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Purification and characterisation of adenosine nucleosidase from *Coffea arabica* young leaves

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Abstract

An adenosine nucleosidase (ANase) (EC 3.2.2.7) was purified from young leaves of *Coffea arabica* L. cv. Catimor. A sequence of fractionating steps was used starting with ammonium sulphate salting-out, followed by anion exchange, hydrophobic interaction and gel filtration chromatography. The enzyme was purified 5804-fold and a specific activity of 8333 nkat mg^{-1} protein was measured. The native enzyme is a homodimer with an apparent molecular weight of 72 kDa estimated by gel filtration and each monomer has a molecular weight of 34.6 kDa, estimated by SDS–PAGE. The enzyme showed maximum activity at pH 6.0 in citrate-phosphate buffer (50 mM). The calculated K_m is 6.3 μM and V_{max} 9.8 nKat.

Keywords: *Coffea arabica* L.; Rubiaceae; Coffee; Enzyme purification; Adenosine nucleosidase; Adenosine; Purine metabolism

1. Introduction

Adenosine nucleosidase (ANase) is an enzyme from the purine metabolic pathway, which hydrolyses adenosine to adenine and ribose, enabling the recycling of both metabolites. The activity of this enzyme has been described in several plant species such as spinach beet (Poulton and Butt, 1976), Jerusalem artichoke (Le Floc'H and Lafleuriel, 1981), yellow lupin (Abusamhadneh et al., 2000), barley (Guranowski and Schneider, 1977), wheat (Chen and Kristopeit, 1981) and tea (Imagawa et al., 1979). Its importance in plants is not well understood but its functioning in catabolizing tissues like cotyledons makes adenine available for transportation and reutilization in other organs (Guranowski and Pawelkiewicz, 1978). The enzyme's ability to de-ribosilate natural cytokinin ribosides indicates its possible role in the inter-conversion of these plant growth regulators (interchange between cytokinin base, riboside and ribotide forms) (Auer, 2002), and consequently in cytokinin transport (Gillisen et al., 2000) and activity regulation (Schulz et al., 2001). Recently Koshiishi et al. (2001) proposed a new pathway for the biosynthesis of caffeine in tea leaves. A chloroplastidial ANase is suggested to be involved in this new pathway. To understand how this enzyme relates to each plant metabolic pathway, it is necessary to purify and characterise the enzyme and to isolate the gene that encodes this protein. The present work describes the purification and characterisation of ANase from young leaves of *Coffea arabica* L.

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2. Results and discussion

2.1. Adenosine nucleosidase activity in young leaves of C. arabica L.

A sample of 1.4 nkat mg^{-1} of protein of ANase (11.26 nkat g^{-1} of fresh weight) was quantified in a crude extract of young leaves of *C. arabica* L., by following the consumption of adenosine at 261 nm. The consumption of adenosine instead of adenine production was chosen due to the higher absorption coefficient exhibited by this substrate (13.8 $mmol^{-1} cm^{-1}$ at 261 nm) in relation to adenine (12.8 $mmol^{-1} cm^{-1}$ at 262 nm). Activity was confirmed when assays with substrate and boiled extract, with extract but without substrate and with substrate, but without extract, revealed no adenosine consumption. Similar results in ANase activity (31.8 nkat g^{-1} of fresh weight) were also obtained by Koshiishi et al. (2001) in crude extracts of young tea leaves.

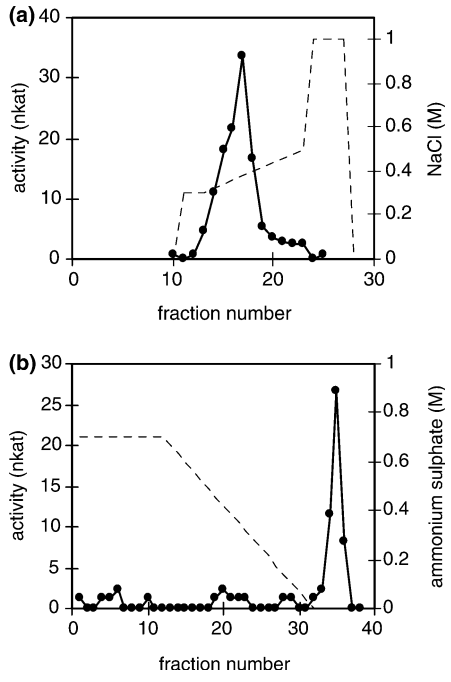

Fig. 1. ANase activity in anion exchange chromatography (a) and hydrophobic interaction chromatography (b). (•) ANase activity. Dotted lines refer to salt gradient profiles.

