Partial purification of trypsin/papain inhibitors from *Hymenaea courbaril* L. seeds and antibacterial effect of protein fractions

Mônica Silva de Brito[1], Mônica Brandão Melo[2], Jamille Perdigão de Andrade Alves[1], Raquel Oliveira dos Santos Fontenelle[2], Marlene Feliciano Mata[3] and Lúcia Betânia da Silva Andrade[1,4]

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ABSTRACT - (Partial purification of trypsin/papain inhibitors from *Hymenaea courbaril* L. seeds and antibacterial effect of protein fractions). The crude extract and protein fractions of *Hymenaea courbaril* L. seeds were investigated for the presence of trypsin and papain inhibitors and antimicrobial activity against *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Escherichia coli*. Protein fractions were obtained from the crude extract after precipitation with ammonium sulfate into three saturation ranges (0-30%, 30-60%, and 60-90%), called Hc030, Hc3060, and Hc6090, respectively. The crude extract and protein fractions inhibited trypsin and papain activity, but to different degrees. Antimicrobial activity was observed in Hc030 and Hc3060 fractions, but only against *V. parahaemolyticus*.The inhibitor isolated from the Hc3060 fraction was more effective in inhibiting trypsin (100% inhibition) than papain (54% inhibition), and showed an apparent molecular mass of 20 kDa. This study shows that *H. courbaril* seeds contain proteins with protease-inhibiting and antibacterial activity, indicating that this species is a source of bioactive compounds.
Keywords: antimicrobial, cystatin, Fabaceae, protease

RESUMO - (Purificação parcial de um inibidor de tripsina/papaína de sementes de *Hymenaea courbaril* L. e atividade antibacteriana de suas frações proteicas). O extrato bruto e frações proteicas de sementes de *Hymenaea courbaril* foram investigados para a presença de inibidores de tripsina e papaína e atividade antimicrobiana contra *Vibrio parahaemolyticus*, *Staphylococcus aureus* e *Escherichia coli*. As frações foram obtidas após precipitação do extrato bruto com sulfato de amônio em três faixas de saturação (0-30%, 30-60% e 60-90%) e denominadas Hc030, Hc3060 e Hc6090, respectivamente. O extrato bruto e as frações apresentaram diferente especificidade em inibir a atividade da tripsina e papaína. A atividade antimicrobiana foi observada nas frações Hc030 e Hc3060 e somente contra *V. parahaemolyticus*. O inibidor isolado a partir de Hc3060 foi mais eficiente na inibição da tripsina (100% de inibição) do que da papaína (54% de inibição), e mostrou uma massa molecular aparente de 20 kDa. Este estudo mostra que as sementes de *H. courbaril* possuem inibidores de proteases e proteínas com atividade antibacteriana, indicando que a espécie é uma fonte de compostos bioativos.
Palavras-chave: antimicrobiano, cistatina, Fabaceae, protease

Introduction

Legume seeds contain abundant proteins, which are synthesized and accumulate during seed development. Some of these proteins serve as reserve of amino acids while others play a role in the defense of the seed against attack by bacteria, fungi, viruses and insects (Shewry *et al*. 1995, Sels *et al*. 2008, Cruz *et al*. 2013).

Seed defense proteins include enzymes, lectins, and protease inhibitors (PIs). These proteins have been studied for their antimicrobial activity against bacteria or fungi that are unrelated to plant diseases, such as animal pathogens (O'Keefe 2001, Santos *et al*. 2010, Wong *et al*. 2010).

Protease inhibitors are a group of molecules that have the ability to form complexes with proteolytic

1. Universidade Estadual Vale do Acaraú, Centro de Ciências Agrárias e Biológicas, Laboratório de Microbiologia, Av. da Universidade, 850, Bairro Betânia, 62040-370 Sobral, CE, Brasil
2. Universidade Estadual Vale do Acaraú, Centro de Ciências Agrárias e Biológicas, Herbário Prof. Francisco José de Abreu Matos, Av. da Universidade, 850, Bairro Betânia, 62040-370 Sobral, CE, Brasil
3. Universidade Estadual Vale do Acaraú, Centro de Ciências Agrárias e Biológicas, Laboratório de Biologia Experimental, Av. da Universidade, 850, Bairro Betânia, 62040-370 Sobral, CE, Brasil
4. Corresponding author: lubetania@yahoo.com.br

enzymes, showing specificity for protease of the four catalytic classes: serine protease, cysteine protease, aspartic protease or metallo-protease, thus reducing or inactivating their catalytic effect (Van der Hoorn 2008, Volpicella *et al*. 2011).

