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ORIGINAL PAPER

Development of a novel pretreatment protocol for the efficient isolation and enrichment of honey proteome using pine honey and the hypopharyngeal glands of *Apis mellifera* L.

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Abstract

A versatile sample pretreatment method for the honey matrix is still needed for any proteomic-based investigations. Invertase and diastase are the most important enzymes in the maturation of pine honey and the origin of these enzymes are attributed to the bee's hypopharyngeal glands (HPG). In our study, we aimed to isolate and enrich these enzymes as model proteins representing the honey proteome in an efficient and practical way. As authenticity comparison, isolating the same enzymes from HPG samples was also accomplished. For yielding pine honey crude protein isolate, as a tandem two-step approach, stirred cell ultrafiltration followed by centrifugal ultrafiltration (CUF) protocol was determined after experimental optimization. HPGs were dissected from the *Apis mellifera* L. and proteins were extracted by using a bead beater followed by concentration using CUF. Protein profiles of pine honey and HPG were compared by SDS-PAGE. The resulting protein concentrations, enzyme activities, and the cleaning efficiencies of the applied techniques were evaluated and optimized using the Bradford assay, modified enzyme activity assays, and sugar profiling method developed at HPLC-RID. The novel pretreatment method provided invertase at 1055.1 U/kg activity and diastase at 693.3 Shade U/g activity with yields of 900.9% and 2432.6%, respectively. The final crude protein isolate can be interpreted as reference material at any authenticity and quality assays of honey. The obtained crude protein extract will pave the way for high throughput proteomic investigations at the honey matrix. Furthermore, this template methodology could be scaled up in the industry for natural enzyme production.

Keywords Pine honey · Protein isolation · Enzyme enrichment · Invertase · Diastase

Introduction

Honey is a natural product produced by honey bees from the nectar of flowers or honeydew. It is a natural sweetener, as it is mostly composed of sugars (60–85%); mainly glucose and fructose but also maltose, sucrose, and other oligosaccharides. Honey's most important physicochemical and nutritional attributes such as viscosity, sweetness, hygroscopicity, specific rotation, and energy value depend on sugar compositions [1, 2]. Honey also contains phenolic compounds, minerals, proteins, free amino acids, enzymes, and vitamins as minor components [3, 4]. The amounts and proportions of these components were pertinent to environmental and climate change and the floral types visited by honey bees [5]. Honey contains small amounts of proteins, approximately 0.1–0.5% of honey dry matter [6, 7] with molecular weights ranging from 22 to 75 kDa [8]. Honey proteins originate from honey bees and/or plants (pollen and nectar) [9]. Lee et al. (1998) revealed that protein quantities are associated with the bee species. For instance, honey protein content from *Apis mellifera* L. is about 0.2–1.6%, whereas the honey by *Apis cerena* contains 0.1–3.3% protein [6]. Al-Sherif et al. (2012) also reported in the amounts of three main honey enzymes; α-glucosidase (invertase), α-amylase (diastase), and glucose oxidase between Egyptian and Carniolan honey bees at different life stages of the worker bees [10]. On the other hand, adulteration, overheat,

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or long-term storage cause a reduction or absence of honey protein content [11].

Enzymes and major royal jelly proteins (MRJPs) are the major components of the honey protein profiles [12, 13]. The main enzymes added to collected nectar are invertase and diastase that converting nectar into honey [14]. Glucose oxidase, acid phosphatase, catalase, and β-glucosidase are the other enzymes that have been found in honey in minor quantities [15]. Invertase and glucose oxidase has an animal origin which is mainly produced in the hypopharyngeal glands (HPGs) of the bees. Worker bees mix collected nectar with their secretions (contain enzymes) from the salivary and HPGs [10, 16]. Some enzymes, such as catalase and acid phosphatase have vegetal origins. These enzymes come from nectar, honeydew, or pollen. Enzymes like diastase could have double origins in the honey. Other probable origins could be honey's microorganisms and some enzymes could come from the plant absorptive insects which produce honeydew [17]. The bees add diastase to honey for converting starch into maltose and dextrins [18]. Honey diastase is a heat-sensitive enzyme [19] and it is used worldwide as a freshness indicator of honey. Diastase concentration in honey is being analyzed through the laboratories that are known as diastase activity (Diastase number, DN). There are legal regulations in Europe [20] and Codex Alimentarius [21] for minimum values of diastase activity of honey. According to the Directive 2001/110/EC, the diastase activity (Schade scale) must not be less than 8 and for some types of honey with low natural enzyme content (e.g. citrus honey) not be less than 3 (in this case the HMF must not more than 15 mg/kg). Invertase hydrolyzes sucrose into fructose and glucose, which are the main sugars in honey [22]. Kubota et al. (2004) purified the three forms of α-glucosidase from *A. mellifera*. They also purified α-glucosidase from honey and immunologically confirmed that honey α-glucosidase is an α-glucosidase III secreted into the honey from the HPG by honey bees [23]. In addition to diastase, invertase is also used as a freshness indicator because its activity decreases in old or heat-treated honey, too [24]. Invertase values in honey can be stated in different units, such as invertase number (Hadorn number, IN) or as invertase per kg (Siegenthaler units, US). Values higher than 10 IN (73.5 US) have been recommended for fresh and not heat treated [25, 26]. Thus, these enzymes are essential for the ripening process of honey and suitable markers for evaluating freshness and detecting adulterations. These origin enzymes can also be preferred in fermentation processes, sugar industry, and starch liquefaction [27].

On the other hand, pine honey is a valuable type of honeydew honey produced by the secretion of an insect called *Marchalina hellenica* as well as *Monophlebus hellecinus* in the classification of aphids [28]. Pine honey is darker than blossom honeys and it is produced in pine forests away from residential areas and environmental pollution [29]. Pine honey has started to be considered among the high-quality honey in recent years due to its superior taste, higher protein, phenolic and mineral contents [30]. At this point, owing to its higher enzyme and protein contents, pine honey was selected as research material in this study. Due to the fact that honey contains low amounts of protein and a sugar-rich environment, it is daunting to isolate proteins and there are still limited studies on its proteome. A versatile protein centric sample pretreatment method is indispensable for any proteomic-based investigations or for the production of natural enzymes on an industrial scale. The ideal protein extraction method should not only be the most efficient and isolate the highest possible number of proteins, but also should maintain the activities of the isolated enzymes/proteins prior to purification or, characterization studies. Another prerequisite, the resulting concentration, and composition of the final isolate should also be sufficiently compatible with the downstream applications such as electrophoresis, immunoaffinity-based applications, mass spectrometry analysis, or protein purification. Recently, there are several extractions, isolation, and enrichment methods present in the literature. Although there are few publications on the isolation and purification of invertase and diastase in honey and HPG, unfortunately, the vast majority of these studies include outdated, labor-intensive, time-consuming non-scalable pretreatment steps. Among these methods; dialysis [23, 31, 32], ultrafiltration [33], and molecular interaction induced extractions using magnetic beads [34], or column chromatography resins are accustomed techniques [35–37]. Moreover, there are also chemical precipitation techniques that are questionable in terms of recovery, such as salt-induced precipitation with ammonium sulfate or sodium chloride [23, 33], isoelectric precipitation with trichloroacetic acid [38], and organic solvent-induced precipitations [39].

