

Purification and identification of hydrolyzed peptides from *Lates calcarifer* by-products and their anti-inflammatory function in lipopolysaccharide-induced RAW 264.7 macrophage cells

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Research Article

Keywords: *Lates calcarifer*, bioactive peptides, by-products, anti-inflammatory response, nitric oxide

Posted Date: July 2nd, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4548969/v1>

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Additional Declarations: No competing interests reported.

Abstract

Fish by-product is considered a sustainable source for obtaining natural protein and hydrolysates with functional and biological activity. The present study explored the potential of barramundi, *Lates calcarifer* by-products as a natural source to generate bioactive peptides with anti-inflammatory properties in LPS-induced RAW264.7 macrophage cells. The results indicated that the highest degree of hydrolysis was achieved at 4% papain at a rate of $26.15 \pm 2.67\%$ in 48 hours. The hydrolyzed peptides had a total amino acid content of 51.55%, with essential and non-essential amino acids accounting for 27.28% and 24.27%, respectively. The active peptides were purified with ultrafiltration and Sephadex G-15 column. Eight peptide exhibited anti-inflammatroy properties were identified by using LC-MS/MS. Evaluation of anti-inflammatory peptides using the PreAIP database revealed high anti-inflammatory scores (0.501–0.659) for peptide sequences LKLLLL, KPKLLL, and LQLLL, and moderate scores (0.433–0.436) for peptide sequences LALDIEIATYR and LQLL, while GPVS, FGVS, and AMSP had lower scores (0.261–0.293). Molecular docking simulations revealed that two peptide sequences, LALDIEIATYR and LKLLLL, can effectively bind to Toll-like receptor (TLR4), with leucine playing a major role in receptor binding. The hydrolyzed peptides from barramundi by-products exhibit potential for improving the inflammatory response, as they effectively inhibited the production of proinflammatory cytokines such as IL-6, IL-1 β , and TNF- α after LPS stimulation and increased the gene expression levels of anti-inflammatory factors such as IL-10 and IL-12. Therefore, the study suggests that the hydrolyzed peptides from barramundi by-products offer a promising therapeutic strategy for the treatment of inflammatory responses.

1. Introduction

Barramundi, *Lates calcarifer* is an aquaculture fish species of high value, cultivated in the regions of Australia and Asia. With the increase in global demand and production, the discards of inedible fish parts have also increased. To many consumers, fish parts such as heads, viscera, blood, and skin are deemed as having little value and are undesirable for consumption. As a result, this contributes to the waste of 30–70% of fish produced, whether through wild catches or aquaculture practices, globally (FAO, 2020). This worsens environmental concerns for both land and sea, including reduced oxygen levels in seawater, the introduction of non-native and invasive species, and the suffocation of living organisms. Moreover, the improper disposal of fish waste on land leads to aesthetic problems and unpleasant odours due to bacterial decomposition (Sheriff et al., 2014). Therefore, from both environmental and economic perspectives, reducing fish waste is vital.

Over the years, fish by-products have gained interest as they are known to contain high amounts of valuable resources beyond their nutritional value, such as high protein, polyunsaturated fatty acids, phospholipids, soluble vitamins, and various bioactive compounds that promote anti-inflammatory and anti-oxidative activities (He et al., 2013). These active compounds are usually obtained through the process of enzymatic hydrolysis. Several studies have reported utilizing fish by-products from enzymatic hydrolysis to recover protein hydrolysates that possess antioxidant activity and the ability to scavenge

hydroxyl radicals, superoxide anion radicals, and hydrogen peroxide (Hsu, 2010, Ahn et al., 2012, Klomklao et al., 2013, Ketnawa et al., 2016, Rocha Camargo et al., 2021). Moreover, peptides from the muscles of Chinese sturgeon, *Acipenser sinensis*, salmon, *Salmonidae* pectoral fins, and *Mytilus coruscus* after pepsin hydrolysis can effectively inhibit inflammation and have anti-inflammatory effects (Ahn et al., 2015, Kim et al., 2016, Gao et al., 2021). In addition, the mixture of by-products of different fish species generated hydrolysis of acid-soluble collagen, which exhibited biological (antioxidant and antimicrobial) and functional activity (solubility, foaming, and emulsifying ability) (Zamorano-Apodaca et al., 2020). Given the diverse nature of fish species, fish by-products may contain enzymes with unique properties. Therefore, exploring the potential benefits of different fish species becomes necessary, not only to provide pharmaceutical health benefits but also to contribute to the reduction of food loss and waste in aquaculture systems. This approach is critical for the transition to sustainable food systems that enhance and maximize the efficient use of fish resources.

The present study uses the by-products of *L. calcarifer* to determine the inflammatory properties of the hydrolyzed peptides. While many recovered peptides from previously studied species have demonstrated antioxidant activity, limited information is available on the anti-inflammatory properties of hydrolyzed peptides obtained from *L. calcarifer*. Inflammation is a natural defense mechanism initiated by the invasion of pathogens or by tissue injury caused by biological, chemical, or physical damage. Typically, the activation of macrophages initiates defensive reactions, which results in the release of inflammatory mediators such as nitric oxide (NO) and proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6, and γ -1 β (Ahn et al., 2012, Chalamaiyah et al., 2018). In addition, the overproduction of NO and proinflammatory cytokines is associated with many human diseases. Given the immunomodulatory benefits of fish by-products, the fish protein-derived bioactive peptides with their antioxidative and inflammatory properties can be utilized to contribute to the host's defense response. Therefore, the objective of this study is to investigate the potential of discarded viscera of *L. calcarifer* as raw material for the production of fish protein hydrolysates exhibiting anti-inflammatory activity in LPS-stimulated RAW264.7 macrophage cells. The findings of this study would not only contribute to the pharmaceutical and food industry but also the complete utilization of fish resources that contribute to the "Blue Transformation," geared towards maximizing the use of aquatic food systems and promotes the development of diverse practices and processes to reduce fish loss and waste.

2. Materials and Methods

2.1. Sample collection and crude protein composition analysis

Barramundi by-product containing viscera, bones, fins, and residual muscles on the bones were collected from a food processing factory located in Pingtung county, Taiwan, and stored at -20 °C until use. The crude protein of fish by-product was determined using the Kjeldahl method described by the Association of Official Analytical Chemist (AOAC).

2.2. Optimal conditions for enzymatic hydrolysis of fish by-products

To establish the optimal degree of hydrolysis (DH) conditions for the Barramundi by-products, the TNBS colorimetry assay was conducted following the procedures described by Adler-Nissen (1979). Samples were prepared by mixing 100g of by-products with papain (9001-73-4, Sigma, Saint Louis, USA) and a commercial tenderizer (08000139, Fly-Horse, Taipei, Taiwan) in varying concentrations to achieve final enzyme concentrations of 3, 4, and 5%. The hydrolysis process was conducted under the conditions of pH 6.0 ± 0.5 , and 55°C for 72 h. The degree of hydrolysis was assessed at 12, 24, 48 and 72 h after the enzyme was inactivated by heating at 100°C for 20 min. Thereafter, 100µL of the supernatant was removed and transferred to a microcentrifuge tube containing 900µL sodium dodecyl sulfate (SDS). The mixture was centrifuged (Universal 320R, Hettich) at 8,000 rpm at 4°C for 10 min, and 15µL sample was transferred to a 96-well plate, and adding 45µL sodium dihydrogen phosphate and 45µL TNBS solution (SIGMA). The hydrolysate was incubated at 50°C and 70 rpm for 1 h. The reaction was stopped by adding 0.1N HCl and the absorbance was measured at 340 nm using a spectrophotometer (SpectraMax 190, Molecular Devices). The α-amino acid, L-leucine was used as the reference standard and the sample concentration was converted by the internal difference method. The hydrolysis rate was calculated according to the formula:

$$DH = (H / H_{\text{tot}}) \times 100\%$$

In which H is the free amino acid content in sample; H_{tot} is the total amino acid content of fish by-products.

2.3. Cytotoxicity Analysis (MTT assay)

Hydrolysates obtained under optimal conditions using papain (4%) at 55 °C for 48 h were used for the MTT assay. After hydrolysis, all resulting hydrolysates were collected, centrifuged, and lyophilized for further experiments.

