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Hydrolysates from Sorghum Crude Kafirin Extract Exhibit Ice Recrystallization Inhibition Activity

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ABSTRACT: Ice recrystallization can damage the microstructure of food products, and one potential solution is the use of molecules with demonstrated ice recrystallization inhibition (IRI) activity. The objective was to determine the IRI activity of sorghum kafirin hydrolysates as affected by succinylation and the dispersing medium. The IRI activity of the sorghum kafirin hydrolysates was analyzed by the splat assay in phosphate-buffered saline (PBS) and 10 mM NaCl. At 2% concentration, sorghum kafirin hydrolyzed by bromelain for 1 h resulted in 55% reduction in ice crystal size in 10 mM NaCl, while this was not observed when the dispersing medium is PBS. Succinylation at 0.25:1 molar ratio (succinic anhydride: amine group of the peptides) yielded modified hydrolysates that were slightly more IRI active in PBS, but the difference was not significant. The data showed the potential application of sorghum kafirin hydrolysates to act as antifreeze molecules to preserve the quality of frozen materials.

KEYWORDS: *sorghum kafirin, hydrolysates, ice recrystallization inhibition, splat assay, bromelain, trypsin*

INTRODUCTION

Sorghum is the fifth-most-produced cereal grain in the world (FAOSTAT 2016). It grows in warmer climates and is resistant to drought conditions, making it a popular cereal grain. Kafirin is the most abundant protein in sorghum making up 77−82% of its endosperm proteins.[1] It is classified into different classes of kafirin by the molecular weight. The major classification, alpha kafirin, makes up 66−84% of the endosperm proteins and has a molecular weight of about 23 to 25 kDa, while beta kafirin accounts for 10−13% of the endosperm proteins and has a molecular weight of about 18 kDa.[1] Kafirin is hydrophobic as it contains more hydrophobic amino acids than wheat and oat proteins.[2,3] Previous works reported the importance of hydrophobic amino acids in ice recrystallization inhibition (IRI);[4,5] hence, the IRI activity of kafirin was explored.

IRI activity is when a molecule can inhibit or slow the growth of ice crystals during freezing.[6] Ice recrystallization occurs due to Ostwald ripening, the growth of large ice crystals at the expense of smaller ice crystals. This process decreases the number of ice crystals and increases the size of each ice crystal.[7] Large ice crystals can physically puncture and damage the microstructure of food products causing changes in color, flavor, and texture after thawing of frozen foods.[8] A potential solution to this problem is the use of antifreeze proteins (AFPs) with an IRI activity. These proteins can inhibit and slow the growth of large ice crystals.[9] The proposed mechanism for IRI activity of AFPs involves amphiphilicity, i.e., proteins' ability to bind to the ice by hydrogen bonding while the hydrophobic domains of the protein repel water molecules preventing ice from growing bigger.[10] Kafirin is a water-insoluble protein; however, it has the potential to be water-soluble through enzymatic hydrolysis and chemical modification, changing its hydrophobicity. By potentially increasing its amphiphilicity, we should observe the IRI activity. Recent studies have shown the importance of the balance of hydrophobic and hydrophilic residues in inhibiting ice growth. For instance, amphiphilic peptoids can inhibit ice recrystallization and result in smaller mean largest grain size than phosphate-buffered saline (PBS) control.[11] In addition, it has been reported that hydrophilic and hydrophobic amino acid residues in silver carp hydrolysates offered synergistic effects in inhibiting ice crystal growth.[12] These studies support the importance of the hydrophobic and hydrophilic balance in designing molecules as antifreeze agents.

In this work, enzymatic hydrolysis was performed to observe the possible IRI activity of kafirin hydrolysates. Enzymes cleave specific peptide bonds and digest the protein into peptides with varying sizes.[13] These peptides will have different functional properties and will possibly improve the functionality of the protein.[14] Another method that can be used to modify the functionality of a protein is succinylation. During this process, succinic anhydride (SA) unfolds and replaces the ϵ-amino group of lysine and hydroxyl groups in proteins with succinyl groups.[15] This modification changes the hydrophilicity of compounds and can change the functional characteristics of the protein.

The objective of this research is to observe the IRI activity of sorghum kafirin hydrolysates and SA-modified hydrolysates. The IRI activity was observed in PBS and 10 mM NaCl solution to evaluate IRI activity in different media utilizing

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splat assay, the most common method of evaluating IRI activity.[16] It is hypothesized that succinylation and the dispersing medium will impact the IRI activity of sorghum kafirin hydrolysates. This work can provide knowledge of the potential application of sorghum kafirin peptides as materials for the preservation of frozen food products.

MATERIALS AND METHODS

Materials. The kafirin was extracted from sorghum flour that was purchased from Bob's Red Mill Natural Foods (Milwaukie, OR). The enzymes used for hydrolysis are Alcalase from *Bacillus licheniformis* (EMD Millipore Corp. Billierica, MA), bromelain (MP Biomedicals, Solon, OH), and trypsin (Alfa Aesar, Ward Hill, MA).

