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Cloning and biochemical characterization of APIT, a new L-amino acid oxidase from *Aplysia punctata*

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Abstract

The purple ink of the sea hare *Aplysia punctata* contains a 60 kDa protein with tumoricidal activity. This *A. punctata* ink toxin (APIT) kills tumor cells within 6–8 h in an apoptosis independent manner by the production of high amounts of hydrogen peroxide which induce a necrotic form of oxidative stress. Here, we describe the biochemical features of APIT associated with its anti-tumor activity. APIT is a weakly glycosylated FAD-binding L-amino acid oxidase that catalyzes the oxidative deamination of L-lysine and L-arginine and thereby produces hydrogen peroxide (H_2O_2), ammonia (NH_4^+) and the corresponding α-keto acids. The tumoricidal effect is completely abrogated in the absence of the amino acids L-lysine and L-arginine. The enzyme is stable at temperatures from 0 to 50 °C. Similar to other FAD-binding enzymes, it is resistant against tryptic digest. Even digest with proteinase K fails to degrade the enzyme. Cloning of the APIT gene and subsequent sequencing revealed a FAD-binding domain followed by a so-called GG-motif, which is typical for L-amino acid oxidases. Strongest homology exists to escapin, aplysianin A precursor, the cyplasins L and S and achacin.

Keywords: L-amino acid oxidase; Flavoprotein; Defensive ink; Cytotoxic; Hydrogen peroxide

1. Introduction

Sea hares lack the protection of a hard outer shell but have developed effective chemical defense mechanisms (Johnson, 1999). In response to an attack by predators like sea anemones, they discharge purple ink that is produced in their mantle gland. Amazingly, even already engulfed animals have been shown to be disgorged undamaged by sea anemones upon ink release (Nolen et al., 1995). Several glycoproteins isolated from the ink of *Aplysia* spec. and *Dolabella* spec. like aplysanin P and dolabellanin P have been described to possess anti-tumor activity (Yamazaki, 1993). However, the mechanisms underlying this activity was unknown. In a recent publication we identified APIT, the *Aplysia punctata* ink toxin, as the tumorlytic substance in the ink of *A. punctata* (Butzke et al., 2004). APIT kills tumor cells via the production of hydrogen peroxide without inducing apoptosis (Butzke et al., 2004).

H_2O_2 is known as intracellular messenger and to be involved in the oxidative burst of phagocytes leading to elimination of invading microorganisms (Nathan and Shiloh, 2000). In addition, H_2O_2 displays diverse effects on cell metabolism including proliferation, growth arrest, apoptosis, and necrosis (Clement and Pervaiz, 2001).

Abbreviations APIT; *Aplysia punctata* ink toxin, LAAO; L-amino acid oxidase, MBTH; 3-methyl-2-benzothiazolone hydrazone hydrochloride.

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Whereas H_2O_2 is unstable and tends to diffuse rapidly, H_2O_2 producing enzymes induce a rather continuous H_2O_2 production. Interestingly, a group of H_2O_2 producing enzymes, the L-amino acid oxidases (LAAOs, EC 1.4.3.2), is frequently found in secretions and venoms. Members of this family of flavoenzymes catalyze the stereospecific oxidative deamination of L-amino acids and thereby produces H_2O_2, ammonia and the corresponding α-keto acids (Du and Clemetson, 2002). The individual LAAOs differ in their substrate specificity: snake venom L-amino acid oxidases (sv-LAAOs) which constitute up to 30% (by weight) of the crude venom (Ponnudurai et al., 1994), possess a clear preference for hydrophobic amino acids. A fish capsule LAAO termed AIP (Apoptosis-Inducing Protein) which is induced by larval nematode infection of *Scomber japonicus* is highly specific for L-lysine (Jung et al., 2000). Achacin, a mucus LAAO from the African snail *Achatina fulica*, metabolizes a very broad range of substrates, including hydrophobic amino acids along with L-lysine, L-arginine, L-cysteine, L-asparagine, and L-tyrosine (Ehara et al., 2002). Similar to AIP and achacin, apoxin 1, the sv-LAAO of the western diamondback rattlesnake has been shown to induce apoptosis in cultured tumor cells within 24 h by an H_2O_2 dependent mechanism (Kanzawa et al., 2004; Murakawa et al., 2001; Torii et al., 1997, 2000).

