JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 5501–5509 5501
DOI:10.1021/jf900524y

Ice-Structuring Peptides Derived from Bovine Collagen

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Antifreeze proteins belonging to structurally diverse families of genetically coded proteins from several living organisms have been isolated and characterized in the past. This paper reports that collagen peptides of a certain molecular size range derived from Alcalase hydrolysis of bovine gelatin are able to inhibit recrystallization of ice in frozen ice cream mix as well as in frozen sucrose solutions in a manner similar to natural antifreeze proteins. The optimum conditions for producing such ice-structuring peptides (ISP) were hydrolysis at pH 9.0 for 30 min at 45 °C and an Alcalase-to-gelatin ratio of 0.176 unit per gram of gelatin. The collagen peptides were fractionated on size exclusion (Sephadex G-50) and ion exchange (sulfopropyl-Sephadex C-25) columns, and the molecular mass distribution of the ice-structuring peptide fractions was determined by matrix-assisted laser desorption ionization−time-of-flight (MALDI-TOF) mass spectrometry. The collagen peptide fractions in the molecular mass range of 600−2700 Da inhibited ice recrystallization in a supercooled ice cream mix and in concentrated sucrose solutions. The cationic collagen peptides within this fraction with molecular mass in the range of 1600−2400 Da were more effective than the anionic peptides in inhibiting ice crystal growth.

KEYWORDS: Collagen peptides; gelatin hydrolysate; antifreeze peptides; ice crystal growth inhibition; Alcalase; ice-structuring peptides

INTRODUCTION

Several living organisms that thrive in subzero temperature environments are known to produce proteins that inhibit recrystallization of ice (*1*−*6*). In addition to inhibiting ice recrystallization, these proteins also depress the freezing point of water in a noncolligative manner without affecting the melting point of ice, a phenomenon known as thermal hysteresis (*7*−*9*). These antifreeze proteins, also known as ice-structuring proteins, could be used for preserving the textural quality of frozen foods (*10*−*13*), improving storage of blood, organs, and tissues, and protecting crops from freezing (*14*) in a freeze−thaw cycle to minimize or prevent damage to cells and tissues. However, the limited availability of these natural antifreeze proteins has restricted their use in such applications.

It is fairly well accepted that the mechanism of inhibition of ice crystal growth by antifreeze proteins involves binding of these proteins to the ice−liquid interface (*15*−*18*). Analyses of the crystal structures of antifreeze proteins from various species have revealed that they typically display a flat hydrophilic side with polar oxygen-containing groups geometrically spaced in a two-dimensional array that mimics the spacing of oxygen atoms in the hexagonal ice lattice (*18*−*20*). This lattice mimicry that is invariably found in most antifreeze proteins is thought to be essential for their ice-binding function (*19*, *21*, *22*). Implicit in this mechanism is that the primary mode of binding is hydrogen bonding, and the other structural elements of the proteins might provide a hydrophobic environment to stabilize the hydrogen bonds and/or provide a steric barrier for water from the liquid phase (*19*−*23*).

The common structural features of the ice-binding face of antifreeze proteins studied to date, despite their structural diversity, suggest that any peptide or polypeptide that can dynamically adapt its backbone conformation to position its oxygen-containing groups in a periodic spacing that matches with that in the ice lattice should be able to bind to the ice surface and inhibit ice crystal growth. If this hypothesis is correct, then peptides derived from gelatin, which has a repeat sequence of −Gly−Xaa−Xaa− and high configurational flexibility and adapatability should exhibit ice-structuring properties. In a preliminary study, we have reported that gelatin hydrolysate produced using papain hydrolysis was able to inhibit ice recrystallization in an ice cream mix (*24*). In the present study, we present partial characterization of ice-structuring collagen peptides derived from Alcalase (from *Bacillus licheniformis*) hydrolysis of bovine gelatin.

MATERIALS AND METHODS

Materials. Ice cream mix was from the dairy plant of the Department of Food Science at the University of Wisconsin—Madison. It contained the following exact composition typical for an ice cream mix: 12.0% milk fat in the emulsified form, 11.0% nonfat milk solids, 16.0% sucrose, 0.1% emulsifier; and the rest as water. In all of the experiments reported here, the original ice cream mix was first diluted 15 wt % with water to bring the total nonfat solids to 23%; to this was added up to 4% gelatin hydrolysate as nonfat solids so that the total nonfat solids was 27% as that of the original ice cream. All experiments were conducted on a single batch of ice cream mix, which was stored at −20 °C in 2 mL aliquots in cryovials.

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 Published on Web 05/29/2009

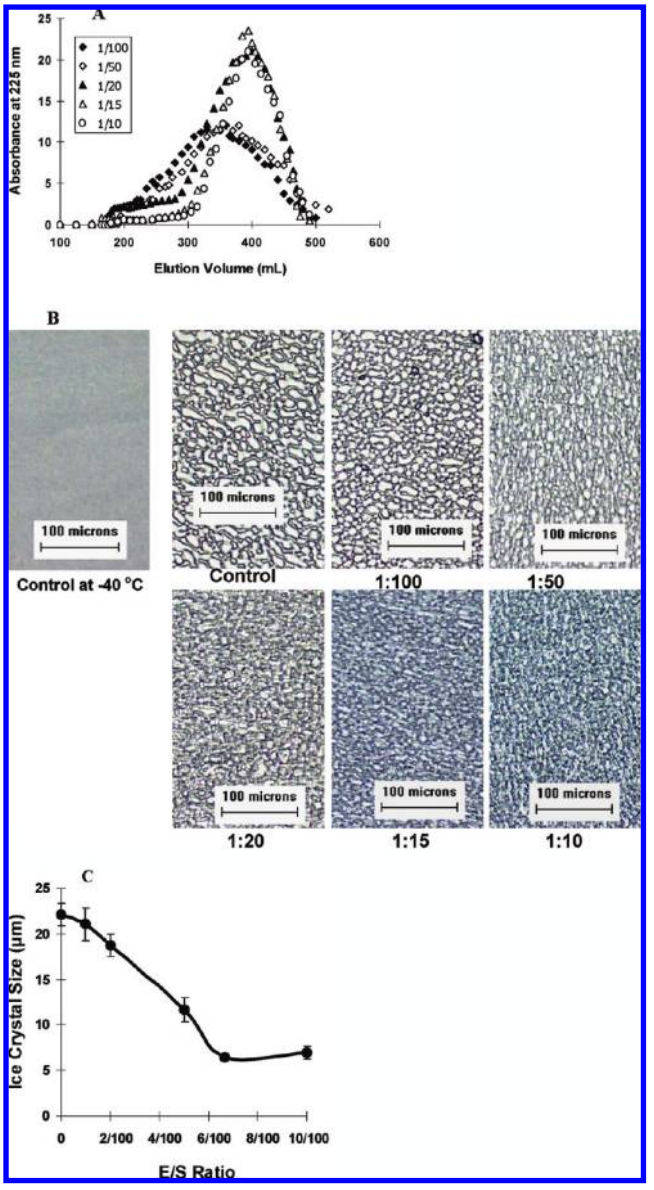

Figure 1. (**A**) Elution profiles of gelatin hydrolysates (pH 9.0, 45 °C, 30 min) obtained at five different alcalase-to-gelatin ratios on Sephadex G-50. (**B**) Effect of gelatin hydrolysates on ice crystal growth in ice cream mix after seven thermal cycles at −14 to −12 °C. The E/S ratios of the hydrolysates are shown at the bottom of each image. The concentration of the hydrolysate was 4 wt %. The image of the control at −40 °C was not subjected to thermal cycling. (**C**) Size distribution of ice crystals in images shown in **B**.