Preparation of Sorghum Protein Kafirin and Its Hydrolysates. Sorghum flour was first defatted with hexanes for 90 min, and the flour was left overnight to dry. The defatted flour was then extracted with 70% ethanol containing 89.7 mM sodium metabisulfite in a shaking water bath at 50 °C for 1 h. Then, the samples were centrifuged for 30 min at 10 000*g* at 4 °C. The supernatant was collected after centrifugation, diluted with deionized water to a concentration of 60% ethanol, and centrifuged again for 30 min at 10 000*g* at 4 °C. The supernatant was collected again and diluted with deionized water to 30% ethanol to allow precipitation of kafirin, a prolamin. This solution was then chilled for 1 h at 4 °C and then centrifuged again under the same conditions. Precipitated protein was collected and freeze-dried.[17] The kafirin was hydrolyzed in a 10% suspension in 50% ethanol by bromelain, trypsin, and Alcalase. High concentrations of ethanol are generally used to solubilize kafirin;[18] however, 50% ethanol was used in this case to maintain the activity of Alcalase and other enzymes as a high concentration of ethanol can decrease enzymatic activity.[19,20] The protein was hydrolyzed for 1 h for Alcalase and bromelain and 4 h with trypsin. The difference in the hydrolysis times for Alcalase (1 h) and trypsin (4 h) is due to the difference in the specificities for these enzymes, Alcalase being a broader endopeptidase which can cut peptide bonds randomly while trypsin is more specific as discussed later in the paper. Furthermore, the three enzymes were selected according to their differences in specificities, as discussed. The enzymes were added at a 1% concentration. The hydrolysis was carried out at pH 8 for Alcalase and trypsin and at pH 6.5 for bromelain. The Alcalase and bromelain hydrolysis was done at 55 °C and trypsin hydrolysis was done at 37 °C. The samples were boiled for 30 min to stop the hydrolysis and remove the ethanol. They were cooled to room temperature before being centrifuged at 10 000*g* for 10 min at 4 °C to separate the supernatant and precipitate, and the supernatant was freeze-dried. Freeze-drying was performed using a VirTis AdVantage Plus EL-85 benchtop freeze-dryer (SP Scientific Inc., Gardiner, NY, USA) at −40 °C and 0.064 MPa.

Modification of the Kafirin Hydrolysates with SA. Kafirin and its hydrolysates were modified with SA. A leucine standard curve was obtained to determine the primary amines in the hydrolysates using the *o*-phthaldialdehyde method. The modification of the hydrolysates by SA was done at 0.25:1 molar ratio of SA and the primary amines in the hydrolysates. The amount of SA was calculated based on the quantity of primary amines in 0.1 g of the sample. The modification reaction was completed at a concentration of 2% sample in 0.1 M ammonium bicarbonate buffer with a pH of 9.0. The reaction continued for 3 h at room temperature, and the pH was monitored to be between 8 and 9. The reaction was stopped by lowering the pH to 6.5 and boiled for 30 min to remove the ammonium bicarbonate buffer.

Protein Profile by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. The molecular weight profile of the samples was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One hundred μL of the sample were mixed with 100 μL of Laemmli buffer containing 5% β-mercaptoethanol and boiled for 10 min. After determining the protein concentration by the Bradford assay, approximately 25 μg of protein was loaded in the SurePAGE Bis-Tris, 10 × 8 gel, and 5 μL Broad Multi Pre-Stained Protein Standard (GenScript, Piscataway, NJ). The Bradford assay was completed by vortexing approximately 1 mg of each sample in deionized water for 1 h and centrifuged for 10 000*g* at 4 °C for 5 min. After centrifugation, the supernatant was collected and diluted 10× with deionized water. The samples and bovine serum albumin (BSA) standard ranging from concentrations of 0−20 μg/mL were plated in a 96-well plate at a volume of 100 μL. One hundred microliters of the Bradford reagent was added to each well containing the samples and standards. The plate was incubated at room temperature for 5 min, and the absorbance was read at 630 nm. Protein concentration was calculated using the generated BSA standard curve. The proteins were separated at 200 V for 35 min. The gel was stained with Coomassie blue G-250 (Bio-Rad, Hercules, CA) overnight while being slowly shaken and destained with deionized water overnight. The gel continued to destain with deionized water until the desired background clarity had been achieved while changing the water periodically.

Average Molecular Weight Determination by Size-Exclusion Chromatography-High-Performance Liquid Chromatography. To analyze the molecular weight distribution of the hydrolysates, size-exclusion chromatography-high-performance liquid chromatography (SEC-HPLC) was performed. Samples were extracted in HPLC-grade water at a concentration of 1 mg/mL and filtered using nylon membrane filters (GE Healthcare Life Sciences). Once the samples were prepared, they were placed in a 1200 Agilent HPLC system (Agilent Technologies, Santa Clara, CA). The HPLC system has an autosampler (G1329A), quaternary pump (G1311A), vacuum degasser (G1322A), a temperature-controlled column (G1316A), and a diode array detector (G1315D). The column used was the BioSep-SEC-S2000 column (300 mm × 7.80 mm, Phenomenex, Torrance, CA). The flow rate was 1.0 mL/min, and the mobile phase was 45% acetonitrile containing 0.1% trifluoroacetic acid. The molecular weight of the samples was calculated using the standard curve made from the standards composed of albumin, aprotinin, glucagon, bradykinin, glutathione, and glycine. The linear regression equation (R^2 = 0.98) from the standards was used to calculate the molecular weight of each peak in the samples. The average molecular weight of each sample was calculated by taking the percent area for each peak and multiplying it by its corresponding molecular weight. The resulting values were summed, and this value was reported as the average molecular weight.

