Recombinant Production and Biochemical Characterization of Ani s 1 from *Anisakis simplex*

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Resumo

Anisakis simplex é um nemátode zoonótico responsável por quadros gastrointestinais e alérgicos em humanos, associados ao consumo de pescado infetado. Entre os seus alergénios, o Ani s 1 destaca-se como o principal antigénio reconhecido por Imunoglobulina E (IgE), altamente estável e resistente ao processamento térmico. Neste trabalho, larvas do terceiro estádio (L3) de Anisakis foram obtidas a partir de *Merluccius merluccius*, o gene ani s 1 foi amplificado a partir de cDNA (ácido desoxirribonucleico complementar) e clonado no vetor pHTP1 para expressão em *Escherichia coli* (*E. coli*.) A análise bioinformática confirmou a presença de um domínio do tipo Kunitz seguido de um motivo repetitivo específico de nematodes ("*worm-specific repeat*"), além de prever potencial atividade antimicrobiana e um peptídeo sinal N-terminal. A expressão recombinante foi testada com e sem região sinal, mas apenas a forma madura foi produzida com sucesso. A proteína expressa acumulou-se exclusivamente na fração insolúvel, sendo necessário proceder à solubilização com ureia (5M), seguida de diálise gradual e liofilização. A SDS–PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) e o dot blot imunológico confirmaram o correto redobramento e manutenção da antigenicidade. Como prova de conceito para aplicação bioanalítica, o Ani s 1 foi utilizado como molde num sensor baseado em polímeros de impressão molecular (MIP), eletropolimerizado em elétrodos de ouro. A caracterização eletroquímica revelou ligação da proteína e remoção parcial, com o H_2SO_4 (ácido sulfúrico) a superar a ureia na etapa de extração. Ensaios preliminares de religação demonstraram redução dependente da concentração no sinal redox, sugerindo reconhecimento específico, embora seja necessária otimização adicional. No conjunto, este trabalho estabelece um protocolo reprodutível para a produção recombinante de Ani s 1 corretamente renaturado e representa os primeiros passos para a sua integração em biossensores MIP como alternativa promissora aos imunodiagnósticos convencionais.

Palavras-chave: *Anisakis simplex*, proteína recombinante, domínio Kunitz, polímero de impressão molecular, biossensor, alergia alimentar, parasitologia.

Abstract

Anisakis simplex is a zoonotic nematode responsible for gastrointestinal and allergic disease in humans through the ingestion of infected seafood. Among its allergenic profile, Ani s 1 is the dominant immunoglobulin E-binding (IgE-binding) protein, highly stable and resistant to processing. In this work, Anisakis third stage larvae (L3) were dissected from *Merluccius merluccius*, and the Ani s 1 gene was amplified via complementary DNA (cDNA) and cloned into the pHTP1 vector for expression in *Escherichia coli* (*E. coli*.) Bioinformatic analysis confirmed that Ani s 1 contains a Kunitz-type domain followed by a worm-specific repeat, and prediction tools indicated antimicrobial peptide potential and a putative N-terminal signal peptide. Recombinant expression with and without the signal peptide was tested, but only the mature form was successfully expressed. The expressed protein was exclusively found in the insoluble fraction, requiring urea-based solubilisation (optimal at ~5 M) followed by gradual dialysis and lyophilisation. SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and immunodot blot confirmed correct refolding and preservation of antigenicity. As a proof of concept for bioanalytical integration, Ani s 1 was then used as template in a molecularly imprinted polymer (MIP) sensor prepared by electropolymerisation on gold electrodes. Electrochemical characterisation demonstrated protein binding and partial removal, with H_2SO_4 (Sulphuric acid) outperforming urea in template extraction. Preliminary rebinding assays revealed a concentration-dependent decrease in redox current, supporting the feasibility of Ani s 1 MIP interaction, though further optimisation is required. Overall, this work establishes a reproducible workflow for the recombinant production of refolded Ani s 1 and provides the first steps toward its integration in MIP-based allergen biosensing, a promising alternative to antibody-dependent diagnostics.

Keywords: *Anisakis simplex*, recombinant protein, Kunitz domain, molecularly imprinted polymer, biosensor, food allergy, parasitology

Table of Contents

List of Tables

List of Figures

Introduction

Fish Consumption and Food Safety Risks

Fish plays a crucial role in global nutrition, providing essential proteins, fatty acids, and micronutrients that are fundamental to human health. However, increased seafood consumption has also increased the risk of exposure to marine-borne parasites. Among these, nematodes of the genus *Anisakis* are particularly relevant, posing both food safety and public health challenges [1].

Portugal stands out as the European Union (EU) country with the highest consumption of fish per capita, exceeding 50 kg per person annually (Figure 1) [2]. The widespread consumption of raw or undercooked fish dishes such as sushi, sashimi, or ceviche further amplifies the probability of parasite ingestion. Although EU legislation (Regulation EC No 853/2004) mandates the freezing of fish products before commercialization, which kills *Anisakis* larvae, the allergenic components of the parasite are not destroyed even after thermal or freezing processing [3][4].

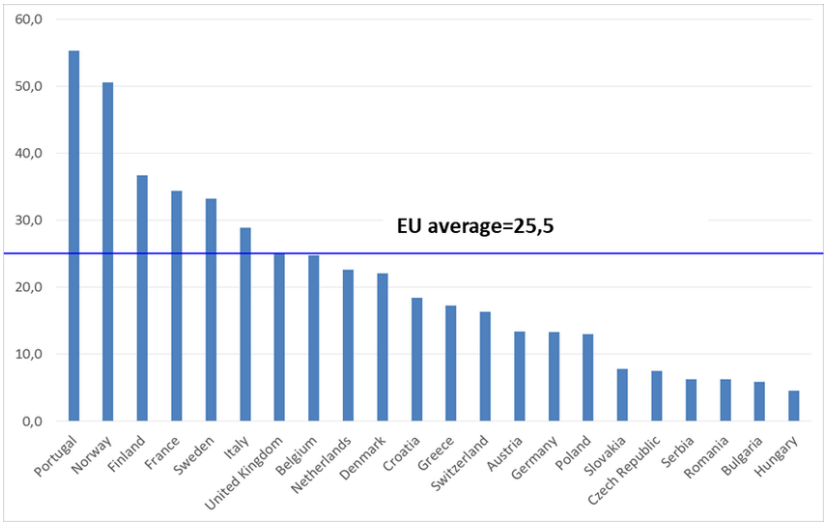


Figure 1- Per capita fish consumption across European Union countries (kg/person/ year), highlighting Portugal as the highest consumer. Adapted from Ivancsó Zs. (2021): Fish in diet of Hungarians: past, present, future.

High infection rates in commercially valuable species such as hake (*Merluccius merluccius*), horse mackerel (*Trachurus trachurus*), and sardine (*Sardina pilchardus*) highlight the necessity for robust detection and prevention strategies. In fact, visual inspection of infected fish often reveals heavy larval infestations in the musculature, posing a major challenge for food safety monitoring. This intersection of public health, marine ecology, and biotechnology has made *Anisakis* a model organism for studying food-borne parasitic risks in Europe [5].

Anisakis simplex: Biology and Life Cycle

Anisakis simplex is a parasitic nematode that belongs to the family Anisakidae. Its life cycle involves several aquatic hosts and multiple developmental stages, from eggs to the infective third-stage larva (L3). Eggs are released through the feces of marine mammals and hatch into free-swimming larvae that are ingested by crustaceans (first intermediate hosts). When these are consumed by fish or squid, the parasite progresses to the L3 stage and migrates into the host's muscle or visceral tissues [6].

Humans are accidental, dead-end hosts who become infected upon consuming fish containing L3 larvae. At this stage, larvae may survive gastric digestion and attempt mucosal penetration, causing local inflammatory responses. Since Anisakis cannot mature in humans, pathology results primarily from tissue invasion and immune activation (Figure 2).

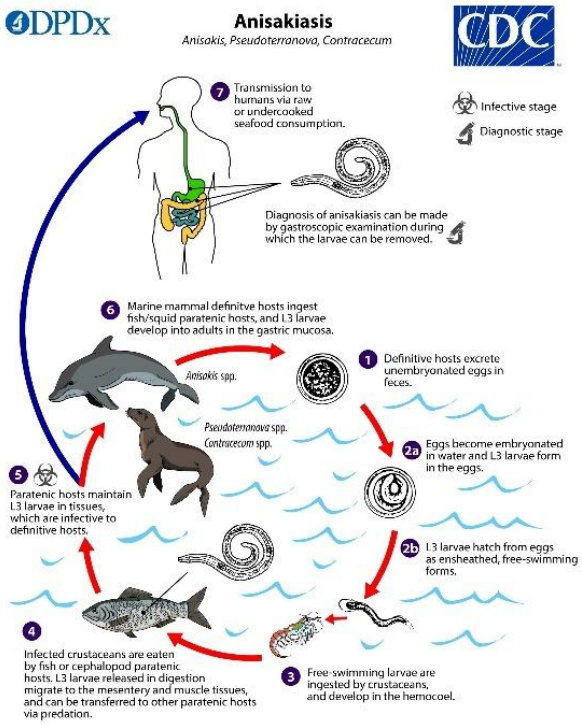


Figure 2- Life cycle of *Anisakis simplex*, involving marine mammals as definitive hosts, crustaceans as first intermediate hosts, fish or squid as paratenic hosts, and humans as accidental hosts. Adapted from CDC DPDx, Division of Parasitic Diseases and Malaria (2023)

Understanding the developmental biology of Anisakis is essential for epidemiological surveillance and experimental work, as antigenic profiles vary across life stages. The L3 larval stage, used in this project, is both the infective form and the main allergenic source [7].

Collection of Parasites for Molecular Studies

The collection and accurate identification of Anisakis larvae are critical prerequisites for any molecular or immunological investigation. Using freshly isolated larvae from naturally infected fish ensures the preservation of nucleic acids and proteins, while also reflecting the natural genetic diversity of wild populations circulating in a particular ecosystem.

The *Anisakis simplex* complex comprises several sibling species, such as *A. simplex sensu stricto*, *A. pegreffi*, and *A. berlandi*, that are morphologically indistinguishable but genetically distinct [8]. These taxa differ in their geographic distribution and host preferences, with *A. simplex s.s* prevailing in northern waters and A. pegreffi dominating in southern regions (Figure 3). Misidentification between the species has historically led to inconsistencies in epidemiological data and allergen characterization [9]. Consequently, molecular confirmation is indispensable for ensuring taxonomic accuracy and experimental reproducibility.

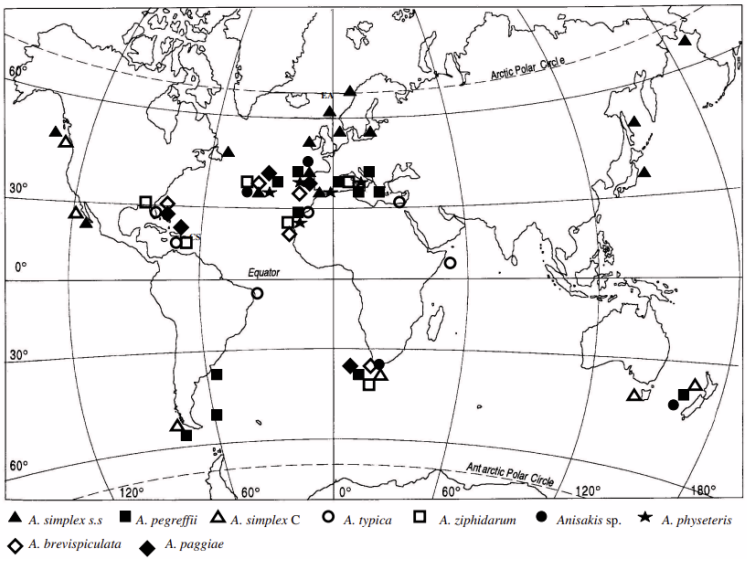


Figure 3- Geographic distribution of sibling species within the *Anisakis simplex* complex, showing prevalence of A. simplex s.s. in northern waters and A. pegreffii in southern regions. Adapted from Mattiucci & Nascetti (2006) [9].

Among the available molecular markers, the mitochondrial cytochrome oxidase subunit II (*cox2*) gene has become the standard for discriminating members of the *A. simplex* complex because of its high interspecific variability and maternal inheritance [10]. Sequencing of *cox2* not only validates the identity of larval specimens but also provides insights into population structure, migration routes and zoonotic potential. By verifying the genetic identity of *A. simplex s.s* prior to downstream characterization, researchers

ensure their work is species-specific, reproducible, and relevant to public health and diagnostic contexts.

Human Health Implications: Anisakis and Allergy

Anisakiasis is an emerging zoonotic disease resulting from accidental human ingestion of viable Anisakis larvae. Two main clinical manifestations are recognized: the gastrointestinal and allergic forms. The gastrointestinal form typically results from larval penetration of the stomach or intestine wall, leading to acute abdominal pain, nausea, and vomiting within hours after ingestion [11]. The allergic form, in contrast, can occur even in the absence of living parasites and is mediated by immune sensitization to heat-stable and enzymatically resistant parasite proteins.

