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Inactivation effect and kinetics of cathepsin L from white shrimp (*Litopenaeus vannamei*) by dense phase carbon dioxide

Ouyang Zheng [a], Xiaojie Cao [a], Yanqing Teng [c], Qinxiu Sun [a,b,*], Shucheng Liu [a,b]

[a] College of Food Science and Technology, Guangdong Ocean University, Guangdong Provincial Key Laboratory of Aquatic Products Processing and Safety, Guangdong Province Engineering Laboratory for Marine Biological Products, Guangdong Provincial Engineering Technology Research Center of Seafood, Key Laboratory of Advanced Processing of Aquatic Product of Guangdong Higher Education Institution, Zhanjiang, 524088, China

[b] Collaborative Innovation Center of Seafood Deep Processing, Dalian Polytechnic University, Dalian, 116034, China

[c] Lunan Technician College, Linyi, 276017, China

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ABSTRACT

The effects of dense phase carbon dioxide (DPCD) pressure and temperature on the activity of cathepsin L in white shrimp (*Litopenaeus vannamei*) were studied. Meanwhile the effects of DPCD and heat treatment on the activity of cathepsin L were compared. The results of inactivation kinetics showed that under the same treatment temperature, the inactivation effect of DPCD on cathepsin L increased gradually with the increase of pressure and time. The effect of DPCD pressure on the activity of cathepsin L accorded with the first-order kinetic model. Under the same treatment pressure, with the increase of temperature, the inactivation effect of cathepsin L was significant at the initial stage of DPCD treatment (rapid inactivation period), and decreased with the extension of time (stable inactivation period). The effect of DPCD treatment temperature on cathepsin L activity accorded with the first-order kinetic model at 35 °C, and two-stage kinetic model at 40–60 °C. The difference of relative enzyme activity between the two treatments showed that the dependence of DPCD on temperature was greater than that of heat. $E_{a,F}$ and $E_{a,S}$ of DPCD were higher than that of heat, which indicated that cathepsin L was more easily inactivated under DPCD treatment.

1. Introduction

Surimi product is one of the main product of high value processing of aquatic products. The quality of the surimi gel depends on the method of induction. The most commonly used method to induce surimi gelation is heating, and the formation of three-dimensional gel network structure of heating induced protein gel is affected by the heating process (Xiong et al., 2022). Gel degradation (modori) easily occurs during the traditional heating process of surimi, resulting in the decline of gel strength and the deterioration of surimi quality (Cao et al., 2020; Kwon and Chang, 2023). The degradation of surimi gel is mainly due to the degradation of myosin by some endogenous proteases (the optimum temperature is 50–70 °C) during heating (Ueki et al., 2019). The endogenous proteases causing gel deterioration mainly include two types: heat stable alkaline protease and cathepsin (Ding et al., 2024). Although alkaline protease is the key enzyme causing the deterioration of surimi gel, the optimum temperature is 60 °C, but it mainly exists in sarcoplasmic protein, most of which can be removed by rinsing. Cathepsin is the most active enzyme at 50–60 °C (Singh and Benjakul, 2018), and it is also the key enzyme causing the deterioration of surimi gel. There are many kinds of cathepsin in surimi, mainly cathepsin B, H, and L. Among them, cathepsin L is the most abundant and active protease in surimi (Chen et al., 2022). Therefore, appropriate measures must be taken to control the activity of cathepsin L in the processing of surimi and surimi products.

At present, it has been found that some non-thermal processing technologies can also make surimi gelate, and its gel quality is better than that of traditional heat-induced gel, such as ultra-high pressure technology (Huang et al., 2023), dense phase carbon dioxide (DPCD) technology (Duan et al., 2024). DPCD technology is a very promising non-thermal processing technology. Supercritical carbon dioxide can be used for gelation of minced meat products because of its non-toxic,

* Corresponding author. College of Food Science and Technology, Guangdong Ocean University, Guangdong Provincial Key Laboratory of Aquatic Products Processing and Safety, Guangdong Province Engineering Laboratory for Marine Biological Products, Guangdong Provincial Engineering Technology Research Center of Seafood, Key Laboratory of Advanced Processing of Aquatic Product of Guangdong Higher Education Institution, Zhanjiang, 524088, China.
E-mail address: sunqx@gdou.edu.cn (Q. Sun).

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non-combustible, low critical temperature (31.1 °C), and medium critical pressure (7.38 MPa) (Tsai and Rizvi, 2016). Besides easy separation of CO_2, its low critical temperature also provides conditions for retaining more heat-sensitive nutrients. In recent years, researchers have found that DPCD could induce surimi gelation, and the gel quality properties were better than those of traditional heat treatment (Zheng et al., 2022). Our previous research also found that DPCD improved the strength of shrimp surimi gel (Zheng et al., 2022). This may be because DPCD has a certain inactivation effect on endogenous protease, thus inhibiting the gel deterioration of surimi products, and enhancing the gel strength. However, at present, the effects of DPCD on enzymes mainly focus on polyphenol oxidase (Zhang et al., 2011; Illera et al., 2019), peroxidase, pectin methylesterase, lipase (Melgosa et al., 2015), β-glucanase (Senyay-Oncel et al., 2023), oxidoreductive enzymes (Marszałek et al., 2019), while the research on cathepsin is rarely reported.

Therefore, the inactivation effect of DPCD treatment pressure and temperature on the cathepsin L of *Litopenaeus vannamei* was investigated, and the changes in cathepsin L enzyme activity during hot processing were compared. The inactivation kinetics of cathepsin L under the same pressure and temperature as DPCD treatment were also analyzed, providing a theoretical basis for DPCD inactivation of cathepsin L in *Litopenaeus vannamei*.

2. Materials and methods

2.1. Chemicals

The fluorescent substrate Z-Phe-Arg-AMC was purchased from Sigma corporation in the United States. Tris, L-Cys, sodium acetate, acetic acid, sodium chloroacetate, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). CO_2 gas (purity 99.9%) was purchased from Zhanjiang Oxygen Plant (Zhanjiang, China), and all reagents are analytical pure.

2.2. Extraction of crude enzyme of cathepsin L

The extraction of cathepsin L refers to the method of Zhu et al. (2022) with little modification. 60 g of fresh shrimp minced meat was weighed, and added into 480 mL pre-cooled 40 mmol/L Tris-HCl buffer (pH 6.0, containing 6 mmol/L L-Cys, 0.4 mmol/L PMSF), which was fully homogenized by tissue mashing machine, and centrifuged with a 3-30 KS centrifuge (SIGMA Corporation, America) at 4 °C, 6624×*g* for 20 min. The supernatant was the crude enzyme solution of cathepsin L, and the specific activity of crude enzyme was 13.9 U/mg.

