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CURRENT PERSPECTIVES OF PROTEOMICS FOR THE IDENTIFICATION AND CHARACTERISATION OF INSECT-DERIVED PROTEINS WITH ALLERGENIC POTENTIAL

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Abstract. Currently, pet owners can easily access a broad array of commercial pet products specifically formulated to meet the unique nutritional needs of their cats and dogs. The trend towards organic pet foods, prioritizing whole ingredients over processed ones, is gaining more and more attention, emphasizing sustainability in sourcing dietary proteins. Insects, rich in essential amino acids, emerge as promising protein substitutes, though they do carry potential risks of triggering adverse food reactions, such as allergies in pets. This, in turn, provides a fertile ground for advanced proteomic analysis, facilitating comprehensive identification and quantification of allergens from edible insects. This includes defining immune epitopes originating from these allergens, thus fostering the discovery of potentially new proteins with allergenic potential and aiding in the in vitro diagnosis of allergen sensitization. This review presents the latest advancements in edible insect-derived allergens and the proteomic investigations conducted to analyse them. We offer insights into classical and advanced proteomic approaches designed for identifying and quantifying these allergens in various food matrices. Additionally, we focus on recent studies concerning the characterization of novel insect allergens, exploring sensitization and cross-reactivity mechanisms, with a specific emphasis on pets.

Key words: proteomics, electrophoresis, liquid chromatography-mass spectrometry, insect allergens, food allergy.

INTRODUCTION

Nowadays, pet owners have access to a diverse array of commercially available products designed to address the specific nutritional requirements of their dogs and cats, considering factors such as breed, age, size, and health predispositions. Cost is a crucial factor in selecting pet food, yet brand reputation, marketing strategies, and past experiences are also very important aspects. Consumers are increasingly favouring pet foods that follow the organic trend, highlighting the inclusion of whole ingredients like meats, fruits, and vegetables, while

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avoiding highly processed ones such as refined grains and other by-products (Buff et al. 2014; Bosch and Swanson 2021).

Dietary protein is essential for providing vital amino acids and stands as one of the most expensive macronutrients, especially when derived from animal sources, thus holding considerable ecological and economic significance. This highlights the necessity of prioritizing sustainability in sourcing dietary protein (Kępińska-Pacelik and Biel 2022). The rising demand for animal protein has driven the intensive exploration of alternative sources, with insects emerging as efficient and high-quality substitutes due to their enrichment in essential and limited amino acids such as lysine, threonine, methionine, and tryptophan (Adamski and Adamska 1996). It should also be noted that they are already successfully used as feed ingredient in fish, swine and poultry farming (Sogari et al. 2023). The rapid growth, short generation time, efficient nutrient utilization, and high reproductive potential of insects facilitate their cost-effective and straightforward breeding (Jensen et al. 2017). Commercially available dry foods typically contain meat meal and by-products from poultry, lamb, beef, pork, and fish. On the other hand, protein in wet foods mainly comes from fresh or frozen meat and animal tissue fragments (Bosch and Swanson 2021). Therefore, incorporating edible insects as an alternative protein source in pet food production shows potential for strengthening sustainable food systems, fostering economic empowerment, enhancing food security, and supporting climate mitigation efforts (Kungl et al. 2007). While acknowledging the above-mentioned advantages, it is important to consider the potential risk of adverse food reactions (AFRs), including hypersensitive responses to various insect proteins and the possibility of cross-reactivity with other food and inhalant allergens. This, in turn, provides a fertile ground for advanced proteomic analysis, enabling extensive identification and quantification of proteins, thereby opening the way for discovering new allergens and identifying proteins with previously documented IgE reactivity, and thus diagnosing allergen sensitization (De Marchi et al. 2021).

