Università di Parma

Dottorato di Ricerca in

Scienze e Tecnologie Alimentari

Ciclo XXVI

Non Proteolytic Aminoacyl Derivatives (NPADs) in cheeses: Synthesis, Origin, Properties

Tutor

Prof. Stefano Sforza

Dottorando

Chiara Bottesini

Coordinatore

Prof. Furio Brighenti

Abstract

The present Ph. D. thesis is focused on the characterization of unusual aminoacidic derivatives (γ-glutamyl amino acids, lactoyl amino acids, pyroglutamyl amino acids), collectively named Non Proteolytic Aminoacyl Derivatives (NPADs), present in Parmigiano Reggiano cheese, considering in particular their structural characterization, the origin and biological-functional properties.

After a general introduction concerning cheese peptidomics approaches, bioactive peptides, peptides synthesis/analysis and previous results about NPADs, the first part deals with the use of NPADs as a marker of cheese ripening, through appropriate statistical analysis.

The enzymatic production and degradation of NPADs are then examined in depth in the third chapter, indicating which enzymes in cheese might be responsible for their production and eventual degradation. Also bioaccessibility was evaluated , demonstrating the resistance of NPADs to gastrointestinal digestion and blood serum. Also the bioavailability of γ-Glutamyl-Phenylalanine, the most important representative of NPADs, was studied using Caco-2 model.

The last part reports the first studies about the immunomodulatory and antioxidant properties of NPADs.

Keywords: Parmigiano Reggiano cheese, Non Proteolytic Aminoacyl Derivatives, γ-Glutamyl-Phenylalanine, Lactoyl-Phenylalanine, marker of ripening, immunomodulatory property, antioxidant capacity, intestinal permeability.

Riassunto

La presente tesi di dottorato riguarda la caratterizzazione di derivati amminoacidici (γ-glutamil amminoacidi, lattoil-amminoacidi, piroglutamil-amminoacidi), denominati Non Proteolyltic Aminoacyl Derivatives (NPADs) presenti nel Parmigiano Reggiano, considerando in particolare la caratterizzazione strutturale, l'origine e le proprietà biologico-funzionali.

Dopo un'introduzione generale riguardante lo studio e l'evoluzione peptidica nel formaggio durante la stagionatura, i peptidi bioattivi, la sintesi/analisi peptidica ed i risultati riportarti in letteratura riguardanti gli NPADs, la prima parte della tesi riguarda l'uso di tali molecole come marker di stagionatura avvalendosi dell'analisi statistica.

La produzione e la degradazione enzimatica degli NPADs sono esaminate nel terzo capitolo, indicando i potenziali enzimi responsabili della loro produzione ed eventuale degradazione. Inoltre, la bioaccessibilità di tali composti è stata studiata nel medesimo capitolo dimostrando la resistenza degli NPADs alla digestione gastrointestinale simulata ed al siero umano. La biodisponibilità della γ-glutamil-fenilalanina, molecola maggiormente rappresentativa degli NPADs, è stata studiata utilizzando un sistema che impiega cellule Caco-2.

L'ultima parte, infine, riporta i primi risultati riguardanti le proprietà immunomodulatorie ed antiossidanti degli NPADs.

Parole chiave: Parmigiano Reggiano, Non Proteolytic Aminoacyl Derivatives, γ-Glutamil-fenilalanina, Lactoil-fenilalanina, marker di stagionatura, proprietà immunomodulatorie, capacità antiossidante, permeabilità intestinale.

Table of content

CHAPTER 1

General introduction

1.1 Cheese peptidomics: the case of Parmigiano Reggiano cheese

Parmigiano Reggiano cheese is a product regulated by a PDO (Protected Designation of Origin, EU regulation 2081/92) produced following traditional norms.

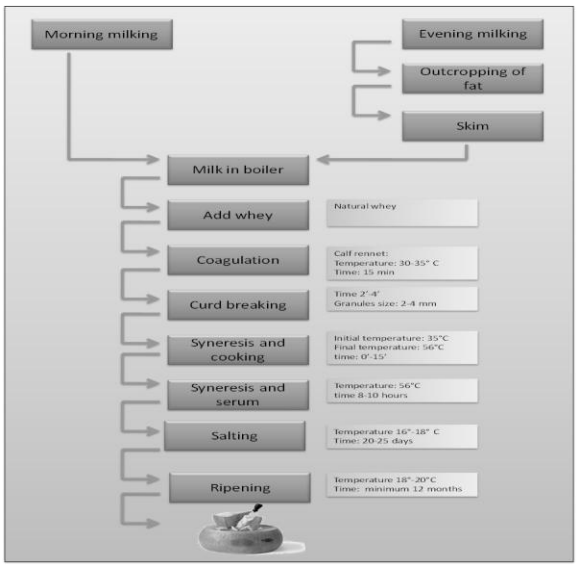

Figure 1: flowsheet of Parmigiano Reggiano cheese production

The skimmed milk from evening milking and from morning milking are used for Parmigiano Reggiano cheese production. The whey is added to the milk in order to select favorable lactic microflora, mostly composed by *L. helveticus, L. delbrueckii sub., bulgaricus, Lactobacillus delbrueckii* spp. lactis (homofermentative) and *L. fermentum* and *L. brevis (*heterofermentative). The acidity of the whey is near 28-35°SH\50ml (pH<4), containing 10^8-10^9 bacteria per ml, leading to a pH reduction.

The curdling is performed through the use of stomach calf rennet containing chymosine and pepsin. The rennet coagulation starts with the

hydrolysis of the peptide bond between phenylalanine and methionine in 105 and 106 k-casein position. The consequence is the formation of macropeptide 106-169 and 1-105 para-k-casein. The k-casein is very important for the caseins structure, because confers hydration and stability, protecting the fraction αs_1, αs_2 and β from the precipitating action of calcium (1,2,3)

The second phase concerns the hydrolysis of the macropeptide, leading to a decrease of micelle hydration, modifying the electric charge causing the micellar aggregation and eventually leading to casein precipitation

Several factors influence this process, such as: casein quality, pH, mineral content, temperature, enzyme properties (type and amounts). The behavior of the enzymatic and aggregative phases are related to the temperature: the first step proceeds better at 5°C, while the coagulation between 30-35 °C.

The syneresis of the serum from the curd granules through the use of the "spino" (a bone stick) is fundamental, and this process is supported by cooking at high temperature (56°C for 4-7 minutes according to the humidity content expected for final product). The cooking allows the selection of thermophilic microflora improving the acidification and removing the lactose. This also induces the modification of the chemical-physical characteristic of the matrix with the accumulation of primary metabolites, such as lactic acid. The purging of the curd is performed in few hours and the cooling is not homogeneous in all parts of the wheel, since in the external part the temperature decrease quicker than the internal part. The curd is then placed in a saline solution in order to complete the serum purging, the increase of salt and the crumble formation, reducing the water activity.

The maturation hold over minimum 12 months, and in this phase several reactions/modifications take place. Cheese ageing is a rather complex process involving lipolysis, glycolysis and proteolysis, and various chemical and biochemical conversions of milk components down to low molecular weight compounds (4,5,6).

The biochemical changes are grouped into primary (lipolysis, proteolysis and metabolism of residual lactose, lactate, citrate) and secondary (metabolism of fatty acids and amino acids). Residual lactose is metabolized to lactate, lypolisis is catalyzed by lipase from milk and cheese microflora, but proteolysis is the most complex biochemical event (13).

In particular the proteolysis, occurs during the ageing, is due to a enzymatic process and exert on the milk protein, especially on casein. The results of the proteolysis are the production of peptides of different size and amino acids. Several proteases degrade the proteins and then peptidases cleave the peptides. The breakages of the peptide bond are performed in a specific site depending by the enzyme.

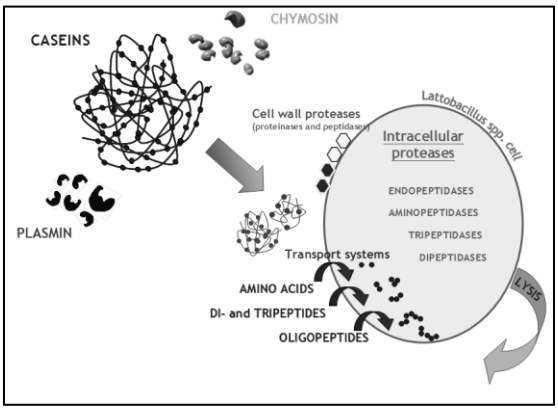

Figure 2: Proteolysis scheme (13)

The proteases in the milk and calf rennet are respectively plasmin and chymosin (6,7).

The peptidases are divided in endopeptidases and exopeptidases, respectively able to cleave external or internal peptide bonds, belonging to starter and non-starer microflora (8,9,10).

Lactic acid bacteria (LAB) hydrolyze the protein in order to produce peptides compatible with the transport through the cellular membrane. The proteolytic system of the LAB is divided in three groups depending the function and the localization: proteases CEP (Cell Envelope Proteinase), system OPP (Oligopeptide Permease System) and peptidases. The lactic acid bacteria possess, also, intracellular oligopeptidases (PepO and PepF) and generic aminopeptidases as PepN, PepC and PepG), a gluamyl-aminopeptidase (PepA), pyrrolidon carboxypeptidase (PCP), leucylcaminopeptidase (PepL), X-prolyldipetidil-aminopeptidas (PepX), prolin-imminopeptidase (PepR), prolidase (PepQ), generic dipeptidas (PepV, PepD, PepDA) a generic tripeptidase (PepT) and a specific system for the amino acids transport.

In general, the peptides generated by chymosin and plasmin are the first substrates for the CEP activity (from *Lactococcus spp*.) in order to obtain smallest peptides. The exocellular proteases exert their action on proline residues and on the hydrophobic regions of caseins. The carboxypeptidase activity is not typical for the LAB, but a similar activity has been detected in *L. helveticus* (11).

Recently, Sforza et al. (2012) (14), studied the cheese peptidomics in Parmigiano Reggiano cheese, where five trends in peptide evolution were outlined during ageing and correlated to the enzymatic activities present in the cheese.

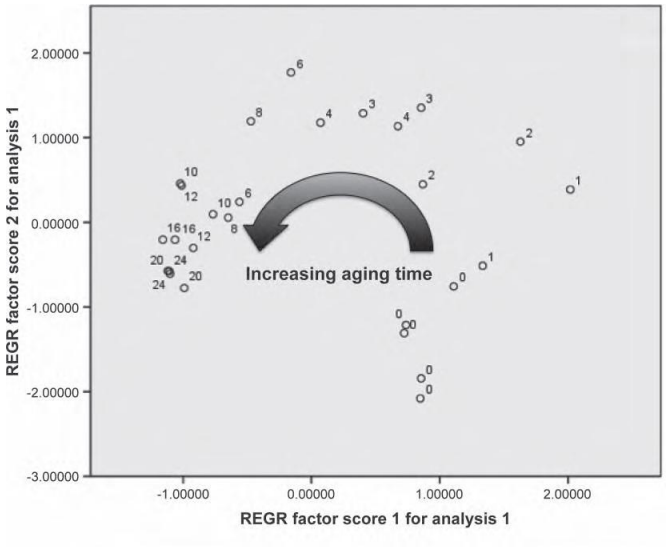

Figure 3: graph of factorial score obtained by considering the peptide amounts in several samples and showing how samples cluster according to their ageing time (14).

In this work, the detected peptides were characterized and semiquantified. The PCA (principal component analysis) showed that samples cluster in accordance with the respective ageing time characterizing the cheese peptide composition.

β-CN 1-23 is the most important peptide belong to the first group, formed by chymosin activity. The decreasing trend is a clear indication that this peptide and others in this group are rapidly degraded during the first hours after curding, and up to the first months, by the CEP of LAB present in the starter culture. The different temperature between internal and external part determines a different bacterial activity and thus different rates of degradation of these peptides.

The second group is composed by peptides formed by LAB proteases using peptides of the first group as substrate.

The third and fourth groups are composed prevalently by phophopeptides produced by NSLAB. The prolonged production in the internal part, leading to a delayed rate of formation of these peptides, indicate that this environment is optimal for enzymatic activity producing these peptides. The fifth and final group is characterized by a constant increase during ageing time, and it mainly include NPADs (Non Proteolytic Aminoacyl Derivatives). The evolution of peptide composition in Parmigiano Reggiano cheese is thus related with the ageing time and the microbiota present in the cheese (14).

1.2 Bioactive peptides

Various food protein sources including fish, milk, egg, soybean, wheat and zein, have been exploited to produce bioactive peptides.

Milk contain several proteins, such as whey proteins, including immunoglobulins, α-lactalbumin, β-lactoglobulin, bovine serumalbumin, immunoglobulin, lactoferrin as well as proteose-peptone fractions and transferrin. Caseins are the main component in the milk, about 80% of the total protein. They exert a physiological role, since are source of amino acids for growth of the neonate and as prevention of pathological calcification of mammary gland.

Much research has been carried out on bioactive peptides from casein hydrolyzates. The peptides are not active within the parent protein but can be released and activated following enzymatic hydrolysis.

The bioactive peptides are defines as a food component that could influence the biological processes and exert a specific action in the human body. Biologically active peptides can be produced from precursor milk proteins in the following ways:

- enzymatic hydrolysis by digestive enzymes;
- fermentation of milk with proteolytic starter culture;
- proteolysis by enzymes derived from microorganisms or plants.

Hydrolysis is a reaction which involves cleavage of the peptide bond fragmenting proteins into smaller peptides or free amino acids. Hydrolysis can be achieved using enzymes or chemicals (15)

Lactic acid bacteria (LAB) use milk proteins as their source of growth. The bioactive peptides can be generated by starter and non starter bacteria used in manufacture of fermented dairy products. In the second

chapter is explained the mechanism of action of proteolytic system of LAB.

The gastrointestinal digestion is considered a physiological process in which bioactive peptides might be produced. Dietary proteins and peptides are subjected to hydrolysis during the various stages, namely ingestion, digestion and absorption. The enzymes involved are pepsin, trypsin and chymotrypsin.

The intestinal brush border membrane is particularly rich in aminopeptidase activity. In addition to these enzymes, the intestinal brush border also contains endopeptidase and dipeptidase activity.

The bioactive peptides can exert several activity: opioid, antioxidant, antimicrobial, immunomodulatory, anti-hypertensive, appetite regulation (16,17).

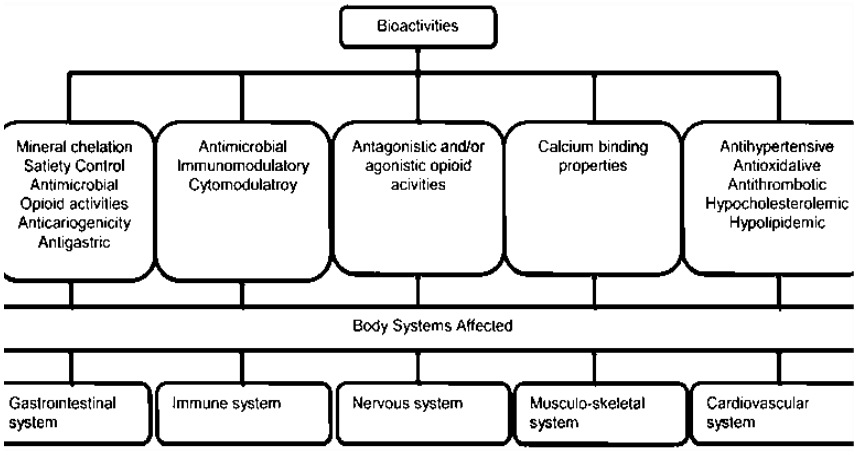

Figure 4: bioactive peptides properties (17)

1.2.1 Anti-hypertensive and anti-thrombotic peptides

The peptides that exert their function on cardiovascular system are peptides with antithrombotic and antihypertensive activity. The first property is defined as the interaction with a coagulating enzyme, such as in the clotting of blood the interaction of fibrinogen with thrombin, casoplatelins (k-casein f106-116, f106-112, f113-116) can prevent blood clotting through inhibition of platelet aggregation.

Antihypertensive peptides influence the can influence the renin angiotensin system inhibiting the ACE (angiotensin converting enzyme). In fact, blood pressure regulation is partially dependent on the renin angiotensin system. Renin acts on angiotensinogen, thus releasing angiotensin I that is further converted into the active peptide hormone angiotensin II, a vasoconstrictor, by the angiotensin converting enzyme (ACE). For example, the sequences into f177-183 and f193-202 of β-casein and f23-24, f23-27 and f194-199 of α_{s1}-casein are considered ACE-inhibitory peptides.

The amino acid sequences Ile-Pro-Pro from k-casein and Val-Pro-Pro from β-casein are also potent antihypertensive peptides.

1.2.2 Opioid peptides

Opioid peptides are defined as peptides like enkephalins that have both affinity for opiate receptor and opiate-like effects which are inhibited by naloxone. The typical opioid peptides all originate from three precursor proteins proopiomelanocortin (endorphins), proenkephalin (enkephalin) and prodynorphin (dynorphins). For example the N-terminal sequence Tyr-Gly-Gly-Phe is a sequence of typical opioid

peptide. The atypical opioid peptides have agonistic or antagonistic activities and are characterized by a distinct N-terminal sequence. For example an opiate like peptide is Tyr-Pro-Val-Glu-Pro-Phe-Thr-Glu.

1.2.3 Immunomodulator and antimicrobial peptides

The human body's defense against invaders mostly relies on the immune system or on the inhibition of pathogenic bacteria.

The first category involves several peptides having the ability to stimulate the immune system, as Val-Glu-Pro-Ile-Pro-Tyr from human β-casein, or f63-68, and f191-193 from bovine β casein.

Peptides derived from α_{s1}-, k-casein have been shown to both stimulate and suppress lymphocyte proliferation. GMP (glycomacropeptide) and its peptic digest were able to promote proliferation and phagocytic activities of human macrophage-like cells. The anti-tumour effects observed in many studies with fermented milk products may be attributed to immunopeptides formed during fermentation

For example, casein phosphopeptides (CPP) are casein derived peptides released in the gastrointestinal tracts which, among other functions also stimulate immunoglobulinA (IgA) production in mice.

The mechanism by which milk-derived bioactive peptides exert their modulatory effects on the immune system is not yet fully understood. In literature is reported the hypothesis that the mechanism is based on the relationship between the immune system and opioid peptides. The immunoreactivity of lymphocytes is connected with the receptor of opioid peptide, because opioid receptors for endorphins are present in T lymphocytes and human phagocytic leukocytes.

The antimicrobial peptides have common characteristics: size 30-60 amino acids, strong cationic (pI 8.9-10.7), heat stable (100°C, 15 min) and hydrophobic nature. These peptides are able to kill target cells rapidly, while having a broad spectrum of activity: they interact with the target site and they interfere with membrane function causing bacterial cell death, but also they inhibit protein, or cell-wall synthesis, interact with DNA or RNA or inhibit some sort of enzymatic activity.

Caseicidin exhibits activity against *Staphylococcus* spp. *Sarcina* spp. *Bacillus subtilis, Diplococcus pneumoniae* and *Streptocusccus pyogenes*; also GMP (Glycomacropeptide) has also been shown to have antimicrobial effects (19,19,20).

1.3.4 Bioavailability and Bioaccessibility

The bioavailability is the fraction of ingested nutrient that is available for the utilization in normal physiologic functions and for storage. The bioaccessibilty is the fraction released from food matrix, available for the intestinal absorption.

In order to define the bioavailable and/or bioaccessible compounds it is necessary to consider the digestion process, the metabolism and the absorption. In short, digestion of proteins begins in the stomach and continues in the luminal phase of the small intestine, which results in a mixture of oligopeptides and free amino acids. The intestinal brush border has an important role, because is rich in amminopeptidase, carboxypeptidase and dipeptidase activity, and has an absorption function.

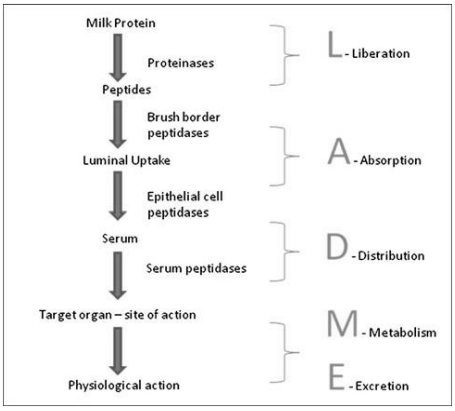

Figure 5: path of peptides and their actions (53)

Peptides, in order to be bioaccessible, must be resistant to gastrointestinal, brush border and serum peptidases; furthermore, to be bioavailable, these peptides must escape hepatic metabolism while making their way to the target organ or site of action.

The intestinal epithelial cells are those devoted to the absorption.

Moreover, the intestine has two other functions: barrier to dangerous compounds/organisms, including detoxification enzymes and efflux transporters inhibiting the invasion by harmful compound into the body, and signal recognition and transduction, because enteroendocrine cells on the intestinal epithelial cells are known to recognize food derived nutrients.