PIs can act by inhibiting enzymes of exogenous origin, such as trypsin (serine protease), papain (cysteine protease) or related enzymes, defending the plant against pathogenic organisms. These proteins are abundant in plants and are classified as serine, cysteine, aspartic or metallo-protease inhibitors, according to the class of proteases that they inhibit. Of these, the serine protease inhibitor (serpins) and cysteine protease inhibitors (cystatins) are the most widely studied (Oliveira *et al*. 2003, Oliveira *et al*. 2007, Volpicella *et al*. 2011, Fluhr *et al*. 2012, Chevreuil *et al*. 2014). Some protease inhibitors are called bifunctional because they inhibit more than one mechanistic class of proteases (Migliolo *et al.* 2010).

Several studies have focused on the antimicrobial activity of purified proteins from seeds, including protease inhibitors (Kim *et al*. 2009, Cândido *et al*. 2011, Costa *et al.* 2014). The screening of protein extracts is the initial step of these studies (Talas-Ogras 2004, Cavalheiro *et al*. 2009).

Hymenaea courbaril L. (Fabaceae, Caesalpinioideae), known as jatobá, is a leguminous tree native to Brazil that occurs mainly in the Cerrado biome. Its fruits are edible and are used in making bread, cakes and cookies (Lorenzi 2002). Although the seeds contain about 10% protein, research into their chemical constituents has emphasized that carbohydrates, mainly xyloglucans, are abundant in this plant. Furthermore, proteases, peroxidases and trypsin inhibition activities have been identified in protein-rich extracts from its seeds (Caramori *et al*. 2004, Matuda & Netto 2005). However, detailed information on the isolation and characterization of these molecules is still sparse, and to our knowledge, to date only one β-galactosidase has been purified from seeds of this species, which appears to be involved in the mobilization of xyloglucans during seed germination (Alcântara *et al*. 2006).

The crude extracts from the genus *Hymenaea* from different plant parts, such as resin and leaves, have been investigated for antiviral and antimicrobial activity (Fernandes *et al*. 2005, Correia *et al*. 2008). These extracts have also been used in folk medicine as an expectorant, antidiarrheal or for the treatment of various human diseases such as anemia, kidney problems and inflammations such as bronchitis and cystitis (Cartaxo *et al*. 2010, Orsi *et al*. 2012). Often these activities are attributed to secondary metabolites such as tannins, flavonoids, terpenes and saponins (Jayaprakasam *et al*. 2007, Cecílio *et al*. 2012). There are no reports of antimicrobial activity in protein fractions of *H. courbaril*.

The aim of this study was to detect and isolate trypsin and papain inhibitors from protein fractions of *H. courbaril* seeds and to investigate their antibacterial activity.

Materials and methods

Preparation of protein-rich fractions - *Hymenaea courbaril* L. seeds (Fabaceae, Caesalpinioideae) were collected from trees in the town of Meruoca, Ceará State. Protein extract was obtained from the seed flour (50 g) stirred for 1 h in 50 mM Tris-HCl pH 7.5 buffer (500 mL) and then centrifuged for 30 minutes at 10.000 g at 4 °C. Ethanol was added to the supernatant to a final concentration of 50% (v:v). After10 minutes at 4 °C to allow precipitation of the reserve polysaccharides, the insoluble material was pelleted (10.000 g, 4 °C, 15 minutes) and the supernatant was dialyzed (cutoff 12 kDa) against the same extraction buffer. To eliminate endogenous proteases, the dialysate was heated for 3 minutes at 100 ºC and after centrifugation (10 minutes, 10.000 g, 4 °C), the supernatant was called crude extract of *H. courbaril* (EHc). This EHc was subjected to ammonium sulfate fractionation into three precipitation ranges (0%-30%, 30%-60% and 60%-90%) and each step was followed by centrifugation at 10.000 g for 30 minutes at 4 °C. The precipitates were termed Hc030, Hc3060 and Hc6090 fractions, respectively, dialyzed against distilled water, lyophilized and stored at - 4 °C until use. The EHc and fractions were used to detect protease inhibitor activity against bovine trypsin (Sigma®) and papain from papaya latex (Sigma®), as well as to detect antimicrobial activity.

