

Supporting Information

Contents:

- 1 Experimental details
 - 1.1 Materials and Reagents
 - 1.2 Immobilization of enzyme
 - 1.3 AGH inhibitors screening by immobilized AGH affinity fishing
 - 1.4 UPLC-QTOF-MS analysis
 - 1.5 AGH inhibition activity assay and kinetics analysis
 - 1.6 Limit of detection (LOD) of this method
 - 1.7 Amount of small molecules retained on the blank sepharose and AGH-immobilized sepharose
 - 1.7.1 Linear relationship of six small molecules
 - 1.7.2 Molecules retained on the blank sepharose
 - 1.7.3 Molecules retained on the AGH-immobilized sepharose
- 2 Figure S4: Lineweaver-Burk plots of EGCG (a), GCG (b) and ECG (c) and inhibitory effects (d) on α -glucosidase
- 3 Discussion about specific elution and non-specific elution in pulldown experiment

1. Experimental details

1.1. Materials and Reagents

α -Glucosidase (E.C. 3.2.1.20) from *Saccharomyces cerevisiae*, albumin from bovine serum (BSA), p-nitrophenyl α -D-glucopyranoside (PNPG) and glutathione (GSH) were purchased from Sigma-Aldrich (St Louis, MO, USA). CNBr-activated SepharoseTM 4B was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Gallic acid (GA) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). (-)-epigallocatechin-3-gallate (EGCG), (-)-gallocatechin-3-gallate (GCG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC) were obtained from Aladdin Industrial Inc. (Shanghai, China). Distilled water was purified by a Milli-Q water purification apparatus (Millipore, Bedford, MA). All other reagents were of analytical grade. Green tea was purchased from Hangzhou West Lake Longjing Industry Co. (Zhejiang, China).

100 mg ground green tea powder was extracted in 20 mL of 50% aqueous methanol by ultrasonic extraction for 30 min. The extract was filtered and freeze-dried, then redissolved in 30 mL affinity buffer (67 mM KH₂PO₄ at pH 6.8) and filtered through a 0.45 μ m membrane as the tested extract sample.

1.2. Immobilization of enzyme

α -Glucosidase (0.20 mg) was dissolved in coupling buffer (0.1 M NaHCO₃ and 0.5 M NaCl at pH 8.3) at a concentration of 2.0 mg protein/mL. The protein solution was mixed with CNBr-activated SepharoseTM 4B gel at room temperature in a ratio of 100 μ L protein solution to 5 mg SepharoseTM powder, which would swell to about 20 μ L of wet gel. The gel slurry was rotated at room temperature for 1 h to ensure the enzyme bound to the beads. After protein coupling, the resident reactive sites on the gel were blocked by reacting with 0.01 M Tris-HCl buffer at pH 8.0 for 1 h at room temperature. After alternatively washed with 0.1 M CH₃COONa buffer (pH 4.0 containing 0.5 M NaCl) and 0.1 M Tris-HCl buffer (pH 8.0 containing 0.5 M NaCl), the gel was kept in affinity buffer for further use. Control supports were prepared in the same manner but with no AGH being added during the immobilization step.

Enzyme immobilization yields were determined by comparison of the amount of free enzyme in the solutions before and after coupling to the gel according to the method described by Bradford using BSA as standard protein, giving 9.5 mg AGH/mL gel.

The specific activity of immobilized AGH was determined as 18.4 U/mg protein (175 U/mL gel).

1.3. AGH inhibitors screening by immobilized AGH affinity fishing

Green tea extracts (400 μL) was mixed with 20 μL immobilized AGH gel and gently stirred at 37°C for 20 min. After incubation with the extracts, the gel were washed with affinity buffer (400 μL) four times to remove any unbound components and then treated with 200 μL desorbed solution (50% methanol aqueous solution at pH 3.3) to release the captured potential inhibitors of AGH. The released components were collected and concentrated to 10 μL under N_2 atmosphere at 40 °C, then applied to UPLC-QTOF-MS for peak identification. Elution from the control supports was collected and analyzed in the same manner.

