

**Alma Mater Studiorum – Università di Bologna**

DOTTORATO DI RICERCA IN  
SCIENZE E BIOTECNOLOGIE DEGLI ALIMENTI

Ciclo XV

Settore Concorsuale di afferenza: 07/F

Settore Scientifico disciplinare: AGR/16

**PRODUCTION OF BIOACTIVE PEPTIDES THROUGH  
SEQUENTIAL ACTION OF *YARROWIA LIPOLYTICA*  
PROTEASES AND CHEMICAL GLYCATION**

Presentata da: **Davide GOTTARDI**

Coordinatore Dottorato

**Chiar.mo Prof. Claudio CAVANI**

Relatore

**Dott.ssa Lucia VANNINI**

Correlatore

**Chiar.ma Prof.ssa  
M. Elisabetta GUERZONI**

**Esame finale anno 2013**

# TABLE OF CONTENTS

## CHAPTER 1. FOOD BY-PRODUCTS

<b>1.1. Food by-products: current production and uses.....</b>	<b>2</b>
<b>1.2. Industrial examples of uses of food by-products.....</b>	<b>4</b>
1.2.1. Vegetable by-products.....	4
1.2.1.1. Starch production by-product.....	4
1.2.1.2. By-products from vegetables canning.....	5
1.2.1.3. By-products from legume.....	5
1.2.2. Animal by-products.....	5
1.2.2.1. Alternative sourcing of gelatin.....	5
1.2.2.2. Beef collagen fibre.....	6
1.2.2.3. Other successful examples on animal by-products.....	6

## CHAPTER 2. PEPTIDES AND FOOD

<b>2.1. Properties of peptides.....</b>	<b>10</b>
2.1.1. Structure and functional properties.....	10
<b>2.2. Sensory properties.....</b>	<b>11</b>
<b>2.3. Peptides bioactivities.....</b>	<b>12</b>
2.3.1. Anti-hypertensive peptides.....	13
2.3.2. Antithrombotic peptides.....	14
2.3.3. Hypocholesterolemic and hypotriglyceridemic peptides.....	16
2.3.4. Antioxidant peptides.....	17
2.3.5. Opioid peptides.....	17
2.3.6. Immunomodulatory peptides.....	18
2.3.7. Anticancer peptides.....	18
2.3.8. Antimicrobial peptides.....	18
2.3.9. Multifunctional peptides.....	21

## CHAPTER 3. GLYCOPEPTIDES

<b>3.1. Structure.....</b>	<b>23</b>
<b>3.2. Antifreezing activity.....</b>	<b>24</b>

<b>3.3. Sensory properties.....</b>	<b>25</b>
<b>3.4. Glycopetides bioactivities.....</b>	<b>25</b>
3.4.1. Antioxidant activity.....	25
3.4.2. Antitumor activity.....	25
3.4.3. Antimicrobial activity.....	26

## **CHAPTER 4. PROTEASES**

<b>4.1. Exopeptidase.....</b>	<b>30</b>
<b>4.2. Endopeptidase.....</b>	<b>31</b>
4.2.1. Serine protease.....	31
4.2.2. Aspartic protease.....	32
4.2.3. Cysteine protease.....	32
4.2.4. Metalloprotease.....	32
<b>4.3. Functions and applications.....</b>	<b>32</b>

## **CHAPTER 5. *Yarrowia lipolytica***

<b>5.1. Taxonomy and morphology.....</b>	<b>35</b>
<b>5.2. Metabolism.....</b>	<b>35</b>
<b>5.3. Industrial relevances for <i>Y. lipolytica</i>.....</b>	<b>36</b>
<b>5.4. Proteases of <i>Y. lipolytica</i>.....</b>	<b>37</b>
5.4.1. Extracellular proteases.....	37
5.4.1.1. Alcaline protease.....	37
5.4.1.2. Acidic protease.....	38
5.4.2. Intracellular protease.....	38
5.4.3. Proteases production.....	38
5.4.4. Industrial applications.....	39

## **CHAPTER 6. OBJECTIVES 41**

## **CHAPTER 7. MATERIAL AND METHODS**

<b>7.1. <i>Yarrowia lipolytica</i> strains and culture conditions.....</b>	<b>45</b>
<b>7.2. Protein matrices.....</b>	<b>45</b>
7.2.1. Total meat protein extraction.....	45

<b>7.3. List of chemicals used.....</b>	<b>45</b>
<b>7.4. Proteolytic activity of <i>Y. lipolytica</i>.....</b>	<b>46</b>
7.4.1. Extracellular proteases recovery and characterization.....	46
7.4.2. Characterization of proteases by Zymography.....	46
7.4.3. Preliminary evaluation of the "cold attitude" of the proteolytic enzymes.....	47
7.4.4. Evaluation of the Proteolytic profiles generated by <i>Y. lipolytica</i> proteases.....	47
7.4.5. SDS-PAGE electrophoresis.....	48
<b>7.5. Improvement of peptides bioactivity.....</b>	<b>48</b>
7.5.1. Model system .....	48
7.5.1.1. Model system preparation and UV spectra collection.....	48
7.5.1.2. LC/MS analysis.....	48
7.5.1.3. Production of hydrolysates.....	49
7.5.1.4. Preparation of Glycated/Glycosylated peptides.....	49
<b>7.6. Chemical characterization of the proteins and hydrolysates.....</b>	<b>50</b>
7.6.1. Degree of hydrolysis (DH).....	50
7.6.2. Size exclusion chromatography.....	50
7.6.3. Determination of peptides and glycopeptides molecular weights by Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI/TOF-MS) .....	50
<b>7.7. Biological characterization of the peptides.....</b>	<b>51</b>
7.7.1. Antioxidant properties.....	51
7.7.1.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.....	51
7.7.1.2 Inhibition of linoleic acid peroxidation .....	51
7.7.2. Angiotensin I-converting enzyme (ACE) inhibitory activity.....	52
7.7.3. Cytotoxicity of the hydrolyzed and conjugated samples on human HepG2 cells	52
7.7.4 Antimicrobial activity .....	53
<b>7.8. Statistical Analysis.....</b>	<b>53</b>

## CHAPTER 8. RESULTS

<b>8.1. Evaluation of the proteolytic activity of <i>Yarrowia lipolytica</i> on different proteins</b>	<b>56</b>
8.1.1. Preliminary characterization of proteases by Zymogram.....	57
8.1.2. "Cold attitude" of the proteolytic enzymes.....	57
8.1.3. Proteolytic profiles generated by <i>Y. lipolytica</i> proteases.....	58
8.1.4. Proteolysis of gelatin.....	59

8.1.5. Characterization of the products of gelatin hydrolysis obtained by <i>Y. lipolytica</i> 1IIYL4A protease.....	60
8.1.6. <i>In vitro</i> bioactivity evaluation of the peptides.....	61
8.1.6.1. DPPH radical scavenging activity.....	61
8.1.6.2. Inhibition of the linoleic acid peroxidation.....	61
8.1.6.3. Cytotoxicity activity in human cells.....	61
8.1.6.4. Antimicrobial activity.....	62
<b>8.2. Improvement of peptides bioactivity.....</b>	<b>62</b>
8.2.1. Model system.....	64
8.2.2. Characterization of wheat gluten and its hydrolyzates.....	65
8.2.3. Chemical evaluation of glycoconjugation of gluten peptides by MALDI/TOF-MS.....	66
8.2.4. <i>In vitro</i> bioactivity evaluation of glycopeptides mixtures .....	67
8.2.4.1. DPPH Radical Scavenging Activity.....	67
8.2.4.2. Inhibition of Linoleic acid peroxidation.....	67
8.2.4.3. Anti-ACE activity.....	67
8.2.4.4. Antimicrobial activity.....	68
8.2.4.5. Cytotoxicity in human cells .....	68
<b>8.3. Tables.....</b>	<b>69</b>
<b>8.4. Figures.....</b>	<b>73</b>
<b>CHAPTER 9. DISCUSSION AND CONCLUSIONS</b>	<b>96</b>
<b>CHAPTER 10. REFERENCES</b>	<b>106</b>
<b>ACKNOWLEDGMENTS</b>	<b>122</b>

**CHAPTER 1.**

**FOOD BY-PRODUCTS**

A by-product is a secondary product derived from food industries that does not represent the primary service produced. In other words, a by-product is the "output of a process that has a minor quantity and/or a net realizable value when compared to the main products". A by-product can be useful and marketable or it can be considered waste.

### **1.1. Food by-products: current production and uses**

The food processing industry, despite the great articulation of its productive sectors, is characterized by a significant production of by-products (Federalimentare, 2006). Because of complexity of the food system, estimating sizes and impacts of these streams is very difficult. However, some estimates can be derived from literature. Wirsenius (2008) estimated the amounts of by-products (used for feed) and wastes of food processing in terms of energetic values (i.e. J which is expressed as energetic value per 100 energetic units food produced). In particular, in Western Europe per 100J food, 56J ends in by-products, while 20J is lost from the food system. According to a survey performed by Awarenet (2004), the European Food processing activities produce about 250 million tonnes per year of by-products and waste along with relevant amounts of high COD effluents. Also in Italy the economic dimension of the management of by-products from food processing industries is significant. The 2-3% of the dry volumes and the 7-10% of the moist volumes produced are by-products. The estimated sale value is around 300 million euro per year (Federalimentare, 2006).

Such waste streams are only partially valorised at different value-added levels (spread on land, animal feed, composting), whereas the main volumes of them are managed as waste of environmental concern, with relevant negative effects on the overall sustainability of the European food processing industry. With increasing disposal costs, alternative uses of co-products are increasingly being sought. The economic value of each product comes from its intrinsic nutritional or applicability value. Moreover for a company the utilization of a by-product can give some benefits from the economical point of view. The erroneous classification of such products as "waste" is doubly negative: from one side, the producers are forced to manage the disposal of the materials even if they are still susceptible to use, from the other side, the potential users are forced to search expensive alternative sources of supply (Federalimentare, 2006).

More serious consequences are reflected on the ecosystem. In fact, disposed by-products would finish in the landfill or through the drains, water courses, creating in this way serious environmental problems (Smith, 1998).

Due to the current promotion of sustainability in the production and consumption of food by governments and international institutions, and, at the same time, considering that food systems must satisfy increasing needs in quantitative and qualitative terms, because of the increment of the world population (Mancini et al., 2011), it is clear that the exploitation and the use of food by-products must be increasingly enhanced.

Although in the industrialized society various former by-products have been upgraded to the food domain (for example whey protein isolates) and feed domain, still vast streams of by-product exist that have potential for the food domain. With an eye on potential handling and application purposes, primary and secondary by-products are distinguished.

*Primary by-products* (e.g. farming by-products such as straw and residues from land management) are traditionally largely used for caring and feeding animals. With an eye on their composition further enhancement of nutrient utilization efficiency for feed (FAO, 2012) and utilization for renewable materials and energy are the most relevant development directions.

Also *secondary by-products* (processing residues) are largely used for feeding purposes. With (historical) industrialisation and scaling up of food processing, the distance between the place of generation and utilisation of the by-products has increased. Consequently, specialized by-products trading companies have developed, who still mainly aim at feed markets. In general wet by-products are transported directly from place of origin to the farmer, whereas parts of the dry by-products are processed to feed concentrates.

Some examples food by-products are reported in **table 1**. Their reutilization can cover the production of: animal and pet food (with sugar beet pulp, corn gluten, cereals used for the production of beer, whey, by-products of the meat processing), pharmaceutical and cosmetic compounds (collagen, gelatin), bio-fertilizer, food ingredients (gluten, germ and fiber), bio-fuels. The chemo-physical properties, the shelf life, the availability and transportability of the by-products are crucial to establish their possible use.

The sugar industry by-products are the easiest to be found and utilized (Smith, 1998). For example the molasses has a sugar content close to the 50% and nowadays it is widely used for the antibiotics, organic acids and bakers' yeast productions (Smith, 1998). Over 20 million tons of animal by-products emerge annually from EU from slaughterhouses, plants producing food for human consumption, dairies and as fallen stock from farms ([http://ec.europa.eu/food/food/biosafety/animalbyproducts/index\\_en.print.htm](http://ec.europa.eu/food/food/biosafety/animalbyproducts/index_en.print.htm)).

In large-scale food industry sectors, with voluminous homogeneous protein-rich by-product streams (like dairy and meat processing), development of application in higher-value domains been very successful in last decades. Some example are summarized in **table 2**. A number of new and



more sophisticated possible exploitation of food processing by-products and waste have been foreseen, tested and sometimes also scaled up (Kosseva, 2009; Galanakis, 2012).

## **1.2. Industrial examples of uses of food by-products**

Notwithstanding the complexity of introducing new foods (or ingredients) based on by-products, various examples of innovations in this field have been successfully introduced in practice.

Current societal focus (and stimulating governmental arrangements) on bio-energy, bio-fuels and other renewable bio-based solutions has resulted in a large number of practical in those areas, which has overwhelmed applications in the food domain. Except for industrial uses of dietary fibres from food by-products (Elleuch *et al.*, 2011), the number of practical examples on innovative use of the by-products presented in scientific literature lately is very limited. Yet, various appealing examples exists. Below a brief summary of some of these successful practical examples in the sector of animal and vegetable by-products and waste is reported; for each example key success factors and obstacles are also briefly outlined.

### **1.2.1. Vegetable by-products**

#### **1.2.1.1. Starch production by-product**

The processing of crops like potato, wheat, rice and corn results in considerable side stream which contain notably potentially valuable proteins. Most prominent example is the food-grade isolation potato protein.

Previously the protein was separated from the potato juice by thermal and acid denaturation, which resulted in an insoluble aggregates with lost functionality, only suited for feed. Recently the Solanic company (AVEBE, Netherlands) has developed a plant that successfully extracts native potato proteins from potato juice (by-product from potato starch production), and the product is marketed to food industries (e.g. bakery, meat, sport supplements). The key success factor is the development of the isolation process combined with the high quality of the derived proteins (solubility, emulsification, foaming and gelling quality, high nutritional value, low allergenicity) compared to other commercial ones of different animal or vegetal origin.

Some other attempts to extract highly functional proteins from other crops are less successful (e.g. extracting gluten from wheat and  $\beta$ -glucans from distillery grains).

#### 1.2.1.2. By-products from vegetables canning

Vegetable canning results in considerable amounts of by-products (peels, rejects, etc.). These are traditionally traded as cattle feed or composted. Value for bio-energy production is limited because of the high water content and limited energetic value. Therefore, interest in application for human food is getting more attention. Recently the company Provalor has developed a process for vegetable juice (natural colorant in food) and fibres extraction from the by-products. The main key success factor for this process is represented by the development of an adequate extraction process with highly appreciable yields. Moreover, also the increasing interest in 'clean labels' (the natural colorant can replace synthetic colorants in food products) and Societal call for sustainable valorisation of by-products have acted as external factors promoting the success of this process.

#### 1.2.1.3. By-products from legume

Grain legumes, also known as pulses, are plants belonging to the family *Leguminaceae*, which are grown primarily for edible grains or seeds. India is the fifth largest legume produced in the world. Among the legumes, the soybean, also classed as an oilseed, is pre-eminent for its high (38-45%) protein. By-products of legume include: hulls, husk, seeds etc. Microcore Research Laboratories (India Pvt Ltd.) has developed a process and patented the technology for the converting husk of Bengal gram to insoluble dietary fibers and micro-crystalline dietary cellulose which can be used in daily diet to control obesity. The main success factors are represented by the development of the adequate technology to obtain the insoluble fiber and the high demand for such a functional component which has strongly increased worldwide.

#### 1.2.2. Animal by-products

Traditionally the major parts of an animal are used as food, feed or materials. As such real waste products from animal slaughter hardly exist. However, there is a continuous technical innovation to maximize the value the refinement and splitting of streams. Stringent regulation change with regard to BSE has been a game changer in these industries for the application of by-products for food and feed.

##### 1.2.2.1. Alternative sourcing of gelatin

Confidence in traditional sources of gelatin (amongst others bovine hides and bones) was seriously damaged by BSE breakout. Increase of gelatin prices has been a trailblazer for alternative

production processes. A successful example is the Dutch company Ten Kate Vetten that developed a production process primarily aiming at extracting fats from pig slaughter by-products. Such a process was innovated so that high-quality gelatin can be isolated from their processing water. The (mild) fat extraction process furthermore enabled valorisation of other protein products in pet feed.

#### 1.2.2.2. Beef collagen fibre

In leather production substantial amounts of animal material occur are cut off and wasted. Hulsh of Protein Technologies in the Netherlands has innovated their process such that these cut offs are kept in food-grade quality and processed to native collage fibres with superior water binding and structuring properties compared to common thermally denatured collagen (due to mild drying and grinding procedures that leave the collagen fibres in their native triple helix structure). The collagen product is Halal certified.

#### 1.2.2.3 Other successful examples on animal by-products

- Insuline from pancreas: pharmaceutical hormone for diabetic patients.
- Mucine from pig bowels: ingredient for synthetic saliva.
- Blood proteins processing and valorisation
- Cholesterol from Lanoline (sheep wool fat).
- Cholesterol as building block for the pharmaceutical industry, cosmetics industry and crucial feed additive for shrimps.

Each of these examples was driven by internal factors: costs of wastage and/or value of the product.

**Table 1.** By-products that could be exploited as substrates for biotechnological processes (Smith, 1998)

<b>Lavorazioni agricole e zootecniche</b>	<b>Lavorazioni forestali</b>	<b>Lavorazioni industriali</b>
Paglia	Idrolisati di scarti legnosi	Melassa
Bagassa	Liquido ricco di solfiti (dalla preparazione della pasta di cellulosa)	Scarti di distillazione
Torsoli di pannocchia	Cortecce, ramaglia, segatura	Siero del latte
Scarti del caffè, del cacao, gusci di noci di cocco	Carta e cellulosa	Reflui dell'industria alimentare (olive, olio di palma, patate, datteri, agrumi, manioca)
Bucce e foglie della frutta	Fibre	Acque di lavaggio industriale (industria casearia, conserviera, dolciaria, bevande analcoliche, industria tessile, industria del malto, macerazione del mais)
Scarti del tè		Sottoprodotti della carne
Pasta di semi oleosi		Rifiuti solidi urbani
Scarti del cotone		Liquami
Crusca		Rifiuti dei macelli
Polpa (pomodori, caffè, banane, ananas, agrumi, olive)		
Deiezioni animali		

**Table 2.** Summary of common and potential uses of by-products in various agro-food sectors.

Sector (by-product)	Historical or common use	State-of-the-art use (and potentials) in food and other high-value applications
Vegetable processing (peels, reject, etc.)	Dairy feed	<p>Food-functional properties of various compounds are broadly recognized.</p> <p>Practical implementation is limited because of technological and/or economic reasons. In a limited number of practical situations, food ingredients are produced out of the by-products (e.g. food-grade trimmings for processed foods, vegetable juices from food-grade peels and rejects).</p> <p>Vegetable wastes such as sugar beet leaves, cauliflower leaves and gram plant with empty pods can serve as a good source of essential vitamins and antioxidants and serve as an organic source of minerals.</p>
Oil seeds (cake)	Protein-rich animal feed	Deriving attractive protein-rich ingredients for food applications will require major changes in oil extraction processes.
Fruits (seed, peel pomace, kernel, wastes)	Feed for livestock; components are used for cosmetics and paints.	<p>Fruit peels have relatively high contents of functional food compounds. For example, apple pomace is a rich source of polyphenols, minerals and dietary fibre (Sudha <i>et al.</i>, 2007), and banana peels have high contents of pectins (including glucose, galactose and xylose).</p> <p>Beyond the food domain, a product like banana peel can be used for biomethanation. Furthermore, it can be used as a sorbent that removes heavy metals from waste water.</p>
Cereals(bran, husks)	Feed for livestock	<p>Because of high contents of dietary fibres, bran is traditionally used in amongst others bakery and products and breakfast cereals.</p> <p>Part of the rice bran is used for rice bran oil; 75% of this oil is used in the food domain, whereas 25% is used for soap manufacturing. Rice bran wax is an important by product of rice bran oil industry. Rice bran wax can be used in the preparation candles, polishes, cosmetics, emulsifiers and other industrial preparations.</p> <p>Relatively new is the application in minced meat, contributing to water binding capacity.</p> <p>Wheat bran could be utilized in solid state, liquid state fermentations and animal feeds.</p>
Dairy by-products (whey, skim milk, butter-milk, etc)	Protein-rich animal feed	<p>Value of whey (powder) as high-value protein product in food is very high.</p> <p>Whey obtained as a by-product of cheese industry has long been utilized in the production of fermented beverages, both alcoholic and non-alcoholic (acidic).</p>

Skim milk is a by-product obtained during the manufacture of cream. It is rich in solids-not-fat content and has high nutritional value. It is regarded as a by-product only when it is either not economically utilized or utilized for derived by-products like casein and related products, coprecipitates, protein hydrolysates etc. E.g.: From by-product of skim milk cultured butter milk and Bulgarian butter milk has been prepared.

Sugar processing by-products (sugarcane bagasse)	Generation of steam and power required to operate the sugar factory	Amongst the alternative valorisation options are use of fibres for paper and boards and bio-ethanol.
Meat processing (bone meal , poultry by-products meal & other animal by-products)	Feed for livestock, pets and aquaculture (rich of essential amino acids, fatty acids, vitamins and minerals)	Because of danger of Bovine spongiform encephalopathy (BSE), stringent limitations have been formulated by governments on use of animal by-products in feeds. Next to feed applications, currently food, pharmacy, pet food, compound feed, fertilisers and technical applications are produced out of meat by-products.

---

**CHAPTER 2.**

**PEPTIDES AND FOOD**

Peptides are short polymers of amino acids present in humans, animals, and plants, and represent an important component of the innate immunity, as they may also participate in the antioxidant, antimicrobial and signalling functions. They can be synthesized *ex novo* (like carnosine or glutathione), or they can be produced after proteolysis. In the first case, the new molecules are synthesized to complete predetermined functions; in the second case, instead, they can follow different pathways, such as the metabolic pathways of amino acids, or remain in a latent state within the protein sequence. The peptides released from proteolytic processes during food processing are related to the functional, nutritional and sensorial properties of the final product.

The desire for functional foods and the need to reduce chemical preservatives are connected with the ever increasing health and nutrition concerns of the consumers (Mills *et al.*, 2011).

The concept that proteins can be tailored and their fragments modelled to achieve a particular function, is now of great interest. In general, peptides generated from food proteins present the great advantage to derive from harmless sources and therefore are considered safe. Moreover, the production of peptides obtained from these sources could bring additional value to food by-products, representing a breakthrough area for the industry of the future (Pellegrini, 2003).

## **2.1 Properties of peptides**

### **2.1.1 Structure and functional properties**

Peptides are polymers of at least two amino acids linked together by covalent bonds, between the carboxyl group of one molecule and the amino group of the other molecule. All the polymers containing more than 50 amino acids are considered proteins, whereas peptides are those containing less than 50 units. Indeed, the complexity of the structure and the degree of activity are supplementary methods to discriminate proteins from peptides (Van De Weert and Randolph, 2012)

The length and the amino acid composition of these molecules will determine their physicochemical properties. Each peptide presents a free amino group in the N-terminal region and a free carboxylic group in the C-terminal with equalised magnetic charges. The elements responsible for the final charge of the peptide, which can be ionized, belong to the inner amino acid side chains. These side chains are very important in the food industry. Also the Maillard reaction, generating colour and flavour compounds during the baking process, relies on the presence of these amino acids side chains. At the same time, the inclusion of a peptide or their *in situ* production, can improve the texture of the food product. For example the development of the texture in the Cheddar cheese during ripening has been thought to depend upon the extent of proteolysis. Because more peptide bonds are broken and more new charged groups (NH<sub>3</sub><sup>+</sup>/COO<sup>-</sup>) are available to compete

for water, the “free” water content of maturing Cheddar cheese curd is reduced (O’Mahony *et al.*, 2005). The foaming capacity is another peptide-dependant property, which has been widely applied in the alcoholic beverages industries (such as beer and sparkling wine) (Sharpe *et al.*, 1981). The liberation of hydrophobic sequences during hydrolysis can lead also to peptides with emulsifying properties (Shimizu *et al.*, 1984). Finally, peptides with antifreezing properties have been reported; they have been obtained or isolated from Antarctic and Arctic fish and they were mainly alanine- and cystine-rich (Wohrmann, 1996). Some others, such as alcalase hydrolizates of bovine gelatine, ranging from 600 to 2700 Da, are also able to inhibit recrystallization of ice in frozen ice cream mix as well as in frozen sucrose solutions (Wang and Damodaron, 2009)

## 2.2 Sensory properties

Peptides can contribute considerably to the final taste of food, in particular cheese and meat products (Hansen-Møller *et al.*, 1997) and they may cover the entire range of taste modalities: sweet, bitter, umami, sour and salty (Temussi, 2012; Seki *et al.*, 1990). Compounds with acidic-rich residues have a sour taste, whereas those rich in hydrophobic residues have a bitter taste, and those with a more balanced composition display little or no taste.

Sequence and conformation can also play an important role in flavour. Nowadays, aspartame (L-aspartyl- phenylalanine methyl ester) is the most extensively used peptide to substitute sugar in beverages. This peptide has the same calories of sucrose, but it is 200 times sweeter; hence, aspartame can be used in a lower concentration, and it can be supplied to diabetics. However, some disadvantages have been detected, such as: low stability at high temperature, low solubility at neutral pH, and high sensitivity to proteolytic reactions. Moreover, phenylalanine, one of its breakdown products, must be avoided by people suffering of phenylketonuria (PKU) (Temussi, 2012). Mazur *et al* (1969) demonstrated that the molecule providing the sweetness to this peptide is from one side the Asp residue, but also a very precise steric structure (H-L-Asp-L-Phe-OMe). All the other possible chiral isomers, i.e. D-L, L-D and D-D H-Asp-Phe-OMe, are bitter.

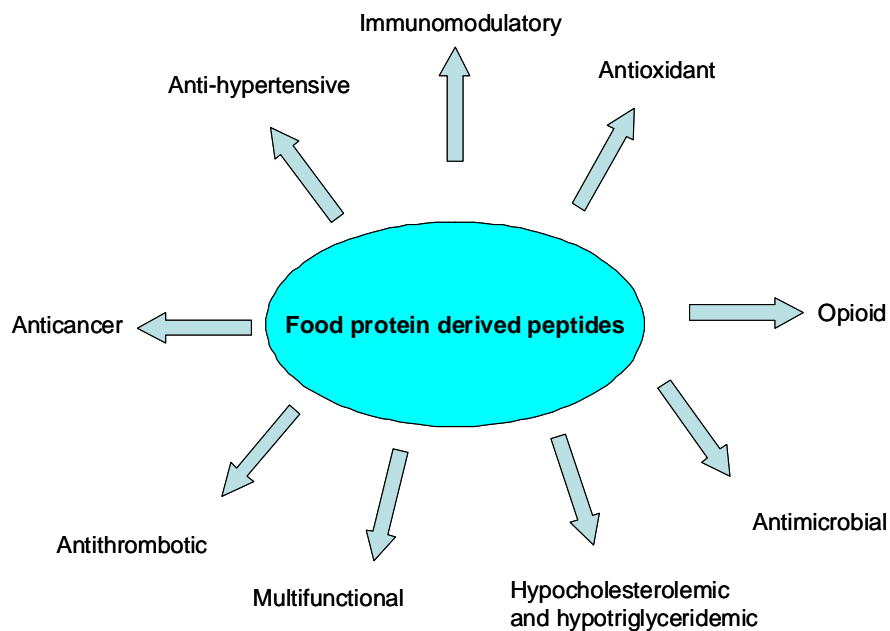
As mentioned above, hydrophobic peptides possess bitter taste. Bitter peptides are prevalent in a wide variety of aged or fermented foodstuffs, because enzymatic hydrolysis frequently generates bitterness; the development of bitter taste in cheese during maturation is a well-studied example (Temussi, 2012). Otagiri *et al.* (1985) reported that a strong bitter taste is observed when arginine is contiguous to proline). Finally, although the umami (taste enhancer) depends mainly to the glutamate, novel "umami peptides" have been studied and isolated. Yamasaki and Maekawa (1987) isolated the "delicious peptide" from a beef soup (H-Lys-Gly-Asp-Glu- Glu-Ser-Leu-Ala-OH), which produces a taste similar to that food product. However, there is no significant evidence



to consider the small umami peptides as an independent class; it is possible that their taste is a consequence of the presence of Asp or Glu. In this way, it is clear that the chemical nature of peptides, particularly their incredible conformational versatility, plays a relevant role in determining many structure–activity relationships, including those connected to food acceptance (Temussi, 2012).

### 2.3 Peptides bioactivities

The study of functional and bioactive peptides has been extensively promoted (Perez Espitia *et al.*, 2012). A peptide is considered bioactive if it can support health through a positive impact on the functions or conditions of living beings (Korhonen and Pihlanto, 2006). The beneficial effects of peptides depend on their antimicrobial (Reddy *et al.*, 2004; Rajanbabu and Chen, 2011), antioxidant (Sarmadi and Ismail, 2010), antithrombotic (Wang and Ng, 1999), anti-hypertensive (Erdmann *et al.*, 2008), opioid and immunomodulatory behaviour (St Georgiev, 1990; Gauthier *et al.*, 2006). The main peptides bioactivities are reported in **figure 1**.



**Figure 1.** Bioactive properties of food protein-derived peptides.

### 2.3.1. Anti-hypertensive peptides

Many of the physiological functions in an organism are mediated by peptides; for instance, blood pressure can be regulated by peptides, like angiotensin-II or bradykinin. The antihypertensive effect is defined by measuring the capability of a putative peptide to inhibit the angiotensin-I-converting enzyme (ACE, EC 3.4.15.1). ACE is a constituent enzyme of the renin-angiotensin system that plays a crucial role in blood pressure regulation and fluid and electrolyte balance (Martínez-Maqueda *et al.*, 2012) These processes are catalysed by two mechanisms: either by the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, or by the degradation of bradykinin, a potent vasodilator, and other vasoactive peptides. Some anti-hypertensive peptides are reported in **table 3**.

Most bioactive peptides generated from milk proteins have demonstrated ACE-I activity. They can be released from two different pathways: milk protein hydrolysis or milk fermentation. A number of peptides with antihypertensive properties have been identified from casein and whey proteins with gastric and pancreatic enzymes.

One of the peptides with proven antihypertensive effect is a  $\alpha_{s1}$ -casein-derived peptide, with sequence FFVAPFPGVFGK. The casein hydrolyzate containing this peptide has been patented and commercialized as an antihypertensive product named Peptide C12®. Moreover  $\alpha_{s1}$ -casein represents a source of bioactive peptides since two other sequences that reduce systolic blood pressure (SBP) have also been identified (RYLGY and AYFYPEL) (Contreras *et al.*, 2009). The use of food-grade enzymes, derived from microorganisms, to release bioactive peptides has also become a common strategy. In nature, the proteolytic activity of lactic acid bacteria during fermentation of dairy products gives off peptides and amino acids, which are used as nitrogen sources necessary for bacterial growth (Martínez-Maqueda *et al.*, 2012). However, some of these peptides, produced by *Lactobacillus helveticus* in combination with *Saccharomyces cerevisiae* during milk fermentation, revealed blood pressure lowering properties (i. e. VPP and IPP). Other strains responsible for the liberation of antihypertensive peptides are *Lactobacillus helveticus* CPN4, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Nakamura *et al.*, 1995)

Eggs are another important source of antihypertensive peptides. Ovokinin (FRADHPFL), resulting from the pepsin hydrolysis, is one of them. These properties can be enhanced by emulsification with egg yolk, because phospholipids promote its absorption and protect the peptide from intestinal peptidases (Fujita *et al.*, 1995). An intriguing discovery about the bioactive egg peptides is that even if they show ACE-I activity *in vivo*, many of them may not work *in vitro*, demonstrating only partial correspondence between *in vitro* and *in vivo* effects.

Due to their optimal extracting conditions, collagen and gelatin are considered as good sources of bioactive peptides. They are obtained mainly from porcine skin and bovine hide, as well as bones, tendons and cartilages. Moreover, studies considering novel sources, such as meat, poultry or fish and marine by-products and waste are already being developed (Martínez-Maqueda *et al.*, 2012). Some previously reported antihypertensive meat peptides are MNPPK and ITTNP, which were released in the thermolysin hydrolysis of porcine muscle myosin. Other peptides were found to be particularly active, such as AVF and VF, from an insect protein digestion, YYRA from chicken bone hydrolysate and KRVIQY from porcine myosin hydrolysate.

Antihypertensive peptides inhibitory to ACE can also be derived from vegetable proteins, such as gluten, zein and hordein (Gobbetti *et al.*, 1997). Indeed, hydrolysed or fermented soybean proteins produced several sequences responsible for the *in vitro* antihypertensive activity (**Tab. 3**).

A daily consumption of a moderate amount of antihypertensive peptides from natural sources could elicit a blood pressure reduction not far from that of synthetic drugs, because the majority of patent drugs available in the market contain similar bioactive peptides to those found in the above mentioned food products (Martínez-Maqueda *et al.*, 2012).

### **2.3.2. Antithrombotic peptides**

Cardiovascular diseases (CVDs) lead to the development of thrombosis, due to the alterations in the coagulation mechanisms. Increased occurrence of thrombosis has been linked to platelet hyperreactivity, high levels of haemostatic proteins (e.g., fibrinogen), defective fibrinolysis and hyperviscosity of the blood (Erdmann *et al.*, 2008). Therefore, antithrombotic drugs are commonly used to reduce platelet aggregation and enhance fibrinolysis. Similarities between the mechanisms of milk clotting, defined by the interaction of  $\kappa$ -casein with chymosin, and blood clotting, defined by the interaction of fibrinogen with thrombin, have been reported. To date, food derived peptides with antithrombotic properties are mainly the result of enzymatic hydrolysis of  $\kappa$ -casein (Erdmann *et al.*, 2008) (**tab. 3**). The dodecapeptide of fibrinogen and the 106-116 sequence of  $\kappa$ -casein show functional homologies. Another peptide, with MAIPPKKNQDK sequence, functions mainly because of the presence of three amino acid residues (Ile108, Lys112, Asp115). It inhibits both the aggregation of ADP activated platelets as well as the binding of human fibrinogen  $\gamma$ -chain to its receptor region on the platelet surface (Smacchi and Gobbetti, 2000).

**Table 3.** Examples of anti-hypertensive and antithrombotic peptides deriving from different food sources.

	sequence	source	reference
Anti-hypertensive	FFVAP	$\alpha$ -CN f23-27	Maruyama et al., 1985
	AVPYPQR	$\beta$ -CN f177-183	Maruyama et al., 1986
	YGLF	$\alpha$ -Laf50-53	Mullaly et al., 1996
	ALPMHIR	$\beta$ -LGf142-148	Mullaly et al., 1997
	KVLPVPQ	$\beta$ -CN f169-174	Maeno et al., 1996
	LVYFPFGPIFNSLFGNIIP	$\beta$ -CN f58-76	Miguel et al., 2006
	LHLPLP	$\beta$ -CN f133-138	Miguel et al., 2007
	YP	$\alpha$ -CN f146-147	Maeno et al., 1996
	FFVAPFPGVFGK	$\alpha$ -CN f23-34	Keshmirian and Nogrady 1988
	RYLGY	$\alpha$ -CN f90-95	Contreras et al., 2009
	AYFYFEL	$\alpha$ -CN f90-96	Contreras et al., 2009
	VPP	$\beta$ -CN f(84-86)	Nakamura et al., 1995
	IIP	$\beta$ -CN f(74-76)	Nakamura et al., 1995
	YRGGLEPINF	Eggwhite	Miguel et al., 2007
	ESIINF	Eggwhite	Miguel et al., 2008
	YAEERYPIL	Eggwhite	Miguel et al., 2005
	RADI-PFL	Eggwhite	Miguel et al., 2006
	IVF	Eggwhite	Miguel et al., 2007
	Hydrolysate	Eggwhite	Miguel et al., 2008
	FRADHPFL	Ovalbumin	Fujita et al., 1995
	QVSLNSGYY	Dhordein	Gobbetti et al., 1997
	SAYPGQITSN	$\alpha$ -zein	Gobbetti et al., 1998
	FNQ	$\alpha$ -zein	Yano et al., 1996
	LAY	$\alpha$ -zein	Yano et al., 1997
	LLP	$\alpha$ -zein	Yano et al., 1998
	LNPA	$\alpha$ -zein	Yano et al., 1999
	LQQ	$\alpha$ -zein	Yano et al., 2000
	AY	$\alpha$ -zein	Yano et al., 2001
	NVGPLV	Soy (glycinin)	Kodera and Nio, 2006
	IAP	Wheat (gliadin)	Motai and Kodama, 2003
		Wheat protein hydrolysate	Kodera and Nio, 2006
	GA(Hyp)GL(Hyp)GP	Chicken leg collagen hydrolysate	Saiga et al., 2008
	GF(Hyp)GP	Porcine skin collagen hydrolysate	Ichimura et al., 2009
	LKP	Meat (chicken muscle)	Fujita et al., 2000
	IKW	Meat (chicken muscle)	Fujita et al., 2001
	LAP	Meat (chicken muscle)	Fujita et al., 2002
	ITNP	Meat (porcine muscle)	Nakashima et al., 2002
	MNPPK	Meat (porcine muscle)	Nakashima et al., 2003
	YYRA	Chicken bone extract hydrolysate	Nakade et al., 2008
	KRVQY	porcine myosin hydrolysate	Muguruma et al., 2009
	DLTDY	Oyster protein hydrolysate	Shiozaki et al., 2010
	DY	Oyster protein hydrolysate	Shiozaki et al., 2011
	Hydrolysate	Oyster protein hydrolysate	Shiozaki et al., 2012
	Hydrolysate	Salmon muscle protein hydrolysate	Ono et al., 2003
	Hydrolysate	Squid skin collagen hydrolysate	Lin et al., 2012
VF	Insects hydrolysis	Vercauteren et al., 2010	
AVF	Insects hydrolysis	Vercauteren et al., 2011	
Antithrombotic	MAIPPKNQDK	k-CN f106-116	Jolles et al., 1986
	KDQDK	k-CN f112-116	Qian et al., 1994
	KRDS	Lactotransferrin f39-42	Qian et al., 1995
		k-CN f106-112	Battazzi, 1996
		k-CN f106-116	Battazzi, 1996
		k-CN f112-116	Battazzi, 1996
	k-CN f113-116	Battazzi, 1996	

### 2.3.3. Hypocholesterolemic and hypotriglyceridemic peptides

A destabilised blood lipids profile (hypercholesterolemia and/or hypertriglyceridemia) is another risk factor for CVDs. It has been reported that dietary proteins with low ratios of methionine/glycine and lysine/arginine, such as soy and fish protein, favour a hypocholesterolemic effect, whereas bovine and casein, having higher amino acid ratios, tend to elevate cholesterol levels (Erdmann *et al.*, 2008). The most studied hypocholesterolemic peptides derive from soy proteins (**tab. 4**). Peptides produced from this source revealed that a hydrophobic region is required for biological activity. Moreover, a proline residue seems to be a key component. Hypotriglyceridemic activities have been also detected in different animal species when hydrolyzed globin has been provided. This effect may depend on the capability of the peptides to decrease intestinal fat absorption and to enhance the lipolysis of triglycerides (Erdmann *et al.*, 2008).