In our study, we aimed at obtaining concentrated pine honey enzymes and proteins with higher yields in a cost effective and practical approach by implementing a novel sample pretreatment methodology. Since the most abundant protein subclass in honey is the enzyme ingredients, we selected the diastase and invertase as model proteins of which performance could be tracked easily by implemented activity assays for the method performance validation. As an origin and authenticity comparison, isolating the same enzymes from HPG samples was also applied within this research.

Materials and methods

Reagents and chemicals

Standards of sugar molecules (fructose, glucose, sucrose, maltose, turanose, trehalose, isomaltose, erlose, melezitose,

maltotriose) at analytical purity level (≥98%), hydrochloric acid (HCl), sodium chloride (NaCl), and acetonitrile (ACN, HPLC grade) were supplied from EMD Chemicals (Gibbstown, NJ, U.S.A.). Calcium chloride ($CaCl_2$), potassium chloride (KCl), and sodium maleate were purchased from Sigma-Aldrich® (St. Louis, MO, U.S.A.). The Tris base (≥99.8%) was purchased from Bio-Rad® Laboratories, Inc. (Hercules, CA, USA). 2-mercaptoethanol (≥98%) (14.2 M) was also supplied from Bio-Rad® Laboratories. Ultrapure water (18.0 MΩ·cm) was obtained using a Milli-Q Plus® system from Millipore® (Bedford, MA, USA).

Honeybee (*Apis mellifera* L.) samples

In this study, *A. mellifera L.* was used as the honey bee species. The collection of worker honey bees was carried out in September–October of 2019 from various hives from the Muğla location where pine honey production continues. They were collected in sterile sample containers (100 mL, Isolab, Interlab, Hadımköy, Istanbul, Turkey) and generally, the collected honey bees used for enzyme extraction were adult worker bees according to forewing size (9 mm). Honey bees were anesthetized with dry ice and kept at − 20 °C until dissection.

Pine honey samples

Pine honey samples were gathered by beekeepers in season of 2019 and researchers worked closely with beekeepers at apiaries during the harvesting season to guarantee the honey's authenticity and for convenient sampling. Pine honey samples were collected from the Aegean region of Turkey, and they were classified according to their honeydew element abundances by performing a melissopalynology assay. All samples were tested for being sure that they were not adulterated with enzyme or sugar syrup addition. For this purpose, authenticity assays like foreign invertase (β-fructofuranosidase), β/γ-amylase, heat-stable amylase, determination of rice syrup markers, C4% analysis (SCIRA method), and physicochemical quality assays like conductivity, humidity, acidity, hydroxymethylfurfural (HMF) content and, sugar content were also investigated. Total invertase activity and total diastase activity of the honey samples were investigated by modification of the official protocols. The analysis results of the selected sample enabled it to be interpreted as pure pine honey and feasible for use in method development studies. The sample was taken in a glass jar, hermetically sealed, and kept at 4 °C until analysis.

Dissection protocol

Dissection of each honeybee was conducted under a binocular microscope (Wild Heerbrugg M12, binocular phase-contrast microscope, Heerbrugg, Switzerland) applying × 100 magnification on a black plate. Each of frozen honeybees was first dissected with the help of forceps (Isolab, Interlab, Hadımkoy, Istanbul, Turkey) to separate the head and abdomen from the thorax. The separated head region was taken into 1–2 mL of water in a petri dish for easy extraction of HPG. After the frozen sample started to thaw (at about 5 °C), the HPG sections were isolated by first opening the occiput behind the head. HPG was reached by placing the mouth facing upwards and meticulously dissecting. They were transferred to microcentrifuge tubes in insect saline buffer (10 mM Tris/HCl, pH 7.4, 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$) and stored at − 80 °C. 80 HPGs were dissected and isolated, and they were aliquoted into four separate microcentrifuge tubes each containing 20 HPGs and including 250 µL of insect saline buffer. In the study to be carried out for the isolation of diastase and invertase enzymes from HPG, the required number of honeybees to be dissected was roughly considered as 80. According to this, 320 HPG samples were dissected and isolated from the collected 320 honeybees. 80 HPGs were stored for the final determined protocol, and the remaining 240 HPG samples were evaluated for method optimization study in terms of extraction and homogenization efficiency.

Total protein assay

The total protein concentration assay of all samples was performed using the Quick Start™ Bradford protein assay kit (Bio-Rad® Laboratories, Inc., Hercules, CA, USA). Sample solutions and all standards (20 µL) were pipetted into clean spectrophotometer cuvettes (Isolab, Interlab, Hadımkoy, Istanbul, Turkey). 1 mL of dye Reagent (1 ×) was added to each cuvette and mixed slightly by pipetting. The absorbance at 595 nm was read at Thermo Scientific™ Evolution™ 350 UV–Vis Spectrophotometer (Thermo Scientific™, Waltham, Massachusetts, USA) against a blank prepared using insect saline buffer and dye reagent (Coomassie stain) mixture after 5 min incubation period.

Diastase activity assay

For the measurement of diastase activity, a modified method based on Megazyme® assay kit (Diastase Activity in Honey Assay, Co. Wicklow, Ireland) was employed. This test works regarding the same scientific principle as Phadebas® assay. The Amylazyme® tablets supplied in the kit are very similar to the Phadebas® tablets but contain cross-linked amylose (linear fraction of the starch molecule) in a dyed structure. As with all standard methods, this kit gives results in Shade units according to the following formulation;

$$\alpha - \text{amylase activity(Diastase number} - \text{DN)}; \text{(Schade Unit/1 gram honey)} = 20.0 \times \Delta\text{Abs}^{*}$$

*ΔAbs; Absorbance differences between reagent blank and honey sample.

According to modifications we made, 1 g of honey sample was weighed into 50 mL centrifuge tubes and 20 mL of 100 mM sodium maleate buffer (pH 5.6) was added to homogenize the sample. The volume of the solution was then made up to 25 mL with same buffer. 1.0 mL of honey solution was transferred to a 15 mL centrifuge tube and the sample was incubated in a hot water bath (Sonorex RK 154 BH, Bandelin Electronic GmbH & Co.KG, Berlin, Germany) at 40 °C for 5 min. An Amylazyme® tablet was added into the tube and the sample was incubated at 40 °C for 10 min. After incubation, 10 mL of trizma base solution (2% (w/v), Sigma®, St. Louis, MO, U.S.A.) was added into the tube to terminate the reaction. Sample was spun at 4000×*g* for 5 min at room temperature. The resulting supernatant was transferred to the spectrophotometer cuvette and its absorbance was measured against its blank at 590 nm at UV–Vis Spectrophotometer. For the preparation of the blank sample, analysis was performed by transferring 1 mL of sodium maleate buffer to a centrifuge tube instead of 1 mL sample. For the fractions derived during sample pretreatment optimization studies, 0.4 mL of the representing liquid sample was transferred into a 15 mL test tube and mixed with 0.8 mL of 100 mM sodium maleate buffer (pH 5.6). Afterward, the procedure was continued as stated above and the measurement was read at 590 nm against the blank sample prepared with sodium maleate buffer.

