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Original scientific paper

Analysis of alcohol dehydrogenase inhibitors from *Desmodium styracifolium* using centrifugal ultrafiltration coupled with HPLC–MS

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Abstract: Alcohol dehydrogenase (ADH) inhibitors play an important role in the treatment of human methanol or ethylene glycol poisoning and the suppression of acetaldehyde accumulation in alcoholics. In this study, centrifugal ultrafiltration coupled with high performance liquid chromatography-mass spectrometry (HPLC–MS) was utilized to screen and identify ADH inhibitors from an ethyl acetate extract of *Desmodium styracifolium* (Osb.) Merr. The experimental conditions of the centrifugal ultrafiltration were optimized. Under the optimum conditions (ADH concentration: 37.5 μg mL^{-1}, incubation time: 90 min, pH 7.0 and temperature: 15 °C), formononetin and aromadendrin were successfully screened and identified from the ethyl acetate extract of *D. styracifolium*. The screening result was verified by ADH inhibition assays. The IC_{50} values of formononetin and aromadendrin were 70.8 and 84.7 μg mL^{-1}, which were in accordance with their degrees of binding. Aromadendrin was for the first time reported to have inhibitory activity on ADH. This method provides an effective way to screen active compounds from natural products.

Keywords: alcohol dehydrogenases; aromadendrin; centrifugal ultrafiltration; *Desmodium styracifolium*; formononetin.

INTRODUCTION

Nowadays, natural products have become the primary source for chemical and pharmaceutical research because of their long history in clinical practice and reliable therapeutic efficacy.[1,2] In the last thirty years, 46 % of the new chemical entities approved as drugs by the US Food and Drug Administration were relevant to natural products.[3] However, the chemical composition of natural products is complex. Compared to the numerous compounds existing in natural pro-

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ducts, the numbers and contents of active compounds are relatively low. The conventional procedures for screening active compounds in natural products are laborious and time-consuming.[4] Accordingly, numerous screening methods were developed, such as cell membrane chromatography, ultrafiltration and equilibrium dialysis.[5–7]

Centrifugal ultrafiltration is a rapid method using centrifugation force and a semi-permeable membrane to retain high molecular weight solutes.[8,9] Compounds with a molecular weight higher than the nominal molecular weight cut-off of the membrane would be retained, while low molecular weight compounds could pass through the membrane.[10,11] Based on this principle, when an extract of natural products was incubated with some enzyme, the active compounds in the natural products would bind to the enzyme and be trapped by the membrane together with enzyme. Combined with high performance liquid chromatography––mass spectrometry (HPLC–MS) analysis, active compounds could be identified by comparing the chromatograms before and after centrifugal ultrafiltration. Therefore, centrifugal ultrafiltration coupled with HPLC-MS could become a simple and powerful tool for discovering active compounds from natural products,[12,13] and many examples have been performed by this method to screen enzyme inhibitors and ligands.[14–19] These studies showed that the screening could be accomplished rapidly and the crude samples could be analyzed without further purification.

Alcohol dehydrogenases (ADH) catalyzes the oxidation of alcohols with the reduction of nicotinamide adenine dinucleotide (NAD^+) in many organisms.[20] In the human body, ADH is also involved in the oxidation of methanol to formaldehyde and ethylene glycol to glycolic and oxalic acids.[21,22]. However, the existence of formaldehyde or glycolic acid is harmful to humans. ADH inhibitors hinder the metabolisms of methanol and ethylene glycol, and, consequently, could be used in the therapies of human methanol and ethylene glycol poisonings.[23,24] Moreover, ADH inhibitors could suppress acetaldehyde accumulation in alcoholics.[25] *Desmodium styracifolium* (Osb.) Merr. is known in China for its heat-clearing and diuretic properties. It is also an important Chinese medicine for the treatments of renal stones and cardio-cerebrovascular disease. The experiments showed that the ethyl acetate extract of *D. styracifolium* showed inhibition on ADH. However, the related detail research is still inadequate.

In this study, ADH inhibitors from *D. styracifolium* were analyzed by centrifugal ultrafiltration coupled with HPLC-MS. The experimental conditions, including enzyme concentration, incubation time, pH and temperature, were investigated and optimized. Two ADH inhibitors were identified under the optimum conditions. Experiment results proved this method could screen and analyze ADH inhibitors without the purification of natural samples and the screening results were reliable.

