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RESEARCH ARTICLE
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OPTIMIZATION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM MILK AND FISH PROTEINS USING RESPONSE SURFACE METHODOLOGY

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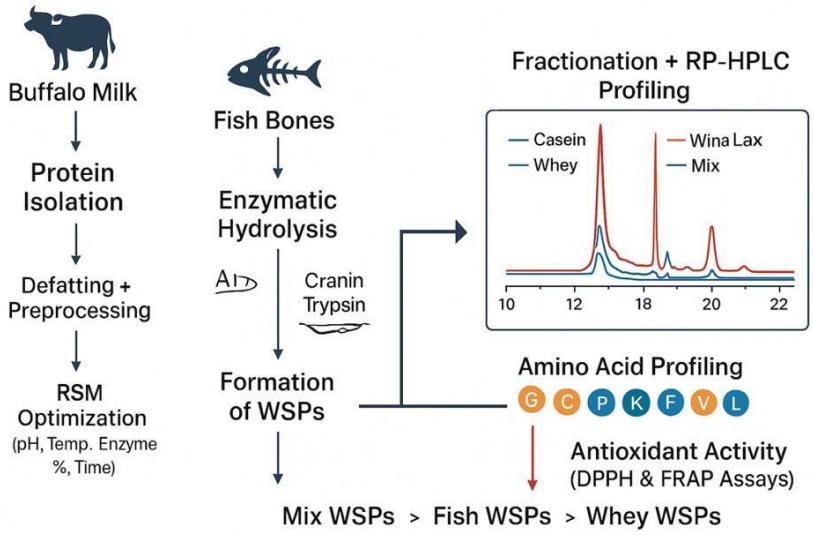
Abstract

Bioactive peptide have a growing interest in nutraceutical and functional food applications, as they possess antioxidant and therapeutic properties. The current research paper entailed the extraction of the water-soluble peptides (WSPs) in buffalo milk casein, whey, and tuna (Thunnus albacares) fish bones, optimization of the process, and characterization of the structure. Alcalase, trypsin, and chymotrypsin enzymatic hydrolysis was performed under controlled physicochemical conditions, and optimization of the hydrolysis parameters was done based on the Response Surface Methodology (Central Composite Design), where Degree of Hydrolysis (DH) was taken as the chief ratio. The optimum conditions varied with the protein sources, which is substrate-specific hydrolytic behavior. The ensuing lyophilized WSPs were fractionated and measured by RP-HPLC, which had a characteristic chromatographic pattern in peptide sources. Mixed WSPs (1:1:1) were associated with the highest peptide concentration with the major peaks eluting at 18-22 min whereas whey WSPs exhibited the least intensity of the peaks. Amino acid profiling showed that there were 17 amino acids and mixed WSPs had high concentrations of essential amino acids, especially phenylalanine and lysine as well as the hydrophobic amino acids including proline and alanine, which indicated better biofunctional potential. The evaluation of antioxidant properties showed that there was significant dose-related enhancement of radical scavenging ability with mixed WSPs possessing much higher DPPH scavenging ability (39.61+ -0.40% at 800 mg/mL) and FRAP values (1.89+-0.40 mM Fe2+/g) than the respective individual sources of WSP. These results suggest a possibility of synergistic effects of combinatorial peptide fractions to increase antioxidant potential. In general, the research defines optimal conditions of extraction of milk- and fish-derived peptides and the high antioxidant capacity of the mixed WSPs, which justifies their use in functional food, nutraceutical, and biologically active ingredients.



GRAPHICAL ABSTRACT

1. **Introduction**

Food proteins have become significantly attractive because of their many physiological functions such as acting as antioxidants, immune modulators and regulators of metabolism, through the production of bioactive peptides (Korhonen and Pihlanto, 2006). These peptides are usually concealed in the original structure of milk or fish proteins and can only be biologically active after being released by activity of enzymatic hydrolysis, fermentation, or digesting in the gastrointestinal tract (Daliri et al., 2018). The rising demand of oxidative-stress-associated diseases and disorders has increased the need to find natural antioxidant peptides as less harmful in terms of cytotoxicity and cancer-causing effects than synthetic antioxidants (Shahidi and Zhong, 2015).

It is known that buffalo milk contains more protein and in particular, casein and whey fractions which contain higher concentrations of hydrophobic and aromatic amino acids that increase radical-quenching capacity during hydrolysis (Saini and Gill, 2022). Casein-derived peptides have also been shown to have metal-chelating, lipid-peroxidation-inhibitory and ROS-scavenging properties with the whey proteins providing cysteine-rich sequences which facilitate glutathione regeneration pathways (Phelan et al., 2009). Controlled proteolysis of milk proteins is therefore a promising technique that may be used to produce potent antioxidant bioactive fractions.

On the same note, the fish processing industry produces high amounts of poorly utilized byproducts such as the bones, which contain protein matrices of collagen (Kim and Mendis, 2006). Marine collagen peptides contain powerful antioxidant, anti-inflammatory, and tissue-regeneration properties, which is why they are very promising as nutraceutical and functional foods (Ngo and Kim, 2013). A good example of this is tuna (Thunnus albacares) bones which are abundant sources of type I collagen and bioavailable minerals which provide a ready, cheap raw material to develop peptide-based products (Nalinanon et al., 2011). The valorization of such byproducts would be consistent with the objectives of the circular bioeconomy because it would reduce the amount of waste, but produce high-value bioactive compounds.

