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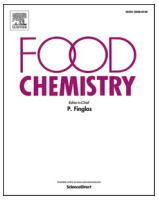

Succinylation of zein and gelatin hydrolysates improved their ice recrystallization inhibition activity

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ABSTRACT

The goal of this research was to enhance the ice recrystallization inhibition (IRI) activity of zein and gelatin hydrolysates (ZH and GH, respectively) by succinylation modification. ZH was prepared by Alcalase treatment for 3 h and then modified by succinic anhydride (SA); whereas GH was made by Alcalase hydrolysis for 0.25 h and succinylated by *n*-octylsuccinic anhydride (OSA). After 0.5 h of annealing at −8 °C at 40 mg/mL, modified hydrolysates decreased the average Feret's diameter of ice crystal from 50.2 μm (polyethylene glycol, negative control) to 28.8 μm (SA modified ZH) and 29.5 μm (OSA modified GH) in comparison to the unmodified hydrolysates, which had the crystal size of 47.2 μm (ZH) and 45.4 μm (GH). Also, the two succinylated samples had altered surface hydrophobicity, which potentially contributed to their enhanced IRI activity. Our results indicate that succinylation of food-derived protein hydrolysates can improve their IRI activity.

1. Introduction

Ice crystal growth during cold storage is a quality issue in frozen foods, and finding effective agents to prevent ice from continuing to grow is an active research topic. Antifreeze proteins (AFPs) are excellent candidates for inhibiting ice crystal growth and are produced in nature by fish, arthropods, winter plants, bacteria, and fungi that have evolved to survive in sub-freezing temperatures. AFPs help reduce cell damage during cryopreservation (Shaliutina-Loginova & Loginov, 2023; Sreter, Foxall, & Varag, 2022). Although the mechanisms of AFPs generally include thermal hysteresis (TH) and ice binding activity, with the latter being considered to be more relevant (Gruneberg, Graham, Eves, Agrawal, Oleschuk, & Davies, 2021), the mechanism of each class of cryoprotective compound may be different (Biggs, Stubbs, Graham, Fayter, Hasan, & Gibson, 2019). Previous studies suggested that hydrogen bonding is the main driving force of ice binding (Damodaran & Wang, 2017). For example, peptides from gelatin, rich in hydroxyl groups, can bind to the prism face of ice by hydrogen bonding (Damodaran & Wang, 2017) and inhibit ice recrystallization and growth. Meanwhile, polyvinyl alcohol (PVA) prevents ice growth through adjacent hydroxyl groups (Wu, Yao, Zhang, & Li, 2021). In addition, hydrophobicity is an important characteristic of antifreeze activity (Hudait, Qiu, Odendahl, & Molinero, 2019). Mutants' substitution experiments indicate that hydrophobic methyl groups of type I and III AFPs contribute to the ice growth inhibition (Zhang, Liu, Fu, Shao, & Cai, 2022; Hudait et al., 2019). Furthermore, the addition of long hydrophobic groups (alkyl chains) significantly increased galactose's ice recrystallization inhibition (IRI) activity (Balcerzak, Febbraro, & Ben, 2013). However, increasing hydrophobicity substantially will result in aggregation and lower solubility, decreasing IRI activity. Therefore, the balance between the hydrophobic and hydrophilic domains is an important feature of IRI active molecules (Biggs et al., 2019; Graham, Fayter, Houston, Evans, & Gibson, 2018). For example, antifreeze glycoproteins (AFGPs) interact with the ice through their hydrophobic peptide backbone while forming hydrogen bonds through their saccharide substituents (Mochizuki & Molinero, 2018).

In order to have a better understanding of IRI activity, gelatin peptides were studied in this work because their amino acid composition is approximately one-third proline and hydroxyproline, both of which contain functional groups capable of hydrogen bonding during the IRI process (Damodaran & Wang, 2017). However, gelatin by itself does not provide an effective hydrophobic moiety and it has high water solubility. On the other hand, zein is rich in non-polar amino acids such as leucine and alanine (Gong, Aguirre, & Bassi, 2016), thus it is more hydrophobic and has traditionally been used to make a variety of bioactive peptides, such as ACE inhibitory peptides (Lin et al., 2011) and anti-

Abbreviations: GH, gelatin hydrolysate; IRI, ice recrystallization inhibition; OSA, n-octylsuccinic anhydride; SA, succinic anhydride; ZH, zein hydrolysate.

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alcoholism peptides (Laniewska-Dunaj, Jelski, Orywal, Kochanowicz, Rutkowski, & Szmitkowski, 2013). It is safe, abundant, and feasible to obtain as a by-product of corn starch isolation and ethanol production (Cheng & Jones, 2017). Thus, zein and gelatin were used as a pair of proteins with different amino acid composition and solubility profiles to study how enzyme hydrolysis and succinylation modification affect their IRI activity.