EXPERIMENTAL

Materials

Dried *Desmodium styracifolium* (Osb.) Merr. was purchased from Beijing Tongrentang Co., Ltd. (Changsha, China). Alcohol dehydrogenase (ADH) was acquired from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Nicotinamide adenine dinucleotide (NAD^+) was obtained from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Acetonitrile, methanol and acetic acid used for HPLC were chromatographic grade and obtained from Tedia Company Inc. (Fairfield, OH, USA). Ultrapure water (18.2 MΩ resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The other chemicals were analytical grade. Formononetin and aromadendrin were isolated and characterized from *D. styracifolium* in the laboratory and their structures were identified by UV, MS and NMR.[26]. Their purities were determined to be over 95 % by normalization of the peak areas detected by HPLC-DAD–MS/MS.

Preparation of Desmodium styracifolium *extract*

Dried *D. styracifolium* (30.00 g) was suspended in 300 mL of ethanol (95 %,) and reflux extracted at 85 °C for 3 h. After cooled at room temperature, the solvent was removed with vacuum rotary evaporation. Then the residue (1.25 g) was dissolved in 150 mL of water and extracted successively with equal volumes of petroleum ether, ethyl acetate and *n*-butanol. The evaporated ethyl acetate extract (0.19 g) was dissolved in 150 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.

Screening of ADH inhibitors by centrifugal ultrafiltration

A mixture of *D. styracifolium* extract (100 μL, 0.5 mg mL^{-1}) and ADH solution (100 μL, 37.5 μg mL^{-1}) was incubated at 15 °C for 90 min. After incubation, the mixture was poured into a centrifugal ultrafiltration device (Nanosep MF Centrifugal Devices, ≤10 kDa, Pall, Port Washington, NY, USA) and centrifuged at 7000 rpm for 30 min at room temperature. The control experiments were performed under the same conditions using denatured enzyme instead of active enzyme. The filtrates were directly analyzed by HPLC–MS without dilution. All the binding assays were performed in triplicate.

HPLC–MS analysis

HPLC was performed on an Acquity™ UPLC system (Waters, Milford, MA, USA) with a cooled autosampler and column oven enabling temperature control of the analytical column. A reversed phase SunFire™ C_{18} (250 mm×4.6 mm i.d., 5 μm, Waters, Milford, MA, USA) column was employed for the analysis. The chromatographic separation was realized using a mixture of solvents, acetonitrile (A) and water containing 0.4 % acetic acid (B). The gradient program for the *D. styracifolium* extract was as follows: 22–33 % A (0–10 min) and 33–40 % A (10–20 min). The flow rate was 1.0 mL min^{-1} and the column temperature was 25 °C. The detection wavelength was set at 254 nm and the injection volume was 20 μL. Triple-quadrupole mass detection was performed on a Micromass® Quattro Micro™ API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in the negative ionization mode. The following settings were applied to the instrument: capillary voltage, 3.00 kV; cone voltage, 40.0 V; extractor voltage, 3.00 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas flow rate, 750 L h^{-1}; cone gas flow rate, 50 L h^{-1} and dwell time, 0.05 s. Nitrogen was used as the desolvation and cone gas. Mass detection was performed in the full scan mode for *m*/*z* in the range 160–800.

All collected data were acquired and processed using MassLynx^{TM} NT 4.1 software with QuanLynx^{TM} program (Waters Corp., Milford, MA, USA).

Enzyme activity assay

A mixture of ethanol (500 μL), NAD^+ (500 μL, 1 mg mL^{-1}) and phosphate buffer solution (1000 μL, pH 7.0, 0.2 M, PBS) were used as the substrate. To initiate the reaction, 10 μL of ADH (37.5 μg mL^{-1}) was added to the substrate at 15 °C. After incubation for 5 min, the increase in absorbance at 340 nm was measured using an UV–Vis spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan). One unit of ADH activity was defined as the amount of enzyme that reduced 1 nmol of NAD^+ per minute under the assay conditions.

When the inhibition of ADH activity was studied, 1000 μL of *D. styracifolium* extract with different concentrations were used instead of PBS. The substrate was incubated for 5 min at 15 °C before the addition of 10 μL of ADH (37.5 μg mL^{-1}). The mixture was incubated for 5 min and the increase in absorbance at 340 nm was measured. The percentage inhibition, *I*%, of the ADH activity was calculated using Eq. (1).

$$I\% = 1 - 100\left(\frac{\Delta A_1}{\Delta A_0}\right) \quad (1)$$

where ΔA_0 is the increase in absorbance at 340 nm and ΔA_1 is the increase in absorbance at 340 nm in the presence of *D. styracifolium* extract. All experiments were conducted in triplicate under the same conditions and the results were obtained as the average of the corresponding experimental values.

