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Isolation and properties of AMP deaminase from jumbo squid (*Dosidicus gigas*) mantle muscle from the Gulf of California, Mexico

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Abstract

Adenosine monophosphate (AMP) deaminase was purified from jumbo squid mantle muscle by chromatography in cellulose phosphate, Q-Fast and 5′-AMP sepharose. Specific activity of 2.5 U/mg protein, 4.5% recovery and 133.68 purification fold were obtained at the end of the experiment. SDS–PAGE showed a single band with 87 kDa molecular mass, native PAGE proved a band of 178 kDa, whereas gel filtration detected a 180 kDa protein, suggesting the homodimeric nature of this enzyme, in which subunits are not linked by covalent forces. Isoelectric focusing of this enzyme showed a p*I* of 5.76, which agrees with p*I* values of AMP deaminase from other invertebrate organisms. AMP deaminase presented a kinetic sigmoidal plot with V_{max} of 1.16 μM/min/mg, K_m of 13 mM, K_{cat} of 3.48 $\mu M.s^{-1}$ and a K_{cat}/K_m of 267 $(mol/L)^{-1}.s^{-1}$. The apparent relative low catalytic activity of jumbo squid muscle AMP deaminase in the absence of positive effectors is similar to that reported for homologous enzymes in other invertebrate organisms.

Keywords: AMP deaminase; Jumbo squid mantle; Purification

1. Introduction

Several methods have been developed to assess the quality of fish and fish products, including sensory, chemical, microbial and physical protocols. Recently biochemical indexes are the most used for fish freshness determination. Changes in ATP and its degradation products [ATP (adenosine triphosphate) → ADP (adenosine diphosphate) → AMP (adenosine monophosphate) → IMP (inosine monophosphate) → INO (inosine) → Hx (hypoxanthine)] are one of the most relevant issues in postmortem biochemistry of marine organisms. The quantification of these metabolites gives a very useful index, called *K*-index or *K*-value, which is a good freshness indicator for a great number of marine species (Ehira & Uchiyama, 1987).

In most invertebrates, ATP is degraded quickly to accumulate AMP, which is later degraded gradually, thus causing a linear increase of *K* value. Marquez-Rios, Morán-Palacio, Lugo-Sánchez, Ocano-Higuera, and Pacheco-Aguilar (2007), studying ATP degradation in jumbo squid mantle (*Dosidicus gigas*), detected very low AMP levels after 24 h of ice storage; however, they also found a high level of Hx, thus giving a *K* value of 74% at the first day of ice storage. These data suggest a high enzymatic activity from enzymes involved in AMP degradation (up to Hx) in this species (Picher, Burch, Hirsh, Spychala, & Boucher, 2003).

ATP degradation pathways change among species. In fish, AMP degradation is mainly by the IMP route due to the action of AMP deaminase, whereas in invertebrates, such as clams and squids, the AMP degradation can be via IMP or Ado (adenosine) route due to the action of 5′-nucleotidase enzyme (Hiltz, Bishop, & Dyer, 1974). Both enzymes in jumbo squid mantle compete for the same

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substrate (AMP) thus causing a fast degradation of this metabolite in postmortem muscle. Degradation of AMP in this organism occurs fast, probably due to the fact that AMP degrading enzymes are very active in squid mantle; as a consequence, it is important to study the 5′-nucleotidase and AMP deaminase enzymes. In this experiment, AMP deaminase, the enzyme that catalyzes the hydrolytic deamination of adenosine-5′-monophosphate to inosine-5′monophosphate and ammonium ion was investigated. The AMP deaminase enzyme, important cellular energy metabolism regulator (Haas & Sabina, 2003a), participates in the intermediary metabolism as a component of the purine nucleotide cycle, governing the AMP, IMP and adenylosuccinate interactions and serves as one conduit for the entrance of catabolized amino acid into the TCA cycle. The AMP deaminase reaction is also critical to signal other aspects of metabolism through competition for available substrate with AMP-preferring cytosolic 5′-nucleotidase (cNT-I). The balance between AMP deaminase and cNT-I determines if the purine rings structure is retained at the nucleotide level as IMP, or metabolized to adenosine, a diffusible catabolite with pleiotropic intracellular and extracellular effects (Haas & Sabina, 2003b). AMP deaminase is an important enzyme in the biochemical postmortem of this species. Hence, the objective of the present research was to isolate and partially characterize this enzyme obtained from jumbo squid flying mantle with the aim to understand why the AMP metabolite is quickly degraded in postmortem jumbo squid muscle.

