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ORIGINAL PAPER

Identification of Novel Proteins from Black Cumin Seed Meals Based on 2D Gel Electrophoresis and MALDI-TOF/TOF-MS Analysis

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Abstract

The amount of cold press oil manufacture is globally rising, which in turn leads to the accumulation of deoiled plant seeds at significant quantities and consequent manufacture of plant protein products. In this study, we made an attempt to analyze the protein profile of black cumin seed protein concentrates prepared by the alkali extraction-acid precipitation technique (AE-IP). The analytical strategy relied on gel-based proteome mapping which included two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF). 14 different protein bands were identified, and in gel-trypsinolysis was carried out for the corresponding gel spots. Using the MASCOT database, current findings on 10 proteins were compared with the existing data. The highest similarity was 46 which was obtained between the highest pI black cumin protein observed here and the cyclin dependent kinase inhibitor of *Arabidopsis thaliana*. The molecular mass of the intact protein was determined by linear MALDI-TOF/TOF-MS as 23,711.2186 Da. The peptide constructs of this protein have been further studied in order to identify potential biological activity. Matching sequences generated bioactive peptides *in silico* such as IR, AL, and SL dipeptides during sequential enzymatic digestion with pepsin and trypsin. Since the majority of bioactivity investigations on black cumin seeds have been related to black cumin oil and its oil soluble components, the structure and bioactivities of black cumin proteins deserve further research.

Keywords Black cumin · Protein concentrates · Proteomic analysis · 2D-gel electrophoresis · In gel trypsinolysis · MALDI-TOF/TOF

Introduction

Plant seeds serve as a significant source of nutrients including proteins that could potentially enhance the well-being of humans. Especially in the field of herbal medicine, seeds are widely utilized due to their various bioactivities. In addition to their bioactive compound contents, seeds also serve as potential resources for the precursors of many pharmacological components or drugs [1].

The plant seed known as black seed or black cumin (*Nigella sativa*) belongs to the botanical family Ranunculaceae. It is native to the East Mediterranean countries, South Europe and Asia Minor [2, 3]. Currently, black cumin is also cultivated in the Middle East, North Africa and Asia [4]. Black cumin has been utilized as a traditional and natural medicine for at least 2,000 years. Its extracts were found to significantly inhibit carcinogenesis *in vivo* [5]. The essential oils obtained from *N. sativa* seeds were previously shown to have anthelmintic activity against tape worms and earthworms [6]. Previous work on *N. sativa* has also shown that seed extracts demonstrated antimicrobial activity against the growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* [7]. Other potential bioactivities of *Nigella sativa* Linn. include prevention against a number of diseases and conditions such as asthma, diarrhea and dyslipidemia; protection against chemical or disease induced nephrotoxicity and hepatotoxicity; antioxidative, anti-inflammatory, analgesic, antipyretic, antimicrobial and antineoplastic activities; and reduction of blood pressure, etc. [8].

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In most cases, however, bioactivities of black cumin seeds listed above were attributed to the components of seed oil such as thymoquinone and volatile oils [9, 10]. However, bioactivities of the protein components of the seeds have been much less explored.

Based on our previous findings, deoiled black cumin meals can be conveniently utilized in protein concentrate production and essential amino acid content of these concentrates accounted for approximately 33% (*w/w*) [11, 12]. These findings were also comparable to that of Babayan et al. [13]. A number of studies were carried out in order to analyze the protein fractions in black cumin seeds. For example, the proteins in the hydro-methanolic fraction of *N. sativa* seeds represented approximately 35–40% of the total dry weight and were found to separate into bands ranging from 10 to 94 kDa on SDS PAGE [14]. The majority of the identified proteins in black cumin seeds were involved in biochemical pathways involving carbohydrate metabolism, amino acid and shikimate pathway, lipid metabolism, nucleotide, cell organization and biogenesis, transport, and defense processes [15]. While detailed studies of the defined proteins were not performed, these investigators have identified a total of 277 proteins using metabolic and proteomic approaches [10]. Further knowledge on the proteins of *N. sativa* could enable conscious consumption of this plant resource as herbal medication or in a regular diet due to its functional or therapeutic effects. Since consumption of the seed is relatively difficult, manufacture of protein ingredients could enhance the edibility characteristics.

