Detection of Fish Freshness Using Immobilized ADP-ase and 5'-Nucleotidase on Polyacrylamide Gel

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ABSTRACT

The presence of ADP and IMP in the muscle of fish could be used as an indicator of its freshness. These metabolites could be detected enzymaticaly using ADPase and 5'-nucleotidase. The aim of this research was to determine the presence of ADP and IMP in the muscle of fish qualitatively, using immobilized ADPase and 5'-nucleotidase on polyacrylamide gel. The results showed that in the non-immobilized form, ADPase has an optimum pH of 6 and stable at pH 5.5-10, while 5'-nucleotidase has two optima pH of 6.5 and 9 and it was stable at pH 7-10. Optimum temperature of ADPase and 5'-nucleotidase was 45 and 50°C, respectively. In the immobilized form, the activity of ADPase was optimum at pH 6 and it was still stable at pH 5.5 - 7.0 after storage at -20°C for 90 days, while 5'-nucleotidase was still stable at pH 7.5-10 after storage at -20°C for 90 days. Both enzymes were more stable in frozen storage than that of chilled storage. Sensitivity of both enzymes to detect the fish freshness during storage was affected by the presence of free inorganic phosphate derived from other phosphate-containing metabolites.

Key words : detection of fish freshness, immobilized ADPase, immobilized 5'nucleotidase.

INTRODUCTION

Criteria for evaluation of seafood quality have been an elusive goal for many years. There have been numerous attempts to find a reliable means of assessing seafood quality. However, many quality tests do not find commercial application owing to a variety of limitations and the inability to measure true total quality during catching, storing, processing, distributing and sale (Jacober and Rand, 1982).

Some metabolites derived from biochemical changes have been used for determination of fish quality such as trimethylamine (TMA), total volatile bases (TVB), lactic acids, volatile reducing substances (VRS), and oxidized lipids (TBA). Unfortunately, these tests appear to signify later stages of freshness (deterioration stages) and some of them have lack of reproducibility (Jacober and Rand, 1982; Ehira and Uchiyama, 1987). These tests could not reflect the quality of seafood at fresh stages.

Soon after death, all living cells will maintain physiological process as in the living conditions using high energy compounds such as ATP and creatine phosphate (Saito *et al.*, 1959; Eskin, 1990). In 1959, Saito *et al.* found that in fish ATP degraded in the following pathway: ATP → ADP → AMP → IMP → Inosine hypoxanthine. Considering this, Saito et al. (1959) proposed that lowering of fish freshness could be followed by increasing of K-value. K-value is defined as [(Inosine + hypoxanthine)/(ATP + ADP + AMP + IMP + Inosine + hypoxanthine)] x 100%. According to Ehira and Uchiyama (1987), K-value was accepted by the Japanese government as an official index of fish freshness. However, this method needs a complicated instrument, time and well-trained technician to measure ATP and its breakdown products.

The aim of this research was to develop a new method to determine fish freshness based on the presence of ADP and IMP in muscle of fish. These two metabolites were choosen due to the fact that in the fresh stage ADP and AMP content are high, while ATP disappeared in a few hours after fish death. On the other hand, IMP content is high after few hours of death and decrease gradually during storage (Saito *et al.*, 1959, Ehira and Uchiyama, 1987; Suwetja, 1988; Marseno, 1993; *Marseno, et al.*, 1994). The presence of ADP and IMP could be detected enzymatically using ADPase and 5'-nucleotidase, respectively. Both enzymes produce

inorganic phosphate, as catalytic product, that could be detected by Chen's reagent producing blue color (Chen, *et al.*, 1956). Considering these finding, we assume that if ADPase and 5'-nucleotidase were immobilized in supporting material it could be used to detect the presence of ADP and IMP in the muscle of fish rapidly which is indirectly reflect the freshness of fish.

MATERIALS AND METHODS

Materials

Purified enzyme ADPase (E.C. 3.6.1.5), 5'-nucleotidase (E.C.3.1.3.5), ADP, IMP and chemicals to prepare polyacrylamide gels were purchased from Sigma Chemical Co. (USA). All other chemicals were of Analytical grade obtained from Merck (Darmstadt, Germany). Fishes were obtained from local market in Yogyakarta.

