

**BIOACTIVE AND FUNCTIONAL PROPERTIES OF FISH PROTEIN HYDROLYSATE FROM
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ABSTRACT

Objective: The present work is focused on the production of biologically active protein hydrolysate from low value by-catch fish, *Leiognathus bindus*; this will be an ideal approach to exploit the underutilized fish to develop a highly nutritious bioactive product. Moreover, fish protein hydrolysates have a broad spectrum of applications ranging from high-value peptones to food ingredients with special functional properties.

Methods: The protein hydrolysate was prepared from *L. bindus* through acidic autolysis processes. The proximate composition, physico-functional, and biochemical properties of hydrolysate and control samples were characterized by standard protocols. Molecular profiling was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis and high-performance liquid chromatography analysis performed to identify the essential amino acid composition in protein hydrolysate.

Results: The results of this study reveals that the protein hydrolysate obtained from the trash fish has relatively high protein (71.01±0.2%) and low lipid (1.57±0.1%) content. The molecular analysis shows low molecular weight peptides are obtained in the hydrolyzed samples which indicate high protein solubility confirmed to have the potential application as an ingredient in the balanced human diet. The chemical score of the hydrolysate indicates that it fulfills adult human nutritional requirements.

Conclusion: This approach also offers the relatively simple and cost-effective process to improve the nutritional characteristics and functional properties of foodstuff, also an opportunity for the conversion of waste fish resources to nutritious products, that can be used in food and pharmaceutical industries.

Keywords: Protein hydrolysate, Emulsifying property, Bioactive, Peptides, Amino acid, Chemical score.

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INTRODUCTION

Fish protein is an essential source of nutrients for many people, especially in developing countries. Fisheries generate renewable natural resource and subject to its own dynamics. Among the various commercial industries, the fisheries occupy a major place in promoting the socioeconomic development of the country. However in worldwide, it was noted that about 25% of the total resource had been wasted in the fishing industry [1]. Moreover, about 38.5 million tons of species of the global marine fish captures are known to be by-catch and discarded due to low market value [2,3].

By-catch is part of fishery catch which may be retained and landed but is usually discarded or released back to the sea in dead or alive. By-catch is a rich bioactive resource with potential application in industries such as pharmaceuticals, food and diet, animal nutrition, and cosmetics [4]. Therefore, a basic understanding of these components is essential to develop new technologies with high yields so that the processing can be economically sustainable [5]. *Leiognathus bindus* are generally considered as important bycatch owing to much less fat content and bones rich in calcium, making them commercially valuable especially for food industries. The fish tissue is known to have increased nutritional significance thereby, can be a valuable dietary module [6]. The preparation of the protein rich hydrolysate from the by-catch fish also serves as a solution for the environmental pollution due to the accumulation of fish waste and utilization of raw material [7].

Protein hydrolysates can be obtained by the hydrolysis process in which the protein is broken down by protease enzymes. Hydrolysis can decrease the peptide size and convert the hydrolysates to obtainable amino acid resource for protein biosynthesis [8]. Fish protein hydrolysates (FPHs) have predictable functional properties. An increase

in the number of peptide molecules and exposed hydrophobic amino acid residues due to hydrolysis of proteins would contribute to the formation of emulsions [9]. Protein hydrolysates generated from fish proteins are good nutritional supplements as bioactive compounds [7] and can be easily absorbed and utilized for various metabolic activities.

From the previous studies, it is evident that the *L. bindus* rich source of protein used for the preparation of nutritious rich health mix powder [10]. However, there have been no reports available in studying the production of potentially bioactive FPH with from *L. bindus*. There exists a need to explore the possibility of developing valuable products from trash fish for the better utilization of these nutritious resources. Hence, this study is focused on the production of FPH from *L. bindus* through novel hydrolysis process and to characterize the biochemical and physico-functional parameters of the FPH.

METHODS**Collection of samples**

The fish samples, *L. bindus*, are identified and were collected from Royapuram, the main fish landing stations in the southern coastal region of Chennai, Tamil Nadu, India. The samples were collected in ice-cold conditions and immediately brought to the laboratory for further analysis. The samples were stored in sterile conditions at ice-cold temperature.