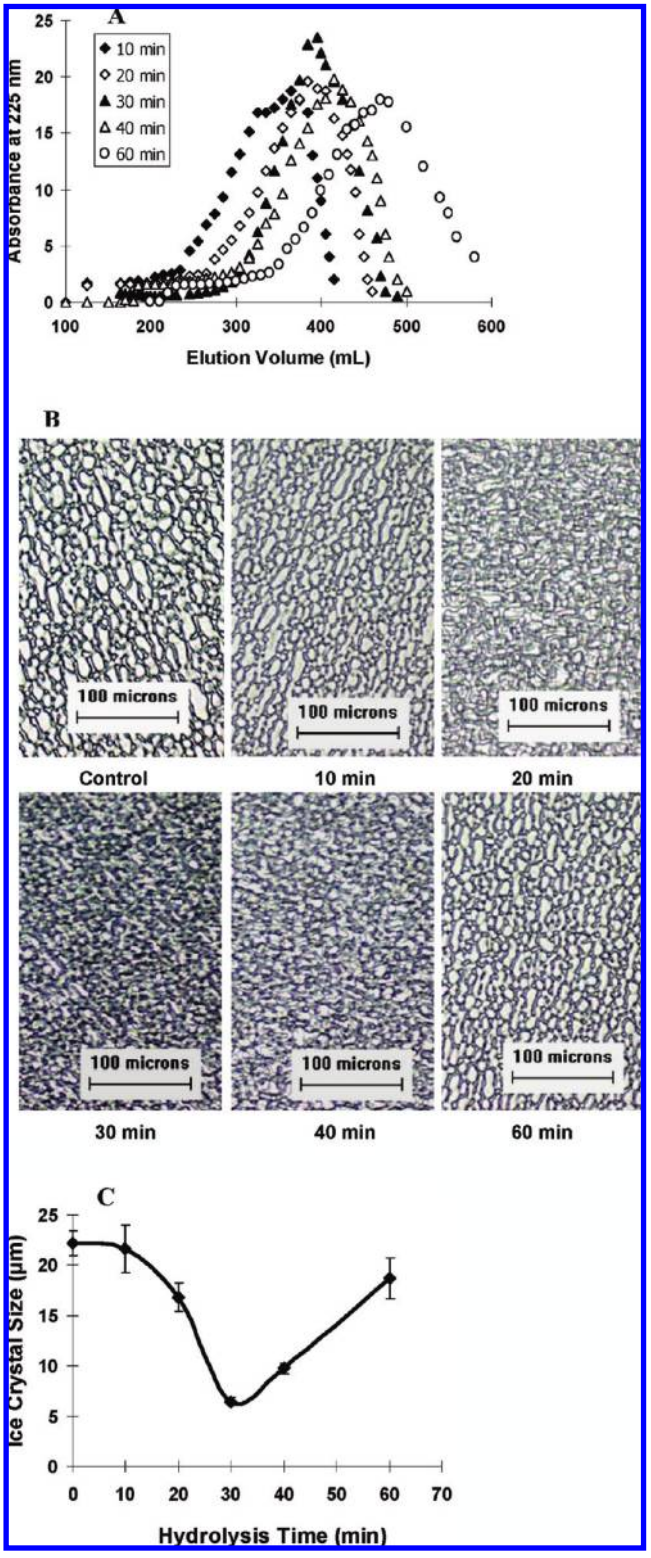

Figure 2. (**A**) Elution profile of gelatin hydrolysates produced at pH 9.0, 45 °C, and Alcalase-to-gelatin ratio of 1:15, and for different hydrolysis times (10, 20, 30, 40, and 60 min) on Sephadex G-50. (**B**) Effect of gelatin hydrolysates on ice crystal growth in an ice cream mix after seven thermal cycles at −14 to −12 °C. The concentration of the hydrolysate was 4 wt %. (**C**) Sze distribution of ice crystals in images shown in **B**.

Sample from one vial was used for each set of experiments, and the unused portion was discarded.

Gelatin (type 225B40, which refers to 225 bloom bovine gelatin with 40 mesh particle size) from bovine was obtained from St. Louis Food Ingredients (St. Louis, MO). Sulfopropyl-Sephadex C-25 and Alcalase (EC 3.4.21.14) from *B. licheniformis* in solution form (2.64 Anson units/g of solution) were purchased from Sigma Chemical Co. (St. Louis, MO). According to the supplier, one Anson unit of enzyme is defined as the amount of enzyme that digests and liberates an amount of TCA-soluble product per minute, which gives the same color with Folin−Ciocalteu phenol reagent as 1 mequiv of tyrosine at 25 °C at pH 7.5. Sephadex G-50 was purchased from Fisher Scientific Co. (Fair Lawn, NJ), and sucrose was purchased from Mallinckrodt Baker Inc. (Paris, KY). All other reagents were of the highest purity available.

Optimization of Gelatin Hydrolysis Conditions. Preliminary experiments were conducted to optimize three reaction parameters: enzyme-to-substrate (E/S) ratio (1:100, 1:50, 1:20, 1:15, and 1:10 w/w); temperature (37, 45, and 50 °C); and reaction time (10, 20, 30, 40, and 60 min).

Gelatin (1.65 g) was dissolved in 6.6 mL of Milli-Q water, and the pH was adjusted with 2.0 M NaOH to an initial pH of 9.0. The solution was preheated in a water bath to a selected temperature, followed by the