2.3. DPCD treatment

DPCD treatment refers to the method of Zheng et al. (2021). The working diagram of DPCD processing equipment is shown in Fig. 1. The DPCD processing kettle is a cylindrical body with a diameter of 5 cm and a volume of 0.6 L. The equipment temperature is controlled by electric heating and water bath circulation. The refrigeration unit and cooling circulation system of the supercritical fluid extraction unit were opened, and the temperature of the treatment kettle was set at the same time to preheat the equipment. After the device was preheated to the set temperature, 3 mL cathepsin L crude enzyme solution was put into 10 mL centrifuge tube, and then placed in the treatment kettle, sealed, and the high-pressure pump was started to pump CO_2. When the pressure rose to the required pressure, the high-pressure pump was closed, the valves at the inlet and outlet of the treatment kettle were closed, and the required pressure and temperature in the treatment kettle were maintained. After static treatment for a period of time, the pressure relief of the vent valve was opened, the sample was taken out, the enzyme solution was gently shaken to discharge CO_2 gas, and the treated enzyme solution sample was placed in a low temperature environment. Then the activity of cathepsin L was measured.

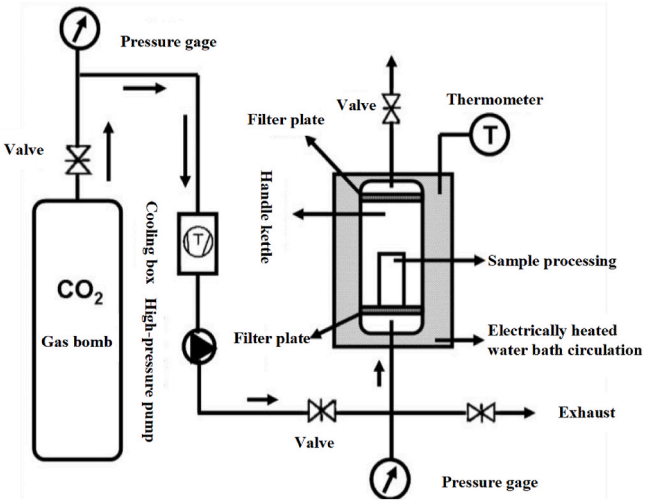

Fig. 1. Dense phase carbon dioxide equipment diagram.

DPCD was designed by double-factor equal-repetitive design. First, the treatment temperature was set at 35 °C, the depressurization rate and pressurization rate were both 2.5 MPa/min, and the pressure was 0.1, 10, 15, 20, 25, 30 MPa for 5, 10, 20, 30, 40, 50, and 60 min, respectively. Secondly, the pressure was set at 25 MPa, the depressurization rate and pressurization rate were both 2.5 MPa/min, and the temperature was 35, 40, 45, 50, 55 and 60 °C for 5, 10, 20, 30, 40, 50, and 60 min, respectively. The physical properties of CO_2 are highly sensitive to temperature and pressure changes, especially density. The ratio of CO_2: enzyme directly affects the enzyme inactivation effect (Benito-Román et al., 2019). Therefore the density of carbon dioxide was obtained through the NIST Chemistry Webbook, and by combining the volume of the enzyme and the volume of the treatment vessel, the CO_2: enzyme solution volume was calculated at different temperatures and pressures (Table 1).

2.4. Determination of cathepsin L activity

The activity of cathepsin L was determined as the method of Cao et al. (2020) with little modification. 100 μL crude enzyme solution was added into a test tube, and 250 μL sodium acetate-acetic acid buffer (pH 4.0, 50 mmol/L) and 50 μL substrate Z-Phe-Arg-AMC (10 μmol/L) were added successively. After mixing evenly in a whirlpool mixer, the temperature was kept at 60 °C for 20 min. Then 400 μL reaction termination solution (pH 4.55, containing 0.1 mol/L sodium acetate, 0.1 mol/L acetic acid, and 0.1 mol/L sodium chloroacetate) was added to terminate the reaction, and the fluorescence intensity was measured by automatic microplate reader. The excitation and emission wavelengths were 344 nm and 436 nm, respectively. Blank control: Before adding 50 μL substrate, 400 μL termination solution was added to cathepsin L crude enzyme solution to inactivate the enzyme. Other operations were the same as described above.

The unit of enzyme activity (U) was defined as the amount of enzyme (1 ng AMC/min) required to hydrolyze the substrate and release 1 ng 7-amino-4-methylcoumarin (AMC) per minute at the optimum reaction temperature (60 °C) and pH (pH 4.0). The calculation of cathepsin L enzyme activity was as formula (1):

$$A = \frac{(F - 0.0602) \times 649.14 \times V}{129.51 \times V_1 \times t} \quad (1)$$

Where A was cathepsin L enzyme activity, U·min^{-1}·mL^{-1}; F was the fluorescence value of the sample; V was the total volume of the reaction system, mL; V_1 was the amount of crude enzyme solution added in the

Table 1
The ratio CO_2: volume of cathepsin L solution at different temperatures and pressures.

Temperature (°C)	Pressures (MPa)	Density CO_2 (g/L)	Ratio CO_2: Enzyme solution (g/mL)
35	0.1	1.7	0.3
	10	712.7	142.5
	15	814.9	163.0
	20	865.5	173.1
	25	901.0	180.2
	30	928.9	185.8
40	0.1	1.7	0.3
	10	628.5	125.7
	15	780.1	156.0
	20	839.6	167.9
	25	879.3	175.9
	30	909.7	181.9
45	0.1	1.7	0.3
	10	498.2	99.6
	15	741.8	148.4
	20	812.6	162.5
	25	857.0	171.4
	30	890.2	178.0
50	0.1	1.7	0.3
	10	384.3	76.8
	15	699.6	139.9
	20	784.2	156.8
	25	834.1	166.8
	30	870.3	174.0
55	0.1	1.6	0.3
	10	325.0	65.0
	15	653.4	130.7
	20	754.4	150.9
	25	810.5	162.1
	30	850.0	170.0
60	0.1	1.6	0.3
	10	289.9	58.0
	15	603.9	120.8
	20	723.5	144.7
	25	786.4	157.3
	30	829.5	165.9

reaction system, mL; t was the reaction time, min.

The relative activity of cathepsin L after DPCD treatment as calculated as formula (2):

$$X(\%) = \frac{A_t}{A_0} \times 100 \tag{2}$$

Where X was the relative enzyme activity of cathepsin L, %; A_t was the activity of cathepsin L after DPCD treatment, U·min^{-1}·mL^{-1}; and A_0 was the activity of cathepsin L before DPCD treatment, U·min^{-1}·mL^{-1}.

2.5. Inactivation kinetics of cathepsin L

Generally, the inactivation law of cathepsin L could be described by the first-order reaction kinetic model, and the expressions are shown in formulas (3) and (4):

$$\log\left[\frac{A_t}{A_o}\right] = -\left[\frac{k}{2303}\right]t \tag{3}$$

$$D = \frac{2.303}{k} \tag{4}$$

Where A_t was the enzyme activity of cathepsin L after DPCD treatment, U·min^{-1}·mL^{-1}; A_0 was the enzyme activity of cathepsin L before DPCD treatment, U·min^{-1}·mL^{-1}; A_t/A_0 was the relative enzyme activity of cathepsin L after DPCD treatment, %; k was the reaction rate constant at certain pressure and temperature, min^{-1}; t was the treatment time, min; D was the exponential decline time, that was, the time required for cathepsin L enzyme activity to decrease by 90% under the set pressure and temperature, min.

