



UNIVERSITI PUTRA MALAYSIA

**THE PRODUCTION OF THREADFIN BREEM (NEMIPTERUS
JAPONICUS) PROTEIN HYDROLYSATE BY HYDROLYSIS WITH
ALCALASE**

NORMAH BT ISMAIL

FSMB 2001 2

**THE PRODUCTION OF THREADFIN BREEM (*NEMIPTERUS JAPONICUS*)
PROTEIN HYDROLYSATE BY HYDROLYSIS WITH ALCALASE**

By

NORMAH BT ISMAIL

**Thesis submitted in Fulfilment of the Requirement for the Degree of
Master of Science in the Faculty of Food Science and Biotechnology
Universiti Putra Malaysia**

November 2001



~ To my friends ~

*Kyaw Zay Ya, Amin Ismail (Dr), Anida, Shita, Leen, Nazri, Manichan,
Mohsin, Yusep, Misnawi, Shida, En. Azman, Kak Jem, En. Halim and Mr
Chan.*



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science.

**THE PRODUCTION OF THREADFIN BREAM (*NEMIPTERUS JAPONICUS*)
PROTEIN HYDROLYSATE BY HYDROLYSIS WITH ALCALASE**

By

NORMAH BTE ISMAIL

November 2001

Chairman: Associate Professor Hajjah Jamilah Bakar, Ph.D.

Faculty: Food Science and Biotechnology

The optimization of hydrolysis conditions in terms of pH, temperature, enzyme-substrate (ES) ratio and hydrolysis time for the production of threadfin bream (*Nemipterus japonicus*) hydrolysate was studied. Alcalase showed a higher percent nitrogen recovery (% NR) than Flavourzyme:Protamex mixture. By using Alcalase, the optimum hydrolysis conditions were pH 8.5, 60°C, ES2% and 120 min hydrolysis time. At least twenty percent degree of hydrolysis (% DH) and 70% NR was achieved. The yield of spray-dried hydrolysate hydrolysed under these conditions was 4%. The hydrolysate was white in color, highly soluble over a wide pH range, high in protein and essential amino acids but low in fat. The hydrolysate also exhibited an improvement in foam ability in comparison to the threadfin bream muscle but the foam was unstable. Hydrolysis with Alcalase also produced hydrolysate with poor emulsifying properties. The major free amino acids in the hydrolysate were glutamic acid,



aspartic acid, lysine, leucine and arginine in which glutamic acid was the dominant amino acid. Bitter amino acids methionine, valine, isoleucine, phenylalanine, leucine, arginine and tyrosine comprised of 42.34% of the total free amino acids. The inosine 5'-monophosphate (IMP) content was higher in the hydrolysate than that in the muscle. SDS-PAGE showed the presence of twenty peptide bands in the muscle having molecular weight between 7.2 to 87.6 kD and thirteen in the hydrolysate with molecular weight between 7.2 to 64 kD. For Sephadex G-75 fractions (FR), no peptide bands were seen in FR I, III, IV, VI, VII and VIII. FR II and V showed the presence of respectively five and seven peptide bands with molecular weight between 11.4 to 54 kD and 10.5 to 51.3 kD. Histamine content in the muscle was found to be 14.45 mgkg¹ but no histamine was detected in the hydrolysate. Sensory evaluation of the hydrolysate indicated that it was fishy in flavor and constitutes three basic tastes namely bitter, umami and salty with umami and bitterness as the dominating tastes.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENGHASILAN HIDROLISAT PROTIN “KERISI” (*NEMIPTERUS JAPONICUS*) MELALUI HIDROLISIS DENGAN MENGGUNAKAN ALKALASE

Oleh

NORMAH BTE ISMAIL

November 2001

Pengerusi: Profesor Madya Hajjah Jamilah Bakar, Ph.D.