Cytotoxicity effects were determined using RAW264.7 macrophage cells following the methods described by Gao et al. (2020) with slight modification. First, using a cell scraper, RAW264.7 mouse macrophage cells were isolated and suspended in a 50 mL centrifuge tube, then centrifuged at 4°C, 4,000 rpm for 30 min to concentrate the cells. The cells were resuspended in fresh DMEM, and the cell count was calculated using a cell counter. Cells were then quantified at a density of 1×10^6 cells/well in a 96-well plate and cultured for 24 h to allow cell adhesion. After cell adhesion, the culture medium was removed, and different concentrations (0, 0.1, 0.25, 0.5, 1, 5 mg/mL) of fish by-product hydrolysate were added to the wells. Cells were co-cultured with the hydrolysate for 24 h. Cell viability was then assessed using the MTT assay by adding 20µL of 0.5% MTT reagent (SIGMA) to each well then incubating in a cell culture incubator (NU-5810, NUAIRE) for 4 hours. The culture medium was then removed, and 200µL of dimethyl sulfoxide (DMSO) (JT Baker®) was added to dissolve the purple crystal formazan. The plate

was covered with aluminium foil to prevent light interference and shaken at 50 rpm for 10 min to ensure complete dissolution. Absorbance was measured at 570 nm using a spectrophotometer, and the cell viability was calculated using the following formula.

Cell viability (%) = (Mean absorbance of sample / mean absorbance of control) x 100%

2.4. Amino acid composition of hydrolysates

Amino acid composition of hydrolysate was analyzed by reverse-phase high performance liquid chromatography techniques (RP-HPLC). Samples weighing 19.5 mg was hydrolyzed under nitrogen gas with 1 ml of 6N HCl containing 1% phenol and placed in an oven at 110°C for 24 h and neutralized with 4N NaOH to pH 7.0. Then, sample was transferred to a vacuum oven at 60°C for drying until the liquid in the ampoule was completely dry. Subsequently, 1 mL of 0.001N HCl was added, mixed, and filtered through a syringe filter (0.22µm PVDF membrane). Following this, ortho-phthalaldehyde (OPA) were mixed in equal amounts at room temperature, and the amino acid content was analyzed by RP-HPLC (JASCO) with an autosampler, a column of Gemini-NX 5u C18 110A 25 cm × 4.6 mm with 5 µm particle size (Phenomenex; Torrance, California, USA), and a fluorescence detector (FP-2020 plus, JASCO, Japan) (excitation wavelength = 340 nm; emission wavelength = 455 nm). Chromatographic conditions were used for the description in the product information of OPA (P0532, Sigma) with some modifications. Additionally, prior to the analysis of methionine and cysteine in the sample, ingredients were pretreated using formic acid (9 parts of 88% formic acid plus 1 part 30% hydrogen peroxide) for protection prior to acid hydrolysis (6 N HCL for 24 h at 110 °C). For tryptophan contents in hydrolysate, sample hydrolysis was done according to the procedure of Çevikkalp et al. (2016) by using NaOH (5 N) solution. OPA-amino acids were also analyzed by a reversed-phase HPLC (PU- 2089 plus, JASCO, Japan) as described above.

2.5. Purification of active peptides from hydrolysates

2.5.1. Ultracentrifugation

Hydrolysate derived from fish by-product was resuspended in sterilized deionized water (10g/L) and the solution underwent individually ultrafiltration with pore sizes of 50 kDa, 30 kDa, 10 kDa, and 5 kDa (Vivaspin, Sartorius, Germany). Centrifugation was performed at 1,500 rpm and 4°C for 10 min, and the resulting samples were separated based on molecular weight into fractions of > 50 kDa, 30–50 kDa, 10–30 kDa, 5–10 kDa, and < 5 kDa. Fractions with anti-inflammatory were concentrated in a freeze-dryer, and powders were stored at -80°C until being used for further purification.

2.5.2. Gel filtration chromatography

The sample with the highest anti-inflammatory activity were dissolved in sterilized deionized water at a concentration of 0.1 g/mL, and then chromatographed on a Sephadex G-15 column (1 × 60 cm, GE Healthcare, Uppsala, Sweden) equilibrated with sterilized deionized water. After equilibration, 1 mL of sample was loaded into the column and later eluted using sterilized deionized water at a flow rate of 0.6

mL/min. Fractions containing anti-inflammatory activity were pooled and concentrated in a freeze-dryer, and the powders were lyophilized and later stored at -80°C for subsequent analysis.

2.6. Analysis of anti-inflammatory activity

2.6.1. Cell culture

RAW264.7 macrophage cells were collected, concentrated, and reconstituted with fresh Dulbecco's Modified Eagle Medium (DMEM). The RAW264.7 macrophage cells were loaded into 90-well plates at a density of 1×10^6 cells/well. Cells were allowed to adhere to the cell wall for 24 h, co-cultured with hydrolysates for 8 h, and then stimulated with LPS (2 µg/mL) for 24 h. Thereafter, the nitric oxide (NO) production and the gene expression of inflammatory cytokines were assessed.

2.6.2. Nitric oxide (NO) free radical inhibition assay

Nitric oxide (NO) is a free radical that plays a crucial role as an inflammatory vector in bacterial endotoxin infection. To evaluate the anti-inflammatory potential of hydrolysed peptides from barramundi by-products, RAW264.7 macrophage cells were stimulated by lipopolysaccharide (LPS) (Sigma) to induce inflammation. The hydrolysed peptides were analysed to inhibit the release of NO from the cells. The quantification of nitric oxide was based on nitrate/nitrite production by using Nitrate/Nitrite Colorimetric Assay Kit. (780001, Cayman, MI, USA) according to the manufacturer's instructions. Briefly, 10 µl supernatant of each sample was mixed with 90 µl assay buffer in a 96-well plate, followed by adding enzyme cofactor and nitrate reductase mixture. After incubation for 1 h, DAN reagent and NaOH were added into each well. The plate was immediately read using a fluorometer (RF-6000, Shimadzu, Tokyo, Japan) at a wavelength of 365 nm and emission wavelength of 430 nm.

2.6.3. RNA extraction and gene expression of inflammatory cytokines

Total RNA RAW264.7 of macrophage cells was extracted using Rezol (PROtech Technologies, Inc.) as per the manufacturer's instructions. The synthesis of cDNA was performed according to the instructions of the M-MuLV Reverse Transcriptase kit (Protech Technology Enterprise CO, Ltd., Taipei, Taiwan). First-strand RNA was adjusted to the same concentration with diethylpyrocarbonate (DEPC) water and accurately quantified with a spectrophotometer. For first-strand complementary (c) DNA synthesis, 1 µg of total RNA from each tissue was used with SuperScript II RNase H- reverse transcriptase (Promega, Madison, WI, USA) to transcribe poly (A) + RNA with oligo d (T)18 as the primer following the conditions recommended by manufacturers.

The RT-qPCR analysis of gene expression was performed according to the method described by Liu et al. (2013) using the ABI PRISM7900 instrument (ABI StepOne Real-Time PCR System, Applied Biosystem, Carlsbad, CA, USA). The analysis of gene expressions was performed using a RT-qPCR with SYBR green. For the normalization of target gene expressions among treatments, an internal control gene, β-actin, was used along with the genes of inflammatory cytokines (Supplementary Table 1). Reactions were

conducted in a 96-well plate, and the conditions of target genes were amplified at 95 °C for 3 min (pre-denaturation) followed by 40 cycles of denaturation at 95 °C for 3 s, and a combined annealing and extension step at 59 °C for 20 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expressions of genes (Livak and Schmittgen, 2001). Results are expressed as the mRNA expression relative to the control.

2.7. Analysis of peptide sequence by LC-MS/MS

The freeze-dried filtrate containing small peptides was reconstituted in 5% acetonitrile (ANC) and 0.1% formic acid (FA) in deionized water. The preparation was then subjected to LC-MS/MS analysis using a Thermo LCQ DECA XP MAX system with an electrospray ionization (ESI) source (Thermo Scientific, Inc., USA). Samples were loaded into C18 columns (150 mm × 2.1 mm, particle size: 5 µm). Elution was performed using a gradient from 5–70% ANC in 0.1% FA over 75 min, with a flow rate of 200 µl/min. MS/MS spectra were acquired using Thermo Xcalibur™ (Thermo-Scientific). The sheath gas flow rate was set to 50 random units. The spray voltage applied for the full mass scan was 4 kV, while the capillary voltage was maintained at 20 V with capillary temperature of 300°C. MS scanning was performed over a mass-to-charge (m/z) range of 100 to 1000. The MS/MS raw data were subsequently converted into MGF files using Mascot Distiller v 2.3.2.0 (Matrix Science, London, UK).