The immune response to Anisakis infection is predominantly Th2-type, characterized by the production of specific IgE antibodies against parasite antigens. Upon re-exposure, these antibodies trigger histamine release from mast cells and basophils [12], causing urticaria, angioedema, or even anaphylaxis (Table 1) [13]. Notably, sensitized individuals may react to both infected fish and processed fish products due to the persistence of allergenic proteins such as Ani s1 and Ani s7 after freezing or cooking [14].

Table 1- Comparison between the gastrointestinal and allergic forms of anisakiasis.

Clinical form	Mechanism	Typical symptoms	Presence of parasite
Gastrointestinal	Tissue invasion (stomach/intestine)	Abdominal pain, nausea, vomiting	Yes (live larvae)
Allergic	IgE-mediated hypersensitivity	Urticaria, angioedema, anaphylaxis	Not required

Epidemiological studies in Spain and Japan report thousands of anisakiasis cases annually, though underdiagnosis is common. The prevalence of anti-Anisakis IgE in coastal populations exceeds 10% in certain areas [15]. Consequently, Anisakis-related allergy has become a significant public health concern in fish-consuming countries, emphasizing the need for reliable detection tools to prevent allergen exposure even in parasite-free products.

Ani s 1: A Major Allergen of unsolved Function

Ani s 1, the first identified allergen from *A. simplex*, is a small (~24kDa) protein recognized as the dominant IgE-binding antigen in human anisakiasis [16]. It belongs to the Kunitz-type serine protease inhibitor family, a group of proteins characterized by a compact β-sheet-rich-fold stabilized by three disulfide bonds formed between six conserved cysteine residues (Figure 5) [17].

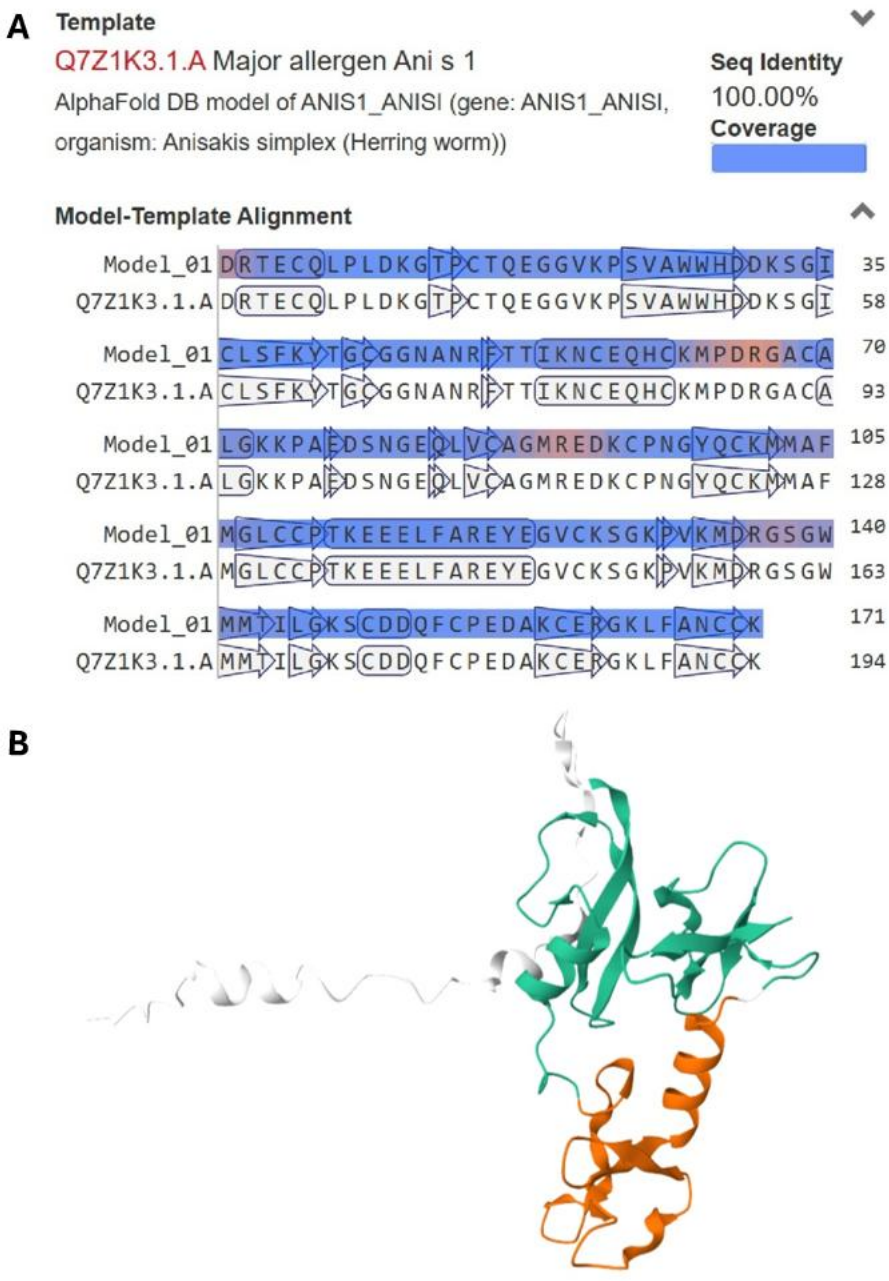


Figure 4- Structure analysis of Ani s 1. A: - Secondary structure of Ani s 1 (Arrows denote beta-sheets; Rectangles denote alpha-helix). B: - Predicted three-dimensional structure of Ani s 1 (SoSSurce: AlphaFold protein structure database; ID:

AF-Q7Z1K3-F1-v6). The different domains are highlighted in colors: Kunitz domain (green), WR1 (worm-specific repeat type I) domain (red).

The biological role of Ani s 1 remains speculative but may be linked to parasitic defense mechanisms against host digestive enzymes. As a serine protease inhibitor, Ani s 1 could help larvae resist proteolytic degradation in the gastrointestinal tract. Additionally, the protein's structural robustness confers resistance to denaturation, explaining its persistence through cooking and gastric conditions. Immunolocalization studies have shown Ani s 1 accumulation in excretory-secretory (ES) glands, suggesting a potential role in modulating host immune responses [18][19].

The combination of immunogenicity, biochemical stability, and resistance to degradation positions Ani s 1 as a critical model for allergenicity studies and as a valuable molecular template for biosensor development aimed at detecting trace allergen presence in processed food samples.

Structural and Biochemical Considerations

The structure of Kunitz-type inhibitors like Ani s 1 contributes to their exceptional physicochemical stability. The three disulfide bridges maintain a compact fold that remains intact under a broad range of pH and temperature conditions, and even after proteolytic digestion. However, these same properties complicate recombinant expression in bacterial hosts like *Escherichia coli* (*E. coli*), where the cytoplasmic environment lacks the oxidative machinery required for disulfide bond formation [20][21].

Misfolded proteins tend to aggregate into insoluble inclusion bodies during bacterial expression. This phenomenon can be partially mitigated by lowering the induction temperature or using weaker promoters to slow protein synthesis, allowing proper folding. Computational modeling of the Ani s 1 primary sequence also predicts a potential N-terminal signal peptide, suggesting it is natively secreted from the parasite's tissues, a feature that was tested in this work by comparing constructs with and without the predicted signal sequence. Understanding how the Ani s 1's structure influences its solubility and folding is fundamental for optimizing recombinant production and preserving its allergenic epitopes during refolding and purification steps.

Recombinant Challenges in *E. coli*

The expression of recombinant eukaryotic proteins in *E. coli* remains one of the most versatile and widely adopted platforms in molecular biotechnology, owing to its rapid growth, well-characterized genetics, and cost-effectiveness. However, the system

presents significant limitations when expressing complex or cysteine-rich proteins, whose correct folding depends on the formation of multiple disulfide bonds. The reducing environment of the bacterial cytoplasm inhibits proper disulfide bridge formation, often leading to the accumulation of misfolded, insoluble aggregates known as inclusion bodies [20][21].

To address these intrinsic challenges, several *E. coli* strains have been engineered to improve the oxidative folding of heterologous proteins. Among them, RNase III-deficient strains such as HT115(DE3) are frequently used for expressing double-stranded RNA or unstable transcripts, as the absence of RNase III enhances mRNA stability and translational efficiency [22][23]. Other engineered hosts, including Origami™ and SHuffle™ strains, provide a more oxidizing cytoplasmic environment conducive to disulfide bond formation, while periplasmic targeting can further facilitate folding in certain contexts [21].

Optimization of expression parameters, including inducer concentration, temperature, and expression time, is also critical for balancing yield and solubility. Lower induction temperatures, for instance, slow protein synthesis rates, allowing the nascent polypeptides more time to fold correctly [24]. Expression of recombinant proteins typically relies on inducible systems such as T7 promoter-based vectors, exemplified by the pHTP1 construct used in this study (Figure 5). This plasmid incorporates a strong T7 promoter, a lac operator for Isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced expression control, and an N-terminal His-tag for affinity purification, representing a standard molecular architecture for bacterial recombinant production systems.

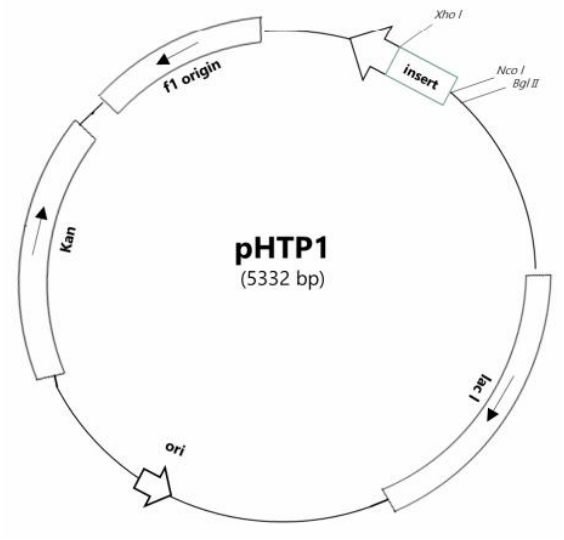


Figure 5- Schematic map of the pHTP1 expression vector (NZYTech), a T7 promoter-based system designed for high-level recombinant protein expression in E. coli hosts. Source: NZYTech, product datasheet for pHTP1 expression vector.

In the case of recombinant allergens, maintaining structural integrity during expression is particularly important, since conformational epitopes largely determine their immunogenicity. These combined biological and physicochemical constraints make their production representative model for understanding the broader challenges of expressing complex eukaryotic proteins.

From Recombinant Protein to Applied Detection

Recovering functional proteins from insoluble inclusion bodies remains one of the most persistent challenges in recombinant protein production. When recombinant proteins misfold in the reducing cytoplasm of *E. coli*, they aggregate into dense, insoluble deposits that require chemical or mechanical solubilization for recovery. The objective is to reestablish the protein's native conformation while preserving its structural and immunological integrity.

Several solubilization approaches have been developed, often involving chaotropic agents such as urea or guanidine hydrochloride to disrupt non-covalent interactions stabilizing aggregates. Urea is widely used due to its ability to denature proteins in a controllable manner and can later be gradually removed through stepwise dialysis, allowing the protein to refold progressively into a near-native conformation. Importantly, conventional refolding methods like dialysis and dilution are often time-consuming and can result in low recovered yields of active protein due to aggregation, frequently making a trial-and-error process necessary [25]. The efficiency of this refolding process depends on fine-tuning parameters such as denaturant concentration, temperature, ionic strength, and redox balance [26]. The addition of low molecular weight additives, such as L-arginine, to the refolding buffer has been shown to significantly improve yields by acting as an aggregation inhibitor [25][27]. Emerging microfluidic platforms now offer a high-throughput alternative, using controlled laminar flow to remove denaturants rapidly and minimize aggregation, thus accelerating process optimization [28].

As an alternative to strong denaturation, milder solubilization strategies have been developed. These aim to preserve the native-like secondary structure that some proteins retain within inclusion bodies, which can dramatically improve subsequent refolding yields [29][30]. For instance, a freeze-thawing method in the presence of low concentrations of urea (e.g., 2 M) has been demonstrated as a highly efficient way to solubilize inclusion bodies for several proteins, leading to a refolding efficiency more than twice that of traditional high-urea methods [30].

Alternative purification routes for recombinant proteins frequently exploit histidine tags, engineered into the constructs, through immobilized metal affinity chromatography (IMAC) systems. Under native conditions, His-tagged proteins bind to nickel–nitrilotriacetic acid (Ni–NTA) resins and are eluted with imidazole gradients. However, in the case of insoluble proteins, denaturing conditions (6–8 M urea or guanidine) are often required during IMAC purification to maintain the target protein in solution. Subsequent refolding through dialysis or rapid dilution remains essential to restore functionality (Figure 6) [31].

Therefore, a balance must be struck between achieving high yield and preserving biological function. For recombinant, disulfide-rich proteins that form inclusion bodies, urea-assisted refolding provides a pragmatic solution. This method enables effective solubilization while aiming to preserve the key conformational epitopes necessary for immunogenicity. Successfully navigating this trade-off is central to producing reliable reagents for downstream applications, such as biosensor immobilization and diagnostic development.

