Food Chemistry 112 (2009) 880–884

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Isolation and properties of 5′-nucleotidase isolated from jumbo squid (*Dosidicus gigas*) mantle muscle from the Gulf of California, Mexico

R. Pacheco-Aguilar [a], J.C. Ramirez-Suarez [a], F.J. Castillo-Yañez [b], E.A. Peña-Ramos [a], E.M. Valenzuela-Soto [a], E. Marquez-Rios [a,*]

[a] Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera a La Victoria, P.O. Box 1735, Hermosillo, Sonora 83000, Mexico
[b] Departamento de Ciencias Químico Biológicas, Universidad de Sonora, Rosales y Niños Héroes S/N, P.O. Box 1819, Hermosillo, Sonora 83000, Mexico

ARTICLE INFO

Article history:
Received 11 January 2008
Received in revised form 18 June 2008
Accepted 26 June 2008

Keywords:
ATP degradation
Jumbo squid mantle
5′-Nucleotidase
Enzyme activity

ABSTRACT

The enzyme 5′-nucleotidase of jumbo squid (*Dosidicus gigas*) mantle was purified and its SDS–PAGE showed a single band of 33 kDa, whereas a protein with a molecular mass of 107 kDa was detected by gel filtration suggesting a homotrimeric nature of this enzyme. Subunits of the named enzyme were not linked by covalent bonds. Isoelectric focusing of this enzyme showed a pI of 3.6–3.8 and presented a hyperbolic kinetics with V_{max} of 1.16 μM/min/mg of protein, K_m of 1.49 mM, K_{cat} of 3.48 μM of P_i s^{-1} and K_{cat}/K_m relation of 356.52 $((mol/L)^{-1}\ s^{-1})$. Purified enzyme preferred AMP as substrate (by 6.7-folds) than IMP, showing a K_m of 6.34 mM, V_{max} of 0.19 μM/min/mg of protein a K_{cat} of 0.3388 mol of P_i s^{-1} and K_{cat}/K_m relation of 53.44 $((mol/L)^{-1}\ s^{-1})$. The low K_m in relation to purified AMP deaminase of the same organism suggested a high contribution of 5′-nucleotidase in AMP degradation in jumbo squid mantle.

1. Introduction

The ATP degradation in ice stored muscle has been studied since the 1950s, showing a fast increase during the 1970s when HPLC methodology appeared and made the work easier (Howgate, 2005). The ATP quantification and the degradation of its products have mainly been focused in getting the K Index (KI), parameter used to determine the freshness of marine products (Ocano-Higuera, Maeda-Martínez, Lugo-Sánchez, & Pacheco-Aguilar, 2006; Saito, Arai, & Matsuyoshi, 1959). The typical pattern of ATP degradation and its products is as follows, however, it can differ depending on the species: ATP (adenosine triphosphate) → ADP (adenosine diphosphate) → AMP (adenosine monophosphate) → IMP (inosine monophosphate) → HxR (inosine) → Hx (hypoxanthine).

The degradation pattern of these metabolites was studied in jumbo squid (*Dosidicus gigas*, d'Orbigny, 1835) muscle and presented a very different behaviour than most of the muscles that were examined. The ATP was quickly degraded during the first 24 h post-mortem (first day on ice storage) up to Hx, causing a high KI value. This value was not found to be a good indicator of this type of analysis and as a consequence the application of the KI value with the purpose of estimating this species freshness should not be used (Marquez-Rios, Moran-Palacio, Lugo-Sanchez, Ocano-Higuera, & Pacheco-Aguilar, 2007).

The APT degradation was across the ATPases, myodenilate kinase, AMP deaminase, 5′-nucleotidase and purine nucleoside phosphorylase. In most organisms that were studied, the ATP was quickly degraded up to AMP which was mainly achieved in fish by IMP route; whereas in invertebrates, such as clams and squids, AMP degradation could be either by the IMP or adenosine (Ado) routes due to 5′-nucleotidase enzyme action (Hiltz, Bishop, & Dyer, 1974). Both enzymes competed in jumbo squid mantle muscle for the same substrate (AMP) causing a fast degradation of this metabolite in post-mortem muscle. The balance between the action of AMP deaminase and 5′-nucleotidase determined if the purine ring structure was retained as IMP at the nucleotide level or converted to adenosine, a diffusible catabolite with pleitrophic intracellular and extracellular effects (Haas & Sabina, 2003).

