PRODUCTION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES FROM RED TILAPIA PROTEIN HYDROLYSATES

MARYAM SHAMLOO

FSTM 2010 19
PRODUCTION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES FROM RED TILAPIA PROTEIN HYDROLYSATES

By

MARYAM SHAMLOO

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

December 2010
To My Parents
Fish proteins are considered as valuable nutrient and a good source of many bioactive peptides such as angiotensin converting enzyme (ACE) inhibitory peptides. Very few reports are available on the ACE inhibitory peptides in freshwater fish hydrolysates. Therefore, this study was carried out with the objective to produce tilapia protein hydrolysates by commercial proteases, named Alcalase, Flavourzyme and Protamex, investigating the ACE (Angiotensin Converting Enzyme) inhibitory activity, the radical scavenging ability and identifying the best enzyme to produce the highest bioactivity; optimizing the production of ACE inhibitory peptides using response surface methodology (RSM); and to fractionate the ACE inhibitory peptides using ultrafiltration membranes. The ACE inhibitory activities were determined using an in vitro method and the IC$_{50}$ (peptide concentration which reduced ACE inhibitory by 50%) was calculated. The result indicated that Alcalase was the best enzyme to
produce tilapia hydrolysates since it had the highest ACE inhibitory activity when compared to Protamex and Flavourzyme. A central composite design (CCD) involving 16 cube points, 8 axial points and 7 center points was employed to study the effect of temperature, time, pH and enzyme-substrate ratio on Alcalase hydrolytic activity. The combined level of 55.8 °C, 259.99 min, pH 7.5 and enzyme-substrate ratio of 3.58 % (w/w) was predicted to provide the most desirable bioactivity, which produce high ACE inhibitory activity in tilapia hydrolysates. The coefficient of determination value ($R^2$) was 0.883 for the experimental data, which indicated a satisfactory adjustment of the reduced response models. The time, temperature and enzyme-substrate ratio of the hydrolysis had significant ($p < 0.01$) effects on the ACE inhibitory activity in tilapia hydrolysates. The most desirable hydrolysates were fractionated using three different molecular weight cut-off membranes (10 kDa, 5 kDa and 2 kDa). Four fractions (> 10 kDa, 10-5 kDa, 5-2 kDa and < 2 kDa) obtained had the ACE inhibitory activity, however, the fraction with molecular weight of < 2 kDa, appeared to have a significantly ($p < 0.05$) lower IC$_{50}$ compared to the unfractionated hydrolysate, and the other fractions.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