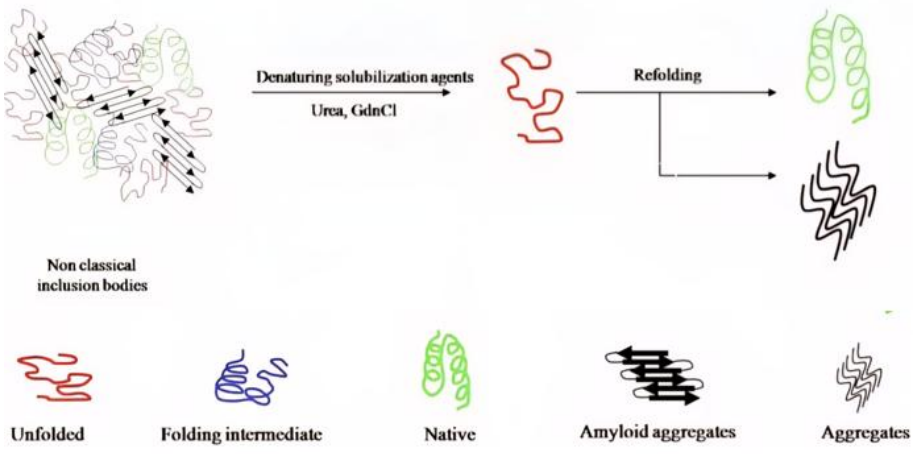


Figure 6- Conceptual workflow illustrating the urea-based solubilization and refolding of inclusion body–derived proteins. Adapted and simplified from Singh & Upadhyay (2016), Microbial Cell Factories, 15(1), 85 [31].

Molecularly Imprinted Polymers (MIPs) as Biosensors

The demand for robust and cost-effective analytical tools for allergen detection has accelerated the development of biosensing technologies that combine sensitivity, selectivity, and stability. Among the most promising synthetic approaches are Molecularly Imprinted Polymers (MIPs), artificial receptors engineered within a polymeric matrix to mimic the affinity and selectivity of natural antibodies.

Introduction to MIPs

Molecularly Imprinted Polymers (MIPs) are synthetic materials engineered to recognize specific target molecules, functioning similarly to antibodies. The principle behind MIPs involves creating polymer matrices that contain tailor-made binding sites complementary in size, shape, and chemical functionality to a particular analyte. This is typically achieved by polymerizing functional monomers in the presence of a template molecule. Once polymerization is complete, the template is removed, leaving behind cavities capable of selectively rebinding the target (Figure 7) [32][33].

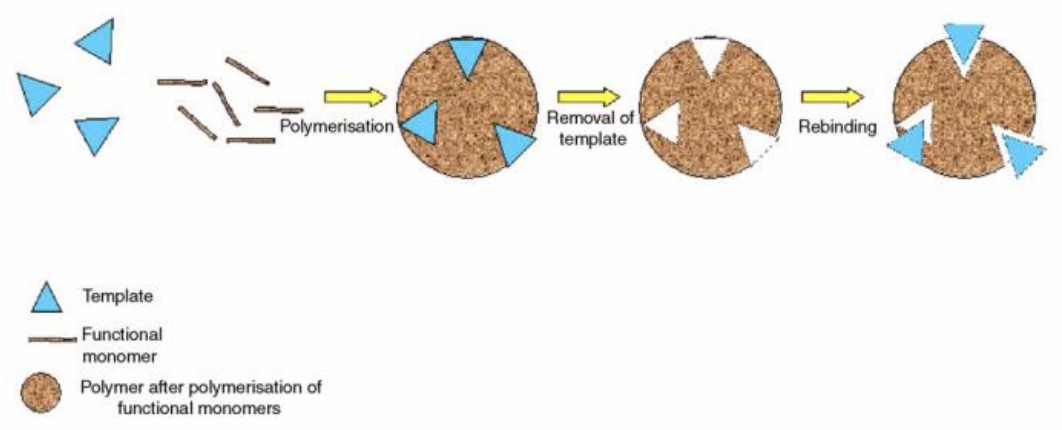


Figure 7- Schematic representation of the molecular imprinting process. Functional monomers self-assemble around the template molecule during polymerisation, forming a cross-linked matrix. After template removal, complementary binding cavities remain within the polymer and can selectively rebind the target analyte. Adapted from Bossi et al. (2006) Molecularly imprinted polymers for the recognition of proteins: The state of the art [33].

Advantages of MIPs Over Natural Antibodies

Compared to natural antibodies, MIPs offer numerous advantages:

•**Stability:** MIPs are resistant to high temperatures, extreme pH, and organic solvents [34][35].

•**Reusability:** They can be reused multiple times without significant loss in performance [36].

•**Cost-efficiency**: Synthesis does not require animal hosts or cell culture systems [36].

•**Shelf life:** MIPs are non-biological and can be stored for long periods without degradation [37].

These features make MIPs particularly attractive for applications in biosensing, where stability, reproducibility, and low-cost manufacturing are essential.

Challenges in Protein Imprinting

Despite their advantages, imprinting proteins might be challenging especially if it has a large size, conformational flexibility, and sensitivity to denaturation. Traditional bulk imprinting methods are often unsuitable for proteins because of poor template removal and heterogeneity in binding sites [33].

To overcome these issues, two strategies have been developed:

•**Epitope Imprinting:** Instead of using the whole protein, stable and solvent-exposed peptide fragments (epitopes) are used as templates, enabling more precise cavity formation [33].

•**NanoMIPs:** These are nanoscale MIPs with binding sites on or near the surface, improving accessibility, diffusion, and binding kinetics [33].

These innovations have significantly improved the performance of MIPs in protein recognition.

Case Studies from University of Porto

Several research groups at the University of Porto have successfully developed MIP-based sensors targeting food allergens:

1.Costa et al. (2022) designed a MIP sensor for the hazelnut allergen Cor a 14 using electropolymerized pyrrole. The sensor achieved high sensitivity and specificity, with detection limits in the femtogram range. Cross-reactivity was minimal, and performance exceeded that of some antibody-based methods [38].

2.Moreira et al. (2023) developed a plasmonic genosensor targeting the Cor a 14 gene. Using gold nanostructures functionalized with DNA probes, the sensor demonstrated attomolar sensitivity and exceptional selectivity against non-target species, including complex food matrices [39].

3.Dias et al. (2024) produced an electrochemical MIP sensor for Gly m TI (soy allergen) using polypyrrole films. The sensor detected allergen concentrations down to the attogram level, maintained functionality in processed foods, and showed superior specificity and robustness [40].

Signal Transduction Mechanisms

MIP-based sensors can utilize various transduction methods:

•**Electrochemical:** Changes in current or impedance reflect binding events. Used in Costa and Dias's studies, this approach offers portability and high sensitivity.

•**Optical (e.g., SPR):** Measures changes in refractive index upon target binding. Moreira's genosensor demonstrates the effectiveness of this label-free technique in nucleic acid detection.

Relevance to this Work

Building on these successful applications, this thesis explores the potential of MIPs in detecting the recombinant *A. simplex* allergen Ani s 1. Given the need for stable, selective, and low-cost diagnostic tools for seafood allergens, MIPs offer a promising alternative to antibody-based immunochemical methods. By applying epitope imprinting or surface imprinting strategies, this project aims to develop a robust biosensor for Ani s 1 detection, contributing to food safety and allergen monitoring.

In summary, the convergence of recombinant protein production and MIP technology sets the stage for next-generation biosensors that combine the precision of molecular design with the durability of synthetic materials.

Aim of this Work

The aim of this thesis was to produce the recombinant Ani s 1 allergen from *A. simplex* by constructing an expression vector, expressing the protein in a bacterial host, and purifying the resulting product. This purified allergen was then characterized biochemically to enable its application as a template in an MIP-based electrochemical biosensor.

The specific objectives were:

•To isolate Anisakis larvae from infected fish and confirm species identity via mitochondrial cox2 gene sequencing.

•To amplify and clone the Ani s 1 gene from *A. simplex* with and without the predicted signal peptide sequence.

•To express recombinant Ani s 1 in *E. coli* HT115 (DE3) under variable induction conditions.

•To optimize solubilization, purification, and refolding protocols.

•To verify recombinant Ani s 1 identity and purity by SDS–PAGE and Western blot.

•To provide purified protein as a molecular template for MIP biosensor development.

1. Materials and Methods

1.1. Parasite Collection and Dissection

Anisakis spp. larvae were obtained from naturally infected European hake specimens, purchased at a local fish market. A total of five fish were processed, each with a weight ranging 0.9-1.1 kg and a total length ranging 21-24 cm. The fish were dissected ventrally under sterile conditions, and, upon opening the visceral cavity, Anisakis larvae were readily observed attached to the internal organs. The muscle tissue was also inspected using a stereomicroscope to confirm the absence of tissue-embedded larvae.

Live Anisakis larvae were manually extracted using sterile tweezers, transferred to 1× phosphate-buffered saline (PBS), and stored on ice until processing. To ensure traceability and enable dual downstream applications, each larva was aseptically bisected using a sterile scalpel. One half was preserved in 100% ethanol at −20 °C for subsequent DNA extraction and molecular identification via mitochondrial cox2 gene barcoding. The remaining half was immediately processed for total RNA extraction, to obtain high-quality template for cDNA synthesis and Ani s 1 gene amplification.

1.2. Species Confirmation via DNA Barcoding

Genomic DNA was extracted from individual larval halves using the NZY Tissue gDNA Isolation Kit (NZYTech, Lisbon, Portugal), according to the manufacturer's protocol. DNA concentration and purity were assessed using a DeNovix DS-11 Series spectrophotometer (DeNovix Inc., Wilmington, DE, USA).

Species identification was performed through amplification of the mitochondrial cox2 gene, using the nematode-specific primers 210 (5′-CACCAACTCTTAAAATTATC-3′) and 211 (5′-TTTTCTAGTTATATAGATTGRTTYAT-3′) [41]. PCR reactions were prepared in a final volume of 25 µL, containing 12.5 µL of NZYTaq II 2× Green Master Mix (NZYTech, Lisbon, Portugal), 1 µL of each primer (10 µM), and approximately 50 ng of genomic DNA. The thermal cycling protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min.

PCR products were separated by agarose gel electrophoresis (1% w/v), stained with GreenSafe (NZYTech, Lisbon, Portugal), and visualized under UV illumination. Reactions showing a single PCR amplicon of expected size (approximately 0.5 kb) were submitted for Sanger sequencing (Stabvida, Caparica, Portugal). The resulting

sequences were analyzed using BLASTN and compared with reference entries in the NCBI GenBank database to confirm species-level identity.

1.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted from the remaining larval halves of *A. simplex* specimens using the NZY Total RNA Isolation Kit (NZYTech, Lisbon, Portugal), according to the manufacturer's instructions. RNA samples were stored at −80 °C for long-term preservation. RNA concentration and purity were measured using a DeNovix DS-11 Series spectrophotometer (DeNovix Inc., Wilmington, DE, USA).

Residual genomic DNA was removed via on-column DNase treatment included in the kit. First-strand cDNA synthesis was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). The resulting cDNA was either used immediately for PCR amplification or stored at −20 °C for short-term use, or at −80 °C for long-term storage.

1.4. Ani s 1 Gene Amplification and Primer Design

The coding sequence for the Ani s 1 gene was obtained from GenBank (Accession Number: AB100095.1) [42]. Specific primers were designed using the Thermo Fisher Scientific online tool, incorporating 20–25 bp overlaps compatible with Gibson Assembly into the pHTP1 expression vector (NZYTech, Lisbon, Portugal) to fuse the sequence in-frame with a vector-provided N-terminal leader sequence encoding a His-tag. Primers were synthesized by STAB VIDA (Caparica, Portugal).

To explore the potential influence of the native signal peptide on recombinant expression, two forward primers were designed: one excluding the signal peptide (targeting the mature protein only), and one including the signal peptide coding region.

PCR amplification of the Ani s 1 gene was performed using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) in a 50 µL reaction containing 10 µL of 5× Phusion HF Buffer, 10 mM dNTPs, 2.5 µM of each primer, 2 µL of cDNA template, and 0.5 µL of enzyme. Thermal cycling conditions were as follows: initial denaturation at 98 °C for 30 s, followed by 5 cycles of 98 °C for 10 s, 62 °C for 20 s, and 72 °C for 30 s, and then, 30 cycles of 98 °C for 10 s and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

Amplification success was confirmed by agarose gel electrophoresis (1% w/v), followed by visualization under UV light. The PCR Ani s 1 amplicon was purified using the

QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following manufacturer's protocol.

1.5. Gibson Assembly and Transformation into Cloning Strain

The Gibson Assembly reaction was prepared in a 10 µL final volume using 0.888 µL of purified Ani s 1 insert (52.986 ng/µL), 1 µL of linearized pHTP1 vector, 1 µL of 10× Reaction Buffer, 0.5 µL of NZYEasy enzyme mix, and 6.61 µL of nuclease-free water. Components were gently mixed by pipetting, avoiding vortexing to preserve enzyme activity. The reaction was incubated in a thermocycler at 37 °C for 60 minutes, followed by 80 °C for 10 minutes and 30 °C for 10 minutes.