Papain inhibition assay - The papain inhibition assay was performed as described by Abe *et al*. (1992) using N_{α}-Benzoyl-DL-arginine β-naphthylamide hydrochloride (BANA) as a substrate. Forty microliters of papain solution (50 μg mL^{-1} in 250 mM sodium phosphate buffer pH 6.0) was incubated for 10 minutes at 37 ºC with 20 μL of an activation solution containing 2 mM EDTA and 3 mM DTT at pH 6.0 and 100 μL of either EHc or each fraction (5 mg protein mL^{-1} in 250 mM sodium phosphate buffer pH 6.0) and 250 μL of 100 mM sodium phosphate buffer pH 6.0. The reactions were started with

100 µL of 1 mM BANA solution prepared in 1% DMSO in 25 mM sodium phosphate buffer pH 6.0. After 20 min at 37 ºC, the reaction was stopped by adding 250 µL of 2% HCl in ethanol. The colored product was developed by the addition of 250 µL of 0.06% *p*-dimethylaminocinnamaldehyde (DMACA) in ethanol (m:v) and measured by absorbance at 540 nm. Blanks were prepared in the same conditions without the addition of substrate, which was added after the stop solution. One unit of inhibitory activity (IU) was defined as the amount of inhibitor that decreased absorbance by 0.01 at 540 nm. All assays were performed in triplicate and expressed as the mean value ± SD.

Trypsin inhibition assay - The trypsin inhibition assay was performed using Nα-Benzoyl-DL-arginine-*p*-nitroanilide (BApNA) as a substrate (Erlanger *et al*. 1966). Forty microliters of trypsin (100 µg mL^{-1} in 1mM HCl) was incubated for 15 minutes at 37 ºC with 560 µL of 50 mM Tris-HCl pH 7.5 and 100 µL of either EHc or each fraction (5 mg protein mL^{-1} in 50 mM Tris-HCl pH 7.5). Reactions were started by the addition of 500 µL of 1.25 mM BApNA solution, prepared in 0.5% DMSO in 50 mM Tris-HCl pH 7.5. After 15 minutes at 37 °C, the reaction was stopped by the addition of 200 µL of 30% acetic acid solution. The enzymatic hydrolysis of the substrate was evaluated by recording the absorbance at 410 nm. The results were expressed as described above for the papain inhibitor.

Protein determination - Protein content was measured according to the procedure of Bradford (1976) with bovine serum albumin as protein standard.

Polyacrylamide gel electrophoresis (SDS-PAGE) - The electrophoresis (12.5%) was performed according to the method described by Laemmli (1970). The gel was stained with 0.1% Coomassie Brilliant Blue R-250.

Partial isolation of the inhibitor - The partial isolation trypsin/papain inhibitor was performed by anion exchange chromatography in a DE52-cellulose column (15.0 × 3 cm) equilibrated with 50 mM Tris-HCl pH 7.5. Thirty mg of Hc3060 was dissolved in 10 mL of the equilibration buffer and applied on the column. Elution was performed at a flow rate of 1.0 mL min^{-1}. Retained peaks were eluted with NaCl at concentrations of 0.1 M, 0.2 M and 0.4 M in stepwise manner. The peaks were assayed for inhibitory activity against trypsin and papain. The peak with inhibitory activity was applied on a trypsin-sepharose affinity column (10 × 1.5 cm) equilibrated with 50 mM Tris-HCl pH 7.5. The retained proteins were eluted with 1 mM HCl solution at a flow rate of 0.75 mL min^{-1}, collected, dialyzed against water and freeze dried. This sample, with anti-tryptic and anti-papain activity, was used for further analysis.