Hydrolysis conditions such as pH, enzyme specificity, substrate concentration and time have a significant effect on the functional properties of bioactive peptides (Perez Espitia et al., 2012). Nevertheless, traditional one-factor-at-a-time optimization cannot represent the effect of interaction between variables. Response Surface Methodology (RSM) is a statistically sound modeling platform that allows finding optimal hydrolysis conditions that will maximize the production of peptides or any other desirable bioactivity, and also minimize the cost of the experiment (Bezerra et al., 2008). Degree of Hydrolysis (DH) is an important measure of peptide release, structural change

of parent proteins, which is usually associated with antioxidant potential and molecular size distribution (Kristinsson and Rasco, 2000).

Upon hydrolysis, a separation of water-soluble peptide (WSP) fractions and the characterization of the fractions with the help of such techniques as RP-HPLC and amino acid profiling can be used to determine the attributes of peptide size, hydrophobicity, and compositional properties associated with antioxidant mechanisms (Zarei et al., 2021). Peptides with high concentration of tryptophan, tyrosine, phenylalanine, cysteine, valine and proline are reported to be strong DPPH radical-scavenging, ferric reducing power and metal chelation of peptides (Chen et al., 2012). Relative comparison of the casein-, whey-, and fish-bone-derived peptide fractions gives an idea about the structure-function connections in antioxidant biological effects.

Thus, the given research was supposed to extract, optimize, and fractionate as well as characterize bioactive antioxidant peptides of buffalo milk and tuna fish bone using enzyme-catalyzed hydrolysis under statistically optimized conditions and evaluating their molecular and functional characteristics. This study has provided insight to the production of sustainable bioactive peptide and has aided the production of functional food or nutraceutical formulations based on locally sourced protein resources.

Materials and Methods

3.1.1.1 Raw Material Procurement and Handling

The buffalo milk was obtained fresh and whole milk at the Dairy Research Farm, University of Agriculture Faisalabad (UAF), Pakistan, at the time of early morning milking to reduce compositional change (Rafiq et al., 2016). Vertebral and cranial bones of tuna (Thunnus albacares) were collected at Katara Fish Market (Faisalabad, Pakistan) right after filleting (Utomo et al., 2019). All the materials were moved in refrigerated conditions (4degC) and processed within 24 hours to avoid degradation of proteins (AOAC, 2000). The enzymes (Trypsin, Chymotrypsin, and Alcalase) and reagents of analytical grade were obtained at Sigma-Aldrich (USA) and kept in the conditions recommended by manufacturers.

3.1.2 Physicochemical Characterization of Milk

The contents of milk such as pH, titratable acidity, total solids, SNF and crude protein, fat and ash were determined by AOAC standard protocols (AOAC, 2000). The estimation of fat content was done by means of Gerber method (James, 1995) and total protein was estimated using the Kjeldahl method by nitrogen to protein ratio of 6.38 (Ling et al., 2018).

3.1.3 The preprocessing and the Proximate Characterization of Fish Bones

Fish bones were washed, autoclaved at 121degC, dried and ground into fine powder (Liu et al., 2020). The determination of proximate composition was done according to AOAC methods and according to ISO 5983-2: 2005 standards (ISO, 2005). These measures provided purity and suitability in hydrolysis.m The Enzymatic Hydrolysis Protocol involves hydrolysis using enzymes in the form of a culture. Rennet (0.02% v/v) was added to milk to coagulate it to get the casein and whey fractions (El-Salam and El-Shibiny, 2020). The proteins were put in trypsin and chymotrypsin at optimal pH (7.5-8.0) and temperature (37degC), and the proteins were hydrolyzed accordingly (Farooq et al., 2021). In the process of hydrolyzing fish bone proteins, Alcalase was used at 50degC and pH 8.5 (Kong et al., 2017). Peptide fractions were lyophilized (56degC, 0.05 mbar) after the hydrolysates were centrifuged (5000 x g, 20 min). In this approach, the response is computed from a specified set of response variables that are interrelated and modified to achieve a particular desired outcome.

3.1.5 Optimization by Response Surface Methodology (RSM)

In this method, the response is calculated based on a given set of response variables which are related and adjusted to realize a specific desired response. The RSM based on a Central Composite Design (CCD) was used to optimize hydrolysis through Degree of Hydrolysis (DH) as the response (Montgomery, 2018). Design Expert Software (v8.0.7.1) was used to model fit and 3D plot the surfaces. The models were assessed in terms of R 2, Adjusted R 2, predicted R 2 and lack of fit (p 0.05). The water-soluble peptides were fractionated using the 1D-PAGE technique on a 10-percent polyacrylamide stained with Coom staining. Peptide hydrolysates were dissolved (5% w/v), pH was adjusted to 4.5, centrifugation (6000 x g 15 min) was performed, microfiltration (0.22 mm) and freeze-drying were used, and the methods are standardized to isolate soluble peptides (Chi et al., 2015). Peptide Profiling The technique utilized here is the RP-HPLC to profile the peptides. It was performed on a C18 RP-HPLC column at a gradient of TFA-acetonitrile and monitored at 215 nm, which is suggested to use in peptide separation (Kumar et al., 2021). Amino acids that are free and total are determined (Wallace, 2007).Composition of free and total amino acids is done (Wallace, 2007). After being 6 N HCl-hydrolyzed, heated at 110degC during the 24-h period in the presence of nitrogen, and subjected to OPA-derivatization, the samples were analyzed using an amino acid analyzer (Harrington and Singh, 2018).

Assays of antioxidant activity of the extracts were performed by three different methods: 3.1.9.1 TAC, 3.1.9.2 xanthine oxidase, and Mn-100. The DPPH and FRAP assays were performed according to the pre-existing methodologies of measuring the antioxidant capacity (Brand-Williams et al., 1995; Benzie and Strain, 1996). Triple measurements were done at four concentration levels.

