS, 10% 2-mercaptoethanol, 0.4% bromophenol blue, pH 7.5; (3) CON loading buffer: 0.5 M Tris,16 M Urea, 4% SDS, 0.4% bromophenol blue, pH 7.5. Afterwards, the mixtures were incubated for 24 h at room temperature, and centrifuged at 10,000 g for 5 min.

TCA method: For the NEM sample, 1 mL of MFP suspension was added with 4 mg NEM, and incubated for 2 h at room temperature. For the MCE and CON samples, the MFP suspensions were added without NEM, and incubated at the same situation. After incubation, 100 μL of 100% trichloroacetic acid (TCA) was added, and incubated in ice for 10 min, followed by centrifugation at 1200 g for 10min. Subsequently, 2 mL of acetone was added to the pellet and centrifuged at 1200 g for 10min. The obtained pellets were dried in the air, and dissolved in 30 μL 0.2 M NaOH. The dissolved samples were then mixed with the loading buffer of 0.25 M Tris, 8 M Urea, 2% SDS, pH 7.5 for NEM and CON treatment, and with the loading buffer of 0.25 M Tris, 8 M Urea, 2% SDS, 5% 2-mercaptoethanol, pH 7.5 for MCE treatment. Afterwards, the mixtures were incubated for 24 h at room temperature, and centrifuged at 10,000 g for 5 min.

SDS-PAGE

10 μL of supernatants were loaded for SDS-PAGE analysis with an electrophoresis system consisting of a continuous 4–20% polyacrylamide gel. Gels were stained with 0.1% coomassie brilliant blue R250 in 45% methanol and 10% acetic acid and then destained with 10% methanol and 10% acetic acid. The gels were captured with ChemiDocTM Imaging System (Biorad, Hercules, CACalifornia, USA).

Results and Discussion

Disulfide protein cross-links of myosin heavy chain (MHC) has been considered as one of the major protein oxidation consequences during meat processing and storage, which show the most important implications in meat quality. The non-reducing and reducing SDS-PAGE have been commonly applied to examine the disulfide protein cross-links [11]. In this technique, extracted myofibrillar proteins are treated with or without MCE, respectively. As a result, the presence of MCE would break the disulfide bond of the oxidized proteins, mainly MHC, whereas the protein samples without MCE treatment would show attenuated MHC bands. However, boiling process is usually used in the sample preparation of SDS-PAGE, and high temperature might introduce disulfide formation and the results would be interfered. Therefore, NEM has been introduced to block the thiol group and prevent the formation of disulfide bonds [11,12,17]. On the other hand, NEM treatment is often ignored in the sample preparation process in many other studies. In the present study, the effect of NEM treatment on the prevention of disulfide formation during the boiling process in the SDS-PAGE procedure was firstly studied. It is expected that NEM could prevent the formation of protein cross-linking, but it did not. Subsequently, different alternative methods for sample preparation were compared to minimize the formation of disulfide artifacts formation.

In this study, both the oxidized myofibrillar proteins via the Fenton oxidation and the non-oxidized ones were used for the SDS-PAGE analysis. These two different protein solutions were boiled in the presence of either NEM, MCE or none during sample preparation. Compared to the non-oxidized myofibrillar proteins, the oxidized proteins showed an attenuated MHC band along with the appearance of a dimer MHC product in the CON sample where NEM and MCE were not present (Figure1), suggesting that covalent cross-linking of MHC due to the oxidation incubation process or, at least partially due to the boiling process in the sample preparation. Furthermore, when the oxidized proteins were treated with MCE, these reduced MHC band was almost recovered, indicating these covalent protein cross-links were mainly attributed to the disulfide formation. This traditional sample method for SDS-PAGE has been commonly applied to detect the disulfide cross-linking during protein oxidation. However, in this traditional method, 5-min of boiling process was applied, which might cause the oxidation of thiol group and thus lead to the formation of disulfide cross-links [11], thus contributing to the attenuated MHC band in the CON sample. Therefore, this artifact might lead to the overestimation of the disulfide formation in the process of protein oxidation. Theoretically, NEM could inhibit the disulfide formation due to its ability to block the thiol group, and thus it was used to treat the samples before non-reducing SDS-PAGE analysis in the present study. It was expected that NEM could prevent the formation of disulfide artifact. However, as revealed in Figure 1, it was shown that the NEM treatment led to more attenuated MHC bands than the CON treatment present in the gels. Meanwhile, in the non-oxidized proteins, slight increase in the density of dimer MHC band in the NEM ample was also observed compared to that of the CON sample. Though the results were unexpected, we then speculated that the high temperature and excess of NEM or the oxidation terminators might be the reasons for the increased cross-linking of MHC in NEM treated oxidized myofibrillar proteins. In this sense, these speculations were testified in the following experiments.

