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Effect of Additives on Subcritical Water Hydrolysis of Whey Protein Isolate

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ABSTRACT: The objective was to examine the effect of the additives acetic acid, lactic acid, sodium bicarbonate, sodium chloride, and sodium hydroxide on the hydrolysis of whey protein isolate with subcritical water. A screening experimental design was used to study the effect of temperature, time, and additives. The most influential additive, sodium bicarbonate, along with temperature and time was used in a second experimental design to predict the treatment conditions to maximize the degree of hydrolysis and production of free amino acids. The maximum degree of hydrolysis achieved was 50% at a concentration of 1.24 M sodium bicarbonate, 291 °C, and 28 min. The highest concentration of total amino acids was 83.0 mg/g of whey protein isolate with 0.83 M sodium bicarbonate at 264 °C for 29 min. Compared to water alone, sodium bicarbonate increased the degree of hydrolysis 4-fold and the production of amino acids by 44% and decreased peptides' molecular weight.

KEYWORDS: *additives, amino acids, degree of hydrolysis, subcritical water, whey protein*

INTRODUCTION

Subcritical water hydrolysis (SWH) is an alternative to traditional acid/base hydrolysis and to enzymatic methods when the cleavage points are not very important. During SWH, water is maintained in the subcritical state, between its boiling (100 °C and 0.10 MPa) and critical point (374 °C and 22 MPa), where it remains as a liquid due to the high pressure.[1] As the temperature and pressure approach the critical point, the ionic product of water increases and, therefore, its potential to act as an acid- or base-like catalyst increases.

In previous research, the highest degree of hydrolysis (DH) attained when whey protein isolate (WPI) was hydrolyzed with subcritical water was 12% for a temperature of 298 °C and a duration of 17 min; and the highest production of total amino acids (AAs) was 57.4 mg/g of WPI at 300 °C for 40 min.[2] Consequently, if the objective is to obtain a higher DH, or a higher production of AA, the conditions of the reaction need modification, for instance, by using additives. Alternatively, the incorporation of modifiers could facilitate these reactions by decreasing the temperature of the reaction for a fixed DH, increasing the DH for a fixed temperature, enhancing the production of AAs, or altering the molecular weight (MW) distributions of peptides.

Other researchers have used modifiers in SWH. The inclusion of nitrogen and carbon dioxide as modifiers has successfully increased the production of AAs during in the case of fish waste and biomass hydrolysis.[3,4] Carbon dioxide alone has also increased hydrolysis rates by promoting acid-catalyzed hydrolysis.[5−8] Degradation products of AAs, such as ammonia, formic acid, acetic acid, and fatty acids, were studied as potential additives because they have been shown to increase AA yields during SWH.[9] Other additives have been used to accelerate the SWH of silk fibroin including sodium hydroxide, strong acids (sulfuric acid and hydrochloric acid), and sodium chloride.[10]

To the extent of our knowledge, the use of modifiers during the hydrolysis of WPI using subcritical water has not been studied. Whey is an abundant byproduct of cheese manufacturing produced at a rate of approximately 145 million metric tons per year in the world, and half ends up in surface water.[11] Because whey is composed of approximately 13% proteins (dry basis),[12] it has intrinsic nutritional and functional value.[13] Furthermore, hydrolyzed whey protein has numerous potential applications because hydrolysis improves heat stability, reduces allergenicity, enhances digestibility, and liberates bioactive peptides.[14−17]

This research has the scientific objective of studying the effect of several additives (acetic acid, lactic acid, sodium bicarbonate, sodium chloride, and sodium hydroxide) on the hydrolysis of whey protein with subcritical water using WPI as material. The goals are to determine the influence of temperature, time, and additives on the DH, production of AA, and MW of peptides.

MATERIALS AND METHODS

Materials. WPI (with 90% protein content) was obtained from Davisco Foods, Inc. (Eden Prairie, MN, USA). Acetonitrile, hydrochloric acid, and methanol were purchased from VWR (Radnor, PA, USA). AA standard kits, MW standard kits, *o*-phthaldialdehyde (OPA) (97% purity), acetic acid (97.7%), disodium phosphate (99%), lactic acid (90%), sodium bicarbonate (100%), sodium chloride (99.5%), and sodium hydroxide (5.4 N) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared with deionized water, and the mobile phases were prepared with HPLC grade water. The OPA derivatizing solution was prepared fresh daily according to the method of Nielsen et al.[18] and used for spectrophotometric and HPLC analyses.

