Original paper

The Effect of High Hydrostatic Pressure on Acrylamide Generation in Aqueous Reaction Systems Using Asparagine and Glucose

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Received October 17, 2018 ; Accepted April 24, 2019

The effect of high hydrostatic pressure (HHP) on acrylamide generation was studied using an equimolar asparagine-glucose model system that was dissolved in ultrapure water or buffer at initial pH values of 5.0 and 9.0. The concentrations of acrylamide and melanoidins were determined after treatment at 120 °C for 60 min within a range of 100 to 300 MPa. The pH prior to and after treatment was also measured. The generation of acrylamide in all model systems was significantly suppressed under elevated pressure conditions. The strength of correlations among pressure value, acrylamide and melanoidins concentration differed depending on the initial pH condition of the model system. It was suggested that the suppression of acrylamide generation occurred due to the different effects of pressure, which depends on the solution pH, on the Maillard reaction involving acrylamide formation. Based on these findings, we propose that application of HHP to heat processing of foods could be effective to reduce acrylamide content.

Keywords: high hydrostatic pressure, Maillard reaction, acrylamide, melanoidins

Introduction

Acrylamide, a potential carcinogen to humans, is unintentionally produced when carbohydrate-rich food materials, like potatoes and cereals, are processed at temperatures of 120 °C or more (Tareke *et al.*, 2002). It has since been reported that some heat-processed foods are contaminated with acrylamide, especially those from Europe and the United States, where potato products such as French fries and potato chips are widely consumed (JECFA, 2011). Consequently, many researchers and food manufacturers have studied methods for reducing acrylamide in processed foods (Becalski *et al.*, 2004; Pedreschi *et al.*, 2004; Palazoglu *et al.*, 2010). In 2003, the Ministry of Agriculture, Forestry and Fisheries of Japan conducted a large-scale survey on the acrylamide content of food products consumed in that country, and reported that acrylamide was present in common processed

foods (i).

The formation of acrylamide occurs during the initial stage of the Maillard reaction when asparagine is heated in the presence of a reducing sugar at high temperatures (Fig. 1, Yaylayan *et al.*, 2003; Zyzak *et al.*, 2003; Stadler *et al.*, 2004; Stadler and Studer, 2015). One of the strategies that have been proposed to control acrylamide levels in food products is to process food materials at low temperatures at which the formation of acrylamide is suppressed (Granda *et al.*, 2004). However, the formation of acrylamide has been confirmed even at temperatures lower than 100 °C, depending on the food materials and heating conditions (Becalski *et al.*, 2011). On the other hand, the Maillard reaction plays an important role in food processing by adding benefits such as flavor improvement and coloring to foods (Ames, 1998). In addition, melanoidins, which are brown-colored polymers and final products of the

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Fig. 1. Formation of acrylamide from asparagine through the Maillard reaction (based on Yaylayan *et al.*, 2003; Zyzak *et al.*, 2003; Stadler *et al.*, 2004; Stadler and Studer, 2015).

Maillard reaction, have beneficial functions for humans, such as antioxidant, antimicrobial and antihypertensive activities (Gomyo and Miura, 1983; Rufián-Henares and Morales, 2007). Therefore, a technology for controlling the Maillard reaction has been needed in the field of food processing in order to suppress the formation of acrylamide in processed foods, while retaining their high quality.

High hydrostatic pressure (HHP) treatment is a food-processing technique employing hydrostatic pressure with a range of tens to hundreds of megapascals (MPa). This technology causes physical changes, not chemical denaturation, by the compressed water in foods because it supplies less energy than heat treatment. Thus, HHP has generally been used as an unheated food processing technique for the purposes of pasteurization (Sonoike, 1997), physical denaturation (Yamazaki *et al.*, 1996; Murokoshi, 2004) and fortification of functional compounds (Sasagawa *et al.*, 2006). In addition, recent studies have focused on the safety and quality of foods processed at high pressure combined with high temperature (Van der Plancken *et al.*, 2012). Previous studies have reported the effects of pressure on the Maillard reaction (Tamaoka *et al.*, 1991a; Hill *et al.*, 1996), including the formation of melanoidins, their intermediates and volatile compounds (Ames, 1998). The effect of pressure on the formation of acrylamide was reported by De Vleeschouwer and coworkers