Fakulti: Sains Makanan dan Bioteknologi

Persekitaran optima dari segi pH, suhu, nisbah enzim kepada substrat dan jangkamasa penghidrolisisan bagi menghasilkan hidrolisat 'Kerisi' (*Nemipterus japonicus*) telah dikaji. Didapati Alkalase menghasilkan peratus nitrogen (% NR) yang lebih tinggi berbanding campuran Flavourzyme:Protamex. Dengan menggunakan Alkalase, persekitaran optima yang diperolehi adalah pH 8.5, 60°C, ES2% dan 120 minit. Sekurang-kurangnya 20% darjah hirolisis (% DH) dan 70% nitrogen (% NR) telah dihasilkan. Penghasilan hidrolisat kering melalui persekitaran ini adalah sebanyak 4%. Hidrolisat yang di hasilkan berwarna putih, mudah larut pada pH yang berbagai, tinggi kandungan protein dan asid amino asas tetapi rendah lemak. Hidrolisat juga menunjukkan penghasilan buih yang lebih baik dibandingkan isi ikan 'Kerisi' akan tetapi buih yang terbentuk adalah tidak stabil. Penghidrolisisan menggunakan Alkalase

juga menghasilkan hidrolisat yang mempunyai sifat-sifat emulsi yang lemah. Asid amino bebas di dalam hidrolisat terdiri daripada asid glutamik, asid aspartik, lisin, liusin dan arginin di mana asid glutamik merupakan asid amino utama. Asid amino pahit iaitu methionin, isoliusin, valin, fenalalanin, liusin, arginin dan tirosin merangkumi 42.34% daripada kandungan keseluruhan asid amino bebas. Kandungan inosin 5'-monophosphate (IMP) adalah lebih tinggi di dalam hidrolisat dibandingkan di dalam isi ikan. SDS-PAGE menunjukkan terdapatnya duapuluh jalur peptida di dalam isi ikan dengan berat molekul di antara 7.2 dan 87.6 kD dan tigabelas jalur peptida dengan berat molekul diantara 7.2 dan 64 kD di dalam hidrolisat. Pecahan (FR) Sephadex G-75 menunjukkan ketiadaan jalur peptida di dalam FR I, III, IV, VI VII dan VIII. FR II dan V menunjukkan terdapatnya lima dan tujuh jalur peptida dengan berat molekul di antara 11.4 dan 54 kD serta 10.5 dan 51.3 kD. Kandungan histamin dalam isi ikan adalah 14.45 mgkg^{-1} dan histamin tidak dapat dikesan di dalam hidrolisat. Penilaian deria mendapati bahawa hidrolisat mempunyai rasa dan bau hanyir di samping mempunyai tiga rasa asas iaitu pahit, umami dan masin di mana umami dan pahit merupakan rasa yang amat ketara.



ACKNOWLEDGEMENTS

Syukur Alhamdulillah to the Almighty Allah s.w.t. for giving me strength, patience and capability to complete this project and thesis write up and salawat and salam to his righteous messenger, prophet Muhammad s.a.w.

First of all, I would like to express my deepest thanks and appreciation to the Chairman of Supervisory Committee, Associate Professor Dr. Hajjah Jamilah Bakar of the Department of Food Technology, Faculty of Food Science and Biotechnology for her guidance, patience, advice and encouragement throughout the course of my study. All the valuable experiences that I gained as one of her graduate students and her continuous commitment towards the success of my study will always be remembered deep in my heart. I would also like to thank my co-supervisors, Associate Professor Dr. Nazamid Saari of the Department of Food Science and Professor Dr. Haji Yaakob bin Che Man of the Department of Food Technology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, for their sincere advice for my self improvement and career development.

My appreciation also goes to all the highly dedicated, friendly, helpful, knowledgeable and very responsible technical staffs of Biochemistry,



Processing and Engineering Labs and to all my fellow graduate students, thank you for sharing your knowledge and ideas and for being very cooperative and supportive throughout my study in UPM.

