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Ciclo XXI

CHARACTERIZATION AND BIOACTIVITY OF OLIGOPEPTIDES IN ASIAGO D'ALLEVO CHEESE

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RIASSUNTO

Gli alimenti funzionali sono degli alimenti che concentengono dei composti in grado di svolgere effetti positivi sulla salute umana. Le proteine del latte presentano un'ampia gamma di attività nutrizionali, funzionali e biologiche che le rendono potenziali ingredenti negli alimenti volti a promuovere la salute. Nel presente lavoro di tesi sono stati identificati oligopeptidi presenti nel formaggio Asiago designato del marchio PDO e si è valutata l'attività biologica di tali oligopeptidi.

La prima parte della tesi rappresenta una visione panoramica degli alimenti funzionali ed in particolare dei prodotti lattiero-caseari come potenziali alimenti in grado di avere un effetto positivo sulla salute degli individui. Inoltre è stata fornita una visione del mercato e della situazione legislativa nel campo degli alimenti funzionali sia nel mondo che nei paesi europei. Infatti, negli ultimi decenni, l'interesse per gli alimenti funzionali ha visto un crescente interesse anche in Europa, sebbene la legislazione dia solo indicazioni molto generali sulle caratteristiche che devono avere tali alimenti. Oltre al ben noto ruolo delle vitamine, dei minerali e di determinati acidi grassi, gli oligopeptidi che si orginano dalla degradazione delle proteine del latte sono considerati come potenziali composti in grado di esercitare funzioni biologiche. Sono inoltre descritte le modalità di formazione di questi oligopeptidi da parte di tagli proteolitici ad opera di enzimi, la loro ulteriore degradazione nello stomaco e nell'intestino dopo ingestione e le principali funzioni fisiologiche che essi possono svolgere. Nella parte finale di questo capitolo, vengono riportare le caratteristiche del formaggio Asiago, nello specifico ci si sofferma sulla descrizione della caseificazione e sulle principali proprietà nutrizionali di questo formaggio italiano.

Questa tesi considera molti campioni di formaggio Asiago d'allevo prodotti in 5 aziende per la produzione di latte localizzate nell'*Altopiano dei Sette Comuni* ed una azienda situata in una zona limitrofa chiamata *Novegno* (Regione Veneto, Italia). Ogni azienda presenta due aree ben distinte: una situata al di sopra dei 1000 m di altitudine ed una seconda a più alte altitudini disponibile solo nel periodo estivo (periodo dell'alpeggio). I formaggi sono stati prodotti con latte crudo secondo il

disciplinare dell'Asiago e sono stati ottenuti in tre diversi periodi sperimentali: *a)* in maggio, le vacche sono in stalla e mangiano *unifeed* (mais e soia); *b)* in luglio, le vacche vengono portate in alpeggio e vengono alimentate con un supplemento (mais, semi di orzo e di soia) con una dose giornaliera di circa 5 kg di sostanza secca per vacca e *c)* in settembre con condizioni di allevamento simili a quelle descritte in *b)*. Tutte le aziende hanno prodotto il formaggio in un caseificio in maggio, mentre durante l'alpeggio, alcune aziende hanno caseificato in piccoli caseifici localizzati in una zona alpina (1500 m di altitudine in media) vicina all'azienda. In ciascun dei tre periodi sperimentali (maggio/stalla; luglio/alpeggio e settembre/alpeggio), un formaggio è stato stagionato per 6, 12 e 18 mesi.

Il secondo capitolo si focalizza sull'identificazione di oligopeptidi presenti nel formaggio Asiago d'allevo prodotto nelle aziende sopra descritte. In questa ricerca sperimentale, sono stati considerati solo campioni stagionati 6 e 12 mesi. Mediante la cromatografia accoppiata allo spettrometro di massa (HPLC/ESI-MS), sono stati identificati 76 peptidi, molti dei quali derivanti dall'α_{s1}- e dalla β-caseina (CN) e solo alcuni derivanti dalla α_{s2}-CN. Alcuni peptidi non sono risultati derivanti da tagli enzimatici a carico della chimosina o degli enzimi delle colture starter, probabilmente il contributo da parte dei batteri non starter è stato determinate nella formazione di questi peptidi. I campioni stagionati 12 mesi sono risultati più ricchi in peptidi rispetto ai campioni stagionati 6 mesi, questo risultato è in accordo con l'azione della proteolisi che determina un progressivo rilascio di oligopeptidi nel formaggio durante la stagionatura. E' stata utilizzata una analisi multivariata delle componenti principali, in quanto si intende proporre i peptidi come potenziali marcatori molecolari per valutare l'autenticità del formaggio. I risultati di questa ricerca confermano che questa analisi statistica basata sul profilo degli oligopeptidi è un approccio potente per valutare la proteolisi nel formaggio e per discriminare il formaggio principalmente in base all'epoca di stagionatura. Il pattern peptidico ha permesso inoltre di differenziare i campioni in base all'utilizzo dello starter durante la caseificazione. Tuttavia, il pattern peptidico ha consentito di mettere in evidenza le

differenze in base all'alimentazione delle vacche e alle condizioni ambientali solo considerando i campioni stagionati 12 mesi. Si può quindi concludere che gli oligopeptidi possono essere considerati utili marker molecolari per determinare l'autenticità del formaggio Asiago PDO in relazione alla stagionatura e all'utilizzo dello starter.

Il terzo capitolo è relativo al secondo contributo sperimentale di questa tesi, ed è improntato sulla valutazione di una possibile attività anti-ipertensiva ad opera degli oligopeptidi presenti nel formaggio Asiago. La pressione sanguigna è regolata da diversi processi chimici, uno dei quali è denominato sistema rennina-angiotensina-aldosterone. L'enzima che converte l'angiotensina (ACE) è un enzima che svolge un ruolo fondamentale in questo processo. A tal proposito, sono stati identificati peptidi derivati dalle proteine del latte in grado di inibire l'enzima ACE consentendo a questi peptidi di ridurre l'elevata pressione sanguigna. In questa ricerca, sono stati prodotti dei formaggi in una azienda in montagna secondo tre diversi sistemi di produzione del formaggio e tre epoche di stagionatura. I peptidi presenti nell'estratto solubile in acqua sono stati ultrafiltrati con membrane aventi cut-off di 10 kDa e 3 kDa in modo da valutare l'attività ACE inibitoria di oligopeptidi a lunga e corta sequenza aminoacidica. Successivamente è stata simulata una digestione gastrointestinale al fine di valutare l'azione degli enzimi digestivi nella formazione di peptidi bioattivi. Dopo apposita trasformazione logaritmica, i dati sono stati sottoposti ad analisi statistica utilizzando un modello misto che includeva gli effetti fissi epoca di stagionatura, sistema di produzione del formaggio, peso molecolare dei peptidi e digestione gastrointestinale. L'effetto sistema di produzione del formaggio non è risultato statisticamente significativo. I formaggi stagionati 6 mesi presentavano una più alta attività inibitoria rispetto ai formaggi stagionati 12 e 18 mesi. Inoltre, la frazione con peptidi aventi peso molecolare inferiore a 3 kDa conferivano una maggiore inibizione rispetto alla frazione con peptidi con peso molecolare inferiore a 10 kDa. La digestione gastrointestinale non ha influito sull'inibizione, anche se si è osservato un aumento di inibizione da parte della

frazione con alto peso molecolare. L'identificazione dei peptidi mediante HPLC/ESI-MS ha permesso l'identificazione di peptidi anti-ipertensivi derivanti principalmente dalla α_{s1}-caseina e dalla β-caseina. Si può quindi concludere da questa prova sperimentale che principalmente i formaggi stagionati 6 mesi presentano una attività ACE inibitoria, seppur debole (circa 12% di inibizione per 100 μg di peptidi), ma durante la stagionatura del formaggio questa bioattività viene meno in conseguenza della probabile degradazione dei peptidi con attività ACE inibitrice.

Il terzo contributo sperimentale trattato nel quarto capitolo si è occupato della valutazione di una potenziale attività antimicrobica di oligopeptidi presenti nel formaggio con peso molecolare inferiore a 10 kDa. L'interesse per questa bioattività è legata ai casi di listeriosi che sono solitamente dovuti a contaminazione del latte. Parallelamente, la contaminazione microbica influenza la sicurezza igienico-sanitaria e le caratteristiche organolettiche del latte, la produzione e l'immagazzinamento del formaggio. Di conseguenza, la presenza di peptidi con attività antimicrobica naturalmente presenti nella matrice del formaggio possono essere un utile strumento per controllare la crescita di batteri patogeni o causarne la morte. I peptidi presenti nel formaggio sono stati incubati con *Listeria innocua* LRGIA 01 e *Listeria monocytogenes* AER 102 a diverse concentrazioni peptidiche. L'attività antimicrobica è stata monitorata per 10 ore di incubazione e dopo 24 ore. In questo studio sono stati utilizzati gli stessi campioni del contributo precedente. I dati sono stati elaborati con un approccio statistico simile a quello utilizzato nel precedente contributo aggiungendo l'effetto tempo di incubazione e l'effetto concentrazione dei peptidi. Considerando gli esperimenti svolti con *L. innocua*, i campioni stagionati 6 mesi hanno mostrato una maggiore inibizione (26%) rispetto ai campioni a più lunga stagionatura (12%, media dei 12 e 18 mesi). Inoltre è stata evidenziata una relazione lineare tra la concentrazione peptidica e l'inibizione osservata. Per quanto riguarda gli esperimenti condotti con *L. monocytogenes*, gli effetti fissi inseriti nel modello non influenzavano in modo statisticamente significativo l'inibizione, che risultava comunque in media inferiore al 12%. Inoltre, in entrambi i batteri oggetto di studio,

la già debole inibizione risultava nulla dopo 24 ore di incubazione suggerendo un'attività batteriostatica da parte degli oligopeptidi presenti negli estratti. Gli estratti sono stati analizzati mediante HPLC/ESI-MS per identificare i peptidi coinvolti nell'attività antimicrobica. Sono stati ritrovati i bene noti peptidi antibatterici α_{s1}-CN *f*(1-23), chiamata isracidina, e α_{s2}-CN *f*(183-207). Anche in questo studio, possiamo concludere che i campioni stagionati 6 mesi presentano degli oligopeptidi che esercitano una attività inibitoria nei confronti di batteri che si possono ritrovare in prodotti lattiero-caseari benché tale attività sia piuttosto debole e non duratura nel tempo.

SUMMARY

Functional food is a food which contains components having a positive effects on human health. Milk proteins are known to exert a wide range of nutritional, functional and biological activities which make them potential ingredients of health-promoting foods. The present PhD thesis concerns the identification and the evaluation of biological function of oligopeptides extracted from the italian PDO Asiago cheese.

The first part is a general review regarding functional foods and especially dairy products. Moreover an overview is given about the market and legislative situation of functional food in the world and in Europe. In fact, in the last decades, the interest on functional food has increased also in the European countries, but it has not been followed by precise and rigid laws regarding functional food. Besides the known role of vitamins, minerals and specific fatty acids, oligopeptides, especially derived from the degradation of milk proteins, are also proposed as potential compounds with biological functions. The peptide generation by the action of enzymatic cleavage, the further degradation in stomach and gastrointestinal tract upon ingestion and the main physiological functions has been described. At the end of this chapter, the description of Asiago was given in order to explain its cheesemaking procedure and its main nutritional properties.

This PhD thesis considered several samples of Asiago d'allevo cheese produced in five dairy farms located in the mountain area so-called *Altopiano dei Sette Comuni* and in a dairy farm situated in another closed mountain area so-called *Novegno* (Veneto Region, Italy). Every farm had two distinctive sites: one in the plateau (above 1000 m a.s.l.) and the other one at higher altitude which is available only in the summer period (alpine grazing season). Cheeses were manufactured with raw milk obtained in three production periods: *a)* in May, the cows were kept in barn and fed a total mixed ration based on hay and concentrate (maize and soybean); *b)* in July, the cows were moved to alpine pasture and fed a supplement (maize, barley and soybean) at daily dose of about 5 kg of dry matter per cow and *c)* in September similar to *b)* condition for the rearing system. All farms produced the cheese in dairy plant in May

meanwhile during grazing season, some of them manufactured cheeses in small dairy plants located whitin the alpine site (1500 m a.s.l. on average) of the farm. In each of the three production periods related to the cheese production system (May/barn; July/alpine grazing and September/alpine grazing), sample was ripened for 6, 12 and 18 months.

The second chapter proposes the identification of oligopeptides in Asiago d'allevo cheeses produced in the dairy farms described above. In this experimental reasearch, we only considered cheeses ripened for 6 and 12 months. Using High Performance Liquid Chromatography coupled on electrospray mass spectrometry (HPLC/ESI-MS), 76 peptides were identified, most of them arose from α_{s1}- and β-casein (CN), and a few arose from α_{s2}-CN. Some peptides do not correspond to known specific cleavages due to chymosin or starter cultures, probably the contribution of non starter bacteria is important to generate these peptides. 12-months aged cheeses had higher peptide content than 6-months-aged ones, this finding is in agreement with the proteolysis event; in fact proteolysis causes a progressive release of oligopeptides in cheese as ripening proceeds. PCA analysis technique was applied in order to propose the peptides as potential molecular marker to assess the authenticity of cheese. The results of this research confirmed that multivariate statistical analysis based on oligopeptidic profiles was an objective and powerful approach for evaluating proteolysis in cheese and discriminate cheeses mainly according to ripening age. Peptide pattern also allowed to differentiate the samples on account of the use of starter. Moreover, the peptide pattern was found different in relation to the cow feeding and the environmental condition effects but only considering the longer maturing period (12 months). Finally, oligopeptides may be considered useful molecular markers for the authenticity of PDO Asiago in relation to both ageing and the use of starter.

The third chapter is related to a second experimental research, with a view to evaluating the potential antihypertensive activity of Asiago water-soluble extracts (WSEs) containing oligopeptides. Blood pressure is affected by several biochemical

pathways, one of them is called rennin-angiotensin-aldosterone system. Angiotensin I-converting enzyme (ACE) is the key enzyme in this pathway. In this regard, it is known that milk-derived peptides are able to inhibit ACE and consequently they could contribute to reduce the high blood pressure. In this research, cheeses were manufactured in a mountain farm according to 3 cheese production systems and 3 ripening periods. The WSEs were ultrafiltrated onto 10 kDa and 3 kDa cut-off membrane in order to evaluate the ACE inhibition of long and small peptides using an enzymatic assay. In addition, a simulated gastrointestinal digestion was carried out to assess the effectiveness of digestive enzymes on the generation of bioactive peptides. After a logarithmic trasformation, data were submitted to a statistical analysis according to a mixed model that included the fixed effects ripening time, cheese production system, molecular weight of peptides and gastrointestinal digestion. The cheese production system had no significant effect. Six-months-aged cheeses had a significantly higher inhibitory potency than 12 and 18 months ones. Moreover, 3 kDa fraction made a more considerable contribution to the ACE inhibitory activity than 10kDa fraction. Simulated digestion did not significantly affect the inhibitory activity even if it seemed to increase ACE inhibitory potency of the 10 kDa permeates. The identification of peptides in WSEs using HPLC/ESI-MS revealed the presence of antihypertensive peptides mainly derived from α_{s1}-casein and β-casein. The main conclusion of this experimental trial is that only 6-months-aged cheeses displayed a weak ACE inhibitory activity (about 12% per 100 μg of peptides) and during cheese ripening this inhibition disappeared probably as conseguence of the breakdown of the bioactive peptpides.

The fourth chapter deals with the evaluation of potential antimicrobial activity of WSEs with a cut-off less than 10 kDa. The concern of this study is related to the outbreaks and sporadic cases of listeriosis which have been associated with contamination of milk. Moreover the bacterial contamination influences milk safety, organoleptic characteristics, production and storage of cheese. Therefore, the presence of antimicrobial peptides naturally present in the cheese may be useful to

control the growth of pathogenic bacterial or to determine their death. WSEs were screened against *Listeria innocua* LRGIA 01 and *Listeria monocytogenes* AER 102 at varying concentrations. The antimicrobial activity was monitored during 10 h of incubation and after 24 h. In this experimental contribute, the analyzed samples were the same of the second trial. Data were submitted to a statistical approach like to the second contribution adding the time of incubation effect and the peptide concentration effect. Regarding the *L. innocua* assay, an higher inhibition was detected in 6-months (26%) than 12 or 18 (19% on average) aged cheeses. Moreover, a linear relationship with the peptide concentration was observed. Considering the *L. monocytogenes* assay, the fixed effects did not affect the inhibition, which resulted below 12% on average. Morover, in both bacteria under investigation, this weak inhibition disappeared after 24 h of incubation suggesting a bacteriostatic activity of Asiago water-soluble peptides. The WSEs were analyzed by HPLC/ESI-MS in order to identify the peptides involved in antimicrobial activity: the antibacterial peptides α_{s1}-CN *f*(1-23), called isracidin, and α_{s2}-CN *f*(183-207) have been identified. Also in this contribution, we can observe that 6 months of ripening determined the production of oligopeptides with a moderate ability to slight inhibite only for some hours the growth of bacteria which can be found in dairy products.

CHAPTER 1

General Introduction

1 Functional food

The term "functional foods" was coined in Japan in the early 1980s (Gibson & Williams, 2000). In fact this country adopted a legal system in relation to allowable claims on functional food. The Japanese government introduced the Food for Specific Health Use (FOSHU) licensing system in 1991 (*Nutrition Improvement Law Enforcement Regulations*; Ministerial Ordinance No. 41, July), in which health claims must be verified through rigorous scientific evidence before FOSHU approval is granted (Arihara, 2006). Representative functional ingredients used for FOSHU products are oligosaccharides, dietary fibers, lactic acid bacteria, soy proteins, glycosides, sterol esters and diacylglycerols. In 2005 there were 537 FOSHU approved products with an estimate retail value worth 6.3 billion US$. As of April 2006, nearly 600 FOSHU products have been approved in Japan.

Regarding the Japanese FOSHU system, a functional food is "*….a food which, according to the knowledge about the relationship between foods or foods constituents and health, may have positive effects on health and is authorised to display a label stating that people using it for a particular purpose can extect to obtain a specific result*".

A product can be labelled as functional if:

- it must be a food (not powder, capsule or tablet);
- it can and should be consumed as an integral part of the daily diet;
- it performs a specific function within the metabolism and serves to regulate biological processes such as:
    - regulation of biological defence mechanism;
    - preventing convalescence after illness;
    - improving convalescence after illness;
    - slowing down the ageing process;
    - regulating the rhythm of physical condition.

Japanese functional food marker is one of the most advanced in the world and is often used as a model for developments in Europe and in the United States (http://www.cspinet.org) as shown in Table 1.1.1.

Table 1.1.1 Functional Foods: an International Comparison of Health Claims Regulation

Japan	FOSHU System (Food for Specified Health Use): Health claims permitted for specific products upon approval of application including scientific documentation demonstrating the medical or nutritional basis for a health claim and ingredient safety information.
US	Food, Drug, and Cosmetic Act: 1) Health claims linking a nutrient to a particular disease or health-related condition. Health claims must be pre-approved by the Food and Drug Administration (FDA). They may also be based on an authoritative statement from another government agency with scientific expertise so long as FDA does not object within a 120-day period after the company notifies FDA of its intent to make a claim. 2) Structure/function claims linking a substance to an effect on a structure or function of the body
UK	Prohibits medicinal claims – claims to treat a disease or restore, correct or modify physiological functions. Health maintenance claims that do not specifically refer to a disease may be lawful. There is no pre-market approval requirement. Enforcement is carried out by local trading standards officers and self-regulating bodies.

