PREPARATION AND INVESTIGATION OF SOME PHYSICOCHEMICAL PARAMETERS IN *ANABAS TESTUDINEUS* (NGA PYAE MA) FISH SAUCE SUPPLEMENTED WITH PAPAIN ENZYME

Nwe Mar Lin[1], Yee Mun Than[2], Myat Kyaw Thu [3]

Abstract

In this study, the fish sauce was prepared using the papain enzyme from the mature green leaves of *Carica papaya* L. The papain enzyme was isolated using ammonium sulphate precipitation (20%–70%). Fish samples of *Anabas testudineus* (Nga Pyae Ma) from the local market in Maubin Township, Ayeyarwady Region, were obtained for this investigation. Fish sauces were produced both with and without the papain enzyme. This study revealed how the physicochemical characteristics of fish sauce containing papain changed during fermentation, including pH (6.2 -7.0), refractive index (1.375-1.386), specific gravity (1.174-1.178), sodium chloride (19.59-20.27 %), total nitrogen (1.34-1.93 %), protein (8.38-12.06 %), formal amino nitrogen (0.60-1.00 %), and degree of hydrolysis (44.78-51.81 %). When compared to those without papain enzyme, it was found that fish sauces containing papain enzyme produced the most favorable outcomes. These results show that the use of papain to speed up the production of fish sauce is important.

Keywords: *Carica papaya*, papain, ammonium sulphate precipitation method, *Anabas testudineus*, fish sauce

Introduction

Climbing perch (*Anabas testudineus)* is a commercially important fish in Asian countries, mostly in Bangladesh, China, India, Malaysia, Pakistan, Sri Lanka, and Thailand. Also, it is used as a valuable food fish in different parts of the world (Hossain *et al*., 2015). Climbing perch is rich in iron and copper, which are essential for haemoglobin synthesis, and also contains easily digestible polyunsaturated fat and essential amino acids. This fish distribution is almost everywhere: in swamp waters, lakes, reservoirs, rivers, and other puddles. This fish can be caught throughout the year, with the spawning season at the beginning of the rainy season (Pahmi and Slamat, 2020).

Fish sauces are clear brown liquids produced by the fermentation of underutilized fish species with high salt contents (Tsai *et al*., 2006). Because of its high salt concentration, fermentation is one of the techniques used to preserve perishable fish. In Thailand, they are known as nampla, patis in the Philippines, kecap ikan or bakasang in Indonesia, badu in Malaysia, nuocnam in Vietnam and Cambodia, and nganpya ye in Myanmar. The process of fermenting traditional fish sauce is conducted by a combination of reactions, which are salting, enzyme hydrolysis, and bacterial fermentation. Fish sauce fermentation can be accelerated by the addition of enzymes (usually papain, bromelain, or bacterial proteases) (Beddows *et al*.,1979). Fish sauce is an enzymatic hydrolysate from fish tissue fully preserved by salt. It provides highly nutritious animal protein, vitamins, minerals, and an umami taste.

The main aim of this research is to isolate the papain enzyme from *Carica papaya* L. leaves, prepare fish sauce samples with and without papain, and investigate the changes in physicochemical parameters in fish sauces during fermentation.

[1] Department of Chemistry, University of Yangon
[2] Department of Chemistry, University of Yangon
[3] Department of Chemistry, University of Yangon

Materials and Methods

Materials

In this research work, Nga Pyae Ma fish samples were collected from local markets in Maubin Township, Ayeyarwady Region. All other chemicals used were of analytical reagent grade.

Sample Preparation

Fish samples were removed from the skin, scales, and bones. The fillets obtained were washed in running tap water and cut into smaller blocks.

Extraction of Papain Enzyme from Papaya Leaves

Papaya leaf samples (44 g) were mixed with 1000 mL of pH (7.4) phosphate buffer and stirred for 2 h at room temperature. And then, solid ammonium sulphate 113.34 g (20 % saturation) were slowly added to this extract on ice. After that, 337.34 g (70% saturation) were slowly added and stirred in ice for 2 h. After standing overnight, the precipitate containing papain enzyme was collected by centrifugation for 30 min at 5000 rpm (Maria, 2016).

