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Transepithelial transport of the bioactive betalain pigments indicaxanthin and betanin across human intestinal Caco-2 cell monolayers

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Abstract

Betalains are bioactive dietary phytochemicals from cactus pear and red beet, which are their main dietary sources. Intestinal absorption mechanism of these phytochemicals and influence of their food matrix on the process were investigated in a model of Caco-2 cells seeded on Transwell inserts, under an inwardly directed pH gradient, simulating luminal and basolateral sides of human intestinal epithelium. Apparent permeability coefficients (P_{app}) for the AP-to-BL trans-epithelial transport suggests a substantial intestinal absorption. Transport of indicaxanthin was not-polarized, linear as a function of time, and unaffected by inhibitors of membrane transporters. Betanin exhibited significantly different bidirectional P_{app} and non-linear efflux kinetics, to be related to a multidrug resistance associated protein 2 (MRP-2)-mediated apical efflux. Neither indicaxanthin nor betanin underwent metabolic transformation. Permeation of both betalains increased remarkably after EDTA treatment of the cell monolayer indicating a paracellular transport for both compounds. Additional trans membrane permeation can be considered for betanin. The food matrix did not affect the AP-to-BL transport of indicaxanthin, but the absorption rate of betanin was reduced, for reed beet more than cactus pear. In conclusion indicaxanthin can freely diffuse through paracellular junctions of intestinal epithelial cells, whereas betanin absorption is limited by a MRP2-mediated efflux and is negatively affected by its own food matrix. Present findings are consistent with the higher bioavailability of indicaxanthin over betanin in humans.

Introduction

Functional foods and nutraceuticals

The idea of functional food and nutraceuticals is in tune with the broad concept of health as a state of complete physical, mental, and social well-being and not merely the absence of disease. Apart from their conventional value as a source of nutrients, functional foods provide benefits for certain body functions, important for maintaining healthy status or reducing disease risk. The concept of functional foods is susceptible of different interpretations relevant to their characteristics, their active components, or their regulatory framework. In recent years, there has been an increasing interest in diets rich in fruits and vegetables, mostly due to the presence of bioactive compounds. Functional food ingredients (**nutraceuticals**) in fruits and vegetables are very different in nature, from small molecular components, such as the secondary plant products, to macromolecular entities, e.g., pectin and cellulose, among others, a large number of polyphenol phytochemicals, whose activity is now considered to be very important to maintain the redox conditions necessary to control cell functions. In fruits, the most common functional ingredients are the color components anthocyanins and carotenoids.

Betalain pigments

Betalain pigments are immonium derivatives of betalamic acid and include a quite modest number of structures. Betalains such as betacyanins and betaxanthins occur in a number of natural sources; however, cactus pear fruit (*Opuntia ficus indica* L. Mill.), Beetroot (*Beta* v*ulgaris* L. ssp. v*ulgaris*), and the fruit from vine cactus are the only foods containing this class of compound and their intensive color is due to the presence of these characteristic pigments. The violet-red betacyanins are conjugates of betalamic acid with either *cyclo*-DOPA or *cyclo*-DOPA glycosyl and acyl derivatives at the C-5 or C-6 positions, whereas the orange-yellow betaxanthins are conjugates with amino acids or amines (Strack *et al*. **2003**)

Beta vulgaris ***Opuntia ficus indica***

Betalains from cactus pear fruits, indicaxanthin and betanin, are adducts of betalamic acid with proline and *cyclo*-DOPA 5-*O*-β glucoside, respectively (**Figure 1**).

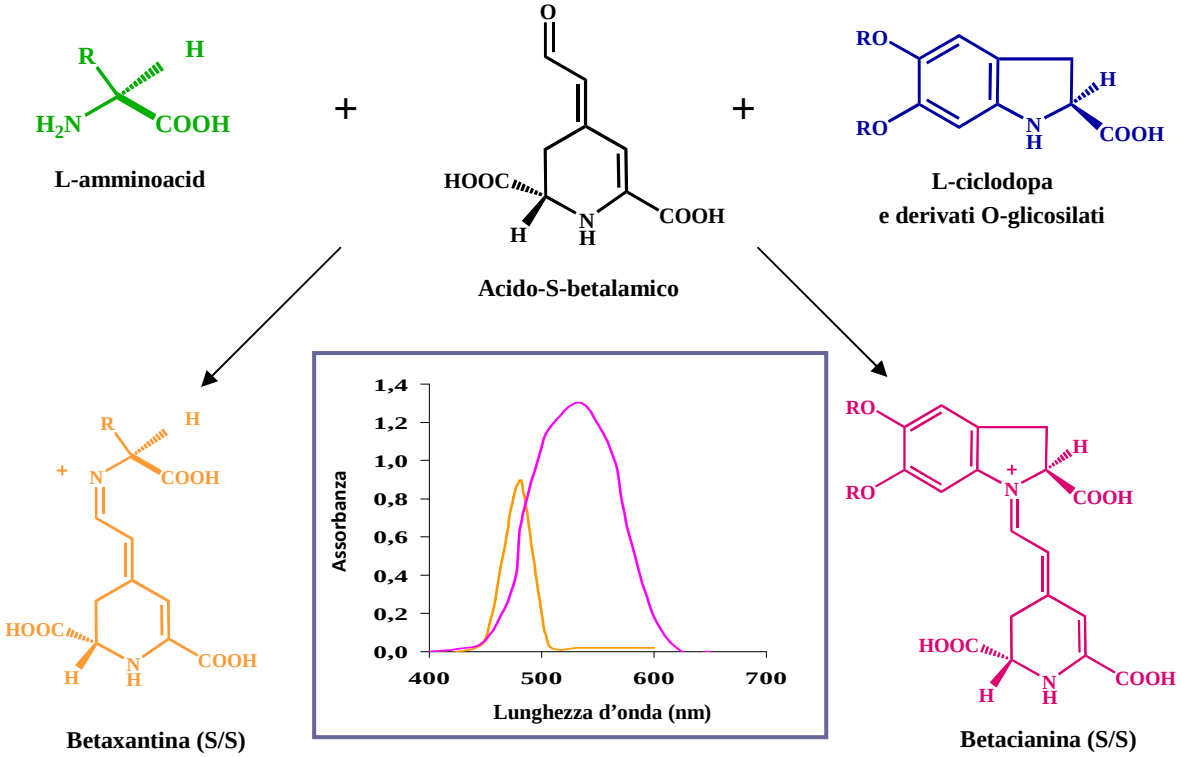


Figure 1 – Betalamic acid and predominant betalains in cactus pear fruit.

The extended conjugation system justifies the high molar extinction coefficients of these compounds in the visible range of the spectrum. The characteristic yellow-orange color ($\lambda_{max} \approx 480$ nm) of betaxantins is due to the presence in their molecular structure of a resonance system of three double bonds, formed by conjugation of an amino acid with betalamic acid. A large batocromic shift (60-70 nm), related to the presence of the aromatic ring portion *cyclo*-DOPA, is responsible for the colouring of betacianins. The intense color, stable in a wide pH range between 3 and 7, and their lack of toxicity (Schwartz D. *et al*. **1983**; Von Elbe JH. **1981**), made betalaines particularly interesting in the food industry. Today some of them are used as natural dyes for weakly acidic food (Francis FJ. **1989**).

Opuntia ficus indica L.

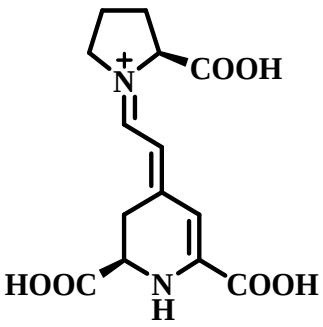
Opuntia ficus indica, also known as prickly pads, represents an important part of the human diet in certain arid zones of the globe. The plant , a member of the Cactaceae family, is an interesting vegetable due the hard environmental conditions where it grows and its resistance to climatic extremes. Prickly pads are grown throughout Mexico and in all North and South America. This cactus grows in many other regions of the World such as Africa, Australia and in the Mediterranean area (de Wit M. **2010**; Corrales GJ. **2003**). In recent years, many countries have increased the production of prickly pears, Sicily ranks second among all countries in the world for producing and exporting prickly pear fruits.

Cactus pads are commonly called “nopales” or “nopalitos” when they are fresh young prickly pads from 3–4 weeks of age. Traditionally “nopales” have been consumed in Mexico and Unites States, using several different preparations or cooking methods. The older stage pads are frequently used as forage, especially when there is shortage of fresh forage due to droughts. This plant is cheap, plentiful and sometimes it has also been used for erosion control (Le Houérou HN. **1996**).

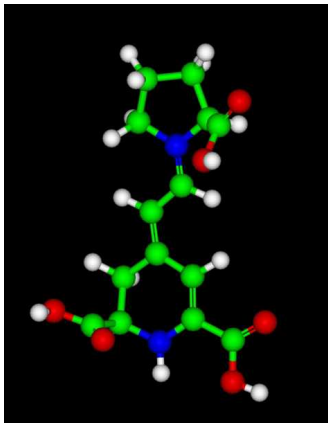
The nutritional properties of the fresh stems (cladodes) have long been known, and prickly pear is used also in traditional medicine for its hypoglycemic and

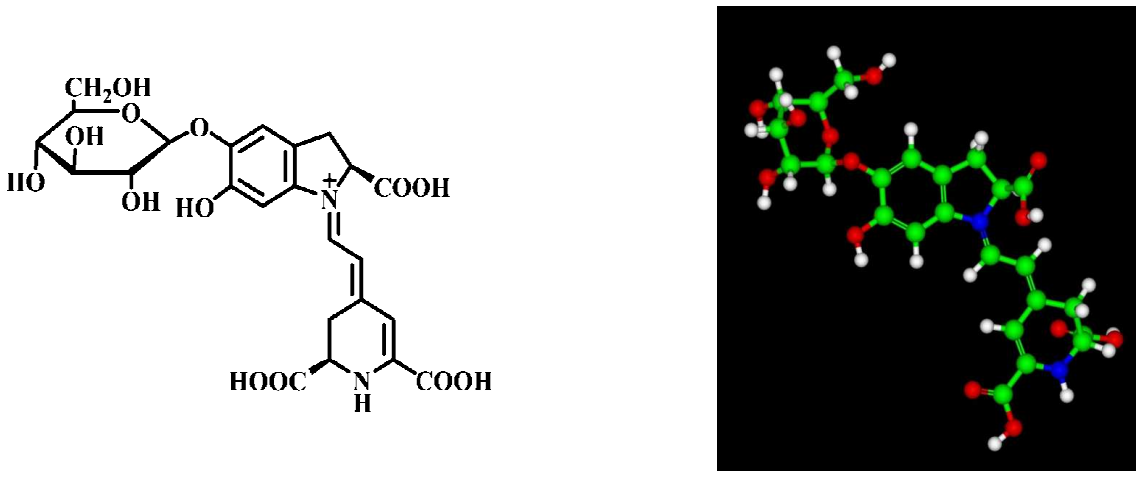
hypolipidemic actions (Reid IR. *et al.* **1995**; Palumbo B. *et al.* **2003**; Feugang JM. *et al.* **2006**; Gebremariam T. *et al.* **2006**). In the industrialized countries of the Mediterranean area, cladodes are not a usual nutritional source for humans, but the fruits are largely consumed. Sweet and tasty fruits of prickly pear, ripening between the end of July and November, may be easily found as spontaneous vegetation, but products from cultivations are now usually marketed. The fruit is juicy and sweet, with a thorny peel and a large number of small and hard seeds. Cactus pear fruits are characterized by various colors due to the combination of two betalain pigments, the purple-red betanin and the yellow-orange indicaxanthin (**figure 2)**.

