SHORT COMMUNICATION

Purification and properties of a novel trypsin inhibitor from ginkgo fruits and its antiproliferative effect in triple-negative breast cancer cells

Xiaohui Zhao\textsuperscript{a,b}, Dayu Zhou\textsuperscript{c,d}, Shiliang Ma\textsuperscript{c}, Kexin Zheng\textsuperscript{c}, Ying Li\textsuperscript{a,b} and Bo Huang\textsuperscript{e,f}

\textsuperscript{a}Department of Oncology, The First Affiliated Hospital of Jinan University, Jinan University, Guangzhou, China; \textsuperscript{b}Department of Oncology, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China; \textsuperscript{c}College of Food Science and Technology, Shenyang Agricultural University, Shenyang, China; \textsuperscript{d}College of Food Science and Engineering, Bohai University, Jinzhou, China; \textsuperscript{e}Department of Surgery, The First Affiliated Hospital of Jinan University, Guangzhou, China; \textsuperscript{f}Department of Surgery, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China

ABSTRACT
A novel low molecular mass ginkgo biloba trypsin inhibitor (GBTI) was isolated from ginkgo fruits (GF) by trypsin inhibitory activity-guided fractionation by using ammonium sulphate precipitation, followed by ultra-filtration, affinity chromatography and RP-HPLC. The molecular mass and amino acid sequence of GBTI was determined using ESI-MS and ESI-MS/MS, respectively. The structure of GBTI was identified as MKNLTVIPPICLFPN, with a molecular mass of 1826 Da. GBTI was stable in the pH range of 4–8 and in the temperature range of 0–80°C for 30 min. However, the inhibitory activity of the GBTI reduced when incubated with various metal ions (K\textsuperscript{+}, Na\textsuperscript{+}, Fe\textsuperscript{2+}, Mg\textsuperscript{2+} and Ca\textsuperscript{2+}). Finally, GBTI exhibited significant antiproliferative effect in human MDA-MB-231 and mouse 4T-1 triple-negative breast cancer cells and without toxicity to MCF-10A normal breast cells. Our results suggest that GBTI could be exploited as a natural and hyperstable anticancer agent for triple-negative breast cancer patients.

ARTICLE HISTORY
Received 29 December 2021
Accepted 21 March 2022

KEYWORDS
Ginkgo fruits; trypsin inhibitor; triple-negative breast cancer; antiproliferation

1. Introduction
Triple-negative breast cancer (TNBC), defined by the lack of the expressions of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor
receptor 2 (HER2), contributes to approximately 15—20% of all breast cancers (Waks and Winer 2020). Most TNBC patients suffer from the high rate of recurrence and metastasis as well as severe side effects after conventional chemotherapies (Aggarwal et al. 2021). Therefore, the effective compounds isolated from natural medicinal plants with specifically targeting recurrence and minimal adverse effects are desirable to be explored (Alesci, Miller et al. 2021; Alesci et al. 2022). The natural compounds from plants as an alternative nutraceuticals with biological properties, including immunostimulant, antioxidant, antiviral and anti-breast cancer activities have widely accepted because of the long term availability and safety (Alesci, Fumia et al. 2021; Alessio et al. 2021; Fumia et al. 2021). In recent years, there has been a growing interest in the search for novel protease inhibitors from natural plants to inhibit TNBC in the biomedical field. Among these proteases, trypsin inhibitors which have been isolated from natural plant tissues possess antiproliferative activity in breast cancer cell lines (Weyburne et al. 2017; Fernandes et al. 2019; Megeressa et al. 2020).