Although regulations for functional foods have not yet been well established in many countries, this situation has not been a significant barrier to the development of novel functional products in the food industry. In the United States and European countries, markets for functional foods have been expanding rapidly. Health-conscious consumers have made functional food the leading trend in the food industry (Arihara, 2006). In 1999, the European market exceed 2 billion US$ representing less than 1% of the European food market. At the moment, the dietary supplements are the leading application market with a 78% share. However, also functional foods and beverages is a fast-growing sector; currently this category has a 13% market share for omega-3. Functional dairy products are the key product sector accounting for sales of around 1.35 billion US$ (Menrad, 2003). The market volume in Germany increased from around 5 million US$ in 1995 to 419 million US$ in 2000, of which 301 million US$ account for pro-, pre-biotic and other functional yoghurts and around 118 million US$ for functional drinks.

Within Europe, Germany, France, the United Kingdom and the Netherlands represent the most important countries within the functional food market. In general, the interest of consumers in functional food in the Central and Northern European countries is higher than in Mediterranean ones (Menrad, 2003).

In Europe, the Commission has recently adopted a Regulation on nutrition and health claims on foods, including food supplements (Regulation EC 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods).

Moreover, in Europe there is an International Life Science Institute (ILSI) which is a global network of scientistis devoted to enhancing the scientific basic for public health decision-making. The European Commission concerted action of *Functional Food Science in Europe* (FUFOSE) in 1998 (http://www.ilsi.org), which was co-ordinately by ILSI Europe, aimed at establishing a science-based approach for concepts in functional food science. In this document the functional food is described by its role: "…*a food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutrition effects, in a way that it is relevant to either an improved state of health and well being and/or reduction of risk of disease. A functional food must remain food and must demonstrate its effects in amounts which can normally be excepted to be consumed in the diet. They are not pills or capsules but part of a normal food pattern*".

A functional food as defined above can be:

- a natural food, a food to which a component has been added;
- a food from which a component has been removed by technological or biotechnological means;
- a food where the nature of one or more components has been modified;
- any combination of these possibilities.

A functional food as defined above might be functional:

- for all member of a population;

- for particular groups of the population which might be defined, for example, by age or by genetic constitution.

The concept of functional food is usually confused with other new terms (Table 1.1.2) such as:

a) *medical foods*, foods with particular nutritional uses and are defined as "…..*foodstuffs which, owing to their special composition or manufacturing process, are clearly distinguishable from foodstuffs for normal consumption, which are suitable for their claimed nutritional purposes and which are marketed in such a way as to indicate such suitability*" and can also be referred to as "clinical foods" or "parnuts". They don't prevent a disease as might be the case for functional foods, but should be useful to treat an ongoing disease or deficiency by a specific nutrition (*e.g.*, in infants or young children in good health (Council Directive May 3, 1989; 89/398/ECC);

b) *nutraceutical*, term composed of the words "nutrition" and "pharmaceuticals", are components which can be considered as food supplements and which can either be added to foods or can be taken separately (*e.g.*, vitamins, minerals, lactoferrin, modified milk proteins). They are usually sold as tables, pills or powders. Nutraceuticals offer protection against diseases or have a positive influence on one or more body functions. A food product with an added nutraceutical is often referred to as a "design food". The homology between designer foods and functional food is very high. For some products, *e.g.*, foods containing "natural plant oestrogens" as a non-medicinal alternative to hormone replacement therapy, the line between medicinal food and functional food becomes slim. The difference between nutraceutical and pharmaceutical is quite clear: the former is taken to reduce risk for desease, the latter to cure disease (Diplock et al., 1999).

Table 1.1.2 List of major differences between different types of food

Discriminative character	Functional Foods	Nutraceuticals	Medical Foods
Purpose	Maintain good health	Improve health	Improve (cure) disease situation
Target population	Total population	Group dependent (age, genetic differences)	Patients
Mode of action	Influence on metabolism	Influence on metabolism	Influencing metabolism related to disease

1.1.2 Functional components in food

Food contains several substances with functional properties. The major natural biological components will be explained in this paragraph.

Polyunsatured fatty acids (PUFAs) are essential components; they confer flexibility, fluidity and selective permability properties to cell membrane. PUFAs contained in membrane phospholipids are precursors for synthesis of prostaglandins and, leukotriens and thromboxanes which bind to specific G-protein-coupled receptor and signal cellular physiological responses to inflammation, vasodilation, blood pressure, pain and fever (Ward & Singh, 2005). Consequently, PUFAs and their derivates and analogues are important nutraceutical and pharmaceutical targets.

Marittime food sources are rich in omega-3 polyunsatured fatty acids (omega-3 FA), as opposed to omega-6 polyunsatured fatty acids (omega-6 FA) found in terrestrial sources. Deep-sea fish contain 0.1-1.2 % omega-3 FA such as eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:5) and therefore they are the main reservoir for humans (Stehr & Heller, 2006). The omega-3 FAs found in fish and fish oils have been reported to have a variety of beneficial effects; the ipolipidic, anti-inflammatory, antithrombotic, anti-atherosclerotic and antiarrhythmogenis effects have been found (Harris, 2007). DHA is one of the most important PUFA; it is a major structural component of the gray matter of the brain and the eye retina and an important component of heart tissue. As a result dietary, DHA has been shown to be

important for proper development of the brian and eye in infants and supports good cardiovascular health (Horrocks & Yeo, 1999).

Conjugated linoleic acid (CLA), which was initially identified as an anticarcinogenic compound in extracts of grilled beef, is composed of a group of positional and geometric isomers of ocadecadienoic acid (Arihara, 2006). Since rumen bacteria convert linoleic acid to CLA by their isomerase, it is most abundant in fat of ruminant origin. Beef fat contains 3-8 mg of CLA per gram of fat. The CLA content in meat is affected by several factors, such as breed, age and feed composition (Dhiman et al., 2005). However, also probiotic bacteria promote the formation of CLA in fermented milk products. The most common CLA isomer found in beef is octadeca-*c*9, *t*11-dienoic acid. Since this fatty acid has anticarcinogenic activity, much interest has been shown in this compound. Recent epidemiological studies have suggested that high intakes of high-fat dairy food and CLA may reduce the risk of colorectal cancer (Ward & Singh, 2005). In addition to its anticarcinogenic property, CLA has antioxidant and immunomodulative properties. CLA may also play a role in the control of obesity, reduction of the risk of diabetes and modulation of bone metabolism (Arihara, 2006).

The vitamins are another group of bioactive compounds found in food and plants. Vitamin E comprises eight different forms, the α-, β-, γ-, and δ-tocopherols and the α-, β-, γ- and δ-tocotrienols. α-Tocopherol (the main vitaminer of vitamin E) is the most important liposoluble antioxidant in biological systems. This anti-oxidizing compound is considered a protecting factor against lipid peroxidation of PUFAs in membrane phospholipidis. Vitamin A is a fat-soluble vitamin found in two forms performed vitamin A, known as retinol, and pro vitamin A, called β-carotene (Shenkin, 2008). Retinol is found only in foods of animal origin while carotenoids, especially α- and β-carotene, are pigments found in about ten plants of livestock interest. Carotenois are considered as natural antioxidants; in fact, they play a role in cell communication and immune function by protecting cells against free radical attack. They can be transferred from forages to milk, therefore higher carotenoid

concentrations in milk contribute to an improvement in the nutritional value of dairy products (Nozière et al., 2006).

Phytochemicals constitute a heterogeneous group of substances and evidence for their role in the protective effect on human health, when dietary intake is significant, is merging (Lee et al., 2002). These compounds have biological properties such as antioxidant activity, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism. The bioavailability of phytochemical can be influenced by intrinsic factors in food and/or in human; in general the substances are little absorbed, largely metabolized and rapidly eliminated. For this reason it is advisable that their consumption is constant in the time to maintain high concentrations of metabolites in the blood (Lee et al., 2002).

Phytosterols are non-nutritive compounds belonging to this family of phytochemicals, with the same basic functions in plant as cholesterol in animals; that is, they regulate the membrane fluidity of plant cells and other physiologic functions associated with plant (Brufau et al., 2008). Phytosterols may be found in a great variety of vegetable oils (corn, palm, sunflower and soybean oil) and vegetable products as fruits (banana, apple and orange) and nuts. Many studies have demonstrated their ability to reduce blood cholesterol levels, in hyper- and normocholesterolemic subjects. Other beneficial effects from phytosterols include anti-inflammatory and antipyretics. Because of their poor water solubility and bioavailability, it was not possible to use them as pharmaceutical agents, and they were consequently abandoned. However, a new interest in phytosterols arone when the esterification of these compounds facilitated their inclusion into some food products (Hendriks et al., 1999; Weststrate & Meijer, 1998).

Among antioxidant substances, polyphenols, naturally occurring in vegetables, fruits and plant-derived beverages such as tea, red wine and extra virgin olive oil, are the most abundant ones. *In vitro* cell culture experiments have shown that polyphenols possess antioxidant properties, and it is thought that these activities can contribute to

the prevention of several oxidative stress-associated disease. In fact, free radical induce numerous diseases by lipid peroxidation, protein peroxidation and DNA damage (Lee et al., 2002).

A great many epidemiological studies indicate that a diet rich in flavonoids, such as catechins and procyanidins, from vegetables and fruits intake appear to be inversely related to coronary heart diseases and cancers mortality in humans (Auger et al., 2004). Flavonoids possess a structure that confers them an antioxidant property which can inhibit the processes leading in the long term to arteriosclerosis and arterial thrombosis. These molecules are the most lipophilic of the natural antioxidant, thus they could be concentrated near to the membranous surface of the low density LDL particles, ready to capture the oxygenated aqueous free radicals (Auger et al., 2004).

In this introduction I will focus on bioactive peptides, another category of functional substances, derived from food proteins especially from dairy products.

1.2 Bioactive peptides

Food proteins contain latent functional peptide sequences, called bioactive peptides (BPs), which are protein fragments having a physiological role in human body. These peptides are hidden in a latent state within the parental protein sequence and they may be released by proteolytic processes during *in vivo* or during food processing (Meisel, 1997). Once released, they may exert opioid, antithrombotic, antibacterial, mineral carrier, antihypertensive, immunomodulating or antigastric activities (FitzGerald & Murray, 2006; Korhonen & Pihlanto, 2007; Lignitto et al., 2007; Meisel, 1997; Pihlanto, 2006; Smacchi & Gobbetti, 2000).

BPs usually contain 3-20 amino acid residues per molecule (sometimes more); in some cases they may act as regulatory compounds with a hormone-like activity, based on their amino acid composition and sequence (Korhonen & Pihlanto, 2003).

Although these exogenic peptides are less active and less specific than their endogenic counterparts, they can be effective after oral administration. They have partial or total resistance to hydrolysis and may reach peripheral blood intact due to their low molecular size and exert systemic effects, or produce local effects in the gastrointestinal tract (Vermeirssen et al., 2004).

1.2.1 Bioactive peptides derived from food proteins

Peptides derived from food may have several biological activities of interest for human health. These peptides are present in the raw materials or are generated during food processing and protein hydrolysis by digestive enzymes or bacterial enzymes. Candidate proteins containing these latent biological activities are found in milk, eggs, meat, fish as well as in different plant protein sources such as soy, wheat and so on (Table 1.2.1) (Hartmann & Meisel, 2007).

The content of peptides in meat increases during *post-mortem* aging or during storage. During these processes, meat proteins are hydrolyzed by muscle endogenous proteases, such as calpains and cathepsin. Although there has been no report on the generation of bioactive peptides in meat during *post-mortem* aging, Arihara (2006) observed a decrease of ACE activity in beef during storage.

Chicken meat proteins contain fragments with immunomodulating (myosin, tropomyosin, collagen), antithrombotic (collagen), antibacterial (collagen), embryotoxic (collagen), antihypertensive (connectin) and also neuroactive (myopsin, collagen, connectin) activity occurring in amino acid sequences. There is a theoretical possibility of release of bioactive fragments from chicken meat proteins by endopeptidases; such possibility especially occurs in the case of hydrolysis by proteinase K (Dziuba et al., 1996).

Recent studies have shown that rice proteins had antidiabetic, antitumor, hypocholesterolemic and immunomodulatory activities (Guan-Hong et al., 2007).

Moreover, there are other bioactive compounds which are not released by protein degradation as explained now. In skeletal muscle and other mammalian tissues, it is possible to find carnosine (N-β-alanyl-L-histidine) which has significant antioxidant properties and is present in higher concentrations in white-type fibres where lactic acid accumulation is more likely (Purchas et al., 2004). In addition, carnosine reduces certain proteolytic reactions associated with cell ageing, and, as a result, has been credited with anti-ageing properties. Coenzyme Q_{10} or ubiquinone (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone), which is involved in the mitochondrial electron transport chain, has antioxidant properties. Meat is an important dietary source of coenzyme Q_{10} and it might be expected that levels in muscle will correlate with the number of mitochondia present (Purchas et al., 2004).

Creatine and its phosphorilated derivative creatine phosphate play an important role in muscle energy metabolism and there is evidence that under some circumstances creatine supplements can enhance muscle performance (McKenna et al., 1999).

Anserine (β-Ala-1-methyl-His) is another dipeptide naturally present in several mammalian tissues including skeletal muscle. This peptide has been studied of its physiological effects, such as antioxidant properties (Arihara, 2006). Glutathione (γ-Glu-Cys-Gly) is another endogenous peptide found in the skeletal muscle of most vertebrates, known to have antioxidant activity (Arihara, 2006).

Table 1.2.1 Examples of bioactive peptides derived from food (modified by Hartmann & Meisel, 2007)

Effect	Origin	Encrypting protein(s)	Name/remark/sequence[a]
ACE-inhibitory/ hypotensive	**Soy**	Soy protein	NWGPLV
	Fish	Fish muscle protein	LKP, IKP, LRP (sardine, bonito..)
	Meat	Meat muscle protein	IKW, LKP
	Milk	α-LA; β-LG	Lactokinins (WLAHK, LRP, LKP)
		α-, β -, κ-CN	Casokinins (e. FFVAP, FALPQY..)
	Egg	Ovotransferrin	KVREGTTY
		Ovoalbumin	Ovokinin (FRADHPPL)
			Ovokinin (2-7) (KVREGTTY)
	Wheat	Wheat gliadin	IAP
	Brocccoli	Plant protein	YPK
Immunomodulatory	**Rice**	Rice albumin	Oryzatensin (GYPMYPLR)
	Egg	Ovalbumin	Peptides not specified
	Milk	α-, β -, κ-CN, α-LA	Immunopeptides (TTMPLW)
	Wheat	Wheat gluten	Immunopeptides
Citomodulatory	**Milk**	α-, β-CN	α-casomorphin (HIQKED(V))
			β-casomorphin-7 (YPFPGPI)
Opioid agonist	**Wheat**	Wheat gluten	Gluten-exorphin
	Milk	α-LA, β-LG	α-lactorphins, β –lactorphin
		α-, β-CN	Casomorphin
Opioid antagonist	**Milk**	Lactoferrin	Lactoferroxins
		κ-CN	Casoxins
Antimicrobial	**Egg**	Ovotransferrin	OTAP-92 f(109-200)[b]
		Lysozime	Peptides not specified
	Milk	Lactoferrin	Lactoferricin
		α-, β -, κ-CN	Casecidins, isracidin, kappacin
Antithrombotic	**Milk**	κ-CN (glycomacropeptide)	κ-CN f(106-116), casoplatelin
Mineral binding, anticariogenic	**Milk**	α-, β-CN	Caseinphosphopeptides
Hypo-cholesterolemic	**Soy**	Glycin	LPYPR
	Milk	β-LG	IIAEK
Antioxidant	**Fish**	Sardine muscle	MY
	Wheat	Wheat germ protein	Peptides not specified
	Milk	α-LA, β-LG	MHIRL, WYSLAMAASDI, YVEEL

[a] The one-letter amino acid codes were used, [b]f: fragment; CN: casein; LA: lactoalbumin; LG: lactoglobulin; ACE: angiotensin converting enzyme.

1.2.2 Bioactive peptides in dairy products

Bovine milk proteins are currently the main source of a wide range of food-derived bioactive peptides which can be released and activated by proteolysis (Meisel, 2005).

Caseins (CNs) and whey proteins (WPs) are the two main protein groups in milk (Table 1.2.2). CNs represent about 80% of the protein content in bovine milk and they constist of four primary proteins, *e.g.*, α_{s1}-, α_{s2}-, β- and κ-CN and all genetic variants differ in their molecular weight (MW) and isoionic pH. WPs represent the remainig content and they are constituted by immunoglobulins, lactoferrin, α-lactoalbumin, β-lactoglobulin and growth factors.

Table 1.2.2 Milk proteins and their characteristics

Protein	% of total milk protein	MW range[b] (Da)	Average MW (Da)	Isoionic pH
Casein	**79 - 83**			
α_{s1}-casein	30 - 36	22066 - 23722	23600	4.91 - 5.35
α_{s2}- casein	8 - 11	25148 - 25388	25200	5.19 - 5.39
β- casein	25 - 28	23939 - 24089	24000	5.11 - 5.85
κ-casein	9 - 10	19005 - 19037	19000/19550	5.37 - 6.07
γ-casein	2 - 4	11600 - 20500	20500	5.8 - 6.0
Whey protein	**17 - 21**			
β-lactoglobulin	9 - 10	18205 - 18363	18300	5.14 - 5.49
α-lactoalbumin	2 - 4	14147 - 14175	15000/14200	4.2 - 4.8
Serum albumin	~ 1	66267 - 69000	66300	4.71 - 5.13
Immunoglobulin	~ 2	153000 - 901000		5.5 - 8.3
Proteose peptone	2 - 4	4100 - 40800		3.3 - 3.7
Miscellaneous	< 2.5			

[b]MW ranges of caseins, β-lactoglobulin, α-lactoalbumin and serum albumin result from differences between genetic variants of the proteins

In recent years it has been recognized that CNs provide a rich source of BPs which can regulate some physiological functions of human body such as opioid, antithrombotic, immunomodulatory, antihypertensive and so on activities (Korhonen & Pihlanto, 2003).

Many milk-derived peptides reveal multifunctional properties, in fact some regions of bovine CNs contain overlapping peptide sequences having two or more different biological activities (Figure 1.2.1). These regions have been considered as "strategic zones" (Fiat & Jollès, 1989) which are partially protected from proteolytic breakdown.

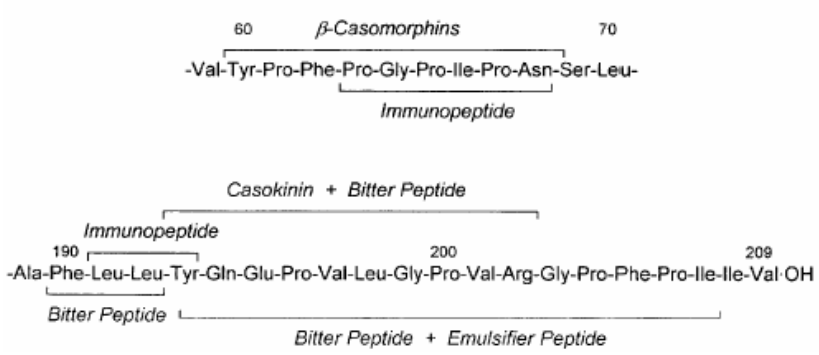


Figure 1.2.1 Schematic representation of the multifunctional activities in milk protein-derived peptides; strategic zones in the primary structure of bovine β-casein (Meisel, 1998)

1.2.3 Biological functions of dairy product-derived peptides

Once produced, bioactive peptides may exert several biological functions as following explained.