Fermentation of Fish Sauce

The prepared fish sample (1 kg) was mixed with 0.3 % w/w papain in a jar. After 2 h 20 % (w/w) sodium chloride was added and then thoroughly mixed (Ooshiro *et al*., 1981). For control, the above procedure was carried out except that enzyme was not added. The prepared fish sauces with papain enzyme and without papain enzyme were stored at room temperature. The sauces were subjected to analysis after 30 days.

Determination of Physical Properties in Fish Sauce Samples

pH values were determined by a pH meter which was first standardized with standard buffer solutions of pH 4.6 and pH 6.8. Refractive indices were determined by a refractometer. The total dissolved solid contents were determined by the oven drying method. An accurately weighed fish sauce sample (5 mL) was put into a crucible and then weighed. The crucible was dried in the oven for half an hour, cooled in a desiccator, and weighed. Then the crucible was placed into the oven for 30 min, cooled in the desiccator, and weighed. The procedure was continued until a constant weight was obtained. From the difference in weights, the amount of total dissolved solids was calculated (AOAC, 1995).

Determination of Chemical Properties in Fish Sauce Samples

Mohr's titration may be used for the determination of chloride in a neutral solution by titration with a standard silver nitrate solution (Vogel, 1969). Total nitrogen and protein contents were determined by the Kjeldahl and Conway methods. (Conway, 1962)

Total nitrogen acts as an indicator of the protein content in fermented fish sauce. By multiplying by 6.25, the protein contents of the fish sauce were obtained. Formol Animo nitrogen was determined by the titrimetric method. The Sorensen method (AOAC, 1995), based on titration with formaldehyde, was used for quantifying the amino nitrogen. The degree of hydrolysis was calculated from amino nitrogen (AN) and total nitrogen (TN) using the Sorensen method (Harrimann *et al*., 2013).

Results and Discussion

Preparation of Nga Pyae Ma Fish Sauce with and without Papain Enzyme

Papain is a proteolytic enzyme from the cysteine protease family. Responsible for breaking down proteins, papain is an enzyme present in papaya and is used for breaking down meat fibres. It is used as a meat tenderizer, since the enzyme can break down muscle fibers in tough meat, leaving it tender (Trivedi *et al.*, 2013). In this study, papain was extracted from mature papaya leaves for the preparation of fish sauce. Papain (1.5 g) was obtained. Fish sauce fermentation began by mixing Nga Pyae Ma fish samples with papain enzyme (0.3%) for 2 h and adding 20 % salt at room temperature. The papain enzyme was not added to the control fish sauce. All volumes of fish sauce increased during fermentation. During fermentation, fish tissue in the presence of papain hydrolyzes more rapidly than that without papain. After fermentation for 180 days, the total volume of fish sauce supplemented with enzyme was 300 mL and found to be greater than that of fish sauce without enzyme (258 mL) (Table 1). During fermentation, fish tissue was gradually hydrolyzed by papain, indicating the activity of proteolytic enzymes.

Table 1. Total Volume of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Total volume (mL) (with papain)	Total volume (mL) (without papain)
180	300	258

Physical Properties in Nga Pyae Ma Fish Sauces

pH is an important parameter that affects the fermentation process (Siti, 2015). The changes in pH values of the fish sauce during fermentation are shown in Table 2 and Figure 1. As can be seen from the table, the pH value of fish sauces was found to increase during the 90-day period of fermentation and then stabilize. Therefore, it can be generally concluded that pH changes as a function of time in the early stages of fermentation. pH was found to be 7.0 in fish sauce supplemented with papain and 7.2 in that without papain after 180 days of fermentation. pH values in fish sauce with enzyme supplements were lower than those without enzymes. These observed values agreed closely with those reported as pH 7 (Koochekian and Moini, 2009), and pH 6.4-6.9 (Koffi-Nervy *et al*., 2011).

The changes in refractive indices of the fish sauce samples with and without papain enzyme during fermentation are shown in Table 3 and Figure 2. As can be seen from the table, the refractive indices of fish sauces were found to slightly increase during fermentation. During fermentation, the increase in the refractive index of fish sauce supplemented with papain was from 1.375 to 1.386 and, without papain, from 1.371 to 1.379. The changes in refractive indices of both fish sauce samples were not noticeable throughout the fermentation.

