

pH-dependent Liquefaction of Thai Fermented Rice Noodles (*khanom jeen*) Associated with Bacterial Amyolytic Enzymes

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Abstract

Khanom jeen is a traditional Thai noodle made from fermented rice flour. It is produced and consumed throughout Thailand. Noodle products in local markets usually maintain their quality without rotting for a few days at ambient temperature. However, producers occasionally suffer the problem of noodle liquefaction. Severe liquefaction occurs unpredictably within a day at the production site. In the present study, liquefaction was induced by treating noodles with McIlvaine buffer at pH 6.0 and pH 8.0, but was not induced when the noodles were treated with distilled water, McIlvaine buffers at pH 4.0, or 1% sodium lactate buffer at pH 4.0. This pH-induced liquefaction was suppressed when chloramphenicol was added to the buffers, suggesting that this phenomenon was associated with bacterial growth. An increase in reducing sugar, presumably derived from oligosaccharides by starch digestion in the noodles, was observed in accordance with α -amylase production under the liquefaction-inducing conditions. Different starch-digesting enzymes in the liquefaction process were observed using zymography. The noodles used in this study contained about 0.03% lactic acid. Maintaining the acidic environment in noodles to prevent bacterial digestion of starch should help retain the *khanom jeen* noodle structure.

Discipline: Food

Additional key words: pH adjustment, starch

Introduction

Khanom jeen is a traditional Thai noodle made from fermented rice flour. It is produced and consumed throughout Thailand, and is a representative part of Thai food culture. Similar types of fermented rice noodles are common in China and Southeast Asian countries such as Laos, Myanmar, Cambodia and Vietnam, where the noodles are known as *mifen*, *khao poun*, *mohingar*, *num banvuchock*, and *bun*, respectively. According to producers, fermented rice flour for *khanom jeen* noodle production is prepared from relatively aged Indica rice grains, including broken rice, which is a byproduct of rice milling production. Therefore, *khanom jeen* production is considered an important value-added system that vitalizes rice production and related Thai industries (Kusano 2017). Although one company produces *khanom jeen* noodles on a large-scale basis for sale nationwide, many small- to medium-sized *khanom jeen* producers

throughout Thailand sell products to local markets, as freshly made *khanom jeen* noodles are preferred by consumers (Kusano 2017). To meet increasing market demand, many of these small- to medium-sized producers now use mass-produced fermented rice flour products purchased from suburban factories, despite having previously prepared fermented rice flour themselves (Kusano 2017).

Fermented rice flour used for making *khanom jeen* noodles is typically produced as follows: The rice materials are first soaked in water and then drained in a sieve container. Wet rice in the container is covered with a plastic sheet and left to stand overnight under ambient conditions to undergo solid-state fermentation. The rice is then washed with water and the same process is repeated for a few days. The fermented rice is then wet-milled and precipitated in an approximately 2% saltwater pool overnight to undergo liquid-state fermentation, after which the supernatant is replaced with fresh salt water. After a few days of liquid-

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state fermentation, excess moisture is squeezed out of the precipitate for preparing fermented rice flour to sell to *khanom jeen* noodle producers. In the noodle factories, the fermented rice flour is heated to achieve partial starch gelatinization and kneaded with a certain amount of boiling water. The dough is then extruded into noodles in boiling water for approximately 1 min., followed by washing in water and draining in a sieve container to sell. *Khanom jeen* noodle products have a unique and preferable flavor attributed to the rice fermentation process. Several lactic acid bacteria species such as *Streptococcus lactis*, *Streptococcus thermophilus*, *Pediococcus acidilactici*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* have been isolated from fermented rice flour products in Thailand, with lactic acid bacteria accounting for 40%-60% of the total isolated bacteria (Uchimura et al. 1991). Another unique characteristic of *khanom jeen* noodles is an elastic texture. The removal of soluble rice proteins during fermentation is involved in achieving the textural properties (Satmalee et al. 2017). The importance of rice starch purity in achieving the unique noodle texture has also been reported for fermented rice noodles in China (Lu et al. 2008).

Generally, *khanom jeen* noodles in local markets retain quality without rotting for a few days at ambient temperature. Refrigeration is not applicable to the noodles because starch retrogradation at cold temperatures ruins the unique texture. However, *khanom jeen* noodle products occasionally suffer from severe liquefaction, which occurs unexpectedly before the products are sent to market. This negatively impacts not only businesses but also consumer trust. Although this serious problem requires an urgent solution, the direct cause has yet to be clarified, with the unpredictability of liquefaction hindering determination of the liquefaction mechanisms. In the present study, we demonstrated the effect of pH on liquefaction induction in *khanom jeen* noodles. We also investigated the role of bacterially produced amyolytic enzymes in pH-induced liquefaction.