1.4. UHPLC-QTOF-MS analysis

UHPLC-MS was performed on an Agilent 1290 Infinity LC-6520 Accurate-Mass QTOF-MS (Agilent Corporation, MA, USA). Analytes eluted from the AGH coupling supports and control supports were separated on a poroshell 120 EC- C_{18} column (3.0 \times 50 mm, 2.7 μm , Agilent Corporation, MA, USA). The solvent system consisted of solvent A (methanol) and solvent B (0.5% formic acid in water, v/v). The solvent A content of the mobile phase was maintained at 5% in 1 min and then gradient increased to 40% linearly in 7 min. The flow rate was set at 0.6 mL/min. Mass analysis was performed in negative ESI ion mode. The operating parameters were as follows: drying gas (N_2) flow rate, 8 L/min; drying gas temperature, 325 °C; nebulizer, 30 psig; capillary, 3500 V; skimmer, 65 V; fragmentor voltage, 120 V. The mass range was set at m/z 100–2000. The system was operated under Agilent MassHunter workstation acquisition software, version B.02.01 (Agilent Corporation, MA, USA).

1.5. AGH inhibition activity assay and kinetics analysis

The AGH inhibitory activity of three captured components (EGCG, GCG, ECG) and three non-captured components (GA, EGC, EC) on AGH was determined spectrophotometrically on 96-well microplate reader. In brief, to a total of 310 μL of reaction mixture containing 175 μL of 67 mM phosphate buffer (pH 6.8), 10 μL of 3 mM GSH, 25 μL of 10 mM PNPG and 100 μL of investigated compounds in the wells was added 10 μL of 0.3 U/mL AGH and mixed. All the reagents used in this assay were dissolved in the same phosphate buffer (67 mM, pH 6.8). After incubation for 10 min at 37 °C, the reaction was stopped by adding 800 μL of 0.1 M Na_2CO_3 solution. Then the absorbance of sample (A_S) at 405 nm was recorded (Sunrise™ microplate absorbance reader, Tecan, Austria). The control was the same mixture except for the investigated sample replaced by the phosphate buffer. The sample blank and control blank were the same mixtures as sample and control, respectively, except AGH was instead with phosphate buffer, respectively. The AGH inhibition activity (%) of test sample on AGH could be calculated as

$$\text{inhibition activity (\%)} = 100\% \times [(A_S - A_{SB}) / (A_C - A_{CB})]$$

where A_S , A_{SB} , A_C and A_{CB} are the absorbance of sample, sample blank, control and control blank, respectively. The measurement was performed in triplicates.

For kinetic analysis of AGH inhibition, a certain concentration of 0.6 U/mL AGH and different contents of sample compounds were incubated with a series of concentrations of substrate. The inhibitory kinetics of the investigated compounds was analyzed using the Lineweaver-Burk plot, double-reciprocal plot of the substrate concentration and velocity.

1.6. Limit of detection (LOD) of this method

The limit of detection (LOD) of our method was measured using a mixture of authentic samples of EGCG, GCG and ECG. The mixture of authentic samples was used as the molecule pool to carry out affinity fishing assay. LOD was documented based on a signal to noise ratio (S/N) of 3:1. The LOD of EGCG, GCG and ECG was established to be 0.48, 0.68 and 0.56 $\mu\text{g/mL}$, respectively, as shown in figure S1.