FOOD ALLERGIES IN DOGS AND CATS

Food allergy refers to the immune system's adverse response to a protein or a specific component within the food. In dogs and cats, food allergies primarily manifest as skin symptoms, often accompanied by gastrointestinal issues, occasionally showing signs in the respiratory and nervous systems. It is crucial to apply the term "food allergies" specifically to reactions involving the immune system, distinguishing them from food intolerances, despite their similar symptoms (Adamski and Adamska 1996). The prevalent food allergens typically consist of low-molecular-weight protein derivatives, found as glycoproteins and polypeptides. These antigens are typically recognized by their IgE reactivity, then categorized as minor or major based on the prevalence of IgE sensitization in response to them (Nony et al. 2016). It should be pointed out that food allergies caused by carbohydrates or fats are extremely rare. Moreover, chemical additives like preservatives, dyes, and fragrances commonly present in pet foods are frequently implicated as potential causes of food allergies (Kungl et al. 2007). The gastrointestinal (GI) tract is considered as a primary site for allergic reactions triggered by food. The GI mucosa acts as a barrier, limiting the passage of substances from the intestinal lumen into the blood. Its anatomical structure, along with biochemical and immunological functions, safeguard the intestinal epithelium, preventing the entry of microorganisms and large antigenic molecules. Bacterial, viral, fungal, and parasitic infections, persistent inflammation, digestive and motility issues, as well as malabsorption, can compromise the integrity of the

intestinal barrier, leading to impaired protective function. This, in turn, triggers an increased passage of antigens through the intestinal wall that initiates a multiorgan process leading to the autoimmune response (Adamski and Adamska 1996). Food allergies are commonly classified as prompt IgE-mediated or delayed non-IgE-mediated reactions, with the former being the most prevalent and classical immune mechanism in food-related allergic responses. Therefore, testing the food allergen-specific IgE levels in serum serves as a diagnostic tool for food allergy, as increased allergen-specific IgE indicates sensitization (Lee 2016).

Food allergies to insects have been previously reported following the ingestion of whiteflies, silkworms, grasshoppers, locusts, cicadas, and bees in humans (de Gier and Verhoeckx 2018). Insect allergens are identified as IgE-binding agents capable of cross-reactivity with antigens found in crustaceans, mollusks, and also nematodes. Cross-reactive allergens encompass alpha-actin, tropomyosin, enolase, arginine kinase, glyceraldehyde 3-phosphate dehydrogenase, and fructose-1,6-bisphosphate aldolase (Jeong and Park 2020). Tropomyosin and arginine kinase are identified as antigens, sharing similarities with homologous proteins present in both crustaceans and house dust mites. It should be pointed out that heat treatment and digestion methods are not entirely effective in eliminating the allergenic properties of insect proteins. While obtaining natural, purified insect allergens remains challenging due to their scarcity, accessing recombinant allergens from cockroaches, silkworms, and whiteflies is more feasible. This accessibility presents opportunities for future research aimed at developing diagnostic allergy tests and potential vaccines (de Gier and Verhoeckx 2018).

FOOD ALLERGEN ANALYSIS: CLASSICAL PROTEIN-BASED METHODS

The most commonly used semi-quantitative methods for analyzing allergenic proteins in foods involve antibody-based techniques, including enzyme-linked immunosorbent assays (ELISA) and lateral flow devices (LFDs). With the ELISA approach, it is possible to assess the presence of either antigens (allergenic food proteins) or specific antibodies such as IgE (found in serum) (López-Pedrouso et al. 2023; Tuppo et al. 2022). Two distinct ELISA types are commonly used to detect one or more allergenic proteins in food. The first one, known as the “direct ELISA”, depends on the attachment of primary antibodies (monoclonal or polyclonal), which are conjugated to fluorophores or enzymes, to an antigens coated on plates. In contrast, the “sandwich ELISA” begins by introducing an antigen to the antibodies already attached to the solid plate. Subsequently, it requires the presence of the enzyme-labeled secondary antibody interacting with a specific antigen (Hayrapetyan et al. 2023). On the other hand, LFDs employ an immunochromatographic approach, conducting the application of the sample and the interaction with the antibodies and conjugates all in a single and rapid process. The antibody-antigen complex migrates across the nitrocellulose membrane toward a testing zone, where a colored line signifies a positive result (Baumert and Tran 2015). As reviewed by Poms and Anklam (2006), these two techniques are widely utilized to detect food allergen proteins, with numerous commercially available test kits showing sensitivities ranging between 0.1 and 5 mg/kg. However, it should be emphasised that antibody-based methods may not accurately detect the target protein when chemical and physical changes occur. These changes can stem from thermal processing or the overall sample preparation methods like extraction or enzymatic hydrolysis. This, coupled with antibody cross-reactivity, can lead to both false positive and false negative outcomes (Hayrapetyan et al. 2023).