Optimization of the screening conditions

The effect of ADH concentration on the screening was evaluated using different concentrations of ADH (12.5, 25.0, 37.5 and 50.0 μg mL^{-1}). Various incubation times (10, 60, 90 and 120 min) were also studied to determine the appropriate incubation time for the assays. To acquire the optimum pH and temperature for screening, the experiments were conducted at different pH value from 5.0 to 9.0 and temperatures in the range from 5 to 45 °C. All experiments were conducted in triplicate and the results were obtained as the average of the corresponding experimental values. The binding strength of compounds to ADH was defined as the binding degree, *BD*%, which can be calculated using Eq. (2):

$$BD\% = 1 - 100\left(\frac{A\mathrm{b}}{A\mathrm{a}}\right) \quad (2)$$

where *A*a and *A*b are the peak areas of a compound in the HPLC chromatograms incubated with denatured and active ADH, respectively.

RESULTS AND DISCUSSION

Optimization of HPLC analysis

The separation of each compound in the extracts of natural products is a challenge for HPLC analysis. Acid in the mobile phase could reduce peak tailing and increase the signal.[27] Therefore, different mobile phases with different flow rates, detection wavelengths and column temperatures were utilized to optimize the HPLC conditions for the analysis of the *D. styracifolium* extract. The results showed that the optimum chromatographic separation was achieved using a mix-

ture of solvents, acetonitrile (A) and water containing 0.4 % acetic acid (B). A gradient program was operated as follows: 22–33 % A (0–10 min) and 33–40 % A (10–20 min). The flow rate was 1.0 mL min^{-1} and the column temperature was 25 °C. The detection wavelength was set at 254 nm and the injection volume was 20 μL. All constituents could reach baseline separation and a relatively short analysis time was achieved. Chromatograms obtained under these conditions are shown in Fig. 1.

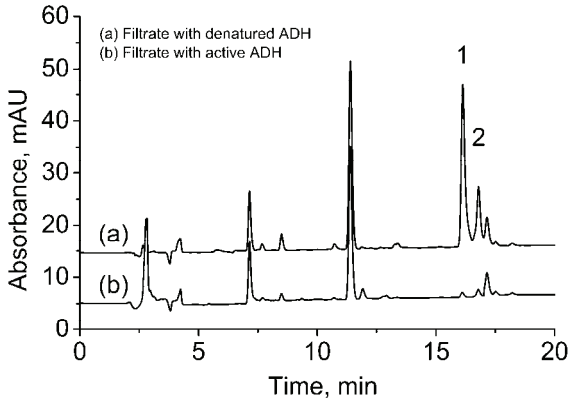


Fig. 1. The chromatograms of filtrates incubated with denatured (a) and active (b) ADH.

Optimization of the screening conditions

Effect of ADH concentration. Different concentrations of ADH (12.5, 25.0, 37.5 and 50.0 μg mL^{-1}) were incubated with *D. styracifolium* extract to investigate the effect of the ADH concentration on binding degree. The chromatograms of filtrates incubated with different concentrations of ADH are shown in Fig. 2A. The peaks of compounds **1** and **2** decreased with increasing ADH concentration. When the concentration of ADH was higher than 37.5 μg mL^{-1}, the

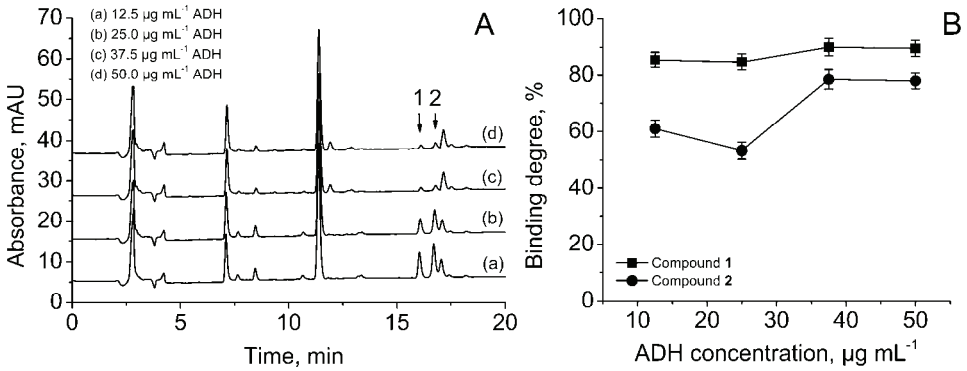


Fig. 2. A) The chromatograms of the filtrates incubated with different concentrations of ADH; B) the effect of the ADH concentration on the binding degrees.

binding degrees remained unchanged (Fig. 2B). As superfluous enzyme is wasteful and would increase the cost of the experiment, an ADH concentration of 37.5 μg mL^{-1} was considered optimal.