Between 5 and 10 µL of the Gibson reaction product were added to 100 µL of chemically competent *E. coli* NZY5α [fhuA2Δ(argF-lacZ) U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17] cells (NZYTech, Lisbon, Portugal), previously thawed on ice. The mixture was gently tapped and incubated on ice for 30 minutes. Heat shock was performed at 42 °C for 40 seconds, after which the cells were returned to ice for 2 minutes. Cells were then recovered by adding 0.9 mL of SOC medium (tryptone 20 g/L, yeast extract 5 g/L, NaCl 0.5 g/L, KCl 0.186 g/L, pH 7.0) and incubating at 37 °C for 1 hour with shaking (225 rpm).

Following recovery, the transformation mixture was centrifuged at 5000 rpm for 1 minute, and 800 µL of the cell-free supernatant were discarded. The remaining 200 µL were resuspended and plated on LB agar plates supplemented with kanamycin (50 µg/mL). The cell suspension was spread using a Drigalski spatula, and plates were incubated inverted overnight at 37 °C

1.6. Colony Screening and Plasmid Verification

Transformed *E. coli* NZY5α colonies were screened by colony PCR to verify the presence of the Ani s 1 insert. Individual colonies were suspended in 20 µL of nuclease-free water and subjected to thermal lysis at 95 °C for 5 minutes. One microliter of the resulting lysate was used as template in a 25 µL PCR reaction containing 12.5 µL of 2× NZYMaster Mix (NZYTech, Lisbon, Portugal), 5.5 µL of nuclease-free water, and 1 µL of each primer (forward F: 5'-GCGAAATTAATACGACTCACTATAGGGG-3' and reverse R: 5'-GGTTATGCTAGTTATTGCTCAGCG-3') synthesized at Stabvida (Caparica, Portugal). Thermal cycling was performed as follows: initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 1 minute, and 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes.

PCR products were analyzed by agarose gel electrophoresis (1.0 %, w/v) to assess the presence and size of the amplified fragment. Colonies showing the expected amplicon size (approximately 0.8 kb) were selected for overnight culture in 5 mL of LB medium supplemented with kanamycin (50 µg/mL), incubated at 37 °C with shaking (180 rpm). Plasmid DNA was extracted using the NZYMiniprep Kit (NZYTech, Lisbon, Portugal) and quantified using a DeNovix DS-11 Series spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Positive clones were further validated by Sanger sequencing using the same primer pair employed for colony PCR.

1.7. Transformation into Expression Strain and Culture Preparation

The verified recombinant plasmid pHTP-Ani s 1 was transformed into *E. coli* HT115 (DE3) [F- mcrA mcrB, IN(rrnD-rrnE)1 rnc14::Tn10(DE3 lavUV5::T7 polymerase)] (purchased from the University of Minnesota, Mineapolis, MN, USA), an expression strain deficient in RNase III. Chemically competent cells were prepared following the standard Hanahan method (1983) [43] and used for heat-shock transformation at 42 °C for 40 seconds. Cells were recovered in 0.9 mL of SOC medium at 37 °C for 1 hour with shaking (225 rpm). After recovery, the suspension was centrifuged at 5000 rpm for 1 minute, and 800 µL of the cell-free supernatant were discarded. The remaining 200 µL were gently resuspended and plated onto LB agar supplemented with kanamycin (50 µg/mL). The cell suspension was spread using a sterile Drigalski spatula, and plates were incubated inverted overnight at 37 °C.

1.8. Recombinant Protein Expression and Induction

A single colony of HT115 (DE3) cells carrying pHTP-Ani s 1 was selected to inoculate 5 mL of LB broth containing kanamycin (50 µg/mL), incubated overnight at 37 °C with shaking (180 rpm). This pre-culture was then used to inoculate two 250 ml-capacity Erlenmeyer flasks containing 20 mL of LB-kanamycin (1:100 dilution), which was grown at 37 °C until reaching an OD_{600} of 0.6–0.8. Protein expression was induced by the addition of IPTG to a final concentration of 0.2 mM and 1 mM, respectively. Two post-induction growth temperatures were also tested: 20 °C and 30 °C. Cultures were shaked at 180 rpm using an Innova 40 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) for approximately 16 hours (overnight), in order to assess the impact of temperature and IPTG concentration on protein expression and solubility. To purify recombinant Ani s 1 protein, inoculated 100-mL LBK50 cultures were used.

After overnight incubation, bacterial cultures were transferred to 50 mL conical centrifuge tubes (Falcon type) and centrifuged at 4000 rpm for 10 minutes at 4 °C to pellet the cells,

using an Eppendorf 5810 R refrigerated centrifuge (Eppendorf AG, Hamburg, Germany). The supernatants were discarded, and the resulting pellets were stored at −20 °C for downstream analysis.

1.9. Cell Lysis and Solubility Assessment

Bacterial pellets were stored at −20 °C for at least 30 minutes prior to lysis to improve extraction efficiency using NZY Bacterial Cell Lysis Buffer (NZYTech, Lisbon, Portugal). Thawed pellets were weighed and resuspended at room temperature by gentle vortexing or pipetting. The lysis buffer was supplemented with lysozyme (50 mg/mL; 2 µL/mL of buffer) and DNase I (2 µL/mL) to facilitate cell wall degradation and reduce viscosity due to genomic DNA. The amount of lysis buffer used was approximately 1.5× the volume recommended by the manufacturer. The mixture was incubated at room temperature for 20 minutes on a rotating platform.

Following lysis, samples were centrifuged at 13,000 rpm for 15 minutes at 4 °C to separate soluble proteins from cell debris. The supernatant (soluble fraction) and pellet (insoluble fraction) were collected and stored at −20 °C for further analysis by SDS–PAGE and Western blot. This step allowed for the initial assessment of Ani s 1 solubility and informed subsequent optimization strategies.

1.10. Assessment of Extracellular Expression by TCA Precipitation

To assess whether Ani s 1, particularly in constructs containing a signal peptide, was secreted into the culture medium, the trichloroacetic acid (TCA) protein precipitation method was used. Briefly, following overnight induction, 1 mL of bacterial culture was centrifuged at 13,000 rpm for 5 minutes at 4 °C to pellet the cells. The supernatant was transferred to a clean microcentrifuge tube, and an equal volume of ice-cold 20 % (w/v) TCA was added. The mixture was incubated on ice for 30 minutes to precipitate proteins. The precipitate was collected by centrifugation at 13,000 rpm for 15 minutes at 4 °C. The resulting pellet was washed with cold acetone, air-dried, and resuspended in SDS–PAGE loading buffer for analysis.

1.11. Affinity Purification Using Ni-NTA Spin Columns

Recombinant Ani s 1 containing an N-terminal His-tag was initially purified by immobilized metal affinity chromatography (IMAC) under denaturing conditions, using the HisPur™ Ni-NTA Spin Purification Kit, 3 mL (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's protocol. Frozen pellets containing the insoluble fraction of cell lysis were resuspended in 6 mL of column equilibration buffer (1× PBS; 8 M urea;

25 mM imidazole; pH 7.5) and incubated for 20 minutes at room temperature with gentle agitation to promote protein solubilization.

The Ni–NTA resin columns were equilibrated to room temperature, uncapped, and the storage buffer was removed by centrifugation at 700 × g for 2 minutes. The resin was equilibrated with two bed volumes of column equilibration buffer, followed by centrifugation under the same conditions.

The clarified lysate was obtained by centrifuging the solubilized extract at 13,000 rpm for 5 minutes at 4 °C. The resulting supernatant was mixed with an equal volume of column equilibration buffer, adjusted to twice the resin-bed volume, and loaded onto the column. Binding was allowed to proceed for 30 minutes at room temperature under gentle end-over-end mixing.

After binding, the column was centrifuged at 700 × g for 2 minutes, and the flowthrough was collected. The resin was washed three times with wash buffer (1× PBS; 8 M urea; 50 mM imidazole; pH 7.5), using two resin-bed volumes per wash, followed by centrifugation at 700 × g for 2 minutes.

Elution was performed in three steps using one resin-bed volume of elution buffer (1× PBS; 8 M urea; 250 mM imidazole; pH 7.5) per elution, each followed by centrifugation at 700 × g for 2 minutes. Collected eluates were analyzed by SDS–PAGE (10 %) and Western blot using anti-His antibody to assess protein recovery and purity.

1.12. Stepwise Solubilization with a Urea Gradient

To solubilize inclusion bodies, the insoluble fraction (pellet) was resuspended in 1× PBS containing increasing concentrations of urea (1 M, 2 M, 4 M, and 5 M). Each sample was incubated at 4 °C with gentle agitation for 1–2 hours using a magnetic stirrer (IBX Instruments HD 1 Series) with centrifugation at 13,000 rpm for 15 minutes at 4 °C after each step. The solubilized fractions were analyzed by SDS–PAGE to determine the optimal urea concentration for Ani s 1 extraction while other contaminants remain insoluble. All fractions were stored at −20 °C until further use.

1.13. Protein Dialysis and Refolding

The urea-solubilized Ani s 1 was refolded by stepwise dialysis using a 10 mL D-Tube™ Dialyzer Mega, 6-8 kDa MWCO (Merck Millipore, Burlington, MA, USA). The sample was dialyzed against refolding buffer (Tris 20 mM; NaCl 150 mM; glycerol 1%; pH 8.0) containing progressively decreasing concentrations of urea (4 M, 2 M, 1 M, and finally a urea-free refolding buffer), with each step lasting 4-6 hours at 4 °C under constant stirring

using a magnetic stirrer (IBX Instruments HD 1 Series) to facilitate controlled renaturation. Following dialysis, samples were centrifuged at 13,000 rpm for 15 minutes at 4 °C to remove any precipitated material. The supernatant, containing the refolded protein, was collected for analysis and stored at −20 °C.

1.14. Lyophilization of Dialyzed Protein Samples

Following dialysis, the recombinant Ani s 1 protein was in a final volume of approximately 10 mL of dialysis buffer. To concentrate the protein for downstream applications, the solution was lyophilized. The dialyzed sample was transferred to a 50-mL conical tube (Falcon) and frozen at –80 °C for 16 hours. Lyophilization was then performed for 48 hours using a Benchtop Pro with Omnitronics™ freeze dryer (SP Scientific, VirTis, Warminster, PA, USA). Dialyzed protein samples were transferred into lyophilization chamber, pre-frozen at −80 °C for 16 hours. The resulting lyophilized protein powder was reconstituted in 2 mL of distilled water, achieving a 5-fold concentration.

1.15. Protein Analysis via SDS-PAGE, Western Blot and Immunodot Blot

Protein samples obtained throughout the workflow (including soluble and insoluble fractions, urea-solubilized extracts, and refolded protein) were analyzed by SDS–PAGE using a discontinuous gel system composed of a 10 % resolving gel and a 5 % stacking gel [44]. Resolving gels were prepared using 30 % acrylamide/Bis stock solution, 1.5 M Tris-HCl (pH 8.8), 10 % SDS, 10 % ammonium persulfate (APS), and TEMED. The stacking gel was formulated with 0.5 M Tris-HCl (pH 6.8) and otherwise identical components. Samples were mixed with 5× loading buffer (NZYTech, Lisbon, Portugal), heated at 95 °C for 5 minutes, and loaded in 15 µL volumes. Electrophoresis was carried out in a vertical gel system (Bio-Rad Laboratories, Hercules, CA, USA) using Tris–glycine–SDS running buffer (pH 8.3) under a constant voltage of 200 V. Gels were stained with BlueSafe protein stain (NZYTech, Lisbon, Portugal) and manually destained in distilled water.

For Western blot analysis, proteins resolved by SDS–PAGE were transferred onto nitrocellulose membranes in Nitrocellulose/Filter Paper Sandwich, 0.45 µm, 8.3 x 7.3 cm (Thermo Fisher Scientific, Waltham, MA, USA) using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Membranes were blocked with 5 % skimmed milk in TBS-T (Tris 20 mM; NaCl 150 mM; Tween-20 0.1%) for 1 hour at room temperature and incubated with an anti-His tag rabbit polyclonal antibody, HRP conjugated (Abbkine Scientific, Atlanta, GA, USA). After washing, bound antibodies were detected using Metal

enhanced DAB substrate kit (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's procedure.

To evaluate antigenic recognition, immunodot blot assays were performed using recombinant refolded Ani s 1 and sera from Anisakis-infected fish. Protein samples were directly spotted onto nitrocellulose membranes, allowed to dry, and blocked with 5 % of skimmed milk in TBS-T. Membranes were incubated with diluted fish sera, washed with TBS-T, and probed using an HRP-conjugated protein A (Thermo Fisher Scientific, Waltham, MA, USA). Reactive spots were visualized using Metal enhanced DAB substrate kit.

1.16. MIP Biosensor

Development of electrochemical biosensor based on MIP for Ani s 1 followed the protocol as in [38]. Briefly, gold screen-printed electrodes (220AT, Metrohm DropSens, Oviedo, Spain) (Figure 8) were first cleaned by thoroughly rinsing with ultrapure water, followed by sequential washing with acetone and ethanol. After each wash, the electrodes were dried using a nitrogen gas stream. Surface activation was then performed by cyclic voltammetry (CV), applying 20 cycles in ultrapure water over a defined potential range, using an Autolab PGSTAT potentiostat/galvanostat (Metrohm Autolab B.V., Utrecht, Netherlands) in a three-electrode configuration (gold working electrode, platinum counter electrode, Ag/AgCl reference electrode).

Figure 8- Gold screen-printed eletrode.