It is expected that peptide modification will improve IRI activity by modulating their hydrophilic and hydrophobic domains. As a result, succinylation was selected in this study because it can change the surface hydrophobicity (Shilpashree, Arora, Chawla, & Tomar, 2015) of most proteins, such as whey protein isolate (Pan, Li, Meng, & Zhang, 2020), to various extents. In addition, it is a generally recognized as safe (GRAS) agent according to the Food and Drug Administration, with no limitations other than good manufacturing practices (Wan, Liu, & Guo, 2018). There are two agents that can be used in succinylation: succinic anhydride (SA) and *n*-octylsuccinic anhydride (OSA). When peptides are modified by SA, carboxylic groups and a negative charge can be introduced to the zein peptides (Yang, Yang, Zhang, & Zhang, 2016), resulting in increased hydrophilicity. Compared to SA, OSA has an octyl group that is hydrophobic, and when it is added to the gelatin peptide, it will increase hydrophobicity. Thus, by comparing the ice crystal size of peptides succinylated with SA or OSA, we may determine the influence of hydrophobic and hydrophilic groups on IRI activity. Our hypothesis is that the balance between hydrophilicity and hydrophobicity of peptides will affect their corresponding IRI activities. Therefore, the objectives of this study were to investigate the effect of enzymatic hydrolysis and succinylation on the IRI activity of gelatin hydrolysates (GH) and zein hydrolysates (ZH), and to determine the effect of concentration and pH on activity. This study is the first to report on the comparative IRI activity of zein and gelatin hydrolysates as affected by succinylation. It is expected that this work will advance our knowledge of how to create peptides with IRI activity from easily accessible protein sources.

2. Materials and methods

2.1. Materials

Zein was purchased from Acros Organics (New Jersey, USA), and the protease from *Bacillus licheniformis* (Alcalase, 3.03 U/mL) was from EMD Millipore Corp (Massachusetts, USA). Knox gelatin was purchased from a local supermarket. Polyethylene glycol 400 (PEG, M_W 380–420 Da) was purchased from Sigma Aldrich (Saint Louis, MO). SA was purchased from Thermo Fisher Scientific (Massachusetts, USA) and OSA was from TCI America (Oregon, USA).

2.2. Preparation of zein and gelatin hydrolysates by Alcalase

In 400 mL of 50 % ethanol, 8 g of zein was dissolved. Then, the pH of zein solution was adjusted to 8.0 with 1 N NaOH. Alcalase was then added at an enzyme-to-substrate ratio of 0.176 Anson units/g zein. The mixture was incubated at 55 ℃ for 1, 3, and 6 h. The hydrolysates were boiled for 1.5 h to remove ethanol and inactivate the enzymes. After centrifugation at 10000 × g for 10 min at room temperature, the supernatant was collected and freeze-dried before use. For the preparation of GH, gelatin (2.5 % w/v aqueous solution) was hydrolyzed using Alcalase at 55 ℃ for 0.25, 0.5, 1, and 2 h. After the pH of the solution was adjusted to 8.0 by 1 N NaOH, Alcalase was added at an enzyme-to-substrate ratio of 0.176 Anson units/g gelatin. The samples were treated the same as above with boiling time of 10 min to inactivate the enzyme.

2.3. Quantifying the degree of hydrolysis (DH) by the o-pthaldialdehyde (OPA) method

Degree of hydrolysis of the hydrolysate samples was analyzed based on the methods of Spellman, McEvoy, O'Cuinn, and FitzGerald (2003) with slight modifications. Fresh OPA reagent was prepared by mixing 25 mL of 100 mM sodium borate, 2.5 mL of 20 % SDS solution, 1 mL of 40 mg/mL OPA dissolved in methanol, 100 μL β-mercaptoethanol, and DI water adjusting the solution to a total volume of 50 mL. The peptide samples were prepared with a concentration of 1 mg/mL in 1× PBS buffer. Ten μL of each sample was placed in a well in a 96-well plate and filled with 200 μL of freshly prepared OPA reagent. Two blank wells were also filled with 1x PBS buffer. The absorbance was read at 340 nm after 5 min incubation. Absorbance was read using a Cambrex ELx808 Microplate Reader. DH values were calculated using the following formula:

$$DH\% = \frac{100n}{N}$$

where n is the OD value of sample, N is the OD value of totally hydrolyzed protein by HCl prepared as follows: briefly, 1 mg nonhydrolyzed protein sample was mixed with 500 μL 6 N HCl and incubated in a 110 ℃ oven overnight. After cooling to the room temperature, 500 μL 6 N NaOH was added to neutralize the system, and the same OPA procedure was applied.

2.4. Determining average molecular weight of gelatin and zein hydrolysates by size exclusion chromatography

The molecular weight distribution for each hydrolysate and protein sample was analyzed using a size exclusion (BioSep-*SEC* -s2000 column Phenomenex, Torrence, CA, USA) HPLC. The mobile phase consisted of a mixture of acetonitrile and ultrapure water in a proportion of 45:55 (v/v), containing 0.05 % trifluoroacetic acid. Isocratic elution was carried out using a flow rate of 1.0 mL/min for 20.0 min with sample injection volume of 20 μL. To determine the molecular weight distribution of the samples, the elution profile was compared to that of mixture of protein standards with known molecular weights (M_w) detected at 214 nm. The retention times and the log of M_w of the standards were used to construct a linear curve fit (R^2 = 0.981) and the established standard curve equation was used to calculate the M_w of each peak in the chromatogram. The average M_w was calculated based on the percentage contribution of each peak as determined by area under the curve. When calculating the average molecular weight, the peak of $M_w < 200$ Da was excluded.

2.5. Measuring the surface hydrophobicity of the hydrolysate samples

The surface hydrophobicity of the hydrolysates and their modified counterparts was determined by fluorescence spectroscopy with 1-anilino-8-naphthalene sulfonate (ANS) as a probe. The hydrolysates and modified samples (5 mg) were mixed with 1 mL of 1xPBS and vortexed for 1 h at room temperature. After centrifugation at 10000 × g for 30 min at 4 °C, the supernatants were collected and diluted with PBS to obtain a range of concentrations between 0.0156 and 0.5 mg/mL. Diluted samples were mixed with ANS reagent on a black polystyrene 96-well microplate. The fluorescence intensity was measured at excitation and emission wavelengths of 390 and 479 nm, respectively (Synergy H1, BioTek, USA). The slope of the curve of the relative fluorescence intensity as a function of protein concentration was reported as the surface hydrophobicity of the sample.