Degree of Hydrolysis Determined by *o*-Phthaldialdehyde Assay. A portion of the sample from SEC-HPLC was used to measure the degree of hydrolysis using *o*-phthaldialdehyde (OPA) assay as previously reported.[21] Ten μL of samples were plated in a 96-well plate in duplicate followed by the addition of 200 μL of freshly prepared OPA reagent. The OPA reagent is composed of 50% 100 mM sodium tetraborate, 42.8% deionized water, 5% 20% (w/v) SDS, 2% 40 mg/mL of OPA in methanol, and 0.2% β-mercaptoethanol. The absorbance was read at 340 nm after 5 min incubation at room temperature.

Surface Hydrophobicity Determined by 8-Anilinonaphthalene-1-sulfonic Acid. The samples were prepared in 1× PBS solution at a concentration of 5 mg/mL and were vortexed for 1 h, followed by centrifugation at 10 000*g* for 30 min at 4 °C, and the supernatant was transferred into new microcentrifuge tubes. From there, each sample went through a serial dilution to the final concentrations of 0.016 to 0.5 mg/mL. A black 96-well plate was used for the analysis by placing 200 μL of each diluted sample in the wells, followed by the addition of 20 μL of 8 mM 8-anilinonaphthalene-1-sulfonic acid (ANS) prepared in 1× PBS. The excitation and emission were performed at 390 and 480 nm, and the surface hydrophobicity was reported as the slope of the line by plotting the fluorescence intensity against sample concentration.

Identification of Peptide Sequences by Mass Spectrometry. The hydrolysates were identified by mass spectrometry (MS). Separate injections (1.0 μL) of each sample were made onto a Bruker Pepsep25 (25 cm long × 75 μm inner diameter × 1.9 μm ReproSil C18AQ; Cat# 1893474; Batch#0000413780). Peptides were eluted and separated from the column with a gradient of acetonitrile

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Table 1. Characterization of Unmodified and Modified Kafirin Hydrolysates[a]

modification	enzymes	hydrolysis (hour)	average MW (kDa)	surface hydrophobicity	degree of hydrolysis, %
0:0	bromelain	1	5.5 ± 0.2[c]	172 826 ± 34 161[a]	13.9 ± 4[b]
	trypsin	1	13.2 ± 0.7[a]	164 329 ± 28 897[ab]	5.9 ± 0.4[c]
		4	11.0 ± 0.3[b]	140 736 ± 44 009[ab]	6.6 ± 0.3[c]
	alcalase	1	2.6 ± 0.1[d]	87 471 ± 16 946[b]	23.9 ± 0.1[a]
0.25:1	bromelain	1	6.3 ± 0.4[a]	44 155 ± 11 204[a]	N/A
	trypsin	1	8.5 ± 3.5[a]	23 118 ± 3 525[ab]	
		4	7.9 ± 1.0[a]	40 310 ± 15 600[ab]	
	alcalase	1	4.6 ± 0.9[a]	16 957 ± 5 189[b]	

[a]Means followed by the same letter within the same modification group are not significantly different at 5% level of significance according to Tukey−Kramer HSD.