The apical cell of the membrane of intestinal epithelial cells has many transporters for several compounds. The nutrients are apically taken by these transporters into the intestinal epithelial for the excretion from the cell to blood circulation. There are three mechanism of transport:

passive diffusion (or paracellular transport), intracellular vesicle (transytosis) and transport mechanism.

Minerals are absorbed by passive diffusion where the transport is regulated by the permeability of the tight junction. High molecular compounds are transported by vesicle mediated transport system.

Amino acids and peptides are transferred across the membrane by transporters, such as PepT-1.

For example, the transport of peptide VPP was studied. The transport by intestinal epithelial cells is likely partly performed by paracellular pathway, because is a non-degrading route. The presence of free valine anyway indicated that part of peptide is degraded due to the enzymes present in brush border membrane, probably intracellular peptidases.

Food intestine interaction influence of course the bioavailability of nutrients, and cell based assays aimed to test this are important in order to study and develop functional foods(21,22,23,53).

1.3 Peptide analysis

Several methods are used in order to analyze the peptides, especially when the peptides are included in a complex matrix, such as a food.
First of all, the peptide fraction extracted from a food matrix needs an analytical technique with high separative power in order to detect every component. The methodologies mainly used are: liquid chromatography (for example reverse phase, ionic exchange and size exclusion) and electrophoretic technics (gels and capillary).
The liquid chromatography with high pressure (HPLC) or ultra high pressure (UHPLC), is characterized by the use of particular columns with variable internal diameter and length, depending by the use. In general, the mainly technique used is the RP-LC, based on the hydrophobic interactions between peptides and apolar stationary phase.
The peptide elution is performed in increasing order of dimension and polarity: from low MW/polar, to high MW/apolar peptides. The elution is due to the mobile phase constituted, in general, by water and acetonitrile.
The liquid chromatography is easily connectable to a mass spectrometer. A Mass spectrometer is an analytical instrument able to determine the ratio mass/charge of ion in gas phase. A mass spectrometer is composed by: source, interface, analyzer and detector. The result of the ionization, ion separation and detection generates a mass spectrum.
The electrospray ionization is very important for the combination with LC/MS and mass spectrometry. In this specific source, the chromatographic flow is sprayed from a little needle in a region with an electric field. The gas and the high temperature applied, induce the solvent evaporation. Droplets containing ions are formed. Multiple

charged gas-phase ions are subsequently formed during the desorption process due to the evaporation of the solvent, and these ions will then enter the mass analyzer.

The quadrupole allows the ion selection with different mass/charge ratio in relation with the variable electric fields in the time. The quadrupole is made by four metal bars, with length 20 cm, that delimit the way of the ions from the ionization room to the detector. At the bars is applied a wavering electromagnetic potential in order to have 2 bars with positive potential and the other 2 with negative potential in an alternating way. The alternating electric field allows only ions having a suitable mass-to-charge ratio compatible with the repulsive fields, and therefore in resonance with the variations of polarity of the bars, to pass through the quadrupole mass filter. The analysis of the spectral data yields several information, such as accurate measure of the molecular mass, which can be defined till the fourth decimal number for high resolution mass spectrometers, and specific fragmentation spectra for every compound useful to fingerprint any given molecule.

Tandem mass spectrometry, or MS/MS, in which intact compounds are fragmented in a collision chamber, is very important for the identification of peptides present in complex mixtures or or molecules with unknown structure. MS/MS is performed in triple quadrupole instruments: in the first quadrupole is possible to select the ion of interest (precursor ion or parent ion), and then this ion is fragmented in the second quadrupole and the yielded ions (daughter ions) are analyzed in the third quadrupole.

Another advantage of MS/MS is the fact that the selectivity applied to the process of selection of precursor ions reduce the signal-to-noise

ration and the ion suppression effects due to the matrix, allowing a better characterization and quantification of compounds.

Among high resolution instruments, LTQ Orbitrap has a peculiar way to measure mass : ions are generated by a ESI source, then go to a linear trap, that further focus the ions and also acts as a first analyzer (full scan or single ion monitoring) and collision cell for the next MS/MS experiment. Then the ions outbound from the trap to go into in the C-Trap, where a continue flow of ions is transformed in a cluster, which is then sent to the orbitrap in a subsequent time.

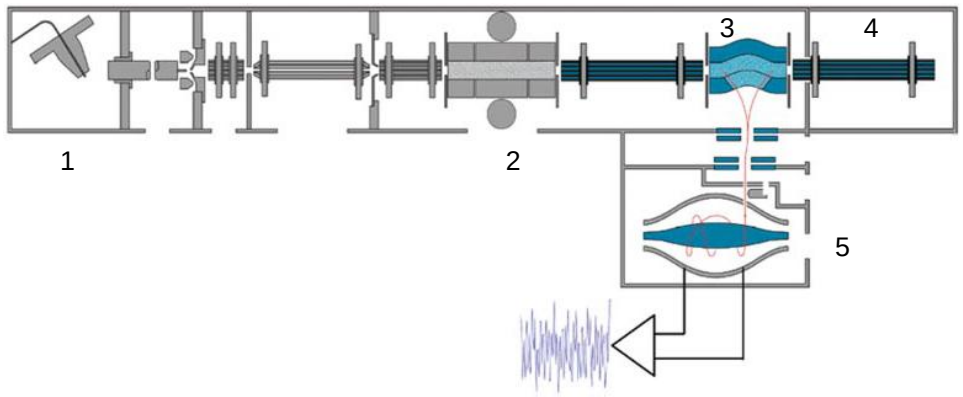

Figure 6: LTQ Orbitrap. Composed by: 1) sourse ESI; 2) linear trap; 3) ottapolo of transfering; 4) C-trap; 5) orbitrap

The ions, in the orbitrap, are then trapped in a electric field and begin to move around a central fuse with an orbital motion on the axes x/y and traslational on the z axis. The motion of all the ions generate a radio frequency. The mathematical algorithm FFT (fast fourier transformation) is then used in order to extract the single radio frequencies of every single m/z species present in the analyzer (24,25,26,27,28).

1.4 The peptide synthesis

1.4.1 Chemical synthesis

Peptides and proteins are composed by the amino acids. The amino acids are compounds containing a carboxyl group, an amino group and different R group substituting the hydrogen on the α-carbon atom. Many amino acids are known, but only 20 α-amino acids, in the L-enantiomeric form are present in the protein.

These compounds are classified in essential and non essential amino acids (according to the their nutritional characteristics) and in non polar/aliphatic , polar/uncharged, positively charged, negatively charged and aromatic, according to the chemicals characteristics derived from the side chain (36,37).

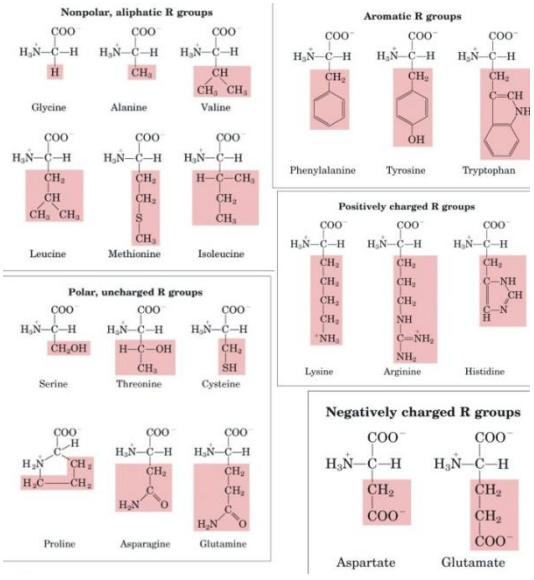

Figure 7: amino acids and their classification

Amino acids undergo reactions linked to the presence of carboxyl and amino groups; for example, the esterification of the carboxyl group, acylation/alkylation of the amino group, and also reactions involving the side chains.

Peptides are oligomers made by amino acids, since two amino acid molecules can be covalently linked through an amide linkage, termed a peptide bond. Peptides are a heterogeneous class of compounds, according to the amino acids composition and length of the chain.

When a peptide is composed by few amino acids is called oligopeptide, whereas the term polypeptide is used when several amino acids are linked (37).

Peptides have at least two functional groups: they have an amino group (basic) and a carboxylic group (acid). The α-amino group free on N-terminal residue has a pKa slightly lower than that of the corresponding free amino acids and the free α-carboxylic group on the C-terminal residue has a pKa slightly higher than the corrisponding free amino acids. Moreover, also the ionizable group from the side chain might contribute to acid-basic properties of peptides.

Peptides of interest can be obtained either by a chemical or by a biological way: chemical synthesis or DNA recombinant technology.

The chemical synthesis includes two strategies: in homogeneous phase and in solid heterogeneous phase. In both cases the reaction begins from the C-terminal side and proceeds to N-terminal side in order to extend the chain. The major breakthrough in this technology was provided by R. B. Merrifield in 1962 (29). His innovation involved synthesizing a peptide while keeping it attached at one end (the C-terminal) of a solid support.

The different strategies of synthesis present advantages and disadvantages. For example in the solid phase case the synthetic steps are more fast, there are no solubility problems and the procedure is easy, but the disadvantage is the lack of purification and of characterization of the intermediate compounds, ending up with several impurities in the final product.

The possibility to purify intermediate compounds and final products is the advantage of the homogenous synthesis, but the very slow procedure and solubility problems makes it less appealing. In general the synthesis in liquid phase is used only for short peptides.

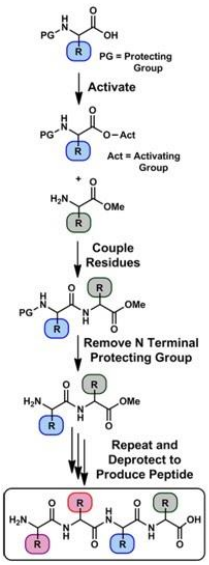

Figure 8: scheme of peptide synthesis

For the purpose of peptide synthesis, amino acids can be considered as having two main functionalities to manipulate,the α-amino and carboxyl groups. Functional groups are also present in the side chains of amino

acids, and these functionalities must be protected so that they do not interfere with the formation of the peptide bond.

Thus, for the synthesis are necessary several steps: protection of the amino group, activation of the carboxyl group of the next amino acid, coupling and selective deprotection of the amino group of the new amino acid inserted (30,31).

A peptide coupling reagent with an appropriate racemization suppressing agent, assures suppression of the undesired racemization and other side reactions, and thus minimizes the loss of the optical integrity at the chiral center (34,38).

Solid phase peptide synthesis (SPPS) can be defined as the process in which a peptide is obtained by the successive addition of the protected amino acids constituting its sequence to the firs C-terminal amino acid, anchored via its C-terminus to an insoluble polymer.

Each amino acid addition is referred to as a cycle consisting of: cleavage of the N^{α}-protecting group, coupling of a protected amino acid, intermediate washing steps. The growing chain bound to an insoluble support. There are two main strategies in SPPS: Fmoc and Boc. The Boc group, labile in acidic environment, has been used during the first 15 years of SPPS.

The introduction of a new type of protecting group has offered more flexibility for the modification of the peptide chain and/or more specificity in the cleavage of the Nα-versus the side-chain protecting groups. The Fmoc strategy employs a 9-Fluorenylmethoxycarbonyl chloride as protecting group, stable in acid environment and which can be removed in alkaline environment through β-elimination. Each strategy is characterized by the use of different protecting groups, reagents and solvents. At the end of the synthesis the peptide is released

from the solid support through the scission of the bond between C-terminal group of the sequence and the resin, in conditions which depend on the linker present on the solid support (32,33).

1.4.2 Recombinant DNA Techniques

Proteins can also be expressed in cell cultures of bacteria, yeasts, molds, mammals, plants or insects, or via transgenic plants and animals. Protein quality, functionality, production speed, and yield are the most important factors to consider when choosing the right expression system for recombinant protein production. The biosynthesis of a foreign gene product (protein) in an organism relies on a recombination of the genetic material of the microorganism with the DNA fragment encoding for the desired protein. The process includes the following steps: isolation of the encoding DNA fragment from the donor organism, insertion of the DNA into a vector, transfection of the vector into the host organism, cultivation of the host organism (cloning), which leads to gene amplification, mRNA synthesis, and protein synthesis and isolation of the recombinant protein. These cell-based, protein manufacturing technologies offer many advantages, producing recombinant important proteins that are safe and available in abundant supply for pharmaceutical and food industry. On the other side, their main drawback, beside the small amount produced, is that unnatural amino acids or unconventional linkings are not possible, being these procedures limited by the biological feasibility (38).

1.5 NPADs:Non Proteolytic Aminoacyl Derivatives

NPADs are synthesized in cheese de novo by an enzymatic activity that uses free amino acids as precursors. The most representative compounds are molecules having an acylic γ-glutamyl portion (47). In Comtè cheese γ-glutamyl-phenylalanine (γ-Glu-Phe), γ-glutamyl-tyrosine (γ-Glu-Tyr) and γ-glutamyl-leucine (γ-Glu-Leu) had been identified in the past. This compounds affect the flavor: for example γ-Glu-Tyr is acid and salty, γ-Glu-Phe present a complex taste close to Umami recently named "Kokumi" (43,44).

In according with the literature, the presence of γ-glutamyl amino acids and peptides such as γ-glutamyl-leucine, γ-glutamyl-valine and γ-glutamyl-cysteinyl-β-alanine in non ripened food such as beans, was also identified. Also in the garlic the presence of γ-glutamyl compounds was determined, and proposed to have the function of storage of nitrogen and sulfur (45).

In the Parmesan ham the presence of γ-Glu-Phe, γ-Glu-Ile and γ-Glu-Leu was also identified and found to increase during the ripening period, and to be connected with the aroma of the final product (46).

Similar compounds, such as pyroglutamyl amino acids, were detected in wheat, mushrooms and hard cheeses. Closely related compounds, lactoyl amino acids, were reported in Parmigiano Reggiano cheese together with the two previous classes of compounds (46,48,49).

Sforza et al., in 2009 (47), demonstrated the amount of these compounds in aged Parmigiano Reggiano cheese can be close to 50mg/100g of ripened cheese.

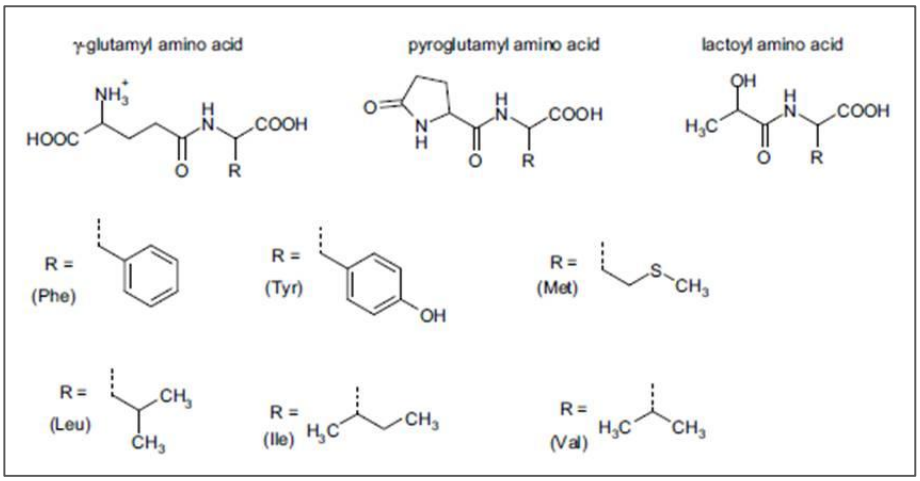

Figure 9: Non Proteolytic Aminoacyl Derivatives (NPADs) (47)

Notably, in Parmigiano-Reggiano cheese, these latter molecules seemed to be evenly distributed in the internal and external part of the wheel, indicating that the enzymes involved are not affected by the different environment present in the different parts of the wheel (47).

1.6 Microbial origin of non proteolytic amino acyl derivatives in long ripened cheeses

(based on E. Sgarbi, C. Lazzi, L. Iacopino, C. Bottesini, F. Lambertini, S. Sforza, M. Gatti ,2013, Food Microbiology, Volume 35, Issue 2, Pages 116-120)

Cheese ripening involves a complex series of biochemical events that contribute to the development of each cheese characteristic taste, aroma and texture. Proteolysis, which has been the subject of active research in the last decade, is the most complex of these biochemical events (40).

However, also aminoacyl derivates of non-proteolytic origin (γ-glutamyl-amino acids and lactoylamino acids) with interesting sensory properties have been identified in cheeses (47) An enzymatic activity producing γ-glutamyl-phenylalanine in Parmigiano-Reggiano water soluble extracts was observed. It was hypothesized that γ-glutamyl-amino acids and lactoyl-aminoacids could be originated by enzymes of bacterial origin. In order to confirm this hypothesis, *Lactobacillus helveticus* and *Lactobacillus rhamnosus* were chosen as representative of starter and non starter microbiota of Parmigiano Reggiano cheese. They were used as model bacteria, in the presence of suitable precursors, to verify their ability to produce γ-glutamyl-phenylalanine and lactoyl-phenylalanine. The eventual abilities of these strains were tested both during growth and after cell lyses. While γ-glutamyl-phenylalanine was produced only by lysed cells, lactoyl phenylalanine was produced either by growing or lysed cells in different amount depending on the species, the cells condition and the time of incubation.

The lactobacilli characterizing Parmigiano Reggiano cheese, *L. helveticus,* main species of natural whey starter and representative of SLAB, and *L.*

rhamnosus, main species found in ripened cheese and representative on NSLAB, were able to produce both γ-glutamyl-phenylalanine and lactoyl-phenylalanine. In vitro, during growth, *L. helveticus* and *L. rhamnosus* produced lactoyl amino acids but they did not produce γ -glutamyl-amino acids. In lysed condition, a great amount of both non-proteolytic aminoacyl derivatives was produced. It is generally known that during cheese production, and ripening, lactose is converted in lactic acid by the action of SLAB. Amino acids, such as glutamic acid, are released by casein degradation, due to SLAB and NSLAB activity mainly occurring after lyses. For this reason, the two precursors of the successive chemical transformation are fully available in cheeses, together with the aminoacyl acceptors of the acyl moieties. Thus, after cells lyses, it is feasible to hypothesize that the enzymes responsible for these activities are released, allowing the production and the accumulation of these non-proteolytic aminoacyl derivatives.

The data contained in the reported study are consistent with the hypothesis that the non-proteolytic aminoacyl derivatives are produced by a bacterial enzymatic activity. In particular lactic acidbacteria, such as *L. helveticus* and *L. rhamnosus*, have been demonstrated to be able to produce γ-glutamyl- amino acids and lactoyl- amino acids in vitro.

Even if the performance of LAB strains appears to be very low, the final amount of ca. 50mg in100 g of cheese (Sforza et al., 2009) (47) is a consequence of the accumulation due to long time of ageing. The enzymatic activity which leads to these compounds production might be useful for the food industry, given their interesting sensory properties. Further studies will be helpful to isolate the responsible enzymes and to identify the optimal conditions to increase the yield.

1.7 References

1. Summer et al. (2005) Influence of milk storing conditions on the loss of coagulable nitrogen. Italian Journal Animal Science 4 (Suppl.2), 233-235
2. Scienza e tecnica lattiero casearia 46, 211-232 (1995)
3. Scienza e tecnica Lattiero-Casearia, 46, 20-35 (1995)
4. M. Rampilli (1993) La coagulazione del latte: aspetti chimico-fisici e tecnologici. In: coagulando. Rassegna storica, scientifica e tecnologica sulla coagulazione del latte. Gist- Brocades Spa, Casteggio (PV), 57,59
5. Scienza e tecnica Lattiero-Casearia,52, 13-22 (2001)
6. B. Battistotti, C. Corradini, (1993). Italian cheese. In P. F. Fox, Cheese: Chemistry, Physics, and Microbiology, Vol 2 221-243.
7. C. Corradini Chimica e tecnologia del latte, Ed. Tecniche nuove, Milano, 1995.
8. E.D. Bastian, R.J. Brown, (1996) Plasmin in milk and dairy products: an update. International Dairy Journal , 6, 435-457.
9. B.M. Prado, S.E. Sombers, B. Ismail, K.D. Hayes (2006) International Dairy Journal 16, 593-599.
10. F. Addeo, L. Moio, C. Stingo (1988) In: “Atti del convegno “Ricerca triennale sulla composizione e su alcune peculiari caratteristiche del formaggio Parmigiano-Reggiano”. Reggio Emilia 28 marzo 1988.
11. M.G. Wilkinson, K.N. Kilcawley (2005) Mechanism of incorporation and release of enzymes into cheese during ripening;. International Dairy Journal 15, 817-830.