2.6. Modification of hydrolysates by succinylation reaction

ZH were modified by SA based on a procedure from previous studies with minor modifications (Shilpashree et al., 2015; Wan et al., 2018). One gram of ZH was dispersed in 125 mL ammonium bicarbonate buffer (pH = 8.0, 0.075 M). SA was added at a molar ratio (based on the reactive amino group) of 1:1. The suspension was stirred vigorously at 700 rpm and sampled periodically, and the samples were incubated in a

water bath at 70 ℃ to evaporate the volatile buffer salt (Smith, 1994). The optimum reaction time was determined by the amount of the residual amino group at various reaction times. The residual amino group was quantified by the OPA method as above. GH was also modified by OSA with the same procedure.

2.7. Determining secondary structure by Fourier-transform infrared spectroscopy (FTIR)

The secondary structure of hydrolysates was analyzed by FTIR (Jiang et al., 2011) using Spectrum Two FT-IR Spectrometer (PerkinElmer Inc., Waltham, MA, US). Hydrolysates and their succinylated forms were prepared in 1 × PBS solution at a concentration of 40 mg/mL. The spectra were recorded with 32 scans in the region between 1800 and 1500 cm^{-1} range with a resolution of 4 cm^{-1} and data were analyzed using PerkinElmer Spectrum IR software. The amide I band (1600–1700 cm^{-1}) in each spectrum was interpreted by Peakfit Version 4.12 (Seasolve Software Inc, San Jose, CA, USA) software to determine peptide's secondary structure.

2.8. Analyzing IRI activity of hydrolysates by splat assay

The splat assay was carried out with sample solutions containing varying concentrations (1–40 mg/mL) prepared by dissolving in 1x PBS buffer, 180 mM HCl, or 180 mM NaOH. Ten μL of the peptide solution was dropped from a 1.5-meter height onto a precooled microscope slide and incubated at −80 ℃. The slide was annealed at −8 ℃ using a cold-stage HCS 302 (Instec Instruments, Boulder, CO) for 10, 20, 30, 40, 50, and 60 min, with pictures taken using the microscope's built-in digital camera (Leica Microsystems, Inc, USA), through the lens of L 20×/0.40. The negative control is 40 mg/mL polyethylene glycol (PEG) (Biggs et al., 2019). All hydrolysates and succinylated samples were prepared in duplicate (trial 1 and trial 2), and each sample was analyzed twice (2 slides). Three pictures were captured from each slide, from which the Feret's maximum diameter was averaged by ImageJ software (Saad, Fomich, Dia, & Wang, 2023).

2.9. Statistical analysis

All data were reported as average ± standard deviation and processed by Excel 2016, and SPSS 22.0, and figures were prepared using Origin 2021 Pro. The statistical significance of the data was determined using one-way analysis of variance (ANOVA) followed by the Duncan test, and the significance was established at $P < 0.05$.

3. Results and discussion

3.1. Degree of hydrolysis (DH) and average molecular weight (M_w) of peptides

During hydrolysis, Alcalase cut peptide bonds randomly, resulting in the production of peptides with various molecular sizes. Therefore, the extent of the enzymatic hydrolysis was assessed by analyzing DH % and average molecular weight of all samples. As shown in Table 1, the DH % increased as hydrolysis time was prolonged while the average M_w decreased. Previous studies have demonstrated that gelatin derived peptides have antifreeze activity, and the molecular weight of these peptides is between 150 and 2500 Da (Cao, Zhao, Zhu, Xu, Yu, & Yuan, 2016; Damodaran & Wang, 2017; W. Wang, Chen, Wu, & Wang, 2015), and our GH reported in this study are within this range. Traditionally, 70–90 % aqueous ethanol is used to dissolve zein (Li, Li, Xia, Zhang, Wang, & Huang, 2012). However, the activity of Alcalase in this concentration range was highly reduced (Simon, Kotorman, Garab, & Laczko, 2001). In order to have a balance between the solubility of zein and protease activity, ethanol concentration was decreased to 50% to prepare ZH. However, the protease activity was lower in such organic solvent, so the DH % of ZH was lower than that of gelatin even if the hydrolysis time was longer. At the same 1 h hydrolysis time, gelatin DH % was 2-fold higher than zein DH % (25.8 % for gelatin and 12.7 % for zein). Furthermore, the molecule fraction representing amino acid residues accounted for nearly 70 % of ZH according to the HPLC chromatogram, but that in GH accounts for <10%. This is because zein with high M_w tends to be less soluble and Alcalase intensively worked more on solubilized protein and peptides. As a result, the small peptide became even more hydrolyzed and possibly producing free amino acids. However, gelatin is soluble in DI water, and does not have this phenomenon.

Table 1
Degree of hydrolysis (DH) and average molecular weight (M_w) of protein hydrolysates generated by Alcalase treatment at different times.

Protein	Hydrolysis time (h)	DH (%) *	Average M_w (g/mol)
Zein	1	12.7 ± 3.8	887.6 ± 9.9^{a}
	3	14.2 ± 2.2	832.6 ± 27.3^{b}
	6	15.4 ± 2.9	814.8 ± 35.7^{b}
Gelatin	0.25	20.3 ± 1.2^{b}	1784.4 ± 71.2^{a}
	0.5	22.0 ± 1.6^{b}	1181.6 ± 108.0^{b}
	1	25.8 ± 0.8^{ab}	973.0 ± 91.7^{c}
	2	29.4 ± 1.8^{a}	937.9 ± 129.1^{c}

*DH: degree of hydrolysis. Different superscripts in the same column indicate significant differences ($P < 0.05$).