2.2. Purification of adenosine nucleosidase

A sequence of purification steps starting from crude extract fractionation by salting-out, followed by anion exchange (Fig. 1(a)) and hydrophobic interaction (Fig. 1(b)), to gel filtration was used to purify ANase (Table 1). The enzyme evidenced high hydrophobic properties, as assessed by the elution from a Superose HR 5/5 column when ammonium sulphate was absent in elution buffer. This step was crucial in ANase purification, since a single peak corresponding to this enzyme was detected during protein elution in absence of ammonium sulphate. The molecular weight of the native enzyme form calculated from gel filtration is 72 kDa. Purified ANase has a specific activity of 8333 nkat mg^{-1} protein (Table 1). The SDS–PAGE of a sample from this last purification step revealed one major protein band of 34.6 kDa (Fig. 2). Considering the native molecular weight estimated by gel filtration, we suggest that the enzyme is composed of two identical subunits. In barley and yellow lupin the native enzyme was also characterised as a dimer, with molecular weight of 66 and 72 kDa respectively, and subunits of 33 kDa (Guranowski and Schneider, 1977; Abusamhadneh et al., 2000). Three enzyme forms were found by Imagawa et al. (1979) from

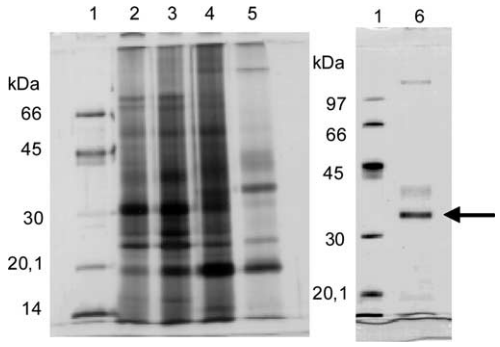

Fig. 2. SDS–PAGE of protein fractions with ANase activity. (lane 1) SDS–PAGE standards of low M_r range, (lane 2) crude extract, (lane 3) 20–60% ammonium sulphate, (lane 4) anion exchange chromatography, (lane 5) hydrophobic interaction chromatography, (lane 6) gel filtration chromatography. Black arrow indicates ANase subunit.

Table 1
Purification of ANase from leaves of *Coffea arabica* L

Step	Total activity (Nkat[a])	Total volume (ml)	Total protein (mg)	Specific activity (nKat mg^{-1} prot)	Purification fold
Crude extract	600	62	418.13	1.4	1
Ammonium sulphate (20–60%)	1200	24	158.45	7.6	5
AAMono Q	1160	22	17.55	66.0	45
Phenyl-superose	800	17	1.44	554.0	389
Superdex 200	252	6	0.03	8333.0	5804

[a] 1 nKat (1 nmol of adenosine consumed per second).

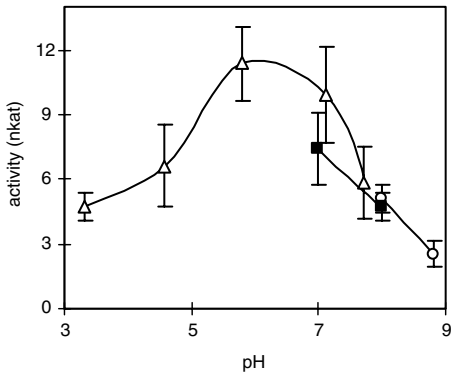

Fig. 3. Effect of pH on ANase activity. (■) Phosphate buffer, (○) Tris–HCl buffer, (△) citrate-phosphate buffer.

tea leaves, by means of CM-cellulose column chromatography. All had the same molecular weight (68 kDa) and similar biochemical properties.

