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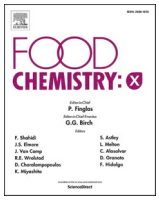

Indoleamine 2,3 dioxygenase 1 immobilization on magnetic nanoparticles for screening inhibitors from coffee

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Chlorogenic acid (PubChem CID 1794427)
Cryptochlorogenic acid (PubChem CID 9798666)
Ferulic acid (PubChem CID 445858)
Isochlorogenic acid A (PubChem CID 6474310)
Isochlorogenic acid B (PubChem CID 5281780)
Isochlorogenic acid C (PubChem CID 6474309)
Neochlorogenic acid (PubChem CID 5280633)
3-O-feruloylquinic acid (PubChem CID 9799386)
5-O-feruloylquinic acid (PubChem CID 73210496)

ABSTRACT

In this study, a ligand fishing method was developed to screen potential indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors from coffee extracts by immobilization of IDO1 enzyme on amino-modified magnetic nanoparticles combined with UHPLC-Q-TOF-MS/MS analysis. Parameters including enzyme concentration, immobilization time, the pH of glutaraldehyde and the amount of magnetic nanoparticles were optimized. The results indicated that immobilized IDO1 could be reused 5 times and was stable during storage for 7 days. Several IDO1 ligands were captured by incubating immobilized IDO1 with coffee extract, of which 10 showed an obvious difference comparing to non-conjugated bare nanoparticles. *In vitro* inhibitory activity was further performed by CE analysis, in which ferulic acid and chlorogenic acid had better IDO1 inhibitory activity, with IC_{50} value of 113.7 μM and 307.5 μM. These results demonstrate that this method provides an effective platform for identifying and screening IDO1 inhibitors from natural products.

Introduction

Depression is a common mental disorder characterized by significant and persistent low mood, and its core manifestations include depressive states, cognitive impairment and anhedonia (Belmaker & Agam, 2008). At present, the pathogenesis of depression is complex, and its pathophysiology is not clear, but a variety of studies have proved the major depression is highly relevant to neuroinflammation (Anisman, 2011; Xie et al., 2014). Plasma levels of numerous pro-inflammatory cytokines such as IFN-α, tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and interleukin-1beta (IL-1β) were significantly elevated in patients with depression (Fujigaki, Yamamoto, & Saito, 2017; Hestad, Tønseth, Støen, Ueland, & Aukrust, 2003; Howren, Lamkin, & Suls, 2009; Kunz et al., 2011; Owen, Eccleston, Ferrier, & Young, 2001). Indoleamine 2,3-dioxygenase 1 (IDO1) is one of the key factors connecting inflammation and depression, and regulating the occurrence of depression (Quak et al., 2014). In inflammatory state, IDO1 is overactivated by pro-inflammatory cytokines, which leads to dysregulation of tryptophan (Trp) metabolism along the kynurenine (Kyn) pathway (Cervenka, Agudelo, & Ruas, 2017; Quak et al., 2014). The significant expression of IDO1 reduces the availability of tryptophan, thus reducing the proportion of tryptophan that can be converted to serotonin (5-HT), further leading to a reduction in 5-HT synthesis (Myint & Kim, 2003; Neumeister, 2003). There were evidences that decreased activity of the

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serotonin system may induce or aggravate depression (Maes, Leonard, Myint, Kubera, & Verkerk, 2011; Miura et al., 2008), and a number of antidepressants are also committed to increasing the availability of serotonin in the synaptic space (Anderson et al., 2008). At the same time, increased levels of kynurenine and its downstream neuroactive metabolites such as 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA) and quinolinic acid (QA) could lead to direct neurotoxicity, and these neurotoxins play a vital role in neurodegeneration, inflammation, and depressive symptoms (Kunz et al., 2011; Maes et al., 2011; Stone & Darlington, 2002). Therefore, all these characteristics and evidences consistently suggest that IDO1 may become a promising drug target for the simultaneous treatment of depression and other coexisting diseases in the future.

Bioactivity-guided screening by free enzymes has been a main stream method in the discovery of active natural products. However, there are still some problems with this method, such as time-consuming and high-cost. More importantly, it cannot be applied to complex sample screening due to its lack of separation ability (Cheng et al., 2019). As a kind of bioactive macromolecule, free enzyme not only has poor spatial conformation stability and catalytic activity, but also is prone to denaturation or inactivation under the influence of temperature, pH, heavy metal salts and other environmental factors (Zhang, Wu, Yang, Yang, & Li, 2019). Immobilization of enzymes on water-insoluble materials can strengthen the robustness of their active conformation, greatly reducing their degradation and aggregation, while protecting the function of the enzyme under biological unfavorable conditions (Wilkerson, Yang, Funk, Stanley, & Bundy, 2018). Compared with traditional free enzymes, the cost of analysis is reduced by using immobilized enzymes because of their good reusability and storage stability (Ye, Zhang, & Cao, 2021). There is no doubt that looking for ideal immobilization support materials and efficient immobilization methods are crucial to the development of enzyme immobilization technology. Therefore, a variety of functionalized solid carriers have been used for off-line analysis of immobilized enzymes, such as magnetic nanoparticles (MNPs), Metal-organic Frameworks (MOFs), Covalent Organic Frameworks (COFs), silica microspheres and cellulose filter paper (CFP) (Xia, Li, Zhong, & Jiang, 2020; Zhang et al., 2019), etc.