Materials and methods

1. pH treatment of *khanom jeen* noodles

Khanom jeen noodles freshly prepared from a commercially produced fermented rice flour were obtained from a noodle factory in Nontaburi Province, Thailand. The product was immediately transferred to a laboratory at the Institute of Food Research and Product Development (IFRPD), Kasetsart University (Bangkok, Thailand), and then subjected to pH treatment under aseptic conditions as follows: Noodles (200 g) were soaked in sterile distilled water (400 mL), 1% sodium

lactate buffer at pH 4.0, or McIlvaine buffers (pH 4.0, 6.0 or 8.0) at room temperature for 10 min., followed by draining and removing excess fluid in a sieve basket. Chloramphenicol (200 µg/mL) was added to each treatment liquid as required. The treated noodles (approx. 20 g) were transferred onto a plastic Petri dish (90 mm in diameter, 15 mm in depth), placed in a zippered plastic pouch, and then incubated at 37°C until used. The incubated noodle samples were cut into pieces approximately 2- to 5-mm long and homogenized. The homogenized noodle samples were stored in a freezer until analysis. Samples (2 g) were mashed with sterile distilled water (4 mL) using BioMasher sp and PowerMasher II (Nippi) instruments, followed by centrifugation at 18,000 ×g for 10 min. at 4°C. The supernatant was collected and used as the sample extract for analysis.

2. Estimation of reducing sugar content

Reducing sugar released in the sample extracts was estimated using the Nelson-Somogyi method. Aliquots (100 µL) of each sample were mixed with equivalent amounts of Somogyi's copper reagent (Sigma-Aldrich) in a microtube, heated in boiling water for 20 min., and then cooled in water for 5 min. Nelson's color reagent (100 µL; Sigma-Aldrich) and water (1 mL) were mixed into the tube, followed by incubation in room temperature for 15 min. The amount of reducing sugar in each sample extract was estimated by measuring the absorbance at 500 nm using a standard curve of 0.2-2 mM glucose for quantification.

3. Detection of starch digestion enzyme activity

The amyolytic activities of the samples were visualized using zymography. The sample extracts were concentrated approximately 20-fold with Amicon Ultra 0.5 mL 100 K centrifugal filters (Merck). The concentrated samples (10 µL) were subjected to polyacrylamide gel electrophoresis using Any kD Mini PROTEAN TGX Precast Protein Gel (Bio-Rad Laboratory) with Tris-Glycine buffer. After electrophoresis, the gel was briefly rinsed with water and then incubated in McIlvaine buffer (pH 6.0) with 1% soluble starch (Nacalai Tesque) for 75 min. at 40°C with gentle shaking. Amyolytic activities in the gel were visualized using the starch iodine reaction with iodine-potassium iodide solution (0.1% I₂/ 0.2% KI).

4. Other methods

The sample extract pH was measured using LAQUA twin compact pH meters (Horiba). The α-amylase activity and lactic acid content were measured using an α-amylase assay kit (Ceralpha method; Megazyme) and a D-/L-lactic acid enzymatic test kit (R-Biopharm), respectively.

Results and discussion

1. pH-induced liquefaction of *khanom jeen* noodles

The liquefaction of *khanom jeen* noodles occurs unpredictably at noodle production sites, causing the *khanom jeen* noodles to lose shape and become watery (Fig. 1). Noodle producers must discard such unsalable products. Furthermore, the fermented rice flour used for noodle production must be replaced, as methods of determining the cause of liquefaction have yet to be developed due to a lack of scientific understanding of the liquefaction mechanism. We hypothesized that liquefaction of the *khanom jeen* noodles was caused by water release associated with digestion of the starch maintaining the noodle structure by rice-derived or bacterial amylolytic enzymes. In general, amylolytic enzymes and enzyme-producing microorganisms have pH preferences for activity. Therefore, the effect of changing pH on *khanom jeen* noodles was observed in the present study. The noodles were treated with distilled water, 1% sodium lactate buffer at pH 4.0, or McIlvaine buffers at pH 4.0, 6.0 or 8.0 as described in the Materials and methods section, followed by incubation at 37°C and daily observation until 5 days after treatment. The pH values of the noodle extracts just after treatment with the aforementioned solutions were 3.7, 4.0, 4.1, 6.0 and 7.7, respectively. No significant pH changes were observed in extracts prepared 1-5 days after treatment under different pH conditions, except for that prepared from noodles incubated for 5 days after treatment with McIlvaine buffer at pH 8.0 without adding chloramphenicol, which was at pH 6.4 (data not shown). In the experiments without adding chloramphenicol, partial moisture transudation was observed in the noodles from 3 days after treatment with McIlvaine buffers at pH 6.0 and pH 8.0 (Fig. 2). Liquefaction was clearly observed in these