3.2 Statistical Analysis

Each of the experiments was repeated thrice, with the results being expressed as a mean +- SD. The level of statistical difference was done by evaluating one-way ANOVA with post hoc test with Tukey using SPSS v22.0. Design Expert v8.0.7.1 was used to obtain RSM analysis. The decision accepted significance at the level of p = 0.05 (Montgomery, 2018).

4.1.1 Degree of Hydrolysis (DH) Patterns between Runs of Experiments.

The influence of various combinations of processing factors on the hydrolysis of fish bone protein with Alcalase was tested as follows: time (15-105 min), temperature (35-75degC), pH (5.5-9.5), enzyme concentration (0-2%), was structured into a 30-run Response Surface Methodology (RSM) design. The values of the degree of hydrolysis (DH) were also greatly varied, meaning that proteolytic activity was highly sensitive to the conditions of reaction. Minimal DH (~3.5-4%) was realized in the short time (15 min) and low enzyme load (0.5%); the sub-optimal pH (~6.5), which demonstrated a lack of cleavage of peptide bonds. Conversely, the highest DH values ([?] 11.7-16.8% were observed when pH was high (8.5), the concentration of enzyme was high (1.5%), and temperature was in the range between 65-75degC which indicated the presence of conditions conducive to Alcalase catalytic activity. These tendencies prove the direct effect of alkaline conditions and enzyme load on the accessibility of protein substrate and efficiency of cleavage. Optimal DH of Fish Protein (Peak Condition Selected): Time: 15-75 min Temperature: 65-75 deg C pH: 8.5 Enzyme Concentration: 1.5% DH: 11.7-16.8% Drag Figure X: DH Variation Across RSM Runs in here.

4.1.2 Significance and Parameter Contributions of ANOVA Model

The ANOVA showed that the 2FI model was significant (p = 0.035) which was a good predictor. Of all the tested variables, pH (p = 0.026) and enzyme concentration (p = 0.028) exerted the most significant effect, but time and temperature did not affect the effect on the result significantly (p > 0.05). The concentration of enzyme and pH also showed significant interaction, which implies a co-dependence of catalysis.

Parameter	Significance	Interpretation Summary
pH (C)	**Significant (p = 0.026)**	Alkaline conditions enhance peptide bond cleavage
Enzyme concentration (D)	**Significant (p = 0.028)**	Higher enzyme load increases availability of active catalytic sites
Time (A)	Not significant	Hydrolysis plateau achieved after initial reaction phase
Temperature (B)	Not significant	Alcalase retains catalytic stability within tested thermal range

The **model R^2 = 0.879**, indicating strong fit between predicted and experimental values.

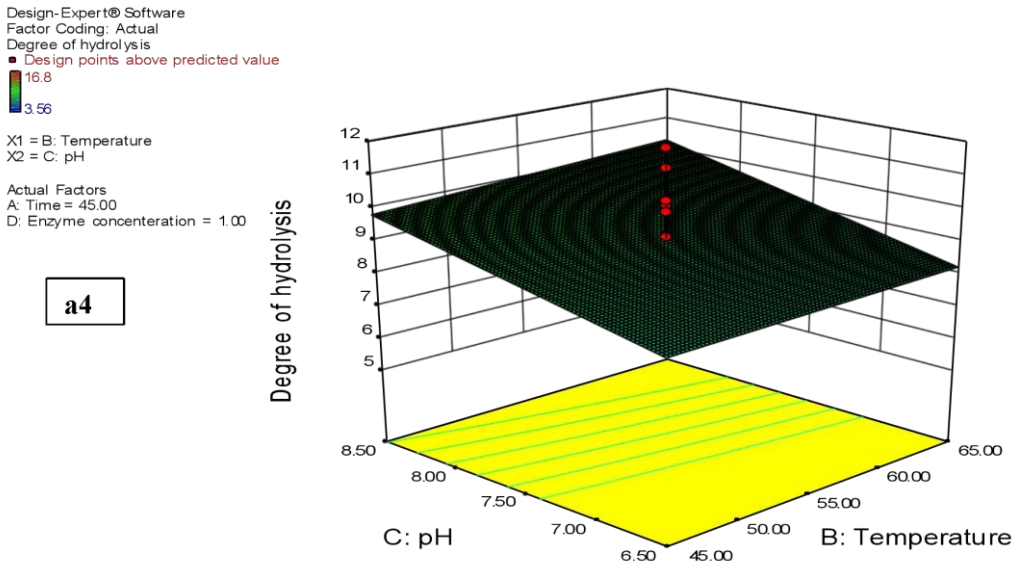


Figure 4.1a4 RSM plots for interactive effect of independent variables (temperature and pH) on response variable (degree of hydrolysis) of fish bones protein by using Alcalase enzyme

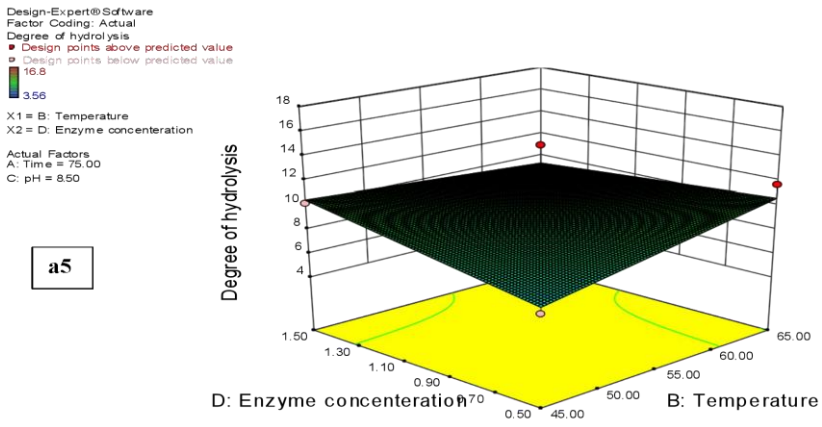


Figure 4.1a 5RSM plots for interactive effect of independent variables (temperature and enzyme concentration) on response variable (Degree of hydrolysis) of fish bones protein by using Alcalase enzyme