in 2010. They reported the effects of high pressure-high temperature (100–115 °C, 400–700 MPa, 0–60 min) on the formation of acrylamide and other Maillard-type reaction compounds in equimolar asparagine-glucose model systems, whereas studies on the effect of pressure less than 400 MPa have not yet been reported, which is more practical in food manufacturing. Although HHP processing at above 400 MPa has been industrially used in Europe and North America, the use of this processing at less than 400 MPa has become common in Japan. One reason for this is that the HHP equipment capable of generating higher pressure is more expensive; therefore, some food manufacturers employ HHP processing at lower pressures to minimize the initial investments. In addition, many heat-processed foods such as canned and retort pouch foods are sterilized by heating under conditions of 120 °C for up to 60 min. Some of these foods generate significant amounts of acrylamide during heat processing (JECFA 2011; i). With regard to heat-sterilized food, the approaches for the reduction of acrylamide generation, i.e., heat treatment at lower temperatures and the addition of asparaginase, might fail to retain the safety and quality of the products. If HHP processing combined with heat sterilization can be applied to food processing, due to suppressed chemical reactions, the generation of acrylamide in heat-sterilized food may be reduced.

Fig. 2. Schematic diagram of the high-pressure/high-temperature reaction system.

The objective of this study is to reduce the generation of acrylamide while retaining food quality by HHP treatment. Here, we measured the formation of acrylamide during heat treatment under HHP (120 °C, 100–300 MPa) in an equimolar asparagine-glucose model system. Additionally, the concentration of melanoidins and the pH value after treatment in model systems were measured as indicators of the degree to which the Maillard reaction occurred. These measurements were used to calculate the correlation coefficient with the concentration of acrylamide generated during HHP treatment.

Materials and Methods

Materials Acrylamide (ultra-pure, >99.9 %) was purchased from Kanto Chemical (Tokyo, Japan) and $^{13}\text{C}_1$ -labeled acrylamide (>98 %) as an internal standard (IS) was obtained from CDN Isotopes (Montreal, Quebec, Canada). JIS special grade L-asparagine monohydrate (99.0 %), D-(+)-glucose (98.0 %), 2-morpholinoethanesulfonic acid monohydrate (MES buffer, >99.5 %), *N*-tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS buffer, 99.0 %), potassium hexacyanoferrate (II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$, Carrez I), zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, Carrez II), sodium chloride, and ethyl acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan), in addition to liquid chromatography-mass spectrometry (LC-MS)-grade ultra-pure water, acetic acid and methanol. Water used for all the reactions was obtained by Auto Pure WT101 UV from Yamato Scientific (Tokyo, Japan).

The heating experiment under HHP was performed with aqueous model systems prepared by mixing equimolar concentrations (0.125 M) of asparagine and glucose in three solvents. To avoid the influence of buffer components on acrylamide formation (Bell, 1997), ultra-pure water was selected as the solvent for an unbuffered model system (pH-

unbuffered sample). The initial pH of this model system was 5.00 ± 0.01 . To minimize the change of pH during HHP treatment, MES and TAPS buffers (0.1 M) in which the pH was stable under pressure (Kunugi, 1991; Taniguchi, 1992) were used in the model systems. The initial pHs of MES and TAPS buffers were adjusted to 5.00 ± 0.01 and 9.00 ± 0.01 , respectively.