Ginkgo biloba has been used for pharmaceutical and medical purposes for hundreds of years (Omidkhoda et al. 2019). The fruits of ginkgo biloba mainly contain sarcotestas and nuts, which have been consumed as food and medicine throughout the world. The extract of ginkgo fruits is a complex mixture of flavonoids and ginkgolic acids as well as active proteases, which have been reported to possess antitumor property and able to restore chemosensitivity to conventional chemotherapeutic drugs of human chemoresistant in TNBC (Iriti et al. 2017; Gao et al. 2020; Zhou et al. 2020). Our previous studies found that the aqueous extracts of GF showed significant antiproliferation in MDA-MB-231 breast cancer cells (Zhao et al. 2013). But their primary trypsin inhibitors and underlying properties are not well understood. And there not have been previously reported any investigations demonstrating the presence of low molecular weight trypsin inhibitor in the aqueous extract of GF. In this study, we used trypsin inhibitory activity-guided processes for the purification of a novel GBTI from GF and investigated the properties and antiproliferation of GBTI in human and mouse breast cancer cells.

2. Results and discussion

This study aimed to isolate a novel low molecular weight trypsin inhibitor from ginkgo fruits. Trypsin inhibitory activity guided processes were performed by using the BAPNA assay (Pontual et al. 2018), which revealed the significant anti-trypsin activity (61.34 ± 2.34) of aqueous extracts from ginkgo fruits in May (Supplementary material, Table S1). These crude extracts were further subjected to ammonium sulphate precipitation and amicon filters as well as affinity chromatography. Under such selected conditions, the purification steps showed a specific inhibitory activity of 21.38 ± 0.51 TIU/mg protein (1.66-fold purification), 40.27 ± 1.07 TIU/mg protein (3.12-fold purification) and 148.80 ± 4.93 TIU/mg protein (11.53-fold purification) respectively, compared to the crude extract (Supplementary material, Table S2). Through trypsin sepharose 4B affinity purification, a peak with the highest trypsin inhibitory activity showed the molecular mass of around 3 kDa visualized by SDS-PAGE (Supplementary material, Figure S1(A,B)). This peak was further purified by RP-HPLC, and the purified GBTI
showed an ideal single peak at the elution time of 33.725 min at 280 nm (Supplementary material, Figure S1(C)). This time GBTI was purified to 15.64 fold with a protein yield of 11.58% and a specific activity of 201.84 ± 7.41 TIU/mg protein (15.64-fold purification) (Supplementary material, Table S2). Then we identified a novel low molecular weight GBTI using ESI-MS/MS with a complete amino acid sequence of GFFKWHPRCGEEHSMWT. The molecular formula of GBTI was established to be C_{85}H_{142}O_{20}N_{20}S_{2} and [M – H]– was observed at m/z 1825.2472.4 by ESI-MS analysis (Supplementary material, Figure S1(D)). By Blast searching the NCBI database, GBTI showed 80% sequence similarity to the Chain B trypsin inhibitor from Helianthus annuus (Supplementary material, Table S3), but contained no intramolecular disulfide bridge. This indicated that GBTI was a novel trypsin inhibitor.

The stability of trypsin inhibitory activity is essential for biotechnological applications, and it is thought to be affected by temperature, pH and metal ion additives (Villalba-Villalba et al. 2013). Some plant trypsin inhibitors have been found to be highly thermal stability and quite active up to 70°C (Jc et al. 2020). As shown in Supplementary material, Figure S2(A), GBTI maintained trypsin inhibitory activity at temperatures ranging from 0 to 80°C after incubation for 30 min. Maximum inhibitory activity of 96 ± 2% was observed at 40°C after which it declined. At 80°C, GBTI retained 79.8 ± 3% of its inhibitory activity whereas at 100°C it only retained 41 ± 5% activity. We also noted that GBTI was highly stable in the pH range of 4–8 after incubation for 30 min and the maximum inhibitory activity of 92 ± 4% was observed at pH 6. However, there was significant decrease in activity at low and high pHs, with the activities of 38 ± 3% and 61 ± 4%, respectively (Supplementary material, Figure S2(B)). At extreme pH values, intramolecular electrostatic repulsion induced by high net charge results in unfolding and swelling of the protease molecules, which exhibit the loss of inhibitory activity (Klomklao et al. 2006). After incubation to a wide range of pH and temperature, trypsin inhibitors are stable due to their stern three dimensional structure and slight conformational changes (Samiksha et al. 2019). Monovalent and divalent metal ions play crucial role in maintaining the structural integrity of trypsin inhibitors that are important for biological activities. As shown in Supplementary material, Figure S2(C), the trypsin inhibitory activity of GBTI was reduced significantly in the presence of K⁺, Na⁺, Fe²⁺, Mg²⁺ and Ca²⁺ at 20 mM with 26–52% loss of activity. Metal ions bound by trypsin inhibitors induce structural modification that will change their conformational stability required for biological activities (Balakrishnan et al. 2011). Collectively, these results indicated that GBTI had a higher trypsin inhibitory activity in a large temperature and pH ranges, whereas the inhibitory activity decreased in the presence of different metal ions.