Opiod peptides

Several milk protein-derived peptides that possess opioid-like activities have been identified (Zioudrou et al., 1979; Bitri, 2004; Smacchi & Gobbetti, 2000). Opioids are substances that exert effects similar to morphin, such as sedation and antinociception (Smacchi & Gobbetti, 2000). The effects of opioids are mainly mediated through opioid receptors (μ, δ, κ) in the central nervous system. The opioid peptides derived from α-CN are named α-exorphins, whereas the opioid peptides originating from β-CN are called β-casomorphins. Peptides that originate from κ-CN are called casoxins. In addition, WPs, *e.g.*, α-lactoalbumin and β-lactoglobulin, contain sequences of opioid peptides in their primary structures (Meisel, 2005). The common structural feature among endogenous and exogenous opioid peptides is the presence of a tyrosine (Tyr) residue at the amino (N-) terminal end (except α-CN opioids) and the presence of another aromatic residue such as phenylalanine (Phe), in the third or fourth position. This is an important structural motif that fits into the binding site of the opioid receptors. Lack of the Tyr residue results in a total absence of bioactivity.

The proline (Pro) residue is crucial for bioactivity in order of its function to maintain the proper orientation of the Tyr and Phe side chains (Meisel, 1997).

Immunomodulatory peptides

CN-derived immunopeptides including fragment from α-CN and β-CN have been shown to stimulate phagoctosis of sheep red blood cells by murine peritoneal macrophages, and to exert a protective effect against *Klebsiella pneumoniae* infection in mice after intravenous treatment (Meisel & Schlimme, 1990). They may stimulate the proliferation and maturation of T-cells and natural killer cells for the defence of the newborn against different bacteria, particularly enteric bacteria (Kayser & Meisel, 1996). Moreover, immunomodulatory peptides might reduce allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract. Nowadays, the mechanisms, structure and activity by which milk protein-derived peptides exert their immunomodulatory effect is still unknown. However, the arginine (Arg) residue at the N- or carbossi (C)-terminal region of the peptide has been suggested to be the leading motif recognizable by specific surface membrane receptors (Silva & Malcata, 2005).

Antithrombotic peptides

Peptides that inhibit blood platelet aggregation and fibrinogen binding (γ-chain) to platelet surface receptors are encrypted within the sequence of glycomacropeptide [κ-CN f(106-169]. Casoplatelins, which are CN-derived peptides display inhibition against both the aggregation of ADP-activated platelets and the binding of human fibrinogen γ-chain to a specific receptor region on the platelet surface. In addition, the fragment κ-CN f(103-111) can prevent blood clotting through inhibiton of platelet aggregation, but is not able to affect fibrinogen binding to ADP-treated platelets (Silva & Malcata, 2005).

Antioxidant peptides

Milk contains several antioxidant factors, like vitamins and enzymes and milk-protein derived peptides are reported to have antioxidative activities. Recent studies have shown that antioxidant peptides can be released from CN in hydrolysis by digestive enzymes and in fermentation of milk with proteolytic LAB strains. Most of them are derived from α_{s1}-CN and they are composed of 5-11 amino acids including hydrophobic amino acis, proline, histidine, tyrosine or tryptophan in the sequence (Pihlanto, 2006). They have been displayed their free radical-scavenging activities and their inhibition against enzymatic and non-enzymatic lipid perodxidation (Korhonen & Pihlanto, 2006).

Antimicrobial peptides

Antimicrobial peptides have been identified from many protein hydrolysates, especially from milk (Minervini et al., 2003; Rizzello et al., 2005; Zucht et al., 1995). The most well-studied are the lactoferricins, which are derived from bovine and human lactoferrin, but a few antibacterial peptides have been also identified from α_{s1}-CN and α_{s2}-CN. These peptides act against Gram-positive and Gram-negative bacteria (*Escherichia*, *Helicobacter*, *Listeria*, *Salmonella* and *Staphylococcus*), yeast and filamentous fungi (Hartmann & Meisel, 2007). They usually contain a high percentage of basic amino acyl residues in an amphipathic structure and this characteristic probably facilitates interaction between the positively charged peptide and the negatively charged bacterial membrane (Malkoski et al., 2001). The disruption of normal membrane permeability is the main action of the antibacterial mechanisms of these peptides.

Antihypertensive peptides

A great number of antihypertensive peptides have been isolated from milk proteins (FitzGerald & Meisel, 2000; Gómez-Ruiz et al., 2002; Gómez-Ruiz et al., 2006; Hayes et al., 2007; Murray & FitzGerald, 2007; Quirós et al., 2001; Saito et al., 2000; Smacchi

& Gobbetti, 1998). They preferentially act against the angiotensin I-converting enzyme (ACE), which plays an important physiological role in regulating blood pressure. ACE catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II increasing the blood pressure in human. ACE inhibitory peptides are generally short-chain peptides, often carrying polar amino acid residues like Pro. For example, the hypotensive peptides valine-proline-proline (Val-Pro-Pro) and isoleucine-proline-proline (Ile-Pro-Pro) can be released from precursor proteins (β-CN and κ-CN) by enzymes from *Lactobacillus helveticus* (Korhonen & Pihlanto, 2003). However, the *in vitro* effect is not directly correlated to the *in vi*vo hypotensive effect. In one side, some peptides can be susceptible to degradation or modification in the gut, in the vascular system and in the liver and they may lose their bioactivity, in the other hand, hypotensive activity of a long-chain candidate peptide can be caused by peptide fragments generated by gastrointestinal enzymes.

Other functionalities

CN-derived phosphorylated peptides, called caseinphosphopeptides (CCPs), enhanced vitamin D-independent bone calcification in rachitic infants (Mellander, 1950). Bovine α_{s1}-, α_{s2} and β-CN contain phosphorylated regions which can be released by digestive enzymes (Silva & Malcata, 2005). CPPs are constituted by a sequence of three phosphoseryl residues, followed by two glutamic acid (Glu) residues such as SerP-SerP-SerP-Glu-GLu (Meisel, 1997). The high concentration of negative charges of phosphate residues makes them resistant to further proteolysis (Clare & Swaisgood, 2000), furthermore they represent the binding sites for minerals. Specific CCPs can form soluble organophosphate sals and lead to enhanced calcium absorption by limiting the precipitation of calcium in the distal ileum. CPPs have benne shown to bind to such macroelements as Ca, Mg and Fe as well as to such oligoelements as Zn, Ba, Cr, Ni, Co and Se. Since CPPs can bind and solubilize mineral, they have been considered physiologically beneficial in the prevention of

osteoporosis, dental caries, hypertension and anemia. However other studies have to be conducted to understand depper the potentiality of CCPs.

The glycomacroeptides (GMP) is formed during the enzymatic cheesemaking process. Rennet or chymosin hydrolyses the peptide bond between residues 105 and 106 of κ-CN, and the resulting molecule, GMP, is eluted in the whey. This fragment κ-CN f(106-169) present a hydrophilic zone in the C-terminal and contains the oligosaccharides that are O-linked to threonine and serine. This peptide can not be absorbed as such, so it has to be broken into small peptides. GMP exhert many beneficial effect upon the nutrition system. It is known to allor absorption of calcium, iron or zinc, it is useful for diets aimed at controlling several liver diseases, in case where branched chain amino acids appear to be used as carbon source (Korhonen & Pihlanto, 2006).

1.2.4 Potential uses in functional foods and pharmaceuticals

Biologically functional dairy proteins and their derivates have gained great interest in recent years due to their potential applications in functional foods and nutraceutical (Korhonen & Pihlanto, 2007). Therefore several approaches have been designed to increase the oral delivery of peptides. First of all, peptides can be chemically modified to increase their oral delivery; secondly, peptides can be produced by genetic engineering in microorganisms and subsequently delivered *in situ* (Korhonen & Pihlanto, 2007).

Recombinant DNA techniques have been employed to produce specific peptides or their precursors in microorganisms. For example, the sequence Arg-Pro-Leu-Lys-Pro-Trp has been introduced into the gene for soybean β-conglycin *α*'subunit. This subunit was expressed in *E. coli*, recovered from the soluble fraction and purified by chromatography. The Arg-Pro-Leu-Lys-Pro-Trp peptide was released from the recombinant containing the subunit after digestion by trypsin and chymotrypsin (Korhonen & Pihlanto, 2007).

A number of studies have addressed development of probiotic cheese; in fact Canestrato Pugliese cheese (Corbo et al., 2001), Cheddar cheese (Dinakar & Mistry, 1994; McBrearty et al., 2001; Philips et al., 2006), Crescenza cheese (Gobbetti et al., 1997), Gouda cheese (Gomes et al., 1995), semi-hard cheese (Bergamini et al., 2006) and white cheese (Kasimoglu et al., 2004) have been developed as probiotic cheeses. The term probiotic is defined as " living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition" (Ross et al., 2002). Probiotic organisms can be added into cheese during manufacture, either as a starter or as adjunct to the starter culture.

If some bioactive peptides are not released under physiological conditions *in vivo*, they could be produced commercially and used as nutraceuticals. CN-derived peptides can be manufactured on industrial scale and adding as dietary supplements and as pharmaceutical preparations (Meisel, 1998). The development of any dairy products with health benefits beyond nutrition requires a deep understanding of the mechanisms underlying the beneficial effect. It is necessary to investigate how these peptides exert their functions.

1.2.5 Bioactive peptides in cheeses

Ripenend-type cheeses contain numerous peptides that originate mainly from CN released as a result of proteolysis during ripening (Durrieu et al., 2006; Gómez-Ruiz et al., 2002; Gómez-Ruiz et al., 2006; Parrot et al., 2003; Rizzello et al., 2005; Saito et al., 2000; Smacchi & Gobbetti, 1998) as reported in Table 1.2.3.

Cheese ripening is a complex process where different enzymes such as native milk enzymes, residual coagulant and the enzymatic systems of starter and non-starter microflora are involved (Paragraph 1.2.6). The presence of several BPs naturally formed in cheese depends on the variety of cheese and on the equilibrium between their formation and degradation exerted by the proteolytic systems involved in cheese ripening.

Meisel et al. (1997) reported that proteolysis increases ACE-inhibitory activity but only to a certain level after which ACE-inhibition index decreases. In fact BPs, released by proteolytic enzymes during the early or middle stages of ripening, may be degraded to inactive fragments as a result of a high level of proteolysis. Consequently, cheeses behave as a dynamic system where peptides are being constantly released, some of these peptides are subsequently hydrolyzed and other accumulate over the ripening process (Gómez-Ruiz et al., 2006).

Table 1.2.3 Identified bioactive peptides from various cheeses

Product	Bioactive peptides	Bioactivity	References
Parmigiano-Reggiano	β-CN *f*(8-16), *f*(58-77) α_{s2}-CN *f*(83-88)	Phosphopeptides; ACE-inhibitory; Precursor of β-casomorphin	Addeo et al., 1992
Italian cheese: Canestrato Pugliese, Pecorino Romano Crescenza, Caprino del Piemonte	α_{s1}-CN *f*(1-23), κ-CN *f*(104-115)	Antimicrobial	Rizzello et al., 2005
Cheddar cheese	α_{s1}-CN and β-CN	Phosphopeptides	Singh et al., 1997
Gouda cheese	α_{s1}-CN *f*(1-9), β-CN *f*(60-68)	ACE-inhibitory	Meisel et al., 1997 Saito et al., 2000
Italian cheese: Mozzarella, Italico Crescenza, Gorgonzola	β-CN *f*(58-72)	ACE-inhibitory	Smacchi & Gobbetti, 1998
Manchego cheese	Ovine α_{s1}-, α_{s2}-β-CN fragments	ACE-inhibitory	Gomez-Ruiz et al., 2002

1.2.6 Production of bioactive peptides in dairy products

Proteolysis is regarded as the most important biochemical event in dairy products (especially in cheese). It is considered the main responsible for the generation of BPs during dairy processing thereby enriching the dairy products (Sousa et al., 2001). Proteolysis contributes to flavour and to off-flavour (*e.g.*, bitterness) of cheese through the formation of free amino acids as well as liberation of substrates for secondary catabolic changes, *e.g.*, desulphuration, catabolism of aromatic amino acid

and reactions of amino acids with other compounds. Furthermore, proteolysis contributes to textural changes of the cheese matrix, due to breakdown of the protein network, decrease in water activity through water binding by liberated carboxyl and amino groups and increase in pH, which facilitates the release of sapid compounds during mastication (Sousa et al., 2001).

The proteolytic digestion of CNs during cheese ripening is believed to take place as multistep reaction, which is initiated by cleavage of intact CNs and thus by the formation of rather large peptides and concluded by the formation of free amino acids and flavour components. The degradation of intact CN is the result mainly from the action of residual rennet and plasmin, whereas the subsequent formation of smaller peptides, free amino acids and flavour components results from the microbial enzymes residing from the starter culture, the secondary starter and the non-starter bacteria (Diagramm 1.2.1). The rennet enzyme is the main responsible of the degradation of α_{s1}-CN in cheese, whereas plasmin is devoted for the degradation of α_{s2}-CN and β-CN during the cheese ripening (Benfeldt, 2006).

To summarize, BPs can be released in different ways: a) through proteolytic enzymes naturally occurring in milk, b) through hydrolysis of proteolytic enzymes derived from bacteria and c) through hydrolysis by digestive enzymes (Korhonen & Pihlanto, 2006).

a) Hydrolysis of proteolytic enzymes in milk

Chymosin (EC.3.4.23.4) is the principal proteinase (88-94%) in the traditional calf rennet used for cheese manufacture. It is an aspartyl proteinase of gastric origin, secreted by young mammals. Chymosin is weakly proteolytic and its principal role in cheese making is to hydrolyse the Phe_{105} – Met_{106} bond of the micelle-stabilizing protein, κ-CN, as a result of which the colloidal stability of the micelles is destroyed, leading to gelation at temperature >20 °C. Creamer et al. (1985) reported that ~6% of chymosin added to milk is retained in the curd, but the amount increased with

decreasing pH at whey draining. During milk coagulation, α_{s1}-, α_{s2}- and β-CN are not degraded, but may be hydrolysed in cheeses during ripening. In cheese, bovine α_{s1}-CN is rapidly split by chymosin at Phe_{23}-Phe_{24} to yield the fragments α_{s1}-CN *f*(1-23) and α_{s1}-CN *f*(24-209). The first fragment is subsequently hydrolysed by starter proteinases. Chymosin fails to cleave susceptible bonds in the C-terminal domain of α_{s1}-CN in cheese (Michaelidou et al., 1998). Also β-CN is degraded by chymosin in several sites, especially near the leucine residues. α_{s2}-CN is relatively resistant to proteolysis by chymosin; moreover hydrolized sites of α_{s2}-CN by chymosin are restricted to hydrophobic region (Michaelidou et al., 1998).

The principal endogenous proteolytic enzyme in milk is the alkaline serine proteinase plasmin, which occurs in milk together with its inactive zymogen, plasminogen. Plasmin and plasminogen enter milk in the mammary gland. This enzyme has affinity for lysine and arginine residues and preferentially cleaves Lys-X and Arg-X bonds (Somma et al., 2008). In milk, plasmin, plasminogen and plasminogen activator are associated with CN micelles in milk and hydrolyse α_{s1}-, α_{s2}- and β-CNs, but κ-CN appears to be resistant. The concentration of plasmin and especially plasminogen in milk increases in late-lactation, lactation number and during mastitic infection (high somatic cell counts) due to the increased permeability of the mammary gland cell walls to blood serum constituens such as plasmin and plasminogen (O'Malley et al., 2000). Plasmin has a strong contribution to primary proteolysis in cheese and its activity depends on cooking temperatue and pH during ripening. Higher plasmin activity has been observed in milk from Holstein-Friesian cows (0.27-0.53 mg L^{-1}) compared to Jersey cows (0.15-0.37 mg L^{-1}). A similar trend was observed in Swedish Fresian and Jersey cattle (Bastian & Brown, 1995) However, when plasmin activity was statistically adjusted for differences in casein content between these breeds, the difference was removed. For this reason, Schaar (1985) concluded that the negative correlation between plasmin activity and casein content is probably caused by competition between casein and the synthetic substrate used to measure plasmin activity.

There are also a number of indigenous enzymes in milk, which originate in somatic cells. It is the case of the acid proteinase cathepsin D originating in lysosomes of somatic cells.

Cathepsin D is an aspartic proteinases and originates in the lysosomes of somatic cells. It is relatively heat labile enzyme in fact it is inactivated at 70 °C for 10 min. Recent studies on heat stability reported that ~8% cathepsin D activity in skim milk survived pasteurization (72 °C for 15 s) suggesting that this enzyme may play a minor proteolytic role in dairy products. Cathepsin D is present in cheese varieties where no rennet was added, but it is hard to quantify its contribution to the ripening of cheese varieties wherein the activity would be masked by far greater level of chymosin (Sousa et al., 2001). Cathepsin D preferentially cleaves α_{s1}-CN, moreover it also cleaves α_{s2}-, β- and κ-CN and α-lactoalbumin with the specificity similar to that of chymosin (Wilkinson & Kilcawley, 2005). In addition to cathepsin D, other proteolytic enzymes are present in lysosomes of somatic cells and may contribute to proteolysis in cheese. One of the principal enzymes found in polymorphonuclear granulocytes (PMN cell or neutrophils) is the serine proteinase, elastase. Elastase has a broad specificity on α_{s1}- and β-CNs and cleaving 25 and 19 sites, respectively. Most of the cleavage sites are located near the N- or C-terminus of the molecules. Therefore it is possible that elastase in milk may play a role in the proteolysis of milk proteins (Considine et al., 1999).

b) Role of starter and non starter lactic acid bacteria in the proteolysis and in the liberation of bioactive peptides

The presence of several BPs may be due to the proteolysis of starter and non-starter cultures in dairy products. In addition, once bacterial lysis occurs, all proteases are free to degrade the CN and the oligopeptides of the dairy products (Sousa et al., 2001).

Lactic acid bacteria (LAB), *e.g.*, *Lactococcus lactis*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, are used as starters microorganisms in dairy

products or endogenous to milk microflora. They have a proteolytic system including cell-bound proteinases (CEPs) and intracellular peptidases which are involved in the peptide formation. CEPs release many different oligopeptides into shorter fragments and amino acids which contribute directly, or as precursors, to flavour. Some of these peptides are bioactive (Gagnaire et al., 2001).

During the maturation of many cheeses, the starter populaton declines and the initially small non-starter lactic acid bacteria (NSLAB) population becomes the dominant bacterial population. The proteolytic activity of NSLAB appears to supplement that of the starter, producing peptides with usually similar molecular weight and free amino acids (Sousa et al., 2001).

Once produced, BPs may play a significant role in cheesemaking not only by selectively inhibiting enzymes of LAB and subsequently affecting cheese quality but also by their potential bioactivity (Smacchi & Gobbetti, 2000).

c) Proteolysis by digestive enzymes: liberation and fate of peptides in vivo

To exert their physiological effect *in vivo*, BPs must reach target sites at the luminal tract or, after absorption, in the peripheral organs.

The gastrointestinal (GI) tract of humans contains several enzymes involved in the hydrolysis of proteins and peptides and they are located in numerous sites (Tomè & Debabbi, 1998).

