Effect of CaCl\textsubscript{2} on the stability and antimicrobial activity of nisin

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Abstract. Nisin is a natural and safe biological preservative which has great potential in cosmetics and food industry. However, the low thermostability of nisin at high pH limits its commercial applications. In this study, the effect of CaCl\textsubscript{2} on the stability and antimicrobial activity of nisin was studied. The results showed that CaCl\textsubscript{2} has a strong protective effect on nisin at weak acid environment (pH of 4.0-6.0) and heat treatment. The results of X-ray diffraction (XRD) and scanning electron microscopy (SEM) showed that the crystalline particles of nisin increased size and decreased peak and a stable structure was formed by CaCl\textsubscript{2} with nisin. The thermogravimetric analysis (TGA) presented that CaCl\textsubscript{2} led to the temperature of initial (T\textsubscript{5}) and complete decomposition (T\textsubscript{95}) of nisin increased by 8 \textdegree C and 13 \textdegree C, respectively, and enhanced the thermostability of nisin. The result of Fourier-transform infrared spectroscopy (FTIR) confirmed that CaCl\textsubscript{2} could promote the formation of hydrogen bonds and increase the stability of proteins. The changes of secondary structure of nisin with CaCl\textsubscript{2} was revealed by circular dichroism (CD) spectroscopy. At pH 4.0, the proportion of α-helix, β-sheet and β-turn were same as to that of pH 2.0 without CaCl\textsubscript{2}. At pH6.0, the proportion of secondary structure changed a little by heat treatment. This indicated that the addition of CaCl\textsubscript{2} protected the secondary structure of nisin.

1. Introduction
Nisin could effectively inhibit the growth of Gram-positive bacteria (such as Bacillus subtilis and Clostridium), and therefore it was widely used in food preservation industry [1]. As a natural antimicrobial peptide, nisin is human non-toxicity and easily hydrolyzed by proteases and carboxypeptidases [2]. Nisin could prevent food from spoiling and without changing the sensory and flavor characteristics of it [3]. Thus, nisin has a wide range of applications and development in food industry.

However, the vulnerable to loss of antimicrobial activity in neutral or alkaline pH environment limited its application [3]. Previous studies demonstrated that the double bond of the unsaturated amino acid could bind nucleophilic substances in neutral or alkaline pH [4] and the force of the molecule led the polymerization of nisin and therefore weaken its antimicrobial activity [5]. Lots of evidences indicate that dehydroalanine (Dha), which is unusual amino acid residues of nisin, is involved in all degradation products of nisin [6]. And in particular, modification of Dha at position 5 (Dha5) leads to a severe loss
of antimicrobial activity [6]. These previous studies indicated that the integrity of unsaturated amino acids was an important factor for the chemical stability of nisin.

It was believed that the unsaturated amino acids residues were easily affected by the heat treatment and high pH environment [5-7]. Previous studies have shown that Ca$^{2+}$ could covalently linked to the side chains of some amino acid residues, such as Asp and Glu, and thus enhanced the stability and activity of proteins [8]. Therefore, the present study will focus on the effect of CaCl$_2$ on the thermal stability and antimicrobial activity of nisin. X-Ray Diffraction (XRD), scanning electron microscopy (SEM), Thermogravimetric Analysis (TGA), Fourier-Transform Infrared Spectroscopy (FT-IR) and Circular Dichroism (CD) were conducted to reveal the protection mechanism of CaCl$_2$ on nisin. The results of this study could be help to increase the thermal stability and antimicrobial activity of and broad the application of nisin during food processing.

2. Materials and methods

2.1. Strains and culture media

*Sarcina lutea* (TCCC12002), which was provided by the Strain Collection Center of Tianjin University of Science and Technology, was used as indicator bacteria for the determination of nisin titer. LB medium contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl with the pH adjusted to 7.0, sterilized at 121°C for 20 min.

Titer detection medium contained 10 g/L Tween 20, 8 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 5 g/L glucose, 2 g/L Na$_2$HPO$_4$ and 16 g/L agar with the pH adjusted to 6.8, sterilized at 121°C for 20 min.

2.2. Preparation of antibiotic nisin solution

50 mg of nisin powder was dissolved in 50 mL PBS (0.01 mol/L). The pH of solution was adjusted by HCl (1.0 mol/L) or NaOH (1.0 mol/L). The solution was vortex mixed and incubated for 30 min at room temperature.