Physical analysis and sample preparation

The samples were collected in ice-cold condition and subjected to physical analysis to understand the freshness of the sample. After analysis of physical condition to evaluate the freshness, the fish samples were rinsed and filleted down. The fillets were packed in polyethylene bags and freeze-stored at -20°C until used. The homogenate was

obtained from mincing filleted tissues with water (in the ratio of 1:3) using high-speed blender for 2 minutes and preserved at -20°C until further analysis.

Preparation of FPH

The frozen homogenate was thawed and then divided into batches for the preparation of hydrolysate for the preliminary studies in order to understand the range of temperature and the pH to be maintained in optimum hydrolyzing condition. The acidic condition was maintained in the presence of sulfuric acid:formic acid. The pH values ranging from 2 to 7 were adjusted, and the autolysis was carried out by maintaining a temperature range of 30-60°C in shaker incubator and samples were withdrawn at a different time interval (0-120 minutes). The hydrolysate was heated to terminate the endogenous enzyme activity and centrifuged at 6500 rpm (revolutions per minute) for 15 minutes to obtain the supernatant and was then freeze-dried and characterized.

Biochemical methods

Proximate composition and degree of hydrolysis (DH)

The total crude protein, total lipid, ash, and moisture in raw materials were determined according to AOAC [11]. DH was defined as the proportion of cleaved peptide bonds in a protein hydrolysate [7]. DH was computed as:

$$\%DH = \left[\frac{10\% \text{ TCA soluble N}_2 \text{ in the sample}}{\text{Total N}_2 \text{ in the sample}} \right] \times 100$$

Protease enzyme assay - (tyrosine standard method)

The proteolytic activities of the crude protein extract were determined based on the casein hydrolysis assay of Kunitz [12] as modified by Walter [13], where Casein was used as a substrate and tyrosine taken as standard to construct the standard curve. One unit of specific activity was defined as the amount of enzyme needed to produce 1 µg tyrosine per minutes per mg soluble protein of enzyme extract (U mg/protein).

Foaming capacity

Foaming capacity was determined by slight modification in the method of Coffman and Garcia [14]. A known quantity of the hydrolyzed sample was mixed with 100 ml of distilled water. The initial volume of the sample is measured and stirred for 10 minutes using a mechanical stirrer. The final volume along with foam formed is measured. The difference of the final volume and the initial volume is the foaming capacity and foaming stability is the volume measured at time intervals of 5, 10, 20, 30, 40, and 60 minutes. Foaming capacity is expressed in percentage (%).

$$\text{Foaming property} = \frac{\text{Final volume after whipping} - \text{Initial volume before whipping}}{\text{Initial volume of the sample before whipping}} \times 100$$

Emulsifying capacity and stability

Emulsion was determined by slightly modified method of Beuchat [15] procedure. 1 g of protein hydrolysate was blended at in high speed in blender with deionized water for 60 seconds and vegetable oil was added in 5 ml portions through continuing to blending. The emulsion so prepared was then allowed to stand in a graduated cylinder, and the volume of water separated in separation phase was observed. EC is expressed as milliliter of oil emulsified per gram of hydrolysate.

$$\text{Emulsifying property} = \frac{\text{Volume of oil released} - \text{Volume of oil released}}{\text{Weight of the sample}}$$

Molecular analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight distribution of hydrolysates SDS-PAGE was carried out according to the method of Laemmli [16], using a 4% stacking gel and a 12% separating gel. Hydrolysate samples (1 µg/µl) were mixed 1:1 (v/v) rationally with the sample buffer (0.5 M Tris-HCl, pH 6.8) and

heated for 2 minutes before loading. A standard wide range marker of 10-200 kDa (purchased from Sigma-Aldrich) was used for the determination of molecular weight.

Amino acid composition

Hydrolyzed samples derivatized using phenyl isothiocyanate precede to high-performance liquid chromatography (HPLC) analysis. The total amino acids were determined by HPLC using a C18 column at the flow rate of 0.5 ml/minute with 338 nm Wavelength of detection (VWD) and reaction temperature of 40°C.