Antimicrobial activity - The antimicrobial activity of the fractions (10 mg mL^{-1} in Tris-HCl 50 mM, pH 7.5) was evaluated by agar diffusion (Fontenelle *et al*. 2007) against *Vibrio parahaemolyticus*, *Staphylococcus aureus* and *Escherichia coli*. Petri dishes 15 cm in diameter were prepared with Mueller-Hinton agar (Difco, Detroit, USA). Wells (6 mm in diameter) were then cut from the agar and 50 µL of each fraction was placed into them. Stock solutions of tetracycline (30 µg mL^{-1}, Sigma Chemical Co., St. Louis, USA) were prepared in aqueous solution and tested as positive controls. The bacterial suspension was inoculated onto the surface of the agar. After incubation for 24 h at 37 °C, all Petri dishes were examined for growth inhibition zones and the diameters of these zones were measured in millimeters (mm). Each experiment was repeated at least twice.

Results

Different abilities to inhibit trypsin and papain were observed for the EHc, Hc030, Hc3060 and Hc6090 fractions. For papain inhibition, the Hc3060 showed the highest inhibitory activity (greater than 50%), whereas the other fractions exhibited low inhibitory activity. The EHc and all fractions showed inhibitory activity against trypsin and 100% inhibitory activity was detected in the Hc3060 fraction, with high specific activity (figure 1).

In addition, to identify antimicrobial activity in the protein fractions, these were evaluated for their ability to inhibit the growth of *S. aureus*, *V. parahaemolyticus* and *E. coli.* The Hc030 and Hc3060 fractions showed antibacterial activity only against *V. parahaemolyticus*, producing inhibition zones of 17 mm and 14 mm, respectively. The control has inhibition zone of 30 mm.

SDS-PAGE of the EHc and fractions showed a profile with a higher concentration of proteins with molecular weights between 20.1 kDa and 30 kDa. In the fractions, these proteins were concentrated in Hc6090 (figure 2).

To isolate the trypsin/papain inhibitor, the Hc3060 fraction, that showed the greatest inhibitory activity, was submitted to anion-exchange chromatography

on DE52-cellulose. The trypsin/papain inhibitory activity was detected at the unretained peak (P_1). The elution with 0.1 M, 0.2 M and 0.4 M NaCl solution yielded three peaks without inhibitory activity (data not shown). The P_1 fraction was then applied on a trypsin-Sepharose affinity column and the retained peak showed 100% inhibition of trypsin (specific activity = 1250 IU/mg protein) and 54% inhibition of papain (figure 3). This peak, called ITHc, was analyzed by SDS-PAGE in the absence of the reducing agent.

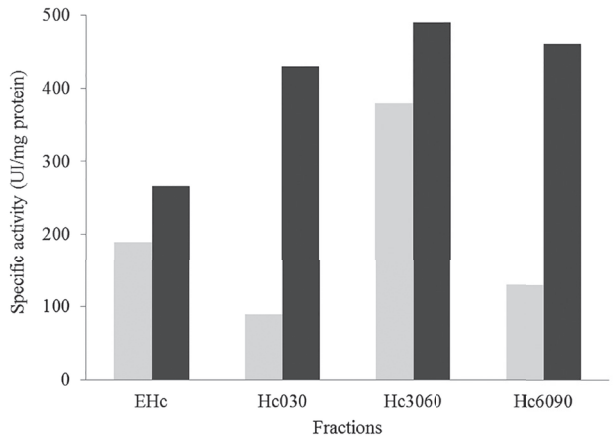

Figure 1. Papain and trypsin inhibitory activity (specific activity = IU*/mg protein) in crude extracts (EHc) and protein fractions of *H. courbaril* seeds. Hc030, Hc3060, and Hc6090 represent the fractions obtained by precipitation with ammonium sulfate at respective saturation ranges of: 0-30%, 30-60% and 60-90%. *One unit of inhibitory activity (IU) was defined as the amount of inhibitor that decreased absorbance by 0.01, under assay conditions. Papain, Trypsin.

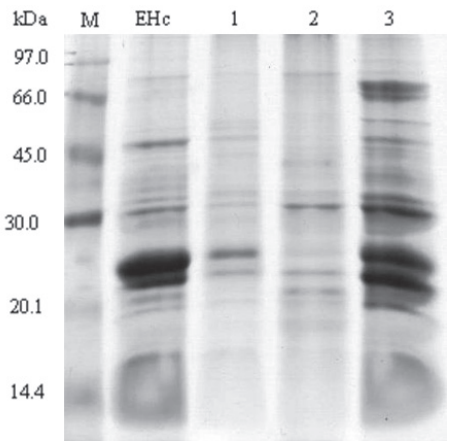

Figure 2. SDS-PAGE of the crude extract (EHc) and Hc030 (1), Hc3060 (2), and Hc6090 (3) fractions of *H. courbaril* seeds. The gel concentration was 3.5% and the running gel concentration was 12.5%. In each well, 40 µg protein was applied. The gel was stained with Coomassie Brilliant Blue R 250. M: Molecular weight markers (LMW-SDS, GE Healthcare).