Methods. *Experimental Designs.* Two experimental designs were used in this research. The first one, a custom 2^3 factorial screening design, was used to determine whether the factors *additive*, *temperature*, and reaction *time* influenced the DH of

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Table 1. Custom Screening Design (2^3) with a Replicate at the Design Center Describing the Factors Additive (x_{1i}), Temperature (x_2), and Time (x_3) and Their Effects on the Degree of Hydrolysis (DH) and pH during Subcritical Water Hydrolysis of Whey Protein Isolate

run	additive[a]	temperature (°C)	time (min)	x_{1i}	x_2	x_3	DH (%)	pH
6	acetic acid	200	0	a	−1	−1	−1.5	3.8
8		200	60	a	−1	+1	5.0	3.4
14		250	30	a	0	0	6.0	4.4
16		250	30	a	0	0	6.6	4.5
17		300	0	a	+1	−1	3.7	4.4
5		300	60	a	+1	+1	4.2	5.1
31	lactic acid	200	0	b	−1	−1	1.3	3.6
21		200	60	b	−1	+1	11.6	4.2
26		250	30	b	0	0	15.1	7.2
35		250	30	b	0	0	13.4	5.9
24		300	0	b	+1	−1	9.7	6.1
23		300	60	b	+1	+1	16.1	9.2
15	sodium bicarbonate	200	0	c	−1	−1	8.8	9.9
3		200	60	c	−1	+1	22.6	9.7
1		250	30	c	0	0	34.4	9.9
11		250	30	c	0	0	39.1	9.8
7		300	0	c	+1	−1	36.6	9.9
13		300	60	c	+1	+1	32.9	9.7
36	sodium chloride	200	0	d	−1	−1	−3.7	6.4
32		200	60	d	−1	+1	7.6	8.7
28		250	30	d	0	0	19.4	9.3
29		250	30	d	0	0	18.0	9.2
25		300	0	d	+1	−1	9.2	9.4
34		300	60	d	+1	+1	14.3	9.6
19	sodium hydroxide	200	0	e	−1	−1	−0.3	7.6
33		200	60	e	−1	+1	4.1	8.9
27		250	30	e	0	0	13.7	9.4
30		250	30	e	0	0	14.1	9.5
22		300	0	e	+1	−1	6.8	9.6
20		300	60	e	+1	+1	12.8	9.6
18	water only (control)	200	0	f	−1	−1	−0.3	7.1
9		200	60	f	−1	+1	6.1	8.9
2		250	30	f	0	0	11.3	9.6
10		250	30	f	0	0	13.2	9.7
4		300	0	f	+1	−1	6.9	9.7
12		300	60	f	+1	+1	12.0	9.3

[a]Final additive concentration = 0.6 M (except sodium hydroxide at 0.13 M).

Table 2. Combination of Factors and Levels in a Central Composite Rotatable Design To Study the Effect of Concentration of Sodium Bicarbonate (x_{1i}), Temperature (x_2), and Time (x_3) on the Degree of Hydrolysis during Subcritical Water Hydrolysis of Whey Protein Isolate

real factor	coded factor	real levels[a]				
concentration (M)	x_{1i}	0.05	0.27	0.61	0.95	1.18
temperature (°C)	x_2	166	200	250	300	334
time (min)	x_3	0	12	30	48	60
	coded levels[a]	−1.682	−1	0	+1	+1.682

[a]Transformations from real to coded levels as follows: x_{1i} = (concentration − 0.61)/0.338; x_2 = (temperature − 250)/50; x_3 = (time − 30)/17.83.

WPI and to identify the approximate range of optimum response (Table 1). The response of the screening design, the DH, was fit to a linear equation (eq 1) as a function of the factors: additives (x_{1i}), temperature (x_2), and time (x_3). i represents the different additives used: acetic acid (x_{1a}), lactic acid (x_{1b}), sodium bicarbonate (x_{1c}), sodium chloride (x_{1d}),

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sodium hydroxide (x_{1e}), and water as the control (x_{1f}). γ_0 is the intercept, and γ_{ij} are the coefficients for the linear, interaction, and quadratic terms.

$$DH = \gamma_0 + \gamma_1 x_{1i} + \gamma_2 x_2 + \gamma_3 x_3 + \gamma_{12} x_{1i} x_2 + \gamma_{13} x_{1i} x_3 + \gamma_{23} x_2 x_3 \quad (1)$$

The most influential factors were used as the parameters in a second experimental design, a central composite rotatable design (CCRD), to determine the conditions that created the highest predicted DH of WPI. The CCRD and its coded levels are shown in Table 2. Experimental runs were randomly conducted.

