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Biochemical Changes in Fermented Rice–Shrimp (*Macrobrachium idella*) Mixture: Changes in Protein Fractions*

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ABSTRACT

Rice–shrimp mixture was allowed to undergo natural fermentation for 10 days at 31°C. Daily biochemical tests were carried out in fermented rice–shrimp mixture for 10 days.

The protein fractions, particularly soluble-nitrogen, increased from the beginning until the end of the fermentation period. The same pattern also occurred with amino-nitrogen formation concomitant with the reduction in molecular weight of shrimp protein. Ammonia-nitrogen was produced in small amounts and reached a maximum on the seventh day. Cathepsin D activity in shrimp had an optimum pH of 3·5 and an optimum temperature of 40°C. The activity of this enzyme decreased with fermentation time.

INTRODUCTION

In general, fermented foods are more attractive to the consumer than the raw ingredients. The fermentation process not only contributes to flavour and aroma in fermented products but also provides an inexpensive method of preserving easily decomposable food materials like shrimp and fish. The flesh of shrimp after death is still very active biochemically. The decomposition or the changes in composition of shrimp may be triggered by several factors as reported by Pedraja (1970). The initial bacterial contamination of shrimp may occur within the sea water before the

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shrimp is caught. After removal of the shrimp from the water and particularly when it is brought aboard the vessel, it is immediately exposed to contamination with micro-organisms of terrestrial origin. Several kinds of bacteria and yeast have been isolated from shrimp (Williams, 1949; Phaff *et al.*, 1952; Vanderzant *et al.*, 1970; Koburger *et al.*, 1975).

Shrimp contains several kinds of enzymes such as cathepsin, peptidases, decarboxylases, etc. Eitenmiller (1974) demonstrated the presence of cathepsin D in the muscle of the white shrimp (*Penaeus stiferus*). As in many protein foods, the protein from shrimp and some portions of protein from the rice in fermented products could be hydrolyzed to peptides and amino acids through the pathway presented by Whitaker (1978). In addition to the major products formed in the pathway, Pedraja (1970) also speculated that some compounds may be formed in the flesh of shrimp as a result of amino acid degradation. The degradation products of protein contribute to the flavor of fermented seafoods (Chung & Lee, 1976), as well as fermented rice–shrimp mixture. The breakdown of protein to amino acids during hydrolysis can be assessed by measuring the amount of amino-nitrogen. Protein hydrolysis also leads to reduction in the molecular weight of protein, as has been found in chicken muscle during ageing (Hay *et al.*, 1973; Rattrie & Regenstein, 1977).

MATERIALS AND METHODS

Preparation of rice–shrimp mixture

Shrimp (*Macrobrachium idella*) were soaked in brine for 3 days; the salted shrimp (containing 13·4 % NaCl on fresh weight basis) was mixed with the salted rice (containing 3 % NaCl) at a ratio of 1:4·5. The mixture was allowed to undergo natural fermentation at room temperature (31 °C) for 10 days. Samples were withdrawn daily for analysis.

Analytical methods

Soluble nitrogen

Ten millilitres of extract (4 g sample per 100 ml) were analyzed for soluble nitrogen by the semi-micro Kjeldahl method (AOAC, 1970). The amount of soluble nitrogen was expressed as milligrams per gram dry weight.

Amino nitrogen

Amino nitrogen content was determined by the formol titration method (AOAC, 1970). The mixture was titrated with 0·2N NaOH to pH 8·6 and the amount of amino nitrogen was expressed as milligrams per gram dry weight.

Ammonia nitrogen

The amount of ammonia nitrogen, expressed as milligrams per gram dry weight, was determined by the magnesium oxide method (AOAC, 1970). Two-and-a-half grams of sample was placed in a distillation flask with 200 ml distilled water and 2 g carbonate-free MgO. A hundred millilitre liquid distillate was collected in a flask containing 20 ml 0·05M H_2SO_4 and then titrated with standard 0·1M NaOH using methyl red indicator.

Molecular weight of shrimp protein

Approximately 2·0 g of shrimp was extracted with 50 ml distilled water. The extracted protein was dialyzed overnight against distilled water and then concentrated in a freeze-drier (Virtis). Protein content of the freeze-dried sample was determined by the method of Lowry *et al.*, (1951) using serum bovine albumin as standard.