Among many solid carriers, magnetic nanoparticles carrying immobilized enzymes have been widely used in ligand fishing assays and enzyme inhibitor screening due to their superparamagnetism, large specific surface area, stable physical properties and versatility (Gessner, Fries, Brune, & Mathur, 2021; Trindade Ximenes, de Oliveira, Wegermann, & de Moraes, 2021; Zhang et al., 2017). More importantly, they can be quickly separated from the reaction mixture by using an external magnet, which makes it easy to operate in the traditional screening analysis and simplifies the reuse process of enzymes (Ramana et al., 2017). However, due to the characteristics of easy oxidation, and the smooth surface without wrinkles, traditional magnetic nanoparticles cannot provide enough sites for immobilized enzymes (Kalantari, Kazemeini, Tabandeh, & Arpanaei, 2012). Moreover, there is a strong magnetic dipole–dipole attraction between magnetic nanoparticles, so that they may easily aggregate in liquid medium (Pan et al., 2009). In order to effectively prevent the aggregation of magnetic nanoparticles in liquid, a functional silica coating can be formed on their surface, while improving their chemical durability and biomaterial compatibility (Chen, Zhao, & Zou, 2009). At the same time, the combination of silica and magnetic carrier will significantly improve the immobilization efficiency of the enzyme. In general, Fe_3O_4@SiO_2 nanoparticles with core–shell structure are often aminated to have a surface modified with an amino group, and then glutaraldehyde is usually used as a cross-linking agent to fix the enzyme on the surface of the amino-modified nanoparticles to improve the fixation efficiency of enzymes and solve the defects of single materials (Akter et al., 2018; Bilal, Zhao, Rasheed, & Iqbal, 2018).

Coffee is the most widely used beverage globally and contains many bioactive compounds, including phenolic compounds, alkaloids and so on (Wu et al., 2022). Recent studies screened out several inhibitors for different enzymes from coffee as active compounds for the potential treatment of related diseases (Budryn et al., 2018; Honda & Masuda, 2016). Our previous study showed that coffee extract had a significant inhibitory effect on IDO1 activity (Liu, Wang, Wang, & Van Schepdael, 2021), but its active ingredients are unclear. Therefore, a simple method for identifying and screening IDO1 enzyme inhibitors from coffee extracts based on Fe_3O_4@SiO_2@GA magnetic nanoparticles was elaborated in this study. Some parameters such as the enzyme concentration, immobilization time, the pH of GA and the amount of magnetic nanoparticles were optimized. Meanwhile, the storage stability and reusability of the immobilized IDO1 were also measured. Furthermore, a UHPLC-Q-TOF-MS/MS method was used to identify the active ingredients in coffee extract and molecular docking was explored to predict the potential interaction. Finally, the inhibitory activity was verified by capillary electrophoresis (CE) based enzyme assay.

Materials and methods

Reagents and chemicals

Human recombinant IDO1 enzyme was obtained from Biovision (San Francisco, USA). Amino-functionalized silica magnetic nanoparticles (40 mg/mL) with a diameter of 300 nm were obtained from Suzhou Beike Nano Technology Co. Ltd. (Suzhou, China). Kynurenine standard was purchased from Aladdin Reagent (Shanghai, China). The internal standard (α-Methyl-DL-tryptophan) was purchased from Sigma (St. Louis, MO, USA). Methylene Blue hydrate was purchased from Alfa Aesar (Shanghai, China). The substrate L-tryptophan (trp), catalase, ascorbic acid, potassium dihydrogen phosphate and glutaraldehyde (GA, 50 % w/v) were purchased from ANPEL Laboratory Technologies, Inc (Shanghai, China). Sodium hydroxide (NaOH) and sodium tetraborate were purchased from Jiangtian Chemical (Tianjin, China). Reference standards for neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, ferulic acid, isochlorogenic acid B, isochlorogenic acid A, isochlorogenic acid C, caffeine, 3-O-feruloylquinic acid and 5-O-feruloylquinic acid were purchased from ANPEL Laboratory Technology (Shanghai) Co., Ltd. and Alta Scientific Co., Ltd. (Tianjin, China). All solutions are prepared daily in deionized water purified by Millipore Water Purification system (Billerica, MA, USA) and filtered through a 0.22 μm polyether sulfone filter prior to injection.