Following analysis, Mascot software (version 2.7.1.0, Matrix Science, London, UK) was used to process all Ms and Ms/Ms data. Then, for amino acid sequence alignment, the discovered peptide sequences were uploaded to the National Center for Biotechnology Information (NCBI). After alignment, the peptide sequences were submitted to the PreAIP Anti-inflammatory Peptide Prediction website (<http://kurata14.bio.kyutech.ac.jp/PreAIP/>) so that a random forest classifier system could predict the anti-inflammatory scores of the peptides. The qualities of several kinds of anti-inflammatory peptides, such as primary anti-inflammatory peptide sequences and evolutionary anti-inflammatory peptides, are combined in this software. With an AUC (Area Under Curve) value of 0.840, the characteristics of several kinds of anti-inflammatory peptides, such as primary anti-inflammatory peptide sequences, evolutionary anti-inflammatory peptide sequences, and structural composition of anti-inflammatory peptide sequences were determined (Khatun et al., 2019).

2.8. Peptide docking analysis with Toll-like receptor 4

To determine the binding affinity of the peptides with TLR4 receptor. The crystal structure of mouse TLR4 and mouse MD-2 complex (2Z64) was downloaded from the RCSB PDB database (Protein Data Bank, <https://www.rcsb.org/>). The docking process was performed using the DS2019 software. Ligands and water molecules were removed from the receptor prior to docking. The coordinates x: y: z = -31.6415, 2.01906, 1.84602 and -31.498, 2.822, -3.498 were given as the optimal docking range, with 2.84Å as the receptor. The peptide composition was mapped using the software as a ligand for molecular docking simulations. After matching the force fields using CHARMM, the combined posture and energy of the two were determined using a docking simulation using CDOCKER. The 3D structure diagram and 2D plan diagram of the complex were obtained using the docking model with the lowest

energy which was later visualized surface annotation of both ligand interactions with the protein via Biovia Discovery Studio Visualizer.

2.9. Statistical Analysis

Statistical analysis was performed by SAS software (SAS Institute, Cary, CA, USA). All statistical analyses with the experimental results were expressed as means $\pm$ standard error (S.E.). One-way analysis of variance and Duncan's multilevel tests were applied for determining significant difference at $p < 0.05$.

3. Results and discussion

3.1. Optimal condition for barramundi by-products protein hydrolysis

The production of biologically active protein hydrolysates from fish by-products requires the use of proteolytic enzymes to break down fish proteins and release active peptides (Chalamaiah et al., 2012). While there are various methods for deriving hydrolysates and peptides, enzymatic hydrolysis is favored for its speed, safety, and ability to prevent the occurrence of toxic chemical residuals under strictly controlled conditions (García-Moreno et al., 2014). The choice of enzyme used is critical as it determines the functional and nutritional properties of the resulting proteins.

In this study, the most effective hydrolysis conditions for processing the by-products of barramundi was investigated by using different concentrations of papain and a commercially available tenderizer. Papain is commonly used in the preparation of protein hydrolysates and has been shown to enhance protein recovery from various fish by-products (Yang et al., 2008, You et al., 2010, Luo et al., 2013, Tacias-Pascacio et al., 2021). Among the different times of assessment (12, 24, 48, and 72 h), the degree of hydrolysis reaction remained constant after 48 h. The highest hydrolysis rate was achieved after 48 hours of 4% papain, with a value of $26.15 \pm 2.67\%$ (Fig. 1). However, there was no significant increase in the rate after 72 hours at $26.99 \pm 2.45\%$. Other studies have also reported varying degrees of hydrolysis depending on the fish species, fish parts used, and the type of enzyme activity applied. For example, Zavareze et al. (2014) obtained 28.5% of hydrolyzed by-product proteins from *Micropogonias furnieri* using Flavourzyme, while Sinthusamran et al. (2019) yielded 25.1–26.9% of protein hydrolysates from salmon frame using Alcalase. The degree of hydrolysis affects protein recovery and other functional properties, such as antioxidative activities. Overall, our findings indicate that hydrolysates obtained using papain provided a high yield.

3.2. Proximate composition of fish by-product

The hydrolysis of proteins is influenced by the composition of amino acids, with essential amino acids being particularly important for proper physiological activity. The proximate composition of barramundi by-products revealed that 51.51% of both essential and non-essential amino acids were present (Table 1). The protein hydrolysate derived from barramundi was found to be enriched in methionine

(10.10%), glutamic acid (7.52%), glycine (5.44%), and alanine (4.53%) of the total amino acids. The higher mass fractions of essential amino acids were found to be 27.28%, whereas non-essential amino acids accounted for 24.27% of barramundi protein hydrolysate. Additionally, the hydrolysate contained hydrophobic amino acids such as phenylalanine (1.57%), valine (2.24%), isoleucine (1.93%), leucine (3.23%), and alanine (4.53%), which are associated with biological and functional properties. Chalamaiah et al. (2018) documented that immunomodulatory peptides are often related to hydrophobic amino acids such as glycine, valine, leucine, proline, phenylalanine, negatively charged amino acid, glutamic acid, and aromatic amino acid, tyrosine. Moreover, other amino acids such as glutamine, glutamic acid, tyrosine, tryptophan, cysteine, asparagine, and aspartic acid, which possess one or more residues of hydrophobic amino acids, facilitate the immunomodulatory activities of food protein-originated peptides. These results suggest that the hydrolysates obtained from barramundi could be utilized as a dietary supplement owing to their high content of essential and hydrophobic amino acids, which can produce highly nutritious products.

Table 1
Amino acid composition of hydrolysate derived from barramundi by-product

Items	Hydrolyzed amino acid composition (%)
Essential amino acids	
Phenylalanine	1.57
Valine	2.24
Threonine	1.8
Isoleucine	1.93
Leucine	3.23
Methionine	10.1
Lysine	2.81
Arginine	3.13
Histidine	0.13
Tryptophan	0.34
Nonessential amino acid	
Aspartate	4.25
Glutamic acid	7.52
Serine	1.33
Glycine	5.44
Alanine	4.53
Tyrosine	1.2
Total	51.55

3.3. Cell viability analysis and inhibition of NO production LPS-induced RAW264.7 cells

The cytotoxic activity of hydrolyzed peptide on RAW264.7 macrophage cells was assessed. The results indicated that cell activity was higher when treated with lower concentrations of hydrolysate compared to cells treated with concentrations exceeding 1 mg/mL (Fig. 2A). The variations in these results may be attributed to the type of enzyme used and the fish species. Similarly, Lee et al. (2012) assessed the effect of enzymatic extracts from *Ruditapes philippinarum* on the viability of RAW264.7 cells using similar concentrations and found that the viability of cells was higher at lower concentrations.

The viability of cells plays a crucial role in supporting the inflammatory process, as activated macrophages of the immune system secrete nitric oxide at the site of inflammation to repair tissue and remove the cause of the inflammation. This study evaluated whether hydrolysates from barramundi could inhibit LPS-induced NO production. The results showed that lower production of NO was observed in cells treated with 0.5 mg/mL hydrolysate compared to 0.1, 0.25 mg/mL and untreated cells, 34.91, 33.46, and 32.02 μ M, respectively (Fig. 2B). Higher concentrations of hydrolyzed peptides demonstrated a significant decrease in NO production in a dose-dependent manner. These findings indicate that barramundi hydrolyzed peptides can inhibit the inflammatory response in macrophage cells. Furthermore, following the purification of NO-inhibitory peptide, fractions of 10–30 kDa molecular weight had a higher NO-inhibitory activity due to lower NO production than the other fractions (Fig. 3A). Therefore, the 10–30 kDa fraction was chosen for lypolization, which yielded significantly lower NO production following the administration of LPS to different concentrations of active peptides compared to the group treated with only LPS (Fig. 3B). Several studies have demonstrated that the production of NO by inducible nitric oxide synthase (iNOS) has been linked to several inflammatory diseases, nitrosative stress, oxidative DNA damage, and the progression of tumors. Inflammatory stimuli such as bacterial lipopolysaccharides (LPS), cytokines, and interleukins can up-regulate iNOS, leading to the overproduction of NO (Surh et al., 2001, Ahn et al., 2015, Kemp and Kwon 2021). Considering this, the ability of active peptides derived from barramundi by-product to inhibit the production of NO highlights the potential benefits of using fish by-products in the pharmaceutical industry.

