Journal of Chromatography B, 995–996 (2015) 64–69

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Analysis of α-amylase inhibitor from corni fructus by coupling magnetic cross-linked enzyme aggregates of α-amylase with HPLC–MS

Liangliang Liu[b,1], Yin Cen[a,1], Fang Liu[a], Jingang Yu[a], Xinyu Jiang[a], Xiaoqing Chen[a,*]

[a] School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China
[b] Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha 410205, China

ARTICLE INFO

*Article history:*
Received 31 December 2014
Received in revised form 1 May 2015
Accepted 14 May 2015
Available online 15 May 2015

*Keywords:*
α-Amylase
Corni fructus
Cross-linked enzyme aggregates
HPLC–MS
Magnetic

ABSTRACT

As a carrier-free immobilization strategy, magnetic cross-linked enzyme aggregates (MCLEAs) showed improved enzyme activity, stability and magnetic response. In this study, MCLEAs of α-amylase (MCLEAs–amylase) was prepared under optimized conditions and characterized with scanning electron microscope and vibrating sample magnetometer. The prepared MCLEAs–amylase showed an amorphous structure and the saturation magnetization was 33.5 emu/g, which was sufficient for magnetic separation. Then MCLEAs–amylase coupled with high performance liquid chromatography–mass spectrometry (HPLC–MS) was utilized to screen and identify α-amylase inhibitors from ethyl acetate extract of corni fructus. The experiment conditions were optimized. At the optimum conditions (incubation time: 10 min, pH: 7.0 and temperature: 20 °C), querciturone was successfully screened and identified with weak non-specific binding. The screening result was verified by inhibition assays and the $IC_{50}$ value of querciturone was 22.5 μg/mL. This method provided a rapid way to screen active compounds from natural products.


## 1. Introduction

Natural products are attracting considerable attention as excellent sources of bioactive compounds due to their long time clinical practice and reliable therapeutic efficacy [1,2]. Among the new chemical entities approved as drugs by the US Food and Drug Administration between 1981 and 2010, a total of 46% are relevant to natural products including 4% natural products, 22% natural-product derivatives and 20% natural mimic compounds [3]. The conventional procedures for finding bioactive components in natural products are to extract and purify compounds followed by subsequent bioassays of the isolated compounds [4]. However, this strategy is time-consuming, arduous, expensive and inefficient [5]. Therefore, it is necessary to develop rapid and effective approaches for screening of active compounds from natural products [6]. In recent years, various screening methods, like cell membrane chromatography, ultrafiltration and functionalized magnetic nanoparticles have been developed to screen active compounds from natural products [7,8].

Since the first report in 2000, cross-linked enzyme aggregates (CLEAs) have been considered as alternative to conventional immobilization on solid supports [9]. As a carrier-free immobilization strategy, CLEAs consist of enzyme precipitation and cross-linking of the resulting aggregates with bifunctional reactive agents [10]. Compared with immobilized enzyme on solid supports, CLEAs showed highly concentrated enzyme activity, low production cost and improved stability toward denaturation by heat or organic solvents [11]. Magnetic CLEAs (MCLEAs) were prepared by addition of amino functionalized magnetic nanoparticles as an additive into enzyme solution, precipitation and cross-linking of enzyme and nanoparticles [12]. Addition of amino functionalized magnetic nanoparticles could not only enhance the stability of CLEAs especially for enzyme with low lysine residue content, but also endow CLEAs with magnetic property for magnetic separation from reaction mixture [13,14]. According to the affinity interaction between enzyme and inhibitor, MCLEAs have promise to be useful materials for specific analysis of active compounds from complex natural products. Besides some reports on the synthesis of MCLEAs and their catalytic activity, there was no report on the screening and analysis of inhibitors using MCLEAs so far [12,15–17].

α-Amylase is an enzyme widely distributed in microorganisms, plants and animal secretions which catalyze the hydrolysis of starch into shorter oligosaccharides [18]. The cleavage of starch by α-

* Corresponding author. Tel.: +86 731 88830833; fax: +86 731 88830833.
*E-mail address:* xqchen@csu.edu.cn (X. Chen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jchromb.2015.05.016
1570-0232/

amylase constitutes the first step in the enzymatic degradation of polysaccharides which is essential in carbohydrate assimilation [19,20]. Inhibitors of α-amylase could delay carbohydrate digestion and prolong overall carbohydrate digestion time, resulting in a reduction in the rate of glucose absorption [21]. Research into α-amylase inhibitors has relevance to several fields like diagnosis of hyperamylasemia disorders, control of diabetes and obesity [22]. Corni fructus, the red fruit of *Cornus Officinalis* Sieb. et Zucc., is an important crude herb used as additive in foods and traditional medicine for herbal therapies in most areas of China, Japan and Korea [23]. It has exhibited biological and pharmacological activities including anti-inflammation, anti-virus, anti-oxidation, invigorating stomach, reducing blood glucose and anti-arrhythmic [24,25]. Our previous researches showed the extract of corni fructus has inhibition on α-amylase. Choi also reported the crude extract of corni fructus showed inhibition on α-amylase (94% inhibition at concentration of 0.2 mg/ml) [26]. However, detailed research of corni fructus is still lacking.

In this study, a rapid screening protocol was developed for elucidation of α-amylase inhibitors from corni fructus by MCLEAs of α-amylase (MCLEAs–amylase). The performance of the MCLEAs–amylase was evaluated. Under the optimum conditions, α-amylase inhibitors from the extract of corni fructus were screened and identified. Experiment results proved the method developed could selectively screen and analyze α-amylase inhibitors without the need for purification of complex samples.