Coffee beans are sourced from supermarkets. For the preparation of the coffee extract, the coffee beans are ground in a grinder and ultrasonically extracted twice in a 70 % ethanol solution for 20 mins each. The centrifuged supernatant was evaporated by rotary evaporation at 45 °C to remove the organic solvent, and the remaining liquid was freeze-dried into powder by vacuum, sealed and stored at −20 °C for future experiment.

Instrumentation

Capillary electrophoresis

The separation and detection of enzyme reaction mixtures was carried out on an Agilent 7100 capillary electrophoresis system equipped with a UV detector (Agilent Technologies, Palo Alto, CA, USA). The analyses were performed in a fused silica capillary with 75 μm i.d. (360 μm o.d.) and a total length of 50 cm (8.5 cm from the detection window to the outlet) (Unimicro Technologies, Shanghai). The cartridge temperature was set to be 25 °C and the detection wavelength was 227 nm. The off-line incubation mixture was injected at a pressure of 35 mbar for 5 s and the product Kyn was separated from substrate trp and α-methyl-DL-tryptophan (internal standard) at a voltage of 21 kV. A new capillary was pretreated with 1 M NaOH for 20 min, then washed with 0.1 M NaOH, deionized water and background electrolyte (BGE) for 10 min, respectively.

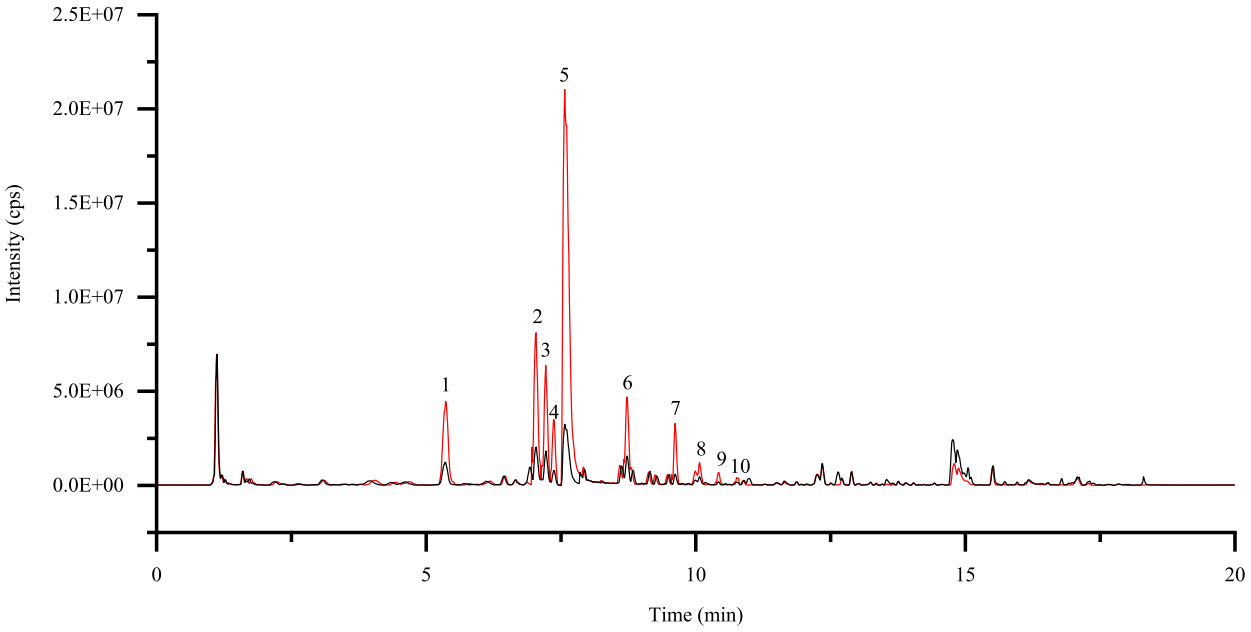

Fig. 1. Screening results of coffee extract by using ligand fishing method. Binding Pattern Chromatogram of the binders screened out from coffee extract. The red solid line represents the binders adsorbed on immobilized IDO1. The black solid line represents the binders adsorbed on blank Magnetic nanoparticles (false positive). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Identification of potential IDO1 inhibitors in coffee extract by UPLC-Q-TOF-MS/MS.