3.4. Expression of inflammatory cytokines

The study investigated whether the purified peptide of 10–30 kDa molecular weight has the ability to regulate anti-inflammatory (such as interleukin-10 (IL-10) and interleukin-12 (IL-12)) and proinflammatory cytokines (such as IL-6, IL-1 β , and TNF- α) after LPS-stimulation in RAW264.7 cells. The results revealed that treating RAW264.7 macrophages cells with 0.1, 0.25, and 0.5 mg/mL of 10–30 kDa barramundi hydrolyzed peptides significantly increased the expression of IL-10 and IL-12 compared to the control group (Fig. 4A and B). These findings suggest that the hydrolyzed peptide of barramundi can regulate cellular inflammatory responses via the induction of anti-inflammatory factors.

Moreover, the expression of all proinflammatory cytokines including IL-6, IL-1 β , and TNF- α was significantly reduced in the barramundi hydrolysate and LPS group compared to the LPS treated cells (Fig. 4C ~ E). Given these findings, hydrolyzed peptide from barramundi by-products may have the potential to act as an anti-inflammatory agent. Similar findings were reported as hydrolysates obtained from salmon by-products showed an anti-inflammatory activity by inhibiting NO production and proinflammatory cytokines (Ahn et al., 2012). Thus, the inhibition of NO is possibly linked to the upregulation of anti-inflammatory cytokines and downregulation of proinflammatory cytokines.

3.5. Gel filtration chromatography and molecular docking

The purification of 10-30kDa hydrolyzed peptides by resulted in two fractions, F1 (74–87) and F2 (88–102) (Fig. 5A). The NO inhibitory activity of both fractions was assessed following stimulation with LPS,

revealing significantly higher inhibitory activity in F2 compared to F1 and LPS-treated cells (Fig. 5B). Furthermore, F2 was analyzed using LC-MS/MS, which identified the peptide sequence LALDIEIATYR as an uncharacterized protein (LOC108897006) associated with keratin, type II cytoskeletal 8. Additionally, seven peptides with probable anti-inflammatory properties were identified, with amino acid sequence lengths ranging from 4 to 11 amino acids (Table 2). The anti-inflammatory properties surrounding the role of bioactive peptides derived from fish-byproducts have been linked to amino acids of short and low molecular weight and containing hydrophobic amino acids. With lower molecular weights, peptides are able to cross the intestinal barrier to promote healthy immunological responses (Kemp and Kwon 2021). Peptides sequences including KPKLLL (Lys-Pro-Lys-Leu-Leu-Leu), LQLLL (Leu-Gln-Leu-Leu-Leu), GPVS (Gly-Pro-Val-Ser), FGVS (Phe-Gly-Val-Ser), and AMSP (Ala-Met-Ser-Pro) were of lower molecular weights and all contain amino acids that possess anti-inflammatory properties. Peptides composed of positively charged amino acids, such as lysine, have been found to possess anti-inflammatory properties. Additionally, hydrophobic amino acids, including phenylalanine, leucine, glycine, valine, and alanine, play a role in the anti-inflammatory response. Proline has also been identified as an important amino acid in antioxidant peptides, with the ability to alleviate some of the negative effects of LPS stimulation (Yu et al., 2018). Highly hydrophobic peptides have the ability to interact with cell membranes and disrupt inflammatory pathway cascades, as reported by Gao et al. (2020). Moreover, Joshi et al. (2016) found that elevated levels of glycine in peptides and hydrolysates can increase anti-inflammatory potential, with short peptides showing better anti-inflammatory activity due to the presence of glycine. Glycine-containing peptides have a high affinity for calcium binding, potentially disrupting Ca^{2+} signaling, which plays a central role in NF- κ B signaling and cytokine production (Tang and Skibsted 2016). Hydrolysates high in radical scavenging peptides, including methionine, have also been found to ameliorate the effects of UVB radiation in mice models by increasing enzyme activity of superoxide dismutase (SOD) and glutathione peroxidase (Gpx) while decreasing IL-1 β , IL-6, TNF- α , inducible nitric oxide synthase (iNOS) (Peng et al., 2020).

Table 2
Identification and score of hydrolyzed peptides derived from barramundi by-product

Identified protein (NCBI Protein ID)	Identified peptides	Molecular weight	Position Start-end	Combined random forest score
Uncharacterized protein LOC108897006 isoform X1 (XP_018551886.2) [<i>Lates calcarifer</i>] (Sequence coverage = 1.2%)	LALDIEIATYR	1276.70	394–404	0.433
Protocadherin-18b isoform X1 (XP_018549920.1) [<i>Lates calcarifer</i>] (Sequence coverage = 0.5%)	LKLLLL	711.53	18–23	0.659
Dendritic cell-specific transmembrane protein (XP_018538040.1) [<i>Lates calcarifer</i>] (Sequence coverage = 1.15%)	KPKLLL	710.51	382–387	0.501
E3 ubiquitin-protein ligase UBR4 isoform X1 (XP_050927096.1) [<i>Lates calcarifer</i>] (Sequence coverage = 0.1%)	LQLLL	598.41	810–814	0.520
Low quality protein: nesprin-2 (XP_050934315.1) [<i>Lates calcarifer</i>] (Sequence coverage = 0.05%)	LQLL	485.32	8541–8544	0.436
Low quality protein: titin (XP_050924851.1) [<i>Lates calcarifer</i>] (Sequence coverage = 0.01%)	GPVS	358.19	27504–27507	0.285
Low quality protein: titin-like (XP_050929955.1) [<i>Lates calcarifer</i>] (Sequence coverage = 0.01%)	FGVS	408.20	27146 – 17149	0.293
Low quality protein: hemicentin-1 (XP_050933008.1) [<i>Lates calcarifer</i>] (Sequence coverage = 0.07%)	AMSP	404.17	886–889	0.261

Among all identified peptide sequences, leucine was presented as a repetitive amino acid in five sequences, suggesting its active role in biological activities. The interaction between the identified peptides was studied using the protein docking method. The analysis revealed that two identified peptides, LALDIEIATYR and LKLLLL, had binding affinity to TLR4 (Figs. 6 and 7). The peptide sequence leucine located at the N-terminus interacts with ASN114, which is known to form stable hydrogen bonds that firmly bind with the protein at the active site. Studies have reported that hydrophobic and branched-chain amino acids such as leucine at the N-terminus have the ability to enhance the antioxidative potential of a peptide (Bashir et al., 2020). These findings suggest that the hydrolyzed peptide from barramundi has the potential to enhance antioxidative activity, along with its anti-inflammatory properties.

The identified peptide, LALDIEIATYR, although confirmed as an uncharacterized protein, is associated with keratin, type II cytoskeletal 8 (CK8). Studies have reported that CK8 plays an important role in binding and regulating TNF α -mediated nuclear factor-kappa (NF- κ B) signaling pathway, which regulates multiple genes involved in different immune and inflammatory responses (Zhang et al., 1991). The NF- κ B signaling may be activated by a diverse range of stimuli, including bacterial and viral products, and cytokines. The findings of this study revealed that the peptide hydrolysate inhibited potent pro-inflammatory markers, such as IL-1 β , IL-6, TNF- α , and NO production, suggesting that peptides purified from barramundi by-products can suppress the NF- κ B pathway and offer a new therapeutic strategy for the treatment of inflammatory response. Similar findings reported downregulation of the NF- κ B pathway in LPS-induced RAW264.7 macrophages following the use of sturgeon peptides (Gao et al., 2021).