The sensitivity of cathepsin L to DPCD pressure was expressed by Z_P value (MPa) and activated volume V_a (cm^3/mol). The Z_P value was the increase in pressure required to reduce the D value by 90%, and its equation was expressed as formula (5)

$$\log\left[\frac{D_1}{D_2}\right] = \frac{P_2 - P_1}{Z_p} \tag{5}$$

The activation volume V_a was expressed by Eyring equations as formula (6):

$$1n\left[\frac{k_1}{k_{21}}\right] = \frac{V_a}{R_p T}(P_2 - P_1) \tag{6}$$

P_1 and P_2 represented the pressures corresponding to D_1 and D_2, k_1 and k_2, Pa; T was the absolute temperature, K; R_P was the gas constant of 8.314 J mol^{-1}·K^{-1}.

The sensitivity of cathepsin L to DPCD temperature was expressed by Z_T value (°C) and activation energy E_a (kJ/mol). The temperature Z_T value was the increase in the temperature required to reduce the D value by 90%, and its equation was expressed as formula (7):

$$\log\left[\frac{D_1}{D_2}\right] = \frac{T_2 - T_1}{Z_T} \tag{7}$$

The activation energy E_a was expressed by Arrhenius equation such as formula (8):

$$1n\left[\frac{k_1}{k_2}\right] = \frac{E_a}{R_T}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{8}$$

T_1 and T_2 denoted the temperature corresponding to D_1 and D_2, k_1 and k_2, K; R_T was the gas constant of 8.314 J mol^{-1}·K^{-1}.

If the inactivation law of cathepsin L does not conform to the first-order kinetic model, it can be further fitted by the two-stage kinetic model. This model divided the inactivation process into two stages: rapid inactivation stage and stable inactivation stage, and these two stages respectively conform to the first-order kinetic model, and their equations wer expressed as formula (9):

$$A_t / A_0 = A_F{*}\exp(-k_F{*}t) + A_S{*}\exp(-k_S{*}t) \tag{9}$$

Where A_t was the activity of cathepsin L after DPCD treatment; A_0 was the activity of cathepsin L before DPCD treatment; A_F and A_S represented the total enzyme activity inactivated in the fast stage and the stable stage, respectively; k_F and k_S denoted the inactivation rates in the fast phase and the stable phase, respectively.

2.6. Statistical analysis

Each experiment was replicated three times, and the data were expressed as mean ± standard deviation. The kinetic model of enzyme activity was simulated by JMP10.0 software, and plotted by Excel, and the confidence interval was 0.05 ($P < 0.05$).

3. Results and discussion

3.1. Effect of DPCD treatment pressure on cathepsin L activity

Pressure is a very important factor in the process of DPCD inactivation. It can be seen from Fig. 2A that compared with heat treatment at the same temperature, DPCD treatment significantly reduced the activity of cathepsin L at the same treatment time ($P < 0.05$), which shows that DPCD had a bigger inactivation effect on cathepsin L than heat treatment. This may be because CO_2 dissolved in water, and reacted with water molecules to form carbonic acid, which was unstable and dissociated hydrogen ions, causing a decrease in the pH of the solution (Duan et al., 2024), and deviating from the optimal activity pH of

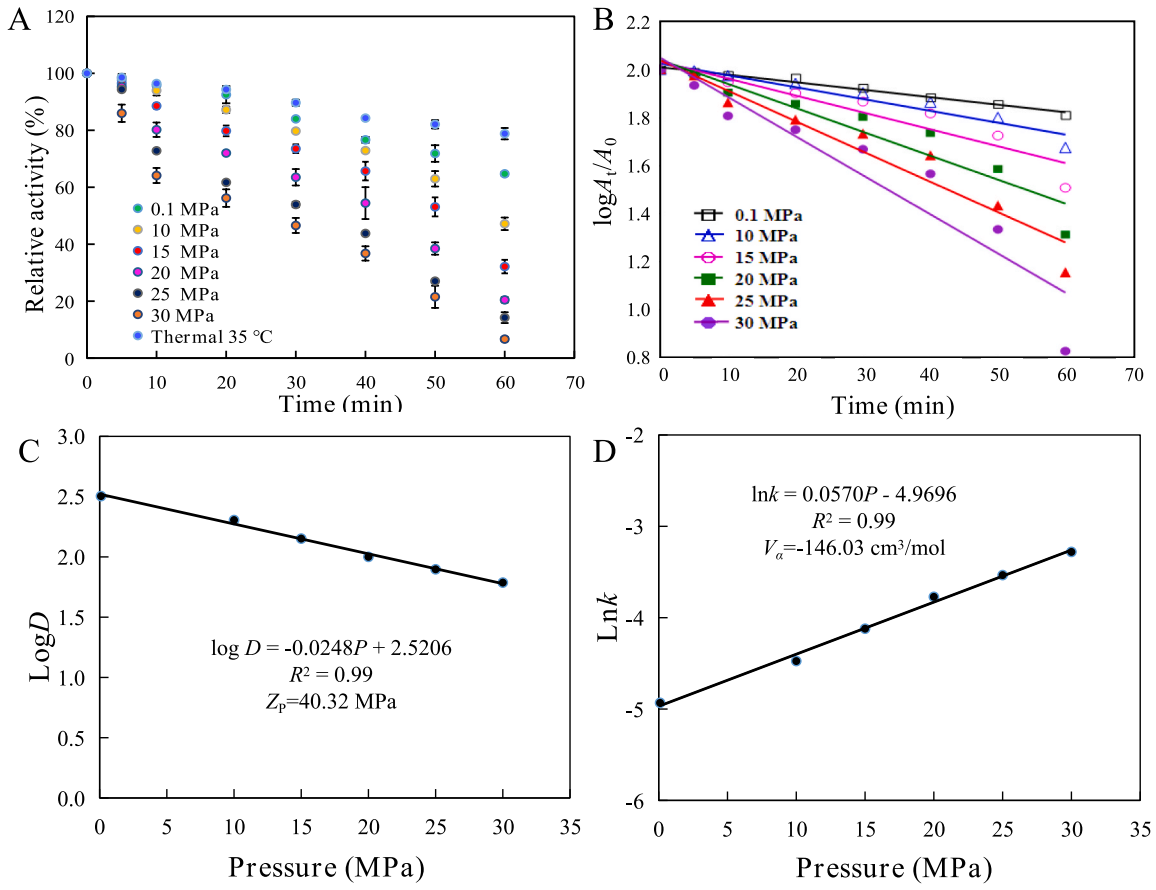

Fig. 2. The relative activity (A, %), Kinetic model of first-order reaction for inactivation (B), Z_p value (C), and V_α value (D) of pressure inactivation of cathepsin L in shrimp surimi treated by heating (35 °C) or HPCD at different pressure.