2.3. Enzyme characterisation

The purified enzyme shows an optimum activity at pH 6.0 (Fig. 3). At this pH the enzyme's K_m for adenosine was 6.3 μM, and V_{max} was 9.8 nkat (Fig. 4), using the Lineweaver–Burk regression. In different plant species, the optimum pH for this enzyme ranges from 4.5 in spinach beet (Poulton and Butt, 1976) to 7.5 in yellow lupin (Abusamhadneh et al., 2000). This enzyme has an unusually broad pH range activity. In our work the enzyme maintained 42% of its maximal activity at pH 3.3. The estimated K_m is within the range of those obtained for the enzyme purified from other plants (Poulton and Butt, 1976; Guranowski and Schneider, 1977; Chen and Kristopeit, 1981; Le Floc'H and Lafleuriel, 1981; Abusamhadneh et al., 2000).

3. Conclusions

The results achieved in this work indicate the presence of a single ANase in young coffee leaves. The optimum pH of this enzyme was determined to be 6 suggesting that its activity is optimal in the cytosol. In this work however, ANase was found to have a broad pH range, maintaining half of its maximum activity in acidic (pH 4.5) as well as in basic (pH 7.7) conditions, which suggests it might be functional in different cell compartments. Koshiishi et al. (2001) refer to the existence of a chloroplastidial ANase (chl-ANase) in tea leaves that metabolises the adenosine pool that is thought to feed the caffeine biosynthetic pathway in chloroplasts. However, this enzyme was not isolated and characterised. In this work any conclusions with respect to the existence of a chl-ANase in coffee leaves and its function in caffeine biosynthesis is speculation. However, if the statements of Koshiishi et al. (2001) are applied also to the coffee plant, there is a possibility of the presence of a chl-ANase isoform in the purified ANase obtained from young coffee leaves. Imagawa et al. (1979) were able to separate three ANase forms using CM-cellulose column chromatography. The enzymes had identical molecular weight and optimum pH between 4 and 4.5. The enzymes showed identical behaviours for other biochemical parameters. The subcellular localisation of the three isoforms was not determined.

A chl-ANase may exist in coffee plant that is implicated in caffeine biosynthesis. The identification of this enzyme as well as the gene that codify the protein will allow studying the exact role of chl-ANase in caffeine biosynthetic pathway.

4. Experimental

4.1. General experimental procedures

All reagents used were 99% of purity grade or superior. Adenine was purchased from Sigma and adenosine from Fluka.

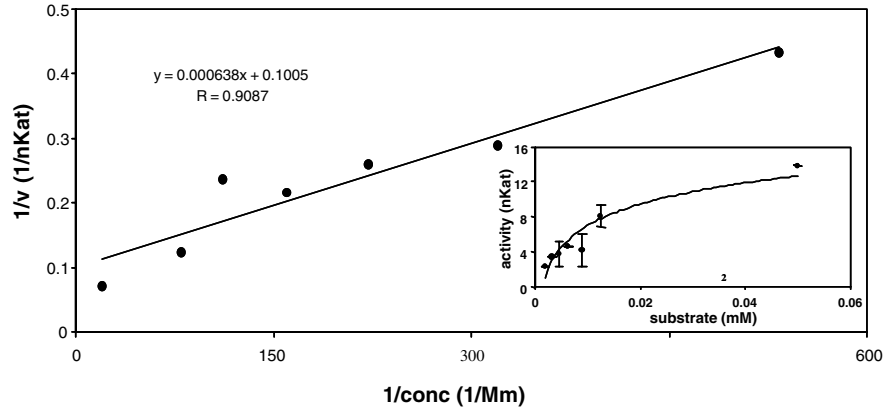

Fig. 4. Lineweaver–Burk plot of ANase activity (v) against adenosine concentration (conc.). Insert: Michaellis–Menten plot with error bars.

4.2. Plant material

Young leaves of *C. arabica* L. cv. Catimor (3–6 cm long) were collected from plants grown at the Centro de Investigaşão das Ferrugens do Cafeeiro, Instituto de Investigaşão Cientíacute fica Tropical (Oeiras, Portugal). After harvesting, leaves were frozen and stored at 80 °C.