12. V. Juillard, H. Laan, E. Kunji, CM (1995) Jeronimus-Strating, A. Bruins, W. Konings (1995). Journal Bacteriology 177, 3472-3478.
13. MJ. Sousa, Y. Ardo, PLH. McSweeney (2001) Advances in the study of proteolysis during cheese ripening, International Dairy Journal 11 327-345.
14. S. Sforza, V. Cavatorta, F. Lambertini, G. Galaverna, A. Dossena, R. Marchelli (2012) Cheese peptidomics: A detailed study on the evolution of the oligopeptide fraction in Parmigiano-Reggiano from curd to 24 months of ageing. Journal of Dairy Science. 95,3514-3526.
15. D.D. Kitts, K. Weiler (2003) Bioactive proteins and peptides from food sources. Applications of bioprocesses use in isolation and recovery. Current Pharmaceutical Design, 9, 1289-1295.
16. S.Mills, R.P. Ross, C. Hill, G. F. Fitzgerald, C. Stantont (2011) Milk intelligence: Mining milk for bioactive substances associated with human health. International Dairy Journal. 21, 377-401.
17. D. Agyei, M.K. Damquah, (2012) Rethinking food-derived bioactive peptides for animicrobial and immunomodulatory activities, 23, 62-69.
18. M. Shimizu (2010) Interaction between Food substances and the Intestinal Epithelium, 74 (2), 232-241, 2010.
19. J. Parada, J.M. Aguilera (2007), Food Microstructure affects the bioavailability of several nutrients. Journal of food science, 72 (2) R21-R32.
20. M. E. Maillard, B. R. Stevens, G. E. Mann (1995) Amino acid transport by small intestinal, hepatic, and pancreatic epithelia Gastroenterology 108, 888-910.

21. R. Nagpal, P. behare, R. Rana, A. Kumar, S. Arora, R. Morotta, S. Jain, H. Yadav (2011) Bioactive peptides derived from milk proteins and their health beneficial potentials: an update, 2, 18-27.
22. B.H. Sarmadi, A. Ismail (2010) Antioxidative peptides from food proteins: A review. Peptides 31, 1949-1956.
23. M. Phelan, D. Kerins (2011) The potential role fo milkderived peptides in cardiovascular disease. Food & Function. 2, 153-167.
24. Makarov (2000) Electrostatic A.xially Harmonic Orbital Trapping: A High-Performance Technique of Mass Analysis; Analytical Chemistry, 72(6) 1156-1162.
25. Q. Hu, R.J. Noll, L. Hongyan, A. Makarov, M. Hardman, R. Graham Cooks (2005) The Orbitrap: a new mass spectrometer. Journal of Mass. Spectrometry 40(4) 430-443.
26. AL. Demain, P. Vaishnav (2009) Production of Recombinant Proteins by Microbes and Higher Organisms. Biotechnology Advances, 27, 297–306.
27. Q. Hu, R. J. Noll, H. Li, A. Makarov, M. Hardman, R. G. Cooks, The Orbitrap: a new mass spectrometer; (2005) Journal of Mass Spectrometry 40:430-443;
28. Makarov, E. Denisov, O. Lange, S. Horning, Dynamic Range of Mass Accuracy in LTQ Orbitrap Hybrid Mass Spectrometer; (2006) American Society for Mass Spectrometry, published by Elsevier.
29. R.B. Merriefield (1963). Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide Journal American Chemical Society 85 (14): 2149–2154.
30. B. Gutte, R.B. Merrifield (1971). The synthesis of ribonuclease A. Journal of Biological Chemistry. 246 (6): 1922–1941.

31. B. Gutte, R. B. Merrifield (1971) Journal of Biological Chemistry. 246 (1971) 1922.
32. S. B. H. Kent, D. Alewood, P. Alewood, M. Baca, A. Jones, M. Schnölzer (1991). 2nd International Symposium on Innovation and Perspectives in SPPS, Canterbury. R. Epton Ed., Intercept Ltd, Andover, 1992.
33. The Bachem practice of SPPS, Bachem.
34. F. Albericio (2004). Developments in peptide and amide synthesis. Current Opinion in Chemical Biology 8:211–221.
35. So-Yeop Han, Young-Ah Kim (2004) Recent development of peptide coupling reagents in organic synthesis. Tetrahedron 60 2447-2467.
36. Lehninger, Biochemistry 2005 4 ed.
37. Brown, Chimica Organica.
38. A.L. Demain, P. Vaishnav (2008) Production of Recombinant Proteins by Microbes and Higer Organism. Biotechnology Advances, 27, 297-306.
39. S.Y. Han, Y.A. Kim (2004) Recent development of peptide coupling reagents in organic synthesis. 60 2447-2467.
40. McSweeney P.L.H., Sousa M.J. (2000) biochemical pathways for the production of flavour compounds in cheese during ripening: a review. Lait, 80, 293-324.
41. Roudot-Algaron F., Kerhoas L., Le Bars D., Einhorn J.,Gripon J.C. (1994). Isolation of γ-glutamyl-peptdies from comtè cheese. Journal of Dairy Science, 77, 1161-1166.
42. Toelstede S., Dunkel A., Hofmann T. (2009). A series of kokumi peptides impart the long lasting mouthfulness of matured Gouda

cheese. Journal of Agricultural and Food Chemistry, 57, 1440-1448.

43. Ueda Y., Tsubuku T., Miyajima R. (1994). Composition of sulfur-containing components in onion and their flavor characters.Biosicence Biotechnology and Biochemistry, 58, 108-110.
44. A. Dunkel, J. Koster, T. Hofmann (2007) Molecular and sensory characterization of gamma-glutamyl peptides as key contributors to the kokumi taste of edible beans Journal of Agricultural and Food Chemistry, 55, 6712–6719.
45. M. Mutsch-Eckner, B. Meier, A. D. Wright, O. Sticher. (1992) gamma-glutamyl peptides from allium sativum bulbs. Phytochemistry, 31-2389-2391.
46. Sforza S., Galaverna G., Schivazappa C., Marchelli R., Dossena A., Virgili, R. (2006). Effect of extended aging of Parma dry-cured ham on the content of oligopeptides and free amino acids. Journal of Agricultural and Food Chemistry, 54, 9422–9429.
47. Sforza S., Cavatorta V., Galaverna G., Dossena A., Marchelli R. (2009). Accumulation of non-proteolytic aminoacyl derivatives in Parmingiano Reggiano cheese during ripening. International Dairy Journal, 19, 582–587.
48. Higaki-Sato N., Sato K., Esumi Y., Okumura T., Yoshikawa H., Tanaka-Kuwajima C., et al. (2003). Isolation and identification of indigestible pyroglutamyl peptides in an enzymatic hydrolysate of wheat gluten prepared on an industrial scale. Journal of Agricultural and Food Chemistry, 51, 8–13.

49. Altamura M. R., Andreotti R. E., Long, Jr. L. (1970). Pyroglutamyl dipeptides in mushroom Agaricus Campestris. Journal of Food Science, 35, 134–139.
50. Mucchetti G., Locci F., Gatti M., Neviani E., Addeo F., Dossena A., Marchelli R. (2000). Pyroglutamic Acid in cheese: presence, orgin and correlation with ripening time of grana padano cheese Journal of Dairy Science, 83,659–665.
51. Mucchetti G., Locci F., Massara P., Vitale R., Neviani, E. (2002). Production of pyroglutamic acid by thermophilic lactic acid bacteria in hard cooked minicheese.Journal of Dairy Science, 85, 2489–2496.
52. Suzuki H., Kajimoto Y., KumagaiSuzuki H. (2002). Improvement of the bitter tast of amino acids through the transpeptidation reaction of bacterial γ-glutamyl transpeptidase. Journal of Agricultural and Food Chemistry, 50, 313-318.
53. O. Power, P. Jakeman, R.J. FitzGerald (2013). Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk derived antioxidative peptides. Amino Acids, 44, 797-820.

CHAPTER 2

Accumulation of NPADs in Parmigiano-Reggiano cheese: determination of the degree of ripening

2.1 Introduction

In cheese industry, the ripening degree is essential in order to determine the commercial value of cheese in the market. The consortium of Parmigiano Reggiano, recently, has introduced three different brands concerning the ripening of cheese, according to the different degree of ripening.

Even if the labelling on the rind carries information on the actual date of production of a given cheese, cheeses, are often grated or cut to a pieces, becoming ingredient for industrial recipe, making impossible to exactly know their production time.

The differences between the price of differently ripened cheeses, could give rise to fraudulent mislabeling of the product. In general, the illegal activity is carried out on grated cheese, where it is not possible to present the crust with the hallmark of cheese. So it is necessary a system for the identification of the correct range of ageing of the final product. (3).

In literature several methods are reported, but not always suitable to the task, for example with the monitoring of the amino acids increase is not possible to discriminate ripening times ranging from 14 to 30 months.

The aim of this work is to study the trend non proteolytic (NPADs) peptides in cheese, which is known to be increasing during the ripening, in order to use them for determining the months of ripening of samples of Parmigiano Reggiano cheese (between 12 and 50 months) from all zones of production.

2.2 Matherial and methods

2.2.1 Samples description

100 samples of Parmigiano Reggiano chesse, between 11 and 48 months of ripening from different province of Emilia Romagna region, were used for this study.

Months of ripening	Number of samples
11	1
12	12
13-17	10
18	13
19-23	9
24	18
25-35	12
36	16
>37	9

Table1: list of samples analyzed

2.2.2 Water soluble extract preparation procedure

At 20 g of grated cheese were added 90 ml of distilled water. The mixture was homogenate for 1.5 minutes with ultraturrax, filtered on paper filter and filter with cut off 0.45 μm.

2.2.3 LC/ESI-MS analysis

Chromatographic analyses were performed with an UPLC chromatographic system (Waters, Milford, MA, USA) coupled to a SQD detector.
A C_{18} BEH Acquity Waters column (2.1x150mm, 1.7 m particles). The eluents were two: water, 0.2% acetonitrile, acidified with 0.1% of formic acid (A) and acetonitrile with 0.1% of formic acid (B). A flow rate was set at 0.2 ml/min. The injection volume was 2 µl. The chromatographic gradient was performed following some steps: 0-7 min isocratic 100% A, 7-50 min linear gradient from 100 % A to 50% A, 50-52.6 min isocratic 50 % A, 53-58 min from 50 % A to 0 % A and reconditioning.
The electrospray parameters in the positive ion mode, are the following: the capillary and the cone voltages were 3.2 kV and 30 V, respectively; the source and the desolvation temperatures were 150 °C and 300 °C, respectively. Cone gas flow (N_2) 100 L/h, desolvation gas (N_2) 650 L/h.

2.2.4 Statistical analysis

Data analysis was done with MassLynx V4.0. The peptide fraction analysis yielded for each sample a TIC (total ion chromatogram). For every signal of interest the most intense ion was extracted, obtaining a XIC (extract ion chromatogram). The area underlying the peak was then determined. The peptides were selected according to the signal intensity and the trend (increasing or decreasing)during ripening.
SPSS 17.0 was used in order to carry out the statistical analysis.

2.3 Results

In the following table, the proteolytic and non proteolytic (NPAD) peptides used for the analysis are reported, together with the amino acid Trypthophan (Trp). It is possible to see that also some acyl amino acids have been identified.

	Name	*Tr*	*Ions*	*MW*	*Kind of molecule*
1	**γ-Glu-Tyr**	13,763	311+182	310	NPAD
2	**pyroglutamyl-Pro**	14,17	227+109+115,8	226	NPAD
3	**γ-Glu-Ile**	16,44	261+132	260	NPAD
4	**γ-Glu-Leu**	17,07	261+132	260	NPAD
5	**Lactoyl-Met**	18	222+176	221	NPAD
6	**γ–Glu-Phe**	18,31	295+166+120	294	NPAD
7	**lactoyl-Val**	18,91	144+190	189	NPAD
8	**piroglytamyl-Ile**	20,21	243+197+132	242	NPAD
9	**γ-Glu-Trp**	20,45	334+205+188	333	NPAD
10	**piroglutamyl-Leu**	21,21	243+197+132	242	NPAD
11	**piroglytamyl-Phe**	22,94	227+231+166+120	276	NPAD
12	**lactoyl-Ile**	23,17	204+158+132	203	NPAD
13	**lactoyl-Leu**	23,49	204+158+132	203	NPAD
14	**butyryl-Val**	24,02	188	187	NPAD
15	**butyryl-Met**	24,04	220	219	NPAD
16	**lactoyl-Phe**	25,09	238+192+166+120	237	NPAD
17	**butyryl-Ile**	28,64	202	201	NPAD
18	**butyryl-Leu**	29,4	202	201	NPAD
19	**butyryl-Phe**	31,23	236	235	NPAD
20	**caproyl-Met**	34,06	248	247	NPAD
21	**caproyl-Val**	34,27	216	215	NPAD
22	**caproyl-Ile**	38,23	230	229	NPAD
23	**caproyl-Leu**	38,82	230	229	NPAD
24	**caproyl-Phe**	40	264	263	NPAD
25	**caproyl-Val**	43,9	244	243	NPAD
26	**tryptophan**	15,82	205+188	204	amino acid
27	**β-CNf(47-52)**	20,39	378,7+494,2+166	755	proteolytic peptide
28	**β-CNf(16-28)3P**	21,8	568,6+1136,5+581,3	1703	proteolytic peptide
29	**β-CNf(14-28)4P**	22,14	667,4+569,0	1999	proteolytic peptide
30	**α_s1-CNf(24-30)4P**	28,38	806,3+562,1+244,9	805	proteolytic peptide

Table 2: Peptides and NPADs considered

2.3.1 Statistical analysis

For the analysis, the area of NPADs, usually increasing with the ageing time, was related to the area of Trp, which remains more constant during the ripening) and four proteolytic peptides.

- NPAD/TRP
- NPAD/β-CNf(47-52)
- NPAD/β-CNf(16-28)3P
- NPAD/β-CNf(14-28)4P
- NPAD/αS_1-CN(24-30)

This procedure was done in order to normalize the content of NPADs, highly variable according to the factory of production even in two samples having the same ageing time.

Two statistical analyses were proposed in order to determine the ageing of the cheese: multiple linear regression and discriminant analysis.

The multiple linear regression (MLR) is a relationship between indipendent and dipendent variables and was expressed by an equation using as dependent variable the month of ageing and independent variables the ratios above reported. (1)

The equation had thus the form:

MONTHS OR RIPENING= a*ratio1+b*ratio2+c*ratio3+…+m,

where a, b, c were the coefficient calculated through statistical elaboration and ratio 1,2,3 were the ratio between the areas of NPAD and the selected peptides. The most accurate equations were the ones making use of the following ratios:

- NPAD/Trp
- NPAD / αS_1-CN(24-30)

They are reported down here.

Months of ripening = (119,812*Lactoyl-Val/Trp) - (18,255*γ-Glu-Leu/Trp) + (904,438* γ-Glu-Trp/Trp) - (83,553* Lactoyl-Leu/Trp) + (1014,589*butiril-Val/Trp) – (855,873* butiril-Ile/Trp) + 17,924

Months of ripening = (2,133*Lactoyl-Val/αS1-CNf(24-30)) - (1,685* Lactoyl-Leu/αS1-CNf(24-30)) + (5,535* pyroglu-Phe/αS1-CNf(24-30)) - (3,453* γ-Glu-Tyr/αS1-CNf(24-30)) - (0,635* γ-Glu-Ile/αS1-CNf(24-30)) + (11,136* γ-Glu-Trp/αS1-CNf(24-30)) + 20,843

Figure 1: Equations giving the best match in the prediction of the months of ageing

All the 100 samples were used in order to build a training set of data, which was used for estimating the error in the determination of the month of ripening, which turned out to be near 15% when using Trp as normalizing factor and about 16% in the case of the other peptide.

A cross validated re-assignment of the months of ageing of the samples showed that 75 samples were correctly assigned, 3 samples differed for 6 months, 2 samples for 8 months of ripening, 6 for 7 months, 11 for 5 months, 1 for 12 months and 2 for 10 months.

A discriminant analysis was also performed. The analysis is based on observed predictors and aims at classifying each sample in finite groups (2).

The samples were clustered in 3 groups: G1 (11-20 months of ripening), G2 (21-30 months of ripening), G3 (31-50 months of ripening).

The variables considered in this analysis were the same ratios used for the multiple regression. A cross validated application of the discriminant analysis confirmed the data obtained by previous statistical elaboration. Tryptophan and proteolytic peptide α_s1-CNf(24-30) were found to be the best normalizing factor for allowing NPADs amount to predict the degree of ripening of Parmigiano-Reggiano cheese.

Months of ripening = (119,812*Lactoyl-Val/Trp) - (18,255*γ-Glu-Leu/Trp) + (904,438* γ-Glu-Trp/Trp) - (83,553* Lactoyl-Leu/Trp) + (1014,589*butiril-Val/Trp) – (855,873* butiril-Ile/Trp) + 17,924

Months of ripening = (2,133*Lactoyl-Val/αS1-CNf(24-30)) - (1,685* Lactoyl-Leu/αS1-CNf(24-30)) + (5,535* pyroglu-Phe/αS1-CNf(24-30)) - (3,453* γ-Glu-Tyr/αS1-CNf(24-30)) - (0,635* γ-Glu-Ile/αS1-CNf(24-30)) + (11,136* γ-Glu-Trp/αS1-CNf(24-30)) + 20,843

Figure 2: Equations giving the best match in the prediction of the motnhs of ageing

The results showed that in the 82% of the cases the statistical system found the correct cheese age group for NPAD/Trp signal, while for NPAD/proteolytic peptide the correct assignment was done in 83% of the cases.

Both methods thus were found to be able to achieve good results in terms of ripening characterization. Then, the WSE of 20 new unknown samples of Parmigiano Reggiano cheese were tested with both methods in a blind analysis and data were analyzed performed following the same procedure as used for previous samples.

	Expected month of ripening (average value)	Uncertainty estimated	Real month of ripening
1	24	4	24
2	17	4	17
3	25	4	31
4	24	4	25
5	19	4	24
6	17	4	24
7	19	4	25
8	41	9	58
9	23	4	26
10	23	4	26
11	24	4	24
12	17	4	19
13	40	9	72
14	20	4	25
15	24	4	36
16	18	4	18
17	22	4	32
18	17	4	13
19	18	4	24
20	17	4	22

Table 3: Results obtained for the 20 unknonwn samples and comparison with real value by applying the linerar regression method to predict the actual month of ageing.

In the table above, the predicted months of ageing obtained by linear regression are shown: in the 70% of the cases the ageing time differed of less than 5 month by the real ripening.

In the discriminant analysis model, the data obtained were used in order to calculate the probability, of each sample, of belonging to a different group, divided as before

- Group 1: samples from 11 to 20 months of ripening
- Group 2: samples from 21 to 30 months of ripening
- Group 3: months from 31 to 50 months of ripening

The discriminant analysis was performed with the method previously developed

	Real group of ripening	Expected group of ripening	% affinity group 1	% affinity group 2	% affinity group 3
1	2	2	9,6	88,2	2,2
2	1	1	98	1,2	0
3	3	2	0,4	93,3	6,2
4	2	2	0,6	98,7	0,7
5	2	1	97,2	2,8	0
6	2	1	98,8	1,2	0
7	2	2	0,7	99,3	0
8	3	3	0	0	100
9	2	2	40,4	44,5	15,1
10	2	1	88,3	6,8	4,9
11	2	1	97,3	2,5	0,2
12	1	1	91,6	8,4	0
13	3	3	0	0	100
14	2	2	3,3	96	0,7
15	3	2	3	75	22
16	1	2	19,3	80,7	0
17	3	1	59	26	15
18	1	1	92	8	0
19	2	2	19,4	80,6	0
20	2	2	35,4	64,6	0

	Real group of ripening	Expected group of ripening	% affinity group 1	% affinity group 2	% affinity group 3
1	2	2	19,9	78,8	1,4
2	1	1	87,4	12,6	0
3	3	2	21,5	77,7	0,9
4	2	2	4,8	94,2	0,9
5	2	1	85,7	14,3	0
6	2	1	92	8	0
7	2	2	36,6	63,3	0,1
8	3	3	0,6	4	95,4
9	2	1	69,2	30,2	0,6
10	2	1	78,1	21,4	0,5
11	2	1	94,8	4,9	0,3
12	1	1	85,4	14,6	0
13	3	3	0,3	2	97,7
14	2	2	33,1	66,5	0,4
15	3	2	28,8	69	2,3
16	1	1	64,3	35,6	0,1
17	3	1	64,3	32	3,7
18	1	1	78,5	21,5	0
19	2	2	48,1	51,8	0,1
20	2	1	59,4	40,6	0

Table 4: results of discrimintant analysis

The tables shows the results by statistical analysis related to cheese samples. In the first table was reported the ratios between the Area of NPAD/Area of Triptophan and in the second one the Area of NPAD/Area of α_{S1}CNf(24-30).
In both cases only about the 50% of identification match with the correct belonging group.