The obtained sample was used as the blank control. 0.832 g CaCl$_2$ was added into the solution of nisin and then subjected to a heat treatment (20 min, 121°C) and was diluted into 250 times and 500 times with PBS (0.01 mol/L). Nisin (10$^6$ IU/mg) used in this study was purchased from Sigma-Aldrich Company (Shanghai, China).

The above sample was freeze dried to yield a powder for subsequent analysis.

2.3. Determination of nisin titer

A modified agar diffusion method was used to determine titer of nisin [9]. The 100 μL nisin solution (the 2 mg/ml nisin solution was diluted 300 times and 600 times by 0.02 mol/L HCl, that was, high and low metering nisin solution) was added to the hole of solid petri dish containing indicator bacteria suspension, and then cultivated at 30°C for 24 h.

The bacteriostatic circle diameter was measured and used to calculate the titer of nisin by using the following formula:

$$\log \frac{C_{SH}}{C_{BH}} = \frac{(X_{SH} + X_{SL}) - (X_{BH} + X_{BL})}{(X_{SH} + X_{BH}) - (X_{SL} + X_{BL})} \times \log k$$

where $k$: high and low dose dilution ratio ($k = 2, \log 2 = 0.301$); $C_{SH}$: sample solution titer (IU/mg); $C_{BH}$: standard solution titer, the unit is international unit per milligram (IU/mg, diluted titer); $X_{SH}$: high-dose sample solution bacteriostatic circle diameter (mm); $X_{SL}$: diameter of the inhibition zone of the low-dose sample solution (mm); $X_{BH}$: high-dose standard solution bacteriostatic circle diameter (mm); $X_{BL}$: diameter of the zone of inhibition of the low-dose standard solution (mm).
2.4. **Characterization of nisin**

The freeze dried samples were sputtered with a gold-palladium mixture for 50 seconds under vacuum, and observation was performed on JSM-5610LV (Tokyo, Japan) with a working voltage of 25 kV. The FT-IR spectra was recorded on a MAGNA-560 infrared spectrometer (NICOLET USA). Sample was prepared by mixing lyophilized nisin sample with KBr and the spectra were recorded with a light source in the range of 4000–400 cm\(^{-1}\). Nisin crystal phase was investigated by XRD analysis. XRD spectra was collected by Philips X’Pert Pro Super diffractometer with Cu-K\(\alpha\) radiation (\(\lambda = 1.54178\ \text{Å, } 2\theta = 10°–70°\)). The weight losses of samples were analyzed using a thermogravimetric analyzer (TGA, Model TA-TGA Q-500, TA Instrument, New Castle, DE, USA). Samples (ca. 3 mg) were heated from 25 to 750 °C with a constant heating rate of 10 °C·min\(^{-1}\) under nitrogen atmosphere. Circular dichroism (CD) spectra was recorded from 260 nm to 190 nm using a MOS 450 spectropolarimeter (Shimadzu, Tokyo, Japan). The temperature was controlled by an ice bath, the concentration was reduced by 10 times, the other same as 2.3.

3. **Results and discussion**

3.1. **Effect of CaCl\(_2\) on nisin antimicrobial activity**

As shown, the titer of nisin decreased 0.00%, 5.60% and 8.78%, respectively, with the increase of pH from 2.0 to 6.0 at 25°C (Fig. 1). Meanwhile, when the temperature was risen to 121°C, the titer of nisin was decrease dramatically. The loss of antimicrobial activity was 25.19% and 56.17% at pH 4.0 and 6.0, respectively. However, when the CaCl\(_2\) was added, the loss of antimicrobial activity of nisin was obviously reduced. The loss of antimicrobial activity of nisin with CaCl\(_2\) dropped from 25.19 to 14.16% and 56.17 to 29.29% at pH 4.0 and 6.0, respectively, compared with that of nisin without CaCl\(_2\). Under heat treatment (121°C), CaCl\(_2\) showed a significant positive effect on nisin. The loss of antimicrobial activity of nisin with CaCl\(_2\) reduced 11.03% and 26.88%, respectively.