HHP equipment and processing Figure 2 shows a schematic of the HHP equipment, which consists of a custom-made pressure vessel (148 mm outer diameter, 49 mm internal diameter, 156 mm height, Echigoseika, Niigata, Japan) equipped with temperature sensors (SCHS1-0 KT128G637; Chino Corporation, Tokyo, Japan) on both the inside and outside, binary hand pumps, i.e., a pressure control pump (high pressure generator 37-5, 76-60-Teflon; High Pressure Equipment Company, USA), a manual pressurization pump (water manual pump WUP-21M; Riken Seiki, Niigata, Japan), and a pressure sensor (Pressure Transmitter P2VA2/5000 bar; Hottinger Baldwin Messtechnik GmbH, Darmstadt, Germany). This equipment allows a computer connected to a data logger (NR-500; Keyence Corporation, Tokyo, Japan) to record pressure and temperature data detected by the sensors. The maximum pressure of the equipment is 400 MPa; this study applied pressures at 100, 200 and 300 MPa.

A custom-made sample holder (Echigoseika) was filled with sample solution and closed with a cap while removing as much air as possible. The sample holder consisted of a 10-mL stainless steel vessel, a stainless steel screw cap with a hole in its center and a silicon seat (1 mm thickness) to mediate pressure. To heat the pressure vessel, it was placed in a preheated oil bath consisting of a custom-made 6.8-L stainless steel chamber with a drain cock (Echigoseika) and an induction heater (KH-PH32; Yamazen Corporation, Osaka, Japan). The pressure vessel and the oil bath were filled with edible

Fig. 3. Illustration of temperature-pressure profiles: temperature (solid line), pressure (dashed lines). The pressure was maintained throughout preheating and for a 60-min time period.

vegetable oil used as the pressure and heating medium. The sample holder was placed in the pressure vessel preheated at approximately 90 °C. The pressure vessel was immediately sealed and pressurized with the manual pressurization pump. After reaching the desired pressure level, the pressure vessel was immediately heated in the oil bath. When the temperature in the pressure vessel was elevated to 115 °C, a reaction period of 60 min was started. After the temperature in the pressure vessel was elevated to 120 °C, the vessel was regulated to retain a temperature of 120 ± 5 °C. The pressure in the pressure vessel was retained at the desired level by controlling the pressure control pump during the period from the start of the heating to the end of the reaction. After the reaction period of 60 min, the hot oil in the oil bath was immediately drained via the drain cock and cold oil at -40 °C was poured into the bath to cool the pressure vessel. After the inside temperature of the pressure vessel cooled to 95 °C, the drain valve attached to this vessel was gradually opened to release the pressure until ambient pressure was achieved. The sample holder was removed from the pressure vessel and was immediately cooled in ice water to stop any further reaction. The time periods in the experiment were as follows: the preheating period from the start of pressurization to the rise in temperature to 115 °C was about 30 min; the reaction period was 60 min; the cooling period from the start of cooling to the completion of pressure release was about 20 min (Fig. 3). Experiments were performed in triplicate. Control experiments were performed by nearly the same method as above, but slightly pressurized (< 5 MPa) to heat the vessel up to 120 °C. Browning and the pH of the

samples were measured after cooling to room temperature and the samples were frozen and stored at -18 °C prior to acrylamide analysis.

Acrylamide extraction Extraction and purification of acrylamide were performed by modifying the method described by Delatour *et al.* (2004). A 1-mL portion of pH-unbuffered sample, MES-buffered sample or TAPS-buffered sample, diluted to the proper concentration, was pipetted into a 30-mL polypropylene centrifuge tube. $^{13}\text{C}_1$ -acrylamide (10 µL of 100 µg/mL) and water (9 mL) were added to the sample and then it was shaken vigorously. One milliliter of 15% (wt/vol) potassium hexacyanoferrate (II) trihydrate solution (Carrez I) and 1 mL of 30% (wt/vol) zinc sulfate heptahydrate solution (Carrez II) were added to the mixture with swirling. The resulting mixture was centrifuged at 15 000 × *g* at 10 °C for 20 min. A 5-mL portion of the supernatant was transferred into a 30-mL glass centrifuge tube containing 1.5 g of sodium chloride and swirled until it dissolved. Ethyl acetate (10 mL) was added to the solution, and then the resulting suspension was stirred vigorously for 1 min. Phase separation was achieved by centrifugation at 1 100 × *g* at room temperature for 10 min (himac CT-15D; Hitachi Koki, Tokyo, Japan). The organic phase was transferred to a 100-mL evaporating flask. Two further extractions of the 5-mL aqueous solution with ethyl acetate (2 × 10 mL) were conducted, and the extracts were collected and mixed with the previous organic phase. The collected organic phase (approx. 30 mL) was evaporated with a rotary evaporator (Tokyo Rikakikai, Tokyo, Japan) at 40 °C, and the residue was dissolved in 2 mL of water. The aqueous