Invertase activity assay

Invertase activity was performed with minor modification of the IHC-2009 method [40]. For pine honey samples 20 g honey was dissolved in 100 mL 0.1 M pH 6.0 phosphate buffer. 0.5 mL portion of this solution was incubated in a water bath at 40 °C for 5 min and 5 mL of substrate solution (0.02 M, 4-Nitrophenyl-β-D-glucopyranoside (pNPG), (Calbiochem®, CA, USA) in 0.1 M pH 6.0 phosphate buffer) was added. The solution was vortexed thoroughly and incubated in a water bath at 40 °C for 20 min for enzymatic hydrolysis reaction. End of the reaction period, 0.5 mL of 3 M trizma base, pH 9.5 was added and agitated for terminating the reaction. Approximately after 15 min incubation, absorbance was measured at 400 nm against its matrix blank sample. Results in U/kg unit was calculated by using the following formulation;

$$\text{Total invertase activity (U/kg)} : 158,94 \times \Delta\text{Abs}^{*}$$

*ΔAbs; Absorbance differences between sample blank and honey sample.

Similarly, the fractions derived from sample pretreatment were used directly without any phosphate buffer dilution. For this, 0.25 mL solution was mixed with 2.5 mL substrate solution and incubated in a water bath at 40 °C for 20 min for the enzymatic hydrolysis. Subsequently, 0.25 mL of 3 M trizma base, pH 9.5 was added and vortexed for the reaction quenching. At the end of the 15 min incubation period, absorbance was measured at 400 nm against its matrix blank sample. Results in U/kg were calculated using the formulation previously mentioned for pine honey.

Determination of sugar composition

The elimination efficiency of small molecules in the developed pretreatment method was measured by following the decrease in the concentration of sugar molecules. Pine honey samples and collected fractions at the method optimization stage were analyzed for sugar composition using the modified DIN 10758:1997-05 method [41]. For pine honey samples (0.5 g) and for fractions (0.5 mL) obtained from method optimization studies were mixed with the 7.5 mL deionized water (D.I.) and 2.5 mL ACN mixture. The samples were vortexed thoroughly for a minute and 2 mL of these solutions were filtrated to amber glass vials for injection using 0.45 µm PVDF syringe filter (Interlab®, Istanbul, Turkey). 5 µL of the standard calibration mixture and samples were injected into the system consecutively. For the external standard calibration purpose, a single-point calibration plot was constituted by means of the injection of the prepared standard mixture. The calibration standard mixture was composed of 36% (w/v) fructose, 27% (w/v) glucose, 3.6% (w/v) sucrose, 3.6% (w/v) maltose, 3.6% (w/v) turanose, 3.6% (w/v) trehalose, 3.6% (w/v) isomaltose, 3.6% (w/v) erlose, 1.8% (w/v) melezitose and 2.0% (w/v) maltotriose. It was prepared by serial dilution of stock solution using D.I. water:ACN (7.5:2.5, v/v). Hydrophilic Interaction Chromatography (HILIC) was applied for carbohydrate separation. The chromatographic separation was achieved using an APS-2 Hypersil™ column (3.0 i.d. × 100 mm length, 5 µm particle size, Thermo Fisher Scientific®, Inc., Waltham, MA, USA) with isocratic flow at Waters® Alliance 2695 High-Performance Liquid Chromatography (HPLC) system (Waters®, Milford, Massachusetts, ABD) equipped with Waters® 2414 HPLC Refractive Index Detector (RID). The flow rate was 0.5 mL/min. Mobile phase composition was ACN:ultra-pure water (83:17 v/v). The column temperature was set to 35 °C during analysis.

Preparation of HPG protein extracts

For the optimization study, 240 HPG samples (12 microcentrifuge tubes each containing 20 HPGs in 250 µL insect saline) obtained after dissection and stored at − 80 °C were brought to room temperature, and 4 pieces of tungsten-carbide beads (Qiagen, Hilden, Germany) were added into each microcentrifuge tube. They have been divided into the 3 equal groups each containing 80 HPGs. Afterward, homogenization and extraction efficiencies were tested by adding 10 µL of protease inhibitor cocktail each microcentrifuge tubes (ProteoGuard EDTA-free protease inhibitor cocktail, Takara Bio Inc., Kusatsu, Shiga, Japan) along with 3 different extraction solutions (RIPA lysis buffer; Thermo Scientific™, Waltham, Massachusetts, USA); ReadyPrep™ 2-D rehydration/sample buffer-1 (7 M urea, 2 M thiourea, 1% ASB-14, 40 mM tris, 0.001% bromophenol blue), (Bio-Rad, Hercules-California, USA); and insect saline buffer in 1.5 mL volume. The resulting mixtures were shaken and homogenized in the Tissue lyzer device (Tissue Lyser LT, Qiagen, Hilden, Germany) at 42 Hz. for 2 min. The homogenization process was repeated as triplicate for maximum recovery. Microcentrifuge tubes were kept on ice for 30 s and cooled in order to prevent denaturation and protein losses due to proteolytic activity, which may occur owing to the heating of the sample between the homogenization replicates. The supernatants attained after homogenization were ultracentrifuged at 15,000×*g* for 20 min at 4 °C. Total protein contents and SDS-PAGE bands of the obtained supernatants were analyzed. After the assessment, since it was seen that RIPA buffer and 2D-rehydration buffer did not exhibit a different extraction efficiency than insect saline buffer, the procedure with insect saline buffer addition was decided as the final protocol to be applied. In the continuation of the process, as a final pretreatment, 4 separate extracts in microcentrifuge tubes each derived from 20 HPGs in insect saline buffer were combined (approximately 7 mL) and filtered supernatant was transferred to a centrifugal filter unit (CFU, Amicon® Ultra-15, 10 kDa MWCO, 15 mL, Merck, Darmstadt, Germany). The total volume was made up to 10 mL with insect saline buffer. The sample, which was spun at 5000×*g* at 4 °C for 40 min was reduced to 500 µL. Subsequently, the volume was completed to 1 mL with insect saline buffer. The obtained final sample can be assumed as a stock, authentic, and reference enzyme extract and it was remained concentrated for SDS-PAGE analysis. Therefore, SDS-PAGE analysis was performed by applying 1/20 dilution prior to sample analysis. Workflow regarding the extraction of the HPG proteins following the dissection of HPGs is given in Fig. 1.

Sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The protein profiles of the HPG extract and pine honey protein isolate were compared by conducting the SDS-PAGE technique. In this context, a 20-fold diluted solution of HPG protein extract and pine honey protein isolate were separated electrophoretically in Mini-PROTEAN® Tetra Cell (Bio-Rad® Laboratories Inc., Hercules, CA, USA) using 4–20% Mini-PROTEAN® TGX™ Precast/stain-free protein gels from Bio-Rad® Laboratories. The Tris/glycine buffer from Bio-Rad® Laboratories was ten-fold diluted from its 10 × stock buffer solution and employed as running