2. Materials and methods

2.1. Experimental animals

Giant squid specimens were captured off the coast of Sonora in the Gulf of California; they were carefully gutted and washed with distilled water. The mantles were placed in polyethylene bags in alternated layers of ice and mantle for transportation. Once in the laboratory, mantles were washed with cold distilled water, placed again in polyethylene bags, and stored to −86 °C until used.

2.2. Enzyme assay

AMP deaminase activity was evaluated quantifying IMP accumulation in a Hewlett Packard high resolution liquid chromatographer (Hewlett-Packard Co. Waldbrom, Germany) with an injection valve of 20 μL capacity loop, equipped with a UV–vis absorbance detector. A Beckman ultrasphere ODS RP 18 reverse phase column (5μm, 25 cm X 3.9 mm i.d.) (Beckman Instruments, Inc., Fullerton, CA) was employed for separation. The mobile phase was 0.1 M phosphate buffer pH 7.0 (0.04 M KH_2PO_4 and 0.06 M K_2HPO_4). Eluate was monitored at 249 nm. The reaction mixture consisted of 50 μL of enzymatic extract, 500 μL 20 mM AMP in 40 mM cacodylate pH 6.0, 0.18 M KCl and 1 mM dithiothreitol (DTT). The assay was carried out at 35 °C for 10 min, stopping the reaction by introducing the reaction mixture tube in boiling water for 3 min. Then, the reaction mixture was centrifuged at 15 000*g*/4 °C/5 min. The supernatant was filtered in a 0.22 μm filter and 20 μL were injected into HPLC equipment. Flow rate was 1.2 mL/min and column temperature of 50 °C. Activity unit (U) was defined as the amount of enzyme which produces 1 μ mole of IMP per min at 35 °C.

2.3. Preparation of crude extract

Tissue was homogenized with 8 volumes of phosphate buffer 0.089 M (KH_2PO_4 0.054 M, K_2HPO_4 0.035 M), containing 0.18 M KCl and 0.1 mM DTT at pH 6.5 (buffer A). The homogenate was stirred for one hour in a plate stirrer at 4 °C, and then was centrifuged at 48 000*g*/20 min/4 °C. The supernatant was taken as the crude extract for the enzyme assay.

2.4. Cellulose phosphate chromatography

Cellulose phosphate (3 g dry weight) was added to crude extract, which was previously washed with 0.5 N KOH, H_2O, 0.5 N HCl, H_2O and finally with 5×10^{-3} M EDTA prior to equilibration with extraction buffer. This suspension was stirred 30 min at 4 °C. The supernatant solution was siphoned off and discarded. The cellulose phosphate slurry was transferred to a column which was washed off with buffer A until no protein was eluted using a flow rate of 30 mL/h. Protein determination was made by the Bradford method (1976). Then, enzyme was eluted at a flow rate of 30 mL/h/4 °C using a linear gradient of KCl concentration (0.18–1.0 M). Collected fractions with enzyme activity were mixed and dialyzed two times for 5 h against 30 volumes of buffer B (40 mM Tris–HCl, pH 7.8).

2.5. Q-sepharose fast flow chromatography

Q-sepharose-fast flow (Amersham Pharmacia Biotech, Uppsala, Sweden) was packed into a column and equilibrated with buffer B. The dialyzed sample was applied to Q-sepharose-fast flow column a flow rate of 10 mL/h/4 °C. After the sample application, the column was washed off with buffer B until no protein was detected in the elution buffer. Proteins adhered to the resin were eluted with a linear gradient of KCl concentration (0.0–1.0 M) in buffer B. Protein determination was made by the Bradford method (1976). Active fractions were dialyzed two times for 5 h against 30 volumes of buffer C (l0 mM Tris–HCl, pH 7.3 and 0.15 M NaCl).

