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HPLC method development for the online-coupling of chromatographic *Perilla frutescens* extract separation with xanthine oxidase enzymatic assay

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ABSTRACT

Enzyme-regulatory effects of compounds contained in complex mixtures can be unveiled by coupling a continuous-flow enzyme assay to a chromatographic separation. A temperature-elevated separation was developed and the performance was tested using *Perilla frutescens* plant extracts of various polarity (water, methanol, ethanol/water). Owning to the need of maintaining sufficient enzymatic activity, only low organic solvent concentrations can be added to the mobile phase. Hence, to broaden the spectrum of eluting compounds, two different organic solvents and various contents were tested. The chromatographic performance and elution was further improved by the application of a moderate temperature gradient to the column. By taking the effect of eluent composition as well as calculated logD values and molecular structure of known extract compounds into account, unknown features were tentatively assigned. The method used allowed the successful observation of an enzymatic inhibition caused by *P. frutescens* extract.

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1. Introduction

The assessment of health-promoting properties of natural extracts with regard to their antioxidative and anti-inflammatory capacity or their capability to regulate the activity of health- or disease related enzymes is a developing field of research. Most studies either focus on the investigation of whole extracts or on single known compounds to assess their impact on physiological parameters [1,2]. Especially the extent of an immunological response or changes in cellular gene expression of health-related enzymes may indicate the benefits of extracts or single compounds. Nevertheless those alterations are not necessarily correlated to the abundance of the proteinaceous gene product or to the activity of the translated enzyme [3]. This realization consequently eventuated in a major interest in the direct assessment of enzyme-regulatory effects of an extract or single compound. The investigation of whole extracts however results in only incomplete information about actual molecules able to inhibit or activate enzymatic catalytic activity. Most researchers therefore initially focus on the study of extract compositions by employing LC-UV or LC–MS techniques, followed by the isolation, purification and identification of single molecules [4]. Subsequently, these compounds can be investigated by introducing them successively to enzymatic assays to determine their regulatory potential.

By employing the so called online coupled continuous flow system, drug screening and determination of enzymatic activity can be combined [5,6]. Therewith the effects of chromatographically separated mixtures on enzymatic activity can directly be assessed. Alterations of substrate degradation and product generation, due to the presence of regulatory compounds, can be observed with mass spectrometric detection. This system has already been utilized in order to determine biochemical interactions [7] and assess IC_{50} values of single compounds [8–10]. In contrast to the injection of single molecules, the injection of mixtures necessarily resulted in the implementation of a chromatographic separation to the system [10]. This setup was thus applied to screen complex extracts for inhibitory molecules affecting the activity of enzymes like cathepsin B [10] or acetylcholine esterase [8,11].

On this basis, the effects of *Perilla frutescens* leave extracts towards xanthine oxidase (XOD) were investigated in the current study. In this regard a suitable HPLC method was developed to enable the direct coupling of extract separation and XOD enzymatic assay.

Abbreviations: XOD, xanthine oxidase; IS, internal standard; ROS, reactive oxygen species; RC, reaction coil.

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Perilla is frequently used as herbal medicine, herb or garnish in the Asian region and has been found to possess a variety of health-promoting effects [12], like working anti-allergic [13], antioxidative [14] and anti-inflammatory [15]. Perilla's advantageous features are related to a plethora of different phenolic compounds, whose individual impact on health has been extensively investigated as well [16,17]. Flavonoids like luteolin, apigenin as well as their glycosides and glucuronides or phenolic acids like caffeic acid and rosmarinic acid are known to possess health-promoting properties (e.g. alleviating the progress of inflammatory processes) [18]. Extract compounds may also be able to avert the exposure of harmful reactive oxygen (ROS) to cells by direct scavenging [19] or by inhibiting enzymes [20] involved in the generation of ROS, like e.g. XOD. Besides its participation in the release of superoxide during substrate degradation [21], XOD is part of the nucleotide metabolism and is also known to be involved in the development of hyperuricemia, in this manner also contributing to the pathogenesis of gout [21]. Molecules able to inhibit the activity of XOD would therefore not only reduce the accumulation of uric acid but also alleviate oxidative stress. Since several extracts as well as natural phenolic compounds have already been shown to regulate the enzyme [22,23], in this study Perilla extracts were screened in order to unveil molecules potentially affecting its activity.

2. Experimental

2.1. Reagent and chemicals

Xanthine oxidase from bovine milk, xanthine, allopurinol, rosmarinic acid, water LC–MS CHROMASOLV®, isopropyl alcohol (IPA) CHROMASOLV® for HPLC, formic acid and ammonium acetate were purchased from Sigma-Aldrich (Steinheim, Germany). Histidine was obtained from Merck (Darmstadt, Germany). Absolute ethanol, acetic acid (#A0820) and ammonia 32% were purchased from AppliChem (Darmstadt, Germany). Methanol HiPerSolv CHROMANORM® for LC–MS and acetonitrile were obtained from VWR (Darmstadt, Germany). *P. frutescens* water extract as well as *P. frutescens* (var. crispa) freeze-dried Perilla leaves were kindly provided by Vital Solutions GmbH and Vital Solutions GmbH (Langenfeld, Germany) and Amino Up Chemicals Co., Ltd (Sapporo, Japan).