Based on an alkali extraction-acid precipitation method, we have manufactured black cumin protein concentrates in order to valorize the deoiled meals from a local manufacturer and a moderate extent of ACE-inhibitory activity was observed in the hydrolyzates of these concentrates [11, 12]. Here, an attempt is made to analyze the individual proteins in the protein concentrates based on analytical methods such as 2D-gel electrophoresis, in gel digestion and MALDI-TOF/TOF-MS. The results were compared to the previous literature and as an example; the protein with the highest pI value was further evaluated via the clarification of matching peptides. Although some studies are present on the proteins of black cumin seeds, the elucidation of their structure and potential bioactivities is still a topic of research interest. The comparison to previous data indicated the novelty of the proteins investigated in this study.

Materials and Methods

Materials

Cold press deoiled meals of black cumin were generously donated by Oneva (Neva Foods Ltd., İstanbul, Turkey), a local manufacturer of cold press oils. In all cases, the maximum temperature observed by the cold press meals was lower than 40 °C.

Preparation of Black Cumin Seed Protein Concentrates

Alkali extraction–isoelectric precipitation (AE-IP) technique was based on the solubilization of protein molecules at basic pH, which was followed by the isoelectric precipitation at acidic pH values. Protein concentrates from deoiled meals of black cumin seeds were produced using the method of Boye et al. [16] with slight modifications. Briefly, 50 g of defatted meal was dispersed in water (1:15, *w/v*) and the pH of the medium was adjusted to pH 9.5 using 1 N NaOH (Sigma Product No. 795429). The dispersions were stirred using a magnetic stirrer operating at 500 rpm for 1 h at room temperature (22 ± 1 °C). Immediately afterwards, the dispersions were centrifuged at a rate of 4,200×*g* (Mixtasel-BL centrifuge, Abrera, Barcelona, Spain) for 30 min. The supernatant containing the solubilized proteins was collected and the medium pH was adjusted to pH 4.5 using 1 N HCl (Sigma Product No. 320331) in order to induce isoelectric precipitation. To ensure the complete separation of precipitating proteins, the supernatant was centrifuged once again at 4,200×*g* for 30 min. The pellet was collected and immediately frozen at −20 °C. Frozen samples were lyophilized using a Teknosem TRS 2/2 V freeze drier (Teknosem Corp., İstanbul, Turkey).

Application of isoelectric precipitation ensured the precipitation of all proteins with a pI value ≥ pH 4.5, which in turn lead to the analysis of a wide variety of proteins. Although as a further step, isoelectric precipitation was also carried out at pH 3, it was not possible to recover significantly higher amounts of proteins in the concentrates. Consequently, some of the non-acid precipitable proteins might be absent from the current samples. To preserve the structural attributes of the AE-IP recovered proteins, no further processing was carried out.

Freeze-dried protein samples were resuspended in 2D-rehydration buffer (Bio-Rad Product No. 162083), which consisted of 8 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris pH 8.5, and 1× protease inhibitor cocktail. 500 μg protein from each sample was cleaned up by precipitation using 2D-Clean up kit (Bio-Rad, USA, ReadyPrep™ 2-D Cleanup Kit). Protein concentrations of each sample was measured by Bradford assay (Bio-Rad, USA, QuickStart™ Bradford Protein Assay).

Two-Dimensional (2D) Gel Electrophoresis

All 2D chemical and supplies were purchased from Bio-Rad, USA. Two hundred and forty μg protein from each protein