![Figure 1. Titer loss rates of nisin with and without CaCl\(_2\) at different pH (2.0, 4.0 and 6.0) and temperature (25 and 121°C).]
As reported, the antimicrobial activity and stability of nisin are closely related to the structure and can be altered by pH. Nisin is more stable at lower pH than higher [10]. The results of our study indicated that the antimicrobial activity of nisin was minimally affected by heat at low pH (2.0). And the antimicrobial activity of nisin during heat treatment was dropped rapidly when the pH raised to 4.0 and 6.0 (Fig. 1). Previous studies demonstrated that the protein was usually protonated at low pH, and which was benefit for maintaining the original conformation of the protein [8]. However, with the increase of the pH, the protonation of nisin was reduced and the polymerization and loss of antimicrobial activity of nisin was occurred antimicrobial [10].

The antimicrobial activity of nisin was obviously altered by the addition of CaCl₂ (Fig. 1). The antimicrobial activity loss rate of nisin with CaCl₂ was lower than that of without CaCl₂. The protective effect of CaCl₂ was more prominent during the heat treatment. The antimicrobial activity loss rate of nisin with CaCl₂ was 11.03% lower than that of nisin without CaCl₂ at pH 4.0. The protective effect of CaCl₂ was more pronounced at pH 6.0, while the loss rate of antimicrobial activity was reduced 26.88% compare to without CaCl₂. As shown in Fig. 2, the diameter of the inhibition zone of nisin with CaCl₂ was obviously larger than that of without CaCl₂ under different pH. The bacteriostatic zone diameter of the nisin with CaCl₂ were not change with pH, and its loss of antimicrobial activity was less than that of without CaCl₂ after heat treatment. These results indicated that CaCl₂ had a positive effect on the thermal stability and antimicrobial activity of nisin.

![Figure 2](image)

**Figure 2.** Inhibition zones of nisin with and without CaCl₂ at different pH (2.0, 4.0 and 6.0) and temperature (25 and 121°C).

3.2. **Effect of CaCl₂ on the morphology of nisin**

The morphology of microparticles were observed by SEM (Fig. 3). The surface of nisin was rougher and uneven. Due to the presence of CaCl₂, the tightly cross-linked structure of nisin was formed and the size was larger than that of without CaCl₂. The previous study showed that CaCl₂ was compatible with proteins because of the electrostatic interactions between Ca²⁺ and nisin [11], and it could bind into some amino acid residues of protein to stabilize protein conformation. This could effectively inhibit the hydrolysis of protein [11–13]. Thus, the results of SEM showed that CaCl₂ was help to maintain the stability of nisin.
Figure 3. Effect of CaCl$_2$ on the morphology of nisin. (a): Lyophilized nisin without CaCl$_2$ (scale bar = 10 μm); (b): Lyophilized nisin with CaCl$_2$ (scale bar = 10 μm).

3.3. Effect of CaCl$_2$ on the thermal stability of nisin.
The thermal stability of nisin with and without CaCl$_2$ was investigated by TGA analysis (Table 1). $T_5$, $T_{50}$ and $T_{95}$ obtained from the TGA curve represent the temperatures of 5%, 50% and 95% mass loss during pyrolysis, respectively. The results showed that the $T_5$ and $T_{95}$ of nisin decreased from 136°C to 127°C and from 681°C to 645°C, respectively, with the pH changed from 2.0 to 6.0 (Table 2).

| Sample          | $T_5$ (°C) | $T_{50}$ (°C) | $T_{95}$ (°C) | $T_d$ (°C) |
|-----------------|------------|---------------|---------------|------------|
| Nisin-pH 2.0    | 136        | 222           | 681           | 162        |
| Nisin-pH 4.0    | 129        | 218           | 668           | 155        |
| Nisin-pH 6.0    | 127        | 216           | 645           | 136        |
| Nisin+CaCl$_2$-pH 4.0 | 137 | 220           | 676           | 160        |
| Nisin+CaCl$_2$-pH 6.0 | 140 | 226           | 678           | 178        |

Note: $^a$ the temperatures of 5% mass loss; $^b$ the temperatures of 50% mass loss; $^c$ the temperatures of 95% mass loss; $^d$ the main decomposition peak temperatures.