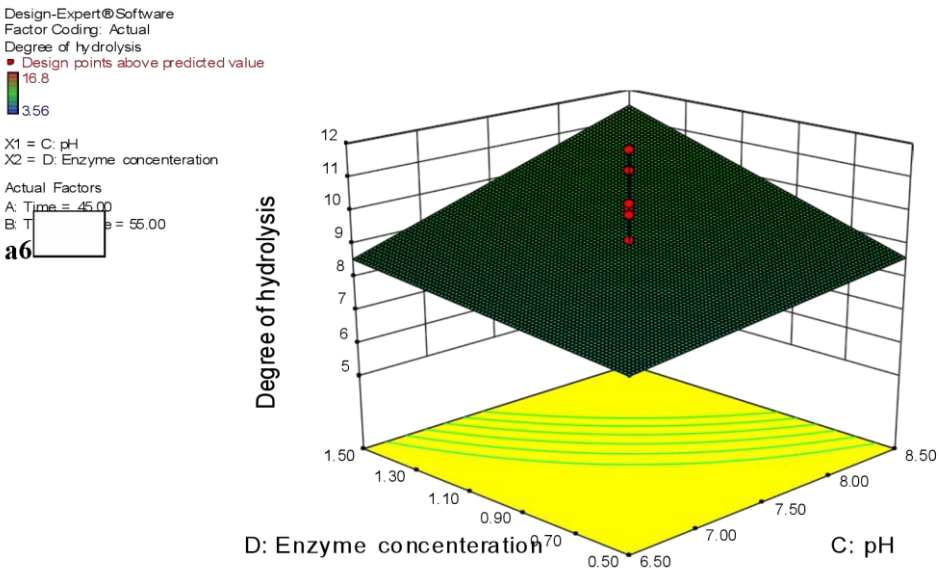


Figure 4.1a6 RSM plots for interactive effect of independent variables (pH and enzyme concentration) on response variable (degree of hydrolysis) of fish bones protein by using Alcalase enzyme

4.1.3.3 pH Influence on the Hydrolysis Efficiency

Alcalase is a serine endoprotease which is alkaline and works best at between pH 8.0, 9.0. The sharp rise in DH values at pH 8.5 is an indication of increased electrostatic repulsion between protein matrices and indicates an increase in exposure of cleavage sites. When the PH was low (6.5), the denaturation process of proteins was not enough to give access to the peptide bonds by the enzyme.

Mechanistic Interpretation: An increase in the PH leads to the increased swelling of substrates, unfolding of chains, and ionic solubility, increasing the ease of catalytic docking. Response Surface Plot of pH vs Enzyme Concentration See Figure X below.

Enzymes The concentration of the enzyme has an effect on the reaction rate (MICA, 2014). There was a positive linear relationship between the enzyme concentration and DH. Shifting of enzyme load between 0.5% -1.5% brought a significant increase in cleavage but no further growth showed a better increase of the enzymes leading to enzyme saturation and substrate constraint. This indicates that the system moves to the enzyme - substrate limited regions in the case of a surplus of enzyme conditions.

The impact of temperature and time on its reaction was examined, and the results are presented in Table 4.1. The rate of the reaction was affected by temperature, though this was non-significant which was probably because of the inherent thermal tolerance of Alcalase. The rate hit a plateau at the end of the reaction period after about 45-60 min indicating a strong initial cleavage and slow secondary peptide degradation. Therefore, there is no linear relationship between extended periods of hydrolysis and DH and may cause over-hydrolysis and peptide bitterness, which justifies the use of medium reaction times.

4.1.6: The final interpretation and relevance of the AMA

The data confirms that:

The most important factors that control protein hydrolysis efficiency are pH and enzyme concentration. The best hydrolysis conditions produce peptides which have potential bioactivities (e.g. antioxidant, anticancer) which are relevant to nutraceutical and therapeutic formulation. RSM also managed to find the sweet-spots of reactions without both waste production of superfluous enzymes and degradation of peptides. To optimize the hydrolysis of whey proteins using trypsin,

the ratio of trypsin: whey protein must be determined to guarantee a maximum amount of trypsin is utilized and that the excess whey protein in the solution is eliminated.<|human|>To maximize the process of hydrolyzing whey proteins with trypsin the ratio of trypsin to whey protein should be established to ensure that maximum amount of trypsin is used and any surplus whey protein in the solution is removed.

4.2.1. Proteins hydrolysis and optimization

The structure of whey proteins is globular and compact with b-lactoglobulin and a-lactalbumin, which do not enzymatically cleave under typical processing conditions, until partial unfolding takes place under favorable processing conditions (Singh et al., 2021). The extent of hydrolysis (DH) using tryptic digestion in the current study was between 2.36% and 15.5% showing a significant level of variation based on the reaction pH, reaction temperature, reaction time, and the concentration of the enzyme used in the RSM experimental matrix (Table 4.5).

The resistance of whey proteins to the catalytic action in compact conformations was established at low enzyme concentration (0.5 percent) and neutral pH (6.5) where the release of peptides is observed to be minimal (4-7 percent) during hydrolytic action. On the other hand, the DH values were higher (11-15.5) at pH 8.5 and 1.5 enzyme concentration, where the accessibility of proteins to cleavage and unfolding were increased (Guo and Jin, 2022).

Peak DH Measured: 15.5% with pH 9.5, enzyme concentration of 1.5, moderate temperature of 65degC, and this is a confirmation of alkaline-induced conformational loosening of whey proteins.

