ChemComm

COMMUNICATION

Screening of α-glucosidase inhibitors from green tea extracts using immobilized enzymes affinity capture combined with UHPLC-QTOF MS analysis†

Cite this: *Chem. Commun.*, 2014, **50**, 2582

Received 21st December 2013, Accepted 9th January 2014

DOI: 10.1039/c3cc49681c

www.rsc.org/chemcomm

Shiren Deng,[ab] Linbo Xia[b] and Hongbin Xiao*[ac]

An applicable affinity capture-based method was developed for screening of enzyme inhibitors from complex plant extracts directly. By elimination of false positives, three non-competitive α-glucosidase inhibitors were fished out from 400 μL green tea extract rapidly, using affinity capture of immobilized α-glucosidase combined with UHPLC-QTOF MS analysis.

Screening of potent enzyme inhibitors from natural products has been of continued importance for drug discovery over the past few decades.[1] Bioassay-guided separation is a classical procedure used to investigate the inhibitory components from complex mixtures. However, the isolation procedure is generally time-consuming, expensive and laborious. High-throughput screening (HTS) is another dominant technique.[2] However, this method would be effective only if a large bank of pure compounds were available. So HTS has also entailed a huge isolation workload and a low efficiency when natural products are used as sources.[3] Therefore, it is quite necessary but challenging to establish an efficient and fast screening method for identifying enzyme inhibitors from a complex natural products extract directly.[4,5]

At present, affinity screening is one of the most efficient methods for direct capture of ligand from complex mixtures.[6–8] However, hitherto, reports about screening of inhibitors using immobilized enzymes are rare, especially from natural products directly. Thongpanchang fished out tight-binding dihydrofolate reductase inhibitors from a mixture of synthesized combinatorial libraries directly.[9] In this case, the components were isolated specifically by enzyme affinity and identified using HPLC-MS. However, the mixture was not a real biological sample but a simple mixture consisting of equimolar amounts of pure synthesized compounds. Recently, Megías screened angiotensin converting enzyme (ACE) inhibitors directly from sunflower extracts by immobilized ACE and HPLC-UV.[10] However, the setup method suffered a dramatic risk of false positives, because the ligands non-specifically absorbed on a support matrix might be regarded as inhibitors. Furthermore, this work could not identify the components using HPLC-UV.

Herein, using α-glucosidase (AGH) and green tea extracts as the model enzyme and the real biological sample, respectively, we developed a novel AGH inhibitor screening method by a combination of immobilized AGH affinity capture and LC-MS analysis for screening and identification of active components directly from green tea extracts. By designing a parallel control supports comparison experiment, the false positive results caused by non-specific absorption were successfully eliminated. As a result, without time-consuming and laborious isolation workload, three AGH inhibitors were correctly fished out from only 400 μL of green tea extracts within only a few hours, with the limit of detection (LOD) of inhibitors being 0.5 μg mL^{-1}, which was proved to be an effective, rapid, economical and sensitive screening method. Furthermore, this rapid fishing method demonstrated its reliability by an inhibitory activity verification test.

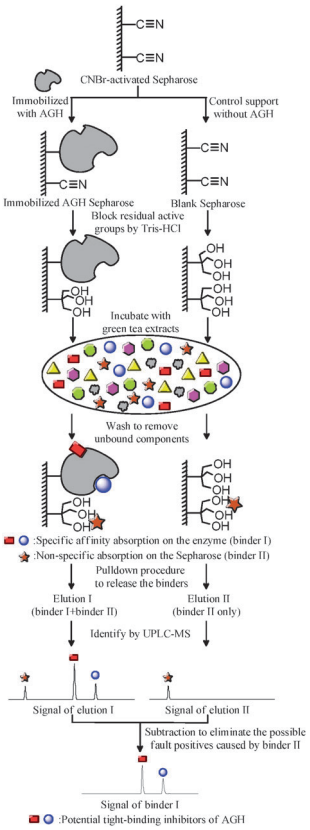
The affinity capture system was established as shown in Scheme 1. Firstly, AGH was immobilized on CNBr-activated sepharose beads, and then the remaining active cyanate ester groups on the matrix were blocked and converted to hydroxyl groups by reacting with a small primary amine (*e.g.* Tris-HCl). Control supports were prepared in the same manner but with no AGH added during the immobilization. Secondly, immobilized AGH sepharose and blank sepharose were incubated with green tea extracts respectively, to launch the affinity absorption. Theoretically, the components retained on the immobilized AGH consist of two parts. The first part is the molecules specifically bound to the AGH protein (binder I) and the second part is the molecules non-specifically absorbed on the sepharose matrix (binder II). However, the components retained on the blank sepharose contain only binder II. Obviously, binder I was the possible AGH inhibitor while binder II was the false positive. Thirdly, to confirm the chemical consistency of binder I, a pulldown experiment was carried out to release the ligands retained on the AGH-coupled sepharose and blank sepharose, leading to elution I (binder I and binder II) and elution II (binder II), respectively. Then both elutions were analysed by

[a] *Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China. E-mail: hbxiao@dicp.ac.cn; Fax: +86-411-8437-9907; Tel: +86-411-8437-9756*

[b] *College of Pharmacy, Liaoning University of Traditional Chinese Medicine, Dalian 116600, China*

[c] *Institutie of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3cc49681c



Scheme 1 General scheme for the screening and identification of AGH inhibitors from natural plants extract by immobilized AGH affinity capture and UHPLC-QTOF MS analysis.