The DH response obtained with the CCRD was fit to a quadratic equation (eq 2) as a function of the codified factors: additive concentration (x_{1i}), temperature (x_2), and time (x_3).

$$DH = \beta_0 + \beta_1 x_{1i} + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_{1i} x_2 + \beta_{13} x_{1i} x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_{1i}^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (2)$$

DH represents the response, β_0 represents the intercept, and β_{ij} represents the coefficients for the linear, interaction, and quadratic terms.

Statistical Analysis. The screening design and the CCRD were analyzed using the JMP program (SAS Institute Inc., Cary, NC, USA) and evaluated using analysis of variance (ANOVA) to determine the fit of the model and significance of factors and interactions. For the screening design and for the CCRD, a first-order polynomial and a second-order polynomial model, respectively, were applied, and the accuracy was determined by the significance of the F test ($p < 0.05$), lack-of-fit test ($p > 0.05$), and the coefficient of determination (R^2). In addition, for the CCRD, a response surface prediction equation was used to determine the best conditions for obtaining the highest DH.

Subcritical Water Hydrolysis. Reactor. Hydrolysis reactions were conducted in a 100 mL, high-pressure reactor made of T316 stainless steel (model 4793, Parr Instrument Co., Moline, IL, USA), outfitted with a 0−20.7 MPa pressure gauge, a 20.7 MPa rupture disk, an inlet valve, a dip tube, a gas release valve, and a fixed thermocouple. The reactor was heated with a 1700 W ceramic-band heater constructed to fit the reactor, and the temperature was controlled with a proportional−integral−derivative temperature controller (CAL Controls, Libertyville, IL, USA).

Additives. Solutions (of acetic acid, lactic acid, sodium bicarbonate, and sodium chloride) were prepared daily at a concentration of 0.6 M, and WPI was added at a ratio of 100 g of WPI/L of water. The concentration of sodium hydroxide was only 0.014 M to avoid hydrolysis before SWH. A preliminary evaluation of WPI with sodium hydroxide is explained under Results, Degree of Hydrolysis. The control consisted of 100 g of WPI/L of water. Solutions were individually stirred for 30 min before use.

Reaction. The reactor was filled with 50 mL of the WPI solutions (100 g/L) with and without additives. The reactor was purged with helium gas that was bubbled via the dip tube for 2 min, and the valves were closed afterward. The reactor, placed in the electrical heater, was heated to the specified temperature previously determined by the screening design or CCRD. When the operational temperature was reached, the reaction time started to count (transient heating was not taken into consideration because each run followed a similar preheating curve); and once the specified time was reached, the reaction was rapidly quenched by submerging the reactor in an ice bath for 5 min. The hydrolysates were removed, centrifuged at 3400g and 4 °C for 30 min, filtered through Whatman no. 4 filter paper, and stored at −20 °C until further analysis.

Degree of Hydrolysis. The DH was determined according to a method described by Nielsen et al.[18] and calculated using eqs 3 and 4.[19] AAs contained in the hydrolysates were derivatized with an OPA solution and measured with a UV−vis spectrophotometer (model UV 1700, Shimadzu Corp., Kyoto, Japan) at 340 nm. The standard, serine (100 mg/L), was used to calculate the DH (eq 4). The blank for SWH samples consisted of water and OPA solution. The blank for samples with additives was an aqueous mixture of water with the respective amount of additive and OPA solution.

$$DH = h/h_t \times 100 \quad (3)$$

$$h = \{[(OD_{sample} - OD_{blank})/(OD_{standard} - OD_{blank}) \times S \times V \times 100/(X \times P)] - \beta\}/\{\alpha\} \quad (4)$$

where DH = degree of hydrolysis (%), h = amount of hydrolyzed bonds determined by absorbance at 340 nm, h_t = total number of peptide bonds per protein equivalent for whey (8.8 mequiv/g),[20] OD = optical density (absorbance) at 340 nm, S = serine concentration (0.9516 mequiv/L) at 100 mg/L, α and β = constants specific for whey protein (1.00 and 0.40, respectively),[20] X = mass (g) of sample, P = protein content (%) of the sample, and V = volume (L) of the sample.