3.2. Modification of zein and gelatin hydrolysates by succinylation reactions

3.2.1. The degree of succinylation reaction

As shown in Fig. 1, the degree of succinylation was determined by measuring the residual amount of free amino group. The reaction of ZH and SA reached a maximum of 83.1 % at 1 h, while the reaction of GH and OSA peaked at 11 h. Based on this, reaction times were set at 1 and 11 h for SA and OSA modifications, respectively, in the subsequent experiments. The reaction time of GH by OSA (GH + OSA) is longer because OSA is hydrophobic and has a lower aqueous solubility than SA, resulting in a slower reaction. In addition, gelatin contains roughly one third of proline and hydroxyproline, which have pyrrolidine ring that can also slow the succinylation reaction of the amino nitrogen (Harrington & Von Hippel, 1962).

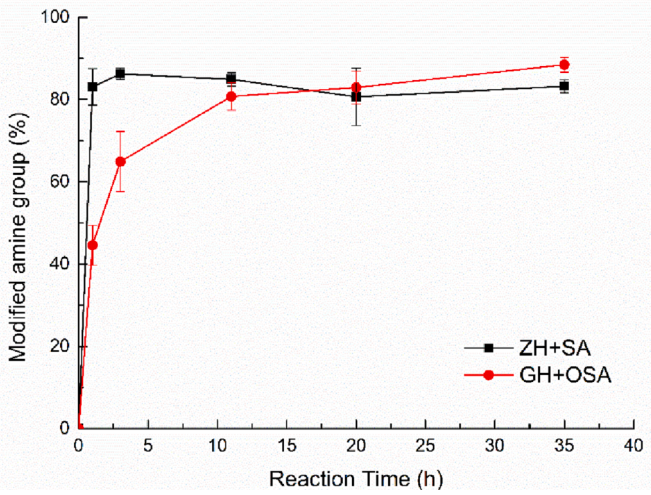

Fig. 1. The degree of modification of zein hydrolysate (3 h) modified by SA (ZH + SA), and gelatin hydrolysates (0.25 h) modified by OSA (GH + OSA) during 35 h reaction at room temperature.

3.2.2. The effect of succinylation reaction on IRI activity

The sizes of the ice crystals in samples with various hydrolysis times, as well as the corresponding succinylated samples, are shown in Fig. 2A–C. In all modified hydrolysates, whether they come from zein or gelatin, the size of the ice crystals is about 40% that of unmodified hydrolysates with the same analyte concentration (40 mg/mL). After modification, the ZH prepared by 3 h Alcalase hydrolysis has the smallest ice crystal size compared to the other two hydrolysates prepared at different times. This difference may be due to the different amino acid sequences or peptide size of the hydrolysates from different enzymatic treatments. The ice crystal size of all GH + OSA, on the other hand, was not significantly different ($P > 0.05$), even though the average M_w decreased significantly (Table 1). This demonstrates that the size of GH after modification has no effect on IRI activity. It may be due to the amino acid composition and sequences of GH. The small peptides in GH have tripeptide repeat sequences (Damodaran & Wang, 2017), and are rich in Pro to form hydrogen bond interactions between the ice crystal surface and water. After modification, OSA provides an alkyl side chain to stabilize the interaction (Wu, Rong, Wang, Zhou, Wang, & Zhao, 2015), and the M_w may not impact such interaction significantly. In contrast, ZH does not have a repeat sequence, and the different hydrolysates may have different amino acid sequences that affect the IRI activity.

Wu et al. (2021) found that some cryoprotectants, such as dimethyl sulfoxide and graphene oxide, lower the freezing point or stick to the surface of ice crystals through hydrogen bonds. Simulation also provided some evidence that hydroxyl groups contribute to the strength of the binding of the AFPs to ice (Hudait et al., 2019). Therefore, the introduction of succinyl groups on ZH contribute to an increase in carboxylic groups (Basak & Singhal, 2022), which helps to control the size of ice crystals.

On the other hand, hydrophobic groups play a key role in ice recrystallization. Type I and III antifreeze proteins bind ice via methyl groups (Zhang et al., 2022; Hudait et al., 2019).When a hydrophobic dimethylfulvene residue was incorporated on the glycan-opposing face, which was hydrophilic, the IRI activity of glycopolymers was significantly increased (Graham et al., 2018). Similarly, the IRI activity of the alkylated galactose derivatives increased when the addition of hydrophobic moieties (alkyl chains) went from seven to nine carbon atoms (Trant, Biggs, Capicciotti, & Ben, 2013). Furthermore, Hudait et al. (2019) used molecular simulations to show that methyl groups slow the movement of water at the ice-binding surface and stabilize the clathrate-like water in the anchored clathrate motif that binds these proteins to ice. Based on this, the addition of an octyl group in OSA, may also contribute to the IRI activity of GH + OSA.

3.3. Physicochemical characteristics of the modified hydrolysates

ZH, treatment by Alcalase for 3 h, had the smallest ice crystal size. Therefore, it was selected for further study. However, the IRI activity of all succinylated GH by OSA is similar; GH made by Alcalase for 0.25 h was chosen for further investigation.