The yellow cultivar exhibits the highest amount of betalains, followed by the red and the white cultivars. The purple-red betanin was negligible in the white fruit, while the ratio betanin/indicaxanthin varied from 1:8 (w:w) to 2:1 (w:w) in the yellow fruit. The yellow one is the main *cultivar* in Sicily, accounting for almost 90% of the plantations, while the red and the white *cultivars* account for 10% and 2% of the plantations, respectively. In addition to betalain pigments, this fruit is rich in sugars, vitamin C and minerals such as Ca, Na, Mg, Zn, Fe, and according to some authors, also Mn and Se (Duro F. **1971**; Gurrieri S. *et al.* **2000**).The nutrients and chemical composition of the prickly pear fruit have been yet reported (El Moghazy *et al.* **1982**; El Kossori RL. *et al.* **1992**).



Indicaxanthin





Betanin

Figura 2 – Betalains in *Opuntia ficus indica*

Spectrophotometric analysis of hydrophilic extracts of fruits, is used to determine the total content of betalains and their ratio in the three main Sicilian *cultivars*, the red, the yellow and white ones(Butera D. *et al.* **2002**).

Taking into account the overlapping of betanin absorbance band on the peak at 482 nm, spectrophotometric quantitation of indicaxanthin can be calculated in accordance with equation (1) :

$$[\text{Indicaxanthin } (\mu M)] = 23.8 \times A_{482} - 7.7 \times A_{536} \qquad (1)$$

This equation was obtained by applying algorithms based on the molar absorbance of indicaxanthin at 482 nm [A_{482} (indicaxanthin) 42 600; (Piattelli M. **1964**)] and of betanin at either 536 nm [A_{536} (betanin) 65 000 ; (Schwartz SJ. *et al.* **1980**)] or 482 nm. [calculated A_{482}(betanin) 30 900]

Radical scavenger activity and biological effects of betalain pigments

Radical-scavenging activity of purified betalains has been assessed using the ABTS colorimetric assay (Butera D. *et al.* **2002)**. Hydrophilic extracts from the three *cultivars* were submitted to the ABTS radical cation decolorization assay, and the activities of the extracts were expressed as Trolox (hydrophilic vitamin E analogue) equivalents. The total radical-scavenging ability of the methanolic extracts from the yellow fruit is significantly higher than the activity of the red and white ones. In fact, indicaxanthin has been reported twice more effective than Trolox, and betanin was demonstrated for up to 20 times more active.

The oxidation potentials of betanin and indicaxanthin were evaluated by Cyclic Voltammetry (**CV**). Differential pulse measurements, which were conducted in order to increase the sensitivity, show three anodic waves for betanin and two anodic waves for indicaxanthin. The peak potentials [Ep(a)s] indicating the reducing power are 404, 616, and 998 mV for betanin, while for indicaxanthin the values are 611 and 895 mV. The shape of the voltammetric waves toward the negative potential indicates that both compounds are irreversibly oxidized and their products can no longer accept electrons. In contrast to other natural pigments such as carotenoids and anthocyanins, the physiological effects of betalains have not studied so extensively. There is rapidly growing information on different bioactivities of betalain pigments from some *in vitro* and *ex vivo* studies.

The antiviral and antimicrobial effects of betalain pigments have been reported (Strack D. *et al.* **2003**). Furthermore, the antioxidant properties of betalains have been demonstrated in a wide range of assays (Zakharova and Petrova, **1998**; Kanner *et al.* **2001**; Butera D. *et al.* **2004**) and it was reported that indicaxanthin and betanin from cactus pear fruits, increased resistance to oxidation, showing antioxidant activity in biological lipid environments from human low-density lipoproteins to cell membranes and whole cells (Tesoriere *et al.* **2005**; Tesoriere L. *et al.* (**B**), **2004**; Tesoriere *et al.* **2003**).

Moreover, both pigments were able to modulate redox-mediate signal transduction pathways involved in activation of endothelial cells by inflammatory cytokines (Gentile *et al*. **2004**). It has recently been documented that betanin can inhibit the cell proliferation of a variety of human tumor cells (Kapadia *et al*. **2011**; Sreekantah, DS. *et al.* **2007**; Reddy MK. *et al.* **2005**), and a role for betalain pigments in the chemoprevention against lung and skin cancers has been reported (Kapadia *et al.* **1996**).

Bioavailability of indicaxanthin and betanin

Although there is evidence that betalain pigments can be absorbed in humans, the information found on their bioaccessibility and bioavailability is limited. The term bioaccessibility is defined as the amount of a compound ingested that is transferred during digestion from the food matrix to the micelles, thus becoming accessible for its absorption in the intestinal tract, it depends mainly on the physical properties of its food matrix. Oral bioavailability, refers to the fraction of a bioaccesible compound that is absorbed from the intestine and becomes available for its use, metabolism, and/or storage by the organism. Studies on the bioavailability of betalains in humans are relevant to indicaxanthin and betanin from cactus pear fruits (Tesoriere L., Butera D. *et al.* **2004**) and betanin from red beet juice (Kanner J. *et al.* **2001**; Frank T. *et al.* **2005**) and seem to indicate that indicaxanthin is about 20 times more bioavailable than betanin(**Figure 3**).

The results of an *in vivo* study showed that daily supplementation with 500 g cactus pear fruit pulp for 2 wk greatly improves the oxidative stress status of healthy subjects. The experimental evidence includes remarkable reductions in plasma markers of oxidative damage to lipids, such as isoprostanes and MDA; an improvement in the oxidative status of LDL; considerably higher concentrations of major plasma antioxidants; and improvement in the redox status of erythrocytes (Tesoriere L. *et al*. (**B**), **2004**).

The *in vitro* measurement of bioaccessibility is considered to be a reliable tool to approach the bioavailability of a dietary compound, taking into account eventual variations due to food source, digestive stability of the molecule, and maximum solubility in the gastrointestinal (GI) medium, as an index of availability for eventual processes of apical uptake by absorptive epithelial cells. Recent studies were carried out to investigate digestive stability and bioaccessibility of a number of betacyanins and betaxanthins from betalain-rich foods. A simulated gastro-intestinal digestion demonstrated that digestive stability and additional factors relevant to the solubilization from food matrix and style of food processing, influence the fraction of soluble betalains in the post-intestinal digesta potentially available for trans-epithelial transfer (Tesoriere L. *et al*. **2008**).

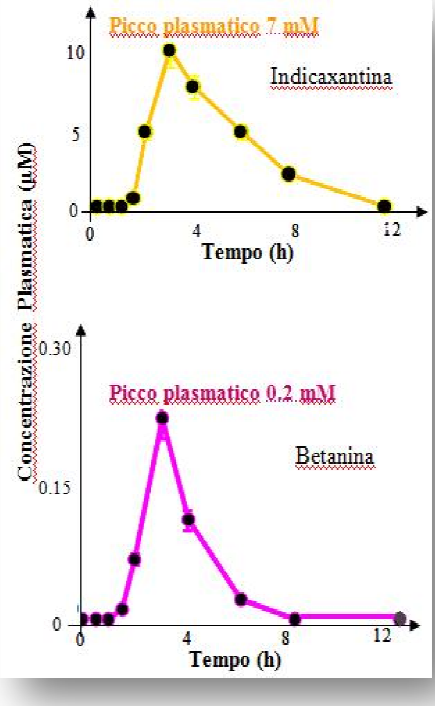


Figure 3. Plasmatic Peaks of betalains after 500g of pulp ingestion (Tesoriere et al. Am. J. Clin. Nutr. 2004)

A minor loss of indicaxanthin, at the gastric-like environment only, and a decrease of vulgaxanthin I through all digestion steps were observed, which was not affected by food matrix. In contrast, food matrix prevented decay of betanin and isobetanin at the gastric-like environment.

Loss of betacyanins, either purified or food-derived, was observed during the small intestinal phase of digestion. Betalamic acid accumulated after digestive degradation of purified pigments, but not of food betalains. Betaxanthins were wholly soluble in the aqueous (bioaccessible) fraction after ultracentrifugation of the postintestinal (PI) digesta, whereas release of betacyanins from the matrix was incomplete. The data suggest that digestive stability controls bioaccessibility of dietary betaxanthins, whereas additional factors, relevant to the food matrix and style of processing, affect betacyanin bioaccessibility (Tesoriere, L. *et al.* **2008**).

Caco-2 cells

Compared to human and animal models that are highly complex, it is easy to control parameters like absorption and metabolism in an *in vitro* study. An *in vitro* assay is simple, more convenient, and less expensive. Caco-2 cells have been the most extensively characterized and useful *in vitro* model in the field of drug permeability and absorption. The differentiated cell monolayers have also been used for the study of unidirectional transport of phytochemicals such as quercetin, epicatechin, proantho cyanidins, and carotenoid and much valuable information has been obtained using this model system.

The Caco-2 cells line is a continuous line of heterogenous human mature epithelial colorectal adenocarcinoma cells. Despite their cancerous origin, Caco-2 cells are unique in their ability to initiate spontaneous differentiation on reaching confluence under normal culture conditions (i.e., in presence of glucose and serum). Consequently, over a period of 20–30 days of post confluent culture, these cells gradually show a morphological polarity and levels of brush border hydrolases that

are both comparable with those of mature intestinal absorptive cells, intercellular tight junctions and apical microvilli, carriers for nutrients as well as efflux proteins. (Artursson P. *et al*. **2001** ; Walgren RA. *et al*. **1998**; Garrett DA. *et al*. **2000**) .
A good correlation between permeability across Caco-2 cell monolayer, solubility and oral bioavailability in humans has been demonstrated (Ungell AL. *et al.* **2004**).

Purpose

In light of the observed bioavailability of betalains in humans, we investigated aspects of the intestinal permeation of indicaxanthin and betanin using Caco-2 cell monolayers seeded on Transwell inserts. In addition, since food matrix can affect absorption, the Caco-2 cell permeation of betalains from various betalainic food preparations submitted to a simulated gastro-intestinal digestion (Bioaccesible fraction, **BF**), was compared with that of from pure compounds.

Materials And Methods

Unless stated otherwise, all reagents and materials were purchased from Sigma Chemical Co. (St. Louis, MO), and solvents were of the highest purity or HPLC grade.

Purification of Indicaxanthin and Betanin.