SDS-disc gel electrophoresis was used to determine the molecular weight of protein. SDS gels were prepared and processed essentially as described by Maizel (1972). Bovine serum albumin, catalase, aldolase, trypsinogen, ribonuclease A and cytochrome C were used as standards for estimating polypeptide molecular weight in 10% SDS-polyacrylamide gels. Proteins were dissolved and dissociated by adding 1% SDS, Tris-HCl buffer (pH 8·3), 10% glycerol, 0·1% 2-mercaptoethanol and 0·001% bromophenol blue adjusted to pH 7–8. The dissociation of proteins was completed by heating at 100 °C for 1–2 min. Fifty microlitres of sample containing 2 mg of protein per millilitre were layered on top of the gels. Electrophoresis was conducted at 25 °C with a constant voltage (100 V). Current was initially set at 4 to 4·5 mA per tube and decreased during the run with constant voltage. Gels were stained with Coomassie Brilliant Blue G for 2·3 h and destained by diffusion in a destaining solution of 7% acetic acid in 50% methanol.

Protein mobilities were calculated from the equation described by Weber & Osborn (1969). Molecular weight was determined from a standard curve.

Cathepsin D activity

The activity of cathepsin D was determined by the procedure of Eitenmiller (1974).

Approximately 25 g of homogenized sample was extracted with 40 ml cold distilled water by shaking for 15 min and then centrifuged at 10000 × *g* for 15 min at 4 °C. The supernatant was filtered through Whatman No. 1 filter paper, the pH adjusted to 3·5 with 1·0N HCl and the volume of the solution was made up to 50 ml with distilled water. The enzyme assay was carried out on the extract immediately after extraction.

The acid denatured haemoglobin used as substrate was prepared by adjusting a 4·4% haemoglobin solution to pH 1·0 with concentrated HCl and allowing the solution to stand at ambient temperature for 2 h. The solution was adjusted to the desired pH with 2·0N NaOH diluted (1:1 v/v) with 0·4M phosphate buffer of the desired pH.

For the pH activity curve, the substrate and buffer at a pH range of 1·4 to 8·5 and of 42 °C incubation temperature were used. For the temperature activity curve, the pH of the substrate and buffer was adjusted to 3·5 (optimum pH) and the temperature was varied in the range 27·5 °C–70 °C.

Cathepsin D activity was determined by incubating equal volumes of substrate and enzyme extract at optimum conditions (pH 3·5; temperature, 40 °C) for 2 h. Following the incubation, 2·0 ml aliquots of the reaction mixture were removed and pipetted into test tubes containing 5·0 ml of 10% TCA. After 30 min, the precipitated protein was removed by filtration through Whatman No. 1 filter paper. Liberated tyrosine in the supernatant was quantitated by the method of Lowry *et al.* (1951) and activity determined from a standard curve was expressed as nmoles tyrosine liberated per gram dry weight per minute.

RESULTS AND DISCUSSION

Soluble-nitrogen

Soluble-nitrogen increased fairly rapidly from the beginning to the end of fermentation (Fig. 1). The increase was due to the degradation of protein into soluble amino acids and smaller peptide chains by the action of shrimp and microbial protease. At the later stage of fermentation the rate of increase was slower because of the reduction of cathepsin D activity to a low level after 5 days (see Fig. 2).

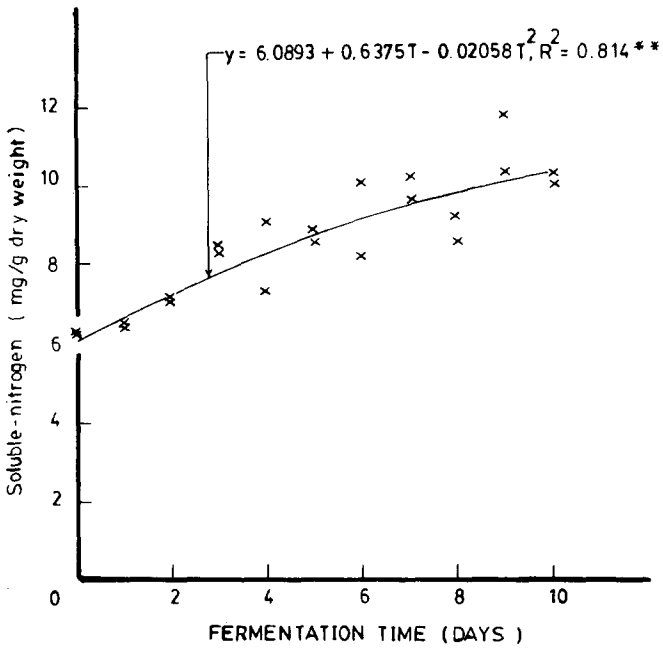

Fig. 1. Soluble-nitrogen changes during fermentation.