A 70 µL aliquot of the Ani s 1 protein solution (100 µg/mL in 20 mM Tris-HCl, pH 8.0) was deposited onto the activated electrode surface and incubated overnight at 4 °C to allow adsorption of the molecular template. Electropolymerization was then performed under the experimental conditions specified in Annex 1. Template removal was carried

out by incubating the polymer-coated electrodes with either 0.5 M H_2SO_4 or 6 M urea, followed by extensive rinsing with ultrapure water.

Electrochemical characterization was performed using square-wave voltammetry (SWV) in a 1:1 (v/v) mixture of 5 mM potassium ferricyanide/potassium ferrocyanide $Fe(CN)6^{3-}/Fe(CN)6^{4-}$ and 20 mM Tris-HCl (pH 8.0). Following template removal, rebinding assays were conducted by incubating the MIP-modified electrodes with 70 µL of Ani s 1 solutions at increasing concentrations, prepared by serial 1:10 dilution (100 µL of previous dilution + 900 µL Tris-HCl buffer per step). Each incubation lasted 10 minutes at room temperature, followed by rinsing with ultrapure water and SWV recording.

2. Results

2.1. Parasite Dissection and RNA Extraction

All five hake specimens were visibly infected with Anisakis larvae. Despite the presence of larvae embedded in muscle tissue, dissection focused exclusively on the visceral cavity, where numerous freely accessible larvae were observed without the need for further processing (Figure 9). Extracting larvae directly from muscle tissue was intentionally avoided to prevent mechanical damage during removal, which would compromise the downstream workflow that required each larva to be bisected for dual DNA/RNA extraction.

Larvae confirmed to belong to the same species were pooled prior to RNA isolation to maximize intra-species diversity within the cDNA sample used for amplification of the Ani s 1 gene.

Figure 9- European hake heavily infested with Anisakis larvae visible within muscle tissue. The photograph was taken by Fernando Atroch (University of Porto, 2024).

2.2. cDNA Synthesis and Ani s1 Gene Amplification

First-strand cDNA synthesis from total RNA was used as a template for PCR. Amplification of the Ani s 1 coding sequence generated single, specific amplicons of the expected sizes: ~567 bp for the construct containing the native signal peptide and ~543 bp for the mature form without the signal peptide. In both cases, analysis by 1% agarose gel electrophoresis confirmed the presence of a discrete band and the absence of primer-dimers or nonspecific products, indicating successful and specific amplification (Figure 10).

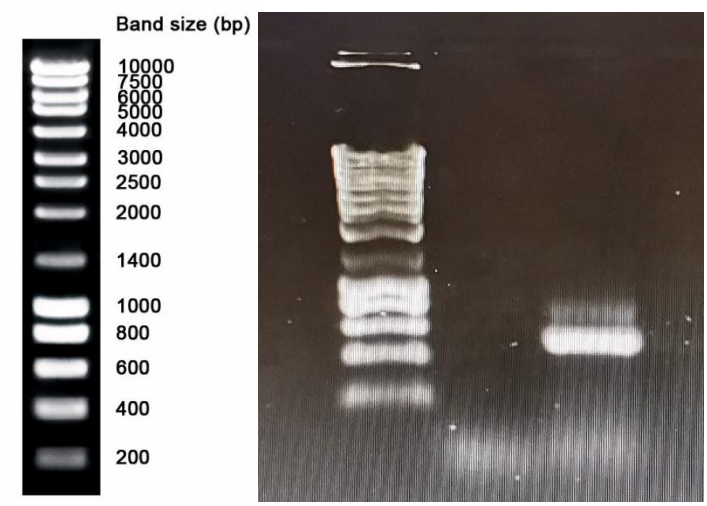


Figure 10- PCR amplification of Ani s 1 coding sequence, showing expected 567 bp band. NZYDNA Ladder III; Lane 1: Negative control; Lane 2: Tested PCR product.

2.3. Gibson Assembly and Cloning Verification

The *Ani s 1* gene was successfully cloned into the pHTP1 expression vector using Gibson Assembly. Following transformation into *E. coli* NZY5α competent cells, robust growth was observed on selective LB agar plates supplemented with kanamycin, indicating successful transformation (Figure 11).

To verify the presence of the insert, colony PCR was performed on three randomly selected transformants. All three colonies yielded amplicons corresponding to the expected size for the *Ani s 1* insert, confirming the successful integration of the gene into the plasmid vector (Figure 12).

Figure 11-Transformed E. coli colonies grown on kanamycin-selective agar plates, confirming successful plasmid uptake.

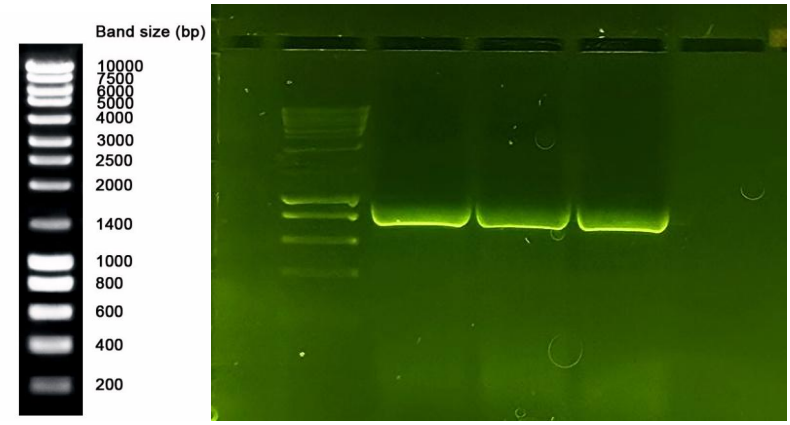


Figure 12- Colony PCR screening of three E. coli NZY5α transformants confirming the presence of the Ani s1 insert. Amplicons of approximately 570 bp were visualized on an agarose gel M: NZYDNA Ladder III; lanes 1–3: tested colonies.

2.4. Sequence Validation and Bioinformatic Characterization

Sanger sequencing of the recombinant pHTP-Ani s 1 plasmid confirmed the successful insertion of the Ani s 1 gene. Sequence alignment revealed 100% nucleotide identity with the reference coding sequence (GenBank: AB100095.1) and verified the correct in-frame insertion and orientation within the vector's multiple cloning site (Figure 13).

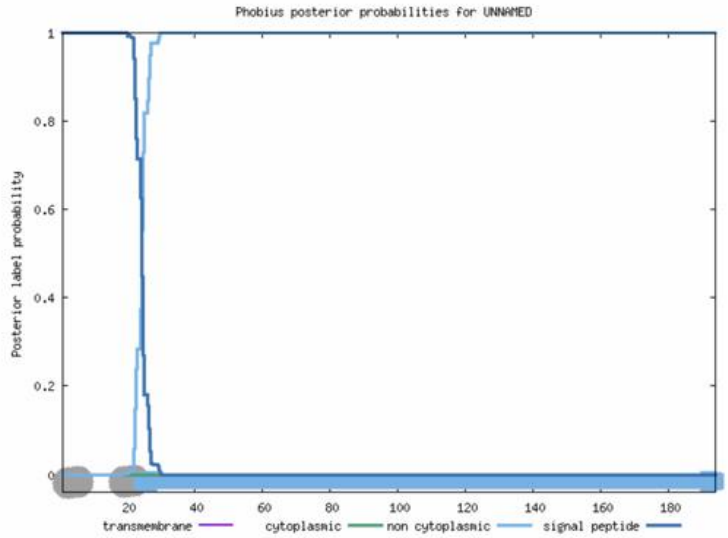
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Anisakis simplex mRNA for 21k allergen, complete cds	Anisakis simplex	1064	1064	100%	0.0	99.66%	1008	AB100095.1

Figure 13- NCBI BLASTn alignment of the sequenced insert against the *Anisakis simplex* Ani s1 reference (GenBank AB100095.1), showing ~99.7% identity and correct reading frame orientation.

Bioinformatic analysis of the deduced amino acid sequence predicted a high probability of an N-terminal secretion signal peptide. SignalP-6.0 software identified a canonical signal peptide with a maximal (Cleavage site) probability score of 0.9995, supporting the initial strategy of including the native signal peptide in one expression construct (Figure 14). However, subsequent small-scale expression trials revealed that constructs containing the signal peptide yielded no detectable recombinant protein, whereas the construct encoding the mature protein (without the signal peptide) showed robust expression. Consequently, all subsequent expression and purification work focused exclusively on the mature, signal peptide-free version of Ani s 1.

Prediction of UNNAMED

```
ID   UNNAMED
FT   SIGNAL       1     23
FT   REGION       1      6       N-REGION.
FT   REGION       7     18       H-REGION.
FT   REGION      19     23       C-REGION.
FT   TOPO_DOM     24    194      NON CYTOPLASMIC.
//
```



Sequence

Prediction: Signal peptide (Sec/SPI)

Cleavage site between pos. 23 and 24: GDA-DR. Probability: 0.6331

Protein type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.9995	0.0005

Figure 14- Output from SignalP 6.0, showing a 0.9995 probability for the presence of an N-terminal secretion signal in Ani s 1, supporting the initial design of two constructs with and without this region.

To investigate potential functional properties, the Ani s 1 amino acid sequence was analyzed for antimicrobial peptide (AMP) motifs using the CAMPR₃ server. All four integrated machine learning classifiers categorized Ani s 1 as an AMP with high confidence, yielding the following prediction scores: SVM (1.000), Random Forest (0.984), Discriminant Analysis (1.000), and ANN (binary classification: AMP) (Figure 15).

Predict Antimicrobial Peptides

Results with Support Vector Machine (SVM) classifier

Seq. ID.	Class	AMP Probability
1	AMP	1.000

Results with Random Forest Classifier

Seq. ID.	Class	AMP Probability
1	AMP	0.984

Results with Artificial Neural Network (ANN) classifier

Seq. ID.	Class
1	AMP

Results with Discriminant Analysis classifier

Seq. ID.	Class	AMP Probability
1	AMP	1.000

Figure 15- CAMP-R3 prediction output for Ani s 1. Results from four machine learning classifiers (SVM, Random Forest, Discriminant Analysis, and ANN) indicate a high probability of Ani s 1 behaving as an antimicrobial peptide (AMP), with AMP scores ranging from 0.984 to 1.000.

Domain architecture analysis of the Ani s 1 coding sequence, performed with the SMART database (EMBL-EBI), identified a conserved Kunitz-type serine protease inhibitor domain (Ku) and a nematode-specific WR1 (Worm Repeat 1) motif (Figure 16). This domain composition is characteristic of secreted proteins in nematodes. The open reading frame (ORF) analyzed was defined using NCBI's ORFfinder tool.

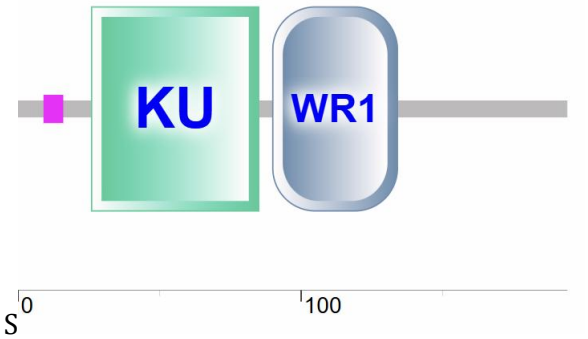


Figure 16- SMART domain architecture of Ani s 1. The analysis detected a Kunitz-type protease inhibitor domain (residues 26–85) and a worm-specific repeat type 1 (WR1) motif (residues 90–134).

2.5. Transformation for Recombinant Expression Optimization

To optimize the production of recombinant Ani s 1, *E. coli* HT115(DE3) cells harboring the pHTP1–Ani s 1 plasmid were induced under various conditions, testing IPTG concentrations from 0.2 to 1 mM and temperatures from 20 to 30 °C. SDS-PAGE analysis of the resulting fractions demonstrated that, irrespective of the induction parameters, Ani s 1 consistently accumulated in the insoluble fraction (cell pellet after lysis). A protein band of approximately 24 kDa, corresponding to the predicted molecular mass of the recombinant His-tagged Ani s 1, was observed exclusively in the insoluble fraction of induced cultures and was absent from the soluble fraction (cell lysate supernatant) and non-induced controls (Figure 17). This indicates that while expression was successful, the recombinant protein was primarily produced as insoluble aggregates.