2.4 Conclusion

The present study represents a first attempt to characterize the degree of ripening of Parmigiano Reggiano cheese using the content of NPADs as molecular marker of the ripening time.

The statistical analysis applied to onormalized NPAD content, obtained by LC/ESI-MS allowed to assess the ageing time with fair accuracy and good precision, although the results were far to be perfectThe reason is likely due to the fact that NPAD content, being of microbial origin, is very much related to the particular microflora developed in the particular cheese factory. Thus, even if the content is always increasing with time, the amount can change for the same ageing among different factories, thus hampering a precise definition of the time. Anyway, they can be used for a preliminary assessment of the ageing time in correlation with other parameters.

2.5 References

1. 1 A. Giacomino, O. Abollino, M. Malandrino, E. Mentasti (2011). The role of chemometrics in single and sequential extraction assays: A review. Part II Cluster analysis, multiple linear regression, mixture resolution, experiemental design and other techniques. Analytica Chimica Acta, 688, 122-139.
2. R.H. Anderson, D.B. Farrar, S.R. Thoms (2009). Application of discriminant analysis with clustered data to determine anthropogenic metals contamination. Science of Total Environments, 408, 50-56.
3. F. Camin, R. Wehrens, D. Bertoldi, L. Bontempo, L. Ziller, M. Perini, G. Nicolini, M. Nocetti, R. Larcher (2012) H, C, N and S stable isotopes and mineral profiles to objectively guarantee the authenticity of grated hard cheeses. Analytica Chimica, Acta, 711, 54-59.

CHAPTER 3

Enzymatic production and degradation of cheese-derived non proteolytic aminoacyl derivatives

Based on**:** Chiara Bottesini, Tullia Tedeschi, Arnaldo Dossena, Stefano Sforza, **Enzymatic production and degradation of cheese-derived non-proteolytic aminoacyl derivatives,** *Amino Acids, 2013, Accepted, in press*

3.1 Introduction

Cheese is a complex mixture of many different compounds, originating from the proteins, the sugars and the fats present in milk modified by several biochemical reactions. One of the most important of these processes is the breakdown of the casein matrix, a phenomenon known as proteolysis. Parmigiano Reggiano (PR-RE) is a hard cheese cooked and long ripened. During the ripening, an intense proteolytic process takes place in this cheese, due to the enzymes present in the milk, calf rennet and starter/non starter lactic acid bacteria (SLAB/NSLAB).(1,2)

As a consequence, the nitrogen fraction of PR-RE cheese is constantly evolving during ageing time. As recently demonstrated, peptides in PR-RE cheese can be classified in four groups, according to their origin and to the ageing period in which they are most present: peptides deriving by action of chymosine (mostly present at the beginning of cheese production), peptides deriving from SLAB (mostly abundant in the first 1-3 months of ageing), peptides deriving from NSLAB (mostly abundant at 6-10 months) and Non Proteolytic Aminoacyl Derivatives (NPAD), characterizing aged cheeses (8). NPADs, dipeptide–like molecules including γ-glutamyl-, lactoyl- and pyroglutamyl-amino acids, have been identified as characterizing the aged cheeses, not only in PR RE, but also in Grana Padano, Asiago, and in Comté cheese, in amounts included between few mg and more than 50 mg per 100g of cheese. (7,9).

Although NPAD properties are still largely unknown, their most studied characteristic is their influence on cheese taste In particular, γ-glutamil-peptides have been shown to have a strong kokumi taste (12). Recently Kuroda et al. (2012) discovered a new γ -glutamyl-peptide (γ-glutamyl-valyl-glycine) in vietnamite fish sauce, a potent kokumi compound present in some samples in amounts over 10 mg/L (4,5). Other biofunctional or technological

properties of these compounds have not been investigated yet, but their abundance in cheese certainly suggests a closer look to their potential.

The origin of these compounds, and particularly the gamma-glutamyl amino acids, the most studied, are still object of a debate. Several papers hinted at their production by GGT (γ-glutamyl-transpeptidase) (3). GGT is an enzyme present in all the organism mammals, plants and bacteria. The role attributed to this ubiquitous enzyme is transfer the γ-glutamyl group, using a γ-glutamyl donor substrate (such as glutamine) to an amino acid, through a transpeptidation reaction.

Recently, we have been able to demonstrate that lactic acid bacteria enzymes produce γ-Glutamyl-Phenylalanine, in vitro condition, starting from glutamic acid, and not from glutamine. This suggest that the enzimatic activity seems to be different by GGT activity, and also that this particular enzymatic activity is strictly related to lactic acid bacteria (10).

Beside the enzymes providing their formation, it would also be interesting to assess if these molecules are susceptible to degradation, both in cheese and, mostly, in the human body, during gastrointestinal digestion and/or in blood serum. This prior knowledge would be essential before starting to study any potential biological activity in vivo.

In this work we performed a screening study, in order to deeper investigate the enzymatic origin of this class of molecules, and also their resistance to enzymatic degradation once formed. In particular several pure enzymes and biological media, including blood serum, Parmigiano-Reggiano cheese extracts and simulated gastrointestinal digestion mixtures, were tested on pure NPADs (γ-glutamyl-phenylalanine and lactoyl-phenylalanine) and on their precursors (phenylalanine, glutamic acid, lactic acid and glutamine), aimed at identifying the conditions favouring bioproduction and biodegradation of these

compounds. These enzymatic activities were investigated also with the help of the isotopically labeled pure compounds.

3.2 Materials and methods

3.2.1 Chemicals

Potassium-dihydrogen-phosphate monohydrate, γ-glutamyltranspeptidase, Carboxypeptidase Y, glutamic acid, D-L lactic acid, L- Lactic acid type VIII A R-amylase from barley malt, uric acid, type III mucin from porcine stomach, urea 98%, D-(þ)-glucose 99.5%, D-glucuronic acid, D-(þ)-glucosamine hydrochloride 99%, bovine serum albumin (BSA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, type III lipase from porcine pancreas, and bovine and ovine bile were pur. Also m-cresol, thioanysole, diisopropylethylamine (DIPEA), methylene chloride were purchased from Sigma (Stuttgart, Germany).

The heavy labeled phenylalanine (D_5) was purchased from Spectra 2000, Rome-Italy (from Cambridge Isotope, Cambridge, UK).

Potassium hydroxide, potassium chloride, sodium chloride, ammonium chloride, 37% hydrochloric acid, potassium dihydrogen phosphate, sodium hydrogen carbonate, and dried calcium chloride were obtained from Carlo Erba (Milan, Italy), potassium thiocyanate, potassium sulfate and sodium sulfate were purchased from Riedel de Haen (Hannover, Germany), magnesium chloride hexahydrate, N^{α}- Boc-L-Glutamic acid-α-^{t}butyl-ester and Hexafluoro phosphate benzotriazlyl Tetramethyl uronium were obtained from Merck (Darmstadt, Germany).

THF dry was obtained by distillation and not stabilized by quinones. Formic acid and trifluoroacetic acids were purchased by Acros Organics (Geel, Belgium).

3.2.2 Parmigiano Reggiano cheese extract (12 months of ripening)

The Parmigiano Reggiano extract was obtained by dilution and homogenization of 20 g of grated cheese in 90 ml of phosphate buffer (50 mM at 7 pH). After centrifugation at 3500 rpm at 4°C (Eppendorf, Hamburg, Germany), the extract was filtered on paper filter and on membrane with 0.45 micron cut off (Millipore, Cork, Ireland), then incubated with deuterated precursors for 24 h at 37 °C. The presence of γ-glutamyl-phenylalanine was determined by LC/ESI-MS analysis.

3.2.3 Chemical synthesis of γ-Glutamyl-Phenylalanine

The standard γ-Glu-Phe was chemically synthesized by using solution methodologies. The compounds were purified by RP-HPLC and characterized by LC/ESI-MS.

The deuterated γ-Glu-Phe was also synthesized by solution methodologies following the same procedure.

Briefly, N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester (0.20 mmols) was dissolved in methylene chloride together with HBTU, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate, (0.19 mmol) and the mixture was stirred at room temperature for 30 min. Phenylalanine-methyl-ester (0.23 mmols) and DIPEA (0.41 mmol) were added and the reaction was left under magnetic stirring for 24 h at room temperature. In order to make the remaining phenylalanine react, 0.10 mmols of N^{α}-Boc-L-Glutamic acid α-^{t}butyl

ester were added and, after 30 minutes of magnetic stirring, also 0.205 mmols of DIPEA. The reaction goes to completion in 16 hours. The reaction was monitored by TLC using ethyl acetate as eluent. The organic solution was washed with a saturated solution of $KHSO_4$, $NaHCO_3$ and NaCl, dried with Na_2SO_4 and filtered.

The solvent was evaporated under reduced pressure and the residue was dissolved in 4 ml of a trifluoroacetic acid / methylene chloride 1:1 solution, containing also 4 % (v/v) of m-cresol and thioanisol as scavengers. The mixture was stirred at room temperature for 1 h and then the solvent was evaporated under reduced pressure. Ethyl ether was added to the residue in order to precipitate the free dipeptide. The precipitate was washed several times with ethyl ether and product dried at rotavapor. The product was diluted in a solution made by water, plus 0.2% of acetonitrile and 0.1% of formic acid. The reaction was analyzed by means of LC/ESI-MS, the experimental procedure described in paragraph 2.4.

The purification was performed with semipreparative HPLC-UV (Water, Milford, MA, USA). The eluents used were: (A) water acidified with 0.1% trifluoroacetic acid and (B) acetonitrile acidified with 0.1% trifluoroacetic acid. The separation gradient began at the 100% of eluent A for 5 minutes, then until 80% of eluent A in 15 minutes, subsequently the gradient followed a cycle of washing with eluent B, and reconditioning. The flow rate was set at 4 ml/min and the column used was Jupiter 5μ C18 300 Å 250x10,00 mm. The purified fraction was dried through rotavapor. The product was diluted in a solution made by water, plus 0.2% of acetonitrile and 0.1% of formic acid. The experimental procedure of LC/ESI-MS analysis of the purified fraction is described in paragraph 2.4.

3.2.4 LC/ESI-MS analysis of NPADs

Chromatographic analyses were performed with an UPLC chromatographic system (Waters, Milford, MA, USA) coupled to a SQD detector.

A C_{18} BEH Acquity Waters column (2.1x150mm, 1.7 μm particles). The eluents were two: water, 0.2% acetonitrile, acidified with 0.1% of formic acid (A) and acetonitrile with 0.1% of formic acid (B). A flow rate was set at 0.2 ml/min. The injection volume was 2 μl. The chromatographic gradient was performed according to the following steps: 0-7 min isocratic 100% A, 7-50 min linear gradient from 100 % A to 50% A, 50-52.6 min isocratic 50 % A, 53-58 min from 50 % A to 0 % A and reconditioning.

The electrospray parameters in the positive ion mode, are the following: the capillary and the cone voltages were 3.2 kV and 30 V, respectively; the source and the desolvation temperatures were 150 °C and 300 °C, respectively. Cone gas flow (N_2) 100 L/h, desolvation gas (N_2) 650 L/h.

Characterization MH^+ (ESI-MS): 295.1 γ-Glu-Phe, 300.1 deuterated-γ-Glu-Phe, 243.1 deuterated-Lac-Phe, and 171.2 for deuterated Phenylalanine.

3.2.5 Formation and degradation in cheese extracts at different pH values

The extracts were obtained with the same procedure previously described but using Phospate Buffer at different pH (4.4; 6; 7; 8). For the experiments aimed at studying formation, a defined amount of extract (900 μl) was incubated with 3.2 mg of heavy labeled Phenylalanine for 24 h at 37 °C. The presence of γ-glutamyl-phenylalanine was determined by LC/ESI-MS by evaluating the ratio between deuterated γ-Glu-Phe /undeuterated γ-Glu-Phe (methods described above).

A similar experiment was set up in order to evaluate the hydrolysis rate of g-Glu-Phe at different pH.: 7 ml of cheese extract were draw adjusted at pH 4-6-7-8 value and equilibrate to 10 ml for all samples. 900 μl of previous extracts were added of deuterated γ-Glu-Phe (1 mM) and incubated for 24 hours at 37°C, evaluating the appearance of deuterated Phe by LC/MS. Again, the ratio between deuterated γ-Glu-Phe /undeuterated γ-Glu-Phe was evaluated, the instrumental analysis was performed as previously reported.

3.2.6 Kinetic of bond formation

In order to evaluate the kinetic of NPAD formation, the soluble fraction of Parmigiano Reggiano cheese was placed in contact with deuterated pheylalanine (4.14 mg) and the sample was analyzed in LC/ESI-MS every 72 minutes for 13 hours in order to evaluate the kinetics of γ-Glu-Phe formation. In addition the extract was incubated 5 hours with different amounts of deuterated phenylalanine (1.6, 3.2, 8 mg) and the enzymatic reaction was stopped with 20 % of acetonitrile. The samples were analyzed by LC/ESI-MS and the ratio between deuterated γ-Glu-Phe /undeuterated γ-Glu-Phe was evaluated.

3.2.7 Evaluation of the potential ability of pure enzymes to produce and degrade NPADs

Carboxypeptidase Y (CPY) and γ-glutamyl-transpeptidase (GGT), were utilized in order to test their ability to form and/or or to cleave the deutered γ-Glu-Phe. The reaction solvent was a phosphate buffer at pH=6 for CPY and at pH 8 for GGT, the reaction was kept overnight at 37 °C with a ratio enzyme:substrate of 1:50. The substrates for the enzymatic reaction were

prepared as follows: glutamine + deuterated phenylalanine, glutamic acid + deuterated phenylalanine, lactic acid + deuterated phenylalanine (all in equimolar amounts 1.175 mmols) and deuterated γ-Glu-Phe.
The production or degradation of γ-Glu-Phe was determined by LC/ESI-MS analysis.

3.2.8 Simulated in-vitro gastrointestinal digestion

Two different experiments of simulated gastrointestinal digestion, differing for the degree of complexity of the simulation, were set up in order to test the behavior of NPADs under these conditions, and also to eventually test the ability of the proteolytic enzymes to form NPADs in presence of the appropriate precursors.
Pepsin, chymotrypsin, trypsin were reacted individually and pooled together with the precursors (solution 2 mg/ml of phenylalanine + glutamic acid) directly added in the optimal buffer used for the enzyme reactions (HCl 10 mM for pepsin, Phosphate Buffer 100 mM at pH 7 for trypsin and chymotripsin), with an enzyme: substrate ratio of 1:100 at 37°C. The samples were analyzed by LC/ESI-MS (6).
The complete gastrointestinal digestion and the preparation of the artificial digestive juices (saliva, gastric juice, duodenal juice, and bile) were performed according to the protocol of Versantwoort (13).
L-glutamic acid and L-phenylalanine-D5 (1:1 stoichiometric ratio 1.175 mmols) and 1 mM of γ-Glu-Phe deuterated in another experiment, were placed in contact with 300 μl saliva and incubated for 5 minutes at 37°C (pH 6.8). After the first step, 600 μl gastric juice at the proper pH (pH 2-3) was added, and reacted for 2 hours, later bicarbonate (100 μl 1M) and duodenal juice-bile, 300 μl, were added and left to react for others 2 hours (pH 6.5-7). The reaction

was incubated at 37 °C and maintained under magnetic stirring. The enzyme reaction was stopped warming at 95°C for 10 minutes and centrifuged at 3500 rpm for 15 minutes. The supernatant was directly injected in LC/ESI-MS.

3.2.9 Stability in blood serum

The γ-Glu-Phe, also, was kept in contact with human blood serum for 1 hours and samples were taken at 2-5-15-30-60 minutes of incubation at 37°C.
The reaction was stopped adding a solution containing 50 % of acetonitrile and after centrifugation (10000 rpm at 4 °C) the outcome was monitored by LC/ESI-MS analysis. A control sample was performed with pure γ-Glu-Phe (without serum) and another control sample was done by replacing the γ-Glu-Phe with a generic peptide (sequence: LQLQPFPQPQLPY) incubated with the serum in the same amounts (0.476 mmols) and conditions.
All the samples were analyzed by LC/ESI-MS.
A similar test aimed at detecting a possible γ-Glu-Phe formation in blood serum was performed with precursors of γ-Glu-Phe: glutamine or glutamic acid o lactic acid and deuterated phenylalanine in ratio 1:1 (1.175 mmols). The samples were monitored at 0-15-60 minutes, the reaction was stopped with acetonitrile and finally analyzed by LC/ESI-MS.

3.3 Results

3.3.1 Enzymatic production and degradation of γ-Glu-Phe and Lac-Phe in cheese aqueous extracts

By using isotopically labelled precursors (in order to discriminate the newly produced γ-Glu-Phe from that already present in the extracts), the presence in

cheese aqueous extracts, obtained at pH=7, of an enzymatic activity able to produce γ-glutamyl-phenylalanine was demonstrated. Deuterated phenylalanine was added to the cheese extracts and the mixture was incubated at 37°C for 24h, by monitoring through LC/ESI-MS the deuterated g-Glu-Phe / undeuterated γ-Glu-Phe ratio. In order to evaluate the kinetic of formation, the production of new γ-Glu-Phe was monitored for 13 hours. As shown in Figure 1, the amount of produced γ-Glu-Phe increased during the time.

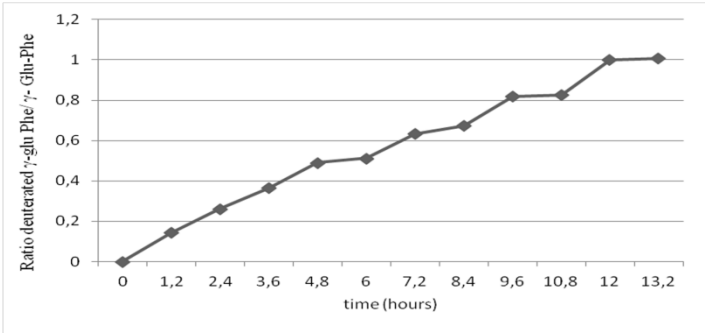

Figure 1: Relative amount of deuterated γ-Glu-Phe formed by incubating deuterated Phe with cheese extract for 13 hours

The extract pretreated at 95 °C for 30 minutes before incubation did not yield the compound after incubation, demonstrating thus the enzymatic origin of γ-Glu-Phe.

In a subsequent experiment, the correlation between the amount of γ-Glu-Phe newly formed and the amount of deuterated Phe added was studied, stopping the reaction after a predefined time (5 hours) with addition of acetonitrile up to 20% (Figure 2).

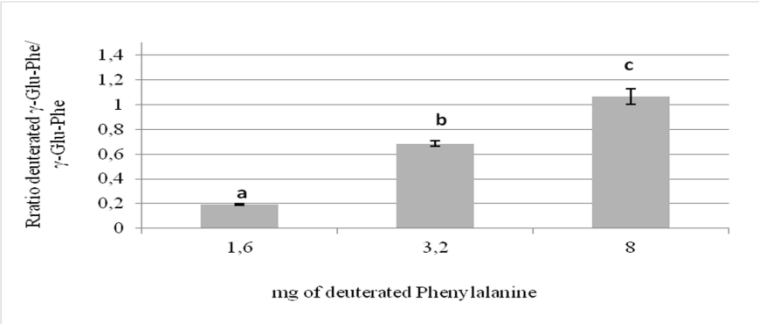

Figure 2: Relative amount of deuterated γ-Glu-Phe formed after 5 hours by incubating increasing amounts of deuterated Phe with cheese extract showing a growth when increasing the amount of precursors

Again, the amount of deuterated γ-Glu-Phe increased according to the increased amount of the precursor, demonstrating that this molecule is actually produced by using phenylalanine as precursor.

Subsequently, experiments aimed at evaluating the efficiency of formation of γ-Glu-Phe at different pH values were performed. An increased amount of deuterated γ-Glu-Phe was obtained by increasing the pH, whereas acidic pH values seemed to inhibit its formation. (Figure 3).

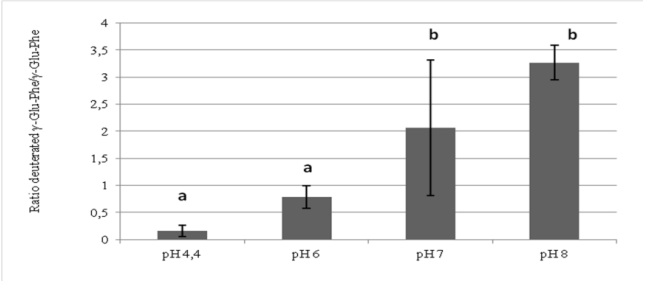

Figure 3: Formation of deuterated γ-Glu-Phe at different pH values., acidic pH values seemed to inhibit its formation

The same experiment was then performed for studying Lac-Phe formation. Quite interestingly, when adding deuterated Phe and monitoring deuterated Lac-Phe formation, a different behavior was observed: no product formation at acidic pH, (figure 4) and then a decreasing trend going from pH=6 to pH=8, differently as seen for γ-Glu-Phe, suggesting that the responsible enzymes in the extracts might be different (Figure 4). Also the efficiency seemed to be lower, as indicated by the lower ratios to the undeuterated compounds measured.