solution was loaded onto a 500 mg-Isolute Multimode cartridge (International Sorbet Technology, Glamorgan, United Kingdom), which was preconditioned with methanol first (3 mL) and then water (6 mL). The eluent was collected in a 10-mL glass vial. A 1-mL aliquot of water was loaded onto the cartridge, eluted, collected and mixed with the previous fraction. The extract was concentrated to 500 μ L under a gentle stream of nitrogen in a thermally controlled water bath at 40 °C. The concentrate was filtered through a 0.20- μ m pore sized filter unit. The purified sample was frozen and stored at -18 °C until analysis.

Acrylamide quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) The reactants were analyzed on a LCMS-8030A (Shimadzu, Kyoto, Japan). The samples (10 μ L injection volume) were separated on a reverse-phase C18 column (Synergi Hydro-RP, 250 mm $\times$ 2 mm i.d., 4 μ m; Phenomenex, USA) at an oven temperature of 40 °C. The mobile phase consisted of methanol and 0.1 vol % acetic acid (2:98, v/v) and the column was eluted at a flow rate of 0.2 mL/min. The eluted acrylamide was ionized using positive electrospray ionization mode (ESI+). The ESI source was operated as follows: nebulizer gas (nitrogen) flow rate, 3.0 L/min; interface voltage, 4.5 kV; desolvation line (DL) temperature, 250 °C; block heater temperature, 400 °C; drying gas flow, 15 L/min; collision-induced dissociation (CID) gas, 230 kPa; detector voltage, 1.74 kV. Acrylamide and IS were detected and identified in multiple reaction monitoring (MRM) mode at m/z 72.10 > 54.95 and m/z 73.10 > 55.95, respectively. The acrylamide concentration was quantified using a standard curve prepared from the peak area ratio of acrylamide (0–1 000 ppb) to IS (1 000 ppb). The limit of detection and the limit of quantification of acrylamide, depending on contamination of the ion source, were 13 ppb and 40 ppb, respectively.

Determination of browning Browning compounds were quantified with an ultraviolet-visible spectrophotometer (UV mini-1240; Shimadzu) by measuring absorbance at 470 nm. The sample was analyzed following dilution to the proper concentration as needed. The corresponding melanoidins concentration, formed from asparagine and glucose, could be calculated from the Lambert-Beer equation using the measured absorbance and an extinction coefficient of 282 L/mol·cm, as described by Knol *et al.* (2005). The concentration of melanoidins can be expressed in terms of the number of glucose molecules incorporated by using the molar extinction coefficient (Knol *et al.*, 2005; Martins *et al.*, 2001). Though melanoidins are complex mixtures of various molecules, a number of studies have reported that melanoidins are quantifiable in this manner (Brands *et al.*, 2002; Martins and Van Boekel, 2003; De Vleeschouwer *et al.*, 2008; De Vleeschouwer *et al.*, 2010).

pH measurement Sample pH was measured with a pH meter (Horiba, Kyoto, Japan) equipped with a glass electrode.

Cooled samples after treatment were measured at room temperature.

Statistical analysis All experiments were performed in triplicate and the results are expressed as means $\pm$ standard deviation. Statistical analysis was performed using a Student's t-test, and linear relationships among all the factors were assessed with Pearson's correlation coefficient using Microsoft Excel 2013. Statistically significant differences were defined at $p < 0.05$ or 0.01.