It is well known that degradation from ATP to AMP is extremely rapid in molluscs. The AMP is a metabolite that is accumulated in the muscle and converted either to IMP or Ado (adenosine) (Seged-hal, Busalmen, Roldan, Paredi, & Crupkin, 1997). However, this behaviour does not occur in jumbo squid until AMP is quickly degraded up to Hx, it may probably be due to a high enzymatic activity of enzymes responsible for the degradation of these metabolites. Marquez-Rios et al. (2007) reported a high K_m value (13 mM) for AMP deaminase while studying the AMP degradation of jumbo squid mantle muscle. Considering that AMP level in fresh jumbo squid mantle muscle was 0.87 μmol/g, data suggested that the main enzyme responsible for AMP degradation in this organism was 5′-nucleotidase. Hence, the objective if this work was to isolate and partially characterise this enzyme from jumbo

* Corresponding author. Tel.: +52 662 289 2400; fax: +52 662 280 0421.
E-mail address: emarquez@guayacan.uson.mx (E. Marquez-Rios).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.06.062

squid mantle muscle trying to understand why the AMP metabolite was quickly degraded in post-mortem mantle muscle, comparing its kinetic parameters with AMP deaminase enzyme of the same source.

2. Materials and methods

2.1. Experimental animals

Giant squid specimens were caught off the coast of Sonora in the Gulf of California. Immediately after capture, they were carefully gutted and washed with distilled water. Mantles were placed in polyethylene bags, stored in alternated layers of ice and transported to the lab, taking the entire sampling procedure a time of 4 h. Once in the lab, mantles were placed again in polyethylene bags and frozen at −86 °C to be used 2 weeks later.

2.2. Preparation of crude extract

According to the methodology described by Marseno, Hori, and Miyazawa (1993a), the crude extract was prepared by homogenising 50 g of jumbo squid mantle muscle with 4 volumes of 40 mM sodium citrate (pH 4.5) containing 20 mM $MgCl_2$, 25 mM NaCl (buffer A) and 0.1 mM PMSF (phenylmethylsulphonyl fluoride). First muscle mantle and buffer were homogenised in a mixer for 2 min in 30 s intervals, second that mixture was centrifuged by 2 h at 48,000*g*/2 h/4 °C, and finally, the supernatant was used as the crude extract for enzyme assay.

2.3. Enzyme assay

In accordance with the methodology described by Fiskie and Subbarow (1925), 40 μL of enzymatic extract were taken and added with 360 μL of 10 mM AMP in 40 mM sodium citrate buffer, 0.1 mM DTT (dithiothreitol), 110 mM NaCl, 20 mM $MgCl_2$, and 20 mM $CaCl_2$ the, 5′-nucleotidase activity was measured by means of inorganic phosphorus quantification. The reaction mixture was incubated for 10 min at 50 °C. The reaction was stopped with ammonium molibdate in 0.5 M HCl and 10% acid ascorbic. The mixture was incubated at 40 °C for 40 min to promote the blue colour formation that was read at 820 nm using a Cary 50 Bio-Rad UV–Vis, spectrofotometer (EL07013465, Varian Instruments, WC, California). The enzymatic unit (U) was defined as μmol of free inorganic phosphorous/min/mg of protein.

2.4. Ammonium sulphate precipitation

A known volume of the crude extract was taken and measured at 55% of saturation with ammonium sulphate. Once the mixture was dissolved, it was centrifuged at 3000*g*/20 min/4 °C. The precipitate was discarded and supernatant was dialysed against 40 mM sodium citrate (pH 4.5) containing 1 mM $MgCl_2$, 110 mM NaCl, 1 mM $CaCl_2$ (buffer B) and 0.1 mM PMSF, using a cellulose membrane (ID × length = 2.7 × 30 cm), keeping molecular weights $\geqslant$12 kDa. Dialysis was performed twice against 20 volumes of buffer B with a period of 5 h for every change.

2.5. Concanavalin A sepharose affinity chromatography

Concanavalin A was washed with 3 volumes of 40 mM sodium citrate (pH 4.5) containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.02% of NaN_3 (TBS–NaN_3 buffer) and with 3 volumes of buffer C, which contained buffer B plus 0.1% triton X-100, both at a flow rate of 10 mL/h. The dialysed fraction was loaded into a concanavalin A column at a flow rate of 10 mL/h, then 3 volumes of buffer C were applied at a flow rate of 20 mL/h until absorbance readings at 280 nm were constant. Next, enzyme was eluted with 60 mL of methyl α-D-manopiranoside in buffer C at a flow rate of 10 mL/h, collecting fractions of 2 mL. Protein concentration was determined by the Coomassie blue method, using bovine serum albumin as a standard (Bradford, 1976).