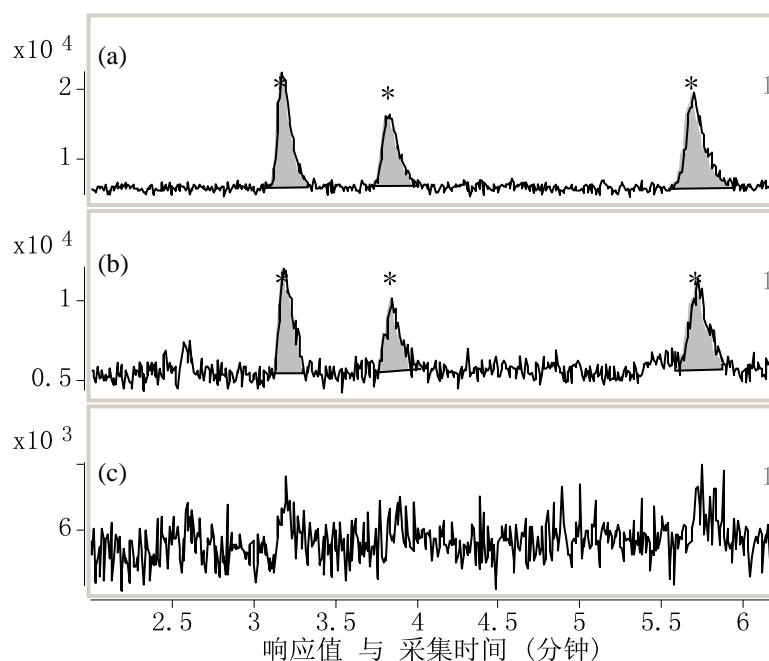


Figure S1. LOD of EGCG, GCG and ECG. (a) A mixture of authentic samples of EGCG, GCG and ECG with concentration of 1 $\mu\text{g/mL}$, with S/N of 7.9, 4.9, 6.6, respectively; (b) A mixture of authentic samples of EGCG, GCG and ECG with concentration of 0.5 $\mu\text{g/mL}$, with S/N of 3.1, 2.2, 2.7, respectively; (c) A mixture of authentic samples of EGCG, GCG and ECG with concentration of 0.1 $\mu\text{g/mL}$.

As a result, we could detect the affinity signal of EGCG even its concentration is as low as 0.5 $\mu\text{g/mL}$ in green tea extracts, which suggested that the setup method was sensitive.

1.7. Amount of small molecules retained on the blank sepharose and AGH-immobilized sepharose in each steps

To measure the content of small molecules retained on the sepharose matrix, the AGH-immobilized sepharose and blank sepharose was incubated with green tea extracts, respectively. Both of the matrix were washed with affinity buffer four times and then treated with desorbed solution (50% aqueous methanol solution at pH 3.3), just as the procedure described in supporting information (section 1.3).

1.7.1. Linear relationship of six small molecules

To quantitatively measure the contents of small molecules, the linear relationship of six components (EGCG, GCG, ECG, GA, EGC and EC) was determined and listed in table S1.

Table S1. linear relationship of six components

compound	Linear range/(ng)	Regression equation	r ²
EGCG	3 - 750	$Y = 3658.3X + 31012$	0.999 6
GCG	0.75 - 75	$Y = 3475.4X + 5648.7$	0.999 1
ECG	0.75 - 300	$Y = 5752.8X - 2990.1$	0.999 2
GA	0.5 - 100	$Y = 6124.3X + 3008.9$	0.999 6
EGC	0.5 - 100	$Y = 7581.4X + 2488.9$	0.999 6
EC	0.5 - 200	$Y = 4201.5X - 613.75$	0.998 9

1.7.2. Molecules retained on the blank sepharose

The amount of small molecules retained on the blank sepharose was calculated by the above regression equation, and the final results were shown in figure S2.

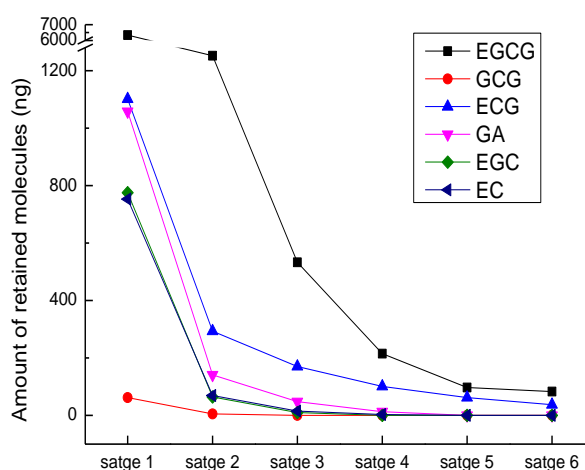


Figure S2. The amount of small molecules retained on the blank sepharose at different steps. Stage 1 was the blank sepharose gel after incubation with green tea; stage 2 to stage 6 corresponded to the matrix washed by affinity buffer for once to for five times, respectively.