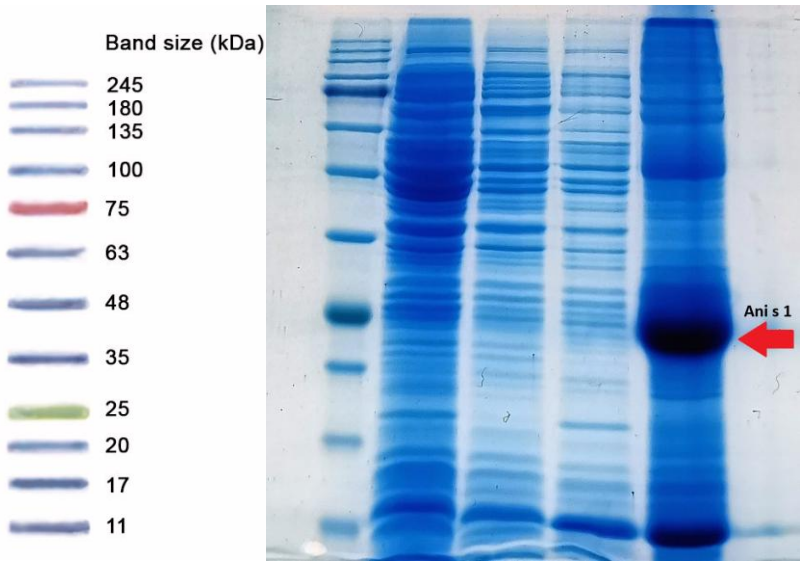


Figure 17- SDS–PAGE analysis of E. coli HT115 (DE3) cultures expressing Ani s 1 without the predicted signal peptide. Lanes: molecular weight marker; non-induced supernatant; induced supernatant; non-induced pellet; induced pellet. The ~24 kDa band (arrow) appears exclusively in the induced pellet.

In contrast to the mature construct, expression of the Ani s 1 variant containing the predicted N-terminal signal peptide yielded no detectable protein in the soluble or insoluble cellular fractions, or in the extracellular medium, as assessed by TCA precipitation and SDS-PAGE (Figure 18). Furthermore, induction with IPTG resulted in pronounced growth arrest, suggesting that the production of the signal-peptide-containing Ani s 1 is either toxic to the host cells or overwhelms its protein processing machinery.

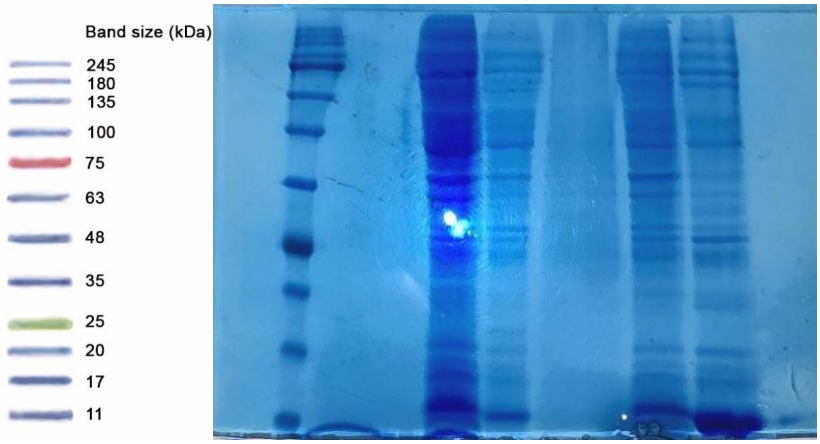


Figure 18- - Expression test of Ani s 1 construct containing the predicted N-terminal signal peptide. No detectable protein was observed in any fraction. Note: the gel was photographed before complete destaining, which causes the blue background.

Due to the absence of detectable expression from constructs containing the native signal peptide, all subsequent work utilized the mature form of Ani s 1 (lacking the signal peptide). This construct yielded robust, IPTG-induced expression; however, the recombinant protein was found exclusively in the insoluble fraction as inclusion bodies.

2.6 Protein Solubility and Western Blot Analysis

Western blot analysis with an anti-His tag antibody confirmed the expression of recombinant Ani s 1 in the insoluble fraction (Figure 19). However, the immunoblot revealed other immunoreactive bands in addition to the full-length protein at ~24 kDa. These additional bands likely represent C-terminal truncation products or misfolded aggregates that retain the N-terminal His-tag. Alternatively, a prominent cross-reactive band may correspond to an endogenous *E. coli* protein that is recognized by the antibody or an Ani s 1 dimer.

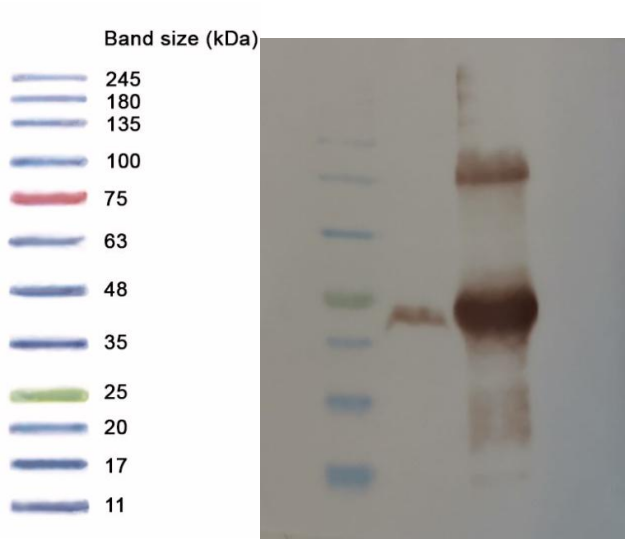


Figure 19- Western blot showing multiple His-reactive bands in the insoluble fraction of recombinant Ani s 1, indicating the presence of truncated or misfolded fragments.

These results indicated that IMAC purification under mild denaturing conditions could lead to co-elution of non-specific, histidine-rich *E. coli* proteins. Consequently, we employed a strategy to first isolate inclusion bodies, followed by urea-based solubilization and refolding to purify Ani s 1.

An initial solubilization screen using 2 M, 4 M, 5 M, and 8 M urea showed that protein extraction efficiency increased with denaturant concentration. The 5 M urea condition yielded the most intense Ani s 1 band with the lowest level of co-solubilized contaminants, as visualized by SDS-PAGE (Figure 20). A second, more refined series (1 M, 2 M, 2 M, 4 M, 4 M, and 5 M urea) was performed, including technical replicates at 2 M and 4 M to assess reproducibility. This optimized step-gradient yielded well-defined target protein bands with significantly reduced background, enabling the selection of the 2 M to 5 M urea fractions for subsequent dialysis and refolding (Figure 21).

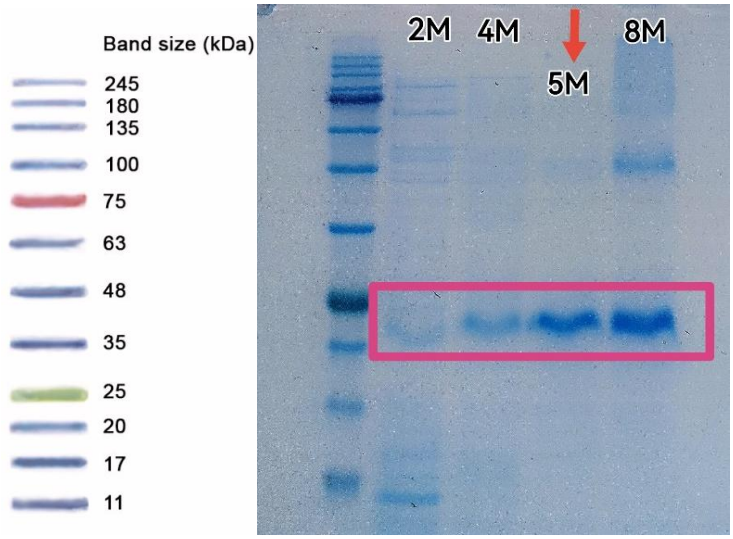


Figure 20- SDS-PAGE of Ani s 1 solubilization gradient (2 M, 4 M, 5 M, and 8 M urea).

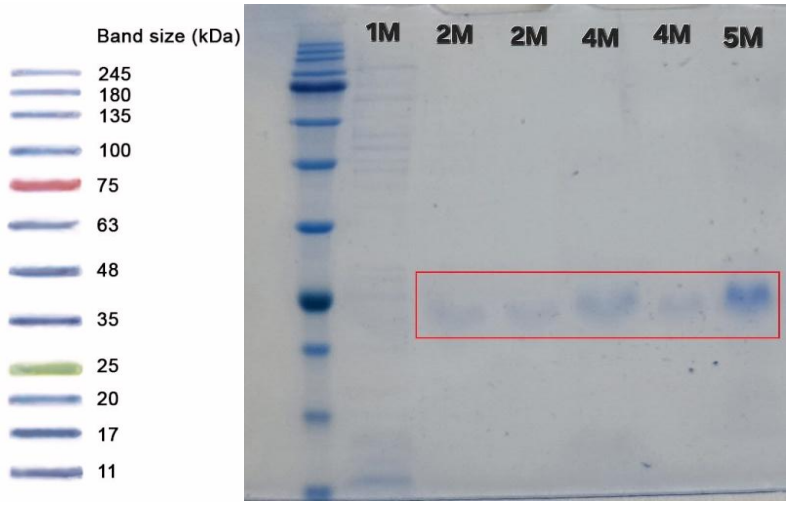


Figure 21- SDS-PAGE of optimized solubilization gradient (1 M, 2 M-1, 2 M-2, 4 M-1, 4 M-2, and 5 M urea) showing defined bands and reduced background noise.

2.7. Protein Refolding by Stepwise Dialysis

To promote correct protein folding, Ani s 1 solubilized in 5 M urea was subjected to stepwise dialysis against buffers with progressively decreasing urea concentrations (4 M, 2 M, 1 M, and finally 0 M). Analysis of the final dialysate by SDS-PAGE revealed a predominant, well-defined band at the expected molecular mass of ~24 kDa (Figure 22), indicating successful refolding and the presence of soluble, monodisperse protein.

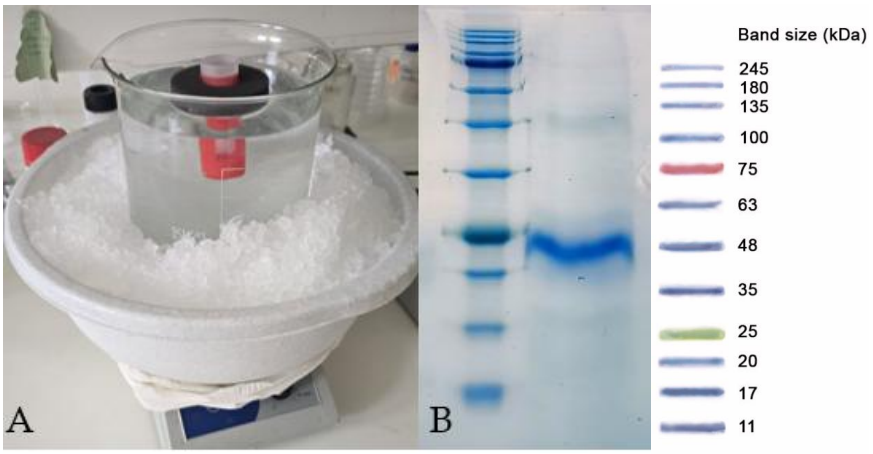


Figure 22- (A) Dialysis membrane immersed in renaturation buffer. (B) SDS-PAGE gel showing Ani s 1 retained after dialysis, indicating preservation of solubility.

To confirm that the refolding process preserved the native antigenic structure of Ani s 1, an immunodot blot was performed using sera from individuals sensitized to Anisakis. The assay demonstrated strong immunoreactivity against the refolded Ani s 1, confirming that the recombinant protein retained its conformational epitopes following in vitro refolding. No signal was detected for the bovine serum albumin (BSA) negative control, confirming the specificity of the antigen-antibody reaction (Figure 23).

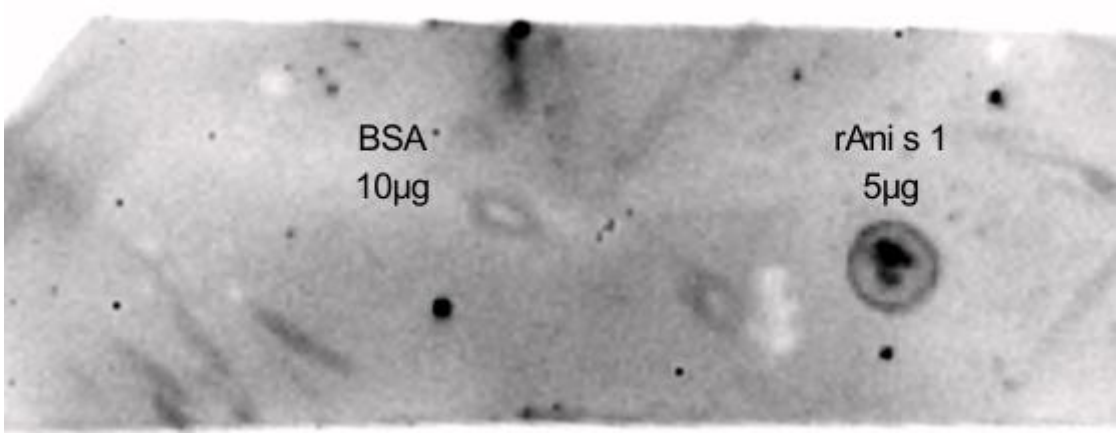


Figure 23- Immunodot blot confirming antigenic recognition of recombinant Ani s 1 using sera from sensitized individuals.

2.8. Lyophilization and Storage

The soluble, refolded Ani s 1 was concentrated by lyophilization. Upon reconstitution in aqueous buffer, all samples remained clear with no visible precipitation, indicating that the protein solubility was maintained through the freeze-drying process. SDS-PAGE analysis of the reconstituted protein confirmed the integrity of the preparation, showing a single, predominant band at the expected molecular mass of ~24 kDa (Figure 24).

