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Effect of pressure-assisted thermal sterilization combining with ε-polylysine on *Bacillus subtilis* spore proteins, nucleic acids and other intraspore substances

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Abstract

Pressure-assisted thermal sterilization (PATS) is a new technology to inactivate bacterial spores and ensure food safety. Little has been known about the effects of PATS combining with ε-PL on the spore's nucleic acid, enzymes and other key substances. This study aimed to investigate the inactivation effect of PATS combining with ε-PL on the spores of *B. subtilis*. The spores were treated with pressure 600 MPa at 25 °C, 65 °C and 75 °C, and ε-PL at 0.1% and 0.3%. After treatment, the survival rate of *B. subtilis* spores, leakage of nucleic acid and protein, the change in the cell membrane ATPase activity, the leakage of dipicolinic acid, and the damage on protein and nucleic acid of the spores were determined. The results showed that PATS combining with ε-PL inactivated more spores, and significantly increased the release of protein and nucleic acid compared to the control. ATPase activity reached the lowest value after the treatment of 600 MPa/75 °C combining with 0.3% ε-PL. The release of dipicolinic acid from the spores was increased by 600 MPa/75 °C combining with 0.3% ε-PL as compared with 600 MPa/75 °C treatment alone. FTIR analysis showed that a combination of PATS with ε-PL changed the spectral features of *B. subtilis* functional groups of proteins and nucleic acids. The PATS treatments when combined with ε-PL were found to shift the symmetric and antisymmetric stretching vibrational absorption peaks of phosphodiester group in nucleic acid molecules (P=O). This change suggested that the combined treatment denatured nucleic acid. The combined treatment also changed the protein from an ordered state to a disordered state, and decreased protein stability. The results improved our understanding on the principle of spore inactivation by PATS combining with ε-PL.

Keywords: pressure-assisted thermal sterilization; ε-polylysine; spore; *Bacillus subtilis*; sterilization.

Practical Application: The combined PATS and ε-PL treatments enhanced spore inactivation and the mechanisms involved many reasons.

1 Introduction

In food science, the research of food sterilization is important to inactivate pathogenic microorganisms in foods and ensure food safety. Food spoilage and food poisoning often occur because of insufficient sterilization intensity (Sadiq et al., 2018; Zhao et al., 2022). More than 70% of food poisoning cases are caused by microorganisms, especially sporogenous bacteria (Ishaq et al., 2021). The inactivation of highly resistant spores is a key issue in the safety of low-acid food (Lopes et al., 2018; Yehia et al., 2022). Pressure-assisted thermal sterilization (PATS), the combination of high pressure and heat, allows a new sterilization technology for producing stable low-acid food, can inactivate bacterial spores and make the food to have better sensory and nutritional quality (Sevenich & Mathys, 2018). However, the most widely used method of spores inactivation in the industry is mainly heat treatment, but PATS can effectively inactivate spores at lower temperatures and in a shorter treatment time than heat treatment alone (Gomes et al., 2022; Luong et al., 2020; Ribeiro & Cristianini, 2020). PATS sterilization technology has lower heat treatment intensity than traditional thermal sterilization technology and can produce higher quality food (Deng et al., 2022; Sevenich et al., 2016). To further reduce the temperature used to inactivate the spores and reduce the impact on the sensory and nutritional values of the food, a combination of PATS combining with ε-polylysine (PL) was used to inactivate the spores. ε-PL is a water-soluble cationic substance and a natural compound that has been used as a food preservative. ε-PL was not only effective against microorganisms, including Gram-positive and Gram-negative bacteria, yeast and mold, but also was heat stable and non-toxic to human body (Liu et al., 2015). ε-PL could be decomposed into lysine and absorbed (Su et al., 2019). At the present, ε-PL has been widely used in food sterilization and preservation. Li et al. (2020); Liu et al. (2015) reported that ε-PL also affected the proteins, nucleic acids and some enzymes of non-spore bacteria, leading to bacterial inactivation. However, to our knowledge, nothing has been reported about the effects of PATS combining with ε-PL on key substances, such as proteins, nucleic acids and enzymes of the spores. The study has found that a combination of PATS with ε-PL treatment significantly affected the proteins, nucleic acids and ATPase of the spores, resulting in a better inactivation effect as compared to the PATS or ε-PL treatment alone. The results from the study may provide a theoretical basis for the application of the combined treatment in the food industry.