After Coomassie Blue staining, a main electrophoretic band with an apparent molecular mass of 20 kDa was observed (figure 3, inset).

Discussion

Hymenaea courbaril is a native tree from Brazil and its fruits and seeds are used in handicrafts and as an ingredient in cakes and cookies prepared by local people. Although the plant is used in food, there are few studies on bioactive compounds from its seeds, especially with regard to the identification of functional properties of their proteins. Jatobá seeds contain approximately 10% protein, so they are a source of natural bioactive molecules (Caramori *et al*. 2004). In this study, we identified and partially isolated papain/trypsin inhibitors from *H. courbaril* seeds and investigated the antibacterial potential of protein fractions.

The presence of storage polysaccharides, mainly xyloglucans and galactomannans, has been reported in jatobá seeds (Clippel *et al*. 2008, Busato *et al.* 2009). Due to the presence these compounds, the crude extract became viscous, so to reduce these polysaccharides, ethanol was added to precipitate them, which also reduced the viscosity of the extract. The alcoholic precipitation is the most usual methods for extraction of xyloglucan from seeds described in the literature (Arruda *et al.* 2015).

In general, Fabaceae seeds have high levels of protease inhibitors and these molecules have been purified from several legume species (Gomes *et al.* 2005*,* Migliolo *et al.* 2010, Paula *et al.* 2012, Cruz *et al*. 2013, Chevreuil *et al.* 2014). For example, in *Pithecelobium dumosum*, two trypsin inhibitors were purified (Oliveira *et al*. 2007) and in *Poincianella pyramidalis* seeds, a Kunitz inhibitor with insecticide effect was isolated (Guimarães *et al.* 2015).

H. courbaril crude seed extracts have been investigated for the presence of trypsin inhibitors and have been found to possess strong inhibitory activity (Caramori *et al*. 2004) but, to our knowledge, this is the first report of a trypsin/papain inhibitor isolated from jatobá seeds. The molecular mass found in ITHc of approximately 20 kDa is similar to the trypsin inhibitors of the Kunitz family, which generally have a molecular mass between 18 and 24 kDa (Oliva *et al.* 2010, Guimarães *et al.* 2015).

The ability of ITHc to inhibit serine (trypsin) and cysteine (papain) proteases suggests this is a bifunctional inhibitor. Similar molecules have been purified from some legume seeds and its bioactivity

has been explored. Namely, PdKI, a trypsin inhibitor purified from *Pithecelobium dumosum* seeds and effective against papain and insect digestive proteinase (Oliveira *et al*. 2007); PTPKI, purified from *Prosopis juliflora* seeds, shown dual activities for papain and trypsin enzymes (Franco *et al*. 2002). Another inhibitor (ApTI) purified from *Adenanthera pavonina* seeds inhibited papain, trypsin and chymotrypsin and had a deleterious effect on *Callosobruchus maculatus* and *Aedes aegypti* larvae (Macedo *et al*. 2004, Sasaki *et al.* 2015).

Because of the versatility in inhibiting different enzymes, bifunctional inhibitors have been investigated to understand their mechanistic inhibitory strategies, in order to elucidate their interaction with their target enzymes (Migliolo *et al*. 2010) and their potential applications as therapeutic drugs to treat cancer and to control pests and crop diseases in transgenic plants (Pandey *et al*. 2007, Mosolov & Valueva 2008, Paula *et al*. 2012, Cruz-Silva *et al.* 2013).

Many studies have been performed with compounds extracted from plants to detect bioactive fractions or substances with antimicrobial activity. In *H. courbaril*, these studies are mainly related to secondary compounds present in ethanol or aqueous extracts of the leaves, fruits and stem bark (Bezerra *et al*. 2013, Costa *et al.* 2014).