Other techniques considered standard for detecting IgE-reacting proteins include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional

electrophoresis (2-DE), followed by immunoblotting. For SDS-PAGE, the quantified protein mixtures are initially boiled in a buffer containing an anionic detergent (SDS) and a reducing agent such as 2-mercaptoethanol or dithiothreitol (DTT). Following this, denaturated proteins are separated on polyacrylamide gels according to their molecular mass, resulting in multiple distinct protein bands (McGuire and Johnson 2001). 2-DE, however, separates proteins based on two distinct properties: the isoelectric point (pI) in the first dimension and the relative molecular weight in the second, emerging in the generation of several thousand of different protein spots on a single gel (Kettenhofen et al. 2008). After separation using either SDS-PAGE or 2-DE, the proteins are transferred onto a nitrocellulose membrane and then incubated with human serum containing IgE antibodies that are produced in response to specific food allergens. The last step entails incubating the proteins on the nitrocellulose membrane with enzyme-labeled antibodies (anti-IgE). When these secondary antibodies bind to IgE, the specific enzyme, such as horseradish peroxidase (HRP), generates a signal, facilitating the final detection process of the target protein (Bouchez-Mahiout et al. 2010).

FOOD ALLERGEN ANALYSIS: ADVANCED PROTEOMICS WORKFLOW

Over the last decade, remarkable progress in proteomics has resulted in vast datasets containing proteins or peptides linked to various biological processes. These advancements have not only facilitated the discovery of new food allergens but also opened promising avenues for diagnostic applications (Croote and Quake 2016; López-Pedrouso et al. 2023). In general, two primary strategies are used to analyse food proteome composition including food allergens: “discovery” and “targeted” (Carbonara et al. 2021).

Discovery proteomics, also termed untargeted proteomics, encompasses a comprehensive analysis of all proteins and peptides present in a given biological sample. A bottom-up proteomics stands as the primary workflow employed in this type of experiments. In such analysis, a complex protein mixture is isolated from the food sample, quantified and separated using gel-based (like gel electrophoresis) or gel-free techniques (such as liquid chromatography). Once resolved, these proteins undergo enzymatic conversion into peptides, often through tryptic digestion. Subsequently, mass spectrometry (MS) is utilized to measure their mass-to-charge ratio (m/z), followed by a database search to identify the proteins (Dupree et al. 2020; Carrera 2021). The gel-based method, especially 2-DE, is increasingly preferred as the primary approach for studying global food protein profiles. In this method, multiple proteins are separated into individual spots, which are visualized using staining techniques like Coomassie Blue, Silver Stain, or fluorescent dyes. These spots are then quantified using bioinformatics tools, allowing the transformation of biological information from the gel into a quantitative, computer-readable dataset. Once the analysis of the 2-D gels is completed, particular spots of interest are manually excised from the gels and analyzed using mass spectrometry (MS) to get the identity of the proteins present in those spots. Protein identification often entails comparing peptides found in a sample to those from related species or utilizing de novo MS sequencing methods (Natale et al. 2011; Carrera et al. 2020). On the other hand, in gel-free techniques, a protein mixture undergoes an initial digestion using a specific serine protease, with trypsin being the most commonly utilized enzyme. This process generates a distinct set of peptides that are subsequently separated through high performance liquid chromatography (HPLC). Overall, in liquid chromatography technique, separation is based on the varying affinities of analytes for the stationary and mobile phases. In studying food proteomes, the most common HPLC modes are reversed-phase (RP), ion