Characterization of non-immobilized ADPase and 5'-nucleotidase

Pure ADPase dissolved with 2 ml of 40 mM Tris HCl (pH 7.5) containing 1 mM $CaCl_2$. The activity of ADPase was measured according to the method of Moodie *et al.*, (1991) with slight modification. The standard assay mixture (0.5 ml) contained 40 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1mM ADP and enzyme. Assay mixture was preincubated at 37°C for 10 minutes. The reaction was started by the addition of ADP and incubated for 20 minutes at 37°C, and it was terminated by the addition of 0.5 ml trichloroacetic acid 10%. The inorganic phosphate (Pi) liberated was measured by the method of Chen et al. (1956). One unit of enzyme activity was defined as the amount of enzyme catalyzing the liberation of 1 µmol Pi from ADP/min and specific activity was defined as unit/mg of protein.

The activity of 5'-nucleotidase was measured according to the method of Marseno *et al.* (1993a). Pure 5'-nucleotidase dissolved with 2 ml of 40 mM Tris-HCl (pH 7.5) The standard assay mixture (0.5 ml) contained 40 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl and enzyme. Assay mixture was preincubated at 37°C for 10 minutes and the reaction was started after the addition of IMP and incubated for 20 minutes at 37°C. The activity of 5'-nucleotidase was terminated by the addition of 0.5 ml of perchloric acid 10%. One unit of enzyme activity corresponds to the release of 1 µmol Pi from IMP/min and specific activity was defined as unit/mg of protein.

Effect of pH on activity and stability of enzyme

The effect of pH on the activity and stability of ADPase and 5'-nucleotidase were determined according to the method of Marseno, *et al* (1993a). The optimum pH was measured using standard assay conditions in the following 40 mM buffers : Sodium-acetate (4.5-7.5), Tris-HCl (7.0-9.0) and Glycine-NaOH (pH 8.0-10.0). The pH value of the reaction mixture was re-estimated at 37°C. The stability of the enzyme at various pH levels was examined by incubating the enzyme in 40 mM buffers as described above at 4°C for 14 hours, and the remaining acticity was assayed at optimum pH. The relative activity is expressed as the percentage of the specific activity of enzyme measured at various pHs relative to that at pH optimum.

Effect of temperature on activity and stability

The optimum temperatures for ADPase and 5'-nucleotidase were determined in the standard assay mixture at various temperatures. Effect of temperature on enzyme stability was examined by incubating the enzyme at various temperatures for 30 minutes and immediately cooling on ice for 10 minutes. The remaining activity was estimated using standard assay condition temperature. The relative activity is expressed as the percentage of the specific activity of enzyme measured at various temperature relative to that at optimum temperature.

Immobilization of ADPase and 5'-nucleotidase on Polyacrylamimed gel

Polyacrylamide gel was made according to the method of Laemli (1973). The standard procedure for immobilization of enzymes are as follows : 10 µl of ADPase (0.8 ng) was immobilized in 1 ml of 7.5% polyacrylamide gel containing 40 mM Tris-HCl (pH 7.5), ammonium persulfate 0.075 %. TEMED 0.05%, 1 mM $MgCl_2$, and 0.2 mM $CaCl_2$. The mixture was put into two-glass plate which have space 1 mm, allowed for 15 minutes, then cut into small pieces (about 0.6 x 0.6 cm). Twenty five µl of 5'-nucleotidase (2.67 ng) was immobilized by the same procedure as described for ADPase in

0.5 ml of 7.5% polyacrylamide gel containing 40 mM Tris-HCl (pH 7.5), ammonium persulfate 0.075 %, TEMED 0.05%, 20 mM $MgCl_2$, 10 mM NaCl. The gels/ were used for the following examinations.

Characterization of immobilized ADPase and 5'-nucleotidase

Effect of pH on the activity and stability of immobilized enzymes was measured by changing the pH of buffer in the standard procedure for immobilization as follows: sodium acetate (pH 5-6.5), Tris-HCl (7.0-8.5) and Glycine-NaOH (9.0-10.0). The gels were stored at -20°C, and at defined time the activity of immobilized enzymes was examined. Effect of temperature on the activity and stability of immobilized enzymes was determined using enzyme which immobilized at optimum pH (pH 6,5 for ADPase and pH 7.5 for 5'-nucleotidase).

Preparation of fish extract

Live *Cyprinus carpio* (ikan Mas) was purchased from local market at Yogyakarta and killed by piercing the medulla oblongata at the point of purchase, to reduce stress or struggle, and brought to the laboratory in an ice bot within 30 minutes. Fish were packed individually and stored at 4°C. At defined time interval, two packs, were withdrawn and extracted as described previously (Marseno, 1993; Marseno, *et al.* 1995).