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2 Materials and methods

2.1 Preparation conditions of spore suspension

The *B. subtilis* spore suspension in this study was prepared with reference to the method of (Li et al., 2021; Liang et al., 2019) with slight modifications. Activated *B. subtilis* (CGMCC 1.3358) was inoculated by scratch onto nutrient agar (DM, Tianjin, China) and supplemented with 47 mg Mn^{2+}/L. After one week at 37 °C, the percentage of sporulated cells was observed by a phase contrast microscope (Nikon, Japan). When at least 99% of the cells were sporulated, the spores on the medium were washed by shaking with sterile deionized water, and collected through a sieve into a sterile centrifuge tube. The collected spore suspension was centrifuged for 15 min (4 °C, 9000 r/min) and repeated three times. Finally, sterile deionized water was added to adjust the concentration of the spore suspension to 1.5×10^9 CFU/mL, and it was stored at 4 °C.

2.2 PATS combining with ε-PL treatment

The 5 mL spore suspensions with 0%, 0.1% and 0.3% ε-PL were transferred to aseptic polyethylene plastic bags, sealed in vacuum and stored at 4 °C. The vacuum bag containing bacterial suspension was placed in an ultra-high pressure equipment, and the sample was pressurized with water as the pressure transfer medium. The pressure device (Baotou, China) can be pressurized to 700 MPa and heated to 90 °C, with the pressure rising rate at 280 MPa/min and the release time less than 4 s. The pressure was set to 600 MPa and held at different temperatures (25, 65 and 75 °C) for 20 min. For each condition, 3 replicates were performed. The treatment time did not include the time needed to boost and relieve pressure. Samples were preheated to the appropriate temperature before PATS treatment. Pressure, time and temperature were monitored and controlled by computer in real time. After decompression, the samples were cooled in an ice bath and stored at 4 °C for 6 h before counting.

2.3 Determination of the number of surviving spores

The number of surviving spores was determined as reported by Xu et al. (2021). Briefly, the spore suspension was serially diluted and spread onto TSA-YE media agar plates. The plates were incubated at 37 °C for 36 h, and the number of colonies that grew at the survival concentration of the spores was then calculated.

2.4 Determination of DPA content in spore suspension

DPA content in filtered supernatants of spore suspensions was determined as reported by Reineke et al. (2013b); Zhang et al. (2012). The spore suspension was centrifuged for 15 min (4 °C, 9000 r/min), and the supernatant was collected and analysed by liquid chromatography after being passed through a 0.22 μm aqueous phase membrane. The chromatographic column was ZORBAXSB-C18 column (150 × 4.6 nm, 5 μm), the injection volume was 10 μL, and the mobile phase was 0.1% phosphoric acid and methanol. The isocratic elution mode was used, the mobile phase gradient was 0.1% phosphoric acid: methanol = 60 : 40 (v/v), with the flow rate at 1.0 mL/min. The detection wavelength was 272 nm, and the running time of the sample was 15 min. The range of the standard curve was 0, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mg/L by external standard method. The leakage amount was calculated according to the DPA content using the following Formula 1

$$\text{Leakage rate}(100\%)=\frac{C_1}{C_2}100\% \quad (1)$$

Where C_1 is the content of DPA in the sample, which was determined in the spore suspension after high pressure sterilization. C_2 is the total content of DPA in the spore suspension. The total DPA content in spore suspensions was determined as described by Reineke et al. (2013b), with some modifications. An appropriate amount of the spore suspension prepared in Section 2.1 was taken and heat-treated at 121 °C for 30 min. The content of DPA in the heat-treated spore suspension was determined by liquid chromatography according to the above method.