3.3.1. Surface hydrophobicity

Fig. 2D depicts the effect of succinylation treatments on surface hydrophobicity. This technique involves the use of ANS as the extrinsic fluorescence probe. After modification, the surface hydrophobicity of modified ZH decreased from 7.8 to 4.2 because of the additional charge (Basak & Singhal, 2022), whereas when the GH was modified by OSA, the surface hydrophobicity increased from 0.6 to 5.3 due to the alkyl chain added. Therefore, all succinylated samples have a better balance between hydrophobic and hydrophilic groups than unmodified hydrolysates. This balance has been proven to correlate with IRI activity (Biggs et al., 2019; Graham et al., 2018). For example, PVA, AFPs, and AFGPs are known to be amphipathic (Biggs et al., 2019; Mitchell, Clarkson, Fox, Vipond, Scott, & Gibson, 2017; Mitchell & Gibson, 2015). Mochizuki and Molinero (2018) also detected the conformation structure of AFGPs and found that they can bind to the ice by hydrophobic methyl group, while hydrophilic side is important to stabilize the unique structure. Based on this, several polymers were developed, including self-assembled triplex metallohelices (Mitchell et al., 2017) and poly (Fulvo) (Graham et al., 2018), to mimic the amphiphilic structure of AFGPs and demonstrate strong IRI activity. Also, it was found that some other IRI active materials had amphiphilic structures on their faces (Mitchell & Gibson, 2015).

3.3.2. Secondary structure of the peptides

The percentage of secondary structure of hydrolysates and their succinylated forms is determined and shown in Table 2, and the FT-IR spectrum at 1600–1700 cm^{-1} is shown in Fig. 3. The band regions at 1610–1640 cm^{-1} and 1670–1690 cm^{-1} were assigned as β-sheet, and the random coil and α-helix ranges were 1640–1650 cm^{-1} and 1650–1660 cm^{-1}, respectively (Yuan et al., 2018). The peaks at 1660–1670 cm^{-1} and 1690–1700 cm^{-1} were also reported to result from β-turn (Jiang et al., 2011; Yuan et al., 2018). Prior to modification, the main secondary structure of ZH and GH is the β-sheet, accounting for 53.1% and 52.3%, respectively. This is consistent with the results of Zhang, Tu, Shen, and Dai (2019): the amide I band of GH from fish scale appeared at 1631 cm^{-1}, indicating that the β-sheet is the main secondary structure of GH. However, after modification, both ZH + SA and GH + OSA showed a decrease in β-turn and an increase in random coil and β-sheet, which means that the conformation become more flexible. This is also consistent with previous findings that SA-induced succinylation of wheat gluten resulted in a transition from β-turn to β-sheet (Liu, Zhang, Li, Yang, Yang, & Wang, 2018). This is because the introduction of succinyl groups increased the electrostatic repulsions by negatively charge (Basak & Singhal, 2022), which then made peptides more flexible, and the peptide chains were more stretched due to the reorganized hydrogen bonding (Wang, Gan, Zhou, Cheng, & Nirasawa, 2017). However, the structure of AFPs and AFGPs are rigid, which mainly based on α-helix and polyproline II helix, respectively (Mitchell et al., 2017; Mochizuki & Molinero, 2018). This is different from the results of this study, which may be because the samples in this study are mixtures of short peptides while those in the previous studies were whole proteins.

3.4. The effect of concentrations of succinylated hydrolysates on IRI activity

As shown in Fig. 4A, the ice crystal size of ZH and PEG solutions was similar at all concentrations. However, ZH + SA showed strong IRI activity, with a crystal size of 28.8 μm compared to that of the negative control sample (PEG), which is 50.2 μm, at the concentration of 40 mg/mL. Even if the concentration was decreased to 1 mg/mL, the ice crystal diameter was still 66.0 μm compared to that of PEG which is 73.8 μm. The IRI activity was significantly increased after modification, especially at higher concentrations. Similarly, although GH had a little IRI activity at a concentration of 40 mg/mL (its crystal size is 45.4 μm, compared to the negative control sample is 50.2 μm), this activity was lost when the concentration decreased. However, the IRI activity of its succinylated version (GH + OSA) is enhanced at all concentrations and still has smaller ice crystals at a concentration as low as 2.5 mg/mL. These endpoint concentrations (1 and 2.5 mg/mL for modified ZH and GH, respectively) are similar to that obtained from previous facially amphiphilic polymers, which slow ice growth in a range from 1 to 10 mg/mL (Biggs et al., 2019). Also, Biggs et al. (2019) found that cryoprotectants with the same mechanism had similar concentrations at which they started to inhibit the growth of ice crystals during preservation. Thus, it also provides some evidence that the IRI activity is from their amphiphilic structure. In addition, the different IRI endpoint concentrations of modified ZH and GH suggest that the intensity of IRI activity is different. For example, type III antifreeze proteins have two isoforms (SP and QAE), and the endpoint concentrations of the latter are