Effect of incubation time. A sufficient period was necessary to achieve ligand–enzyme equilibrium. Hence, different incubation periods ranging from 10 to 120 min were studied to investigate the effect of time on the binding degree. The binding degrees of compounds **1** and **2** at different incubation times were calculated and are shown in Fig. 3A. The binding degrees of compounds **1** and **2** became the highest when the incubation time reached 90 min, and did not increase on prolongation of the incubation time. The results manifested that 90 min incubation time was sufficient in screening experiments.

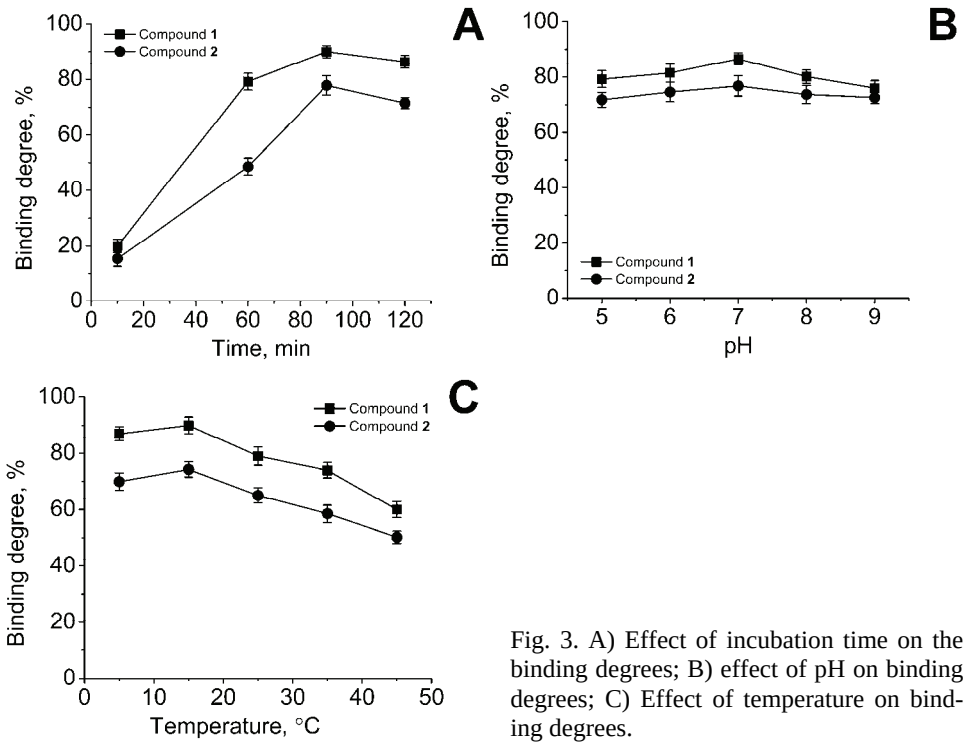


Fig. 3. A) Effect of incubation time on the binding degrees; B) effect of pH on binding degrees; C) Effect of temperature on binding degrees.

Effect of pH. The pH value affects the status of an enzyme and its activity. The effect of pH on the binding degree was studied at different pH values ranging from 5.0 to 9.0. As shown in Fig. 3B, the maximum binding degrees of compounds **1** and **2** were obtained at pH 7.0. As reported in the literatures, ADH showed optimum activity at pH 7.0, which was in accordance with the obtained experiment results.[28] Therefore, the screening pH value was set at 7.0.

Effect of temperature. The effect of temperature on binding degree was investigated and the results are shown in Fig. 3C. It was found that the highest binding degrees were achieved at 15 °C. The enzyme is thermally sensitive in general and its activity would decrease in a high temperature environment.[29] Thus, the temperature was set at 15 °C in order to maintain the enzyme active during the experiments.