**Table 4.** Examples of antioxidant, hypocholesterolemic and opioid peptides deriving from different food sources.

	sequence	source	reference
Antioxidant	MY	Fish (sardine muscle)	Erdmann et al., 2006
	LLPHH	Soy ( $\beta$ -conglycinin)	Chen et a., 1995
	YFYPEL	Milk (casein)	Suetsuna et al., 2000
	MHIRL	Milk ( $\beta$ -lactoglobulin)	Hernandez-Ledesma et al., 2005
	YVEEL	Milk ( $\beta$ -lactoglobulin)	Hernandez-Ledesma et al., 2006
	WYSLAMAASDI	Milk ( $\beta$ -lactoglobulin)	Hernandez-Ledesma et al., 2007
	YAEERYPIL	Egg (egg white)	Davalos et al., 2004
		$\beta$ -CN f98-105	Rival et al., 2001
		$\beta$ -CN f177-183	Rival et al., 2002
		$\beta$ -CN f169-176	Rival et al., 2003
	$\beta$ -CN f170-176	Rival et al., 2004	
	k-CN f96-106	Kudoh et al., 2001	
Hypocholesterolemic	LPYPR	Soy (glycinin)	Yoshikawa et al., 2000
	IAVPGEVA	Soy (glycinin)	Pak et al., 2005
Opioid	RYLGYLE	$\alpha$ -CN f90-96	Loukas et al., 1983
	YPIQYVLSR	k-CN f25-34	Chiba et al., 1989
		$\beta$ -CN f60-63	Meisel, 1997
		$\beta$ -CN f60-70	Fiat et al., 1993
	GYYP	gluten	Fukudome and Yoshikawa, 1992 Fukudome and Yoshikawa, 1993 Fukudome et al., 1997
	GGT		
	GGT		
	GGT		
YFSL			

#### 2.3.4. Antioxidant peptides

The use of antioxidants as preservatives has been systematically applied in the food industry. Recently, the use as health products of food-derived peptides has also attracted interest, because they can supplement endogenous antioxidants against oxidative stress (Fang *et al.*, 2002). Recent studies have shown that peptides with antioxidant properties can be released from food sources such as milk casein, whey protein, egg and soy protein and food by-products (Erdmann *et al.*, 2008; Bougateg *et al.*, 2010). Some examples of bioactive peptides derived from different protein sources are listed in **table 4**.

Some suggested mechanisms influencing the antioxidant properties of peptides have been: metal ion chelation, scavenging or quenching of reactive oxygen species (ROS), inhibition of enzymatic (lipoxygenase-mediated) and nonenzymatic peroxidation of lipids and essential fatty acids (Udenigwe and Aluko, 2012). The antioxidant activity has been attributed to certain amino acid sequences, in particular histidine, proline, cysteine, methionine, and aromatic amino acids. Chen and co-workers reported that histidine residues of peptides are able to chelate metal ion, quench active oxygen, and scavenge OH (Chen *et al.*, 1998) through its imidazole group, which can participate in hydrogen atom transfer and single electron transfer reactions (Chan and Decker 1994). This activity can be increased adding hydrophobic amino acids (such as proline and leucine) to the N-terminus of a dipeptide His-His. This hydrophobic part is important because it can lead the antioxidant peptides to interact with hydrophobic cellular targets (Chen *et al.*, 1998). Although selected information about the specific activities of single peptides has been reported, it is not clear how protein hydrolysates contribute in these processes. Li *et al.* (2008) noticed that there is a dose-dependent relationship between hydrolysate concentration and antioxidant activity. The highest antioxidant activity was found in peptides between 500–1500 Da.

#### 2.3.5. Opioid peptides

Opioid peptides are small molecules naturally produced in the central nervous system (CNS) and in various glands throughout the body. They contribute to some behaviours, such as motivation, emotion, and attachment, the control of food intake and the response to stress and pain. They work in the same way of classic alkaloid opiates (such as morphine and heroin) (Froehlich, 1997). Exorphins, a class of peptides with opioid activity, were found in pepsin hydrolysates of wheat gluten and soy  $\beta$ -protein (Fanciulli *et al.*, 2003; Zioudrou *et al.* 1979) (**tab. 4**) and others, called cytochrophins and hemorphins, have been produced from enzymatically treated bovine blood (Brantl *et al.*, 1986).

### **2.3.6. Immunomodulatory peptides**

Immunomodulatory peptides are able to enhance the functions of immune system, including regulation of cytokine expression, antibody production, and ROS-induced immune functions (Hartmann and Meisel 2007; Yang *et al.*, 2009). It has been reported that a tryptic digest of rice protein can promote phagocytosis and increase superoxide anion production in human leukocytes (Takahashi *et al.*, 1994). In addition, egg-derived peptides are used during cancer immunotherapy to increase immune functions (Mine and Kovacs-Nolan, 2006). (Udenigwe, 2012) Chicken meat proteins, especially myosin, tropomyosin and collagen, contain bioactive peptide fragments with immune-stimulating properties which, theoretically, could be released through the activity of proteinase K (Dziuba *et al.*, 1996).

### **2.3.7. Anticancer peptides**

A number of studies on anticancer peptides have been focused on soy peptides, in particular in Lunasin (Hernandez-Ledesma *et al.*, 2009). The anticancer property of lunasin is predominantly against chemical and viral oncogene-induced cancers, and based on the modulation of histone acetylation and deacetylation pathways specifically. The final effects are repression of cell cycle and promotion of apoptosis in cancer cells (Hernandez-Ledesma *et al.*, 2009). Recently, 2 large peptides (Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr) from tuna dark muscle by-product hydrolyzed with papain and protease XXII were isolated by Hsu and co-workers (2011). These peptides exhibited dose-dependent antiproliferative activities against cultured breast cancer (MCF-7) (Hsu *et al.*, 2011). This result demonstrates the potential of meat products and meat by-products as valuable sources of bioactive peptides for incorporation into functional foods (Ryan *et al.*, 2011).

### **2.3.8. Antimicrobial peptides**

One of the first lines of defence against infections used by animals, plants and insects is the production of antimicrobial peptides. According to data already reported, antimicrobial peptides should be relatively short (12 to 100 amino acids), positively charged (to interact with lipids) and amphiphilic (to enter into the cell membrane). Despite their similar properties, antimicrobial peptides have very limited sequence homologies with a wide range of secondary structures. To date, hundreds of such peptides have been identified and their different mechanisms of action have been elucidated (Jenssen *et al.*, 2006). There are at least four major groups (Mackintosh *et al.*, 1998):

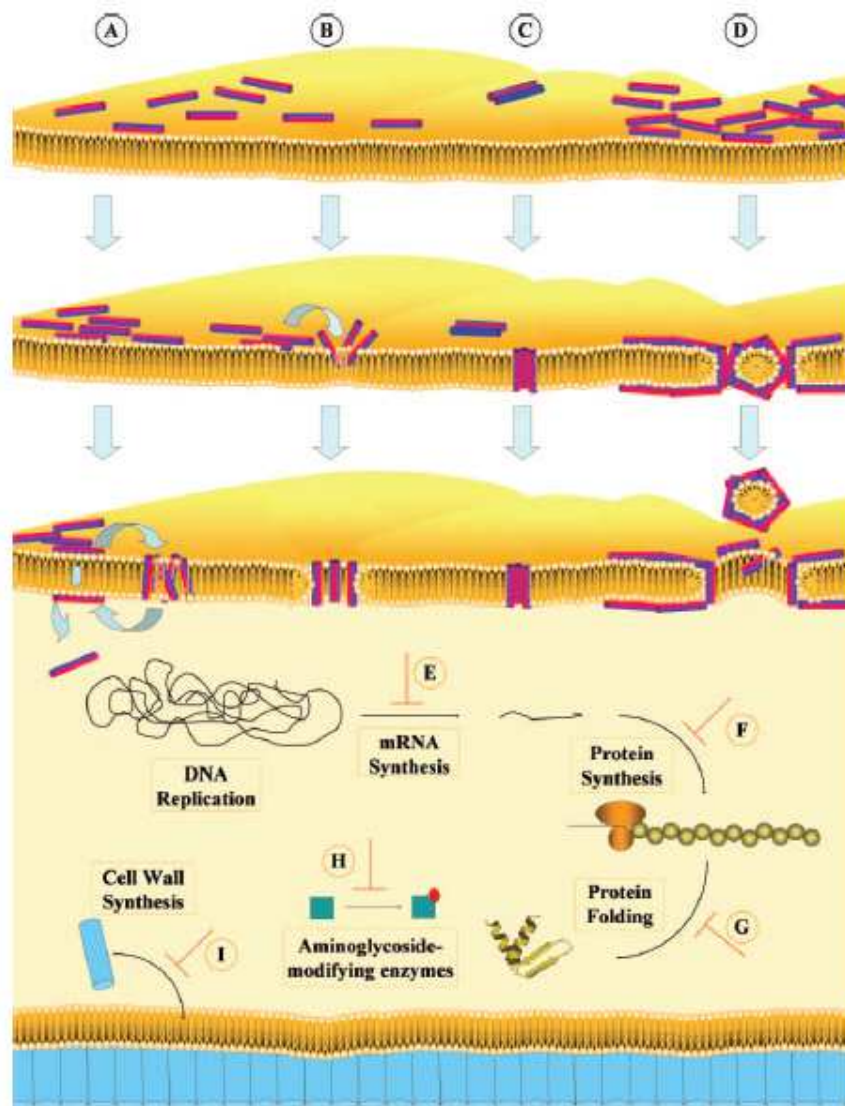
- 1) Linear helical peptides without cysteine, such as the cecropins from insects and pigs (Boman, 1995);
- 2) Peptides with an antiparallel  $\beta$ -sheet structure stabilized by two or three intramolecular disulfide bonds, such as defensins (Ganz, 2003);
- 3) Peptides with one intramolecular disulfide bond, such as bactenecin (Romeo *et al.*, 1988);
- 4) Linear peptides containing high proportions of one or more amino acids, in particular tryptophan-, arginine- and proline-rich peptides, like drosocin (Bulet and Stöcklin, 2005).

Jennesen *et al.* (2006) reported that the antimicrobial action in peptides is given by permeabilization through the microbial cells. In such case, a model has been proposed to describe the mode of action (**fig. 2**): first, in the “aggregate” model (A), there is a reorientation of the peptides spanning the membrane and forming an aggregate; then, on the “toroidal pore” model (B), peptides form pores; in the “barrel-stave” model (C), peptides are inserted in a perpendicular orientation to the plane of the bilayer, form the “staves” in a “barrel”-shaped cluster, and in the “carpet” model (D), peptides aggregate in parallel to the lipid bilayer, showing a detergent-like activity. Peptides that do not act by permeabilising the membrane can work at different levels: inhibiting DNA and RNA synthesis (E), decreasing protein synthesis (F), reducing the enzymatic activity (G), modifying aminoglycosides (H), or forming structural components (I) that can disturb the normal bacterial growth. Bacteria antimicrobial peptides (bacteriocins) are among the first ones to be isolated and characterized. They are produced as defence from other bacteria that might compete in the same environment. One of the main studied bacteriocins is nisin, a lanthionine containing a peptide, used for nearly 50 years as a food preservative (Jenssen *et al.*, 2006).

Even though it has not been as well-studied as the antioxidant capacity of food peptides, the hydrolysis of food proteins can generate new and potent antimicrobial peptides (**tab. 5**). The most investigated food-derived antimicrobial peptide is lactoferricin, the fragment 17-41 of the iron-binding glycoprotein lactoferrin. This peptide has antimicrobial activity against Gram-negative bacteria and *Candida albicans* (Farnaud and Evans, 2003).

Peptides released from milk proteins are the richest source of antimicrobial peptides (AMPs). Peptides obtained from bovine  $\alpha_{s1}$ -casein and produced by *Lactobacillus acidophilus* DPC6026, are active against both Gram-positive and Gram-negative bacteria; these have been proposed as bioprotective compounds if supplied in milk food products (Hayes *et al.*, 2006)





**Figure 2.** Mechanisms of action of antibacterial peptides proposed by Jenssen *et al.*, 2006.

Muscle foods as source of antimicrobial peptides are less documented. A peptide obtained from bovine meat (GLSDGEWQ) inhibited the growth of both Gram-positive and Gram-negative pathogens. It was also reported that GFHI and FHG inhibited the growth of the pathogen *P. aeruginosa* (Ryan *et al.*, 2011). Moreover, a cysteine rich antimicrobial peptide was produced from the digestion of oyster muscle, using a combination of alcalase and bromelin (Liu *et al.*, 2008). This peptide also resulted active inhibiting the growth of the fungi *Botrytis cinerea* and *Penicillium expansum* (Ryan *et al.*, 2011)

**Table 5.** Examples of antimicrobial peptides deriving from different food sources.

	sequence	source	reference
Antimicrobial	YQEPVLGPVRGPFPIIV	colostrum	Birkemo et al., 2009
	YQEPVLGPVRGPFPI	colostrum	Birkemo et al., 2010
	RPKHPIKHQGLPQEVLNENLLRF	colostrum	Birkemo et al., 2011
	IKHQGLPQE	casein	Hayes et al., 2004
	VLNENLLR	casein	Hayes et al., 2005
	SDIFNPIGSENSEK	casein	Hayes et al., 2006
	GLSDGEWQ	beef sarcoplasmic protein hydrolysate	Ryan et al., 2011
	GFHI	beef sarcoplasmic protein hydrolysate	Ryan et al., 2012
	FHG	beef sarcoplasmic protein hydrolysate	Ryan et al., 2013

### 2.3.9. Multifunctional peptides

The functions described above can be detected in different peptides, but sometimes one peptide show multiple activities at the same time. In this case it is defined as a multifunctional peptide (Udenigwe and Aluko, 2012). A hexapeptide (TTMPLW) derived from  $\alpha_{S1}$ -casein (f194–199), through trypsin-catalyzed digestion, exhibited both ACE-inhibitory and immunomodulatory activities (Meisel, 2004); in addition, a  $\beta$ -lactoglobulin-derived  $\beta$ -lactorphin (YLLF) inhibited ACE activity and also possessed opioid-like activity (Mullally *et al.*, 1997). Moreover, crude chemotryptic  $\alpha$ -casein hydrolysates displayed several *in vitro* bioactivities such as ACE inhibition, antioxidant,  $Zn^{2+}$ -binding, and antibacterial activities (Srinivas and Prakash, 2010). Four peptides (GFHI, DFHING, FHG, and GLSDGEWQ) present in beef sarcoplasmic protein hydrolysates have been reported to possess anticancer, antimicrobial, and ACE-inhibitory properties (Jang *et al.*, 2008).

**CHAPTER 3.**  
**GLYCOPEPTIDES**

Glycopeptides are molecules present and produced in nature. The study of sugars bounded to amino acids has become increasingly interesting, since these structures have been showed to play important roles in fertilization, the immune system, brain development, the endocrine system and inflammation (<http://en.wikipedia.org/wiki/Glycopeptide>). Some glycopeptides, isolated from microorganisms, are already used as antibiotics (Kahne *et al.*, 2005), while some others instead are produced by the insects as the first defence against bacterial infections (Bulet and Stöcklin, 2005). The synthesis of novel glycopeptides can be useful to elucidate glycan function in nature, but the current major challenge is to uncover their therapeutic and biotechnological applications in food (<http://en.wikipedia.org/wiki/Glycopeptide>).

### 3.1. Structure

Glycopeptides are short sequences of amino acids (minimum 7-8) bound to one or more mono-, di- and oligosaccharides. The function of the sugar group has not been fully elucidated; even though the absence of that sugar inside the chain can reduce around 100 times the glycopeptide bioactivity (Otvos *et al.*, 2002). In general it could be hypothesized that the presence of the sugar determines a chemical and physical rearrangement of the peptide conformation, similarly to what occurs in proteins, as described above.

Different studies that tried to design non-glycosylated analogues of glycopeptides in order to explore the role of glycosylation have been published. Kaur *et al.* (2006), for example, reported that it is possible to obtain a functionally equivalent non-glycosylated analogue from a native glycosylated peptide just by performing strategic modifications of the sequence. This means that the presence of the sugar provides the peptide with a new spatial distribution. At the same time, the peptide, and in particular the type of amino acid, can influence the special sugar conformation once it is linked (Hindley *et al.*, 2005).

Some of the most studied glycopeptides belong to insects and they are produced as a mechanism of defence against bacterial invasion. These peptides, most of them rich in proline, can undergo glycosylation with one, two, or three glycan residues, forming a O-glycosylated substitution with a conserved threonine residue (Gobbo *et al.*, 2002). The first characterized glycopeptide was Drosocin, a O-glycosylated peptide, consisting of 19 amino acid residues (of which nearly one third are proline), and three characteristic Pro-Arg-Pro motifs. Drosocin is glycosylated on Thr 11 by either a monosaccharide (such as N-acetyl-galactosamine) or a disaccharide. The presence of the disaccharide in the middle of the molecule may open the turn comprising residues 10-13 to a more extended conformation, thus helping drosocin to assume the

most suitable orientation to bind to its putative intracellular target. In many cases the integrity of the carbohydrate side chain is also necessary for the maximum activity of the glycopeptides (such as formaecin and dipteridin); however, it has also been reported that unglycosylated peptides, like pyrrolicin, appear to be more potent than the native glycosylated form. To date, it has not been possible to define a specific structure for "the" glycopeptide, and in particular many studies are still in progress (Gobbo *et al.*, 2002).

A well-studied glycopeptide, already patented as a potent drug is Vancomycin. It was first isolated in 1953 from a soil sample and it is produced by the bacteria *Amycolatopsis orientalis*. It is a 1449.3 Da molecule and from its structure several novel compounds were isolated, produced and designed. The common structure in all, called "vancomycin-related", contains a homologous heptapeptide scaffold. Five out of the seven residues in vancomycin are aromatic, and the remaining two residues are modified tyrosines, with chlorine at the meta position of the aromatic ring, and an OH substitute at the benzylic carbon of the side chain. The electron-rich side chains of these aromatic amino acid residues facilitate the oxidative crosslinking, leading to a rigid architecture of the heptapeptide scaffolds. Finally, the peptide framework is glycosylated, by a mono- or disaccharide on residue 4 (Kahne *et al.*, 2005). Another glycopeptide with bioactive properties was isolated by Yang *et al.* (2009) from a fermentation broth of *Penicillium* sp. M03. This molecule is a 1017 Da and contains five aminoacids (Ala, Glu, Gly, Asp and Ile) plus two monosaccharides (glucose and xylose). PGY is also a low molecular-weight glycopeptide; it was isolated by Wu and Wang (2009) from the fruit *Ganoderma lucidum* was. This molecule possess a peptide part with a Ser-Arg-[(Ala)<sub>2</sub>(Gly)<sub>2</sub>] sequence, and a carbohydrate part, coupled by O-linkage via Ser, constituted of a backbone of (1 → 3)-β-glucan with (1 → 6)-linked Araf branches. The carbohydrate moiety, especially the side chains of terminal α-L-Araf residues, is essential for the preservation of the activity of this class of glycopeptides.

### **3.2. Antifreezing activity**

It is already well known that many O-glycan-rich glycoproteins, especially isolated from arctic fish, act as *in vivo* "antifreezer", preventing nucleation of ice and allowing them to survive at temperatures of -2 °C (Gamblin *et al.*, 2009). In fact, antifreeze glycopeptides (AFGP) and peptides have been isolated from 37 species of Antarctic fish. The glycopeptides are made up of a tripeptide repeat (alanine-alanine-threonine), with a disaccharide moiety attached to the threonyl residues (Wohrmann, 1996). Their molecular weights range from 2600 Da to 34000 Da (Wohrmann, 1996). These compounds kinetically depress the temperature at which ice grows, in a non-colligative

manner, and hence exhibit thermal hysteresis, i.e. a positive difference between the equilibrium melting point and the ice growth temperature (the temperature at which seed ice crystals will grow in the solution) (Harding *et al.*, 2003)

### **3.3. Sensory properties**

Certain glycopeptides and peptides provide an improved taste function, in particular for the kokumi taste function. These molecules could be used in a wide range of industrial applications and enhance the basic tastes, as well as impart thickness, spread, continuity, and unity of the final product (<https://usgene.sequencebase.com/patents/US20060083847>).

### **3.4. Glycopetides bioactivities**

Just like peptides, the glycopeptides have revealed different properties and bioactivities. The main studied property is the antimicrobial one. Nevertheless, their additional properties have been reported to a lesser extent.

#### **3.4.1. Antioxidant activity**

Wu and Wang (2009) isolated from the fruiting bodies of *G. lucidum* a new water-soluble glycopeptide (PGY) with higher levels of antioxidant properties against superoxide radicals, compared with a selected antioxidant (BHT). This activity strictly depends on the carbohydrate moiety, while the peptide moiety appears to be nonessential for their antioxidant activity. Furthermore, they demonstrated that PGY provides higher levels of antioxidant characteristics against superoxide radicals, in comparison with that of a selected antioxidant (BHT).

#### **3.4.2. Antitumor activity**

Natural ecosystems play an important role as a source of new antitumor compounds. The antitumor bleomycin (BLM) was first isolated from *Streptomyces verticillus*; it provided the name to a class of natural antitumor glycoconjugates, made of complex polypeptide aglycones with a variety of mono-, di-, and tetra-saccharides attached. The BLM, clinically known as Blenoxane (Bristol-Myers Squibb), treats several types of tumours, such as testicular cancer (90% efficiency), Hodgkin's lymphoma, and carcinomas of the skin, head and neck. However, the cytotoxic therapeutic efficacy of BLM is limited, due to induction of lung fibrosis. The mechanism of action of BLM depends on a metal-dependent oxidative cleavage of DNA and RNA, which leads to cell

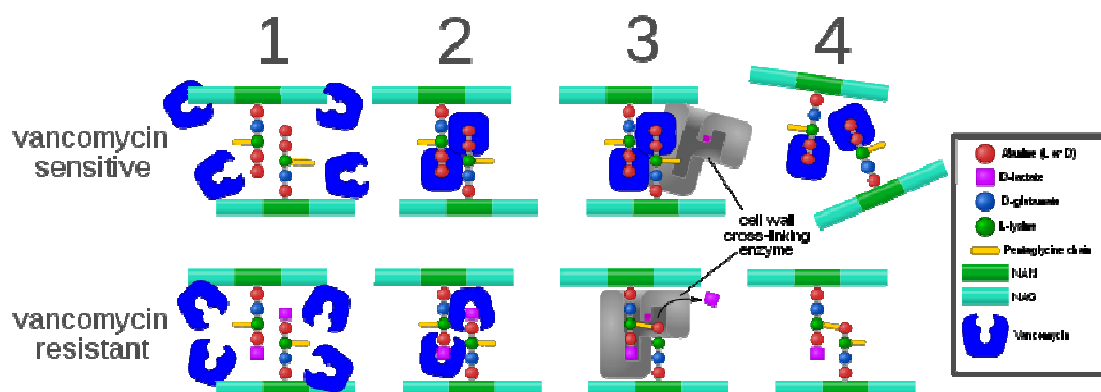
death. Three functional domains can be recognized inside the structure of BLM: the N-terminal domain, represented by pyrimidoblastic acid (PBA) along with  $\beta$ -hydroxy-histidine, is the metal-binding domain and enables the activation of molecular oxygen; the C-terminus is the DNA-binding domain, and it is formed from a bisthiazole moiety with a cationic tail; and the hydroxyl group of b-OH-His, which that participates in cell recognition and uptake and in metal ion coordination. NC0604 is a BLS analogue isolated from *Streptomyces verticillus* var. *pingyangensis*, with enhanced antitumor activity and lower pulmonary toxicity (La Ferla *et al.*, 2011).

Other natural antitumor glycopeptides have been found in macromycetes (macro-fungi). PSP and PSK, from the macro-fungus *Coriolus versicolor*, for example, are well known for their immunomodulatory and antitumoral activities. Both glycopeptides stimulate the T-cell activation, and induce cytokine production *in vitro* and *in vivo*, enhancing “killer” cytotoxic activity against tumours; they are considered biological response modifiers. The glycopeptides 220-GP and 120-GP obtained from pronase- treated ovomucin (an egg-white glycoprotein) were found to have high direct antitumor activity at low concentrations; both glycopeptides have shown to promote complete rejection of direct tumours and slight growth inhibition of distant tumours. A number of muramyl glycopeptides (fragments of murein, a bacterial peptidoglycan from the cell wall) are known to cause growth inhibition and necrosis of experimental tumours. For example, glucosaminylmuramyl dipeptide (GMDP) was reported to enhance tumoricidal activity of macrophages, by inducing the secretion of TNF- $\alpha$ , one of the key players of macrophage cytotoxicity against tumoural cells. GMDP also augmented the cytotoxic effect on tumour cell lines, when combined with TNF-a and cisplatin; in this case, 100% tumour cells were killed (La Ferla *et al.*, 2011).

### 3.4.3. Antimicrobial activity

Vancomycin was the first glycopeptide antibiotic to be discovered. Despite its toxicity profile, it gained prominent use to face the problem of the emergence of a large-scale resistant *S. aureus*. The mechanism by which vancomycin exerts its action depends on the inhibition of cell wall synthesis in Gram-positive bacteria, as opposed to the Gram-negative, because the outer membrane in the Gram-negative bacterial membrane keeps the glycopeptides from reaching their targets at the periplasmic face of the cytoplasmic membrane (Kahne *et al.*, 2005). The large hydrophilic molecule is able to form hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides (**fig. 3**). In this way, it prevents the transglycosylation and subsequently affects the transpeptidation, which is essential for bacterial cell wall cross-linking (Sujatha and Praharaj, 2012). Several studies attempting to develop new molecules mimicking vancomycin have been reported (Chen *et al.*, 2002). Hence, glycopeptides having effect on Gram-

negative microorganism have been isolated as well. In fact, Drosocin, a glycopeptide isolated from insects, works in a low micromolar range of concentration, and it is mainly active towards Gram-negative bacteria (Bullet and Stocklin, 2005).



**Figure 3.** Mechanisms of action of Vancomycin towards sensitive and not sensitive bacteria (<http://en.wikipedia.org/wiki/Vancomycin>).

The relatively slow-killing kinetics of drosocin and the observation that an all-D analogue is 50 to 150-fold less active than the native isomer suggested that the peptide do not seem to have a membrane permeabilization mechanism, which is thus markedly dissimilar to that of lytic peptides such as cecropins.

As already indicated, deglycosylation significantly reduces the antimicrobial activity of drosocin. The syntheses of differently glycosylated peptide analogues showed that the antimicrobial activity against several Gram-negative bacteria is affected by the type of sugar and the type of glycosidic linkage, particularly in the case of *E. coli* D21 and the *K. pneumoniae* strains (Gobbo *et al.*, 2002).

The mechanism of action of these proline-rich glycopeptides could depend on the interaction with the lipopolysaccharides (LPS) of Gram-negative bacteria and/or with the bacterial chaperone/heat shock proteins GroEL and DnaK; such an interaction inhibits protein folding (Kragol *et al.*, 2001). The fact that some of these glycopeptides (i.e. pyrrolicin analogues) do not show selectivity towards Gram-negative or Gram-positive strains, has confirmed that their toxicity for bacteria is not strongly related to membrane binding. At the same time, the specificity to certain bacterial strains may derive from altered binding to DnaK (Otvos *et al.*, 2000). An additional effect of glycopeptides is the protection of host cells from pathogen adhesion. The anti-adhesive capacity of the glycopeptides was reported by Yang and co-workers when  $\beta$ -conglycinin hydrolysates were produced. These glycopeptides have both a D-mannosyl residue and a hydrophobic region; it was



suggested that the presence of the mannose substructure of glycopeptides prevents *E. coli* and *Salmonella* adhesion to the intestinal epithelium (Yang *et al.*, 2008). Glycopeptides with antibiofilm properties have been also reported. In particular, peptides with an amino acid sequence bound to a casein or a fragment of casein, comprising at least one glycosylated amino acid, can prevent dental caries, gingivitis and periodontitis (<http://www.freepatentsonline.com/y2012/0283174.html>).

**CHAPTER 4.**

**PROTEASES**

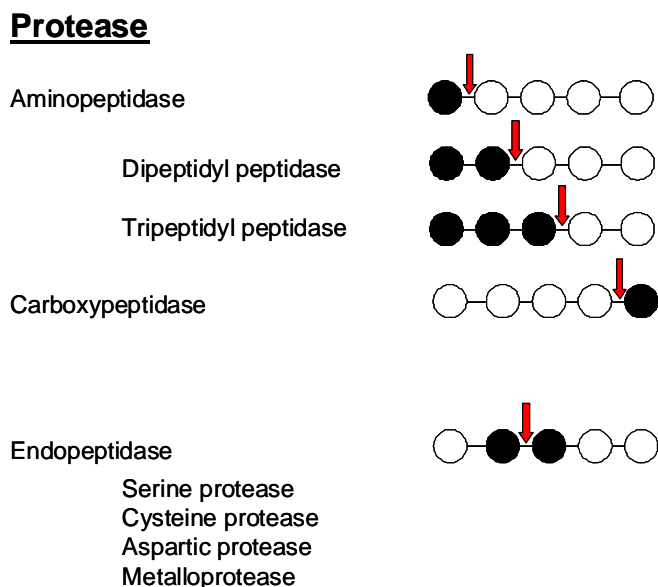
According to the Enzyme Commission (EC) classification, proteases belong to the hydrolase family (group 3), which hydrolyse peptide bonds (sub-group 4). The processing of many food products depends on proteolytic activities, because they can enrich the characteristics of the final product or generate innovative food and ingredients (Sumantha *et al.*, 2006). Until fairly recently, proteases were considered primarily to be protein-degrading enzymes. However, this view dramatically changed when their important roles in physiological processes such as generating signalling and/or functional molecules was discovered. Consequently, they immediately attracted the interest for commercial applications, becoming one of the three largest groups of industrial enzymes, and accounting for approximately the 60% of the total worldwide sales of enzymes. (<http://acd.ufrj.br/proteases/ProteaseApres.htm>). In food processing, proteases can be derived from the food matrix, generated by microorganisms or supplied as additives. Because of their wide applicability, the microbial proteases represent the 40% of the commercial enzymes currently present in the market (Rao *et al.*, 1998).

Proteases can be separated into two major groups based on their ability to cleave peptide bonds: when they act at the terminus of polypeptide chains (amino- or carboxy- peptidases), they are classified as **exopeptidases** (EC 3.4.11-19); if they act internally, they are considered **endopeptidases** or proteinases (EC 3.4 21-99). The exopeptidases are used for their debittering action and they are becoming increasingly available in the industrial enzyme market (Raksakulthai and Haard, 2003). Additionally, proteases can also be classified according to: their optimal pH (as acidic, neutral or alkaline); substrate specificity (collagenase, keratinase, elastase, etc.); or their homology with well-studied proteins (trypsin-like, pepsin-like, etc.) (Sumantha *et al.*, 2006).

A more detailed classification is presented in **Figure 4**.

#### **4.1. Exopeptidases**

The exopeptidases act only near the ends of the polypeptide chains at the N or C terminus. The aminopeptidases act at on the free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide; in general, the aminopeptidases are intracellular enzymes. The commercial Aminopeptidase I and II have been generated from *Escherichia coli* and *Bacillus licheniformis*, respectively. In contrast, the exopeptidases act on the free C terminus, to liberate a single amino acid or a dipeptide; they have been isolated mainly from *Penicillium* spp., *Saccharomyces* spp., and *Aspergillus* spp. Additionally, the omega peptidases are enzymes working on isopeptide bonds, i.e. an amide bond not present on the main chain of the protein (Rao *et al.*, 1998).



**Figure 4.** Classification of proteases. Arrows show the sites of action of the enzyme.

## 4.2. Endopeptidases

The endopeptidases work preferentially on the peptide bonds away from the N and C termini. The presence of free  $\alpha$ -amino or  $\alpha$ -carboxyl groups has a negative effect on the activity of the enzyme. According to the reactive groups at the active site involved in catalysis, they can also be divided into serine- (EC 3.4.21), cysteine- (EC 3.4.22), aspartic-peptidases (EC.3.4.23) and metallo-peptidases (EC 3.4 24). Among these, the alkaline proteases are the most industrially significant (Sumantha *et al.*, 2006).

### 4.2.1. Serine proteases

They are characterized by the presence of a serine group in their active site, and they are the most conserved proteases among eukaryotic and prokaryotic organisms. Even if the primary structure is different among the members of this class, their general catalytic reaction depends on three common amino acids, i.e. serine (nucleophile), aspartate (electrophile), and histidine (base). They are active at neutral and alkaline pH (between pH 7 and 11), and they have molecular masses between 18 and 35 kDa. Subtilisin is one of the most well-studied serine proteases isolated from *Bacillus subtilis*, although other enzymes have also been discovered in *S. cerevisiae* (Mizuno and Matsuo, 1984) *Y. lipolytica* (Li *et al.*, 2009), *Conidiobolus* spp. (Phadattare *et al.*, 1993) and *Aspergillus* spp. (Hajji *et al.*, 2008). Some of these enzymes have already been produced for

industrial purposes, such as Alcalase® (from *Bacillus licheniformis*), Savinase® (from *Bacillus lentus*), Esperase® (*Bacillus* spp.), etc. (Novozymes, Denmark) (Rao *et al.*, 1998).

#### **4.2.2. Aspartic proteases**

The aspartic proteases owe their name to the presence of aspartic acid residues necessary for their catalytic activity. The active-site aspartic acid residue is situated within the motif Asp-X-Gly, where X can be Ser or Thr. Because most of the aspartic proteases show a maximal activity at low pH (pH 3 to 4), they are also known as acidic proteases; their molecular masses are in the range of 30 to 45 kDa. Most of the acidic proteases have been studied in fungi and yeasts (Rao *et al.*, 1998). A significant property of aspartic proteases is the ability to coagulate milk, as it is evidenced by their widespread application in the dairy industry to coagulate casein during the manufacturing of cheese (Yegin *et al.*, 2010).

#### **4.2.3. Cysteine proteases**

The activity of cysteine proteases depends on the presence of a dyad of Cys and His in the catalytic site. These kinds of enzymes occur in both prokaryotes and eukaryotes and have an optimal neutral pH. Papain (from *Carica papaya*), bromelain (from *Ananas comorus*), ficin (from *Ficus* sp.) are the most studied proteases from food origin; Clostripain and Streptopain are produced by *Clostridium histolyticum* and *Streptococcus* spp., respectively (Sumantha *et al.*, 2006).

#### **4.2.4. Metalloproteases**

Enzymes belonging to this class are the most heterogeneous proteins according to the catalytic site structure. The only common characteristic among them is the requirement for a divalent metal ion to perform their activities; thus, they can be endo- or exopeptidases. Collagenase, a member of this family, has been discovered in bacteria (*Clostridium histolyticum* and *Achromobacter iophagus*), yeasts (*Candida albicans*, Lima *et al.*, 2009) and fungi, and it is very specific for the proteolysis of collagen and gelatine (Rao *et al.*, 1998).

### **4.3. Functions and Applications**

The estimated value of the worldwide sales of industrial enzymes is \$1 billion a year, with proteases accounting for about 60 % the total (Godfrey and West, 1996). Proteases have been widely applied, mainly in the detergent and food industries. For example, acidic proteases are used in the dairy industry for their ability to coagulate milk protein (casein) to form curds, from which

cheeses are prepared (Neelakantan *et al.*, 1999). Alkaline proteases, instead, are useful in baking processes (for biscuits, crackers and cookies, for instance), because they can hydrolyse flour proteins and thus enhance the texture, flavour, and colour of the final product. Commercial enzymes commonly used in the food industry include Alcalase®, Neutrase®, and Novozym®. In addition, mixtures of enzymes are available, such as Flavourzyme™ and Kojizyme™. These are fungal complexes of exopeptidases and endoproteases derived from the fermentation of soy sauce by *Aspergillus oryzae*. Proteases from *Bacillus subtilis* have been used to deproteinize crustacean waste and obtain chitin (Yang *et al.*, 2000)

Other fields where proteases have been successfully applied are in pharmaceutical, cosmetic and bioremediation processes. Even though applied biotechnological methods have significantly improved the production of these enzymes in the past few years, the search for innovative sources of enzymes, revolutionary production techniques, and novel applications of such enzymes in unexplored fields has continued uninterrupted (Sumantha *et al.*, 2006).