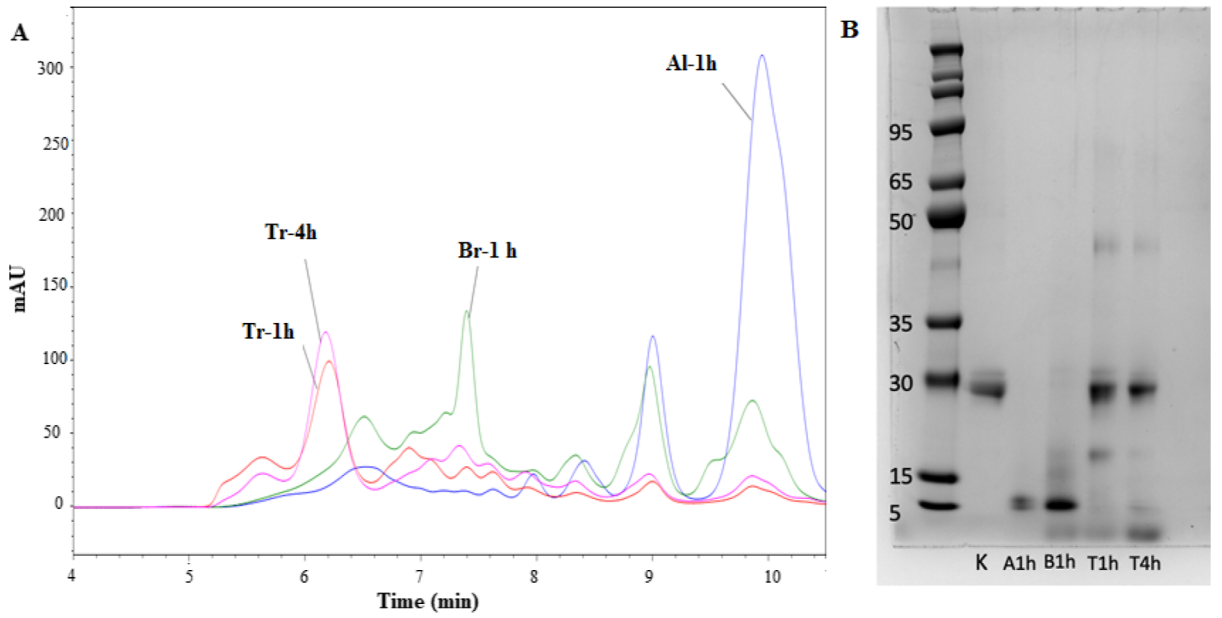

Figure 1. Protein profile and molecular weight distribution of kafirin hydrolysates. (A) Overlaid chromatograms of the kafirin hydrolysates from SEC-HPLC. The peak labeled Tr-1h is the trypsin 1 h, Tr-4h is the trypsin 4h, Br-1h is the bromelain hydrolysate, and Al-1h is the Alcalase hydrolysate. (B) Electrophoresis profiles of kafirin and kafirin hydrolysates. The lane abbreviations: MW is the molecular weight marker, K is the unhydrolyzed kafirin, A1h is the Alcalase 1 h hydrolysate, B1h is the bromelain 1 h hydrolysate, T1h is the trypsin 1 h hydrolysate, and T4h is the trypsin 4 h hydrolysate.

at 300 nL/min. The Bruker nanoElute system is attached to a Bruker timsTOF-Pro2 mass spectrometer via a Bruker CaptiveSpray nanospray source. Initial conditions were 2% B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by 20 min gradient to 40% B; ramp to 95% B over 0.5 min, and hold at 95% B for 8 min. The total run time was 28.5 min. MS data were collected in positive-ion data-dependent PASEF mode over an m/z range of 100 to 1700. One MS and ten PASEF frames were acquired per cycle of 1.17 s (~1 MS and 120 MS/MS). Target MS intensity for MS was set at 10 000 counts/sec with a minimum threshold of 250 counts/s. A charge-state-based rolling collision energy table was used from 76 to 123% of 42.0 eV. An active exclusion/reconsider precursor method with release after 0.4 min was used. If the precursor (within mass width error of 0.015 m/z) was > 4× signal intensity in subsequent scans, a second MS/MS spectrum was collected. Isolation width was set to 2 m/z (<700 m/z) or 3 m/z (800−1500 m/z). Realtime database searches were performed with Bruker PaSER2023 using default parameters: trypsin, Alcalase (C-terminal to AEFKLMSWY), or bromelain (C-terminal to DEKR) as enzyme, two missed cleavages allowed; 20 ppm mass error on precursor, 0.1 Da mass error on CID MS/MS fragments; oxidized Met as variable modification. Data were then filtered as follows: all identified peptides were filtered for a 1% false discovery rate. A *Sorghum bicolor* protein FASTA-formatted reversed database (145 564 entries, including common contaminants) was downloaded from NCBI and indexed for searching (including common contaminants and reversed entries) using the PaSER database utility. Peptide identification was predicted by uniport at https://www.uniprot.org/peptide-search. Only the peptides related to sorghum were considered in the results.

Determination of IRI Activity by Splat Assay. The IRI activity of the samples was measured by splat assay using polarized light microscopy (Leica, DM2700M, Wetzlar Germany). The cooling stage used was the HCS 302 (Insect instrument, Boulder, Colorado) and a digital camera (Leica, DMC4500, Wetzlar, Germany) was used on the microscope. The hydrolysates and poly(ethylene glycol) (PEG) were solubilized in 1× PBS and 10 mM NaCl at concentrations of 2, 1, and 0.5% (w/v). Splat assay was conducted in two different dispersing media to determine the effect of salt concentrations and describe the potential mechanism of action. Furthermore, the 10 mM NaCl concentration was chosen as it provides salt concentration high enough to avoid false positive results as previously reported.[22,23] These samples were dropped from a syringe with a diameter of 0.90 mm onto glass slides that were previously stored in a −80 °C freezer. Once the sample was dropped, it was placed into the microscope stage, which was kept at a temperature of −8 °C. The ice crystals in the sample were captured after 30 min. Three pictures were taken at different parts of the drop, and a second drop of the same sample was evaluated. The diameters of the ice crystals were calculated using

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Table 2. Percent Feret's Diameter (% FD) of Unmodified and Modified Kafirin Hydrolysates Tested at 2% Concentration[a]

modification	enzymes	hydrolysis (hour)	% FD in PBS	% FD in 10 mM NaCl
0:0	bromelain	1	101.5 ± 4.2^{aA}	45.0 ± 4.3^{bD}*
	trypsin	1	108.2 ± 6.7^{aA}	77.0 ± 11.4^{aC}*
		4	100.7 ± 7.1^{aA}	63.0 ± 4.5^{aC}*
	alcalase	1	92.1 ± 9.1^{aA}	64.4 ± 4.2^{aC}*
0.25:1	bromelain	1	85.3 ± 13.6^{aA}	109.6 ± 4.5^{bB}
	trypsin	1	83.0 ± 16.7^{aA}	78.1 ± 7.6^{cC}
		4	85.0 ± 11.1^{aA}	78.1 ± 3.5^{cC}
	alcalase	1	95.4 ± 0.3^{aA}	138.0 ± 3.7^{aA}*

[a]Means followed by the same letter are not significantly different at 5% level of significance according to Tukey–Kramer HSD. Lowercase letters indicate the significance within each modification separated by solvent. Uppercase letters indicate significance between all modifications within the same solvent. Asterisk (*) indicates significant differences between solvents by *t*-test.