Fig. 1. Example photo of *khanom jeen* noodle liquefaction observed at a producer site in Bang Khun Thian district, Thailand

noodles 5 days after pH treatment (Fig. 2). In contrast, liquefaction was not observed in noodles treated with water, 1% sodium lactate buffer at pH 4.0, and McIlvaine buffer at pH 4.0 throughout the observation period (Fig. 2), or in those treated with chloramphenicol (data not shown). These results indicated that *khanom jeen* noodle liquefaction was induced, accompanied by bacterial growth in products in which the pH had been adjusted by buffers at pH 6.0 or 8.0. Furthermore, bacteria-mediated noodle liquefaction could be prevented by maintaining the acidic conditions of the noodles where the extract pH was around 4.0 or lower. Lactic acid produced in the fermented flour should be the major factor determining the pH of *khanom jeen* noodles, as lactic acid was the major organic acid detected in Cambodian fermented rice noodles (Ikeda et al. 2010). *Khanom jeen* noodles used in the present study contained approximately 0.03% lactic acid, with equivalent amounts of D- and L-lactic acid. The lactic acid levels in noodles treated with distilled water were maintained throughout the observation period in the present study (data not shown). Acidic conditions created by lactic acid in the noodles should be important to prevent liquefaction.

2. Amylolytic activities under pH-induced liquefaction conditions

To demonstrate the involvement of starch digestion in noodle liquefaction, reducing sugar released in the sample extracts was measured using the Nelson-Somogyi method. An increase in the reducing sugar content was observed in noodles treated with McIlvaine buffers at pH 6.0 and 8.0, from 3 and 2 days after treatment, respectively (Fig. 3 (A)), but was under the detection limit throughout the observation period in noodles treated with distilled water, 1% sodium lactate, and McIlvaine buffer at pH 4.0 (data not shown). Reducing sugar detected under the liquefaction-inducing pH conditions should be water-soluble oligosaccharides derived largely from starch digestion in the noodles. Although the reducing sugar content in noodles treated with buffer at pH 6.0 increased from 3 to 5 days, it decreased in those treated with buffer at pH 8.0 after 3 and 4 days of treatment, followed by a significant increase during the next day. This suggested a difference in the microbial carbohydrate metabolism properties between these pH conditions. α -Amylase activity in the noodles treated with buffers at pH 6.0 and pH 8.0 increased from 2 days and 1 day after treatment, respectively (Fig. 3 (B)), while it was not detected in noodles treated with the buffer at pH 4.0 throughout the observation period (data not shown). Overall, increasing the noodle pH above 6.0 activated amylolytic bacteria to cause *khanom jeen* noodle liquefaction. A transient

