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Inhibition of Ice Crystal Growth in Ice Cream Mix by Gelatin Hydrolysate

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The inhibition of ice crystal growth in ice cream mix by gelatin hydrolysate produced by papain action was studied. The ice crystal growth was monitored by thermal cycling between −14 and −12 °C at a rate of one cycle per 3 min. It is shown that the hydrolysate fraction containing peptides in the molecular weight range of about 2000 −5000 Da exhibited the highest inhibitory activity on ice crystal growth in ice cream mix, whereas fractions containing peptides greater than 7000 Da did not inhibit ice crystal growth. The size distribution of gelatin peptides formed in the hydrolysate was influenced by the pH of hydrolysis. The optimum hydrolysis conditions for producing peptides with maximum ice crystal growth inhibitory activity was pH 7 at 37 °C for 10 min at a papain to gelatin ratio of 1:100. However, this may depend on the type and source of gelatin. The possible mechanism of ice crystal growth inhibition by peptides from gelatin is discussed. Molecular modeling of model gelatin peptides revealed that they form an oxygen triad plane at the C-terminus with oxygen−oxygen distances similar to those found in ice nuclei. Binding of this oxygen triad plane to the prism face of ice nuclei via hydrogen bonding appears to be the mechanism by which gelatin hydrolysate might be inhibiting ice crystal growth in ice cream mix.

KEYWORDS: Ice cream mix; ice crystal growth inhibition; gelatin; gelatin hydrolysate; ice nucleation; cryoprotectants; antifreeze peptides; antifreeze proteins

INTRODUCTION

Water is one of the major, if not the predominant, components of food products. Consequently, changes in the physical state of water in food systems and/or the extent of its interactions with other food components during storage cause structural and textural changes in foods, which, in some cases, are detrimental to their quality. This is particularly a problem in frozen foods, such as meat, fish, desserts, and frozen fruits and vegetables (*1*). During freezing, which is a process of ice crystallization from supercooled water, nucleation of ice occurs first, followed by recrystallization of ice (*2*). The size distribution of ice crystals formed during this recrystallization stage has a strong influence on the texture of frozen foods (*3*, *4*) and the structural integrity of cell membranes (*5*, *6*). For instance, although ice crystals in the range of 15–20 μm bestow a desirable smooth texture to the ice cream, those that are larger than 40 μm impart an unacceptable coarse and grainy texture to the ice cream (*7*, *8*). Temperature fluctuations during storage and handling of frozen foods promote ice crystal growth. The crystal growth rate is very slow at lower storage temperatures, especially when the product is stored below its glass transition temperature (*9*–*11*). For ice cream, the glass transition temperature is typically in the range of −30 to −40 °C, depending on the sugar ingredient used (*12*). Above the glass transition temperature, the greater molecular mobility of water leads to faster growth of ice crystals. Because the typical average storage temperature in household freezers is well above −20 °C and fluctuates because of automatic defrost cycles (*13*), formation of large ice crystals and deterioration of textural qualities of frozen foods is a common occurrence under household conditions (*14*). Thus, one of the major challenges faced by frozen foods manufacturers is developing appropriate technological conditions and ingredient formulations that can inhibit ice crystal growth during storage and handling.

Addition of hydrocolloids, such as gums and polysaccharides, to frozen foods retards the rate of ice crystal growth (*15*); this has been attributed to increased viscosity of the serum phase, which slows down molecular mobility of water (*16*–*19*), and to a possible increase of the glass transition temperature (*7*). Available evidence indicates that hydrocolloids have no or only a marginal effect on heterogeneous nucleation temperature of supercooled water, but they have a measurable effect on ice crystal growth (*19*). However, there is no consensus on the mechanism because results from various studies have been contradictory (*19*–*22*).

Thus, there is a need for developing alternative ingredients for controlling ice crystal growth in frozen foods during storage. Previously, it was reported that enzymatic modification of gelatin with L-leucine n-dedecyl ester through the plastein reaction using papain resulted in a product that exhibited antifreeze properties (*23*–*25*). The L-leucine n-dodecyl ester derivative of gelatin

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hydrolysate was found to decrease the freezing point of water to about −7 °C in the presence of the heterogeneous nucleation inducer AgI, whereas the control hydrolysate decreased the freezing point only to about −4 °C (*23*). Other than these initial reports, to the best of our knowledge, no other study has been reported in the published literature. There are several unanswered questions in the previous studies (*23*, *24*); first, as recently demonstrated (*26*), it is almost nearly impossible to eliminate the influence of dust particles under normal crystallization conditions. Thus, experimentally determined ice nucleation temperature is not always very reliable (*26*). It is also not clear whether the L-leucine n-dodecyl ester residues were, in fact, essential to impart the antifreeze properties to gelatin hydrolysate.