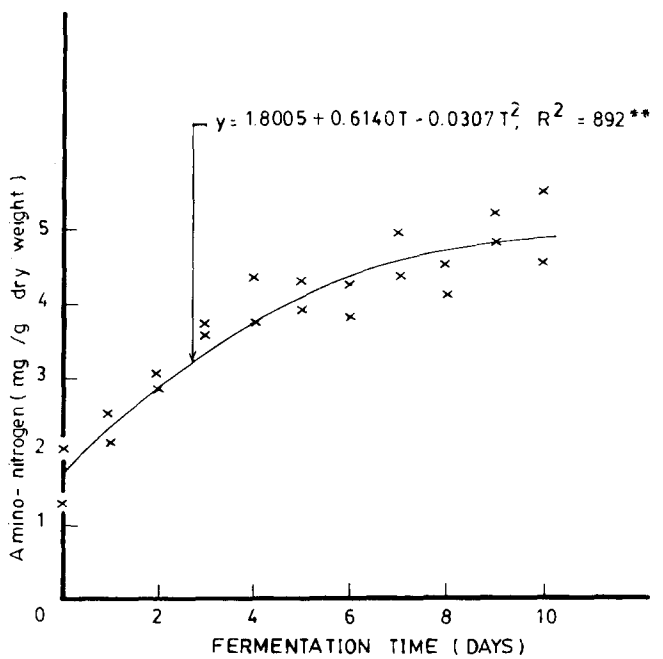

Fig. 2. Amino-nitrogen changes during fermentation.

Amino-nitrogen

Protein from shrimp and some portions of protein from rice in the fermenting mixture are hydrolyzed to peptides and amino acids during the fermentation process. There was a rapid increase during the first 4 days, followed by a gradual decrease in rate until fermentation was terminated (Fig. 2). The changes in amino-nitrogen were related to those of soluble-nitrogen, cathepsin D activity and molecular weight of protein.

Ammonia-nitrogen

The increase in this undesirable component was comparatively slight. Ammonia-nitrogen content increased as the fermentation progressed and reached a maximum on the seventh day, followed by a gradual decrease until fermentation was terminated (Fig. 3). Ammonia might be formed by the action of deaminase which is suspected to remain in shrimp after the brining process through the pathway proposed by Whitaker (1978). Ammonia could be produced by lactic bacteria from the amino acid arginine (Sharpe, 1962; Sharpe *et al.*, 1966) or by the action of contaminated micro-organisms which can grow at the initial phase of

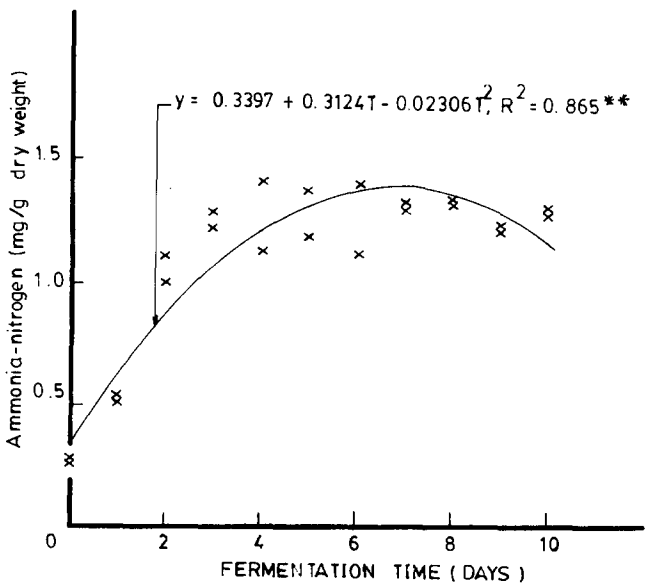

Fig. 3. Ammonia-nitrogen changes during fermentation.

fermentation. After prolonged incubation, salt (NaCl) and acid formed during fermentation may inhibit undesirable micro-organisms and enzyme activity. The reaction of ammonia with acid also resulted in the reduction of ammonia-nitrogen levels.