2.5 Determination of leaking materials of the spores

The content of leaking materials of the spores was measured as described previously (Su et al., 2019). The spore suspension before and after treatment was centrifuged at 9,000 × g, 4 °C for 15 min, and the supernatant was collected. The absorbance values at 260 nm (nucleic acid) and 280 nm (protein) were determined by an ultraviolet spectrophotometer. The untreated spore suspension was used as the control group, and the aseptic water was used as the blank control.

2.6 Analysis of ATPase activity

The activity of Na^+/K^+-ATPase was measured as described previously (Zhang et al., 2022). The spore suspension was treated with ultrasonic cell breaker in an ice bath for 3 s (the gap was 10 s) and repeated for 30 times. After ultrasound treatment, the sample was centrifuged (9000 × g, 4 °C, 15 min), and the supernatant was taken. The protein content in the supernatant was determined by Coomassie Brilliant Blue method. Na^+K^+-ATPase activity was determined according to the instructions of the assay kit (A070-2, Nanjing Jiancheng Bioengineering Institute, China). The test tube and control tube were set up in each treatment group. Each assay was replicated 3 times and results reported as mean ± standard deviation.

2.7 FT-IR spectral analysis

The suspension of *B. subtilis* treated by PATS was freeze-dried, and the freeze-dried sample was analysed by Fourier transform infrared spectrometer (Spectrum Two, PerkinElmer Corporation, USA) at room temperature. The sample was mixed with KBr with 100 times the mass of the sample and then fully ground. The mixed abrasive was poured into the pressing machine (JYP-15, Jiaxinhai Machinery Company, Tianjin, China) to form a transparent sheet, and the blank KBr sheet was used as a control. All the samples were placed on the infrared spectrometer and scanned in the range of 400-4000 cm^{-1} with 32 scanning times and a resolution of 4 cm^{-1}. The baseline was corrected by PeakFitv 4.12 software in the band range (amide I band 1600-700 cm^{-1}),

then deconvolution with Gaussian and second derivative fitting were conducted, and the residual error was minimized by multiple fitting. The secondary structure contents of the spore protein of *B. subtilis* were calculated according to the peak area. The infrared data of 1300-900 cm^{-1} nucleic acid band were analysed using Origin 2020, the data were normalized and the second order derivative calculated.

2.8 Statistical analysis

Data were analyzed by one way ANOVA using SPSS 19 software and plotted by Origin 2021 software. All experiments were repeated at least 3 times, and the results were expressed as mean ± standard error. $P < 0.05$ was regarded as the threshold for significant difference.

3 Results and discussion

3.1 Number of surviving spores

The initial count of *B.subtilis* spores before treatment was 1.5×10^8 CFU/mL, after 600 MPa/25 °C treatment for 20 min, the viability of the spores of *B. subtilis* decreased by 0.04 log. The pressure treatment at 25 °C had no significant inactivation effect on the spore. These results are consistent with the results reported by Reineke et al. (2011) showing that *B. subtilis* spores were very resistant to high pressure (for example at 600 MPa/37 °C). The spores of *B. subtilis* treated at 600 MPa-65 °C/75 °C decreased by 4.87 log and 5.36 log respectively, indicating that as the temperature increased, the number of spores inactivated by PATS increased under the same pressure (600 MPa) conditions. As shown in Figure 1, ε-PL decreased the survival of the spores. *B. subtilis* spores were inactivated by 0.72 log, 5.41 log, 6.34 log when treated with 0.3% of ε-PL combining with 600 MPa at 25 °C, 65 °C and 75 °C, respectively. Thus PATS combining with ε-PL inactivated more *B. subtilis* spores.