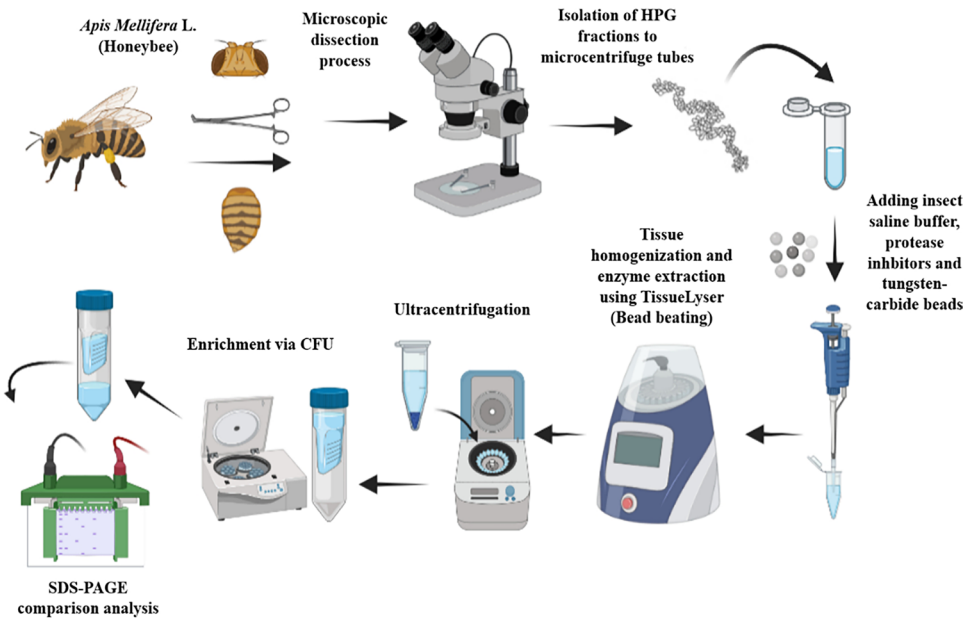

Fig. 1 *Apis mellifera* L. dissection and protein extraction from the isolated HPGs. Created with BioRender.com

buffer. 60 µL from each sample was mixed with 20 µL of SDS-PAGE sample buffer that contains 10% of 2-mercaptoethanol (BME) as reducing agent and 90% of 4 × Laemmli sample buffer. 45 µL aliquots from the samples were loaded to gels along with an unstained protein ladder (Precision Plus Protein™ Unstained Protein Standards, Strep-tagged recombinant, Bio-Rad® Laboratories, Inc., Hercules, CA, USA). Data visualization was accomplished at Gel Doc™ EZ Imager system through Image Lab software (Bio-Rad® Laboratories Inc., Hercules, CA, USA).

Preliminary study for the preparation of pine honey protein concentrates

Stirred cell concentration of honey

The whole honey proteome was tried to be extracted, enriched, and isolated from the matrix environment. Sequential ultrafiltration has been applied for the higher and purer protein yields at the first step of the method optimization studies. For the assessment of the enzyme yields and corresponding losses at each step of the workflow, we have collected a total of 15 representative samples. These 15 samples were tested in terms of their enzyme activities, sugar compositions and total protein amounts. With these results, recoveries of the sample preparation steps were calculated to verify the efficacies. Initially, 50 g of honey was dissolved in homogenization buffer (insect saline buffer; 10 mM Tris/HCl, pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$) in 1:8 proportion (w/w). 2 mL of dissolved honey was aliquoted for measuring enzyme activities, sugar profiles and protein contents and kept at − 80 °C until analysis *(Sample 1)*. The dissolved honey solution was then centrifugated for 20 min at 4000×*g* and + 4 °C (Hettich®, Rotina 35R Type, Tuttlingen, Germany) to abolish any pollen and other major insoluble particulates. The supernatant was transferred to stirred cell ultrafiltration (SCU) device for further ultrafiltration and enrichment steps. Due to the low abundance of protein concentrations, a dissolved honey sample was concentrated via Amicon® stirred Cell (400 mL volume, Merck EMD Millipore®, Billerica, MA, USA) coupled with pre-conditioned Poly (ether sulfone) (PES) ultrafiltration disc membrane, 76 mm for stirred cell (Biomax® 10 kDa Molecular weight cut-off in dalton (MWCO), (EMD Millipore®, Billerica, MA, USA) for the first ultrafiltration/enrichment step. During the ultrafiltration process, positive pressure using nitrogen flow and continuous stirring using a magnetic stirrer (IKA, C Mag HS 7, Staufen, Germany) has been utilized in a refrigerated incubator (INCU-Line®, VWR, Pennsylvania, USA) at + 4 °C condition to prevent potential protein degradation and/or aggregation assuming both the protein loss and membrane clogging drawbacks. At the end of the concentration process, all retentate and filtrate were collected individually. 2 mL portion of both retentate *(Sample 2)* and filtrate *(Sample 3)* was aliquoted to perfom performance assays (enzyme activities, sugar content and total protein content). The retentate phase (roughly 50 mL) was divided into the 2 mL microcentrifuge tubes (Interlab®, Arnavutköy, Istanbul, Turkey) and ultracentrifuged (Sigma® Laborzentrifugen GmbH, Osterode am Harz, Germany) at 15,000×*g* for 10 min at + 4 °C. After the centrifugation, supernatants were combined in a 15 mL centrifuge tube and 2 mL of the resulting solution *(Sample 4)* was aliquoted and stored again to conduct performance assays later on. The remaining combined solution was filtrated using a 0.45 µm polyvinylidene difluoride (PVDF) syringe filter (Interlab®, Arnavutköy, Istanbul, Turkey). Similarly, representive sample after filtration *(2 mL; Sample 5)* for performance assays was also collected at this method step. Next, approximately 40 mL resulting filtered sample was attained and was divided into two parts (7.5 mL for dialysis approach and the remaining 32 mL was divided to 3 equal volumes for centrifugal ultrafiltration (CUF) approach using centrifugal ultrafiltration units (CFU). At the ensuing steps, the effectiveness of dialysis and centrifugal ultrafiltration equipment was investigated based on the removal efficiency of low molecular weight interfering molecules but also their protein/enzyme yields.

Dialysis approach

Dialysis was performed with 7.5 mL of an ultrafiltered sample using Slide-A-Lyzer™ Dialysis Cassettes, 20 kDa MWCO, 12 mL, (Thermo Scientific™, Waltham, Massachusetts, USA) at 4 °C for 24 h against 2 L of D.I. water as dialysis buffer. 2 mL samples were aliquoted from deionized waters (dialysis external buffer) for performance analysis during dialysis buffer exchange stages (after 2 h later; *Sample 6* and end of the second 2 h**;** *Sample 7*) and at the end of the dialysis process (*Sample 8*). 1.5 mL of the sample was also aliquoted from the retentate of the dialysis cassette as the final product (*Sample 9*). The remaining dialyzed sample (6 mL) was transferred to 10 kDa MWCO, Amicon® Ultra-15 CUF and then it was centrifugated for 40 min at 5000×*g* at 4 °C. 2 mL portion of filtrate (*Sample 10*) was collected for performance assays and approximately 500 µL sample left in the CUF was homogenized with 1.5 mL homogenization buffer and this final sample (*Sample 11*) was also kept until performance analysis.

CUF approach

A 10 mL portion from the concentrated sample was directly transferred to CFU device 10 kDa MWCO, Amicon® Ultra-15 which was preconditioned with insect saline buffer for further enrichment. The sample was spun for 40 min at

5000×*g* and at 4°C. and 2 mL from the resulting filtrate (*Sample 12*) was aliquoted for performance tests. 12 mL of insect saline buffer was added to the concentrated protein solution in CFU and centrifugation was repeated. This stripping phase (buffer exchange) and diafiltration were repeated two more times. End of the each washing step, 2 mL was aliquoted from the filtrate again (*Sample 13* and *Sample 14*). Retentate, approximately 500 µL was homogenized with 1.5 mL of homogenization buffer and completed to volume of 2 mL. The final sample (*Sample 15*) was stored at -80°C until the assessment of performance for analysis. The whole experimental design that was utilized for the determination of the final procedure was constructed as in Fig. S1 (S.I.) and the determined sample pretreatment protocol was decided according to the obtained analytical performance values during these experiments.

