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Determination and stability of divicine and isouramil produced by enzymatic hydrolysis of vicine and convicine of faba bean

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ABSTRACT

The aglycones of vicine and convicine, divicine and isouramil, are the causative agents of favism and, therefore, should be analysed along with vicine and convicine in research seeking to eliminate them. This study investigated the stability of the aglycones produced by hydrolysis with β-glucosidase. Reversed-phase, high-performance liquid chromatography (HPLC) with UV detection was shown to be able to observe both aglycone formation and further reactions in isolated fractions and extract made from faba bean and in faba bean suspension. Divicine and isouramil were unstable and degraded almost completely in extract in 60 min and completely in fractions in 120 min at a pH of 5 at 37 °C. Adding sodium ascorbate delayed degradation of divicine. Divicine was more stable at 20 °C than at 37 °C. Being able to show formation and degradation of the aglycones, the proposed method allows monitoring of the vicine and convicine detoxification process.

1. Introduction

The current demand for plant protein for food and feed use in Europe is much higher than the production. As much as 70% of protein-rich feed needed for livestock production is imported soybean (Bues et al., 2013; De Visser, Schreuder, & Stoddard, 2014). Cultivated areas in Europe are mostly used for cereal crops such as maize, rice and wheat (FAO, 2013) and only 1.8% of arable land is used for cultivation of protein crops (Bues et al., 2013). New, protein-rich plants are needed to improve self-sufficiency and diminish imports. Potential options to replace soybean are legumes such as pea, faba bean and lupine. Protein content in faba bean (*Vicia faba*) is approximately 25–30% in European grown cultivars (Lizarazo et al., 2015), and it is adapted to the relatively demanding Boreal cropping systems.

Although legumes have a valuable nutritional composition, they also contain antinutrients that limit their use. In faba bean, these include vicine (2,6-diamino-4,5-dihydroxypyrimidine 5-β-D-glucopyranoside) and convicine (2,4,5-trihydroxy-6-aminopyrimidine 5-β-D-glucopyranoside), both of which can be hydrolysed in food processing and *in vivo* to their corresponding aglycones: divicine and isouramil (Fig. 1). These aglycones cause oxidative stress in the red blood cells in cases of a genetic disorder that is expressed as a deficiency in the glucose-6-phosphate dehydrogenase (G6PD) enzyme (Cappelini & Fiorelli, 2008). Oxidative agents transform glutathione (GSH) to its oxidized form (GSSG). The G6PD enzyme protects the red blood cells by regenerating GSH, which maintains the essential activity of cell-protecting enzymes. Otherwise, an overload of GSSG leads to fatal structural changes such as denaturation of haemoglobin and disulfide bond aggregates (Heinz bodies) in the red blood cells, which leads to haemolytic anemia, called favism (Baker, Bosia, Pescarmona, Turrini, & Arese, 1984; McMillan, Bolchoz, & Jollow, 2001). Favism caused by digestion of the faba bean has many variants, and its prevalence is the highest in Asia, the Mediterranean area and Africa (Cappelini & Fiorelli, 2008).

Faba bean cultivars with one-tenth the wild-type concentrations of vicine and convicine have been bred (Duc, Crepon, Marget, & Muel, 2004; Khazaei, O'Sullivan, Jones, Pitts, Sillanpaa, Parssinen, & Stoddard, 2015), but the low-vicine-convicine gene, vc-, has not been bred into commercially available cultivars for the Boreal region. Processing methods such as soaking, heating, germinating, and fermenting have been investigated for removal of vicine and convicine (Cardaror-Martinez et al., 2012; Coda et al., 2014; Donath & Kujawa, 1991; Goyoaga et al., 2008; Jamalian & Ghorbani, 2005; McKay, 1992). Even though reduction or elimination of vicine and convicine has been confirmed using

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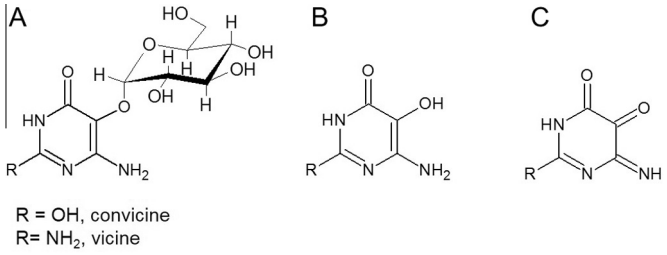

Fig. 1. Chemical structures of (A) vicine and convicine, (B) their aglycones, divicine and isouramil and (C) suggested oxidized aglycones.

these methods, the corresponding aglycones, divicine and isouramil, may still be present, as a result of enzyme- or acid-induced cleavage of the glycosidic bond. Because the aglycones are the actual favism-inducing compounds, their elimination must be confirmed in detoxification processes of vicine and convicine.

To enable the elimination of divicine and isouramil, their stability and reactivity must be understood comprehensively. In previous studies, the aglycones have mainly been prepared by crystallization after acid hydrolysis of vicine and convicine and studied for their stability in different solvents. Divicine and isouramil have specific UV-absorption maxima, and changes in absorption maxima have indicated their further reaction when measured with UV spectrophotometry or HPLC with UV detectors that were not able to record the spectra (Baker et al., 1984; Chevion, Navok, Glaser, & Mager, 1982; Marquardt, Arbid, & Frohlich, 1989; Marquardt, Frohlich, & Arbid, 1989; McMillan, Schey, Meier, & Jollow, 1993).