Results

Acrylamide is formed during the Maillard reaction, and we attempted to inhibit its generation by applying HHP.

Figure 4A, B and C show the concentrations of acrylamide in samples of the pH-unbuffered (initial pH 5.0), MES-buffered (initial pH 5.0) and TAPS-buffered systems (initial pH 9.0), which were heated at a temperature of 120 °C for 60 min under HHP up to 300 MPa. The generation of acrylamide in the pH-unbuffered system (A) was suppressed by higher pressure. In the MES-buffered system (B), while a small amount of acrylamide was generated in the control treated at less than 5.0 MPa, lower levels of acrylamide were generated in samples pressurized at 200 and 300 MPa. On the other hand, while the generation of acrylamide in the TAPS-buffered system (C) was significantly reduced under pressure, it was over 10 times higher than those in the pH-unbuffered and MES-buffered systems. In addition, the amounts of acrylamide at 100 and 300 MPa were almost the same, but lower than at 200 MPa.

Figure 4D, E and F show the concentrations of melanoidins in samples of each aqueous reaction system after the above-described treatment. Sample solutions changed from colorless to light brown in water and MES buffer or to dark brown in TAPS buffer. A tendency to reduce melanoidins generation by an increase in pressure was observed, similar to that of acrylamide in the pH-unbuffered and MES-buffered systems (D and E). These results indicated that the Maillard reaction including acrylamide formation was suppressed under higher pressure at pH 5.0 with or without buffering. However, the MES buffer had no apparent effect on the enhancement of acrylamide and melanoidins formation. In the TAPS-buffered system (initial pH 9.0, F), the level of melanoidins was not significantly different between pressurized samples and control, while it was slightly increased at 300 MPa. Similar to the result of acrylamide, the generation of melanoidins in the TAPS-buffered system was over 10 times higher than that in the pH-unbuffered and MES-buffered systems.

Figure 4G, H and I show the pH values of treated samples of each aqueous reaction system. The degree of increase in pH in the pH-unbuffered and MES-buffered systems (G and H) was suppressed depending on the pressure and was the lowest at 300 MPa. In the TAPS-buffered system (I), the pH values dropped upon increasing pressure.

Table 1A, B and C show the correlations among the above-

Fig. 4. Effect of pressure on asparagine-glucose model systems in water (A, D, G), MES buffer (B, E, H) (0.1 M, initial pH 5.0) or TAPS buffer (C, F, I) (0.1 M, initial pH 9.0) heated at 120 °C for 60 min. (A, B, C) Acrylamide formation; (D, E, F) Melanoidins formation; (G, H, I) pH values of the treated samples. Control was treated under < 5.0 MPa. Dashed lines in G and H indicate the initial pH of 5.0 of model systems in water and MES buffer, respectively. * $p < 0.05$, ** $p < 0.01$ compared with control.

mentioned outcomes in the pH-unbuffered, MES-buffered and TAPS-buffered systems, respectively. The correlations were similar for the pH-unbuffered and MES-buffered systems (Table 1A and B). Pressure was negatively correlated with acrylamide concentration and melanoidins concentration. In addition, there were positive correlations between acrylamide and the melanoidins concentration. The pressure value in these systems correlated stronger with acrylamide concentration than with melanoidins concentration. In the TAPS-buffered system (Table 1C), a negative correlation was found between pressure and acrylamide concentration. In addition, although negligible differences in the level of melanoidins were observed between pressurized samples and control (Fig. 4F), a positive correlation

was found between the pressure value and melanoidins concentration. This difference between these results was due to the difference in the statistical methods employed. Compared with the pH-unbuffered and MES-buffered systems, the pressure value was weakly correlated with acrylamide and melanoidins concentration in this system. Moreover, acrylamide concentration was not correlated significantly with melanoidins concentration in the TAPS-buffered system.