Verification experiment for the determined sample preparation procedure

The confirmation study as the final procedure was utilized. As in the previous optimization process, a portion of the samples at critical steps was subjected to Bradford total protein assay, sugar content analysis, diastase, and invertase activity tests to validate the protocol. The experimental model generated for this study is depicted in Figs. 2 and 3.

The confirmation procedure in detail was carried out, is as follows;

- 50 g of pine honey sample was dissolved in insect saline buffer and completed to 400 mL to obtain a homogeneous solution.
- The solution was divided into 8×50 mL centrifuge tubes and spun at 5000×*g* for 20 min. 2 mL was taken from the supernatant and labelled as verification sample-1 (*VS-1*). Sample was concentrated until roughly 15 mL remains in the stirred cell. 2 mL of sample was separated from the filtrate (*VS-2*) and the resulting retentate (*VS-3*).
- The concentrated sample was transferred to microcentrifuge tubes and ultracentrifuged for 10 min at 15,000×*g* and at 4 °C. The supernatants were combined and filtered through a PVDF syringe filter, leaving up to 2 mL of sample for measurements (*VS-4*).
- The filtered sample (approx. 12 mL) was transferred to the preconditioned CFU. It was centrifuged for 40 min at 5000×*g* at 4 °C. 2 mL sample was taken from the filtrate (*VS-5*).
- For the first washing, 12 mL of insect saline buffer was added onto the retentate and the centrifugation was

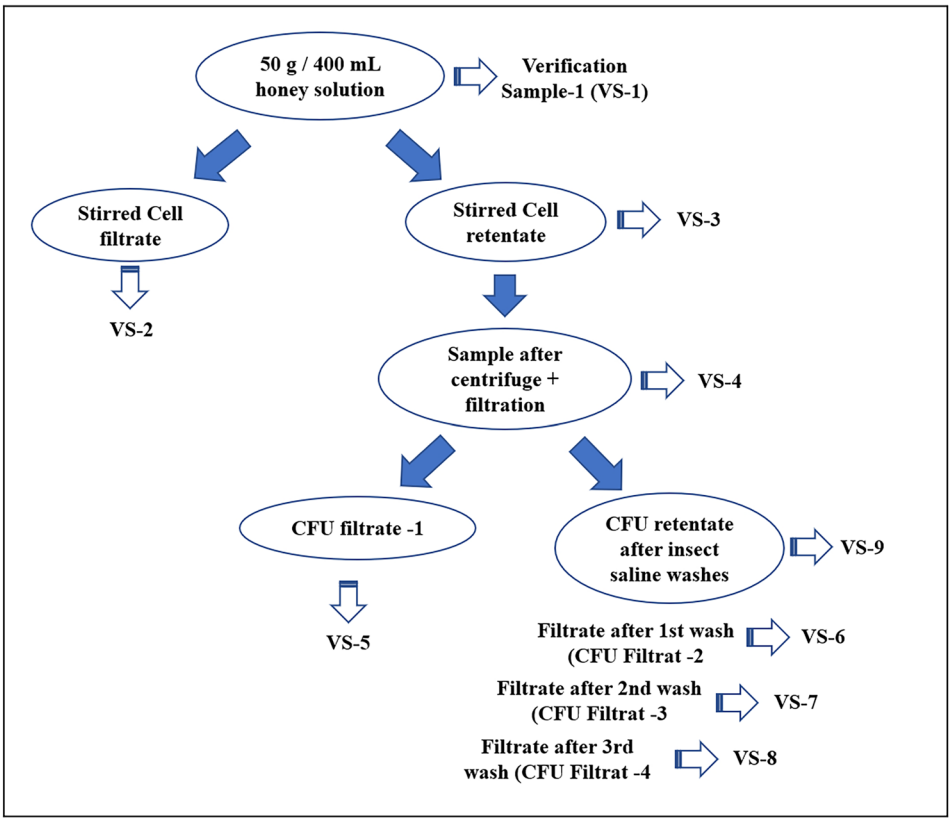

Fig. 2 Experimental model for the confirmation of the pretreatment study

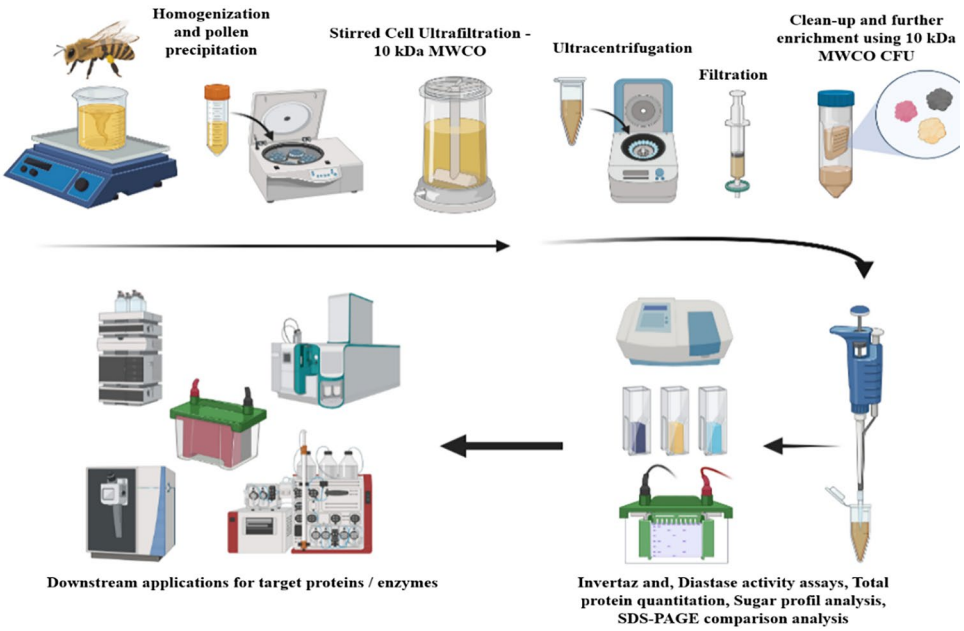

Fig. 3 Summary of the determined protocol for the total protein extraction from pine honey. Created with BioRender.com

applied for 40 min at 5000×*g*, at 4 °C. A sample of 2 mL was aliquoted from the filtrate (*VS-6*).

- Washing step was repeated by adding another 12 mL buffer and new filtrate sample was aliquoted (*VS-7*).
- Washing step was repeated by adding another 12 mL buffer and 2 mL sample was taken again from the filtrate formed at the bottom (*VS-8*). (The number of washings was increased by adding a third step washing to the 2 repeated washings in the optimization study).
- At the end of the washes, the upper retentate (approximately 250–300 µL concentrated enzyme and protein solution) was completed to 3.5 mL with insect saline buffer. The sample was stored at − 80 °C (*VS-9*).