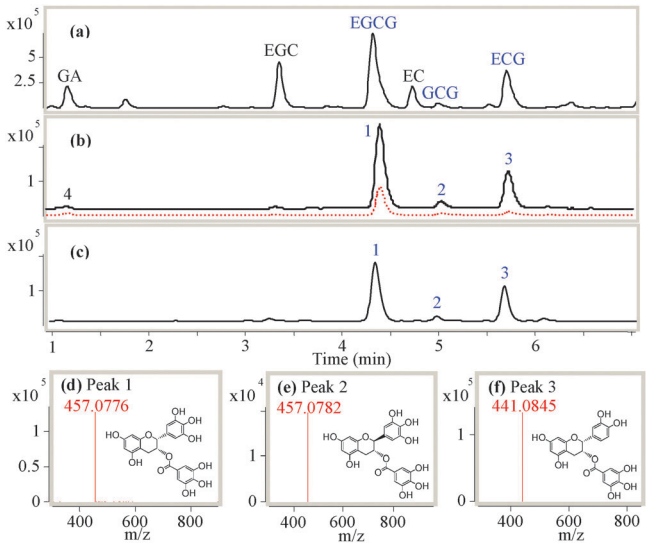


Fig. 1 (a) TIC of green tea extract. The components of GA, EGC, EGCG, EC, GCG and ECG were deduced by their mass spectra and confirmed by comparing retention times with their standards. (b) TIC of the binders screened out from green tea extract. The black solid line represents "elution I", *i.e.*, binder I and binder II. The red dotted line represents the "elution II", *i.e.*, binder II. (c) TIC of the binder I, which was made by subtracting TIC of elution II from TIC of elution I. (d) The structure and accurate mass spectrum of peak 1 (EGCG). (e) The structure and accurate mass spectrum of peak 2 (GCG). (f) The structure and accurate mass spectrum of peak 3 (ECG).

UHPLC-QTOF MS, a more powerful analysis protocol with higher resolution, faster analysis speed and higher accuracy compared with the traditional HPLC-MS, to obtain the signal of elution I and elution II. Finally, by subtracting the signal of elution II from that of elution I, the false positive signal was eliminated and the structural information of binder I was identified.

During the immobilization, the CNBr-activated sepharose was chosen as the coupling support due to its low non-specific adsorption, convenient coupling method and high binding capacity.[11] Yeast AGH was immobilized on the matrix stably *via* the reaction between cyanate ester groups in sepharose and primary amino groups in the enzymes,[12] resulting in a protein capacity of 9.5 mg mL^{-1} sepharose and specific activity of 175 U mL^{-1} sepharose. Furthermore, the remaining active cyanate ester groups must be blocked after the coupling. Otherwise, the residual active groups will probably cause serious non-specific absorption.

Using AGH and green tea extracts as the model enzyme and the real biological sample, respectively, the above affinity capture-based method was investigated. AGH inhibitors, which can suppress postprandial hyperglycemia, are used to treat type II diabetes.[13] Although strong synthetic AGH inhibitors (*i.e.*, acarbose) are available, they usually cause significant adverse events.[14] So naturally occurring AGH inhibitors are considered to be viable, low toxicity alternatives. The green tea was chosen because its aqueous extracts and catechins were found to have potent AGH inhibitory effects.[15]

Screening results are shown in Fig. 1. The green tea extracts consisted of epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epicatechin gallate (ECG), gallic acid (GA), epicatechin (EC) and epigallocatechin (EGC). Among them, peaks 1, 2, 3 and 4 (Fig. 1b) were captured by the immobilized AGH sepharose as "elution I". By subtracting the signal of elution II from that of elution I, binder I was successfully recognized as peaks 1, 2 and 3 (Fig. 1c). With the aid of QTOF MS accurate molecular mass measurements (Fig. 1d–f), the three peaks were identified to be EGCG, GCG and ECG, which were considered to be potential tight-binding AGH inhibitors. In contrast, some other components such as EGC and EC were not captured although their amounts were abundant in extracts, which were thought to be weak or non-AGH inhibitors due to their relatively weak affinity to AGH. It is worth noting that peak 4 (GA) would probably have been recognized as an AGH binder (Fig. 1b) if there was no parallel control investigation. By comparison of the signal of elution II, GA was finally recognized as a false positive (binder II) and was retained only on the sepharose matrix, although it was captured by immobilized AGH.