exchange (IEC), affinity, size-exclusion (SEC), and hydrophilic interaction liquid chromatography (HILIC) (Cavaliere et al. 2018). The separated peptides undergo fragmentation, and subsequent MS/MS spectra are recorded for each fragmented peptide. These spectra are then utilized to search protein databases for identification purposes. HPLC-based separation coupled with electrospray ionization mass spectrometry (ESI-MS) analysis of peptide mixtures has emerged as the standard and the most popular method in untargeted proteomics experiments (Neagu et al. 2022).

On the other hand, targeted proteomics serves as a technique to validate a preselected group of peptides and/or proteins revealed in the discovery phase, offering high sensitivity, precise quantification, and reproducibility. Within a targeted workflow, the mass spectrometer (MS) is set to identify precise peptide ions originating from the proteins being investigated. As extensively reviewed by Borràs and Sabidó (2017) targeted proteomics methods primarily encompass selected reaction monitoring (SRM) and parallel reaction monitoring (PRM). It should be highlighted that MS-based proteomics stands out as a promising alternative to the aforementioned methods in analyzing allergenic proteins. Its distinction lies in being the most comprehensive and sensitive approach for quantitatively profiling proteins, regardless of the complexity of food matrices (Croote and Quake 2016).

PROTEOMIC ANALYSIS OF INSECT-DERIVED FOOD ALLERGENS: STATE OF ART

As recently reviewed by Jackson (2023) several animal- and plant-based products such as beef, dairy, chicken, and wheat emerged as primary culprits for adverse food reactions in dogs, while beef, fish, and chicken were found to be predominant allergens in cats. It is postulated that allergy following insect ingestion could emerge due to either primary sensitization or cross-reactivity with other allergens, where IgE antibodies recognize similar allergenic molecules, thus inducing an immune response. This phenomenon is referred to as the panallergen concept, denoting families of allergenic proteins that exhibit a high sequence identity (Premrov Bajuk et al. 2021). In this context, proteomic tools have been extensively used to identify and characterize proteins derived from insects that may be acknowledged as a putative allergens. For instance, Barre et al. (2021) employed a combination of SDS-PAGE and nano-LC-MS/MS (nano-liquid chromatography-tandem mass spectrometry) to explore the array of proteins found in commonly consumed edible insects, such as silkworms (*Bombyx mori*), crickets (*Acheta domesticus*), African migratory locusts (*Locusta migratoria*), yellow mealworms (*Tenebrio molitor*), red palm weevils (*Rhynchophorus ferrugineus*), and giant mealworm beetles (*Zophobas atratus*). The authors emphasized that the majority of these potential allergens are panallergens, which are prevalent not only in insects but also in other arthropods. Nonetheless, the study also revealed several allergens specific only for insects, including chemosensory proteins (CSP), odorant or pheromone-binding proteins (OBP), and hexamerin, which is the primary storage protein in insect fat bodies. Furthermore, proteins such as apolipophorin III, larval cuticle proteins, and receptors for activated protein kinases exhibited notable specificity for edible insects, being either absent or less prevalent in other arthropods, mollusks, and nematodes. According to Barre et al. (2021), due to their ability to potentially induce allergic reactions in sensitized individuals, these specific proteins could serve as reliable markers for detecting insect-derived proteins in various food products. Premrov Bajuk et al. (2021) conducted a similar study utilizing SDS-PAGE followed by LC-MS/MS to identify proteins from yellow mealworm (*Tenebrio molitor*) larvae, which are presently part of commercially available dog