Molecular weight of shrimp protein

Proteolysis of protein leads to the formation of free amino acids and peptides of various sizes (Schromuller, 1968). SDS-gel electrophoretic patterns showing molecular weight (MW) changes are illustrated in Figs 4 to 6. Fresh shrimp protein exhibited bands on the gels varying in molecular weight from $1{\cdot}45 \times 10^4$ to 9×10^4 daltons. After the brining

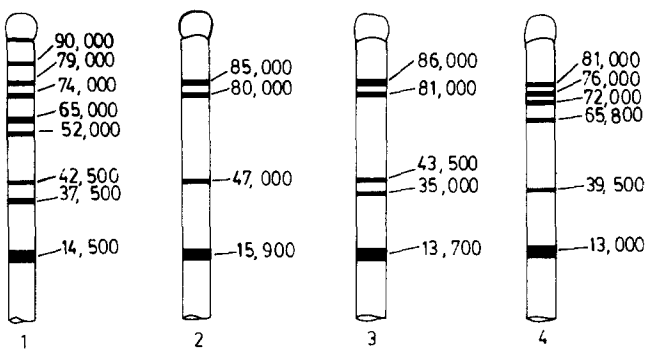

Fig. 4. Molecular weight changes during fermentation. 1 = Fresh shrimp. 2 = Brined shrimp (0 day). 3 = 1 day. 4 = 2 days.

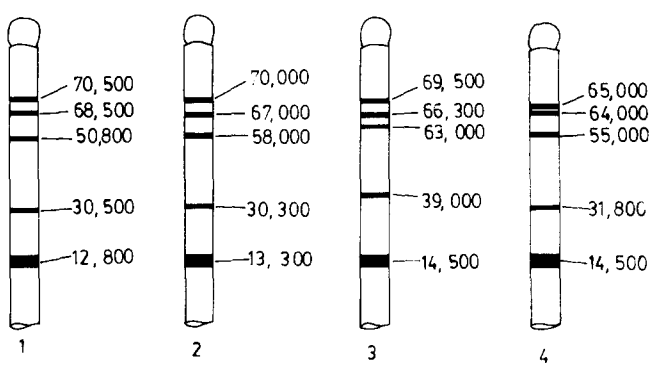

Fig. 5. Molecular weight changes during fermentation. 1 = 3 days. 2 = 4 days. 3 = 5 days. 4 = 6 days.

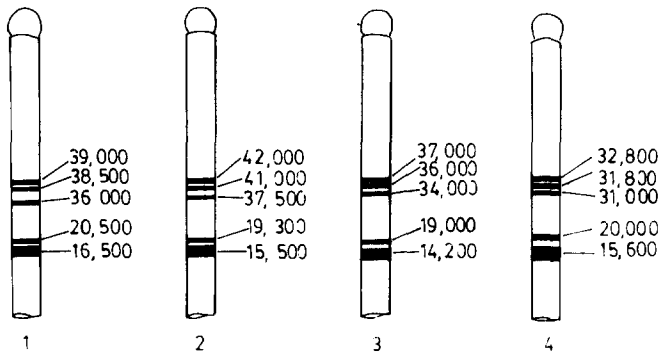

Fig. 6. Molecular weight changes during fermentation. 1 = 7 days. 2 = 8 days. 3 = 9 days. 4 = 10 days.

process only four bands appeared on the gel and the molecular weights were reduced, indicating that during the diffusion of salt into shrimp flesh protease activity was strong and protein hydrolysis resulted in the reduction of molecular weights (MW). As the fermentation progressed the highest MW of shrimp protein was reduced from $8{\cdot}5 \times 10^4$ daltons at day 1 to $3{\cdot}28 \times 10^4$ daltons on the tenth day. It has been shown that, although the proteolytic activity decreased with the fermentation time, the remaining activity proceeded to hydrolyze the protein molecule into smaller peptide chains.