Betanin and indicaxanthin were isolated from cactus pear fruits as previously reported (Butera *et al.* **2002**) Prickly pear fruits, collected in September-November in Sicily (Italy), were obtained from a local market and were processed within 48-72 h of collection. Four different lots of fruits, at comparable ripening stages, were analyzed for each cultivar. The fruits were peeled and finely chopped. The pulp was separated from the seeds and weighed, and 100-g pulp samples were homogenized with 100 mL of methanol. The mixtures were allowed to stand for 60 min at 4 °C before centrifugation (10 min at 3000g). The organic layer was then recovered and the extraction repeated with the same volume of methanol. The combined extracts

were subjected to rotary evaporation to remove the organic solvents. All the samples were portioned and stored at -80 °C. Betanin in the methanolic pulp extracts was spectrophotometrically evaluated by the absorbance at 536 nm, using a molar extinction coefficient of 65 000 (Schwartz SJ. *et al.* **1983**). Owing to the overlapping of betanin absorbance with the absorbance of indicaxanthin at 482 nm [calculated A_{482}(betanin)) 30 900], the indicaxanthin concentration in crude extracts containing both pigments was calculated according to eq (1). Betanin and indicaxanthin were separated by gel filtration on a Sephadex G-25 column (40 cm x 2.2 cm) according to Kanner et al. (Kanner J. *et al.* **2001**), with minor modifications. Briefly, aliquots of methanol extracts from 2 g of fresh pulp were eluted with 1% acetic acid.Fractions (2.5 mL) were collected and tested spectrophotometrically at 482 and 536 nm for the presence of indicaxanthin and betanin, respectively (Piattelli M. *et al*. **1964**).The eluition profile provided evidence that complete separation of the two pigments was achieved. The isolated pigments were submitted to high-performance liquid chromatography analysis on a Varian Microsorb C-18 column (125 cm, Varian, Palo Alto, CA), eluted with a 20-min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) at a flow rate of 2 mL/min. Spectrophotometric revelation was at 536 and 482 nm for betanin (12.4 min) and indicaxanthin (13.6 min), respectively. A minor peak in the HPLC chromatogram, tentatively identified as isobetanin (13.3 min) (Stintzing FC. *et al.* **2002**), accounted for 0.5% of the isolated betanin. The elution volumes relevant to indicaxanthin and betanin were collected. Samples after cryoessiccation were resuspended in PBS at suitable concentrations for experimental measurements.

Physicochemical Properties

Molecular descriptors of the betalains such as ClogP, ClogD were computed by Qikprop 3.1 predict program (Schrodinger, LLC, New York, NY). The non-polar surface area (NPSA) was obtained as the difference of the molecular surface area (MSA) and polar surface area (PSA), calculated by CODESSA PRO software

(Katritzky AR *et al* **1996**). pK_a values of indicaxanthin were obtained by two different approaches, i.e. the semi-empirical partial charge related and the Hammet and Taft linear free-energy relationships. Semi-empirical calculations were carried out by means of Marvin Sketch 5.0.6.1 prediction program (ChemAxon, Budapest, Hungary), based on the calculation of Mulliken partial charge of atoms in the molecule. The Hammet and Taft linear free-energy relationships were calculated by Epik 1.6 software (Schrödinger, LLC, New York, NY), which adopts the combination of Hammet and Taf methods in conjunction with ionization and tautomerization tools. The pK_a values of betanin were obtained from literature (Gliszczynska *et al.* **2006**).

Cell Culture.

Caco-2 cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 1% non-essential amino acids, 10 mM HEPES, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin and were maintained at 37 °C in 5% CO_2, and 95% humidity. Medium was changed 2-3 times per week. Caco-2 cells were used between passages 27 and 31.

In vitro simulated digestion and preparation of the bioaccessible fraction from betalainic food

Fresh cactus pear fruits (*O. ficus indica* L. Mill.) from yellow and red Sicilian cultivars and red beet roots (*B.* V*ulgaris* L. ssp V*ulgaris*) from Tuscany (Italy) cultivations, were obtained from a local market. Red beet juice (Biotta AG, CH-8274, Tagewiler, Switzerland) was purchased in a healthy food store.

Cactus pear fruit juice was prepared after brief homogenization of the pulp in a kitchen-type blender and filtration through a colander (0.2 mm mesh size).

Aliquots (20 g of each food preparation) was chewed 10 times by a single investigator and subsequently expelled in a tared beaker. The post-oral material was briefly homogenized in Hanks' balanced salt solution, pH 7.4 (HBSS, 1:2, w:v) for 2 min in a laboratory blender (Waring, New Hartford, CT), acidified at pH 2.0 with HCl, and 8 mg/mL porcine pepsin (3200-4500 units/mg) was added. After incubation in a shaking (100 rpm) water bath (type M 428-BD, Instruments s.r.l., Bernareggio, Mi, Italy) at 37 °C, for 2 h to simulate the gastric phase, the pH of the sample was immediately increased to 7.4 with 0.5 M $NaHCO_3$. The small intestinal phase of digestion was started after the addition of 2.4 mg/mL porcine bile extract and 0.4 mg/mL pancreatin. After incubation in the shaking water bath as above, for 2 h at 37 °C, the post-intestinal digest was centrifuged at 167000*g*, for 35 min at 4 °C in a Beckman Optima TLX ultracentrifuge, equipped with an MLA-55 rotor (Beckman Instruments, Inc., Palo Alto, CA), to separate the aqueous bioaccessible fraction (BF) from particulate material. Digestive enzymes were removed by ultracentrifugation through YM-10 membranes, and the betalain content of BF measured by HPLC (see below). Food BF were stored at -80 °C until use.

Cytotoxicity of the BFs on Caco-2 cells was checked in pilot studies. Caco-2 cells were seeded at $5x10^5$ cells well in a six-well flat-bottom plate, and the medium was changed three times a week. After 15 days from confluence, differentiated monolayers were washed three times with HBSS, then 2 mL of food BF was added. The BFs were filtered through a Millex HV 0.2 µm filter (Millipore, Billerica, MA) immediately before the use. After a 90 min incubation cells were washed and 50 µl HBSS containing 5 mg/mL MTT were added. The medium was discarded after a 4 h incubation at 37 °C, and formazan blue formed in the cells was dissolved in DMSO. The absorbance at 540 nm of MTT-formazan of untreated cells was taken as 100% viability. In addition, viability of the cells after treatment was determined by trypan blue exclusion and microscopy examination. Neither treatment caused cell toxicity.

Trans-epithelial transport

Transport experiments were carried out using Transwell^{R} inserts (polycarbonate membrane, 0.4 μm pore size, 24 mm diameter, Corning Inc., Corning, NY).

Inserts were placed in 6-well plates. Caco-2 cells were seeded at $5x10^4$ cells per cm^2 on the membrane insert with 1.5 mL of medium in the apical /luminal side (AP) and 2.5 mL of medium in the basolateral /serosal side (BL). Cells reached confluence within 5 days post-seeding. Culture medium was changed three times a week. On day 15 after reaching confluence, the DMEM was removed and the cells were rinsed three times with HBSS. The integrity of Caco-2 cell monolayers was evaluated by measuring the transepithelial electric resistance (TEER), according to Hidalgo et al. (Hidalgo IJ. *et al*. **1989**). TEER values across the cell monolayers were measured using a Millicell-ERS voltohmeter (Millipore Corp., Bedford, MA). Only monolayers with TEER > 300 Ω /cm^2 were utilized. After washing of the cells with HBSS as reported above, either indicaxanthin or betanin or food BF in HBSS were added to the donor compartment and HBSS to the acceptor one. HBSS in the apical compartment was buffered at pH 6.0 with 20 mM 2-(N-morpholine) ethanesulfonic acid (MES).When necessary, HBSS solutions of various either inhibitors or substrates of membrane transporters were added in the AP side.

Cultures were incubated (37°C, 5% CO_2) and the acceptor medium was collected at 15 min time-intervals between 0 and 90 min, and replaced with an equal volume of HBSS. The acceptor medium was centrifuged at 1000 g for 10 min at 4°C and submitted to HPLC analysis of betalains. In parallel experiments flux of marker compounds phenolsulfonaphtalein (phenol red, 5 mM) and testosterone (100 μM) were evaluated by spectrophotometric and HPLC analysis, respectively (Behrens I *et al*. **2001**).

The effect of either purified betalains or food BF on the barrier integrity of Caco-2 cell monolayers was assessed by checking the TEER values at the end of each transport experiment. In addition, the transfer of phenol red from the AP-to-BL compartment was also measured.

Under the conditions applied, treatment with either betalains or BF did not significantly modify the monolayer resistance or barrier integrity. The apparent permeability coefficients (P_{app}) were calculated according to the equation

$$P_{app} = \frac{V}{AC_o} \frac{dC}{dt} \text{ cm s}^{-1} \qquad \text{Eq.1}$$

where V = the volume of solution in the receiving compartment, A = the membrane surface area (4.71 cm^2), C_o = the initial concentration in the donor compartment, and *d*C/*d*t the steady-state flux across the monolayer calculated as the slope of the curve betalain concentration in the receiving compartment versus time (Artursson P. **1990**).

The mass balance, calculated as the percentage of the original pigment mass accounted for at the end of the trans-epithelial transport experiments, was assessed by evaluating the compound recovered in the donor and receiving chambers, and associated with cells. To this end the cell monolayer was washed three times with 2 mL of HBSS containing 5 mmol/L sodium taurocholate, to remove the pigment adhered to cell surface. Rinsed cells were scraped in methanol and each well was washed three times with the same solvent. Cells with washing solvent were immediately extracted, sonicated in ice bath for 5 min and centrifuged at 2000 g for 5 min. Methanol supernatant was collected and the cells were re-suspended in methanol and extracted again as above. The methanol extracts were gathered and reconstituted in suitable solvent for HPLC analysis of betalains.

Transport after alteration of the Tight Junction barrier

A 10 μM EDTA solution was prepared using PBS solution without Ca^{2+}/$Mg^{2+}$and applied to the apical and basolateral sides of Caco-2 cell monolayers for 5 min at 37°C (Artursson P *et al*. **1990**).

After the solution was removed, cells were washed three times with the PBS without Ca^{2+}/Mg^{2+}, before adding of either betalains (100 μM), or phenol red (5 mM), or

testosterone (100 μM), at the apical compartment, under pH-gradient conditions (pH 6.0/7.4; AP/BL).

HPLC analysis of betalains

HPLC measurements of betalain pigments were performed as reported (Butera D. *et al*. **2002**) using a RP-18e Performance column (100 x 4.6 mm; Merck, Darmstadt, Germany), equipped with RP-18e Chromolith guard cartridge (5 x 4.6 mm, Merck), eluted with a 20-min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile), at a flow rate of 1 mL/min. Spectrophotometric detection of indicaxanthin (Rt 9.3 min) or betanin (Rt 12.5 min) was at 482 nm or 536 nm, respectively. Quantitation was by reference to curves constructed with 1 to 100 ng of the purified compounds, and by relating the amount of compound under analysis to the peak area.

Statistical Analysis of Data

All data are expressed as means ± SD. Three independent observations were carried out for each experiment. All experiments have been replicated two to three times, to have $6< n <9$. Statistical difference were calculated using unpaired *t*-test. Significance was accepted if the null hypothesis was rejected at the $P < 0.05$ level. Calculations and graphs were obtained by Instat-3 statistical software (GraphPad Software Inc., San Diego, CA).

Results

Physicochemical parameters

Physicochemical parameters and ionization constants calculated for indicaxanthin and betanin are reported in Table 1. Indicaxanthin and betanin are cationized molecules, with a positive charge localized in proximity of the N1 nitrogen (Fig. 1). Both molecules possess a number of ionizable carboxyl groups, with pk_a between 2.0 and 5.4 from our calculations and literature data. In addition, betanin bears a phenol

hydroxyl at the cyclo Dopa, the pk_a of which has been reported more acidic than expected (Gliszczynska *et al.* **2006**). In accordance to the measurements, indicaxanthin mainly exists as a bis-anion both at pH 6.0 and 7.4, whereas betanin must shift towards a tris-anion around pH 7.0 (Frank T. *et al.* **2005**; Gliszczynska *et al.* **2006**).