3.2 DPA content in spore suspension

DPA is a substance unique to bacterial spores, accounting for 5-15% of the dry matter content of the spores. DPA is released when the spores germinate or their structural integrity is damaged (Aldrete-Tapia & Torres, 2021). It was reported that the leakage mechanisms of DPA were different at medium pressure (200-500 MPa) and high pressure (> 500 MPa) (Liang et al., 2019; Reineke et al., 2013b). Under moderate pressure conditions, pressure-activated germination receptors (GRs) may induce spores to germinate and release DPA. However, higher pressure (> 500 MPa) may directly open the DPA channel SpoVA and may not activate GRs. After the release of DPA, the nucleoid of the spores was hydrated, which caused the loss of heat resistance of the spores (Winter & Jeworrek, 2009), and the spores with loss of heat resistance were eventually inactivated by a combination of pressure and heat. As shown in Figure 2, the DPA release amount (38.6%) at 600 MPa/25 °C increased to 64.4% at 600 MPa/75 °C. Under the same pressure (600 MPa) conditions, the increase of temperature significantly promoted the release of DPA, which confirmed a previous report showing that the synergism between pressure and temperature was reduced by the pressures over 600 MPa, and the treatment temperature alone affected DPA release (Reineke et al., 2013a).

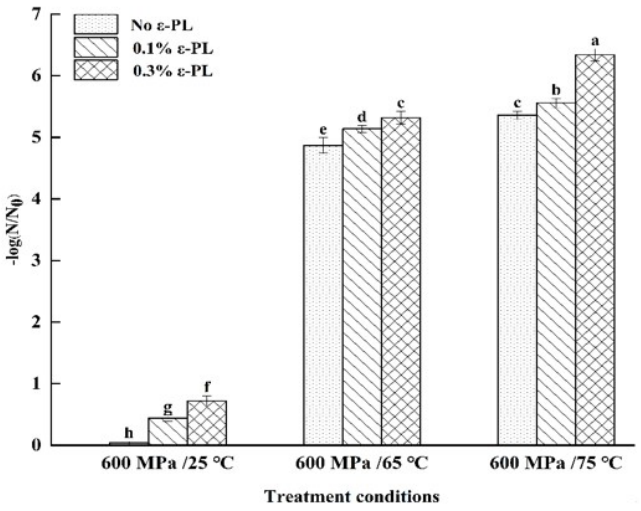

Figure 1. The effect of PATS treatment alone and PATS treatment combined with ε-polylysine on the survival concentration of *B. subtilis* spores. Each measuremnet was replicated 3 times. Different letters indicate significant differences ($P < 0.05$).

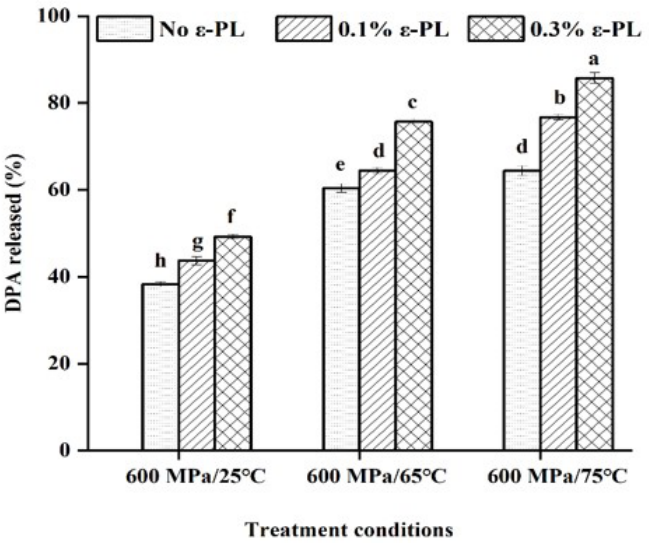

Figure 2. The effect of PATS treatment alone and PATS treatment combined with ε-polylysine on DPA leakage from *B. subtilis* spore suspensions. Each measurement was replicated 3 times. Different letters indicate significant differences ($P < 0.05$).

The DPA release of *B. subtilis* spores from PATS combining with ε-PL treatment further increased, with the DPA release amount at 600 MPa/75 °C-0.3% ε-PL treatment reaching 85.7%. DPA leakage led to the loss of resistance of *B. subtilis* spores to pressure and heat, which in turn affected the inactivation of the spores. This coincided with the results of spore inactivation.