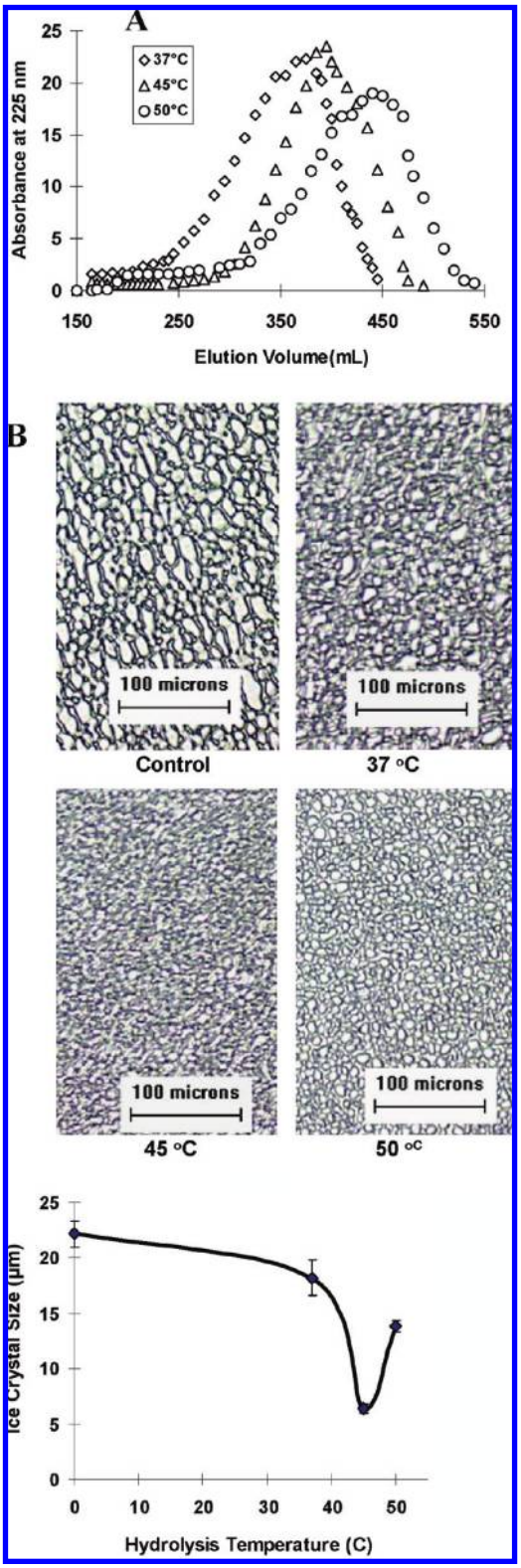

Figure 3. (**A**) Elution profile of gelatin hydrolysates on Sephadex G-50 produced at an Alcalase-to-gelatin ratio of 1:15, pH 9.0, 30 min, and different hydrolysis temperatures (37, 45, and 50 °C). (**B**) Effect of gelatin hydrolysate on ice crystal growth in an ice cream mix after seven thermal cycles at −14 to −12 °C. The concentration of the hydrolysate was 4 wt %. (**C**) Size distribution of ice crystals in images shown in **B**.

addition of the enzyme. The hydrolysis was carried out at the selected experimental temperature at various E/S ratios and hydrolysis times. Hydrolysis was stopped by boiling samples for 10 min. The samples were then cooled under cold flowing water and centrifuged at 14000*g* for 10 min. The supernatants were collected and assayed for antifreeze activity. Unused samples were discarded after 1 week at 4 °C.

Fractionation of Gelatin Hydrolysate. The gelatin hydrolysate (2.5 mL of 20% solution) was fractionated on a Sephadex G-50 gel filtration column (2.6 cm diameter × 100 cm length) using deionized water at pH 7.0 as the eluent. The flow rate was set at 2 mL/min, and 5 mL/tube fractions were collected. The elution profile was determined by measuring the absorbance at 225 nm using a Shimadzu spectrophotometer (model UV-1601 PC, Shimadzu Corp., Columbia, MD). The tubes corresponding to various molecular mass ranges were pooled and lyophilized.

The fractions exhibiting antifreeze activity were pooled separately and fractionated further on a Sulfopropyl-Sepadex C-25 (SP-Sephadex) cation-exchange column (2.0 cm diameter × 55 cm length) previously equilibrated with the starting buffer (0.01 M phosphate buffer, pH 7.0). Initially, the sample was eluted with the starting buffer at a flow rate of 0.5 mL/min. After the elution of unadsorbed (anionic) peptides, the column was eluted with a gradient of NaCl (0−0.5 M) in the same buffer. The elution profile was determined by measuring the absorbance at 225 nm. The tubes corresponding to unadsorbed (SP1) and adsorbed (SP2) fractions were pooled, desalted using a combination of dialysis at 4 °C for 48 h using a 500 Da molecular mass cutoff membrane, and chromatographed on a Sephadex G-10 column. The desalted fractions were lyophilized, and their ice-structuring activity was evaluated.

Mass Spectrometry. The molecular weight distribution of gelatin peptide fractions was analyzed by matrix-assisted laser desorption ionization−time of flight (MALDI-TOF) mass spectrometry (Applied Biosystems, Foster City, CA) at the Biotechnology Center of the University of Wisconsin—Madison.

Ice-Structuring Activity Determination. Ice crystal growth in the ice cream mix and in 23% sucrose solution was studied using a cold stage (model HCS302, Instec Scientific Instruments Ltd., Boulder, CO) mounted on a Nikon Eclipse microscope (E200, Nikon Inc., Japan) as described by Damodaran (*24*). The images were analyzed using IMAGE-PRO PLUS software (Media Cybernetics, Silver Spring, MD). In a typical experiment, a small drop (5 μL) of ice cream or sucrose solution (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 40 °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 cooling and warming process, the sample was first converted to a glass at −40 °C; as the sample was slowly warmed to −14 °C (i.e., above its glass transition temperature), it underwent irruptive recrystallization of ice, 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 seven cycles was employed because these temperature cycling conditions represent approximately 2 months of storage of ice cream in a typical household freezer according to industry sources. Microscopic images of the sample were recorded at the end of the seven cycles at a magnification of 220×. The average size of ice crystals formed after seven cycles was determined using the IMAGE-PRO PLUS software (Media Cybernatics, Silver Spring, MD).

Viscosity Determination. The viscosity of the ice cream mix samples was measured at 25 °C using a calibrated viscometer tube (size 100, Fisher Scientific). Pure water, 100% original ice cream, 15 wt % diluted ice cream, 4 wt % gelatin hydrolysate in water, and 4% gelatin hydrolysate in 15 wt % diluted ice cream (total nonfat solids content same as in 100% original ice cream) were prepared for measurements. The resistance to flow was measured by the time taken for a given volume (10 mL) of sample to flow under gravity between two points in a calibrated viscometer tube. The viscosity of the sample was determined by using the equation

$$\eta_{\text{sample}} = \eta_{\text{water}} \frac{\rho_{\text{sample}}}{\rho_{\text{water}}} \frac{t_{\text{water}}}{t_{\text{sample}}}$$