Results and discussion

Although a few types of research in literature have been conducted on the identification and characterization of the proteins in honey and HPG, these researches do not include easy-to-apply and time-efficient protein extraction and isolation procedures. Thus, a versatile sample pretreatment method at honey matrix is needed for any proteomic-based downstream investigations. Within this described novel protein extraction and enrichment procedure, we have met key criterias in terms of providing the crude protein extract of honey in a native and enriched way.

Authenticity and quality assessment of pine honey samples

Quality assessment of honey has great significance to know about its nutritional value as well as the suitability of the storage. Additionally, authenticity analyses can address if there is any fraud at honey. In this study, for the purpose of the authentic pine honey sample election, physicochemical quality and adulteration assays were performed. The pine honey used in method development was selected according to the analysis results and the acquired values for the selected research material are given in Table 1.

Optimizations for stirred cell protein concentration workflow

Three different weights (100 g, 150 g, 200 g) of pine honey were chosen as initial amounts but these weights caused membrane clogging due to the high viscosity of the pine honey solution. Thereby, the quantity was reduced, and 50 g was decided as the starting amount by weight. According to the literature findings, enzymes targeted for the validation purpose of this study have an average molecular weight of 70 kDa and 55 kDa for invertase and diastase, respectively [37, 42]. As a rule of thumb, the appropriate membrane molecular weight cut-off (MWCO) value should be at least two times smaller than the molecular weight of the solute

Table 1 Authenticity and physicochemical analysis results of selected pine honey

Moisture (%)	16.8
HMF (mg/kg)	9.1
Acidity (meq/kg)	26.8
Conductivity (µS/cm)	0.96
C4%	1.2
Diastase (Shade U/g)	28.5
Invertase (U/kg)	117.1
β-Fructofuranosidase (U/kg)	NA
β/γ amylase (U/kg)	NA
Rice Syrup markers	NA
Heat stable amylases	NA
Fructose + Glucose (%)	57.7
Total disaccharides (w/v, %)[a]	4.9
Total trisaccharides (w/v, %) DP = 3[b]	1.5

[a]Total dissacharides: Sucrose + maltose + turanose + trehalose + isomaltose

[b]Total trissacharides: Erlose + melezitose + maltotriose

DP degree of polymerization, *NA* not available

that is being retained. Hence, the Amicon® stirred cell combined with 10 kDa MWCO, PES ultrafiltration disc membrane was employed for protein concentration. This enabled small molecules to be eliminated by passing through the pores while concentrating the proteins without any loss. Regenerated cellulose disc membrane was also tested for comparison purposes but there was no difference observed between ultrafiltration efficiencies. Owing to the samples' low protein concentration and stability concern of the enzymes, the cooled ultracentrifugation process was applied for further precipitation and elimination of the insoluble, non-proteinaceous substances. This allowed us to transfer concentrated enzymes to the next step without any loss of recovery and it also prevented the dialysis and CFU membranes from clogging. The supernatant was filtered through a PVDF filter to attain a cleaner sample. The results demonstrated that cellulose acetate syringe filter caused protein loss due to the non-specific binding activity of the material. Thereby a 0.45 µm PVDF type filter was decided to be used. Before the concentration process, invertase and diastase enzyme activities, sugar composition, and total protein quantity of dissolved honey (50 g/400 mL) were measured as initial performance values. At the end of the process, these parameters were measured again on retentate and filtrate samples. The protein concentration, which was 0.06 mg/mL at the beginning, was observed as 0.48 mg/mL at the end of the stirred cell procedure, and this value enhanced the recovery value, which was initially set as 100%, to 777.7%, giving a 7.77-fold increase in protein concentration. Regarding the enzyme activity gains, diastase activity was obtained from 28.5 Shade U/g to 236.5 Shade U/g with 830.6% recovery, while invertase activity was attained from 117.1 to 308.2 U/kg with a recovery of 263.1%. Analysis results of representative samples collected after all the mentioned stages are given in Table 2.

Table 2 Results of the protein enrichment and clean up steps

Pretreatment	Sample	Total sugar content % (w/v)	Total protein (mg/mL)	Total diastase activity (Shade Unit/g = DN)	Total Invertase activity (U/kg)	Invertase yield (%)	Diastase yield (%)	Protein yield[a] (%)
Stirred cell concentration	1	64.1	0.06	28.5	117.1	100	100	100
	2	67.8	ND	ND	ND	–	–	–
	3	62.2	0.56	237.5	305.4	260.8	833.1	909.1
	4	59.4	0.52	236.5	307.1	262.2	829.8	814.1
	5	60.1	0.48	236.8	308.2	263.1	830.6	777.7
Dialysis clean up + CFU concentration	6	0.1	ND	ND	ND	–	–	–
	7	ND	ND	ND	ND	–	–	–
	8	ND	ND	ND	ND	–	–	–
	9	0.1	0.39	158.9	116	99.2	557.5	633.5
	10	0.1	ND	ND	ND	–	–	–
	11	1.5	1.10	297.4	307.3	262.4	1043.5	1779.1
CFU concentration and clean up	12	64.1	ND	ND	ND	–	–	–
	13	27.6	ND	ND	ND	–	–	–
	14	10.8	ND	ND	ND	–	–	–
	15	9.8	1.12	450.1	641.5	547.7	1578.6	1808.5

[a]The protein yields were obtained by calculating the protein in mg, taking into account the volume of the relevant solution