Table 2. Effect of CaCl$_2$ on the typical FT-IR peaks of nisin.

| Peak wave number (cm$^{-1}$) | Characteristic peaks | Reference |
|------------------------------|----------------------|-----------|
| pH 2.0 | pH 4.0 | pH 6.0 | pH 4.0-CaCl$_2$ | pH 6.0-CaCl$_2$ |          |
| 3395 | 3397 | 3421 | 3392 | 3406 | O-H stretching vibration peak | [17] |
| 2932 | 2932 | 2935 | 2929 | 2927 | Moved to the right of 5 | [17] |
| 1624 | 1629 | 1633 | 1627 | 1630 | Moved to the right of 3 | [17] |
| 1232 | 1236 | 1260 | 1229 | 1246 | Moved to the right of 14 | [18] |
| 1033 | 1033 | 1053 | 1015 | 1032 | Moved to the right of 18 | [17] |

Note: The peak wave number (cm$^{-1}$) of nisin with and without CaCl$_2$ at different pH (2.0, 4.0 and 6.0).
The decrease of $T_3$ and $T_{95}$ verified the negative effect of increasing pH on the thermal stability of nisin. A similar observation has been reported previously, where Hurst reported that there was no loss of nisin activity after 15 minutes at 121°C, at pH 2.0, but there was 40% loss of activity at pH 5.0 and 90% loss at pH 6.8 [14]. Retention of nisin activity after heating for 15 min at 121°C is highly dependable on pH [5].

With the addition of CaCl$_2$, the $T_3$ of nisin was 137°C and 140°C at pH 4.0 and 6.0, respectively, and the main decomposition peak temperatures (Tp) of nisin with CaCl$_2$ was 160°C and 178°C, respectively, which were obviously higher than that of the without CaCl$_2$, and so as to $T_{95}$. Meanwhile, the temperature of $T_{95}$ of nisin with CaCl$_2$ was also increased from 668°C to 676°C at pH 4.0 and from 645°C to 678°C at pH 6.0. Those results suggested that the addition of CaCl$_2$ could increase the thermal decomposition temperature of nisin and enhance its thermal stability.

3.4. Effect of CaCl$_2$ on the FT-IR of nisin

Table 2 showed the FT-IR spectral peak changes of nisin with and without CaCl$_2$ at different pH. The FT-IR spectrum of nisin showed characteristic absorption bands at 3395 cm$^{-1}$ (O−H stretching vibrations), 2932 cm$^{-1}$ (C−H stretching vibrations), 1624 cm$^{-1}$ (COO-asymmetric stretching vibrations), 1232 cm$^{-1}$ (O−H stretching vibrations) and 1033 cm$^{-1}$ (C−O−C stretching vibration) [15–17]. It could be found that the wave number of C−O−C declined from 1033 and 1053 cm$^{-1}$ to 1015 and 1032 cm$^{-1}$, and the wave number of O−H declined from 3397 and 3421 cm$^{-1}$ to 3392 and 3406 cm$^{-1}$ respectively, at pH 4.0 and pH 6.0 with the addition of CaCl$_2$, which could be due to the increase of hydrogen bonding of nisin [15,17]. Similar observations have been reported, where Hosseini et al. entrapped nisin with sodium alginate (Alg) and alginate-resistant starch (Alg-RS) microparticles, and the addition of resistant starch to alginate formulation caused a decrease in the wave number O−H from 3430 to 3411 cm$^{-1}$. These changes are consistent with an increase in the hydrogen bonding of the particles [15]. These results further enhanced our original assumptions that the addition of CaCl$_2$ protected the original molecular structure and activity groups of nisin and increased its spatial structure stability [18,19].

3.5. Effect of CaCl$_2$ on X-ray diffraction of Nisin.

The X-ray diffraction pattern of nisin with and without CaCl$_2$ was shown in Fig. 4. At 25°C, the crystallization peaks of CaCl$_2$ were at $2\theta$=14.82°, 19.28°, 20.68°, 21.18°, 29.52°, 32.02°, 32.16°, 43.02° (Fig. 4a) [20]. Meantime, a remarkable peak at 31.7–31.8° of nisin was detected (Fig. 4b). This corresponding to the characteristic diffraction pattern of sodium chloride (NaCl), which was a component of nisin [21].