The intestine is the principal site of protein absorption, in fact the brush-border membrane of the enterocyte is characterized by a mixture of peptidases manly belonging to four classes: endopeptidase, aminopeptidases, carboxypeptidases and dipeptidases. Therefore, the polypeptides are further cleaved by pancreatic proteases such as α-chymotrypsin, elastase and carboxypeptidases at more alkaline pH. This event generates a mixture of oligopeptides and free amino acids *in situ*. The free amino acids are absorbed as such into the enterocytes across the brush border membrane via distinct amino acid transport systems (Vermeirssen et al., 2004). The oligopeptides undergo further hydrolysis by the action of brush border peptidases,

resulting in a mixture of free amino acids and di- and tripeptides. In the apical membrane of enterocytes there is a peptide transporter, called PepT1, which uses a transmembrane electrochemical proton gradient as the driving force and has broad substrate specificity. However this transport mechanism carries only di- and tripeptides, in fact oligopeptides with more than four residues are hardly recognized by this transporter system (Satake et al., 2002). Once inside the enterocyte, these small peptides are usually hydrolysed to free amino acids in the cytoplasm by various intracellular peptidases. Via specific amino acid transport systems, the amino acids cross the basolateral membrane of enterocyte and reach the portal circulation.
However, some transporter routes which allow the transport of intact peptides and proteins have been identified. Three different transport routes, namely paracellular, passive diffusion and adsorptive transcytosis, may participate in oligopeptide transport across the intestinal epithelium (Figure 1.2.2) (Gardner, 1988). The contribution of each route must be different among the peptides, depending on the molecular size and other structural properties such as hydrophobicity.

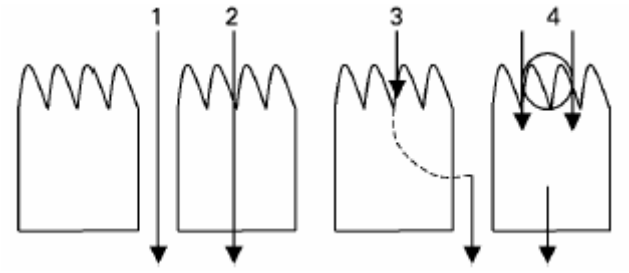


Figure 1.2.2 Mechanisms for intestinal transport of peptides: (1) paracellular; (2) passive diffusion; (3) endocytosis; (4) carrier-mediated transport (Vermeissen et al., 2004)

It seems that intact peptides use both paracellular and transcellular routes for passage across the intestinal epitelium, but there is still debate as to the relative importance of these. Large water-soluble peptides pass via the tight junctions between cells; highly lipid-soluble peptides appear to be able to diffuse via the transcellular route. Peptides may also enter the enterocytes via endocytosis, which entails membrane binding and vesciculisation of the material (Ziv & Bendayan,

2000). The intestinal basolateral membrane also possesses a peptide transporter, which facilitates the exit of hydrolysis-resistent small peptides from the enterocyte into the portal circulation (Gardner, 1988).

To investigate wheter biopeptides resist gastrointestinal digestion, they are usually subjected *in vitro* to hydrolysis by pepsin, trypsin, α-chymotrypsin or pancreatin. It is interesting that proline- and hydroxyproline containing peptides are generally resistant to degradation by digestive enzymes (FitzGerald & Meisel, 2000).

BPs generated in the diet can be absorbed intact through the intestine and produce biological effects at the tissue level. However, the potency of the administered peptides decreases as the chain-length increases. As in infants, the GI barrier is not yet completely mature, intact peptides and proteins are much better absorbed in infants than in adult. Together with the limited GI proteolysis in infants, this explains why milk containing several bioactive proteins and peptides has a physiological role in the young (Vermeissen et al., 2004). Finally, once peptides reach the peripheral blood they may be broken by peptidase enzymes widely distributed in this tissue. The half-life of certain peptides in plasma is usually very short; for example, angiotensin II degradation occurs ever within seconds. Thus the fate of the peptides in the blood is not easy.

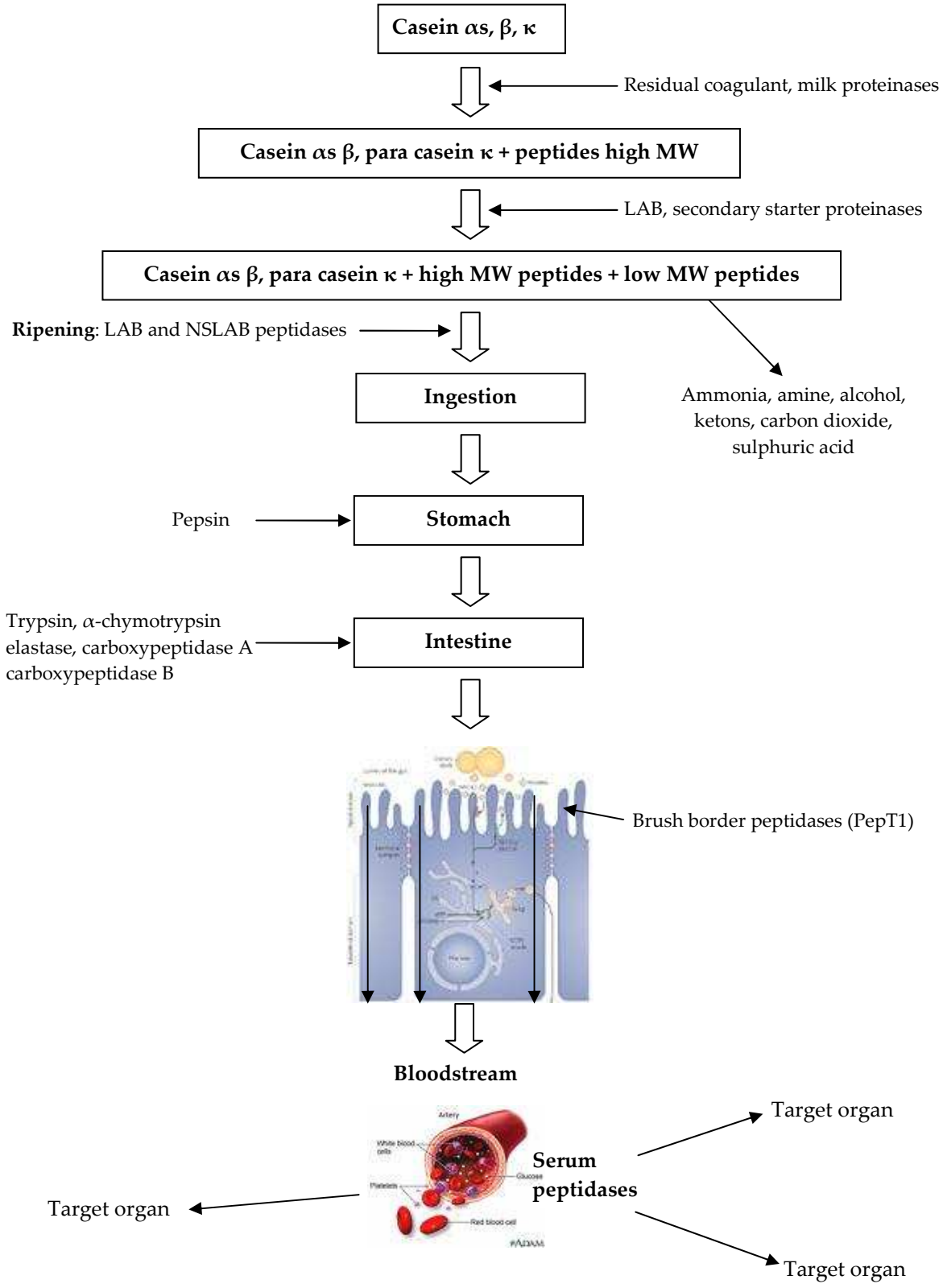


Diagram 1.2.1 Generation of oligoeptides, peptides and free amino acids (MW: molecular weight)

1.3 ASIAGO CHEESE

1.3.1 Production of Asiago cheese

Asiago is a semi-cooked cheese made only from cow's milk and produced in the north-eastern region of Italy. Asiago has been certified as Controlled Designation of Origin (DOC) cheese since 21st December 1978, when a law established the limits of the geographical area within which the milk used to produce this variety of cheese could be collected and where production traditionally takes place. The officially-recognised zones for Asiago cheese are defined as the entire provinces of Vicenza and Trento and two areas in the lowland provinces of Treviso and Padova. Asiago cheese obtained the "Protected Designation of Origin" (PDO) certification on 12th June 1996 (EEC Reg. 1107/96); it is marked with two distinct flavours: pressato and allevo, which correspond to the fresh and ripened varieties, respectively.
In the following Table 1.3.1, the production of Asiago cheese is displayed:

Table 1.3.1 Characteristics of Asiago varieties

Cheese type	***Asiago d'allevo*** **(ripened variety)**	***Asiago pressato*** **(fresh variety)**
Seasoning	Max 2 years Mezzano(medium variety): 4-6 months	20 days
Form	Cylindrical, with straight sides, and flat or almost flat upper and lower surfaces	Cylindrical, with smooth, convex sides and flat or almost upper and lower surfaces
Size	30-36 cm diameter, 9-12 cm high	30-40 cm diameter, 11-15 cm high
Weight	8-12 kg	11-15 kg
Crust	Smooth and regular	Fine and elastic
Appearance and consistency	Small holes, straw colour, crumbly	Larger holes, light color, soft
Flavour	Distinct piquant taste	Sweet, delicate taste

In the year 2000 production of Asiago cheese exceeded 20000 tonnes (+33% with respect to the previous three-year periods) and an invoice turnover of about 75

million Euro was recorded for the same year. These figures raised the Asiago brand to the fifth position in the Italian national market listings for PDO cheeses.
Asiago can obtain the added value of "mountain product" if it is produced above 600 m of altitude, thus this cheese has been produced with milk from animals feeding on mountain pastures. For the production of this type of Asiago, the ripening period have to be at least of 30 days for pressato and 90 days for the allevo.

Asiago pressato - Fresh variety

This variety of Asiago is obtained exclusively with whole cow's milk, procured from 1 or 2 milking operations. For the production of Asiago pressato, it is possible to use milk derived from one or two milkings, raw or pasteurized at 82 °C for 15 seconds following the laws in force.
The bovine enzyme (rennet) is added to stimulate coagulation of the milk at temperatures generally ranging from 35 to 40 °C. The curd is cut as soon as it reaches a suitable consistency. This operation is performed with a curd-knife when the granules have grown to the size of a small nut. The half-cooking process continues until the mass of curd being worked reaches at temperature of 44±2 °C. After the half-cooking process, the curd is extracted and placed in the moulds for forming. The working of the curd which has just been extracted is the important phase in the production of fresh Asiago and includes the operations of repeated cutting of the curd, salting and turning the cheese; the curd is then placed in the moulds, sometimes after previous portioning, and is compacted by manual or pneumatic presses. The presalting operation takes place while the cheese is kept at controlled temperature (13-15 °C) and relative humidity (85%) for periods varying from 36 to 96 hours. The salting operation can be carried out in two ways: either with the surface dry-salting technique or with brine (20±2 °B). The final phase is the ageing or maturing period, during which the cheeses are left in storage bays to ripen. The important factors are the storage temperature (10–15 °C) and relative humidity (80-85%).

Asiago d'allevo – Ripened variety

This variety is manufactured by processing milk obtained from 1 or 2 milking operations. After collection and before the cheese-making process is begun, the milk undergoes partial skimming treatment. Coagulation occurs by means of the addition of a coagulating enzyme of bovine origin (rennet) at the temperature of 35±2 °C. The half-cooking process is continued until the mass being worked reaches the temperature of 47±2 °C, which takes about 18-21 minutes. Following heating, and after the cutting operation, or 'spinatura', in which the mass of cheese curd is broken up into granules, the curd is extracted and the cheese is placed in moulds for forming. It is transferred onto a sloping table in order to improve the drainage of whey (3-5 h), obtained by turning the wheels several times. The product is then sent to the presalting area, where the removal of whey is completed and the forms are marked with the seals of the regulatory board. The whole process is completed in three to five days, during which time the forms are turned several times. The salting operation is then carried out; this can be done in two ways: either with the surface dry-salting technique or with brine. The final phase is the ripening period, in which the cheeses are left in storage bays to ripen.

1.3.2 Nutritional properties of Asiago d'allevo cheese

Similar to other cheeses, Asiago has high nutritional value according to lipidic and proteic bioactive compounds.

Lipids

Asiago cheese contains a large number of different fatty acids (FAs), some of which may be of potential benefit to human health, including CLA and PUFA (Polyunsaturated FA) of the n-3 FA group (Balzan et al., 2007). It is recommended, therefore, that consumers increase their intake of these compounds. Many factors affect the FA composition, including breed, season, geographical location, access to

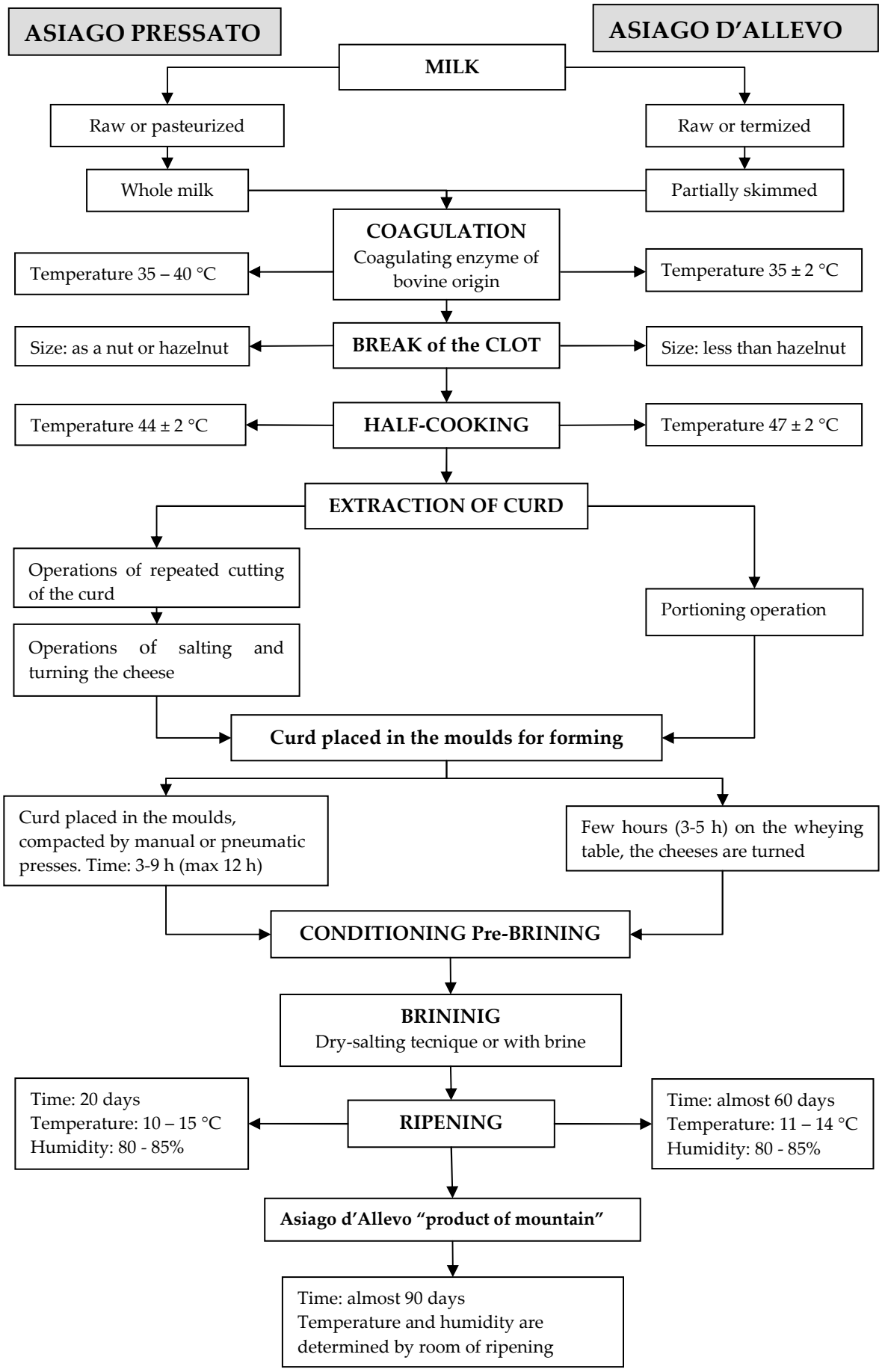
fresh grazing, silage type, cereal feeding and oli supplementation of feed. In this regard, it is well-known that grazing alpine pasture is able to affect FA profile of milk, and consequently in cheeses, enhancing the amount of long-chain PUFA, of n-3 and of CLA (conjugated linoleic acid), and reducing medium and long chain saturated FA (Collomb et al., 2008). This effect is mainly due to pasture high content of PUFA and, in particular of linoleic and linolenic acid (Bailoni et al., 2005). The increase of CLA as a function of herbage intake in Asiago cheese is probably mainly due to the concentration of vaccenic acid as a result of a higher ruminal activity of cellulosolitic microorganism (Segato et al., 2007).

Asiago d'allevo is a natural source of vitamin E (around 8.5 mg per 100 g ss) (Novelli et al., 2006). Experimental evidence suggests that oral vitamin E has anti-tumorigenis activity, it can interfere with inflammation either by scavenging reactive oxygen and nitrogen species, by modulating the synthesis of inflammatory lipid mediators, or by influencing signal tranduction and the expression of specific cytokines, chemokins and other inflammatory molecules (Zingg, 2007). In fact the nutrient composition of dairy products is strongly influenced by the nature of the diet ingested by the cows, in particular the grass-based diets affected the concentration of this tocopherol (Calderón et al., 2007). Grazing alpine pasture enhances milk pigments content such as α-tochopherol. In fact, in Asiago cheese, during the alpine grazing, the concentration of vitamin E increased twofold (Novelli et al., 2006) suggesting that the type of forage was probably the main factor of variation of vitamin E. Moreover the content of vitamin E remained constant during Asiago ripening period (6, 12 and 18 months) as suggested by Hartmann and Dryden (1965).

Asiago d'allevo is also a good source of vitamin A (Elia et al., 2006). It seems that the low altitude affect positively the vitamin A concentration. Vitamin A exhibits important biological actions as antioxidant. β-carotene is particularly involved in prevention of photo-oxidation as it absorbs light in a concentration-dependent manner. Carotenoids function as singlet oxygen scavengers and may also react with other reactive oxygen species (Nozière et al., 2006). Milk carotenoids are transferred

into cheeses with minimal lossess and thus contribute to their yellow coloration. In Europe, the yellow colour of dairy products is generally seen as a positive trait that contributes to consumers' preference for dairy products produced in summer (*e.g.*, derived from fresh grass based diet) (Nozière et al., 2006). Because carotenoids are fat-soluble, the yellow coloration is a function of both fat colour and concentrations, and fat colour is a function of the carotenoid concentration in the fat. The yellow coloration is also higher when cows are fed pasture or grass silage that hay based, grain or maize silage diets (Martin et al., 2005). In fact, pasture feeding has been associated with high β-carotene concentrations in milk for a long time, with high variations related to season. Carotenoids are sensitive to different physico-chemical factors including air, oxidizing agents and ultraviolet ligh; their degradation is accelerated by increasing temperature, consequently, technological treatment applied during cheese manufacture, are likely to degrade these micronutrients and influence the vitamin potency. During cheese manufacturing, between 800 and 950 g/kg if the retinol and carotenoids in the original milk are recovered in the curd. In many studies, little or no change in the concentration of these components has been observed during ripening for up to a year. These results suggest that β-carotene is very stable (Nozière et al., 2006). In fact, also in Asiago d'allevo cheese the vitamin A content was constant during the aging period. (Elia et al., 2006)

Favaro et al. (2005) reported the presence of terpenes and sesquiterpenes in Asiago produced with milk from animal feeding on mountain pastures. In particular, sesquiterpenes are present only in Asiago mountain cheese. Therefore the authors proposed the sesquiterpenes as marker of mountain origin. Systematic qualitative analysis revealed several sesquiterpenes in mountain herbage and milk, in particular β-caryphyllene and α-humulene, in Asiago mountain cheese, confirming sesquiterpenes as markers of cheese produced form animals grazing on mountain pastures.



