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Inactivation of lipoxygenase and cyclooxygenase by natural betalains and semi-synthetic analogues

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ABSTRACT

Betalains are natural pigments characteristic of plants of the order Caryophyllales. In this work, the role of betalains in the anti-inflammatory activity described for plant extracts is analysed in terms of the inactivation of the enzymes involved in the biochemical response (lipoxygenase and cyclooxygenase). Pure natural betalains and semi-synthetic analogues are demonstrated to promote a significant reduction of the enzymes activity. Reactions were followed spectrophotometrically and by HPLC-DAD. Phenethylamine-betaxanthin was the most potent in the inactivation of cyclooxygenase, with a reduction of 32% of the control activity at 125 μM, while the natural pigment betanidin and a betalain analogue derived from indoline resulted as the most potent inactivators of lipoxygenase, with IC_{50} values of 41.4 and 40.1 μM, respectively. Molecular docking studies revealed that betalains interact with the lipoxygenase amino acids involved in substrate binding and with Tyr-385 and Ser-530 close to the cyclooxygenase active site, interfering in enzyme catalysis.

1. Introduction

Betalains are nitrogen-containing pigments of hydrophilic nature responsible for the bright colouration of plant species belonging to the order Caryophyllales (Gandía-Herrero & García-Carmona, 2013). They are divided into two groups: the violet betacyanins and the yellow betaxanthins. Betalains substitute anthocyanins in leaves, flowers, and stems, but also in edible parts. This is the case of the roots of red beet (*Beta vulgaris*) (Hempel & Böhm, 1997), the fruits of cactus *(Opuntia ficus indica)* (Felker et al., 2008), and the berries from *Rivina humilis* (Khan, Harsha, Giridhar, & Ravishankar, 2012). Betalain-containing plants of the Amaranthaceae are also consumed cooked or fresh (Sang-Uk, Buk-Gu, Yong-Seo, Dong-Kwan, & Shela, 2009).

Betalains have, in recent years, shown their bioactive potential. The strong free radical scavenging capacity of these pigments and its modulation by different structural factors is well documented (Gandía-Herrero, Escribano, & García-Carmona, 2010; Gliszczyńska-Świgło, Szymusiak, & Malinowska, 2006). Studies in cell lines have also shown the potential of betalains in the chemoprevention of cancer (Sreekanth et al., 2007). Interestingly, this activity has also been demonstrated *in vivo* using very low concentrations of dietary pigments which were able to inhibit the formation of tumors in mice (Lechner et al., 2010). In humans, plasma concentration of betalains after ingestion is high enough to promote their incorporation into LDL and red blood cells, protecting them from oxidative damage and hemolysis (Tesoriere, Butera, Allegra, Fazzari, & Livrea, 2005). In addition, species belonging to the order Caryophyllales have been traditionally used to ameliorate the symptoms of inflammatory processes (Ibrahim, Sowemimo, van Rooyen, & van de Venter, 2012; Neto et al., 2005). In recent years, the anti-inflammatory activity of betalain-containing extracts from Caryophyllales has been characterised for *Opuntia* fruits (Loro, del Rio, & Pérez-Santana, 1999) and *Gomphrena* inflorescences (Silva et al., 2012). The use of plant extracts has avoided the identification of active compounds and has limited the conclusions on the mechanisms involved and possible therapeutic applications.

At the biochemical level, the enzyme cyclooxygenase (COX) plays a pivotal role in the inflammatory response by catalysing the formation of prostaglandin H_2 from free arachidonic acid (Hata & Breyer, 2004) (Fig. 1A). Prostaglandin H_2 is the precursor molecule involved in the generation of derived prostaglandins and thromboxanes in mast cells, macrophages, and dendritic cells. The roles of the mediator molecules include bronchoconstriction, eosinophil infiltration, vasodilatation, and platelet shape change and infiltration (Hata & Breyer, 2004). Although there are two

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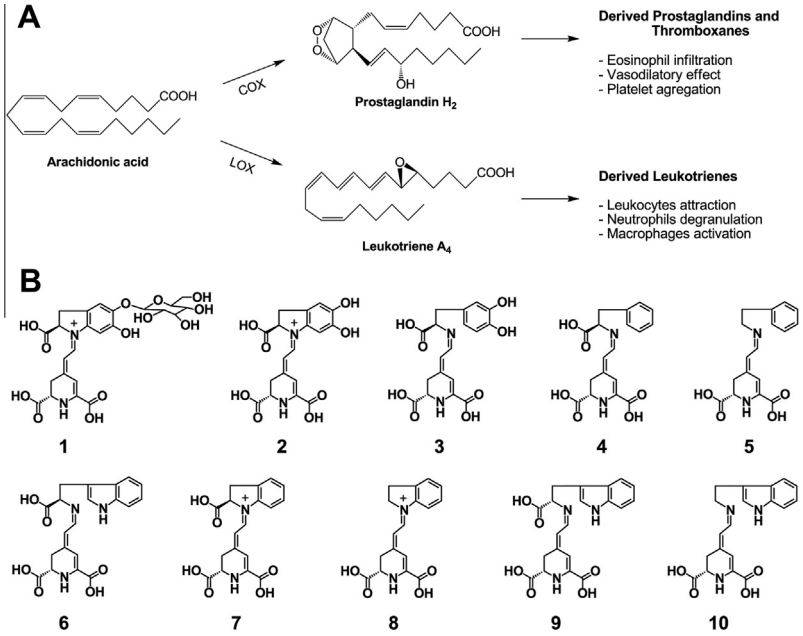