feed formulations. The authors also utilized the AllermatchTM tool to assess the allergenic potential of proteins, considering them hypersensitive if they share more than 35% identity with a known allergen within an 80 amino acid sequence window or beyond. As a result, Premrov Bajuk et al. (2021) identified 12 proteins considered as a potential allergens according to the AllermatchTM algorithm. These proteins encompass tropomyosins, Tm-E1a cuticular protein, odorant-binding protein 14, apolipophorin-III, glucose dehydrogenase, alpha-amylase, hexamerin 2, serpin1, 86 kDa early-stage encapsulation inducing protein, cockroach allergen-like protein, and larval cuticle protein F1. On the other hand, Stobernack et al. (2023), employed a workflow that integrated both untargeted (LC-MS) and targeted (immunoaffinity enrichment) proteomics strategies to first select peptide targets derived from dried silkworm pupae and then to precisely identify several peptides characteristic of these insects. The authors have identified 37 abundant and reliably detectable peptides corresponding to eleven proteins, including low molecular 30 kDa lipoproteins PBMHP-6, PBMHP-12, PBMHPC-21, and PBMHPC-23, sex-specific storage protein 2, cuticular proteins hypothetical 16 and 3, larval cuticle protein, uncharacterized protein, fibroin light chain, and sex-specific storage protein 1. The authors subsequently selected two peptides, DHFEAFGQK (derived from sex-specific storage protein 2) and LYNSILTGDYDSAVR (from low molecular mass lipoprotein PBMHP-12), as potential targets due to their possession of favourable immunogenic and mass spectrometric properties. Furthermore, Stobernack et al. (2023) demonstrated that the IAE-based targeted analysis of these selected peptide markers enabled the precise identification of silkworm pupae at a concentration as low as 0.05% in different feed types for aquaculture, poultry and swine animals. Another study conducted by Varunjikar et al. (2022) further confirms that proteomic workflow and associated bioinformatics methodologies can effectively serve as valuable tools in detecting and differentiating insect proteins present in both feed and food. This study aimed to develop an analytical LC-MS/MS proteomics assay dedicated for analyzing insect meal from five different species (black soldier fly larvae, yellow mealworm, lesser mealworm, house cricket, morio worm), focusing on identifying both shared and distinct insect species-specific proteins, and detecting potential allergens within these species. The findings from this study revealed new markers crucial for developing targeted MS analyses to detect insect species in food and feeds. Additionally, Varunjikar et al. (2022) pointed out the consistent presence of well-known allergens like arginine kinase, tropomyosin and troponin C across in all analysed insect species. The findings from the previous studies are further corroborated by recent research from Kamemura et al. (2019), who employed LC-MS/MS techniques to identify tropomyosin as the main allergen in both crickets (*Gryllus bimaculatus*) and shrimps, highlighting its capacity to induce allergic reactions in individuals with crustacean allergies. This, in turn, has led to an additional research inquiry: whether different processing methods, like heating and protease treatment, could diminish its immunoreactivity. Addressing this, Hall and Liceaga (2021) employed selective precipitation techniques to extract tropomyosin from tropical crickets (*Gryllus sigillatus*) exposed to varied heating and protease treatments. To assess the alterations in tropomyosin, the researchers employed AllermatchTM tool to predict 31 epitope regions. Simultaneously, proteomic analysis (SDS-PAGE combined with LC-MS/MS) showed a reduction in intact epitope regions notably in microwave-heated and protease-treated cricket samples. Additionally, the authors assessed tropomyosin's immunoreactivity using classical methods like immunoblotting and ELISA techniques, revealing a noteworthy decrease in IgE and IgG reactivity subsequent to protease treat-