In the present investigation, we studied the efficacy of low molecular weight peptides derived from papain-mediated hydrolysis of gelatin on the inhibition of ice crystal growth in ice cream mix assuming that peptides from gelatin may bind to ice nuclei and crystals.

MATERIALS AND METHODS

Gelatin (Type 225B40 from bovine) was obtained from Sanofi Bio-Industries (Waukesha, Wisconsin). Papain (EC 3.4.22.2) from papaya latex was purchased from Sigma Chemicals Co. (St. Louis, MO). According to the supplier, the specific activity of papain used in this study was 2.3 units/mg. One unit of enzyme is defined as hydrolysis of 1 μmol of benzoyl-L-arginine ethyl ester (BAEE) substrate per min at pH 6.2 at 25 °C. All other reagents were of analytical grade from Sigma Chemicals Co. (St. Louis, MO). Ice cream mix from a local commercial source was used in these studies. Although the exact composition of the ice cream mix was not available, it was assumed to contain the following gross composition typical for an ice cream mix: 12% milk fat in the emulsified form; 9–12% nonfat milk solids; 10–16% sucrose; 0.1% emulsifier; 0–0.4% stabilizer; and the rest as water. All studies reported here are on a single batch of ice cream mix, which was stored at −20 °C in 2 mL aliquots in cryovials. The sample from one vial was used for each set of experiments, and after the experiment the unused portion of the ice cream mix was discarded.

Gelatin Hydrolysis. A 20 wt% solution of gelatin was hydrolyzed using papain at 37 °C for 10 min at an enzyme-to-gelatin weight ratio of 1:100 in the presence of 10 mM cysteine under various pH conditions. Hydrolysis was stopped by incubating the solution for 5 min in boiling water. The gelatin hydrolysate was fractionated on a Sephadex G-50 gel permeation column (100 cm length and 2.6 cm diameter) using water at pH 7.0 as the eluent. Fractions (5 mL) were collected at a flow rate of 2 mL/min. The elution profile was determined by measuring the absorbance at 225 nm using a spectrophotometer. The tubes corresponding to various molecular weight ranges were pooled and lyophilized. The protein content of lyophilized samples was determined by the Biuret method using bovine serum albumin as the standard. The molecular weight versus elution volume relationship of the Sephadex G-50 column was calibrated using α-lactalbumin (14.200 Da), myoglobin (17,000 Da), trypsin inhibitor (20,100 Da), trypsinogen (24,000 Da), and carbonic anhydrase (29,000 Da). The void volume of the column was determined using blue dextran. The molecular weight versus elution volume (V_e) relationship followed eq 1.

$$\log \mathrm{MW} = -0.0034V_e + 5.03 \quad (1)$$

Equation 1 was used to estimate the apparent molecular weight (MW) range of the gelatin hydrolysate fractions.

Ice Crystal Growth in Ice Cream Mix. Ice crystal growth in ice cream mix was studied using a cold stage (Model THMS600, Linkham Scientific Instruments, Ltd., Surrey, UK) mounted on a Leitz Laborlux S microscope (W. Nuhsbaum, Inc., McHenry, IL). In a typical experiment, a small drop (~5 μL) of ice cream (with or without gelatin hydrolysate) was placed on a microscope glass slide and covered with a glass coverslip. The glass slide was placed inside the thermal stage and quickly frozen by decreasing the temperature from ambient to −40 °C at the rate of 20 °C/min. The sample was held at −40 °C for 5 min, and a microscopic image of the sample at −40 °C was recorded. The temperature of the sample was then gradually increased from −40 to −14 °C at the rate of 1 °C/min and then cycled between −14 and −12 °C at a rate of 1 cycle/3 min. During this freezing process, the sample is first converted to a glass at −40 °C and, as the temperature is slowly increased to −14 °C, the sample undergoes irruptive recrystallization, producing a cloud of ice nuclei or very fine ice crystals, which further grow during thermal cycling at −14 to −12 °C. A decrease in the number as well as the size of ice crystals in the presence of an additive after a given number of thermal cycles would be indicative of the additive's ability to inhibit ice crystal growth. In these studies, a minimum of 7 cycles was employed, and microscopic images of the sample were recorded at the end of the 7 cycles at a magnification of 320×.