Trans- epithelial transport of pure betalains

The bidirectional trans-epithelial transport of indicaxanthin and betanin across Caco-2 cell monolayers was investigated under a pH gradient (6.0/7.4; AP/BL). Functional characteristics of the monolayer were preliminarily checked using phenol red and testosterone as markers for paracellular and trans-cellular permeation, respectively. The values of $P_{app\ (AP\text{-}to\text{-}BL)}$ calculated for these compounds were $0.28 \pm 0.01 \times 10^{-6}$ cm s^{-1} and $30.1 \pm 0.6 \times 10^{-6}$cm s^{-1} respectively. All transport experiments were done with 100 μM of either betalains and the amount of pigment in the receiving compartment was monitored at 15 min time-intervals for 90 min, under initial rate conditions.

The P_{app} measured for indicaxanthin across the Caco-2 cell monolayer in the absorptive AP-to-BL direction ($4.2 \pm 0.4 \times 10^{-6}$ cm s^{-1}) was comparable with that measured in the secretive BL-to-AP direction ($4.4 \pm 0.4 \times 10^{-6}$ cm s^{-1}, Fig. 2), indicating a non polarized transport.

On the other hand, the calculated P_{app} of betanin was significantly higher in the absorptive AP-to-BL direction ($3.2 \pm 0.33 \times 10^{-6}$ cm s^{-1}) than in the efflux direction ($2.5 \pm 0.23 \times 10^{-6}$ cm s^{-1}, Fig. 2). Due to the pk_a value of the –OH phenol group near to the pH in the donor BL-compartment, variation of the ratio between bis anion and tris anion species in favor of the latter could negatively affect the efflux of betanin, resulting in an asymmetric flux of the phytochemical.

At the end of each experiment a complete mass balance of both compounds was verified on the basis of the sum of the cumulative amounts of the phytochemicals recovered in the receiving chamber and the residual compound in the donor one.

Only traces of the molecules were found in cells. These data ruled out metabolism of betalains by intestinal cells as well as endocellular accumulation.

The cumulative amounts of either indicaxanthin or betanin transported into the receiving chamber as a function of time are shown in Fig. 3. The amount of indicaxanthin crossing the monolayer increased linearly with time within 90 min, in both the absorptive and efflux direction, with comparable transport rates (Fig. 3, A). For betanin, the relationship was linear in the absorptive direction, whereas a quite different relationship was observed in the efflux transport (Fig. 3, B). On the whole, the data suggested a simple diffusion process for indicaxanthin, but a more complex transfer for betanin, across the Caco-2 cell monolayer.

In the presence of the proton gradient applied, H^+-dependent influx carriers were considered. Then, the AP-to-BL trans-epithelial transport of betanin was measured in the presence of substrates for the monocarboxylate transporter (MCT1) and the organic anion transporting polypeptide (OATP2B1), as potential competitors. Permeation of betanin was unaffected by either ferulic acid or acetate, both substrates of MCT1 (Konishi Y. *et al.* **2003**, Thwaites DT. *et al.* **2007**), and by pravastatin, a substrate for OATP2B1, whereas valproate, a substrate for both transporters (Thwaites DT. *et al.* **2007**), caused an unexpected increase of P_{app} (Table 2). The AP-to-BL trans-epithelial transport of betanin was also investigated in the presence of either verapamil or indomethacin as specific inhibitors of the efflux proteins P-glycoprotein and multidrug resistance associated protein 2 (MRP2), respectively (Shugarts S. *et al.* **2009**). Indomethacin, but not verapamil, caused a significant increase of betanin permeation (Table 2), indicating that absorption of betanin across the cell monolayer was negatively influenced by an MRP2-mediated efflux. As a corollary, these data provided an explanation to the increase of P_{app} evaluated for betanin in the presence of valproate, since valproate has been shown to be a substrate of MRP2 (Ogawa K. *et al.* **2006**). Finally. the absorption of glycosylated phytochemicals could involve the Na^+-dependent glucose transporter of the brush border membrane (Walgren RA. *et al*. **2000**). When 100 μM betanin was co-

incubated in the apical side with excess glucose, the permeation rate was not significantly modified (Table 2).

In parallel experiments, the AP-to-BL permeability of indicaxanthin was measured in the presence of the mentioned competitors for H^+-dependent carriers and efflux transporters, to rule out that the apparent absence of a carrier-mediated transport actually resulted from a false negative. None of the compounds caused variations of the calculated P_{app} of indicaxanthin (not shown), ruling out effects of the pigment on the transporters considered.

The P_{app} of the AP-to-BL transfer of indicaxanthin and betanin across Caco-2 cell monolayers was evaluated after treatment of the monolayer with EDTA, that affects paracellular permeability *via* loosening of the tight junctions (Artursson P. **1990**), in comparison with compounds markers for paracellular and trans-cellular pathways. The P_{app} value of indicaxanthin increased around 11-fold, and of betanin 7-fold (Fig. 4). As expected, an effective transfer of phenol red across the epithelial cell layer was observed only after loosening of the TJs, whereas the permeability of testosterone, the transfer of which occurs *via* a passive trans-cellular pathway, was almost unaffected by the EDTA treatment of the cells (Fig. 4). It was concluded transport of both betalains substantially occurred through paracellular permeation.

Transport of betalains from food digesta

The absorption of betalains from digested betalainic food was evaluated. Cactus pear fresh fruits and juice as a source of indicaxanthin and betanin, as well as raw red beet and red beet juice, as a source of betanin, were processed through a simulated *in vitro* digestion, and the bioaccessible fractions so obtained were placed at the apical side of Caco-2 cells layered on a Transwell insert, after measuring their betalain content. Permeation of indicaxanthin and betanin is reported in Table 3. Whereas permeation of indicaxanthin from cactus pear either fruit or juice was quite comparable with that of the pure pigment, the food matrix reduced the transport rate of betanin, red beet more than cactus pear (Table 3).

DISCUSSION

Bi-directional transfer of indicaxanthin and betanin through Caco-2 cell monolayers

The intestinal absorption of two bioavailable dietary betalains, betanin and indicaxanthin, has been investigated using Caco-2 cell monolayers grown on permeable polyester membranes as a model for the small intestinal mucosal epithelium, and the influence of the betalainic food matrix on the absorption process evaluated. Different tracts of the gastro-intestinal lumen are characterized by different pH values, and further pH changes are measured during digestion (Dressman JB. *et al*. **1990**). The pH in the upper gastro-intestinal tract under fasting conditions ranges from 5.0 to 6.5, and it has been reported to be 5.8 to 6.3 just above the absorbing epithelial cell layer (Lucas M. **1983**). We then measured the flux of betalains across the Caco-2 cell monolayer under conditions of an inwardly directed pH gradient (pH 6.0/7.4) approaching the pH microclimate at the luminal and serosal sides of human intestinal cells, with maintaining first order conditions to mimic physiological absorption circumstances at the small intestine. In addition, the experiments were carried out using a betalain concentration which approached the amount in the intestinal digesta from one serving of either cactus pear fruit pulp or raw red beet (Tesoriere L. *et al*. **2008**), when considering an intestinal volume of 600 mL (Mahe S. *et al*. **1992**).

Like for xenobiotics, the intestinal absorption of phytochemicals may occur passively, through trans-cellular permeation or paracellular route in accordance with molecular mass and physicochemical characteristics, and could involve either influx or efflux membrane transporters. Our computational analysis provided solubility parameters and the polar and non-polar surface area of indicaxanthin and betanin, as well as dissociation constants of the indicaxanthin carboxyl groups. According to our calculations and literature data (Frank T. *et al*. **2005**, Gliszczynska *et al.* **2006**) both compounds mainly occur as bis-anions at pH 6.0. The octanol/water partition coefficients Clog*P* and Clog*D* (pH 6.0) indicated that indicaxanthin is moderately less polar than betanin. Finally, the calculated NPSA provided evidence that both

betalains have a quite large non-polar surface, accounting for around 50% of surface area, which substantiated previous observations on the ability of these molecules to interact with lipid environments from membranes (Kanner J. *et al*. **2001**; Tesoriere L. *et al.* **2005**; Turco Liveri M. *et al*. **2007**; Turco Liveri M. *et al*. **2009**) to low density lipoproteins (Tesoriere L. *et al.* **2003**; Tesoriere L. *et al*. (**A**), **2004**; Tesoriere L. *et al*. **2007**).

Generally, unless utilizing transporters in the epithelial membrane, charged solutes of a suitable molecular mass should diffuse through the paracellular route, and be transported passively by solvent drag (Adson A. et al. **1994**; Knipp GT. *et al*. **1997**). Quite consistent with the physicochemical features of indicaxanthin and betanin our study shows that both compounds may cross the epithelial cell layer by passive diffusion *via* the paracellular pathway, without any metabolic transformation. Some findings, however, suggest a mixture of paracellular and trans-cellular transport for betanin.

The effects of perturbation on the permeation of solutes are used as criteria to determine the preferred route of intestinal transport (Konishi Y. *et al*. **2003**, Gan LS. *et al.* **1993**). If the permeation is significantly affected by perturbation of cellular tight junctions (TJs), then the permeation is considered to occur predominantly by the paracellular route. We observed that an opening of TJs with EDTA induced a remarkable incease of the indicaxanthin and betanin influx, consistent with a paracellular transport of both compounds across the Caco-2 cell monolayer. Indeed, the trans-epithelial transfer of indicaxanthin did not appear to be polarized, nor the absorption was varied by inhibiting membrane transporters, in accordance with the simple diffusion-solubility criteria governing paracellular transports. On the other hand, the absorption of the glycosylated betanin appeared polarized, and the cumulative transfer in the efflux direction was not linear with time, suggesting the involvement of saturation-dependent efflux systems. Our experiments in the presence of various competitors revealed a role for the apical efflux transporter MRP2. Notably however, under our experimental set-up mimicking physiological conditions,

the permeation rate of betanin was higher in the absorptive than in the secretive direction, which should assure that absorption at the intestine will prevail. It may be worthwhile to mention that our experiments have been done with amounts of betalains consistent with the intestinal content after a dietary intake (Tesoriere L. *et al.* **2008**), which may be critical when dealing with carrier-mediated processes.

The involvement of MRP2 in the transport discloses interesting features of the betanin ability to migrate through the Caco-2 cell monolayer. While indicating that the bioavailability of this phytochemical is limited by an absorption barrier, our findings suggest that trans-cellular transfer of betanin could occur in parallel with the paracellular one. Partition of various ionized species into chemical bilayers has been determined (Avdeef A. *et al*. **1998**), and trans-cellular transport of ionized species across Caco-2 cell monolayers has been suggested in other studies (Palm K. *et al.* **1999**; Menez C. *et al*. **2007**). Moreover, previously performed chemico-physical partition studies in our laboratory provided evidence that betanin locates at the phospholipid core of the bilayer in an aqueous phosphatidyl-choline liposomal system (Turco Liver ML. *et al*. **2007**). Then it seems reasonable to suppose betanin permeation of the cell membrane at the apical side, with access to the apical membrane efflux transporter, and possibly lateral diffusion to the basal side of the cell and release to the basolateral compartment.