2.6. 5′-AMP sepharose chromatography

5′-AMP sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden) was washed off with 200 mL of buffer C and then was loaded into the column at a flow rate of 5 mL/h/4 °C. Then the column was washed off with

3 volumes of buffer C until no protein was detected in the elution buffer. AMP deaminase was eluted with 20 mM AMP in buffer C at flow rate of 10 mL/h/ 4 °C. Protein determination was made by the Bradford method (1976).

2.7. Polyacrylamide gel electrophoresis

Non-reducing SDS–PAGE was carried out according to the method of Laemmli (1970) to determine the purity and subunit molecular weight of the isolated enzyme on a 14% polyacrylamide slab gel. The proteins bands were stained with silver nitrate (Copeland, 1994). The same procedure was used for native PAGE, but on 8% polyacrylamide slab gel.

2.8. Protein determination

Protein was measured by the Coomassie blue method, using bovine serum albumin as a standard (Bradford, 1976).

2.9. Molecular weight (MW)

The native molecular weight of the enzyme was estimated by gel filtration using a superdex 200 HR 10/30 (1x30 cm) packed column. (Amersham biosciences, Piscataway, NJ). The standards used were: thyroglobulin (669 kDa), equine ferritin (440 kDa), bovine catalase (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 flow rate of 5 mL/h, and 0.5 mL fractions were collected.

2.10. Electrofocusing

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

2.11. Kinetic parameters

The initial velocity of the enzymatic reaction was evaluated at 35 °C by varying AMP substrate concentration between 1 and 45 mM. Kinetic constants K_m and V_{max} were determined by means of Hill plots and 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. AMP deaminase purification

Purification of AMP deaminase was carried out in a first step according to Smiley, Berry, and Suelter (1967) by means of cellulose phosphate chromatography with a 34.8% recovery, which is very similar to the 36% reported by these authors (Table 1). In our study, AMP deaminase was eluted with 0.50 M KCl (Fig. 1), while in a study by Ogasawara, Goto, and Yamada (1983) using rabbit muscle, they eluted the same enzyme and an isoform using cellulose phosphate with 0.4 M NaCl and 0.35 M NaCl from white and red muscle, respectively. In another study, Swieca, Rybakowska, Koryziak, Klimek, and Kaletha (2004) eluted the enzyme from hen stomach using 1.45 M KCl. All these studies indicate differences in elution profile of AMP deaminase due to the effect of saline concentration, which depends of the enzyme source, being this an inherent characteristic of each enzyme.

Dutka et al. (2004) and Smiley et al. (1967) have reported the enzyme purification using a one step cellulose phosphate chromatography only. However, in the present study, the single use of this type of chromatography showed the eluted fraction, where AMP deaminase is supposed to be pure, was impure as demonstrated by SDS–PAGE analysis (Fig. 2a). Same results were obtained by

Table 1
AMP deaminase purification from jumbo squid flying mantle

Purification step	Protein (mg/mL)	Total act (units)	Esp. act (U/mg)	Yield (%)	Purification (Fold)
Crude extract	2.22	33.24	0.018	100	1
Cellullose phosphate	0.43	11.58	0.314	34.8	16.57
Q-Fast	0.45	6.16	0.662	18.53	35.40
5′-AMP sepharose	0.05	1.50	2.500	4.50	133.68

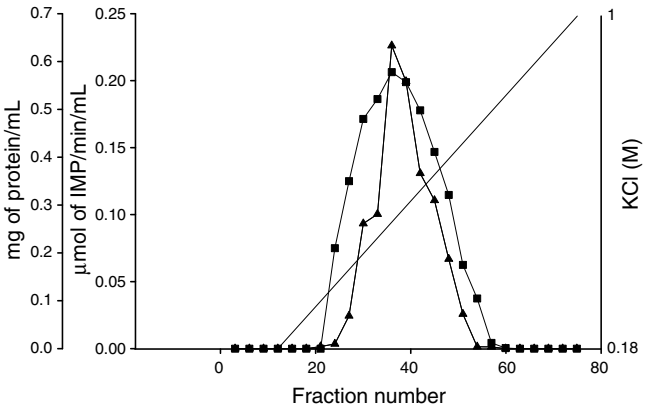

Fig. 1. Cellulose phosphate chromatography. Elution profile using a linear gradient with KCl concentration increment from 0.18 to 1.0 M. Protein (■), AMP deaminase activity (▲), KCl (-).