2.6. 5′-AMP sepharose affinity chromatography

Active fractions obtained by Con A-affinity chromatography were pooled and twice dialysed against 30 volumes of buffer D (40 mM Tris–HCl, pH 7.4 and 0.15 M NaCl) with buffer changes every 5 h. The 5′-AMP sepharose 4B affinity column (Amersham Pharmacia Biotech, Uppsala, Sweden) was washed first with 200 mL distilled water and then with 30 mL TBS–NaN_3 buffer (0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.02% of NaN_3). Finally, it was equilibrated with 30 mL of buffer D. Dialysed fraction was loaded into the column at a flow rate of 5 mL/h and run with buffer D (same flow rate) until absorbance readings at 280 nm were constant. The enzyme was eluted with 20 mM AMP in buffer D at a flow rate of 5 mL/h. Protein concentration was carried out according to Bradford (1976). To remove AMP, the active fractions were pooled and twice dialysed against 30 volumes of buffer D for 10 h (Marseno et al., 1993a).

2.7. Polyacrylamide gel electrophoresis

According to Laemmli (1970), SDS–PAGE was carried out on a 14% polyacrylamide slab gel in order to determine purity and subunit molecular weight of isolated enzyme. Gels were stained with silver nitrate (Copeland, 1994; Whitaker, 1994).

2.8. Molecular weight (MW) determination

Molecular weight of native enzyme was estimated by gel filtration using a superdex 200 HR 10/30 packed column (1 × 30 cm) (Amersham biosciences, Piscataway, NJ). Standards used were: thyroglobulin (669 kDa), horse ferritin (440 kDa), bovine catalasa (232 kDa), bovine lactate dehydrogenase (140 kDa) and bovine serum albumin (66 kDa) (Amersham biosciences, Piscataway, NJ). The column was pre-equilibrated with 40 mM Tris–HCl pH 7.0. Sample and standard proteins (200 μL) were applied at a flow rate of 5 mL/h, and 0.5 mL fractions were collected.

2.9. Electrofocusing

The isoelectric point (pI) of the isolated enzyme was evaluated by analytical electrofocusing in a thin layer polyacrylamide flat gel (LKB-Ampholine PAG-Plate) containing ampholynes over a pH range 3.5–9.5. An isoelectric focusing calibration kit containing 11 proteins of known pI was used as a reference (polyacrylamide flat gel and calibration kit were of Sigma–Aldrich, Mexico). Proteins were stained with silver nitrate (Westermeier et al., 1997).

2.10. Kinetic parameters

Michaelis–Menten constant (K_m), maximum velocity (V_{max}) and catalysis constant (K_{cat}) were determined. The initial velocity of the enzymatic reaction was evaluated at 50 °C by varying AMP substrate concentration between 0.2 and 15 mM. K_m and V_{max} were evaluated by non-linear regression analysis after plotting the velocity against substrate concentration. Turnover number or K_{cat} was obtained by dividing V_{max} by enzyme molar concentration, which was estimated using its molecular weight as determined by SDS–PAGE (Copeland, 2000).

3. Results and discussion

3.1. 5′-Nucleotidase purification

A crude extract with 3.55 mg protein/mL, 1.93 total units and specific activity of 0.0054 U/mg was obtained. Protein of the extract was precipitated with ammonium sulphate up to 50% saturation; the precipitate was discarded and the supernatant, with the highest concentration of adenosine/min/mL, was collected. The supernatant had a concentration of 0.48 mg protein/mL, with specific protein activity of 0.0062 U/mg, having a recovery of 85.49% and purification of 1.14-fold (Table 1). The supernatant was submitted to dialysis and then loaded onto a concanavalin A sepharose affinity chromatography column (Fig. 1a). Chromatography is one of the most common processes used to purify this enzyme due to the affinity of this resin with proteins rich in carbohydrates, as it is the case of 5′-nucleotidase (Marseno, Hori, & Miyazawa, 1993b).

A pool of fractions between 10 and 25 was made. It had an activity of 1.05 total units, corresponding to 63.63% of all the units loaded into the column. It also presented a specific activity of 0.77 U/mg protein increasing purification fold to 142.59. A second step concanavalin A chromatography, similar to the one used in this study (but as a first step) was employed by Finni et al. (2003) to purify 5′-nucleotidase from bull plasma. They recovered 68% of the total units loaded into the column and increased the specific activity from 8 to 20.6 U/mg of protein. Grondal and Zimmermann (1987) purified 5′-nucleotidase from torpedo electric organ recovering 56% of the total units loaded into concanavalin A, and increasing the specific activity from 0.24 to 0.87 U/mg of protein. Experimental results in the present study, as well as information reported in the literature, indicate that purification on concanavalin A was a good tool to purify this enzyme. The previous information was corroborated by means of SDS–PAGE, detecting few bands in this purification step.