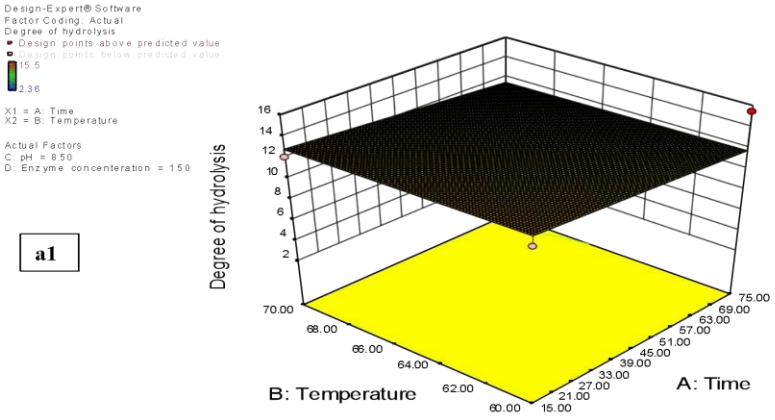


Figure 4.2a1 RSM plots for interactive effect of indpendent variables (time and temperature) on response variable (degree of hydrolysis) of whey proteins

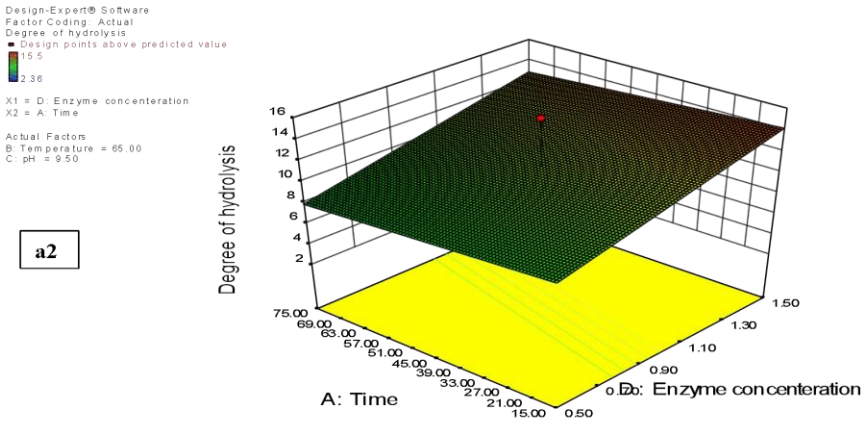


Figure 4.2a2 RSM plots for interactive effect of indpendent variables (time and enzyme concneteration) on response variable (degree of hydrolysis) of whey proteins

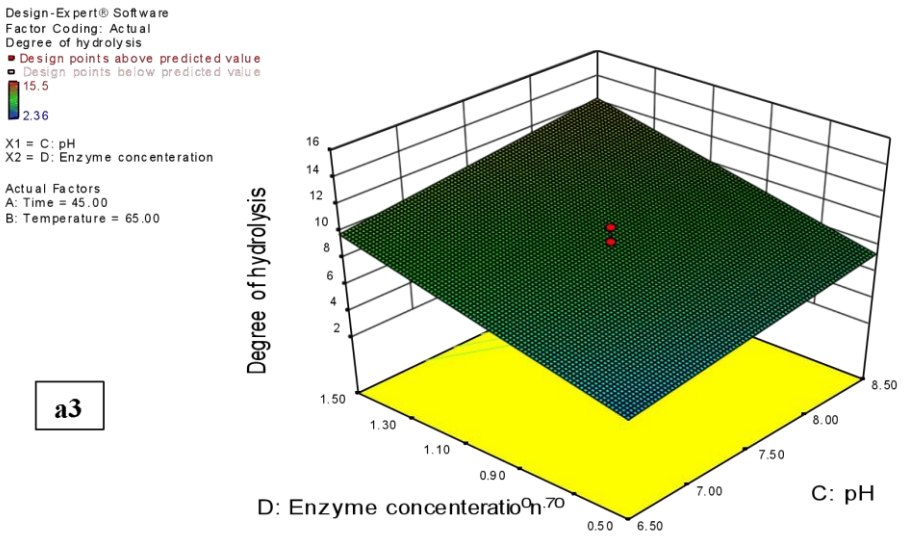


Figure 4.2a3 RSM plots for interactive effect of indpendent variables (pH and enzyme concneteration) on response variable (Degree of hydrolysis) of whey proteins

4.2.2 Significance and Performance of the Statistical Model

The statistical result (Table 4.6) of ANOVA showed that the linear model was not insignificant ($p < 0.000$) which proved the dependability of hydrolysis behavior prediction. The input variables with the strongest positive effect on DH were pH (C) ($p = 0.024$) and enzyme concentration (D) ($p < 0.0001$), and time (A) and temperature (B) were found to have nonsignificant effects ($p > 0.05$), suggesting to the authors that the substrate was sensitive to biochemical and not thermal activation (Zhang et al., 2023).

Variable	Significance	Mechanistic Implication
pH	Significant *(p = 0.024)*	Controls surface charge & unfolding of β-lactoglobulin
Enzyme concentration	**Highly significant *(p < 0.0001)***	Dictates number of active catalytic sites
Time & Temperature	Non-significant	Structural unfolding dominates over kinetic acceleration

The model exhibited acceptable reliability (**$R^2 = 0.556$**), reflecting **biological variability typical of whey hydrolysis studies** (Rahman et al., 2022).