pool was loaded onto immobilized 11 cm, pH gradient strips (IPG) (pH 3–10NL) by passive rehydration. Separations based on isoelectric points were achieved using a Protean isoelectric focusing (IEF) cell (Bio-Rad, USA). The following conditions were used for IEF: 20 min at 250 V with rapid ramp, 2 h at 4,000 V with slow ramp and 2.5 h for 4000 V with rapid ramp until a total of 32,000 V/h was reached at 20 °C. After isoelectric focusing, strips were then washed with buffer I (6 M Urea, 375 mM Tris-HCl pH 8.8, 2% SDS, 20% glycerol, 2% (*w/v*) DTT) for 30 min and with buffer II (6 M Urea, 375 mM Tris-HCl pH 8.8, 2% SDS, 20% glycerol, 2.5% iodoacetamide (*w/v*)) for 30 min at room temperature and subjected to SDS-PAGE for the second dimention separation using TGX precast gels in a Dodeca gel running system (Bio-Rad, USA). After the separation, the gels were fixed in 40% methanol, 10% acetic acid and then stained with Collaidal Coomassie Blue G250 (Bio-Rad, USA).

Image Analysis

Gel images were captured using VersaDoc MP 4000 Imaging System (Bio Rad, USA). PDQuest Advance 2D-analysis software (BioRad, USA) was used for the analysis of protein spots. Quantity of each spot was normalized by linear regression modelling. Selected gel spots were excised using ExQuest Spot-cutter (Bio-Rad, USA) for protein identification.

Protein Identification

Protein identification experiments were performed at Kocaeli University DEKART proteomics laboratory (http://kabiproteomics.kocaeli.edu.tr/) by using AB SCIEX MALDI-TOF/TOF 5800 system. In gel tryptic digestion of 14 different protein spots was performed using an in-gel digestion kit following the recommended protocol (Pierce, USA, Product no. 89871). The data obtained from MALDI-TOF/TOF were searched against MASCOT server v.2.5.1 (October 2016) (Matrix Science) by using a streamline software, ProteinPilot (ABSCIEX, USA). A database was created using the entries in NCBI-NR databank (https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins) and implemented into the MASCOT search engine [17] including the following search criteria: enzyme of trypsin; at least five independent peptides matched; at most one missed cleavage site; MS tolerance set to ±50 ppm and MS/MS tolerance set to ±0.4 Da; fixed modification being carbamidomethylation (Cys) and variable modification being oxidation (Met); peptide charge of 1+ and being monoisotopic. Only significant hits, as defined by the MASCOT probability analysis ($p < 0.05$), were accepted. The analytical scheme of the study was summarized (Fig. 1).

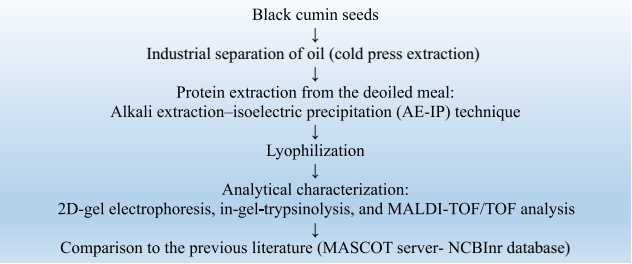

Fig. 1 The analytical scheme of the current study

Results and Discussion

In the previous studies from our group, protein concentrate manufacture was carried out from deoiled meals of black cumin based on alkali extraction-acid precipitation (AE-IP) method and protein content of the concentrates was quantified using Kjeldahl analysis as 54.7 ± 0.5% [11, 12].

In this study, further analysis of the protein concentrates was carried out. Once lyophilization was complete, analytical characterization and comparison to the previous literature data was carried out.

Firstly, protein dispersions were analyzed for their molecular weight and pI distribution using 2D-electrophoresis [12]. The pH range utilized in the analysis was 3–10 and the molecular weight range was 20–200 kDa. While there were some protein spots between 20 and 25 and 25–40 kDa, as observed also by Haq et al. [18], there were some spots around ≥65 kDa and finally around 200 kDa. The pI values of a variety of proteins were ≥ pH 7. Amino acids with high pI such as arginine (pI = 10.76), lysine (pI = 9.47) and histidine (pI = 7.59) were found to account for approximately 17.2% of all the amino acids in the black cumin protein concentrates [12].