From figure S2, we could find out the amount of molecules retained on the blank sepharose was decreasing with the increase of washing times. It is worth noting in figure S2 that this decreasing was not significant after washing for four times (stage 5), which means the unbound components retained on the blank sepharose were considered to be removed as much as possible after four times of washing. Therefore, a four times of washing was chosen to remove unbound components.

1.7.3. Molecules retained on the AGH-immobilized sepharose

The amount of small molecules retained on the AGH-immobilized sepharose was calculated by the above regression equation, and the final results were shown in figure S3.

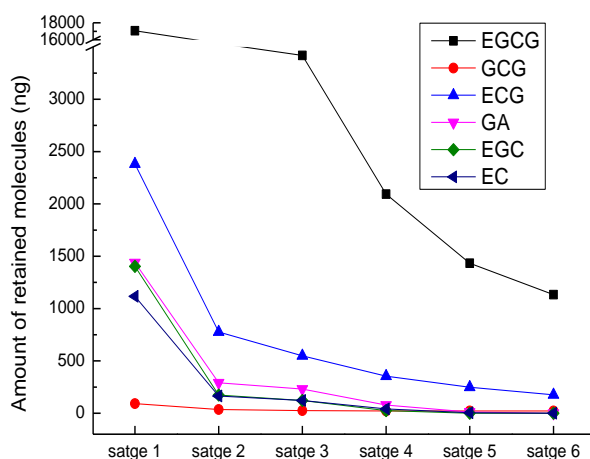


Figure S3. The amount of small molecules retained on the AGH-immobilized sepharose at different steps. Stage 1 was the AGH-immobilized sepharose after incubation with green tea; stage 2 to stage 6 corresponded to the matrix washed by affinity buffer for once to for five times, respectively.

From figure S2 and figure S3, we could find out the amount of molecules retained on the AGH-immobilized sepharose was more than that on the blank sepharose in each steps, which was due to the affinity absorption by the AGH enzyme. After washing for four times (stage 5), the unbound components retained on AGH-immobilized sepharose and on the blank sepharose were considered to be removed as much as possible. Meanwhile, too much washing would possibly influence the enzyme activity and lower the method sensitivity. Therefore, a four times of washing was finally chosen.

The retained amounts of small molecules were listed in table S2.

Table S2. Retained amounts of small molecules after four times of washing (ng)

compound	AGH-immobilized sepharose	blank sepharose
EGCG	1433	97
GCG	22	0
ECG	249	62
GA	28	28
EGC	0	0
EC	4	3