Chemical score

The chemical score of the prepared protein hydrolysate sample was computed according to Ovissipour [17]. It was calculated relative to the essential amino acid (EAA) profile in a standard reference protein as described by FAO/WHO [18] and NRC [19]. Briefly, the chemical score was calculated based on the following equation:

$$\text{Chemical score} = \frac{\text{EAA in sample protein} \left(\frac{\text{g}}{100\text{g}} \right)}{\text{EAA in standard reference protein} \left(\frac{\text{g}}{100\text{g}} \right)} \times 100\%$$

Statistical analysis

The data obtained were subjected to one-way analysis of variance using SPSS 12 the statistical software. Duncan's new multiple range test was performed to determine the significant differences between samples at the 5% probability level.

RESULTS AND DISCUSSION

The protein hydrolysate obtained is a complex mixture of peptides of different chain length together with free amino acids. Autolytic method is also reported for the recovery of proteins from underutilized fish [20] or fish processing waste [21]. However, a few studies [22] have shown hydrolysate could be obtained through autolysis with comparable yield. Moreover, due to the simplicity of the operation and no enzyme costs involved the process is still used for the preparation of protein hydrolysate.

In this study, proximate composition of unhydrolyzed raw material and hydrolysate protein prepared in autolysis process is shown in Table 1. From the results, it was evident that the protein content of FPH is high (71.1%), whereas in unhydrolyzed sample, the percent of protein content was comparatively low, i.e., 49.0±0.3%. The results coincide the results of previous studies where the protein content ranged from 63.4% to 89% [20,23-26]. In contrary to protein content, the lipid level exhibits a converse range in hydrolyzed sample (1.57±0.1%) when compared to unhydrolyzed (27.4±0.1%) raw sample mainly due to the interference of acid hydrolysis. In acidic hydrolysis, free lipids cross-linked to proteins and as well break-up protein particles so that most lipids are accessible to solvent. As reported earlier [23,27], the reduction in lipid content in FPH in the present may be due to the exclusion in centrifugation and mostly due to lipid oxidation. However, the elimination of lipid can improve the stability and quality FPH product.

Table 1: Proximate composition (%/ml) of control and FPH^a samples

Composition (%)	Contol	FPH
Protein	49.0±0.3	71.0±0.2
Lipid	27.4±0.1	1.57±0.1
Moisture	10.7±0.1	4.76±0.02
Ash	12.8±0.1	22.65±0.1

^aValues represented mean±SE (n=3). FSH: Fish protein hydrolysate, SE: Standard error

Protein recovery

The results of protein recovery obtained by different pH and time are presented in Fig. 1. There was a significant difference ($p < 0.05$) in protein recovery was noted among the different hydrolyzing conditions. From the results, it is evidence of the concentration of protein increase with an increase in time and gradually reduced after the optimum condition. For instance, the concentration of protein at pH 4 is $60.68 \pm 0.3 \mu\text{g}/\mu\text{l}$ at 60 minutes, whereas at 90 minutes, it increases to $63.60 \pm 0.9 \mu\text{g}/\mu\text{l}$. In hydrolysates high protein recovery ranged from 63% to 71% and has been increased with increasing incubation time, similar trend was also reported by Nemati *et al.* [25], Ovissipour *et al.* [28]. It is due to the ionic strength or hydrophobicity and also indicates that maximum peptide bonds are broken, and free amino acids are released.

DH

According to Souissi [29], DH increase with an increase in hydrolysis time and correlates with pH in reaction mixture. In this study, the DH exhibits a bell-shaped pattern and the related result was also reported by Souissi *et al.* [29], Amiza *et al.* [30] in silver catfish frame hydrolysis. The effect of pH and time on DH is normally in bell-shaped pattern with increases in steady state at 90 minutes in pH (2-7) is clearly shown in Fig. 2. In contrast, at 120 minutes declining status in DH is due to higher DH, due to a maximum rate of peptide cleavage [28]. The values of DH in may be useful in preparing hydrolysates with certain functional properties, since the DH is the principle determinant of protein hydrolysate properties. The result was in accordance to [28] in which DH of yellow tuna fish increased with increase in hydrolysis time.