The cytotoxic effect of GBTI in the MDA-MB-231, 4 T-1 and non-tumorigenic MCF-10A cell lines was investigated. We found that GBTI exhibited the dose-dependent cytotoxic effect in MDA-MB-231 and 4 T-1 cells with the IC₅₀ values of 0.37 μM and 0.28 μM, respectively (Supplementary material, Figure S3(A)). The cytotoxicity of GBTI was less than 5% below 0.1 μM concentration and it had no cytotoxicity in MCF-10A cells. Furthermore, colony formation assay showed that GBTI significantly inhibited cells proliferation at concentrations ranging from 0.05 to 0.1 mM and led to about 68% and 72% reduction of colony formation at concentration of 0.1 μM in MDA-MB-
231 and 4 T-1 cells, respectively (Supplementary material, Figure S3(B)), indicating that GBTI may be responsible for the antiproliferation of triple-negative breast cancer cells in the GF aqueous extract. Together, these findings suggested the low molecular mass and physicochemical stability of GBTI from natural plant might offer the advantage of being easily used in pharmaceutical applications.

3. Conclusion

In summary, we demonstrated that a novel low molecular weight GBTI was isolated from the GF extract by trypsin inhibitory activity guided processes and exhibited nearly high temperature and neutral pH stability properties as well as antiproliferation in MDA-MB-231 and 4 T-1 cells. It significantly decreased its inhibitory activity when incubated with metal ions. This novel GBTI may help in the development of new anti-cancer agents for TNBC treatment.

Conflict of interest statement

The authors have declared no conflict of interest.

Funding

This work was supported by 10-100-1000 Program for High-End Talents Introduction of Liaoning Province in China [grant number 521082403-880303-88030312004].

References

Aggarwal S, Verma SS, Aggarwal S, Gupta SC. 2021. Drug repurposing for breast cancer therapy: old weapon for new Battle. Semin Cancer Biol. 68:8–20.

Alesci A, Fumia A, Cascio PL, Miller A, Cicero N. 2021. Immunostimulant and antidepressant effect of natural compounds in the management of Covid-19 symptoms. J Am Coll Nutr. 1–15.

Alesci A, Miller A, Tardugno R, Pergolizzi S. 2021. Chemical analysis, biological and therapeutic activities of Olea europaea L. extracts. Nat Prod Res. 1–14.

Alesci A, Nicosia N, Fumia A, Giorgianni F, Santini A, Cicero N. 2022. Resveratrol and immune cells: a link to improve human health. Molecules. 27(2):424.

Alessio A, Pergolizzi S, Gervasi T, Aragona M, Cascio PL, Cicero N, Lauriano ER. 2021. Biological effect of astaxanthin on alcohol-induced gut damage in Carassius auratus used as experimental model. Nat Prod Res. 35(24):5737–5743.

Balakrishnan B, Chellappan S, Basheer S, Elyas KK, Bahkali AH, Chandrasekaran M. 2011. Protease inhibitor from Moringa oleifera leaves: isolation, purification, and characterization. Process Biochem. 46(12):2291–2300.

Fernandes J, Mehdad A, Valadares NF, Mourão C, Freitas S. 2019. Crystallographic structure of a complex between trypsin and a nonapeptide derived from a Bowman-Birk inhibitor found in Vigna unguiculata seeds. Arch Biochem Biophys. 665:79–86.