3.3 Release of UV-absorbing substances from spores

The leakage of nucleic acids and proteins from the spores was determined by measuring the ultraviolet absorption intensity at 260 nm/280 nm (Su et al., 2019). As shown in Figure 3, the

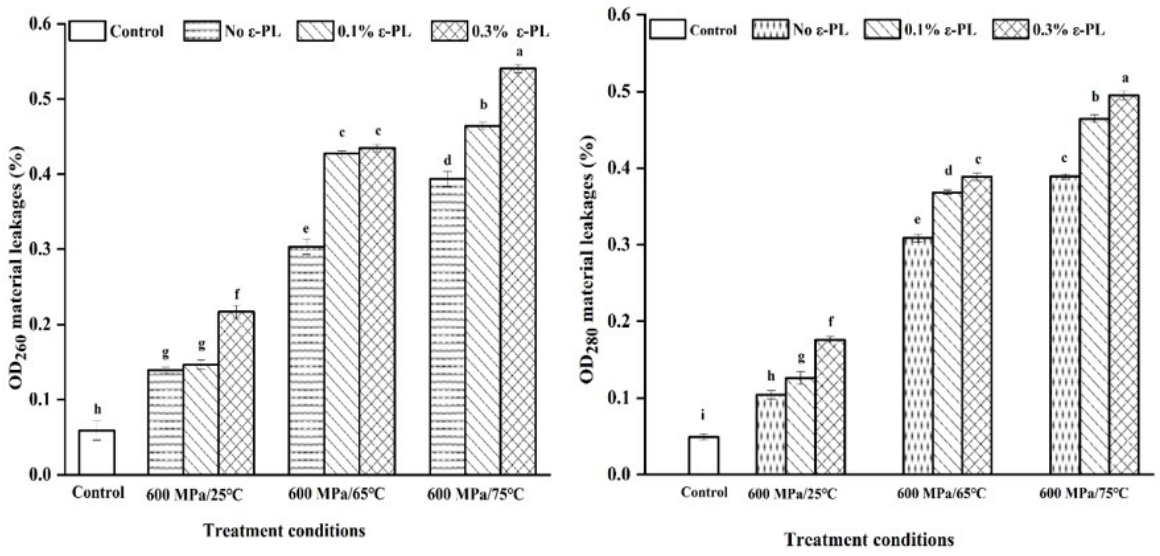

Figure 3. The effect of PATS treatment alone and PATS treatment combined with ε-polylysine on the leakage of UV absorbing substances from *B. subtilis* spores. Each measurement was replicated 3 times. Different letters indicate significant differences ($P < 0.05$).

leakage of nucleic acids and proteins from the spores increased significantly with the increase in the temperature under PATS treatment, and the maximum leakage was observed at 600 MPa/75 °C, with the values of OD_{260} and OD_{280} of 0.394 and 0.389, respectively. The leakage of nucleic acids and proteins from *B. subtilis* spores treated by PATS combining with ε-PL increased further under the conditions of 600 MPa-75 °C combining with 0.3%ε-PL, with the values at OD_{260} and OD_{280} of 0.540 and 0.495, respectively. The results showed that ε-PL increased the release of proteins and nucleic acids from the spores under PATS treatment. Previous studies have found that more nucleic acids and proteins were released with increasing ε-PL concentrations (Storia et al., 2011; Ye et al., 2013). Liu et al. (2020); Su et al. (2019) also reported that ε-PL caused the release of proteins and nucleic acids from the cells.