Bacterial LPS has been commonly used to study inflammation, due to the abundance of inflammatory effects that it generates through TLR4 signaling. TLRs are primarily associated with the innate immune response with the role of detecting pathogen-associated molecular patterns known as PAMPs and host-derived molecules known as damage-associated molecular patterns (DAMPs) (Mahapatra et al., 2023). In particular, the TLR4 has been identified to detect LPS (Nie et al., 2018). Furthermore, TLR4 has a integrated structure composed of leucine-rich repeats that initiates a cascade of protein-protein interactions leading to the production of pro-inflammatory cytokines and interferons, thus initiating the inflammatory and immune responses (Kuzmich et al., 2017). The study provided promising insights into the potential benefits of the by-products of barramundi. Specifically, two identified peptides containing leucine were found to effectively bind to TLR4, highlighting their potential pharmacological benefits. Additionally, all identified peptides were composed of amino acids with anti-inflammatory properties, which warrant further investigation.

4. Conclusions

The findings of this study revealed that peptides hydrolysate derived from barramundi by-products exhibited antioxidative and immunological properties by producing anti-inflammatory cytokines, including IL-10 and IL-12, while inhibiting the production of proinflammatory cytokines, such as TNF- α , IL-6, and IL-1 β , in LPS-induced RAW264.7 macrophage cells. The enzymatic hydrolysis of the fish by-products resulted in the successful yield active peptides which demonstrated the ability to bind to TLR4, a receptor involved in the regulation of innate immunity. Furthermore, this study not only highlights the potential of utilizing and transforming fish waste into functional compounds with biological activities but also presents an opportunity to contribute to zero-waste. Further studies on barramundi by-products are necessary to identify other functional compounds, their biological activities, and their subsequent practical applications. The identification of additional functional compounds and their potential applications could result in the development of new products that could benefit various industries, including the pharmaceutical and food industries.

Declarations

Acknowledgments

This study was supported by a grant from the National Science and Technology Council, Taiwan (110-2313-B-020-006-MY3).

Author Contribution

Ann-Chang Cheng: Conceptualization, Data curation, Reviewing and Editing; Hua Yi Liang: Conceptualization, Investigation, Data curation; Rolissa Ballantyne: Reviewing and Editing; Chun-Hung Liu: Conceptualization, Resource, Data curation, Writing-original draft; Reviewing and Editing, Funding acquisition.

Funding

The research leading to these results has received funding from the National Science and Technology Council, Taiwan (110-2313-B-020-006-MY3).

Conflict-of-interest statement

The authors declare no conflicts of interest.

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Figures

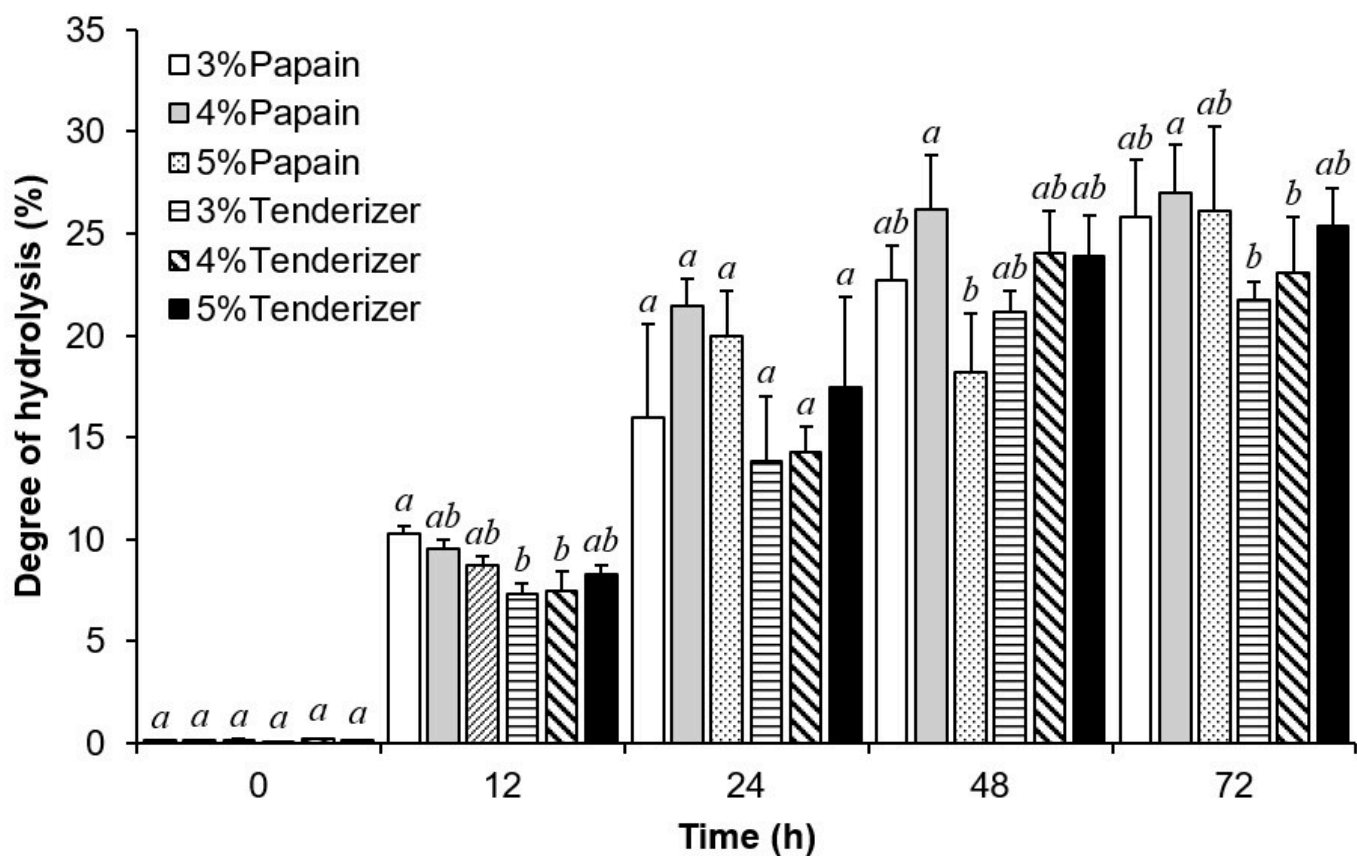


Figure 1

Degree of hydrolysis of barramundi by-product treated with papain and tenderizer at different concentrations. Data represent the mean $\pm$ standard deviation of three samples. Different letters indicate significant differences among groups at the same time point ($p < 0.05$).