PENGHASILAN PEPTIDA PERENCAT ENZIM PENUKAR ANGIOTENSIN DARIPADA PROTEIN HIDROLISAT TILAPIA MERAH

Oleh

MARYAM SHAMLOO

Disember 2010

Pengerusi : Profesor Jamilah bt. Bakar, PhD

Fakulti : Sains dan Teknologi Makanan

Protein ikan dianggap sebagai nutrien yang berharga dan sumber peptida bioaktif yang baik seperti peptida perencat enzim penukar angiotensin (ACE). Laporan mengenai peptida perencat ACE daripada hidrolisat ikan air tawar sangat sedikit. Oleh sebab itu, kajian ini dijalankan dengan objektif untuk menghasilkan hidrolisat protein tilapia menggunakan proteasa homersil, iaitu Alcalase, Flavourzyme dan Protamex, mengkaji aktiviti perencat ACE, aktiviti pemeranghapan bilasan radikal bebas dan mengenalpasti jenis enzim yang dapat menghasilkan bioaktiviti tertinggi, mengoptimumkan penghasilan peptida perencat ACE berdasarkan kaedah permukaan respons (RSM), dan mengfraksinasikan peptida perencat ACE dengan menggunakan membran ultrafiltrasi. Kaedah in vitro digunakan untuk menentukan aktiviti perencatan ACE dan pengiraan nilai IC50 (kepekatan peptida untuk mengurangkan perencat ACE sebanyak 50%). Keputusan menunjukkan bahawa Alcalase merupakan
enzim yang terbaik untuk menghasilkan hidrolisat tilapia dengan aktiviti perencat ACE yang tertinggi. Reka bentuk komposit memusat (CCD) yang melibatkan 16 titik kubus, 8 titik paksi dan 7 titik pusat telah digunakan untuk mengkaji kesan suhu, masa, pH dan nisbah enzim-substrat terhadap aktivitis hidrolisat Alcalase. Gabungan suhu, masa, pH dan nisbah enzim-substrat pada 55.8 °C, 259.99 minit, 7.5 dan 3.58 % (berat/berat) masing-masing telah ramalkan dapat menghasilkan aktiviti perencat ACE yang tinggi. Nilai koefisien hubung-kait yang tinggi ($R^2 = 0.883$) menunjukkan bahawa model regresi yang dihasilkan menerangkan variasi data dengan memuaskan. Masa, suhu dan nisbah enzim-substrat terhadap aktiviti hidrolysis telah menunjukkan kesan yang ketara ($p <0.01$) ACE keatas aktiviti perencatan pada hidrolisat tilapia yang dihasilkan. Hidrolisat telah di ultrafiltrasi dengan menggunakan tiga jenis membrane dengan cut-off berat molekul yang berbeza (10 kDa, 5 kDa dan 2 kDa). Keempat fraksi (> 10 kDa, 10-5 kDa, 5-2 kDa dan <2 kDa) yang diperolehi telah menunjukkan aktiviti perencatan ACE, walau bagaimanapun fraksi dengan berat molekul <2 kDa nampaknya telah menunjukkan nilai IC50 yang lebih rendah ($p < 0.05$) dari fraksi yang lain.
ACKNOWLEDGEMENTS

There have been a lot of supports for the present study, of which the majority has come from professional, knowledgeable and experienced individuals from the Department of Food Science and Technology. Many people I came to know through professional contact have become true friends, which I cannot express my word enough for their valuable friendship.

Firstly, I would like to gratefully acknowledge my supervisor, Prof. Dr. Jamilah Bakar. Her leadership, friendship and consistent support have guided me through difficulties both academic and personal. She is a dear friend and a wonderful supervisor and she has taught me how to be good student.

My sincere appreciation also goes to the other members of the Supervisory Committee: En. Dzulkifly Mat Hashim and Dr. Alfi Khatib for their invaluable guidance during this study.

I am thankful to my dear friend, Bita Forghani. Her love, friendship and support cannot be grateful enough.

Appreciation is extended to the Government of Malaysia and Universiti Putra Malaysia for granting me Graduate Research Assistantship to carry out the project.

Finally, I would like to express a special note of appreciation to my beloved husband, my dear parents and my lovely sister for their help, advice and support throughout the duration of the project.
I certify that an Examination Committee has met on 23/12/2010 to conduct the final
examination of Maryam Shamloo on her Master of Science thesis entitled
“Production of Angiotensin Converting Enzyme Inhibitory Peptides From Red
Tilapia (Oreochromis Niloticus) Hydrolysates” in accordance with Universiti
Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia
(Higher Degree) Regulations 1981. The Committee recommends that the candidate be
awarded the relevant degree. Members of the Examination Committee are as follows:

TAN CHIN PING, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

ABDULKARIM SABO MOHAMMED, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

NAZAMID SAARI, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

MAMOT SAID, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the supervisory Committee were as follows:

**Jamilah bt. Bakar, PhD**  
Professor  
Faculty of Food Science and Technology  
Universiti Putra Malaysia  
(Chairperson)

**Alfi Khatib, PhD**  
Faculty of Food Science and Technology  
Universiti Putra Malaysia  
(Member)

**Dzulkifly Mat Hashim, M.Sc.**  
Faculty of Food Science and Technology  
Universiti Putra Malaysia  
(Member)

---

**HASANAH MOHD GHAZALI, PhD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
DECLARATION

I declare that the thesis is my original work except for quotations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