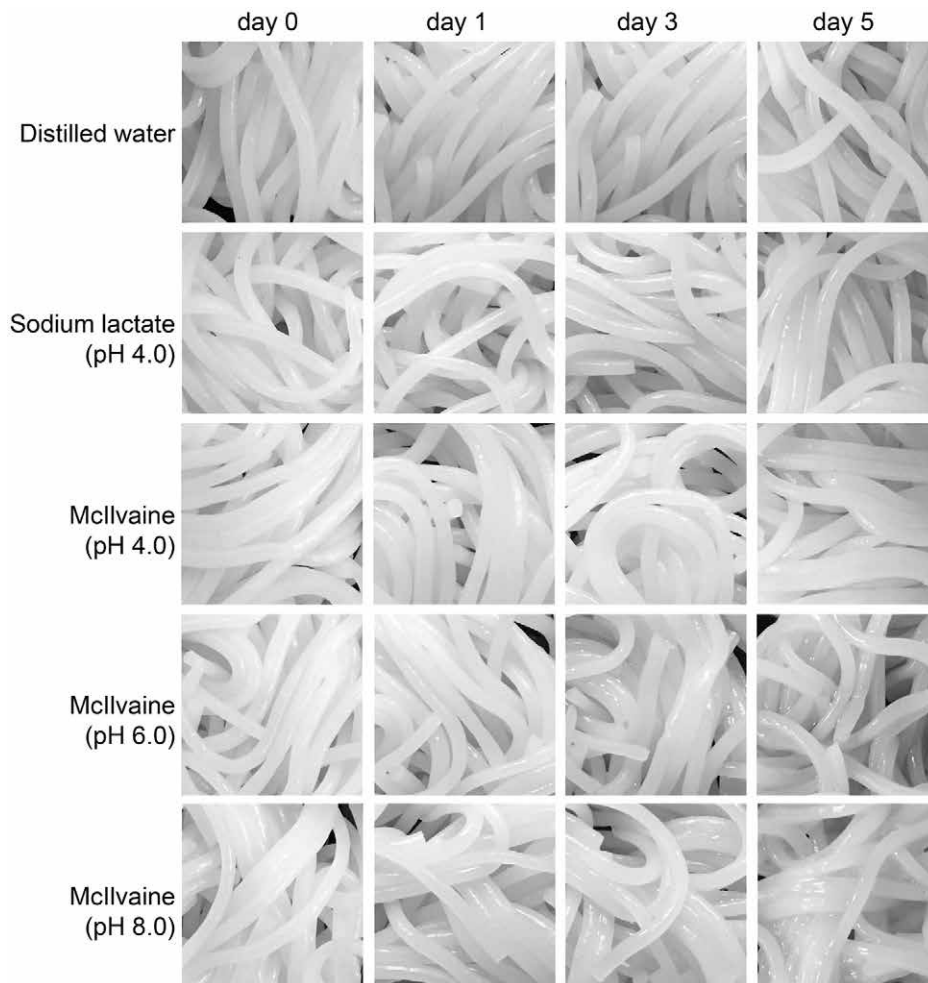


Fig. 2. Time-dependent change in appearance of *khanom jeen* noodles treated with buffers at various pH levels

Noodles treated with distilled water and the indicated buffers were photographed daily during incubation at 37°C until 5 days after treatment. The appearance of representative noodles at each observation point is presented.

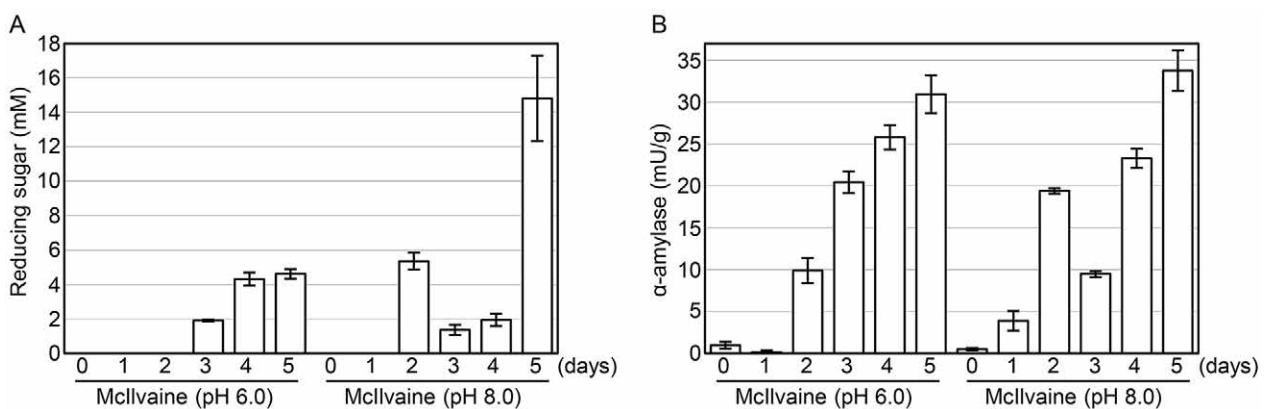


Fig. 3. (A) Reducing sugar and (B) α -amylase activity profiles under pH-dependent *khanom jeen* noodle liquefaction conditions

(A) Reducing sugar accumulation and (B) α -amylase activity in *khanom jeen* noodles treated with Mcllvaine buffers at pH 6.0 and pH 8.0 were monitored daily during incubation at 37°C until 5 days after treatment. Mean values of three replicated measurements are presented with standard deviation.

decrease in the activity observed 3 days after pH 8.0 buffer treatment (Fig. 3 (B)) might be due to carbon catabolite repression of bacterial amylolytic enzymes, or a change in the bacterial profiles, including amylolytic species. The different periods required to initiate amylase production under the pH 6.0 and pH 8.0 conditions suggested there were differences in the amylolytic bacteria at different pH levels.