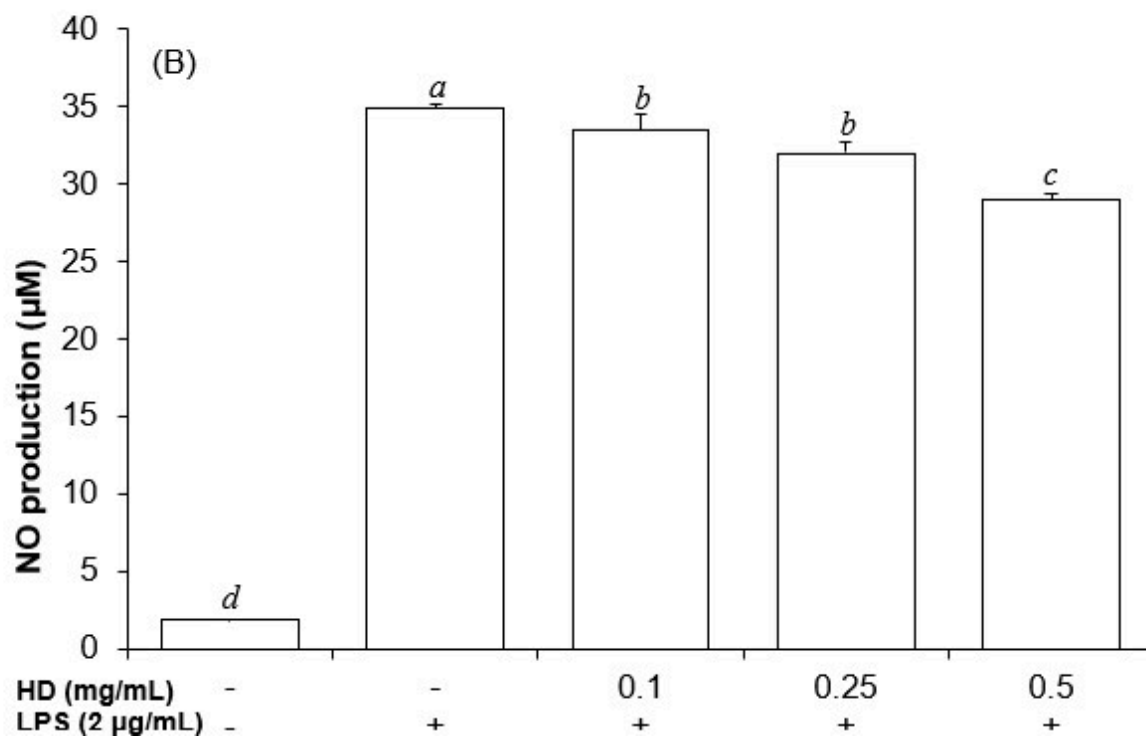
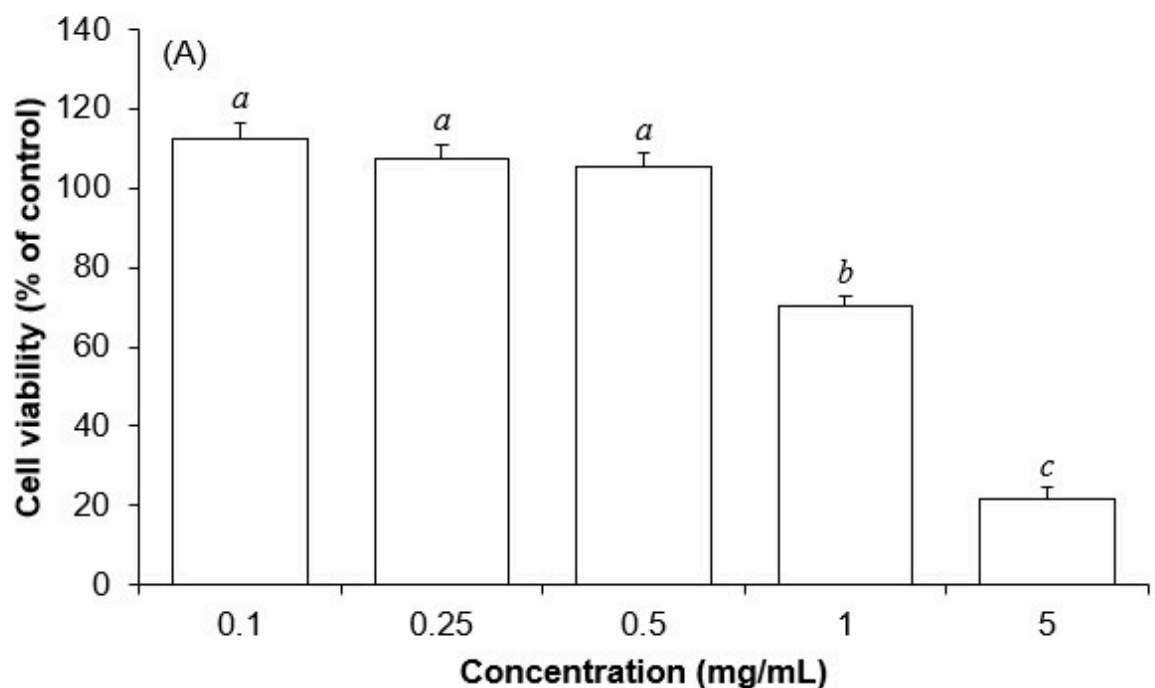


Figure 2

Cytotoxicity analysis in RAW264.7 macrophage cells treated with different concentrations of hydrolysates derived from barramundi by-product (A). Nitric oxide production (NO) in LPS-stimulated RAW264.7 macrophage cells. The cells were treated with different concentrations of hydrolysate (HD) derived from barramundi by-products and 2µg/mL of LPS (B). Data represent the mean $\pm$ standard deviation of six samples. Different letters indicate significant differences among groups ($p < 0.05$).

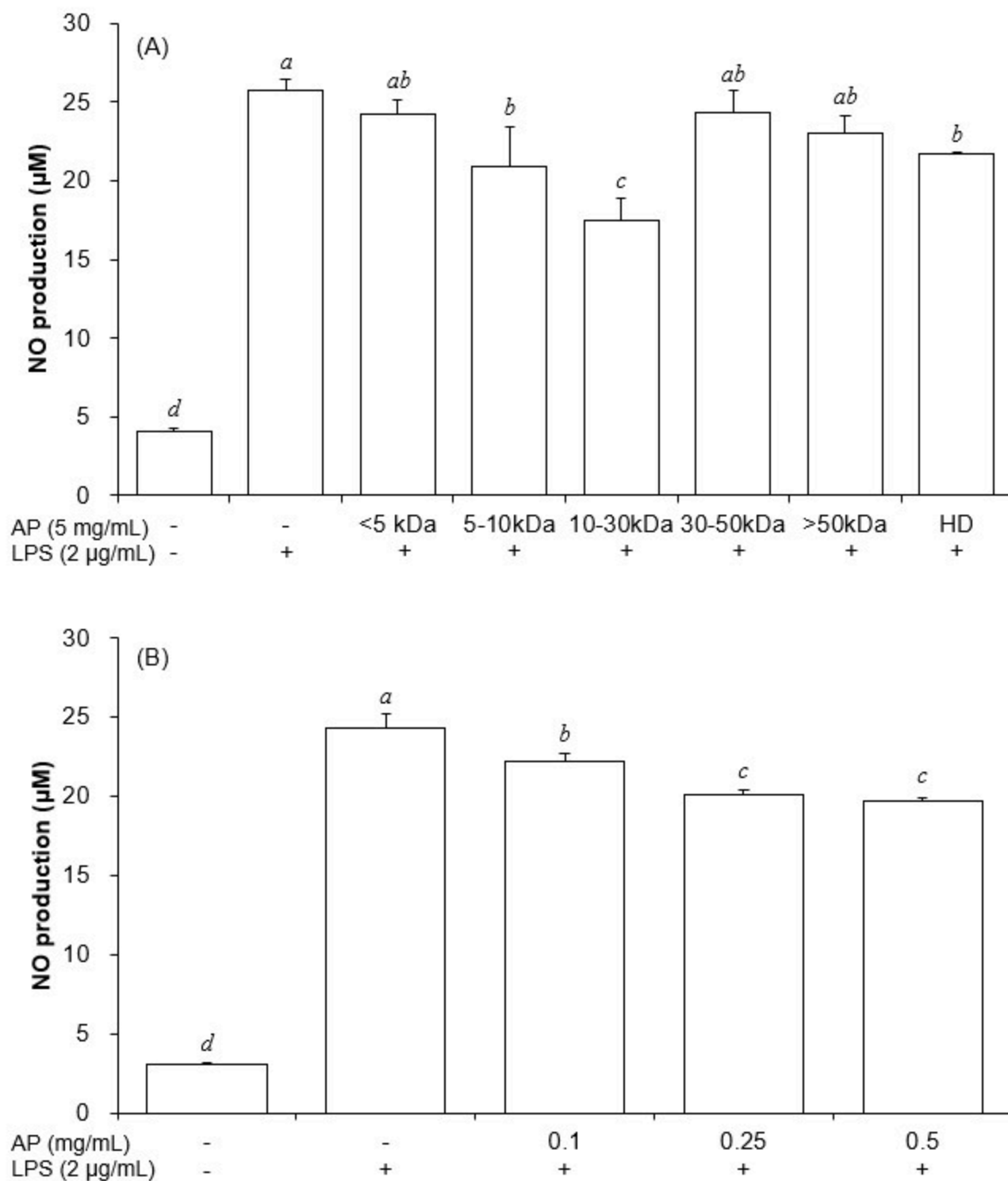


Figure 3

Nitric oxide (NO) production in LPS-stimulated RAW264.7 macrophage cells. The cells were treated with active peptides (AP) derived from barramundi by-product of different molecular weights, crude extract (HD) and 2 µg/mL of LPS (A); The cells were treated with 10-30 kDa of active peptides (AP) derived from barramundi by-product at different concentrations and 2µg/mL of LPS (B). Data represent the mean ±

standard deviation of six samples, and different letters indicate significant differences among groups ($p < 0.05$).