Detection of fish freshness

Immobilized ADPase and 5'nucleotidase were dipped in the fish extract for 20 minutes at room temperature (28°C), then washed in distilled water for 5 minutes followed by dipped in the Chen's reagent (Chen *et al.*, 1956) for 30 minutes.

RESULTS AND DISCUSSIONS

Characterization of free form of ADPase and 5'-nucleotidase

Figure 1 shows the effect of temperature on the activity of ADPase and 5'-nucleotidase in non immobilized condition. The optimum temperature of ADPase and 5'-nucleotidase were 45°C and 50°C, respectively. Both enzymes have the same temperature stability at 4 - 45°C. In case of 5'-nucleotidase in this study, it has same temperature optimum and stability with membrane-bound 5'-nucleotidase (Marseno *et al.*, 1993a) and cytosol 5'-nucleotidase from fish *Sebastes inermis* muscle (Marseno *et al.*, 1993b).

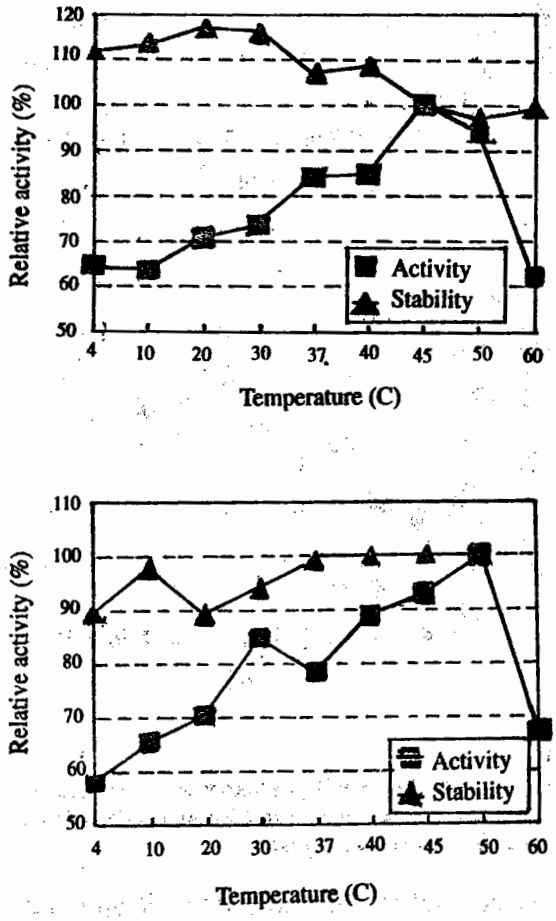


Figure 1. Effect of temperature on activity (■) and stability (▲) of ADPase (upper) and 5'-nucleotidase (lower) in the non-immobilized form.

Figure 2. shows the effect of pH on the activity and stability of ADPase and 5'-nucleotidase. Enzyme 5'-nucleotidase has two optimum pH, 6.5 and 9.0 and stable at pH 7-10. The presence of two optimum pH was found also in 5'-nucleotidase from carp muscle (Tomioka and Endo, 1984), pig intestinal smooth muscle (Burger and Lowenstein, 1970). On the other hand, one optimum pH was found in membrane-bound (pH 8.3) and cytosol (pH 8.1) 5'-nucleotidase from fish *Sebastes inermis* muscle (Marseno *et al.*, 1993a; Marseno *et al.*, 1993b).