The research we have done suggests that Ca$^{2+}$ bound to the small molecule peptide of nisin, and it caused the change of protein conformation at pH4.0 (Fig. 4c). At this point, based on the weakening of the characteristic peak of nisin in Fig. 6b, and it can be inferred that nisin as small molecule peptides combined with Ca$^{2+}$ by ionic interactions, thus which promoting the compatibility of the two substances and disrupting the polymerization of nisin (Fig. 4c) [15]. When the two substances were combined, the characteristic peak of CaCl$_2$ was largely absent and the characteristic peak of nisin at positions 3, 4 and 5 were wider (Fig. 4c), the amorphous structural features were evident, which was caused by the excessive amount of nisin. These results were similar to those obtained by Bastarrachea et al., where the intensity of the crystalline peaks decreased due to the increase of nisin concentration. The particles exhibit an amorphous structure when the concentration of nisin reaches 5000 IU/cm$^2$. It was concluded that nisin as a foreign substance disrupts the formations of polymorphic crystals [22].

At pH 6.0, the intensity of the characteristic peak of nisin with CaCl$_2$ at the same position was weaker than that of pH 4.0. The kurtosis at positions 3, 4, and 5 became wider, and positions 4 and 5 basically disappeared (Fig. 4d). This suggested that the increased of pH made the binding of Ca$^{2+}$ and nisin more tightly due to ion-to-protein interactions and electrostatic interactions [18,19].

As shown in Fig. 4e and Fig. 4f, it can be seen that the characteristic peaks and the amorphous peaks of nisin with CaCl$_2$ did not change significantly. There were no new peaks generated after heat treatment.
at weak acid environment (pH of 4.0−6.0). It was shown that the stable structures of nisin with CaCl\textsubscript{2} was not destroyed by heat treatment, which suggested that CaCl\textsubscript{2} could effectively protect the stable conformation of nisin.

![Figure 4](image_url)

**Figure 4.** Effect of CaCl\textsubscript{2} on X-ray diffraction spectra of nisin. (a): CaCl\textsubscript{2}, 25°C treatment; (b): nisin, 25°C treatment; (c): nisin with CaCl\textsubscript{2}, pH 4.0, 25°C treatment; (d): nisin with CaCl\textsubscript{2}, pH 6.0, 25°C treatment; (e): nisin with CaCl\textsubscript{2}, pH 4.0, 121°C treatment; (f): nisin with CaCl\textsubscript{2}, pH 6.0, 121°C treatment.

3.6. Effect of CaCl\textsubscript{2} on nisin CD

The previous study suggested that the interaction between Ca\textsuperscript{2+} with Asp and Glu residues could modify the secondary structures of protein and enhance the stability of nisin [23]. Therefore, the effect of CaCl\textsubscript{2} on the structure of nisin was investigated by CD [24].

As shown, the \(\alpha\)-helix was not detected by CD at 25°C and different pH, (Table 3). The proportion of \(\beta\)-sheet, \(\beta\)-turn and irregular curl of nisin was 10.80%, 28.40%, 60.80%, respectively, at pH 2.0, and there were no changed when the pH increased to 4.0. At pH 6.0, the proportion of \(\beta\)-turn and \(\beta\)-sheet increased to 28.90% and 11.80%, and the proportion of irregular curl decreased from 60.80% to 59.30%.
However, when CaCl$_2$ was added at pH 6.0, the proportion of $\beta$-sheet, $\beta$-turn and irregular curl of nisin was 10.80%, 28.40%, 60.80%, respectively, which was same as to that of pH 2.0 without CaCl$_2$. These results indicated that the addition of CaCl$_2$ was help to keep the secondary structure of nisin free of pH influences.

| Sample                      | $\alpha$-Helix | $\beta$-Sheet | $\beta$-Turn | Irregular curl |
|-----------------------------|----------------|---------------|---------------|---------------|
| Nisin (pH 2.0)-25°C         | 0.00%          | 10.80%        | 28.40%        | 60.80%        |
| Nisin (pH 4.0)-25°C         | 0.00%          | 10.80%        | 28.40%        | 60.80%        |
| Nisin (pH 6.0)-25°C         | 0.00%          | 11.80%        | 28.90%        | 59.30%        |
| Nisin+CaCl$_2$ (pH 4.0)-25°C| 0.00%          | 10.80%        | 28.40%        | 60.80%        |
| Nisin+CaCl$_2$ (pH 6.0)-25°C| 0.00%          | 10.80%        | 28.40%        | 60.80%        |
| Nisin (pH 2.0)-121°C        | 0.00%          | 10.80%        | 28.40%        | 60.80%        |
| Nisin (pH 4.0)-121°C        | 1.80%          | 36.80%        | 29.80%        | 31.60%        |
| Nisin (pH 6.0)-121°C        | 4.80%          | 38.20%        | 30.80%        | 26.20%        |
| Nisin+CaCl$_2$ (pH 4.0)-121°C| 0.00%         | 10.80%        | 28.40%        | 60.80%        |
| Nisin+CaCl$_2$ (pH 6.0)-121°C| 0.00%         | 15.35%        | 28.80%        | 55.85%        |