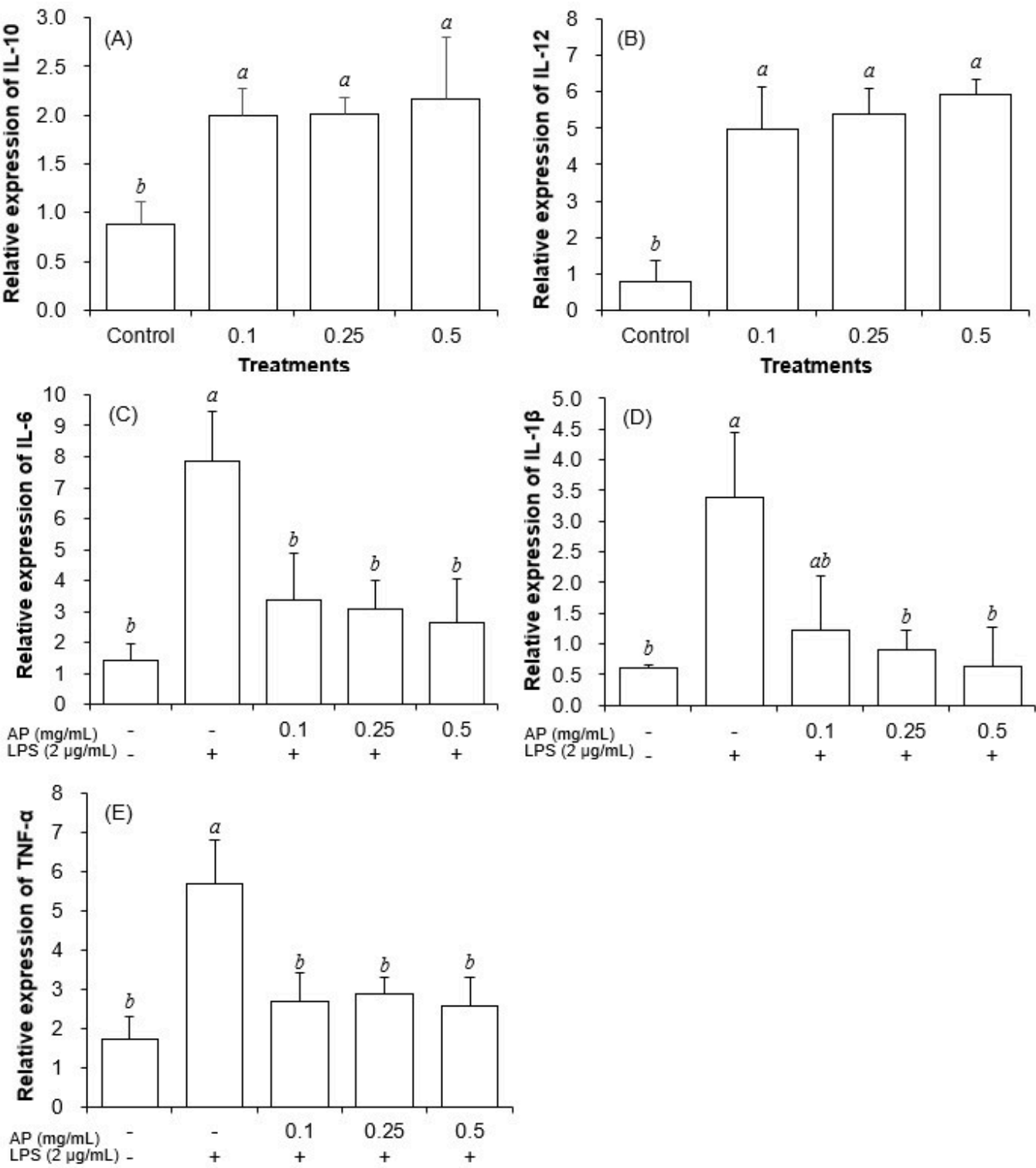


Figure 4

Relative expression of cytokines in LPS-induced RAW 264.7 macrophage cells with 10-30 kDa active peptides derived from barramundi by-product. (A) IL-10, (B) IL-12, (C) IL-6, (D) IL-1 β , and (E) TNF- α mRNA

expression levels are shown. Data represent the mean $\pm$ standard deviation of six samples, and different letters indicate significant differences between groups ($p < 0.05$).

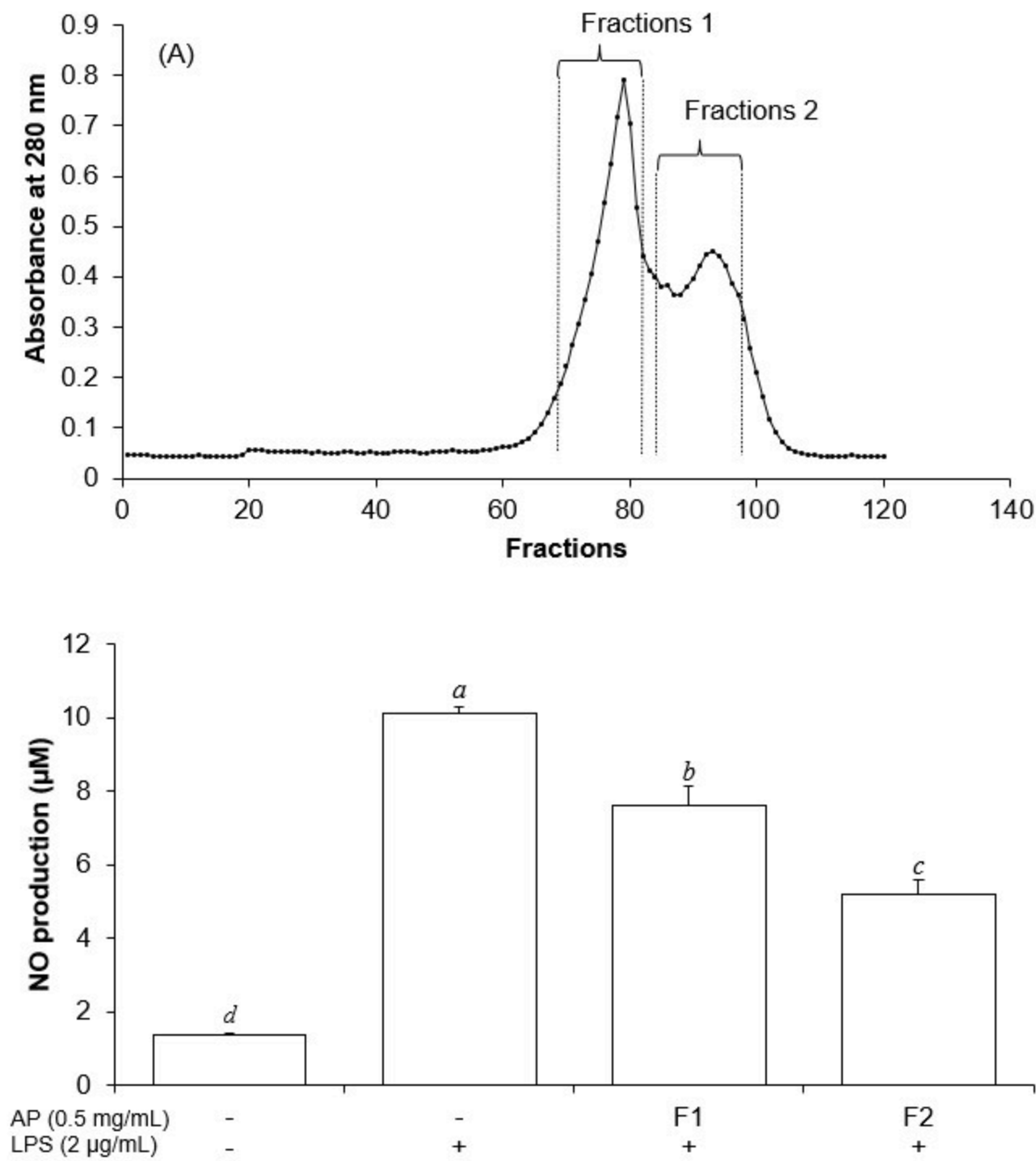


Figure 5

Elution profile of active peptides derived from barramundi by-product by using Sephadex G-15 column (1 $\times$ 60 cm). Fractions 2 (88~102) with the highest inhibition of nitric oxide (NO) production were pooled for the peptide identification (A). NO production in cells were treated with active peptides (AP) obtained from

fraction peaks at a concentration of 0.5mg/mL and 2µg/mL of LPS. Data represent the mean ± standard deviation of six samples, and different letters indicate significant differences between groups ($p < 0.05$).