Peptide Molecular Weight. The MWs of the hydrolysates larger than 2 kDa were determined by high-performance size exclusion chromatography (HP-SEC). After filtration through 0.45 μm syringe filters (Sigma-Aldrich, St. Louis, MO, USA), 200 μL of each hydrolyzed sample was mixed with 600 μL of 0.2 M disodium phosphate buffer solution at pH 7, and a 5 μL aliquot was injected into the liquid chromatograph. The separation was performed with a 9.4 mm i.d. × 25 cm Zorbax GF-250 column (Agilent, Santa Clara, CA, USA) at a flow rate of 1.0 mL/min with detection at 214 nm.[21] The MW standards used to generate a calibration curve were albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa).

MWs below 2 kDa were measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) at the Statewide Mass Spectrometry Facility (University of Arkansas, USA). Hydrolysates were desalted by insertion into a Micro Dispodialyzer (Harvard Apparatus, Holliston, MA, USA) and then conditioned overnight as specified by the manufacturer's instructions. After desalted samples were retrieved, they were mixed with 1 M solution of dihydroxybenzoic acid (DHB) in 90% methanol at a 1:1 ratio. A 1 μL sample of the mixture was spotted onto a ground, stainless steel MALDI target for analysis using a Bruker Reflex III MALDI-TOF-MS (Billerica, MA, USA) with a N_2 laser set at 337 nm.

Amino Acid Content. AAs were analyzed by reversed-phase HPLC using a Shimadzu HPLC system (Shimadzu Corp.) similar to the method described by Henderson et al.[22] Hydrolyzed WPI samples were filtered through 0.25 μm syringe filters and derivatized with OPA. The hydrolysates, OPA solution, and borate buffer solution were mixed (for 2 min) and then injected (10 μL) onto a Zorbax Eclipse AAA column (4.6 × 150 mm) equipped with a 4.6 × 12.5 mm guard column (Agilent). Analytes were separated with a binary gradient consisting of sodium phosphate buffer at pH 7.8 (eluent A) and acetonitrile/methanol/water (45:45:10 v/v/v) (eluent B). The gradient program was the following: 0−1.9 min at 0% B; 1.9−20 min with a linear gradient to 76% B; 20−21 min with a linear gradient to 100% B; 20−24 min at 100% B; 24−28 min with a linear gradient to 0% B; 28−30 min at 0% B. AAs were identified and quantified using the AA standard kit described under Materials and Methods.

RESULTS AND DISCUSSION

Degree of Hydrolysis. The custom 2^3 screening design was conducted to find the most influential factors and region of highest response for the DH. For practical applications, a lower DH provides greater functionality; however for a scientific approach and ease of discussion, obtaining the highest DH was our objective. According to the ANOVA, the F ratio (3.03) was significant for a $p < 0.05$, indicating that the first-order model fit the responses, and 85% of the variance could be explained by the model. The lack-of-fit test was significant ($p = 0.0002$), which indicated either an omission of higher order effects (like a second-order model) or the need for exact replication of factor combinations in the model. Of all the additives, only