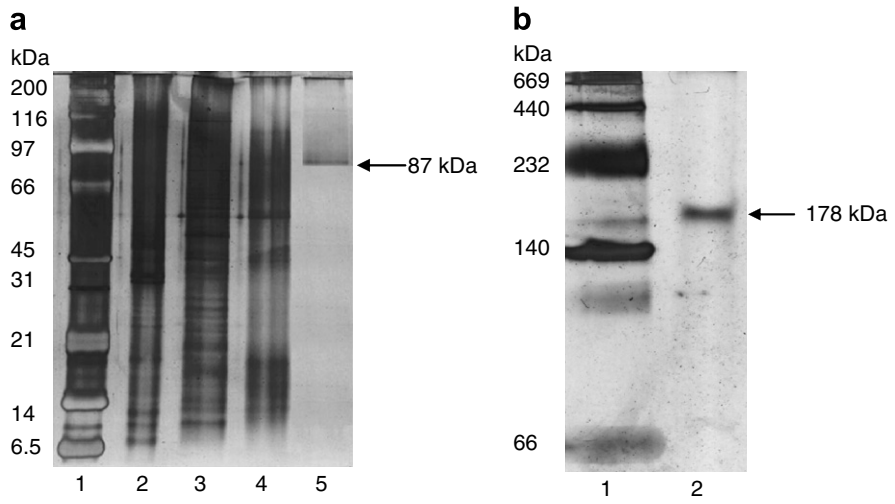

Fig. 2. (a) SDS–Polyacrylamide gel electrophoresis (PAGE) throughout purification of AMP deaminase. Polyacrylamide Gel to 14%, protein bands were stained with silver nitrate. Line 1, broad range Standard (Bio-Rad); line 2, crude extract; line 3, cellulose phosphate chromagraphy pool; line 4, Q-sepharose Fast flow chromatography pool; line 5, 5′-AMP sepharose pool. (b) Nondenaturing polyacrylamide gel electrophoresis (PAGE) of affinity 5′-AMP sepharose chromatography pool. Polyacrylamide Gel to 8%, protein bands were stained with silver nitrate. Line 1, high molecular weight (Amersham); line 2, affinity 5′-AMP sepharose chromatography pool.

Thakkar, Janero, Yarwod, Sharif, and Hreniuk (1993) who demonstrated that cellulose phosphate was not enough to purify AMP deaminase due to the fact that during this purification step did not obtained one protein band on SDS–PAGE. These results indicate the need to apply complementary alternatives of purification. Thakkar et al. (1993) established a purification procedure for AMP deaminase from rabbit heart using cellulose phosphate chromatography, followed by gel filtration and finally affinity with 5′-AMP sepharose chromatography. In the present study, this same approach was followed except that ionic interchange chromatography was used instead of gel filtration.

Fractions 27–45 (4 mL per fraction) from cellulose phosphate chromatography were pooled, dialyzed and loaded into Q-sepharose fast flow. From this chromatographic step, fractions 6–11 were eluted with 0.35 M KCl (Fig. 3) and pooled (0.45 mg/mL final protein concentration), showing an activity of 6.16 total units and a specific activity of 0.662 U/mg, indicating a yield of 18.5% and a purification fold of 35.4 (Table 1).

