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Neohesperidin dihydrochalcone: Presentation of a small molecule activator of mammalian alpha-amylase as an allosteric effector

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ABSTRACT

Flavonoids and their precursor *trans*-chalcone have been reported as inhibitors of mammalian alpha-amylase. With regard to this background, neohesperidin dihydrochalcone (NHDC) effect was investigated toward porcine pancreatic alpha-amylase (PPA), and found to be an activator of the enzyme. The maximal activation (up to threefold) was found to occur at 4.8 mM of NHDC, which could be considered to have a high activation profile, with regard to the alpha and beta parameters (alpha < 1 < beta). NHDC is a non-essential activator of the enzyme and based on the results obtained from modeling tools, it is suggested to interact with PPA at a hydrophilic site located at the N-terminal, far from the active site of the enzyme.

1. Introduction

Mammalian pancreatic α-amylases are endo-type enzymes responsible for the hydrolysis of internal α-(1,4)-glucosidic bonds in starch and related compounds. The hydrolysis occurs toward the non-reducing end of the substrates [1], which are then processed by other enzymes, finally resulting in an increase in blood glucose in mammals [2]. Inhibition of this enzyme is thus a potential way to lower blood glucose, and may find therapeutic application in the diabesity state [3]. This may explain why the majority of studies that concern effectors of this enzyme focus on its inhibitors.

In the few reports that have been published on activators of alpha-amylases, anions effects have been extensively documented. Chloride was the first anion reported as an allosteric activator of human pancreatic alpha-amylase (HPA) [4]; further studies revealed the existence of a wide range of chloride-dependent alpha-amylases and the fact that the three essential residues binding to chloride (R195, N298, and R337 in HPA) [5–8], are highly conserved in animal alpha-amylases and some Gram-negative bacteria [9]. Other anion activators include nitrite, nitrate, and azide [4,6].

Additives including triton X-100, poly ethylene glycol (PEG) and poly vinyl alcohol (PVA) [10], as well as xanthine compounds [11] have also been found to activate alpha-amylase, but mostly in a non-dose dependent manner.

The chalcone scaffold, synthesized by the help of plant chalcone synthase, is the precursor of the natural products flavonoids and isoflavonoids [12]. Based on the fact that flavonoids had been reported as non-carbohydrate based inhibitors of mammalian alpha-amylase [13], we started a project on screening inhibitory effect of compounds with similar structure to flavonoids. Based on the fact that HPA and porcine pancreatic alpha-amylase (PPA) possess high similarity [14,15], PPA was used as a model enzyme. This study resulted into observation of the inhibitory effect of *trans*-chalcone on (PPA) [16] and prompted us to further explore other chalcone derivatives effect on this enzyme. The present report concerns the unexpected activator effect that was found for neohesperidin dihydrochalcone (NHDC) (Scheme 1). NHDC, a synthetic glycoside chalcone, is added to various foods and beverages as a low caloric artificial sweetener; a delayed onset and a long lingering menthol-licorice like sweetness have limited its use [17,18] and it is used in industry blended with other sweeteners [19]. NHDC binding site on the human sweet taste receptor has been identified, and overlaps with the one of cyclamate [20]. Since structural variations of sweet taste receptors may be involved in the physiopathology of metabolic diseases (such as diabetes) [21], and targeting the "ingestive behavior" of patients (via taste receptors) has been proposed as a therapeutic approach [22], artificial sweeteners may be considered as potential drugs. Furthermore,

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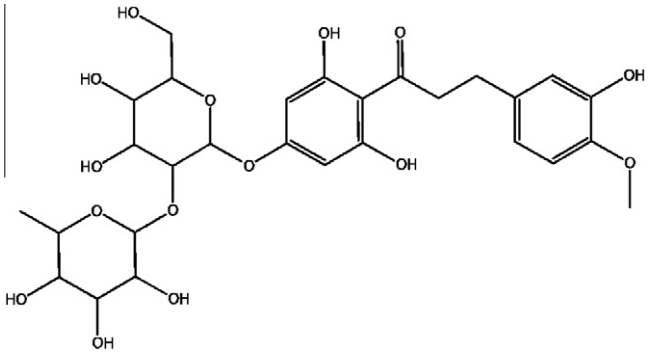


Scheme 1. Neohesperidin dihydrochalcone (NHDC).

NHDC possess other potential therapeutic properties, since its antioxidant [23] and anti-ulcer [24] activities have been observed.

2. Materials and methods

2.1. Material

Porcine pancreatic alpha-amylase (E.C.3.2.1.1) (DFP Treated, Type I-A), neohesperidin dihydrochalcone, acarbose, 4-nitrophenyl α-D-maltohexaoside, 4-nitrophenol, and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), soluble starch, maltose and other chemicals were obtained from Merck (Darmstadt, Germany).