Discussion

The effect of pressure on the generation of acrylamide in the Maillard reaction was studied by heat treatment under HHP using model systems in which equimolar mixtures of

Table 1. Correlation coefficient of each factor [A, water (initial pH 5.0); B, 0.1 M MES buffer (initial pH 5.0); C, 0.1 M TAPS buffer (initial pH 9.0)].

A			
	Pressure (MPa)	Acrylamide (ppm)	Melanoidins (mM)
Pressure (MPa)	1		
Acrylamide (ppm)	−0.897*	1	
Melanoidins (mM)	−0.840*	0.900*	1

B			
	Pressure (MPa)	Acrylamide (ppm)	Melanoidins (mM)
Pressure (MPa)	1		
Acrylamide (ppm)	−0.852*	1	
Melanoidins (mM)	−0.724*	0.909*	1

C			
	Pressure (MPa)	Acrylamide (ppm)	Melanoidins (mM)
Pressure (MPa)	1		
Acrylamide (ppm)	−0.651*	1	
Melanoidins (mM)	0.631*	−0.301	1

*Significant correlation at the 0.05 level.

asparagine and glucose were dissolved in water (initial pH 5.0), MES buffer (initial pH 5.0) or TAPS buffer (initial pH 9.0). All these experiments resulted in the suppression of acrylamide generation by the application of HHP. These suppression mechanisms seemed to be different in acidic (pH 5.0) and alkaline (pH 9.0) conditions because the strength of correlations among the outcomes differed based on the initial pH. As shown in Fig. 1, the initial stage of the Maillard reaction starts with the reaction of an amino acid with a carbonyl compound, such as a reducing sugar, to give a Schiff base. The Schiff base is converted to a stable Amadori compound by Amadori rearrangement, which is regarded as essentially irreversible, followed by the generation of melanoidins via the intermediate stage and the complex advanced stage (Martins *et al.*, 2001; Nursten, 2005). The major pathway for acrylamide formation goes through decarboxylation of the Schiff base (Zyzak *et al.*, 2003; Stadler *et al.*, 2004). There is no difference in the mechanism with or without the application of elevated pressure for Maillard browning (Hill *et al.*, 1996). Therefore, our results indicated that elevated pressure suppressed acrylamide generation, not by giving rise to the reaction for other compounds but by retarding part of the initial stage of the Maillard reaction including acrylamide formation.

In the pH-unbuffered and MES-buffered models, i.e., initial pH of 5.0, the generation of both acrylamide and melanoidins was suppressed by an increase in pressure. It is inferred from this result that pressure mainly affected the condensation reaction between asparagine and glucose, a common reaction

in the generation of both compounds.

In pH-unbuffered models, this retardation of the first condensation reaction can be explained by the pH drop in the solution caused by the application of HHP. Higher pH is favorable for the acyclic sugar and the unprotonated amino group that are regarded as the reactive forms (Martins *et al.*, 2001); thus, the pH drop results in their decreased reactivity. The dependence of the Maillard reaction on pH can be explained by these reactants pH-dependent reactivity (Martins *et al.*, 2001). Furthermore, the dissociation of ionizable substances into ions (e.g., salts, acids, bases, polyelectrolytes and water) is enhanced by pressure (Martinez-Monteagudo and Saldaña, 2014). A reversible and temporary change in pH is caused by formed ions and is restored by pressure release (Martinez-Monteagudo and Saldaña, 2014); for example, the pH of water drops between 0.39 and 0.73 units per 100 MPa (Hayert *et al.*, 1999). Kunugi (1991) and Taniguchi (1992) individually published the molar volume changes for the proton dissociation of water and buffering acids in solution. They reported that a larger change in molar volume leads to a bigger change in acid dissociation constant, pK_a , by pressure, followed by a larger shift in pH (Kunugi, 1991; Taniguchi, 1992). They also reported that under pressure, the pHs of MES and TAPS buffers change only slightly owing to the smaller changes in their molar volumes, in contrast with the pH of water, which declines markedly owing to the larger volume change (Kunugi, 1992; Taniguchi, 1992). Asparagine exhibits a change in dissociation equilibrium by pressure in the same manner as the above solutions. Under mildly acidic conditions, proton