In Fig. 2, a zoom of the 2D-gel is demonstrated between pH 5–9, since the majority of black cumin proteins were concentrated in this pH range. The marked spots on the gel (Fig. 2) were subjected to in gel-trypsinolysis and consequently analyzed using MALDI-TOF/TOF-MS as ions with the highest intensities were sequenced via MS/MS (Fig. 3). The results were compared to the previous literature using MASCOT database and similarities to the previously identified proteins were determined (Table 1, Supplementary Data). Among the 14 different proteins that were labelled on the 2D-gel, 10 matching proteins were identified and the extent of similarity between black cumin proteins and literature data listed in the MASCOT library ranged between approximately 10 and 50% (Table 1). Based on MASCOT search results, matching peptides for all proteins were determined and listed on Table 2 (Supplementary Data).

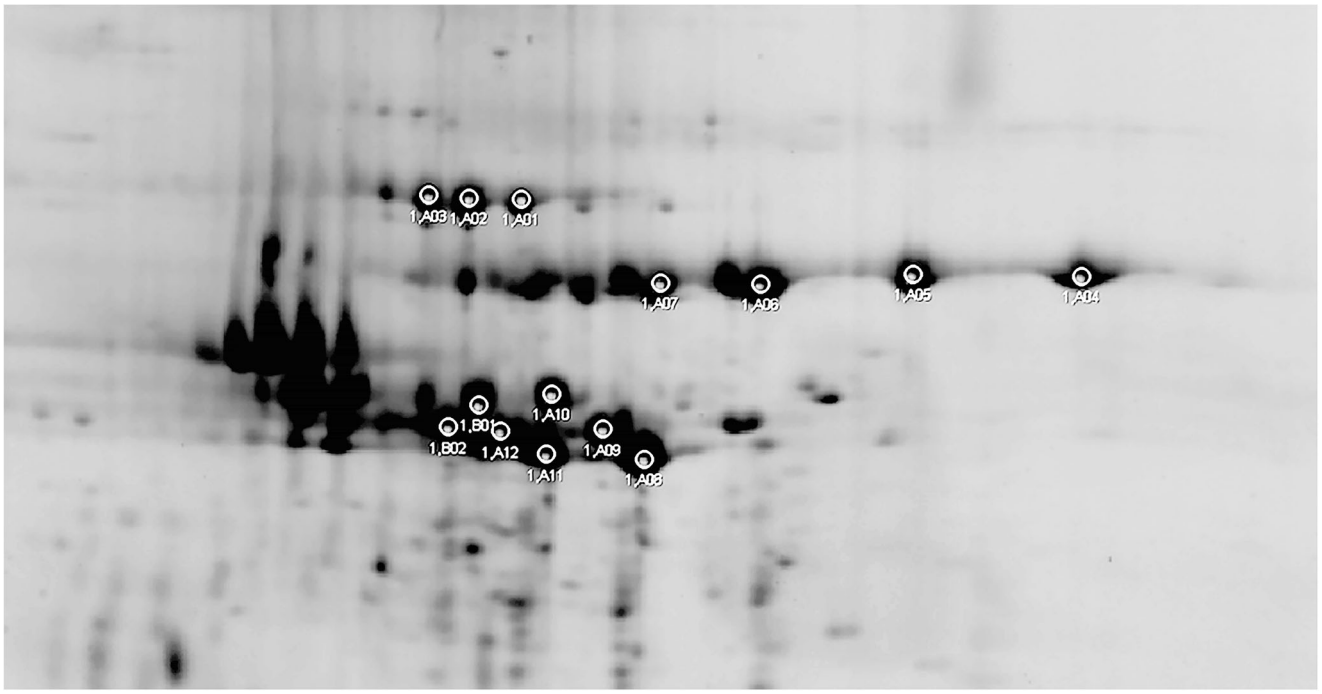

Fig. 2 2D-electropheresis of black cumin protein concentrates prepared from the industrial by-products of cold press deoiled meals. The pH range was 5–9 (from left to right). The circled spots were trypsinized in further experiments

The longest match between the sequences of A4 protein (Spot ID. A4 on Fig. 2) and and cyclin-dependent kinase inhibitor 4 from *Arabidopsis thaliana* (KRP4_ARATH) were marked on Fig. 3, while the peptides identified by sequencing accounted for 15% similarity between these two proteins (Fig. 3b). In terms of amino acid length, this match corresponded to 46 amino acids. Similarities to KRP4_ARATH was marked directly on the amino acid sequence of the A4 protein (Fig. 3b). The high extent of similarity in the primary sequences could also represent similarities in the structure and function of both proteins. Interestingly, A4 protein was also the highest pI protein in the black cumin protein concentrates, since its calculated pI was 9.61 based on MALDI-TOF/TOF-MS analysis. For all the other similar proteins, the similarity score was >30 amino acids.