Cellpose as reported previously.[24] The diameters of the ice crystals were compared to that of the PEG sample as the negative control.

Statistical Analysis. All experiments were completed in three replicates. The statistical analysis was done using SAS Version 9.4 (SAS Institute Inc., Cary, NC) considering the data collected follow normal distribution, which is important in parametric assumption. The data were evaluated to determine significant differences using ANOVA with Tukey as the posthoc test and correlation with a *P*-value of 0.05. Type III tests of fixed effects were done with ANOVA. For the effect of dispersing medium, a *t*-test was done with the SAS Institute Inc. (Cary, NC).

RESULTS AND DISCUSSION

Hydrolysates Yield, Average Molecular Weight, and Protein and Peptide Profile of Kafirin Hydrolysates. Kafirin, the major protein in sorghum, was hydrolyzed by Alcalase, bromelain, and trypsin. Alcalase hydrolysis for 1 h resulted in 47.5 ± 3.8% of the starting kafirin extract going to the soluble supernatant, while hydrolysis with bromelain for 1 h only yielded 21.5 ± 5.2%. Trypsin hydrolysis resulted in an increased yield from 1 h hydrolysis of 17.3 ± 0.6% to 4 h hydrolysis of 26.6 ± 1.5%. The difference in yield can be associated with the differences in the specificity of the enzymes: Alcalase being a random protease resulted in the highest yield (more soluble peptides in the supernatant), while trypsin only cleaves peptide bonds associated with carboxyl group of lysine and arginine giving the least amount of hydrolysates for 1 h of hydrolysis time. The average molecular weight by SEC-HPLC is shown in Table 1. The Alcalase hydrolysate had the lowest average molecular weight at about 2.6 kDa, followed by bromelain, with an average molecular weight of about 5.5 kDa. The trypsin hydrolysates had the highest average molecular weights. The chromatogram of the overlaid peaks from SEC-HPLC is shown in Figure 1A. This shows the difference in the retention time of hydrolysates. The trypsin hydrolysates had the lowest retention time, indicating the highest molecular weight, and the bromelain hydrolysate followed with the second lowest retention time. The Alcalase hydrolysates had the highest retention time, indicating its low molecular weight. To confirm the result from the SEC-HPLC, the molecular weight profile of kafirin and its hydrolysates were analyzed by SDS-PAGE, shown in Figure 1B. The unhydrolyzed kafirin had a dark band just below the 30 kDa molecular weight marker. This agrees with literature as kafirin has been found to have an average molecular weight of about 23–25 kDa (1). The Alcalase hydrolysate has the lowest molecular weight with only one band at the 5 kDa marker; this agrees with the SEC-HPLC data. The bromelain hydrolysate has a darker band at the 5 kDa marker and also a faint band at the 15 kDa marker. This shows that it has a larger molecular weight than the Alcalase hydrolysate, confirming the SEC-HPLC. Lastly, the trypsin hydrolysates have the highest molecular weight. The trypsin 1 h hydrolysate has a band at the 30 kDa molecular weight marker. The trypsin 4 h hydrolysate has a band at the 30 kDa marker and a band below the 5 kDa marker. This indicates that the trypsin 1 h hydrolysate is much less hydrolyzed than the trypsin 4 h hydrolysate. The Alcalase hydrolysates having the lowest average molecular weight suggest that these hydrolysates were the most easily obtained. Alcalase has a broad specificity as it hydrolyzes most peptide bonds.[25] The bromelain hydrolysate being the second most hydrolyzed hydrolysate agrees with the fact that it is the second most specific enzyme. Bromelain has specificity for cleaving peptide bonds of alanine, leucine, and glycine.[26] The trypsin hydrolysates having the highest molecular weight suggests that they are the least hydrolyzed and this is due to the enzyme having higher specificity as trypsin cleaves the carboxylic sides of lysine and arginine.[27]

Degree of Hydrolysis of Kafirin Hydrolysates. The degree of hydrolysis of the hydrolysates was determined by an OPA assay to further confirm the level of hydrolysis by each enzyme. As shown in Table 1, the Alcalase hydrolysate had the highest degree of hydrolysis, and bromelain hydrolysates had the second highest degree of hydrolysis. The trypsin hydrolysates had the lowest degree of hydrolysis. These results agree with the molecular weight profile from SEC-HPLC and SDS-PAGE.

Surface Hydrophobicity of Kafirin Hydrolysates. The surface hydrophobicity of the hydrolysates is shown in Table 1. The bromelain hydrolysate had the highest surface hydrophobicity, followed by the trypsin hydrolysates, and the Alcalase hydrolysate had the lowest surface hydrophobicity. From these results, it is inferred that both enzyme specificity and molecular weight contribute to the surface hydrophobicity of peptides.