## **CHAPTER 5.**

### *Yarrowia lipolytica*

A "non-conventional" yeast is defined as "a microorganism easily distinguished from the well-studied and widely used *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*". Among the "non-conventional" ones, *Yarrowia lipolytica* is one of the most studied as a model system in physiology, genetics, dimorphism, gene manipulation, protein expression, and lipid accumulation research works (Bankar *et al.*, 2009). Since a few amount of sugar is needed for its growth, it has been isolated from diverse environments rich in lipids and proteins; its natural habitats include oil-polluted environments, rivers and foods such as cheeses, yogurt, kefir, shoyu, meat, and poultry products (Bankar *et al.*, 2009). It is considered a non-pathogenic organism and it is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA) (Cohelo *et al.*, 2010). Isolated for the first time in 1928 by Nannizzi, it just reached industrial interest by the late '40s, especially for the dairy industry and later as a producer of citric acid.

## 5.1. Taxonomy and morphology

*Y. lipolytica* is a yeast belonging to the Fungi kingdom, division *Ascomycota*, class *Saccharomycetes*, order *Saccharomycetales*. The wild form of *Y. lipolytica* presents different morphology ranging from smooth and shiny to wrinkled colonies. These characteristics depend on the growth conditions (oxygen, carbon, ammonia availability), but also on the genotype and strain (Rodriguez and Domínguez, 1984). It is a dimorphic fungus that can come in the form of single cells, pseudo-hyphae, or septate hyphae (Barth and Gaillardin, 1997). The yeast-to-mycelium transition is associated with unipolar growth, asymmetric division, large polar-located vacuoles, and repression of cell separation after division. It is believed that yeast dimorphism is related to a defence mechanism against adverse conditions, such as temperature and nutritional changes (Cohelo *et al.*, 2010). The name *Y. lipolytica* was given in 1980 by van der Walt and von Arx, but it is still possible to find it with the previous nomenclature, as follows: *Mycotorula lipolytica* (1928), *Candida lipolytica* (1942), *Candida olea* (1949), *Azymoprocandida lipolytica* (1961), *Candida paralipolytica* (1963) *Candida pseudolipolytica* (1973) (Kurtzman and Fell 1998).

## 5.2. Metabolism

*Y. lipolytica* is a unique, strictly aerobic yeast, with the ability of efficiently degrade hydrophobic substrates such as n-alkanes, fatty acids, fats and oils, for which it has specific metabolic pathways (Fickers *et al.*, 2005). Even if, from the genome point of view, it is related to



*Saccharomyces cerevisiae*, it has significantly different genetic mechanisms. Particularly, the genome displays an expansion of protein families and genes involved in hydrophobic substrate utilization (Cohelo, 2010).

### 5.3. Industrial relevance of *Y. lipolytica*

There are two reasons for *Y. lipolytica* to be extensively studied: its secreted metabolites and enzymes, and its activities during the cell growth. Without a doubt, the main interesting feature about this microorganism is the expression of its extracellular and cell-bonded lipases (Ota *et al.*, 1982). Lipases are lipolytic enzymes able to hydrolyze the triglycerides in glycerol and fatty acids, and they are also exploitable for several applications in the detergent (substituting chemical surfactants), food, and environmental industries. Some of these lipases possess also the capability to work under cold conditions (Parfene *et al.*, 2011) and this aspect is progressively getting interesting for industrial applications (Joseph *et al.*, 2007). Being capable to consume n-alkanes, isoprenoids and aromatic hydrocarbons as the group of naphthalenes and the group of phenanthrene, *Y. lipolytica* could be also applied in bioremediation of contaminated environments (Bankar *et al.*, 2009) or for the treatment of olive mill wastewaters (Lanciotti *et al.*, 2005).

*Yarrowia lipolytica* is recognized as one of the most frequent species associated with milk (Lanciotti *et al.*, 2004; Gardini *et al.*, 2006) and meat products (Patrignani *et al.*, 2011 a; 2011b), due to its enzymatic activities, it has been regarded as a good candidate for accelerating ripening (Guerzoni *et al.*, 1998; van den Tempel and Jakobsen, 2000; Lanciotti *et al.*, 2005; Patrignani *et al.*, 2007). In fact, thanks to its high proteolytic and lipolytic activities, some yeast species may play an important role in the production of aroma precursors from amino acids, fatty acids and esters (Suzzi *et al.*, 2001).  $\gamma$ -Decalactone is a peach-like aroma compound, reported in several food and beverages, that can be produced biotechnologically, e.g. by *Y. lipolytica* which is able to biotransform ricinoleic acid (12-hydroxy-octadec-9-enoic acid) into the lactone (Cohelo, 2010).

Another important application of this yeast is the production of citric acid. Currently, the conventional procedure depends on the bioconversion of molasses into citric acid by *Aspergillus niger*. This production is estimated to be approximately 1.6 million tons per year (Sauer *et al.*, 2007) However, the use of *Y. lipolytica* may bring some more benefits: a larger substrate variety (n-paraffin, fatty acids, glucose), a smaller sensitivity to low dissolved oxygen concentrations and heavy metals, and higher product yields. One potential disadvantage of this process is the secretion of isocitric acid (ICA) as by-product; when it is above 5% of the citric acid

concentration can generate problems in the crystallization of CA during the purification process (Cohelo, 2010).

## **5.4. Proteases of *Yarrowia lipolytica***

The production of proteases by *Y. lipolytica* and their possible applicability are two aspects that need to be studied in depth. *Y. lipolytica* is a natural secretor of proteins, and it was used especially for this property as eukaryotic host for secretion of heterologous proteins. In common with many strains of yeast, *Y. lipolytica* expresses also proteolytic enzymes (extracellular and intracellular proteases). In particular, an acidic extracellular protease (AXP) and an alkaline extracellular protease (AEP) have been mainly studied (Young *et al.*, 1996). From 1 to 2% of the total cell proteins belong to AEP; hence, over 1 g of AEP per litre has been estimated (Matoba *et al.*, 1988) at high cell densities.

### **5.4.1. Extracellular proteases**

#### **5.4.1.1. Alkaline protease**

The gene *xpr2*, coding for the AEP, has been cloned and sequenced (Davidow *et al.*, 1987). It has a functional promoter region greater than 700 bp, maybe as a result of the complex regulation of the gene. The regulatory region, consisting in a TATA box and other two major activation sequences, is deeply influenced by the environment (Young *et al.*, 1996). In fact, the gene is transcribed during nitrogen or sulphur starvation, lack of carbon sources, and the presence of extracellular proteins (Davidow *et al.*, 1987). According to the mRNA sequence, AEP is originally synthesized in an immature form, having some pro-regions added (Hernández-Montañez *et al.*, 2007). Most probably a 55 kDa precursor is synthesized first, and then after different cleavages and maturing steps, 52, 44, and 36 kDa polypeptides are generated. In the final step, in which an intracellular protease participate, the 32-kDa mature AEP is formed (Matoba *et al.*, 1988). It has been also reported that the pro-region is glycosylated (Young *et al.*, 1996). Studies regarding the AEP sequence showed a strong homology with the subtilisin family serine proteases. The highest homologies belong to the region referring to the active-site of the enzyme (Davidow *et al.*, 1987); in particular, a 32% homology with the *Bacillus subtilis* DY subtilisin (Nedkov *et al.*, 1983), 32% homology to *Thermoactinomyces vulgaris* thermitase (Meloun *et al.*, 1985), and 42.6% homology with the *Tritirachium album* proteinase K (Jany *et al.*, 1986). Once the enzyme is in its mature form, it has been reported that the AEP secretion process is rapid.

#### 5.4.1.2. Acidic protease

Initially, three acidic proteases were reported (Yamada and Ogrydziak, 1983), but upon additional studies and the development of more sophisticated techniques, it was demonstrated that *Y. lipolytica* produces just one acidic protease under specific growth conditions. The AXP1 gene encoding the acid protease has been characterized and genetic control of the synthesis has been reported (Young *et al.*, 1996; González-López *et al.*, 2002). As the AEP, the AXP is also synthesised as a pro-enzyme, having 397 residues and a molecular weight of 42 kDa. The mature 37 kDa enzyme is produced by cleavage of the precursor between Phe<sub>44</sub> and Ala<sub>45</sub>. In this case no glycosylation of the pro region has been reported (Young *et al.*, 1996). According to the final primary sequence, AXP shows a 44.94% identity with a 36 amino acid overlap with Endothiapepsin, an aspartyl protease from the chestnut blight fungus, *Cryphonectria parasitica* (Razanamparany *et al.*, 1992), and Candidapepsin, the aspartic endopeptidase of *Candida albicans* (Young *et al.*, 1996).

#### 5.4.2. Intracellular proteases

Although there is scarce evidence, the presence of some intracellular proteases in *Y. lipolytica* has been reported. An aminopeptidase (yylAPE), belonging to the metalloprotease group, has been intracellularly detected in a soluble form, whereas a dipeptidyl aminopeptidase (yylDAP) activity has been reported both in the soluble and in the membrane fractions. The membrane form of yylDAP is a serine protease maybe involved in the maturation process of AEP; in contrast, the soluble form yylDAP is a metalloprotease probably involved in the dimorphic transition of the yeast. Finally, no specific functions have been described for the discovered soluble carboxypeptidase (yylCP) belonging to the serine protease family (Hernández-Montañez *et al.*, 2007).

#### 5.4.3. Proteases production

The main element that discriminates the production of AEP or AXP is the environmental pH. In general, neutral and high pH values (6-9) lead to AEP production, while low pH (2-6) favours AXP (Ogrydziak, 1993).

Also carbon and sulphur compounds, as well as nitrogen sources (ammonium ions, amino acids) can play a role in repressing the protease production. On the other hand, their production can be induced if a protein source is present in the growth medium (Nelson, 1986). Moreover, it has been reported that an extra carbon source (glucose) reduces AEP production (Akpınar *et al.*,

2011). Thus, if the type of synthesized protease is strictly dictated by the environmental pH, both proteases are similarly induced at the end of the exponential phase on complex media (González-López *et al.*, 2002).

#### **5.4.4. Industrial applications**

Due to its lipolytic and proteolytic activities, *Y. lipolytica* has a high industrial potential. In fact, its use as a co-starter for the production of some cheese varieties has already been proposed by several Authors (Bintsis and Robinson, 2004; Lanciotti *et al.*, 2005). The extracellular proteases have important commercial value and multiple applications can be found in various industrial settings. Although there are many microbial sources available for protease production, only few are recognized as commercial producers. Since *Yarrowia lipolytica* is a GRAS microorganism it could be used in different industrial processes; however there is missing evidence referring to the applicability of *Y. lipolytica* proteases. One of the first studies by Nelson and Young (1986) considered the use of these enzymes in the brewing industry as a chill-proofing agent for beer. Additional work reported the use of *Y. lipolytica* enzymes for meat tenderisation; hence Krasnowska *et al.* (2006) showed that yeast preparations had better proteolytic (and collagenolytic) activities than pepsin. All enzyme preparations showed different activity against beef meat proteins according to the pH. Instead, Patrignani *et al.* (2007) demonstrated that the surface-inoculated *Y. lipolytica* did not have a deep effect on the immediate proteolysis of dried fermented sausage when compared to *Debaryomyces hansenii*.

**CHAPTER 6.**  
**OBJECTIVES**

Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolysis products (Kalisz, 1988). At the same time, the extracellular proteases have also been widely commercially exploited to assist protein degradation in various industrial processes (Kumar and Takagi, 1999; Outtrup and Boyce, 1990; Gupta *et al.*, 2002).

Commercial protease preparation usually consist of a mixture of various enzymes. They are largely utilised in food processing to

- improve the workability of dough (as backing enzymes particularly for crackers and biscuits);
- optimize and control the aroma formation of cheese and milk products;
- improve the texture of fish products;
- tenderize meat;
- stabilize beer

Due to their large consumption and applications commercial preparations do not have a specific action apart from the protease used for the production of hypoallergenic foods. In Europe there are about forty commercial protease preparations on the market. Fifteen of these preparation are produced with genetically modified *Aspergillus* and *Bacillus* spp. Their optimal activity is between 35 and 50 °C.

The resource that I have explored to identify proteases with different promising attitudes has been the collection of about 112 strains of *Yarrowia lipolytica*, isolated from different ecosystems (cured meat, Po river alkanes contaminated waters, commercial chilled foods, cheeses, irradiated poultry meats or light butter) and characterized during the last 20 years. All the strains belong to the DISTAL Alma Mater Studiorum, University of Bologna. Preliminary research on 112 strains showed great strain-related differences in the protein breakdown profiles (Badiali, 2004). In particular, the cluster analysis of electrophoretic profiles obtained when  $\alpha$ - and  $\beta$ -caseins had been separately exposed to cell free supernatants of the various strains showed that the profiles of strains isolated from the same ecosystem clustered together. On the basis of these results I selected for each cluster one strain having the typical proteolytic profile representative of the group. In particular, I selected the following strains of *Y. lipolytica*: 1IIYL4A and 1IIYL8A, both isolated from speck, but

characterized by different RAPD-PCR profiles (Badiali, 2004); 16B from irradiated poultry meat; PO19 from Po river water; CLCD from salami; Y16 from commercial chilled food.

The main objectives of my research on these strains have been to:

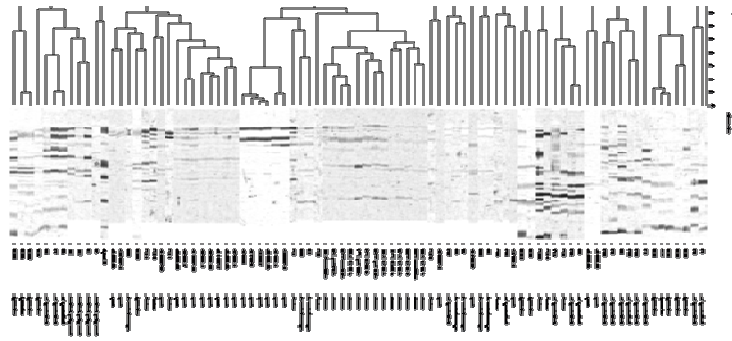
1) evaluate the suitability of proteases released by *Y. lipolytica* to hydrolyse proteins of different origins available as industrial food by-products. In particular I have taken into consideration gluten, gelatin, milk and meat proteins;

2) identify proteases with "cold attitude", i.e. proteolytic activity at temperature  $\leq 10$  °C, in order to save energy consumption during industrial processes. In fact, the main technological criterium to assess the suitability of a biological process to exploit by-products regards the energy consumption during all the phases. Therefore the enzyme to be selected for the biotransformation should be active within a temperature range between 4 and 20°C;

3) obtain and characterize peptides having specific biological activity (namely antioxidant, anti-hypertensive, antimicrobial, cytotoxic)

4) improve the bioactivity of the peptides through a novel process based on their glycation/glycosylation.

The sequence of the activities performed on the selected strains can be summarized in the following scheme:

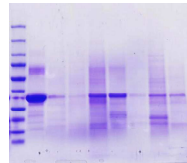


*Selection of 7 different strains and hydrolysis of different matrix*



**Protease characterization**

**Gelatin**



**Gluten**

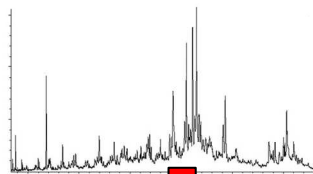
**Caseins**

**Meat extract proteins**

*Selection of one strain and hydrolysis of one matrix*

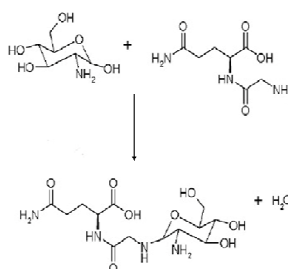


**Gelatin**



**Bioactivities**

*Improvement of bioactivities of peptides through glycation/glycosilation*



**Bioactivities**



**CHAPTER 7.**

**MATERIAL AND METHODS**

## **7.1. *Yarrowia lipolytica* strains and culture conditions**

The strains of *Yarrowia lipolytica* used in this Ph.D. thesis belong to the Department of Agri-Food Science and Technologies (DISTAL) - Alma Mater Studiourum, University of Bologna.

The strains selected have different isolation habitats: salami (CLCD), speck (1IIYL4A, 1IIYL8A), irradiated poultry meat (16B), commercial chilled foods (Y10, Y16) and superficial waters of Po river (PO19). The strains were cultivated in Sabouraud broth (Oxoid, Basingstoke, England) at 27°C for 72-96 h.

## **7.2. Protein matrices**

The proteolytic activity of *Y. lipolytica* was evaluated on proteins of different origin also available as industrial food by-products, and namely gelatin, meat protein, dairy proteins (skim-milk,  $\alpha$ - and  $\beta$ -caseins), and gluten from wheat.

In particular,  $\alpha$ - and  $\beta$ -caseins were purchased from Sigma-Aldrich, skim-milk from a local dairy processing plant, gelatin and meat proteins were extracted from meat by-products used for the production of salami.

### **7.2.1. Total meat protein extraction**

Total proteins were extracted from 2 g of sample (i.e. meat by-products) with 40 ml of 1.1 M potassium iodide, 0.1 M sodium phosphate, pH 7.4 buffer (Cordoba, 1994). The sample was homogenized for 3 minutes and then centrifuged (Beckman Coulter Avanti J-10, Fullertn, CA, USA) at 8000 x g for 15 min at 4°C. The supernatant was filtered with 0.22  $\mu$ m filter and then used for the enzymatic activity assay (paragraph 7.4.4.).

## **7.3. List of chemicals used**

Alcalase (Alc, EC 3.4.21.14, from *Bacillus licheniformis*) and Flavourzyme (Flv, from *Aspergillus oryzae*), Glycine-Glutamine (Gly-Gln), Glucosamine hydrochloride (GlcN), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Transglutaminase (TGase) from guinea pig liver, Hanks' Balanced Salt Solution (HBSS) were purchased from Sigma-Aldrich (St. Louise, MO). All chemicals used in SDS-PAGE electrophoresis, Size Exclusion Chromatography (SEC), LC-MS/MS and MALDI-TOF were of HPLC grade supplied by Sigma-Aldrich (St. Louise, MO), whereas other

chemicals were of analytical grade. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

## **7.4. Proteolytic activity of *Y. lipolytica***

### **7.4.1. Extracellular proteases recovery and characterization**

For the production and the recovery of extracellular proteases, the protocol used by Vannini et al. (2001) was followed with some modifications. The strains of *Y. lipolytica* were pre-cultured for 72 h at 27°C in Sabouraud Broth under agitation (200 rpm). After centrifugation at 8000 x g (Beckman Coulter Avanti J-10, Fullerton, CA, USA) at 4°C for 15 min the supernatants were collected and were used as the source of the protease enzymes and thus referred to the “supernatants”.

A preliminary characterization of the extracellular proteases released by the various strains was made by analyzing the cell-free supernatants with the technique of substrate-incorporated polyacrylamide gel electrophoresis (zymography). Such a technique was used to separate and characterize the enzymes produced by cultures of *Y. lipolytica* and to evaluate their activity on substrates (i.e. gelatine and casein) incorporated into the gel.

### **7.4.2. Characterization of proteases by Zymography**

The characterization of the extracellular proteases was performed by the technique of substrate-incorporated polyacrylamide gel electrophoresis (Zymography). In particular two different precast Zymogram gels, containing gelatin or caseins as substrate incorporated into the gels (Bio Rad), were used to see if proteases showed a different specificity for the substrates. 50 µL of the cell-free supernatants of the various strains of *Y. lipolytica* were mixed with an equal volume of Zymogram sample buffer 2X (Bio Rad) and let to rest for 10 min at room temperature. 35 µL of each sample was loaded on the gels. A 10 µL aliquot of Precision Plus Protein Standard All Blue (Bio Rad) was used as standard. Gels were run in a Mini Protean Cell System with a Tris-Glycine SDS Running Buffer at 100 V for the first 10 min and at 120 V for 1 h. After running, the gel was soaked into the Zymogram Renaturing Buffer (Bio Rad) and incubated with a gentle agitation for 40 minutes at room temperature. Subsequently, the Zymogram Renaturing Buffer was washed before (30 min) and then replaced with the Zymogram Developing Buffer (Bio Rad). The gel was incubated in this buffer overnight at 37°C to allow enzymatic digestion of the protein.

Gel was stained for 1 hour with the Staining solution (0.5 % Bromophenol blue, 50 % methanol and 7% glacial acetic acid) and de-stained for 2 hours with the Destaining solution (40% Glacial acetic acid, 10% methanol).

#### **7.4.3. Preliminary evaluation of the "cold attitude" of the proteolytic enzymes**

The ability of the extracellular proteases to hydrolyse the different proteins was evaluated both at 27 and 6°C. In particular, the supernatants of the various strains were tested on gluten agar, skim-milk agar and gelatin.

Gluten agar was prepared according to Wiese (1995) with some modifications (1.5 % gluten, 1 % glucose, 0.5 % universal peptone, 1.7 % agar from Oxoid). The media was boiled in stirring conditions and then poured into Petri plates. Similarly, skim-milk agar (1 % skim-milk and 1.7 % agar from Oxoid) was boiled in stirring conditions and then poured into the Petri plates. Both the media contained suspended solid particles which made the agar plates cloudy.

Gelatin liquefaction was estimated preparing a solution of 12 % gelatin, 1 % glucose, 0.5 % universal peptone. After autoclaving it was dispensed into glass tubes.

Aliquots of 10 µL of the cell-free supernatants were spotted on the centre of the agarized media. The plates were incubated at 6 or 27°C in order to assess enzyme activity at cold and control temperatures, respectively. After 3 days (when incubation was at 27°C) or 10 days (for the cold conditions) plates were checked in respect to growth, contaminants and enzymatic digestion of the proteins. In particular proteolytic activity resulted in the formation of clear zones onto the agarized media (for skim milk and gluten) or liquefaction (for gelatin). In general, the proteolytic activity was carried out at least in triplicate.

#### **7.4.4. Evaluation of the proteolytic profiles generated by *Y. lipolytica* proteases**

Enzymatic activities of the extracellular proteases of the various strains were determined by using the following substrates:  $\alpha$ -casein and  $\beta$ -casein from bovine milk (Sigma), commercial gelatine and meat protein extract.

The assay mixture contained *Y. lipolytica* supernatants, sterile distilled water (1:2) and 1% (w/v) of gelatin or meat protein extract, or 0.1% (w/v) of caseins. The hydrolysis was performed in stirring conditions. After 48, 72 and 96 hours of incubation at 27°C, the reaction was stopped by heating at 100 °C for 5 min and the collected samples were stored at -20°C before their use for protein analysis by SDS-PAGE electrophoresis (paragraph 7.4.5), chemical (paragraph 7.6) and biological (paragraph 7.7) characterization.

### **7.4.5. SDS-PAGE electrophoresis**

The hydrolysis of the proteins was detected by sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis. For the denaturation, 70  $\mu$ L of each sample were mixed with an equal volume of Laemmli Sample Buffer 2X (Bio-Rad Laboratories, Milan, Italy) containing  $\beta$ -mercaptoethanol. The mixture was incubated at 100 °C for 10 min and then charged on a precast gel. Different gels (Bio Rad) were used according to the protein analysed: a gradient 4-20 % gel was used for the casein samples and a 4-15% gel for the meat protein and the gelatin samples. 10  $\mu$ L of Precision Plus Protein Standard All Blue (Bio Rad) was used as standard, and 35  $\mu$ L of each sample were loaded on the gels. Gels were run in a Mini Protean Cell System with a Tris-Glycine SDS Running Buffer at 100 V for the first 10 min and at 200 V for 1 h.

Gels were stained for 1 hour with the Staining solution (0.1 % Bromophenol blue, 50 % methanol and 7% glacial acetic acid). Subsequently, 2 hours of Destaining solution (40% Glacial acetic acid, 10% methanol) were performed.

## **7.5. Improvement of peptides bioactivity**

### **7.5.1. Model System**

#### **7.5.1.1. Model system preparation and UV spectra collection**

A model system consisting of a dipeptide Gly-Gln (0.03 M) and GlcN with different molar ratios of 1:1, 1:3 and 1:10 was prepared. Samples were dissolved in 0.05 M  $(\text{NH}_4)\text{HCO}_3/\text{NH}_4\text{OH}$  buffer at pH 8.8 with or without TGase (2 unit/g). TGase was activated with 5 mM calcium chloride solution. In addition, 0.03 M and 0.12 M solutions of GlcN in the absence of the peptide were prepared separately as control. These solutions were treated in the same way as the mixtures of peptide and GlcN. About 5 mL of the mixture was poured into screw-cap tubes (15 mL) and incubated at 25 and 37°C with shaking (200 rpm) for 0, 3.5 and 7 h. All aliquots were kept at -20°C upon incubation for further use. The UV-Vis absorbance spectra (250 - 500 nm) of the samples were measured using a spectrophotometer SpectroMax M3 plate reader (Molecular devices, Sunnyvale, CA) in order to create the UV profiles.

#### **7.5.1.2. LC/MS analysis**

Samples prepared as above described were diluted in aqueous 25 % (v/v) acetonitrile and 0.2 % (v/v) formic acid, and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific, Mississauga, ON) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific, Mississauga, ON). Nanoflow chromatography and electrospray ionization were accomplished by

using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100  $\mu\text{m}$  inner diameter (300  $\text{\AA}$ , 5  $\mu\text{m}$ , New Objective). Samples were injected onto the column at a flow rate of 3000 nL/min and resolved at 500 nL/min using 30 min linear acetonitrile gradients from 5 to 50 % (v/v) aqueous acetonitrile in 0.2 % (v/v) formic acid. Mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60 000 and m/z range of 100–2000.

#### 7.5.1.3. Production of hydrolysates

Exactly 5 % (w/v) of gluten was suspended in 0.05 M  $(\text{NH}_4)\text{HCO}_3/\text{NH}_4\text{OH}$  buffer, the final pH of the mixture was pH 7.5 – 8. It was homogenised with an Ultra-Turrax homogenizer, model T 25 (IKA Works, Inc. Staufen, Germany) at 10,000 rpm for 1 min. Then it was heated at 80  $^\circ\text{C}$  for 10 min. The gluten mixture was cooled to 50  $^\circ\text{C}$  before adding Alc or Flv, respectively at 1:10 volume ratio of enzyme to buffer, followed by incubation in a shaker (50  $^\circ\text{C}$ , 3.5 h, 200 rpm). Incubation was terminated at 80  $^\circ\text{C}$  for 10 min, the hydrolysates were centrifuged at  $10,000 \times g$  (10  $^\circ\text{C}$ ) for 15 min and filtered by using Whatman No. 1 filter paper. The filtrate was collected, lyophilized and stored at -18  $^\circ\text{C}$  before their chemical and biological characterization.

#### 7.5.1.4. Preparation of Glycated/Glycosylated peptides

Samples of the lyophilized hydrolysate powders (from Alc or Flv respectively) were weighed at 1.5 g each and added to GlcN at the weight ratio of 1:1. Each of the weighed powders was dissolved in 30 mL of 0.05 M  $(\text{NH}_4)\text{HCO}_3/\text{NH}_4\text{OH}$  buffer (pH  $7.0 \pm 0.5$ ) and each incubated at 25  $^\circ\text{C}$  and 37  $^\circ\text{C}$  for 3.5 h. Samples with GlcN were subjected to incubation with or without TGase (2 unit/g of lyophilized hydrolysate) at pH 7.5. 5 mM calcium chloride were added prior to use in order to activate TGase. Controls consisting of the lyophilized hydrolysate were incubated at the same temperature without GlcN. At the end of incubation, all the mixtures were passed through a 0.2  $\mu\text{m}$  nylon syringe filter (13 mm, Mandel, Ontario) followed by ultra-filtration with a molecular weight cut-off membrane of 10 kDa ( $3,900 \times g$ , 20 min, 10  $^\circ\text{C}$ , Amicon Ultra Centrifugal filters (Millipore, Cork, Ireland)). Excess of GlcN was removed by dialysis membrane with a molecular weight cut-off membrane of 100 – 500 Da (Spectrum Laboratories, TX). The retentates were collected and lyophilized, samples were then stored at -18  $^\circ\text{C}$  before their chemical and bioactive characterization.

## **7.6. Chemical characterization of the proteins and hydrolysates**

### **7.6.1. Degree of hydrolysis (DH)**

The measurement of DH was carried out according to the OPA method as stated by Nielsen *et al.* (2001) by using serine as a standard for hydrolysis determination. Protein contents of gluten samples were assessed. The percent of DH was calculated according to Alder-Nissen (1986).

### **7.6.2. Size exclusion chromatography**

After filtration with 0.2 nm filters, the samples were subjected to size exclusion chromatography using a 120 mL HiLoad 16/60 Superdex 200 pg column (GE Healthcare Amersham Biosciences) connected to a fast protein liquid chromatography (GE Healthcare Amersham Biosciences). A sample volume of 100  $\mu$ L (1 mg/mL of the freeze-dried peptide mixture) was injected and eluted isocratically at a flow rate of 0.5 mL/min with 50 mM phosphate buffer containing 0.15 M NaCl. Eluted molecules were detected at 215 nm (for gelatin) or 280 nm (for gluten). The mass calibration was performed using a protein mixture (200 to 12.4 kDa) of  $\beta$ -Amilase, Alcohol Dehydrogenase, Albumin, Carbonic Anhydrase and Cytochrome C (Sigma Aldrich).

### **7.6.3. Determination of peptides and glycopeptides molecular weights by Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI/TOF-MS)**

For the MALDI-TOF-MS analysis, the protein hydrolysate samples were diluted tenfold in 50 % (v/v) acetonitrile/water + 0.1 % (v/v) trifluoroacetic acid/water. One microliter of each sample was mixed with 1  $\mu$ L of  $\alpha$ -cyano-4-hydroxycinnamic acid (matrix) (4-HCCA, 10 mg/ml in 50 % acetonitrile/water + 0.1 % trifluoroacetic acid/water). One microliter of the sample/matrix solution was then spotted onto a stainless steel target plate and allowed to air dry. All mass spectra were obtained using a Bruker Ultraflex MALDI/TOF-MS (Bruker Daltonic, GmbH). Ions were analyzed in positive mode after acceleration from the ion source 174 by 25 kV. External calibration was performed by use of the following standard peptide mixture ((M+H)<sup>+</sup> monoisotopic masses are reported in Da): Bradykinin fragment 1-7 (757.3997), Angiotensin II (human) (1,046.5423), synthetic peptide (1,533.8582), ACTH fragment 18-39 (human) (2,465.1989), Insulin oxidized B chain (bovine) (3,494.6513), Insulin (bovine) (5,730.6087) (Sigma Aldrich).

## 7.7. Biological characterization of the peptides

### 7.7.1 Antioxidant properties

#### 7.7.1.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Yen and Wu (1999) and modified by Hsu (2010). Exactly 200  $\mu\text{L}$  of each sample (10 mg/mL for gelatin and meat extract or 1 mg/ml for caseins, referring to the starting protein utilized) were mixed into a test tube containing 400  $\mu\text{L}$  of 0.5 mM DPPH and 1.4 mL of 99.5 % methanol. L-ascorbic acid (0.1 mg/mL) was used to replace sample and referred to as positive control. The whole mixture was mixed thoroughly and incubated for 30 min in the dark at room temperature. Then the absorbance at 517 nm was measured using a spectrophotometer. The percentage of inhibition of radical scavenging activity was calculated as follows:

$$\% \text{ DPPH scavenging activity} = ((\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}) \times 100$$

where:  $\text{Abs}_{\text{control}}$  is the absorbance of reference solution containing only DPPH and water, and  $\text{Abs}_{\text{sample}}$  is the absorption of the DPPH solution with sample after 30 min. Methanol was used as a blank. A lower absorbance represents a higher DPPH scavenging activity.

When reported, the EC<sub>50</sub> value (concentration required for 50 % reduction of activity) was calculated through software Prism 5.

#### 7.7.1.2. Inhibition of linoleic acid peroxidation

Linoleic acid was oxidised in a model system according to Mendis *et al.* (2005) with modifications from Li *et al.* (2007) Reagent mixtures consisting of 1.5 mL of 0.1 M sodium phosphate buffer (pH 7.0), 1.5 mL of ethanol containing 50 mM linoleic acid and 2 mL of 10 mg/mL of gelatin or gluten hydrolyzed samples were prepared. For the positive control, peptide mixture samples were replaced with 2 mM alpha-tocopherol and 2 mM butylated hydroxytoluene (BHT); for the negative control, samples were replaced by water. All the mixtures were kept sealed and incubated in the dark at 40 °C. The oxidation level was measured every 24 h over 7 days by using a ferric thiocyanate method as described by Osawa and Namiki (1981). Exactly 50  $\mu\text{L}$  of the mixture were mixed with 100  $\mu\text{L}$  of 1 M HCl, 50  $\mu\text{L}$  of 30 % (w/v) aqueous ammonium thiocyanate, 50  $\mu\text{L}$  of 20 mM ferrous chloride in 3.5 % HCl and 2.25 mL of 75 % ethanol. The mixture was gently agitated and incubated at room temperature for 5 min before reading the optical



density at 500 nm. The linoleic acid peroxidation was monitored for 7 days following the increase of the absorbance at 500 nm.

### **7.7.2. Angiotensin I-converting enzyme (ACE) inhibitory activity**

The hydrolysis of N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FA-PGG), which represents a substrate for angiotensin converting enzyme, was used to assess the angiotensin converting enzyme inhibitory activity (Bunning *et al.*, 1983). The assay was performed according to Vermeirssen *et al.* (2002) and Shalaby *et al.* (2006). Buffers were made according to Hou *et al.* (2003) and the volumes were reduced to fit into 96-well plates. FA-PGG was dissolved at a concentration of 1.75 mM in 50 mM Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl. The ACE solution (0.25 units/mL) was freshly prepared by adding purified water to a vial containing 0.25 units of enzyme (Sigma). The assay was performed in a 96-well, clear, flat-bottomed polystyrene plate. Exactly 10  $\mu$ L of ACE solution and 10  $\mu$ L of protein hydrolysate samples were placed separately in the well at room temperature. 150  $\mu$ L of pre-heated (37 °C) substrate solution (FA-PGG) was added quickly to each well with an eight channel pipette to start the reaction. The plate was immediately transferred into a SpectroMax M3 plate reader. Enzyme activity at 37 °C was based on the initial linear rate of change in absorbance at 340 nm, recorded every 3 min for 30 min. The control contained all reaction components, and water instead of the protein hydrolysate sample. Blanks with no enzyme (substituted by water) or with no substrate (substituted by 50 mM Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl) were used. The ACE activity was expressed as the slope (m) of the decrease in absorbance at 340 nm and the ACE inhibition (%) was calculated from the ratio of the slope in the presence of sample to the slope obtained without added sample, according to the formula:

$$\% \text{ ACE inhibition} = (1 - (m_{\text{sample}} / m_{\text{control}})) \times 100$$

### **7.7.3. Cytotoxicity of the hydrolyzed and conjugated samples on human HepG2 cells**

HepG2 cells were grown in growth medium (EMEM supplemented with 10 % Fetal Bovine Serum - FBS), 50 units/mL penicillin and 50  $\mu$ g/mL streptomycin) and were maintained at 37°C and 5 % CO<sub>2</sub> as described by Wolfe and Liu (2007). Cells used in this study were between passages 5 and 10. Cytotoxicity was determined using the protocols of the Water Soluble Tetrazolium Salts (WST1) assay by using protein hydrolyzed samples at 3 different concentration, i.e. 0.1, 0.5 and 1 mg/ml. The inhibition of cell growth by the tested protein hydrolyseate samples was expressed as

the percentage of cell viability with respect to control. Concentrations of samples that decreased the absorbance by >10 % when compared to the control were considered to be cytotoxic.

#### **7.7.4. Antimicrobial activity**

The antimicrobial activity of the protein hydrolysate samples was determined by the modified micro-dilution technique against some bacteria, i.e. *Escherichia coli* 555, *Listeria monocytogenes* 56Ly, *Bacillus subtilis* FAD 110 and *Salmonella enteritidis* 155, and yeasts, i.e. *Pichia membranaefaciens* OC70, *P. membranaefaciens* OC71, *Pichia anomala* CBS 5759 and *P. anomala* DBVPG 3003. The bacterial strains were grown at 37°C in Brain Heart Infusion broth (BHI, Oxoid), while those of yeasts at 27°C in Sabouraud (Oxoid). The microbial cultures were prepared at 10<sup>4</sup> cfu/mL in the 96-well microplates. Diluted sample solutions of the protein hydrolysates were dispensed into the wells providing final concentrations in the range of 16 to 0.06 mg/ml for gelatin and 40 to 0.097 mg/ml for gluten. The same tests were performed simultaneously to check for the growth in control conditions (media + microorganism) using water instead of the samples, and sterility control (media + the protein hydrolysate samples tested). The final volume in each well was 200 µL; plates were incubated at the optimal growth temperatures typical for each microorganism for 24 or 48 hours. The MIC values were defined as the lowest concentrations preventing any discernible growth. From the same MIC well, 10 µL of the suspension was plated in Luria Broth LB agar plate and incubated at 37 °C for 24 h (for bacteria) or 27°C for 48 h (for yeasts). The absence of growth in the plate represented the MBC value, thus the capacity to completely kill all the bacteria or yeasts. All the MIC and MBC values were evaluated in triplicate.

In order to obtain information on the antimicrobial activity of protein fractions with specific molecular weights, samples of gelatine, hydrolysed with the strain 111YL4A and collected after 96 hours, were also ultra-filtered with a molecular weight cut-off membrane of 3 kDa (3,900 × g, 20 min, 10 °C) (Amicon Ultra Centrifugal filters (Millipore, Cork, Ireland)). The retentates corresponded to the fraction > 3 kDa, whereas the filtered part corresponded to the < 3kDa. Both the fractions were used for MIC analysis.