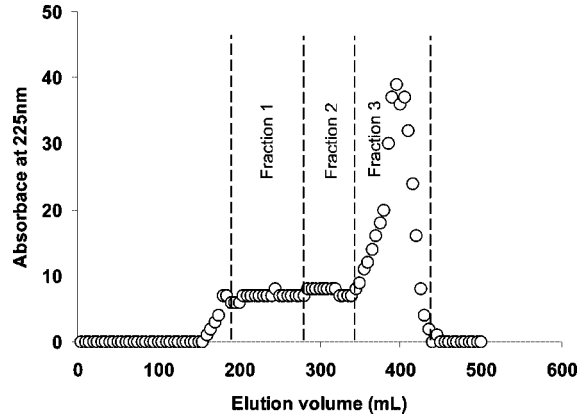

Figure 1. Elution profile of gelatin hydrolysate on a Sephadex G-50 gel permeation column. Hydrolysis was performed on 20% gelatin (225B40) in 1 M Na_2CO_3, pH 9.0, containing 10 mM cysteine, for 10 min at 37 °C at a papain to gelatin ratio of 1:100. Five milliliter fractions were collected at a flow rate of 2 mL/min.

Computational Analysis. Computational analysis of model gelatin peptides was performed using the ChemSite Pro Molecular Modeling and Analysis software and the MOPAC energy minimization application (CambridgeSoft, Cambridge, MA).

RESULTS

Papain is a nonspecific protease with an optimum pH in the range of 6–7 and optimum temperature at 65 °C. The products of hydrolysis of gelatin by papain generally consist of peptides with a range of molecular weight, and the molecular weight distribution of the peptides produced can be manipulated by varying the pH and temperature of the hydrolysis reaction.

Figure 1 shows the elution profile of gelatin hydrolysate obtained by treating a 20 wt% gelatin in 1 M Na_2CO_3 containing 10 mM cysteine at pH 9.0 and 37 °C for 10 min with papain at an enzyme-to-gelatin ratio of 1:100. Based on equation 1, the molecular weight of peptides in the hydrolysate ranged from approximately 3,200 to 22,400 Da. The 5 mL fractions were pooled into three major fractions, as shown in **Figure 1**, and the pooled fractions were lyophilized. It should be noted that the viscosity of 4% gelatin hydrolysate in water was less than 1.3 mPa.s, and therefore its effect on the viscosity of the ice cream mix was assumed to be negligible.

Figure 2 shows the effects of the total hydrolysate (i.e., unfractionated) and the three fractions on ice crystal growth in ice cream mix after seven thermal cycles between −14 and −12 °C. At the 4 wt% level, the total gelatin hydrolysate was unable to inhibit ice crystal growth in ice cream mix during thermal cycling. This was also the case with fractions 1 and 2, although the size of ice crystals was smaller than those in the control ice cream mix with no added gelatin hydrolysate. In contrast,