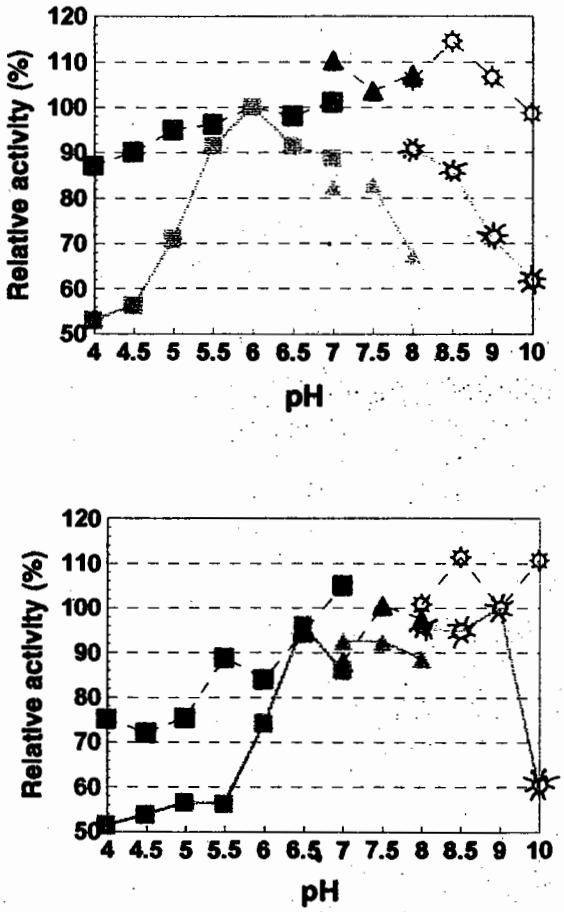


Figure 2. **Effect of pH on activity (dotted line) and stability (solid line) of ADPase (upper) and 5'-nucleotidase (lower) in the non-immobilized form.**

Enzyme ADPase has only one pH optimum at 6.0 and stable at pH 5-10. Although both ADPase and 5'-nucleotidase are classified as membrane-bound enzyme, they have different characteristic on pH. The data show that enzyme ADPase was active in acidic condition, while 5'-nucleotidase was active at basic condition.

Characterization of immobilized ADPase and 5'-nucleotidase

The effect of substrate concentration on immobilized enzyme activity is shown in Figure 3. The enzyme activity increased in concomitant with increased substrate concentration. This fact was reflected by increase in the blue color intensity at higher concentration of substrate.

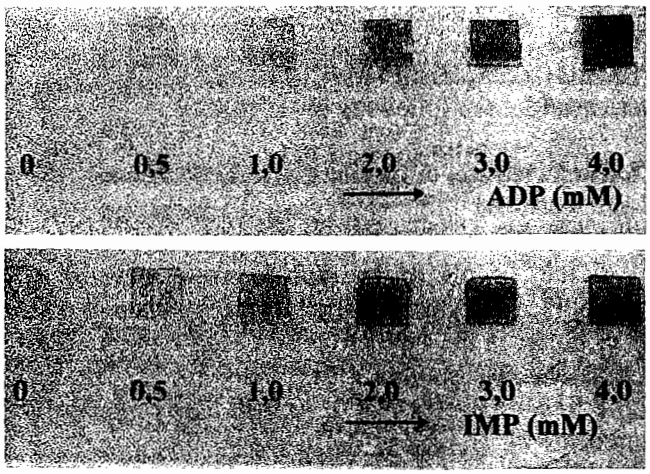


Figure 3. **Effect of substrate concentration on activity of immobilized ADPase (upper) and 5'-nucleotidase (lower).**

The effect of acrylamide concentration on enzyme activity as entraping agent was examined. The data showed that concentration of acrylamide at 7.5 - 12.5% has no effect on the activity of both ADPase and 5'-nucleotidase (Figure 4). Considering this result, both enzymes were immobilized in 7.5% polyacrylamide gel for further studies.

The effect of temperature in activity and stability of immobilized ADPase and 5'-nucleotidase are shown in Figure 5. At 4°C, the activity of both ADPase and 5'-nucleotidase are very low. The activities of ADPase and 5'-nucleotidase are high at 27 - 45°C, and above 45°C the activity of these enzymes decreased gradually. Stability of immobilized ADPase and 5'nucleotidase during chilled (4 - 8°C) and frozen (-20°C) were examined. The data in Figure 5 show that, until 90-th days of storage, either ADPase and 5'-nucleotidase are more stable in frozen storage than that of chilled storage.

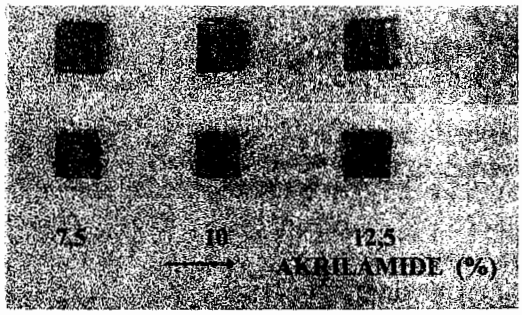
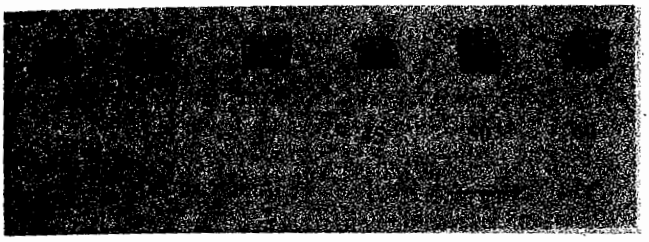