#### **7.8. Statistical Analysis**

Data were reported as means ± standard deviation of at least triplicates. Means obtained were analysed by one way analysis of variance (ANOVA), separated by Duncan test using STATISTICA statistics software (ver. 6.0). Means were considered significant when  $p < 0.05$ . As

for model study, the results from UV–Vis spectra were analysed by using bidimensional hierarchical clustering analyses (heat map) as reported by Hrynets *et al.* (2013).

# **CHAPTER 8.**

## **RESULTS**

## 8.1. Evaluation of the proteolytic activity of *Yarrowia lipolytica* on different proteins

Due to its physiological attributes and biotechnological potentialities, *Yarrowia lipolytica* is one of the most studied "non-conventional" yeasts and it is classified as "generally recognized as safe." It is able to grow in different environments and many strains have been isolated from oil-polluted environments, rivers and foods such as refrigerated foods, cheese, irradiated meats, sausages, salami and other products (Bankar *et al.*, 2009).

In addition to its potential use as adjunct as ripening agent of dairy and meat products (Suzzi *et al.*, 2001; Lanciotti *et al.*, 2005; Patrignani *et al.*, 2007; 2011a; 2011b), *Y. lipolytica* has been exploited also for the production of single cells proteins for feeds and the treatment of olive mill wastewaters (Lanciotti *et al.*, 2005). Without any doubt, the most studied features of this microorganism are the production of lipases (Ota *et al.*, 1982), citric acid and  $\gamma$ -decalactone (Cohelo, 2010). In particular some strains have been described as producers of "cold" lipases (Parfene *et al.*, 2011) and this aspect is progressively getting interesting for industrial applications (Joseph *et al.*, 2007). Proteases are another class of enzymes that are produced and secreted by *Y. lipolytica*. In particular, an acidic extracellular protease (AXP) and an alkaline extracellular protease (AEP) have been mainly studied (Young *et al.*, 1996). The main element that discriminates the production of AEP or AXP is the environmental pH. In general, neutral and high pH (6-9) lead to AEP production, while low pH (2-6) favours AXP (Ogrydziak, 1993). However, both proteases are similarly induced at the end of the exponential phase (González-López *et al.*, 2002). The screening of novel enzymes that are capable of generating compounds with specific and improved functional bioactivities is constantly necessary. In particular this kind of research can have a relevance for the food industry as a possible strategy to transform cheap food by-products into active compounds with an increased market value.

In my research, seven strains of *Y. lipolytica* have been used to evaluate the capability of the extracellular proteases to produce bioactive peptides from different matrices. In particular gelatin, gluten, milk and meat by-product proteins have been considered. The possibility to obtain biologically active peptides, endowed with technological properties from different matrices through the use of lactic acid bacteria has been reported. But on the other hand the biotechnological properties of yeast concerning this aspect have been not explored. The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Proteases from *Aspergillus oryzae* have been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. An asparaginase isolated from *E. coli* is used to eliminate

asparagine from the blood stream in various forms of leukaemia. In addition microbial proteases have been used for the synthesis of dipeptides (Barros *et al.*, 1999) and tripeptides (So *et al.*, 2000) Nutritionally enhanced meat products have been obtained by using microbial proteases able to restructure and improve appearance, texture and nutritional quality of animal parts of poorer technological quality (Marques *et al.*, 2010)

### 8.1.1. Preliminary characterization of proteases by Zymogram

The Zymograms of the 72 h old supernatants of the 7 strains obtained both on casein and gelatin are shown in figures 5A and 5B, respectively. In **figure 5A**, two main bands were detected, one at low molecular weight (MW) nearby 37 kDa (1IIYL4, 16B, Y10) and one at high MW higher than 200 kDa (Y16, 1IIYL8 and Y10). While the first one corresponds to the acid protease typical of *Y. lipolytica* reported by many Authors (Young *et al.*, 1996), the other one presents a band with an unusual MW never reported in this species. The strains 1IIYL4 and 16B were characterized by an intense protein clearness zone nearby 37 kDa, while the strain PO19 showed a weak band suggesting either a lower activity or a lower release of the protease. The strain CLCD, isolated from salami, did not show any apparent activity. A similar behaviour was observed in the presence of gelatin although the bands were less intense (**fig. 5B**).

### 8.1.2. "Cold attitude" of the proteolytic enzymes

In order to evaluate the cold attitude of the proteases, the supernatants were spotted onto agar plates containing gluten or skim milk as substrates for the enzymatic reactions. The activity was evaluated on the basis of the clearance of the media after 10 days of incubation at 6 °C. A 72 h sample incubated at 27 °C was used as a control. In addition, the ability to hydrolyse gelatin in the same conditions was assessed in tubes measuring the depth of liquefaction.

At the optimal temperature (27 °C), 1IIYL4A, 16B, Y16 and 1IIYL8A were the strains with the highest ability to hydrolyse the skim milk and gluten, whereas CLCD was not active under these conditions. The incubation at 6 °C did not affect the final capability of all the strains to work at this low temperature, except for 1IIYL8A on gluten agar (**tab. 6a** and **6b**)

Regarding gelatin, similar behaviours were observed at 27 °C for all the strains, except for Y16 and 1IIYL8A which showed a reduction of their activity. On the other hand, the strain Y10, which was unable to hydrolyze both skim milk and gluten, showed a significant activity. The reduction of the temperature moderately affected the final degree of the proteolysis (**tab. 6c**). In fact the strains 1IIYL4 and 16B presented a band at 37 kDa while the 1IIYL8A and Y16 showed an

intense band at 200 kDa. By the comparison of these results with the two Zymogram it is evident that the two proteins visualized display their activity both on gelatin and casein.

### 8.1.3. Proteolytic profiles generated by *Y. lipolytica* proteases

Four different protein matrices (meat proteins,  $\alpha$ -casein,  $\beta$ -casein and gelatin) were resuspended in water and incubated with the supernatants of the 7 strains of *Y. lipolytica* for 72 h at 27 °C. The proteolytic profiles were evaluated by SDS-PAGE electrophoresis.

**Figure 6** reports the proteolysis of  $\alpha$ -casein.  $\alpha$ -casein includes two main isoforms  $\alpha$ s1 and  $\alpha$ s2 that migrate in the SDS PAGE electrophoretogram with a final MW of around 34 kDa. After incubation with *Y. lipolytica* supernatants, a significant proteolytic activity was detected. In particular, 11IYL4A, 16B and Y10 significantly reduced the intensity of the casein bands. A partial hydrolysis, with the formation of several detectable bands having different low MWs, was observed with CLCD, PO19 and Y16. Comparing these strains, CLCD and PO19 were the least active since both the bands of  $\alpha$ s1- and  $\alpha$ s2- caseins were still present. Taking into consideration the bands present in the control, the bands at 30 and 34 kDa were still visible in the samples of the strains 11IYL4A CLCD, PO19 Y16 and 11IYL8A while they disappeared in 16B and Y10. The band of 24 kDa disappeared in all the samples except for strains PO19, whereas the band at 22 kDa was detected in CLCD, PO19 and Y16. New bands at 20 and 21 kDa were formed with PO19 and Y16, and a band at 18 kDa was detected with CLCD, Y16, 11IYL8A and PO19. It can be noticed that the strains 11IYL4A and 16B, which showed in the zymograms a 37 kDa protease, were characterized by similar breakdown profiles and a strong proteolysis. On the other hand the strains Y16 and 11IYL8A, which displayed in SDS-PAGE electrophoretogram a lower proteolytic activity, were characterized in the zymograms by a protease having a MW higher than 200 kDa.

In **figure 7** the  $\beta$ -casein profile of hydrolysis is reported. Also against this protein, the strains 11IYL4A and 16B, characterized by the 37 kDa protease, displayed a very strong proteolytic activity. In fact, not only the bands of  $\beta$ -casein including the principal one at 31 kDa but also all the bands having MWs ranging from 25 to 15 kDa disappeared. Also the strain PO19, having a weaker 37 kDa protease in the zymogram, displayed a good proteolytic activity also if at lower extent than the former. The comparison of all the proteolytic profiles of  $\alpha$ - and  $\beta$ -caseins demonstrated that a strong activity was displayed by all the strains of *Y. lipolytica*, except for Y10, against  $\beta$ -casein. In fact, also the strains Y16 and 11IYL8A, characterized by the protease > 200 kDa, showed a strong proteolytic activity against  $\beta$ -casein.

In **figure 8**, the meat protein breakdown profiles obtained with the supernatants of the various strains are showed. With this protein matrix, two different behaviours can be identified: the

strains 1IIYL4A, 16B and PO19, characterized by the 37 kDa protease, displayed a strong proteolysis with the formation of peptides having MW lower than 10 kDa and the fading of higher MW peptides. In particular, the 75, 70, 47, 26, 17 kDa MW bands faded, whereas those at 65, 37 and 24 kDa disappeared. On the contrary CLCD, Y10, Y16, 1IIYL8A showed only a weak hydrolytic activity as most of the bands were as intense as the control samples.

#### 8.1.4. Proteolysis of gelatin

The strains having the highest activity in gelatin agar plate (tab. 6), were incubated with gelatin for 72 h and the corresponding hydrolysed samples were analysed through SDS-PAGE gel electrophoresis (**fig. 9**).

**Figure 9** showed that not all the strains were able to hydrolyze gelatin at the same extent. Looking at the higher MW, above 100 kDa, it can be observed that the intensities of the two typical bands of gelatine were reduced in the samples 16B and 1IIYL8A, while they were completely absent in 1IIYL4A. As far as the bands with the intermediate MWs (75-20 kDa), none of the samples had the 37 kDa band. Moreover some differences can be detected in the sample 16B, where the band at 23 kDa disappeared, while the 25 kDa one became more intense. The same behaviour was partially shown by the sample Y16. Comparing all the samples together only the sample 1IIYL4A showed a general increment in the intensities of the bands in the range 37-20 kDa. Instead, molecules below 10 kDa were generated by all the samples, also if with different extents.

In order to have a wider overview on these profiles, size exclusion chromatography was applied to the gelatin hydrolyzed samples. The chromatograms reported in **figure 10** showed how in many samples, the peak corresponding to gelatin (having an elution volume of 40 mL) reduced compared to **figure 10 A**, which represents the gelatin before its hydrolysis. As the extent of the hydrolysis increased, more peaks having lower MWs appeared on the left side of the chromatogram with higher elution volumes. In some samples, such as CLCD, Y10 and PO19 (**fig. 10 D, F, G**) there was mainly an increment of the peaks eluting between 50 and 100 mL, whereas peaks eluting between 90 and 140 mL represented the main one for the samples 1IIYL4A, 16B and 1IIYL8A (**fig. 10 B, C, E**). Among all these conditions, the sample that showed the best hydrolysis with the formation of smaller MWs was the 1IIYL4A. An overlay of the two chromatograms (before and after hydrolysis) is reported in **figure 11**.

On the basis of the comparative results of the size exclusion chromatograms the strain 1IIYL4A was chosen for a more detailed characterization of the peptides obtained.



### 8.1.5. Characterization of the products of gelatin hydrolysis obtained by *Y. lipolytica* 111YL4A protease

The gelatin hydrolysis by proteases from *Y. lipolytica* 111YL4A was followed over time (time 0, 48, 72, 96 hours) in stirring conditions (useful for the enzyme activity). In **figures 12** the SDS-PAGE electrophoretograms (**A**) and size exclusion chromatograms (**B**) of the hydrolysates collected over time are reported, respectively. In particular, **figure 12 B** represents a closeup of the final elution volume of the size exclusion analysis in order to show better the progressive increment of the lowest MWs (lower than 10 kDa). It is possible to see that three main regions are distinguishable: the first one, ranging between 90-115 mL, the second one between 115-125 mL and the third one between 125-135 mL. These three areas were integrated and the relative abundances are reported in the **figure 13**. Among the three areas, the first one, ranging between 90-115 mL, reached its maximum abundance already at 48 h and it seemed constant at 72 and 96 h. However, looking at **figure 12B** it is possible to see that even if the final area of 72 h and 96 h samples is the same, the peak at 48 h is smaller and broader. This latter aspect has to be taken into consideration because it suggests that different MWs are present in that area. On the contrary, in the peaks of 72 and 96 h samples ranging between 90-115 mL, the MWs should be more similar among them. A sensible decrease in the abundance of the second peak (ranging between 115-125 mL) and a sensible increase of the third peak (ranging between 125-135 mL) was observed over time.

The following analysis were performed only on the peptides with low MWs because it has been reported that many peptides having antioxidant, anti-hypertensive, antimicrobial, and cell modulating activities are characterized by MWs below 10 kDa (Li *et al.* 2008; Ryan *et al.*, 2011). However, the size exclusion technique is not able to separate and identify the exact MW of the peptides, and particularly those lower than 10 kDa. For this reason the samples were analysed also with MALDI-TOF after ionization (**fig. 14 A and B**). The reduction of MWs is well evidenced by a shift of the signals towards the left side of the spectrum (**fig. 14 B**). In particular all the peaks ranging between 7 and 3.3 kDa disappeared, while new signals mainly ranging from 4 to 1 kDa appeared.

Taking into consideration all the signals detected, **table 7** reports the flow over time of the peptide MWs. In this table the evolution of some peptides, present in at least two sampling times, is reported. All the red numbers derive from the time 0, in particular from the native gelatin added with the supernatant of *Y. lipolytica* 111YL4A; in green the peptides produced after 48 h, and in blue that ones released at 72 h.

The MALDI-TOF results (**fig. 14, tab. 7**) are comparable with the SDS-PAGE image (**fig. 12A**) where it was highlighted that strong differences in the hydrolytic profiles occurred only after

how 72 h. Moreover, at 96 h the main peptides are either newly originated or deriving from those detected at 72 h, although the number of peptides identified at 72 and 96 h was similar.

### 8.1.6. *In vitro* bioactivity evaluation of the peptides

Preliminary investigations on the biological properties of the peptides obtained were performed on the gelatin hydrolysates produced by extracellular protease of *Y. lipolytica* 111YL4A.

#### 8.1.6.1. DPPH radical scavenging activity

A DPPH assay was performed to establish if the degree of proteolysis, and in particular the mixture of peptides whose MWs had been determined with MALDI-TOF analysis, affected the antioxidant properties of the hydrolysates collected after 0, 48 72 and 96 h of incubation.

According to **figure 15** a progressive increase of the DPPH activity with the hydrolysis time was observed. In particular the antioxidant activity, which was  $19.7 \pm 6\%$  at time 0 with respect to the ascorbic acid chosen as a reference sample, attained values of  $39.4 \pm 1.4$  and  $48.3 \pm 0.5\%$  after 72 and 96 h, respectively. The latter differences were significant ( $p < 0.05$ ).

#### 8.1.6.2. Inhibition of the linoleic acid peroxidation

The capability to inhibit the peroxidation of linoleic acid by the peptide mixture collected over time was also evaluated. As previously shown by the DPPH assay, the progressive incubation time gave rise to a significant increase ( $p < 0.05$ ) of the antioxidant activity in particular for the samples at 72 and 96 h (**fig. 16**). On the other hand the sample collected at time 48 h showed a behaviour much more similar to that at time 0. The  $\alpha$ -tocopherol, chosen as a reference for the inhibition of the peroxidation, has been used at 0.89 mg/ml. Its activity was higher than that of the peptide mixture, but it is necessary to outline that I compared the antioxidant properties of a pure compound with a mixture of peptides of 20 mg/ml (freeze-dried powder/water) having a peptide content of about 1.3 mg/ml. Moreover, it can be assumed that only few peptides included in the mixture were endowed with antioxidant properties.

#### 8.1.6.3. Cytotoxicity activity in human cells

The assessment of the cytotoxicity is a prerequisite for the use of a new ingredients, such as peptide mixtures having technological properties, in food formulations. On the basis of the **figure 17** potential cytotoxic molecules are generated only when the proteolysis was extended over 72 h and the cells HepG2 were exposed to a concentration of 1 mg/ml.

#### 8.1.6.4. Antimicrobial activity

The bacteria species used for evaluation of the antimicrobial activity were *Escherichia coli* 555, *Salmonella enteritidis* 155, *Listeria monocytogenes* 56Ly, *Bacillus subtilis* FAD 110, *Pichia membranaefaciens* OC71, *P. membranaefaciens* OC70, *Pichia anomala* CBS 5759, and *P. anomala* DBVPG 3003. None of the bacteria resulted sensitive to the compounds at any concentration tested. However, it has been observed a progressive inhibition of the growth of some strains of *Pichia membranaefaciens* in relation to the degree of hydrolysis. In particular MIC values of 8 mg/ml were observed for gelatin hydrolysates collected at 48 and 72 h. The extension of the hydrolysis up to 96 h resulted in a two fold reduction of the MIC value. Moreover, when the sample at 96 h was separated into fractions with MWs higher than 3 kDa, a MIC value of 2 mg/ml was detected. On the contrary, no activity was observed for the fraction below 3 kDa.

## 8.2. Improvement of peptides bioactivity

The technological attitudes of the peptide mixtures can be regarded as promising, but need to be improved. A new frontier to be explored, to enhance the properties of peptides, is their transformation into glycopeptides. The enhancement of the functional and biological properties in the glycoproteins with respect to the native form has been explored (Bielikowicz *et al.*, 2010; Liu *et al.*, 2012) and the importance of this peptide transformation on some physiological processes (such as immune-system, inflammation, brain development, endocrine system and fertilization) has been emphasised (Spiro, 2002).

The reaction involving sugars and amino acids can be triggered either through enzymes or spontaneously occur under specific conditions. Glycosylation, as an enzyme driven process, is one of the main processes occurring in eukaryotic and prokaryotic cells. In fact, it is known that the majority of proteins are subjected to post-translational modifications, such as the attachment of glycans. This reaction is crucial in many biological pathways (Spiro, 2002). On the contrary, "glycation" is the term universally used to define the chemical bounding of sugars with proteins or peptides. This second reaction is spontaneous, and can occur both in human body and in food systems (Liu *et al.*, 2012). As reviewed by Oliver *et al.* (2006), glycation via the Maillard reaction can improve several important functional properties of food proteins (Liu *et al.*, 2012). One of the main requirements for the glycation through the Maillard reaction is the use of high temperatures or a prolonged heat treatment, in order to favour the reaction between sugars and aminoacids. A controlled Maillard-induced glycosylation is fundamental to limit the progress of the reaction into undesired advanced stages; advanced Maillard reactions may result in reduced food digestibility

(Erbersdobler *et al.*, 1981), formation of mutagenic compounds (Brands *et al.*, 2000), development of off-flavors (Moor and Ha, 1991), and excessive browning (Guerra- Hernandez *et al.*, 2002). (Wang and Ismail, 2012). Due to the low reactivity of sugars and amino acids, high temperatures are necessary to promote the reaction (Wang and Ismail, 2012). On the other hand, amino-sugars demonstrated to have a higher reactivity comparing to normal sugars (Kraehenbuehl *et al.*, 2008). The amino-sugar glucosamine (GlcN) can be obtained through the hydrolysis of chitosan, the main by-product from shrimp and other crustacean shells processing. The acetylated form of GlcN is fundamental for the building the bacterial cell wall and the particular cartilage in the human body (Wang SX *et al.*, 2007).

Aminosugars binding, exploited through the transglutaminase (TGase) enzymatic process, has been already proposed, in particular between GlcN and peptides. However, results have been inconclusive (Jiang and Zhao, 2010). The above mentioned enzyme (TGase), discovered in eukaryotic and prokaryotic cells, and extensively used in food processing, is responsible for the acyl-transfer reaction between the  $\gamma$ -carboxamide group of glutamine residue and the primary amino group. Moreover, this reaction can be driven towards the formation of inter- and intra-molecular cross-linkages (if the primary amino group derives from other amino acids, such as lysine) or deamidation (in absence of primary amino groups, a molecule of H<sub>2</sub>O is used). The first two types of bonds are stable and resist to proteolysis (Greenberg *et al.*, 1991)

Wheat gluten, a by-product of the wheat starch industry, is massively produced worldwide. Due to its modest price, it can industrially compete with milk and soy proteins, as an economic protein source (Kong *et al.*, 2007). Because of their functional properties, such as solubility, foaming and emulsifying capacity, the wheat gluten peptides obtained by hydrolysis attracted the interest of food industries (Kong *et al.*, 2007, Wang *et al.*, 2007) focused on the preparation of hypoallergenic nutritional mixtures (Daya *et al.*, 2006). Additional studies have reported antioxidant properties of gluten hydrolyzates, including the capability to inhibit the linoleic acid peroxidation or to quench DPPH radical. Moreover, Koo *et al.*, (2011) demonstrated that gluten hydrolysates exhibited taste-enhancing properties. Wang and Ismail (2012), instead, reported the production of some partially glycosylated wheat protein. My strategy to improve the bioactivity of peptides obtained by enzymatic proteolysis of gluten and other proteic by-products was based on glycosylation/glycation. The experiment has been developed in two steps:

- 1) a single peptide was employed to prove the occurrence of the glycation (through a mild Maillard reaction) and glycosylation (TGase-mediated), and to optimize the main reaction parameters to be pursued (such as concentration, temperature). This process is totally new and never ever exploited before,

2) a more complex system, based on gluten hydrolysates, was taken into consideration and the bioactivities of the glycopeptides mixture obtained was assessed.

### 8.2.1 Model system

To establish the novel conjugation method, and in order to determine the most appropriate conditions for the reaction, a simplified model system involving glucosamine (GlcN) and the dipeptide glycine-glutamine (Gly-Gln) was considered. It has been reported that GlcN at high temperature is able to undergo rearrangements, auto-condensation processes and Maillard reaction (in presence of amino-acids), with a yield higher than with the simple sugars. Hence, in the first part of this model, different concentrations of GlcN were tested to detect the threshold conditions for the formation of its side-products and hence to avoid their production.

The reaction involving the peptide Gly-Gln (chosen because of its simple structure and the presence of glutamine) and GlcN (molar ratio 1:1, 1:3 and 1:10, dipeptide/glucosamine) was monitored over time (0, 3.5 and 7 hours) at 25 or 37°C. As control, GlcN alone was incubated at the same conditions and monitored over time. The UV-spectra (280 - 420nm) of the samples during incubation are shown in **figure 18**. The temperature and the sugar concentration played a fundamental role in both the increased and progressive shift of absorbance to high wavelength values. The highest absorbance was revealed with the 1:10 ratio at 37°C (**fig. 18 A**). As the sugar ratio increased, its augmented by-products created interferences when monitoring the sugar-peptide interaction and evolution. On the other hand, the ratios 1:3 and 1:1 at 25 or 37 °C promoted a suitable arrangement (**fig. 18 B** and **18 C**, respectively). At 1:3 ratio, an increase in the absorbance, due to the sugar auto-condensation and rearrangement, was reported. However, these absorbance increases were < 1 after 3.5 hours (compared to 1:10, where Abs ranged from 1 to values higher than 2.5). To monitor the reaction behaviour, the wavelengths corresponding to the typical Maillard markers were selected, as follows: 280 nm for furosine, 320 nm for the soluble pre-melanoidin compounds, and 420 nm for the melanoidins (Rada-Mendoza *et al.*, 2002; Wijewickreme *et al.*, 1997). The results are summarized in a heat map (**fig. 19**). The conditions able to generate significant increases of the optical densities at 280, 320 and 420 nm clustered together and were characterised by a high amount of sugar (1:10 molar ratio peptide/sugar), long incubation time (> 3.5 h) and high temperature (37°C). The sugar concentration (1:10 peptide/sugar ratio) promoted the highest increase in the selected wavelengths, independently from the peptide presence. For instance, following the sample N37C or C37C over time, a progressive increase was initially observed in the absorbance at 280 nm, and then at 320 and 420 nm, from the 3.5 h time point. In contrast, N37B\_7 and C37B\_7 reached a comparable absorbance at 280 nm after 7 h. This results

confirms that glucosamine generates either Maillard reaction intermediate products (MRP) or condensation products; thus, less native sugar would be available for the conjugation. Temperature reduction to 25°C required a longer incubation time (7 h) to show an increase of the absorbance (which was detectable in N25C\_7 and C25C\_7). Samples with a low peptide/sugar ratio (1:3 and particularly 1:1) had lower or no detectable glucosamine degradation/rearrangement products. Therefore, the lowest peptide/sugar ratio (1:1) and the lowest incubation time (3.5 h) were selected as the most suitable conditions to carry on the glycosylation reaction.

To confirm the conjugation (both glycation and glycosylation), the peptide was incubated with and without TGase in the presence of glucosamine, and then analysed using Orbitrap-LC/MS. The spectrum of peptide plus glucosamine and TGase after incubation is reported in **Figures 20**. The MW of reactants are indicated as follows: m/z 180.08 for GlcN and m/z 204.09 for Gly-Gln. To calculate the glycosylated form of the peptide, the typical release of H<sub>2</sub>O in the Maillard reaction was considered. Hence, a mass shift of +161 Da (i.e. 179-18 Da) from the original MW of the peptide was predicted. One mole of ammonia was released from TGase-catalysed reactions (Ramos *et al.*, 2001). Thus, the MW of glycopeptides resulting from glycosylation of peptides with GlcN, in the presence of TGase, can be predicted based on a mass shift of +162 Da (179 - 17 Da).

In **figure 20**, products of both glycation and glycosylation are reported: m/z 366.15 is related to the MW of the glycosylated glycopeptide, whereas m/z 365.16 corresponds to the glycosylated glycopeptide. The Orbitrap-mass spectrum shows also a protonated ion at 162.07 m/z, which is a typical adduct of glucosamine. As showed in **figures 20**, the intensity of glycoconjugates produced are low compared to the intensity of the reagents. However, this result does not reflect the actual concentration of glycopeptides produced. In fact, according to Itoh *et al.* (2009), glycopeptides are poorly ionized in mass spectrometry. Moreover, their MS signal can be reduced or suppressed if a mixture of peptides and glycopeptides is analysed. The final hypothesised mechanism of the glycosylation and glycation involving GlcN and Gly-Gln is summarised in **figure 21**.

### **8.2.2. Characterization of wheat gluten and its hydrolyzates**

The first step of the process was the gluten hydrolysis. In this phase, focused on the optimisation of the process, I preferred to use commercial proteases (Alcalase and Flavourzyme) instead of *Yarrowia lipolytica* enzymes which have not been purified yet. The chromatograms obtained by size exclusion analysis of the hydrolysates confirmed the enzyme activities. In **figure 22**, it is possible to observe the shift of the peaks from high to low molecular weights after

hydrolysis. The estimated degree of hydrolysis (DH) by Alcalase was  $4.70 \pm 0.2\%$ , whereas the Flavourzyme DH was  $11.85 \pm 1.2\%$ .

### 8.2.3. Chemical evaluation of glycoconjugation of gluten peptides by MALDI-TOF/TOF-MS

After the glycation/glycosylation process, the MW profiles of the samples were determined using MALDI-TOF-MS. The analysis of the controls (GAH and GFH) revealed different profiles when Alcalase or Flavourzyme were used. The Alcalase peptides ranged from 500 to 3000 Da, whereas those in Flavourzyme were from 300 to 4100 Da. This distribution was expected, because Alcalase is an endopeptidase that generates a homogeneous distribution of MW, while Flavourzyme produces a more scattered profile including low and higher MW, due to its mixture of endo- and exo-peptidases.

To estimate the glycation of the peptides, all the signals present in the spectra have been checked for their mass before and after the reaction. In particular the MWs of glycopeptides were calculated adding a mass shift of 161 Da (or any multiple of it, in case of additional sugars attached) to the original peptide MWs, due to the production of 1 mole of water. Hrynets *et al.*, (2013) proposed that a potential glycation reaction, with an initial release of water, and a subsequent ammonia molecule, may occur with an additional mass shift of 144 Da to be added to the peptide MW. Hence, a mass shift of 162 Da in the MW of the peptides was considered to identify the glycosylated peptide in the presence of TGase.

The total amount of glycopeptides estimated when the ratio peptide:GlcN was 1:1 (both at 25 and 37°C) was 5 fold higher than the amount of glycopeptides observed at 1:3 ratio (5 vs. 25); in general, GAT25 (1:3) and GFT25 (1:3) presented a minor number of glycopeptides. This would be because GlcN tends to auto-condensate resulting in a lower free GlcN amount available for the glycation/glycosylation process when present at higher concentrations (Zhu *et al.*, 2007). Among the samples with the lowest (1:1) ratio, at least three different glycopeptides were produced through glycation, independently from the temperature used (**fig. 22 and 23**). Only one glycosylated peptide was estimated in GFT37 (**fig. 24**). The MW profiles of the glycopeptides were different if their origin was considered: gluten Alcalase glycopeptides were mainly constituted of 1322 and 1676 Da glycopeptides, whereas gluten Flavourzyme glycopeptides presented MWs ranging from 1775 to 3112 Da.

## 8.2.4. *In vitro* bioactivity evaluation of glycopeptides mixtures

### 8.2.4.1. DPPH Radical Scavenging Activity

The quenching activity of the samples against DPPH was assessed and the EC<sub>50</sub> values estimated are reported in **table 8**. As shown in **table 8**, all the samples that underwent glycation improved their antioxidant activity, because neither GAH nor GFH (peptides not glycated) had a concentration-dependent activity. Among GA samples, GAC37 was the most active, followed by GAT25, GAT37 and GAC25. Nevertheless the final EC<sub>50</sub> values were not significantly different amongst them. GFT37 was the most active among GF samples. Anyway all the samples incubated with TGase (GFT25 and GFT37) had a EC<sub>50</sub> lower than the corresponding control with no enzyme added (GFC37 and GFC25). The reaction mixture with 1:3 peptide/sugar ratio, where glycopeptides had not been generated, was used as control, in order to assess if other Maillard reaction products could prevent oxidation. None of the tested samples showed any activity similarly to GAC37 and GFC37, as reported in **table 8**.

### 8.2.4.2. Inhibition of Linoleic acid peroxidation

The capability of the samples to inhibit linoleic acid peroxidation was evaluated in comparison to  $\alpha$ -tocopherol, generally used as positive control. The results obtained are reported in **Figures 25** and **26**. On the basis of the monitoring of the absorbance over time, GAC25 (1:1) was the only sample with a low lipid oxidation level (**fig. 25**). It was observed that during the first three days of the trial, no differences were detectable between GAH and GAC25. However, the oxidation curves significantly diverged over time ( $p < 0.05$ ), reflecting the improved capability of the conjugated sample to inhibit the linoleic acid peroxidation. All the other GA samples (both 1:1 and 1:3 ratio) did not show any significant antioxidant activity ( $p < 0.05$ ).

The GF samples (1:1) (**fig. 26**) not only did not show any antioxidant activity ( $p < 0.05$ ), but also one of them, GFC25, resulted as the most significantly pro-oxidative sample. In fact, it reached the highest absorbance after three days, followed by a rapid decline associated with the decomposition of (hydro) peroxide.

### 8.2.4.3. Anti-ACE activity

Samples were tested for their anti-hypertensive activity, measuring the capacity to inhibit the ACE enzyme (**fig. 27**). In the samples GAH and GFH (with a concentration of 10 mg/ml) the enzyme inhibition was  $64.1 \pm 1.95$  % and  $50.3 \pm 1.00$ %, respectively. The glycation had negative effects in almost all the samples, in particular in those corresponding to the GF. In fact, a half



reduction in the final activity was measured in all the GF samples except for the GFC37, whereas in the GAC37, GAT25 and the GAT37 a significantly lower reduction was detected ( $p < 0.05$ ).

#### 8.2.4.4. Antimicrobial activity

The antimicrobial activity of the samples, before and after glycation/glycosilation, was tested against two bacterial strains: *Escherichia coli* and *Bacillus subtilis* (Gram negative and Gram positive, respectively). The peptide mixtures before the treatment (GAH and GFH) did not show any antimicrobial activity. However, despite the absence of activity in the 1:3 samples, the majority of the 1:1 ratio samples resulted active against *E. coli* (**tab. 9**). Specifically, all the conjugated GF samples inhibited the cell growth at 40 mg/ml, and GFC25 and GFT37 were also bactericidal. Among the GA samples, GAT37 was both inhibitor and bactericidal (40 mg/ml) for *E. coli*. None of the samples (1:1) was active against *B. subtilis*.

It can be outlined that the peptide mixture includes peptides not glycated and about three glycopeptides. Therefore the MICs values are overestimated and presumably the MIC really effective against *E. coli* are lower than that obtained.

#### 8.2.4.5. Cytotoxicity in human cells

The cytotoxicity of the peptides and glycopeptides (1:1 peptide/sugar ratio) was tested against human hepatocyte HepG2, using three different concentrations (1 mg/ml, 0.5 mg/ml and 0.1 mg/ml). When comparing GAH vs. GFH, no effects on cell viability were shown on human carcinoma cells (**fig. 28**), even at 1 mg/ml ( $p < 0.05$ ). However, enhanced cytotoxicity was detected in all the conjugated GF samples, resulting in a significant reduction of the cell viability of approximately 50% at 1mg/ml ( $p < 0.05$ ). High cytotoxicity was detected in GFT25 and GFT37 at 0.5 mg/ml. Similarly GAT37 showed a strong activity at 1 mg/ml ( $p < 0.05$ ), while the cytotoxicity of the other GA samples was not significant.

## 8.3 Tables

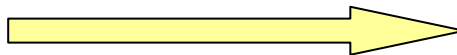
**Table 6.** Proteolytic activity of different strains of *Y. lipolytica* towards three different matrices (**a**, milk proteins; **b**, gluten; **c**, gelatin) at two different temperature (27 and 6 °C). The proteolytic activities on skim milk and gluten agar were evaluated measuring the clear zone formed. The proteolytic activity on gelatin was estimated measuring the volume of liquefied gelatin. The values were obtained after three (for 27 °C) or ten (for 6 °C) days of incubation.

strain	(a) skim milk agar		(b) gluten agar		(c) gelatin	
	27 °C	6 °C	27 °C	6 °C	27 °C	6 °C
<b>CLCD</b>	0.5*	0.5*	0.5*	0.5*	0.5**	0.5**
<b>1IILY4A</b>	3	2.6	2.3	2.3	1.5	0.8
<b>16B</b>	2.5	2.3	2	2	1.8	0.7
<b>PO19</b>	0.5	0.5	1.3	0.5	0.5	0.5
<b>Y10</b>	0.5	0.5	0.5	0.5	1.9	0.5
<b>Y16</b>	3.2	2.3	2.3	2.5	0.8	0.5
<b>1IIYL8A</b>	3	2.3	2	0.5	0.5	0.5

\* refers to the clear zone diameter expresses in cm; \*\* refers to liquefied volume in a glass tube expresses in cm

**Table 7.** The evolution of some peptides, present in at least two sampling time, are reported. All the red cells correspond to the time 0, in particular to the native gelatine added with the supernatant of *Y. lipolytica* 111YL4A; the green ones correspond to the peptides produced after 48 h, the blue ones correspond to those released at 72 h.

MW+ (Da)	Y 0	Y 48	Y 72	Y 96
1143				
1449				
1606				
1748				
2078				
2224				
2766				
3298				
3371				
3535				
3766				
3839				
3921				
4678				
4842				
5059				



**Table 8.** DPPH scavenging activity of Alcalase (GAH) and Flavourzyme (GFH) hydrolysates (control samples not conjugated) and the corresponding conjugated peptides obtained without enzyme (GAC25, GAC37, GFC25, GFC37) or with enzyme (GT25, GAT37, GFT25, GFT37). The EC50 values (mg/ml) are reported. All these samples refer to the mixture obtained incubating peptide/glucosamine in a 1:1 ratio. Since all the 1:3 ratio did not show any activity, GAC37 1:3 is just reported as an example.

	Alcalase			Flavourzyme		
	sample	EC50 (mg/ml)	R <sup>2</sup>	sample	EC50 (mg/ml)	R <sup>2</sup>
Before glycation	<b>GAH</b>	nd *		<b>GFH</b>	nd	
After glyaction with higer ammount of sugar (1:3)	<b>GAC37</b>	nd		<b>GFC37</b>	nd	
After glycation	<b>GAC25</b>	27.45	0.98	<b>GFC25</b>	32	0.99
	<b>GAC37</b>	20.25	0.99	<b>GFC37</b>	35.3	0.97
After +Tgase	<b>GAT25</b>	21.82	0.99	<b>GFT25</b>	22.1	0.99
	<b>GAT37</b>	25.15	0.99	<b>GFT37</b>	10	0.64

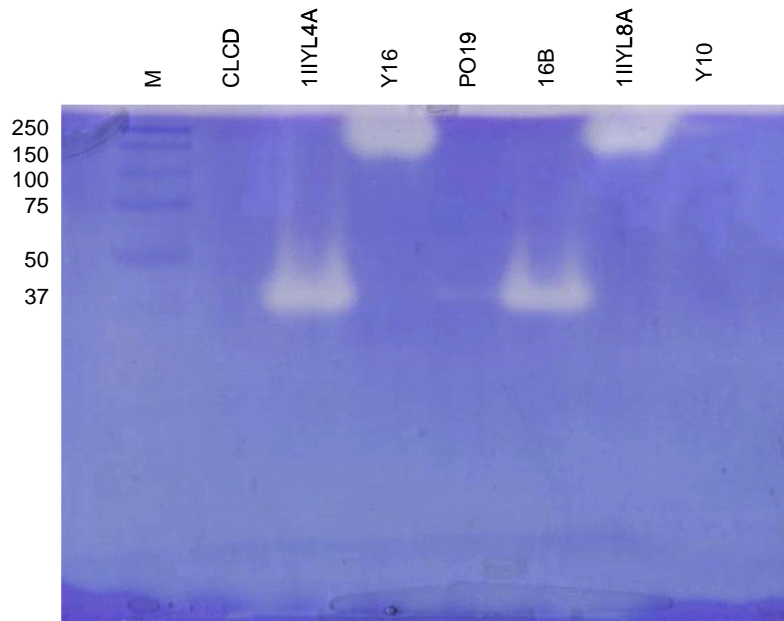
\* not detectable.