The taxonomic information for the corresponding plant (*A. thaliana*) was indicated along with the variable modifications, cleavage and sequence coverage data. Corresponding molecular sizes and start-end points in the sequence were specified and the Mascot score histogram was given for other partly similar proteins (Fig. 3b). As shown by the histogram, *A. thaliana* match was by far stronger than all other matches. A MASCOT score of ≥25 was regarded as significant in this study.

The molecular mass of the intact A4 protein was determined by linear MALDI-TOF/TOF-MS (Fig. 3c) as 23,711.2186 Da in the absence of trypsin treatments. Based on amino acid sequence profiling ionization, the longest matching sequence was found to be the ESTPCSLIRRPEIMTTPGSSTK peptide. During sequential enzymatic digestion with pepsin and trypsin *in silico*, this peptide fragmented into the ESTPCSL - IR - R - PEIMTTPGSSTK peptides yielding the multi-functional (*i.e.*, ACE-inhibitory, DPP-IV inhibitory, hypotensive, antioxidative, etc.) IR peptide [19]. Similarly, the other matching peptides in this protein yielded DPP-IV inhibitory (AL, SL) peptides [16], after sequential pepsin and trypsin treatments.

Conclusions

In this study, an attempt was made to analyze the protein profile of black cumin seed protein concentrates prepared from cold press deoiled meals. Using in gel-trypsinolysis and MALDI-TOF/TOF, similarities between black cumin proteins and proteins listed in the literature were investigated. Detailed analysis of the protein with the highest isoelectric point (pI) was performed and its peptide constructs have been further studied. Currently, analysis of the potential bioactive peptide sequences in this valuable protein source is underway in our labs, including ACE-inhibitory characteristics [12]. Since the majority of bioactivity investigations on black cumin seeds were related to black cumin oil and oil soluble components, we think that the bioactivity of black cumin proteins have been underinvestigated and deserve further research.