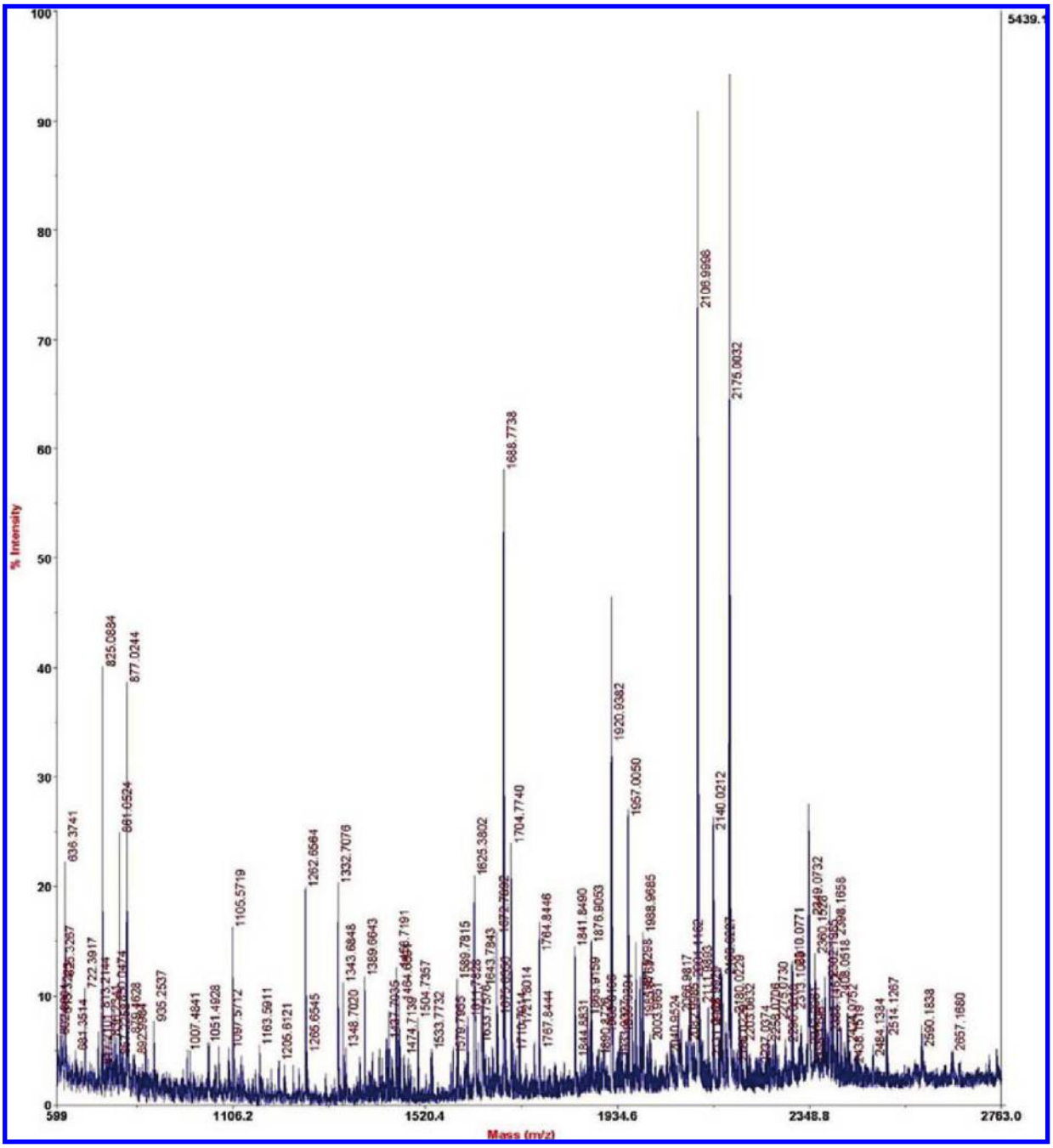

Figure 4. Molecular mass distribution of gelatin hydrolysate (pH 9.0, 45 °C for 30 min, and Alcalase-to-gelatin ratio of 1:15) by MALDI-TOF.

where η is the viscosity, ρ is the density, and t is the time taken for a given volume of the liquid to flow through the viscometer tube.

Statistical Analysis. All experiments were done in triplicate. Data were subjected to analysis of variance (ANOVA). When significant treatment effects ($P < 0.05$) were found, their means were separated by Duncan's multiple-range tests using a computerized statistical package.

RESULTS

Alcalase is a serine endoproteinase from a selected strain of *B. licheniformis*. It has a broad specificity and a preference for large uncharged amino acid residues in the P1 position. The optimal temperature and pH ranges for Alcalase activity are between 50 and 65 °C and between 7.5 and 10.0, respectively. In preliminary experiments, we found that gelatin hydrolysates produced at pH 9.0 were more effective in inhibiting ice recrystallization than those obtained at pH 7.0 (data not shown). Therefore, optimal hydrolysis conditions for producing gelatin hydrolysates with best ice-structuring activity were investigated by fixing the pH at 9.0 and manipulating three reactions parameters, that is, the enzyme-to-substrate (E/S) ratio, temperature, and reaction time.

Figure 1A shows elution profiles on Sephadex G-50 of gelatin hydrolysate obtained by treating a 20 wt % gelatin in pure water at pH 9.0 with Alcalase at various E/S ratios for 30 min at 45 °C. The E/S ratios are expressed in weight ratios of enzyme in liquid form to dry weight of gelatin. The elution profile of the hydrolysate shifted to the right as the E/S ratio was increased, indicating that the average molecular mass of peptides liberated in the hydrolysate decreased with increasing E/S ratio. **Figure 1B** shows the effects of total (i.e., unfractionated) gelatin hydrolysates on ice crystal growth in the ice cream mix after seven thermal cycles between −14 and −12 °C. At the 4 wt % level, the total gelatin hydrolysate obtained at an enzyme-to-gelatin ratio of 1:100 was not able to inhibit ice crystal growth during thermal cycling (**Figure 1B**). At higher E/S ratios, the ice-structuring activity of

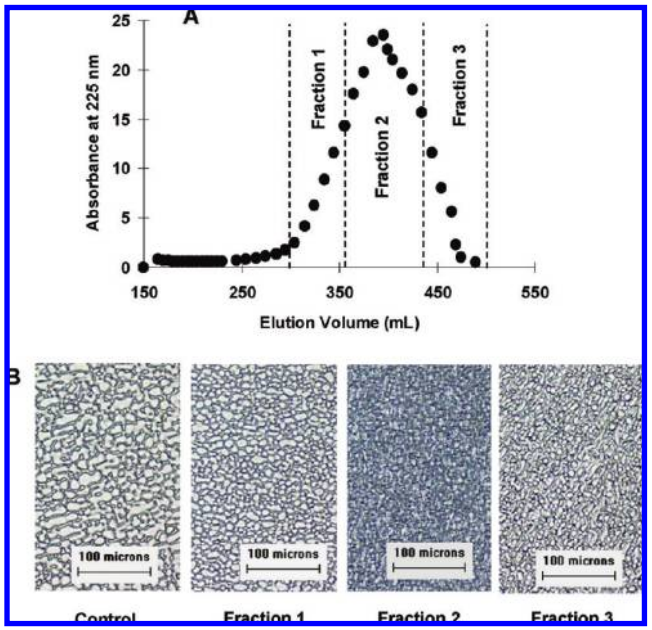

Figure 5. (**A**) Elution profiles of gelatin hydrolysate (pH 9.0, 45 °C, 30 min, E/S ratio 1:15) on Sephadex G-50 column. The elution profiles was divided into three fractions as shown by the vertical dotted lines. (**B**) Effect of fractions 1−3 from Sephadex G-50 chromatography on ice crystal growth in an ice cream mix after seven thermal cycles at −14 to −12 °C. The concentration of the hydrolysate fractions was 4 wt %.

hydrolystates increased with increasing E/S ratio. The relationship between the average size of ice crystals formed after seven thermal cycles and the E/S ratio is shown in **Figure 1C**. The hydrolysate obtained using an E/S ratio of 1:15 seemed to be the most active in terms of reducing the average size of ice crystals. This ratio was selected for optimizing other reaction parameters.

Figure 2A shows the effect of hydrolysis time (10, 20, 30, 40, and 60 min) on the elution profiles of gelatin hydrolysates produced by treating a 20 wt % gelatin in pure water at pH 9.0, 45 °C, and E/S ratio 1:15 on Sephadex G-50. The elution profile shifted to the right as the hydrolysis time increased from 10 to 60 min, indicating that the average molecular mass of peptides in the hydrolysates decreased with increase of hydrolysis time. **Figure 2B** shows the effect of hydrolysis time on the ice-structuring activity of the hydrolysates. At the 4 wt % level, the inhibition of ice recrystallization increased initially up to 30 min of hydrolysis time and then decreased thereafter. The relationship between hydrolysis time and the average size of ice crystals formed after seven thermal cycles is shown in **Figure 2C**. The 30 min hydrolysate had the maximum activity in retarding ice crystal growth.