Sincere gratitude is also dedicated to the financial support provided by the IRPA fund which was awarded to Associate Professor Dr. Hajjah Jamilah Bakar. Finally, I would like to thank Bahagian Latihan dan Pembangunan Staf, Universiti Teknologi Mara (UiTM) for providing me with the scholarship to pursue the degree. To all my colleagues at Food Technology Department, Faculty of Applied Science, UiTM, thank you for your support and encouragement.



TABLE OF CONTENTS

		Page
DEDICATION.....		ii
ABSTRACT.....		iii
ABSTRAK.....		v
ACKNOWLEDGEMENTS.....		vii
APPROVAL.....		ix
DECLARATION.....		xi
TABLE OF CONTENTS.....		xii
LIST OF TABLES.....		xv
LIST OF FIGURES.....		xvii
LIST OF PLATES.....		xviii
LIST OF ABBREVIATIONS.....		xx
CHAPTER		
I	INTRODUCTION.....	1
II	LITERATURE REVIEW.....	5
	Hydrolysate and Its Production.....	5
	Acid Hydrolysis.....	6
	Alkaline Hydrolysis.....	8
	Enzymatic Hydrolysis.....	9
	The Utilization of Proteases in Hydrolysate Production.....	11
	Fish Protein Hydrolysate.....	12
	Quality of Hydrolysate.....	16
	Taste, Flavor and Color of Hydrolysate.....	17
	Functional Properties and Factors Affecting Functional Properties.....	20
	Applications of Hydrolysate.....	26
	Hydrolysate as A Flavoring Agent.....	26
	Hydrolysate as A Protein Ingredient.....	29
III	OPTIMIZATION OF THE HYDROLYSIS CONDITIONS FOR THE PRODUCTION OF THREADFIN BREAM (<i>Nemipterus japonicus</i>) HYDROLYSATE BY ALCALASE.....	31
	Introduction.....	31
	Materials and Methods.....	33
	Materials.....	33
	Enzymatic Hydrolysis of Threadfin Bream (<i>Nemipterus japonicus</i>).....	34



Effect of pH.....	35
Effect of Temperature.....	36
Effect of Enzyme-Substrate Ratio and Hydrolysis Time.....	36
Percent Nitrogen Recovery (% NR) and Percent Degree of Hydrolysis (% DH).....	36
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	38
Statistical Analysis.....	39
Results and Discussion.....	40
Effect of pH.....	40
Effect of Temperature.....	42
Effect of Hydrolysis Time and Enzyme-Substrate Ratio on % DH.....	44
Effect of Hydrolysis Time and Enzyme-Substrate Ratio on % NR.....	46
Effect of Hydrolysis Time and Enzyme-Substrate Ratio on SDS-PAGE.....	49
Conclusion.....	55

IV	PHYSICO-CHEMICAL AND FUNCTIONAL PROPERTIES OF THREADFIN BREAM (<i>Nemipterus japonicus</i>) HYDROLYSATE.....	56
	Introduction.....	56
	Materials and Methods.....	57
	Materials.....	57
	Preparation of Hydrolysate.....	58
	Proximate Composition and Colour Measurement.....	61
	Functional Properties of Hydrolysate.....	63
	Sephadex G-75 Fractions of Threadfin Bream Hydrolysate.....	66
	Determination of Inosine 5'-Monophosphate (IMP).....	68
	Amino Acids Composition.....	71
	Molecular Weight Determination – SDS-PAGE... ..	71
	Determination of Histamine Content.....	74
	Statistical Analysis.....	75
	Results and Discussion.....	75
	Yield and Proximate Composition.....	75
	Functional Properties.....	79
	Taste Characteristics of Sephadex G-75 Fractions.....	84
	IMP Content in Sephadex G-75 Fractions.....	88
	Amino Acids Composition of the Muscle, Hydrolysate and Sephadex G-75 Fractions.....	89