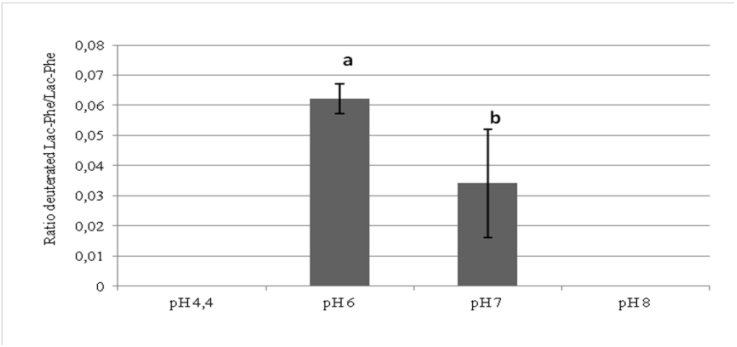

Figure 4: Formation of deuterated Lac-Phe at different pH values. the efficiency seemed to be lower, as indicated by the lower ratios to the undeuterated compounds measured

The samples analyzed were complex non homogeneous mixtures, so it is reasonable to expect that the amount of enzyme extracted was not the same in every repetition of the experiment. Thus, the high errors observed are likely due to the natural variability of the biological system. The bars bearing different letters resulted significantly different by Tukey's test ($p<0.05$).

In order to evaluate the condition of γ-Glu-Phe degradation in cheese acqueous extracts, deuterated g-glutamyl-phenylalanine was added to the extract,

followed by incubation at 37 °C for 24 hours, and monitoring again the ratio deuterated γ-Glu-Phe / undeuterated γ-Glu-Phe. The experiment showed a clear decrease of the added deuterated γ-glutamyl-phenylalanine, as compared to the amount of γ-Glu-Phe naturally present in the samples, indicating that in cheese extracts an enzymatic activity able to degrade γ-Glu-Phe is also present. The degradation of the target molecule at different pH values indicated an increased degradation rate when increasing the pH, suggesting that the producing and the degrading enzyme have the same optimal pH and might possibly be the same enzyme (Figure 5).

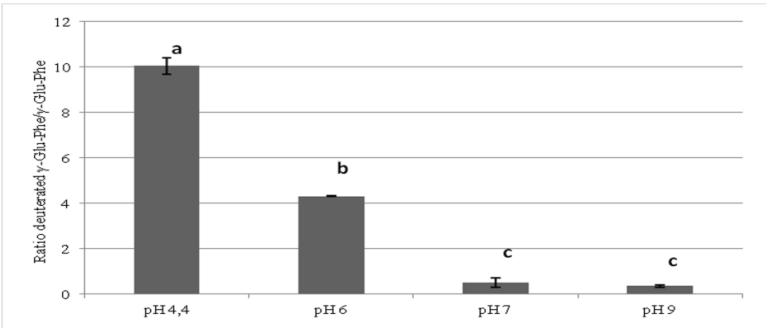

Figure 5 : Degradation of deuterated γ-Glu-Phe at different pH values, The degradation of the target molecule at different pH values indicated an increased degradation rate when increasing the pH

The bars bearing different letters are significantly different by tukey's test ($p<0.05$) all the data represent means and standard deviation.

3.3.2 Enzymatic production and degradation of γ–Glu-Phe and Lac-Phe by pure enzymes

In order to assess the ability of pure enzymes to produce and degrade NPADs, different enzymes were considered, with different precursor (Gln, Glu, Lac, Phe) or full γ-Glu-Phe (when studying degradation) . The results of these experiments are briefly summarized in Table 1.

Precursors	γ-Glutamyltranspeptidase	Carboxypeptidase Y
Bond formation GLN+PHE(D_5)	Yes	No
Bond formation GLU+PHE-(D_5)	Yes	No
Bond formation LAC+PHE-(D_5)	No	Yes
Bond degradation γ-Glutamyl-Phenylalanine-(D_5)	Yes	No

Table 1: Enzymatic production and degradation of γ-Glu-Phe and Lac-Phe by pure enzymes

First, common endoproteolytic enzymes common in the human digestive tract (trypsin, chymotrypsin and pepsin) were tested, both singularly and in combination. However, γ-Glu-Phe demonstrated to be totally resistant to their action. Even a simulated complete gastrointestinal digestion in more physiological conditions (details in the experimental section) resulted to be totally ineffective in degrading the compounds, demonstrating their extreme resistance in the human digestive tract. The same enzymes were also incubated with the precursors of the above molecules, in order to possibly test the ability of these digestive enzymes to produce them, but no γ-Glu-Phe and

Lac-Phe were formed, demonstrating that these enzymes are uneffective also for production, beside degradation, of NPADs.

Caroxypeptidase Y, a common exopeptidase, was then tested both with γ-Glu-Phe and Lac-Phe. Again, no degradation of both molecules was outlined. On the other hand, when incubated with the suitable precursors, a production of Lac-Phe by carboxypeptidase Y was observed, but not of γ-Glu-Phe.

Supplemental test was carried out incubating Parmigiano Reggiano cheese extract with Lactoyl-Phenenylalanine precursors (Phenylalanine deuterated, lactic acid) and a commercial generic inhibitors of proteases. The results obtained showed the inhibition of Lactoyl-Phenylalanine production, monitored as deuterated-Lactoyl-Phenylalanine. The natural presence of a large amount of glutamic acid in the cheese extract and deuterated-Phenylalane added has a consequence the formation of deuterated γ-Glu-Phe. γ-glutamyl-phenylalanine was detected in all samples with and without inhibition. It was possible conclude that only the enzyme responsible of Lactoyl-Phenylalane production could be blocked by a non-specific proteases.

This Lac-Phe production was totally inhibited when carrying the experiment in presence of protease inhibitors (Sigma FAST Protease Inhibitor Tablets). On the other side, when incubating cheese extracts with protease inhibitors, deuterated γ-Glu-Phe was anyway formed, suggesting that only the enzyme responsible of Lactoyl-Phenylalanine production is a protease.

γ-glutamyl-transpeptidase (GGT) was then tested for γ-Glu-Phe production, being usually indicated as the enzyme able to originate γ-Glu-Phe in foods. As expected, when using glutamine as precursor, which is a known substrate for GGT (11), g-Glu-Phe was produced in high yield, but even when using glutamic acid small amounts of γ-Glu-Phe could be detected. As expected, GGT was also found to be able to cleave the bond of γ-Glu-Phe degrading it.

3.3.3 Enzymatic production and degradation of γ-Glu-Phe and Lac-Phe in blood serum

In order to test the possibility of enzymatic production of γ-Glu-Phe and Lac-Phe in blood serum, the aminoacidic precursors (L-phenylalanine and L-glutamic acid or glutamine or lactic acid) were placed in contact with a blood serum for 1 hour at 37°C. In all cases no formation of the target molecules was outlined by LC/ESI-MS.

In order to test the resistance at degradation in blood serum, γ-Glu-Phe was placed in contact with blood serum and monitored for one hour. As control, the degradation of a standard peptide in serum was monitored over the same time.

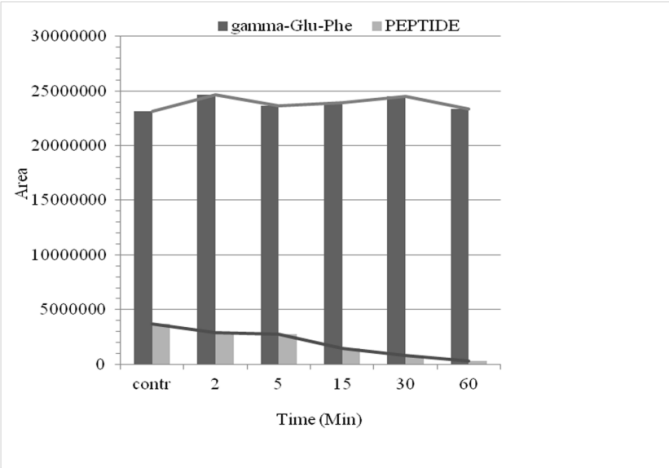

Figure 6: : Degradation of γ-Glu-Phe and a generic peptide in blood serum, γ-Glu-Phe, unlike the control peptide, was not degraded by the proteolytic activities present in blood serum, showing a perfect resistance over one hour time

The figure 6 shows that, γ-Glu-Phe, unlike the control peptide, was not degraded by the proteolytic activities present in blood serum, showing a perfect resistance over one hour time.

3.4 Discussion

γ-Glu-Phe and Lac-Phe are produced by yet unknown one or more enzymes, already demonstrated to be of bacterial origin, present in cheese. A degrading activity of these compounds is also present in cheese, possibly due to the same enzymes, an observation consistent with the hypothesis that these enzymes might be proteases. Anyway, the different efficiency of production at different pH hints for γ-Glu-Phe and Lac-Phe being produced by different enzymes. With the aim of testing the hypothesis that proteolytic enzymes are responsible for the formation of γ-Glu-Phe and Lac-Phe, common endoproteases (trypsin, pepsin, chimotrypsin) and one exoprotease (carobxypeptidase Y) were tested with the precursors. Only carboxypeptidase Y was found to be able to produce small amounts of Lac-Phe, but not of γ-Glu-Phe. On the other hand, γ-Glu-Phe was produced by GGT, as expected, also, quite surprisingly, when glutamic acid is used as precursor. Therefore, a combined action of GGT and exoproteases might be at the origin of these compounds in cheese.

γ-Glu-Phe and Lac-Phe were also tested for their resistance to gastrointestinal digestion and in blood serum, in order to define their bioaccessibility, and they were found to be perfectly resistant, indicating their potential absorption.

More studies will be needed in order to outline their ability to be absorbed in the gastrointestinal tract and their eventual biological and nutritional functionalities.

3.5 Acknowledgements

Ajinomoto Inc. is gratefully acknowledged for its support to this research through the Ajinomoto Amino Acids Research Program ("Identification of microrganisms and conditions for optimal bioproduction of gamma-glutamyl aminoacids as new flavouring agents").

3.6 References

1. Fallico V, McSweeney PLH, Horne J, Pediliggieri C; Hannon JA; Carpino S, Licitra G (2005) Evaluation of bitterness in Ragusano cheese. J Dairy Sci 88:1288-1300. doi: 10.3168/jds.S0022-0302(05)72795-8
2. Forde A, Fitzgerald GF (2000) Biotecnological approaches to the understanding and improvement of mature cheese flavour. Curr Opin Biotechnol 5, 484-9. doi: 10.1016/S0958-1669(00)00130-0
3. Hu X, Legler PM, Khavrutskii I, Scorpio A, Compton JR, Robertson K L, Friedlander AM, Wallqvist A (2012) Probing the Donor and Acceptor Substrate Specificity of the γ-Glutamyltranspeptidase. Biochemistry 51:1199−1212. doi: 10.1021/bi200987b
4. [a]Kuroda M, Kato Y, Yamazaki J, Kageyama N, Mizukoshi T, Miyano H Eto Y (2012) Determination of -glutamyl-valyl-glycine in raw scallop and processed scallop products using high pressure liquid chromatography–tandem mass spectrometry. Food Chem 134:1640-1644. doi: 10.1016/j.foodchem.2012.03.048
5. [b]Kuroda M, Kato Y, Yamazaki J, Kai Y, Mizukoshi T, Miyano H, Eto Y (2012) Determination of -glutamyl-valyl-glycine in commercial fish sauces. J. Agric. Food Chem 60: 7291−7296. doi: 10.1021/jf3012932
6. Prandi B, Bencivenni M, Tedeschi T, Marchelli R, Dossena A, Galaverna G, Sforza S, (2012) Common wheat determination in durum wheat samples through LC/MS analsysis of gluten peptide. Anal Bioanal Chem 403(10):2909-2914. doi: 10.1007/s00216-012-5731-2
7. Roundot-Algaron F, Kerhoas L, Le Bars D, Einhorn J, Gripon JC (1994) Isolation of -glutamyl peptides from Comtè cheese. J Dairy Sci 77:1161-1166. doi: 10.3168/jds.S0022-0302(94)77053-3

8. Sforza S, Cavatorta V, Lambertini F, Galaverna G, Dossena A, Marchelli R (2012) Cheese peptidomics: A detailed study on the evolution of the oligopeptide fraction in Parmigiano-Reggiano cheese from curd to 24 months of aging. J Dairy Sci 95:3514–3526. doi: 10.3168/jds.2011-5046
9. Sforza S, Cavatorta V, Galaverna G, Dossena A, Marchelli R, (2009) Accumulation of non-proteolytica aminoacyl derivatives in Parmigiano Reggiano cheese during ripening. Int Dairy J 19:582-587. doi: 10.16/j.idairyj.2009.04.009
10. Sgarbi E, Lazzi C, Iacopino L, Bottesini C, Lambertini F, Sforza S, Gatti M (2013) Microbial origin of non proteolytic aminoacyl derivatives in long ripened cheeses. Food Microbiology 35:116-120. doi: 10.1016/j.fm.2013.02.013
11. Suzuki H, Kajimoto Y, Kumagai H (2005) Improvement of the bitter taste of amino acids through the transpeptidation reaction of bacterial ?-glutamyltranspeptidase. J. Agric Food Chem 50:313-318. doi: 10.1021/jf010726u
12. Toelstede S, Hofmann T (2009) Kokumi-Active Glutamyl Peptides in Cheeses and their biogeneration by Penicillium roqueforti. J Agric Food Chem 57: 3738-3748. doi: 10.1021/jf900280j
13. Versantwoort CHM; Oomen AG, Van de Kamp E; Rompelberg CJM, Sips AJAM (2005) Applicability of an in vitro digestion model in assessing the bioaccesibility of mycotoxins from food. Food and Chem Toxicology 43:31-40. doi: 10.1016/j.fct.2004.08.007

CHAPTER 4

Bioavailability of NPADs

4.1 Introduction

Non proteolytic aminoacyl derivatives (NPADs) are molecules identified in several food and biological system. For example, γ-glutamyl-valyl-glycine, γ-glutamyl phenylalanine and lactoyl phenylalanine have been recently reported in fish sauce, ripened cheesees. (1,2)

Sgarbi et al. 2013 confirmed the microbial origin of these compounds, demonstrating the production of NPADs. by starter lactic acid bacteria and non starter lactic acid bacteria (*L. helveticus* and *L. rhamnosus*), both during the growth and after cell lysis.(3)

Since these compounds are contained in large amount in foods, their bioavailability and bioaccessibility is to be investigated. Bioaccessibility is the ability of a given compound to be resistant to the digestive enzymes and conditions found in the gastrointestinal tract, whereas with bioavailability is defined the possibility of the compound to reach the target organs , so to be absorbed by the intestinal cells. Both are actually important factors for the evaluation of functional foods and substances (4).

We already showed in a previous chapter the behavior of γ-glutamyl phenylalanine in presence of different enzymes, demonstrating that the compound is totally resistant to the simulated gastrointestinal digestion and blood serum enzymes, so bioaccessible. In order to test effective bioavailability it is necessary to determine also their ability to cross the cell membranes, particularly those of the intestinal cells.

Molecules can use different methods in order to cross the intestinal barrier: passive or paracellular transport, carrier mediated, transcytosis.

The transport of peptides in the intestine is due to transporter, such for example the Pept-1, which transports into the cells small peptides. Often, the peptides are hydrolyzed to amino acids by cellullar peptidase, but in other

cases the paracellular pathway allows the absorption of intact small peptides (4).

Caco-2 (polarized human colon carcinoma cells line) monolayer is a cell culture often used as model of the intestinal epithelium. Since, after cells differentiation, tight junctions form between adjacent Caco-2 cells, similar to those in the small intestine epithelium, it is assumed that whatever can get through Caco-2 cells, is bioavailable (5, 6).

The aim of the work presented in this chapter is to evaluate the ability of NPADs to be transported thorugh cell membranes, placing in contact γ-glutamyl-phenylalanine with HUVEC (Human Umbelical Vein Endothelial Cells) and Caco-2 cells in order to study the transport, the potential cytotoxicity and the proliferation.

4.2 Materials and methods

4.2.1 γ-glutamyl-Phenylalanine syntesis (γ-Glu-Phe)

The standard γ-Glu-Phe was chemically synthesized by using solution methodologies. The compounds were purified by RP-HPLC and characterized by ESI-MS. The deuterated γ-Glu-Phe was also synthesized by solution methodologies following the same procedure, in according with Bottesini et al. 2013 (11).

Briefly, N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester (0.20 mmols) was dissolved in methylene chloride together with HBTU (0.19 mmol) and the mixture was stirred at room temperature for 30 min. Phenylalanine-methyl-ester (0.23 mmols) and DIPEA (0.41 mmol) were added and the reaction was left under magnetic stirring for 24 h at room temperature. ½ eqivalent of each reagent, not phenylalanine, was added in the same order, to carried out the complete reaction in other16 hours. The reaction was monitored by TLC using ethyl acetate as eluent. The organic solution was washed with a saturated solution of $KHSO_4$, $NaHCO_3$ and NaCl, dried with Na_2SO_4 and filtered.

The solvent was evaporated under reduced pressure and the residue was dissolved in 4 ml of a trifluoroacetic acid / methylene chloride 1:1 solution, containing also 4 % (v/v) of m-cresol and thioanisol as scavengers. The mixture was stirred at room temperature for 1 h and then the solvent was evaporated under reduced pressure. Ethyl ether was added to the residue in order to precipitate the free dipeptide. The precipitate was washed several times with ethyl ether product dried at rotavapor. It was not possible to calculate the yields because the product was obtained as a gel. The product was diluted in H_2O CH_3CN (0.2%) HCOOH (0.1%) and analyzed by means of HPLC-MS.

4.2.2 LC/ESI-MS analysis of NPADs

Chromatographic analyses were performed with an UPLC chromatographic system (Waters, Milford, MA, USA) coupled to a SQD detector.

A C_{18} BEH Acquity Waters column (150 mm, mm, μm particles). The eluents were two: water, 0.2% acetonitrile, acidified with 0.1% of formic acid and acetonitrile with 0.1% of formic acid. A flow rate was set at 0.2 ml/min. The injection volume was 2 μl.

The electrospray parameters in the positive ion mode, are the following: the capillary and the cone voltages were 3.2 kV and 30 V, respectively; the source and the desolvation temperatures were 150 °C and 300 °C, respectively.

Characterization MH^+ (ESI-MS): 295.1 γ-Glu-Phe, 300.1 deuterated-γ-Glu-Phe, 243.1 deuterated-Lac-Phe.

4.2.3 Caco-2 cell culture

The Caco-2 cells used in this study were grown at 37°C, 5% of CO_2, in DIMEM (pH7.4) containing 1% of L-glutamine, 10% of fetal bovine serum, 1% penicillin/streptomycin. The medium was changed every day. Until getting at 80% of confluence, cells were trypsinized with trypsin-EDTA 2.5 mg/ml (Lonza, Switzerland) solution and resuspended in medium. The cells quantification was performed using the neubauer chamber. At 100 ul of cell solution were added 900 ul of medium and placed in the camber.

The cells are seeded in polycarbonate membrans (millipore) and the cell grow for 21 days.

The TEER (transepithelial electrical resistance) was measured with microelectrodes (Millipore) at different times in culture. order to evaluate the good condition of the cell membrane.

4.2.4 Transport study

Solutions of γ-glutamyl-phenylalanine diluted in HBSS buffer (10% HBSS 10X, 1.3% $CaCl_2$, 2.2% $MgCl_2$ and 86.5% of steril water) at different concentration: , Atenolol, metaprolol, digoxine as a control at 10 uM in HBSS buffer and solutions of PSC833 and lorepamide 10 uM in HBSS were prepared. The solutions control were always tested and the other solutions were tested singularly or in combination.

The Caco-2 cell monolayer were rinsed two times with HBSS supplemented with 5 mM of glucose. The samples and controls were added to the apical, and basal compartmentsfor the bidirectional analysis. The inserts were incubated at 37°C, 5% CO_2, for 2 hours.

Following this incubation, the solution from the apical and basolateral compartments were collected for LC/MS analysis. The collection was performed for all compartments to carry out evaluate the bidirectional analysis. The samples where were added the compound were drawn 25 ul diluted 1:1 with HBSS, for the other compartements were collected 50 ul.

In order to evaluate the cells layers integrity, the LY (lucifer yellow) assay was performed. The fluorescence was determined for LY by exitation at 485 nm and emission 530 nm. The transoport buffer is HBSS, was added to the apical compartment. The HBSS applied to the basolateral compartments were analyzed. The apparent permeability was calculated considering: Papp=(V/(A _ Ci)) - (Cf/T), where V is the volume of the basal chamber(μL), A is the area of the membrane insert (cm^2), Ci and Cf are the initial and final concentrations of, respectively, and T is the assay time (s).