Characterization of SA-Modified Kafirin Hydrolysates. The molecular weights of the modified kafirin hydrolysates are shown in Table 1. The average molecular weights are similar to the unmodified hydrolysates. However, there are some increased and decreased molecular weights of the hydrolysates after the modification. The change in average molecular weight is due to the succinylation reaction. This reaction involves a nucleophilic substitution reaction that specifically attacks the ε-amino group of lysine and hydroxyl groups in proteins.[15] These groups are replaced with succinyl carboxyl groups, forming an amide bond after the opening of the anhydride ring during the reaction. These added groups

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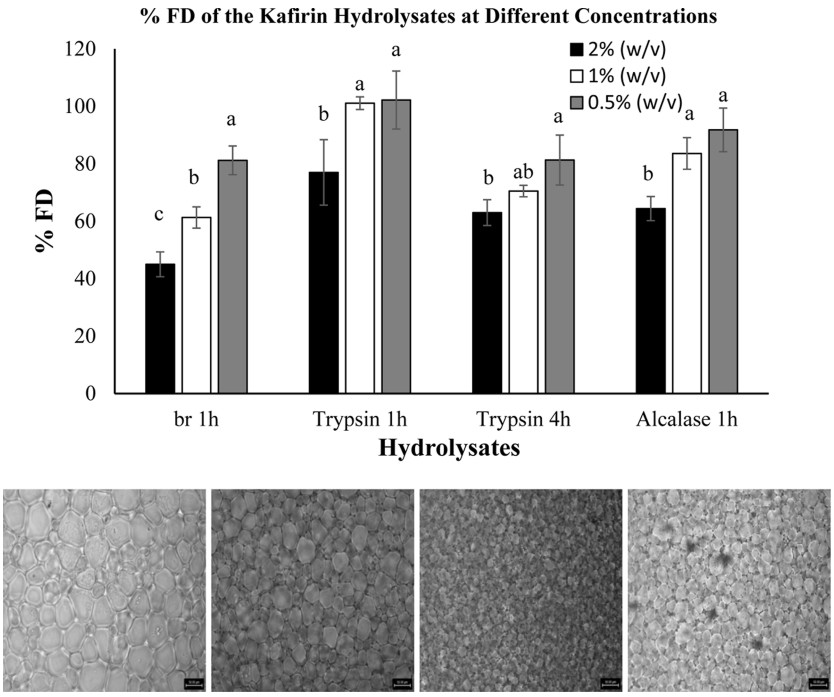

Figure 2. (A) IRI activity of kafirin hydrolysates as affected by different concentrations [2, 1, and 0.5% (w/v)] in 10 mM NaCl. Means followed by the same letter are not significantly different at a 5% level of significance according to Tukey−Kramer HSD within each hydrolysate. (B) Representative ice images from left to right of 2% PEG in PBS, 2% PEG in 10 mM NaCl, 2% kafirin Br 1h in 10 mM NaCl, and 1% kafirin Br 1h in 10 mM NaCl.

increase the electronegativity of the protein and could change the average molecular weight of the hydrolysates.[25] The reduction in the molecular weight of modified trypsin hydrolysates is not explainable. The surface hydrophobicity of the modified hydrolysates decreased after modification as shown in Table 1. This implies that the succinylation reaction decreased the surface hydrophobicity of the hydrolysates. This can be explained by the increase in the electronegativity of the hydrolysates because of the addition of succinyl carboxyl groups.[28] The decrease in the surface hydrophobicity after modification confirms that the succinylation reaction did occur.

IRI Activity of Kafirin Enzymatic Hydrolysates and SA-Modified Hydrolysates. The IRI activity of the unmodified kafirin hydrolysates was determined by the splat assay in 1× PBS, and the results are shown in Table 2. The hydrolysates had high percent Feret's diameters (% FD) relative to PEG in PBS, meaning they were not IRI active. The % FD of the succinylated hydrolysates (0.25:1) in PBS had % FD values much less than 100%, indicating that they are IRI active. The IRI activity of the hydrolysates slightly increased after modification when compared to the unmodified hydrolysates. This can be explained by the decrease in surface hydrophobicity of the hydrolysates after succinylation, indicating that the amphiphilicity of the hydrolysates may have increased. Amphiphilic proteins have been shown to be IRI active in past studies, explaining the increase in IRI activity of the hydrolysates after modification.[29] Statistical analysis shows that there is no correlation between surface hydrophobicity and % FD. This suggests that low % FD is not correlated with hydrolysates having either low or high surface hydrophobicity, supporting the explanation of needing a balance of both hydrophobic and hydrophilic residues for IRI activity. Representative ice crystal images of the splat assay in PBS can be seen in Supporting Information, Figure 1.

IRI Activity of Kafirin Hydrolysates in 10 mM NaCl. Because past studies have found differences in IRI activity of proteins in different salt concentrations, the kafirin hydrolysates were also analyzed in 10 mM NaCl by the splat assay.[23,30] The results in Table 2 show that the unmodified hydrolysates were significantly more IRI active in 10 mM NaCl, with the unmodified bromelain hydrolysate having the highest IRI activity. An increase in IRI activity in 10 mM NaCl can be explained by the "salting-in" theory suggesting that low salt concentration increased the solubility of the protein. An increase in solubility leads to an increase in the protein surface area available to absorb into the ice/water interface and therefore increasing the IRI activity of the protein.[30] Previous studies have shown the production of IRI active materials after enzymatic hydrolysis. For instance, hydrolysis of soy protein isolates by Alcalase, trypsin, and pancreatin all resulted in IRI active materials with Alcalase hydrolysates being most active after gel filtration chromatography.[31] Furthermore, Alcalase hydrolysis of bovine collagen resulted in peptide mixtures capable of inhibiting ice recrystallization and this was affected by Alcalase hydrolysis time.[32]