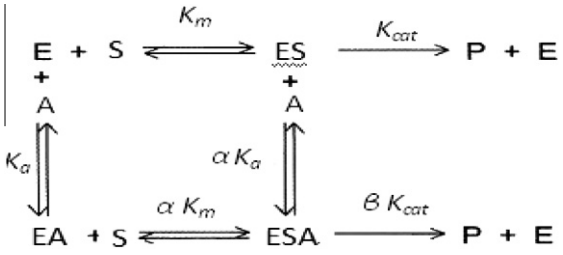
2.2. Methods

2.2.1. Enzyme assay

Each compound effect was tested on PPA with the use of two substrates, namely 4-nitrophenyl α-D-maltohexaoside (synthetic substrate) and starch. Assays were performed in phosphate buffer, pH 7.2 ± 0.01, at 42 °C for 4-nitrophenyl α-D-maltohexaoside and 25 °C for starch. Compounds were incubated with the enzyme at 25 °C and stirred gently for 10 min previous to activity assay. Continuous kinetic method was used for the synthetic substrate [25] and Bernfeld method [26] was applied when using starch. The activity of alpha-amylase was defined as produced millimoles (mM) of *p*-nitrophenol and maltose as the products for the synthetic and natural substrates respectively. Absorbance measurements were made with the use of a Shimadzu UV-1800 spectrophotometer and activities calculated by the UV-Probe software. PPA activity with the use of starch and 4-nitrophenyl α-D-maltohexaoside was 560 (IU ml^{-1}) and 2580 (IU ml^{-1}), respectively. Substrates concentrations were 0.8 (g 100^{-1}) for starch and 3 mM for 4-nitrophenyl-α-D-maltohexaose. Relative activity was defined as a percentage by comparison with a control sample activity, measured in the absence of the compounds. All tests were repeated at least three times. SD and CV were calculated, and results with CV ⩽ 5.5 (4-nitrophenyl α-D-maltohexaoside), and CV ⩽ 6 (starch) considered acceptable.

2.2.2. Kinetic analysis

The kinetic model for non-essential activators (Scheme 2) resembles the one of non-linear partial inhibitors but in opposite direction. K_m is the Michaelis–Menten constant, K_a stands for activator dissociation constant and k_{cat} is the catalytic constant [27–29]. α is defined as the magnitude of interaction between substrate and moderator binding site and β is the magnitude of increase of catalytic constant [30]. In this study, all kinetic parameters were obtained with consideration to this scheme by the use



Scheme 2.

of the Linweaver–Burk (L–B) plots; catalytic constants (k_{cat}) were calculated applying Cleland method [31,32] based on Eq. (1). Eq. (1) refers to the following modified Michaelis–Menten equation,

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot \frac{\left(1+\frac{[A]}{K_a}\right)}{\left(1+\frac{\beta \cdot [A]}{\alpha \cdot K_a}\right)} + [S] \cdot \frac{\left(1+\frac{[A]}{\alpha \cdot K_a}\right)}{\left(1+\frac{\beta \cdot [A]}{\alpha \cdot K_a}\right)}} \quad (1)$$

where V_{max} is the maximum velocity in absence of moderator, [*S*] and [*A*] are substrate and activators concentrations, respectively, and v shows the initial velocity in the absence of activator [30]. K_a, α and β values were obtained by Linweaver–Burk plotting and replotting [28,30].

2.2.3. Docking

Docking was performed with Auto dock vina [33]. The 3L2M pdb (www.pdb.org/) file was first processed with the use of MOE 2010.10 (Chemical Computing Group Inc., Montreal, Canada). Additional molecules to alpha-amylase, were deleted and the protonation state of the structure was adjusted for neutral pH. Grid box of 60 × 72 × 64 points was used with a spacing 1.0 Å, and the grid box center was put on $x = 37.318$, $y = 31.139$, and $z = 44.183$. Gasteiger charges were assigned to protein and ligand molecules. Exhaustiveness was set on 20 and a computer with eight processors was utilized for the computation. Hundred poses were generated for NHDC and preparation of the image representing the best pose was done with MOE 2010.10. An additional blind docking was also performed with the use of the Swissdock web server (http://swissdock.vital-it.ch/) [34].

3. Results and discussion

3.1. Probing the activation effect of NHDC

Both natural (starch polymer) and synthetic substrate (4-nitrophenyl-α-D-maltohexaoside) were used to test the effect of 5–1000 μM concentrations of NHDC on PPA. As shown in Fig. 1A, with the artificial substrate, the enzyme relative activity rises by increasing NHDC concentration, and finally reaches a maximum of 130% in presence of 1 mM of NHDC. The activating effect of similar amounts of NHDC is more pronounced with the use of starch as substrate, where relative activity of the enzyme activity attains 170%, and with increasing the amount of NHDC, activity keeps on increasing to reach a maximum of three-fold by 4.8 mM of NHDC (Fig. 1B). The substrate nature could influence inhibitors behavior, which may show different inhibition types in the case of PPA [35]. This effect could be attributed to the size and location of substrate binding. More specifically, we had a similar observation relative to the non-dose dependent xanthine activators, whose effects were more apparent in presence of starch [11]. The (remote) possibility that NHDC could act as a substrate was also taken into account, but incubation of the enzyme with NHDC did not result in any significant activity (results not shown).