3. Starch-digestion enzymes involved in *khanom jeen* noodle liquefaction

Starch digestion activities during pH-induced *khanom jeen* noodle liquefaction were visualized as clear bands in acrylamide gel by zymography using approximately 20-fold concentrated extracts prepared from noodle samples in which liquefaction was induced by McIlvaine buffers at pH 6.0 or 8.0 (Fig. 4). Under the pH 6.0 inducing conditions, bands appeared in samples from 2 to 5 days after buffer treatment (bands a-c), whereas band a was faint after 2 days. Under the pH 8.0 inducing conditions, 2-5 clear bands were observed in the samples 1-5 days after buffer treatment (bands d-j). In correlation with the starch digestion and amylase activity profiles shown in Figure 2 (A) and (B), clear zymography bands appeared earlier under the pH 8.0 conditions than under the pH 6.0 conditions (Fig. 4). Although the range of amylase activity measured in the present study was equivalent under the pH 6.0 and pH 8.0 inducing conditions (Fig. 3 (B)), the band intensities were stronger at pH 8.0 than at pH 6.0 (Fig. 4). This might be due to differences in the stability and substrate specificity of enzymes involved in liquefaction at each pH level. Moreover, the multiplicity of the starch digesting

enzymes and possible diversity in the enzyme-producing bacteria under each liquefaction-inducing pH condition is of scientific interest for improving methods of producing *khanom jeen* noodles and fermented rice flour to control the risk of *khanom jeen* noodle liquefaction. The isolation and characterization of amylolytic bacteria involved in pH-dependent noodle liquefaction of various *khanom jeen* noodle products from local manufacturers around Thailand are thus being considered.

Conclusions

The identification of pH-induced *khanom jeen* noodle liquefaction in the present study enabled analysis of the enzymatic digestion of starch, which is the main component maintaining the noodle structure. Measuring the reducing sugar content in the noodles allowed the initiation of liquefaction to be detected before it was visibly observed. Liquefaction was prevented by chloramphenicol treatment under the experimental conditions, indicating that amylolytic bacteria were involved in liquefaction. Further study is needed to fully understand the diversity and involvement of amylolytic bacteria in liquefaction to alleviate this problem. Our results showed that noodle liquefaction could be prevented by inhibiting bacterial growth under acidic conditions in noodles where the extract pH was around 4.0 or lower. Monitoring and managing the pH value and/or lactic acid contents in *khanom jeen* noodles and fermented rice flour products should effectively control the liquefaction problem. It might also be recommendable for producers to optimize and use commercially available sodium lactate buffer as a pH stabilizer to ensure their products are under acidic conditions.

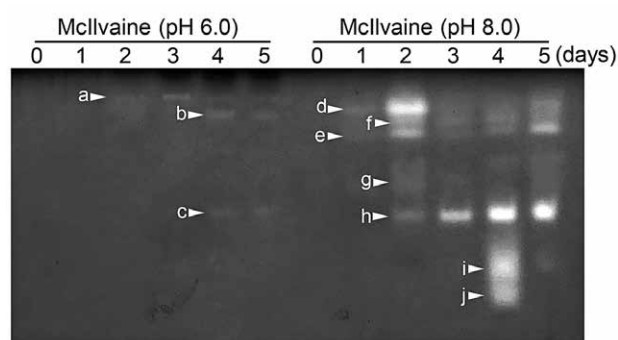


Fig. 4. Variation in amylolytic enzymes during pH-dependent *khanom jeen* liquefaction progression
Amylolytic activities in *khanom jeen* noodles treated with McIlvaine buffers at pH 6.0 and pH 8.0 were monitored daily by zymography during incubation at 37°C until 5 days after treatment. The band showing amylolytic activity in each mobility group is indicated by lowercase letters with arrowheads.

References

- Ikeda, M. et al. (2010) Characterization of the composition and bacterial manufacturing process for rice noodles in Cambodia. *J. Home. Econ. Jpn.*, **61**, 91-99.
- Kusano, E. (2017) Export and production of rice noodles in Thailand. *Kaihatugakukenyu (J. Agric. Dev. Stu.)*, **28**, 32-38 [In Japanese with English summary].
- Lu, Z. et al. (2008) Effect of fermentation metabolites on rheological and sensory properties of fermented rice noodles. *J. Sci. Food. Agric.*, **88**, 2134-2141.
- Satmalee, P. et al. (2017) Removal of soluble proteins during fermentation process for improving textural properties of traditional Thai rice noodles, *khanom-jeen*. *JARQ*, **51**, 325-329.
- Uchimura, T. et al. (1991) Identification of lactic acid bacteria isolated from fermented rice noodle "Khanom Jeen" of Thailand. *Nippon shokuhin kogyo gakkaiishi*, **38**, 465-475 [In Japanese with English summary].