Although there was an increase in IRI activity at low salt concentrations for the unmodified hydrolysates, this trend was not seen with the modified hydrolysates. Representative images of the ice crystals from the splat assay in 10 mM NaCl can be seen in Supporting Information, Figure 2. Due to the high IRI activity of the unmodified hydrolysates in 10 mM NaCl at a 2% (w/v) concentration, the IRI activity of the hydrolysates at 1 and 0.5% (w/v) was studied. Figure 2A shows the % FD of the hydrolysates in 10 mM NaCl at 2, 1, and 0.5% concentrations. The bromelain hydrolysate had the most IRI activity at 2% and it was still IRI active at 1% as both % FDs were significantly

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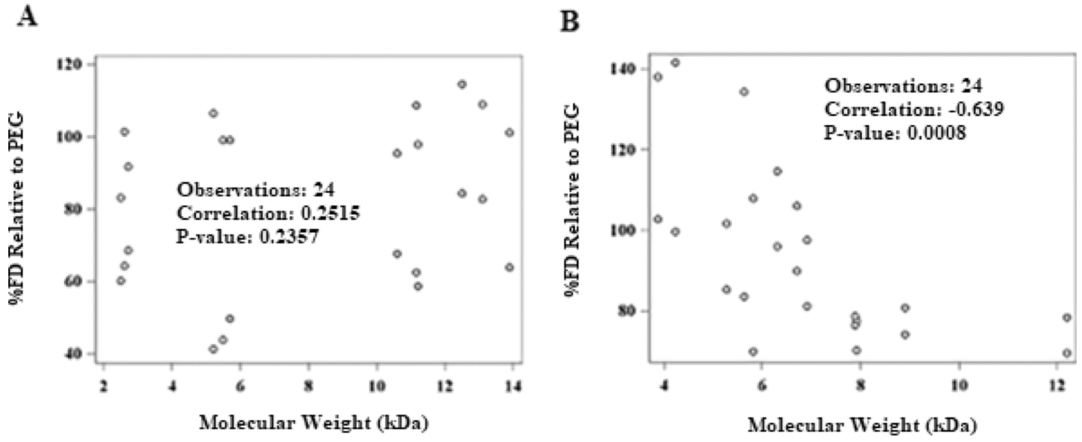

Figure 3. (A) No significant correlation between % FD and the average molecular weight of the unmodified hydrolysates. (B) Significant correlation between % FD and average molecular weight of the modified hydrolysates.

lower than the % FD at 0.5%. The trypsin 1 h hydrolysate was significantly more IRI active at 2% compared with the other concentrations. The trypsin 4 h hydrolysate had % FDs that were not significantly different at 2 and 1% concentrations, showing that the hydrolysate had similar IRI activity at both concentrations. The Alcalase 1 h hydrolysate had the most IRI activity at 2%. Overall, most of the hydrolysates had a maximum IRI activity at 2% and most maintained the IRI activity at 1% concentration. Representative images of ice crystals from splat assay at the 1 and 0.5% concentrations can be seen in Supporting Information, Figures 3 and 4, respectively. In addition, Figure 2B delineates the effect of 1× PBS, 10 mM NaCl, and bromelain 1 h hydrolysate at 2 and 1%. It can be seen that the ice crystals in 10 mM NaCl are smaller than in PBS, and the addition of bromelain hydrolysate for 1 h in 10 mM NaCl further reduced the ice crystal size, especially at 2%, indicating the IRI activity of kafirin extract hydrolysate.

The correlation between the average molecular weight of the unmodified and modified hydrolysates and % FD in both solvents is shown in Figure 3A,B. There was no significant correlation between the molecular weight of the unmodified hydrolysates and % FD (Figure 3A); however, there was a negative correlation between the modified hydrolysate molecular weight and the % FD (Figure 3B). This suggests that low % FD values, meaning higher IRI activity, are correlated with higher molecular weight of the peptides. This supports the findings as the bromelain and trypsin-modified hydrolysates had the highest molecular weights and had the most IRI activity. The type III fixed effects of the % FD from unmodified and modified hydrolysates and in both solvents are shown in Table 3. These data show the significant differences in enzyme treatment, modification, and the effect of solvent. It confirms that there was a significant difference in the IRI activity of the modified and unmodified from both solvents. It also shows that the different solvents caused significant differences in the IRI activity in hydrolysates and that the enzymatic treatment had a significant impact on the differences in IRI activity of the hydrolysates in the different solvents.

Identification of Peptides in the Hydrolysates. The peptide sequences found in the hydrolysates are listed in Table 4. The trypsin 1 h hydrolysate identified lipid transfer protein, alpha-kafirin, and beta-kafirin as the major proteins. The trypsin 4 h hydrolysates had alpha-kafirin, beta-kafirin, and

Table 3. Type III Fixed Effects of ANOVA % FD[a]

effect	P-value
Enzyme	0.0006
Modification	<0.0001
solvent	<0.0001
enzyme × modification	<0.0001
enzyme ×solvent	0.0007
modification × solvent	<0.0001
enzyme × modification × solvent	0.0005

[a]Interaction values were found by Tukey−Kramer HSD with a 5% significance level.