**Table 9.** Antimicrobial activity of Alcalase (GAH) and Flavourzyme (GFH) hydrolysates (control samples not conjugated) and the corresponding conjugated peptides obtained without enzyme (GAC25, GAC37, GFC25, GFC37) or with enzyme (GT25, GAT37, GFT25, GFT37) against *E. coli*. MIC and MBC (both expressed in mg/ml) refer to effect exerted by the mixture obtained incubating peptide/glucosamine in a 1:1 ratio.

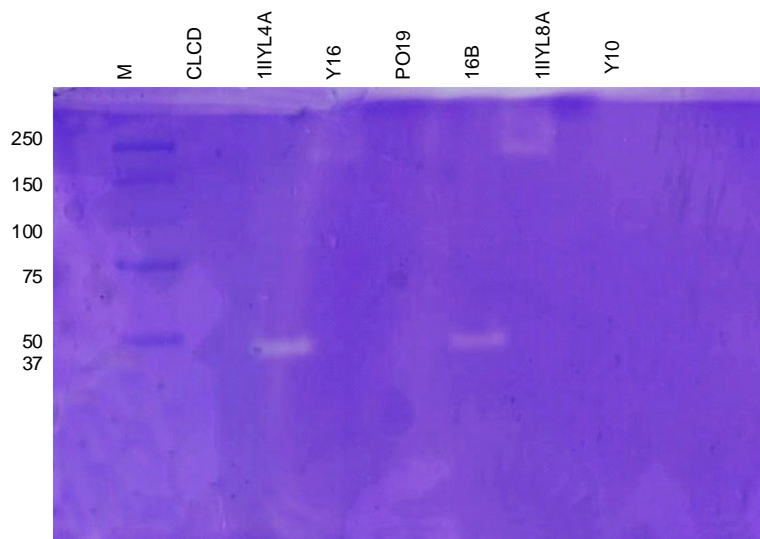
		<i>E. coli</i>				
	sample	MIC	MBC	sample	MIC	MBC
Before glycation	<b>GAH</b>	-	-	<b>GFH</b>	-	-
After glycation	<b>GAC25</b>	-	-	<b>GFC25</b>	40	40
	<b>GAC37</b>	-	-	<b>GFC37</b>	40	-
After +Tgase	<b>GAT25</b>	-	-	<b>GFT25</b>	40	-
	<b>GAT37</b>	40	40	<b>GFT37</b>	40	40

"-" no activity was observed for concentration  $\leq 40$  mg/ml

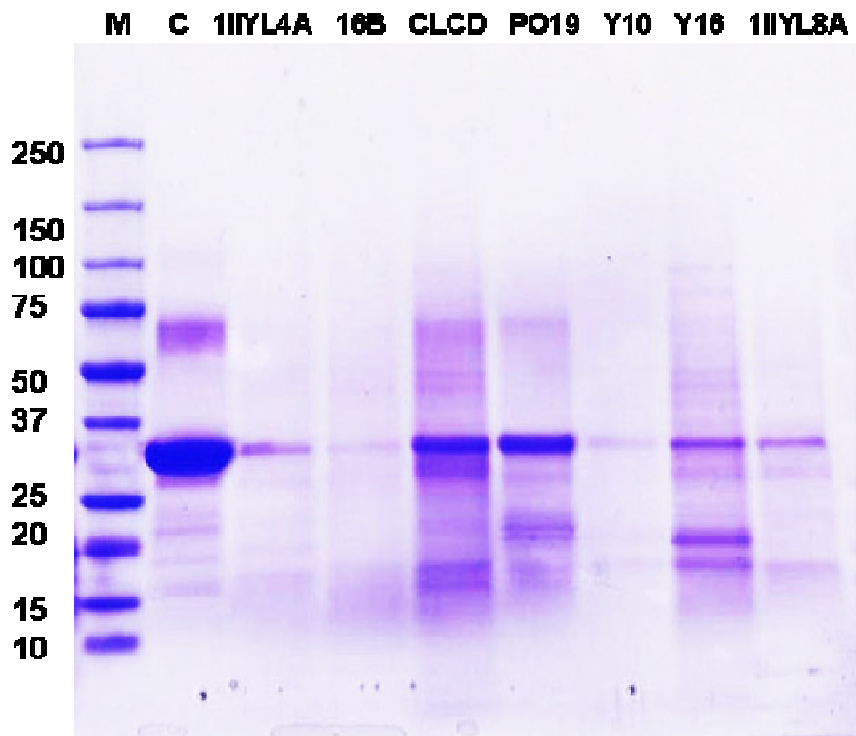
## 8.4 Figures



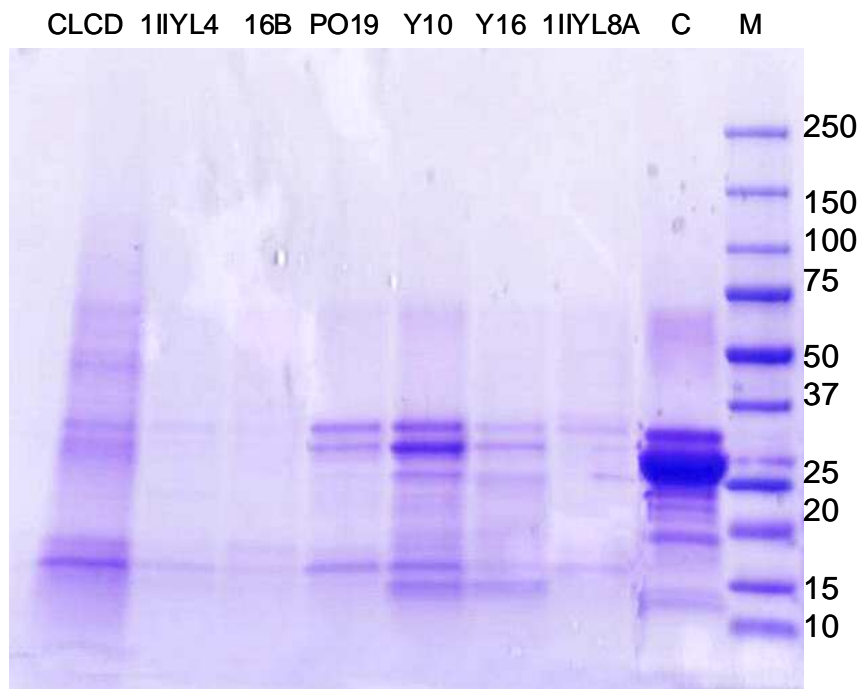
**Figure 5A.** Casein zymography of different *Y. lipolytica* culture supernatants. Culture supernatants were recovered by centrifugation of a 4 days culture. The label of the strains is reported above each lane. M: Molecular weight markers are reported in kDa.



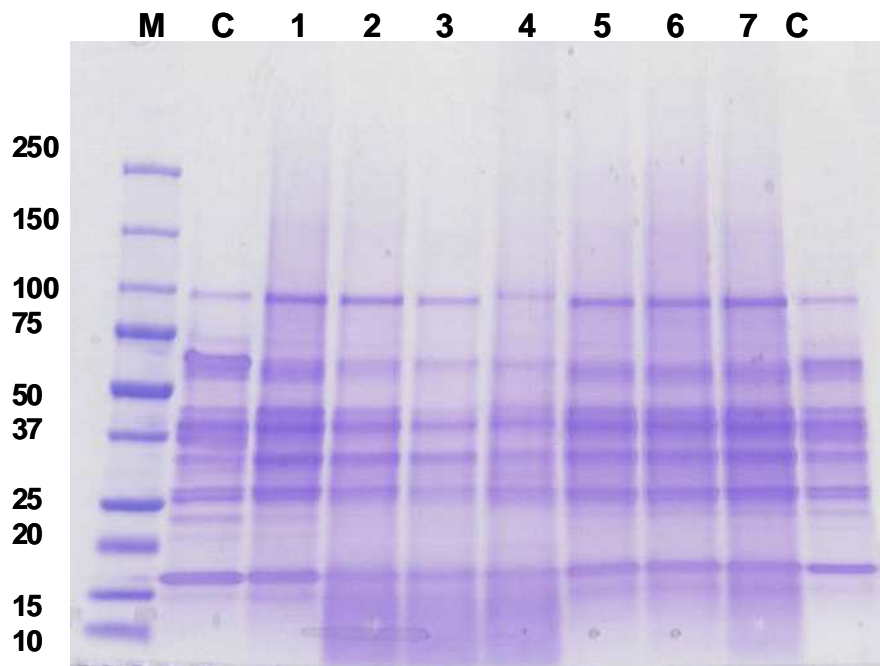
**Figure 5B.** Gelatin zymography of different *Y. lipolytica* culture supernatants. Culture supernatants were recovered by centrifugation of a 4 days culture. The label of the strains is reported above each lane. M: Molecular weight markers are reported in kDa.



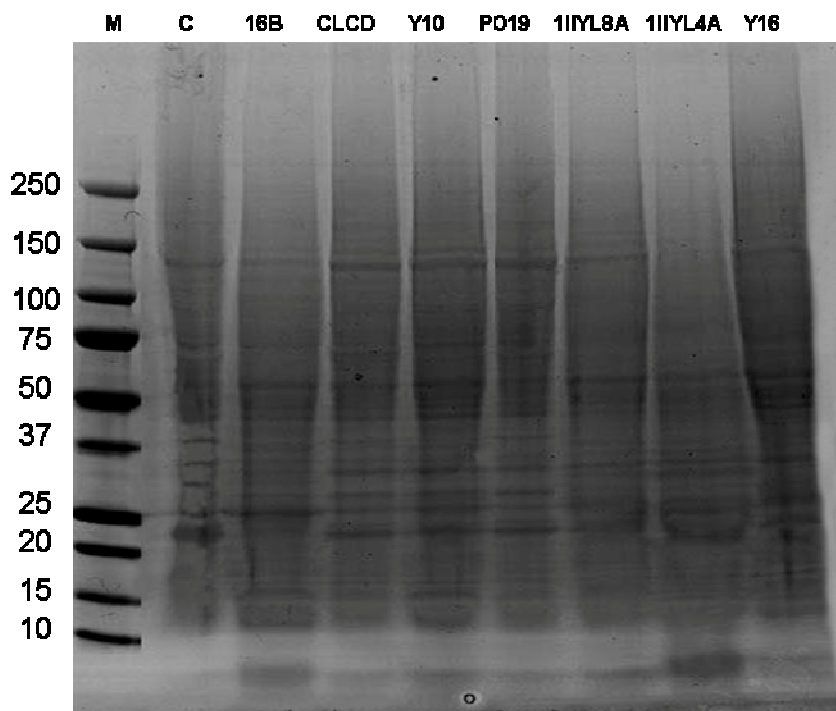
**Figure 6.** SDS-PAGE electrophoresis of  $\alpha$ -casein hydrolyzed by different strains of *Y. lipolytica* at 72 h of incubation. C:  $\alpha$ -casein alone, M: Molecular weight markers are reported in kDa.



**Figure 7.** SDS-PAGE electrophoresis of  $\beta$ -casein hydrolyzed by different strains of *Y. lipolytica* at 72 h of incubation. C:  $\beta$ -casein alone, M: molecular weight markers were marked in kDa.

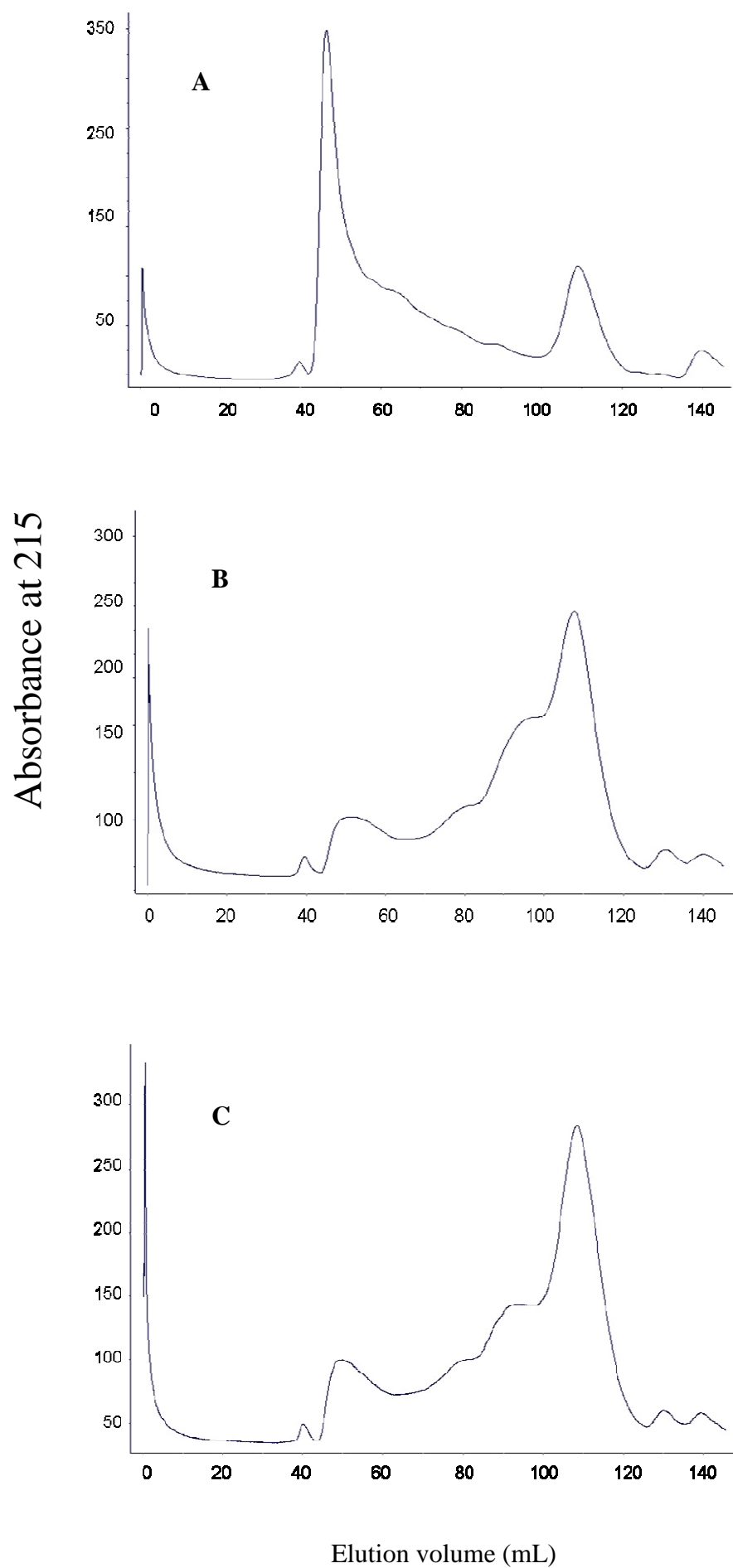


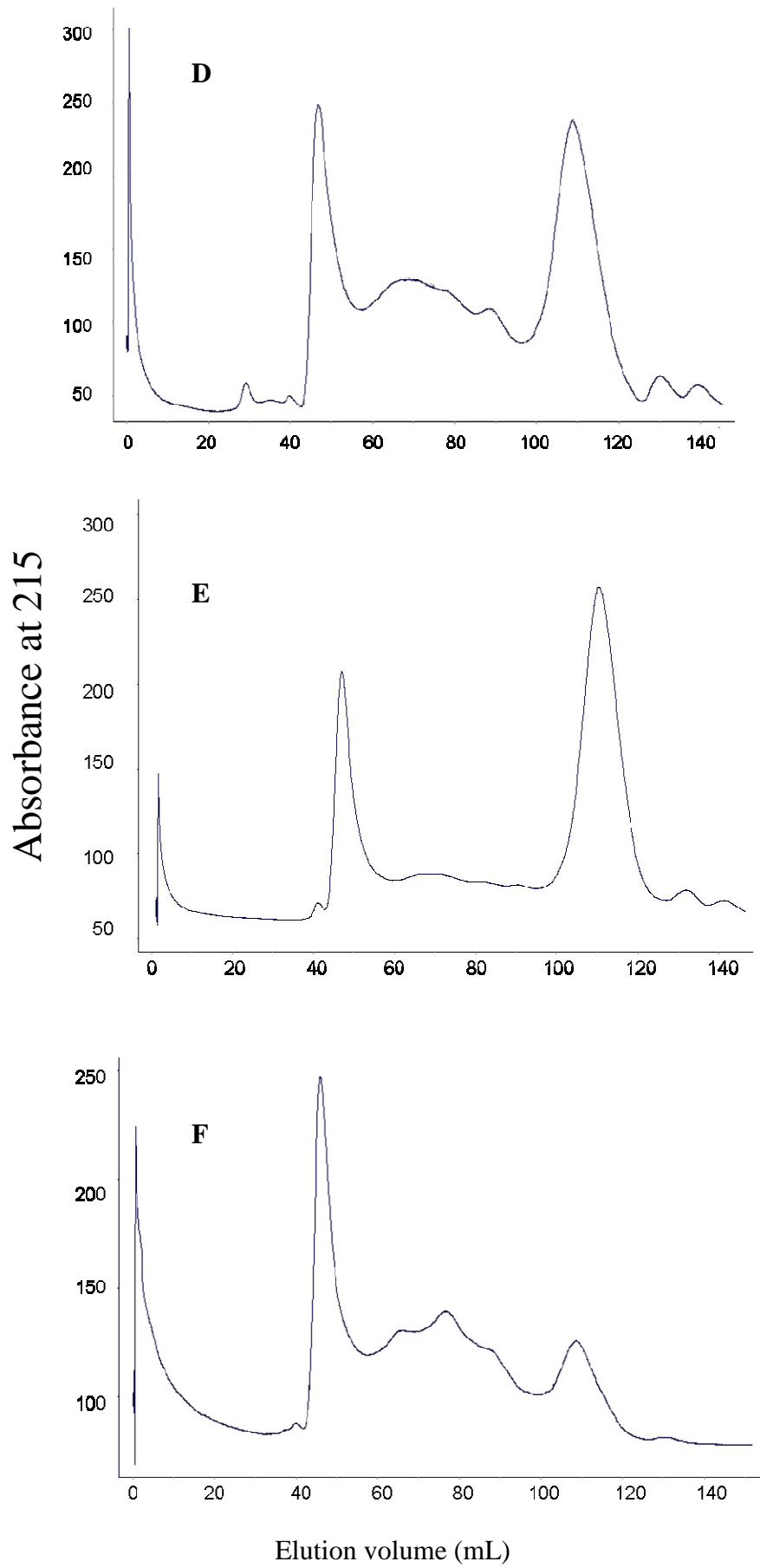
**Figure 8.** SDS-PAGE electrophoresis of meat protein extracts hydrolyzed by different strains of *Y. lipolytica* at 72 h of incubation. C: meat protein extracts alone, M: molecular weight markers were marked in kDa. 1, CLCD; 2, 1IIYL4A; 3 16B; 4, PO19; 5, Y10; 6, Y16; 7, 1IIYL8A.

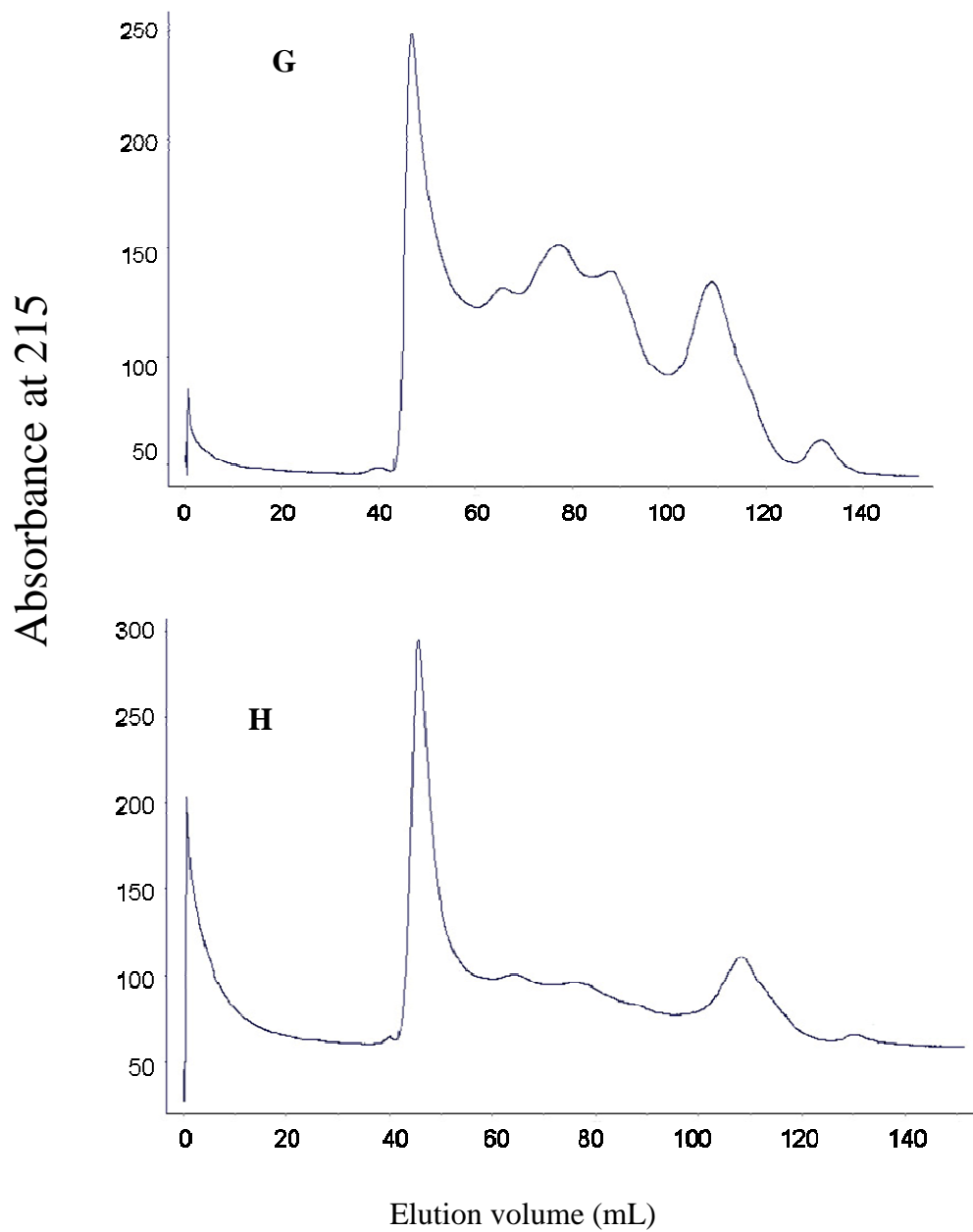


**Figure 9.** SDS-PAGE electrophoresis of gelatin hydrolyzed by different strains of *Y. lipolytica* at 72 h of incubation. C: gelatin alone, M: molecular weight markers were marked in kDa.

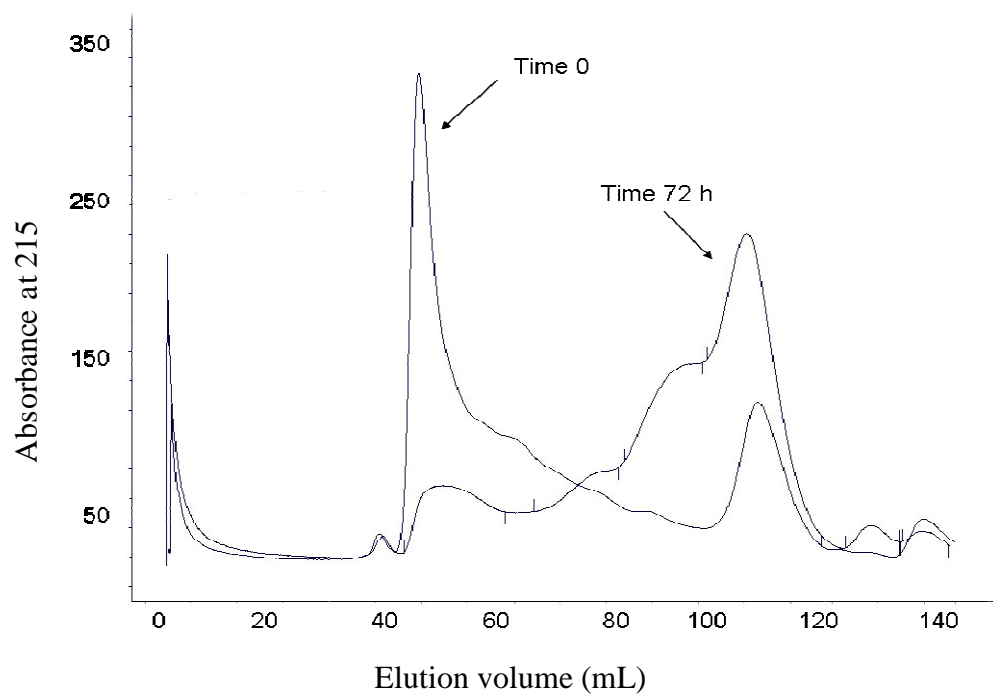




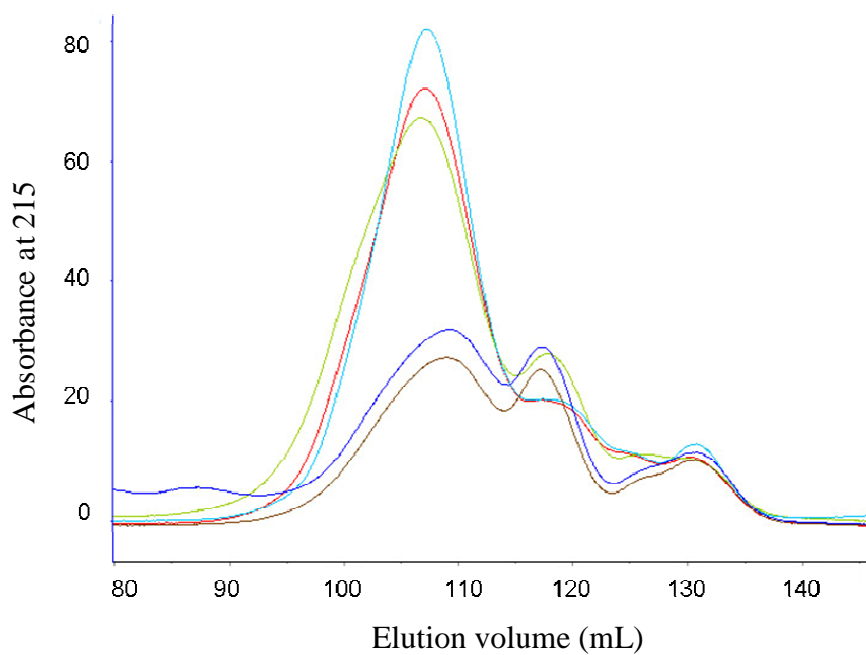
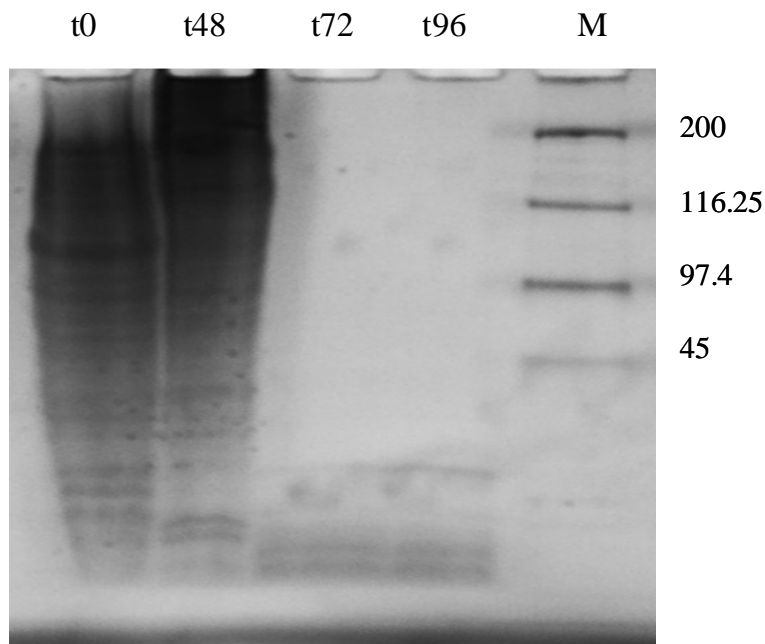




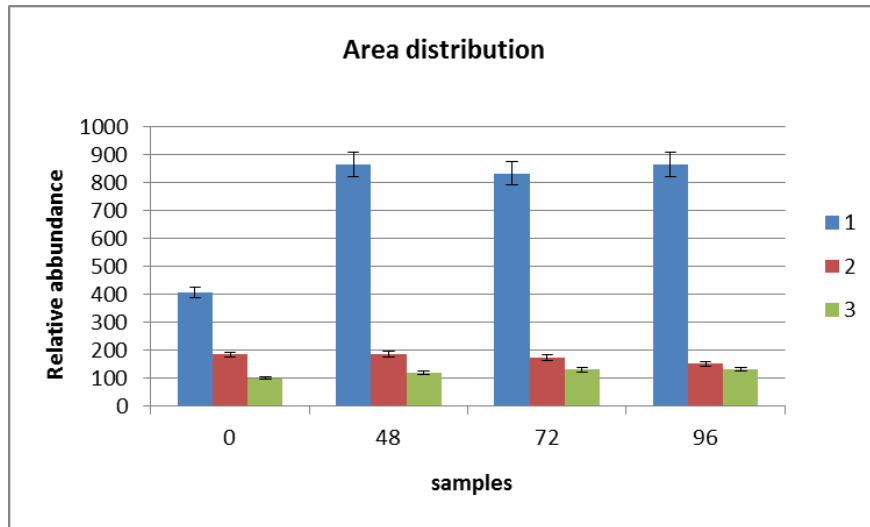
**Figure 10.** Size exclusion chromatograms of gelatin incubated for 72 h with the supernatant of different strains of *Y.lipolytica*: Gelatin alone (A), 111YL4A (B), 16B (C), CLCD (D), 111YL8A (E), Y10 (F), PO19 (G), Y16 (H).



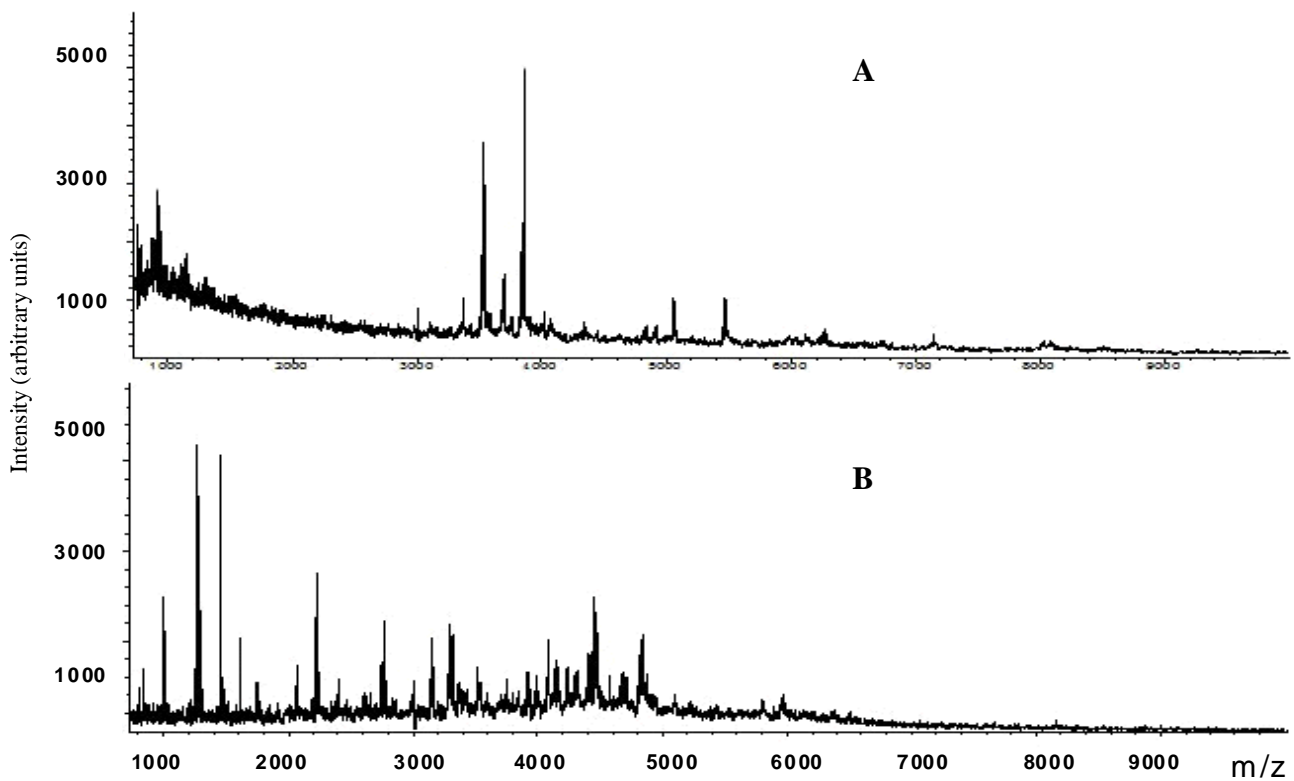
**Figure 11.** Overlay of two size exclusion chromatograms representing the gelatin at the starting point (time 0) and after hydrolysis with the supernatant of 1H4L4A (time 72 h).



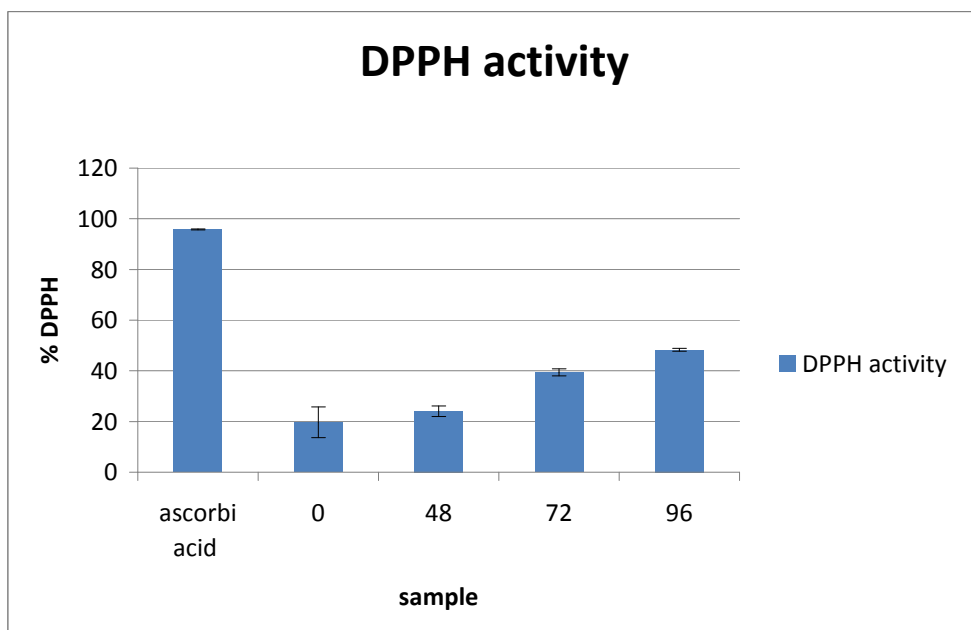
**Figure 12.** Gelatin hydrolysed by *Y. lipolytica* 11IYL4A collected over time (time 0, 48, 72 and 96 h). (A) SDS-PAGE electrophoretogram of the samples after different incubation times. M: molecular weight markers are marked in kDa. (B) Overlay of size exclusion chromatograms of the gelatin hydrolysed by *Y. lipolytica* 11IYL4A collected over time time 0 (blue and brown), 48 (green), 72 (red), 96 h (light blue). In particular a closeup of the MW < 25 kDa (estimated on the basis of a standard mixture previously injected) is shown.



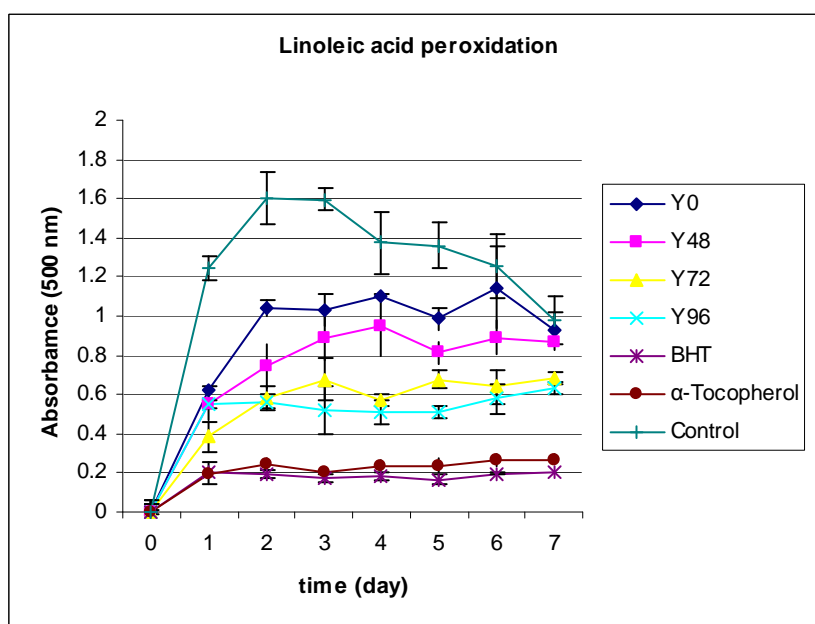
**Figure 13.** Relative abundance of the three main area referred to the figure 8B. In particular, range between 90-115 mL (1), 115-125 mL (2) and 125-135 mL (3) for each chromatogram were considered.