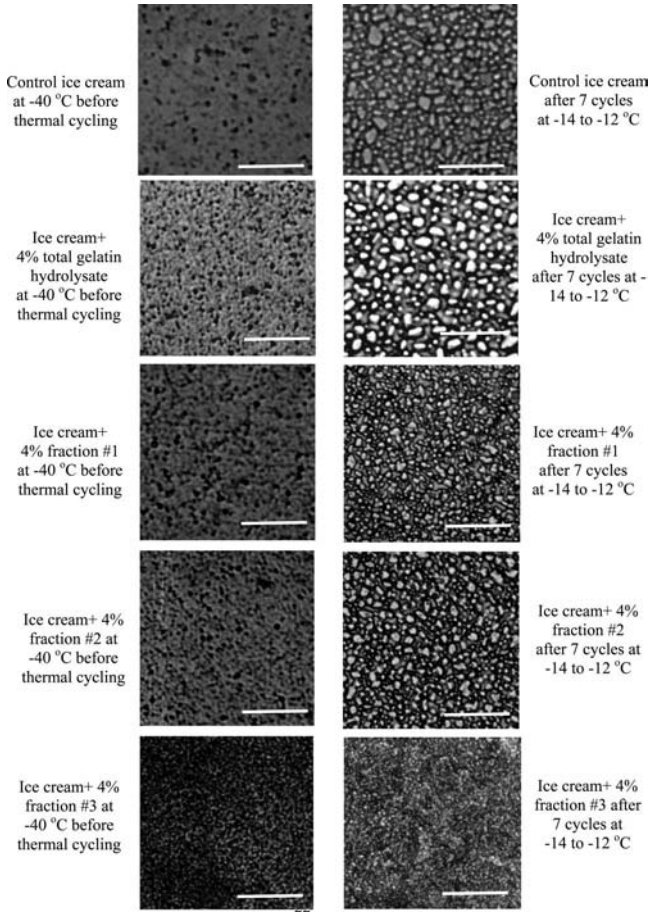

Figure 2. Effect of gelatin hydrolysate fractions on ice crystal growth in ice cream. Total hydrolysate represents unfractionated gelatin hydrolysate obtained under the hydrolysis conditions described in **Figure 1**. Fractions 1, 2, and 3 are lyophilized pooled fractions from a Sephadex G-50 column elution profile, shown in **Figure 1**. Bar scale = 40 μm.

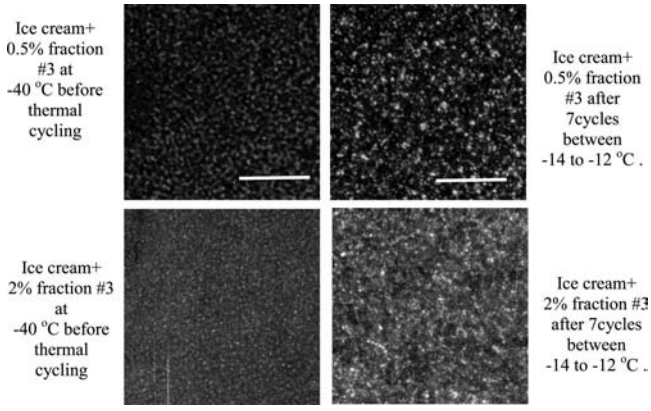

Figure 3. Inhibition of ice crystal growth in ice cream by fraction No. 3 at 0.5 and 2% levels. Bar scale = 40 μm.

fraction 3 was very effective in inhibiting ice crystal growth even at the 0.5 wt% level, as shown in **Figure 3**.

Taken together, the results shown in **Figures 1** and **2** indicate that gelatin polypeptides greater than 7000 Da had poor ability to inhibit ice cyrstal growth in ice cream mix, whereas polypeptides with molecular weights less than 7000 Da possessed inhibitory activity on ice crystal growth. To determine if peptides smaller than 3000 Da were relevant for the inhibitory effect of fraction No. 3 on ice crystal growth, fraction No. 3 was dialyzed overnight against water using a 3000 Da nominal molecular weight cutoff dialysis membrane. The retentate was

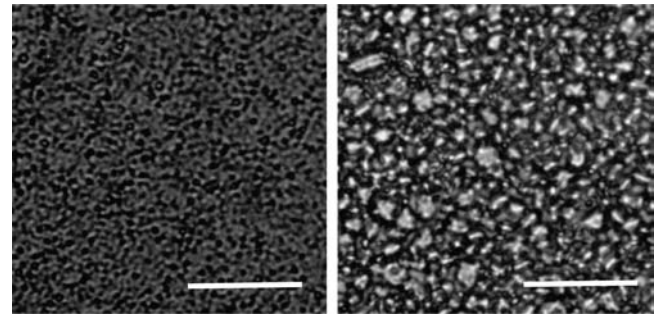

Figure 4. Effect of dialysis (3000 Da nominal cutoff membrane) on the inhibitory activity of fraction #3 on ice crystal growth in ice cream. Left panel: ice cream+ 4% dialyzed fraction #3 (see **Figure 1**) at −40 °C before thermal cycling. Right panel: ice cream +4% dialyzed fraction #3 after 7 cycles at −14 to −12 °C. Bar scale = 40 μm.