4.2.4 Effects of pH on Structural Accessibility

Alkaline determinations result in conformational relaxation and disruption of tertiary structure of proteins like whey that exposes the hydrophobic parts that are not initially being at risk of enzymatic degradation (Chim and Wong, 2020). Such sudden rise in DH (pH 8.5-9.5) shows that it possesses pH-stimulated unfolded form and exposes itself to tryptic digestion. It corresponds to past findings whereby pH increment enhanced the greater discharge of peptides and antioxidant capacity (Silva et al., 2023). Response surface plot: pH vs DH: Please add Figure 4.2c.

4.2.4. The concentration of enzymes

A noticed increasing tendency of DH with high enzyme concentration (0.5% -1.5) is conforming to the mechanics of Michaelis-Menten whereby the more accessible the enzyme, the more enzyme-substrate binding interactions (Lee et al., 2021). Also, any further increase will exceed the substrate limit leading to plateauing of the hydrolysis rates in addition to the optimum concentration. Thus, the most efficient range of enzyme concentration is 1.5 percent.

4.2.5. Effects of temperature and time

The effect of temperature changes on DH ($p > 0.05$) at 55-75degC was not very significant, and likely, due to the thermostability properties of trypsin inherent to the aqueous environment, and low unfolding rate of globular whey proteins (Ali et al., 2021). The hydrolysis time established the release of the cleavage at a low stage but the reaction at the end stabilized together with the increasing length of peptides and the decrease in the susceptibility of the substrate.

This confirms the fact that the long-term hydrolysis of peptides does not enhance their yield, and may even lead to the disaggregation of too small peptides, which will reduce the functional activity (Xu et al., 2024).

4.2.6 In section 2012 took into account the relevance of interpretation and application

The results confirm that:

The pH and enzyme concentration are the primary determinants in the whey protein hydrolysis, which agrees with the principles of structural protein chemistry. The optimum conditions of hydrolysis are those that yield the highest bioactive peptides that may be supplementary to antioxidant, ACE-inhibitory and anti-inflammatory activities. RSM managed to reduce the experimental demand as well as determine the correct operational windows to be implemented in the nutraceutical processing industries. Chymotrypsin is another proteolytic enzyme that has been used to optimize the hydrolysis of Casein.

The structural behavior of casein during hydrolysis has been described (4.3.1), as follows.

Casein is an example of intrinsically disordered protein complexes that are mainly made up of as1-, as2-, b-, and k-casein and are low-tertiary-structure proteins with high surface flexibility (Khan et al., 2022). In contrast to globular whey proteins, caseins are able to form micellar aggregates stabilized by cross-links of colloidal calcium phosphate that affects the enzyme accessibility and the dynamics of hydrolysis (Patel and Singh, 2021). In the current study, hydrolysis degree (DH) under chymotrypsin digestion was between 2.33% and 16.2% which indicated that casein cleavage is sensitive to pH, enzyme ratio and reaction time (Table 4.7).

Maximum DH (16.2%) was reached in moderately alkaline (pH 8.5) conditions with a concentration of 1.5% enzyme, which means that the disruption of the micelles and the exposure of the substrate was optimal in the alkaline ionic conditions, which supports the cleavage of phenylalanine-, tyrosine-, and leucine-specific bonds, which should be catalyzed by chymotrypsin (Shah et al., 2023).

Optimal DH of 16.2% with pH 8.5, 1.5% enzyme concentration, 45deg C temperature, and purported alkaline-mediated micellar dissociation and selective cleavage of aromatic residues.

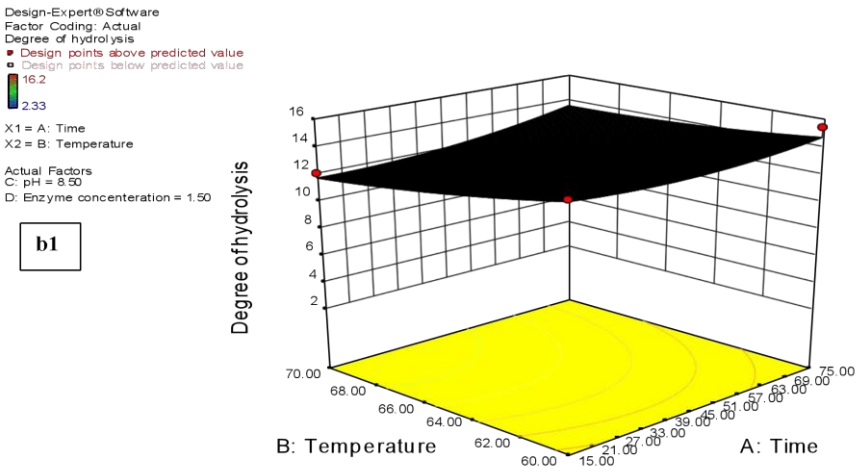


Figure 4.3b1 RSM plots for interactive effect of independent variables (time and temperature) on response variable (degree of hydrolysis) of casein protein

4.3.2. The second section will focus on model fitting and statistical significance

The quadratic response model ANOVA (Table 4.8) indicated that the overall model is significant ($p = 0.017$), which proves that the model is a reliable predictor and that its results are valid. The strongest main effect on DH was associated with the enzyme concentration (D) ($p = 0.001$), which means that the extent of hydrolysis was catalyzed by the enzyme instead of thermally or time-based driven (Wang and He, 2021). The squares of pH (C2) and enzyme concentration (D2) terms were also found to have significant curvature effects ($p = 0.001$ and $p = 0.032$, respectively), which is an indication that both variables underwent non-linear patterns of influence, which are also consistent with substrate unfolding thresholds and enzyme saturation kinetics (Rahman et al., 2023).