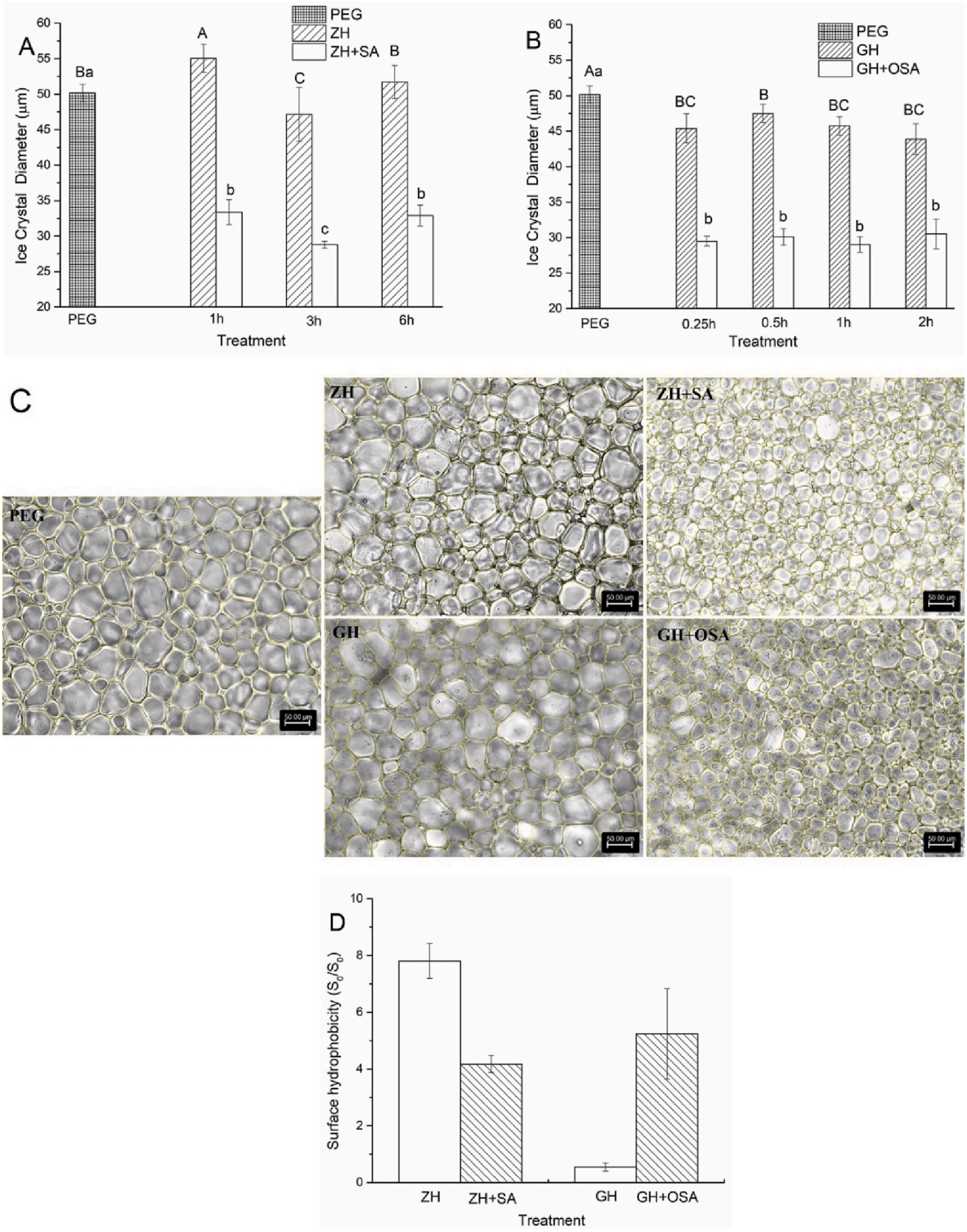

Fig. 2. (A–B) Effect of hydrolysis time and succinylation modification on average Feret's diameter of ice crystal as affected by 40 mg/mL zein (A) and gelatin (B) and their succinylated forms. Different lowercase letters represent significantly different means of unmodified hydrolysates and capital letters represent significantly different means of succinylated samples ($P < 0.05$). (C) Microscopic images of PEG, ZH (3 h), ZH + SA, GH (0.25 h), and GH + OSA solution at 40 mg/mL concentration after annealing for 30 min at −8 ℃ under 20x/0.40 lens (scale = 50 μm). (D) The surface hydrophobicity of hydrolysates and their corresponding modified samples. The data were shown as the ratio of hydrolysates or modified hydrolysates to their corresponding protein.

Table 2
Secondary structure profiles of different hydrolysates as determined by FTIR.

samples	Secondary structure (%)			
	β-sheet	random coil	α-helix	β-turn
ZH	53.1 ± 0.1	8.1 ± 2.6	16.8 ± 2.3	20.3 ± 2.7
ZH + SA	61.7 ± 0.8	10.1 ± 0.5	19.0 ± 1.9	9.3 ± 2.0
GH	52.3 ± 1.1	1.3 ± 0.1	18.6 ± 0.7	28.3 ± 2.4
GH + OSA	63.4 ± 0.7	6.8 ± 0.2	14.7 ± 0.5	15.2 ± 0.4

ZH: zein hydrolysates made by Alcalase for 3 h, ZH + SA: zein hydrolysates made by Alcalase for 3 h and then succinylated by SA, GH: gelatin hydrolysates made by Alcalase for 0.25 h, GH + OSA: gelatin hydrolysates made by Alcalase for 0.25 h and then modified by OSA.

half those of the former, which showed that the IRI activity of SP isoforms is weaker than that of QAE (Gruneberg et al., 2021). They also found that the different IRI activity is due to different ice planes' binding activities due to the structure changes. Furthermore, Hudait et al. (2019) demonstrated that both hydrogen-bonding and hydrophobic groups contribute equally but differently to ice binding. It is possible that the balance between the hydrophobic and hydrogen-bonding groups in ZH + SA and GH + OSA have contributed to their enhanced and different IRI activities.