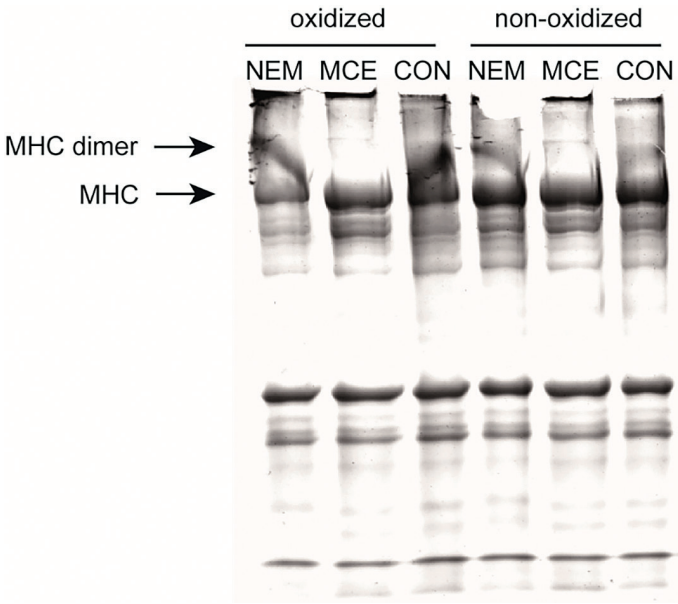

Figure 1. SDS-PAGE patterns of non-oxidized and oxidized myofibrillar proteins prepared by the traditional method.
Note: NEM, in the presence of N-ethylmaleimide during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation

Base on the previous trial, the enhanced MHC covalent cross-links might be attributed to some unknown cross-linking reactions induced by high temperature of the boiling process. Therefore, a non-heating sample preparation process was examined where Urea was included in the sample buffer to denature proteins at room temperature. As shown in Figure 2, similar to the traditional method, a weaker MHC band was observed in CON sample compared to the MCE sample for oxidized myofibrillar proteins, while no such observations were found for non-oxidized proteins. However, the NEM treated oxidized proteins presented even weaker bands than the CON treatment of the oxidized proteins, suggesting more covalent bonds were still formed due to the presence of NEM. Therefore, high temperature in the traditional method seems not to be reason for the increased cross-linking of MHC in oxidized myofibrillar proteins during sample preparation.

In the next trial, to exclude the interference of excess NEM or the oxidation terminators on the formation of MHC cross-linking during sample preparation process, TCA was used to precipitate the proteins after the NEM treatment. As shown in Figure 3, though the NEM sample

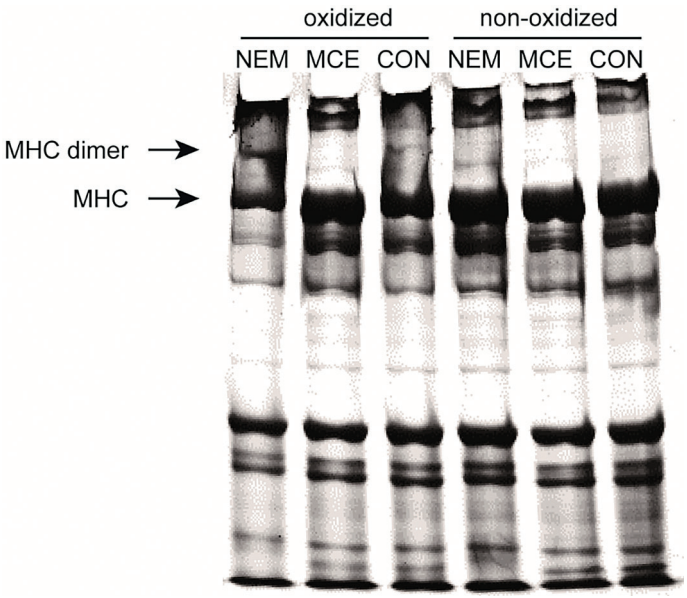

Figure 2. SDS-PAGE patterns of non-oxidized and oxidized myofibrillar proteins prepared by non-heating method. Note: NEM, in the presence of N-ethylmaleimide during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation

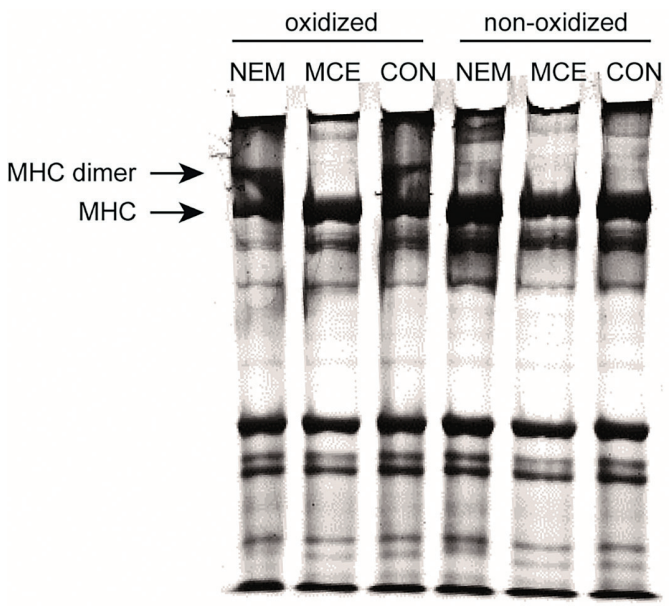

Figure 3. SDS-PAGE patterns of non-oxidized and oxidized myofibrillar proteins prepared by TCA method. Note: NEM, in the presence of N-ethylmaleimide during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation

had similar intensity of MHC band to that of the CON sample, but a more stronger dimer MHC was shown, suggesting increased formation of MHC cross-linking occurred in samples treated with NEM. Therefore, this finding indicated that the excess of NEM or the oxidation terminators were not neither the reason for the diminished MHC bands in the non-reducing SDS-PAGE gels of NEM treated samples. Interestingly, a previous study has reported that whey protein exhibited an increase in the disulfide crosslinking in the SDS-PAGE gels after incubation with NEM for a period of time, and the authors has pointed out that NEM could somehow promote protein cross-linking though it is most extensively used to block the thiol groups and thus to prevent the disulfide formation [18]. Furthermore, a possible explanation was proposed that the formation of covalent crosslinks through non-natural amino acids due to the presence of NEM, as in lysinoalanine [19]. Such cross-links cannot be cleaved with MCE, and therefore should be presented as dimer MHC in reducing SDS-PAGE gels. However, as illustrated in Figure 4, the formed dimer MHC band (lane "NEM") as a result of NEM treatment disappeared (lane "M+N") when MCE was included at the same time in the sample buffer, indicating that the formation of dimer MHC band due to NEM treatment could be attributed to the disulfide type of protein cross-linking.

Covalent disulfide cross-linking of protein molecules can be brought by sulfhydryl oxidation into disulfide bonds and/or sulfhydryl group/disulfide bond interchange reaction [20]. In a previous study, the disulfide cross-link between two polypeptides of rhodopsin was broken in preparation for non-reducing SDS-PAGE and a new disulfide bond was re-formed in one of the subunits of rhodopsin via the sulfhydryl group/disulfide bond interchange reaction,