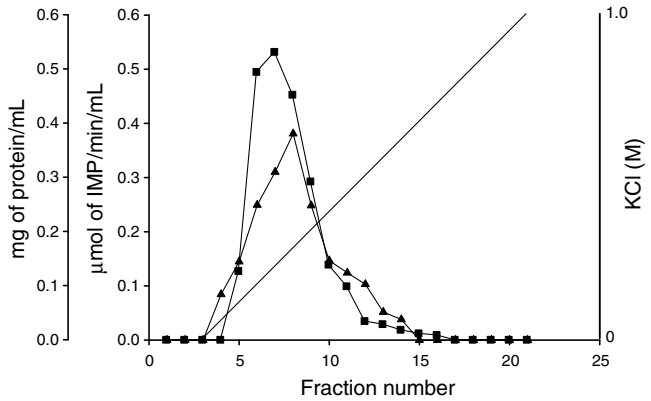

Fig. 3. Q-sepharose fast flow chromatography. Elution profile using a linear gradient with KCl concentration increment from 0 to 1.0 M. Protein (■), AMP deaminase activity (▲), KCl (-).

Affinity chromatography on 5′-AMP sepharose has had little application in AMP deaminase purification. In the present study, this last chromatography (Fig. 4) showed good results, eluting the enzyme with 20 mM AMP. Enzyme activity analysis resulted in 1.5 total units of activity, 2.5 U/mg specific activity and a 4.5% yield as final result of the purification process, increasing its purification fold from 35.4 to 133.

3.2. Purity and molecular weight

Purification process of the enzyme was evaluated with non-reducing SDS–PAGE. After 5′-AMP sepharose chromatography, a single protein band was obtained with molecular weight (MW) of 87 kDa, indicating the enzyme purity (Fig. 2a). When purified sample was analyzed by native electrophoresis another single protein band of 178 kDa was observed (Fig. 2b). Both results indicate the homodimeric nature of the enzyme, where subunits association is without covalent interactions. A study by Stankiewicz, Spychala, Skladanowski, and Zydowo (1979) who purified AMP deaminase from perch (*Sander lucioperca*) reported it had a MW of 280 kDa and proposed a homotetrameric structure with 70 kDa subunits. In another study, Ranieri-Raggi, Martini, Sabbatini, Moir, and Raggi (2003) purified this enzyme from rabbit skeletal muscle and reported a MW of 85 kDa in SDS–PAGE. It is necessary to mention that most of the AMP deaminases purified from different sources are reported to have a MW of 70 kDa approximately, though the gene that codifies for the same (AMPD1) would generate a 86–87 kDa protein (Haas & Sabina, 2003a; Merkler, Wali, Taylor, & Schramm, 1989;

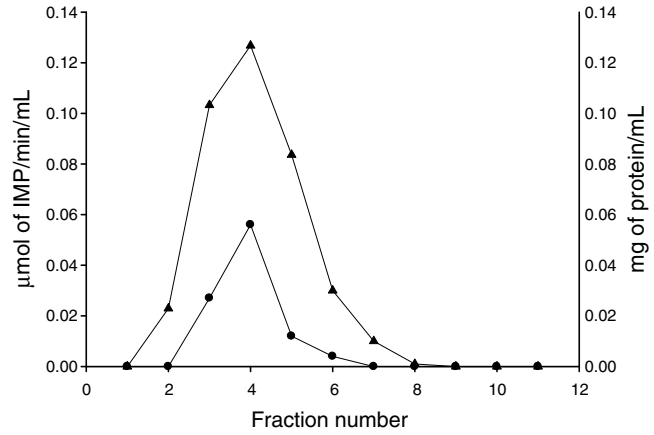

Fig. 4. Affinity 5′-AMP sepharose chromatography. Elution profile using 20 mM AMP. Protein (■), AMP deaminase activity (▲).

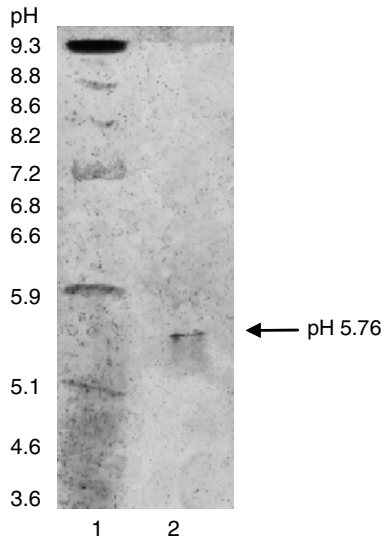

Fig. 5. Isoelectrofocusing of AMP deaminase. Line 1, protein markers (3.6–9.3); line 2, affinity 5′-AMP sepharose chromatography pool.