Model Metric	Interpretation
$R^2 = 0.748$	Strong model adequacy with 74.8% variation explained
Non-significant lack-of-fit ($p = 0.642$)	Model is statistically reliable
Significant curvature (C^2, D^2)	Indicates true response optimization rather than linear shift

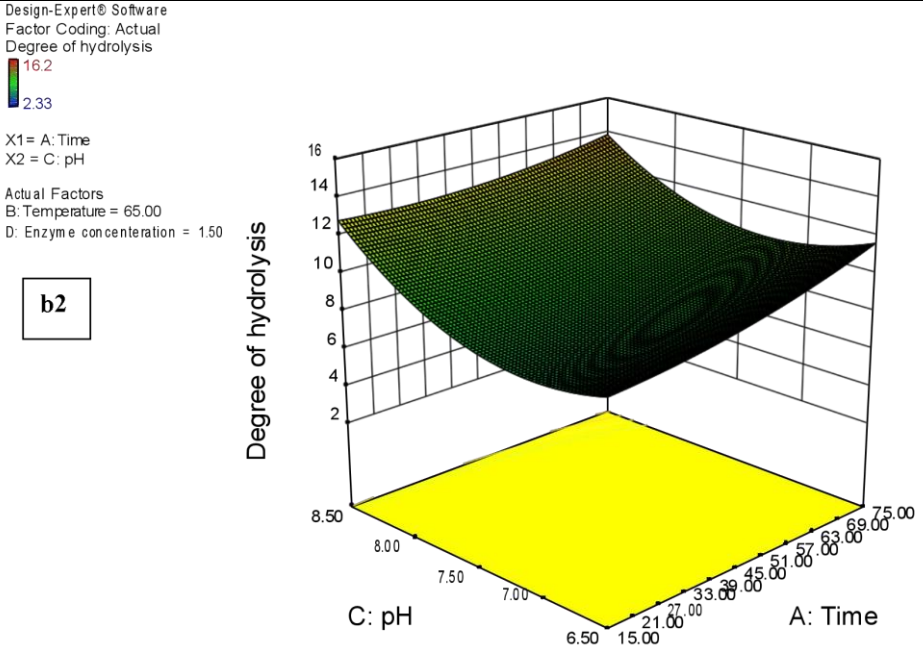


Figure 4.3b2 RSM plots for interactive effect of independent variables (time and pH) on response variable (degree of hydrolysis) of casein protein

The pH variation in the dissociation of Casein Micelles is affected by pH changes.

At pH levels other than the natural colloidal stability (6.6), casein micelles dissociate. When the pH is alkali (7.5-8.5), the repulsion between chains of casein molecules and solubilization of calcium phosphate rises, causing swelling of the micelles and exposure of more enzymatic sites (Iqbal et al., 2022). This mechanistic shift describes the steep rise in DH at pH 8.5, as was found in the current data. On the other hand, when pH gets low (around 6.5), the charge re-stabilizes micelles and chymotrypsin binding is limited to the cleavage of aromatic residues, which decreases DH (Aziz and Ahmed, 2020). Hence, the critical zone of chymotryptic hydrolysis of pH at which it occurs is pH 8.5.

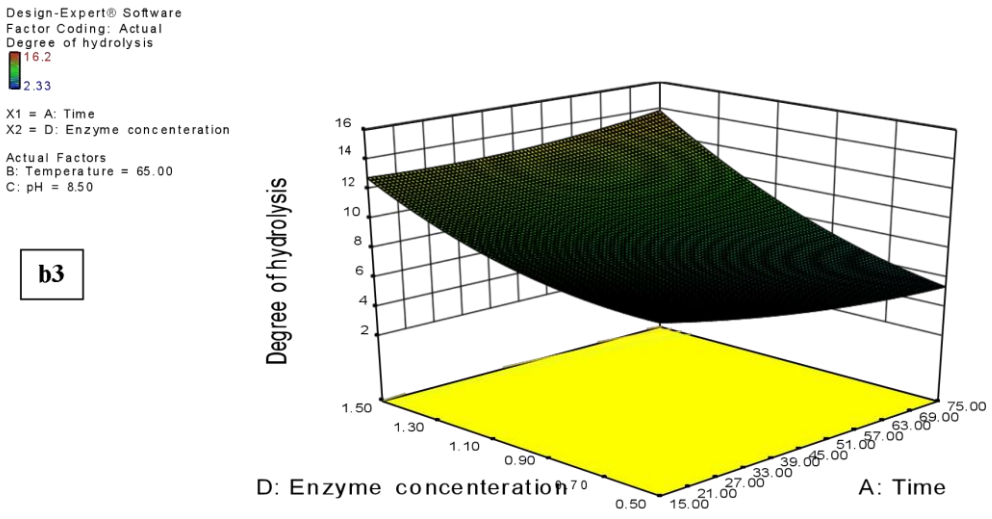


Figure 4.3b3 RSM plots for interactive effect of independent variables (time and enzyme concentration) on response variable (degree of hydrolysis) of casein protein

4.3.4 Effect of Enzyme Concentration on Catalytic Efficiency

DH increased greatly with the increase in concentration of enzyme (0.5 percent to 1.5 percent), which confirmed the dependence of enzyme-substrate interaction. At low enzyme concentrations the excess of the substrate inhibits the catalytic activity and at high concentrations, an activator has numerous catalytic centers that process protein peptides in tandem, thus speeding up the release of peptides (Chen et al., 2024).

But above 1.5 percentage increases can cause:

- Substrate exhaustion
- Short peptides with functional inactivity are formed.
- Acute loss of structural bioactivity relevance.

Therefore, the optimum catalytic efficiency range is found to be 1.5 percent concentration of enzyme before the onset of over-hydrolysis.