The pool obtained from concanavalin A chromatography was dialysed and loaded onto 5′-AMP sepharose. Fig. 1b shows the elution pattern of 5′-nucleotidase. We applied to the column 1.05 activity units, recovery only 0.25 (23.8% of applied units), thus obtaining 12.9% yield, a specific activity of 5.15 U/mg and a purification of 953.7-fold. Chromatography on 5′-AMP sepharose is one of the most common techniques used in final stages of purification process for this enzyme. Tkacz-Stachowska, Lechward, and Skladanowski (2005) purified 5′-nucleotidase from pigeon breast muscle increasing, by means of this chromatography, the specific activity increased from 0.60 to 4.3 U/mg of protein, the purification from 18- to 131-fold and obtaining a 20.6% final yield.

3.2. Purity and molecular weight

SDS–PAGE was used to verify the enzyme purification process (Fig. 2a). During this process, the efficiency of concanavalin A chromatography was observed, noticing few protein bands (line 4). Further purification with 5′-AMP sepharose chromatography showed just one protein band corresponding to 33 kDa molecular weight. The pool, resulting from the last chromatography, was applied onto a gel filtration column to obtain the native enzyme MW, which was estimated to be of 107 kDa (Fig. 3). These results suggested that 5′-nucleotidase of jumbo squid mantle is a homotrimeric enzyme that defers from values of different enzyme sources reported in the literature. Finni et al. (2003) working with bull plasma and dog heart, reported a trimer with native MW of 200 kDa and 66 kDa subunits and a tetramer of 166 kDa with 43 kDa subunits, respectively. On the other hand, Höglund and Reichard (1990) reported a native MW of 45 kDa with subunits of 22 kDa, proposing a homodimeric nucleotidase in human placenta. Results in this study and the available information, indicate that 5′-nucleotidase is a multimeric enzyme of varied molecular weight and subunits number.

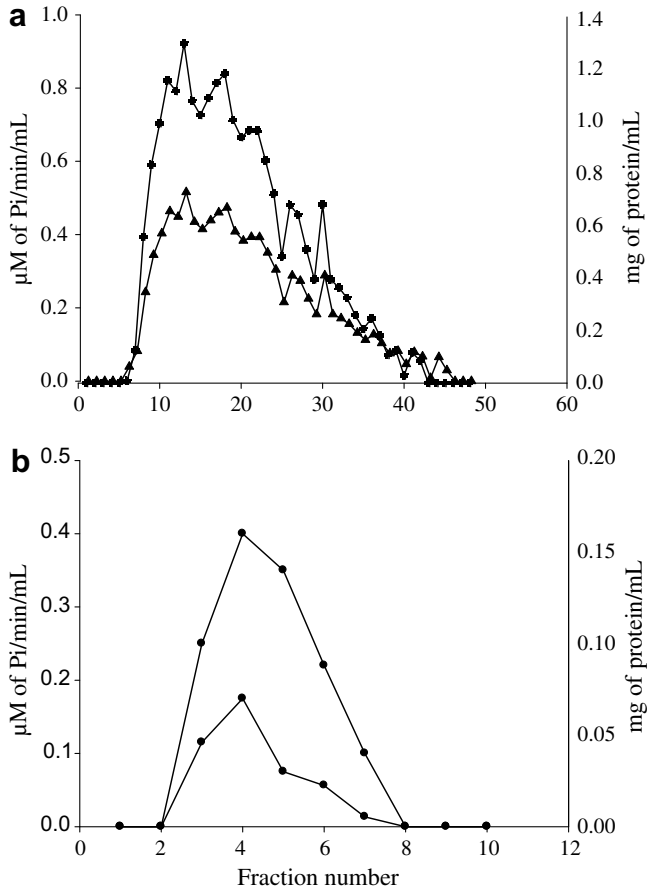

Fig. 1. (a) Concanavalin A sepharose affinity chromatography. Elution with 0.5 M of α-D-methyl manopyranose. Protein (•), 5′-nucleotidase activity (▲). (b) 5′-AMP sepharose affinity chromatography. Elution with 20 mM AMP. Protein (■), 5′-nucleotidase activity (▲).