	Molecular Weight of Muscle, Hydrolysate and Sephadex G-75 Fractions.....	96
	Histamine Content.....	100
	Conclusion.....	102
V	SENSORY CHARACTERISTICS OF THREADFIN BREAM (<i>Nemipterus japonicus</i>) hydrolysate.....	104
	Introduction.....	104
	Materials and Methods.....	106
	Materials and Preparation of Hydrolysate.....	106
	Preparation of Rice Porridge.....	106
	Preparation of Samples for Sensory Evaluation..	106
	Sensory Evaluation of Threadfin Bream Hydrolysate.....	107
	Panelists Response to the Taste and Aroma of Threadfin Bream Hydrolysate.....	108
	Statistical Analysis.....	110
	Results and Discussion.....	111
	Sensory Evaluation of Threadfin Bream Protein Hydrolysate Incorporated into Rice Porridge and the Determination of the Ideal Concentration.....	111
	Sensory Evaluation of Threadfin Bream Protein Hydrolysate Incorporated into Rice Porridge in Comparison to Commercial Flavorings.....	116
	Sensory Evaluation of Threadfin Bream Protein Hydrolysate in Comparison to Other Hydrolysates.....	116
	Taste and Aroma Characteristics of Threadfin Bream Hydrolysate.....	118
	Conclusion.....	121
VI	SUMMARY AND CONCLUSIONS.....	123
	REFERENCES.....	126
	APPENDICES	138
	BIODATA OF THE AUTHOR.....	183



LIST OF TABLES

Table		Page
1	Proximate compositions and Hunter Color values of threadfin bream muscle and its spray-dried hydrolysate.....	77
2	Protein solubility of threadfin bream hydrolysate at pH 2.5 to 11.....	80
3	Functional properties of threadfin bream muscle and hydrolysate.....	82
4	Inosine 5'-monophosphate (IMP) content of threadfin bream hydrolysate, muscle and threadfin bream hydrolysate fractions collected from Sephadex G-75 and panelist response to the taste of each fraction.....	87
5	Amino acids composition of threadfin bream muscle and its spray-dried hydrolysate.....	90
6	Amino acids composition of threadfin bream hydrolysate and Sephadex G-75 fractions(FR).....	93
7	Pearson Correlation coefficient for the score for bitterness responded by trained panelists.....	95
8	Pearson Correlation coefficient for the score for umami responded by trained panelists.....	95
9	R _f values for peptide bands in muscle, spray-dried threadfin bream hydrolysate and Sephadex G-75 fractions.....	101
10	Score for odor, color, taste and overall acceptability of spray-dried threadfin bream (<i>Nemipterus japonicus</i>) hydrolysate reconstituted hydrolysate.....	112
11	Pearson Correlation coefficient between odor, color, taste and overall acceptability for spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) reconstituted to 0% and applied to rice porridge at 10% (v/w).....	112



12	Pearson Correlation coefficient between odor, color, taste and overall acceptability for spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) reconstituted to 3% and applied to rice porridge at 10% (v/w).....	113
13	Pearson Correlation coefficient between odor, color, taste and overall acceptability for spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) reconstituted to 6% and applied to rice porridge at 10% (v/w).....	113
14	Pearson Correlation coefficient between odor, color, taste and overall acceptability for spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) reconstituted to 9% and applied to rice porridge at 10% (v/w).....	114
15	Pearson Correlation coefficient between odor, color, taste and overall acceptability for spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) reconstituted to 12% and applied to rice porridge at 10% (v/w).....	114
16	Pearson Correlation coefficient between odor, color, taste and overall acceptability for spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) reconstituted to 15% and applied to rice porridge at 10% (v/w).....	115
17	Score for odor, color, taste and overall acceptability of spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) , fish sauce and soy sauce.....	115
18	Score for odor, color, taste and overall acceptability of spray-dried threadfin bream hydrolysate (<i>Nemipterus japonicus</i>) (TFB) , Flavourzyme treated rice hydrolysate (RF), Alcalase treated rice hydrolysate (RA) and vegetable hydrolysate (VEGE).....	117
19	Quantitative descriptive (QDA) for fishy aroma and taste characteristics.....	117