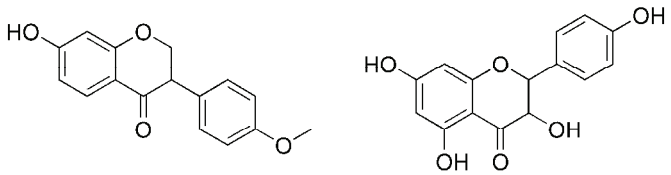
Screening and identification of ADH inhibitors

The ethyl acetate part of the *D. styracifolium* extract showed ADH inhibition with an IC_{50} value of 36.2 μg mL^{-1}, while the IC_{50} values of the petroleum ether and *n*-butanol parts were greater than 500 μg mL^{-1}. These results suggested that there were compounds that inhibited ADH in the ethyl acetate part of the *D. styracifolium* extract. Therefore, the ethyl acetate part of the *D. styracifolium* extract was selected as the screening sample and was analyzed by centrifugal ultrafiltration combined with HPLC–MS.

Screening experiments with denatured ADH were performed to exclude the possibility of nonspecific adsorption between the compounds and enzyme. The chromatograms of the filtrates incubated with denatured and active ADH are seen in Fig. 1. Two compounds marked as **1** and **2** clearly showed decreases in peak areas after centrifugal ultrafiltration with active ADH. The chemical structure of these two compounds was identified according to their UV and HPLC–MS spectroscopic data. According to previous reports, the data shown in Table I were in agreement with literature values.[30,31] Therefore, these two compounds were identified as formononetin and aromadendrin. Their chemical structures are shown in Fig. 4. The screening results indicated formononetin and aromadendrin had potential inhibitory activities on ADH.

TABLE I. The identification, UV, and MS characteristics of compounds in *D. styracifolium*

Peak	Formula	UV (λ_{max} / nm)	Proposed ion	Structural assignment	Ref.
1	$C_{16}H_{12}O_4$	248.3	$[M+H]^+$ 269	Formononetin	30
2	$C_{15}H_{12}O_6$	225.3	$[M+H]^+$ 289	Aromadendrin	31



Formononetin Aromadendrin

Fig. 4. Chemical structures of the screened compounds.

Inhibition analysis of the screened compounds

In order to confirm the screening results, the inhibitory activities of authentic formononetin and aromadendrin were analyzed. The authentic samples of formononetin and aromadendrin exhibited inhibitory activities on ADH, and their IC_{50} values were 70.8 and 84.7 μg mL^{-1}, respectively. In addition, the binding degrees of formononetin and aromadendrin under the optimum conditions were 90.0 % and 86.5 %, respectively. These results showed that the compound with high binding degree had the lower IC_{50} value. The binding degree could indicate not only the potential inhibitory activity of compound, but also the degree of inhibition. Based on current literature, the inhibitory activity of formononetin on ADH has been reported,[32] while, the inhibitory activity of aromadendrin on ADH is reported herein for the first time. This demonstrated that screening utilizing centrifugal ultrafiltration combined with HPLC-MS was effective and conclusive.

CONCLUSIONS

A facile screening method based on centrifugal ultrafiltration combined with HPLC-MS was established for analyzing ADH inhibitors from *D. styracifolium.* The experimental conditions were optimized. Finally, formononetin and aromadendrin were screened, identified and analyzed as ADH inhibitors. This method proved to be rapid and effective for the identification of active compounds in natural products.

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ИЗВОД

АНАЛИЗА ИНХИБИТОРА АЛКОХОЛНЕ ДЕХИДРОГЕНАЗЕ ИЗ *Desmodium styracifolium* ПРИМЕНОМ ЦЕНТРИФУГАЛНЕ УЛТРАФИЛТРАЦИЈЕ СПРЕГНУТЕ СА HPLC-MS

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Инхибитори алкохол дехидрогеназе (ADH) имају значајну улогу у третирању тровања метанолом и етиленгликолом и супресији акумулације ацеталдехида код алкоголичара. У овом раду, центрифугална ултрафилтрација је спрегнута са високоефикасном течном хроматографијом–масеном спектрометријом (HPLC–MS), ради скрининга и идентификације ADH инхибитора из етилацетатног екстракта *Desmodium styracifolium* (Osb.) Merr. Оптимизовани су услови центрифугалне ултрафилтрације. При оптималним условима (концентрација ADH: 37,5 μg mL^{-1}, време инкубације: 90 min, pH 7,0 и температура: 15 °C), формононетин и аромадендрин су успшно идентификовани из

етилацетатног екстракта *D. styracifolium*. Резултати скрининга су верификовани тестом ADH инхибиције. Вредности IC_{50} за формононетин и аромадендрин су биле 70,8 и 84,7 μg mL^{-1}, што је сагласно са афинитетом везивања ових једињења. Инхибиторна активност на ADH је први пут публикована за аромадендрин. Ова метода обезбеђује ефикасан начин за скрининг активних једињења у природним производима.

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