ND not detected

Dialysis and CUF method considerations

Up to this step in the process carried out, the protein was concentrated but the small molecules at bulk quantities such as sugars and organic acids have not been eliminated from the pine honey proteome. Therefore, optimization studies were continued with additional processes for the elimination of these small molecules but also for further enrichment of the proteins. Dialysis and CUF are the methods that can be utilized for the elimination of small molecules. The sugar analysis was chosen to evaluate the cleaning efficiency by dialysis and ultrafiltration because the working matrix inherently has plethora of carbohydrates. It has been interpreted as the elimination of carbohydrates using size exclusion theory and calculating the carbohydrate residues will be a good indicator of the effective elimination of other small and low amount molecules such as organic acids, amino acids, minerals, vitamins, phenolics, salts. According to the results given in Table 2, total sugar amounts were negligible in *Sample 9, 10, 11.* Both dialysis and CUF were effective at sugar-freeing. The total sugar concentration, which was 64.1% at the beginning, decreased to 0.11% at the end of the dialysis process *(Sample 9).* Nevertheless, low recovery particularly at invertase has been occurred at dialysis treatment. Similarly, a decrease in diastase activity and loss of total protein were also observed. While the diastase activity value was obtained with 236.8 shade U/g activity at the end of the stirred cell process, it was observed as 158.9 shade U/g at the end of the dialysis process. On the other hand, the invertase activity decreased from 308.2 to 116 U/kg. When the recovery values obtained with these numbers were compared with the recoveries acquired at the end of stirred cell ultrafiltration, a distinct decrease was observed from 830.6 to 557.5% and from 263.1 to 99% for diastase and invertase, respectively. Considering the total protein content, the 777.7% recovery value obtained at the end of the stirred cell ultrafiltration procedure and the protein content at a concentration of 0.48 mg/mL decreased to a concentration of 0.39 mg/mL at the end of dialysis, and this value corresponded to 633.5% in terms of percentage recovery. As a result of the dialysis process, sugar elimination was achieved effectively, but there was a decrease in diastase and invertase activities and total protein amount. This may be a consequence of high dialysis duration. In order to see the enrichment efficiency of CUF after the dialysis process and to recover the loss in dialysis, two techniques were incorporated and the sample in the dialysis cassette was transferred to CFU, and additional concentration was performed. It can be said that CUF is effective in terms of enrichment since it was seen that the enzyme activities lost in the previous dialysis process were recovered at a rate close to two to threefold *(Sample 11).* Cleaning (passive ultra-diafiltration) and concentrating with only CUF yielded very effective results in terms of the concentration of protein isolates. The *Sample 15* obtained after only CUF treatment provided the notably highest enzyme activity and protein concentration with minimal recovery loss. At the end of two consecutive washes with CFU, the total sugar concentration of 9.8% (w/v) was measured in the retentate sample obtained. 64.1% attained from the filtrate formed as a result of the first ultrafiltration process. 27.6% and 10.8% total sugar percentages were obtained at the end of the first and second washing processes, respectively. That pinpointed the washing step should be continued and thus sugar elimination equivalent to dialysis can be achieved. Concordantly, it was decided to perform one more washing step in the CUF process, which is the procedure decided upon. No protein ingredient was detected in the filtrates in all CUF processes, including the washing steps. With 547.7% invertase, 1578.6% diastase, and 1808.5% total protein recovery values, the CUF method paved the way to gain high enzyme and protein content free from small molecules. Consequently, dialysis is a very time-consuming and tedious process with a 36 h analysis time, and obtained performance values were not satisfying. Upscaling of the suggested methodology could also be problematic for dialysis. The CUF process can be completed in 2.5 h, including all washing steps. This can be considered time effective, rather than an application such as dialysis that takes an average of 1.5 days. It was decided in the direction of the analysis data, it would be more plausible to follow CUF procedure during the preliminary preparation process. Hence, we excluded the dialysis and continued the preliminary studies employing solely the CUF in the latter step. A confirmation-oriented preliminary study (verification experiment) was performed with this procedure again, and analysis reproducibility and recovery were validated.

Verification experiment

In the previous preliminary study, at the end of the concentration process with stirred cell, 40 mL of sample was obtained as retentate, 7.5 mL of which was subjected to dialysis, and the remaining part was subjected to CUF application. In the verification study, the SCU process was continued until approximately 15 mL of the sample remained on the membrane as retentate. In this respect, it was expected that invertase activity, diastase activity, and total protein amount would be prominently higher compared to the previous study. While the total diastase activity value gained in the preliminary study was 450 Shade U/g for the CUF procedure, it was determined as 693.3 Shade U/g in the verification study. Invertase activity, on the other hand, increased from 641.5 to 1055 U/kg. The total protein concentration was found to be 1.25 mg/mL, which was found to be 1.12 mg/mL in the previous CUF procedure. Considering the data obtained, the recoveries for diastase, invertase,

and total protein after the verification study were found as 2432.6%, 900.9%, and 2149.8%, respectively. Test results are given in Table 3 and images of the enzyme activity assays are given in Fig. 4.

By verification experiment results, approximately 24.3-fold enrichment for diastase, 9 fold enrichment for invertase, and 21.4-fold enrichment for total protein were achieved *(VS-9)*. The low recovery in invertase, compared to diastase and total protein may be due to the fact that the enzyme is less resistant against temperature compared to diastase, and is more unstable against pretreatment times and the number of steps. The results showed us that CUF presented an effective clean-up process without loss of protein. Sugar elimination was improved at each washing steps. It was seen that, if CUF was preferred, the analysis time will be significantly reduced compared to dialysis with the same clean-up efficiency.

Protein extraction from HPGs of *Apis mellifera* L. and comparison of protein profiles with pine honey

Extraction technique, compositions of the extraction buffers and the number of HPG to be extracted were optimized in order to get enzymes at a concentration that can be identified from isolated HPG samples. The HPGs gained at the end of the dissection were stored at − 80 °C in insect saline buffer in order to provide physiological pH and electrolyte concentration to ensure the stability of the enzymes until homogenization and enzyme extraction [37]. It has been deduced that various numbers of HPG samples are used in the literature in protein/enzyme extraction and isolation studies. Ohashi

Table 3 Results of the protein enrichment and clean up steps at verification experiment

Pretreatment	Sample	Total sugar content % (w/v)	Total protein (mg/mL)	Total diastase activity (Shade Unit/g = DN)	Total Invertase activity (U/kg)	Protein yield[a] (%)	Diastase yield (%)	Invertase yield (%)
Stirred cell concentration	VS-1	64.1	0.06	28.5	117.1	100	100	100
	VS-2	63.9	ND	ND	ND	–	–	–
	VS-3	62.1	1.11	451.2	591.3	1906.5	1583.1	504.6
	VS-4	58.8	1.06	444.1	601.2	1821.2	1558.2	513.2
CFU concentration and clean up	VS-5	63.7	ND	ND	ND	–	–	–
	VS-6	22.5	ND	ND	ND	–	–	–
	VS-7	1.2	ND	ND	ND	–	–	–
	VS-8	ND	ND	ND	ND	–	–	–
	VS-9	ND	1.25	693.3	1055.1	2149.8 (21.4 fold)	2432.6 (24.3 fold)	900.9 (ninefold)

[a]The protein yields were obtained by calculating the protein in mg, taking into account the volume of the relevant solution

ND not detected, *VS* verification sample

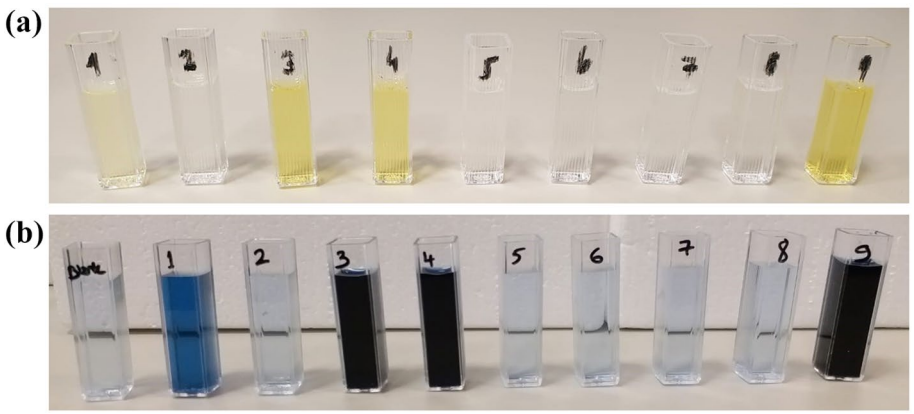

Fig. 4 Images of enzyme activity results for each step of sample pretreatment. **a** Images of invertase activities (yellow colors show the presence of invertase enzyme in direct proportion to the concentration) and high invertase activity was observed in samples 1, 3, 4, and 9. **b** Images of diastase activities (the dark blue colors show the presence of diastase enzyme in direct proportion to the concentration) and high diastase activity was observed in samples 1, 3, 4, and 9