dissociation of the carbonyl group of asparagine is promoted by pressure, contributing to the pH drop (Ames, 1998; Hill *et al.*, 1996). In addition, asparagine releases ammonia upon its deamidation, which leads to an increase in the pH of the solution (De Vleeschouwer *et al.*, 2008). As shown in Fig. 4G, increases in pHs of pH-unbuffered samples were suppressed with an increase in pressure, indicating that pressure decreased the resulting ammonia by changing the equilibrium of the deamidation reaction of asparagine. The deamidation of asparagine reduces its concentration, however, it might cause an increase in the reactive asparagine, which has the unprotonated amino group, by increasing the pH. To summarize, we concluded that a substantial retardation of the Maillard reaction with an increase in pressure in pH-unbuffered systems was ascribed to the change in dissociation equilibrium of the solution compounds.

Thus, pressure causes a pH shift and noticeably affects the progression of the Maillard reaction. Since the Maillard reaction consists of highly complicated chemical reactions including various stages and pathways, each of the individual reactions within the Maillard reaction might be differently affected by high pressure (De Vleeschouwer *et al.*, 2010). Martinez-Monteagudo and Saldaña (2014) reviewed recent studies in which the effect of pressure on the individual reactions within the Maillard reaction was expressed as activation volume ($\Delta V^\ddagger$). According to their review, pressure accelerates the reactions having negative $\Delta V^\ddagger$, i.e., sugar mutarotation, condensation and melanoidins formation, while it inhibits the reactions having positive $\Delta V^\ddagger$, i.e., Amadori rearrangement, sugar dehydration and fragmentation (some products such as 3-deoxyglucosone can be used for acrylamide formation). Based on the results of the pH-unbuffered system, we inferred that the suppression effect of the pressure-induced pH shift on acrylamide formation is stronger than the enhancement effect of pressure, which activates the sugar and promotes the condensation reaction.

In the MES-buffered system in which pH is stable against pressure (Kunugi, 1991; Taniguchi, 1992), the retardation of the first condensation reaction can be explained in the same manner as the pH-unbuffered system, i.e., the decrease in dissociation of the compounds is caused by the application of HHP. As shown in Fig. 4G and H, the pH stability of samples treated with higher pressure might indicate that pressure suppressed heat-induced ammonia release from the asparagine side chain, retarding the Maillard reaction by suppressing the pH increase. Alternatively, the equilibrium between protonated and deprotonated amino groups ($-\text{NH}_3^+ \rightleftharpoons -\text{NH}_2 + \text{H}^+$) may have shifted slightly to the protonated form by pressure (Ames, 1998; Hill *et al.*, 1996), and therefore protonation of asparagine amino groups, namely nonreactive asparagine, might be promoted slightly and lead to a suppression of the reaction with glucose.

On the other hand, in the TAPS-buffered system, pressure

suppressed the generation of acrylamide while it had no pronounced effect on that of melanoidins. Moreover, weaker or no correlations in this system were observed among pressure and the levels of acrylamide and melanoidins as compared with the acidic systems. Under alkaline conditions, it is possible that pressure retards not the condensation between asparagine and glucose but the reactions following this condensation or that pressure accelerates the degradation/polymerization of the formed acrylamide. Pressure may directly retard elimination reactions releasing acrylamide from all three precursors, as shown in Fig. 1, because activation volumes of chemical reactions containing bond cleavage are generally positive values (Tamaoka *et al.*, 1991b). This direct effect of pressure might occur not only in alkaline systems but also in acidic systems, which is not dominant. However, an increase in the rate of color development ensues following an increase in the pH of a system (Hill *et al.*, 1996) and the Maillard reaction brings about a considerable lowering of the pH (Nursten, 2006). Our results in the alkaline system showed the generation of significant amounts of melanoidins and the considerable lowering of pH, agreeing with their reports (Hill *et al.*, 1996; Nursten, 2006). The pH decrease induced by the Maillard reaction affects this reaction further, thereby bringing the more complicated consequence. In addition, the obtained results showed no pronounced effect of pressure (≤ 300 MPa) on melanoidins generation under alkaline conditions. In contrast, it has been reported that pressure (600 and 400 MPa) promotes the Maillard reaction under alkaline conditions (Ames, 1998; Hill *et al.*, 1996; Nursten, 2005). Based on these reports and the results of this study, it could be concluded that the generation of melanoidins will increase with increasing pressure, which will induce a further equilibrium shift for each reaction.