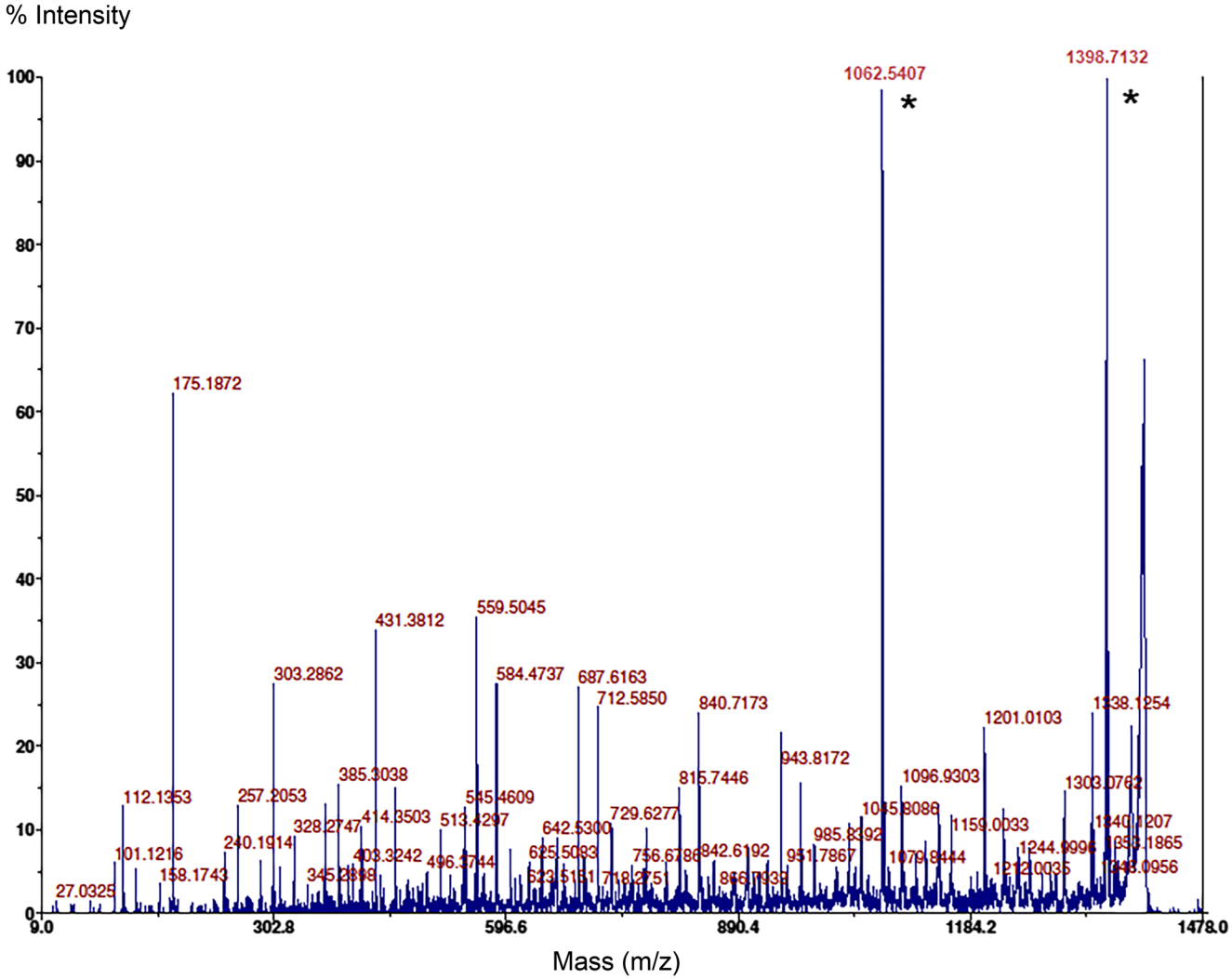

Fig. 3 Characterization of black cumin seed protein (Spot ID. A4) with the highest pI and match number by MALDI-TOF/TOF-MS. **a** MALDI-TOF/TOF-MS of tryptic peptides derived from the A4 protein. * represents the longest matching peptides between A4 and KRP4_ARATH. **b** Mascot data obtained by analysis of the tryptic peptides are demonstrated in detail. The matching peptides between the cyclin dependent kinase inhibitor 4 (KRP4_ARATH) from the mouse ear cress (*Arabidopsis thaliana*) and the highest pI protein from black cumin (A4 protein) were shown in Bold Red along with the taxonomic data for *A. thaliana*. Corresponding molecular sizes and start-end points in the sequence were also specified. Finally, Mascot score histogram was given. **c** MALDI-TOF/TOF-MS determination of the ionization characteristics of the intact A4 protein, where no prior trypsin treatment was administered

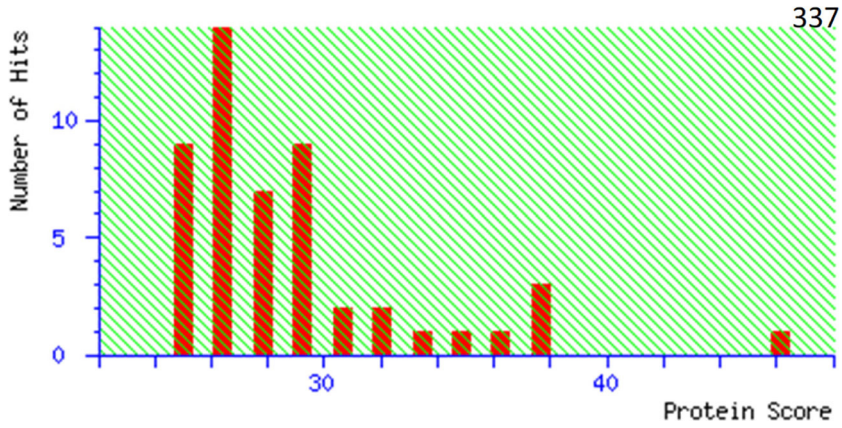

Fig. 3 continued.