The changes in specific gravities of the fish sauce samples with and without papain enzyme during fermentation are shown in Table 4 and Figure 3. Specific gravities of fish sauces with and without papain were 1.174 and 1.172, respectively. However, after fermentation for 180 days, the specific gravities of all fish sauces were found to be comparable. Specific gravities of fish sauces with and without papain were 1.178 and 1.176, respectively. The increased specific gravity was due to the greater solubility of amino acids and small peptides at higher temperatures. In this research, the specific gravity of fish sauce supplemented with papain was slightly higher than without the papain enzyme.

In this study, total dissolved solids in Nga Pyae Ma fish sauces with and without papain were 29.2 % and 28.4 %, respectively, after 30 days of fermentation. Total dissolved solids in fish sauce supplemented with papain were higher than those without the enzyme. After 180 days of fermentation, total dissolved solids in fish sauces with and without papain were, respectively, 33.8 % and 32.2 % (Table 5 and Figure 4). It can be seen that the total dissolved solids content of the fish sauce supplemented with papain was slightly higher than that of the fish sauce without enzyme.

Table 2. Changes of pH of Nga Pyae Ma Fish

Fermentation time (Days)	pH (with papain)	pH (without papain)
30	6.2	6.3
60	6.4	6.5
90	6.6	6.7
120	6.8	7.0
150	6.9	7.2
180	7.0	7.2

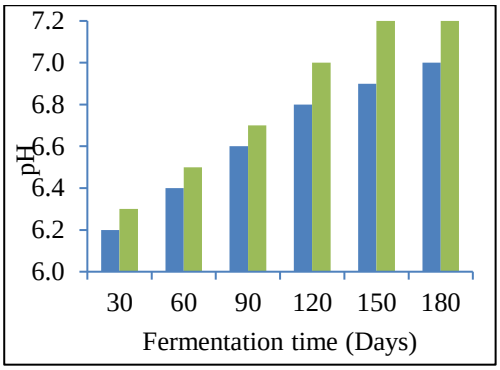

Figure 1. Changes of pH of Nga Pyae Ma fish sauces during fermentation

Table 3. Changes of Refractive Index of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Refractive index (with papain)	Refractive index (without papain)
30	1.375	1.371
60	1.377	1.373
90	1.379	1.373
120	1.380	1.375
150	1.382	1.376
180	1.386	1.379

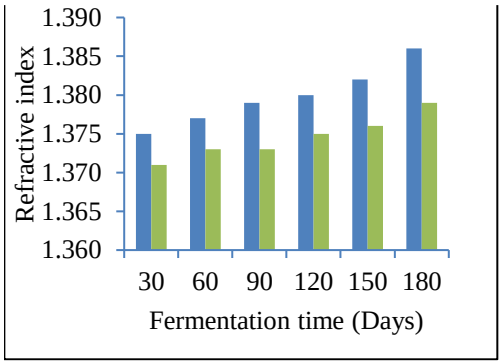

Figure 2. Changes of refractive index of Nga Pyae Ma Fish sauces during fermentation

Table 4. Changes of Specific Gravity of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Specific gravity (with papain)	Specific gravity (without papain)
30	1.174	1.172
60	1.174	1.173
90	1.175	1.174
120	1.176	1.175
150	1.178	1.176
180	1.178	1.176

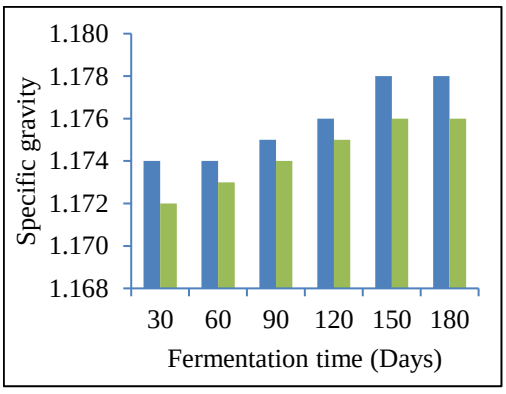

Figure 3. Changes of specific gravity of Nga Pyae Ma fish sauces during fermentation