Flow diagram for the manufacture of Asiago cheese

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Web-bibliography

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CHAPTER 2

Proteolytic oligopeptides as molecular markers of cheesemaking procedure and ageing in Asiago d'allevo cheese

ABSTRACT

The present study aimed at the identification of oligopeptides in 6 and months-aged Asiago d'allevo cheeses produced in six mountain dairy farms and manufactured with different cheesemaking procedures. The water-soluble peptides were analyzed by using High Performance Liquid Chromatography coupled on electrospray mass spectrometry. Among the 76 peptides identified, most of them arose from α_{s1}- and β-casein, and a few arose from α_{s2}-casein. However some peptides do not correspond to known specific cleavages due to chymosin or enzymes of starter cultures, indicating also a contribution of non starter bacteria proteinases to generate these peptides. PCA analysis technique was applied in order to propose the use of peptides as potential molecular marker to assess the authenticity of cheese. Results showed that this approach easily allowed for the discrimination of cheeses based on ripening times and the use of starter cultures in Asiago.

Introduction

Asiago is an Italian semi-hard cheese included in the list of Italian cheese having Denomination of Origin (Regulatory Board Reg. 1107/96) a definition that considers technological characteristics and geographical restrictions. Asiago is produced in a restricted area in the north-eastern region of Italy; the geographical area includes both lowland and mountain zone, for which an integrating appellation of "Product of the Mountain" (> 600 m a.s.l.) is possible. Asiago is manufactured from bovine milk and Asiago d'allevo is the aged variety cheese which is matured for a period ranging from 6 to 18 (and sometimes more) months.

Semi-hard cheeses are characterized by an intensive proteolytic process that influences the main organoleptic traits depending on the ripening time (Ardo., 2001). In fact, as ageing proceeds, milk curd proteins (α_{s1}-, α_{s2}-, β- and κ-caseins) undergo an extensive degradation. Different proteolytic agents such as endo- and exoproteases of raw milk (plasmin and cathepsin D), the rennet, starter and non

starter proteinases are involved in producing peptides and free amino acids. On account of this activity, nitrogen components of aged cheese consist of a mixture of native caseins (CNs), high, medium and low molecular weight peptides and free amino acids (Sousa et al., 2001).

A powerful way to gain more information on the proteolytic process in cheese is to identify the peptides produced throughout the ripening. Moreover, by knowing the identities of the peptides that typically accumulate in a specific cheese during ripening enhance the possibilities to control the ageing process by the actions taken during cheese production. This knowledge is also highly useful when assuring the authenticity of the cheese (Ardö et al., 2007).

In this study, several samples of Asiago d'allevo aged 6 and 12 months and manufactured in 6 dairy farms located in two mountain areas with different management practises were tested. In summer, cows were kept in alpine grazing and they were fed a pasture and concentrates as supplements. Cheesemaking procedure was carried out *in situ*, thus the existing microflora was autochthonous and originated from the environment and the milk. These samples were compared also with other cheeses obtained in May, a period of the year in which cows stayed in barn and were fed a total mixed ration; as regard this latter cheese production system samples were produced in a dairy plant.

The aim of the study was to assess the proteolysis process of Asiago d'allevo cheese to test the potential use of the peptide pattern or of some of them as molecular markers of production system and age of ripening. According to this purpose, the identification of peptides was carried out by using high-performance reverse-phase liquid chromatography/electronspray ionization source-mass spectrometry (RP-HPLC/ESI-MS) and the cluster of different experimental thesis was made by using principal component analysis (PCA) technique.

Materials and methods

Cheesemaking procedure

The samples of Asiago d'allevo cheese used for this study were manufactured with raw partially skimmed milk according to the disciplinary regulation, both in dairy plant and directly in the alpine site farm (*in situ*). The morning milk was added to the evening skimmed milk, previously left at room temperature, and warmed in the vat to 35 °C. The coagulation was induced by addition of starter culture (optional) and rennet. In the case of 4 dairy plants, starter culture contained *Streptococcus thermophylus* strain, meanwhile *in situ* it was a mixture of *Streptococcus thermophylus* and *Lactobacillus* strains. The other two farms did not use the starter cultures in both seasoning period. After coagulation, the curd was broken into small granules and the temperature was raised to 47±2 °C within 18-21 min. The curd was then extracted, placed in moulds and transferred onto a sloping table, for the first drainage of the whey (3-5 h), assisted by turning the forms (round in shape) several times. The wheels were then sent to the presalting area, where the removal of whey was completed before starting with brining. In 4 farms, the salting procedure was accomplished in two days with brining made up of 24% salt while in the other two farms the salting was dried. The cheeses obtained by both management practises were ripened in the same storage bay at 11 °C and 80-85% of relative humidity.

Cheese samples

This study considered 28 samples of Asiago d'allevo cheese produced in five dairy farms located in the mountain area so-called *Altopiano dei Sette Comuni* and in a dairy farm situated in another closed mountain area so-called *Novegno* (Veneto region, Italy). Every farm had two distinctive sites: one in the plateau (above 1000 m a.s.l.) and the other one at higher altitude which is available only in the summer period (alpine grazing season). Cheeses were manufactured with milk obtained in three production systems: *a)* in May, the cows were kept in barn and fed a total mixed ration (TMR) based on hay and concentrate (maize and soybean); *b)* in July, the cows

were sent in alpine pasture and fed concentrate as supplement (maize, barley and soybean) at daily dose of about 5 kg of dry matter per cow and *c)* in September similar to *b)* condition for the rearing system. The cheesemaking condition were the follow: farms A, B, D and F used the starter culture (*Streptococcus thermophylus* strain, meanwhile *in situ* it was a mixture of *Streptococcus thermophylus* and *Lactobacillus* strains), while farm C and E did not use any kind of starter. All farms produced the cheese in a dairy plant in May, meanwhile during grazing season, they manufactured cheeses in a small dairy plant located in the alpine site (1450 m a.s.l. on average) of the farm (*in situ)*. In each of the three experimental periods related to the cheese production system (May/barn; July/alpine grazing and September/alpine grazing), sample was ripened for 6 and 12 months. Every wheel was sliced and stored under vacuum at -80 °C until use.

Table 1 Techniques and cheesemaking parameters in the different farms considered in this study

Farm	Altitude (m) a.s.l.[a]	Starter	Cooking (°C)	pH curd[b]	Salting procedure
A	1100-1300	Yes	45.0	6.35	brining
B	1250-1450	Yes	45.5	6.30	Dry salting
C	1600-1900	No	48.0	6.30	brining
D	1100-1200	Yes	45.0	6.35	brining
E	1600-1800	No	47.5	6.50	Dry salting
F	1400-1700	Yes	46.0	5.85	brining

[a] Altitude related to the alpine grazing site; [b] at the end of the cooking step

Extraction and concentration of the oligopeptide fraction

Ten grams of finely grated cheese were suspended in 45 mL of 0.1 N HCl. (L,L)-phenylalanine (Phe-Phe) was added as internal standard (2.5 ml of a 1 mM water solution, Sigma-Aldrich, St. Louis, MO, USA). The suspension was homogenized for 1 min by an Ultra Turrax T50 (IKA-Werke, Staufen, Germany) and then centrifuged at 5000 x *g* for 45 min at 4 °C in an ALC 4237R centrifuge (Thermo Electron,

Waltham, MA, USA). The supernatant was filtered through filter papers and then extracted three times with 40 mL of ethyl ether in order to completely discard the lipophylic component. The aqueous solution was filtered with a Millipore 46 mm Sterifil Aseptic system through 0.45 mm HVLP Millipore filters (Millipore, Billerica, MA, USA). 3 mL of the resulting solution were dried and redissolved in 900 μL of a 0.1% formic acid solution (pH 3). The solution was diafiltered at 2800 x *g* through Millipore PLGC filters (nominal molecular cut-off 10000 Da) using an Amicon Micropartition system MPS-1 (Millipore, Billerica, MA, USA). The filtrate was dried under nitrogen, redissolved in 500 μL of H_2O containing 0.1% formic acid (HCOOH) and analyzed by HPLC/MS.

Liquid chromatography/mass spectrometry analysis of the oligopeptide fraction

The HPLC equipment consisted of a Waters Alliance 2695 (Waters, Milford, MA, USA) separation module equipped with a Gemini (Phenomenex, Torrance, CA, USA) C18 column (5 μm, 300 Å, 250 x 4.6 mm) connected to a Micromass ZMD mass spectrometer (Micromass, Manchester, UK). Eluent A: H_2O containing 0.2% acetonitrile (CH_3CN) and 0.1% HCOOH; eluent B: CH_3CN containing 0.2% H_2O and 0.1% HCOOH; elution: 0–15 min isocratic 100% A, 15–60 min linear gradient from 100% A to 67% A, 60–69 min isocratic 67% A, 69–70 min from 67% to 60% A, 70–80 min from 60% A to 0% A, 80–85 min isocratic 0% A, plus reconditioning (85-90 min to 100%A. The column flow was splitted 10:1 before the ESI interface. The mass-spectrometry equipment consisted of an ES ionization in positive ion mode and single quadrupole analyzer: capillary voltage 3200 V, cone voltage 30 V, source temperature 100 °C, desolvation temperature 150 °C, cone gas (N_2) 100 L h^{-1}, desolvation gas (N_2) 400 L h^{-1}. Acquisition of total ion chromatograms (TIC, 100–1900 m z^{-1}), scan time 4 s. Data were acquired by the software MassLynx 4.0. By using a suitable software developed by Sforza et al. (2003), the most abundant peptides were identified according to their molecular weights and their significant fragment ions. For every single peptide, all the possible isobaric sequences contained in the caseins

compatible with its molecular weight were first calculated. Among these possible peptides, the software then gave molecular mass of the fragments generated by the loss of the first N- or C-terminal amino acid, allowing to compare them with the fragments actually observed and thus leading to the peptide identification. All identified peptides were semiquantified by comparison with the internal standard Phe-Phe. The oligopeptides were semiquantitated by measuring the ratio between the peptide area in the extract ion chromatogram (XIC) and the area of the Phe-Phe in its relative XIC.

Statistical analysis

Statistical treatment of data of the semiquantification peptides obtained from chromatographic analyses was performed using SPSS 15.0 statistical software (2007). Principal component analysis (PCA) was performed by factorial dtata reduction extracting 2 factors, with an unrotated factor solution in the space of the observed variables. The first and second PCA components were calculated as independent variables on data by linear regression analysis. Discriminant Analysis (DA) was performed with the Wilks lambda method, with a stepwise strategy: at each step, the variable that mostly contribute to the separation of the groups was entered into the discriminant functions. Wilks lambda is used to test the null hypothesis that the populations have identical means on discriminant function. The criteria for entry and removal was based on probability of full hypothese (entry p = 0.05, removal p= 0.1) and were set as defaults. Month of production was chosen as discriminant factor

Results and discussion

Identification of the proteolytic peptides

The peptides originated by the proteolysis process in Asiago cheese were analysed by using HPLC-ESI-MS: a typical TIC (Total Ion Current) chromatogram is reported in Figure 1 for a 12-month aged sample. From this chromatogram for every single

peak (peptide) the corresponding characteristic m z^{-1} values of higher intensity (characteristic peaks) were obtained. On the base of the MS spectrum, the software is able to recalculate the molecular weight of the peptide. By using a suitable software developed by Sforza et al. (2003), for every peptide we obtained a list of all the possible sequences contained in the caseins that were compatible with that particular weight. By applying another software, it was possible to calculate the MW of the fragments generated by all possible peptides from the loss of the first N- or C-terminal amino acids, thus to identify the peptides.

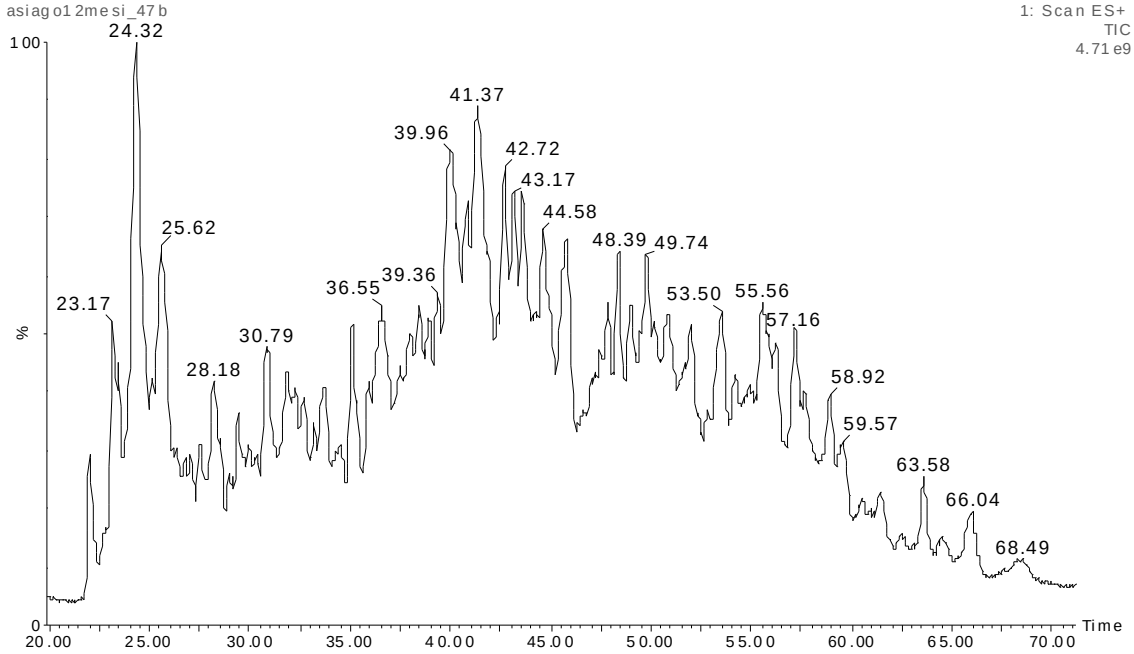

Figure 1 HPLC/ESI-MS chromatogram (TIC, total ion chromatogram) of a Asiago d'allevo cheese (12 months)

In this study, 76 peptides were found in the sample extracts: 50 of them were identified and are reported in Table 2. Other 15 peptides did not derive from casein degradation but they were probably produced by enzymatic activities which act toward free amino acids. For the remaining peptides the assignment of the correct amino acidic sequences was not possible. Taking into account the protein regions from which the identified peptides originated, we found that most of them derived by the N-terminal part of α_{s1}-CN and from both the N-terminal and the C-terminal part of β-CN. No peptides were found derived from κ-CN.

Table 2. List of 50 peptides identified in Asiago d'allevo cheese

MW	Sequence	MW	Sequence
389	β *f*(4-6)	1601	β *f*(4-18)
487	α_{s1} *f*(18-21)	1665	α_{s1} *f*(1-14)
488	α_{S1} *f*(27-30)	1708	α_{s1} *f*(24-38)
502	β *f*(3-6)	1721	α_{s1} *f*(2-16)
542	α_{s1} *f*(10-14)	1781	β *f*(193-208)
545	β *f*(10-14)	1790	β *f*(15-28) 3P*
601	α_{s1} *f*(17-21)	1835	α_{s1} *f*(2-17)
608	α_{s1} *f*(192-196)	1877	α_{s1} *f*(1-16)
631	β *f*(2-6)	1880	β *f*(193-209)
741	β *f*(8-14)	1991	α_{s1} *f*(1-17)
754	α_{s1} *f*(10-16)	2332	α_{s1} *f*(189-207)
755	β *f*(47-52)	2340	β *f*(11-28) 4P*
787	β *f*(1-6)	2347	α_{s1} *f*(1-20)
805	α_{s1} *f*(24-30)	2461	α_{s1} *f*(1-21)
855	β *f*(7-14)	2617	α_{s1} *f*(1-22)
964	α_{s1} f(80-87)	2708	α_{s1} *f*(6-28)
1156	β *f*(84-93)	2764	α_{s1} *f*(1-23)
1236	α_{s1} *f*(24-34)	2780	α_{s1} *f*(89-110)
1255	β *f*(83-93)	2908	α_{s1} *f*(1-24)
1283	α_{s1} *f*(3-13)	3116	α_{s2} *f*(183-207)
1380	α_{s1} *f*(2-13)	3133	β *f*(98-124)
1509	α_{s1} *f*(2-14)	3217	α_{s2} *f*(182-207)
1510	β *f*(82-95)	4024	β *f*(59-96)
1536	α_{s1} *f*(1-13)	4063	β *f*(57-93)
1590	α_{s1} *f*(17-28) 3P*	4235	α_{s1} *f*(1-36)

* P means phosphate groups, bound to serine residues

The percentage distribution of the identified peptides as a function of the different casein sequences from which they derive is reported in Figure 2.

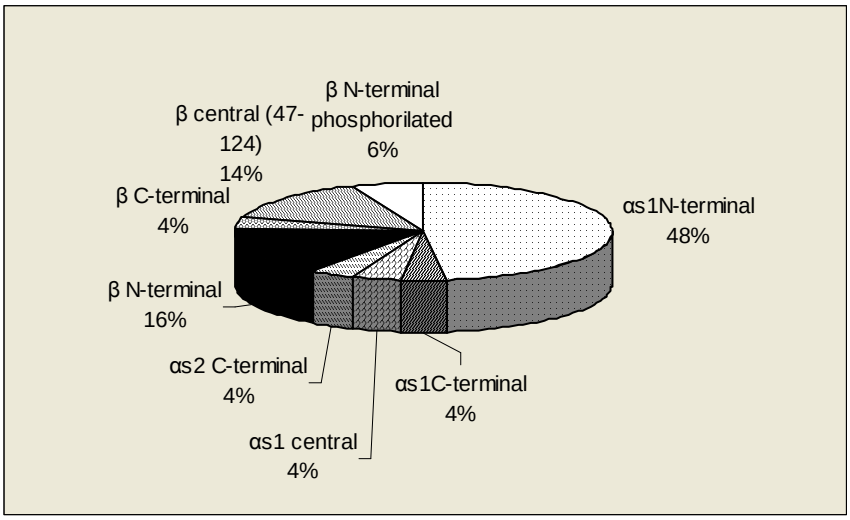

Figure 2. Percentage distribution of the identified peptides respect to the casein sequences.