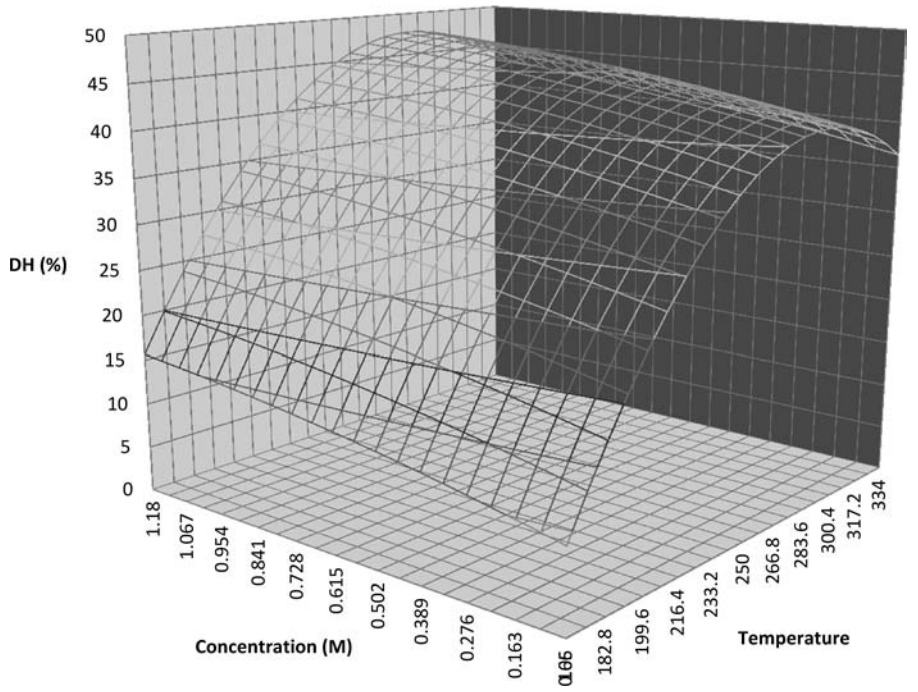

Figure 1. Predicted surface response of the degree of hydrolysis following subcritical water hydrolysis of whey protein isolate as affected by sodium bicarbonate concentration and temperature with a reaction time of 28 min.

sodium bicarbonate had a significant effect on the DH. There was a positive interaction between sodium bicarbonate (x_{1c}) and temperature (x_2), and both temperature and time (x_3) had a positive effect on the DH (eq 5). The linear prediction equation from the screening design data is as follows:

$$\mathrm{DH} = 11.9 + 17.1x_{1c} + 4.3x_2 + 3.0x_3 + 5.2x_{1c}x_2 \qquad (5)$$

A preliminary evaluation of WPI with sodium hydroxide (x_e) at 0.014, 0.034, and 0.25 M generated pH values of 8.3, 12.1, and 13.4, respectively. Although treatment with 0.25 M generated the highest DH, the high pH could potentially hydrolyze proteins prior to treatment;[23] therefore, the lowest concentration (0.014 M) was chosen for the hydrolysis reactions, which resulted in a nonsignificant increase in the DH. Kang and Chun[10] observed a 4-fold increase in hydrolysis when adding sodium hydroxide to SWH using 250 °C for approximately 30 min; however, they did not discuss accounting for prereaction hydrolysis. The addition of lactic acid (x_b) and sodium chloride (x_d) also increased the DH, but they did not significantly affect the DH over all treatment conditions. Kang and Chun[10] improved the AA production during SWH of silk fibrin using sodium chloride, but it was not significant when compared to water. In this study, the addition of acetic acid reduced the DH compared to water alone, which is most likely due to the acidic pH at the end of the reaction (Table 1) or due to recombination of peptides after the reaction had been quenched, which was noted before by Kang et al.[10] The negative DH values observed in a few experimental conditions (Table 1) are possibly the result of the unavailability of lysine's ε-amine group to interact with OPA. Heat treatment of the standards may reduce the number of available ε-amine groups, and the negative values would occur when the DH of standards is subtracted from the DH of samples, according to Nielsen et al.[18] In addition, Maillard reaction products (background color) may interfere with the measurement.

Unlike the other additives, sodium bicarbonate (x_3) had a positive effect on the DH at all the conditions evaluated, with the highest DH or region of highest response at 250 °C for 30 min (Table 1); thus, sodium bicarbonate was the additive selected for the CCRD experiment.

The CCRD response was fit to a quadratic equation (eq 6) with a significant ($p < 0.05$) F test (27.3), and 93% of the variation could be explained by the model. The lack-of-fit ($p = 0.0001$) was significant, indicating that higher order effects may be missing. The quadratic prediction equation from the CCRD with sodium bicarbonate concentration (x_{1c}), temperature (x_2), and time (x_3) as the independent variables is shown in eq 6:

$$\mathrm{DH} = 37.7 + 9.2x_{1c} + 8.2x_2 + 2.1x_3 - 2.6x_{1c}^2 - 5.4x_2^2 - 3.0x_3^2 \qquad (6)$$

The concentration of sodium bicarbonate, reaction temperature, and reaction time exhibited significant first- and second-order effects on the DH. With the addition of 1.18 M sodium bicarbonate and treatment at 250 °C for 30 min, the DH was 49%, which was significantly greater than the DH (12.3%) observed without the additive under the same conditions. The predictive model (Figure.1) indicated a 50% DH could be attained with the addition of 1.24 M sodium bicarbonate and treatment at 291 °C for 28 min. Interestingly, the predictive model and second-order effects suggest that conditions beyond 1.24 M, 291 °C, and 28 min may promote degradation of peptides and AA.