Formation of oxidation products from the aglycones has been confirmed by being able to regenerate them to the respective aglycones using reduction. The absorption maxima for pure divicine and isouramil underwent a rapid decline during oxidation and were restored by adding a reducing agent such as sodium borohydride, cysteine or dithiothreitol (DTT) (Chevion et al., 1982; Marquardt, Frohlich, et al., 1989). Furthermore, adding the reducing agent after exposure to oxygen restored the divicine peak in an HPLC chromatogram (Marquardt, Arbid, et al., 1989; Marquardt, Frohlich, et al., 1989; McMillan et al., 1993). Fig. 1 shows the suggested structures for oxidized divicine and isouramil.

The decomposition pathways of the aglycones are poorly known. A reaction product that could not be restored to the aglycones after adding reducing agents has also been reported. Its absorption maximum wavelength was less than 230 nm for both the aglycones (Chevion et al., 1982). An absorption maximum of 250 nm has also been reported for this type of product from divicine (Marquardt, Frohlich, et al., 1989). Decomposition of the aglycone ring has been suggested to ultimately lead to loss of UV absorptivity (Chevion et al., 1982).

Degradation has occurred fast for divicine and isouramil in studied solutions. Oxidized divicine has formed quickly after hydrolysis of vicine, and both divicine and oxidized divicine have disappeared rapidly. UV spectra of the aglycones changed in less than 10 min at neutral pH in the presence of oxygen (Chevion et al., 1982). On the other hand, nitrogen saturated air stabilized divicine (Chevion et al., 1982; Marquardt, Frohlich, et al., 1989; McMillan et al., 1993) and the aglycones were more stable in acidic conditions than in neutral or alkaline conditions (Baker et al., 1984; Chevion et al., 1982; Marquardt, Frohlich, et al., 1989). However, stability of the aglycones has been studied only in simple solutions, and it is not known how sample matrix affects their stability. Stability of the aglycones and the possibility of eliminating them by degradation must be studied in conditions relevant to food applications and in a real sample matrix.

The main aims of the study were to investigate the stability of the vicine and convicine aglycones and to evaluate the suitability of the method developed for vicine and convicine analysis (Pulkkinen et al., 2015) to monitor aglycone formation and degradation. The aglycones were produced by enzymatic hydrolysis of vicine and convicine in faba bean extract and by hydrolysis of isolated vicine and convicine. Stability of the vicine aglycone was studied under various conditions. In addition, suitability of the method for detecting the aglycones in faba bean suspension was investigated.

2. Materials and methods

2.1. Extraction of vicine and convicine from faba beans

Vicine and convicine were extracted from faba beans (cultivar Kontú) grown in Finland in 2011 (Lizarazo et al., 2015). Beans were dehulled (Jiang et al., 2015), milled with a Retsch mill (ZM 200) to a particle size of 0.5 mm and stored at -20 °C. Beans contained 6.07 mg/g of vicine and 3.45 mg/g of convicine (Pulkkinen et al., 2015).

The extraction method was modified from that used by Arbid and Marquardt (1985a). Faba bean flour (25 g) was mixed with a 70% ethanol-water solution (100 ml) for 30 min with a shaker (Heidolph promax 2020, Germany) at room temperature. The mixture was centrifuged for 15 min at 4 °C with 15,000*g* (Sorvall RC5C centrifuge, USA) and the supernatant was filtered through Whatman 42 filter paper. The filtered supernatant was concentrated 6-fold with a rotavapor, and pH of the concentrated supernatant was set to pH 4 with formic acid. The solution was again centrifuged with 13,000*g* at 4 °C for 10 min and used after dilution to MilliQ-water from the MilliQ Plus system (0.22 μm, Millipore Corporation, Bedford, MA, USA) in a 1:10 ratio to produce the pure vicine and convicine fractions (Section 2.2) and directly for a hydrolysis experiment (Section 2.3). The extract contained 660 μg/ml of vicine and 380 μg/ml of convicine.

2.2. Production of pure vicine and convicine fractions

Vicine and convicine were separated from the extract by preparative liquid chromatography (LC) equipment with mass spectrophotometric (MS) detection (Waters Corp., Milford, MA, USA) (Pulkkinen et al., 2015). This preparative LC–MS consisted of an automatic sample manager (Waters 2767) and a binary gradient module (Waters 2545) coupled with single quadrupole MS (Waters micromassZQ) and photodiode array (PAD) (Waters 2996) detectors. An Atlantis prepT3 column (10 × 250 mm, particle size 5 μm) was used to separate the vicine and convicine isocratically with MilliQ-water containing 0.1% formic acid as an eluent for 20 min. The column was washed with gradient elution; the proportion of acetonitrile containing 0.1% formic acid was increased to 80% (Pulkkinen et al., 2015). Flow rate was 3.5 ml/min, and before the detectors the flow was split with an active flow splitter at a split ratio of 1:100. Data were collected with MassLynx 4.1 software (Waters Corp., Milford, MA, USA). The injection volume was 500–950 μl, and vicine and convicine were collected according to *m*/*z* values 305 and 306, respectively. The UV absorption was simultaneously monitored at 273 nm. Concentration of the vicine fraction was approximately 50 μg/ml and that of the convicine fraction was 10 μg/ml.

2.3. Enzymatic hydrolysis of vicine and convicine to aglycones

The extract and isolated fractions of the vicine and convicine were hydrolyzed with β-glucosidase (EC 3.2.1.21, from almonds,