4.3. Protein extraction and quantification

According to Negishi et al. (1988), 52 g of frozen leaves were reduced to powder and homogenised in cold acetone, filtered and washed with cold acetone containing ascorbic acid (0.1% w/v). Proteins were extracted in 520 ml Tris–HCl (100 mM) (pH 7.5) with 10 mM 2-mercaptoethanol, 0.5% (w/v) ascorbic acid (extraction buffer), Polyvinylpolypyrrolidone (PVPP) (2% w/v), and centrifuged at 20,300 *g*, for 40 min at 4 °C. Supernatant was brought to 80% saturation with ammonium sulphate and centrifuged in the same conditions. The pellet was dissolved in 30 ml extraction buffer and loaded onto a Hiprep 26/10 desalting column (Amersham Biosciences) with Tris–HCl (20 mM) (pH 8.0) (buffer A). The chromatography was carried out in a FPLC system with a 5 $\mathrm{ml\,min^{-1}}$ elution rate. Protein elution was monitored at 280 nm. Protein was quantified in an Ultrospec 4000 (Amersham Biosciences) at 280 nm using bovine serum albumin (BSA) as a standard.

4.4. Enzyme purification

The crude extract was successively fractionated with ammonium sulphate at 20%, 40%, 60% and 80% of saturation. Fractions with ANase activity were loaded onto a Mono Q HR 5/5 column (Amersham Biosciences) equilibrated with buffer A. Protein elution was achieved increasing the concentration of NaCl to 1 M in buffer A, with an elution rate of 0.5 $\mathrm{ml\,min^{-1}}$. Fractions with ANase activity were mixed with equal volume of ammonium sulphate (1.4 M) in buffer A. With this procedure the final ammonium sulphate concentration in protein sample was below the lowest concentration of this salt used for salting-out, allowing an increase of protein hydrophobicity without achieving precipitation. Protein fractions were loaded onto a Phenyl superose HR 5/5 column (Amersham Biosciences) equilibrated with Tris–HCl (20 mM) with ammonium sulphate (0.7 M) (pH 8.0). Protein elution (0.5 $\mathrm{ml\,min^{-1}}$) was obtained by decreasing the concentration of ammonium sulphate in buffer A until zero. ANase fractions were concentrated and loaded onto a Superdex 200 HR 10/30 column (Amersham Biosciences). Buffer A with 150 mM NaCl was used at an elution rate of 0.2 $\mathrm{ml\,min^{-1}}$. Superdex 200 HR 10/30 was calibrated with the protein standards ferritin (440 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and trypsinogen (24 kDa). All chromatographies were performed in a FPLC system (Amersham Biosciences) and protein fractions detected at 280 nm.

4.5. Assay for ANase activity

Adenosine and adenine absorption coefficients (ε) were determined as the ratio between the absorbance at the wavelength of maximum absorption for the molecule (UV range 200–400 nm), and the molecule concentration. The range of concentrations tested varied from 0.005 to 0.045 mM. Compounds were dissolved in Tris–HCl (50 mM) with 1 mM $MgCl_2 \cdot 6H_2O$ (pH 8.0).

Enzyme assays were performed in an Ultrospec 4000 at 261 nm. The reaction was started by adding 20–25 μl of sample to the reaction mixture composed of Tris–HCl (50 mM), $MgCl_2 \cdot 6H_2O$ (1 mM) and adenosine (0.16 mM) (pH 8.0) (final volume of 500 μl). Tris–HCl was used instead of phosphate buffer to avoid the activity of adenosine deaminase and adenosine phosphorylase. The slope of adenosine decay was converted to total activity (nKat) using the Eq. (1):

$$\mathrm{nKat} = \frac{\mathrm{slope}}{\varepsilon} 1.67 \times 10^4 \left(\mathrm{nmol\,s^{-1}}\right) \quad (1)$$

The 'slope' refers to the absorbance variation as a function of time ($\mathrm{abs\,min^{-1}}$), ε to the absorption coefficient for adenosine ($\mathrm{mmol\,cm^{-1}}$), and 1.67×10^4 to the constant that converts the result to $\mathrm{nmol\,s^{-1}}$. Three assays were used to confirm the activity, one excluding the substrate, another excluding the extract, and a third with a boiled extract.

4.6. SDS–PAGE

Protein electrophoresis was carried out according to Laemmli (1970). Acrylamide gels (12%, w/v) were silver stained as described by Blum et al. (1987). Protein standards of low molecular weight (Amersham Biosciences) were used for protein molecular weight determination.

4.7. Enzyme characterisation

Optimum pH, K_m and V_{max} were determined from Superdex 200 HR 10/30 purified fractions; three replicates were made for each assay. The adenosine concentration ranged between 1.875 and 50 μM for K_m and V_{max} determination.

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