3.5. Effect of pH on the IRI activity of SA and OSA modified zein hydrolysates

In order to analyze the effect of charge on the IRI activity of peptides, NaOH and HCl were used because the peptides will carry a relatively more positive charge in acidic solutions and a more negative charge in alkaline solutions. The ionic concentration also needed to be controlled similarly to 1xPBS, so 180 mM NaOH and 180 mM HCl solutions were used, and their pH values are 12.46 and 1.20, respectively. Fig. 4C–E show the ice crystal size of PEG solution remains similar in different pH solutions, but that in ZH and ZH + SA decreased from 60.1 and 41.8 μm in PBS to 46.0 and 26.6 μm in 180 mM NaOH. The ice crystal size increased to 91.1 and 55.7 μm in 180 mM HCl for ZH and ZH + SA, respectively, after 60 min of incubation. It is shown that two zein hydrolysates (ZH and ZH + SA) have higher IRI activity in alkaline solutions when all of them have a net negative charge. Similarly, this phenomenon was also shown in a previous study, in which the ice-binding protein from *Marinomonas primoryensis* (MpIBP), with a pI of 4.1, only exhibited IRI activity between pH 6 and pH 12, when it carried a net negative charge (Delesky, Thomas, Charrier, Cameron, & Srubar, 2021). Also, Burkey, Riley, Wang, Hatridge, and Lynd (2018) found that polymers with hydroxyl groups, like poly(ethylene oxide) (PEO), linear polyglycerol (PG), and poly(vinyl alcohol) (PVA), have higher IRI activity at high pH solution. They speculated that this might be because the pH affects hydrogen bonding in solutions.

4. Conclusion

In summary, succinylation is a feasible and safe way to enhance the IRI activity of ZH and GH by changing their hydrophobic and hydrophilic balance. ZH hydrolyzed by Alcalase for 3 h and then succinylated by SA had the highest IRI activity, while OSA modified GH made by

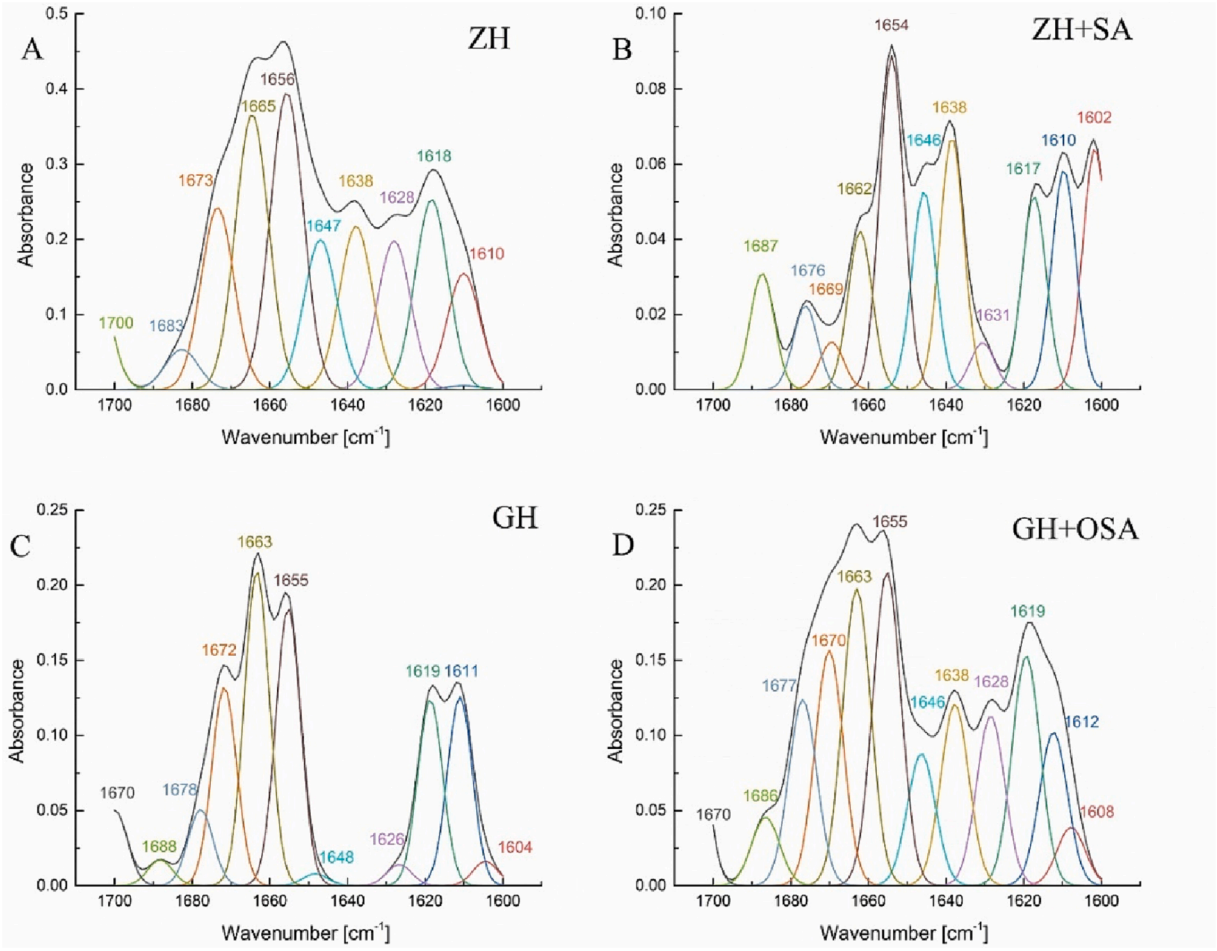

Fig. 3. The FTIR spectra of amide I band (1700–1600 cm^{-1}) of ZH (A), ZH + SA (B), GH (C) and GH + OSA (D) in 1 × PBS buffer at 40 mg/mL concentration and at room temperature.