ments coupled with microwave heating (Hall and Liceaga 2021). Similarly to humans with crustacean allergies potentially developing hypersensitivity upon cricket consumption (Kamemura et al. 2019), dogs allergic to mites may also clinically exhibit cross-reactivity with mealworm proteins (Premrov Bajuk et al. 2021). However, in a study conducted by Premrov Bajuk et al. (2021), intending to investigate the connection between *Tenebrio molitor* proteins and the immune response in dogs displaying clinical allergy symptoms and sensitivity to storage mites, the authors did not observe a distinct association when compared to healthy dogs. While the authors confirmed the binding of canine serum IgEs to several mealworm proteins, the differences between allergic and healthy dogs were not statistically significant. Nonetheless, within the protein extracts of yellow mealworms, they detected numerous allergen-specific IgE antibodies using immunoblotting. These antibodies are recognized for their ability to cross-react in humans who have allergies to either crustaceans or dust mites (Premrov Bajuk et al. 2021).

Currently, there's a lack of scientific research on the utilization of proteomics-based techniques to comprehend the pathology of allergic diseases in dogs and cats caused by insect-protein, with a predominant focus on dogs. Further comprehensive research is required for precise identification and characterization of allergens, thorough allergen quantification, and enhanced molecular diagnostics, including the identification of efficacy biomarkers in this field.

CONCLUSIONS

In conclusion, the current focus on proteomic workflows investigating insect-induced food allergies predominantly centers around humans and, to a lesser extent, canines. This highlights a pressing necessity for broader research into the pathology of allergic diseases in felines and canines. Nonetheless it is clear that the advancements in proteomics have significantly transformed the study and management of food allergies, unveiling new allergen isoforms in various food matrices through high-throughput methods. Anticipated technological advancements in this field hold promise for rendering feed products healthier and safer for both cats and dogs.

From the literature analysis, tropomyosin and arginine kinase stand out as the primary allergens triggering cross-reactions in individuals allergic to both shellfish and insects like crickets and dust mites. However, several new potential candidates have emerged, including chemosensory proteins, odorant or pheromone-binding proteins, hexamerin, apolipophorin III, larval cuticle proteins, and receptors for activated protein kinases. These could potentially become reliable markers for identifying insect-derived proteins in pet food products. Additional research is required to precisely identify, characterize and quantify insect allergens, and further develop molecular diagnostics, including the identification of biomarkers for diagnosis and prediction of disease course. These advancements will significantly contribute to improving our comprehension of allergic diseases in pets, ultimately ensuring their well-being and health in the future.

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AKTUALNE PERSPEKTYWY ZASTOSOWANIA PROTEOMIKI DO IDENTYFIKACJI ORAZ CHARAKTERYSTYKI BIAŁEK POCHODZENIA OWADZIEGO WYKAZUJĄCYCH POTENCJAŁ ALERGIZUJĄCY

Streszczenie. Obecnie właściciele kotów i psów mają dostęp do szerokiej gamy produktów, takich jak karmy suche, mokre i specjalistyczne formuły dietetyczne, umożliwiających zaspokojenie konkretnych potrzeb żywieniowych ich pupili. Globalny trend w żywieniu zwierząt towarzyszących karmami organicznymi, opartymi na naturalnych składnikach zamiast przetworzonych, zyskuje coraz większe uznanie. Podkreśla się też potrzebę wykorzystywania alternatywnych źródeł białka do ich produkcji. W tym kontekście owady wydają się obiecującą alternatywą, choć mogą wiązać się z potencjalnym ryzykiem występowania niepożądanych reakcji pokarmowych, w tym również alergii. Zaawansowane analizy proteomiczne mogą stać się kluczowym narzędziem w identyfikacji, charakterystyce oraz ilościowej analizie białek pochodzenia owadziego wykazujących potencjał alergizujący, umożliwiając tym samym późniejszą diagnozę nadwrażliwości u psów i kotów. Niniejsza praca poświęcona jest ocenie aktualnego stanu wiedzy w tym zakresie.

Słowa kluczowe: proteomika, elektroforeza, chromatografia cieczowa połączona ze spektrometrią mas, białko owadzie, alergia pokarmowa.