In order to investigate novel molecules with antimicrobial activity, some proteins fractions have been evaluated for the presence of antibacterial and antifungal compounds (Santos *et al.* 2008, Kandappa *et al*. 2015). In this context, Hc030 and Hc3060 protein fractions inhibited the growth of *V. parahaemolyticus*, an important gram-negative pathogen that causes human gastroenteritis associated with seafood consumption without appropriate cooking (Pereira *et al*. 2007, Su & Liu 2007). So, plant-derived proteins would be a good source of antibacterial compounds.

The electrophoretic profile of Hc030 and Hc3060 showed protein diversity in these fractions, thus not allowing the identification of which molecule is responsible for the antibacterial activity. Given the low yield of the isolated inhibitor (ITHc), it was impossible to evaluate its antibacterial effect. It is worth noting that although there are reports of the purification of many proteins with antibacterial activity, the antimicrobial effect might not be due to the isolated compound, but to the combined action of several molecules acting synergistically.

In conclusion, the results revealed the presence of trypsin/papain inhibitor and antimicrobial proteins in

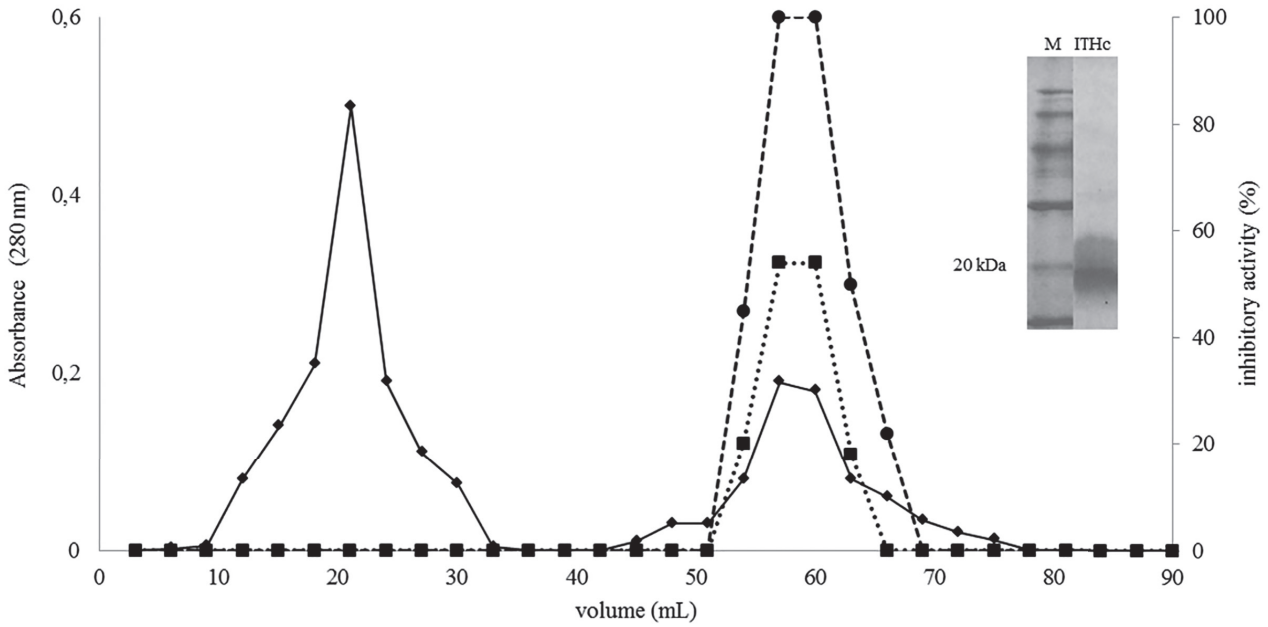

Figure 3. Elution profile in a trypsin–Sepharose affinity column of P_1 from the Hc3060 fraction of *H. courbaril* seeds. The column was equilibrated with 50 mM Tris-HCl pH 7.5. The retained peak (ITHc) was eluted with 1 mM HCl. The protein concentration was monitored at 280 nm (—) and the fractions were assayed against trypsin (--●--) and papain (···■···). Figure 3 inset: 12.5% SDS-PAGE. (ITHc) trypsin/papain inhibitor, (M) Molecular weight markers. The protein band was stained with Coomassie Brilliant Blue R-250.

H. courbaril seeds. Therefore, further investigations are needed for the isolation, accumulation and identification of all bioactive molecules present in these protein fractions.

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