Table 5. Changes of Total Dissolved Solids of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Total dissolved solids (%) (with papain)	Total dissolved solids (%) (without papain)
30	29.2	28.4
60	31.0	29.2
90	31.8	30.0
120	33.2	31.6
150	33.2	31.6
180	33.8	32.2

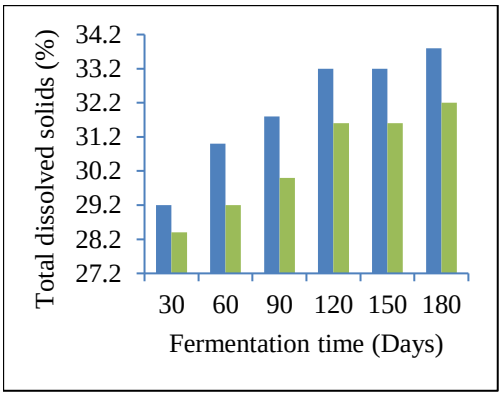

Figure 4. Changes of total dissolved solids of Nga Pyae Ma fish sauces during fermentation

Sodium Chloride Content in Nga Pyae Ma Fish Sauce Samples

The fish-to-salt ratio is another factor affecting fish sauce quality. Salt controls the type of microorganisms and retards or kills some pathogenic microorganisms during fermentation. In this research, the salt content was found to increase during a 90 - day period of fermentation. After that, the sodium chloride content in both sauces was found to decrease. The decrease may be due to the increase in volume of fish sauce. Sodium chloride contents were 20.27 % and 20.60 % in fish sauces with and without papain enzyme, respectively (Table 6 and Figure 5). In this research, higher sodium chloride contents were found in the control than those in the enzyme supplement sauce.

Total Nitrogen in Nga Pyae Ma Fish Sauce Samples

The soluble nitrogen components, including proteins, peptides, and amino acids, were generated by the activities of the proteolytic enzyme. Nitrogenous compounds in fish sauce are composed of protein and nonprotein nitrogenous compounds such as free amino acids, nucleotides, peptides, ammonia, urea, and TMAO. Total nitrogen increased dramatically during the fermentation period, especially in the first four months, and then remained relatively constant in the following six months (Brillantes *et al*., 2002). In this research, the total nitrogen content was found to increase during fermentation. After 180 days of fermentation, the total nitrogen of fish sauces with and without papain enzyme was found to be 1.93 % and 1.81 %, respectively (Table 7 and Figure 6). Total nitrogen in fish sauce with enzyme was higher than in fish sauce without enzyme. The total nitrogen content in liquid is also one of the most important quality factors for fish sauce and is used as an indicator to determine the price of fish sauce in Thailand (Lopetchart *et al.*, 2001).

Table 6. Changes of Sodium Chloride Contents of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Sodium chloride contents (%) (with papain)	Sodium chloride contents (%) (without papain)
30	19.59	19.89
60	20.19	20.49
90	21.39	21.70
120	21.09	21.39
150	20.60	20.87
180	20.27	20.60

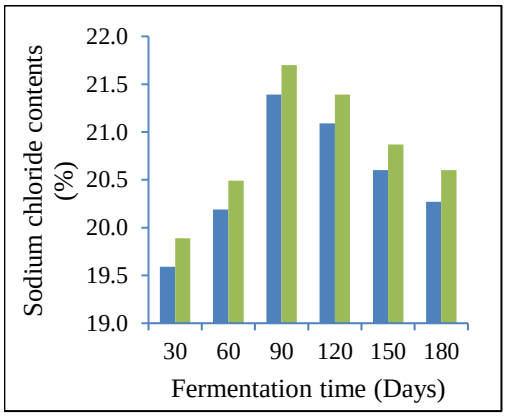

Figure 5. Changes of sodium chloride contents of Nga Pyae Ma fish sauces during fermentation

Table 7. Changes of Total Nitrogen Contents of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Total nitrogen contents (%) (with papain)	Total nitrogen contents (%) (without papain)
30	1.34	0.82
60	1.66	0.89
90	1.78	1.34
120	1.81	1.66
150	1.90	1.77
180	1.93	1.81