Peak	Compound	Formula	t_R (min)	$[M + H]^+$	Fragments
1	Neochlorogenic acid	$C_{16}H_{18}O_9$	5.37	355.1167	337.1034,163.0455,145.0342, 135.0502
2	Chlorogenic acid	$C_{16}H_{18}O_9$	7.04	355.1162	337.1070,181.0567,163.0452, 135.0497,145.0337
3	Cryptochlorogenic acid	$C_{16}H_{18}O_9$	7.21	355.1162	337.1088,181.0540,163.0453, 145.0334,135.0491
4	3-O-Feruloylquinic acid	$C_{25}H_{24}O_{12}$	7.37	369.1317	391.1158,351.1021,177.0608, 149.0643,145.0345,134.0452
5	Caffeine	$C_8H_{10}N_4O_2$	7.57	195.0886	138.0550
6	5-O-Feruloylquinic acid	$C_{25}H_{24}O_{12}$	8.71	369.1325	391.1175,351.1221,177.0618, 149.0652,145.0384,134.0416
7	Ferulic acid	$C_{10}H_{10}O_4$	9.61	195.0714	177.1188,145.0352,149.0639, 134.0970
8	Isochlorogenic acid B	$C_{25}H_{24}O_{12}$	10.07	517.1551	499.1431,555.1116,539.1371,355.1150,337.0986,181.0561,163.0451, 145.0340,135.0478
9	Isochlorogenic acid A	$C_{25}H_{24}O_{12}$	10.42	517.1537	499.1416,539.1368,555.1095,355.1129,337.1033,181.0567,163.0454, 145.0324,135.0496
10	Isochlorogenic acid C	$C_{25}H_{24}O_{12}$	10.76	517.1542	499.1435,555.1109,539.1365,181.0556,163.0455,145.0346,135.0487

Table 2
Docking results of identified compounds with IDO1.

Compounds	Total Score	Amino acid residues of IDO1, in close contact with compound
Neochlorogenic acid	6.12	SER267, SER167, Q5PF502
Chlorogenic acid	5.69	ASP274, GLN271, SER267, GLY265, ALA264, ARG343
Cryptochlorogenic acid	6.07	SER267, ALA264, Q5PF502, TYR126, GLU171
3-O-Feruloylquinic acid	6.99	ALA264, ARG343
Caffeine	3.84	–
5-O-Feruloylquinic acid	7.09	ALA264, ARG343
Ferulic acid	5.97	SER267, TYR126, GLU171
Isochlorogenic acid B	6.66	ASP274, SER267, LEU384
Isochlorogenic acid A	6.39	GLU171, SER267, TYR126, SER167
Isochlorogenic acid C	5.57	TYR126, GLU171, SER167, ARG343, GLY265, ASP274, SER267

UHPLC-Q-TOF-MS/MS

Coffee extract, as well as the MNP eluent, were analyzed using a UHPLC-Q-TOF-MS/MS system with a TripleTOF 6600 Mass Spectrometer (SCIEX, USA) connected to the ExionLC AD UHPLC system (SCIEX, USA). Using 0.1 % formic acid aqueous solution as mobile phase A and acetonitrile as mobile phase B, the separation was carried out with a C_{18} analytical column (Agilent ZORBAX C_{18}, 2.1 × 100 mm, 1.8 μm). During the whole study, the optimized gradient is as follows: 0–2 min, 5 % B; 2–10 min, 5–30 % B; 10–15 min, 30–80 % B; 15–15.5 min, 80–95 % B; 15.5–16.5 min, 95 % B; 16.5–16.6 min, 95–5 % B; 16.6–20 min, 5 % B. The column temperature was set at 35 °C, the flow rate was 0.2 mL/min, and the injection volume was 1 μL.

For the ESI-MS/MS analysis, the mass spectrometer is operated with an electrospray ionization source (ESI) in positive ion mode. The parameters of mass spectrometric analysis were set as follows: ionspray voltage at 5500 V, curtain gas at 30 psi, ion source gas 1 at 50 psi, ion source gas 2 at 50 psi, temperature at 500 °C. The scanning range of precursor ions is *m/z* 70–1000, and the MS/MS acquisition scanning range is *m/z* 50–1000.