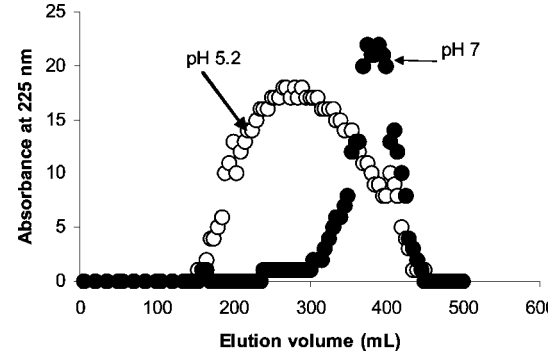

Figure 5. Elution profiles of gelatin hydrolysates produced at pH 5.2 and 7.0. Other digestion conditions were 20% gelatin (type 225B40) in water temperature, 37 °C; hydrolysis time of 10 min; and a papain-to-gelatin ratio of 1:100.

lyophilized and its inhibitory activity on ice crystal growth was checked. The results are shown in **Figure 4**. Removal of peptides smaller than 3000 Da eliminated the inhibitory activity of fraction No. 3 on ice crystal growth, which strongly suggests that the antifreeze properties of gelatin hydrolysate might arise predominantly from peptides smaller than 3000 Da. It should be noted, however, that a 3000 Da nominal molecular weight cutoff dialysis membrane would permit, to some extent, leaching of peptides in the 3000–4000 Da range, and it is therefore possible that some peptides in the 3000–4000 Da range also might possess inhibitory activity on ice crystal growth.

To further confirm if the molecular size of gelatin peptides was critical for their inhibitory effect on ice crystal growth, papain digestion of gelatin was performed in water at pH 5.2 and at pH 7.0 to generate peptides with different molecular weight distribution profiles. After digestion and inactivation of papain by heat, the pH of the hydrolysates was adjusted to 7.0 and lyophilized. The elution profiles of these hydrolysates on a Sephadex G-50 gel permeation column are shown in **Figure 5**. The pH 5.2 hydrolysate contained polypeptides with a broad molecular weight distribution ranging from 3300 to 31 000 Da, with a maximum distribution around 12 000 Da. On the other hand, the pH 7 hydrolysate contained a narrow molecular weight distribution of polypeptides ranging from 3300 to 8900 Da, with a maximum distribution around 5300 Da. The elution profile of pH 7 hydrolysate was similar to that of fraction No. 3 (**Figure 1**). Ice crystal growth inhibition in ice cream by these hydrolysates is shown in **Figure 6**. The pH 5.2 hydrolysate did not exhibit significant inhibitory activity on ice crystal growth even at the 4% level (**Figure 6A**). Its behavior was very similar to that of fractions 1 and 2 (see **Figure 1**), presumably because it

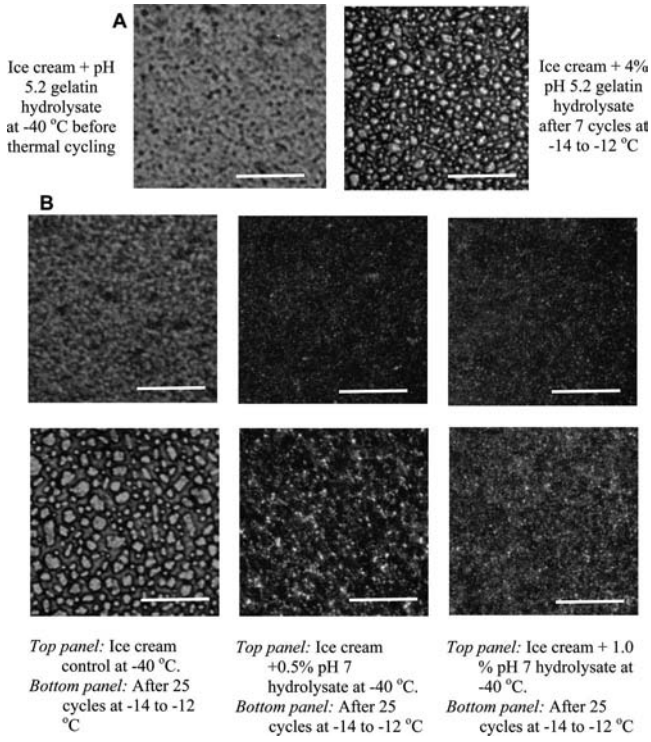

Figure 6. Effects of gelatin hydrolysate produced at pH 5.2 (**A**) and gelatin hydrolysate produced at pH 7.0 (**B**) on ice crystal growth inhibition in ice cream. Bar scale = 40 μm.

contained more of the high molecular weight peptides, similar to those found in fractions 1 and 2, and less of low molecular weight peptides, similar to fraction No. 3, on a mass basis. On the other hand, the pH 7.0 hydrolysate exhibited very remarkable inhibitory activity on ice crystal growth, even at 0.5 and 1.0 wt% levels and for over 25 thermal cycles between −14 and −12 °C (**Figure 6B**).