Fig. 1. (A) Scheme for the conversion of arachidonic acid to mediator molecules of the inflammatory process by lipoxygenase and cyclooxygenase. (B) Betalains and semi-synthetic analogues used in this study.

forms of the enzyme, only COX-2 is expressed in response to inflammatory stimuli and thus it is considered to be the relevant isoform in the inflammatory process and the target for drug exploration and design. Lipoxygenase-5 (LOX-5) catalyses the transformation of arachidonic acid into the leukotriene A_4 by dioxygenation (Fig. 1A). This molecule is the starting point for the biosynthesis of a wide variety of leukotrienes, which display different roles in the inflammatory response (Werz, 2007). They are potent chemoattractants for leukocytes, cause the degranulation of neutrophils (releasing superoxide anions) and mediate in vascular leakage and leukocyte adhesion to endothelial cells, amongst other activities. Due to the close structural and functional similarities, the readily available lipoxygenase-1 enzyme from soybean is extensively used as a model enzyme for LOX activity in mammals (Pontiki & Hadjipavlou-Litina, 2008).

This paper aims to explore the possible inactivation of the key enzymes involved in the inflammatory response (LOX and COX), by pure betalains. The significance of structural elements is discussed in terms of structure–activity relationships. Semi-synthetic analogues were obtained according to the information provided by the natural compounds and assayed under the same conditions.

2. Materials and methods

2.1. Chemicals

Enzymes, chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) and solvents from Merck Chemicals Ltd. (Dorset, England). HPLC-grade acetonitrile and methanol were purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Betalains

Betanin and dopaxanthin were extracted and purified from roots of *Beta vulgaris* and yellow flowers of *Lampranthus productus*, respectively as described earlier (Gandía-Herrero, Escribano, & García-Carmona, 2005a). Betanidin was obtained from violet flowers of *L. productus* (Gandía-Herrero, Escribano, & García-Carmona, 2007). Other compounds were obtained by a combined procedure for the release of betalamic acid from purified betanin and condensation with the following amines and amino acids: indoline, (*S*)-indoline-2-carboxylic acid, (*S*)-phenylalanine, 2-phenylethylamine, tryptamine, (*S*)-tryptophan, and (*R*)-tryptophan. Semi-synthesis followed a previously described method (Gandía-Herrero, García-Carmona, & Escribano, 2006). All extracted and semi-synthetic compounds were purified by anionic exchange chromatography and solid phase extraction, and characterised spectrophotometrically, chromatographically, and by electrospray ionisation mass spectrometry (ESI-MS) as detailed below.

2.3. Anionic exchange chromatography

Anionic exchange chromatography of betalains was performed in an Äkta purifier apparatus (Amersham Biosciences, Uppsala, Sweden) using a 25 × 7 mm, 1 ml Q-Sepharose Fast Flow column (cross-linked agarose with quaternary ammonium as exchanger group, 90 μm of particle size). The solvents used were sodium acetate buffer 10 mM, pH 5.0 (solvent A), and sodium acetate buffer 10 mM, pH 5.0 with NaCl 2 M (solvent B) for betanidin purification. Sodium phosphate 10 mM, pH 6.0 (solvent A), and sodium phosphate 10 mM, pH 6.0 with NaCl 2 M (solvent B) were used for the rest of the pigments. After sample injection, the elution process was as follows: 0% B from beginning to 3 ml, and then a linear gradient was developed from 0% B to 35% B in 20 ml. For betanidin, the same gradient was used with an initial 6 ml washing with 0% B. In all cases, the flow rate was 1 ml min^{-1}. 1 ml fractions were collected and injection volume was 1 ml. 50 μl were injected for retention times comparison. Elutions were followed at 280, 480 and 536 nm (Gandía-Herrero, Escribano, & García-Carmona, 2010).

2.4. Solid phase extraction

1 ml C-18 cartridges (Waters, Milford, MA, USA) were conditioned with 5 ml of ethanol followed by 10 ml of purified water. Aqueous solutions of extracted pigments and semi-synthetic betalains were injected and bound to the minicolumn. Salts and buffers