2.2. Preparation of P. frutescens extracts

P. frutescens freeze-dried and milled leaves were extracted with ethanol (EtOH)-water (50:50, v/v) or methanol (MeOH)-water-formic acid (FAc) (90:9.5:0.5, v/v/v), respectively. For this purpose 500 mg freeze-dried and milled leaves were weighed, followed by the addition of 5 mL extraction solvent. After thorough mixing, the Perilla-solvent mixture was sonicated for 10 min at 4 °C and centrifuged afterwards for 20 min at 1500 rpm. The supernatant was transferred to another tube and the extraction procedure was repeated twice. The collected supernatant was evaporated to dryness (miVac Duo concentrator, GeneVac, Ipswich, England) and stored at −20 °C until use. Perilla water extract was provided in form of extracted and evaporated powder. Extracted Perilla was redissolved as required, whereby water extract was redissolved in water, 50% EtOH extract in EtOH-water (50:50, v/v) and 90% MeOH, 0.5% FAc extract in 100% EtOH.

2.3. Instrumentation and experimental setup

The online coupled continuous flow setup is comprised of three traces, each of which delivering either enzymatic assay components (Fig. 1, upper and bottom trace) or potentially regulatory compounds introduced to the system individually or as chromatographically separated mixture (Fig. 1, middle trace).

Enzyme solution was provided in a syringe (2.5 mL, Hamilton-Bonaduz, Switzerland) located in a syringe pump (Model 11 Plus, Harvard Apparatus, Hugo Sachs Elektronik, Hugstetten, Germany), which was set to a flow rate of 25 μL/min (Fig. 1, upper trace). Substrate solution was filled in a superloop (volume 10 mL, Amersham Biosciences, Uppsala, Sweden) and introduced to the system with a flow rate of 50 μL/min (Fig. 1, bottom trace). Samples were injected (Injector, Rheodyne, California, sample loop volume: 2 μL) into a continuous flow of 25 μL/min delivered by a quaternary HPLC pump (1100 series, Agilent Technologies, Waldbronn, Germany) and thus taken along (Fig. 1, middle trace).

Pumps were controlled by ChemStation software (version B.04.03, Agilent Technologies). Reaction coils (RC) 1 and 2 (Teflon, 0.25 mm ID) were knitted as described in literature [24] to provide a sufficient mixing of enzyme with the sample injected (RC 1, reaction time ~1.3 min) or of the enzyme-sample mixture with the substrate (RC 2, reaction time ~2.9 min), respectively. XOD and histidine stock solution were prepared in ammonium acetate (NH_4Ac) (pH 7.4, 10 mM), whereas xanthine stock was dissolved in water-NH_3 (0.1 M) (70:30, v/v), due to its low solubility in neutral aqueous solutions. Initial concentrations of the assay components were 0.032 U/mL for the enzyme XOD along with 80 μM histidine, latter serving as internal standard, (Syringe, Fig. 1, upper trace) and 50 μM xanthine (Superloop, Fig. 1, bottom trace). Due to the confluence of continuously delivered flows (Fig. 1, upper, middle and bottom trace), final concentrations of assay components in RC2 were 0.008 U/mL of XOD, 20 μM of histidine and 25 μM of xanthine, respectively.

2.3.1. Separation of P. frutescens extracts

Dried Perilla extracts were redissolved to a final concentration of 0.5 g extracted freeze-dried leaves/mL and introduced to the system equipped with chromatographic column (Fig. 1, middle trace, injection valve with 2 μL sample loop). The chromatographic performance of Luna PFP(2) column (100 Å, 2 mm IDx100 mm length, Phenomenex, Aschaffenburg, Germany) was tested with an isocratic elution using different low organic solvent concentrations and the application of a moderate temperature gradient up to 70 °C (Table 1), thus without conflict to the column specifications. The column was installed into a column oven (HT HPLC 2000, Scientific Instruments Manufacturer GmbH, Oberhausen, Germany) and equipped with a SecurityGuard Cartridge System (Phenomenex) with PFP(2) material precolumn (Phenomenex). It was flushed after each extract injection using a gradient up to Acetonitril (ACN):water (50:50, v/v), followed by the reconstitution to the respective eluent.

The temperature gradient was started simultaneously with the Perilla extract injection. The heated chromatographic eluent flow was cooled down to 30 °C before it was mixed with the flow containing the enzyme (Fig. 1, RC1). Chromatographic separation was performed using either EtOH—NH_4Ac (pH 7.4, 10 mM) (5:95, v/v), EtOH—NH_4Ac (pH 7.4, 10 mM) (10:90, v/v) or isopropyl alcohol (IPA)-NH_4Ac (pH 7.4, 10 mM) (5:95, v/v). In case of a chromatographic separation performed with 5% EtOH or 5% IPA, the enzyme was solved in NH_4Ac solution (pH 7.4, 10 mM)-the respective solvent (95:5, v/v). This led to an organic solvent concentration of 5% in RC1. The substrate was solved in NH_4Ac solution (pH 7.4, 10 mM)-respective solvent (85:15, v/v), resulting in a final organic solvent concentration of 10% in RC2 (Fig. 1). For a chromatographic separation using 10% EtOH, XOD as well as xanthine substrate were solved in NH_4Ac (pH 7.4, 10 mM)-EtOH (90:10, v/v), resulting in a solvent concentration of 10% in RC1 and RC2. Final organic solvent proportion of 10% in RC2 was chosen for all experiments to maintain a

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