Note: The secondary structure ($\alpha$-Helix, $\beta$-Sheet, $\beta$-Turn and irregular curl) proportion of nisin with and without CaCl$_2$ at different pH (2.0, 4.0 and 6.0) and temperature (25 and 121°C).

After heat treatment (121°C), the proportion of $\alpha$-helix, $\beta$-sheet and $\beta$-turn of nisin increased and irregular curl decreased obviously, and these changes were enhanced with the increasing of pH. However, when CaCl$_2$ was added, the change of secondary structure was obviously declined. At pH 4.0, the proportion of $\alpha$-helix, $\beta$-sheet and $\beta$-turn were same as to that of pH 2.0 without CaCl$_2$. At pH 6.0, the proportion of $\alpha$-helix, $\beta$-sheet and $\beta$-turn were decreased from 4.80%, 38.20% and 30.80% to 0.00%, 15.35% and 28.80%, and the proportion of irregular curl increased from 26.20% to 55.85% compared with the nisin without CaCl$_2$, at 121°C. This indicated that the addition of CaCl$_2$ made the secondary structure of nisin less damaged by heat treatment.

It was reported that the antimicrobial activity and stability of nisin were related to the secondary structure, which showed the inversely proportional linear correlation with the pH and temperature, while the influencing factors of secondary structure are multiple factors ($\alpha$-helix, $\beta$-sheet, $\beta$-turn and irregular curl). It was reported that the irregular curl and $\beta$-turn of nisin protein molecules with the stability and antimicrobial activity have good consistency [25,26]. As shown in Table 3 and Fig. 1, the antimicrobial activity basically no lost at pH 2.0-4.0, which main reason was the proportion of irregular curl not changed at 25°C. At pH 6.0, the about 10% loss of antimicrobial activity was caused by those changes that the proportion of $\beta$-turn increased from 28.40% to 28.90%, and the proportion of irregular curl declined from 60.80% to 59.30%. However, nisin with CaCl$_2$, the proportion of irregular curl and $\beta$-turn were the same as pH 2.0, at 25°C and pH 6.0. The results described above showed that CaCl$_2$ could protect the secondary structure, and therefore enhanced the antimicrobial activity of nisin.

After heat treatment (121°C), at pH 2.0, the secondary structure of nisin was not damaged by the high temperature (Table 3), and nisin had stable structure and antimicrobial activity. When the pH increased from 4.0 to 6.0, the change of secondary structure caused an extreme change in the antibacterial activity of nisin, which was caused by the polymerization between nisin at a higher pH and temperature [4]. However, when CaCl$_2$ was present, the proportion of irregular curl $\beta$-sheet, $\alpha$-helix and $\beta$-turn structures were obviously declined, and the thermal stability and antimicrobial activity of nisin with CaCl$_2$ was enhanced. These results demonstrated that the addition of CaCl$_2$ caused little change of the secondary structure of nisin, and could protect the secondary structure of nisin under heat treatment at pH 4.0 and 6.0.
4. Conclusion
The effect of CaCl$_2$ on the stability and antimicrobial activity of nisin was investigated and clarified in this study. The results showed that the thermal stability and antimicrobial activity of nisin could obviously be improved by CaCl$_2$. The SEM and XRD analysis showed that CaCl$_2$ could enhance the structure of nisin at pH 4.0 and 6.0. The TGA analysis showed that the addition of CaCl$_2$ during heat treatment at weak acid environment improved the chemical thermal stability of nisin. Furthermore, the FT-IR results showed that the addition of CaCl$_2$ could protect the hydrogen bonds of nisin, and therefore enhanced the stability of nisin. The CD analysis showed that CaCl$_2$ had a protective effect on the secondary structure of nisin.

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