Peptide Analysis. *High-Performance Size Exclusion Chromatography.* As treatment temperature and time increased, there was an increase in the area of late-eluting peaks (smaller MW peptides) at the expense of the reduction in area of the earlier eluting (larger MW peptides) shown by the size exclusion chromatography analysis. However, there was a loss of all peptides when treatment temperature and time were greater than 300 °C and 48 min, respectively. Figure 2

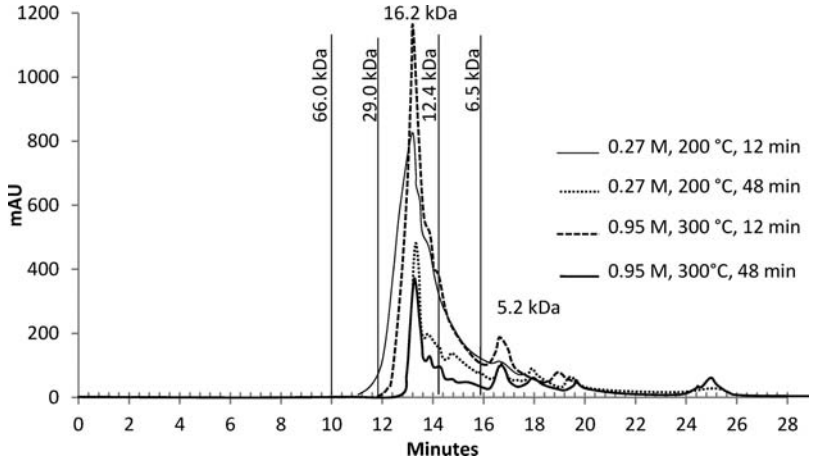

Figure 2. High-performance size exclusion chromatogram of whey protein isolate hydrolyzed by subcritical water with 0.27 or 0.95 M sodium bicarbonate at different temperatures and for different durations. Vertical lines indicate molecular weight markers.

demonstrates the effect of temperature, reaction time, and sodium bicarbonate concentration, respectively, on peptide MWs. For discussion purposes, only the peaks eluting at approximately 13.1 min (MW ~ 16.2 kDa) and 16.4 min (MW ~ 5.2 kDa) are presented. As treatment temperature increased from 166 to 250 °C, the peptides with MW around 16.2 and 5.2 kDa increased; however, when treatment temperature increased from 250 to 334 °C, the peptides of both MWs decreased. The production and subsequent decomposition of proteins with increasing treatment temperature and time during SWH were also observed with hydrolysis of yeast cells.[24]

Reaction time patterns also show a second-order effect on hydrolysis at 300 °C, but not at 200 °C. As sodium bicarbonate concentration increased (Figure 2), the 16.2 and 5.2 kDa peptides increased as well, which would be indicative of a positive effect of the additive on hydrolysis. Also, when SWH without additives was compared to SWH with additives (chromatograms not shown), a sodium bicarbonate concentration as low as 0.05 M increased the 16.2 kDa peptide by approximately 58%. The increase of peptides at 16.2 kDa was most evident at low to medium temperatures and times, but at high temperatures and long reaction times, the effect of the additive diminished (Figure 2). Roglinski et al.[25] also observed that additive influence decreases with increasing temperature when monitoring AA yield from SWH of bovine serum albumin using carbon dioxide.