4.2.5 WST-1-based cytotoxicity assay

Caco-2 monolayer cell monolayers were maintained in Dulbecco's modified Eagle's medium with phenol red (DMEM, Cellgro, Mediatech, VA) supplemented with 10% fetal bovine serum incubated at 37 °C
under 5% CO2 in a humidified incubator. Caco-2 cells were harvested
using commercial trypsin-EDTA 2.5 mg/ml and viable cell concentrations were determined by neubauer quantification. About 3×10^6 viable cells were added to each well of a 96-well tissue culture plate and incubated overnight at 37 °C under 5% CO_2 in a humidified incubator to allow cells to attach to wells of 96-well cell culture plates. The cells were usable at 80% of confluency.
The WST-1 cell cytotoxicity assay kit (Roche Applied Science) was used following manufacturer's instructions.
Solutions of γ-glutamyl-phenylalanine diluted 1:5 were prepared with a concentration range between 10 mM-0.0128 uM.
100 ul of sample were diluted with 1.9 ml of medium. 100 ul of samples were placed in contact with the cells and were used a triton solution as a positive control and medium as a negative control. The samples were incubated for 2 hours at same conditions as before.
In another test the same experiment was executed changing the time of incubation before the test, 24 and 48 hours, and the cell confluency. The cells were used at 50% of confluence.
The absorbance was measured in a microplate reader (Constar) at 450 nm and the percent cytotoxicity was calculated as above. The WST-1 reagent incubation time of 2 h was found to be optimum since the color intensity did not change after 2 h (data not shown), and this condition was used in all subsequent assays.

4.3 Results

First, γ-glutamyl-phenylalanine was determined not to be cytotoxic for Caco-2 cells after 2 hours of incubation. The figure below represents the results obtained by WST-1 assay, showing that the cells remains fully viable alos after the contact with the molecule at different concentrations.

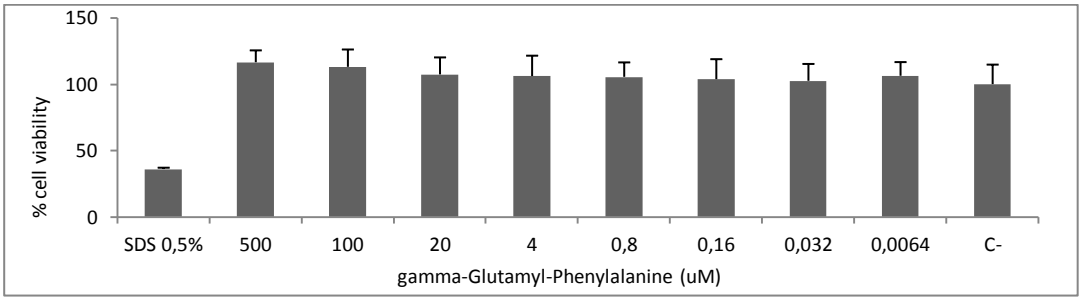

Figure 1: WST-1 assay

The permeability coefficients have then been determined for both directions o ftransport: apical to basolateral (A–B) and basolateral to apical (B–A),to investigate whether the transport is passive.

γ-glutamyl phenylalanine was shown to pass the Caco-2 monolayer through a Pg-P transporter. The figure show that the molecule shows the same behavior of the digoxine, a standard known to be trasported through the Pg-P transporter.

The apparent permeability (P app) is the parameter used in order to define the affinity at permeation or transport. High values of P app in Apical-Basal direction determine a high permeation across the intestinal barrier. If the P

app is low it is necessary the efflux ratio. High apparent permeability in direction Basal-Apical direction and high efflux ratio, identify a Pg-P substrate.

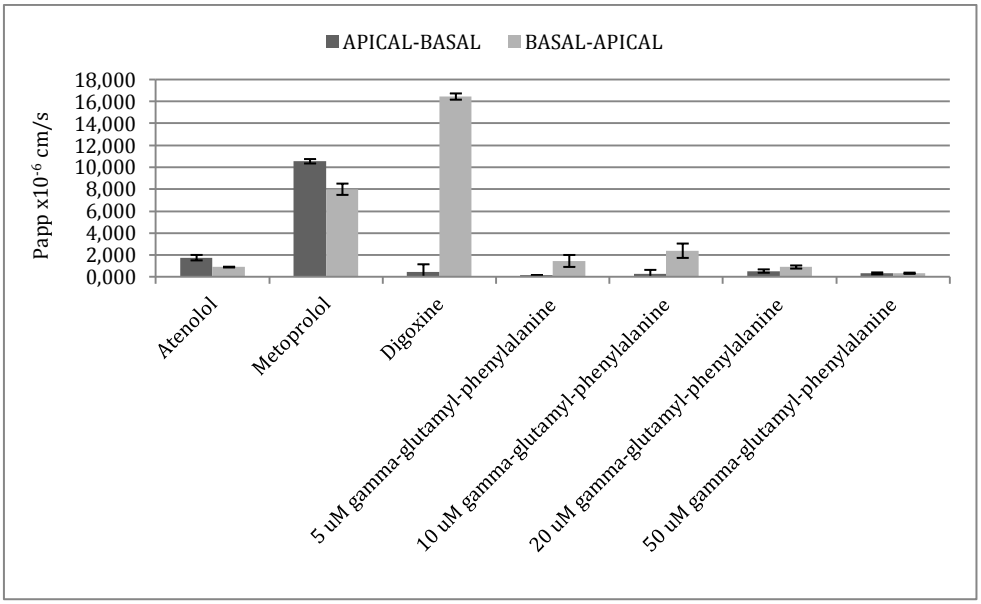

Figure 2: Permeability test performed with several concentration of γ-Glu-Phe (from 5 to 50 μM)

The four concentration tested of γ-glutamyl-phenylalanine showed that the optimum was 10 μM concetration, since higher values the samples might saturate the Pg-P, inhibiting the transport.

Often, the method used in order to evaluate if the target molecule is a Pg-P substrate is place in contact in the same transwell inhibitor and competitor of Pg-P substrate. (7), so in another experiment we placed in contact an inhibitor and a competitor of Pg-P transporter (respectively PSC833, and lorepamide) with γ-glutamyl-phenylalanine (Figure 3).

The trend of γ-Glu-Phe in presence of inhibitor and competitor was different by the compound alone. In presence of PSC833 and digoxine, the apparent permeability in basolateral-apical case was diminished.

The situation with lorepamide showed an increase of apparent permeability of apical-basolateral permeation. These findings confirm that γ-glutamyl-phenylalanine is a Pg-P substrate.

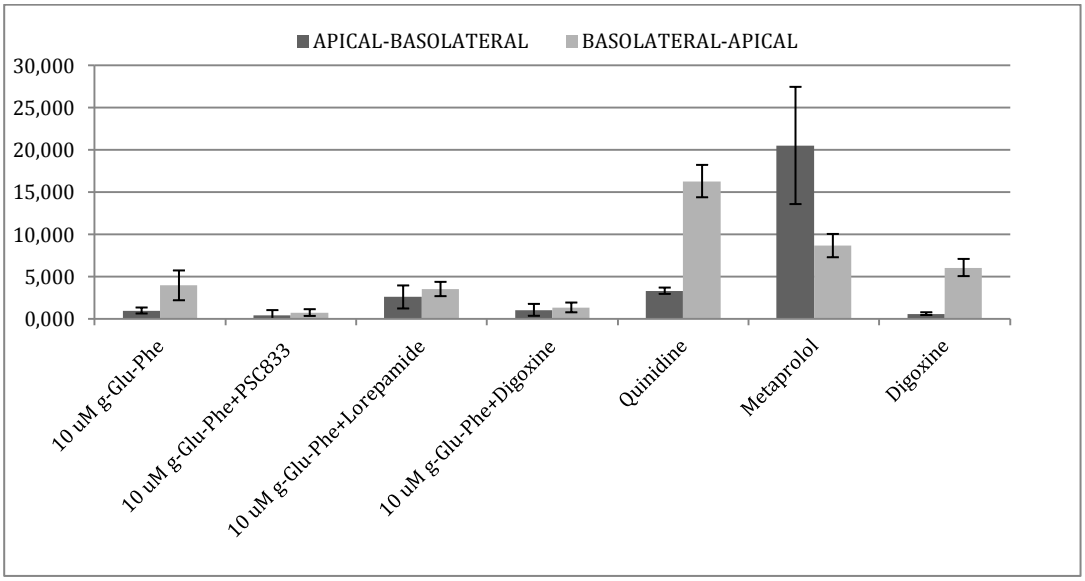

Figure 3: Permeability test in presence of inihibitor and competitor of Pg-P substrate

4.4 Conclusion

Cytotoxic and proliferation test demonstrated that in presence of γ-Glutamyl-Phenylalanine, the cells remains viable and can continue the growth, so the molecule seems not to be cytotoxic.

The results obtained by permeability test, indicate that the molecule cross the membrane thought a transporter, which in this case was the Pg-P, expressed in Caco-2 cells. These results were confirmed by the fact that γ-Glu-Phe transport competes with lorepamide and is somehow inhibited by PSC833.

It is anyway to be remembered that studies reported in literature show the presence of peptidases and endopeptidase in the brush border membrane which can hydrolyze peptide bonds on the N-terminal side of hydrophobic amino acids. (8) Even if NPADs seem to be resistant to peptidases, Smith et al described the active transport of amino acid by γ-glutamyl transpeptidase (GGT) and DPP IV through Caco-2 cell monolayers. The enzymes is involved to the amino acids transport, but also contribute to further peptide hydrolysis. (9, 10) As we have demonstrated in previopus chapters, γ-Glu-Phe is sensitive to the action of GGT, thus might be hydrolyzed by GGT

In conclusion, γ-glutamyl phenylalanine can be transported across the intestinal membrane through a transporter to the blood circulation and possibily become bioavailable for the target organs.

4.5 References

1. S. Sforza, V. Cavatorta, G. Galaverna, A. Dossena, R. Marchelli. (2009) Accumulation of non-proteolytic aminoacyl derivatives in Parmigiano Reggiano cheese during ripening. International Dairy Journal, 19, 582-587.
2. M. Kuroda, Y. Kato, J. Yamazaki, Y Kai, T. Mizukoshi, H. Mirano, Y. Eto (2012) Determination and quantification of g-glutalmyl-valyl-glycine in commercial fish sauces. Journal of agricoltural and food chemistry, 60, 7291-7296.
3. E. Sgarbi, C. Lazzi, L. Iacopino, C. Bottesini, F. Lambertini, S. Sforza, M. Gatti (2013) Microbial originm of non proteolytic aminoacyl derivatives in long ripened cheeses. Food microbiology, 35, 116-120.
4. M. Shimizu (2010) Interaction between food substances and the intestinal epithelium. Bioscience Biotechnology Biochemistry, 74, 232-241.
5. J. Parada, J.M. aguilera (2007) Food microstructure affects the bioavailability of several nutrients. Journal of Food Science, 72, R21-R32
6. Chua, HL Jois, S. Sum, M. K. Go, M. Lin (2009). Transport of angiotensin peptides across Caco 2 monolayer. Peptides, 25.
7. H. Sun, K. Sandy Pang (2008). Permeability, transport and metabolism of solutes in Caco 2 cell monolayer: a theoretical study. Drug Metabolism and Disposition, 36, 102-123.
8. R. Ano, Y. Kimura, M. Urakami, M. Shima, R. Matsuno, T. Ueno, M. Akamatsu (2004). Relationship betweene structure and permeability of dipeptide derivatives containing trhyptophan and related compounds across human intestinal epithelium Caco Cells. Bioorganic and medicinal chemistry, 12, 249-255.

9. T.K. Smith, C. L. Gibson, B.J. Howling, J. M. Pratt (1991). Active transport of amino acids by g-glutamyltranspeptidase throught Caco-2 cell monolaye. Biochemical and Biophysical Research Communications, 178, 1028-1035.
10. S. Howell, A. J. Kenny, A. J. Turner (1992). A survey of membrane pepdidase in two human colonic cell lines, Caco 2 and HT 29. Biochemical Journal, 284, 595-604.
11. C. Bottesini, T. Tedeschi, A. Dossena, S. Sforza (2013) Enzymatic production and degradation of cheese-derived non-proteolytic aminoacyl derivatives. Amino Acids, accepted, in press

CHAPTER 5

First studies on the biological properties of NPADS: immunomodulatory and antioxidant properties

5.1 Immunomodulatory properties

Proteins may contain sequences of amino acids that once hydrolyzed, due for example to microbial fermentation or gastrointestinal digestion, can yield biologically active peptides. These peptides can derive from protein-containing food materials, both of animal and plant source. Their properties might be very diverse, such as antioxidant, cytomodulatory, antimicrobial, mineral carrier, antihypertensive, immunomodulatory. Immunomodulatory peptides have positive effects on the promotion of the human health and disease risk reduction, playing an important role in the proliferation and maturation of T cells and natural killer cells, thus helping the protection against a large number of bacteria.

A specific example is represented by the stimulation of immunoglobulin A production in mice by CCP (casein phosphopeptides). Isradicin, the conventional name of αS1-casein f(1-23) possesses antibacterial activity against *S. aureus*, *C. albicans* and also gives protection against mastitis. (1,2)

A particular interesting property in immunomodulation is the stimulation of innate immunity. Innate immunity is the first line of defense used by the organisms in order to fight microbial colonization and tissue damage. Host Defense Peptides (HDPs) form this line of defense against the microbes. The expression of these HDPs increase during infection and inflammation, directly killing the invaders or modulating the innate immune response of the host. Defensins and cathelicidins are the major classes of humans HDPs. Defensins have identified in epithelial cells, tissue macrophages, small intestinal epithelial cells and cardiomyocytes.

Cathelicidins are expressed by myeloid precursor cells, and in mature circulating neutrophils and neonatal lymphoid tissue in some species. The active forms of cathelicidins are released in the extracellular circulation in

response to an external stimulation . LL-37 is a major representative of this family of antimicrobial peptides and it is involved directly in the disruption of microbial membranes and in other processes such as angiogenesis, chemotaxis, cytokine production. (3)

Several peptides derived from bovine casein proteins present immunomodulatory activity, such as, for example: β-CNf(63-68), β-CNf(191-193), β-CNf(66-60). (2)

The interest about antimicrobial peptides is also due to the possible substitution of antibiotics:molecules which increase the host innate immunity could be useful in order to avoid the problem of antibiotic resistance. So the specific induction of Host Defence Peptides might be considered an approach to control and prevention infectious diseases without the use of anitbiotics.

In this work, we wanted to study for the first time the potential immunomodulatory activity of γ-glutamyl-phenylalanine through immunofluorescence analysis.

5.2. Matherials and methods

5.2.1 Chemical synthesis of γ-glutamyl-phenylalanine

The standard γ-Glu-Phe was chemically synthesized by using solution methodologies. The compounds were purified by RP-HPLC and characterized by ESI-MS. The deuterated γ-Glu-Phe was also synthesized by solution methodologies following the same procedure.

Briefly, N^{α}-Boc-L-Glutamic acid α-^{t}butyl ester (0.20 mmols) was dissolved in methylene chloride together with HBTU (0.19 mmol) and the mixture was stirred at room temperature for 30 min. Phenylalanine-methyl-ester (0.23 mmols) and DIPEA (0.41 mmol) were added and the reaction was left under

magnetic stirring for 24 h at room temperature. ½ eqivalent of each reagent, not phenylalanine, was added in the same order, to carried out the complete reaction in other16 hours. The reaction was monitored by TLC using ethyl acetate as eluent. The organic solution was washed with a saturated solution of $KHSO_4$, $NaHCO_3$ and NaCl, dried with Na_2SO_4 and filtered.

The solvent was evaporated under reduced pressure and the residue was dissolved in 4 ml of a trifluoroacetic acid / methylene chloride 1:1 solution, containing also 4 % (v/v) of m-cresol and thioanisol as scavengers. The mixture was stirred at room temperature for 1 h and then the solvent was evaporated under reduced pressure. Ethyl ether was added to the residue in order to precipitate the free dipeptide. The precipitate was washed several times with ethyl ether product dried at rotavapor. It was not possible to calculate the yields because the product was obtained as a gel. The product was diluted in H_2O CH_3CN (0.2%) HCOOH (0.1%) and analyzed by means of HPLC-MS.

5.2.2 Immunofluorescence analysis

Human intestinal ephitelial cells were stimulated with γ-glutamylphenylalanine and the cells were fixed in paraformaldehyde 4% PBS at room temperature for 10 min for LL-37 peptide. Non-specific background was blocked by incubation with a solution composed by: 1% serum bovine albumine, 10% fetal bovine serum, 0.3% of glycine, 0.1% Tween all reagents were diluted in PBS.

Sections were incubated at room temperature for 1 h with first antibody against peptide LL-37 (ratio 1:250), 3 washing with PBS and another incubation with anti-goat antibody (ratio 1:200) for 60 minutes at room temperature. After four washing with PBS, the sample was incubated for 5 minutes at 4°C with 5 ug/ml of 2-(4-amidinophenyl)-1H -indole-6-

carboxamidine (DAPI). Subsequently, slide was rinsed, mounted and visualized on a fluorescence microscope.

5.3 Results

The figure 1, shows the results obtained by immunofluorescence analysis of human intestinal ephitelial cells which were stimulated with γ-glutamylphenylalanine. The treated and non treated cells were stained with a fluorescent reagent reacting with the antibodies to LL-37 peptides, thus cells which have been immunostimulated appeared to be fluorescent. The two firsts black squares represent the controls, the last two the treated cells.

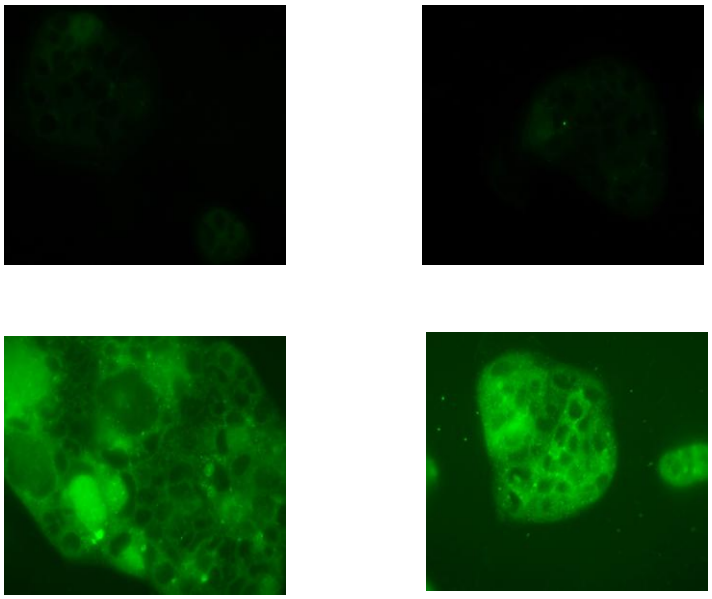

Figure 1: Images deriving by fluorescence microscope
(in the upper part the control experiment, in the lower part the cells stimulated with 200 micrograms of γ–glutamyl phenylalanine)

It is evident that 200 micrograms of the compound exert a evident stimulation of LL-37 production, indicating that the innate immune activity is stimulated by these compounds.

In order to confirm these results, the amount of LL-37 was also determined by ELISA test, in presence or absence of γ-Glu-Phe, or in presence of an agonist of LL-37 (Figures 2 and 3)

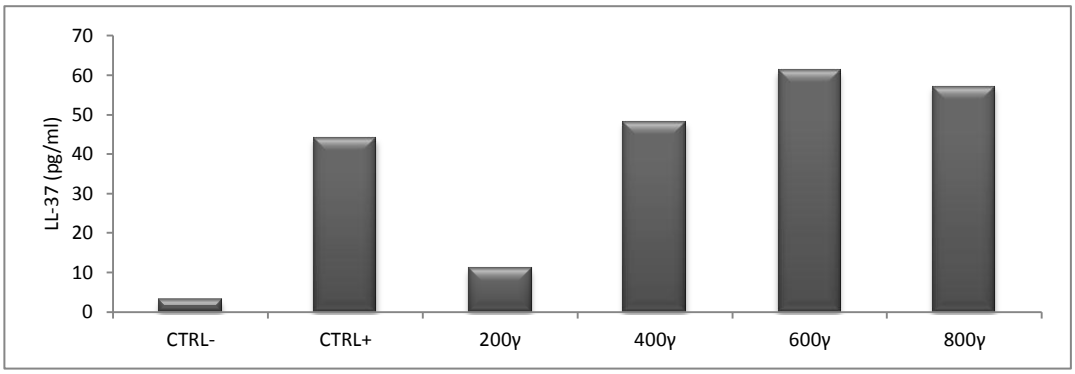

Figure 2: LL-37 levels in Caco-2 supernatants measured by ELISA. CTRL-: cells without stimulation ; CTRL +: cells stimulated with an agonist of LL-37; cells stimulated with 200, 400, 600, 800 micrograms (γ) of γ-Glu-Phe, respectively.