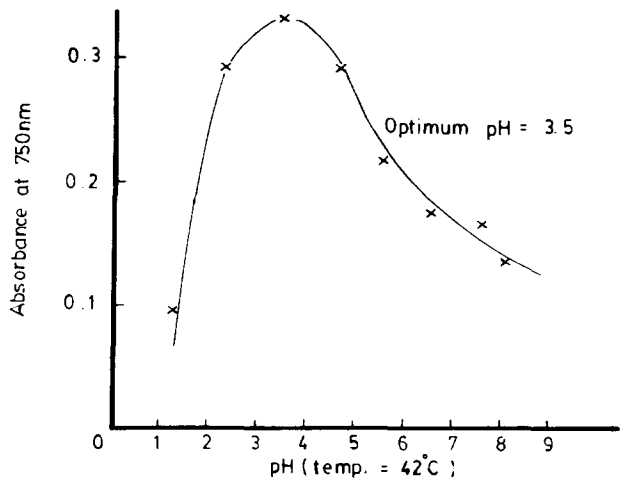

Fig. 7. pH activity curve of cathepsin D.

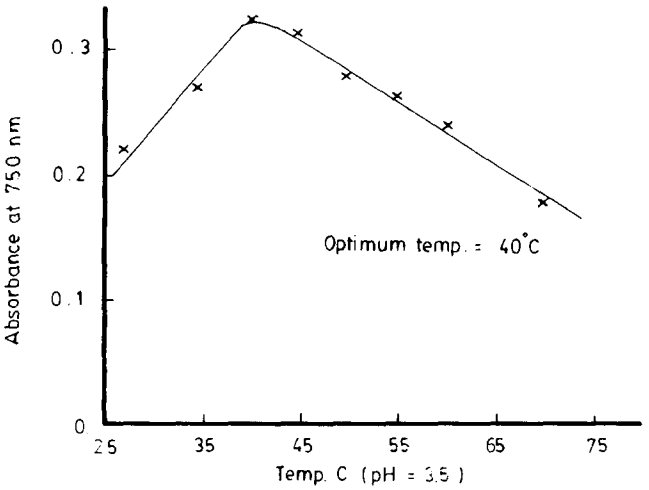

Fig. 8. Temperature activity curve of cathepsin D.

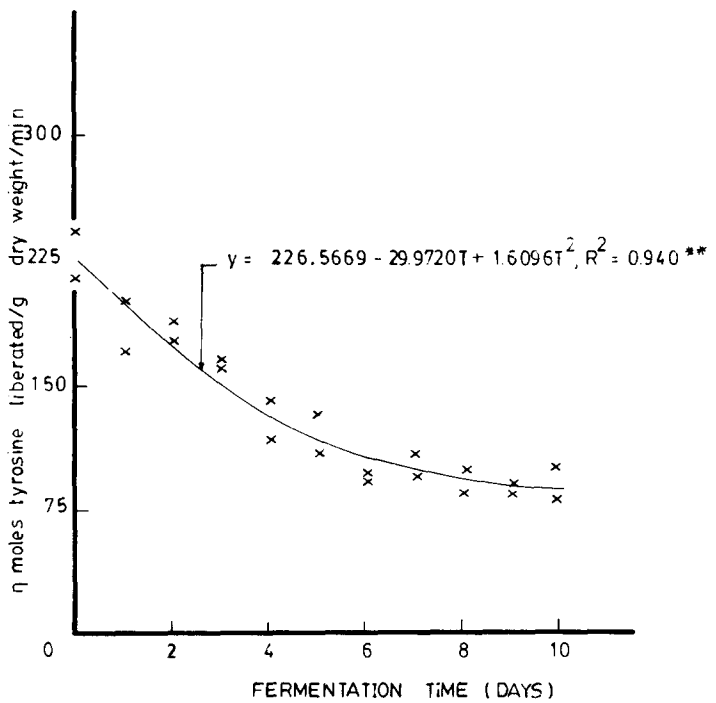

Fig. 9. Cathepsin D activity changes during fermentation.

Cathepsin D activity

Cathepsin D activity of shrimp extracts was optimum at pH 3·5 (Fig. 7) and 40 °C (Fig. 8). With the increase in fermentation time the activity of cathepsin D decreased rapidly up to the seventh day of fermentation and remained fairly constant until fermentation was terminated (Fig. 9). This is probably due to the denaturing effect of acid formed during fermentation on some part of this enzyme. In addition, the prolonged incubation of enzyme in high salt (NaCl) concentration (13·4% in the shrimp portion) also reduces the enzyme activity. The pattern of changes indicates that shrimp was the main source of proteolytic enzyme.

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