cathepsin L, resulting in a decrease in enzyme activity. In addition, CO_2 is a nonpolar solvent, which can interact with hydrophobic amino acid residues of proteins to expose them. By changing the balance of water-protein interaction, DPCD caused conformational changes of proteins (Htwe et al., 2023), thus promoting inactivation of proteases (cathepsin L). What's more, the changes in the properties of carbon dioxide in and around its critical point may affect its ability to interact with enzyme molecules, thereby affecting their rate of denaturation (Sheikh et al., 2023b). Saini, and Sharma (2023) thought that as a non-polar solvent, DPCD has zero surface tension and strong diffusion ability, which can quickly wet and penetrate into protein molecules, and interfere with protein local conformation, thus affecting the structure of protein molecules. Under the same treatment time, the inactivation effect of DPCD on cathepsin L increased with the increase of treatment pressure. At 35 °C for 60 min, the pressure increased from 0.1 MPa to 30 MPa, and the activity of proteinase L decreased from 64.72% to 6.68%. Hua (2013) found that DPCD treatment effectively inactivated the activities of polyphenol oxidase and peroxidase in fresh-cut lotus roots compared with heat treatment, and the enzyme activities gradually decreased with the increase of DPCD pressure and temperature. This is because under a fixed temperature, increasing the pressure caused solubility of CO_2 in water to increase. The amount of CO_2 dissolved in water increased, thus form more HCO^{3-}, CO_3^{2-}, and H^+, lowered the pH of the solution, leading to a gradual decrease in enzyme activity. On the other hand, the increase of pressure can increase the density of CO_2, thereby increasing the ratio of CO_2 to enzymes (Table 1), therefore increase the penetration of CO_2 (Duan et al., 2023), so that CO_2 could enter the enzyme molecule and come into contact with ammonia. The basic group and hydrophobic group of basic acid residues interacted with each other, which changed the conformation of enzyme, and led to inactivation of enzyme. In addition, compression due to increased pressure may enhance the binding affinity of some folded chains (Duong and balaban, 2014), mask the active sites of enzymes, or change the structure of enzymes, thus promoting the inactivation of cathepsin.

In addition, treatment time is also an important factor of DPCD inactivation. It can be seen from Fig. 1A that under the same treatment pressure and temperature, the inactivation effect of DPCD on cathepsin L increased with the increase of treatment time ($P < 0.05$). At 35 °C and 30 MPa, the relative activity of cathepsin L decreased by 79.29% when the treatment time increased from 10 min to 60 min. This is because with the increase of reaction time, more CO_2 molecules penetrated into cathepsin L solution (Htwe et al., 2023), and more CO_2 molecules interacted with cathepsin to promote the change of protease structure. Therefore, the inhibitory effect of DPCD on cathepsin L was time-dependent (0–60 min) and pressure dependent (0.1–30 MPa), which was superior to heat treatment.

3.2. First-order kinetics of DPCD pressure inactivation of cathepsin L activity

According to the first-order kinetic equations (3) and (4), the data in Fig. 2 were linearized and the kinetic parameters were calculated. As illustrated in Fig. 2B, within the pressure range of 0.1–30 MPa, the coefficient of determination (R^2) of the first-order kinetic model was around 0.90, indicating that the first-order kinetic model could basically be used to describe the inactivation process of cathepsin L by DPCD treatment pressure. It can be seen from Table 2 that with the increase of pressure, the inactivation rate constant k increased, and the value D

Table 2
Kinetic parameters of first-order reaction for inactivation of cathepsin L by DPCD treatment at 35 °C.

Pressure (MPa)	k (min^{-1})	D (min)	R^2 ($P < 0.05$)
0.1	0.0072	319	0.98
10	0.0114	202	0.93
15	0.0162	142	0.89
20	0.0230	100	0.90
25	0.0292	79	0.93
30	0.0376	61	0.88

decreased. This indicates that the higher the pressure, the faster DPCD could inactivate tissue cathepsin L, and the shorter the time required to achieve the same inactivation effect.

According to equations (5) and (6), the data in Table 2 were processed and plotted, and the sensitive pressure Z_P value and activation volume V_a value were calculated, respectively. The results are shown in Fig. 2C. The Z_P value was the increase in pressure when the D value decreased by 90%, indicating the sensitivity of the D value to pressure. From Fig. 2C, it can be seen that under the treatment temperature of 35 °C, the Z_P value of DPCD inactivated cathepsin L was 40.32 MPa. V_a was related to the rate constant k and pressure, and the smaller the V_a value, the easier it was for cathepsin L to be inactivated. According to the eyring equation, the V_a value of DPCD inactivated cathepsin L was −146.03 cm^3/mol, and its linear fitting was good. The average coefficient R^2 was 0.99, indicating that the first-order reaction kinetics formula and eyring equation could be used to analyze the kinetics of DPCD pressure inactivation cathepsin L. Chen et al. (2013) also found that the inactivation of many enzymes by DPCD accords with the first-order kinetic model. The Z_P value and V_a value were 31.07 MPa and −168. 15 cm^3/mol, respectively, which is very similar to the results of this study. Liu et al. (2008) treated polyphenol oxidase in red beet by DPCD, and the results also accorded with the first-order reaction kinetics model.

3.3. Effect of DPCD treatment temperature on cathepsin L activity

It can be seen from Fig. 3A that the general trends of inactivation of cathepsin L activity at different treatment temperatures were the same, and the cathepsin L activity was reduced with the extension of treatment time. This is because the treatment time is prolonged, and the interaction between CO_2 and tissue to enzyme molecules is more sufficient, which strengthens the structural changes of cathepsin and inhibits cathepsin activity. After being treated at DPCD 35 °C for 5 min, the relative enzyme activity of cathepsin L decreased by 5.65%. From 5 min to 60 min, the relative enzyme activity of cathepsin L decreased by 77.13%. After treatment at DPCD 60 °C for 5 min, the relative enzyme activity of cathepsin L decreased by 76.85%. From 5 min to 60 min, the relative enzyme activity of cathepsin L decreased. These indicated that under the same treatment time, the inactivation effect of DPCD on