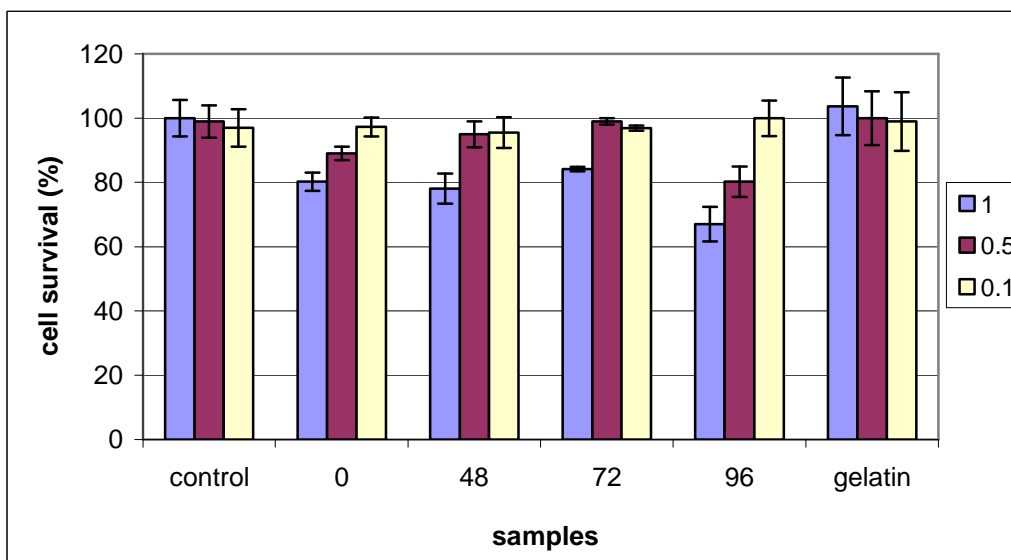
**Figure 14.** MALDI-TOF spectra of ultrafiltered (cut-off < 10 kDa) gelatin hydrolysed by *Y. lipolytica* 1IIYL4A. The spectrum A refers to time 0, the spectrum B refers to time 96 h.



**Figure 15.** DPPH radical scavenging activity of gelatin incubated with the supernatant of *Y. lipolyica* 111YL4A and collected over time (0, 48, 72 and 96 h). Ascorbic acid (0.1 mg/ml) was used as reference standard. Values are expressed as means  $\pm$  SD in triplicate experiments.



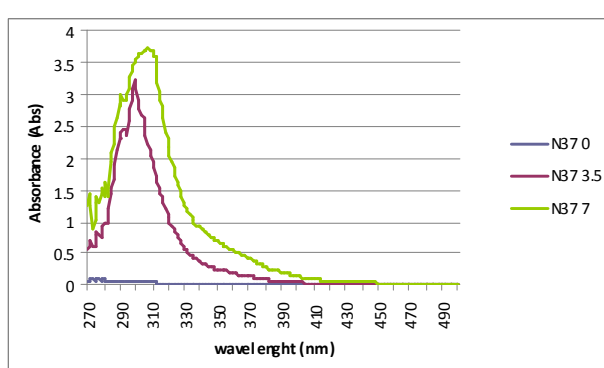
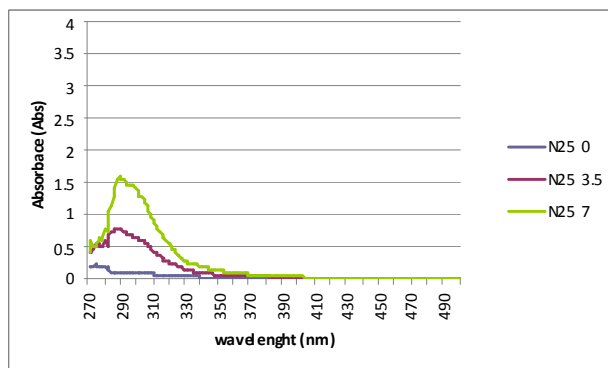
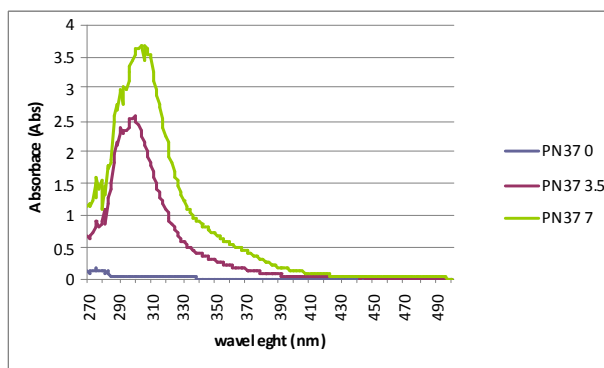
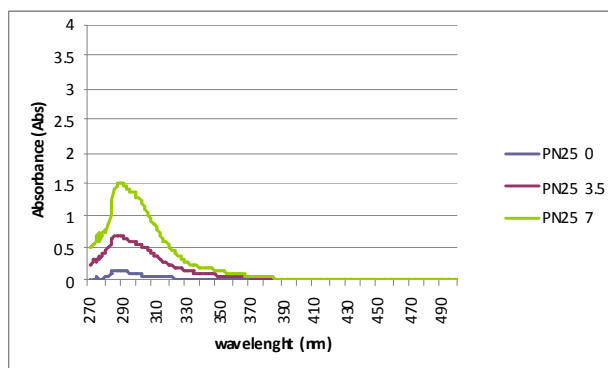
**Figure 16.** Antioxidant activity, in a linoleic acid oxidation model system, played by gelatin hydrolysates by *Y. lipolyica* 111YL4A protease collected over time (Y0, 48, 72, 96 h). BHT and  $\alpha$ -tocopherol were used as reference standards. Control was prepared with linoleic acid and water instead of the sample. Values are expressed as means  $\pm$  SD in triplicate experiments.



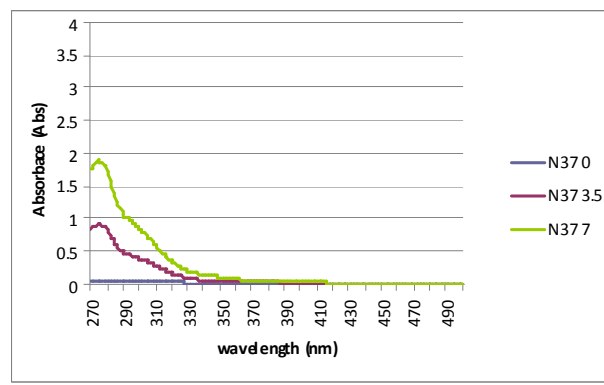
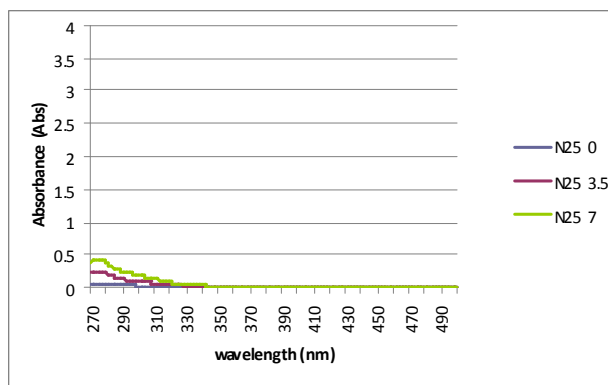
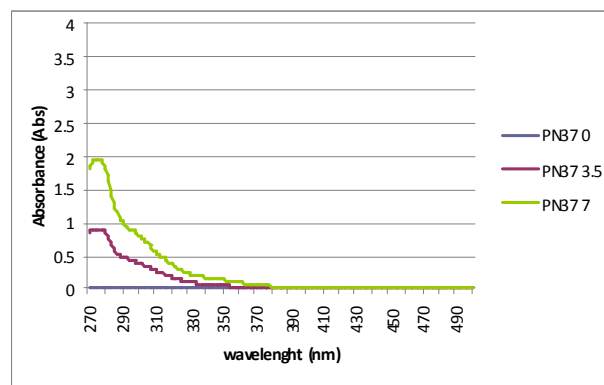
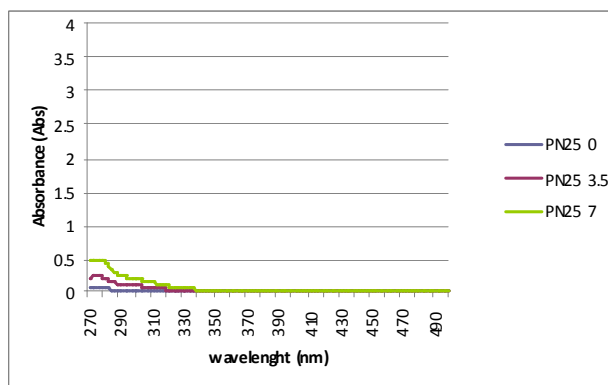
**Figure 17.** The cytotoxic effect of the gelatin hydrolysates, obtained at different times, against Hepatocellular carcinoma (HepG2). Cells were treated with different concentrations of the gelatin hydrolyzed (1, 0.5 ad 0.1 mg/ml). Values are expressed as means  $\pm$  SD in quadruplicate experiments. Control refers to untreated cells; gelatin refers to cells treated just with gelatin.



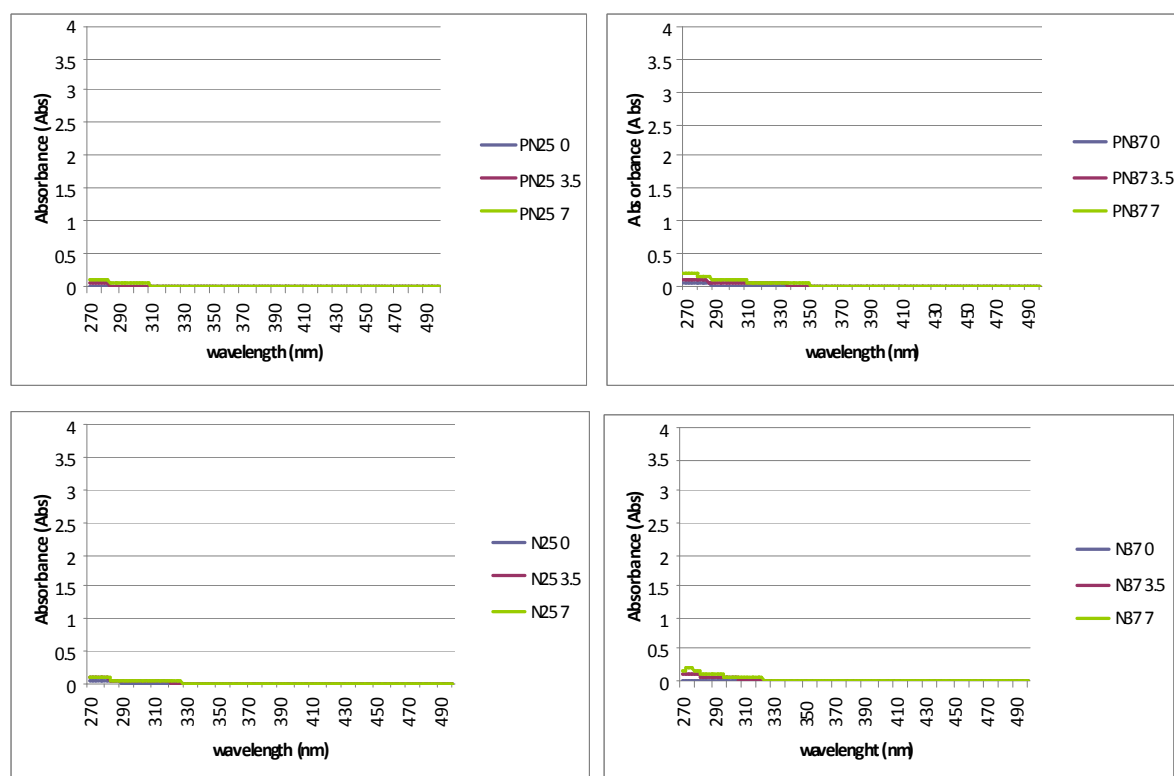
# A



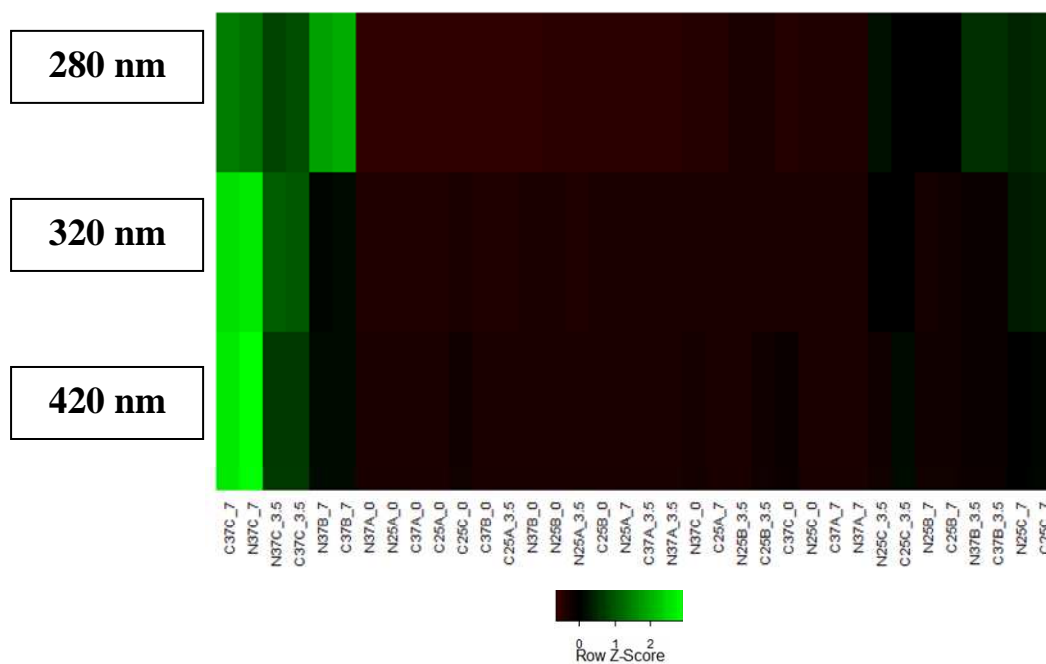
# B



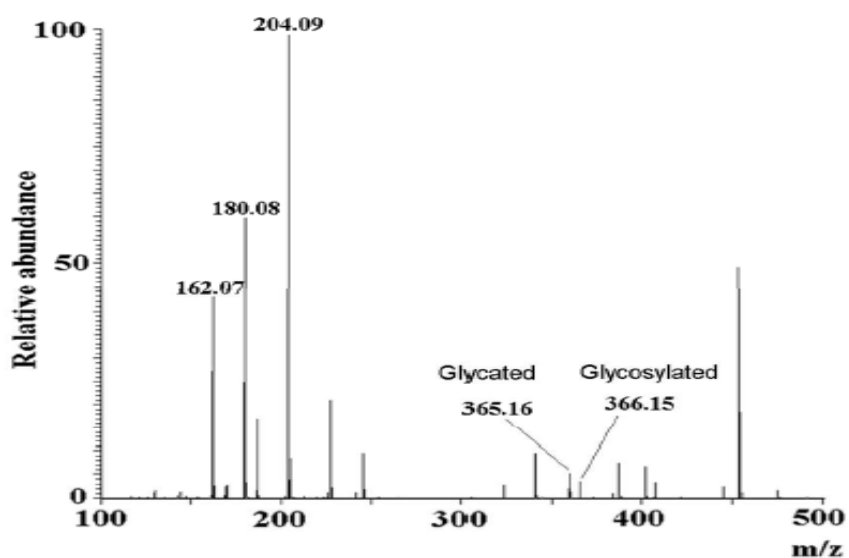
C



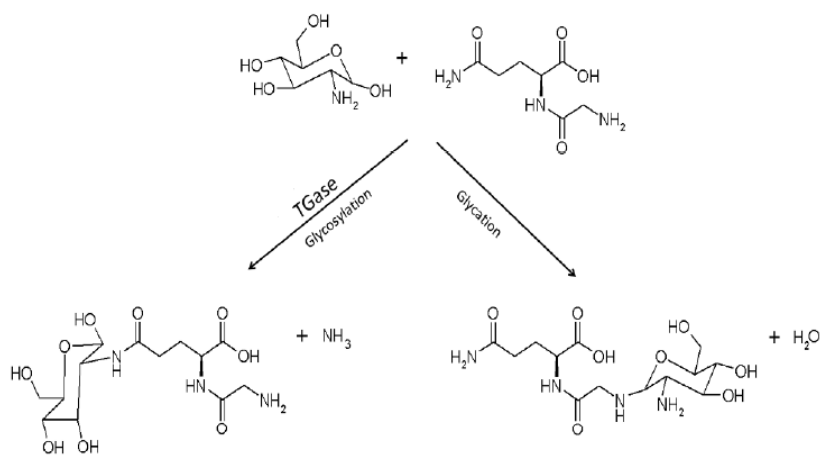
**Figure 18.** UV-spectra of samples absorbance (Abs) of the Glucosamine (N) and peptide plus glucosamine (PN) were monitored during incubation at two different temperatures (25 or 37°C), for different incubation times (0, 3.5 and 7h). A, B and C represents the 1:10, 1:3 and 1:1 molar ratio of peptide/GlcN or GlcN alone, respectively. Values are expressed as triplicate experiments.



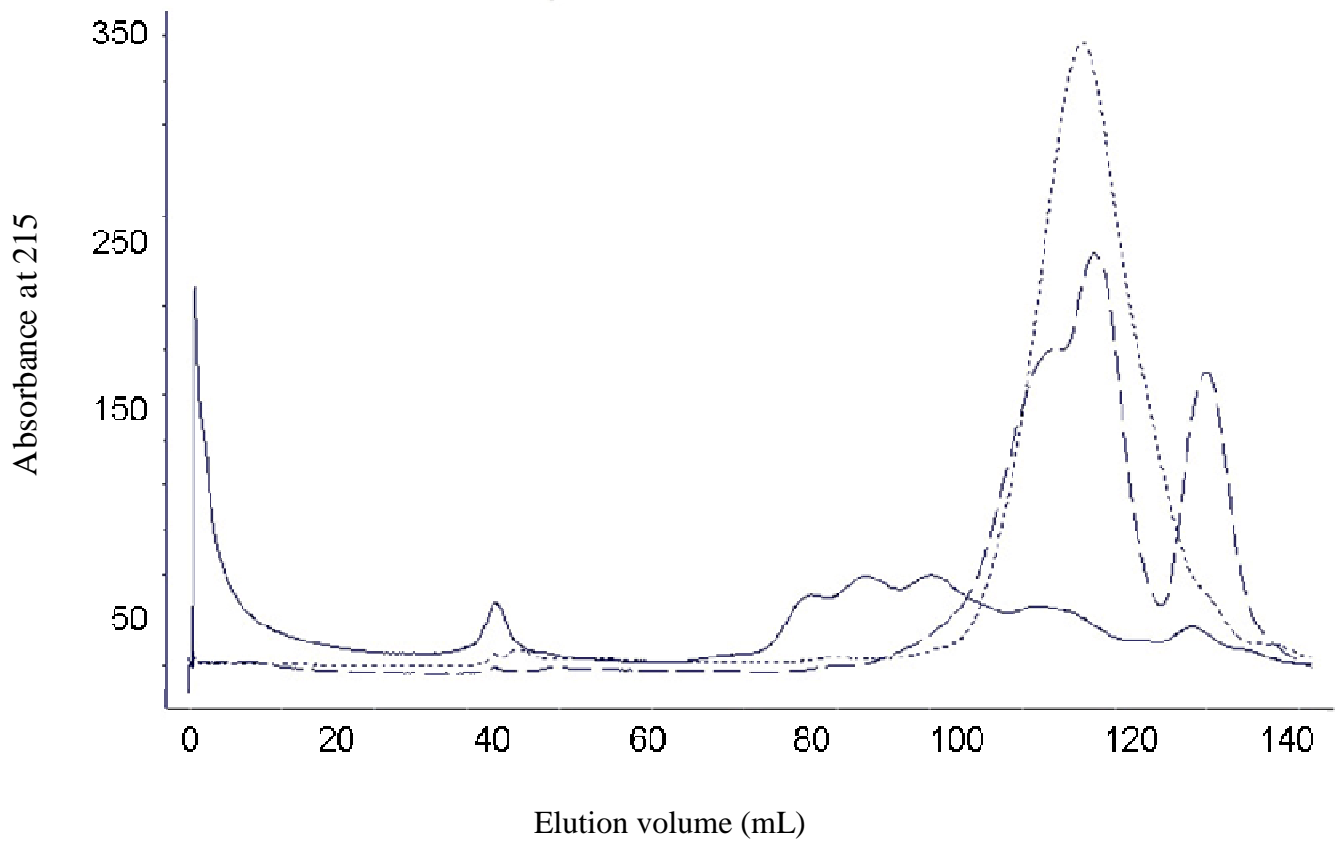
**Figure 19.** Hierarchical clustering of peptide–sugar (or just sugar) UV–Vis absorbance data for Gly-Gln and glucosamine (GlcN). The vertical axis represent individual wavelengths (280, 320 and 420 nm). The horizontal axis represents individual glycation treatments. The colours in each cell indicate the absorbance of a particular sample relative to the mean level from all samples for the specific wavelength. The colour scale extends from bright green (maximum absorbance) to bright red (minimum absorbance). The samples GlcN (N), GlcN plus peptide (C) were followed during the incubation, under different temperatures (25 or 37°C), different peptide:GlcN ratios (1:1 (A) , 1:3 (B) or 1:10 (C)) and incubation times (0, 3.5 and 7h).



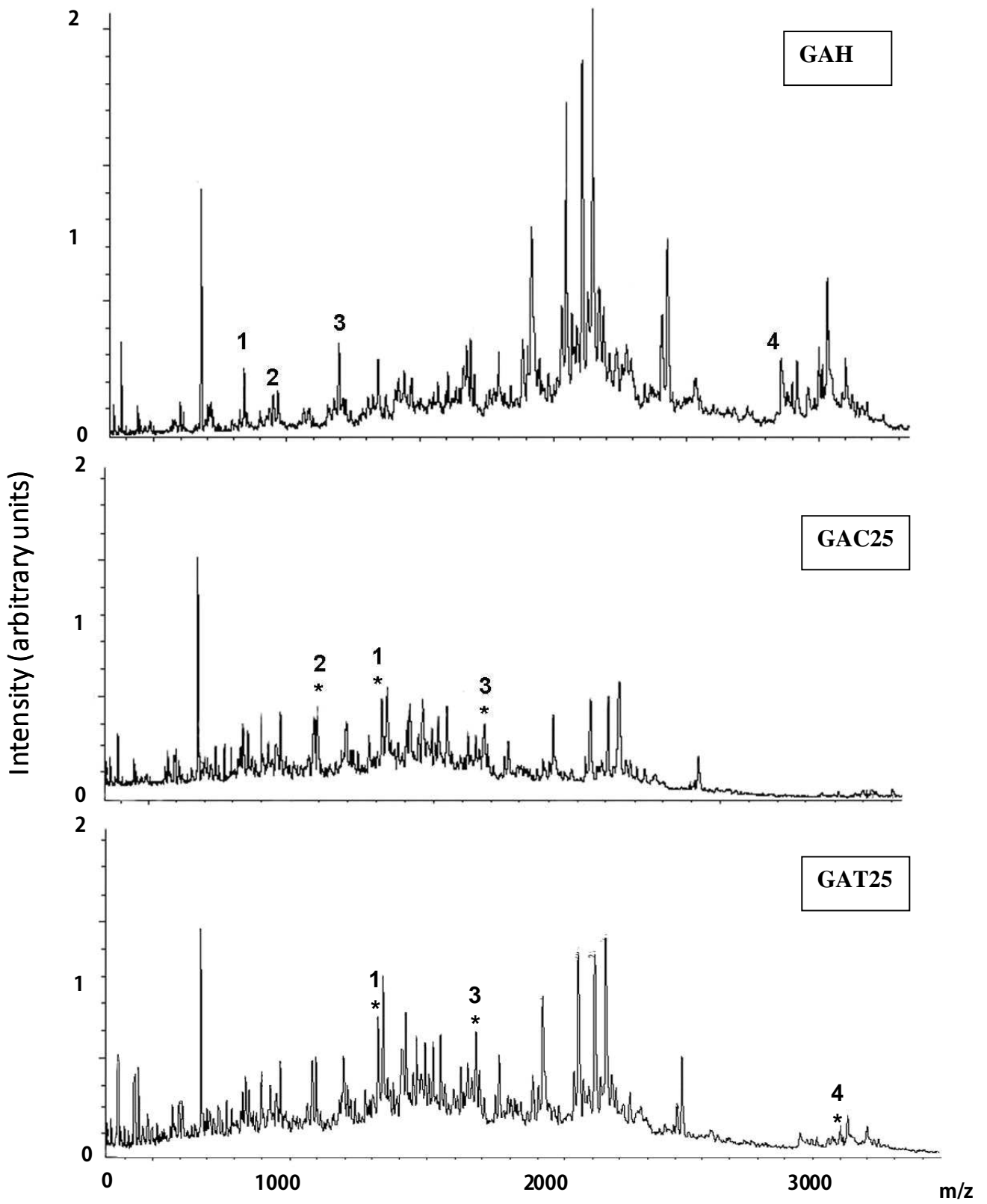
**Figure 20.** Orbitrap LC-MS spectrum of products resulting from TGase mediated Glycosylation of peptides with GlcN.

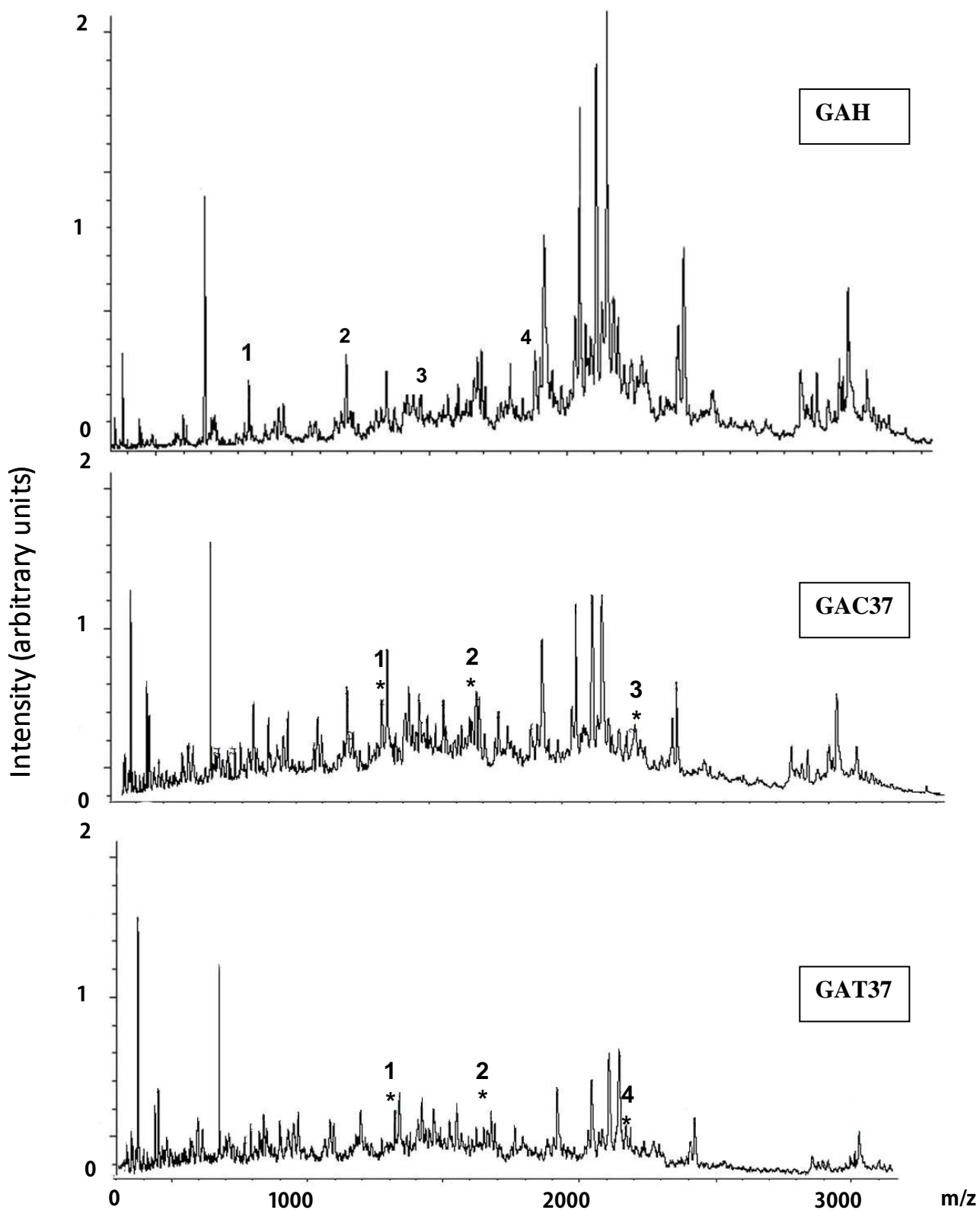


**Figure 21.** Proposed mechanism of reaction at 37 °C for 3.5 h: glycosylation and glycation

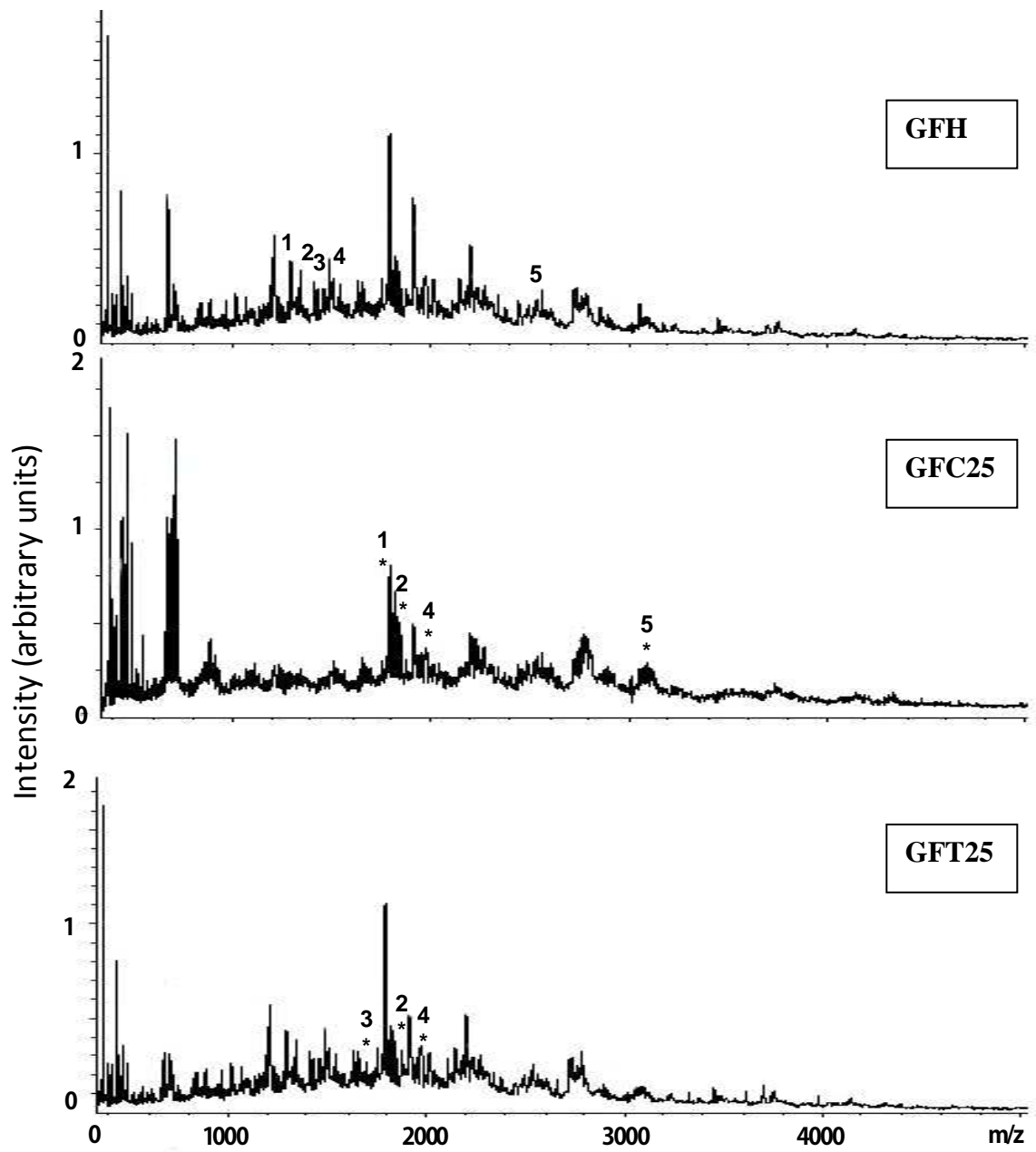


**Figure 22.** Size exclusion chromatogram of gluten (solid line) and the hydrolysates subjected to Alcalase (GA, dots) and Flavourzyme (GF, dotted line) hydrolysis, respectively.

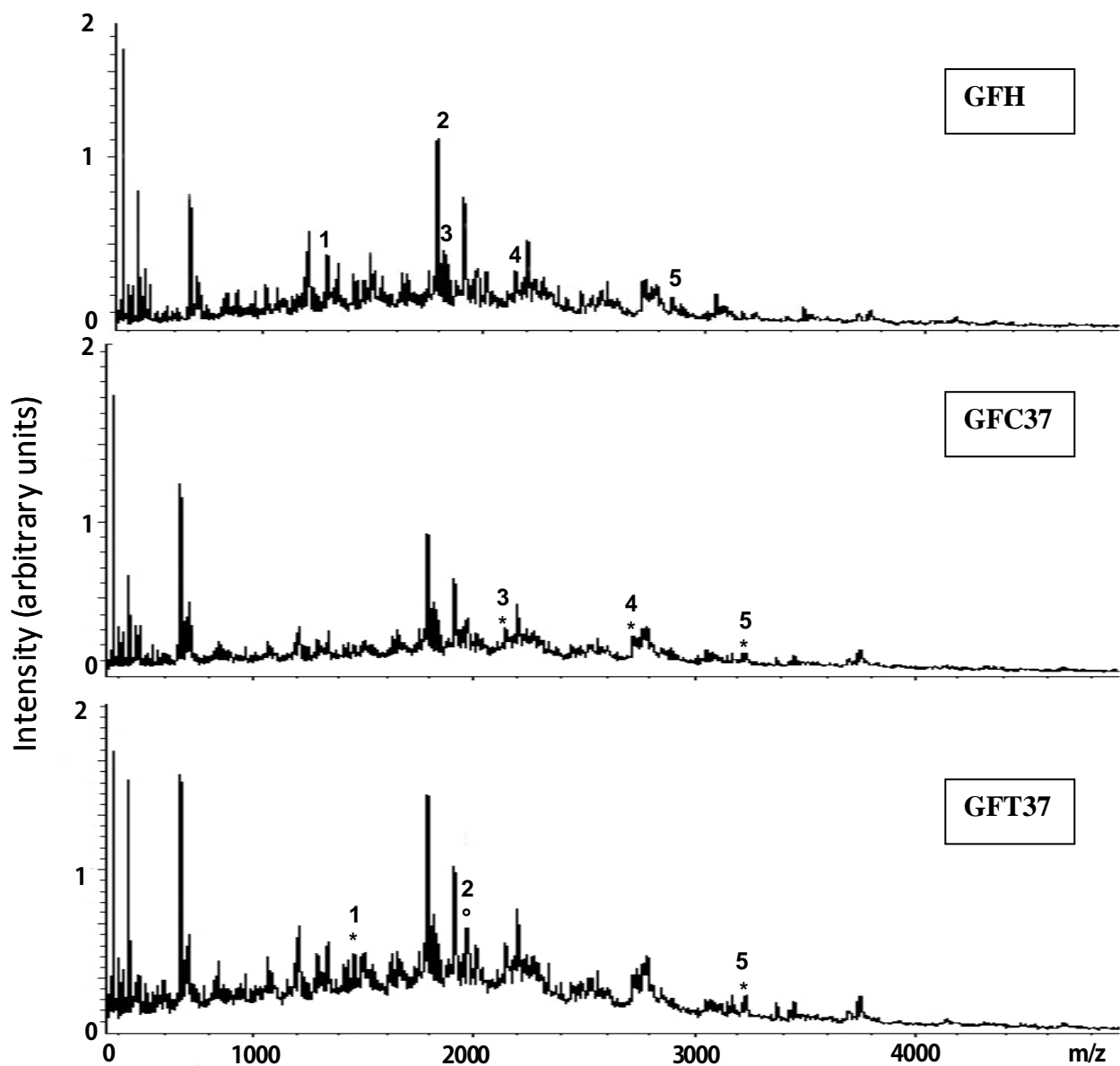




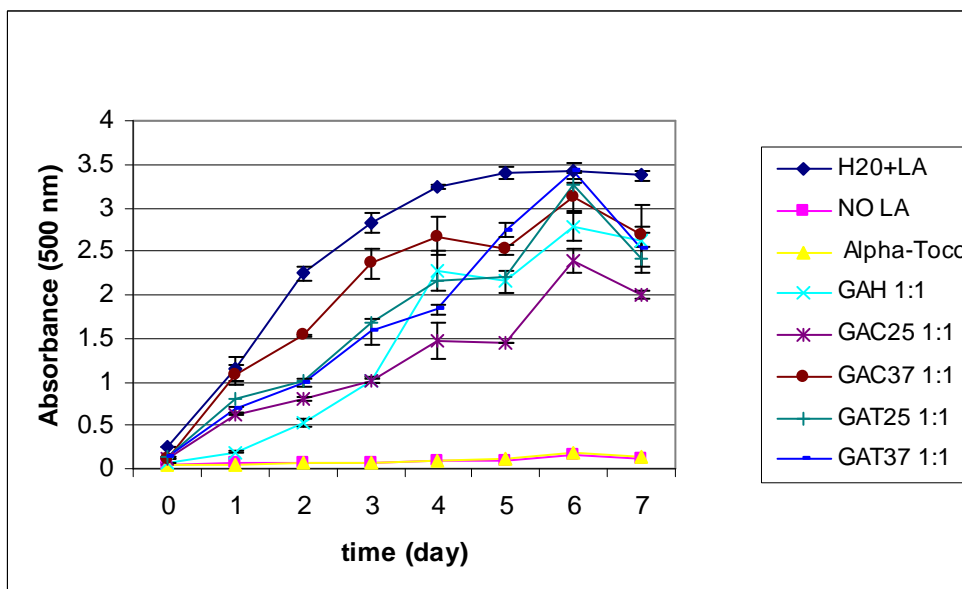
**Figure 23.** MALDI-TOF-MS spectra of gluten hydrolysed by Alcalase (GAH), gluten hydrolysate glycosylated at 25 or 37 °C (GAC25 and GAC37), gluten hydrolysate enzymatically glycosylated at 25 and 37 °C (GAT25 and GAT 37). Glycosylated peptides are marked with an asterisk (\*), while glycosylated peptides are marked with a filled circle (•).