2. Figure S4: Lineweaver-Burk plots of EGCG (a), GCG (b) and ECG (c) and inhibitory effects (d) on α -glucosidase

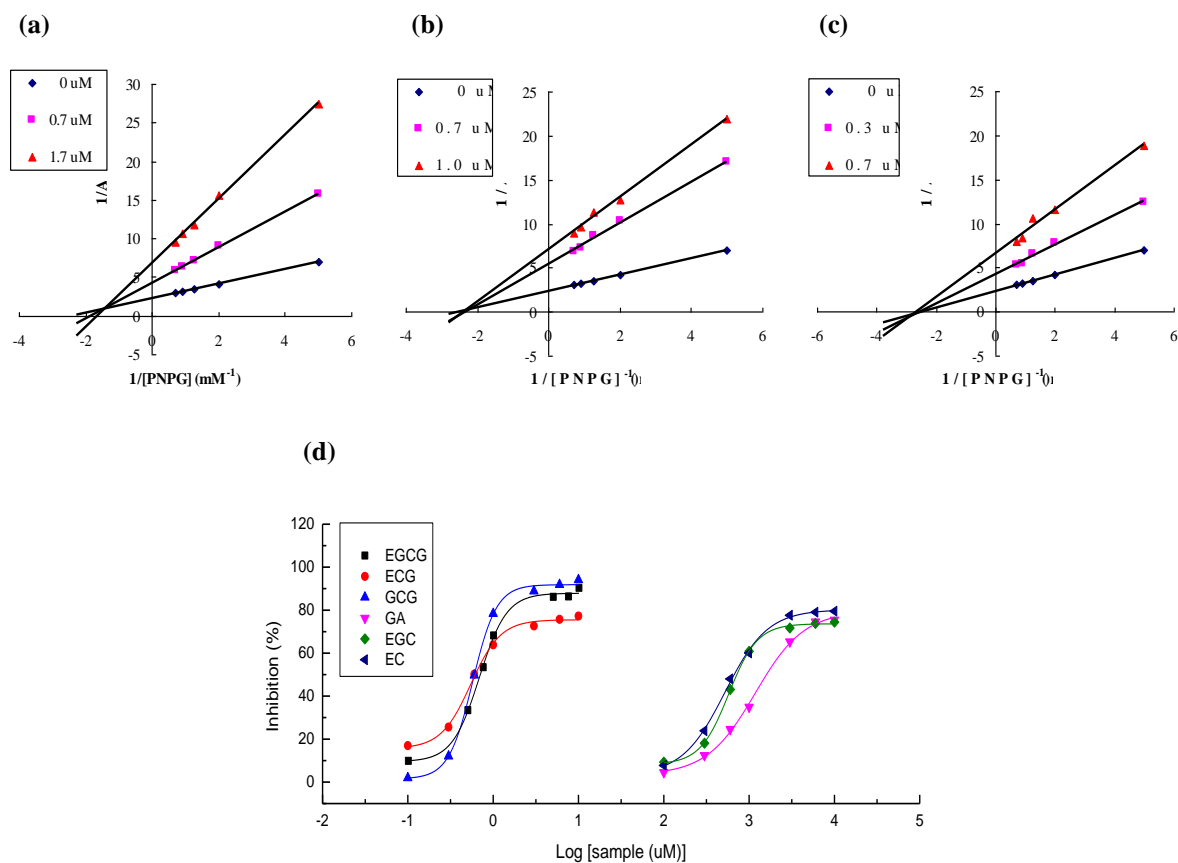


Figure S4. Lineweaver-Burk plots of EGCG (a), GCG (b) and ECG (c) and inhibitory effects (d) on α -glucosidase. The equilibrium dissociation constant (K_i) of EGCG, GCG and ECG could be calculated from figure S4a, S4b and S4c, with the values of 0.50, 0.44 and 0.40 μM , respectively. The results showed that the three captured compounds had strong affinity abilities to the AGH enzyme. The inhibitory activities of six compounds were also investigated in dose-dependent manners (figure S4d). The measured results demonstrated that the fishing out components captured by the immobilized AGH sepharose specifically (i.e. EGCG, GCG and ECG) are strong AGH inhibitors indeed. Furthermore, the Lineweaver-Burk plots indicated that all of the three inhibitors were non-competitive inhibitors.

3. Discussion about specific elution and non-specific elution in pulldown experiment

The pulldown procedure in normal affinity chromatography was usually carried out by two main types of elution, called specific elution and non-specific elution, as shown in Fig S5.

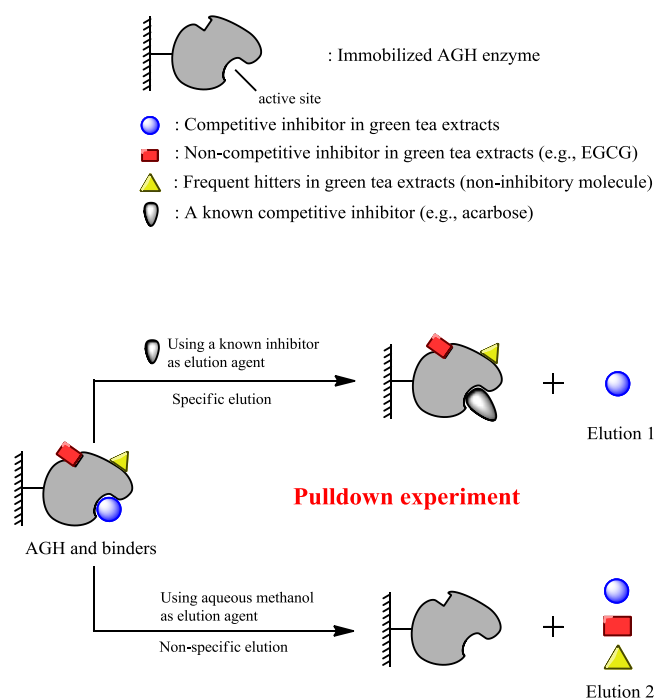


Figure S5. General scheme of specific elution and non-specific elution

In specific elution, the pulldown experiment usually applied a known competitive inhibitor as elution agent, which could replace the new compound to be identified from the active site of enzyme, and consequently screening out competitive inhibitors (binding at the active site of enzyme) from green tea extracts., as elution I (blue circle in Fig S5). However, this elution could not release non-competitive inhibitors (red square in Fig S5), because non-competitive inhibitors do not interact with the enzyme at the active site.