Figure 3A shows the elution profiles of gelatin hydrolysates obtained at three different reaction temperatures in water at pH 9.0 for 30 min at an E/S ratio of 1:15. The effects of these hydrolysates on the inhibition of ice recrystallization are shown in **Figure 3B**. The shift in the elution profile toward higher elution volume with increasing temperature indicated a decrease in the average molecular mass of peptides liberated in the hydrolysate. **Figure 3C** shows the effect of hydrolysis temperature on the average size of ice crystals formed after seven thermal cycles. Maximum inhibition of ice recrystallization occurred with the hydrolysate produced at 45 °C.

Taken together, the results shown in **Figures 1−3** indicate that the size distribution of gelatin peptides was critical for their ice-structuring activity. Only the gelatin peptides with a molecular mass range corresponding to an elution volume in the range of 300−475 mL on the Sephadex G-50 column used in this study were able to inhibit ice recrystallization in the ice cream mix. Gelatin peptides having higher or lower than this molecular mass range were less effective in inhibiting ice recrystallization. The optimum conditions to produce a gelatin hydrolysate with maximum ice-structuring activity seem to be hydrolysis of a 20% gelatin solution at pH 9.0 for 30 min and 45 °C using an Alcalase to gelatin ratio of 0.176 Anson unit per gram of gelatin (which corresponds to a 1:15 weight ratio).

Table 1. Mean Ice Crystal Size in Ice Cream Mix Containing Gelatin Hydrolysate[a]

sample	no. of cycles	av ice crystal diameter ± SD (μm)
15% diluted ICM (control)	7	22.16 ± 1.21 A
15% diluted ICM + 4% total gelatin hydrolysate	7	6.43 ± 0.39 C
15% diluted ICM + 4% fraction 1	7	17.96 ± 1.13 A
15% diluted ICM + 4% fraction 2	7	5.02 ± 0.55 D
15% diluted ICM + 4% fraction 3	7	8.39 ± 0.58 B
15% diluted ICM + 4% SP1	7	9.16 ± 0.89 B
15% diluted ICM + 4% SP2	7	3.35 ± 0.46 E

[a] Values expressed as mean ± SD. Values with different letters in the same column differ significantly ($P < 0.05$). SD, standard deviation; ICM, ice cream mix; fractions 1−3 are fractions from the Sephadex G-50 column as shown in **Figure 5**; SP1 and SP2 are fractions from the SP-Sephadex C25 column.

The MALDI-TOF mass spectrum of the peptides in the hydrolysate produced under the above conditions is shown in **Figure 4**. The molecular mass of the peptides was in the range of 600−2500 Da. To elucidate which peptides within the molecular mass range of 600−2500 Da were greatly responsible for the ice-structuring activity, the gelatin hydrolysate produced under optimal conditions (pH 9.0 at 45 °C, E/S ratio of 1:15, and 30 min hydrolysis time) and fractionated on Sephadex G-50 gel filtration column was pooled into three major fractions as shown in **Figure 5A**. The ice-structuring activities of these three fractions are shown in **Figure 5B**, and the average size of ice crystals produced in the ice cream mix after seven thermal cycles is presented in **Table 1**. Among these three fractions, fraction 2 (from the middle portion of the elution profile) exhibited the most ice-structuring activity with an average ice crystal size of 5.02 ± 0.55 μm. Although the ice-structuring activity of fraction 3 (8.39 ± 0.58 μm) was statistically poorer than that of fraction 2, it was significantly better than that of fraction 1 (17.96 ± 1.13 μm) and the control (22.16 ± 1.21 μm). The data in **Table 1** tentatively suggest that the ice-structuring activity of total hydrolysate (6.43 ± 0.39 μm) might emanate mainly from fractions 2 and 3. Shown in **Figure 6** is the MALDI-TOF mass spectrum of fraction 2. Although fraction 2 contained numerous peptides, those corresponding to molecular masses of 1625.40, 1672.86, 1688.84, 1921.02, 1957.09, 2107.09, 2175.10, 2280.15, and 2296.14 Da were the dominant peptides in the mixture. The relative intensities of these peptides in different batches of fraction 2 were very similar (data not shown), suggesting that although Alcalase is essentially a nonspecific enzyme, its cleaving pattern is basically the same as long as the hydrolysis conditions are the same.

Fraction 2 from Sephadex G-50 was further fractionated on an SP-Sephadex C-25 cation-exchange column using a 0−1.0 M NaCl gradient in 0.01 M phosphate buffer, pH 7.0 (**Figure 7A**). Two fractions were obtained, one corresponding to unadsorbed (anionic) peptides (SP1) that were eluted with 0 M NaCl and one corresponding to adsorbed (cationic) peptides (SP2) that were eluted by the 0−1.0 M NaCl gradient. The SP1 and SP2 fractions were pooled separately, exhaustively dialyzed (for 48 h at 4 °C) using a 500 molecular weight cutoff (MWCO) membrane, and lyophilized. The ice-structuring activities of SP1 and SP2