Fumia A, Cicero N, Gitto M, Nicosia N, Alesci A. 2021. Role of nutraceuticals on neurodegenerative diseases: neuroprotective and immunomodulant activity. Nat Prod Res. 1–18.

Gao X, Jiao Q, Zhou B, Liu Q, Zhang D. 2020. Diverse bioactive components from Ginkgo biloba fruit. Therm Sci. 24:48–48.
Cotabarren J, Broitman DJ, Quiroga E, Obregón WD. 2020. GdTI, the first thermostable trypsin inhibitor from Geoffroea decorticans seeds. A novel natural drug with potential application in biomedicine. Int J Biol Macromol. 148:869–879.

Iriti M, Kubina R, Cochis A, Sorrentino R, Varoni EM, Kabaa-Dzik A, Azzimonti B, Dziedzic A, Rimondini L, Wojtyczka RD. 2017. Rutin, a quercetin glycoside, restores chemosensitivity in human breast cancer cells. Phytother Res. 31(10):1529–1538.

Klomklao S, Benjakul S, Visessanguan W, Kishimura H, Saeki H. 2006. Trypsins from yellowfin tuna (Thunnus albacares) spleen: purification and characterization. Comp Biochem Physiol Part B Biochem Mol Biol. 144(1):47–56.

Megeressa M, Siraj B, Zarina S, Ahmed A. 2020. Structural characterization and in vitro lipid binding studies of non-specific lipid transfer protein 1 (nsLTP1) from fennel (Foeniculum vulgare) seeds. Sci Rep. 10(1):21243.

Omidkhoda SF, Razavi BBM, Hosseinzadeh H. 2019. Protective effects of Ginkgo biloba L. against natural toxins, chemical toxicities, and radiation: a comprehensive review. Phytother Res. 33(11):2821–2840.

Pontual E, Pires-Neto D, Fraige K, Higino T, Carvalho B, Alves N, Lima T, Zingali R, Coelho L, Bolzani V, et al. 2018. A trypsin inhibitor from Moringa oleifera flower extract is cytotoxic to Trypanosoma cruzi with high selectivity over mammalian cells. Nat Prod Res. 32(24):2940–2944.

Samiksha Singh D, Kesavan AK, Sohal SK. 2019. Purification of a trypsin inhibitor from Psoralea corylifolia seeds and its influence on developmental physiology of Bactrocera cucurbitae. Int J Biol Macromol. 139:1141–1150.

Villalba-Villalba AG, Ramirez-Suarez JC, Valenzuela-Soto EM, Sanchez GG, Ruiz GC, Pacheco-Aguilar R. 2013. Trypsin from viscera of vermiculated sailfin catfish, Pterygoplichthys disjunctivus, Weber, 1991: its purification and characterization. Food Chem. 141(2):940–945.

Waks AG, Winer EP. 2020. Breast cancer treatment: a review. JAMA. 321(3):288–300.

Weyburne ES, Wilkins OM, Sha Z, Williams DA, Pletnev AA, Bruin GD, Overkleeft HS, Goldberg AL, Cole M, Kisselev AF. 2017. Inhibition of the proteasome β2 site sensitizes triple-negative breast cancer cells to β5 inhibitors and suppresses Nrf1 activation. Cell Chem Biol. 24(2):218–230.

Zhao XD, Dong N, Man HT, Fu ZL, Zhang MH, Kou S, Ma SL. 2013. Antiproliferative effect of the Ginkgo biloba extract is associated with the enhancement of cytochrome P450 1B1 expression in estrogen receptor-negative breast cancer cells. Biomed Rep. 1(5):797–801.

Zhou D, Jiang C, Fu C, Chang P, Yang B, Wu J, Zhao X, Ma S. 2020. Antiproliferative effect of 2-hydroxy-6-tridecylbenzoic acid from ginkgo biloba sarcotestas through the aryl hydrocarbon receptor pathway in triple-negative breast cancer cells. Nat Prod Res. 34(6):893–897.