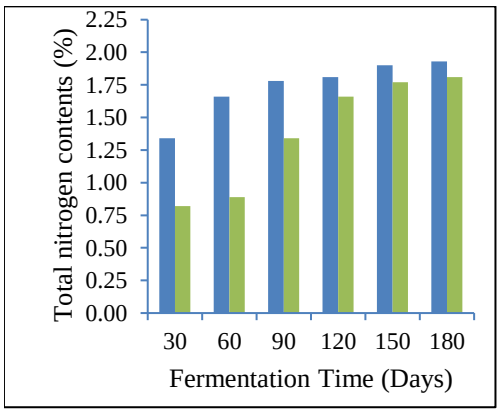

Figure 6. Changes of total nitrogen contents of Nga Pyae Ma fish sauces during fermentation

Protein Percentage in Nga Pyae Ma Fish Sauce Samples

The protein content of fish sauces was found to increase during fermentation and to become more stabilized in the later stages, i.e., after 180 days (Table 8 and Figure 7). The protein contents of fish sauce with papain enzyme were increased to 8.38 % - 12.06 % and without papain enzyme to 5.13 % - 11.31 % during fermentation. The protein contents of fish sauce with an enzyme supplement were higher than those of fish sauce without an enzyme. These observed values agreed closely with those reported to have a protein content of 12.3 % (Gildberg *et al*., 2007) and a protein content of 10.17–10.51% (Poernomo *et al*., 1984).

Formol Amino Nitrogen in Nga Pyae Ma Fish Sauce Samples

Ammonia nitrogen is suitable as a relational index for fish sauce to better understand its quality and character. Amino nitrogen is usually used as an indicator of the degree of fermentation (Lopetcharat *et al.*, 2001). The amino nitrogen represents the number of amino groups in fish sauce. An increase in amino nitrogen concentration is related to the degradation of

the polypeptide (Tungkawachara *et al.,* 2003). In this work, the formal amino nitrogen contents of fish sauce with papain enzyme were increased to 0.60 – 1.00 % and without papain enzyme to 0.32 – 0.82 % during fermentation. The formal amino nitrogen of fish sauce supplemented with enzyme was higher than that of fish sauce without enzyme (Table 9 and Figure 8).

Table 8 Changes of Protein Contents of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Protein contents (%) (with papain)	Protein contents (%) (without papain)
30	8.38	5.13
60	10.38	5.56
90	11.13	8.38
120	11.31	10.38
150	11.88	11.06
180	12.06	11.31

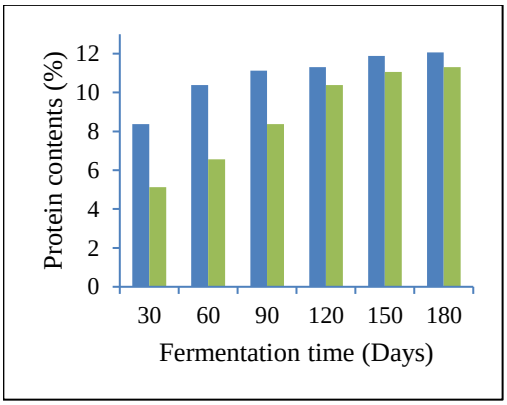

Figure 7. Changes of protein contents of Nga Pyae Ma fish sauces during fermentation

Table 9 Changes of Formol Amino Nitrogen Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Formol amino nitrogen (%) (with papain)	Formol amino nitrogen (%) (without papain)
30	0.60	0.32
60	0.76	0.36
90	0.82	0.54
120	0.86	0.67
150	0.93	0.75
180	1.00	0.82

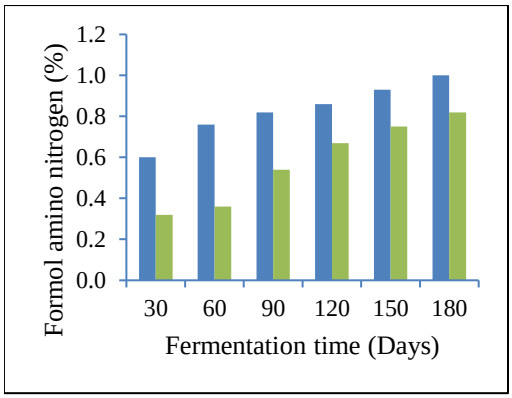

Figure 8. Changes of formol amino nitrogen of Nga Pyae Ma fish sauces during fermentation