Table 1
5′-Nucleotidase purification from jumbo squid flying

Purification step	Protein (mg/mL)	Total act (units)	Esp. act (U/mg)	Yield (%)	Purification
Crude extract	3.55	1.93	0.0054	100	1
Ammonium sulphate Precipitation	0.48	1.65	0.0062	85.49	1.14
Concanavalin A	1.02	1.05	0.77	54.40	142.59
5′-AMP sepharose	0.05	0.25	5.15	12.95	953.70

U: μmol of adenosine formed per minute per mg of protein.

3.3. Isoelectric point

The isoelectric point of 5′-nucleotidase obtained from 5′-AMP sepharose chromatography showed two protein bands (Fig. 2b) that corresponded to isoelectric points of 3.6 and 3.8. It should be due to the possible existence of isoforms of this enzyme, since gel filtration showed just one protein peak (Fig. 3) and SDS–PAGE just one protein band (Fig. 2a), as was shown earlier. On the other hand, there was a possibility that the native enzyme could be denatured during isoelectrofocusing analysis and unfolded into

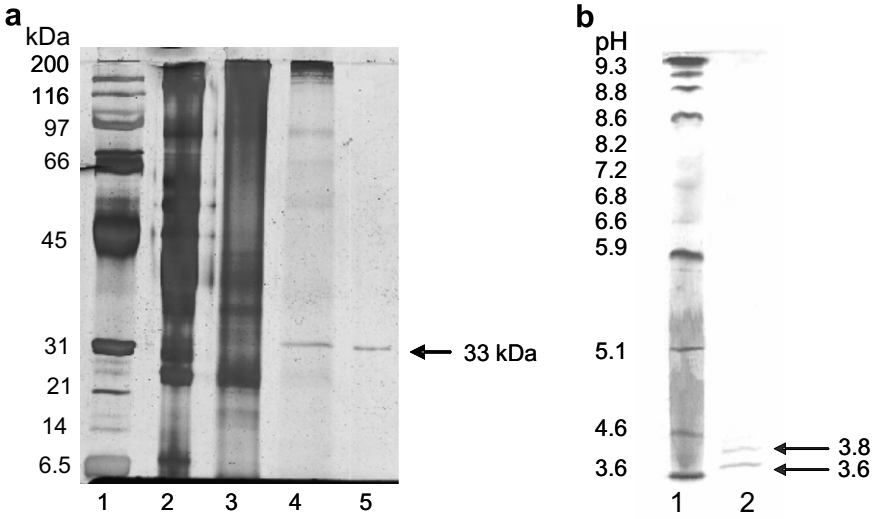

Fig. 2. (a) SDS–polyacrylamide gel electrophoresis (PAGE) throughout purification of 5′-nucleotidase. Polyacrylamide Gel to 14%, protein bands were stained with silver nitrate. Line 1, broad range standard (biorad); line 2, crude extract; line 3, dialysed; line 4, concanavalin A chromatography; line 5, 5′-AMP sefarose pool. (b) Isoelectrofocusing of 5′-nucleotidase. Line 1, protein markers (3.6–9.3); line 2, 5′-AMP sepharose affinity chromatography.

its subunits; therefore, it could show both, one isoelectric point for the native form and another for its subunits.

Isoelectric points in the acid zone indicated the predominance of acidic amino acids like glutamic and aspartic acids in relation with those basic amino acids. Garcia et al. (1997) detected a different isoelectric point value for 5′-nucleotidase from *Escherichia coli*, reporting an isoelectric point of 4.6. In another study, Finni et al. (2003) reported isoelectric points of 6.58, 6.33, 6.19 and 6.07 for 5′-NT-1, 5′-NT-2, 5′-NT-3 and 5′-NT-4 isoforms, respectively.

As it was previously mentioned, 5′-nucleotidase is a species dependant enzyme of varied molecular weight that presents great variations in its primary structure. This could bring about differences in their isoelectric points, which explain the discrepancies of results in this study with those reported in the literature.

3.4. Kinetic

The initial velocity in the substrate range from 0 to 15 mM showed a hyperbolic behaviour that obeyed Michaelis–Menten's kinetic (Fig. 4). The 5′-nucleotidase enzyme from jumbo squid mantle showed a K_m of 1.49 mM, and a V_{max} of 0.29 μM/min/mg of protein. These results indicated a high affinity of the enzyme for its substrate in comparison with AMP deaminase enzyme purified from the same organism (Márquez, 2006); nevertheless the K_m value obtained in this study differs from at reported in the literature for other marine species. Marseno et al. (1993b), studying a ecto-5′-nucleotidase from rock fish (*Sebastes inermes*), reported a K_m of 21 μM; Grondal and Zimmermann (1987) reported a K_m value of 38 μM for 5′-nucleotidase from ray muscle, whereas Senger, Pacheco, Dutka, Reis, and Bonan (2004) found a K_m of 135 μM for 5′-nucleotidase from zebra fish muscle (*Danio rerio*).