DISCUSSION

Several antifreeze proteins have been found in microorganisms (*27*, *28*), fungi (*29*), plants (*30*, *31*), insects (*32*, *33*), and fish species (*34*, *35*). The fish antifreeze proteins, which are the most studied ones, fall into two groups: antifreeze proteins (AFP) and antifreeze glycoproteins (AFGP). The structure and properties of these proteins and the mechanism of inhibition of ice crystal growth have been reviewed (*33*, *34*, *36–41*). These proteins are present in millimolar concentrations in the plasma of Antarctic fishes and up to 35 mg/mL in some cases. In addition to inhibiting ice nucleation, these proteins inhibit ice crystal growth and change the crystal habit of ice by binding to small ice crystals (*31*, *42–45*). The molecular weight of type 1 and type 3 AFPs range from 3.3 to 6.0 kDa and are rich in alanine and threonine residues. In winter flounder AFP, which is a single α-helix with a sequence of Asp-Thr-Ala-Ser-Asp-$(Ala)_6$-Leu-Thr-$(Ala)_2$-Asn-Ala-Lys-$(Ala)_3$-Glu-Leu-Thr-$(Ala)_2$-Asn-$(Ala)_7$-Thr-Ala-Arg, there are 4 ice-binding regions, shown in italics (*46*). The amino acid residues in these ice-binding regions simultaneously form 5−6 hydrogen bonds each with water molecules in the prism face of ice crystals. One of the common features among these antifreeze proteins is that their ice binding face is flat, and the distance between oxygen atoms on this face is about the same as that in ice nuclei, that is, about 4.52 Å (*45*, *47*).

The ice crystal growth inhibition by gelatin peptides might follow a mechanism similar to that of AFP and AFGP. Gelatin is a very unique protein. On an average, it contains 33% Gly, 33% Pro (and hydroxyproline), and the remaining other amino acid residues. Generally, the amino acid sequence of gelatin is often depicted as -$(Gly\text{-}Pro\text{-}X)_n$-, where X is any one of the twenty amino acid residues. However, in the primary structure of collagen, where the first position of this tripeptide repeat is always occupied by Gly, the second position is not always occupied by Pro or Hyp. Thus, in addition to -Gly-Pro-X- segments, there are several segments with sequences -Gly-Z-X- (where Z is any amino acid residue) within the collagen sequence. However, it can be visualized that -Gly-Pro(Hyp)-X- and -Gly-Z-X- would be the major repeating units in the peptide fragments of the hydrolysate. These repeating units are different from the -Ala-Ala-Thr- repeating sequence found in AFGP of fish (*48*), but it is very similar to the Gly-X-X repeating sequences found in two antifreeze proteins from snow fleas with molecular weight of 6.5 and 15.7 kDa (*49*). However, the antifreeze proteins from snow fleas contain 1–2 disulfide bonds that are essential for their antifreeze activity, whereas the gelatin peptides do not contain any cysteine residues. Nevertheless, the amino acid sequences of gelatin peptides and the snow flea antifreeze proteins are strikingly similar, and it is conceivable that the -Gly-Pro-X- and -Gly-Z-X- tripeptide repeat sequences in gelatin hydrolysate might play a role in their ice crystal growth inhibitory property. Because of the lack of conformational/steric constraints, the Gly-Pro(Hyp)-X- and Gly-Z-X- segments in small peptides of gelatin hydrolysate may adopt a flat face with the oxygen atoms of the carbonyl groups geometrically aligned with the oxygen−oxygen distance in ice nuclei.

To examine this possibility, the energy-minimized structures of several model gelatin peptides were analyzed using ChemSite Pro Molecular Modeling software. We selected model gelatin peptides of the type Gly-Pro-X-Gly-Pro-Z-Gly for the structural analysis. The rationale for selecting these model peptides was as follows; glycine residues occur at every third residue in the primary sequence of gelatin. Because papain is a nonspecific endoprotease, it is reasonable to assume that there is a 33% probability of having glycine at the C-terminal of peptides in gelatin hydrolysate. Futhermore, papain prefers a bulky hydrophobic residue at the P2 position (*50*), and therefore, the presence of a bulky chain at the X position of the -Gly-Pro(Hyp)-X- tripeptide repeat also would result in liberation of peptides predominantly with -Gly-Pro(Hyp)-X-Gly as the C-terminal segment.