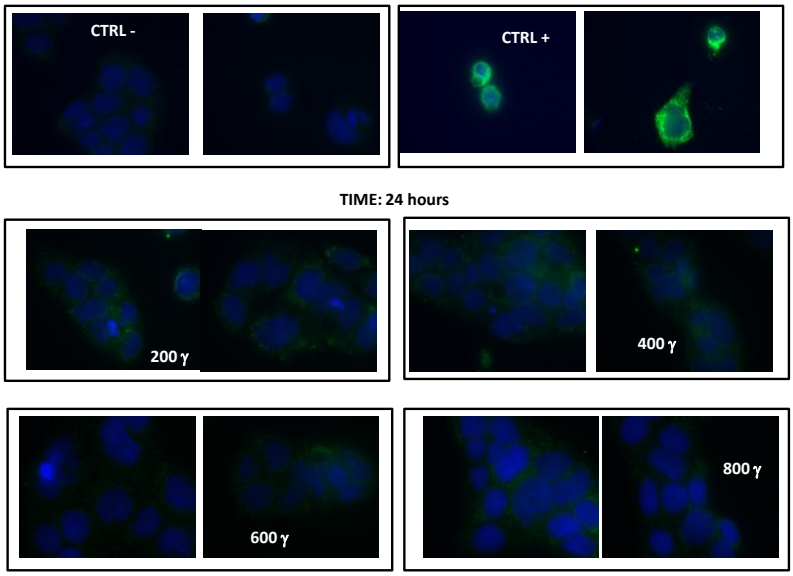

Figure 3: Images deriving by fluorescence microscope
(in the upper part the negative and positive control, in the lower part the cells stimulated with 200, 400, 600 800 micrograms of γ–glutamyl phenylalanine at 24th hour)

Since the biological activity of NPADs has never been studied, this is the first evidence of an immunomodulatory effect of γ-glu-phe, which thus might be able to act against infections, promoting immunoregulation and stimulation of intestinal mucosal tropism.

5.4 Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese: the potential role of NPADs

Based on: Chiara Bottesini, Sara Paolella, Francesca Lambertini, Gianni Galaverna, Tullia Tedeschi, Arnaldo Dossena, Rosangela Marchelli, Stefano Sforza *Int J Food Sci Nutr.* 2013 64:953-958.

5.4.1. Introduction

Parmigiano Reggiano is a typical high quality Italian cheese of high nutritional value. The cheese has been included in the list of Protected Designation of Origin (PDO, EU regulation 2081/92) foods. This regulation guarantees the way of production, the quality and the typical zone of origin, in a few provinces of northern Italy. Parmigiano Reggiano is a hard cheese made from cow's milk and is characterized by a long ageing period, at least 12 months of ripening (Production norm of Parmigiano Reggiano cheeses; 30). During the ripening time a complex set of biochemical reactions take place: proteolysis, lipolysis, lactic and propionic acid fermentation (20). The lipolysis in Parmigiano-Reggiano contribute to cheese aroma, through compounds produced by lipases and esterases, enzymes from lactic acid bacteria. As a matter of fact, free fatty acids are produced by lipase hydrolysis of triglycerides, and then they might be esterified again, by esterase enzymes, in order to obtain flavor compounds (18, 19). Proteolysis involves different enzymes and influences the texture, the bitter flavor and the release of sapid compound (26). Proteolysis in cheese mostly affects caseins, cleaved by the enzymes of the calf rennet, including chymosin and bovine pepsin, and by mik plasmin. The peptides produced are also cleaved by other proteolytic enzymes that came from starter and non-starter microflora of cheese. Among them, an important role is assigned to intracellular peptidases, which are released after lysis of the cells, since they are responsible for amino acid production through degradation of small peptides (27). Free amino acids actually increase during cheese ripening, through the action of bacterial aminopeptidases (10, 24).

Many peptides are biologically active, exerting functions beyond nutrition, such as antimicrobial, antioxidant, anti-hypertensive and opioids (13, 16, 21). Antioxidant peptides might play a significant role in food systems, since they

reduce the autoxidation and the hydroperoxide content of fatty foods (11). Such peptides might also play a role in protecting the organism from ROS, reactive oxygen species (9). Actually, the balance between antioxidant and oxidant compounds is important in order to protect the cell from oxidation, and this ratio is influenced by the oxidative stress or antioxidant uptake (15).

Gupta et al. reported in Cheddar cheese a steady increase in antioxidant capacity during the first period of the ripening time, until a maximum, whereas during the subsequent period of ageing a decrease in antioxidant capacity was observed, suggesting that eventual antioxidant peptides were not resistant to further proteolysis (12). In another study, fractions of water soluble extracts of Cheddar cheese showed a high antioxidant capacity. In a specific fraction showing high antioxidant capacity, different peptides were identified including residues of proline, thus it was concluded that this amino acid influence the antioxidant capacity (14). Peptide containing also methionine, tyrosine, tryptophan and hystidine show antioxidant capacity (22), and this activity is influenced by the structure, the position and the hydrophobicity of the amino acids (8, 17, 24). Anyway, ingested peptides might be degraded by human digestive enzymes after ingestion, reducing their bioaccesibility and their antioxidant potential, even if of course free amino acids might still exert antioxidant capacity.

In this work we present an investigation concerning the antioxidant capacity of water soluble extracts of Parmigiano Reggiano cheese (WSEs) at different months of ripening, measured by ABTS assay. The WSEs were also fractionated by semipreparative HPLC-UV and the antioxidant activities of the single subfractions were determined. The amino acids/peptide composition of the total WSEs and of the subfractions were studied by LC/ESI-MS, in order to identify the compounds mostly contributing to the antioxidant capacity.

Moreover, the antioxidant capacity was also determined after simulated *in vitro* gastro-intestinal digestion of the extracts.

5.4.2. Materials and Method

5.4.2.1 Chemicals

All solvents and reagents were HPLC grade and used as commercially available without any further purification; deionized water was obtained by Millipore Alpha Q system (Millipore Corporation, Billerica, MA, USA); formic acid 99% were from ACROS Organics (New Jersey, USA). ABTS 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) were purchased from Fluka (Sigma, St. Louis, MO, USA). Potassium persulfate ($K_2S_2O_8$), Trolox (6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid), tryptophan, pepsin from swine gastric tissue, □-chymotripsin from swine pancreas, trypsin from swine pancreas, potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl), sodium cloride (NaCl), were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Hydrochloric acid 37 % were purchased from Carlo Erba (Rodano, Mi, Italy). Qubit-fluometer (Invitrogen, UK). AccQ*Fluor reagent kit was obtained from Waters (Milford, MA, USA).

5.4.2.2 Samples

Parmigiano Reggiano cheese (PDO) is well know Italian hard cheese, long ripened, made from raw and partially skimmed cow's milk. The cheese making procedure starts with the addition of a natural whey starter, heating at 33 °C and the addition of the calf rennet. The characteristic of Parmigiano Reggiano cheese technology are: use of natural whey culture as starter obtainded from

previous day's cheese constituted by thermophilus Lactic Acid Bacteria; the high temperature of curd cooking and a long maturation period. In this work were used six wheels of Parmigiano Reggiano cheese at 7, 12, 24, 36, 41 months of ripening. In order to reduced the natural variability the samples were withdraw from the same cheese factory in the province of Mantova (Italy).

5.4.2.3 Water Soluble Extracts of Parmigiano Reggiano cheese

90 ml of distilled water were added to 20 g of Parmigiano-Reggiano and the mixture was homogenized for 1.5 minutes and filtrated on paper filter.

5.4.2.4 Protein content of WSEs and subfractions

The protein content of WSEs and subfractions was performed with a fluorimetric protein quantitation assay (Qubit-fluometer, Invitrogen, UK).

5.4.2.5 LC/ESI-MS of Water Soluble Extract of Parmigiano Reggiano cheese

900 µl of WSEs were filtered with Millipore system 0.45 µm and injected in LC/ESI-MS system. UPLC/ESI-MS (WATERS, Milford, MA, USA) analysis conditions: eluent A: H_2O (0.2% CH_3CN and 0.1 % HCOOH); eluent B CH_3CN (0.1 % HCOOH); Gradient elution was performed according to the following steps: 0-7 min isocratic 100% A, 7-50 min linear gradient from 100 % A to 50% A, 50-52 min isocratic 50 % A, 53-58 min from 50 % A to 0 % A and reconditioning. Column: AQUITY UPLC Beh C_{18} (1.7 um, 2.1x150mm). Flow rate: 0.2 ml/min. MS conditions: ESI, positive ions, single quadrupole analyzer.

Capillary voltage 3.2 kV, cone voltage 30 V, source temperature 100 °C, desolvation temperature 200 °C, cone gas flow (N_2) 100 L/h, desolvation gas (N_2) 650 L/h, acquisition 100:2000 m/z.
All data were acquired and processed by the software MassLynx 4.0 (Waters, Milford, MA, USA).

5.4.2.6 Subfraction purification by semipreparative LC

Mobile phases for chromatographic elution were 0.1% formic acid and 0.2% acetonitrile in water (eluent A); 0.1% formic acid in acetonitrile (eluent B). The flow rate was 5 ml min^{-1}. Elution conditions: isocratic 100% A until 12 min, then a linear gradient up to 50% of A in 81 minutes. The eluent B was then brought in 9 minutes at 100% for rinsing the column, followed by 10 minutes in 100% A in order to re-equilibrated the column. The column (Jupiter 5µ C18 300Å 250x10 mm, Phenomenex) was maintained at room temperature. The UV-detector (Waters 486, Tunable Dedector Absorbance) was set at 214 nm.
For the first fractionation, 500 µl of WSE was injected in the semipreparative LC system and 18 subfractions were collected sampling every 5 minutes.
For the second fractionation 5 subfractions weree collected every 2 minutes in the the first 10 minutes of run, using the same conditions as above. Every subfraction was dried under vacuum.

5.4.2.7 Simulated gastrointestinal digestion of WSEs

WSEs were acidified with HCl 1M until pH=2.2. Pepsin was added and the solution was incubated at 37 °C for 3 hours. The solution was then neutralized with ammonium carbonate up to pH 7.5 and trypsin and chimotrypsin were

then added. The solution was incubated at 37 °C for 4 hours. The enzymatic activity was stopped by adding 19 % of acetonitrile. The solution was dried under nitrogen flow and maintained at -20 °C until the analysis. All three enzymes were used according to an enzyme:protein ratio of 1:100.

5.4.2.8 Antioxidant Capacity

The antioxidant capacity was performed by ABTS assay according with the methods proposed by Re et al.(1999) with some modifications. The ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid, Fluka) and potassium persulfate ($K_2S_2O_8$, Sigma-Aldrich) were dissolved in deionized water, in order to obtain a concentrations respectively 70 mM and 2 mM. The stock solution containing ABTS radical cation was generated by adding 100 µl of $K_2S_2O_8$ to ABTS solution and keeping the sample overnight in the dark at room temperature. The working solution was prepared by diluting 2 mM ABTS in phosphate buffer solution (PBS 8 mM) in order to obtain an absorbance of about 0.7±0,02. The PBS was composed to: 2.5 mM KCl, 125 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and the pH was adjusted to 7.4 with H_3PO_4

The WSEs and digested WSEs were diluted 1:100 and 200 µl of sample were directly put in a cuvette (Plastibrand), added of 1.8 ml of ABTS working solution and incubated for 1 hour at room temperature. The control solution was done by 1800 µl of work solution and 200 µl of PBS pH 7.4.

The cuvettes of sample and control were inserted in the spectrophotometer (Lambda Bio 20, Perking Elmer) and the absorbance at 734 nm was monitored. The antioxidant capacity of the subfractions purified by HPLC was performed by the same method, but the samples were diluted 4 times (subfraction with more antioxidant capacity) or 2 times (subfraction with less antioxidant capacity).

Trolox (Sigma-Aldrich), the water-soluble vitamin E analogue, was used as standard in order to provide antioxidant capacity in terms of TEAC (Trolox Equivalent Antioxidant Capacity, expressed in μmols of Trolox for mg $^{-1}$ of protein and ml $^{-1}$ of extract). Trolox was analyzed with the same procedure of the sample. The inhibition percentage of every sample was plotted against the equivalent nmols of trolox attaining the same inhibition, in order to obtain the TEAC of the sample. All samples and standards were analyzed in duplicate.

5.4.2.9 Amino acids analysis

Some subfractions purified by semipreparative LC were analyzed for their amino acid content.

The dried fraction was diluted with 200 μl of deionized and stirred for 1 minutes. 10 μl of sample solution were placed in a new tube, with buffer and fluorescent derivatizing reagent (AccQ*Fluor reagent kit, WATERS). After stirring, the solution was maintained at 55 °C for 10 minutes.

The HPLC analysis was performed on an Alliance 2695 separation system (Waters, Milford, MA, USA) with a HPLC column ACCQ-Tag C 3.9x150 mm at 37 °C, at a flow rate of 1 ml min^{-1} and 2 μl of volume injected. The eluents used were composed by: eluent A phosphate buffer AccQ*Tag diluted in water and eluent B CH_3CN:H_2O in ratio 60:40. The gradient elution was performed with different step: from 100 % elutent A to 67 % in 51 minutes, followed by column washing and reconditioning.. The detection was carried out by Waters 470 Fluorescence detector (λ excitation 250 nm and λ emission 395 nm).

5.4.2.10 Tryptophan quantification

Tryptophan pure standard was diluted in water in order to get a final concentration of 2 mM. The concentration range from 0.1 mM to 2 mM of tryptophan was considered for the calibration curve. The quantification was carried out in WSEs and subfractions by LC/ESI-MS, applying the same method described above. The signal of tryptophan was obtained by the eXtract Ion Chromatogram (XIC) method, followed by integration of the peak area. Quantitation was performed according to the external standard method.

5.5. Results and discussion

5.5.1 Antioxidant capacity of Parmigiano-Reggiano WSEs at different ageing times

From six samples of Parmigiano-Reggiano the Water Soluble Extracts (WSEs) were obtained and their antioxidant capacity was measured by standard ABTS test. In particular, wheels of Parmigiano Reggiano produced in the same cheese factory at different ageing times were chosen, in order to reduce the natural variability existing between different cheese factories and only considering the variability introduced by the ageing time.

The WSEs were obtained by dilution and homogenization in deionized water as detailed in the experimental part and, after filtration on paper filter, the antioxidant capacity was directly analyzed. The results were expressed in terms of Trolox Equivalent Antioxidant Capacity (TEAC). The protein content of the extracts was also measured by the Qubit assay, and the TEAC was then calculated as related to the apparent protein content, in μmols of TROLOX per μg or protein in WSE. (Figure 1).

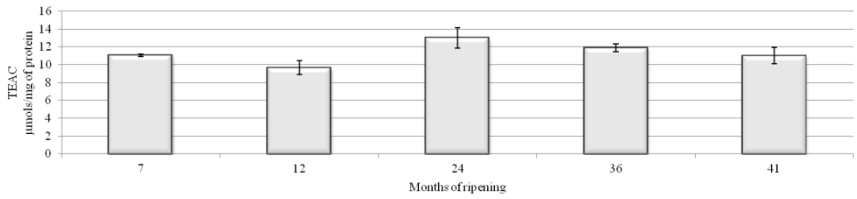

Figure 1: Trend of antioxidant capacity during ageing time in Parmigiano-Reggiano cheese

The values given in Figure 1 indicate an antioxidant capacity comparable to the results already reported in the literature: actually, the behavior of the TEAC of WSEs of cheddar cheeses during ripening time reported in literature (12) showed a steady increase until the fourth month of ageing time (9.81 μM/mg of protein), but upon further ripening the TEAC decreased. In the case of Parmigiano-Reggiano, the TEAC values seemed to remain quite constant during ripening time, suggesting that the molecules responsible for it are not particularly affected by the biochemical processes taking place during the ageing time. In particular, this means that the amino acid side chains responsible for the antioxidant capacity maintain almost the same capacity either when they are included in peptides and proteins or when they are present in free amino acids.

5.5.2 Molecular characterization of the WSEs and identification of the main antioxidant components

The WSEs, were also analyzed by UPLC/ESI-MS in order to assess their composition. The analysis of the chromatograms, and of the mass spectra associated to the main peaks, allowed to identify in the chromatograms free amino acids, oligopeptides and proteins, eluting at different retention times (Figure 2).

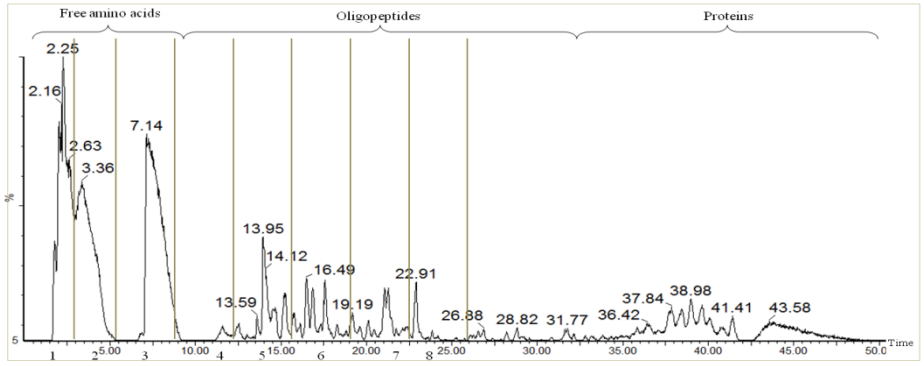

Figure 2: Typical UPLC/ESI-MS chromatogram of WSEs from Parmigiano Reggiano cheese, which indicated the elution zones of amino acids, oligopeptides and proteins. Fractions from 1 to 8 collected by semipreparative HPLC are also indicated.

WSEs from samples having 24, 36 and 41 months of ageing were then fractionated by semipreparative HPLC (UV detection at 214 nm), using the same eluents and the same gradient as UPLC/ESI-MS analysis, suitably modified for the different semipreparative column. Eighteen fractions were

obtained sampling every 5 minutes; all fractions were then subjected to measurement of antioxidant capacity by ABTS assay as indicated before, protein quantification by Qubit assay and molecular characterization by UPLC/ESI-MS.

The initial screening of the fractions showed a high antioxidant capacity in fractions 1, 2 and 5 and also some capacity in fraction 6, and no clear correlation was observed between these activities and the ageing time, whereas the antioxidant capacity was negligible from fraction 7 to 18 (figure 3).

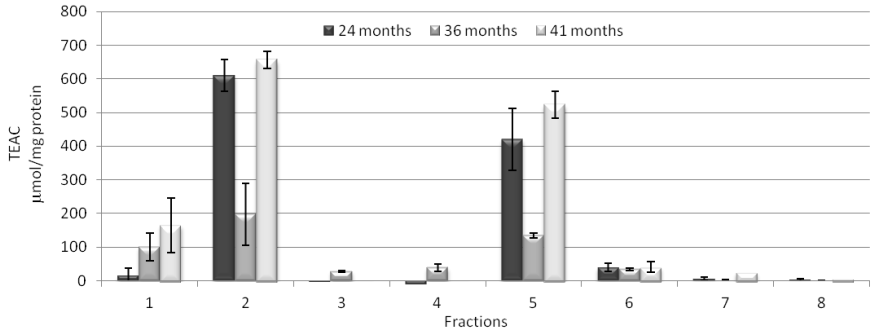

Figure 3: Antioxidant capacity (TEAC µmols/mg protein) in the fractions considered.

All fractions were subjected to UPLC/ESI-MS analysis in order to identify the molecules most responsible for the antioxidant capacity. In fraction 5, the most abundant compound was tryptophan.

This is hardly surprising, since Estevão et al., already demonstrated the ability of the indole ring to act as radical scavenger. They studied different molecules containing the indole ring, including tryptophan. The molecules with free

amine in the side chain as well as indolic nitrogen had the highest radical scavenging capacity. The indole nucleus is reactive with the radical species, due to the resonance stabilization of the intermediate radical formed (8). Actually, among the amino acids having a radical scavening capacity, in literature tryptophan is reported as having a high radical scavenging property, exactly because of the indole group, allowing for electron delocalization. (29)
In order to confirm these finding, tryptophan was quantified in the fraction 5 with the external standard method. The concentration showed value at 24 months of 0.53 mM, of 0.24 mM at 36 months, and of 0.84 mM at 41 months. It is easy to notice that the trend of quantification was directly connected with the trend of the antioxidant capacity in fraction 5 (Figure 4).