With solutes of like charge paracellular permeability is a function of the molecular mass and decreases with the increase of molecular weight [Adson A. *et al*. **1994**; Karlsson J. *et al.* **1994**; Knipp GT. *et al.* **1997**). Since indicaxanthin and betanin have a comparable ionic charge at pH 6.0, the molecular mass should favor the paracellular permeation of indicaxanthin. Noteworthy, that in the presence of indomethacin, i.e. blocking the MRP2 efflux transporter, the P_{app} for the absorption of betanin has appeared as high as that of indicaxanthin may be an indirect evidence that additional trans-membrane transport of betanin does occur. The intestinal absorption of xenobiotics is considered negligible if the $P_{app} < 0.1 \times 10^{-6}$ cm s^{-1} and essentially complete if the trans-epithelial $P_{app} > 5.0 \times 10^{-6}$ cm s^{-1} (Hillgren KM. *et al*. **1995**;

Artursson P. *et al*. **1991**). The permeability coefficients of indicaxanthin and betanin, with the trans-epithelial gradient of the betalains at the intestinal lumen after ingestion and their continuous removal by the bloodstream at the serosal side, could account for a significant intestinal transport. Present data indeed are consistent with and appear to validate the high fraction of dietary indicaxanthin absorbed *in vivo* (Tesoriere L. *et al*. (**A**), **2004**). Bioavailability of betanin in humans has been shown much lower than indicaxanthin (Tesoriere L. *et al*. (**A**), **2004**; Kanner J. *et al*. **2001**). According to previous studies (Tesoriere L. *et al*.(**B**), **2004***).* around 50% of betanin is lost during the digestive process, however its recovery in human urine was found no more than 3 % of the compound ingested with various foods (Tesoriere L. *et al*.(**A**), **2004**; Kanner J. *et al*. **2001**). Present findings on the intestinal processing and the calculated P_{app} do not fully consist with the bioavailability measurements. The complexity of the *in vivo* system could involve other significant losses, possibly hydrolytic processes by glycosidases of the intestinal microflora, and/or some oxidation of the pigment in the body (Tesoriere L. *et al*. **2009**). Other factors, including the food matrix, could play additional roles.

Influence of betalainic food matrix on the intestinal permeation of the pigments

The absorption efficacy of phytochemicals can be affected by the mixture of their food matrix (Boyer J. *et al*. **2004**). With respect to the pure compounds, the trans-epithelial transport rate of betanin from the soluble fraction of betalainic food digesta was strongly reduced, whereas that of indicaxanthin was not. Phenolic groups of phytochemicals may be involved in hydrogen bonding with protein moiety (Hagerman AE. *et al*. **1981**), then complexes between betanin and soluble protein fragments in the post-intestinal digesta could prevent a fraction of the pigment to be absorbed. Eventual competition by other betalainic food components for the paracellular pathway could also be hypothesized.

Present observations that betanin from cactus pear appeared more readily absorbed than from red beet are quite in accordance with previous studies in humans showing

higher bioavailability of betanin from dietary cactus pear fruit than red beet juice (Tesoriere L. *et al.* (**A**), **2004**;Kanner J. *et al*. **2001**). This may deserve consideration for nutritional purposes.

Conclusive remarks

Dietary bioactive phytochemicals are now considered potential nutraceuticals / pharmacological molecules (Konishi T. **2011**), then analyzing mechanisms and factors affecting intestinal absorption transfer could help to predict their eventual effects in the body. Definite evidence has been provided that the very high bioavailability of dietary indicaxanthin in humans (Tesoriere L. *et al*. (**A**), **2004**) results not only from relatively high stability of the molecule to the digestive process (Tesoriere L. *et al*. **2008**), but also from favorable intestinal absorption through paracellular route by solvent drag, and easy release from food.

Betanin bioavailability, instead, appears to be limited by digestive instability (Tesoriere L. *et al*. **2008**) and by an intestinal efflux mechanism reducing absorption by around 35%. Furthermore, its intestinal permeation may negatively be influenced by its food matrix. Importantly, indicaxanthin and betanin do not need metabolic transformations to be released in plasma and circulate as unconjugated molecules.

Taking all these facts into consideration, beneficial effects of dietary betalain pigments, as well as impact of betalainic foods on human health (Tesoriere L. *et al.*(**B**), **2004**) may be considered and results from appropriately planned *in vitro* studies may be interpreted to suggest real effects *in vivo*. Present data may provide a basis for research on the potential health effects of these substances, eventually to be orally administered as purified compounds.

Table 1. Physicochemical parameters of indicaxanthin and betanin

	MW	logP[a]	logD[b]	PSA	NPSA	pK_a	
				(Å^2)		(COOH)[c]	(phenol-OH)[c]
Indicaxanthin	309	0.362	-7.25	126.92	161.11	$5.0_{(2)}$;$3.7_{(11)}$;$2.6_{(13)}$[d]	
Betanin	551	-1.767	-15.63	246.53	229.26	$<3.4_{(2)}$;$3.4_{(15)}$;$3.4_{(17)}$[e]	$<7.4_{(6)}$[e]

The molecular surface is described by the polar surface area (PSA) and the non-polar surface area (NPSA).
[a]octanol/water partition coefficient
[b]octanol/buffer pH 6.0 partition coefficient
[c]C atom in bracket
[d]theoretically calculated pK_a values (see Methods)
[e]pK_a values from Gliszczynska *et al*. **2006**

Table 2. Influence of various compounds on betanin transport rate across Caco-2 cells monolayers

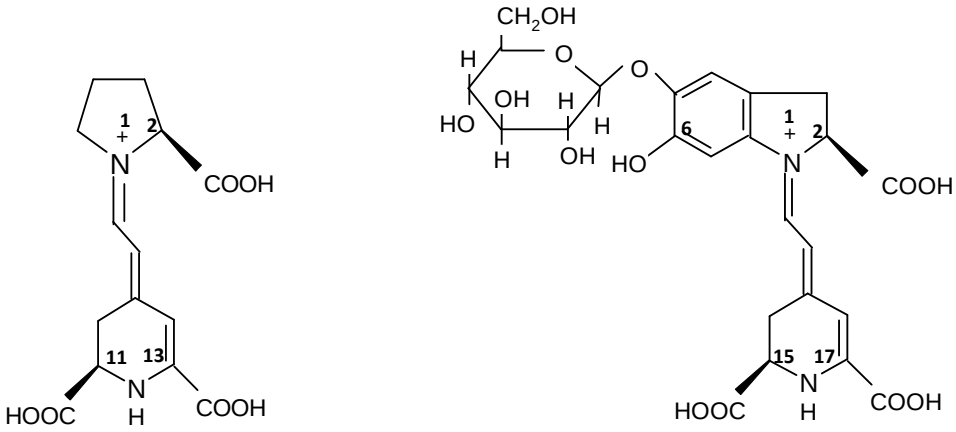
Compound	Concentration (mM)	$P_{app\ (AP-BL)}$ ($x10^{-6}cms^{-1}$)	n
Control		3.21±0.30	4
Ferulic acid	10	3.17±0.29	2
Acetic acid	5	3.25±0.31	2
Pravastatin	5	3.11±0.35	2
Valproic acid	10	4.51±0.38*	3
Verapamil	5	3.09±0.31	2
Indomethacin	10	4.93± 0.33*	3
Glucose	10	3.15±0.28	2

Betanin (100 μM) was added to the apical (AP) compartment of monolayers in the absence (control) or in the presence of each compound at the indicated concentration. Transepithelial transport experiments were carried out under gradient pH conditions (pH6.0/7.4; AP/BL) at 37°C for 60 min as described in Methods. Data are the means ± SD of n separate experiments carried out in triplicate. *Statistically significant vs control with $p<0.005$ (Student's *t* test).

Table 3. Apparent permeation coefficient (P_{app}) across Caco-2 cell monolayers of betalains recovered in the bioaccessible fraction (BF) from betalainic foods.

Betalain	BF Source	concentration μM	P_{app} (10^{-6} cms^{-1})
Indicaxanthin	cactus yellow fruit	30.5±2.3	4.32±0.35
	cactus yellow fruit juice	29.8±3.2	4.15±0.41
Betanin	raw red beet	62.5±5.3	1.98±0.21*
	red beet juice	11.7±1.1	1.82±0.20*
	cactus red fruit	12.2±1.5	2.78±0.34**
	cactus red fruit juice	13.1±1.2	2.61±0.31**

Preparation of BF from foods and incubation conditions of Caco-2 monolayers were as reported in methods. Values are the mean ± SD of three determinations carried out on two different food samples.
Significantly different from P_{app} of the relative pure compound, *$p<0.001$, **$p<0.05$ (Student's *t*-test).



Indicaxanthin **Betanin**

Fig. 4.

Molecular structure with charge delocalization in indicaxanthin and betanin

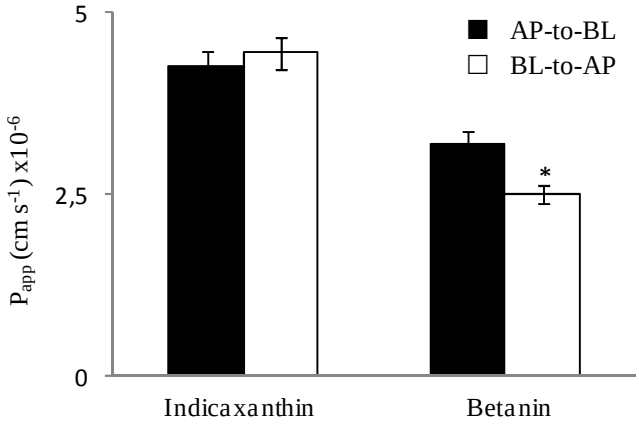


Fig. 5.

Bidirectional apparent permeability coefficient (P_{app}) of betalains across Caco-2 cell monolayers under inwardly pH gradient (AP pH 6.0/ BL pH 7.4). Transepithelial transport AP-to-BL (black bars) and BL-to-AP (white bars) and P_{app} measurements were as reported in methods. Values are the mean of three separate experiments carried out in triplicate. *Statistically significant vs the relevant AP-to-BL direction with $p<0.001$(Student's *t* test).

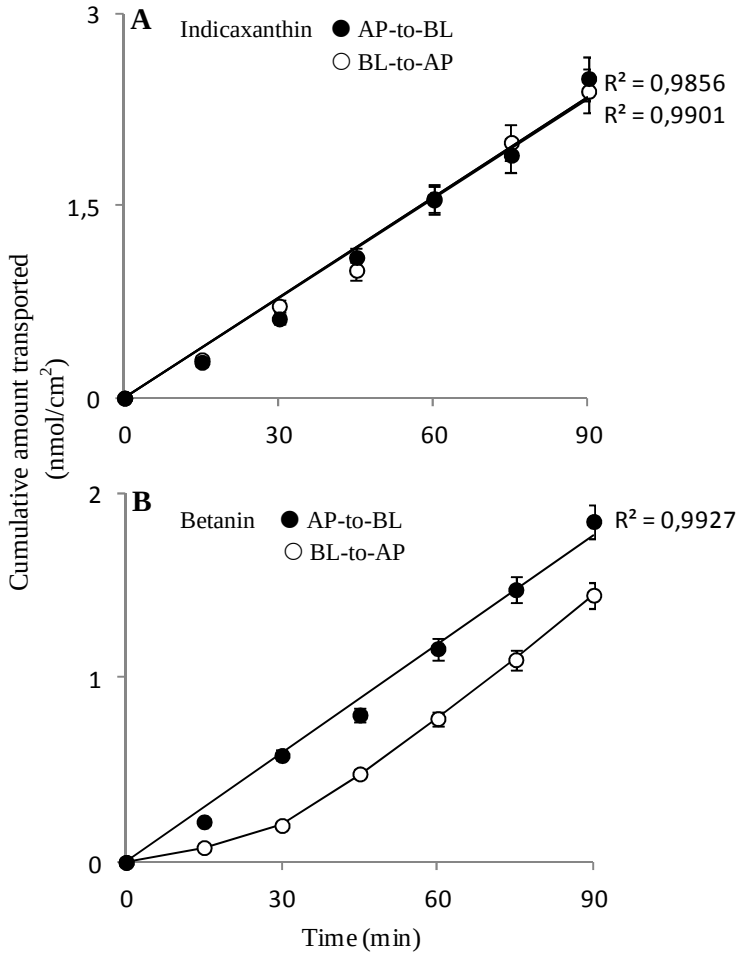


Fig. 6.