Mass Spectrometry. WPI peptides (<3.0 kDa) hydrolyzed by SWH with sodium bicarbonate were also analyzed by MALDI-TOF; however, the discussion is limited to the presence of peaks in certain MW ranges due to the lack of a standard. Generally, as sodium bicarbonate concentration, temperature, and time of SWH increased, the amount of peaks decreased in the higher MW ranges (≥1.0 kDa) and increased in the lower MW ranges (300−900 Da). Changes in treatment temperature appeared to have the greatest effect on MW. With a constant concentration (0.6 M) and time (30 min), the majority of the peaks were less than 2.5, 0.9, and 0.4 kDa at treatment temperatures of 166, 250, and 334 °C, respectively. With constant concentration (0.6 M) and temperature (250 °C), the majority of the peaks were less than 1.8, 0.9, and 0.5 kDa at reaction times of 0, 30, and 60 min, respectively. In contrast, there was no observable pattern with changes in additive concentration when temperature and time were held constant.

The importance of peptide MW lies in the specific application for which it is produced. For incorporation into food, there are many factors to consider, such as allergenicity, bitterness, digestive ease, and functionality. Using hydrolysis conditions of 250 °C for at least 30 min provides the greatest peptide MW distribution near 0.9 kDa and, according to Gonzalez-Tello et al.,[26] hydrolysates with peptide MWs from 0.5 to 1.0 kDa possessed fewer bitter peptides, prevented allergies, and improved digestibility. Additionally, Severin and Xia[27] demonstrated that the solubility of protein samples increased with decreasing MW; however, foaming and emulsifying properties decreased at lower MWs. On the basis of the MALDI-TOF data, there is potential to generate lower MW peptides with relative specificity by SWH with sodium bicarbonate, which may prove valuable for hydrolyzing whey proteins for specific applications after further analysis of safety and functionality.

Amino Acid Content. The total AA content of the CCRD data fit a second-order polynomial model (eq 7) with a significant ($p < 0.05$) F test (4.34), and 65% of the variance could be explained by the model. The lack-of-fit test was significant ($p = 0.0001$), indicating that some higher order effects may be left out. There was a significant interaction between temperature and time and second-order effects for sodium bicarbonate concentration (x_{1c}), temperature (x_2), and time (x_3).

$$\mathrm{DH} = 34.1 + 35.6x_{1c} + 0.08x_2 + 0.09x_3 - 87.3x_3^2 - 0.005x_2^2 - 0.03x_3^2 - 0.013x_2x_3 \quad (7)$$

The treatment condition that generated the greatest concentration of total AAs (70.9 mg/g WPI) was 250 °C for 30 min with 1.81 M sodium bicarbonate. At 250 °C for 30 min with water alone, the highest production of total AAs was 54.3 mg/g WPI.[24] The predicted maximum AA content for this design was 83.0 mg/g WPI using 0.83 M sodium bicarbonate at 264 °C for 29 min. Yoshida et al.[28] also found the greatest AA yields at ~265 °C during SWH of fish trimmings.

Because the AAs respond differently to each reaction temperature and reaction time[29] and possibly to the different additive concentrations, the low R^2 value (0.65) may be due to the inability to incorporate the effect of different conditions on individual AA into the predictive equation.[30] By analyzing the effects of sodium bicarbonate concentration, temperature, and

Table 3. Predicted Conditions (Sodium Bicarbonate Concentration, Temperature, and Time) of Subcritical Water Hydrolysis of Whey Protein Isolate for Obtaining the Maximum Concentration of Amino Acids

		conditions		
amino acid	total (mg/g WPI)	concentration (M)	temperature (°C)	time (min)
alanine	14.9 ± 2.2	0.79	244	36
arginine	0.11 ± 0.07	0.05	170	1
aspartic acid	7.83 ± 4.3	1.18	166	0
glutamic acid	2.54 ± 1.1	1.18	160	0
glycine	7.52 ± 0.87	0.88	254	31
histidine	0.33 ± 0.10	0.25	175	0
isoleucine	5.29 ± 1.81	1.18	202	60
leucine	17.20 ± 2.55	1.86	242	34
lysine	21.3 ± 3.29	0.05	334	60
methionine/valine	5.68 ± 1.31	1.18	238	51
phenylalanine	11.39 ± 2.75	1.04	223	54
serine	1.21 ± 0.19	0.78	225	35
threonine	1.16 ± 0.18	0.71	231	32
tryptophan	3.11 ± 0.44	1.00	242	36
tyrosine	6.23 ± 1.15	1.30	237	42

time on the concentration of each AA, the R^2 value increased (>0.8) and the F tests were significant for alanine, glycine, histidine, isoleucine, leucine, lysine, methionine/valine, phenylalanine, serine, threonine, tryptophan, and tyrosine. However, only alanine, glycine, and serine had a nonsignificant lack-of-fit test. Table 3 displays the conditions that would produce the maximum predicted concentration of each of the AAs.