et al. (1999) extracted HPG tissue from 500 bees in their research to learn how glucose oxidase and diastase expression levels change with the age of worker honey bees [37]. Al-Sherih et al. (2017) dissected 70 HPGs and investigated the honey ripening enzyme activities of HPG samples in 2 subspecies of *A. mellifera* L. [43]. Another group examined protein expressions by extracting 50 HPG tissues in insect saline buffer [42]. In 2002, Pontoh and Low have used 25 HPG samples for β-glucosidase purification [36]. As can be seen, variable number of tissue samples have been studied, and in line with these findings, it has been envisaged that 80 HPGs for the extraction of diastase and invertase will be above the literature average and can provide sufficient enzyme levels. For the extraction process, 320 honeybees were dissected and HPG samples were collected. 80 HPGs were used for the experiment, and 240 HPG samples were evaluated for method optimization by including different solvents and volumes to be used in extraction and homogenization efficiency research studies. After determining the required HPG number theoretically, it was affirmed by experimental studies that the foreseen number gave sufficient enzyme activity. Three different extraction buffers were tested (RIPA buffer, 2D-rehydration buffer and insect saline buffer). EDTA, which is frequently added to the lysis buffers, was not employed in this study due to the metalloenzyme structure of α-amylase. The role of calcium ions is worth noting for the stability of the enzyme. Chelating agents such as EDTA can bind to the metalloprotein part of the enzyme, causing its separation from the apoenzyme part and denaturation of the enzyme, thus inhibiting the activity. It was figured out that the RIPA buffer and 2D-rehydration buffer did not exhibit a different extraction efficiency than the insect saline buffer. Targeted bee enzymes are located in the extracellular matrix and have a high water-soluble structure. For this reason, harsh extraction reagents with the addition of detergent and chaotropic agents did not contribute significantly to extraction recovery. It was observed that the three buffers presented the same extraction efficiency and therefore we chose the insect saline buffer as a mild and non-denaturating media. The protein profiles of the 1/20 dilutions of the concentrated pine honey sample and HPG extract gave similar bands according to SDS-PAGE analysis. The comparison result is given as SDS-PAGE electrophoresis image in Fig. 5.

As a result of the preliminary preparation, the concentrated enzyme/protein SDS-PAGE profile obtained from pine honey provided major bands at approximately 70 kDa and 55 kDa values, similar to the protein contents extracted from honey bee HPG samples. Pine honey enzymes, which have been confirmed to be invertase and diastase with both their activities and molecular weights, show similar activity and electrophoretic mobility with diastase and invertase in HPG extracts, confirming the isolation of authentic enzymes. In addition, other distinct protein bands were also observed in the HPG protein extract in the range of 150 kDa, 230 kDa, and 20–25 kDa. Many proteins and peptides from HPG glands can be expressed extracellularly. Many structural and signaling proteins, their fragments and precursors can be found in tissue protein extracts. The tissue lyzer extraction performed in this study may not be considered a lysis process since agents such as lysozyme and/or detergents are not used in the homogenization buffer. Assumed that the cytosolic and structural proteins of the HPG cells will not be present in the final extract. But enzymes such as acid-phosphatase, glucose oxidase, catalase, major royal jelly proteins (MRJP 1–9), and many yet unidentified proteins

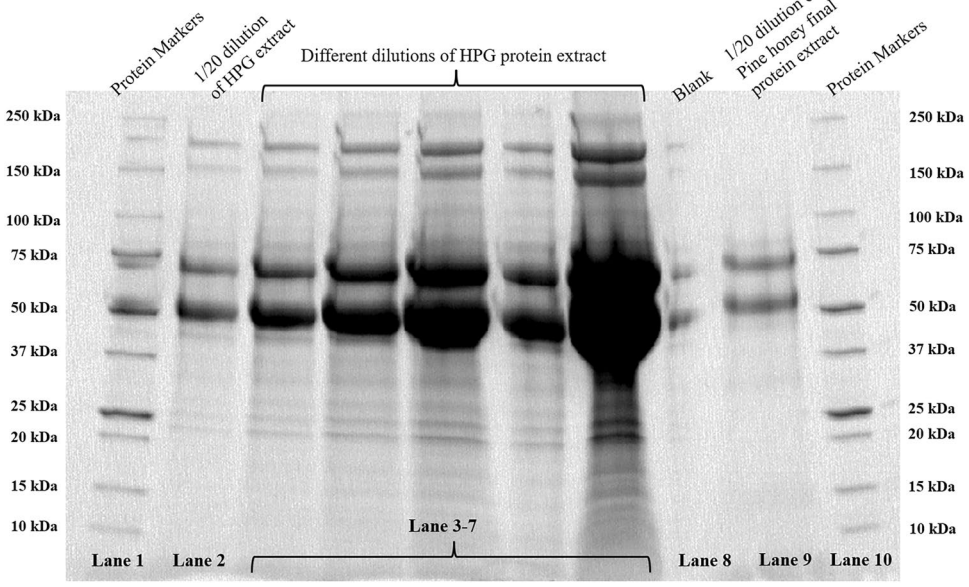

Fig. 5 SDS-PAGE illustration of the HPG and pine honey protein extracts. Lane 1 and Lane 10; protein ladders, Lane 2; 1/20 dilution of final HPG extract, Lane 3–7; varying dilutions of the final HPG protein extract, Lane 8; blank, Lane 9; 1/20 dilution of the prepared pine honey protein extract. Distinct bands approximately at 55 kDa and 70 kDa indicate the activity confirmed bee diastase and invertase enzymes respectively

and peptides, as well as their potential oligomeric variants, could be found in the extracellular matrix. For this reason, the identification of these protein bands, except diastase and invertase, whose presence has been confirmed after activity and electrophoretic molecular weight determination, should be considered as a separate research topic. The developed isolation and enrichment protocol from the HPGs provides great convenience in terms of identification of these proteins.

Conclusion

As an overview, high-throughput protein isolation is a complex, multi-step process. There are plenty of technical challenges in the course of this process that are not experienced when purifying a single protein. Among the most challenging is the high-throughput protein concentration and buffer exchange, which is not merely labor-intensive but can also result in significant losses of target proteins. In this research, we described an effective pretreatment method for isolating pine honey proteins. High recoveries, and ease of use with novel sample pretreatment methodology have been defined for selected enzymes which was stated as model proteins. The obtained protein profile was compared with the protein profiles extracted from *A. mellifera* L. and the authenticity of the enriched enzymes was affirmed by electrophoresis as well as their activities. Verification results demonstrated considerably high enzyme activities and clean-up efficiencies as well as acceptable recoveries and satisfactory enrichment folds were reported. Additionally, by making use of SCU combined with CUF rather than dialysis or chemical precipitation techniques, novel method highlighted the possibility to mitigate sample preparation times and workloads for protein extraction and concentrating. The established sample pretreatment method in this study will provide convenience for any downstream applications such as protein identification, purification, and characterization at honey matrices. The obtained isolate provides honey proteins in their native and active structures besides their high concentrations. Therefore, they can be interpreted as a reagent of enzymatic assays or a reference material at analytical approaches such as authenticity or quality tests at honey or at any product developments workflows in the food industry. Moreover, the given method would be also an effective preliminary isolation technique for the production of natural enzymes that may be an alternative to enzymes that are currently used in the industry after implementing the scaling-up process. Furthermore, the presented two-step analytical method is also applicable to different food matrices with low protein content and may form a basis for proteome-based applications.

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Declarations

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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