During cheese ripening, CNs are hydrolyzed into peptides and amino acids by a broad range of enzymes. However, some of the peptides accumulate because they are not easily further broken down by the enzymes present in the cheese. Although a number of enzyme activities are common to many cheess, the peptides composition at different ages is characteristic of the cheese variety (Ardö, 2001)

The peptides that typically accumulate in semi-hard cheese are produced by microbial proteolytic activity on the rennet derived α_{s1}-CN *f*(1-23) and from β-CN (Sousa et al., 2001). In fact, an intensive degradation from N-terminal part of α_{s1}-CN was observed in Asiago cheese; α_{s1}-CN was rapidly hydrolysed by chymosin, at the Phe_{23}-Phe_{24} to yield α_{s1}-CN *f*(1-23) and other fragments were found such as α_{s1}-CN *f*(1-13), *f*(1-14), *f*(2-13), *f*(2-14), *f*(3.13) and *f*(1-17). The pattern of these released peptides showed that they were themselves progressively degraded with the current loss of one or more residues from their N-terminal end as observed in Emmental cheese (Gagnaire et al., 2001), whereas the C-terminal part of the molecule, *e.g.*, α_{s1}-CN *f*(24-199) was almost not hydrolyzed. In contrast to α_{s1}-CN, β-CN was degraded more evenly over the whole sequence, moreover, a recurrent degradation pattern

was observed as shown by the formation of fragment β-CN f(83-93) and β-CN f(84-93). Only some peptides were released from α_{s2}-CN; the low number of identified peptides from this casein can be attributed to its content in the caseins and therefore a higher difficulty in detecting peptides arising from it. A large amount of small phosphopeptides derived from α_{s1}- and mainly from β-CN have been identified in Asiago as well as in other semi-hard cheese (Ardö et al., 2007).

Proteolytic peptides as molecular markers of authenticity

The peptidic pattern of each sample is particularly rich and complex and largely different within the different samples. Chemometric analysis of this pattern may be used to better understand cheese proteolysis and which factors have the highest effect (milk physico-chemical traits, use of starter, seasoning time and condition, etc.). All identified peptides were semiquantified by comparison with an internal standard (Phe-Phe). The oligopeptides were semiquantitated by measuring the ratio between the peptide area in the extract ion chromatogram (XIC) and the area of the Phe-Phe in its relative XIC. Thus, the semiquantification data were analysed by PCA in order to assess differences among the samples as a function of the investigated cheesemaking processes.

As cheese producers move to an optimization of the maturing period and condition, the need emerges to develop appropriate molecular markers that are sensitive and specific of cheese production system and/or ageing time. The score plot including all the analyzed samples and deriving from the calculation of the factor score of every sample for each component is reported in the Figure 4. The score plot referred to all samples, displays two separate clusters which could be put in clear in relation with the months of ripening 6 and 12 months of ageing which turned out to be the main effect able to differenziate the samples. This difference is qualitative and quantitative too: in fact, there are not only some sequences characteristic of each time of ripening but as far as the total amount of peptide concernes, samples of 12 months showed higher amount of peptides than those at 6 months, with a 5.7 times increment. This

findings are a consequence of the proteolysis that caused a progressive release of peptides as ripening proceeds (Meisel, 1997). The proteolytic pattern is completely different among the samples. PCA analysis shows (from the loading plot-here not shown) the most representative peptides at 6 months of ageing are β-CN *f*(10-14), α_{s1}-CN *f*(17-21), β-CN *f*(8-14), α_{s1}-CN *f*(10-16) and α_{s1}-CN *f*(3-13) and at 12 months β-CN *f*(193-209), α_{s2}-CN *f*(189-207), α_{S1}-CN *f*(6-28) and α_{s2}-CN *f*(183-207). Figure 4 reports the histogram of these peptides in relation to the months of ripening.

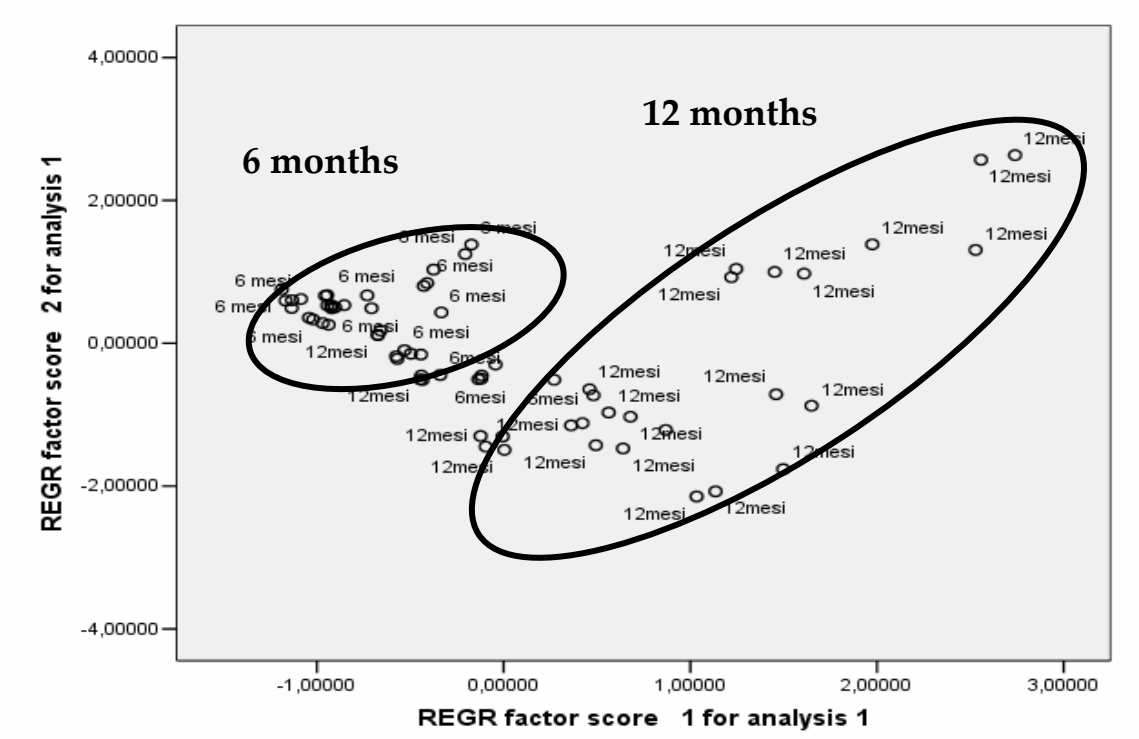

Figure 3 Score plot of the Asiago cheese analyzed samples, labelled and regrouped according to the ageing period (expressed in months). Component 1 explains the 32.33% of the variance, component 2 the 15.70%

PCA shows also other significant differences as a function of the cheesemaking procedure utilized by the farms. In Figure 5 samples were labelled based on the farm of origin and a net separation in two groups is visible in accordance with the use of the starter (with or without, respectively) within the ageing time based distinction. The four experimental combinations were perfectly distinguishable according to ripening time (6 vs. 12, on the left of line vs. on the right of the line) and to the

production technology: group 1 used microbial starter, whereas group 2 didn't use it. This evidence confirms that this approach could contribute to profile cheese according to specific attribute (*i.e.*, use of starter, ripening time) of cheeses produced over a wide range of cheesemaking procedure even if within the disciplinary regulation of the PDO.

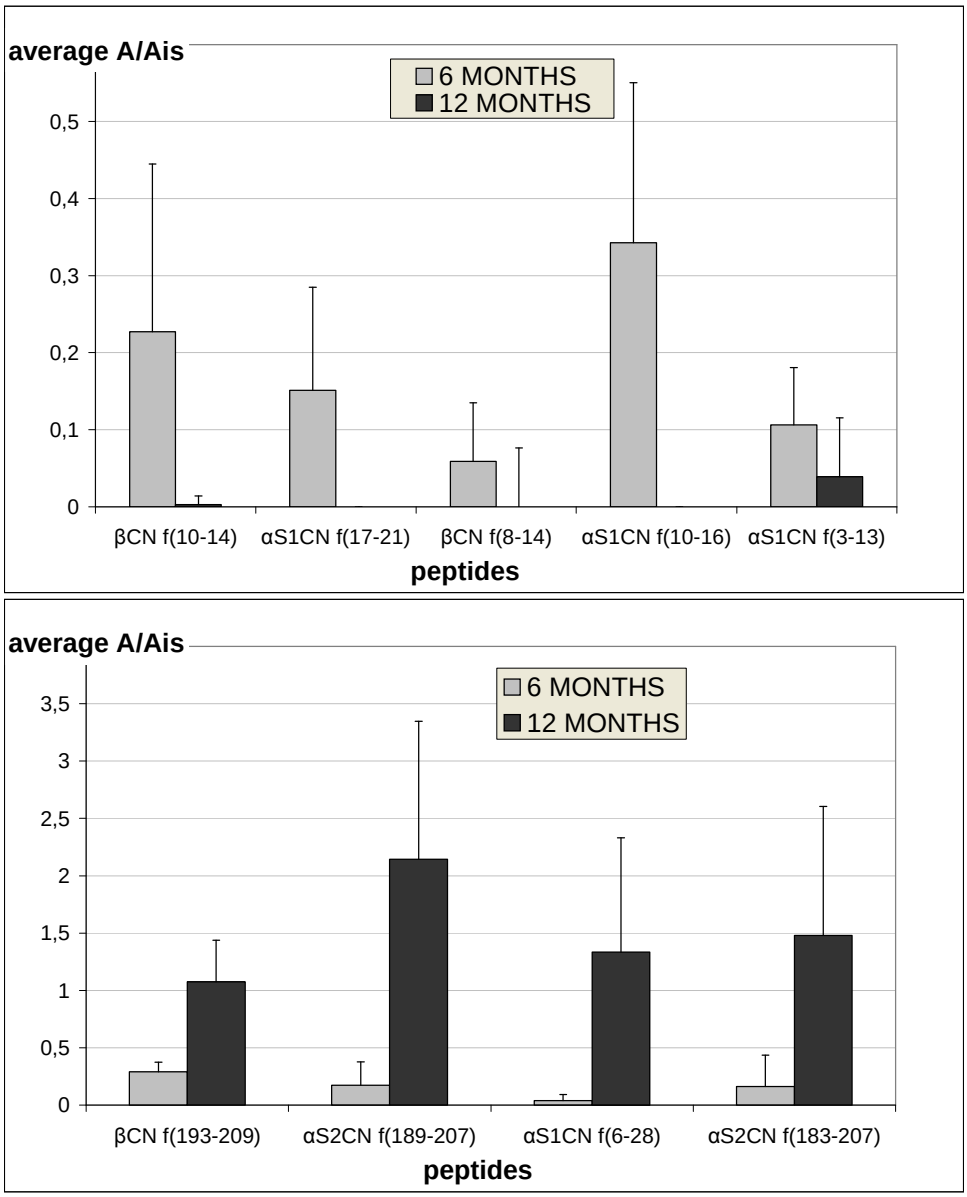

Figure 4 Amounts of the mainly representative identified peptides characteristic of cheeses aged 6 (left) and 12 (right) months. Legend: grey= 6 months; black= 12 months cheese samples

As far as the peptides characteristics of the two different technologies are concerned, the peptides α_{S1}-CN *f*(27-30), β-CN *f*(47-52), α_{S1}-CN *f*(2-14) are typical of the cheeses obtained using starters, whereas α_{S1}-CN *f*(1-17), α_{S1}-CN *f*(1-20), α_{S1}-CN *f*(1-21) and α_{S1}-CN *f*(1-22) are typical of the technologies without starters (Figure 6).

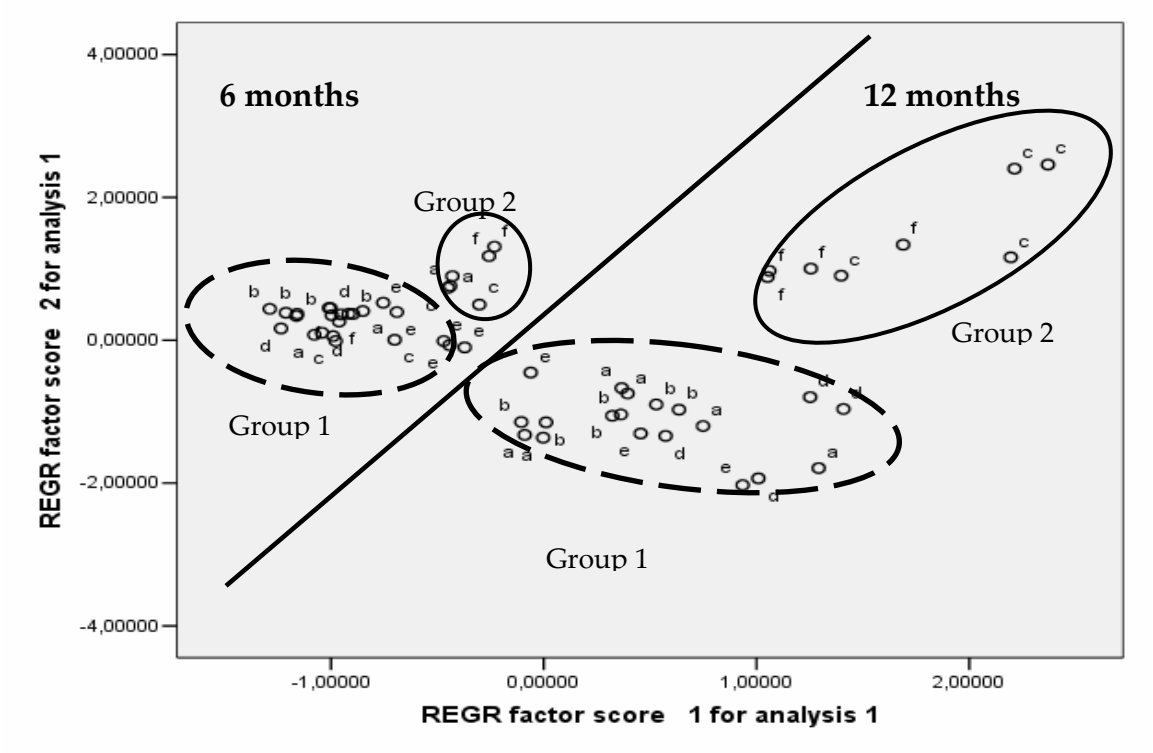

Figure 5. Score plot with groups based on ripening time (left vs. right respect for the oblique line) and of the production technology (group 1 used the starter, group 2 did not use starter)

It is worth noting that all the peptides, apart from α_{s1}-CN *f*(1-17), do not seem to derive from known endopeptidase activity, but from a carboxypeptidase activity on α_{S1}-CN *f*(1-23). Being the carboxypeptidase activity almost rare in cheeses, these peptides may derive from enzymatic activity of non lactic microflora, favoured by higher pH values of the production without starters (Figure 6).

PCA did not show any significant differences among samples as a function of the cheese production system which implies differences in feeding of cows (TMR vs. alpine grazing) and the environment condition (*i.e.*, autochthonous microflora) in the dairy plant, whereas PCA revealed that the proteolytic pattern was more influenced by ageing time and by the use of starter.

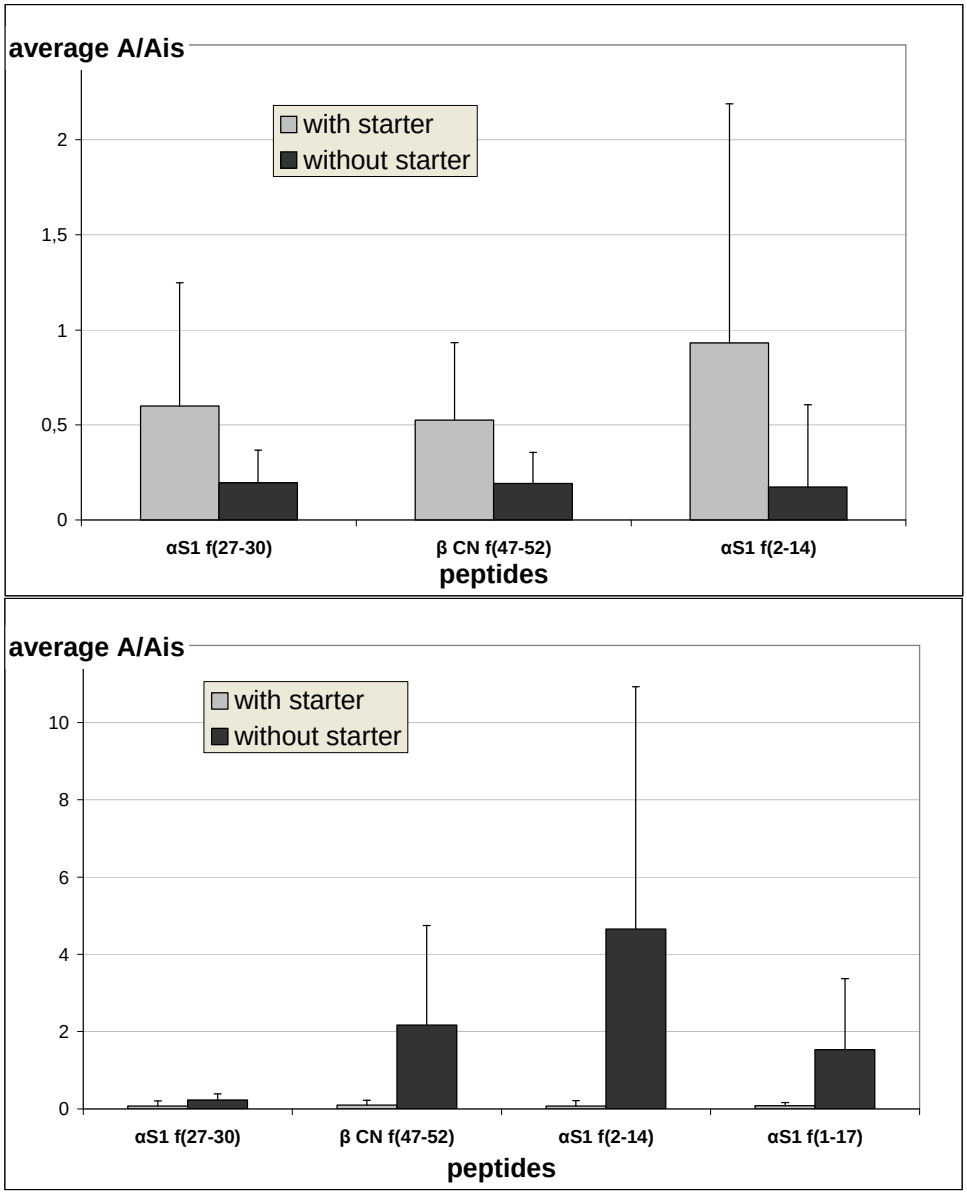

Figure 6 Amounts of the mainly representative identified peptides characteristic of productive technology. Legend: grey= starter; black= without starter

Nevertheless, a discriminant analysis (DA) was also performed in order to classify the samples according to the different production technologies. This analysis is used to classify or assign object (*e.g.*, individual cheeses) to a small number of groups (i.e. ageing time or caseification month) that are predicted by a number of variables that should be uncorrelated, in order to achieve classification. The attempted classification was based on production month, an effect that in PCA results was probably covered by the other two faxtors, ageing time and use of starter. The discriminant analysis within the 12 months samples is perfectly able to discriminate

samples according to the cheese production system, allowing for a correct classification on the base of some discriminant peptides (Figure 7).