Characteristics of the transepithelial transport of betalains across Caco-2 cell monolayers. Transport of indicaxanthin (A) or betanin (B) across cell monolayers both in AP-to-BL and BL-to-AP direction was measured as reported in methods. Data are the means ±SD of three separate experiments carried out in triplicate. Trend lines from linear regression analysis fitted through zero showing good linearity (R^2>0.95) are reported

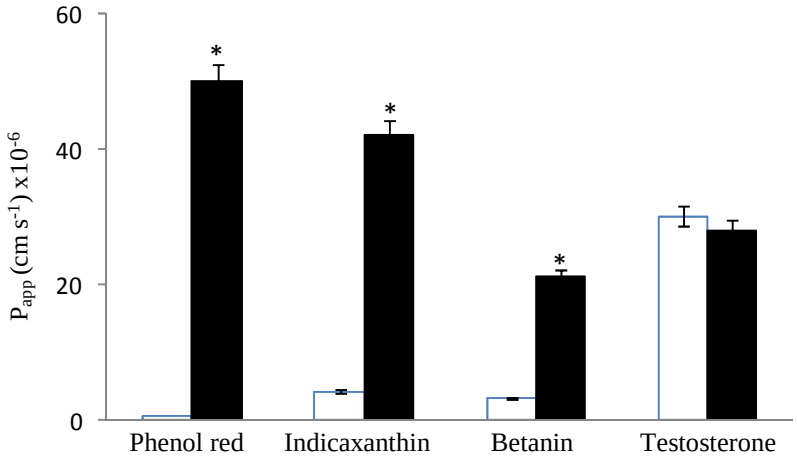


Fig. 7.

Apparent permeability coefficient (P_{app}) for red phenol, betalains and testosterone across Caco-2 cell monolayer with tight intact (without EDTA) (open bars) and after opening of tight junctions with 5 mM EDTA (black bars). Data are the means ± SD of two separate experiments carried out in triplicate. For each compound, bars labeled with (*) are statistically different with $p<0.001$ (Student's *t*-test).

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APPENDIX

FOREIGN EXPERIENCE

Baltimore

March - September 2011

An archaeal molecular model to test human chaperonins II associated with human diseases

Abstract

Mutations in CCT subunits 4 and 5 have been found to cause disease but little is known on the pathogenetic mechanisms at the molecular level, and on the impact of the mutations upon the subunits' structure and functions. This absence of information is mostly due to the lack of a suitable prokaryotic experimental model, simpler than the eukaryotic cell itself but still representative of what happens in it. We have developed such an experimental model using the archaeon *Pyrococcus furiosus* (Pf), which has a single CCT subunit but forms octamers and hexadecamers like the human counterparts. We have focused on the human CCT5 mutation His147Arg, and have standardized the Pf model accordingly by using bioinformatics, computer-assisted modeling, and molecular biological and biochemical analyses. We have studied the structural and biochemical properties of purified recombinant Pf-Cpn at various temperatures, assessing the formation of oligomers, and chaperoning properties, and the effect of mutations on them. Using structural modeling we have identified the position that in the Pf chaperonin (Pf-Cpn) matches His147 in the human ortholog, and studied the effect of mutations at this position on 3D structure, oligomerization, and chaperoning properties. The data show that the archaeal model is reliable and amenable to the manipulations necessary to generate a strategy to mimic pathological features.

Introduction

Molecular chaperones constitute multifunctional cellular systems that assist folding and assembly of newly synthesized proteins, translocation of unfolded proteins across membranes, as well as refolding and degradation of misfolded and aggregated proteins (Okochi *et al.* **2005**). Chaperonins (Cpns) are large, hollow complexes that promote correct folding of a wide range of unfolded, misfolded or partially folded proteins (Ranson *et al.* **1998**). Chaperonopathies constitute a large group of diseases in which defective chaperones play a pathogenetic role (Macario, A.J.L. *et al.* **2005**). Since chaperones are essential mediators of cellular protein folding in eukaryotes, defective chaperones may affect a variety of cellular processes unrelated to protein quality control(Cappello *et al.* **2008**; Macario A.J.L. *et al*. **2010**).

Unchecked protein aggregation and misfolding are recognized as the root cause of a large and diverse collection of diseases termed 'protein misfolding' or 'protein conformational' disease (Chiti *et al.* **2006**). These diseases, which include amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's, Huntington's and other polyglutamine diseases, arise when certain proteins adopt non-native conformations that endow them with a tendency to aggregate and form intra- and/or extra-cellular deposits. In all these cases, protein misfolding results in 'gain-of-function' proteotoxicity, whereby misfolding confers newly-gained cytotoxicity onto the disease protein, by promoting inappropriate interactions that are detrimental to the cell. Misfolding and aggregation of the disease protein may also confer some degree of loss of function, which may additionally contribute to disease pathogenesis (Lim J. *et al*. **2008**;Schiffer N.V. *et al*. **2008**).

Although considerable progress has been achieved in the study of chaperonopathies from the genetic and clinico-pathologic standpoints, little is known about the molecular mechanisms by which a defective chaperone affects cell physiology and causes tissue and organ pathology. This scarcity of knowledge on the intrinsic molecular mechanisms driving the physiopathology of chaperonopathies is

partly due to the difficulties inherent in the study of complex molecular assemblies and their interactions with others, which are characteristic of chaperones.

In humans (*Homo sapiens*), for example, the number of the chaperonin of type II genes is as high as 14, including canonical and non-canonical CCT molecules, and to this number one must add the variations in the proteins from these genes(Mukherjee, K. *et al.* **2010**). There is, therefore, a need for developing experimental models that are simpler than those afforded by human cells, or any eukaryotic cell, but still representative of what occurs in those cells.

Archaea are simple prokaryotes that nonetheless are quite similar to eukaryotes in a number of features, among which the chaperoning system, and are thus good model candidates (Macario A.J. L. *et al*. **2004**). In this regard, bacteria are also simple but are evolutionarily distant from eukaryotes, more distant than archaea and, with rare exceptions still under scrutiny, do not have some of the key chaperones present in human cells like, for example, the chaperonins of type II (Macario A.J. L. *et al*. **2004**).

We are developing a model system for analysing the structure and the functions of CCT subunits using as model the archaeon *Pyrococcus furiosus*, which has only one CCT homolog (Pf-Cpn) and forms oligomers similar to those typical of the human counterparts (Luo H. *et al*. **2009**, Luo H. *et al*. **2011**).

Here we report our results pertaining to Pf-Cpn as a model system to study *Homo sapiens* CCT subunits, CCT5 (Hs-CCT5) in particular. Mutations in this subunit have been implicated in the pathogenesis of some distal sensory-motor neuropathies (Bouhouche A. *et al.* **2006** (**A**); Bouhouche *et al.* **2006** (**B**); OMIM entry http://omim.org/entry/256840).

Materials and Methods

Cloning and expression of Pf Cpn and mutants

E.coli strains used in this study were DH5α for the initial cloning of the Pet33b expression plasmid and Rosetta cells for recombinant protein expression. Restriction enzymes, polymerase, and ligase were purchased from New England Biolabs (Beverley, MA). All other chemicals were of analytical grade from Sigma-Aldrich (St Louis, MO). All solutions were made up in ultra-pure water. The native gel protein marker was NativeMark^{TM} unstained molecular weight protein standard from InVitrogen (Carlsbad, CA). Glutamate, ATP, EDTA, DTT, β-NADH, and NAD were purchased from Sigma- Aldrich (St Louis, MO). Bovine glutamate dehydrogenase (GDH) was purchased from Amresco (Solon, OH).

Purification and preparation of Cpn wild-type and mutants

Pf Cpn was prepared as previously reported (Luo *et al.* **2009**). Genes for Pf Cpn mutants were amplified by PCR with the corresponding primers listed in Table 1. The mutants were cloned, expressed, and lyzed using the protocol for Pf Cpn (Luo *et al.* **2009**) with modification. The supernatant extracts were heated at 85°C for 30 min, then they were purified to homogeneity by two steps of anion exchange: HiTrap^{TM} Q HP cartridge from Biorad (Hercules, CA) and Bio-Scale^{TM} macro-prep high Q cartridge from GE healthcare (Uppsala, Sweden). The protein concentration was determined by the Bradford assay.

ATP hydrolysis activity

The standard reaction mixture contained 25 mM HEPES-KOH, pH7.2, 300 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml Cpn, and 100 μM ATP in a final volume of 90 μl. The reaction mixture was incubated at the indicated temperatures for 3 min before adding ATP to initiate ATP hydrolysis. The reaction was performed for 15 min, followed by 2% perchloric acid addition to quench the reaction. The liberated Pi was determined

by the Malachite Green assay at 630 nm (Baykov *et al*. **1988**; Geladopoulos *et al.* **1991**; Yoshida *et al*. **1997**).

Bovine Glutamate Dehydrogenase (GDH) Protection Assays

Chaperone activities of Pf Cpn WT and mutants were compared by their protection of Bovine GDH at 50°C. GDH samples (2.5 mg/ml) were incubated in buffer A (25 mM HEPES-KOH, pH8.0, 300 mM KCl, and 1 mM $MgCl_2$) at 50°C in the presence or absence of Cpn (0.2mg/ml) and 4 mM ATP. Thermostability was assayed by measuring the enzyme activity after indicated interval. GDH activity was determined by oxidative dearmination measuring the glutamate-dependent reduction of NADP at 340 nm as previously described (Laksanalamai *et al.* **2009**). The reaction mixture contained 90 mM EPPS, pH8.0, 2 mM L-glutamate, 0.5 mM NADP, and enzyme in a total volume of 325 µl.

Size determination of complexes of Pf-Cpn mutants

The oligomeric status of mutants were determined by 3-8% non-native gradient gel.

Molecular modeling

Direct and comparative modeling; secondary and higher order structures of wild type and mutant molecules.