LIST OF FIGURES

Figure		Page
1	pH profile for the hydrolysis of threadfin bream (<i>Nemipterus japonicus</i>) muscle with Alcalase and Flavourzyme:Protamex (F : P) mixture.....	41
2	Temperature profile for the hydrolysis of threadfin bream (<i>Nemipterus japonicus</i>) muscle with Alcalase and Flavourzyme:Protamex (F : P) mixture.....	43
3	Effect of hydrolysis time and enzyme-substrate ratio 0, 0.5, 1, 2, 3 and 4% on percent degree of hydrolysis (% DH).....	45
4	Effect of hydrolysis time and enzyme-substrate ratio of 0, 0.5, 1, 2, 3 and 4% on percent nitrogen recovery (% NR)	48
5	Elution pattern of threadfin bream hydrolysate from Sephadex G-75 column.....	86



LIST OF PLATES

Plate		Page
1 & 2	SDS-PAGE profile of the control and hydrolysed threadfin bream (<i>Nemipterus japonicus</i>) hydrolysed at 0, 30, 60, 120, 180 and 240 min at ES0% and 0.5%.....	50
3 & 4	SDS-PAGE profile of the control and hydrolysed threadfin bream (<i>Nemipterus japonicus</i>) hydrolysed at 0, 30, 60, 120, 180 and 240 min at ES1% and 2%.....	51
5 & 6	SDS-PAGE profile of the control and hydrolysed threadfin bream (<i>Nemipterus japonicus</i>) hydrolysed at 0, 30, 60, 120, 180 and 240 min at ES3% and 4%.....	52
7a	Fresh threadfin bream (<i>Nemipterus japonicus</i>).....	59
7b	The minced muscle of threadin bream (<i>Nemipterus japonicus</i>) collected after deboning.....	59
7c	Homogenized muscle obtained after the addition of water and homogenization in a waring blender.....	60
7d	Hydrolysis mixture obtained after the termination of hydrolysis process.....	60
7e	The soluble fraction (supernatant) collected following centrifugation at 5000 rpm, 4°C, 20 min.....	62
7f	Spray-dried hydrolysate of threadfin bream (<i>Nemipterus japonicus</i>).....	62
8	Sample of tray containing five reference solutions consisting of bitter, sour, sweet, salty and umami in sensory cups and Sephadex G-75 fractions in ependorf tubes presented to panelists.....	69
9	SDS-PAGE profile of the control and hydrolysed threadfin bream (<i>Nemipterus japonicus</i>).....	97



10	SDS-PAGE profile of Sephadex G-75 fractions of threadfin bream (<i>Nemipterus japonicus</i>) hydrolysate.....	99
11	Sample of tray presented to panelists.....	109



LIST OF ABBREVIATIONS

Au/g	Anson Unit per gram
ES	enzyme-substrate ratio
% NR	percent nitrogen recovery
% DH	percent degree of hydrolysis
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
D	Dalton
kD	kilo Dalton
RH	Relative Humidity
ID	Internal Diameter
RP	Reverse Phase
QDA	Quantitative Descriptive Analysis
IMP	Inosine 5'- monophosphate
FR	Fraction
meq/g	microequivalent per gram
nm	Nanometer
FAO/WHO	Food and Agriculture Organization/World Health Organization
HPLC	High Pressure Liquid Chromatography



CHAPTER I

INTRODUCTION

Hydrolysis of protein by the application of enzyme to produce fish protein hydrolysate is another tool for recovering protein from underutilized fish species (Hoyle and Merritt 1994; Quaglia and Orban 1987a; Shahidi et al. 1994). Hydrolysate is a modified form of protein consisting of a mixture of peptides of varying sizes and can be partially concentrated to form a liquid, spray-dried or freeze-dried hydrolysate to obtain a product with a highly concentrated protein (Skanderby, 1994). Hydrolysate is produced for use as milk replacer, food ingredients, flavor enhancer or as protein supplement (Rebeca et al. 1991; Yanez et al. 1976). The potential source of raw material for fish protein hydrolysate production are underutilized fish, by-catch as well as wastes from processing operation such as head, tail, bone, skin and viscera (Kim et al. 1994; Kim et al. 2000).