Figure 4. **Effect of acrylamide concentration on activity of immobilized ADPase (upper) and 5'-nucleotidase (lower).**

A. Activity

ADPase

5'-nucleotidase



B Stability

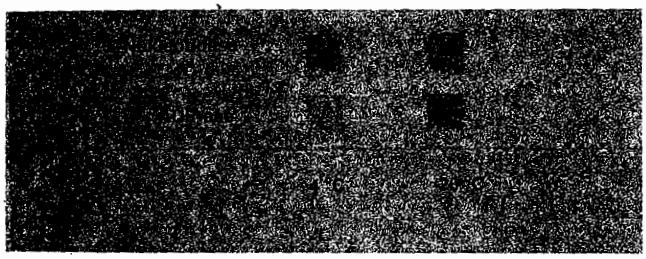


Figure 5. Effect of temperature on acticity (A) and stability (B) of immobilized ADPase and 5'-nucleotidase.

The effect of pH on activity and stability of immobilized ADPase and 5'-nucleotidase are shown in Figure 6. It is clearly shown that ADPase was still active and stable at acidic condition (pH 5.7-7.0) during storage at -20°C for up to 80 days, while 5'-nucleotidase was active and stable at basic condition (pH 7.5-10). Considering this data, it could be stated that there were no differences on pH stability between free and immobilized form of both enzymes. For further experiments, ADPase was immobilized in 7.5% polyzacrylamide gel at pH 6.5, while 5'nucleotidase was immobilized in 7.5% polyacrylamide gel at pH 7.5.

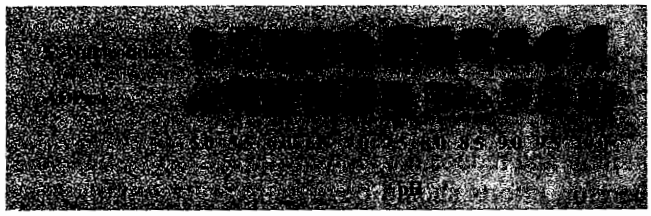


Figure 6. Effect of pH on activity and stability of immobilized ADPase and 5'-nucleotidase after 80 days storaged at -20°C.

Detection of fish freshness

Based on fact that various concentrations of ADP and IMP could be detected by immobilized ADPase and 5'-nucleotidase, respectively, thus, we suggest that the freshness of fish could be detected through ADP and IMP content in fish muscle. In fresh stage, ADP content is high and IMP content is low. On the other hand, in deteriorate stage, both ADP and IMP content are low. The data in Figure 7 show that in the firs few hours of *post morten* ADP and IMP content were high and gradually decreased in concomitant with time of storage. ADP elapses within less than 4 hours, while IMP could not be detected after 12 hours of *post morten'*.

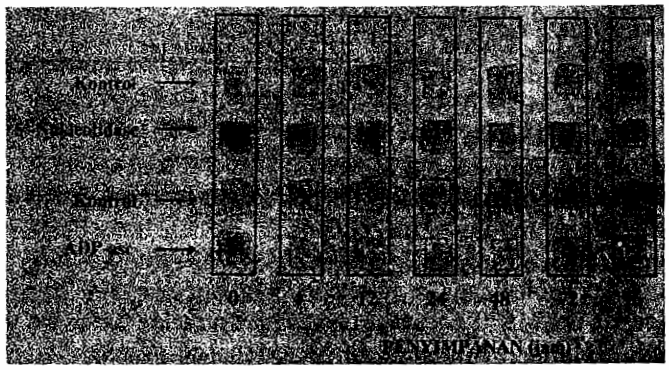


Figure 7. Detection of ADP and IMP content in fish muscle during storage 4°C using immobilized ADpase and 5'-nucleotidase.

CONCLUSIONS

Changes of ADP and IMP content in fish muscle could be detected by ADPase and 5'nucleotidase that immobilized on polyacrylamide gel and this finding can be used to develop a simple method to detect firsh

freshness. However, the presence of free inorganic phosphate derived from othe phosphate-containing compounds such as NADP and creatine phosphate will interfere and reduce the reproducibility of this method. Therefor, efforts should be done in order to eliminate free inorganic phospate.

ACKNOWLEDGEMENT

This work was funded by the Directorate General of Higher Education, Department of Education and Culture, Indonesia. We would like to thank Miss B. Sri Utami and Mr. Srianta for their assistance in laboratory work.

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