In non-specific elution, the pulldown experiment included changes in ionic strength, pH or solvent hydrophobicity, which could theoretically release all the binders, such as competitive inhibitors (blue circle), non-competitive inhibitors (red square) and 'frequent hitters' (yellow triangle) 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. At the same time, non-specific elution was carried out by using hydrophobic solvent (aqueous methanol). The results were shown as Fig S6.

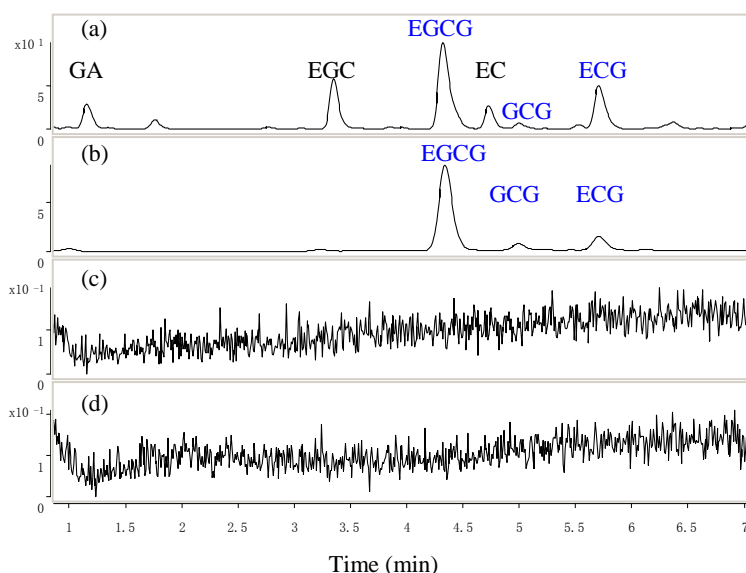


Figure S6. (a) UHPLC-MS TIC of green tea extract. (b) UHPLC-MS TIC of non-specific elution by aqueous methanol. (c) UHPLC-MS TIC of specific elution by 500 mM acarbose. (d) TIC of specific elution by 500 mM voglibose.

As a result, we could not detect any obvious affinity signal during the non-specific elution (Fig S6c and S6d). The results suggested that AGH competitive inhibitors were probably absent in green tea extracts. On the other hand, some potent AGH inhibitors (e.g. EGCG) existed 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 agent. However, these non-competitive inhibitors could be successfully fished out by non-specific elution as shown in Fig S6b. Therefore, the pulldown experiment was finally carried out by using non-specific elution.

There is a crucial issue involved with "binder distinguishment" when using non-specific elution. Just as shown in Fig S5, not all the binders released from the protein by non-specific elution are inhibitors, because some of binders are just "frequent hitters", unselectively clogging the protein by hydrophobic interaction without any inhibitory. So it is very important to distinguish inhibitory molecules (blue circle and red square) from "frequent hitters" (yellow triangle) during inhibitor screening. To do this, we carried out the following validation experiments.

1. The authentic samples of all the released binders (EGCG, GCG and ECG) were collected.
2. Inhibitory of all the binders was investigated by traditional enzyme inhibition assay. The results showed all the three binders were indeed potent AGH inhibitors ($IC_{50} < 10^{-6}$ M).
3. Lineweaver-Burk plots indicated that the three binders were all non-competitive inhibitors (Figure S1a-S1c in the Supporting Information).

The above validation experiment successfully distinguished between inhibitory binders and non-inhibitory binders. The results showed that the three binders (EGCG, GCG and ECG) screened out from green tea extracts were all AGH inhibitors.