In this study, the experimental K_{cat} value and the relation K_{cat}/K_m were 0.5171 mol of P_i s^{-1} and 356.62 $((mol/L)^{-1}\ s^{-1})$, respectively. Similar results were reported by Tkacz-Stachowska et al. (2005), who isolated and characterised a cytosol 5′-nucleotidase from pigeon breast muscle with a K_{cat} value of 0.1065 mol of P_i s^{-1} and a catalytic efficiency of 57 $((mol/L)^{-1}\ s^{-1})$. In another study, Hunsucker, Spychala, and Mitchell (2001) worked with 5′-nucleotidase from human, finding a K_{cat} value of 5.13 mol of P_i s^{-1} and a catalytic efficiency of 1734 $((mol/L)^{-1}\ s^{-1})$.

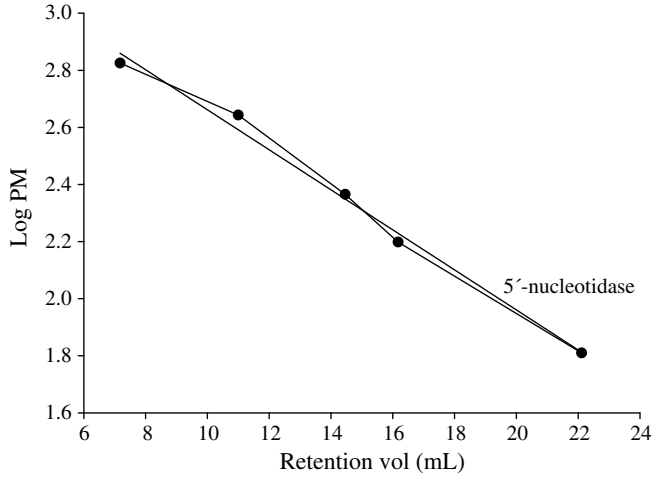

Fig. 3. Estimation of native molecular weight of purified 5′-nucleotidase on superdex 200 gel filtration. The logarithm of the molecular weight was plotted against volume of retention. The peaks eluted were determinated by optical density (280 nm) and enzymatic activity was measurement as described under Section 2.

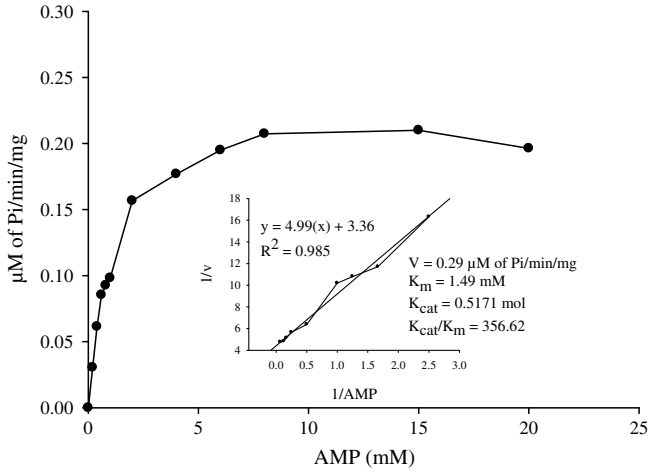

Fig. 4. Saturation curve for 5′-nucleotidase kinetics. AMP concentrations from 0.2 to 15 mM; enzyme concentration: 2 μg; buffer: 40 mM sodium cacodylate, pH 4.5, 20 mM $MgCl_2$, 20 mM $CaCl_2$ and 200 mM NaCl at 50 °C.

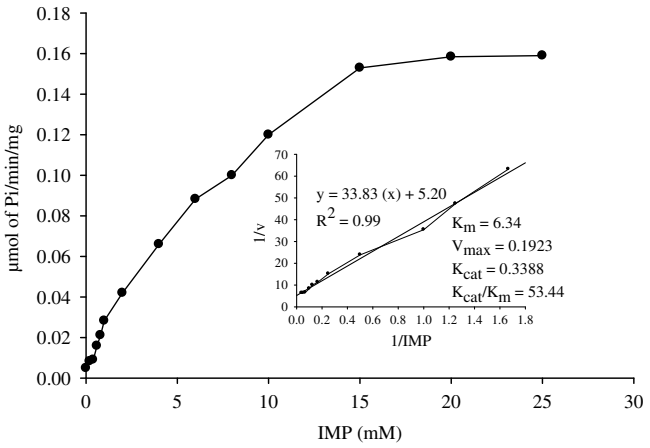

Fig. 5. Saturation curve for 5′-nucleotidase kinetics. IMP concentrations from 0.2 to 25 mM; enzyme concentration: 2 µg; buffer: 40 mM sodium cacodylate, pH 4.5, 20 mM $MgCl_2$, 20 mM $CaCl_2$ and 200 mM NaCl at 50 °C.