Shown in **Figure 7** is the energy-minimized structure (using the MOPAC computation application) of the peptide Gly-Pro-Pro-Gly-Pro-Ala-Gly. In this energy-minimized structure, the oxygen atoms O[38] (C-terminal carbonyl group), O[34] (carbonyl group of Ala residue), and O[27] (carbonyl group of Pro residue) lie on a flat face (**Figure 7B**). In this configuration, the distance between O[34] and O[38] is 4.538 Å, and that between O[34] and O[27] is 4.552 Å. These distances are very close to the 4.52 Å found in the prism face of ice nuclei. This region of the molecule is highly hydrophilic, and no other discernible oxygen grouping is apparent in the molecule. Extension of the peptide chain length of the gelatin model peptide by one repeat unit does not significantly change the configuration of this flat face. The molecule essentially assumes a collagen-type helix structure as the chain length is increased. For instance, the energy-minimized structure of Gly-Pro-Pro-Gly-Pro-Ala-Gly-Pro-Ala-Gly is shown in **Figure 8**. The configuration of the oxygen-containing flat face is essentially the same as that of Gly-Pro-Pro-Gly-Pro-Ala-Gly, and no other

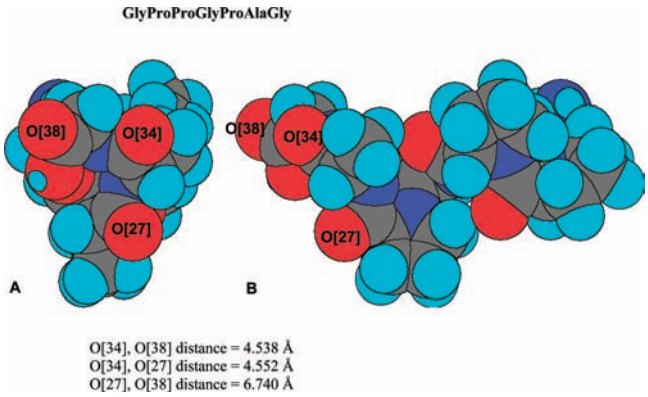

Figure 7. Energy-minimized structure of model gelatin peptide Gly-Pro-Pro-Gly-Pro-Ala-Gly. (**A**) 0° projection and (**B**) 90° projection. The positions of O[34] (carbonyl oxygen of Ala), O[38] (carbonyl oxygen of C-terminal Gly), and O[27] (carbonyl oxygen of Pro), which form an oxygen triad plane, are shown. The distance between O[34] and O[38] is 4.538 Å, and that between O[34] and O[27] is 4.552 Å. The distance between O[38] and O[27] is 6.74 Å. The atoms in the structures are: carbon, grey; nitrogen, blue; oxygen, red; hydrogen, cyan.

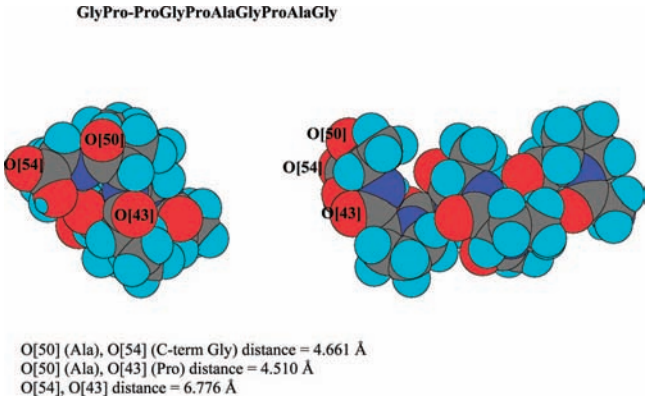

Figure 8. Energy-minimized structure of model gelatin peptide Gly-Pro-Pro-Gly-Pro-Ala-Gly-Pro-Ala-Gly. (**A**) 0° projection and (**B**) 90° projection. The oxygen atoms in the oxygen triad plane and the O-O distances are shown.

hydrophilic region with O−O distance and configuration similar to that of ice nuclei is found on other parts of the molecule. We hypothesize that these three oxygen atoms, which lie on a plane, constitute the ice binding face of these gelatin peptides.