Fish protein hydrolysate can be prepared by the hydrolysis of either the fatty or non-fatty fish species. However, the preparation of hydrolysates from fatty-fish species requires either the removal or stabilization of fat prior to the hydrolysis process (Ritchie and Mackie 1982; Hoyle and Merritt 1994; Liceaga-Gesualdo and Li-Chan 1999). Although enzymatic



hydrolysis of fish protein produces hydrolysate with high protein content and excellent solubility, bitterness and fishy off-flavor are not an uncommon problem in fish protein hydrolysate (Shahidi et al. 1995; Hevia and Olcott 1977; Hoyle and Merritt 1994). Several studies have been carried out to overcome these problems such as partial deodorization by passing through steam containing orthophosphoric acid and ethanol extraction (Hoyle and Merritt 1994; Venugopal and Lewis 1981).

By-catch refers to the non-targeted fish species that are caught incidentally with the targeted species such as the shrimp or commercial fish. They are heterogeneous in composition consisting of species that are very small, bony, dark flesh and toxic while others having strong flavor and unattractive appearance and texture. These factors caused sorting difficulties and due to some of the aforementioned factors in addition to the wide market price gap in comparison to the shrimp price, the fish gain less popularity and therefore are often discarded at sea (Venugopal and Shahidi 1995; Wan Ismail and Abdullah 1983). The wastage of by-catch has been a major problem in the world fisheries. Statistics between 1988 to 1990 indicated that out of 77 million tons global marine catches, 35% were discarded by-catch which were mostly from the shrimp trawlers and Malaysia's shrimp trawler was listed as one of the top twenty fisheries with the highest recorded discard (Alverson et al. 1994). In the study of species



classified as by-catch, threadfin bream was listed among the largest species identified (Wan Ismail and Abdullah, 1983). In a more recent study reported by Matsushita and Ali (1997) during the on-deck sorting selectivity studies in Kuala Kedah, Malaysia showed that threadfin bream was grouped as small or low-price ('trash fish') in addition to slender shad (*Ilisha elongata*) and squid. In Malaysia, by-catch are used in fish meal production, some have been cured into dried salted fish or used in fish noodles production for human consumption (Wan Ismail and Abdullah, 1983). They have also been used for marinated and breaded product in Brunei, surimi production in Thailand and as mechanically deboned such as in India (Mariani et al. 1996; Suwanrangsi 1988; Venugopal and Shahidi 1995).

Studies regarding fish protein hydrolysate involved the optimization of the hydrolysis conditions, physico-chemical and functional properties, its potential use in fish sauce production, removing fishy off-flavor and determining the nature of bitter components (Diniz and Martin 1996; Shahidi et al. 1997; Beddows and Ardeshir 1979; Hoyle and Merritt 1994, Hevia and Olcott 1977). The production of threadfin bream (*Nemipterus japonicus*) hydrolysate has not been carried out thus far. Therefore, the purposes of this study are 1) to compare the activity of Alcalase and Flavourzyme:Protamex mixture on the hydrolysis of threadfin bream and

to determine the optimum hydrolysis conditions of the selected enzyme or enzyme mixture namely the pH, temperature, enzyme-substrate ratio and reaction time, 2) to determine the physico-chemical, functional properties and sensory characteristics of Alcalase produced threadfin bream hydrolysate and 3) to study the taste and intensity characteristics of the hydrolysate (five basic tastes namely bitter, sweet, sour, salty and umami).