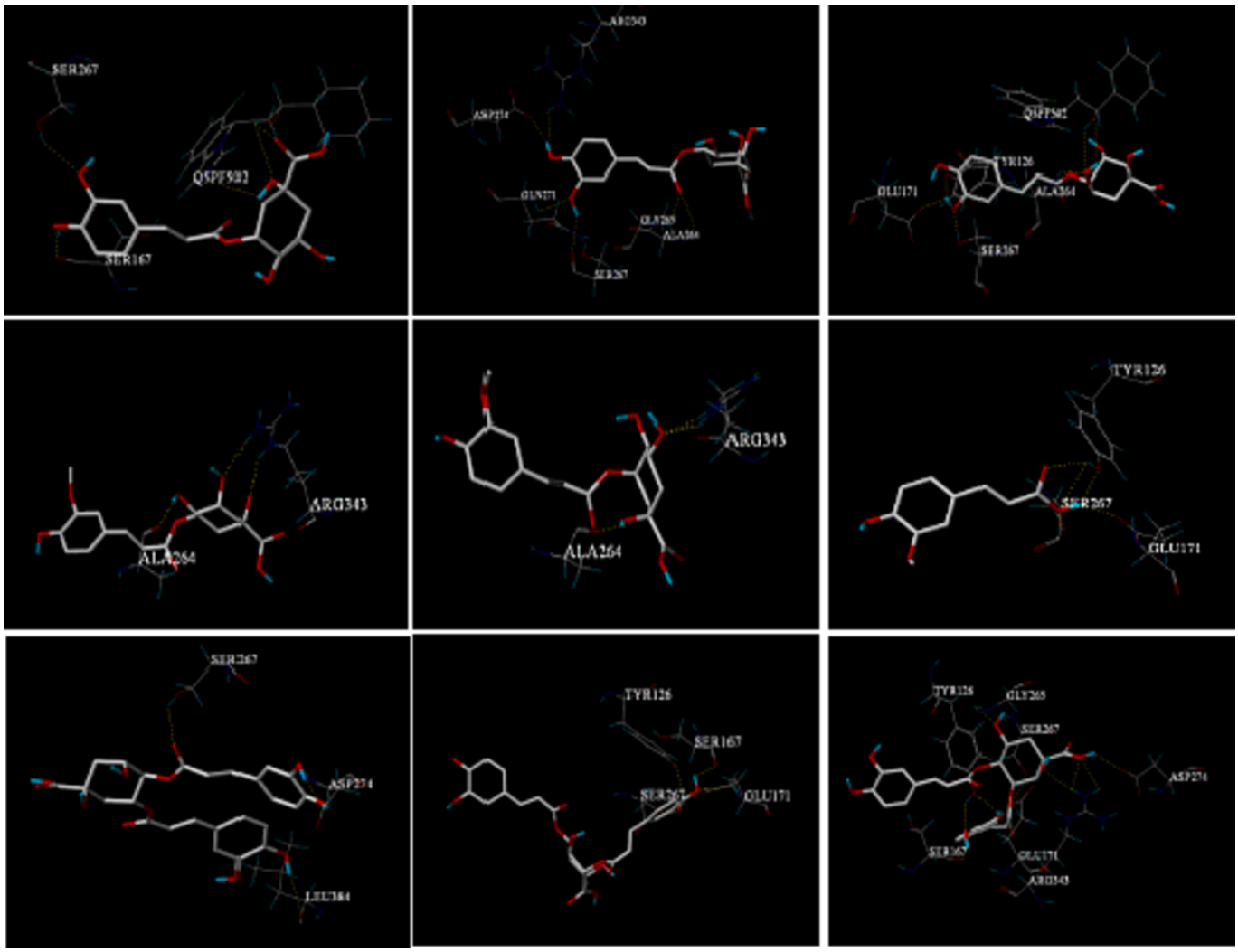

Fig. 2. The binding modes with interaction effects of Neochlorogenic acid (A), Chlorogenic acid (B), Cryptochlorogenic acid (C), 3-O-feruloylquinic acid (D), 5-O-feruloylquinic acid (E), Ferulic acid (F), Isochlorogenic acid B (G), Isochlorogenic acid A (H), Isochlorogenic acid C (I) to IDO1.

Table 3
Percent Inhibition for Natural Compounds.

Compounds(100 μM)	Inhibition(%)
Ferulic acid	66.09
Chlorogenic acid	30.52
Isochlorogenic acid B	19.11
3-O-feruloylquinic acid	16.36
5-O-feruloylquinic acid	12.73
Cryptochlorogenic acid	11.82
Isochlorogenic acid C	10.91
Neochlorogenic acid	10.89
Isochlorogenic acid A	0
Caffeine	0

Immobilization of IDO1

The method for immobilizing IDO1 enzyme was established as follows. First, commercial amino functionalized silica magnetic nanoparticles (40 mg/mL) were vortexed for 1 min and then washed five times with potassium phosphate buffer (PPB, 50 mM, pH 6.5). Magnetic nanoparticles were modified with glutaraldehyde as a crosslinking agent to ensure successful covalent fixation of IDO1 enzyme on the surface of magnetic nanoparticles. Briefly, the glutaraldehyde solution (50 %) was diluted to a concentration of 5 % in the PPB, and then the magnetic nanoparticles were added to an equal volume of glutaraldehyde solution (5 %, pH 6.5) for 1 min of ultrasonication. After reaching a completely uniform dispersion, the mixture was placed in a constant thermostatic oscillator and incubated with oscillations (200 rpm) at room temperature for 4 h. Subsequently, the aldehyde modified material was washed five times with PPB to remove excess glutaraldehyde. The desired concentration of IDO1 enzyme solution (including 20 mM ascorbic acid, 20 μM methylene blue and 200 μg/mL catalase) was added to the Fe_3O_4@SiO_2@GA particles (2 mg/mL), and the mixture was oscillated and incubated at 37 °C for 1 h, after vortexing for 1 min. Finally, the magnetic nanoparticles with bound IDO1 enzyme were collected and washed three times with PPB to remove the non-specifically bound free enzyme, and then dispersed in 100 μL PPB, and stored at 4 °C for subsequent experiments. For all the washing steps, magnetic nanoparticles are magnetically separated using external magnets.