Protease activity

During hydrolysis, the fish tissue is degraded by endogenous and exogenous protease enzymes and these enzymes hydrolyze protein into smaller peptide units or free amino acids. According to Barrett [31], protease is proteolytic enzymes which are a group of enzymes whose catalytic function was to hydrolyze proteins. From the Fig. 3, it was evidence the protease activity increase at the time interval of 90 minutes. However, the hydrolysis of the fish was characterized by an initial rapid phase, during which a large number of peptide bonds were hydrolyzed. The protease activity red sea bream *Pagrus major* [32] and *Takifugu obscurus* [33] also initiate hydrolysis of the intact protein as it was observed in the present study.

Foaming capacity and emulsifying property

The foaming capacity of protein hydrolysate showed a significant difference in different pH. A good foaming capacity might attribute to an increase in the surface activity, which is due to partial proteolysis that produced the greatest number of polypeptide chains and therefore allowed more air to be incorporated. The protein hydrolysate obtained in the present study prepared at pH 5 gave highest high foaming capacity. The foaming capacity is improved on limited proteolysis. DH had a higher whippability, which was even better than the undigested protein. Foam formation decreased with increasing DH and also with increasing pH values and related to the result reported by Amiza *et al.* [30] (Fig. 4).

Environmental conditions such as pH, ionic strength, and temperature have an effect on the emulsification properties. It was found that the emulsion capacity is optimized when the protein had a balance between hydrophilic and hydrophobic residues [34]. In this study, the high range of emulsifying capacity was observed at pH 5 at 60 minutes and 57.14% and decline rate at 90 minutes. There are many different factors that may account for the difference observed between hydrolysates in both the ability to form an emulsion and emulsion stability, such as peptide molecular characteristics and peptide chain length show the major impact [23] (Fig. 5).

Molecular profile

The molecular profile of the samples has been notified through SDS-PAGE, and the results show that the samples have a low molecular weight. The banding pattern in L2 and L3 lane indicate that the samples possess low molecular weight protein as the bands coincides with

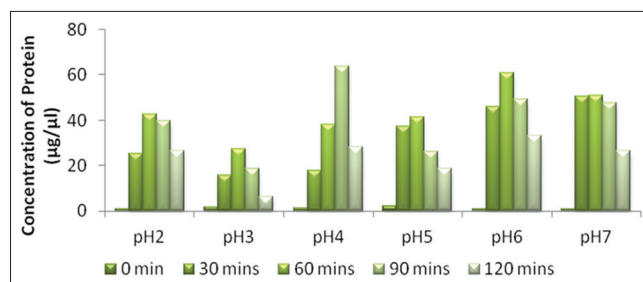


Fig. 1: Results for protein content (mg/ml) in *Leioagnathus bindus* fish protein hydrolysate prepared at different condition

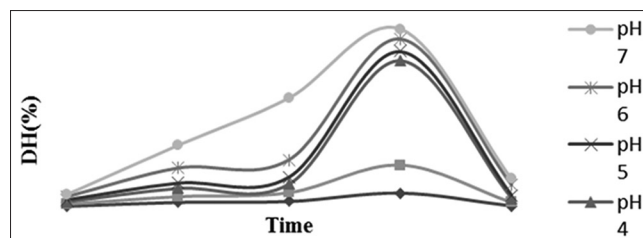


Fig. 2: Degree of hydrolysis (%/ml) in *Leioagnathus bindus* fish protein hydrolysate prepared at different hydrolyzing condition (time and temperature)

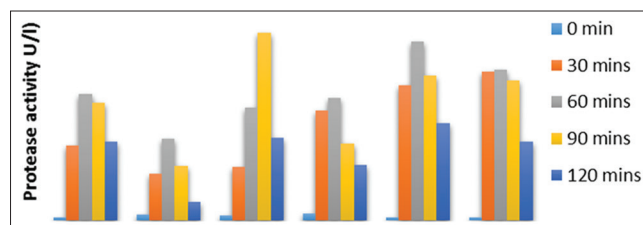


Fig. 3: Protease activity (U/l) in fish protein hydrolysate from *Leioagnathus bindus* in different hydrolyzing condition