Here, we report the cloning and biochemical characterization of a new LAAO from the ink of the sea hare *A. punctata* that induces oxidative necrosis in tumor cells. APIT exhibits a tight substrate specificity and is resistant against digest with trypsin and proteinase K. The homology of APIT to other glycoproteins from *Aplysia* spec. as escapin, aplysianin A and cyplasin L and S, points to a similar biological function of these proteins.

2. Materials and methods

2.1. Animals

A. punctata were obtained from the Station Biologique Roscoff, Bretagne, France. Crude ink was prepared as previously described (Butzke et al., 2004). In order to dissect mantle gland, nidamental gland, digestive gland and opaline gland animals were relaxed by injection of 5–10 ml sterile $MgCl_2$ solution (380 mM). Isolated tissues were frozen immediately in liquid nitrogen.

2.2. Cells, media and reagents

Jurkat T-cells were cultured in RPMI medium containing 10% FCS (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Media with defined amino acid composition were prepared by supplementing HEPES buffered modified Krebs Ringer (KRG: 25 mM HEPES (pH 7.4), 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 6 mM glucose, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$) with 10% FCS, 2 mM glutamine, vitamins (Invitrogen), non-essential amino acids (Invitrogen), or single essential amino acids in concentrations equivalent to RPMI medium (Invitrogen). Media were adjusted to pH 7.4 and filter sterilized. In addition, the following reagents were used: horseradish peroxidase from Roche; luminol, ABTS, MBTH and H_2O_2 from Sigma; anti-CD95 antibody clone CH11 from Immunotech.

2.3. Purification of APIT

Crude ink was filtered and concentrated using Ultrafree-15 Units (exclusion weight 30 kDa) followed by three washing steps with 20 mM Tris–HCl (pH 8.2). Ink was concentrated 20–60-fold and then applied to a Source 15Q 10/40 anion exchange column equilibrated with 20 mM Tris–HCl (pH 8.2). Proteins were eluted using a linear gradient from 0 to 800 mM NaCl. The fractions containing cytotoxic activity were pooled, concentrated and loaded onto a Superose 12 HR 10/30 column (Pharmacia). Proteins were eluted with 100 mM potassium phosphate buffer (pH 7.2) at a flow rate of 0.4 ml/min. In order to monitor flavins, spectra were recorded in the range of 320–550 nm.

2.4. Glycostaining

The staining of the carbohydrate residues of APIT was performed by the 'DIG Glycan/Protein Double Labelling Kit' (Boehringer Mannheim) according to the manufacturer's instructions. In a first experiment for optimization of the non-glycosylated creatinase was used as control and as expected, showed no labeling at all. In order to test the specificity of the method, the mandatory oxidation of carbohydrates with periodate was blocked in parallel experiments by prior destruction of periodate with sodium disulfite.

2.5. Preparation of apo-glucose oxidase and cofactor reconstitution assay

1.5 ml of a saturated ammonia sulfate solution (pH 1.4) was stirred at 0 °C and 150 μl of a glucose oxidase solution (8 mg/ml) was added slowly. After centrifugation (15 min, 4 °C, 13,000 rpm), the pellet was dissolved in 150 μl potassium phosphate (100 mM, pH 7.2). Precipitation was repeated twice. The colorless apo-enzyme was washed twice with potassium phosphate (100 mM, pH 7.2) on a Microcon-column (30 kDa) and dissolved to a final concentration of 100 μg/ml. In order to separate the unspecified cofactor of APIT, purified APIT (100 μg/ml) was heated to 95 °C for 5 min and centrifuged. 10 μl of the supernatant were incubated for 1 h with 10 μl of the apo-enzyme of glucose oxidase at room temperature and tested for enzymatic activity assay in the presence of glucose. Pure FAD was added as control.