In squid mantle stored in ice the ATP degradation up to Hx occurred during the first 24 h post-mortem (Marquez-Rios et al., 2007). In this period, AMP levels fluctuated between 0.87 and 1.14 mM, concentrations in the order of the K_m value for the 5′-nucleotidase. Since K_m of the AMP deaminase from the same organism was found to be of 13 mM (Márquez, 2006), the previous information suggested a major production of adenosine in relation to the IMP formation. Thus, considering the AMP low levels in post-mortem muscle during the first 24 h post-mortem, AMP deaminase enzyme might have low impact over AMP degradation.

Experimental results of this study indicated that the catalytic properties of 5′-nucleotidase from squid mantle were similar to those from other invertebrates. On the other hand, AMP deaminase properties differed from other marine organisms (Márquez, 2006). Therefore, our results agreed with others in the literature, where it is shown that 5′-nucleotidase enzyme were more active that AMP deaminase in invertebrate organisms, as jumbo squid. Thus, it is probable that AMP degradation in squid mantle occurs mainly via the 5′-nucleotidase enzyme.

The 5′-nucleotidase enzyme is characterised for acting on 5′-ribo and 5′-deoxynucleotides. In the present study, the catalytic properties of this enzyme were determined using IMP as substrate (Fig. 5). Results indicated that enzyme from squid mantle acted more efficiently on AMP than on IMP-like substrates; therefore, the purified 5′-nucleotidase favours AMP-like substrates, which was the fundamental tool to distinguish between nucleotidases cN-I and cN-II (cN-II had a preference for IMP as substrate). The 5′-nucleotidase from jumbo squid mantle showed a K_m value of 6.34 mM, V_{max} of 0.19 µM/min/mg of protein and a K_{cat} of 0.3388 mol of P_i s^{-1} (Fig. 5) using IMP-like as substrate.

The catalytic efficiency is the ideal tool that helped us estimate or compare the efficiency of one enzyme on a different substrate. The catalytic efficiency of this enzyme, using AMP as substrate, was of 356.62 $((mol/L)^{-1}\ s^{-1})$, whereas its acting over IMP was of 53.44 $((mol/L)^{-1}\ s^{-1})$, thus being its efficiency 6.7 times higher over AMP. This demonstrated that the nucleotidase isolated in the present study is a cN-I.

4. Conclusions

The 5′-nucleotidase enzyme was purified from jumbo squid mantle. According to results, it seems to be an important enzyme in AMP degradation in this invertebrate due to its low K_m value in relation to that reported for AMP deaminase enzyme from the same source. The catalytic efficiency of 5′-nucleotidase enzyme was very similar to that isolated from the same organism. However, the AMP concentration in squid mantle is closed to K_m value of 5′-nucleotidase, therefore, this enzyme seems to be determinant in the AMP degradation route in this organism. On the other hand, in squid mantle post-mortem existed adequate levels of AMP for 5′-nucleotidase action. Therefore, this is a key enzyme involved in AMP degradation during ice storage from jumbo squid.

References

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Analytical Biochemistry, 72*, 248–254.

Copeland, R. A. (1994).*Methods for protein analysis*. New York, NY: Chapman & Hall. pp. 39–58.

Copeland, R. A. (2000). *Enzymes: A practical introduction to structure mechanism and data analysis* (2nd ed.). New York: Wiley-VCH. pp. 188–265.

Finni, C., Talamo, F., Cherri, S., Coli, M., Floridi, A., Ferrara, L., et al. (2003). Biochemical and mass spectrometric characterization of soluble ecto-5′-nucleotidase from bull seminal plasma. *Biochemistry Journal, 372*, 443–451.

Fiskie, C. H., & Subbarow, Y. (1925). The colorimetric determination of phosphorous. *Journal of Biological Chemistry, 66*, 375–400.