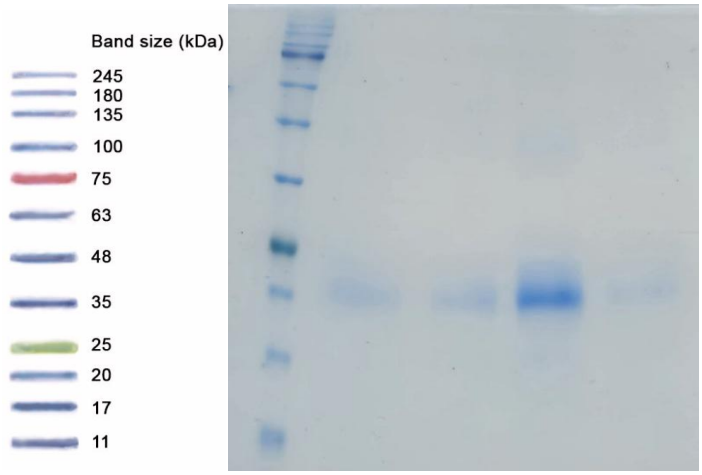


Figure 24- SDS–PAGE showing Ani s 1 bands obtained after resuspension of lyophilized samples.

Equivalent fractions were pooled, yielding a final volume of 10 mL of purified Ani s 1. The protein concentration was determined to be approximately 650 µg/mL by spectrophotometric measurement (NanoDrop), confirming a successful concentration and recovery following lyophilization and reconstitution. Analysis by SDS-PAGE indicated a final purity exceeding 90%, with a single predominant band observed at the expected molecular mass (Figure 24).

2.9. MIP-Based Biosensor Development

The development of the MIP sensor was monitored by square-wave voltammetry (SWV) before and after template removal. Following elution with 0.5 M H_2SO_4, a significant increase in the SWV peak current was observed, indicating successful creation of accessible binding cavities. The peak current after template removal recovered to approximately 50% of the intensity recorded for the initial, unmodified electrode (Figure 25).

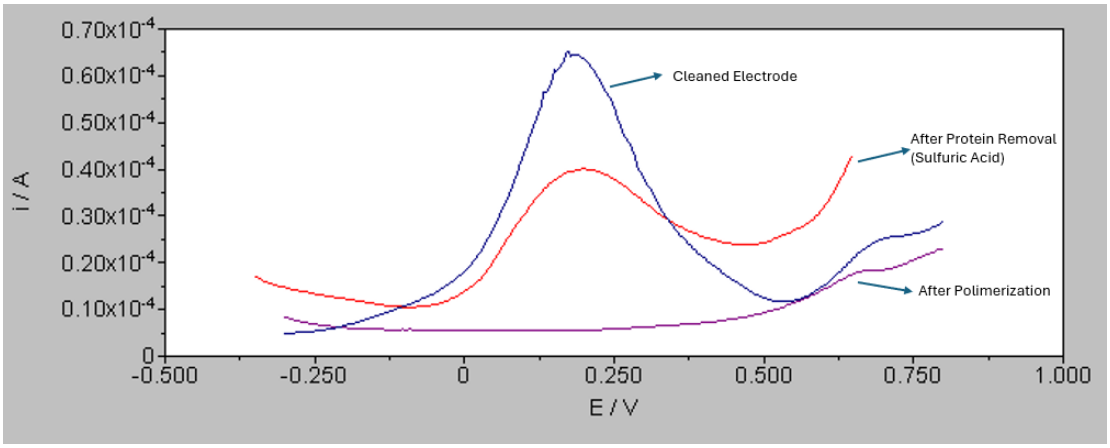


Figure 25- Square-wave voltammetry (SWV) analysis of the MIP-modified electrodes before and after template removal. The highest peak corresponds to the bare gold electrode prior to polymerisation. The intermediate peak reflects the partially restored redox signal after template removal using 0.5 M H_2SO_4, reaching ~50 % of the original current. The nearly flat line represents the polymerised electrode before template extraction, indicating successful surface blocking by the imprinted polymer layer.

In contrast to the acid-based method, an alternative template removal assay using 6 M urea did not yield a significant increase in the SWV signal compared to the post-polymerization state, indicating ineffective elution under these conditions (Figure 26).

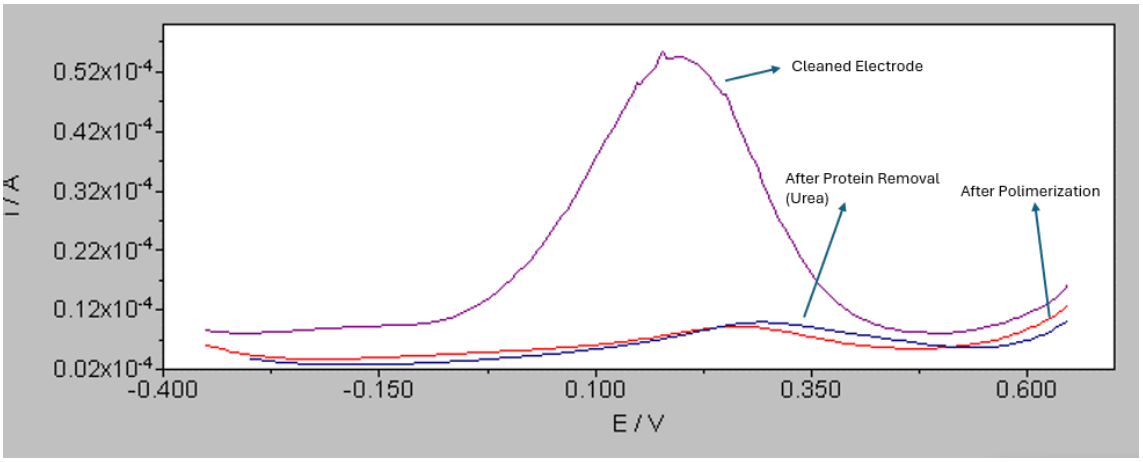


Figure 26- SWV response of the MIP-modified electrode before and after template removal using 6 M urea. The voltammogram shows no appreciable recovery of the redox peak following urea treatment. The post-removal signal remains nearly identical to the polymerised electrode trace, indicating ineffective template extraction under these conditions.

The analytical functionality of the MIP sensor was subsequently evaluated through rebinding assays. Upon exposure to increasing concentrations of Ani s 1 (10-12 M to 10-2 M), a progressive decrease in the SWV peak current was observed. This signal attenuation is consistent with the target protein occupying the imprinted cavities, thereby hindering the diffusion of the redox probe to the electrode surface. The highest tested

concentration resulted in the most significant signal suppression, demonstrating a concentration-dependent response (Figure 27).

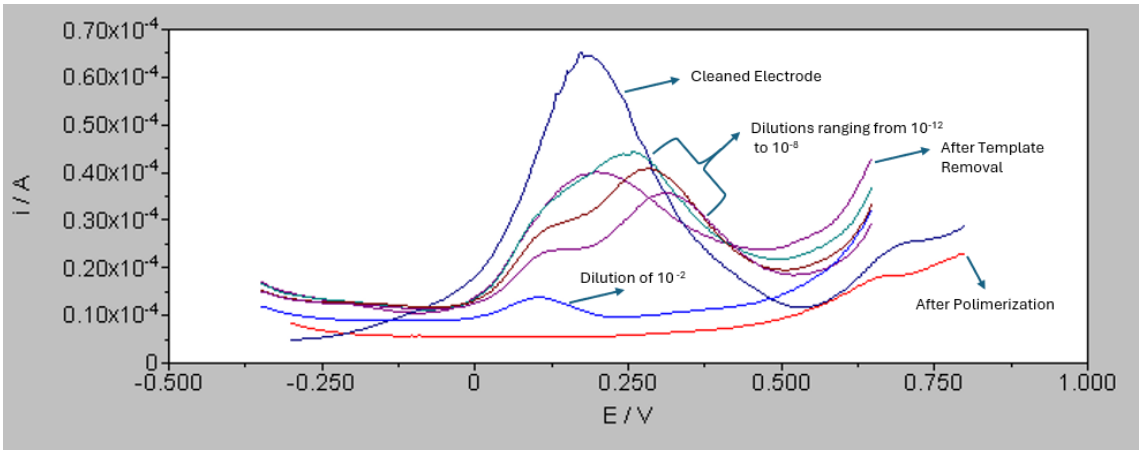


Figure 27- SWV rebinding response of the MIP-modified electrode exposed to increasing concentrations of Ani s 1. Redox peak intensity progressively decreased with rising analyte concentration (10^{-12} M → 10^{-10} M → 10^{-8} M), reflecting specific rebinding within the imprinted cavities. The highest concentration tested (10^{-2} M) resulted in the most pronounced current suppression, indicating near-complete surface blocking by the protein. For reference, the plot also includes the initial, unmodified electrode trace (highest peak) and the post-polymerization signal prior to rebinding (lowest baseline line).

3. Discussion

3.1. Molecular Cloning and Bioinformatic Characterization of Ani s1

The cloning of the Ani s 1 gene was confirmed by Sanger sequencing, which revealed almost 100 % nucleotide identity to the reference sequence available in GenBank. This validation ensures that all subsequent recombinant expression, purification, and immunological assays were performed using an accurate and functional version of the intended target gene.

Bioinformatic analysis using SignalP 6.0 predicted, with a probability greater than 0.99, the presence of an N-terminal signal peptide in Ani s 1. This result suggests that in its native context, the protein is likely secreted via the classical secretory pathway. This is consistent with prior studies reporting that Ani s 1 is excreted by *A. simplex* and associated with extracellular vesicles, where its structure is stabilised by a rich network of disulphide bonds [18][19][45].

Structural annotation additionally revealed two relevant domains within the Ani s 1 sequence: a Kunitz-type serine protease inhibitor domain (residues 26–85) and a worm-specific repeat type 1 (WR1) domain (residues 90–134). Kunitz domains belong to the I2 family of serine protease inhibitors and are characterised by small α/β folds stabilised by three disulphide bridges, typically functioning extracellularly and conferring resistance to host digestive proteases [17]. The WR1 domain, originally described in several Caenorhabditis elegans proteins, is likewise rich in conserved cysteines and frequently co-occurs with Kunitz domains, suggesting complementary roles in structural stabilisation, host interaction, and immune evasion [46]. The presence of the WR1 domain alongside the Kunitz domain is a feature shared with other nematode proteins. This architecture is exemplified by the Ascaris trypsin inhibitor (ATI), a serine protease inhibitor whose structure is stabilized by five disulfide bonds, an adaptation thought to enhance its resilience in the hostile gut environment of the host [47]. Therefore, this domain architecture in Ani s 1 may represent a key evolutionary adaptation for survival and pathogenicity within the host.

Interestingly, Ani s 1 showed similitude with AMPs on the CAMP-R3 database using machine learning models (SVM, Random Forest, Discriminant Analysis, and ANN) with high confidence. Other Kunitz-type inhibitors have been reported to display dual protease inhibitory and antimicrobial properties [48]. The high-confidence prediction of antimicrobial properties for Ani s 1 is particularly intriguing. As a secreted protein in a

parasite that must navigate microbe-rich host environments, this suggests a potential dual role for Ani s 1: not only in evading host proteases but also in modulating the local microbiome or protecting the parasite from secondary infection. This hypothetical, dual protease-inhibitor and antimicrobial function, while requiring experimental validation, would significantly expand our understanding of Ani s 1's role in the complex biology of host-parasite interactions.

3.2. Recombinant expression and solubility challenges

The recombinant production of Ani s 1 in *E. coli* required the testing of two constructs: one including the native N-terminal signal peptide and another comprising only the mature protein. No detectable expression was observed for the construct containing the signal peptide, a result that may reflect the toxicity or misrouting of the protein within *E. coli*, resulting in arrested growth without completely blocking cellular viability as shown in previous studies [49] as evidenced by the presence of residual non-specific protein bands on SDS–PAGE. Similar observations have been reported for other secretory parasitic proteins expressed heterologously, where signal peptide-containing versions impair host machinery or accumulate in misfolded states [48].

Conversely, the construct lacking the signal peptide yielded high expression levels, although exclusively in the form of insoluble inclusion bodies, a behaviour expected for cysteine-rich and disulphide-bonded proteins expressed in a prokaryotic cytoplasm lacking an oxidative folding environment [20][21]. This is consistent with reports of other Kunitz-type inhibitors and nematode proteins requiring specialised folding systems or redox-active periplasmic conditions for solubility [50]. The exclusive formation of inclusion bodies places Ani s 1 in the same category as other disulfide-rich allergens like the peanut allergen Ara h 2, which also requires denaturing conditions for solubilization when expressed in *E. coli* [51]. The consistent insolubility of disulfide-rich allergen proteins in standard *E. coli* systems underscores a fundamental bottleneck in the production of recombinant diagnostic and immunological reagents for parasitology. Our results reinforce the necessity of denaturing purification and refolding strategies as a default approach for this class of proteins and highlight the potential value of exploring more advanced expression systems, such as *E. coli* strains with enhanced disulfide bond formation or eukaryotic hosts like Pichia pastoris, for future high-throughput production.