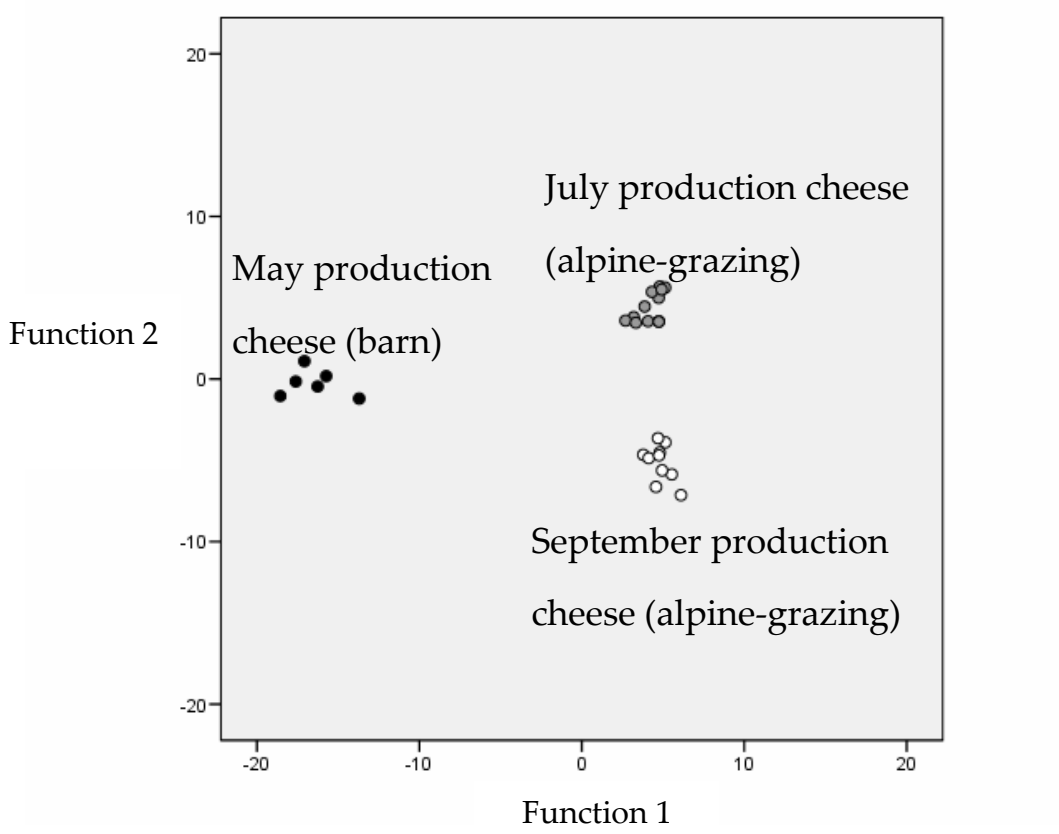

Figure 7 PCA score plot for peptides distribution from the 24 Asiago samples aged for 12 months. Each point is marked with a cheese production system: dot black, barn and TMR/May; dot grey, alpine grazing/July; dot white, alpine grazing/September

Classification was obtained on the base of 12 peptides identified as the most discriminant ones among them the most differencing peptides were α_{S2}-CN *f*(183-207), α_{s1}-CN *f*(2-14) and β-CN *f*(7-14). All these three peptides did not correspond to known enzymatic cleavage sites of the most important proteolytic enzymes, suggesting the presence of enzymatic activities due to non starter bacteria characteristics of the environmental and milking conditions linked to the production period.

Conclusions

Several peptides originating from α_{s1}- and β-CN were identified and an increasing content was observed as ageing proceeds. The results of this research confirmed that multivariate statistical analysis based on oligopeptidic profiles was an objective and powerful approach for evaluating proteolysis in cheese and discriminate cheeses mainly according to ripening age. Peptide pattern also allowed to differentiate the samples on account of the use of starter. Moreover, the peptide pattern was found different in relation to the cow feeding and the environmental condition effects but only considering the longer maturing period. Finally, oligopeptides may be considered useful molecular markers as a tool of authenticity of PDO Asiago in relation to both ageing and the use of starter.

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CHAPTER 3

Angiotensin-converting enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese

ABSTRACT

The angiotensin-converting enzyme inhibitory (ACEI) activities of water-soluble extracts (WSEs) from Asiago cheeses have been assayed according to cheese production systems and having different ripening times. The WSEs were ultrafiltrated onto 10 kDa and 3 kDa cut-off membrane in order to evaluate the ACEI of long and small peptides. The cheese production systems had no significant effect on the ACEI, whereas six-months-aged cheeses had higher inhibitory potency than the others. Moreover, the fraction lower than 3 kDa made a more considerable contribution to the ACEI activity than the fraction lower than 10kDa, suggesting an inhibitory effect due to small peptides. Peptides derived from α_{s1}-casein and β-casein were identified by using RP-HPLC coupled to mass spectrometric detection. A simulated gastrointestinal digestion was carried out in order to evaluate the effect of digestive enzymes on the generation of bioactive peptides, but it did not significantly affect the inhibitory activity.

Introduction

Hypertension is a medical condition in which blood pressure is chronically elevated. It is a major risk factor for cardiovascular diseases, such as coronary heart disease, congestive heart failure and stroke (Otte et al., 2007). The blood pressure is regulated by different biochemical pathways, one of them is called rennin-angiotensin-aldosterone system. Angiotensin I-converting enzyme (ACE) is the key enzyme in this pathway (Muguerza et al., 2006). ACE is ubiquitously distributed in many tissues such as lung, kidney, heart, gastrointestinal system and prostate, with highest levels in the surface of vascular endothelium. ACE catalyzes the conversion of angiotensin I, an inactive decapeptide, into angiotensin II, an octapeptide with a potent vasoconstrictor action. Moreover ACE catalyses the degradation of bradykinin, which has an important vasodilation activity (Takano, 1998). By these actions, ACE elevates blood pressure. Several trials have demonstrated that ACE

inhibitory (ACEI) substances in various foods are responsible for decreasing the high pressure in humans (Alonso et al., 2006; Burke et al., 2001). Some epidemiological evidences suggest that consumption of milk and other dairy products is associated with a reduction of blood pressure (Conlin et al., 2000; López-Fandiño et al., 2006). In fact, several milk protein-derived biologically active peptides have been identified as ACE-inhibitors. These peptides are the products of the proteolysis of milk proteins especially by the action of microbial enzymes (Korhonen & Pihlanto, 2006).

The structure-activity relationship of ACEI peptides from food proteins has not yet been established, however, some general features have been found (FitzGerald et al., 2004; Meisel, 1997). ACEI peptides usually contain 2–20 amino acid residues, although active peptides with up to 27 amino acids have been identified (Saito et al., 2000; Yamamoto et al., 1994). ACE seems to prefer substrates or competitive inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal position; the presence of lysine or arginine, with a positive charge on the ε-amino group, also seems to contribute to the inhibitory potency (Murray & FitzGerald, 2007).

The most potent ACEI activities have been measured for tripeptides isoleucine-proline-proline (Ile-Pro-Pro; IPP) and valine-proline-proline (Val-Pro-Pro; VPP), which have also been reported to lower blood pressure in animals and humans (Hata et al., 1996; Sipola et al., 2001).

To exert their function in vivo, ACEI peptides have to reach their target sites without being degraded or inactivated by the action of gastrointestinal or plasma peptidases and pH conditions. Thus gastrointestinal enzymes and enzymes localized on the surface of enterocytes could cleave the oligopeptides in smaller peptides which could increase or lose their bioactivity. The main digestive enzymes (pepsin, chymotripsin, pancreatin) are involved in this process. For example, α_{s1}-casein (CN) *f*(23-27) and α_{s1}-CN *f*(104-109), two potent ACE inhibitors in vitro, were shown to have no hypotensive effect in vivo (FitzGerald & Meisel, 2000; Maruyama et al., 1989). On the other side, proline- and hydroxyproline-containing peptides are generally resistant to

degradation by digestive enzymes (FitzGerald & Meisel, 2000). Therefore these peptides are able to survive gastrointestinal system, be absorbed and reach the cardiovascular system in an active form to exhibit their bioactivity.

Antihypertensive-food-derived peptides have been isolated from fermented milk (Chen et al., 2007), from different cheese varieties such as Gouda (Saito et al., 2000), Cheddar (Ryahanen et al., 2001), Manchego (Gómez-Ruiz et al., 2002) and also from some Italian cheeses such as Parmigiano-Reggiano (Addeo et al., 1992), Crescenza, Gorgonzola, Mozzarella and Italico (Smacchi & Gobbetti, 1998).

Asiago is a Protected Designation of Origin (PDO) semi-hard cheese of the north-eastern region of Italy and is manufactured from bovine raw milk partially skimmed according to the specifications of CE Regulation No. 1107/96. Asiago d'allevo is the variety most ripened which takes from 6 to 18 (and sometimes more) months of maturation.

In this study, several samples of Asiago d'allevo cheese manufactured in two different ways were tested. During the summer, cows were moved to alpine grazing and they grazed on pasture and received concentrates as supplements. Cheesemaking procedure was carried out *in situ*, thus the existing microflora was autochthonous and originated from the milk and from the environment. In the remaining period of the year cows stayed in barn, they were fed a total mixed ration and cheese was produced in a dairy plant.

The aim of this study was to evaluate the ACEI activity of water-soluble extracts (WSEs) of Asiago d'allevo cheese having different ages of ripening and manufactured with two management practises explained above. The main oligopeptides in these extracts were characterized by means of LC/ESI-MS methodology. Moreover, the molecular composition of the WSEs was also investigated after treatment with proteolytic enzymes involved in gastrointestinal digestion, in order to assess if new ACEI peptides can be potentially generated after ingestion in vivo.

Materials and methods

Cheesemaking procedure

The samples of Asiago d'allevo cheese used for this study were manufactured with raw partially skimmed milk according to the disciplinary regulation, both in dairy plant and alpine farmhouse (*in situ*). The morning milk was added to the evening skimmed milk, previously left at room temperature, and warmed in the vat to 35 °C. The coagulation was induced by addition of starter culture and rennet. In the case of dairy plant, starter culture contained of *Streptococcus thermophylus* strain, meanwhile *in situ* it was a mixture of *Streptococcus thermophylus* and *Lactobacillus* strains. After coagulation, the curd was broken into small granules and the temperature was raised to 47±2 °C within 18-21 min. The curd was then extracted, placed in moulds and transferred onto a sloping table, for the first drainage of the whey (3-5 h), assisted by turning the forms (round in shape) several times. The wheels were then sent to the presalting area, where the removal of whey was completed before starting with brining. The salting procedure was accomplished in two days with brining made up of 24% salt concentration at 17 °C and natural humidity. The cheeses obtained by both management practises were ripened in the same storage bay at 11 °C and 80-85% of relative humidity.

Cheese samples

This study considered nine (n=9) samples of Asiago d'allevo cheese obtained from milk produced in a farm located in a mountain area (above 1000 m a.s.l.) so-called *Altopiano dei Sette Comuni* (Veneto Region, Italy) that reared around 50 lactating Holstein-Friesian cows. The cheeses were produced in two seasons corresponding to two different productive systems. Three (3) were produced during the period of indoor feeding (May), in which the cows were kept in barn and fed a total mixed ration based on hay and concentrate (maize and soybean) and relative cheeses were manufactured in a commercial dairy plant near the farm. The other six (6) were produced in summer (3 in July and 3 in September) during the alpine grazing period

and they were directly manufactured in an artisanal dairy plant belong to the farm (*in situ*). In this outdoor feeding period, cows were kept in the alpine site (1350 m a.s.l in average) of the farm and they were fed on pasture plus concentrate (based on maize, barley and soybean meal) as supplement at daily dose of around 4.5 kg of dry matter per cow. In each of the three seasonal periods related to the cheese production system (May/barn, July/alpine grazing and September/alpine grazing), one sample was ripened for 6, 12 and 18 months. Sliced samples were storied at -80 °C until the extraction of water-soluble peptides.

Preparation of water-soluble extracts (WSEs)

WSEs were obtained according to the procedure described by Kuchroo and Fox (1982) with some modifications. Twenty grams of cheese were resuspended in 40 mL of water and the pH was adjusted to 2.0 using 1 *N* HCl. The suspension was homogenized for 10 min at room temperature at 20500 rpm using an IKA Ultra-Turrax T25 (Werke GmbH & CO.KG, Germany). Internal standard (2.5 mL, Phe-Phe, 2 mM in MilliQ water, Sigma-Aldrich, St. Louis, MO, USA) was added for the semiquantification of peptides. The homogenate was then heated to 40 °C and held under gentle stirring for 1 h. Centrifugation at 10,000 x *g* for 30 min at 4 °C formed a pellet of insoluble material on the bottom, a lipid solution in the upper part while the water phase was intermediate. The WSEs were recovered and filtered first through a glass fibre prefilter (Millipore, Ireland) and then through a 0.45 μm filter (Sartorius Biotech GmbH., Germany). Three extractions with ethyl-ether were then carried out in order to completely discard the lipophylic component. WSEs were finally ultrafiltered through a 10 kDa cut-off membrane in a stirred ultrafiltration cell module (Millipore, Bedford, MA, USA). Ultrafiltrated permeates were both analysed as such and after a second step of fractionation through a 3 kDa molecular weight (MW) cut-off filter (Amicon-Ultra15, Millipore, MA 01821, USA) with centrifugation (3200 x *g* for 40 min at 15 °C). The permeates were kept at -20 °C until use.

Simulated gastrointestinal digestion

Pepsin (from porcine stomach mucosa, EC 3.4.23.1, 1:60,000), pancreatin (from porcine pancreas, activity equivalent to 8 x U.S. Pharmacopeia specifications), casein from bovine milk, sample buffer, standard molecular mass markers and Coomassie Brilliant Blue were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The enzymatic digestion of WSEs (<10 kDa and <3 kDa fractions) was performed according to the method of Parrot et al. (2003) with some modifications. Enzyme solutions were prepared in 0.01 M HCl (0.1 g pepsin L^{-1}) or water (0.2 g pancreatin L^{-1}) and stored as aliquots at -20 °C. The hydrolysis was conducted also on a casein solution considered equally to control sample. Casein was dissolved to 20 g L^{-1} in water. The pH of the suspension was adjusted to 2 with 1 M HCl.

Digestion experiments were carried out in a stirrer with orbital oscillating plate at 160 movements per min (Elettrofor, Roma, Italy) to simulate the gastrointestinal conditions. Five mL of WSEs were first hydrolysed with 0.5 mL pepsin solution [the enzyme to substrate ratio (E/S) was 1:200 w/w when using casein as substrate] for 30 min at 37 °C at pH 2.0. The reaction was stopped by increasing pH to7.5 with 40% w/w NaOH without diluting the solution. Pancreatin solution (0.125 mL) was then added to the pepsin pre-digested mixture. The E/S ratio was 1/400 w/w. After 240 min of incubation, the reaction was stopped by heating the mixture at 85 °C for 5 min in water bath in order to inactivate digestive enzymes. The gastrointestinal digestion was done in triplicate and each sample was stored at -20 °C until analysis. To evaluate the effective digestion, SDS-PAGE was carried out according to Laemmi's method with a 4% acrylamide stacking gel and a 15% acrylamide separating gel. The digested WSEs were mixed 1:1 with sample buffer (0.125 M Tris-HCl buffer, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.004% bromophenol blue) heated at 95 °C for 5 min and let ride under constant voltage. Proteins in the gel were stained with Coomassie Brilliant Blue. Standard molecular mass markers (6.5-66 kDa) were used for molecular mass estimation of proteins bands on the gels.

Determination of ACE-inhibitory (ACEI) activity

ACEI activity was assayed in triplicate by the method of Cushman and Cheung (1971) with some modifications. This assay was performed on WSEs (two cut-off permeates: <10 kDa and < 3 kDa, before and after digestion). This method is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (Hip-His-Leu; HHL) (Sigma-Aldrich, St. Louis, MO, USA) catalysed by the ACE. Briefly, each assay mixture contained 200 μL HHL solution (5mM HHL, 100 mM sodium borate buffer, 300 mM NaCl, pH 8.3), ACE solution (0.1 U mL^{-1}, EC 3.4.15.1, from rabbit lung, Sigma-Aldrich) and 80 μL WSE. After 30 min of incubation at 37 °C, the reaction was stopped by adding of 0.25 mL 1 HCl. The hippuric acid formed was extracted with 1.7 mL ethyl acetate, and the organic phase was transferred to a test tube and evaporated. The residual containing hippuric acid was dissolved in 1 mL deionised water and the solution was measured using ULTROSPEC3000 visible spectrophotometer (Amersham Pharmacia Biotech, New Jersey, USA) at 230 nm against deionised water as a blank. The amount of hippuric acid liberated from Hip-His-Leu under test conditions, but in the absence of an inhibitor, is defined as 100% ACE activity. Captopril (1mM, Sigma-Aldrich) was used as an inhibitory reference in the assay.

The percentage of inhibition (IACE) was calculated using the formula:

$$IACE = (A-B)/(A-C) \times 100 \quad (1)$$

where A is the optical density at 230 nm with ACE but without WSE, B is the optical density in the presence of both ACE and WSE, and C is the optical density without ACE and WSE.

Peptide concentration (c_{pep}), expressed as mg per mL, was estimated by the ultraviolet absorbance method using the following equation (Stoscheck, 1990):

$$c_{pep} = [(1.55 \times A_{280}) - (0.76 \times A_{260})] \quad (2)$$

where A_{280} and A_{260} means the absorbance at 280 nm and at 260 nm respectively.
The inhibition was then expressed as the percentage of inhibition per 100 μg of peptides ($IACE_{100}$) as follows:

$$IACE_{100} = (IACE \times 100)/(c_{pep} \times WSE_{80}) \quad (3)$$

where WSE_{80} is the volume (μL) of the water-soluble extract added to the ACE assay mixture.

HPLC/ESI-MS analysis

The WSE with molecular masses below 10 kDa and 3 kDa, before and after digestion, were analysed by HPLC (Alliance Waters 2695 separation module, Waters, Milliford, MA) by using the Jupiter (Phenomenex, Torrance, CA, USA) C18 column, 250 x 4.6 mm. The injection volume was 10 μL. Gradient elution was carried out with a mixture of two solvents (solvent A: 0.2% acetonitrile and 0.1% formic acid in water, solvent B: 0.2% water and 0.1% formic acid in acetonitrile, v/v). Proteins and peptides were eluted as follows: 0-15 min, 100% A; 15-60 min, linear gradient from 100% A to 67 % A; 60-69 min, isocratic 67% A; 69-70 min, from 67% to 60% A; 70-80 min, from 60% to 0% A; 80-85 min, isocratic 0% A. The flow rate was 1 mL min^{-1}. The column was maintained at 30 °C. The column flow was splitted 10:1 before the ESI interface. The mass-spectrometry equipment consisted of an ES ionization in positive ion mode and single quadrupole analyzer: capillary voltage 3200 V, cone voltage 30 V, source temperature 100 °C, desolvation temperature 150 °C, cone gas (N_2) 100 L h^{-1}, desolvation gas (N_2) 400 L h^{-1}. Acquisition in total ion mode (100-1900 m z^{-1}), scan time 4 s. Data were acquired by the software MassLynx 4.0. By using a suitable software developed by Sforza et al. (2003), the most abundant peptides were identified according to their molecular weights and their significant fragment ions. For every single peptide, all the possible isobaric sequences contained in the caseins compatible with its molecular weight were first calculated. Among these possible

peptides, the software then gave molecular mass of the fragments generated by the loss of the first N- or C-terminal amino acid, allowing to compare them with the fragments actually observed and thus leading to the peptide identification. All identified peptides were semiquantified by comparison with the internal standard Phe-Phe. The oligopeptides were semiquantitated by measuring the ratio between the peptide area in the extract ion chromatogram (XIC) and the area of the Phe-Phe in its relative XIC.