Screening for ligands from the extract of coffee using immobilized IDO1

The freeze-dried coffee powder was dissolved in PPB (50 mM, pH 6.5). The constructed Fe_3O_4@SiO_2@GA@IDO1 was used to screen potential IDO1 ligands from coffee extract. Ligand fishing experiment steps are as follows. First, 100 μL coffee extract (20 mg/mL) was incubated with 100 μL of the above immobilized IDO1 enzyme at 37 °C for 30 min. After incubation, magnetic separation was carried out with an external

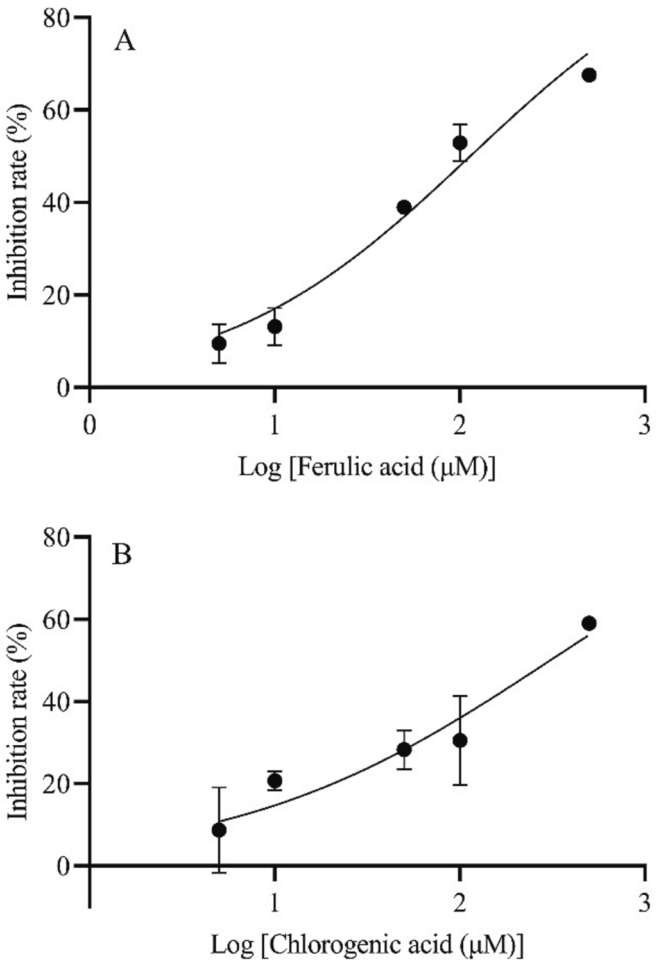

Fig. 3. The dose–response curves of ferulic acid (A) and chlorogenic acid (B).

magnet and the supernatant was collected. Subsequently, the immobilized enzyme was washed 3 times with 100 μL PPB to remove the non-specific components that have no affinity for IDO1. Finally, 100 μL of 50 % acetonitrile was added to the immobilized enzyme followed by 5 min waiting time to release potential ligands bound to IDO1, which was repeated four times. Then, the combined liquids were collected and freeze-dried, reconstituted with 100 μL of 50 % acetonitrile solution, and analyzed by UHPLC-Q-TOF-MS/MS. The non-specific binding control experiment was carried out in the same way with magnetic nanoparticles without IDO1. By comparison of the signals intensity of the components in both eluents, the stronger signals captured by the immobilized IDO1 were identified as the potential ligands.

Molecular docking

Surflex-Dock module in SYBYL-X 2.0 molecular docking software was used to explore the potential interaction between compounds identified from coffee extract and IDO1 enzyme. The crystal structure of the known complex of IDO1 enzyme with NLG919 (PDB ID: 5EK4) was obtained from RCSB Protein Data Bank (https://www.rcsb.org/pdb). In order to improve the reliability of molecular docking, unnecessary original ligands, water molecules and B chains in 5EK4 were removed prior to docking, and then the protein was further modified by adding hydrogen atoms and filling the missing side chains. Meanwhile, the 3D structures of all experimental compounds docked with IDO1 are from PubChem. Taking the binding site of the original ligand as the active site, each compound was docked with IDO1 target protein for scoring. In general, the total score is used to predict its binding efficacy. The higher the total score, the stronger the binding affinity between the compound and the target.