Results

Pf-Cpn hydrolyzes ATP, has exceptional stability, forms a functional chaperonin complex, and is amenable to mutations that affect function, (**Figures 1- 3**). Hs-CCT5 and Pf-Cpn are very similar in sequence and structure, (**Figure 4**). Pf-Cpn "humanized" mutants Ile138His and Ile138Arg, particularly the latter, have impaired functional ability as compared with the Pf-Cpn wild-type, (**Figures 5 - 7).**

Conclusions

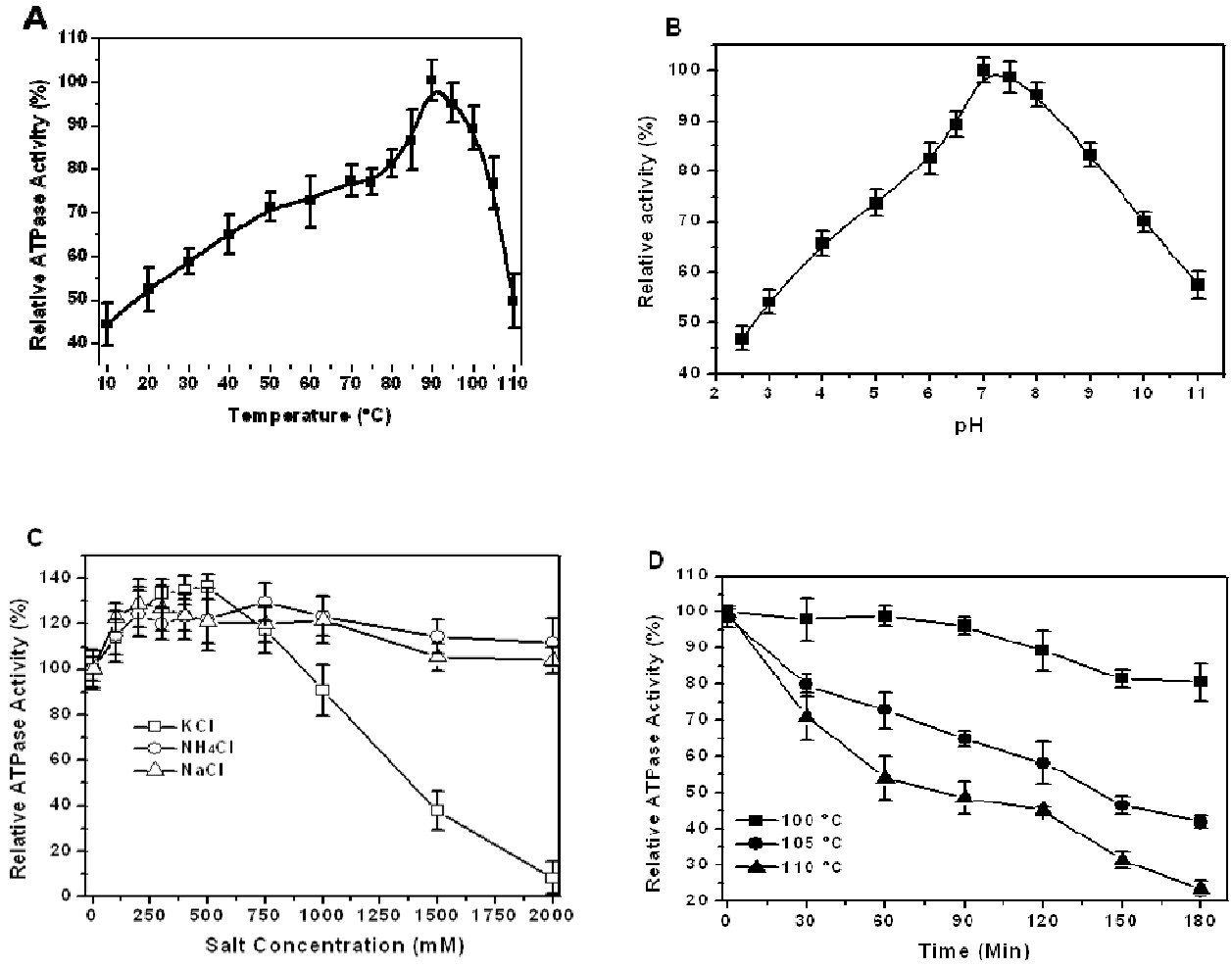
Sequence comparisons between the wild-type human CCT-5 (Hs-CCT5) and archaeal (Pf-Cpn) proteins, using various complementary methods such as alignments of entire sequences and of selected portions of them, and comparison of 3D molecular models showed that they are very similar in all their aspects;

Molecular modeling of wild-type and mutant Hs-CCT5 and comparison of the models with one another by various complementary and sequential methods showed that the mutation His147Arg has little impact on overall structure;

The position in Pf-Cpn equivalent to the Hs-CCT5 His147 is Ile138;

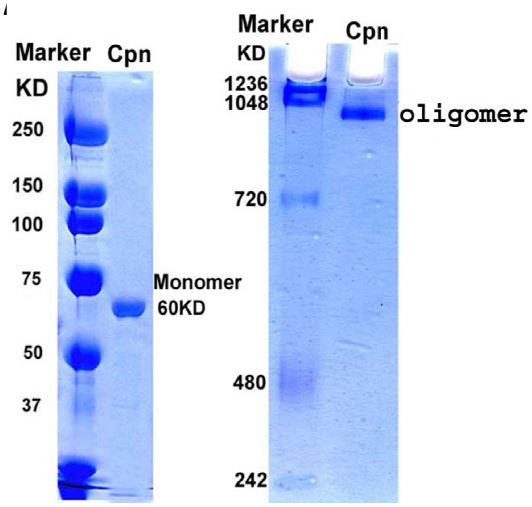
Laboratory testing of wild-type and mutants to assess ATP-binding and hydrolysis, protection of proteins from denaturation by stressors, and formation of homoligomers, showed that Pf-Cpn mutants Ile138His (matching Hs-CCT5 wt) and Ile138Arg (matching Hs-CCT mutant that is implicated in disease) impair function. The Ile138His mutation has a slight effect on function and stability, whereas Ile138Arg causes significant defects in function.

Figure 1. Pf-Cpn hydrolyzes ATP and has exceptional stability.



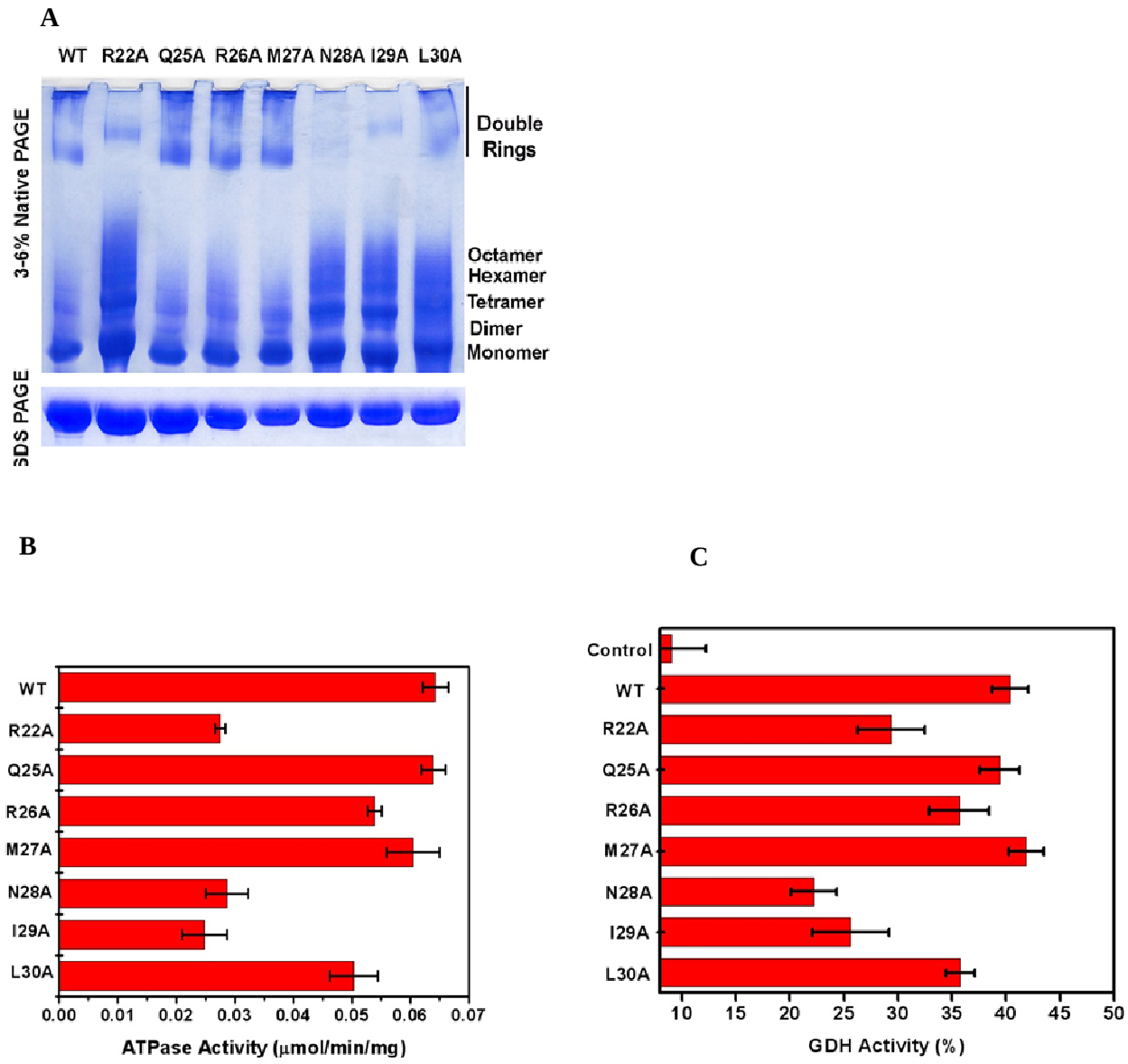
A. Temperature profile of ATPase acitivity. **B.** ATPase activities of Pf-Cpn at various pH levels. **C.** ATPase activities of Pf-Cpn in different salt concentrations. **D.** Thermal stability of the recombinant Pf-Cpn at high temperatures. The samples were heated at the shown temperatures for the specified times and then assayed for their ATPase activities at 90°C. Data reflect the average values from three experiments.

Figure 2. Pf-Cpn is a functional chaperonin complex



SDS (left) and Native (right) PAGE of the purified Pf-Cpn.

Figure 3. Identification of mutations impairing Pf-Cpn functions



A. Size determination of N-terminal deletion mutants by native gel electrophoresis. **B**.ATPase activities of the mutants. **C.** Protection of GDH by the mutants.