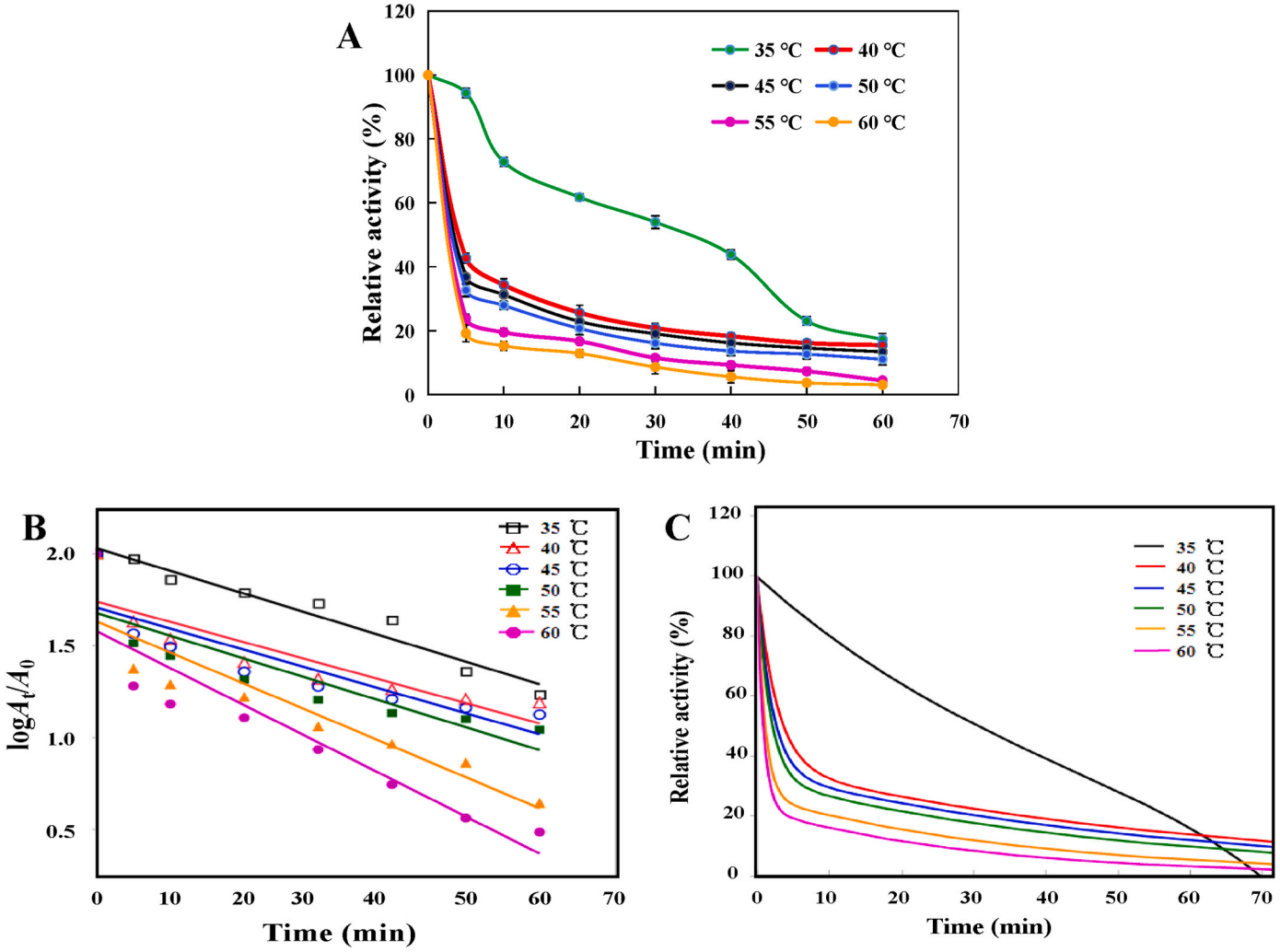

Fig. 3. The relative activity (%), kinetic model of first-order reaction for inactivation (B), and kinetic model of second-order reaction for inactivation (C) of cathepsin L in shrimp surimi by DPCD at different temperatures.

cathepsin L was increased with the increase of treatment temperature. This may be because an increase in temperature promoted the reaction between CO_2 and some amino acid residues, thereby promoting protein denaturation and reducing enzyme activity of cathepsin L. The most basic amino acids like arginine (pI 10.8), lysine (pI 9.5), and histidine (pI 7.6) are the most commonly found in CO_2 protein binding sites (Monhemi et al., 2015). Although serine (pI 5.7) and threonine (pI 5.9) are far less basic than Arg, His, and Lys, they show relatively large affinities for CO_2. DPCD can also interacts with the secondary structure of proteins, promoting protein denaturation. Our previous research (Liu et al., 2017) also found that β-sheet show a marked preference for CO_2 binding compared to α-helice. In addition, as a hydrophobic molecule, CO_2 can interact with the hydrophobic groups of proteins, thereby promoting the conformational and structural changes of proteins (Mitsuda et al., 1975). For groups with the same treatment temperature, increasing the treatment time enhanced the ability of DPCD to inactivate the activity of cathepsin L. This may be due to the thermal effect of DPCD treatment. The increase of temperature promoted the molecular movement of CO_2, and increased the contact opportunity with enzyme molecules (Tang et al., 2021). On the basis of thermal effect, CO_2 dissolved in water, reduced the pH of solution, which led to the gradual decrease of enzyme activity (Arbal et al., 2024). In addition, the dissociation of carbonic acid into bicarbonate, carbonate and hydrogen ion also helps to reduce the electrostatic force on the surface of protein molecules and promote the aggregation and denaturation of protein (Lima et al., 2019). Ortuño et al. (2013) also found that increasing DPCD treatment time promoted the inactivation of polyphenoloxidase and lypoxygenase in in feijoa puree.

3.4. First-order kinetics of DPCD temperature inactivation of cathepsin L

The data in Fig. 3A were linearized, and the kinetic parameters were calculated according to the first-order kinetic equations (3) and (4). As can be seen from Fig. 3B, the logarithm of cathepsin L relative enzyme activity was linearly related to heating time. It can be seen from Table 3 that the determination coefficient (R^2) was 0.95 at 35 °C, which indicated that the change of cathepsin L activity at 35 °C may conform to the first-order kinetic model. In the range of 40–60 °C, with the increase of treatment temperature, the rate constant k increased and the exponential decline time D decreased, which indicated that the higher the temperature, the easier it was to inactivate cathepsin L. The determination coefficient (R^2) was less than 0.9 in the range of 40–60 °C, which indicated that the first-order kinetic model could not be used to describe the inactivation process of cathepsin L by different DPCD treatment temperatures. Therefore, a two-stage dynamic model (Equation (9)) was needed for simulation.

3.5. Two-stage kinetics of DPCD temperature inactivation of cathepsin L

The data in Fig. 3A was simulated, plotted, and calculated using a two-stage dynamic model (Equation (9)). It can be seen from Fig. 3C that the two-stage kinetic model process of DPCD inactivation of cathepsin L could be divided into rapid inactivation period and stable inactivation period in the temperature range of 40–60 °C. It can be seen from Table 4 that the two-stage kinetic model fitted the experimental data well in the temperature range of 35–60 °C, and the determination coefficient (R^2) reached 0.99, but the model coefficient A_F (total enzyme activity inactivated in rapid stage) fitted at 35 °C was negative, which was not realistic. Therefore, in the range of 40–60 °C, the two-stage kinetic model was basically used to describe the inactivation process of cathepsin L by DPCD treatment temperature. With the increase of temperature, k_F and k_S values increased, while D_F and D_S values decreased. k_F values in rapid inactivation period were significantly higher than k_S values in stable inactivation period, which indicated that cathepsin L was inactivated rapidly in rapid inactivation period, and the dependence of stable inactivation period on temperature was higher than that in rapid inactivation period.