Activity assay of Fe_3O_4@SiO_2@GA@IDO1

The activity of immobilized IDO1 enzyme was measured with trp as substrate. Briefly, the prepared immobilized IDO1 magnetic nanoparticles (25 nM) were ultrasonically treated for 1 min, 100 μL immobilized IDO1 magnetic nanoparticles was incubated with 50 μL of PPB (50 mM, pH 6.5) with or without inhibitors at 37 °C for 5 min. Subsequently, 50 μL of trp (200 μM) was added to initiate the reaction and the reaction mixture was maintained at 37 °C for 20 min. Subsequently, the magnetic nanoparticles were separated from the reaction mixture using an external magnet, and 20 μL trichloroacetic acid (30 %) was added to the sample to end the enzymatic reaction. After incubation at 65 °C for 15 min, the sample was centrifuged and 75 μL of the supernatant was added to 25 μL of the internal standard solution before CE analysis.

The percentage of inhibition (%) was calculated according to the following formula:

$$\text{Inhibition } (\%) = (A_{control} - A_{sample}/A_{control}) \times 100\ \%$$

A_{sample}: the corrected peak area measured in the presence of different concentrations of inhibitors; $A_{control}$: the corrected peak area measured without inhibitors.

Statistical analysis

The IC_{50} value was calculated using Prism 8 (GraphPad software, San Diego, CA) software. Other data analysis was calculated using origin 2019 (OriginLab, Northampton, MA, USA) software.

Results and discussion

Optimization of the immobilization conditions

Effect of the pH value of GA solution

The effect of the pH value of GA solution in the range of 6–8 on the activity of immobilized IDO1 was investigated. As can be seen from Fig. S1A, when the pH is increased from 6 to 6.5, the amount of crosslinking between glutaraldehyde and the carrier has increased dramatically, whereby the number of immobilized enzyme molecules increases. Under lower pH conditions, the amino group is protonated, and the crosslinking reaction of the GA monomer and the amino group is unstable. Therefore, the acidic pH is not conducive to increasing the crosslinking amount of GA. Previous studies (Pan et al., 2009; Wine, Cohen-Hadar, Freeman, & Frolow, 2010) have shown that GA monomer can be polymerized under neutral or alkaline conditions and its stability is improved. The results show that the extent of enzymatic activity obtained after immobilization, referred to below as activity of immobilized enzyme, in the range of pH 6.5–8 is generally higher than pH 6. The activity of immobilized enzyme reached the highest value when the pH of the GA was 6.5, which may be because the immobilized enzyme is just saturated under this condition.

Effect of the enzyme concentration

In order to evaluate the effect of enzyme concentration on immobilization, magnetic nanoparticles were immobilized with different concentrations of IDO1 enzyme in the range of 3.125–50 nM. As shown in Fig. S1B, the activity of immobilized enzyme increased gradually as the enzyme concentration increased from 3.1 nM to 25 nM. The activity of immobilized enzyme reached the highest level at 25 nM, when the binding amount of the enzyme was sufficiently saturated. When the enzyme concentration is less than 25 nM, the amount of IDO1 enzyme fixed to the magnetic nanoparticles is less, which eventually leads to lower activity of immobilized enzyme. However, when the IDO1

concentration was increased to 50 nM, the activity of immobilized enzyme decreased significantly. This situation may be due to the excessive aggregation of IDO1 enzyme, which leads to steric hindrance between enzyme molecules blocking the binding between substrate and active site.

Effect of immobilization time

The effect of different immobilization time (0.5–4 h) on immobilized enzyme quantity was studied. As shown in Fig. S1C, the IDO1 enzyme has been successfully fixed on the carrier and the enzyme amount reached the optimal level when immobilized for 1 h. Beyond that, with the extension of immobilization time, the activity of immobilized enzyme began to decline gradually. The reason could be that the active site of the immobilized enzyme is hidden by the overloaded enzyme.

Effect of the amount of magnetic nanoparticles

The number of magnetic nanoparticles is related to the covalent binding efficiency of the IDO1 enzyme immobilization. The IDO1 enzyme was immobilized on magnetic nanoparticles with different concentrations in the range of 0.5–10 mg/mL. As shown in Fig. S1D, with an increase of the amount of magnetic nanoparticles, the extent of binding of IDO1 enzyme also increased, with the highest activity of immobilized enzyme at 2 mg/mL. An excessive number of magnetic nanoparticles will not only block the exposure of enzyme active sites, but may also cause the aggregation of magnetic nanoparticles, which ultimately leads to the reduction of the activity of immobilized enzyme. Therefore, a concentration of magnetic nanoparticles of 2 mg/mL was selected for subsequent experiments.

Reusability and storage stability of the immobilized IDO1

To assess the reusability of the immobilized enzyme, the immobilized enzyme was recovered from the reaction mixture using an external magnet and washed three times with PPB, waiting to be incubated again with the same substrate. As shown in Fig. S2A, the immobilized enzyme remained stable for 5 consecutive runs, with approximately 20 % decrease in activity observed after 7 consecutive repetitions. As the number of uses increases, a possible reason for the decrease of enzyme activity is enzyme shedding from the carrier. The results showed that the immobilized IDO1 had great reusability, which was helpful to reduce the experimental cost.