Figure 4. Selective alignment Hs-CCT5 and Pf-Cpn

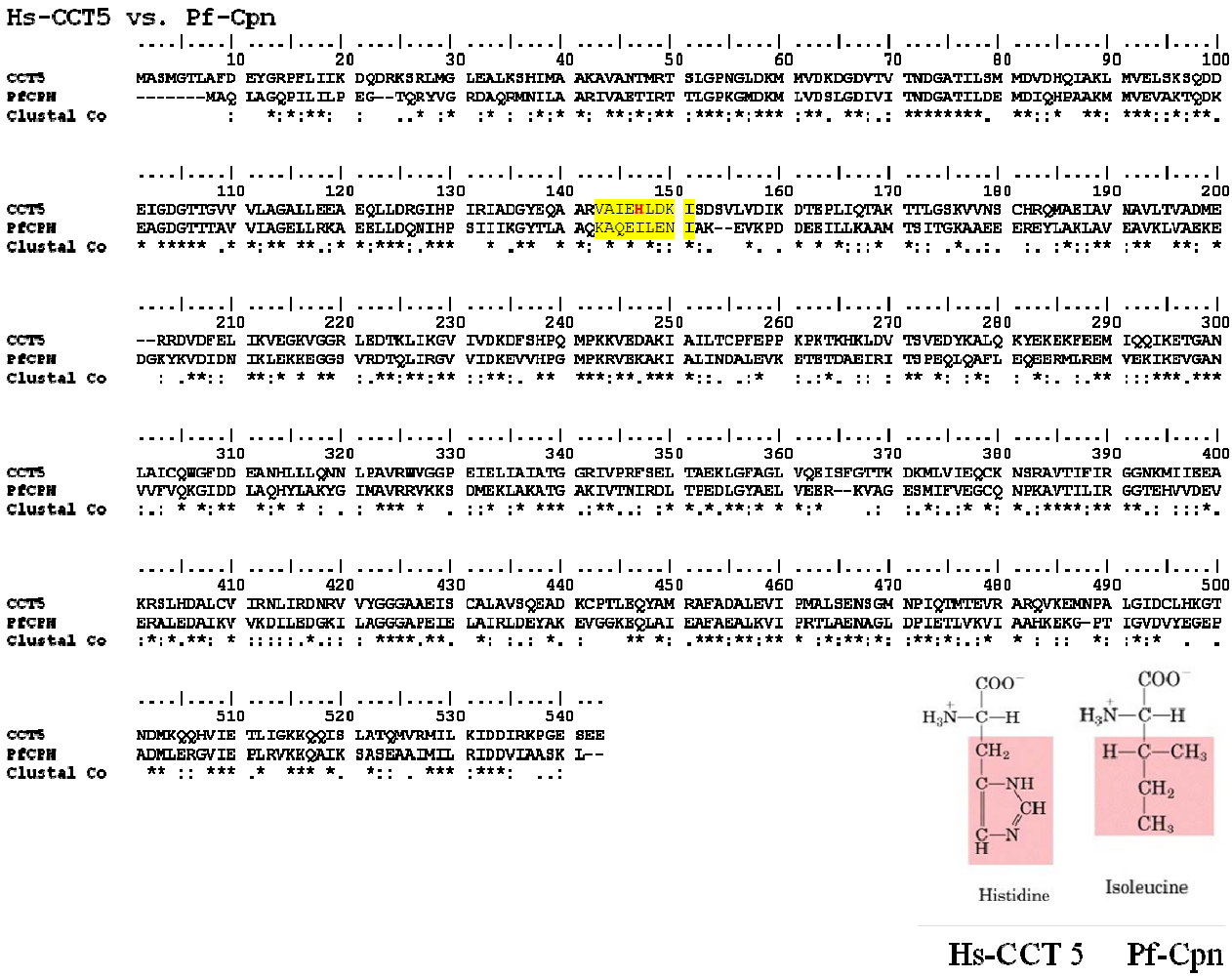


Figure 5. ATPase activity of purified mutants H and R, and wild type (WT) Pf-Cpn proteins

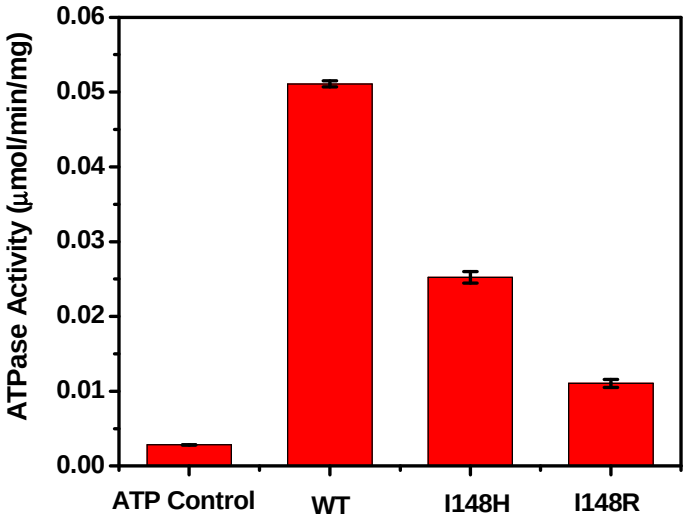


Figure 6. Protection of GDH against heat (52 ° C) denaturation by Pf-Cpn Wt and H and R mutants

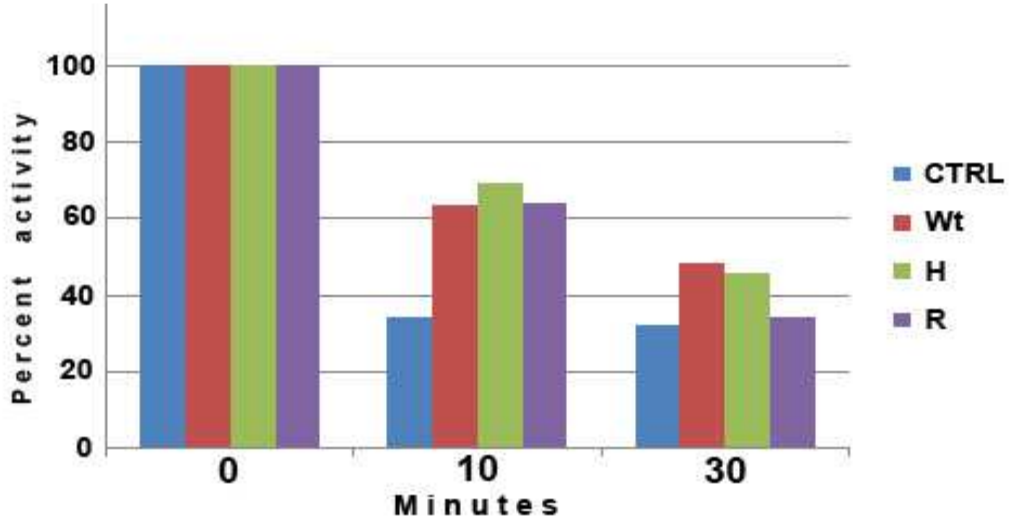
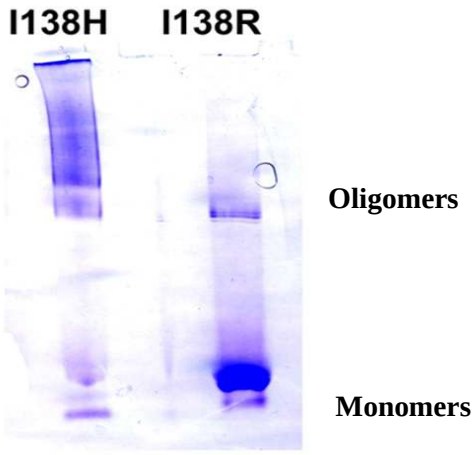


Figure 7. Oligomerization capacity of purified Pf-Cpn mutants H and R



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Malta

September – December 2011

Immunohistochemical detenction of receptors and neuronal markers in paraformaldehyde fixed nervous tissues

Introduction

The Lateral Habenula

The lateral habenula (LHb) is part of the habenular complex, which is situated in the dorsal diencephalon of all vertebrates on both sides of the third ventricle The LHb is involved in the response to stress,(Chastrette N. et al. 1991; Wirtshafter D. *et al.* **1994**; Timofeeva E. *et al*. **2001**; Amat J. *et al.* **2001**; Villareal JS. *et al*. **2002**) maternal behavior, (Corodimas KP. *et al*. **1993**; Corodimas KP. *et al*. **1992**; Felton TM. *et al*. **1998**; Kalinichev M. *et al.* **2000**) nociception,(Nagao M. *et al.* **1993**; Cohen SR. *et al.* **1985**; Cohen SR. *et al.* **1986**; Cohen SR. *et al*. **1993**; Terenzi MG. *et al.* **1990**) circadian rhythms,(Guilding C. *et al*. **2007**; Tavakoli-Nezhad M. *et al.* **2005**; Zhao H. *et al.* **2005**; Hattar S. *et al.* **2006**; Qu T. *et al.* **1996**) learning, (Villareal JS. *et al.* **2002**; Lacourtier L. *et al.* **2007**) and reward (Sutherland RJ. *et al.* **1981**; Morissette MC. *et al.* **2008**; Vachon MP. *et al.* **1992**; Nakajima S. *et al.* **1984**).Most recently, it has been discovered that the LHb conveys negative reward information to the dopaminergic midbrain and therefore plays a crucial role in reward learning(Ullsperger M. *et al.* **2003**; Matsumoto M. *et al*. **2007**). Due to its prominent interactions with the dopaminergic and serotoninergic system the LHb has been suggested to be involved in the development and progression of psychiatric diseases, including major depression, schizophrenia, and drug addiction (Sutherland RJ. **1982**; Ellison G. **1994**; Kelly PH. **1998**; Lecourtier L. *et al*. **2004**; Shepard PD. *et al.* **2006**; Morris JS. *et al.* **2001**).

In contrast to the surprising diversity of behaviors, in which the lateral habenular complex is thought to be involved, there is an astonishing lack of information regarding its cellular organization, its neuronal circuits, and the neurophysiological mechanisms, which may provide the physiological and molecular basis for its diverse biological functions. Recently, a detailed subnuclear organization has been described

(Andres *et al*. **1999**). Available criteria, however, can be applied to Cryostat or Vibratome thin sections to allow identification and delineation of subnuclei of the lateral habenular complex.

Materials and Methods

Paraformaldehyde perfusion

Rats were fixed by transcardial perfusion under deep chloral hydrate anesthesia (800 mg/kg) with 100 mL phosphate buffer (0.1 M, pH 7.4) followed by 500 mL of a paraformaldehyde solution (4% in phosphate buffer). Brains were removed and postfixed overnight in the same fixative, they were then washed and cryoprotected in phosphate-buffered saline (12 mM phosphate buffer, pH 7.2, with 0.9% NaCl) containing 30% sucrose.

Subsequently sections were stabilized with Agar blocks, cut at 40 μm following the frontal plane on a Vibratome (VT 1000S, Leica, Deerfield, IL) at 40 μm and collected free-floating in PBS .

Histochemistry

For immunohistochemistry, free-floating sections were washed in PBS (3 x 10 minutes), to remove all traces of paraformaldehyde and sucrose that interfere in the results of analysis. After that they were incubated for 15 minutes in a 1% H_2O_2/50% ethanol solution if used for a peroxidase reaction, washed in PBS (3 x 10 minutes), and incubated in PBS containing Triton X-100 and 5% donkey serum for 45 minutes. Sections were then incubated overnight at room temperature in PBS with Triton X-100, 1% donkey serum, and primary antibody. Triton X-100 was used at 0.5%, except for glutamic acid decarboxylase (GAD) immunohistochemistry, for which it was lowered to 0.2% to reduce process staining and facilitate cell body identification.

Sections for peroxidase reaction were washed in PBS (3 x 10 minutes), incubated with a biotinylated donkey antirabbit secondary antibody (Amersham Biosciences, Arlington Heights, IL; 1:200 in PBS, 0.5% Triton X-100, 1% donkey serum) for 1 hour and 30 minutes, washed in PBS (3 x 10 minutes), and incubated with PBS containing the avidinbiotin-peroxidase complex (ABC) (ABC Elite, 0.2% A and 0.2% B; Vector) for 1 hour and 30 minutes. After washing in Tris-HCl buffer (0.05 M, pH 7.5; 3 x 10 minutes), bound peroxidase was revealed either by incubation in 0.025% 3.3x- diaminobenzidine tetrahydrochloride (DAB), 0.0006% H_2O_2 (Sigma) in Tris-HCl buffer or by using the peroxidase substrate Vector SG kit (Vector). Sections were incubated for approximately 10 minutes and washed again. Finally, sections were mounted on glass slides, coverslipped and analysed with a Leica CS-SL confocal microscope.

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