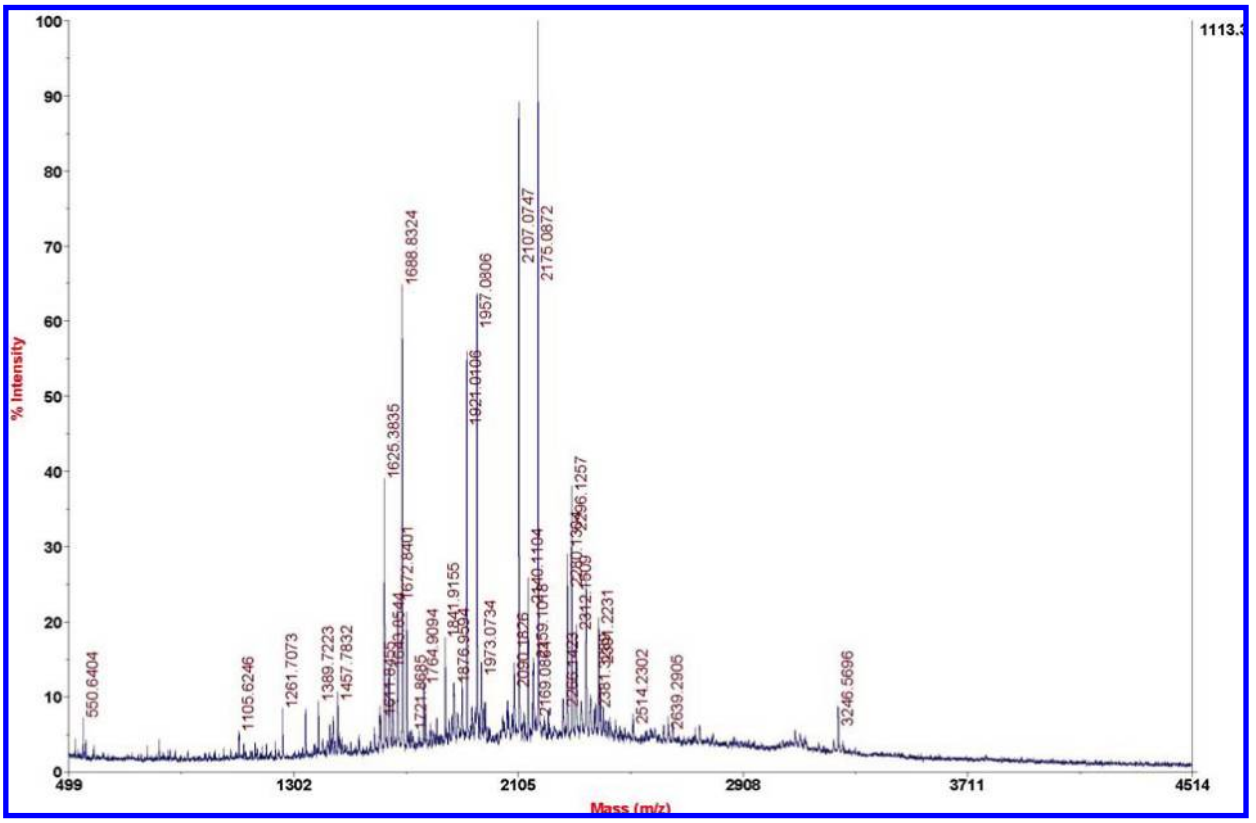

Figure 6. MALDI mass spectrum of fraction 2 from **Figure 5**.

fractions are shown in **Figure 7B**. It should be noted that the SP2 fraction containing cationic peptides was more effective than the anionic SP1 fraction in inhibiting ice recrystallization. The average size of ice crystals formed after seven thermal cycles in SP1- and SP2-containing ice cream mix is presented in **Table 1**. In the presence of the SP2 fraction the average size of ice crystals was about $3.35 \pm 0.46\ \mu$m compared to $22.16 \pm 1.21\ \mu$m in the control and $9.16 \pm 0.89\ \mu$m in the SP1-containing sample. The SP2 fraction was able to significantly retard ice crystal growth even after 25 thermal cycles (**Figure 8**; **Table 2**). These results indicate that, in addition to size distribution, the charge characteristics of gelatin peptides also play a critical role in their ice-structuring activity: the cationic gelatin peptides are more effective than the anionic peptides in inhibiting ice recrystallization.

The MALDI-TOF mass spectra of the SP1 and SP2 fractions are shown in **Figure 9**, panels **A** and **B**, respectively. The molecular mass distributions of both the anionic SP1 and cationic SP2 fractions were essentially in the range of 600−2600 Da. The SP1 fraction contained about six major peptides (i.e., 1389.60, 1654.69, 1956.93, 1972.92, 2139.94, and 2295.95 Da) with percent intensity >25%. Among these peptides, the 1956.92 Da peptide was the most abundant one. On the other hand, the cationic SP2 fraction contained about nine major peptides with m/z values 1625.39, 1643.85, 1672.84, 1688.83, 1921.00, 1957.07, 2107.07, 2176.08, and 2280.13 Da; the peptide with a mass of 2107.07 Da was dominant among these peptides.

To elucidate whether the inhibition of ice recrystallization by gelatin peptides was related to specific molecular level interactions of these peptides with ice nuclei or to nonspecific effects of viscosity increase resulting from the addition of the hydrolysate, the viscosities of 100% original ice cream containing 27% total soluble solids (TSS), 15 wt % diluted ice cream (23% TSS), 15 wt % diluted ice cream with 4% SP2 (27% TSS), and 4 wt % gelatin hydrolysate in water were measured (**Table 2**). The viscosity of 4% gelatin hydrolysate in water was ∼1.3 mPa·s, which was not statistically different ($P > 0.05$) from the viscosity of pure water. The viscosity of 100% original ice cream (7.81 mPa·s) was not significantly different ($P > 0.05$) from that of 15 wt % diluted ice cream with 4% SP2 (8.17 mPa·s). On the other hand, the viscosity of 15% diluted ice cream mix was 4.78 mPa·s, which was statistically different ($P < 0.05$) from the viscosity of the above two samples. **Figure 10** shows ice crystal growth in these samples after seven thermal cycles. The average sizes of ice crystals formed in 100% original ice cream mix and the 15% diluted ice cream mix were very similar (**Table 3**) even though these samples had different viscosities. In contrast, the average size of ice crystals formed in the 15% diluted ice cream mix containing 4% SP2 was significantly smaller ($P < 0.05$) than that in the 100% ice cream mix control (**Table 3**), even though the viscosities of these samples were similar. These data clearly indicate that the inhibition of ice recrystallization in the SP2-containing sample must arise from specific interaction of gelatin peptides with ice nuclei.

The ice-structuring activity of fraction 2 (from Sephadex G-50 chromatography) and the SP2 fraction (from SP-Sephadex G-25 chromatography) in sucrose solution, as opposed to ice cream mix, is shown in **Figure 11**. In the absence of SP2, ice crystal growth was evident in both 23 and 27% sucrose solutions. Addition of either fraction 2 of SP2 at 4% level significantly inhibited ice crystal growth. As in the case of ice cream mix, the SP2 fraction was more effective than fraction 2 in inhibiting ice crystal growth (**Table 4**).

DISCUSSION

The results presented here seem to support the hypothesis that a polypeptide that can dynamically adapt its backbone conformation to position its oxygen-containing amide carbonyl groups in a periodic spacing that matches with that in the ice lattice should be able to bind to the ice surface and inhibit ice crystal growth. Collagen (gelatin)-derived peptides seem to possess the

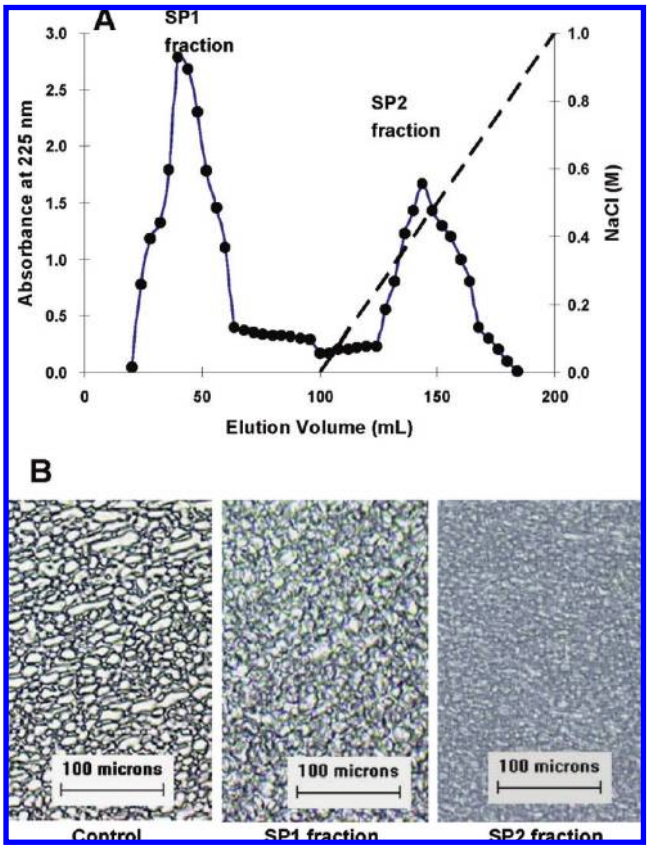

Figure 7. (**A**) Cation-exchange chromatography of fraction 2 (from **Figure 5**) on the sulfopropyl (SP) Sephadex C-25 column. The column was eluted with a 0−1.0 M NaCl gradient in 20 mM phosphate buffer, pH 7.0. (**B**) Effect of the unadsorbed fraction SP1 and adsorbed fraction SP2 from SP-Sephadex C-25 chromatography on ice crystal growth in an ice cream mix after seven thermal cycles at −14 to −12 °C. The concentration of the peptide fractions in the ice cream was 4 wt %.

appropriate conformational flexibility and adaptability needed for this function.