Ranieri-Raggi et al. 2003). This discrepancy in MWs could be assumed due to a proteolytic action from enzymes present during the purification procedures, as it was reported by Thakkar et al. (1993). Our results may suggest that AMP deaminase did not suffer this suggested hydrolysis since its MW (SDS–PAGE) was 87 kDa, very close to the reported for the enzyme. However, this claim is very risky, because little or nothing is known about the gene coding for the enzyme AMP deaminase in jumbo squid mantle *D. gigas*, therefore it is necessary to conduct molecular biology studies about the identity of this gene. Moreover it is important to mention that in preliminary studies the enzyme AMPD was purified in presence and absence of protease inhibitors obtaining the same results, in addition in our research group we have been working hard with squid mantle, finding nil or negligible proteolytic activity during storage in ice for a 15 days period (Ibarra 2006).

3.3. Electrofocusing

The isoelectric point (p*I*) of AMP deaminase was of 5.76 (Fig. 5), which is in agreement with the reported by Stankiewicz (1982) from prawn *Procambarus clarkia* (5.8–6.1). Stankiewicz and Spychala (1981) investigated the p*I* for this enzyme in rabbit white muscle, hen white and red muscle, rat skeletal muscle, perch skeletal muscle and frog skeletal muscle, reporting values of 4.79, 5.02, 4.93, 4.33, 4.55 and 4.80, respectively, arguing vertebrates enzymes had a minor p*I*. Boosman and Chilson (1976) quantified AMP deaminases amino acids from hen and rabbit muscle, reporting a predominance of acid amino acids like aspartic and glutamic over the basic lysine and arginine. These data explains the lower p*I* values (on the acid region) for these AMP deaminases. Probably, our enzyme slightly differs from the amino acid composition reported for vertebrates. Further studies need to be done in order to elucidate this. Isoelectric focusing confirmed the purity of AMP deaminase isolated from jumbo squid mantle.

3.4. Kinetic parameters

The study of catalytic properties of an enzyme is very useful in the knowledge of its activity in its natural environment. In this study, kinetic studies were made to determine the intrinsic parameters of this enzyme, such as K_m, V_{max} and K_{cat}. The initial rates were determined from 0 to 45 mM range of substrate concentration, obtaining a sigmoidal plot behavior, typical for multimeric enzymes like AMP deaminase (Fig. 6). Coffee and Solano (1977) who worked with this enzyme in rat skeletal muscle also obtained a sigmoidal behavior using 5 mM KCl, whereas at 100 mM KCl its kinetic behavior changed to hyperbolic with very similar K_m and V_{max}. Literature reports that the sigmoidal behavior obtained for AMP deaminase could be transformed to hyperbolic in the presence ATP and ADP

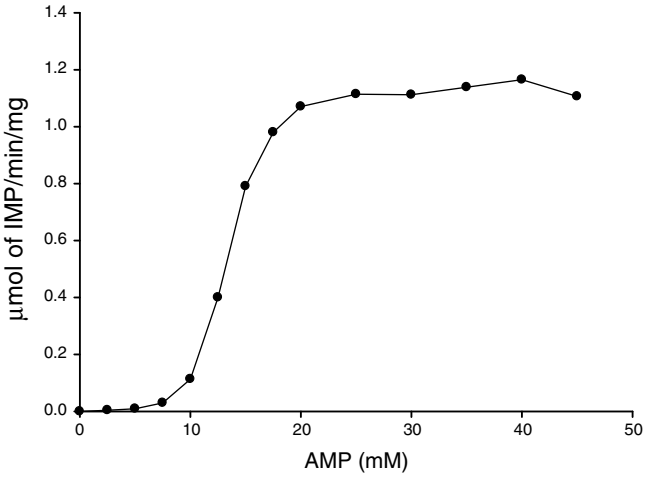

Fig. 6. Saturation curve for AMP deaminase kinetics. AMP concentrations from 0 to 45 mM; enzyme concentration: 2.5 µg; buffer: 40 mM sodium cacodylate, pH 6.0, 0.18 M KCl to 35 °C.

to obey the Michaelis–Menten kinetics (Kaletha & Nowak 1988). This kinetic behavior is easy to analyze by means of the transformation of the data into its reciprocal doubles to obtain K_m and V_{max}.