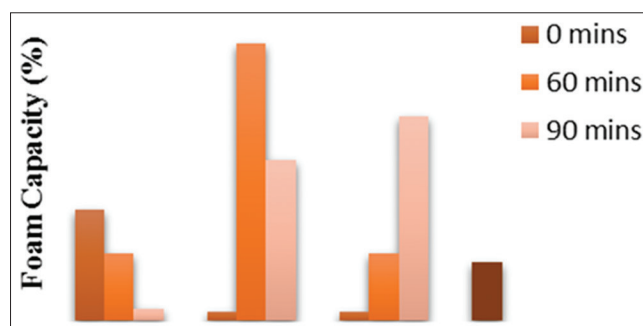


Fig. 4: Foaming capacity (%/ml) in *Leioagnathus bindus* fish protein hydrolysate in different pH and time interval

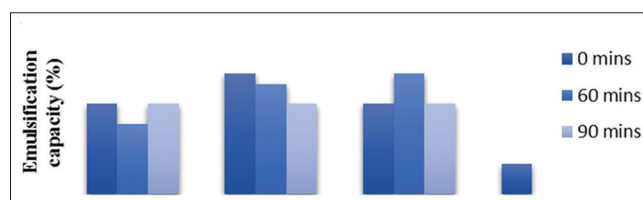


Fig. 5: Emulsification capacity (%/ml) in *Leioagnathus bindus* fish protein hydrolysate and control samples

6th to 7th bands of the marker. This indicates the molecular weight of the samples lie in the range of 14-20 kDa due to the extent of protein

hydrolysis was supported by the disappearance of large proteins in the electrophoretic profile of the protein hydrolysates (Fig. 6). The low molecular weight peptides in the protein hydrolysates indicating the efficacy of these protein hydrolysates as a source of bioactive peptides [24]. The recovery of protein was very high thus, making it a soluble peptide.

Amino acid composition

In this study, there was significant ($p < 0.05$) result for EAA. It was observed that hydrolysates sample has EAA threonine, methionine, isoleucine, lysine, and phenylalanine; this indicates the hydrolysate is nutritionally rich products shown in Table 2 and Fig. 7. Indeed, protein hydrolysates developed by acid analysis were rich EAA, which includes leucine, arginine, histidine and lysine, but lower in tyrosine, aspartic acid and alanine as compared to fish muscle. This finding was close to the report of Klompong *et al.* [6] which showed that yellow stripe trevally (*Selaroides leptocephalus*) protein hydrolysates had a higher ratio of EAAs to non-EAAs compared to unhydrolyzed flesh. Shahidi *et al.* [20] also reported autolytic method can be suggested for recovery of proteins either from underutilized or fish processing waste. However, some studies [35] have shown hydrolysate could be obtained through autolysis with comparable yield.

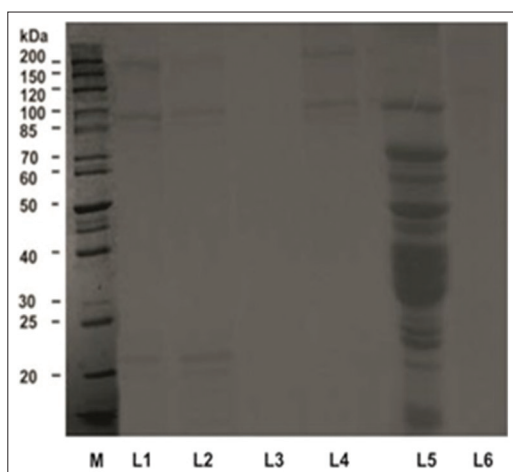


Fig. 6: Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of *Leioagnathus bindus* fish protein hydrolysates conditions. Lane M - Wide range standard marker, L1 - Hydrolysate with pH 6, L2 - Hydrolysate with pH 5, L3-Hydrolysate with pH 3, L4- Hydrolysate with pH 4, L5- control sample, L6- Unhydrolyzed sample

Moreover, the amino acid compositions in peptides make a significant biological function [36]. Besides, amino acids such as Arg and His are also establishing biological function like cytotoxic effect. The results shown in Table 2 indicate that protein hydrolysate prepared from *L. bindus* possess positively charged amino acids in with low molecular weight bioactive peptides. It is notable that a good proportion of His in the total amino acids which might have potential cytotoxicity effect [37].

In addition, due to the simplicity of the operation and no enzyme costs involved the process is still used for the preparation of protein hydrolysate. The nutritive value of the protein hydrolysate was evaluated through the chemical score, which can be used to compare the levels of EAAs in standard protein and sample. In the present experiment, chemical scores are computed based the reference of FAO/WHO [18] for adults and amino acid requirements especially of the EAAs represent a high proportion among the total [38,39].