Using the above screening method, three binders (EGCG, GCG and ECG) were identified to be tight-binding ligands. However, not all the binders bound to the enzyme are inhibitors, because some of binders are just "frequent hitters",[16] unselectively clogging the protein by hydrophobic interaction without any inhibitory effects. To distinguish between inhibitory binders and "frequent hitters", inhibitory activity verification tests of three binders were investigated using a traditional enzyme inhibition assay. As shown in Table 1, EGCG, GCG and ECG (positive inhibitors) showed high AGH inhibitory activities with

Table 1 Inhibition activities of positive and negative potential inhibitors against α-glucosidase

	Inhibition[a] (%)	IC_{50} [a,b] (μM)	K_i (μM)
EGCG	54.01 ± 1.40[c]	0.67 ± 0.02	0.50
GCG	55.49 ± 2.13[c]	0.59 ± 0.02	0.44
ECG	69.65 ± 2.85[c]	0.48 ± 0.06	0.40
GA	16.47 ± 0.35[d]	1235 ± 58	1430
EGC	43.10 ± 1.38[d]	560 ± 25	790
EC	48.01 ± 1.28[d]	526 ± 6.8	900

[a] Results are the average of three determinations ± standard deviation. [b] Concentration required for 50% inhibition of the enzyme activity. [c] Inhibition by 1 μM samples. [d] Inhibition by 500 μM samples.

IC_{50} values of 0.67, 0.59, and 0.48 μM, while GA, EGC and EC (negative inhibitors) showed weak inhibitory activities with the values of 1235, 560 and 526 μM, respectively. The results demonstrate that the specific affinity-captured components are indeed strong AGH inhibitors.

To validate the binder affinity, the equilibrium dissociation constants (K_i) of positive and negative inhibitors were investigated (Table 1). EGCG, GCG and ECG showed strong affinity for the AGH enzyme, with K_i values of 0.50, 0.44 and 0.40 μM, respectively, determined using Lineweaver–Burk plots[17] (Fig. S4a–c, ESI†). In contrast, GA, EGC and EC showed weak affinity, with K_i values of about 1000 μM. These results were also in agreement with the "principles of affinity chromatography",[12] *i.e.*, the K_i value between ligand and target enzyme should be less than 100 μM in order to achieve efficient adsorption during typical affinity purification. Furthermore, the Lineweaver–Burk plots indicated that all of the three inhibitors were non-competitive inhibitors.

To optimize the washing procedure, the amounts of small molecules retained on the blank sepharose and AGH-coupled sepharose were measured quantitatively at different washing steps. As a result, the unbound components retained on AGH-coupled sepharose and on the blank sepharose were considered to be removed as much as possible after four times of washing (Fig. S2 and S3, ESI†). Besides, too much washing would possibly influence the enzyme activity and lower the method sensitivity. Therefore, four times of washing was finally chosen.

The pulldown procedure in normal affinity chromatography was usually carried out by two main types of elution, called specific elution and non-specific elution, respectively (Fig. S5, ESI†). Specific elution usually applied a known competitive inhibitor as an elution agent, which could replace the new compound to be identified from the active site of the enzyme, and consequently screen out competitive inhibitors. Non-specific elution included changes in ionic strength, pH or solvent hydrophobicity, which could theoretically release all the binders (such as competitive inhibitors, non-competitive inhibitors, frequent hitters *etc.*) from the protein. We tried specific elution to fish out competitive AGH inhibitors from green tea extracts by using acarbose and voglibose (well-known AGH commercial competitive inhibitors) as elution agents. However, we could not detect any obvious affinity signal during the experiments (Fig. S6, ESI†). The results suggest that AGH competitive inhibitors were probably absent in green tea extracts. On the other hand, some potent AGH inhibitors (*e.g.* EGCG) existing in green tea extracts could not be fished out using this method, which may be because EGCG is a non-competitive inhibitor and could not be replaced and eluted by specific elution agents (Fig. S5, ESI†). However, these non-competitive inhibitors could be successfully fished out by hydrophobic solvent elution as shown in Fig. 1. Therefore, the pulldown experiment was finally carried out by using non-specific elution.

In natural products chemistry, sample limitation is one of the main challenges. Different concentrations of authentic sample mixtures of EGCG, GCG and ECG were used as the molecule pool to carry out affinity fishing assays. We could acquire an obvious affinity signal of EGCG when its concentration was as low as about 0.5 μg mL^{-1} (Fig. S1, ESI†). The limit of detection (LOD) of EGCG, GCG and ECG was calculated to be 0.48, 0.68 and 0.56 μg mL^{-1}, respectively.

In conclusion, we have developed a novel immobilized enzyme-UHPLC-MS method for rapid screening of AGH inhibitors directly from the complex natural plant extracts. By parallel control supports comparison, the fault positive caused by gallic acid was successfully eliminated. As a result, without time-consuming and laborious isolation workload, three AGH inhibitors could be fished out from only 400 μL of green tea extracts within only a few hours, with a LOD of EGCG 0.5 μg mL^{-1}, which suggested that the screening method was rapid, economical, sensitive and feasible. In addition, the screened compounds were proved to be strong AGH inhibitors by a traditional enzyme inhibition assay, demonstrating the reliability of the method. Furthermore, this method could be modified and has the potential for use in *e.g.* screening of natural products extracts against some new targets by immobilizing some other biomacromolecules (*i.e.*, enzymes, nucleic acids and receptors).

This work was supported by National Natural Science Foundation of China (81202857), and the China Postdoctoral Science Foundation (20100471484).

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