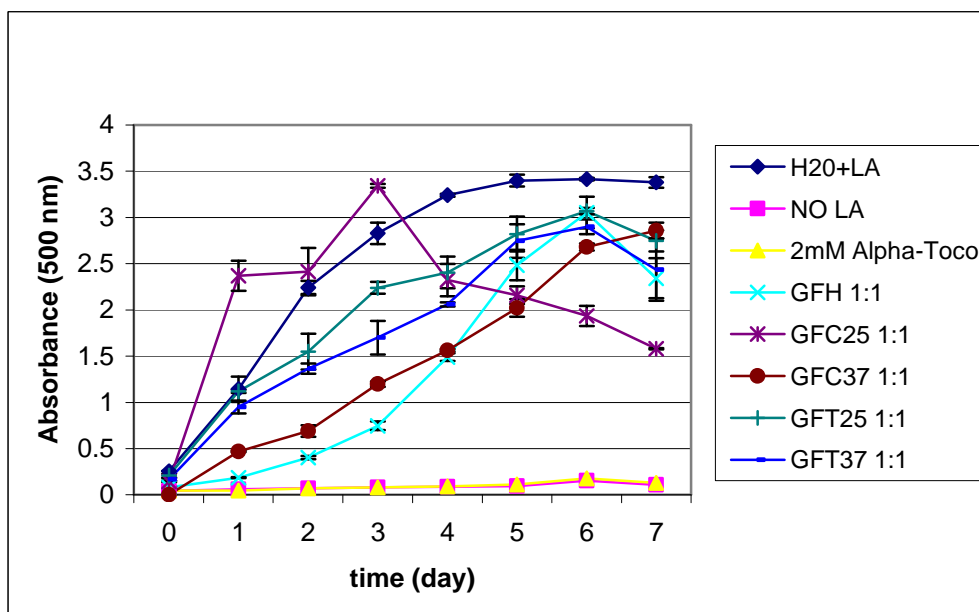




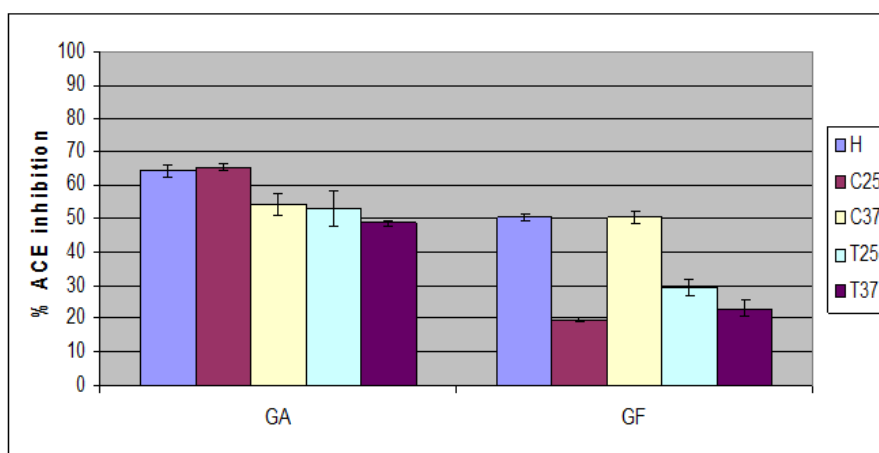
**Figure 24.** MALDI-TOF-MS spectra of gluten hydrolysed by Flavourzyme (GFH), gluten hydrolysate glycosylated at 25 or 37 °C (GFC25 and GFC37), gluten hydrolysate enzymatically glycosylated at 25 and 37 °C (GFT25 and GFT 37). Glycosylated peptides are marked with an asterisk (\*), while glycosylated peptides are marked with a filled circle (•).



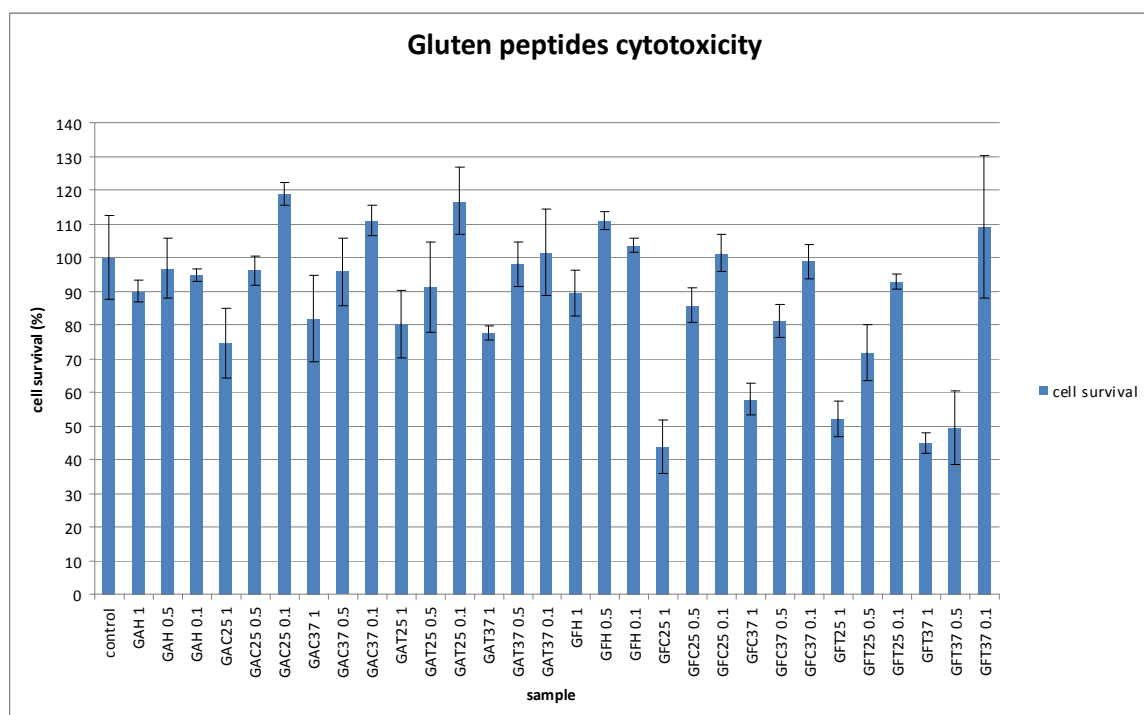
**Figure 25.** Antioxidant activities of Alcalase gluten hydrolysate and its glycyated/glycosylated samples in a linoleic acid oxidation system. The antioxidant activity was measured on the basis of the ability to keep constant the absorbance at 500 nm over time.  $\alpha$ -tocopherol was used as reference positive control. Linoleic acid plus water was used as negative control. Values are expressed as means  $\pm$  SD of at least triplicate experiments.



**Figure 26.** Antioxidant activities of Flavourzyme gluten hydrolysate and its glycyated/glycosylated samples in a linoleic acid oxidation system. The antioxidant activity was measured on the basis of the ability to keep constant the absorbance at 500 nm over time.  $\alpha$ -tocopherol was used as reference positive control. Linoleic acid plus water was used as negative control. Values are expressed as means  $\pm$  SD of at least triplicate experiments.



**Figure 27.** Anti-ACE activity of gluten samples hydrolysed with Alcalase (GA) and Flavourzyme (GF). The samples were incubated with GlcN (C) and glucosamine + TGase (T) at 25 and 37°C. Values are expressed as means  $\pm$  SD of at least triplicate experiments.



**Figure 28.** The cytotoxic effect of Alcalase (GAH) and Flavourzyme (GFH) hydrolysates (control samples not conjugated) and the corresponding conjugated peptides obtained without enzyme (GAC25, GAC37, GFC25, GFC37) or with enzyme (GT25, GAT37, GFT25, GFT37) against Hepatocellular carcinoma (HepG2). Cells were treated with different concentrations (1, 0.5, 0.1 mg/ml) of the mixture obtained incubating peptide/glucosamine in a 1:1 ratio. Values are expressed as means  $\pm$  SD in quadruplicate experiments. Control refers to cells not treated.

**CHAPTER 9.**

**DISCUSSION AND CONCLUSIONS**

The extracellular proteases are widely studied in *Y. lipolytica*, in particular genes encoding for an alkaline protease (AEP) and an acidic protease (AXP) have been cloned and sequenced (Hernández-Montañez *et al.*, 2007). However, since *Y. lipolytica* is an attractive host for the production of foreigner proteins, its proteolytic system must be further studied further because unspecific proteases may degrade the heterologous proteins or in many case protolytic processing is necessary for the expression of activities such as aminopeptidase, or dipeptidil-aminopeptidase by the host cells. Young *et al.* (1996) cloned and sequenced the gene and potential regulatory region of the extracellular acid protease of a strain of *Y. lipolytica* and showed that the transcription of both the genes for acid and alkaline is regulated by the pH of the medium. Investigation relative to the strain diversity, the effect of the temperature or the occurrence of dimeric or complex forms of the enzymes are lacking.

A novel finding resulting from my experimental results is the detection of a new high MW protease in strains of *Y. lipolytica*. On the basis of the results some of the tested strains released prevalently or only a protease having MW higher than 200 kDa active both on casein and gelatin, while others released only protease, active on gelatin and casein, having a MW of 37 kDa. Both the proteins are released at the end of the exponential phase. Proteases having MW higher than 150 kDa have never been reported in *Y. lipolytica* and other yeasts, except for an extracellular protease having a MW of approximately 200 kDa has been described in *M. pulcherrima* (Reid *et al.*, 2012). Reid *et al.*, (2012) reported a protein, or a protein complex, having a protease activity characterized by 180-200 kDa MW. Moreover Chen *et al.*, (1997) characterized, in *Cryptococcus* spp., an extracellular proteolytic activity *in vitro* as a serine proteinase and found it associated with proteins of approximately 200 kDa. Goodley and Hamilton (1993) isolated a 200 kDa proteinase from the filtrate of *C. neoformans*.

It has been reported that the initial size of intracellular and extracellular enzymes can change over time. In fact the MW of a protease can change because of the removal of a molecular fragment that obstruct the catalytic size. This has been documented as a common activation step of some mammalian proteases (Nagase *et al.*, 1990). In order to avoid dangerous degradation of intracellular proteins, proteases are often synthesised with an additional segment that obstructs the catalytic site. This latent form (called either pro-enzyme or zymogen) displays up to 60% of the activity of the fully active molecule (Woessner, 1995). Outside the cells the fragment is cleaved off and full activity is achieved. This hypothesis does not fit the experimental results. In fact, generally the MW of the removed fragment did not significantly change the MW of the active protease (Hoffman and Decho, 2000). Moreover an activity of 37 kDa was not observed in the supernatant of strains showing proteolytic activity at 200 kDa, and vice versa.

Microorganisms evolved in stressful environments, like marine ones, produce an array of enzymes having different MWs. In particular the marine bacterium *Pseudomonas atlantica* releases high MW (103 kDa) proteases during the exponential phase. These enzymes are degraded over time and new low MW enzymes (34, 31, 75, 69 kDa) are formed (Hoffman and Decho, 2000).

Also this temporal evolution of released enzymes does not fit my data because both the enzymes at 200 and 37 kDa are alternatively produced in samples processed the same time, stored and run under the same conditions and using the same reagents. The possibility of artefacts can be considered negligible.

In *Y. lipolytica* the aminoacid sequence, deduced by Young *et al.*, (1996) from the nucleotide sequence encoding for AXP, consists of 353 aminoacids and a MW of 37 kDa. According to Young *et al.* (1996), the mature extracellular enzyme is produced from a propeptide by cleavage between Phenylalanine and Alanine. Likewise *Y. lipolytica* releases an alkaline extracellular protease from a 55 kDa precursor produced after cleavage of a 15 aminoacid signal peptides. This precursor is then processed by a diaminopeptidase to generate a 52 kDa proprotein which is subsequently cleaved to give the mature 32 kDa secreted protein. Generally a propeptide is inactive and can be converted to mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide.

If, as suggested by Young *et al.* (1996), *Y. lipolytica* is characterized by just one specific gene for an acidic protease of a MW of 37 kDa, it can be hypothesised that the band having proteolytic activity and having a MW higher than 200 kDa corresponds to a complex of the acid proteases. However, further studies would be necessary to demonstrate why a such a complex of proteins is active. Moreover all the proprotein or the obstructed proteases generally present a significantly lower MW. In any case a proteolytic activity of a protein or protein aggregate having MW higher than 200 kDa has not been reported in *Y. lipolytica* and in genetically related yeast yet (Rao *et al.*, 1998). Moreover our preliminary results showed that the two hypothetical acid proteases (with a MW of 37 and higher than 200 kDa), associated to different strains, generated different profiles from caseins, meat extract and gelatin. It could be wondered why a protease coded by the same gene from the same organism produces different profiles on the same protein. *Y. lipolytica* strains used in my thesis presented different RAPD profiles and different physiological characteristics as well as different ecological origins. Therefore it can be postulated that, due to subsequent environment-induced mutations, the protease acquires a different substrate specificities compared to the starting one (Craik *et al.*, 1985). It must be considered that the genome of *Y. lipolytica* has been sequenced only in the strain *Y. lipolytica* CLIB122 provided by the Genolevures

Consortium. Therefore it can be hypothesised that other strains of the same species harbour different genes and protein/enzymes not described yet.

Concerning the biotechnological properties, most of the strains used in my work were endowed with proteolytic activity on gelatin, skim milk and gluten at T 6°C. The intracellular and extracellular proteolytic systems of *Y. lipolytica* have been object of several investigations and exploited for different technological uses (González-López *et al.*, 2002). Different strains of *Y. lipolytica* used as cheese and dried sausage ripening adjuncts (Guerzoni *et al.*, 1998, Vannini *et al.*, 2001, Lanciotti *et al.*, 2005; De Wit *et al.*, 2005; Patrignani *et al.*, 2007) gave rise to different proteolysis patterns as well as strain dependent sensory and texture properties.

In any case the physiological attribute that mostly distinguishes *Y. lipolytica* or its ecological biotypes from other yeasts species having biotechnological potential such as *Debaryomyces hansenii*, *Geotrichum* spp., *Candida* spp., (Vakhlu and Kour 2006; Bankar *et al.*, 2009) is the ability to produce cold active enzymes. The possible application of cold active enzymes in the food industries are numerous (Gerday *et al.*, 2000). Cold proteases offer potential economic benefits particularly through substantial energy saving in large scale processes that would not require the expensive heating of reactors. The typical example is the industrial peeling of leather by proteases which can be done with cold enzymes at temperature under 10°C instead of 37°C. In general the cold active enzymes have a more flexible structure and undergo the conformational changes necessary for catalysis with a lower energy demand. More recently enzymes from psychrotrophic species have become interesting for industrial applications partially because of the ongoing efforts to decrease energy consumption during industrial processes. When enzymes from psychrotrophic species are used it becomes feasible to develop for instant laundry applications that can be performed at lower temperatures.

Concerning the valorisation of meat by-products, the enzymes currently used are characterized by mesophilic or thermophilic characteristics. The quest for valuable proteases with distinct specificity for industrial applications is always a continuous challenge. Proteolytic enzymes from plant and fungal sources have received special attention for being active over a wide range of temperatures and pH. In general the commercial enzymes currently used present an optimal activity near 50-60°C.

The strains of *Y. lipolytica* taken into consideration are characterized by cold attitude, versatile proteases and diversity as far as the peptides profiles obtained on all the protein sources considered.

One of the strains taken into consideration has been selected, due to its intense proteolytic activity in the range from 4 to 28°C, to evaluate its performance in the production of peptides

having antioxidant, antimicrobial and cytotoxic, anti-ACE properties. The selected protein was gelatin due to its large availability as industrial by-product. The whole peptide mixture obtained during proteolysis over time were characterized by an increasing antioxidant activity. The peptides playing this effect are formed during the last 72 and 96 h. The latter samples were characterized by the presence of peptides having prevalently a MW lower than 3 kDa.

The selection of strains having good biotechnological potential is a fundamental tool to identify natural active compound. However, wild strains and reactions performed with their enzymes generally give rise to a low level of active molecules. In order to increase the bioactivities of the peptides produced by the enzymes released by *Y. lipolytica*, I developed a chemico/enzymatic approach that has only been suggested in literature, but not explored yet. I would like to highlight that this process can be exploited not only for the peptides produced by proteases from *Y. lipolytica*, but also for peptides obtained from other protein hydrolysates of food by-products, thus increasing their added-value.

The formation of a bound between a sugar and an amino acid occurs through enzymatic (glycosylation) and non-enzymatic (glycation) reactions. Although various techniques are available to prepare synthetic glycoproteins or glycopeptides (Caer *et al.*, 1990; Christopher *et al.*, 1980; Colas *et al.*, 1993; Hattori *et al.*, 1996; Kitabatake *et al.*, 1985), the glyco-conjugates of proteins and polysaccharides obtained by the Maillard reaction have received much attention only in recent years (Liu *et al.*, 2012). In fact the role of Maillard reaction on the improvement of functional properties of food proteins has been only recently proposed (Oliver *et al.*, 2006). The main disadvantage of the Maillard reaction is the use of high temperature or prolonged heat treatments, to obtain the glycation (Liu *et al.*, 2012). In my work, I proposed a novel procedure to conjugate sugars and peptides, exploiting the particular behaviour of the amino-sugar GlcN, and the use of temperature not exceeding 37 °C. In the model system development the conditions and the validation of the glycation between GlcN and Gly-Gln at 25-37°C were shown. Further, the same model system was used to demonstrate the feasibility of using GlcN as a substrate for TGase, and for the production of glycosylated peptides. TGase is a universal enzyme capable of modifying proteins through the incorporation of amines; it is used in the food industry to improve the texture of vegetable and animal proteins and to create innovative food products with different properties (Seguro *et al.*, 1996).

Based on the results of the UV spectra, the experimental conditions optimal to reduce, to a feasible extent the formation of GlcN side-products generated by its high reactivity, were determined. Lanciotti *et al.* (1999) reported that Maillard reactions (> 90°C) between sugars and aminoacids produced antimicrobial and antioxidant compounds. For this reason, even though lower



absorbance curves were detected in 1:1 ratio samples, 1:3 ratio was subsequently considered to assess the possible interference with the final activities. In general, conjugation leads to the formation of compounds with different properties, compared with the native structure. For example, it has been reported that the presence of sugars bounded to the peptides enhances the antimicrobial effect of the native peptide by approximately 100 times (Otvos *et al.*, 2002).

Once proved the conjugation in a model system, I tried to transfer the process into a more complex mixture of peptides. Wheat gluten, a by-product of the wheat industry, is a heterogeneous mixture of peptides with more than 60 different molecular weights ranging from 30 to 90 kDa. In particular, the main abundant forms are represented by the polymeric glutenin and the monomeric gliadin (Wang J. *et al.*, 2007). Glutenin can also rearrange forming disulfide bonds and create structures with MW of 50 to 2000 kDa (Bietz and Wall, 1972). In my work, gluten was employed after proteolysis with Alcalase and Flavourzyme.

The estimated degree of hydrolysis (DH) was not elevated for Alcalase. In fact higher DH for wheat gluten has been previously reported (Kong *et al.*, 2007). On the contrary, the Flavourzyme generated a more common DH already described for protein hydrolysis. Even if differences in DH may depend on the selected method for its assessment, the low solubility of gluten at neutral pH could have also played an important role (Takeda *et al.*, 2001).

The hydrosylated samples obtained were then employed for the conjugation. All the samples were mainly glycosylated rather than glycosylated, and at least three glycopeptides were present in all samples. The excess of GlcN in the 1:3 ratio samples did not interfere with the tested activities. In these samples very low glycopeptides were detected and no interferences, due to possible GlcN side-products, were induced. Hence, it can be suggested that the reactivity of glucosamine at higher concentrations can form auto-condensed and rearrangement products that were not suitable for the glycation/glycosylation process. On the other hand, low amounts of sugar (1:1) generated more glycopeptides, and showed enhanced modulation of the final bio-activities.

The assessed bioactivities were: DPPH scavenging activity, inhibition of linoleic acid peroxidation, anti-ACE, antimicrobial and cytotoxicity. As already mentioned, DPPH measures the free radical scavenging capacity of a sample, based on a combination of hydrogen atom and electron transfer reactions (Huang *et al.*, 2005). With this assay all the conjugated samples showed an improvement of the final activity compared to the control ones (GAH and GFH). In particular GFT37, the sample having a glycopeptide obtained through glycosylation, was the most active. I would like to outline that in all the samples the contribution of the classical Maillard products can be regarded as negligible. These activities may depend on the sugar moiety of glycopeptides, which could be both electron donors and electron acceptors (van Boekel, 2001). The 1:3 peptide/sugar

ratio was also tested to determine whether the increased sugar content in the solution could influence the reducing power. However, no activity was detected in any sample when comparing GAH and GFH (data not shown) because of the lower content or absence of glycation of the peptides. It was also confirmed that under these reaction conditions, the possible formation of some Maillard Reaction Products (MRPs) did not influence the antioxidant activity.

The linoleic acid assay measures the capability of a specific sample to inhibit or reduce the lipid oxidation. However, the antioxidant reactions that occur in this case are different from those in the DPPH analysis. Zhang *et al.* (2008) reported that hydrophobic amino acids represent a key element to protect against lipid derived-radicals, due to their ability to interact with lipids (Ajibola *et al.*, 2011). In contrast, the presence of the sugar moiety within a glycopeptide (glycoprotein) could lead to an increase on the overall hydrophilicity (Wang and Ismail, 2012). This could explain the pro-oxidative activity found in GFC25, the highest glycated sample as compared to the others. On the other hand, the antioxidant activity of GAC25 may depend on the relatively lower MW (1095 Da), with respect to all the other glycated peptides. Li *et al.* (2008) reported that protein hydrolysates prepared from corn gluten meal had increased effective antioxidant capacity when the MWs of peptides were between 500 - 1500 Da. Hence, glycopeptides with low MW may also be more effective in the linoleic acid assay.

The ability of a peptide to inhibit ACE was also tested. This activity is strongly linked to the peptide amino acid composition and their primary sequence. Anti-ACE gluten peptides have been already reported (Kim *et al.*, 2004); in particular, the main activity of the hydrolyzates can be attributed to the low molecular weight peptides. In fact, after fractionation of the samples, the inhibitory activity increased by four-fold. Kinoshita *et al.* (1993) reported that high molecular weight peptides fraction reduced the blood pressure in hypertensive rats. The conjugation of gluten peptides had shown negative effects in almost all the samples. Je *et al.* (2004) described that the presence of hydrophobic amino acids in the sequence enhances the ACE-inhibitory ability of the peptides. As a result, the perturbation of the final hydrophobicity of the peptide (once glycation has occurred) can have a negative effect in all activities depending on the proportion of hydrophobic/hydrophilic region inside the molecule.

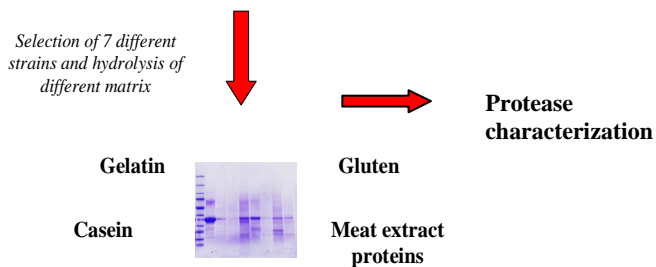
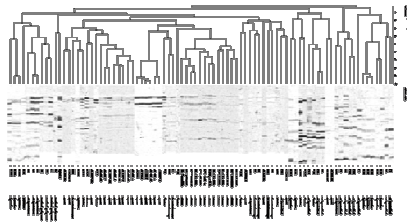
The antimicrobial activity of these samples demonstrated that the glycation was essential for the improvement of the antimicrobial activity. In particular, this effect is more relevant in GF than GA. Although the antimicrobial activity of glycopeptides has been reported, (Kahne *et al.*, 2005; Bullet and Stocklin, 2005) the mechanisms of action have not been completely described. Glycopeptides active specifically against Gram-negative bacteria have been reported; for example, Drosocin, a well-studied glycopeptide isolated from *Drosophila melanogaster*, is a small proline-

rich peptide (around 2-4 kDa) with a disaccharide (galactose and N-acetylgalactosamine) (Bulet and Stocklin, 2005). Gluten, on the other hand, is mainly constituted by glutamine/glutamic acid (around 40%), and proline (around 17-20%) (Wieser, 2007). The sugar moiety enhances the antimicrobial effect of the native peptide by approximately 100 times in Drosocin (Otvos *et al.*, 2002). Thus, the peptide sequence and the presence of a hydrophilic part in a specific position of the peptide are suggested to be important for the antimicrobial action. Kragol *et al.* (2001) reported that these glycopeptides may inhibit the protein folding through a mechanism involving the bacterial chaperonine/heat shock proteins GroEL and DnaK, once entered into the cells.

The bioactivity of the samples was also assessed in relation to human tumor cells (HepG2). The enhanced cytotoxicity detected in the conjugated peptides implies that the formation of glycopeptides through a glycation process can be responsible for the main production of molecules with higher cytotoxicity. The low TGase-dependent effect could depend on the low/absent glycosylation reported or on the low affinity of the peptides for TGase. The cytotoxicity of glycopeptides has been previously described; although the role of sugar remains unknown, the carbohydrate moiety is likely to be involved in cell recognition, cellular uptake and DNA binding (La Ferla *et al.*, 2011). Indeed, Brahim *et al.* (2008) demonstrated that the deglycosylated Bleomycin-A2 (a cytotoxic glycopeptide) was less toxic than its native form, due to the inability to induce ROS formation. Therefore, the two main elements influencing the studied properties were: 1) the type of starting peptides (which are affected by the enzyme used for the hydrolysis and the MWs of the resulting peptides); 2) the reduction of the final hydrophobicity of the peptides.

Although it was demonstrated the formation of glycopeptides through TGase both in the model system and in the gluten system, these results pointed out that the enzymatic process was not able to create sufficient glycosylated gluten peptides with improved activities. Indeed, studies regarding TGase and gluten have been reported. Dekking *et al.* (2008) demonstrated that transglutaminase can deamidate gluten peptides by introducing negative charges. This process was responsible for the enhanced immunogenicity of gluten. Similarly, Elli *et al.* (2012) reported the use of TGase as a tool to incorporate lysine, the first limiting amino acid in wheat products, into gluten. Cross-linking with glutamine using TGase promoted lysine availability. However, the incorporation of molecules to "protect" glutamines as a way to reduce the toxicity of gluten has not been exploited yet. The attachment of GluN can represent a potent tool in this direction. In this way, the conjugation method could be highly effective to modify peptides. The use of GluN and TGase can be employed to create innovative functional peptides or mixtures of peptides, using a mild temperature process. Additional studies have to be performed to improve the final yield of glycation/glycosylation process and to test the stability of these new compounds over time.

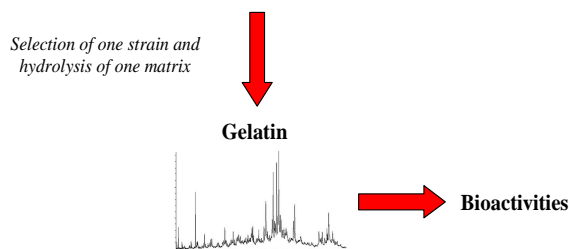
I can summarize the results of my thesis taking into consideration the steps included in the original project.



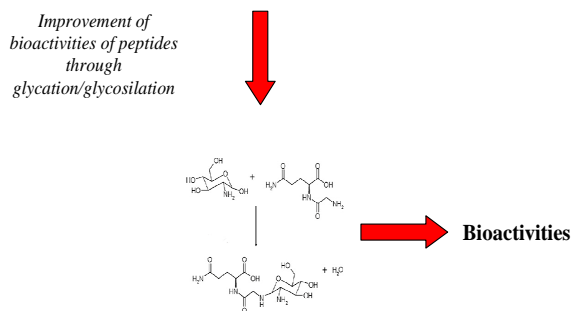
Detection of proteases with 2 MWs

Different proteolytic profiles

Enzymes able to work at 6 °C



Production of peptide mixtures having antioxidant and antimicrobial activities



Development of a new method to glycate/glycosylate peptides

Improved activities of the peptide mixtures after glycation

My research can not be regarded as exhaustive. In fact several points need to be further elucidated and developed: i.e. the nature of the high MW protease release by *Y. lipolytica*, the identification of the most active peptides in relation to their specific bioactivities, and the optimisation of the glycation reaction in relation to the food by-products and their hydrolysis when proteases released by *Y. lipolytica* are employed. However, I tried to integrate the selection and development of strains endowed with valuable biotechnological potential with a chemico/technological approach aimed at enabling the enhancement of the bioactivities of the resulting compounds.

**CHAPTER 10.**

**REFERENCES**

- Adler-Nissen J (1986) Methods in food protein hydrolysis. In "Enzymic hydrolysis of food proteins", Adler-Nissen J (Ed.), Elsevier Applied Science Publishers: (New York), USA, pp. 116-124.
- Ajibola CF, Fashakin JB, Fagbemi TN, Aluko RE (2011) Effect of Peptide Size on Antioxidant Properties of African Yam Bean Seed (*Sphenostylis stenocarpa*) Protein Hydrolysate Fractions. *Int J Mol Sci*, 12: 6685-6702.
- Akpınar O, Uçar F, Yalçın HT (2011) Screening and regulation of alkaline extracellular protease and ribonuclease production of *Yarrowia lipolytica* strains isolated and identified from different cheeses in Turkey. *Ann Microbiol*, 61(4): 907-915
- Awarenet (2004) Handbook for the Prevention and Minimisation of Waste and Valorisation of By-products in European Agro-Food Industries. Waldron K. (Ed.).
- Badiali F (2004) Biodiversità e proprietà tecnologiche di *Yarrowia lipolytica* in relazione all'origine ambientale. Ph.D: Thesis in Food Biotechnology, Alma Mater Studiorum, Università di Bologna.
- Bankar AV, Kumar AR, Zinjarde SS (2009) Environmental and industrial applications of *Yarrowia lipolytica*. *Appl Microbiol Biotechnol*, 84: 847–865.
- Barth G, Gaillardin C (1997) Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. *FEMS Microbiol Rev*, 19: 219-237.
- Bielikowicz K, Wojtacha P, Kostyra E, Iwan M, Jarmołowska B, Kostyra H (2010) Influence of glycation of wheat albumins and globulins on their immunoreactivity and physicochemical properties. *Pol J Food Nutr Sci*, 60(4): 335-340.
- Bietz JA, Wall JS (1972) Wheat gluten subunits: Molecular weights determined by sodium sulfate-polyacrylamide gel electrophoresis. *Cereal Chem*, 49: 416-430.
- Bintsis T, Robinson RK (2004) Study of the effects of adjunct cultures on the aroma compounds of Feta-type cheese. *Food Chem*, 88: 435–441.
- Boman HG (1995) Peptide antibiotics and their role in innate immunity. *Annu Rev Immunol*, 13: 61-92.
- Bougatef A, Nedjar-Arroume N, Manni L, Ravallec R, Barkia A, Guillochon D, Nasri M (2010) Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. *Food Chem*, 118: 559–565.
- Brahim S, Bettaieb A, Kenani A (2008) Deglycosylated bleomycin triggers apoptosis in laryngeal carcinoma cells in a caspase and reactive oxygen species independent manner. *J Oral Pathol Med*, 37(6): 352–357.

- Brands CMJ, Alink GM, Boekel MA, Jongen WMF (2000) Mutagenicity of heated sugar-casein systems: effect of the Maillard reaction. *J Agr Food Chem*, 48: 2271-2275.
- Brantl V, Gramsch C, Lottspeich F, Mertz R, Jager KH, Heltz A (1986) Novel opioid peptides derived from hemoglobin: hemorphins. *Eur J Pharmacol*, 125: 309-310.
- Bulet P, Stöcklin R (2005) Insect Antimicrobial Peptides: Structures, Properties and Gene Regulation. *Prot Pep Lett* 12: 3-11.
- Bunning P, Holmquist B, Riordan JF (1983) Substrate specificity and kinetic characteristics of angiotensin converting enzyme. *Biochem*, 221, 110-116.
- Caer D, Baniel A, Subirade M, Gueguen J, Colas B (1990) Preparation and physicochemical properties of glycosylated derivatives of pea legumin. *J Agr Food Chem*, 38: 1700-1706.
- Chan KM, Decker EA (1994) Endogenous skeletal muscle antioxidants. *Crit Rev Food Sci Nutr*, 34:403–26.
- Chen HM, Muramoto K, Yamauchi F, Fujimoto K, Nokihara K (1998) Antioxidant properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *J Agric Food Chem*, 46:49–53.
- Chen LC, Pirofski L, Casadevall A (1997) Extracellular Proteins of *Cryptococcus neoformans* and Host Antibody Response. *Infect Immun*, 65(7): 2599–2605.
- Chen Z, Eggert US, Dong SD, Shaw SJ, Sun B, LaTour JV, Kahne D (2002) Structural requirements for VanA activity of vancomycin analogues. *Tetrahedron*, 58(32): 6585–6594.
- Christopher P, Stowell P, Lee YC (1980) Neoglycoproteins. The preparation and application of synthetic glycoproteins. *Adv Carbohydr Chem Bio*, 37: 225-281.
- Coelho MAZ, Amaral PFF, Belo I (2010) *Yarrowia lipolytica*: an industrial workhorse. In “Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology”, Méndez-Vilas A. (Ed.), 2: 930-940, Formatex, Badajoz (Spain)
- Colas B, Caer D, Fournier E (1993) Transglutaminase-catalyzed glycosylation of vegetable proteins. Effect on solubility of pea legumin and wheat gliadines. *J Agr Food Chem*, 41: 1811-1815.
- Contreras MM, Carron R, Montero MJ, Ramos M, Recio I (2009) Novel casein-derived peptides with antihypertensive activity. *Int Dairy J*, 19: 566–573
- Córdoba JJ, Antequera T, Ventanas J, López-Bote C, García C, Asensio MA (1994) Hydrolysis and loss of extractability of proteins during ripening of Iberian ham. *Meat Sci*, 37(2): 217-27.
- Craik CS, Largman C, Fletcher T, Roczniak S, Barr PJ, Fletterick R, Rutter WJ (1985) Redesigning trypsin: alteration of substrate specificity. *Science*, 237: 291-297.