lipid transfer proteins identified as the major ones. The bromelain hydrolysate and Alcalase hydrolysate had alpha-kafirin as the majority. To confirm and determine the potential mechanism of IRI activity of these peptides, it is suggested that the synthesis of the identified peptides in the bromelain hydrolysates should be conducted. The differences on the peptide profile found in each of the hydrolysates may be responsible for the differences in the IRI activity observed when these hydrolysates were assessed using splat assay. For instance, we show that bromelain hydrolysis for 1 h resulted in the most IRI active sorghum kafirin hydrolysates reducing ice crystal size by up to 55% in 10 mM NaCl. On the other hand, the same hydrolysates did not show this activity when tested in 1× PBS. Statistical analysis (Table 3) shows that there is a significant interaction between enzyme and solvent (dispersing medium) in affecting IRI activity. One potential explanation for these observed differences on the IRI activity as affected by the low concentration of salt in the dispersing medium is the increased crowding in molecules. At 10 mM NaCl, the amount of unfrozen water at −8 °C is lower than that in 1× PBS that could potentially result in more crowding of the hydrolysates, resulting in higher IRI activity. Also, at lower salt concentration, hydrolysates could have higher solubility owing to the "salting-in" effect making them more completely dissolved in the dispersing medium, hence more activity. It has been previously reported that when the protein surface area available to absorb into the ice/water interface is increased, the antifreeze activity of the molecules is also increased.[33,34] Also, the ion composition of the dispersing medium could affect the antifreeze activity of the materials. Changing the anions with the same cation and vice versa affected the mean ice crystal

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Table 4. Peptide Sequences Identified in Kafirin Hydrolysates by Liquid Chromatography with Tandem Mass Spectrometry

hydrolysate	sequence	protein
bromelain 1h	LGAVSPATFWPQQQLLR	alpha-kafirin
	GATLPSSPLSD	SnoaL-like domain-containing protein
	ELAAVPSR	alpha-amylase inhibitor 4
	ALPCPKEE	uncharacterized, *Sorghum bicolor*
	TGGISALAHPWSLKNPD	polymerase/histidinol phosphatase N-terminal domain-containing protein
	VVGPGEAVTE	cytochrome P450 78A3
	ICLEAESE	RING-CH-type domain-containing protein
	IFRSLLSRAGFSLTYE	MATH domain-containing protein
	IPPVPD	NAC domain-containing protein
trypsin 1h	SLNSAAR	nonspecific lipid-transfer protein
	GISGLNAGNAASIPSK	nonspecific lipid-transfer protein 1
	QAAAGYQPLNADAIR	nonspecific lipid-transfer protein
	LGAVSPATFWPQQQLLR	19kD-like alpha kafirin B3
	MMDMQSR	beta-kafirin
	SLNSAASSTADKR	nonspecific lipid-transfer protein
	VSGLNAGNAASIPSK	nonspecific lipid-transfer protein
trypsin 4h	VNPVVAANPLAAAFLQQQQLLPFNQISLMNPAFSWQQPIVGSAIF	alpha kafirin
	LGAVSPATFWPQQQLLR	19kD-like alpha kafirin B3
	MMDMQSR	beta-kafirin
	GPFVPLPVLK	bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain-containing protein
	YVNSPNAR	bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain-containing protein
	SLNSAAR	nonspecific lipid-transfer protein
	GISGLNAGNAASIPSK	nonspecific lipid-transfer protein 1
	QLTGGMR	bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain-containing protein
	GYEETMPPLEK	bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain-containing protein
	QAAAGYQPLNADAIRDLPK	nonspecific lipid-transfer protein
	YVMFPTNPK	bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain- containing protein
	SLLAQANNTPDRR	bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain-containing protein
	NVANGANGSDTYINR	bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain-containing protein
	NPSLLWQQPIVGGAIF	alpha kafirin
alcalase 1h	QQQQRLTVNPL	alpha kafirin
	QQPIFGGDIF	alpha kafirin
	LQQPIIRGA	uncharacterized protein
	TYIPQPHCS	19kD-like alpha kafirin B1
	PHHMPA	uncharacterized protein
	KHPPTRQRDPNTQK	uncharacterized protein

size, indicating that the Hofmeister series has an influence in the process of ice recrystallization.[35,36]

In conclusion, the combination of both enzymatic hydrolysis and chemical modification with SA was successful in increasing the IRI activity of sorghum kafirin in PBS. Bromelain hydrolysis for 1 h resulted in the most active unmodified sorghum kafirin hydrolysates when tested in 10 mM NaCl dispersing medium, maintaining activity as low as 0.5%. Our findings show the potential of abundantly available food proteins to be enzymatically derived to produce hydrolysates that can be used as preservation materials that can delay ice growth in food products intended for frozen storage. On the other hand, our current study did not look at the potential ability of sorghum kafirin hydrolysates to bind ice crystals which could potentially show how these molecules can impact ice shaping at conditions of high sucrose concentrations and low temperature storage used in sucrose sandwich assay. In addition, since our samples are mixtures of peptides, it could be difficult to determine which one of the components of the mixture is responsible for such ice shaping activity. Also, we did not look at other properties of the hydrolysates that could impact their use as novel food ingredients, such as nutritional quality and sensory properties. In the future, these aspects should be addressed, not only for sorghum kafirin hydrolysates but also for other food-derived hydrolysates showing antifreeze activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.4c00312.

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Ice crystals from splat assay when treated with different concentrations of sorghum kafirin hydrolysates in two dispersing media of 1× PBS and 10 mM NaCl (PDF)

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Notes

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