It was also evident that the molecular size distribution of collagen peptides was critically important for their ice-structuring activity. In this respect, collagen peptides in the mass range of 600−2700 Da exhibited the greatest inhibitory activity on the recrystallization of ice. In addition, among the peptides of this molecular mass range, the cationic peptides displayed significantly greater inhibitory activity than the anionic peptides. It is also likely that not all cationic peptides in the molecular mass range of 600−2700 Da are equally active and that only a few peptides in the hydrolysate might be mostly responsible for the observed activity. Isolation of those peptides or partial purification of the hydrolysate to enrich the concentration of the active peptides would likely reduce the concentration needed for effective inhibition of ice recrystallization. Unlike papain, Alcalase is not known to catalyze plastein-type reaction products. Thus, it is apparent that the ice-structuring activity of gelatin hydrolysate observed in a previous study (*24*) using papain was not due to some unique peptides formed as a result of plastein-type reaction catalyzed by papain. Moreover, although the peptide fragmentation pattern in the papain-hydrolyzed sample (i.e., 700−1600 Da in fraction 2) (*24*) was very different from that in the Alcalase-hydrolyzed sample (i.e., 1100−2500 Da in fraction 2), the ice recrystallization inhibition activity was very similar. This suggests that the gross molecular mass range, that is, 700−2500 Da, is more important than specific peptide fragmentation pattern for recrystallization inhibition activity.

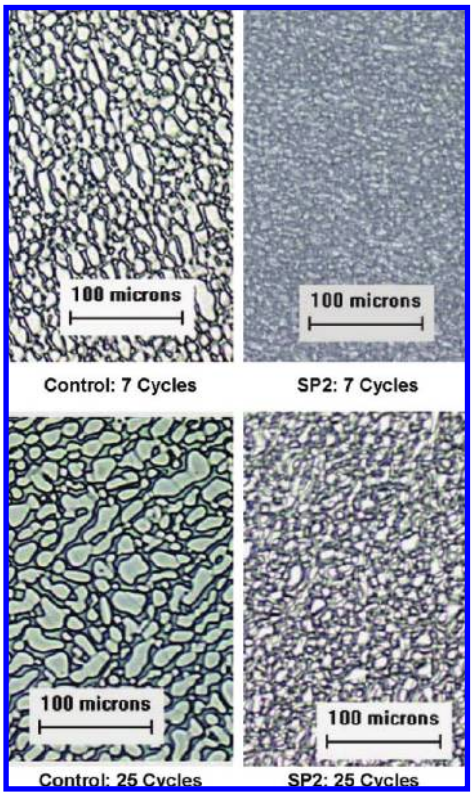

Figure 8. Effect of SP2 fraction from SP-Sephadex C-25 column on ice crystal growth in an ice cream mix after 7 and 25 thermal cycles at −14 to −12 °C. The concentration of the peptide fractions in the ice cream was 4 wt %.

Table 2. Mean Ice Crystal Size in Ice Cream Mix Containing Fraction SP2 from the SP-Sephadex C25 Column

sample	no. of cycles	av ice crystal diameter ± SD (μm)
15% diluted ICM (control)	7	22.16 ± 1.21 B
15% diluted ICM + 4% SP2	7	3.35 ± 0.46 D
15% diluted ICM (control)	25	46.61 ± 2.89 A
15% diluted ICM + 4% SP2	25	10.32 ± 1.86 C

[a] Values expressed as mean ± SD. Values with different letters in the same column differ significantly ($P < 0.05$). SD, standard deviation; ICM, ice cream mix; fractions 1−3 are fractions from the Sephadex G-50 column as shown in **Figure 5**; SP1 and SP2 are fractions from the SP-Sephadex C25 column.

Although the mechanism of inhibition of ice crystal growth by collagen peptides is not clear, it is likely that it might follow a mechanism similar to that of the ice-structuring activity of antifreeze proteins found in fishes, insects, and plants that live in subzero temperatures (*1*−*6*, *25*). It is well accepted that the mechanism of inhibition of ice crystal growth by antifreeze proteins involves binding of these proteins to the ice−liquid interface. Analyses of the crystal structures of antifreeze proteins from various species have revealed that they typically contain a flat face containing polar oxygen-containing groups in a two-dimensional array that mimics the spacing of oxygen atoms in the hexagonal ice lattice (*18*−*20*, *26*). This lattice mimicry has been proposed to be essential for their ice-binding function (*19*).

Antifreeze proteins that have been isolated and characterized up to now from various living species are polypeptides belonging to structurally diverse families of genetically coded proteins: some of them are α-helix-type proteins and others β-helix-type proteins. The molecular masses of these proteins range from 3 to