- Davidow LS, O'Donnell MM, Kaczmarek FS, Pereira DA, DeZeeuw JR, Franke AE (1987) Cloning and sequencing of the alkaline extracellular protease gene of *Yarrowia lipolytica*. *J Bacteriol*, 169(10): 4621-4629.
- Daya L, Augustina MA, Bateyb IL, Wrigleyb CW (2006) Wheat-gluten uses and industry needs. *Trends Food Sci Technol*, 17(2): 82–90.
- De Wit M, Osthoff G, Viljoen BC, Hugo A (2005) A comparative study of lipolysis and proteolysis in Cheddar cheese and yeast-inoculated Cheddar cheeses during ripening. *Enzyme Microb Technol*, 37: 606–616.
- Dekking EHA, Van Veelen PA, de Ru A, Kooy-Winkelaar EMC, Gröneveld T, Nieuwenhuizen WF, Koning F (2008) Microbial transglutaminases generate T cell stimulatory epitopes involved in celiac disease. *J Cereal Sci*, 47(2): 339–346.
- Dziuba J, Minkiewicz P, Plitnik K (1996) Chicken meat proteins as potential precursors of bioactive peptides. *Pol J Food Nutr Sci*, 5/46 (4): 87-98
- Elleuch M, Bedigian D, Roiseux O, Besbes S, Blecker C, Attia H (2011) Dietary fibre and fibre-rich by-products of food processing: Characterisation, technological functionality and commercial applications: A review. *Food Chem*, 124(2): 411-421.
- Elli L, Roncoroni L, Hils M, Pasternack R, Barisani D, Terrani C, Vaira V, Ferrero S, Bardella MT (2012) Immunological effects of transglutaminase-treated gluten in coeliac disease. *Hum Immunol*, 73(10): 992-997.
- Erbersdobler HF, Brandt A, Scharrer E, Wangenheim B (1981) Transport and metabolism studies with fructose amino acids. *Prog Food Nutr Sci*, 5: 257-263.
- Erdmann K, Cheung BW, Schröder H (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J Nutr Biochem*, 19(10): 643-54.
- Fanciulli G, Dettori A, Fenude E, Demontis MP, Alberico E, Delitala G, Anania V (2003) Intravenous administration of the food-derived opioid peptide gluten exorphin B5 stimulates prolactin secretion in rats. *Pharmacol Res*, 47(1): 53-58.
- Fang YZ, Yang S, Wu G (2002) Free radicals, antioxidants, and nutrition. *Nutrition*, 18: 872–879.
- FAO (2012) Crop residue based densified total mixed ration – A user-friendly approach to utilise food crop by-products for ruminant production, by Walli TK, Garg MR, Harinder PS Makkar. *FAO Animal Production and Health Paper No. 172*. Rome (Italy).
- Farnaud S, Evans RW (2003) Lactoferrin - a multifunctional protein with antimicrobial properties. *Mol Immunol*, 40: 395–405.
- Federalimentare, (2006) I sottoprodotti dell'Industria alimentare, 1-2

- Fickers P, Benetti PH, Waché Y, Marty A, Mauersberger S, Smit MS, Nicaud JM (2005) Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. *FEMS Yeast Res*, 5: 527-543.
- Froehlich JC (1997) Opioid peptides. *Alcohol Health Res World*, 21(2): 132-136
- Fujita H, Sasaki R, Yoshiwaka M (1995) Potentiation of the antihypertensive activity of orally administered ovokinin, a vasorelaxing peptide derived from ovalbumin, by emulsification in egg phosphatidylcholine. *Biosci Biotechnol Biochem*, 59: 2344–2345
- Galanakis CM (2012) Recovery of high added-value components from food wastes: conventional, emerging technologies and commercialized applications. *Trends Food Sci Technol*, 26(2): 68-87.
- Gamblin, D.P.; Scanlan, E.M.; Davis, B.G. (2009) Glycoprotein Synthesis: An Update. *Chem Rev*, 109: 131-163.
- Ganz T (2003) Defensins: Antimicrobial peptides of innate immunity. *Nat Rev Immunol*, 3(9): 710-720.
- Gardini F, Tofalo R, Belletti N, Iucci L, Suzzi G, Torriani S, Guerzoni ME, Lanciotti R (2006) Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiol*, 23: 641–648.
- Gauthier SF, Pouliot Y, Saint-Sauveur D (2006) Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *Int Dairy J*, 16(11): 1315–23.
- Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis MA, Feller G (2000) Cold-adapted enzymes: from fundamentals to biotechnology. *Trends Biotechnol*. 18(3): 103-107.
- Gobbetti M, Smacchi E, Corsetti A, Bellucci M (1997) Inhibition of proteolytic enzymes from *Pseudomonas fluorescens* ATCC 948 and Angiotensin I-converting enzyme by peptides from zein, hordein and gluten hydrolyzates. *J Food Protect*, 60: 499-504.
- Gobbo M, Biondi L, Filira F, Gennaro R, Benincasa M, Scolaro B, Rocchi R (2002) Antimicrobial Peptides: Synthesis and Antibacterial Activity of Linear and Cyclic Drosocin and Apidaecin 1b Analogues. *J Med Chem*, 45: 4494-4504.
- Godfrey T, West S (1996) In "Industrial Enzymology", Macmillan Publishers Inc., New York (USA).
- González-López CI, Czabo R, Blanchin-Roland S, Gaillardin C (2002) Genetic control of extracellular protease synthesis in the yeast *Yarrowia lipolytica*. *Genetics*, 160: 17–427.

- Goodley JM, Hamilton AJ (1993) A novel extracellular high molecular weight proteinase from *Cryptococcus neoformans*. In “proceedings of the 2nd International Conference on *Cryptococcus* and *Cryptococcosis*”, P1-3.
- Greenberg CS, Birckbichler PJ, Rice RH (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB J*, 5(15): 3071-3077.
- Guerra-Hernandez E, Gomez CL, Garcia-Villanova B, Sanchez NC, Gomez JMR (2002) Effect of storage on non-enzymatic browning of liquid infant milk formulae. *J Sci Food Agric*, 82: 587-592.
- Guerzoni ME, Gobbetti M, Lanciotti R, Vannini L, Chaves lopez C (1998) *Yarrowia lipolytica* as potential ripening agent in milk products. In “Proceedings of the IDF Symposium on Yeasts in Dairy Industry: Positive and Negative Aspects” Jakobsen M, Narvhus J, Viljoen BC (Eds.), Copenhagen, Denmark, 1996. International Dairy Federation, Brussels, Belgium, pp. 23–33.
- Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol*, 59: 15-32.
- Hajji M, Rebai A, Gharsallah N, Nasri M (2008) Optimization of alkaline protease production by *Aspergillus clavatus* ES1 in *Mirabilis jalapa* tuber powder using statistical experimental design. *Appl Microbiol Biotechnol*, 79(6): 915-923.
- Hansen-Møller J, Hinrichsen L, Jacobsen T (1997) Evaluation of Peptides Generated in Italian-Style Dry-Cured Ham during Processing. *J Agric Food Chem*, 45 (8): 3123–3128.
- Harding MM, Anderberg PI, Haymet ADJ (2003) ‘Antifreeze’ glycoproteins from polar fish. *Eur J Biochem*, 270: 1381–1392.
- Hartmann R, Meisel H (2007) Food-derived peptides with biological activity: from research to food applications. *Curr Opin Biotechnol*, 18: 163–9.
- Hattori M, Aiba Y, Nagasawa K, Takahashi K (1996) Functional improvement of alginic acid by conjugating with  $\beta$ -lactoglobulin. *J Food Sci*, 61: 1171-1176.
- Hayes M, Ross RP, Fitzgerald GF, Hill C, Stanton C (2006) Casein-Derived Antimicrobial Peptides Generated by *Lactobacillus acidophilus* DPC6026. *Appl Environ Microbiol*, 72(3): 2260–2264.
- Hernandez-Ledesma B, Hsieh C-C, de Lumen BO (2009) Lunasin, a novel seed peptide for cancer prevention. *Peptides*, 30: 426–30.
- Hernández-Montañez Z, Araujo-Osorio J, Noriega-Reyes Y, Chávez-Camarillo G, Villa-Tanaca L (2007) The intracellular proteolytic system of *Yarrowia lipolytica* and characterization of an aminopeptidase. *FEMS Microbiol Lett*, 268(2): 178-86.

- Hindley ML, Lee KC, Davis JT (2005) The Peptide Can Influence Sugar Conformation in Small Asn Glycopeptides. *J Carbohydr Chem*, 24: 261–273.
- Hoffman M, Decho AW (2000) Proteolytic enzymes in the marine bacterium *Pseudoalteromonas atlantica*: post-secretional activation and effects of environmental conditions. *Aquat Mic E*, 23(1): 29-39
- Hou CW, Chen HJ, Yaw-Huei L (2003) Antioxidant peptides with angiotensin converting enzyme inhibitory activities and applications for angiotensin-I-converting enzyme purification. *J Agr Food Chem*, 51: 1706-1709.
- Hrynets Y, Ndagijimana M, Betti M (2013) Non-enzymatic glycation of natural actomyosin (NAM) with glucosamine in a liquid system at moderate temperatures. *Food Chem*, 139: 1062–1072.
- Hrynets Y, Ndagijimana M, Betti M (2013) Non-enzymatic glycation of natural actomyosin (NAM) with glucosamine in a liquid system at moderate temperatures. *Food Chem*, 139: 1062–1072.
- Hrynets Y, Ndagijimana M, Betti M (2013) Non-enzymatic glycation of natural actomyosin (NAM) with glucosamine in a liquid system at moderate temperatures. *Food Chem*, 139: 1062–1072.
- Hsu K (2010) Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product. *Food Chem*, 122: 42-48.
- Hsu K, Li-Chan ECY, Jao C (2011) Antiproliferative activity of peptides prepared from enzymatic hydrolysates of tuna dark muscle on human breast cancer cell line MCF-7. *Food Chem*, 126: 617–622.
- Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. *J Agric Food Chem*, 53(6): 1841-1856.
- Itoh S, Takakura D, Kawasaki N, Yamaguchi T (2009) Glycopeptide analysis using LC/MS and LC/MSn. Site-specific glycosylation analysis of glycoprotein. In “The Protein Protocols Handbook”. Walker J.M. (Ed.), Third edition, Humana Press, New York (New York), pp., 1419-1430.
- Jang A, Jo C, Kang KS, Lee (2008) Antimicrobial and human cancer cell cytotoxic effect of synthetic angiotensin converting enzyme (ACE) inhibitory peptides. *Food Chem*, 107: 327-336.
- Jany KD, Lederer G, Mayer B (1986) Amino acid sequence of proteinase K from the mold *Tritirachium album* Limber. *FEBS Lett*, 199:139-144.

- Je JY, Park PJ, Kwon JY, Kim SK (2004) A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *J Agr Food Chem*, 52: 7842-7845.
- Jenssen H, Hamill P, Hancock REW (2006) Peptide Antimicrobial Agents. *Clin Microbiol Rev*, 19(3): 491–511.
- Jiang SJ, Zhao XH (2010) Transglutaminase-induced cross-linking and glucosamine conjugation in soybean protein isolates and its impacts on some functional properties of the products. *Eur Food Res Technol*, 231(5): 679-689.
- Joseph B, Ramteke PW, Thomas G, Shrivastavan N (2007) Review Cold-active Lipases: a versatile tool for industrial applications. *Biotech Mol Biol Rev*, 2: 39–48.
- Kahne D, Leimkuhler C, Lu W, Walsh C (2005) Glycopeptide and Lipoglycopeptide Antibiotics. *Chem. Rev*, 105: 425-448.
- Kalisz HM (1988) Microbial proteinases. *Adv Biochem Eng Bio-technol*. 36: 1-65.
- Kaur KJ, Pandey S, Sakunke DM (2006) Design of a functionally equivalent nonglycosylated analog of the glycopeptide antibiotic formaecin I. *Prot Science*, 16: 309-315.
- Kim J, Whang J, Suh H (2004) Enhancement of angiotensin I converting enzyme inhibitory activity and improvement of the emulsifying and foaming properties of corn gluten hydrolysate using ultrafiltration membranes. *Eur Food Res Technol*, 218(2): 133-138.
- Kinoshita E, Yamakoshi J, Ikuchi M (1993) Purification and identification of an antiotensin I-converting enzyme inhibitor from soy sauce. *Biosci Biotech Biochem*, 57: 1107-1110.
- Kitabatake N, Cuq JL, Cheftel JC (1985) Covalent binding of glycosyl residues to  $\beta$ -lactoglobulin: Effects on solubility and heat stability. *J Agr Food Chem*, 33: 125-130.
- Kong X, Zhou H, Qian H (2007) Enzymatic hydrolysis of wheat gluten by proteases and properties of the resulting hydrolysates. *Food Chem*, 102: 759–763.
- Koo SH, Bae IY, Lee S, Lee DH, Hur BS, Lee HG (2011) Evaluation of wheat gluten hydrolyzates as taste-active compounds with antioxidant activity. *J Food Sci Technol*, 1-8.
- Korhonen H, Pihlanto A (2006) Bioactive peptides: production and functionality. *Int Dairy J*, 16(9): 945–60.
- Kosseva MR (2009) Processing of Food Wastes. In “Advances in Food and Nutrition Research”, Taylor S.T. (Ed.), Academic Press, 58:, pp. 57-136.
- Kraehenbuehl K, Davidek T, Devaud S, Mauroux O (2008) Basic and acidic sugars as flavour precursors in the Maillard reaction. Expression of Multidisciplinary Flavour Science. In “Proceedings of the 12th 491 Weurman Symposium: Expression of multidisciplinary flavor

- science”, Blank I, Wust M, Yeretian C. (Eds.), Zurich University of Applied Sciences: Interlaken, Switzerland, pp. 267-270.
- Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos L Jr (2001) The Antibacterial Peptide Pyrrolicorin Inhibits the ATPase Actions of DnaK and Prevents Chaperone-Assisted Protein Folding. *Biochem*, 40: 3016-3026.
- Krasnowska G, Rudownik E, Hyliński A, Nowak A (2006). Forming the tenderness of beef using of elected enzymatic preparations of microbiological origin. *EJPAU* 9(4): #27. <http://www.ejpau.media.pl/volume9/issue4/art-27.html>.
- Kudoh Y, Matsuda S, Igoshi K, Oki T (2001). Antioxidative peptide from milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* IFO13953. *Nippon Shokuhin Kagaku Kaishi*, 48: 44–55.
- Kumar CG, Takagi H (1999) Microbial alkaline proteases: from abioindustrial viewpoint. *Biotechnol Adv*, 17: 561-594.
- La Ferla B, Airoidi C, Zona C, Orsato A, Cardona F, Merlo S, Sironi E, D’Orazio G, Nicotra F (2011) Natural glycoconjugates with antitumor activity. *Nat Prod Rep*, 28: 630-648.
- Lanciotti R, Anese M, Sinigaglia M, Severini C, Massini R (1999) Effects of Heated Glucose-fructose-glutamic Acid Solutions on the Growth of *Bacillus stearothermophilus*. *LWT - Food Sci Technol*, 32: 223-230.
- Lanciotti R, Chaves-López C, Patrignani F, Paparella A, Guerzoni ME, Serio A, Suzzi G (2004) Effects of milk treatment with dynamic high pressure on microbial populations, and lipolytic and proteolytic profiles of Crescenza cheese. *Int J Dairy Technol*, 57(1): 19-25.
- Lanciotti R, Gianotti A, Baldi D, Angrisani R, Suzzi G, Mastrocola D, Guerzoni ME (2005) Use of *Yarrowia lipolytica* strains for the treatment of olive mill wastewater. *Biores Technol*, 96(3): 317–322.
- Lanciotti R, Vannini L, Chaves Lopez C, Gobbetti M, Guerzoni ME (2005) Evaluation of the ability of *Yarrowia lipolytica* to impart strain-dependent characteristics to cheese when used as a ripening adjunct. *Int J Dairy Technol*, 58: 89–99.
- Li B, Chen F, Wang X, Ji B, Wu Y (2007) Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chem*, 102: 1135-1143.
- Li J, Chi Z, Wang X, Peng Y, Chi Z (2009) The selection of alkaline protease-producing yeasts from marine environments and evaluation of their bioactive peptide production. *Chin J Oceanol Limnol*, 27(4): 753-761.

- Li XX, Han LJ, Chen LJ (2008) In vitro antioxidant activity of protein hydrolysates prepared from corn gluten meal. *J Sci Food Agric*, 88: 1660-1666.
- Lima CA, Rodrigues PMB, Porto TS, Viana DA, Lima Filho JL, Porto ALF, Carneiro da Cunha MG (2009) Production of a collagenase from *Candida albicans* URM3622. *Biochem Eng J*, 43(3): 315–320.
- Liu J, Ru Q, Ding Y (2012) Glycation a promising method for food protein modification: Physicochemical properties and structure, a review. *Food Res Int*, 49: 170–183.
- Liu ZY, Dong SY, Xu J, Zeng MY, Song HX, Zhao YH (2008) Production of cysteine-rich antimicrobial peptide by digestion of oyster (*Crassostrea gigas*) with alcalase and bromelin. *Food Control*, 19: 231–235.
- Mackintosh JA, Veal DA, Beattie AJ, Gooley AA (1998) Isolation from an Ant *Myrmecia gulosa* of Two Inducible O-Glycosylated Proline-rich Antibacterial Peptides. *J Biol Chem*, 273(11): 6139-6143
- Mancini L, Lettenmeier M., Rohn H., Liedtke C (2011) Application of the MIPS method for assessing the sustainability of production-consumption systems of food. *J Econ Behav Organ*, 81(3): 779–793.
- Martínez-Maqueda D, Miralles B, Recio I, Hernández-Ledesma B (2012) Antihypertensive peptides from food proteins: a review. *Food Funct*, 3: 350-361
- Matoba S, Fukayama J, Wing RA, Ogrzydziak DM (1988) Intracellular precursors and secretion of alkaline extracellular protease of *Yarrowia lipolytica*. *Mol Cell Biol*, 8(11): 4904-4916.
- Mazur RH, Schlatter JM, Goldkamp AH (1969) Structure–taste relationships of some dipeptides. *J Am Chem Soc*, 91: 2684–2691.
- Meisel H (1997) Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers*, 43: 119–128.
- Meisel H (2004) Multifunctional peptides encrypted in milk proteins. *BioFactors*, 21: 55-61.
- Meloun B, Baudys M, Kostka V, Hausdorf G, Frommel C, Hohne WE (1985). Complete primary structure of thermitase from *Thermoactinomyces vulgaris* and its structural features related to the subtilisin-type proteinases. *FEBS Lett*, 183: 195-200.
- Mendis E, Rajapakse N, Kim S (2005) Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J Agric Food Chem*, 3: 581-587.
- Mills S, Stanton C, Hill C, Ross RP (2011) New Developments and Applications of Bacteriocins and Peptides in Foods. *Ann Rev Food Sci Technol*, 2: 299-329

- Mine Y, Kovacs-Nolan J (2006) New insights in biologically active proteins and peptides derived from hen's egg. *Worlds Poult Sci J*, 62: 87–95.
- Mizuno K, Matsuo H (1984) A novel protease from yeast with specificity towards paired basic residues. *Nature*, 309: 558–560.
- Moor CV, Ha EYW (1991) Off-flavors of whey protein concentrates: a literature review. *Int Dairy J*, 1: 1-11.
- Mullally MM, Meisel H, FitzGerald RJ (1997) Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. *FEBS Lett*, 402: 99-101.
- Nagase H, Enghild JJ, Suzuki K, Salvesen G (1990) Stepwise activation mechanisms of the precursor of matrix metalloproteinase and (4-aminophenyl) mercuric acetate. *Biochem*, 29: 5783-5789.
- Nakamura Y, Yamamoto N, Sakai K, Okubo A, Yamazaki S, Takano T (1995) Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J Dairy Sci*, 78(4): 777-783.
- Nedkov P, Oberthur W, Braunitzer G (1983) Die primärstruktur von Subtilisin DY. *Hoppe-Seyler's Z Physiol Chem*, 364: 1537-1540.
- Neelakantan S, Mohanty AK, Kaushik JK (1999) Production and use of microbial enzymes for dairy processing. *Curr Sci*, 77: 143–148.
- Nelson G (1986) Yeast extracellular proteases and their applications in the brewing industry. Ph.D. thesis, University of Birmingham.
- Nelson G, Young TW (1986) Yeast extracellular proteases for chill-proofing beer. *J Inst Brew*, 92: 599-603.
- Nielsen PM, Petersen D, Dambmann C (2001) Improved method for determining food protein degree of hydrolysis. *J Food Sci*, 66: 642-646.
- O'Mahony JA, Lucey JA, McSweeney PLH (2005) Chymosin-Mediated Proteolysis, Calcium Solubilization, and Texture Development During the Ripening of Cheddar Cheese. *J Dairy Sci*, 88: 3101–3114
- Ogrydziak DM (1993) Yeast extracellular proteases. *Crit Rev Biotechnol*, 13: 1-55.
- Oliver CM, Stanley RA, Melton L D (2006) Creating proteins with novel functionality through the Maillard reaction. *Cr Rev Food Sci Nutr*, 46: 337–350.
- Osawa T, Namiki M (1981) A novel type of antioxidant isolated from leaf wax of Eucalyptus leaves. *Agric Biol Chem*, 45: 735-739.



- Ota Y, Gomi K, Kato S, Sugiura T, Minoda Y (1982) Purification and some properties of cell-bound lipase from *Saccharomyces lipolytica*. *Agr Biol Chem*, 46: 2885-2893
- Otagiri K, Noshio Y, Shinoda I, Fukui H, Okai H (1985) Studies on a model of bitter peptides including arginine, proline and phenylalanine residues. I. Bitter taste of di- and tripeptides, and bitterness increase of the model peptides by extension of the peptide chain. *Agric Biol Chem*, 49(4): 1019-1026.
- Otvos L Jr, O I, Rogers ME, Consolvo PJ, Condie BA, Lovas S, Bulet P, Blaszczyk-Thurin M (2000) Interaction between Heat Shock Proteins and Antimicrobial Peptides. *Biochem*, 39: 14150-14159.
- Otvos L, Bulet P, Varga I, Hoffmann R (2002) Antibacterial insect glycopeptides: Synthesis, structure and activity. Peptides frontiers of peptide science. In "Proceedings of the Fifteenth American Peptide Symposium", Tam PJ, Kaumaya TP (Eds.), Vol 5 Session IX, June 14-19, 1997, Nashville (Tennessee), 2002, pp. 703-704.
- Outtrup H, Boyce COL (1990) Microbial proteinases and biotechnology. In "Microbial enzymes and biotechnology", Fogarty CT, Kelly K (Eds.). Elsevier (London), pp 227-254.
- Parfene G, Hornicar VB, Bahrim G, Vannini L, Gottardi D, Guerzoni ME (2011) Lipolytic activity of lipases from different strains of *Yarrowia lipolytica* in hydrolysed vegetable fats at low temperature and water activity. *Rom Biotech Lett*, 16(6): 46-52
- Patrignani F, Iucci L, Vallicelli M, Guerzoni ME, Gardini F, Lanciotti R (2007) Role of surface-inoculated *Debaryomyces hansenii* and *Yarrowia lipolytica* strains in dried fermented sausage manufacture. Part 1: Evaluation of their effects on microbial evolution, lipolytic and proteolytic patterns. *Meat Sci*, 75(4): 676-686.
- Patrignani F, Vannini L, Gardini F, Guerzoni ME, Lanciotti R (2011a) Variability of the lipolytic activity and volatile molecules production by a strain of *Yarrowia lipolytica* in pork fat and its dependence on environmental conditions. *Meat Sci*, 89(1): 21-26.
- Patrignani F, Vannini L, Gardini F, Guerzoni ME, Lanciotti R (2011b) Variability of the lipolytic activity in *Yarrowia lipolytica* strains in pork fat. *Meat Sci*, 88(4): 689-693.
- Pellegrini A (2003) Antimicrobial Peptides from Food Proteins. *Curr Pharm Des*, 9(16): 1225-1238.
- Perez Espitia PJ, de Fátima Ferreira Soares N, dos Reis Coimbra JS, de Andrade NJ, Souza Cruz R, Alves Medeiros EA (2012) Bioactive Peptides: Synthesis, Properties, and Applications in the Packaging and Preservation of Food. *Compr Rev Food Sci*, 11(2): 1541-4337.

- Phadatare SU, Srinivasan MC, Deshpande VV (1993) High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): enzyme production and compatibility with commercial detergents. *Enzyme Microb Technol*, 15: 72–76.
- Rada-Mendoza M, Olano A, Villamiel M (2002) Furosine as indicator of Maillard reaction in jams and fruit-based infant foods. *J Agric Food Chem*, 50: 4141-4145.
- Rajanbabu V, Chen JY (2011) Applications of antimicrobial peptides from fish and perspectives for the future. *Peptides*, 32(2): 415–20.
- Raksakulthai R, Haard NF (2003) Exopeptidases and Their Application to Reduce Bitterness in Food: A Review. *Crit Rev Food Sci Nutr*, 43(4): 401-445.
- Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiol Mol Biol Rev*, 62(3): 597–635.
- Razanarnparany V, Jara P, Legoux R, Delrnas P, Msayeh F, Kaghad M, Loison G (1992) Cloning and mutation of the gene encoding endothiapepsin from *Cryphonectria parasitica*. *Curr Genet*, 21: 455-461.
- Reddy KVR, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. *Int J Antimicrob Agents*, 24(6): 536–47.
- Reid VJ, Theron LW, du Toit M, Divol B (2012) Identification and Partial Characterization of Extracellular Aspartic Protease Genes from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384. *Appl Environ Microbiol*, 78(19): 6838–6849.
- Rival SG, Boeriu CG, Wichers H. J (2001) Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *J Agr Food Chem*, 49: 295–302.
- Roberts PR, Burney JD, BlackKW, Zaloga GP (1999) Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion*, 60: 332–337.
- Rodriguez C, Domínguez A (1984) The growth characteristics of *Saccharomycopsis lipolytica*: morphology and induction of mycelium formation. *Can J Microbiol*, 30(5): 605-612.
- Romeo D, Skerlavaj B, Bolognesi M, Gennaro R (1988) Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. *J Biol Chem*, 263(20): 9573–9575
- Ryan JT, Ross RP, Bolton D, Fitzgerald GF, Stanton C (2011) Bioactive Peptides from Muscle Sources: Meat and Fish. *Nutrients*, 3(9): 765-791.
- Sarmadi BH, Ismail A (2010) Antioxidative peptides from food proteins: a review. *Peptides*, 31(10): 1949-1956.
- Sauer M, Porro D, Mattanovich D, Branduardi P (2007) Microbial production of organic acids: expanding the markets. *Trends Biotechnol*, 26(2): 100-108

- Seguro K, Nio N, Motoki M (1996) Some Characteristics of a Microbial Protein Cross-Linking Enzyme: Transglutaminase. *Acs Sim Ser*, 21: 271–280
- Seki T, Kawasaki Y, Tamura M, Tada M, Okai H (1990) Further study on the salty peptide, ornithyl-beta-alanine. Some effects of pH and additive ions on the saltiness. *J Agric Food Chem*, 38(1): 25–29.
- Shalaby SM, Zakora M, Otte J (2006) Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. *J Dairy Res*, 73: 178-186.
- Sharpe FR, Jacques D, Rowsell AG, Whitear AL (1981) Rapid methods of measuring the foam-active nitrogenous components of worts and beers. In “Proceedings of the the European Brewery Convention Congress, 18th Congress, Copenhagen (Denmark), pp. 607- 614.
- Shimizu M, Lee SW, Kaminogawa S, Yamauchi K (1984) Emulsifying properties of an N- terminal peptide obtained from the peptic hydrolyzates of  $\alpha_{s1}$ -casein. *J Food Sci*, 49:1117-1120.
- Smacchi E, Gobbetti M (2000) Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiol*, 17(2): 129-141.
- Smith JE (1998) *Biotechnologie*. Zanichelli (Bologna), pp. 22-25.
- Spiro RG (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiol*, 12: 43R–56R.
- Srinivas S, Prakash V (2010) Bioactive peptides from bovine milk  $\alpha$ -casein: isolation, characterization and multifunctional properties. *Int J Pept Res Ther*, 16: 7-15.
- St Georgiev V (1990) Immunomodulatory activity of small peptides. *Trends Pharmacol Sci*, 11(9):373–8.
- Sudha ML, Baskaran V, Leelavathi K (2007) Apple pomace as a source of dietary fibre and polyphenols and its effect on the rheological characteristics and cake making. *Food Chem*, 104: 686-692.
- Sujatha S and Praharaj I (2012) Glycopeptide Resistance in Gram-Positive Cocci: A Review. *Interdiscip Perspect Infect Dis*, 2012: 1-10.
- Sumantha A, Larroche C, Pandey A (2006) Microbiology and industrial biotechnology of food-grade proteases: a perspective. *Food Technol Biotechnol*, 44(2): 211-220.
- Suzzi G, Lanorte MT, Galgano F, Andrighetto C, Lombardi A, Lanciotti R, Guerzoni ME (2001) Proteolytic, lipolytic and molecular characterisation of *Yarrowia lipolytica* isolated from cheese. *Int J Food Microbiol*, 69(1-2): 69-77.
- Takahashi M, Moriguchi S, Yoshikawa M, Sasaki R (1994) Isolation and characterization of oryzatensin: a novel bioactive peptide with ileum-contracting and immunomodulating activities derived from rice albumin. *Biochem Mol Biol Int*, 33: 1151–1158.

- Takeda K, Matsumura Y, Shimizu M (2001) Emulsifying and Surface Properties of Wheat Gluten under Acidic Conditions. *J Food Sci*, 66(3): 393-399.
- Temussi PA (2012) The good taste of peptides. *J Pept Sci*, 18: 73–82.
- Tong LM, Sasaki S, McClements DJ, Decker EA (2000) Mechanisms of the antioxidant activity of a highmolecular weight fraction of whey. *J Agric Food Chem*, 48: 1473–1478.
- Udenigwe CC, Aluko RE (2012) Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. *J Food Sci*, 71(1): 11-24.
- Vakhlu J, Kour A (2006) Yeast lipases: Enzyme purification, biochemical properties and gene cloning. *Electron J Biotechnol*, 9: 69-85.
- van Boekel MAJS (2001) Kinetic aspects of the Maillard reaction: a critical review. *Nahrung*, 45: 150-159.
- Van De Weert M, Randolph TW (2012) Physical Instability of peptides and proteins. In: “Pharmaceutical Formulation Development of Peptides and Proteins”, Hovgaard L, Frokjaer S, Van De Weert M (Eds), 2nd ed, CRC Press, pp 107-130.
- van den Tempel T, Jakobsen M (2000) The technological characteristics of *Debaryomyces hansenii* and *Yarrowia lipolytica* and their potential as starter cultures for production of Danablu. *Int Dairy J*, 10: 263-270.
- Vannini L, Baldi D, Lanciotti R (2001) Use of Fourier transform infrared spectroscopy to evaluate the proteolytic activity of *Yarrowia lipolytica* and its contribution to cheese ripening. *Int J Food Microbiol*, 69(1–2): 113–123.
- Vermeirssen V, Van Camp J, Verstraete W (2002) Optimisation and validation of an Angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *J Biochem Biophys Methods*, 51: 75-87.
- Wang HX, Ng TB (1999) Natural products with hypoglycemic, hypotensive, hypocholesterolemic, antiatherosclerotic and antithrombotic activities. *Life Sci*, 65(25): 2663–2677.
- Wang J, Zhao M, Zhao Q, Bao Y, Jiang Y (2007) Characterization of Hydrolysates Derived from Enzymatic Hydrolysis of Wheat Gluten. *J Food Sci*, 72(2): C103-C107.
- Wang Q, Ismail B (2012) Effect of Maillard-induced glycosylation on the nutritional quality, solubility, thermal stability and molecular configuration of whey protein. *Int Dairy J*, 25: 112-122.
- Wang S and Damodaron S (2009) Ice-Structuring Peptides Derived from Bovine Collagen. *J Agric Food Chem*, 57: 5501–5509.

- Wang SX, Lavery S, Dumitriu M, Plaas A, Grynopas MD (2007) The effects of glucosamine hydrochloride on subchondral bone changes in an animal model of osteoarthritis. *Arthritis Rheum*, 56(5): 1537-1548.
- Wiese B (1995) Die Mikroflora von Weizenvorteigen und ihr Einfluss auf die Aromabildung. Dissertation Universität Hannover, Fachbereich Biologie.
- Wieser H (2007) Chemistry of gluten proteins. *Food Microbiol*, 24(2): 115-119.
- Wijewickreme AN, Kitts DD, Durance TD (1997) Reaction conditions influence the elementary composition and metal chelating affinity of nondialyzable model Maillard reaction products. *J Agric Food Chem*, 45: 4577-4583.
- Wirsenius S (2008) The Biomass Metabolism of the Food System: A Model-Based Survey of the Global and Regional Turnover of Food Biomass. *J Ind Ecol*, 7(1): 47-80.
- Woessner J Jr (1995) Quantification of matrix metalloproteinases in tissue samples. In: "Methods in enzymology-proteolytic enzymes: aspartic and metalloproteinases", Barrett AJ (Ed.), Academic Press, San Diego (CA), pp. 510–528.
- Wohrmann APA (1996) Antifreeze glycopeptides and peptides in Antarctic fish species from the Weddell Sea and the Lazarev Sea. *Mar Ecol Prog Ser*, 130: 47-59.
- Wolfe KL, Liu RH (2007) Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods and dietary supplements. *J Agric Food Chem*, 55: 8896-8907.
- Wu Y and Wang D (2009) A New Class of Natural Glycopeptides with Sugar Moietydependent Antioxidant Activities derived from *Ganoderma lucidum* Fruiting Bodies. *J Proteome Res*, 8(2): 436–442.
- Yamada T, Ogrzydziak DM (1983) Extracellular acid proteases produced by *Saccharomyces lipolytica*. *J Bacteriol*, 154: 23–31.
- Yang B, Zhang X, Bao X, Lv Y, Zhang J, Guo S (2008) Glycopeptide derived from soybean b-conglycinin inhibits the adhesion of *Escherichia coli* and *Salmonella* to human intestinal cells. *Food Res Int*, 41: 594–599.
- Yang JK, Shih IL, Tzeng YM, Wang SL (2000) Production and purification of a protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme Microb Technol*, 26: 406–413.
- Yang R, Pei X, Wang J, Zhang Z, Zhao H, Li Q, Zhao M, Li Y (2010) Protective effect of a marine oligopeptide preparation from chum salmon (*Oncorhynchus keta*) on radiation-induced immune suppression in mice. *J Sci Food Agric*, 90: 2241–2248.

- Yang WH, Zhang WC, Lu XM, JiangGS and Gao PJ (2009) Characterization of a novel antibacterial glycopeptide produced by *Penicillium* sp. M03. *Lett Appl Microbiol*, 48: 393–397.
- Yegin S, Fernández-Lahore M, Guvenc U, Goksungur Y (2010) Production of extracellular aspartic protease in submerged fermentation with *Mucor mucedo* DSM 809. *Afr J Biotechnol*, 9(38): 6380-6386.
- Yen G, Wu J (1999) Antioxidant and radical scavenging properties of extracts from *Ganoderma tsugae*. *Food Chem*, 65: 375-379.
- Young TW, Wadeson A, Glover DJ, Quincey RV, Butlin MJ, Kamei EA (1996) The extracellular acid protease gene of *Yarrowia lipolytica*: sequence and pH-regulated transcription. *Microbiol*, 142(10): 2913-2921.
- Zhang SB, Wang Z, Xu SY (2008) Antioxidant and antithrombotic activities of rapeseed peptides. *J Am Oil Chem Soc*, 85: 521:527.
- Zhu A, Huang JB, Clark A, Romero R, Petty HR (2007) 2,5-Deoxyfructosazine, a D-glucosamine derivative, inhibits T-cell interleukin-2 production better than D-glucosamine. *Carbohydr Res*, 342: 2745-2749.
- Zioudrou C, Streaty RA, Klee WA (1979) Opioid peptides derived from food proteins: the exorphins. *J Biol Chem*, 254: 2446-2449.

## Web references

[http://ec.europa.eu/food/food/biosafety/animalbyproducts/index\\_en.print.htm](http://ec.europa.eu/food/food/biosafety/animalbyproducts/index_en.print.htm)

<http://en.wikipedia.org/wiki/Glycopeptide>

<http://en.wikipedia.org/wiki/Vancomycin>

<http://www.freepatentsonline.com/y2012/0283174.html>

<https://usgene.sequencebase.com/patents/US20060083847>

## ACKNOWLEDGMENTS

Another step of my life has arrived at the end. Such a long way since I arrived at the Food Microbiology Lab but it is quite satisfactory to see the outcome of my adventure. I would like to acknowledge myself because I reached this result but I have to admit that everything was possible just because of the assistance of the following people.

I am particularly grateful to my supervisor, Prof. Maria Elisabetta Guerzoni, who dared to trust in me and whose incredible creativity and support gave me feedback and inspirations during my entire PhD career. Her time and continuous guidance are hugely appreciated.

I am also thankful to my other supervisor, Dott.ssa Lucia Vannini, who always believed in me and gave me complete freedom as well as her support to conduct my research.

My deep appreciation is for my Canadian supervisor Prof. Mirko Betti, who gave me the chance to live the most professional experience of my life. Without his support and his trust in me I could not have been able to further my investigation. I will always keep with me all his suggestions.

A sincere thanks to Dr. Maurice Ndagijimana for all the scientific discussions we had and his constant and respectful presence, constantly next to me with precious suggestions.

Prof. Fausto Gardini and Prof.ssa Rosalba Lanciotti, my gratitude is also extended to you.

I would like to thank all my friends and colleagues of the Food Microbiology Lab at the University of Bologna: Diana Serrazanetti, Chiara Montanari, Giulia Tabanelli, Pamela Vernocchi, Francesca Patrignani, Giorgia Gozzi, Lucia Camprini, Lorenzo Siroli, Rodrigo Troncoso, Lucilla Dei Più and Danielle Taneyo. We lived, worked and suffered together and we always supported each other.

A personal thanks to "my" technician Luciana Perillo, that followed my "first steps" in the microbiology lab and she has always supported me during these years.

I would also like to show my appreciation to Danka Bukvicki and Amit Kumar Tyagi because they became some of my best friends rather than colleagues, and they have continuously provided me with their consideration as well as with moral support through the distance.

Since I spent almost one year in Canada, I have to offer my gratitude for the support and friendship received from all my friends and colleagues at the University of Alberta, in Prof. Betti's

Lab: Pui Khoon (Kimi) Hong, Zied Khiari, Yulija Hrynets, Lihui (Mavis) Do, Henan Wang; and those in the Microbiology lab: Alma Fernanda Sanchez Maldonado, Petya Koleva and Xiaoji (Christine) Liu. A thanks goes also to all the other people that I met in Edmonton and that made my permanence there as familiar as possible in some way: Lynda and Alan Jones, Sam Cheng, Soledad Urrutia, Maricruz Ormeño Urrutia, Sahar Navidghasemizad and also all the "Chinese basketball players".

My old friends Andrea Bonora, Luca Vanini, Luca Brochetto, Caterina Villani who have always encouraged and granted me with their friendship.

A special and affectionate thanks to Emma Hernandez Sanabria, because from the beginning she has been patient and supportive with me, giving me strength and love.

I would like to express my sincere admiration to my grandparents, but most importantly to my parents, who have been incredibly encouraging along my Ph.D. They all have undoubtedly made sacrifices and it is due to them that I have found the motivation and resolution to continue straight and committed.