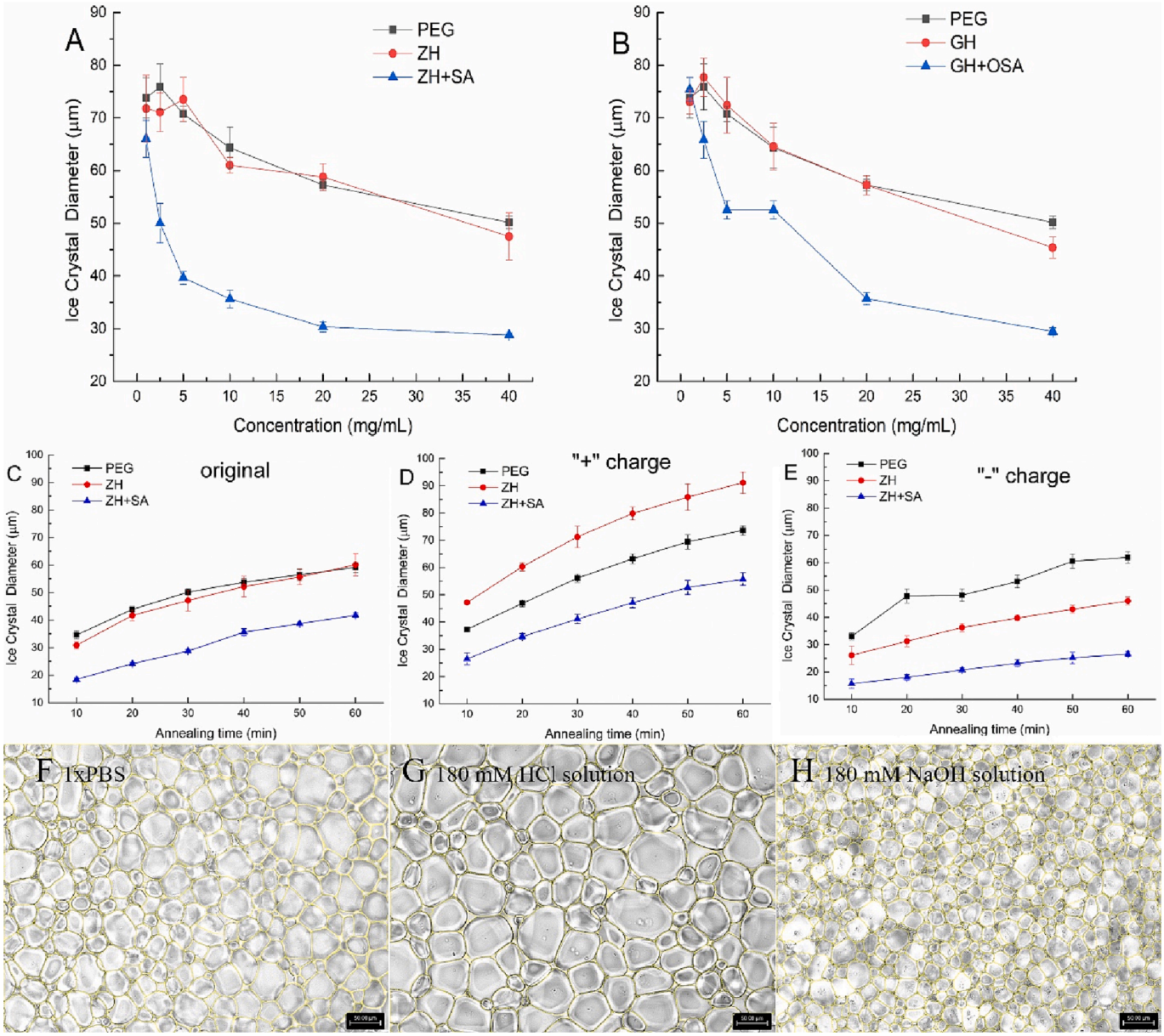

Fig. 4. (A-B) Average Feret's diameter of ice crystals affected by succinylated ZH (3 h) and gelatin (0.25 h) hydrolysates by SA (A) and OSA (B) analyzed by splat assay in 1xPBS at different concentrations after 60 min at −8 °C annealing. (C-E) Average Feret's diameter of ZH and ZH + SA ice crystals measured by splat assay in 1xPBS (pH = 7.3) (C), 180 mm HCl (pH = 1.20) (D), and 180 mM NaOH (pH = 12.46) (E) solutions at 40 mg/mL (w/v) for 60 min. (F-H) Microscope images of ZH + SA in 1xPBS (pH = 7.3) (F), 180 mm HCl (pH = 1.20) (G), and 180 mM NaOH (pH = 12.46) (H) solution (c = 40 mg/mL) after annealing for 60 min at −8℃ under 20x/0.40 lens (scale = 50 μm).

Alcalase for different times have similar IRI activity. Modified peptides showed strong IRI activity at the concentration as low as 2.5 mg/mL. Furthermore, the pH of the solution affected the IRI activity of the modified ZH, which shows that the overall negative charge can contribute in controlling ice crystal growth.

CRediT authorship contribution statement

Yuan Yuan: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Maddy Fomich:** Investigation, Writing – review & editing. **Vermont P. Dia:** Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition. **Tong Wang:** Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition.

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

Data will be made available on request.

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