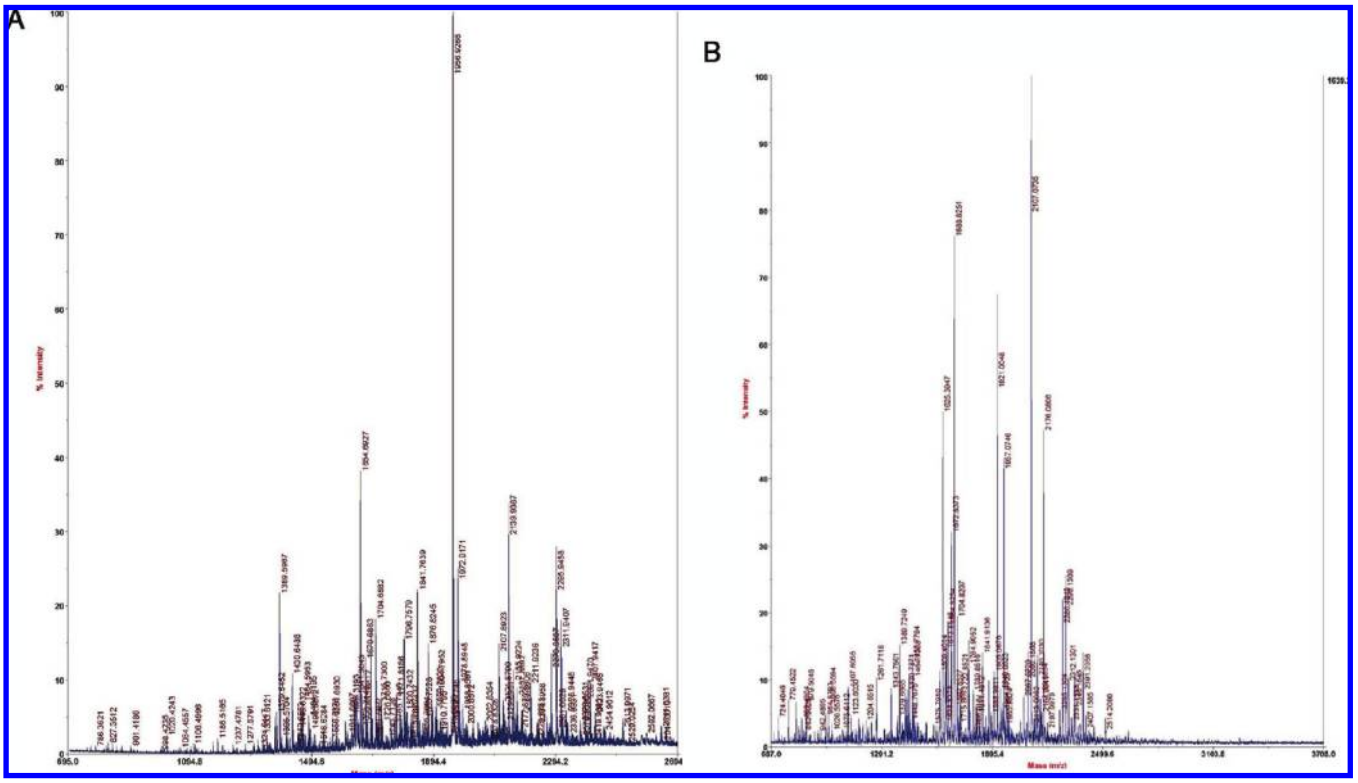

Figure 9. MALDI-TOF mass spectrum of SP1 (**A**) and SP2 (**B**) fractions from **Figure 7**.

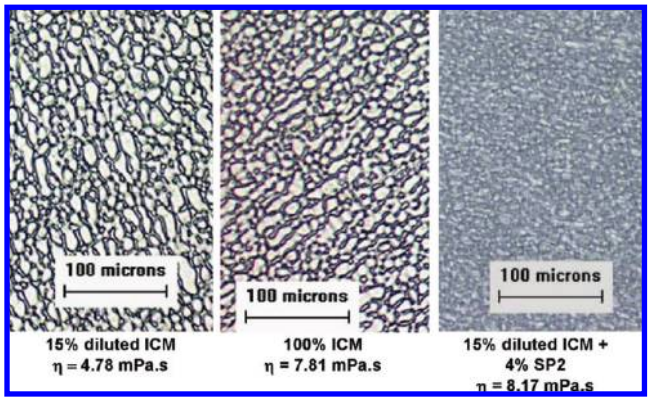

Figure 10. Effect of soluble solid contents on ice crystal growth in an ice cream mix after seven thermal cycles at −14 to −12 °C: (left to right) 15% diluted ice cream mix after seven cycles; 100% ice cream mix after seven cycles; 15% diluted ice cream mix + 4 wt % SP2 fraction after seven cycles.

Table 3. Viscosity of Ice Cream Mix with and without Gelatin Peptides and Its Effect on Ice Crystal Growth

sample	viscosity (mPa·s)	av ice crystal diameter ± SD (μm)
water	1.00 ± 0.02 C	
100% ICM	7.81 ± 0.03 A	20.66 ± 1.58 a
15% diluted ICM	4.78 ± 0.07 B	22.16 ± 1.21 a
4% SP2 in water	1.26 ± 0.01 C	
15% diluted ICM + 4% SP2	8.17 ± 0.04 A	3.35 ± 0.46 b

[a] Values with different letters in the same column differ significantly ($P < 0.05$). SD, standard deviation; ICM, ice cream mix; SP2, fraction SP2 from the SP-Sephadex C25 column.

36 kDa. There are no reports in the literature on the ice-structuring of activity of peptide fragments derived from native proteins.

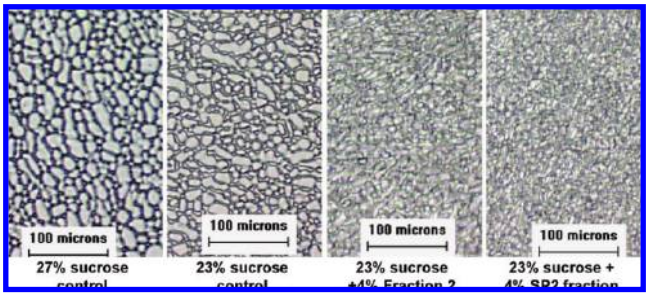

Figure 11. Effects of fraction 2 from Sephadex G-50 chromatography (**Figure 5**) and SP2 from SP-Sephadex C-25 ion-exchange chromatography (**Figure 7**) on ice crystal growth in sucrose solution after seven thermal cycles at −14 to −12 °C.

Table 4. Ice Crystal Growth in Sucrose Solution Containing Gelatin Peptides

sample	no. of cycles	av ice crystal diameter ± SD (μm)
23% sucrose solution (control)	7	24.32 ± 1.67 A
27% sucrose solution	7	22.03 ± 1.25 A
23% sucrose solution + 4% fraction 2	7	7.86 ± 0.83 B
23% sucrose solution + 4% SP2	7	4.97 ± 0.58 C

[a] Values expressed as mean ± SD. Values with different letters in the same column differ significantly ($P < 0.05$). Fraction 2, fraction 2 from the Sephadex G-50 column; SP2, fraction SP2 from the SP-Sephadex C25 column.

Collagen/gelatin is a very unique protein. On average, it contains 33% Gly and 30% Pro + Hyp. Generally, the amino acid sequence of gelatin is depicted as $-(Gly-Xaa-Xaa)_n-$, where Xaa is any amino acid residue. The second position in this tripeptide repeat is more often than not occupied by Pro or Hyp. This tripeptide repeat sequence provides a very high degree of molecular flexibility to collagen as well as to gelatin, which is a partially hydrolyzed product of collagen. Interestingly, this

tripeptide repeat sequence is found in two glycine-rich antifreeze proteins with molecular masses of 6.5 and 15.7 kDa recently isolated from snow fleas (*4*). However, these snow flea antifreeze proteins contain two intramolecular disulfide bonds; reduction of these disulfides in the presence of reducing agents destroys their antifreeze activity (*4*, *18*, *26*). Although the amino acid composition of the snow flea antifreeze proteins is not similar to that of bovine collagen/gelatin (e.g., bovine collagen is devoid of cysteine), the tripeptide repeat sequence is strikingly similar. It is possible that the snow flea antifreeze protein could have evolved as a result of mutations of a collagen gene. Nonetheless, the similarity of the −Gly−Xaa−Xaa− tripeptide sequence between collagen and the snow flea antifreeze protein gives credence to the fact that some of the collagen (gelatin) polypeptide fragments might possess antifreeze activity. The results of this study indicate that the size of such peptides is in the range of 600−2700 Da. As pointed out earlier, within this molecular mass range, some collagen peptides might be more active than others, depending on certain unique amino acid sequence patterns.

Although isolation and structural characterization of collagen peptides showing high antifreeze activity remain to be studied, it is likely that the mechanism of inhibition of ice recrystallization by these collagen peptides might be similar to that of the snow flea antifreeze protein (*18*, *26*). However, one of the major advantages of collagen-derived antifreeze peptides is that unlike other natural antifreeze proteins, the antifreeze activities of collagen peptides are not destroyed by thermal treatments.

ACKNOWLEDGMENT

We are grateful to St. Louis Food Ingredients (St. Louis, MO) for providing the gelatin samples used in this study.

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Received February 13, 2009. Revised manuscript received April 29, 2009. Accepted May 12, 2009. This research was supported by the U.S. Department of Agriculture National Research Initiative Program (Grant 2006-35503-16998).