Garcia, L., Chalet, L., Kettlun, A. M., Collados, L., Chiang, M., Traverso-Cori, A., et al. (1997). Kinetic characteristics of nucleoside mono-, di- and triphosphatase activities of the periplasmic 5′-nucleotidase of *Escherichia coli*. *Comparative Biochemistry and Physiology, 117B*, 135–142.

Grondal, E. J. M., & Zimmermann, H. (1987). Purification, characterization and cellular localization of 5′-nucleotidase from torpedo electric organ. *Biochemistry Journal, 245*, 805–810.

Haas, A. M., & Sabina, R. L. (2003). Expresión, purification and inhibition of in vitro proteolysis of human AMPD2 (isoform L) recombinant enzymes. *Protein Expression and Purification, 27*, 293–303.

Hiltz, D. F., Bishop, L. J., & Dyer, W. J. (1974). Accelerated nucleotide degradation and glycolysis during warming to and subsequent storage at −5 °C of prerigor quick-frozen adductor muscle of the sea scallop (*Placopecten magellanicus*). *Journal of the Fisheries Research Board of Canada, 31*, 1181–1187.

Höglund, L., & Reichard, P. (1990). Cytoplasmic 5′(3′)-nucleotidase from human placenta. *Journal of Biological Chemistry, 265*, 6589–6595.

Howgate, P. (2005). A review of the kinetics of degradation of inosine monophosphate in some species of fish during chilled storage. *International Journal of Food Science and Technology, 40*, 1–13.

Hunsucker, S. A., Spychala, J., & Mitchell, B. S. (2001). Human cytosolic 5′-nucleotidase: I. Characterization and role in nucleoside analog resistance. *The American Society for Biochemistry and Molecular Biology, 276*, 10498–10504.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head bacteriophage T4. *Nature, 227*, 680–685.

Márquez, R. E. (2006). Caracterización de la ruta metabólica de degradación de adenosina monofosfato en manto de calamar gigante (*Dosidicus gigas*) del Golfo de California. Tesis de doctorado en ciencias, Centro de Investigación en Alimentación y Desarrollo, A.C. Coordinación de Tecnología de Alimentos de Origen Animal.

Marquez-Rios, E., Moran-Palacio, E. F., Lugo-Sanchez, M. E., Ocano-Higuera, V. M., & Pacheco-Aguilar, R. (2007). Postmortem biochemical behaviour of giant squid (*Dosidicus gigas*) mantle muscle stored in ice and its relation with quality parameters. *Journal of Food Science, 72*(7), 356–362.

Marseno, D. W., Hori, K., & Miyazawa, K. (1993a). Purification and properties of membrane-bound 5′-nucleotidase from black rockfish (*Sebastes inermis*) muscle. *Journal of Agriculture and Food Chemistry, 41*, 863–869.

Marseno, D. W., Hori, K., & Miyazawa, K. (1993b). Purification and properties of cytosol 5′-nucleotidase from black rockfish (*Sebastes inermis*) muscle. *Journal of Agriculture and Food Chemistry, 41*, 208–1212.

Ocano-Higuera, V. M., Maeda-Martínez, A. N., Lugo-Sánchez, M. E., & Pacheco-Aguilar, R. (2006). Postmortem biochemical and textural changes in the adductor muscle of catarina scallop stored at 0 °C. *Journal of Biochemistry, 30*, 373–389.

Saito, T., Arai, K., & Matsuyoshi, M. (1959). A new method for estimating the freshness of fish. *Bulletin of the Japanese Society of Scientific Fisheries, 24*, 749–752.

Segedhal, A., Busalmen, J. P., Roldan, H. A., Paredi, M. E., & Crupkin, M. (1997). Post-mortem changes in adenosine triphosphate and related compounds in mantle of squid (*Illex argentinus*) at different stages of sexual maturation. *Journal of Aquatic Food Product Technology, 6*(4), 43–55.

Senger, M. R., Pacheco, E., Dutka, R., Reis, M., & Bonan, C. D. (2004). Ecto-5′-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology, 139B*, 203–207.

Tkacz-Stachowska, K., Lechward, K., & Skladanowski, A. C. (2005). Isolation and characterization of pigeon breast muscle cytosolic 5′-nucleotidase-I (cN-I). *Acta Biochimica Polonica, 52*, 789–796.

Westermeier, R., Fichmann, J., Gronau, S., Schickle, H., Theßeling, G., & Wiesner, P. (1997). *Electrophoresis in practice* (2nd ed.). VCH Wiley Company.

Whitaker, J. R. (1994). *Principles of enzymology for food sciences* (2nd ed.). New York: Marcel Dekker Inc.. pp. 63–115.