The amino acid composition of protein hydrolysate in this study revealed that the hydrolysate contains higher EAA compared to the suggested pattern by FAO/WHO for the adult humans requirements. This is an interesting novel work focus on the nutritional composition, especially the composition in EAAs is a determining factor in human and animal feeding. From this study, it is evident that protein hydrolysate from *L. bindus* can potentially serve as a good source of desirable peptides and EAAs thus it can be used as a functional ingredients food industry.

CONCLUSION

The outcome of this study shows the potential usage of wasted fish resource for the production of FPH through novel and efficient technology. FPHs obtained from waste fish consist of EAAs and functionally active compounds, which can serve as a potential source for nutritional and pharmaceutical applications. Moreover, this approach offers a relatively simple and cost-effective process in the conversion of waste material. Fish hydrolysate can also serve as another beneficial protein nutritional supplement which can bring economic advantages for the food industry. As the protein hydrolysates prepared in the present study consist of all EAA recommended by FAO/WHO for the adult humans, hence it can serve as a substitute to intact protein in dietary formulations to sustain the nutritional requirements for few populations.

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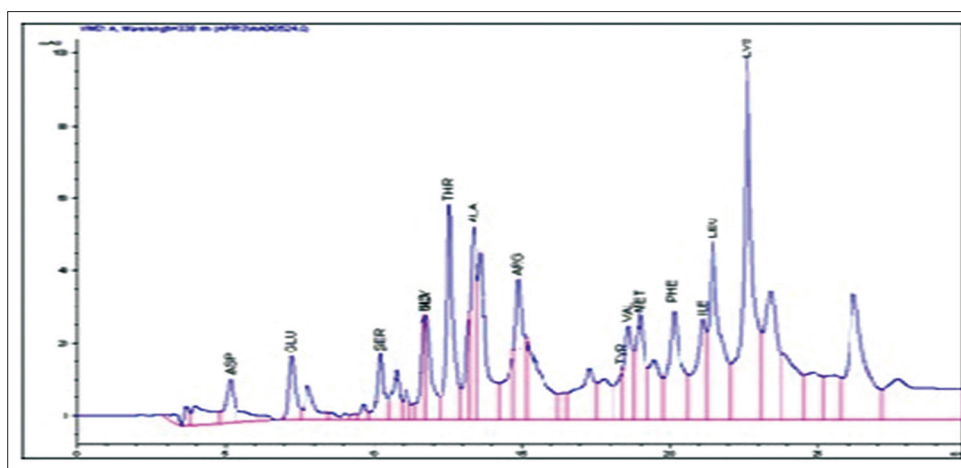


Fig. 7: High-performance liquid chromatography of amino acid composition in fish protein hydrolysates

Table 2: Comparison amino acid composition and chemical score in FPH (g/100 g) with FAO/WHO and NRC reference protein

Amino acid	FPH	Chemical score		Chemical score	
		RP1 (Adult) ^a	RP2 (Poultry) ^b	RP1	RP2
Arginine	1.22		0.7		1.74
Threonine	1.91	0.9	0.47	2.1	4.06
Tyrosine	0.32	-	-	-	-
Valine	1.83	1.3	0.7	1.4	2.61
Methionine	1.81	1.7	0.3	1.06	6.03
Phenylalanine	2.22	3.8	0.83 ^c	0.58	2.67
Isoleucine	1.78	1.3	0.65	1.33	2.06
Leucine	2.37	1.9	0.82	1.24	2.89
Lysine	2.99	1.6	0.69	1.86	4.33
Histidine	1.92	1.6	0.17	1.2	11.29
Alanine	1.21				
Aspartic acid	0.72				
Glutamic acid	2.14				
Serine	2.14				
Glycine	0.98				

RP1: Chemical score calculated with FAO/WHO reference protein as the base. RP2: Chemical score calculated with amino acid requirements as per NRC (1993). ^aSuggested EAA profile requirements in adults (FAO/WHO, 1990). ^bEAA requirements for poultry, according to NRC (1993). FPH: Fish protein hydrolysate, EAA: Essential amino acid

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