MARYAM SHAMLOO
Date: 23 December 2010
# TABLE OF CONTENT

<table>
<thead>
<tr>
<th>TABLE OF CONTENT</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>viii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## CHAPTER

### 1 INTRODUCTION

1

### 2 LITERATURE REVIEW

2.1 Definition and Application of Bioactive Peptides 5
2.2 Food Sources of bioactive peptides 6
2.2.1 Marine Sources 6
2.2.2 Non-Marine Sources 10
2.3 Types of bioactive peptides 12
2.3.1 Antihypertensive Peptides 12
2.3.2 Antioxidant Peptides 13
2.3.3 Antimicrobial Peptides 14
2.3.4 Antithrombotic Peptides 14
2.3.5 Opioid Peptides 15
2.3.6 Mineral-binding Peptides 15
2.2.7 Immunomodulatory Peptides 16
2.2.8 Obesity Control 16
2.4 Methods of Producing Bioactive Peptides 17
2.4.1 The Chemical Hydrolysis Process 17
2.4.2 Biochemical Processes 18
2.5 Angiotensin Converting Enzyme (ACE) 26
2.6 Mechanisms and Structural Properties of Angiotensin Converting Enzyme Inhibitory Peptides 29
2.7 Isolation Methods of Protein and Peptides 33
2.7.1 Chromatography Methods 33
2.7.2 Non-Chromatography Methods 34

### 3 ENZYMATIC PRODUCTION AND CHARACTERIZATION OF RED TILAPIA (*Oreochromis niloticus*) PROTEIN HYDROLYSATES

3.1 Introduction

38
3.2 Materials and Methods
  3.2.1 Materials 40
  3.2.2 Production of Protein Hydrolysate 41
  3.2.3 Degree of Hydrolysis (DH) 43
  3.2.4 Kinetic of Degree of Hydrolysis 44
  3.2.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 45
  3.2.6 ACE inhibitory Activity Assay 45
  3.2.7 DPPH Radical Scavenging Ability 47
  3.2.8 Determination of Amino Acid Composition 47
  3.2.9 Statistical Analysis 49
  3.3 Results and Discussion 49
    3.3.1 Enzymatic hydrolysis of Tilapia Muscle 49
    3.3.2 SDS-PAGE of Tilapia Protein Hydrolysates 52
    3.3.3 ACE Inhibitory Activity 54
    3.3.4 2,2-Diphenyl-1 picryl -hydrazyl(DPPH) Radical Scavenging Activity 56
    3.3.5 Amino Acid Composition 58
  3.4 Conclusions 62

4 OPTIMIZATION OF PRODUCTION OF ACE INHIBITORY PEPTIDES FROM ALCALASE TILAPIA HYDROLYSATE
  4.1 Introduction 63
  4.2 Materials and Methods 65
    4.2.1 Materials 65
    4.2.2 Tilapia Hydrolysis 65
    4.2.3 ACE Inhibitory Activity Assay 66
    4.2.4 Degree of Hydrolysis (DH) 66
    4.2.5 Experimental Design 66
    4.2.6 Statistical Analysis 69
    4.2.7 Optimization and Validation Procedures 70
  4.3 Results and Discussion 71
    4.3.1 Effect of Hydrolysis Parameters on ACE and DH 71
    4.3.2 Statistical Analysis 71
    4.3.3 Reduced Response Surface Model 74
    4.3.4 Optimization Procedure 79
    4.3.5 Validation of the final reduced model 79
  4.4 Conclusions 81

5 FRACTIONATION AND CHARACTERIZATION OF ACE INHIBITORY PEPTIDES IN HYDROLYSATES BY ULTRAFILTRATION MEMBRANE
  5.1 Introduction 82
  5.2 Materials and Methods 84
6 
SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

REFERENCES 100
APPENDICES 111
BIODATA OF STUDENT 121