The Z_T and E_a values of the two stages were calculated by processing the data in Table 4 according to Equations (7) and (8), respectively. As can be seen from Fig. 4B and D, the values of $Z_{T,\ F}$ and $E_{a,\ F}$ were 40.8 °C and 21.23 kJ/mol, respectively. As can be seen from Fig. 4A and C, the values of $Z_{T,\ s}$ and $E_{a,\ s}$ in stable inactivation period were 64.1 °C and 13.45 kJ/mol, respectively. The values of $Z_{T,\ F}$ in the fast inactivation stage were lower than those in the stable inactivation stage, which indicated that the dependence of cathepsin L in the fast inactivation stage on temperature was greater than that in the stable inactivation stage. In many studies, it has been found that the inactivation of many enzymes by DPCD accorded with the two-stage kinetic model. Liu et al. (2013) used DPCD to treat polyphenol oxidase in watermelon juice, and found that the two-stage kinetic model could be used to describe its inactivation process. Zhi et al. (2008) used DPCD to treat pectin methylesterase in apples, and also used two-stage kinetic model to describe its inactivation process.

3.6. Comparison of the effects of heat treatment and DPCD temperature treatment on cathepsin L activity

As shown in Fig. 5 that when the temperature was 35 °C, the difference between the relative enzyme activity of heat treatment and that of DPCD treatment gradually increased with the increase of time, indicating that DPCD has a significant inactivation effect on cathepsin L when the temperature was 35 °C. This may be because the temperature of 35 °C was low, and heat treatment could not inactivate the enzyme, therefore, even with the increase of time, the effect of heat treatment on cathepsin activity has little change. While carbon dioxide at the same temperature mutated in supercritical state, which had zero surface tension, could wet and penetrate into protein molecules (Li et al., 2021; Liu et al., 2021), interfere with its local conformation, and destroy the structure of natural protease, and the penetrate content of carbon dioxide increased with the prolongation of reaction time, thus promoting the inactivation of cathepsin L. In addition, carbon dioxide also reacted with amide groups of some amino acid residues in protein chain to form amino bonds, thus changing the structure of protease (Sheikh et al, 2023a, Saini, & Sharma, 2023). At low temperature (35 °C), the reaction between carbon dioxide and protein gradually increased with the increase of reaction time, thus promoting the inactivation of cathepsin L. The solubility of carbon dioxide increased in the supercritical state (Heidari et al, 2023, Aleosfoor, Mohammadi, & Esmaeilzadeh, 2023b), and it combined with water to form carbonic acid, which dissociated into bicarbonate, carbonate and hydrogen ion, and this reaction increased with the increase of reaction time. This was beneficial to reduce the electrostatic force on the surface of protein molecules, promote the aggregation and denaturation of protein molecules, and then lead to the decrease of cathepsin L activity (Ding et al., 2020). When the temperature ranged from 40 °C to 60 °C, the relative enzyme activity difference between heat treatment and DPCD increased at first and then decreased with the increase of time, which indicated that DPCD had obvious inactivation effect on cathepsin L in a short time, but with the extension of time, both heat treatment and DPCD could effectively inactivate cathepsin L, and when the treatment time was 60 min, the relative enzyme activity of the two treatments approached zero. This

Table 3
Kinetic parameters of first-order reaction for inactivation of cathepsin L by DPCD treatment at different temperature.

Temperature (°C)	k (min^{-1})	D (min)	R^2 ($P < 0.05$)
35	0.0284	81	0.95
40	0.0253	91	0.78
45	0.0265	87	0.76
50	0.0286	80	0.76
55	0.0390	59	0.82
60	0.0465	50	0.83

Table 4
Kinetic parameters of second-order reaction for inactivation of cathepsin L by DPCD treatment at different temperature.

Temperature (°C)	A_F	k_F (min^{-1})	D_F (min)	A_S	k_S (min^{-1})	D_S (min)	R^2 ($P < 0.05$)
35	−0.1731	−0.0694	−33	99.7962	0.0217	106	0.98
40	63.5405	0.3773	6	36.4162	0.0163	141	0.99
45	65.4289	0.4940	5	34.5579	0.0178	129	0.99
50	67.9371	0.5743	4	32.0583	0.0199	116	0.99
55	73.6508	0.9355	3	26.3492	0.0266	87	0.99
60	77.7859	1.1249	2	22.2141	0.0327	70	0.99

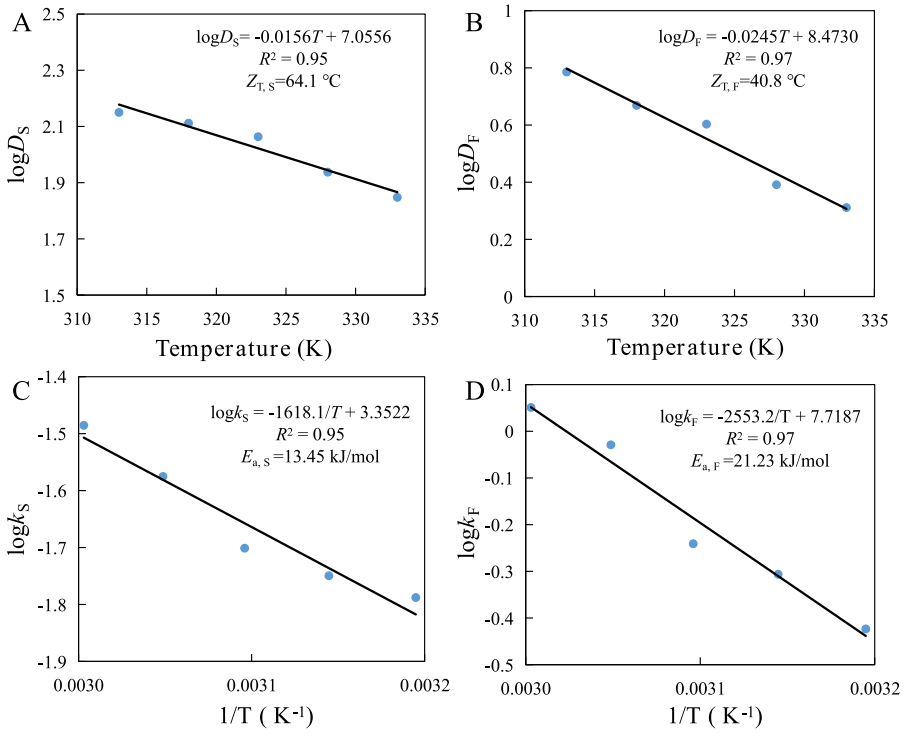

Fig. 4. $Z_{T,S}$ value (A), $Z_{T,S}$ (B), $E_{a,S}$ (C), and $E_{a,F}$ (C) value of pressure inactivation of cathepsin L at different temperature during DPCD treatment.