3.4 Change of ATPase activity

As shown in Table 1, the ATPase activity of *B. subtilis* spores was significantly reduced by 600 MPa/75 °C alone. It was reported that after *E. coli* O157:H7 was treated with 400 MPa for 5 min, the relative activity of Na^+/K^+-ATPase was only 43.90% of that of the untreated sample (Ma et al., 2019). In this study, it was interestingly found that under the same pressure conditions, the activity of Na^+/K^+-ATPase was reduced by the increase of temperature. This may be because the increase of temperature denatured Na^+/K^+-ATPase, resulting in a decreased activity. After PATS combining with ε-PL treatment (600 MPa/75 °C/0.3% ε-PL), the Na^+/K^+-ATPase activity of *B. subtilis* spores reached the lowest value (3.72 U/mg protein). It has been previously reported that ε-PL inhibited the activity of Na^+/K^+-ATPase (Liu et al., 2015).

3.5 FT-IR spectra of PATS combining with ε-PL treatment B. subtilis spores

FT-IR can detect vibrational absorption peaks of molecules with polar bonds (C=O, P=O, N-H) contained in proteins, nucleic acids and lipids of bacteria (AlMasoud et al., 2021; Alvarez-Ordóñez et al., 2011). Therefore, FT-IR was used to determine the changes in lipids (3000-2800 cm^{-1}), proteins (1700-1600 cm^{-1}) and nucleic acids (1300-900 cm^{-1}) of bacteria after treatment (Georget et al., 2014). FTIR was performed for the control, 600 MPa/75 °C, 600 MPa/75 °C/0.1% ε-PL and 600 MPa/75 °C/0.3% ε-PL, respectively. The wavelength range of FTIR detection was 4000-600 cm^{-1}. The data of the original spectrum were further analyzed by using second derivative processing. The second derivative spectrogram can distinguish the differences in the original spectrogram, which is beneficial to the analysis of the spectrogram. Nucleic acids (1300-900 cm^{-1}) were selected for the second derivative analysis.

Table 1. The effect of PATS treatment alone and PATS treatment combined with ε-polylysine on ATPase activity of *B. subtilis* spore cell membrane.

Conditions	ATPase activity (U/mg protein)		
	Not added	0.1% ε--PL	0.3% ε-PL
Control	25.63 ± 1.76	-	-
600 Mpa-25 °C	21.76 ± 1.02	21.13 ± 0.65	19.48 ± 0.35
600 Mpa-65 °C	10.35 ± 0.45	8.11 ± 0.21	7.36 ± 0.37
600 Mpa-75 °C	6.61 ± 0.29	4.17 ± 0.16	3.72 ± 0.11

3.6 Second derivative spectrogram of spores treated by PATS combining with ε-PL

Figure 4 shows the infrared second derivative spectrum of *B. subtilis* spores in the 1300-900 cm^{-1} band before and after PATS combining with ε-PL treatment. This region mainly reflects the symmetrical and antisymmetric stretching of the nucleic acid phosphodiester backbone (Al-Qadiri et al., 2008; Wang et al., 2019). The P=O symmetric and antisymmetric stretching vibrational bands of the phosphodiester group of nucleic acid molecules in the untreated *B. subtilis* spores were located at 1071 cm^{-1} and 1230 cm^{-1}. After PATS treatment, the absorption peak shifted to 1075 cm^{-1} and 1235 cm^{-1}, suggesting that PATS denatured nucleic

acids in the spores. Meng et al. (2016) found that 500 MPa/60 °C treatment caused the antisymmetric and symmetric P=O stretching vibrations of the *B. subtilis* spore nucleic acid phosphodiester backbone to shift, indicating the denaturation of nucleic acids. It was reported that 200 MPa treatment for 8 min condensed nucleic acids (Mañas & Mackey, 2004). Moussa et al. (2007) also reported a similar effect of high pressure on *E.coli* nucleic acid. After the treatment of PATS (600 MPa/75 °C) combining with 0.3% ε-PL, the P=O symmetric and antisymmetric stretching vibrational absorption peaks of the phosphodiester group of nucleic acid molecules were shifted to 1084 cm^{-1} and 1242 cm^{-1}, and the intensity of the absorption peak decreased significantly, indicating the denaturation of nucleic acids in the spores. The higher the concentration of ε-PL was added, the more significant the change in nucleic acids was, which may be due to the damage of DNA by ε-PL. (Ye et al., 2013) also found that ε-PL entered the cytoplasm due to membrane breakage and interacted with DNA, resulting in DNA damage.