The initial solubility profile was further investigated via Western blotting of soluble and insoluble fractions, which revealed multiple lower molecular weight bands reactive to the anti-His antibody. These bands likely represent partially degraded or truncated forms of

Ani s 1 retaining the N-terminal His-tag, a phenomenon that has been frequently described for proteins with compact disulphide-rich domains expressed under denaturing or proteolytically stressful conditions [51]. Cross-reactivity with endogenous histidine-rich bacterial proteins or co-purified contaminants cannot be excluded [52]. The lack of a clear single band in the soluble fraction further supported the decision to proceed with denaturing extraction and urea-based solubilisation as an alternative recovery method.

3.3. Urea-based solubilisation, refolding, and antigen preservation

Due to the exclusive accumulation of Ani s 1 in inclusion bodies, urea-based solubilisation was implemented to recover the protein under denaturing conditions. Initial solubilisation trials demonstrated that protein yield increased with increasing urea concentration, with 5 M urea yielding a strong and well-defined band at the expected molecular weight. At higher concentrations, non-specific extraction became more evident, resulting in excessive background staining on SDS–PAGE.

A refined solubilisation series using intermediate urea concentrations (1–5 M) generated cleaner extracts, allowing downstream refolding through stepwise dialysis. Final dialysed fractions showed a single band at the expected molecular weight with no visible precipitation, indicating successful refolding and restoration of soluble structure. The success of the stepwise dialysis in yielding a monodisperse, soluble protein, as evidenced by the single band on SDS-PAGE and lack of precipitation, strongly suggests that the compact, disulfide-stabilized Kunitz fold of Ani s 1 is capable of spontaneous reformation upon gentle denaturant removal. This inherent structural robustness is a defining feature of this allergen family and is likely the very property that confers stability in the harsh gastrointestinal environment, making it a notoriously persistent allergen.

The antigenic properties of the refolded protein were evaluated using an immunodot blot with sera from naturally infected fish. A clear immunoreactive signal was obtained, demonstrating that key epitopes were retained following denaturation and refolding. Most critically, this immunoreactivity confirms that the refolding process successfully restored not just solubility, but also key conformational epitopes. This is a non-trivial achievement for a disulfide-rich protein and validates our denaturing approach. It confirms that the recombinant product is a biologically relevant surrogate for the native allergen, making it suitable for the downstream immunological applications that were a primary aim of this work. It is also consistent with its reported resistance to thermal and chemical denaturation during parasite infection [53].

3.4. Preliminary electrochemical sensing using molecularly imprinted polymers (MIPs)

The use of Ani s 1 as a molecular template for electrochemical sensing was explored through the development of a protein-based MIP. This work constitutes an initial attempt to evaluate the feasibility of detecting a structurally complex allergen using a polymer-based recognition layer deposited on gold electrodes. The approach was not intended to test selectivity or analytical performance parameters, but rather to determine whether Ani s 1 could be successfully imprinted, removed, and rebound in a detectable, concentration-dependent manner.

Template removal from the MIP layer was first assessed using two denaturation strategies: 0.5 M H_2SO_4 and 6 M urea. The electropolymerised protein layer partially recovered its redox permeability following H_2SO_4 treatment, as reflected by a ~30 % restoration of the SWV peak relative to the pre-imprinting signal. By contrast, template removal with 6 M urea resulted in only minor recovery of the electrochemical signal. This may indicate insufficient disruption of the protein–polymer interface under these conditions, potentially due to the high structural stability of Ani s 1 and its resistance to denaturation, as previously reported for this allergen [54]. This differential efficacy in template removal is a critical finding; it suggests that harsh chemical treatments (acid) may be necessary to disrupt the strong non-covalent interactions formed by this stable protein, a consideration that will directly inform the development of robust regeneration protocols for future sensor devices. The superior performance of acidic template removal over urea is consistent with findings from chromatin-protein separation studies [55], where acid treatment caused "a marked structural change (denaturation)" in the proteins. This contrasted with other treatments, such as using high salt or salt and urea, which were less disruptive and thus recommended for studies aiming to preserve biological function.

Rebinding experiments using serial dilutions of Ani s 1 demonstrated a progressive decrease in SWV signal intensity, consistent with increased protein adsorption at the MIP surface. The lowest signals were recorded for the highest concentration (100 µg/mL), whereas more moderate peak suppression was observed with lower concentrations (10^{-8}–10^{-12} M). Although these results do not establish specificity or binding kinetics, they confirm that Ani s 1 can interact with the MIP layer in a measurable and concentration-dependent manner. This successful proof-of-concept is significant as it demonstrates that a complex, disulfide-stabilized allergen can generate a quantifiable

electrochemical signal upon rebinding, overcoming a major hurdle in transitioning from simple molecular targets to full-protein biomarkers in MIP-sensor development.

These observations support the feasibility of developing a MIP-based electrochemical system for Ani s 1 detection, but further optimisation is required. Future work will need to include the use of a non-imprinted polymer (NIP) control, testing of cross-reactive proteins, and formal calculation of imprinting factors and analytical parameters such as sensitivity, selectivity, and limit of detection. Additional optimisation of polymer composition, electro-polymerization conditions, and template extraction may improve signal recovery and dynamic response. Nevertheless, the present data confirm that Ani s 1 can be both removed from and rebound to the polymer, with measurable electrochemical consequences.

Conclusion

This work successfully achieved the recombinant production and initial characterisation of the *Anisakis simplex* allergen Ani s 1, combining molecular cloning, expression, solubilisation, refolding, and immunological validation. The Ani s 1 gene was cloned and confirmed by Sanger sequencing, with full identity to the reference sequence. Bioinformatic analysis revealed the presence of functionally relevant domains, including a Kunitz-type serine protease inhibitor domain and a worm-specific repeat type 1 (WR1) region. In addition, computational predictions indicated a high likelihood of secretion through a signal peptide and antimicrobial potential, although these functional hypotheses require experimental validation.

Recombinant expression in *E. coli* was achieved only for the construct lacking the native signal peptide, with detectable protein accumulating exclusively in inclusion bodies. Solubilisation and refolding through a stepwise urea gradient enabled the recovery of a soluble form of Ani s 1 that preserved antigenic properties, as shown by recognition in immunodot blots using sera from naturally infected fish. This confirms that antigenically relevant conformations of the protein can be recovered following denaturation and refolding.

A preliminary proof-of-concept was also carried out using Ani s 1 as a molecular template for electrochemical detection via molecularly imprinted polymers (MIPs). Although the system is still in its early stages, the template could be successfully removed, rebound, and detected through measurable changes in the electrochemical signal. These results demonstrate that Ani s 1 can interact with the MIP matrix in a concentration-dependent manner. However, further optimisation is required, including the use of non-imprinted controls, improved template removal, and full analytical validation.

In summary, this work establishes a reliable basis for the biotechnological exploration of Ani s 1 as a recombinant allergen and as a target for biosensing. Future studies should focus on solubility optimisation, assessment of the predicted biological activities and refinement of the MIP sensing platform toward selective and reproducible detection.

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Annex 1

Development of an electrochemical biosensor based on molecularly imprinted polymers for the identification of Trypsin inhibitors (soy) in food matrices

Brief Introduction of the work

In recent years, food allergies have been considered a very important public health problem, affecting a significant portion of the global population. The growing awareness of the relevance of food allergies and the need to protect sensitized/allergic individuals from accidental exposures to food has led industry and regulatory agencies to test and monitor food for the presence of allergens.

The Kunitz family of soybean trypsin inhibitors are present in a wide range of plant species and has been most characterized in soybeans. This Kunitz inhibitor has been reported to induce anaphylaxis. Soy lecithin is commonly used as an emulsifier in processed foods and cosmetics.

The main objective of this work will be the construction and development of a biosensor for the detection of the soybean trypsin Inhibitor allergen and subsequent application in food matrices. Molecular imprinting is a technique used to prepare polymers with predetermined selectivity for the target molecule obtained by applying voltammetric techniques and which lead to the formation of cavities in the polymer matrix with affinity for the Imprinted molecule (MIP).

The non-molecularly imprinted polymer - NIP - is distinguished from MIP by the lack of the target molecule in the polymerization solution.

Recommended Literature: https://www.sciencedirect.com/science/article/pii/S0IXI3267021011363

1. Preparation of solutions:

1.1 0.5 M sulfuric acid:

Measure a volume of 5.6 mL to a final volume of 200 mL.

Concentrated H_2SO_4: 96%, M= 98.99 g/mol, d=1.840 g/mL at 25ºC

1.2 PB buffer solution:

Weigh 0.6 g of NaH_2PO_4 and 0.5 g of Na_2HPO_4 in a volume of 100 mL and adjust the pH until reaching a pH of 8.0.

$M(Na_2HPO_4)$=119.98 g/mol; $M(NaH_2PO_4)$= 141.96 g/mol

1.3 Hexaaminoruthenium chloride II redox probe

C= 5 mM $(Ru[NH_3]_6]^{2+/3+}$

M= 30S.61 g/mol

Weigh 0.0062 g of Hexaaminoruthenium chloride II and add 4 mt PB.

1.4 Soybean trypsin Inhibitor (protein):

Stock solution with concentration 1.0 mg/mL.

1.5 MIP Printing Solution

Cf Soybean inhibitor = 100 µg/mL

Pipette 100 µL of the protein from the stock solution with C = 1.0 mg/mL, add 0.7 µL of Pyrrole and make up with PB to a total of 1 mL (899.3 µL PB) resulting in a concentration of 0.01 mol/L In pyrrole and 100 µg/mL protein.

1.6 Electrodes

Commercial gold electrodes (gold screen-printed electrodes, Au-SPE) (DropSens, 220AT) will be used. These electrodes are printed on a ceramic substrate, the auxiliary and working electrodes (d = 4 mm) are composed of gold, while the reference electrode is composed of sliver.

2. Soybean trypsin inhibitor standards

For the calibration curve (assessment of analytical performance): Serial dilution from the initial solution of Soybean trypsin inhibitor (C = 1.0 mg/mL) (see Table 1). Table 1: Method of preparation of standard solutions.

Table 1: Preparation method of standard solutions.

	Stock solution Soybean trypsin inhibitor mg/mL	1.0	Vf /µL	1000
		V(PBS)/ µL	C_{final}_mg/mL	Vf/µL
1	100 µL stock solution	900	1.0×10^{-1}	1000
2	100 µL standard solution #1	900	1.0×10^{-2}	1000
3	100 µL standard solution #2	900	1.0×10^{-3}	1000
4	100 µL standard solution #3	900	1.0×10^{-4}	1000
5	100 µL standard solution #4	900	1.0×10^{-5}	1000
6	100 µL standard solution #5	900	1.0×10^{-6}	1000
7	100 µL standard solution #6	900	1.0×10^{-7}	1000
8	100 µL standard solution #7	900	1.0×10^{-8}	1000
9	100 µL standard solution #8	900	1.0×10^{-9}	1000
10	100 µL standard solution #9	900	1.0×10^{-10}	1000
11	100 µL standard solution #10	900	1.0×10^{-11}	1000
12	100 µL standard solution #11	900	1.0×10^{-12}	1000
13	100 µL standard solution #12	900	1.0×10^{-13}	1000
14	100 µL standard solution #13	900	1.0×10^{-14}	1000

3. Biosensor preparation and characterization procedure:

3.1 SPE cleaning

Wash the electrode surface with acetone, ethanol and finally with ultrapure water.

3.2 Electrochemical cleaning

Add 70 µL of 0.5 mol/L H_2SO_4 to the SPE, and using cyclic voltammetry, perform 20 cycles in the potential range between 0/-0.1/-1.2 V.

3.3 Electropolymerization (MIP preparation)

Add 70 ML of the MIP printing solution (prepared in point 1.5) and perform cyclic voltammetry.

Experimental parameters: 1 scan starting at 0 V/-0.2 V/ 1.0 V speed 100

mV/s. At the end, wash abundantly with water.

3.4 Electropolymerlzafion (NIP)

Prepare a pyrrole solution In PB (without protein) with a concentration of 0.01 mol/L. To do this, add 0.7 µL of pyrrole and make up with PB to a total volume of 1000 µL (V PB = 999.3 µL).

3.5 Protein extration

Place the electrode immersed in a 25 mM SDS (sodium dodecyl sulfate) + methanol (10:1) v/v solution and leave for 13h.

The next day, wash thoroughly with ultrapure water.

Add 70 µL of PB and perform a set of 5 cycles In the potential range (0 /-0.5 V/0.1 V} —

to remove solution and portions of the protein that may remain in the polymer matrix.

4. Evaluation of the analytical performance of the biosensor — Calibration curve

Calibration will be performed using the square wave voltammetry (SW) analytical technique and the protein incubation method.

4.1 Before performing the study with the standards, place the redox probe to perform the blank study, after finishing washing the electrode and drying It with nitrogen.

4.2 Add 70 µL of the P14 standard, let It Incubate for 10 min. Wash and dry the electrode and place the Ru|NH3]6]2+/3+ redox probe. Then perform the measurements using cyclic voltammetry and SW.

4.3 CAUTION: start the Incubation with the solution with the lowest concentration.

4.4 Wash the electrode thoroughly with ultrapure water and repeat the same procedure for the other standards.

• **Equipment**

All measurements will be performed using the AutoLab device controlled by the Nova
2.1.3 software. The electrodes are connected to a DropSens box (DRP-DSC), allowing
Interfacing with Autolab.