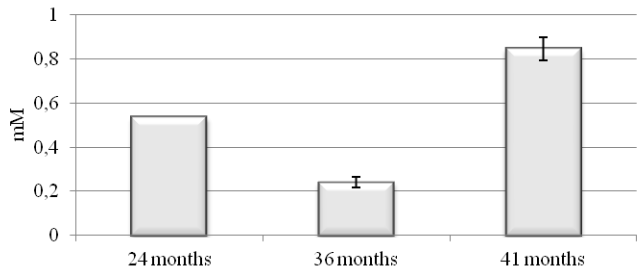

Figure 4: Tryptophan quantification in the fraction 5.

Lactoyl-tirosine, a NPAD containing tyrosine, was detected as one of the major component in fraction 6. This molecule is synthetized de novo in cheeses by unknown enzymatic activies (25); it is possible to hypothesize that lactoyl-tyrosine might be the molecule most responsible for the radical scavenging capacity in this fraction, due to the ability of its phenolic group to act as hydrogen donor. (23)

In order to better identify the most antioxidant molecules in the fractions 1 and 2, those fractions were subjected to qualitative amino acids analysis. The amino acids analysis confirmed the presence of all amino acids in fraction 1 in all samples, whereas, in fraction 2, tyrosine was always present in all samples and occasionally (sample having 36 months of ageing) methionine was also identified.

In order to better confirm the actual amino acids most responsible for the antioxidant capacity, the sample having 41 months of ageing was also subjected to further subfractionation in the first minutes of elutions by semipreparative HPLC in the same condition, obtaining 6 further subfractions collected every 2 minutes. The six subfractions obtained were analyzed by ABTS assay and characterized by LC/ESI-MS. As shown in figure 5 only subfraction 3 and 4 presented antioxidant capacity. LC/ESI-MS confirmed that these subfractions were constituted by the amino acids tyrosine and methonine.

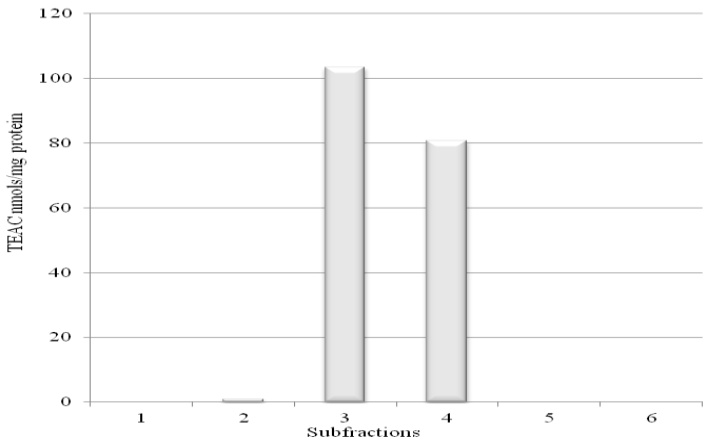

Figure 5: Antioxidant capacity (TEAC nmols/mg protein) in the subfractions of further fractionation.

5.5.3 Antioxidant capacity after simulated gastrointestinal digestion

In order to evaluate if the antioxidant capacity was maintained after gastrointestinal digestion (GI), simulated *in vitro* GI was performed. The gastrointestinal tract was modeled by performing pepsin digestion at acidic pH (stomach) and trypsin and chimotrypsin digestion at neutral pH (intestine) (31).

The antioxidant capacity was measured on the digested samples in the same conditions of Water Soluble Extracts (Figure 6).

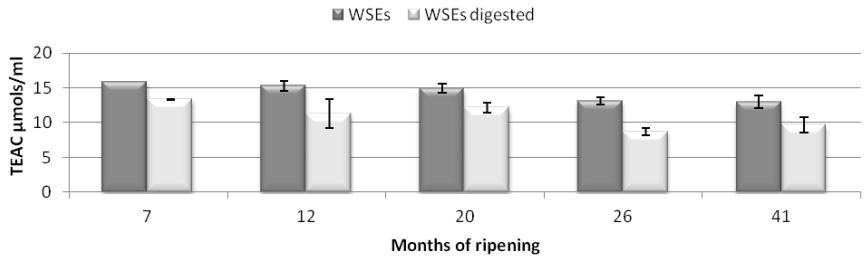

Figure 6: Different between antioxidant capacity values of digested Water Soluble Extracts and Water Soluble Extracts

About 80% of the natural antioxidant capacity of Parmigiano Reggiano WSEs was retained also after digestion, demonstrating that the degradation of shorter peptides to free amino acids and of longer peptides to shorter peptides did not affect the antioxidant capacity, in agreement with the fact that the already present free amino acids are mostly responsible for the observed capacity. The slight decrease observed might be due to the degradation of

tryptophan caused by the acidic environment of the simulated gastric digestion.

The fact that some free amino acids and small peptides are able to exert antioxidant properties, is interesting both for the fact that they might exert local antioxidant effects in the gut, but also in view of their bioavailability. The amino acids are actually absorbed through a specific transport system, and peptides as well, although with a different specific independent transport system.(32). The blood transports the amino acids resulting from the peptide digestion and those coming from luminal absorption in all organs throughout the body (33). Moreover, some peptides can resist to the proteasic degradation in blood after absorption and thus might exert an antioxidant function in vivo. (34).

5.6 Conclusions

The measured antioxidant capacity of water soluble extract of Parmigiano Reggiano cheese (WSEs) at different ageing time indicated a quite high radical scavenging capacity, nearly unaffected by ageing time and simulated *in-vitro* gastrointestinal digestion.

Fractionation experiments demonstrated that antioxidant capacity in WSEs from Parmigiano-Reggiano cheese is mostly due to antioxidant free amino acids, in particular tyrosine, methionine and tryptophan, and only in minimal part to antioxidant peptides, also including NPADs.

The *in vitro* approach used in this study used the ABTS assay (an electron transfer model based on a single reaction between the antioxidant and a free radical), reported in literature as a one of numerous method commonly used. Other commonly used methods are based on the hydrogen atom transfer, involving several reactions between radicals and the oxidizable probe.

Anyway, in order to assess the translability of these results *in vivo*, further studies will have to be performed in living organisms identifying biomarkers of biological efficacy in preventing oxidative damages (35).

5.7 References

1. S. Mills, R.P. Ross, C. Hill, G. F. Fitzgerald, C. Stanton (2011) Milk intelligence: Mining milk for bioactive substance associated with human health
2. D. Agyei, M.K. Danquah, (2012) Rethinking food-derived bioactive peptides for animicrobial and immunomodulatory activities. Trends in Food Science& Technology 23, 62-69.
3. S. V. Silva, F. X. Malcata (2005) Caseins as source of bioactive peptides, International Dairy Journal 15, 1-15.
4. L. Steinsstraesser, U. Kranenburg, F. Jacobsen, S. Al-Benna (2011), Host defense peptides and their antimicrobial-immunomodulatory duality. Immunobiology 216, 322-333.
5. W. Jiang, L. T. Sunkara, X. Zeng, Z. Deng, S. M. Myers, G. Zhang, (2013), Differential regulation of human cathelicidin LL-37 by free fatty acids and their analogs. Peptides 50, 129-138.
6. J. Schauber, R. L. Gallo (2008), Antimicrobial peptides and the skin immune defense system. Journal of Allergy and Clinical Immunology 122 (2), 261-266
7. A.M. Van Der Does, P. Bergman, B. Agerberth, L. Lindbom, Induction of the human cathelicidin LL-37 as a novel tratment against bacterial infections. Journal of Leukocyte-Biology-2012 92:735-742.
8. Estevao, MS, Carvalho LC, Ribeiro D, Couto D, Freitas M, Gomes A, Ferreira LM, Fernandes E, Marques MMB. (2010). Antioxidant capacity of unexsplored indole derivatives: synthesis and screening, Eur J Med Chem 45: 4869-4878.

9. Finley JW, Kong AN, Hintze KJ, Jeffery EH, Ji LL, Lei XG. (2011). Antioxidants in Foods: State of the Science Important to the Food Industry, J Agr Food Chem 59: 6837-6846.
10. Gatti M, De Dea Lindner J, Gardini F, Mucchetti G, Bevacqua D, Fornasari ME, Neviani E. (2008). A model to assess lactic acid bacteria amminopeptidase activities in Parmigiano Reggiano cheese during ripening, J Dairy Sci 91: 4129-4137.
11. Gill I, Lopez-Fandino R, Jorba X, Vulfson EN. (1996). Biologically active peptides and enzymatic approaches to their production. Enzyme Microb Tech 18 (3): 163-183.
12. Gupta A, Mann B, Kumar R, Sangwan RB.(2009). Antioxidant capacity of Cheddar cheeses at different stages of ripening. Int J Dairy Technol, 62 (3): 339-347.
13. Hartmann R, Meisel H. (2007). Food-derived peptides with biological capacity: from research to food applications. Curr Opin Biotech 18: 163-169;
14. Hernandez-Ledesma B, Miralles B, Amigo L, Ramos M, Recio I. (2005). Identification of antioxidant and ACE-inhibitory peptides in fermented milk, J Sci Food Agric, 85: 1041-1048.
15. Himaya SWA, Ngo DH, Ryu BM, Kim SK. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) capacity and cellular oxidative stress, Food Chem 132: 1872-1882.
16. Korhonen H (2009). Milk-derived bioactive peptides: From science to application, J Funct Foods, 1: 177-187.
17. Li Y W, Li B, He J, Qian, P. (2011). Quantitative structure-capacity relationship study of antioxidative peptide by using different sets of amino acids descriptors, J Mol Struct 998: 53-61.

18. Liu SQ, Holland R, Crow VL. (2004). Esters and their biosynthesis in fermented dairy products: a review. Int Dairy J 14: 923-945.
19. Malacarne M, Summer A, Franceschi P, Formaggioni P, Pecorari M, Panari G, Mariani P. (2009). Free fatty acid profile of Parmigiano-Reggiano cheese throughout ripening: Comparison between the inner and outer regions of the wheel. Int Dairy J 19: 637-641.
20. McSweeney PLH. (2004). Biochemistry of cheese ripening, Int J Dairy Technol 2-3: 127-144.
21. Muro Urista C, Alvarez Fernandez R, Reira Rodriguez F, Arana Cuenca A, Tellez Jurado A. (2011). Review: Production and Functionality from active peptide from milk, Food Sci Technol Int 17: 293-317.
22. Nimalaratne C, Lopes-Lutz D, Schieber A, Wu J. (2011). Free aromatic amino acids in egg yolk show antioxidant properties, Food Chem 129 (1): 155-161.
23. Saito K, Jin DH, Ogawa T, Muramoto K, Hatakeyma E, Yasuhara T, Nokihara K. (2003). Antioxidative properties of tripeptide libraries prepared by the compbinatorial chemistry, J Agr Food Chem 51: 3668-3674.
24. Sarmadi BH, Ismail A. (2010). Antioxidative peptides from food proteins: A review, Peptides 31: 1949-1956.
25. Sforza S, Cavatorta V, Galaverna G, Dossena A, Marchelli R. (2009). Accumulation of non-proteolytic aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening, Int Dairy J 19: 582-587.
26. Smit G, Smit BA, Engels WJM. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products, FEMS Microbiol Rev 29: 591-610.
27. Sousa MJ, Ardo Y, McSweeney PLH. (2001). Advances in the study of proteolysis during cheese ripening, Int Dairy J 11: 327-345.

28. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (1999). Antioxidant capacity applying an improved ABTS radical cation decolorization assay, Free Radical Bio Med 26 (9-10): 1231-1237.
29. Tsopmo A, Romanowski A, Banda L, Lavoie JC, Jenssen H, Friel JK (2011). Novel anti-oxidative peptides from enymatic digestion of human milk, Food Chem 126: 1138-1143.
30. Zannoni M (2010). Evolution of the sensory characteristics of Parmigiano-Reggiano cheese to the present day, Food Qual and Prefer 21: 901-905.
31. Cavatorta V, Sforza S, Aquino G, Galaverna G, Dossena A, Pastorello E.A, Marchelli R. (2010). In vitro gastrointestinal digestion of the major peach allergen Pru p 3, a lipid transfer protein: Molecular characterization of the products and assessment of their IgE bindings abilities, Mol Nutr Food Res 54:1452-1457.
32. Walter E, Kissel T, Amidon, G.L. (1996). The intestinal peptide carrier: A potential transport system for small peptide derived drugs, Adv Drug Deliver Rev 20:33-58.
33. Maillard M.E, Stevens B. R, Mann G.E. (1995). Amino acids transport by small intestinal, hepatic, and pancreatic epithelia, Gastroenterology 108:888-910.
34. Picariello G, Iacomino G, Mamone G, Ferranti P, Fierro O, Gianfranti C, Di Luccia A, Addeo F. (2013) Transport across Caco-2 monolayers of peptides arising from in vitro digestion of bovine milk proteins, Food Chem 139: 203-212.
35. Power O, Jakeman P, FitzGerald R.J. (2013). Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides. Amino Acids 44:797-820.

GENERAL CONCLUSION

Conclusion and perspectives

The present Ph.D. thesis concerns the study of the synthesis, the origin and the properties of Non Proteolytic Aminoacyl Derivatives (NPADs) in cheeses.

Parmigiano Reggiano cheese is a complex matrix containing about one third of proteins, about one third of fat, about one third of humidity. Ripening usually involves several phenomena, mostly affecting changes in the nitrogen fraction at the molecular level. The caseins, present in milk, are subjected to proteolytic enzyme action, enzymes deriving from milk (such as plasmin), rennet (chymosin) and Starter and Non Starter Lactic Acid Bacteria (LAB). This yields many peptides and free amino acids, which evolve along the ageing time following characteristical trends.

One of the major breakthroughs in the molecular characterization of the nitrogen fraction of Parmigiano Reggiano cheese in the last years has been the discovery of Non Proteolytic Aminoacyl Derivatives (NPADs), peptide-like compounds not deriving from proteolytic events, but de novo synthesized by unknown enzymes starting from the aminoacidic precursors formed during the proteolysis. This class of molecules includes γ-glutamyl amino acids, lactoyl amino acids and, in minor amount, pyroglutamyl amino acids. In this thesis, also acyl amino acids formed with fatty acid residues have been preliminarly identified. In a previous work, Starter and Non Starter lactic acid bacteria, such as *L. helveticus* and *L. rhamosus*, have already been demonstrated to be able to produce γ-glutamyl-phenylalanine and lactoyl-phenylalanine starting from lysed cells, and lactoyl-phenylalanine in growing conditions.

This thesis was aimed at increasing the so far scarce knowledge on this new class of compounds.

First a detailed study on their presence in Parmigiano Reggiano cheese was performed in many samples coming from different factories at various stages of ageing, providing for the first time a complete and comprehensive overview

of their presence in cheeses. Then, the conditions in which they are formed, the enzymes possibly responsible for their formation, and also the conditions in which they are degraded were throuhgly investigated. For doing this, synthetic procedures for obtaining these compounds, also in isotopically enriched form, were first devised, allowing to obtain them by pure chemical synthesis. In this context, also their resistance to biological fluids, namely in human serum and in conditions simulating the gastrointestinal digestion, have been investigated, posing the basis for assessing their bioaccessibility. As a natural prosecution of this study, bioavailability was then tested, measuring their ability to be transported throught Caco-2 cells. Finally, preliminary studies have started on their potential biofunctional acitivities, namely immunomodulatory and antioxidant.

Their general presence in all Parmigiano-Reggiano samples was definitely demonstrated, as well as the fact that they show an accumulating trend along the ageing time. This allowed to use them as molecular markers for assessing the ageing time. The chemometric approach, using multiple linear regression and linear discriminanting analysis allowed to determine the range of ripening with good approximation.

The production, but also the degradation of γ-glutamyl phenylalanine and lactoyl-phenylalanine by enzymatic activities present in Parmigiano Reggiano cheese extracts was confirmed. Different efficiency of production at different pH hints for γ-Glu-Phe and Lac-Phe being produced (and degraded) by different enzymes. The hypothesis that the responsible enzyme(s) might be protease(s) was tested incubating common endoproteases (trypsin, pepsin, chimotrypsin), one exoprotease (carobxypeptidase Y) and also gamma-glutamyltranspeptidase (GGT) with the precursors. Only carboxypeptidase Y was found to be able to produce small amounts of Lac-Phe, but not of γ-Glu-Phe. On the other hand, γ-Glu-Phe was produced by GGT, as expected, and also,

quite surprisingly, when glutamic acid instead of glutamine was used as precursor. Therefore, the combined action of GGT (for γ-Glu-Phe) and exoproteases (for Lac-Phe) might be at the origin of these compounds in cheese using glutamic acid and lactic acid as precursors, together with the other amino acids present in the pools of free amino acids.

In order to study the bioaccessibility and bioavailability of these compounds, γ–Glu-Phe were also tested for its resistance to gastrointestinal digestion and in blood serum, and it was found to be perfectly resistant. its intestinal permeability was analyzed by the Caco-2 permeability model. The results obtained shows that the molecule cancross the membrane thought a transporter,iIn this case Pg-P, expressed in Caco-2 cells.

These experiments taken together allow to hypothesize that the γ-glutamyl phenylalanine can be absorbed intact, transported to the blood circulation and thus becoming bioavailable for the target organs.

Anyway, the biological activity NPADs had never been studied before. According to the results presented in this thesis, an evidence that γ-glu-phe can have immunoregulatory properties, stimulating the innate immune reponse, was found.

The antioxidant capacity was also measured on Parmigiano Reggiano cheese extracts with ABTS assay, and a possible contribute of Lactoyl-tyrosine, albeit minor if compared to free amino acids, was also outlined.

This Ph.D. thesis lays the foundation for the study of NPADs, because the results here presented for the first time outline their ubiquituous presence in all Parmigiano Reggiano cheese samples, their bioavailability and their bioaccessibility and their potential biological properties. More studies will be needed in order to further improve knowledge regarding their use as markers, their biological activity and their applicability in food industry.

Author

Chiara Bottesini was born in 1984 in Parma.
chiara.bottesini@gmail.com

Studies:

2011-2013: Ph.D. in Food Science Technology. Department of Food Science. University of Parma, Italy.

NOV 2012-MAR 2013: Experience abroad, LEITAT technological center Bioinvitro Unit, Barcelona, Spain.

2003-2009: Master degree in Food Science and Technoloty, Faculty of Agronomy, University of Parma, Italy.

Seminars:

LC/MS 2012 Advanced course on the coupling of mass spectrometry with separation techniques in liquid phase.

Pubblication List

Enzymatic production and degradation of cheese-derived non proteolytic aminoacyl derivatives
Chiara Bottesini, Tullia Tedeschi, Arnaldo Dossena, Stefano Sforza
Amino Acids 2013 DOI 10.1007/s00726-013-1637-3 ACCEPTED

Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese

Chiara Bottesini, Sara Paolella, Francesca Lambertini, Gianni Galaverna, Tullia Tedeschi, Arnaldo Dossena, Rosangela Marchelli, Stefano Sforza*

International Journal of Food Sciences and Nutrition. Volume 64, Issue 8, (2013), 953-958

Microbial origin of non proteolytic aminoacyl derivatives in long ripened cheeses

Elisa Sgarbi, Camilla Lazzi, Luca Iacopino, Chiara Bottesini, Francesca Lambertini , Stefano Sforza, Monica Gatti

Food Microbiology, Volume 35 (2013) 116-120

Antimicrobial activity of poultry bone and meat trimmings hydrolyzates in low-sodium turkey food

Pier Paolo Zanello, Stefano Sforza, Arnaldo Dossena, Francesca Lambertini, Chiara Bottesini, Ilya V. Nikolaev, Olga Koroleva, Tecla Ciociola, Walter Magliani, Stefania Conti and Luciano Polonelli

Food and Function DOI: 10.1039/c3fo60454c ACCEPTED

Growth promotion of Bifidobacterium and Lactobacillus species by proteinaceous hydrolysates derived from poultry processing leftovers

Camilla Lazzi,Federica Meli,Francesca Lambertini,Chiara Bottesini,Ilya Nikolaev,Monica Gatti,Stefano Sforza,Olga Koroleva,Vladimir Popov,Erasmo Neviani,Arnaldo Dossena

International Journal of Food Science & Technology, volume 48, issue2, pages 341–349, 2013

Assessing allergenicity of different tomato ecotypes by using pooled sera of allergic subjects: identification of the main allergens

Mariangela Bencivenni Andrea Faccini Chiara Bottesini Rosa Rao Aikaterini Detoraki Erminia Ridolo Gianni Marone Pier Paolo Dall'Aglio Arnaldo Dossena Rosangela Marchelli Stefano Sforza European Food Research Technology (2011) DOI 10.1007/s00217-011-1640-4

Oral Comunications

Antioxidant activity of peptide fraction of Parmigiano Reggiano cheese at different ageing time - Euro Food Chem XVI –Gdansk 6th-8th July 2011

Non Proteolytic Aminoacyl Derivatives (NPADs): Synthesis, biochemical, biofunctional properties - XVIII Workshop on the developments in the italian PHD research on food science technology and biotechnology - Conegliano 25-27 september 2013