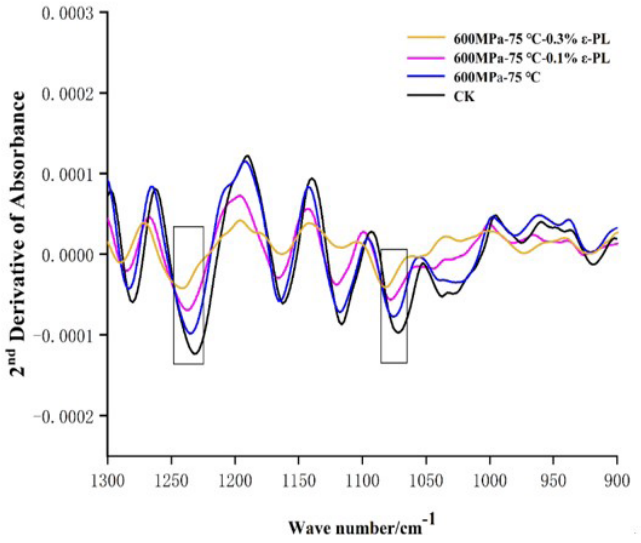

Figure 4. The second derivative spectra of *B. subtilis* in the range of 1300-900 cm^{-1} after PATS and PATS combined with ε-polylysine treatments.

3.7 Effect of PATS combining with ε-PL on the secondary structure of bacterial protein

Figure 5 shows the fitting figure of the deconvolution of the protein amide I band before and after the PATS combining with ε-PL treatment on *B. subtilis* spores. The FT-IR absorption peak in the amide I band located in the 1600-1700 cm^{-1} band was associated with the C=O stretching vibration. The second derivative of *B. subtilis* spore amide I band in the control group, the PATS group, and the PATS combined with ε-PL group was processed by curve fitting. The peak areas of each sub-peak and the total peak were calculated according to the fitting diagram to obtain the content of each secondary structure (Georget et al., 2014). Each sub-peak can be classified as protein α-helix (1650-1660 cm^{-1}), β-folding (1610-1640 cm^{-1}), β-rotation angle (1660-1670 cm^{-1}) or random coil (l640-1650 cm^{-1}) (Baltacıoğlu et al., 2017).

As shown in Table 2, the untreated *B. subtilis* protein amide I band had high contents of ordered secondary structures α-helix