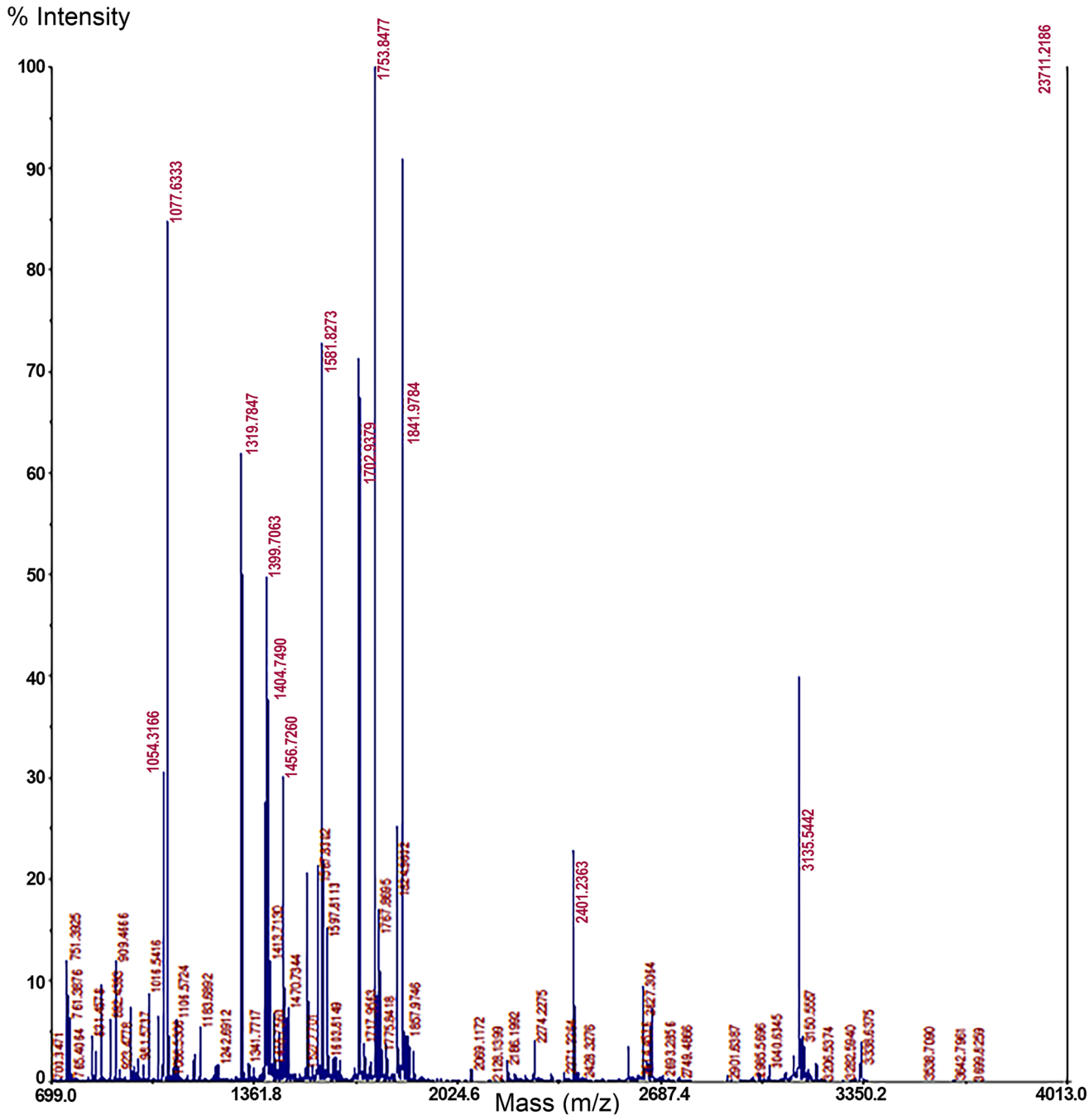

Fig. 3 continued.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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