In this study, we measured the pH of sample solutions after HHP treatment. The degree to which the Maillard reaction occurred can be known from the pH, because the pH drop in the treated samples is derived from organic acids formed in this reaction (De Vleeschouwer *et al.*, 2010). In the pH-unbuffered and MES-buffered systems, however, the pH values after treatments were higher than that before treatment, and these pH increases were suppressed with increasing pressure. The increasing pH in pH 5.0 systems is considered to be due to the deamidation of asparagine to aspartic acid (De Vleeschouwer *et al.*, 2008) as described above. The increase in pressure in these systems inhibited the release of ammonia from asparagine, affecting the generation of acrylamide and melanoidins following suppressed pH increase. Increased amounts of asparagine deamidation might be greater than organic acids generation in acidic systems, resulting in the highest pH values of controls. In the TAPS-buffered system, the pH value after treatment was lower than that before treatment and decreased with increasing pressure. We considered that the TAPS-buffered system promoted the

Maillard reaction more than the pH-unbuffered and MES-buffered systems and caused the generation of organic acids in excess of the buffer concentration, leading to a large pH drop.

These results suggested that heat treatment at 120 °C under HHP of 100 to 300 MPa is able to reduce acrylamide generation as compared with heat treatment at the same temperature under atmospheric pressure. Although the applied HHP in our study was lower, our results were in agreement with the report (De Vleeschouwer *et al.*, 2010) in which the maximal acrylamide concentrations generated during a 60-min high-pressure and high-temperature treatment of 600 MPa and 115 °C (up to 1 700 ppb) are much lower than a corresponding heat treatment (up to 6 500 ppb). De Vleeschouwer and coworkers (2010) also reported that two types of buffers, i.e., phosphate and MES buffers, used in their study had temperature stability and pressure stability, respectively, and that the acrylamide concentration generated in the MES-buffered system was much higher than that in the phosphate-buffered system. De Vleeschouwer and coworkers (2010) concluded that the difference between both buffers is due to changes in pH induced by temperature and/or pressure. Our finding that the suppression effect of HHP on acrylamide generation is due to changes in pH with regard to the pH-unbuffered and MES-buffered systems is consistent with their conclusions (De Vleeschouwer *et al.*, 2010). However, it must be noted that the differences between our model systems and theirs are pH conditions and buffer components. Accordingly, it is not clear whether the differences in the HHP levels between 400–700 MPa and 100–300 MPa affect acrylamide generation.

Conclusion

This study revealed that HHP of up to 300 MPa, which has been used in the food industry, is able to suppress acrylamide generation during heat treatment under both acidic and alkaline conditions. The obtained results also suggested that the suppression effect of pressure on acrylamide generation differed among initial pH conditions. Under acidic conditions, the pressure works on the first condensation reaction, while under alkaline conditions, the pressure is related to the processes of acrylamide generation after condensation. These findings suggest that high-pressure high-temperature treatment is beneficial for the reduction of acrylamide generation in food processing.

Acknowledgements The authors thank Dr. Y. Nomi from the Niigata University of Pharmacy and Applied Life Science for assistance with the LC-MS/MS analysis. We are grateful to Dr. T. Konishi from the Niigata University of Pharmacy and Applied Life Science for his guidance.

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