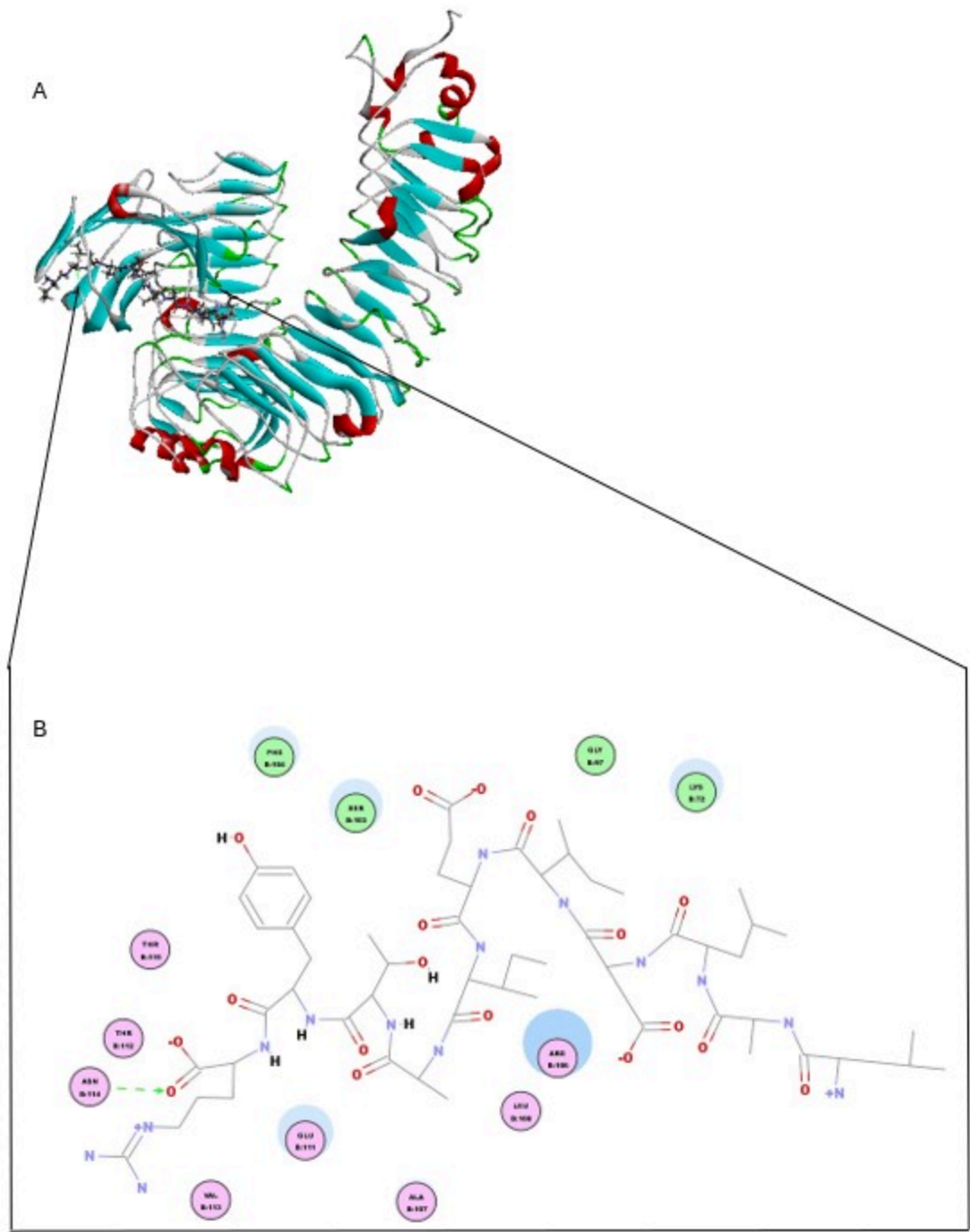


Figure 6

Docking of peptide sequence LALDIEIATYR with TLR4: (A) 3D Structure diagram of the interaction; (B) Interaction with TLR4 activation site residues.

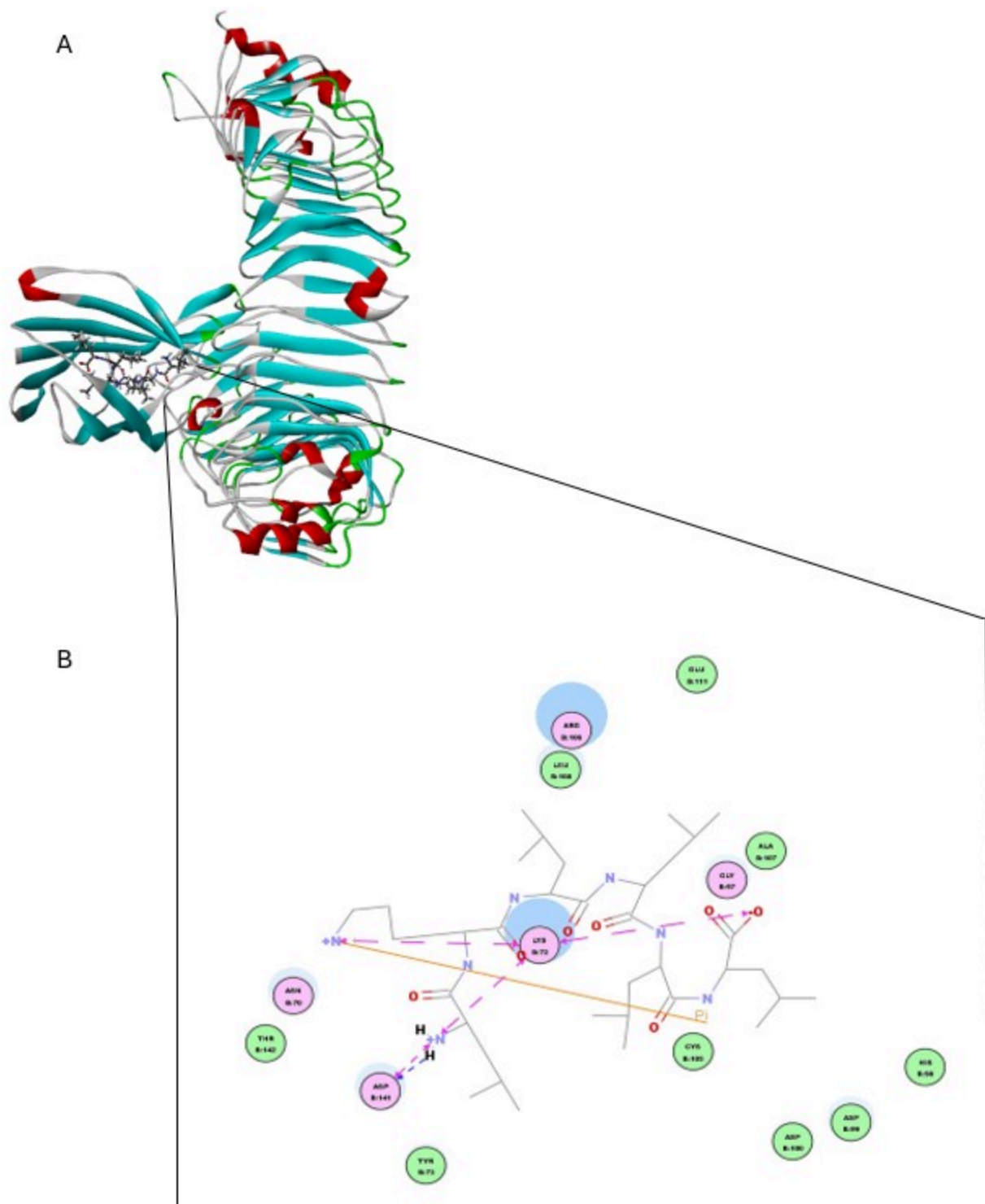


Figure 7

Docking of peptide sequence LKLLL with TLR4: (A) 3D Structure diagram of the interaction; (B) Interaction of LKLLL with TLR4 activation site residues.

Supplementary Files

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- [SupplementaryTable1.docx](#)