Degree of Hydrolysis in Nga Pyae Ma Fish Sauce Samples

The degree of hydrolysis is defined by the proportion of cleaved peptide bonds in the protein hydrolysate. The degree of hydrolysis represents the extent of the hydrolytic degradation of the protein in the sample (Siti *et al*., 2015). During fermentation, the degree of hydrolysis in fish sauce with papain enzyme, increased from 44.78 % to 51.81 %, and without papain enzyme, it increased from 39.02 % to 45.31 % (Table 10 and Figure 9). The degree of hydrolysis increases during fermentation. A high degree of hydrolysis indicates a much greater amount of a complex compound will be cut down into a smaller compound (Siti *et al*., 2015). The degree of hydrolysis was greater in fish sauce supplemented with papain enzyme than in fish sauce without papain.

Table 10. Changes of Degree of Hydrolysis of Nga Pyae Ma Fish Sauces during Fermentation

Fermentation time (Days)	Degree of hydrolysis (%) (with papain)	Degree of hydrolysis (%) (without papain)
30	44.78	39.02
60	45.78	40.45
90	46.07	40.30
120	47.51	40.36
150	48.95	42.37
180	51.81	45.30

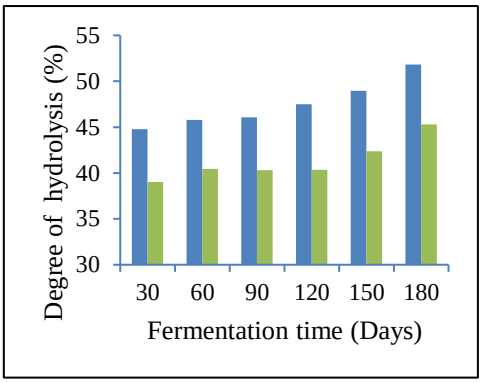

Figure 9. Changes of degree of hydrolysis of Nga Pyae Ma fish sauces during fermentation

Quality of the Prepared Fish Sauce

The prepared fish sauce samples were found to be clear, brown liquids that were rich in salt. Some properties of fish sauce samples prepared in this study were compared to the reported value of fish sauce (Table 11). The pH values of prepared fish sauces were neutral, and commercial fish sauces were slightly acidic. The salt contents of the prepared fish sauce were lower than those of commercial fish sauces. Total nitrogen, protein contents, formal amino nitrogen, and degree of hydrolysis in fish sauce supplemented with papain were found to be the highest among the others.

Table 11. Comparison of the Physicochemical Properties of Nga Pyae Ma Fish Sauce and Commercial Fish Sauces

Properties	Fish sauce (with papain)	Fish sauce (without papain)	Mingalar Daung	Shwe Pwar	Sein Gae
pH	7.0	7.2	5.8	5.3	5.7
Sodium Chloride (%)	20.27	20.6	25.91	27.12	25.01
Total Nitrogen (%)	1.93	1.81	1.69	0.77	1.6
Protein (%)	12.06	11.31	10.56	4.81	10.5
Formol Amino Nitrogen (%)	1.00	0.82	0.75	0.27	0.71
Degree of Hydrolysis (%)	51.81	45.3	44.38	35.06	42.26

Conclusion

The effect of papain supplements on the preparation of fish sauce was investigated in this research. Papain (EC 3.4.22.2) was isolated from mature green *Carica papaya* L. leaves by ammonium sulphate precipitation. During fermentation, pH values gradually increased and stabilized after 150 days. The refractive index and specific gravity slightly increased throughout the fermentation, but the increase was negligible whether the fish sauces were supplemented with papain or not. Higher sodium chloride contents were found in the control than those in the enzyme supplement sauce. Total dissolved solids, total nitrogen, formol nitrogen, and protein contents in fish sauce with enzyme supplements were higher than those without enzymes. The degree of hydrolysis in fish sauce with papain was found to be higher than without papain. Compared to commercial fish sauces, the prepared fish sauces with papain showed the most satisfactory results.

Based on these findings, fish sauces with papain enzymes were produced in less time than those without papain. This study showed that the prepared papain enzyme could be used as a meat tenderizing agent and to improve the quality of the fish sauce.

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