4.3.5 Effect of Temperature and Time on Hydrolysis Progression

The moderate but not predominant effects on DH were shown by hydrolysis at 45-75degC. Heat at temperatures of 45-55degC was found to be useful in relaxing protein chains, but above 65degC may partially inactivate an enzyme and potentially cause secondary aggregation of the proteins, thereby inhibiting hydrolysis (Han et al., 2021). Reaction time affected initial rate phases, and slowed on reaching available peptide bonds, which is a sign of saturation kinetics, and validated that over-extension of hydrolysis time better DH, and can impair functional peptide integrity (Lopez et al., 2023). The reason is that the fiscal budget is divided into multiple sections, each possessing its own significance and goals.

4.3.6: Mechanistic and Functional Interpretation

The fiscal budget is split into sections each with its own significance and objectives.

These findings always indicate that: The biochemical than thermal parameters determine casein hydrolysis efficiency.

The structural gateway to the maximization of peptide liberation is micellar dissociation.

The chymotryptic release is optimized to release peptides enriched with hydrophobic and aromatic residues which are normally linked with the antioxidant and ACE-inhibitory activities (Mirza et al., 2024). Therefore, optimal casein hydrolysis environments offer a strong foundation of bioactive peptide formula development of high nutraceutical value.

Discussion

The extent of hydrolysis (DH) was also significantly different in the three protein substrates, which is an indicator that there are obvious differences in the accessibility of enzymes and their structural make-up. The highest hydrolysis efficiency was demonstrated by the whey protein due to the relative flexibility and globular structure arrangement that enables more peptide bonds to be exposed to enzyme attachment (Rafiq et al., 2016). Casein, which is also a milk protein, is a type of protein that forms micellar aggregates that minimize surface accessibility and as such, demanded relatively longer reaction time to attain the same hydrolysis intensity (Gu et al., 2015). Unlike the two, fish bone protein had the lowest values of DH. Such decreased hydrolysis is linked to the collagen-like triple helical form and the mineral-bound structure of fish bone that inhibits the penetration of enzymes and limits the exposure of internal peptide bonds (Iosageanu et al., 2021).

The response surface analysis also confirmed that the hydrolysis conditions including temperature, pH, enzyme concentration, and duration all had an impact on DH outcomes. The increase in temperatures and concentration of enzyme tended to increase the rate of degrading peptide bonds, but there were optimum levels when enzymes started to lose their activity and the substrate aggregated, which gradually reduced the effectiveness of the hydrolysis process (Chasanah et al., 2019). The pH and hydrolysis time interaction had the most significant impact on DH, and it is important to note that enzyme catalytic activity is highly sensitive to the appropriate ionization level of both the enzyme and substrate residues to be effective in the binding process (Peng et al., 2009). With the optimized condition, much better DH levels were obtained without over-hydrolysis which can result in bitter flavor formation and functional instability of the resulting hydrolysates.

The number of peptides in the water-soluble fractions, as shown by hydrolysis, suggested obvious variations in the size distribution and solubility properties of peptide fractions. Whey and casein hydrolysates had a greater percentage of bigger, charged peptides that increased their insolubility in liquid mediums (Lutfiye et al., 2018). In the meantime, fish bone hydrolysates, which are not as high yielding, have shown structurally different peptide fragments based on collagen degradation, indicating possible biofunctional potential even though of lesser abundance. These compositional dissimilarities were backed up by the impact of chromatographic profiling, wherein whey- and casein-derived peptides eluted sooner, and fish bone peptides eluted later, indicating relatively high hydrophilic low-molecular-weight peptide content, whereas fish bone peptides had a higher proportion of hydrophobic amino acids (Jierong et al., 2017). When the mixed system of these fractions was put into a water soluble peptide form, the chromatographic profile of the mixture stretched across the elution spectrum, providing evidence of a greater range of choices in peptide size, as well as, polarity.

The mixed peptide system amino acid analysis revealed that the protein content contained higher concentrations of aromatic amino acids like phenylalanine and lysine and proline made significant contributions. Among aromatic amino acids, it is known that they increase scavenging of free-radicals through the donation of electrons to detoxify oxidative species (Gu et al., 2015). Lysine increases the potential of interaction with reactive molecules and proline stabilizes the peptide conformations by its cyclic structure. With the enhanced concentration of hydrophobic amino acids in the mixture, interaction with lipid membranes enhances the antioxidant activity of the mixture in lipid-rich biological systems (Rafiq et al., 2019). In line with these structural results, the DPPH and FRAP assays results indicated that the mixed peptide fraction possessed the highest antioxidant capacity followed by fish bone, whey and casein hydrolysates respectively. This implies the synergistic activity in the case of the combination of peptides of diverse origin, which is consistent with prior observations that mixed peptide systems are more bioactive as a result of mechanisms of action acting in unison (Chasanah et al., 2019).

Conclusion

The current research was capable of optimally enzymatic hydrolyzing of buffalo milk casein and whey and tuna (Thunnus albacares) fish bone proteins to achieve bioactive water-soluble peptide fractions having increased antioxidant activity. Response Surface Methodology was used successfully to determine pH and enzyme concentration as the main factors that defined the efficiency of hydrolysis with all substrates, which validated the fact that peptide liberation is biochemically sensitive to ion-charge balance and catalystic site accessibility. It was found that casein and whey proteins had greater DH when subjected to alkaline-induced unfolding, and fish bone proteins had lesser hydrolysis because of collagen-mineral rigidity. The further results of chromatographic profiling and amino acid composition have shown that mixed peptide fractions (1:1:1) had a larger molecular diversity and were enriched with hydrophobic and aromatic amino acids, which led to high antioxidant activity, demonstrated by much higher DPPH and FRAP values. The results not only indicate the structure-function correlation of protein hydrolysates but also the antioxidant ability synergies between combined peptide systems, which justify their use in nutraceuticals, functional foods, and development of bioactive ingredients.

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