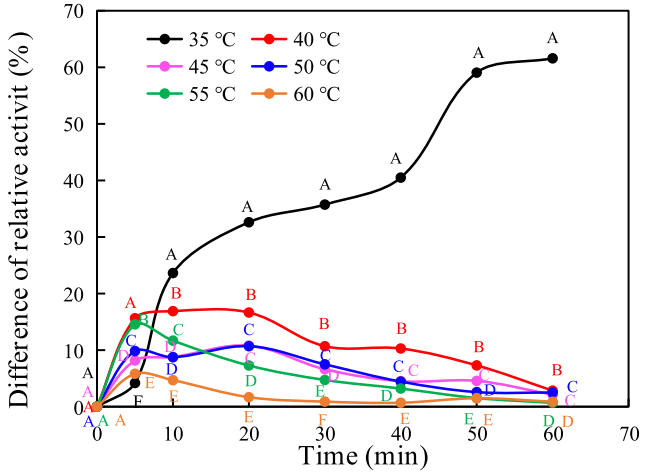

Fig. 5. Difference of cathepsin L relative activity (%) between heating treatment and DPCD treatment at different temperature. Different capital letters indicate significant differences between groups with the same treatment time and different treatment temperatures ($P < 0.05$).

maybe because the enzyme inactivation caused by DPCD treatment includes thermal effect caused by temperature, mechanical damage caused by pressure, and molecular effect caused by supercritical CO_2 (Sheikh, Saini and Sharma, 2023a). With the increase of treatment temperature, the proportion of enzyme inactivation caused by thermal effect increased, so the difference between heat treatment and DPCD treatment on enzyme activity decreased gradually.

It can be seen from Table 5 that the activation energy of cathepsin L inactivated in the two stages of DPCD treatment was higher than that of heat treatment. Only from the perspective of heat energy, heat treatment was easier to inactivate cathepsin L than DPCD treatment. However, DPCD was a non-heat treatment technology. In addition to thermal energy effect, the molecular action of DPCD was more important in inactivating enzymes. It can also be seen from Table 5 that the Z_T values of cathepsin L in both stages of DPCD treatment were lower than those of heat treatment, which showed that cathepsin L was more sensitive to temperature under DPCD treatment than under heat treatment. That was, at the same temperature, DPCD treatment was easier to inactivate

Table 5
Kinetic parameters of second-order reaction for inactivation of cathepsin L by heating treatment at different temperature.

Treatment	$Z_{T,F}$ (°C)	$E_{a,F}$/kJ·(mol^{-1})	$Z_{T,S}$ (°C)	$E_{a,S}$/kJ·(mol^{-1})
Heating	158.7	5.5	71.9	12
DPCD	40.8	21.23	64.1	13.45

cathepsin L than heat treatment. This may be mainly because the molecular action of CO_2 under high pressure changed the structure of cathepsin L, which made it more sensitive to temperature. By combining the effects of heat treatment and DPCD temperature on cathepsin L activity, it can be concluded that DPCD has better inactivation effect on cathepsin L at the same treatment temperature and time.

4. Conclusions

The effects of DPCD treatment pressure and temperature on cathepsin L activity were studied. The results showed that the inactivation of cathepsin L by DPCD treatment pressure accorded with the first-order kinetic model. The inactivation of cathepsin L by DPCD treatment at 35 °C accorded with the first-order kinetic model and at 40–60 °C accorded with the two-stage kinetic model. Cathepsin L in DPCD treatment was more dependent on temperature than in heat treatment. Compared with heat treatment, cathepsin L was more easily inactivated by DPCD treatment.

CRediT authorship contribution statement

Ouyang Zheng: Methodology, Writing, Writing – original draft. **Xiaojie Cao:** Conduct experiments. **Yanqing Teng:** Conceptualization. **Qinxiu Sun:** Writing – review & editing. **Shucheng Liu:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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References

Arbal, A., Ghangale, D., Wadje, P., Kumar, M.K., Byresh, T.S., Sengar, A.S., Sunil, C.K., 2024. Dense phase carbon dioxide (DPCD) inactivation of microorganisms and enzymes, and its application in food: A review. Food Chem. Adv. 5, 100782.

Benito-Román, Ó., Sanz, M.T., Melgosa, R., de Paz, E., Escudero, I., Beltrán, S., 2019. Studies of polyphenol oxidase inactivation by means of high pressure carbon dioxide (HPCD). J. Supercrit. Fluids 147, 310–321.

Cao, H., Zhu, H., Wang, Q., Fan, D., Zhang, H., 2020. Intervention on activity and structure of cathepsin L during surimi gel degradation under microwave irradiation. Food Hydrocolloids 103, 105705.

Chen, J.L.C., Zheng, X.J., Dong, J., Song, L.J., 2013. Effect of dense phase carbon dioxide on inactivation of polyphenol oxidase and peroxidase in hami melon juice. Adv. Mater. Res. 634–638, 1441–1448.

Chen, N., Gao, P., Jiang, Q., Yu, X., Li, P., Xu, Y., Yu, D., Yang, F., Xia, W., 2022. Effects of deheading and rinsing pretreatment on the quality of white leg shrimp (*Litopenaeus vannamei*) surimi based on endogenous proteases. Food Res. Int. 160, 111678.

Ding, L., Zhao, Q., Zhou, X., Tang, C., Chen, Y., Cai, Z., 2020. Changes in protein structure and physicochemical properties of egg white by super critical carbon dioxide treatment. J. Food Eng. 284, 110076.

Ding, N., Sun, X., Yu, Q., Hong, H., Luo, Y., Tan, Y., 2024. Unlocking the secrets of crude myofibril-bound serine protease from grass carp: the role in degrading myofibrillar proteins. Food Chem. 437.

Duong, T., balaban, M., 2014. Optimisation of the process parameters of combined high hydrostatic pressure and dense phase carbon dioxide on enzyme inactivation in feijoa (*Acca sellowiana*) puree using response surface methodology. Innovat. Food Sci. Emerg. Technol. 26, 93–101.

Duan, W., Qiu, H., Htwe, K.K., Sun, Q., Han, Z., Wang, Z., Liu, Y., Wei, S., Liu, S., 2024. Investigation of the relationship between gel strength and microstructure of surimi gel induced by dense phase carbon dioxide based on quantitative analysis. Food Hydrocolloids 146, 109209.

Duan, W., Qiu, H., Htwe, K.K., Wang, Z., Liu, Y., Wei, S., Xia, Q., Sun, Q., Han, Z., Liu, S., 2023. Correlation between water characteristics and gel strength in the gel formation of golden pompano surimi induced by dense phase carbon dioxide. Foods 12 (5), 1090.

Heidari, S., Aleosfoor, A., Mohammadi, M., Esmaeilzadeh, F., 2023. Solubility prediction of CO_2 in ionic liquids under subcritical and supercritical carbon dioxide conditions by the adaptive neuro-fuzzy inference system. Case Studies in Chemical and Environmental Engineering 7, 100317.

Htwe, K.K., Duan, W., Wei, S., Sun, Q., Wang, Z., Han, Z., Liu, Y., Liu, S., 2023. Quantitative analysis of the correlation between gel strength and microstructure of shrimp surimi gel induced by dense phase carbon dioxide. Food Res. Int. 174, 113623.

Hua, Z., 2013. Effect of desen phase carbon dioxide on enzyme activity of fresh-cut lotus root. Food & Machinery.

Huang, P., Cheng, Y., Hsieh, H., Ko, W., Lu, W., Li, P., 2023. Effects of transglutaminase on the physicochemical properties of surimi and kamaboko prepared by thermal and low level-pressure treatments. LWT–Food Sci. Technol. 183, 114863.