In addition, the storage stability of immobilized IDO1 was investigated by measuring the enzyme activity of immobilized IDO1 stored at 4 °C. As shown in Fig. S2B, the enzyme remained at its initial activity after one week. The immobilization of IDO1 on magnetic nanoparticles has good stability performance for the enzyme, which allows for long-term storage and large-scale practical application.

Ligand fishing assay for coffee extract

In order to demonstrate the usefulness of magnetic nanoparticles with immobilized IDO1 enzyme, they were applied to salvage potential IDO1 ligands from coffee extract. As shown in Fig. 1, the signal intensity of ten compounds captured by blank Fe_3O_4@SiO_2@GA magnetic nanoparticles was much less than that of immobilized IDO1. Therefore, these ten compounds were considered to be potential ligands of IDO1. It is worth to mention that, besides specific ligand-enzyme interactions, some limited non-specific binding might occur between potential ligands and aminated MNPs through electrostatic acid base interaction.

Based on the mass spectrometry data of the respective references (Asamenew et al., 2019; Nemzer, Abshiru, & Al-Taher, 2021) and the retention time of the standards, 10 compounds were initially identified as neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3-O-feruloylquinic acid, caffeine, 5-O-feruloylquinic acid, ferulic acid, isochlorogenic acid B, isochlorogenic acid A and isochlorogenic acid C. The detailed identification results are listed in Table 1.

Molecular docking

It is well known that molecular docking is one of the virtual screening techniques for predicting and studying the interaction of biologically active molecules and protein receptors. Therefore, this study predicted their binding affinity to IDO1 enzyme by using the molecular docking technique. As shown in Table 2, the total scores of the docking and bound amino acid residues were summarized. The docking results showed that except for caffeine, the total score of the remaining 9 compounds was greater than 5, and the higher the total score of the dock, the higher the affinity between compounds and IDO1 enzyme, indicating that they have good binding affinity with IDO1. As shown in Fig. 2, the identified 9 compounds can interact with a variety of amino acid residues of IDO1 through hydrogen bonding, which improves the binding efficiency. In particular, the amino acid residues SER267, ALA264, ARG343 and TYR126 of IDO1 have close contact to ligands, which was consistent with literature reports (Cai et al., 2020; Yu et al., 2021). In summary, the 9 compounds identified in this study are highly likely to be potential IDO1 inhibitors. Considering the uncertainty of molecular docking, their inhibitory activity needs to be further verified using pure bioactive substances.

Inhibitory activity of the compounds on IDO1

In order to verify the inhibition activity of 10 identified compounds against IDO1, inhibitor screening experiments were performed at 100 μM concentration. The inhibition rates of compounds are shown in Table 3, and the results indicate that 8 natural compounds exhibit inhibitory activity, except for caffeine and isochlorogenic acid A. Ferulic acid and chlorogenic acid had good inhibitory activity and represented the active ingredients in coffee extract. The dose–response curves of ferulic acid and chlorogenic acid constructed from standard substance are shown in Fig. 3A and B. The IC_{50} value of ferulic acid was 113.7 μM, which was similar with that reported in the literature (Singh, Kaur, & Goel, 2017). The IC_{50} value of chlorogenic acid was 307.5 μM, which was reported for the first time in this work.

Conclusion

In this study, the IDO1 enzyme was successfully immobilized on the surface of amino-functionalized silica magnetic nanoparticles using glutaraldehyde as a crosslinking agent. The prepared Fe_3O_4@SiO_2@-GA@IDO1 has good reusability and storage stability. A novel ligand fishing strategy based on immobilized IDO1 was established, and 10 compounds were identified from coffee extract, with the help of UHPLC-Q-TOF-MS/MS. Molecular docking results showed that all of the nine compounds, except for caffeine, bound to the amino acid residues of IDO1, which may thus be potential inhibitors of IDO1. The inhibitory activity of 10 compounds was further analyzed. In contrast to caffeine and isochlorogenic acid A, the other 8 compounds showed IDO1 inhibitory activity. Among them, ferulic acid and chlorogenic acid have a significant inhibitory effect, with an IC_{50} of 117.3 μM and 307.5 μM, respectively. The specificity of the described protocol is related to the sequential combination of a fishing step with an IDO1 enzyme inhibition test. In summary, this approach provides new opportunities for discovering bioactive ingredients acting against IDO1 from complex natural products.

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CRediT authorship contribution statement

Zhuoting Liu: Methodology, Validation, Formal analysis, Writing – original draft. **Yunfang Ping:** Methodology. **Lumei Zhang:** Methodology. **Jingran Zhang:** Resources. **Xu Wang & Ann Van Schepdael:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100591.

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