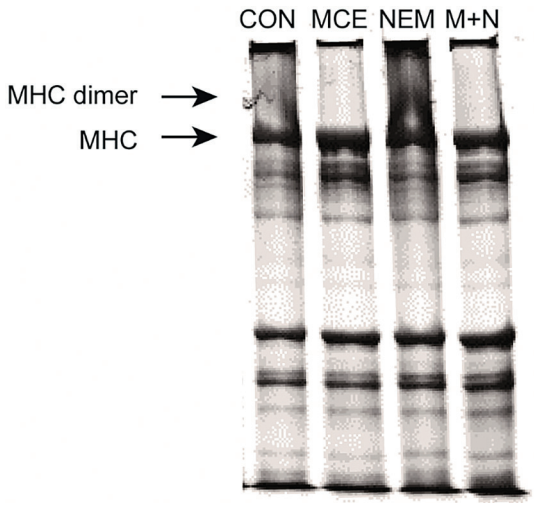

Figure 4. SDS-PAGE patterns of oxidized myofibrillar proteins prepared by the TCA method. Note: CON, neither N-ethylmaleimide nor β-mercaptoethanol were present during sample preparation; MCE, in the presence of β-mercaptoethanol during sample preparation; NEM, in the presence of N-ethylmaleimide during sample preparation; M+N, in the presence of both β-mercaptoethanol and N-ethylmaleimide

however, the presence of NEM could prevent the occurrence of these events due to its blocking of sulfhydryl group of an adjacent cysteine residue in this subunit [21]. Along with this observation, a possible mechanism was proposed to explain why oxidized myofibrillar proteins treated with NEM exhibited higher degree of disulfide cross-linking of MHC in SDS-PAGE gels under non-reducing conditions, as observed in the present study. (a): In oxidized myofibrillar proteins, an MHC dimer is formed through disulfide cross-linking between two MHC monomers during Fenton oxidation reaction; (b): In the sample preparation for SDS-PAGE, upon protein denaturation either by

the boiling treatment or the non-heating Urea treatment, a sulfhydryl group of an adjacent cysteine residue in one of the MHC monomer reacts with the disulfide bond, and thus a sulfhydryl group/disulfide bond interchange reaction occurs, leading to the broken of the already formed disulfide bond and the formation of a new intramolecular disulfide bond. Consequently, more MHC monomers and less MHC dimers will appear in the non-reducing SDS-PAGE gels; (c): In contrast, when the oxidized proteins are treated with NEM during sample preparation, the sulfhydryl group of that adjacent cysteine is modified by NEM. Therefore, the sulfhydryl group/disulfide bond interchange reaction described in (b) will not happen, and the disulfide bond is preserved. As a result, more MHC dimers will be observed in the non-reducing SDS-PAGE gels accompanied by less amounts of MHC monomers.

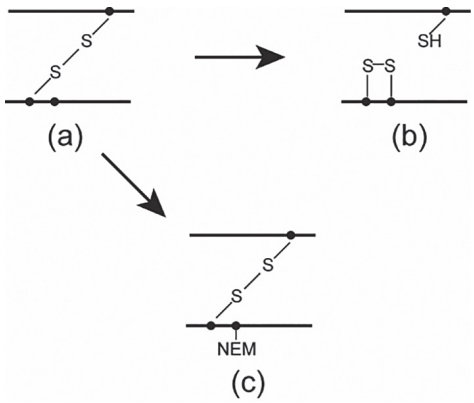

Figure 5. Possible role of NEM in the prevention of cleavage of disulfide bonds in the dimer MHC during sample preparation for SDS-PAGE. Notes: (a), MHC dimers formed as a result of protein oxidation; (b), broken of disulfide bonds due to protein denaturation in the sample preparation for SDS-PAGE; (c), MHC dimers after sample preparation in the presence of NEM; a dot indicates a cysteine residue; a line indicate a monomer MHC

Conclusion

In conclusion, the present study was designed to investigate the effect of NEM on the evaluation of disulfide formation in oxidized myofibrillar proteins during the sample preparation of the non-reducing SDS-PAGE procedure. The results demonstrated that inclusion of NEM could lead to more cross-linked MHC in the non-reducing SDS-PAGE gels, indicating that the disulfide bonds formed as a result of protein oxidation could be preserved during sample preparation. On the other hand, it is speculated that when treated without NEM, these formed intermolecular disulfide bonds might be broken, which can lead to underestimation of protein cross-linking via disulfide formation. Therefore, it is suggested to include NEM in the sample preparation for SDS-PAGE analysis in the studies that examine the formation of disulfide protein cross-linking in myofibrillar proteins and its consequence in meat quality. In addition, whether the boiling process in sample preparation for non-reducing SDS-PAGE could introduce disulfide artifacts were not testified in this study. However, considering the presence of these oxidation terminators, the oxidation of sulfhydryl groups into disulfide bonds might not occur or develop to a great extent.

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All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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