Illera, A.E., Sanz, M.T., Beltrán, S., Benito-Román, O., 2019. Effect of high pressure carbon dioxide on polyphenoloxidase from *Litopenaeus vannamei*. *LWT-Food Science & Technology*, 109, 359–365.

Kwon, C.W., Chang, P.S., 2023. Role of endogenous cathepsin L in muscle protein degradation in olive flounder (*Paralichthys olivaceus*) surimi gel. Molecules 26 (7), 1901.

Li, X., Shi, J., Scanlon, M., Xue, S.J., Lu, J., 2021. Effects of pretreatments on physicochemical and structural properties of proteins isolated from canola seeds after oil extraction by supercritical-CO_2 process. LWT–Food Sci. Technol. 137, 110415.

Lima, J.C., Seixas, F.A.V., Coimbra, J.S.R., Pimentel, T.C., Barão, C.E., Cardozo-Filho, L., 2019. Continuous fractionation of whey protein isolates by using supercritical carbon dioxide. J. CO2 Util. 30, 112–122.

Liu, S., Liu, Y., Luo, S., Dong, A., Liu, M., Ji, H., Gao, J., Hao, J., 2017. Molecular dynamics simulation of the interaction between dense-phase carbon dioxide and the myosin heavy chain. Journal of CO_2 Utilization 21, 270–279.

Liu, X., Gao, Y., Peng, X., Yang, B., Xu, H., Zhao, J., 2008. Inactivation of peroxidase and polyphenol oxidase in red beet (*Beta vulgaris L*) extract with high pressure carbon dioxide. Innovat. Food Sci. Emerg. Technol. 9 (1), 24–31.

Liu, Y., Hu, X., Zhao, X., Zhang, C., 2013. Inactivation of polyphenol oxidase from watermelon juice by high pressure carbon dioxide treatment. J. Food Sci. Technol. 50 (2), 317–324.

Liu, Y., Kang, N., Cheng, H., Chu, X., Sun, Z., Xi, C., 2021. Preparation and characterization of whey protein isolate nanoparticles in supercritical CO_2. LWT–Food Sci. Technol. 144, 111227.

Marszałek, K., Doesburg, P., Starzonek, S., Szczepańska, J., Woźniak, Ł., Lorenzo, J.M., Skąpska, S., Rzoska, S., Barba, F.J., 2019. Comparative effect of supercritical carbon dioxide and high pressure processing on structural changes and activity loss of oxidoreductive enzymes. J. CO2 Util. 29, 46–56.

Melgosa, R., Sanz, M.T., G. Solaesa, Á., Bucio, S.L., Beltrán, S., 2015. Enzymatic activity and conformational and morphological studies of four commercial lipases treated with supercritical carbon dioxide. J. Supercrit. Fluids 97, 51–62.

Mitsuda, H., Kawai, F., Yamamoto, A., Nakajima, K., 1975. Carbon dioxide-protein interaction in a gas-solid phase. in this experiment. This adsorption was found to be almost specific to carbon dioxide gas. J. Nutr. Sci. Vitaminol. 21 (3), 151–162.

Monhemi, H., Housaindokht, M.R., Nakhaei, P.A., 2015. Effects of natural osmolytes on the protein structure in supercritical CO_2: molecular level evidence. J. Phys. Chem. B 119, 10406–10416.

Ortuño, C., Duong, T., Balaban, M., Benedito, J., 2013. Combined high hydrostatic pressure and carbon dioxide inactivation of pectin methylesterase, polyphenol oxidase and peroxidase in feijoa puree. J. Supercrit. Fluids 82, 56–62.

Senyay-Oncel, D., Kimiz-Gebologlu, I., Yesil-Celiktas, O., 2023. New developments in supercritical fluids as a green technology: processing of β-glucanase with sub- and supercritical carbon dioxide. Biochem. Eng. J. 195, 108934.

Sheikh, M.A., Saini, C.S., Sharma, H.K., 2023a. Structural modification of plum (*Prunus domestica L*) kernel protein isolate by supercritical carbon-dioxide treatment: functional properties and in-vitro protein digestibility. Int. J. Biol. Macromol. 230, 123128.

Sheikh, M.A., Saini, C.S., Sharma, H.K., 2023b. Supercritical carbon dioxide treatment of plum (*Prunus domestica L*) kernel protein isolate: impact on structural, thermal, physical, and functional properties. Sustainable Chemistry and Pharmacy 32, 100979.

Singh, A., Benjakul, S., 2018. Proteolysis and its control using protease inhibitors in fish and fish products: a review. Compr. Rev. Food Sci. Food Saf. 17 (2), 496–509.

Tang, Y., Jiang, Y., Jing, P., Jiao, S., 2021. Dense phase carbon dioxide treatment of mango in syrup: microbial and enzyme inactivation, and associated quality change. Innovat. Food Sci. Emerg. Technol. 70, 102688.

Tsai, W.C., Rizvi, S.S.H., 2016. Liposomal microencapsulation using the conventional methods and novel supercritical fluid processes. Trends Food Sci. Technol. 55, 61–71.

Ueki, N., Matsuoka, Y., Wan, J., Watabe, S., 2019. Quality improvement of thermally induced surimi gels by extensive washing for dressed white croaker to remove contamination by body surface mucus proteases. Fisheries Science 85 (5), 1–11.

Xiong, Z., Shi, T., Jin, W., Bao, Y., Monto, A.R., Yuan, L., Gao, R., 2022. Gel performance of surimi induced by various thermal technologies: a review. Crit. Rev. Food Sci. Nutr. 1–16.

Zhang, L., Liu, S., Ji, H., Zhang, C., Gao, J., 2011. Inactivation of polyphenol oxidase from Pacific white shrimp by dense phase carbon dioxide. Innovat. Food Sci. Emerg. Technol. 12 (4), 635–641.

Zheng, O., Luo, S., Sun, Q., Liu, S., Wei, S., Xia, Q., Ji, H., Hao, J., Deng, C., 2021. Radial adsorption behaviour of high pressure carbon dioxide in shrimp surimi. Innovat. Food Sci. Emerg. Technol. 72, 102744.
Zheng, O., Sun, Q., Dong, A., Han, Z., Wang, Z., Wei, S., Xia, Q., Liu, Y., Ji, H., Liu, S., 2022. Gelation process optimization of shrimp surimi induced by dense phase carbon dioxide and quality evaluation of gel. Foods 11 (23), 3807.
Zhi, X., Zhang, Y., Hu, X., Wu, J., Liao, X., 2008. Inactivation of apple pectin methylestera induced by dense phase carbon dioxide. J. Agric. Food Chem. 56 (13), 5394–5400, 2008.
Zhu, C., Chen, L., Zeng, X., Sun, Y., Jiao, D., Liu, M., Zheng, M., Liu, J., Liu, H., 2022. Effects of ultra high pressure-magnetic field treatment on protein properties and quality characteristics of stored shrimp (*Litopenaeus Vannamei*). LWT–Food Sci. Technol. 170, 114070.