HPLC analysis following OPA derivatization resulted in a baseline separation of all AAs except methionine and valine, which coeluted at 14.6 min. Proline and cystine/cysteine peaks were not analyzed because, according to Spellman et al.,[31] unstable reactions may occur between these AAs and OPA that would generate large variability within sample replications.

Each AA was affected differently by the factors or factor interactions. For example, the interaction of sodium bicarbonate, temperature, and time negatively affected serine.

The interaction of sodium bicarbonate concentration and temperature positively affected arginine and histidine, whereas the interaction of temperature and time negatively affected alanine, glycine, isoleucine, leucine, methionine/valine, phenylalanine, tryptophan, and tyrosine. Sodium bicarbonate alone positively affected glutamic acid, glycine, isoleucine, methionine/valine, phenylalanine, serine, tryptophan, and tyrosine. When compared with water alone,[24] alanine, aspartic acid, glutamic acid, histidine, leucine, phenylalanine, serine, threonine, and tyrosine concentrations also increased in the presence of sodium bicarbonate. Because of the positive effect sodium bicarbonate has on certain AA, it may be advantageous to explore SWH with this additive for feed supplementation. For example, chickens require several of the AAs that sodium bicarbonate had a positive hydrolysis effect on, especially methionine.[32] Also, branched-chain AAs (isoleucine, leucine, and valine) are seen as popular components of supplements.[33] An increase in AA production by SWH with additives was also observed by Kang and Chun.[10] The authors postulated that extreme acidity and alkalinity during SWH of proteins may increase AA yields. Further research may be of interest to find other additives that affect specific AA for potential application.

Temperature had a second-order effect on all AAs, except lysine. The greatest concentrations of arginine, aspartic acid, glutamic acid, histidine, isoleucine, and phenylalanine were produced when the temperature was held below 200 °C. In contrast, the greatest concentrations of alanine, glycine, leucine, methionine/valine, serine, threonine, tryptophan, and tyrosine were produced when the treatment temperatures were 200–250 °C. Abdelmoez et al.[30] also found that the arginine, aspartic acid, glutamic acid, and threonine were sensitive to temperatures around 230 °C during SWH.

Lysine was an exception to the other AAs because the concentration of lysine increased with higher temperatures and decreased with higher concentrations of sodium bicarbonate. In fact, the greatest amount of lysine was produced at a treatment temperature around 334 °C. Sato et al.[29] also found lysine to be the least liable AA at higher temperatures. The negative effect of additive on the concentration of lysine can be seen when samples hydrolyzed at the same conditions with and without sodium bicarbonate are compared. Hydrolyzed WPI samples had a lower concentration of lysine in the presence of sodium bicarbonate. This may be the result of an increase in pH when sodium bicarbonate is present in the hydrolysate, which may increase Maillard reactions. Nielsen et al.[18] explained that Maillard reactions may occur at the ε-position of lysine, which interferes with the reaction of OPA, thereby decreasing the reported lysine content.[18]

The interaction of sodium bicarbonate, temperature, and time significantly affected the DH with a 4-fold increase when compared with water alone. In addition, an increase in additive concentration decreased the MWs of peptides, especially at low temperatures over time. Also, the interactions of additive concentration, temperature, and time affected each AA differently, which could lead to further study on individual AAs for specific applications. Overall, the employment of sodium bicarbonate proved to lower the temperature and time needed to achieve the same yields as SWH alone. It is not known, however, if sodium bicarbonate would be a suitable additive for industrial hydrolysis of whey products due to potential corrosion of the reactor and high levels of sodium in the final hydrolysate. Further research would help to elucidate some of these issues.

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Notes

The authors declare no competing financial interest.

■ ABBRIEVIATIONS USED

AA, amino acid; CCRD, central composite rotatable design; DH, degree of hydrolysis; MW, molecular weight; HP SEC, high-performance size exclusion chromatography; HPLC, high-performance liquid chromatography; MALD-TOF-MSI, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; OPA, *o*-phthaldialdehyde; SWH, subcritical water hydrolysis; WPI, whey protein isolate.

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