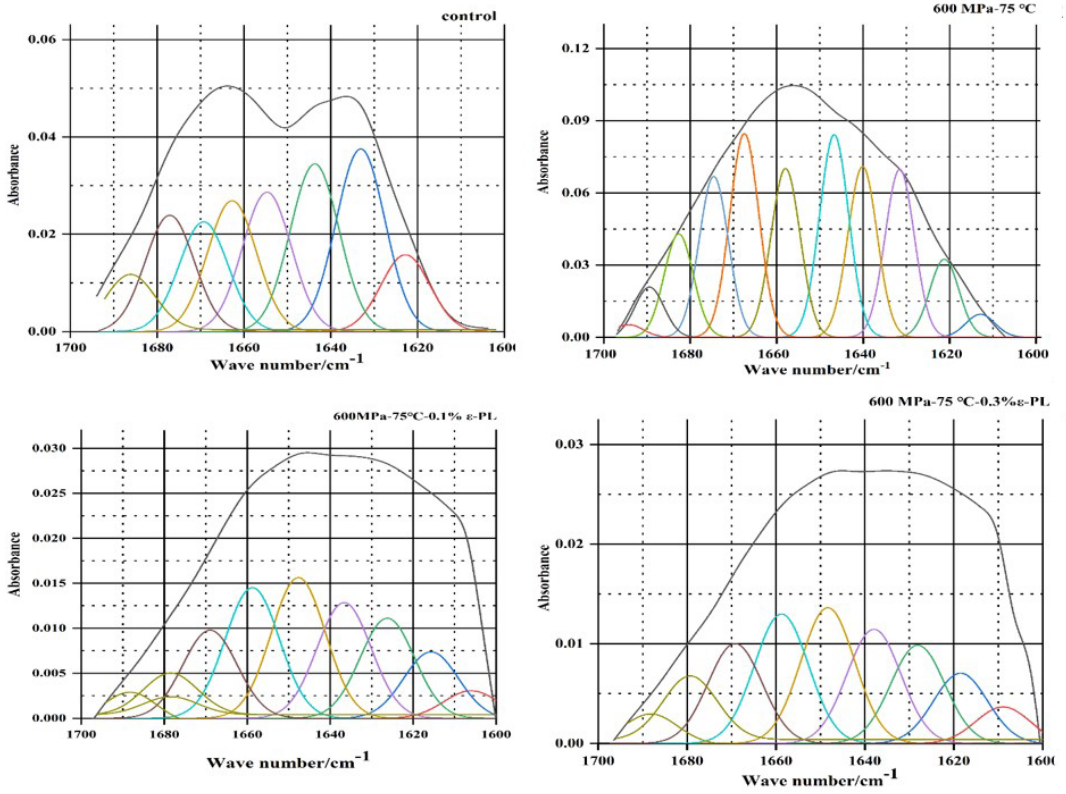

Figure 5. Fitted curves of the amide I band (1700-1600 cm^{-1}) of *B. subtilis* spores that have undergone PATS and PATS combined with ε-PL treatments.

Table 2. The effect of PATS treatment and PATS treatment combined with ε-polylysine on protein secondary structure of *B. subtilis* spores.

Protein secondary structure		α-helix	β-folding	β-rotation angle	random coil
Control		33	30	20	17
PATS	600 Mpa, 25 °C	26	28	28	18
	600 Mpa, 65 °C	22	27	25	23
	600 MPa, 75 °C	21	30	30	25
	600 MPa, 25 °C, 0.1% ε-PL	26	29	27	26
	600 MPa, 25 °C, 0.3% ε-PL	24	21	29	30
	600 MPa, 65 °C, 0.1% ε-PL	21	20	27	38
PATS combining with ε-PL	600 MPa, 65 °C, 0.3% ε-PL	18	19	28	33
	600 MPa, 75 °C, 0.1% ε-PL	18	18	31	31
	600 MPa, 75 °C, 0.3% ε-PL	17	19	32	29

and β-folding. The contents of β-rotation angle and random coil of *B. subtilis* spores treated with PATS increased, while the contents of α-helix and β-folding decreased, indicating that the amide I band of *B. subtilis* spore's protein changed from an ordered state to a disordered state after PATS treatment. The α-helix structure was sensitive to pressure treatment, and the transformation of the secondary structure from ordered state to disordered state may lead to the decrease in protein stability and further affect the metabolic process of the cells. After the treatment of PATS combining with ε-PL, the β-rotation angle content increased, while the contents of α-helix and β-folding significantly decreased, the transition from ordered state to disordered state was further strengthened, and the protein stability significantly decreased. These results indicated that the combined treatment significantly reduced protein stability in the spores.

4 Conclusion

A combination of PATS and ε-PL significantly reduced the survival of *B. subtilis* spores. Compared with PATS alone, a combination of PATS with ε-PL increased the release of DPA, protein and nucleic acid, decreased the activity of Na^+/K^+-ATPase, and denatured the nucleic acids in *B. subtilis* spores. The second derivative fitting results showed that after the combined treatment, the contents of α-helix and β-fold significantly decreased, the transition of protein from ordered state to disordered state was further strengthened, and the protein stability significantly decreased. The combined treatment significantly affected proteins, nucleic acids and ATPase in the spores, resulting in the inactivation of the spores. The combination of PATS and ε-PL can be used for food sterilization.

Conflict of interest

All authors declared there are no conflicts of interest.

Funding

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