Variations in amino acid residues at the X and Z positions do affect the configuration of the ice binding face. For instance, the energy-minimized structure of Gly-Pro-Thr-Gly-Pro-Leu-Gly displays three oxygen atoms in a plane, but the O−O distances are 4.670 and 4.349 Å (**Figure 9**), both of which are not very compatible with the O−O distance in ice nuclei. On the other hand, the energy-minimized structure of Gly Pro-Hyp-Gly Pro-Ala-Gly exhibits an oxygen plane with O-O distances of 4.565 and 4.542 Å (**Figure 10**), which are about the same as for Gly-Pro-Pro-Gly-Pro-Ala-Gly (**Figure 7**) and O−O distances in ice nuclei.

Analyses of the energy-minimized structures of various model gelatin peptides suggested that sequences with small amino acid residues, such as Ala, Ser, Thr, Pro, Hyp, and Gly at X and Z positions invariably exhibited an oxygen triad plane (flat face) at the C-terminus with two O−O distances of close to 4.52 Å. Because no other hydrophilic surface with O−O configurations similar to that of ice nuclei is apparent in other parts of the molecule, we hypothesize that the oxygen triad plane may be the ice binding region of the molecule, and several of these

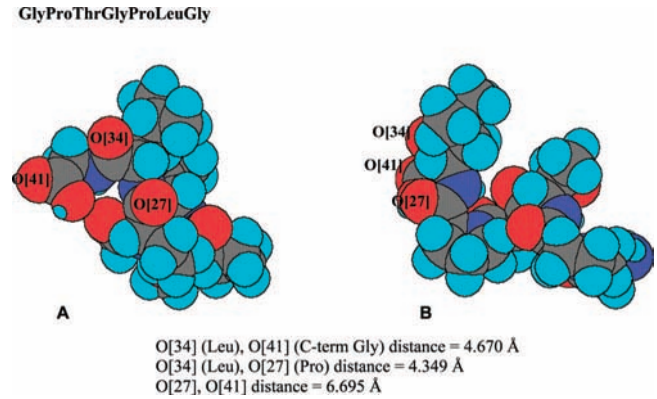

Figure 9. Energy-minimized structure of model gelatin peptide Gly-Pro-Thr-Gly-Pro-Leu-Gly. (**A**) 0° projection and (**B**) 90° projection. The oxygen atoms in the oxygen triad plane and the O−O distances are shown.

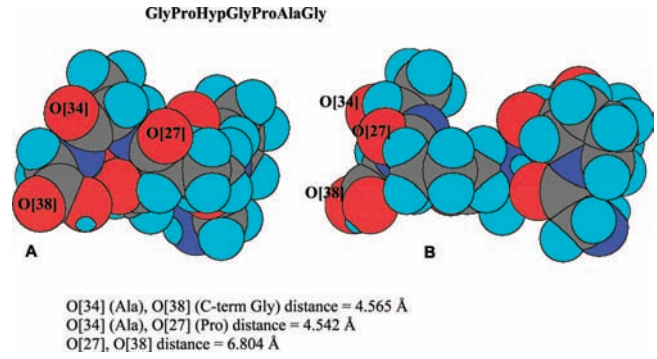

Figure 10. Energy-minimized structure of model gelatin peptide Gly-Pro-Hyp-Gly-Pro-Ala-Gly. (**A**) 0° projection, (**B**) 90° projection. The oxygen atoms in the oxygen triad plane and the O−O distances are shown.

flat-faced peptides with the dog-shaped structure may bind to the prism face of ice nuclei via hydrogen bonding, thereby inhibit their growth. The aliphatic side chains of proline and alanine residues may provide a partial nonpolar environment to stabilize such hydrogen bonding interactions against competition from ice–water hydrogen bonding interactions. Gelatin peptides with molecular weights less than 4000 Da may be able to form stiff collogen-type helix rods, which may favor stacking of these rods on ice nuclei with the oxygen triad plane facing the prism face of ice nuclei. On the other hand, gelatin peptides greater than 4000 Da may loosen their stiffness and, as a result, steric hindrances may prevent proper stacking of the peptides on the prism face of ice nuclei, thus decreasing their ability to inhibit ice crystal growth.

Further elucidation of the molecular interactions responsible for ice crystal growth inhibition by peptides from gelatin hydrolysate may lead to rationale designing of peptide cryoprotectants with greater antifreeze activity.

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