The AMP deaminase from jumbo squid flying muscle showed a K_m of 13 mM, a V_{max} of 1.1 μM/min/mg and Hill's coefficient of 2.9. The initial rate change (V_i) with regard to the substrate concentration (from 0 to 10 mM range) was low (Fig. 6), which indicates a low affinity of the enzyme for its substrate giving as result a high K_m value compared with other AMP deaminases. Lushchak, Smirnova, and Storey (1998) studying AMP deaminase from marine scorpion found a K_m of 0.5 mM, whereas Martini, Ranieri-Raggi, Antonietta, and Raggi (2001) working with AMP deaminase from rabbit muscle reported a K_m value of 0.5 mM.

Hill's coefficient indicates the cooperative degree of one multimeric enzyme with relation to its substrate. Values > 1 indicate cooperativity among the enzyme subunits, which means that the union of a molecule of substrate to a subunit affects the union of another molecule of substrate to another subunit (Copeland, 2000). The kinetic enzymatic results were in agreement with those expected for a multimeric enzyme, with a typical sigmoidal behavior of an allosteric enzyme. Hill's coefficient was of 2.9, indicating a high cooperativeness among both subunits of the enzyme. This high Hill's coefficient should be due to the very low activity of this enzyme to substrate concentrations below its K_m. Mosharov, Vitvitsky, and Ataullakhanov (1998) found a sigmoidal behavior for this enzyme in human erythrocytes, reporting a K_m value of 4 mM, a Hill's coefficient of 2.3 and V_{max} of 1.6 μM/min/mL. On the other hand Haas and Sabina (2003a) studied three AMP deaminase isoforms in human which showed a sigmoidal behavior with Hill's coefficients of 2.5, 2.5 and 3.2.

The K_{cat} value and K_{cat}/K_m relation (catalytic efficiency) for jumbo squid muscle AMP deaminase were 3.48 IMP $M.s^{-1}$ and 267.7 $[(mol/L)^{-1}.s^{-1}]$, respectively. Notably AMP deaminase from marine scorpion muscle exhibits a K_{cat} of 205.3 $M.s^{-1}$ and a high catalytic efficiency of 369,603 $[(mol/L)^{-1}.s^{-1}]$ due to its low K_m value (Lushchak et al. 1998). In another study, Leray, Raffin, and Winninger (1979), reported a K_{cat} value of 38.75 $M.s^{-1}$ and a catalytic efficiency of 5613 $[(mol/L)^{-1}.s^{-1}]$, for AMP deaminase from rainbow trout gills (*Salmo gairdneri*). Thus, compared to these marine vertebrate organisms, jumbo squid muscle AMP deaminase has a relative low catalytic activity in the absence of positive effectors, which is consistent with what is observed in other invertebrate organisms (Fujisawa & Yoshino, 1987). This may suggest that adenylate catabolism in jumbo squid muscle proceeds predominantly through 5′-nucleotidase. However, additional characterization of AMP deaminase in the presence of small molecule regulators is necessary in order to understand its physiological role in this marine invertebrate organism.

4. Conclusions

AMP deaminase from jumbo squid mantle is an allosteric enzyme with low catalytic activity in the absence of positive effectors. Given the low concentration of AMP in squid muscle, these results suggest that 5′-nucleotidase may have a more prominent role in the adenylate catabolism of this invertebrate organism. However, further characterization of AMP deaminase in the presence of small molecule effectors is needed to clarify its physiological significance in jumbo squid muscle.

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