## 2. Experimental

### 2.1. Chemicals and reagents

α-Amylase (from porcine pancreas, Type VI-B, ≥10 units/mg), 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate tetrahydrate, soluble starch, 3-aminopropyltriethoxysilane and glutaraldehyde were acquired from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All of other chemicals were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Querciturone was isolated and characterized from corni fructus in our laboratory and their structures were identified by UV, MS and NMR. The purity of it was determined to be over 97% by normalization of the peak areas detected by HPLC–DAD–MS/MS.

### 2.2. Sample preparation

Dried corni fructus was purchased from Hunan Sanxiang Chinese Medicine Pieces Co., Ltd. in 2014. Dried corni fructus (10.00 g) was suspended in 100 mL of 90% (v/v) ethanol solution, reflux extracted for 3 h, and then cooled at room temperature. The solvents were removed with vacuum rotary evaporation to yield 2.41 g of yellow residue. The residue was dissolved in 100 mL of water and submitted to liquid–liquid fraction using equivalent volume of petroleum ether, ethyl acetate and *n*-butanol with increasing polarities. The ethyl acetate fraction (0.28 g) was then dissolved in 100 mL of water, filtered through a 0.45 μm membrane (Acrodisc® Syringe Filter, Pall, Port Washington, NY, USA) and stored at 4 °C for further experiments.

### 2.3. Preparation and characterization of MCLEAs–amylase

The $Fe_3O_4$–$NH_2$ nanoparticles were synthesized according to our previous work [27]. 1.35 g of ferric chloride, 3.60 g of sodium acetate and 1.00 g of polyethylene glycol were dissolved in 40 mL of ethylene glycol under stirring. The mixture was sealed in a teflon lined stainless steel autoclave and heated at 200 °C for 8 h. The resulting $Fe_3O_4$ nanoparticles were washed with water and dried under vacuum. 100 mg of $Fe_3O_4$ nanoparticles were dispersed in 200 mL of ethanol under mechanical stirring. 2 mL of 3-aminopropyltriethoxysilane was added and the mixture was stirring at room temperature for 6 h. The prepared $Fe_3O_4$–$NH_2$ nanoparticles were washed three times with water and dried at 50 °C in vacuum.

5 mg of $Fe_3O_4$–$NH_2$ nanoparticles were mixed with 1 mL of α-amylase (5 mg/mL, 0.1 M phosphate buffer and pH 7.0) and shaken for 15 min at 25 °C. 4 mL of saturated ammonium sulphate solution was added and shaken at 4 °C for 30 min. After precipitation, glutaraldyhyde was added to the final concentration of 50 mM and stirred for 2 h at 25 °C. The resulting MCLEAs–amylase was separated using bar magnet, washed for three times with phosphate buffer and stored at 4 °C.

The surface morphology of MCLEAs–amylase was investigated using a scanning electron microscope JSM-6360LV (SEM, JEOL, Tokyo, Japan). Magnetic property of MCLEAs–amylase was performed on a Lakeshore 7407 vibrating sample magnetometer (VSM, Westerville, Ohio, USA) at room temperature.

### 2.4. Enzyme activity assay

Enzyme activity was determined using soluble starch as substrate. The mixture containing 1 mL of enzyme (1 mg/mL for free amylase or 5 mg/mL for MCLEAs–amylase) and 2 mL of soluble starch were incubated at 25 °C for 3 min. The reaction was stopped by adding 1 mL of DNS reagent and subjected to boiling water for 10 min. Then, the solution was diluted with 10 mL of water before measuring the absorbance at 540 nm on UV-2450 UV–vis Spectrophotometer (Shimadzu, Kyoto, Japan). A blank experiment was performed under the identical conditions but without enzyme. One amylase activity unit (U) was defined as the amount of enzyme capable of producing 1 μmol of maltose per minute under assay conditions.

To determine the pH activity profiles of free amylase and MCLEAs–amylase, activities were measured in phosphate buffer (0.2 M, pH 5.0–9.0) at 25 °C. Effects of temperature on the activities of free amylase and MCLEAs–amylase were conducted over the temperature range of 20–100 °C at pH 7.0. The activity was expressed as relative form (%) with the maximal value of activity at a certain pH or temperature set as 100%.

### 2.5. Screening of amylase binders from corni fructus

The procedure of screening for amylase binders was roughly illustrated in Fig. 1. 20 mg of MCLEAs–amylase was added in 1 mL of sample solution and shaken at 20 °C for 10 min. After incubation, MCLEAs–amylase was collected by magnetic separation and washed three times with 1 mL of phosphate buffer (0.1 M, pH 7.0). Then, 1 mL of methanol was added to MCLEAs–amylase and the mixture was shaken for 5 min to elute the bound binders. Finally, the supernatant was filtered with 0.45 μm filter membrane and stored at 4 °C for analysis.

### 2.6. HPLC–MS analysis

The HPLC analysis was carried out on an Agilent 1260HPLC system (Agilent Technologies, Santa Clara, California, USA) which consisted of a G1311C quaternary pump equipped with on-line vacuum degasser, a G1329B auto-sampler, a G1316A column oven and a G1314B variable wavelength detector. Chromatographic separations were performed on a reversed phase SunFire™ $C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm, Waters, Milford, MA, USA). The mobile phase consisted of water containing 0.4% (v/v) acetic acid (A) and acetonitrile (B). A gradient elution program was used