Statistical analysis

To approximate the normal distribution, the percentage of ACE-inhibition per 100 μg of peptides ($IACE_{100}$) was recalculated using a logarithmic transformation as: [$\log_{10}(IACE_{100}) + 2$]. After this log adaptation, data were normally distributed (PROC UNIVARIATE and Shapiro-Wilk test). A general linear random model procedure was performed to evaluate the potential inhibitory activity of water-soluble extracts:

$$Y_{ijklm} = \mu + R_i + S_j + M_k + D_l + MD_{kl} + C_{ij} + \varepsilon_{ijkl} \quad (4)$$

where Y is the response for ACE inhibition, μ is the overall mean, R_i is the ripening time effect (3 levels: 6, 12 and 18 months), S_j is the cheese production system effect (3 levels: May/barn; July/alpine-grazing; September/alpine-grazing), M_k is the molecular weight effect (2 levels: <10 kDa and <3 kDa), D_l is the simulated gastrointestinal digestion effect (2 levels: digested, not digested), C_{ij} is the random effect of each cheese form nested within R and S, MD_{kl} is the interaction between M and D and ε is the error. The other interactions among fixed effects were not introduced in the model because they were not statistically significant. The degrees of freedom of R effect were used in the two orthogonal contrasts (6 vs. 12+18 and 12 vs. 18). When an effect was significant ($P < 0.05$), differences among levels or treatment means were determined using the PDIFF option along with Bonferroni adjust. All statistical analyses were carried out by using SAS (2002).

Results

ACE-inhibitory (ACEI) activity of Asiago d'allevo cheeses

In the first experiment, the ACEI activity of samples based on the ageing time and two different filtrations (3 kDa and 10 kDa nominal cut off fractions) were tested (n=18). Since the ACEI activity of these WSEs can be related to their peptide content, the inhibitory indices were expressed as the percentage of inhibition per 100 μg of peptides, as described in Materials and Methods. In Table 1, it can be observed that 6-months-aged cheeses had higher inhibitory activity (11.9% per 100 μg of peptides) than 12 and 18-months-ripened cheeses (3.6% and 3.4% per 100 μg of peptides, respectively). Concerning the molecular weight (MW) of peptides, 3 kDa permeates had 9.5% inhibition per 100 μg of peptides, while 10 kDa permeates displayed 3.1% inhibition per 100 μg of peptides.

Table 1 Influence of ripening time, molecular weight (MW) and gastrointestinal digestion on the average, median and coefficient of variation (CV) of the ACE inhibition (IACE, %) per 100 μg of peptides of Asiago WSEs

	Ripening time (months)			**Molecular Weight (kDa)**		**Gatrointestinal digestion**	
	6	12	18	< 3	< 10	before	after
Samples (n)	12	12	12	18	18	18	18
Average±SD	11.9±9.8	3.6±3.5	3.4±4.2	9.5±8.3	3.1±5.0	3.6±2.6	9.0±9.6
Median	8.4	2.1	1.8	6.7	1.6	7.5	2.9
CV (%)	82.4	97.2	123.5	87.4	161.3	72.2	106.7

SD = standard deviation; CV = (SD/average)x100

However, in the absence of normal distribution, a logarithmic transformation of data was adopted in the statistical model. Therefore values were expressed as $[\log_{10}(IACE_{100}) + 2]$. WSEs from cheeses ripened for 6 months showed a significantly higher inhibitory activity than WSEs from 12- and 18-months-aged cheeses (6 vs. 12+18, $P < 0.05$) as displayed by the two orthogonal contrasts (Figure 1). The cheese production system was found to be not influent ($P = 0.87$) on the inhibitory response. The ACEI activity differed significantly between cut-off permeates ($P < 0.001$) as

showed in Figure 2. It can be seen that the higher value of inhibition corresponded to the peptide fraction with small MW (< 3 kDa).

The simulated digestion did not influence the ACEI activity (2.26 vs. 2.43; $P = 0.46$). However, a significant interaction ($P < 0.05$) between the MW and the gastrointestinal digestion (GD) effect was observed, as reported in Figure 3. It seemed that the GD increased the ACEI activity of permeates with high MW peptides (<10 kDa) ($P < 0.05$), whereas in the <3 kDa permeates the inhibition slightly decreased after simulated digestion.

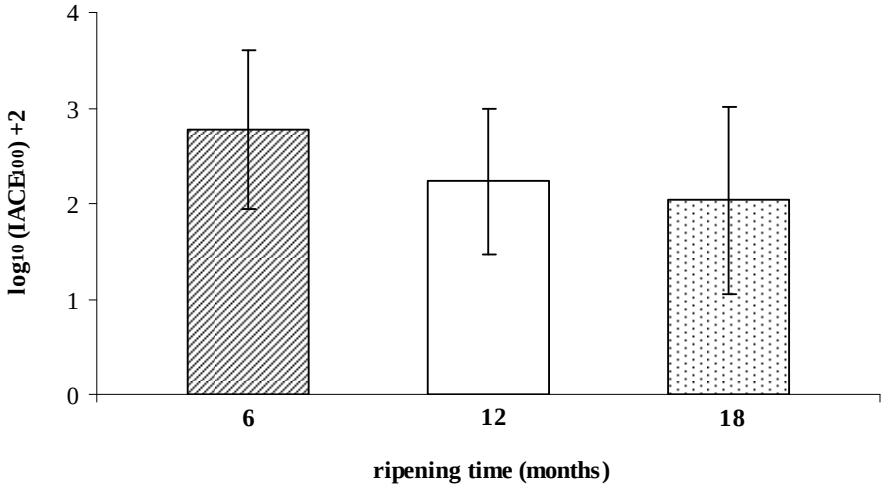

Figure 1 ACE inhibitory activity (ACEI) of water-soluble extracts of 6-, 12- and 18-months-aged cheeses. Values (means ± SEM) are expressed as [$\log_{10}(IACE_{100}) + 2$], in relation to the ripening time (6 vs. 12+18; $P < 0.05$) (12 vs. 18; $P = 0.51$)

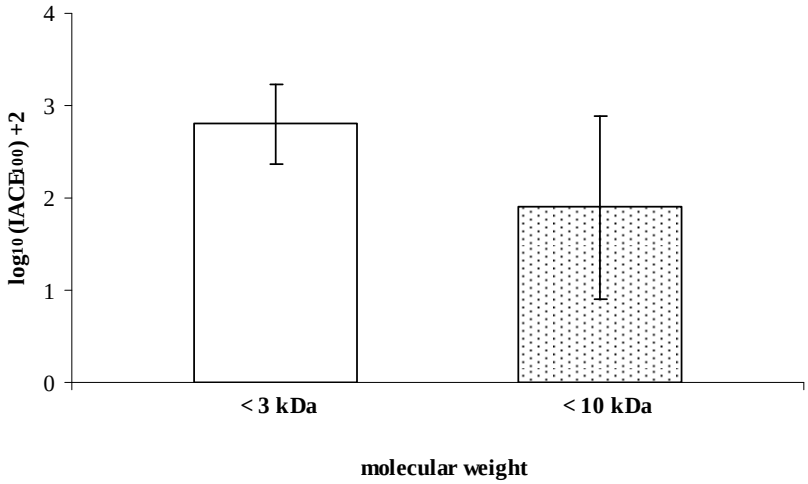

Figure 2 Values (means ± SEM) of the ACE-inhibitory indices (IACE), expressed as [$\log_{10}(IACE_{100}) + 2$], in relation to the molecular weight of the peptides ($P < 0.001$)

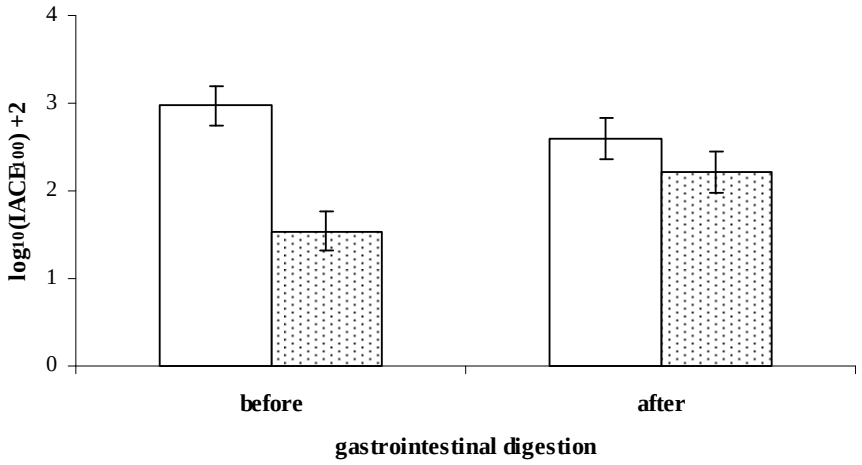

Figure 3 Effect of the interaction ($P < 0.05$) between the molecular weight (□, <3 kDa; ▒, <10 kDa) of peptides and gastrointestinal digestion (before and after digestion) on ACE-inhibitory indices (IACE), expressed as [$\log_{10}(IACE_{100}) + 2$]

Identification of peptides in 10 kDa and 3 kDa permeates and survival to simulated gastrointestinal digestion

The presence of antihypertensive peptides in the WSEs was investigated by HPLC-MS analysis. The identification of peptides was carried out by applying a method described by Sforza et al. (2003) as explained in Materials and methods. Among the identified peptides, in Table 2 we reported some peptides already known in literature as having bioactivity and others deriving from the cleavage of the fragment α_{s1}-CN *f*(1-23) and β-CN with none detected bioactivity yet.

Two samples, that showed the highest ACEI activity after digestion and in both permeates, were characterized by mass spectrometry, in order to study the resistance of ACE-inhibitors to gastrointestinal enzymes and to investigate the potential generation of new bioactive peptides after GD. By comparison with the chromatograms of the same samples not digested, the digestion increased the amount of amino acid residues and the loss of some biopeptides was observed. For example, the fragments α_{s1}-CN *f*(1-23), *f*(1-36), *f*(24-32), β-CN *f*(1-6) and *f*(59-96), abundant before digestion, were present in minimal concentration after digestion,

while other peptides such as α_{s1}-CN *f*(1-13), *f*(2-14), *f*(24-30) and β-CN *f*(193-209) were still found in higher amount.

Table 2 Brief list of water-soluble peptides isolated in the 10 kDa and 3 kDa WSEs of Asiago d'allevo cheese before and after gastrointestinal digestion with demonstrated or unknown bioactivity (peptides not found in digested samples are underlined)

MW [a] (Da)	Sequence	Amino acid sequence [b]	Bioactivity	Reference
489	α_{s1}-CN *f*(27-30)	PFPE	ACE-inhibition	Ong et al., 2007
755	β-CN *f*(47-52)	DKIHPF	ACE-inhibition	Gómez-Ruiz et al., 2006
805	α_{s1}-CN *f*(24-30)	FVAPFPE	ACE-inhibition	Gómez-Ruiz et al., 2002
855	β-CN *f*(7-14)	NVPGEIVE	ACE-inhibition	Gobbetti et al., 2000
787	β-CN *f*(1-6)	RELEEL	ACE-inhibition	Gómez-Ruiz et al., 2002
1052	α_{s1}-CN *f*(24-32)	FVAPFPEVF	ACE-inhibition	Ong et al., 2007
1881	β-CN *f*(193-209)	YQEPVLGPVR GPFPIIV	ACE-inhibition	Yamamoto et al., 1994
631	β-CN *f*(2-6)	ELEEL	unknown	-
1509	α_{s1}-CN *f*(2-14)	PKHPIKHQGLPQE	unknown	-
1536	α_{s1}-CN *f*(1-13)	RPKHPIKHQGLPQ	unknown	-
1665	α_{s1}-CN *f*(1-14)	RPKHPIKHQGLPQE	unknown	-

[a] MW: molecular weight; [b] The one-letter amino acid codes was used

Discussion

In this work aimed at investigating the possible ACEI effect of WSEs of Asiago cheese, we first explored the effects due to cheese productive systems and ripening times, considering also two different molecular cut-off permeates. No inhibitory effect due to the cheese production system was observed, indicating that the inhibitory peptides are generated by the same mechanisms with both technologies. Probably the combined effect of lactic acid bacteria (starter) proteases and the action

of chymosin with the mild heating (45 °C) treatment of milk allowed the intense proteolysis of caseins into bioactive peptides in all cheeses (Rizzello et al., 2005). In fact, as suggested by Gobbetti et al. (2004), the type of lactic acid bacteria starter used is one of the main factors that influence the synthesis of hypotensive peptides in dairy products.

Samples aged 6-months showed higher ACEI activity than 12 and 18 months-aged ones. These results would suggest that ACEI peptides are formed in Asiago d'allevo cheese during the first steps of ageing and are active for a limited period before splitting into other (inactive) peptides and amino acids as ripening proceeds. Although the peptide content increased during the ripening, the ACE inhibition did not follow the trend of proteolysis index, likely indicating that small peptides generated by starter culture could be responsible for the observed effects. These findings are partly consistent with those found by Meisel et al. (1997), who observed higher ACEI activities in middle-aged Gouda cheese than in short-termed or long-termed ripened cheese. In fact, the ACEI activity of cheeses increased as proteolysis progressed, however, the bioactivity decreased when proteolysis exceeded at a certain level (free/peptide-bound amino acids >0.5). In our study, ACEI peptides were not apparently released anymore during the late stages of ripening (12 and 18 months) and/or could be further hydrolysed into inactive fragments. As observed in Manchego cheese (Gómez-Ruiz et al., 2006), also Asiago d'allevo cheese seems to be prone to a dynamic system where peptides are being constantly released, some of them are subsequently hydrolysed and other accumulate over the ripening process. Thus the presence of active peptides that are naturally formed in cheese depends on a complex equilibrium between their formation and their degradation by the proteolytic systems involved in cheese ripening. Proteolysis caused a progressive release of polypeptides and potential biological peptides, encrypted within the primary structure in cheese, but these biopeptides may further be broken by microbial fermentation or enzymatic digestion and lose their biological functions as proteolysis progressed (Meisel, 1997).

With regard to the molecular weight, consistently higher inhibitory activity was found in the 3 kDa permeates than in the 10 kDa permeates. This reveals that small peptides made a considerable contribution to the ACEI activity of the Asiago WSEs as suggested by literature (Bütikofer et al., 2008; Gómez-Ruiz et al., 2002; Quirós et al., 2007).

Many peptides have been identified through the HPLC/ESI-MS analysis in Asiago d'allevo cheeses and most of them derived from the N-terminal region of α_{s1}-CN *f*(1-23) and from β-CN. Bovine α_{s1}-CN can be rapidly hydrolysed by chymosin at the Phe_{23}-Phe_{24} to yield α_{s1}-CN *f*(1-23). During ripening, this fragments may be hydrolysed rapidly by bacterial enzymes to several small peptides resulting in the formation and accumulation of fragments α_{s1}-CN *f*(1-9) and *f*(1-13) that were found in Asiago d'allevo cheese. Regarding β-CN fragments, a progressive degradation both of N-terminal and C-terminal ends of β-CN was observed.

In this study, 6 peptides (below 3 kDa) were identified which had been previously described as ACE-inhibitors (Table 2). They derive from α_{s1}-CN and β-CN, they have a marked hydrophobic character and low molecular weight. Specifically, they are rich in the amino acid proline (Pro), which contributes to the correct location of the peptide in the active site of the ACE, probably due to the rigid structure of this residue. In addition, the Pro at the C-terminus generally contributes to enhance the ACEI activity (Saito et al., 2000). Some peptides derived from known lactic acid bacteria proteolytic action such as α_{s1}-CN *f*(1-13), *f*(1-14) and *f*(1-17). These peptides are produced from the chymosin-derived peptides α_{s1}-CN *f*(1-23) by starter bacteria.

ACEI peptide α_{s1}-CN *f*(24-30) has been described in Manchego cheese (Gómez-Ruiz et al., 2002) and it has been found also in Asiago cheese although the bovine sequence, FVAPFPE, differs from ovine sequence, VVAPFPE, for the residues in the N-terminal part. Also bovine β-CN *f*(1-6), RELEEL, shares several residues with the ovine ACEI fragment β-CN *f*(1-6), REQEEL.

Peptides that exhibit apparent in vitro ACEI activity can fail to show in vivo antihypertensive activity if these peptides are also substrates of gastrointestinal

enzymes (Vermeirssen et al., 2004). Therefore, in vitro simulated GD can be useful to detect the active form of the amino acid sequence. In this study, WSEs were subjected to a two-stage hydrolysis process that simulated physiological digestion in order to study the resistance of peptides to enzyme and to evaluate whether digestive enzymes generated peptides with higher ACEI activity. Only in the case of 10 kDa permeates, the ACEI activity in digested fractions tended to be higher than that observed in the undigested ones. Differently from the investigation conducted by Parrot et al (2003), our results seemed to suggest that GD of 10 kDa permeates may generate further short peptide sequences with rising ACEI activity. This finding was not observed in the 3 kDa permeates.

We noted that digested samples had some peptides in common with the undigested samples; these peptides were α_{s1}-CN *f*(1-13), β-CN *f*(7-14), *f*(47-52), and *f*(193-209). It means that these peptides may be partially protected from proteolytic breakdown. On the other side, some ACEI peptides were broken by digestive enzymes, in fact the fragments α_{s1}-CN *f*(24-32) and β-CN *f*(1-6) were no more found in the digested samples.

Conclusion

In conclusion, the presence of moderate ACEI activity for many of the considered cheeses was observed. This activity was essentially concentrated in the 6 months-old WSEs and imputable to compounds of low molecular mass (< 3 kDa), but it was not affected by different cheesemaking procedures. The simulated gastrointestinal digestion did not influence the ACEI, even if it seemed to increase inhibitory potency of the 10 kDa permeates. Several peptides known in the literature for having ACEI activity were identified in the WSEs, namely α_{s1}-CN *f*(24-30), α_{s1}-CN *f*(27-30), α_{s1}-CN *f*(24-32), β-CN *f*(7-14), β-CN *f*(47-52) and β-CN *f*(193-209). Of course, other peptides not yet reported in the literature as ACE inhibitory may be responsible for the observed activities. Moreover, the sequences of some peptides found in the WSEs

may suggest the presence of other biological activities in these samples. For instance α_{s1}-CN *f*(24-34) contains the ACEI peptide α_{s1}-CN *f*(24-30) and the peptide α_{s2}-CN *f*(182-207) contains the antimicrobial peptide α_{s2}-CN *f*(183-207).

It may be interesting to investigate the peptide fractions in order to identify the actual peptides responsible for the inhibitory potency. It may be also considered the use of other digestive enzymes to understand if they may generate different peptides with ACEI potency than that found in this study.

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CHAPTER 4

Preliminary evaluation of anti-listerial activity of water-soluble extracts in Asiago d'allevo cheese

ABSTRACT

Antimicrobial activity in nine water-soluble extracts (WSEs) obtained with a cut-off less than 10000 Da of Asiago d'allevo cheeses was investigated. WSEs were screened against *Listeria innocua* LRGIA 01 and *Listeria monocytogenes* AER 102 at varying concentrations. WSEs were obtained from cheeses having different times of ripening and two different cheese production systems (barn vs. alpine grazing). The WSEs were analyzed by RP-HPLC coupled to mass spectrometry detection in order to identify the peptides involved in antimicrobial activity. Many peptides deriving from α_{s1}-casein (CN) and β-CN were identified and some of them are known from the literature to have antibacterial activity such as the fragment α_{s1}-CN *f*(1-23), called isracidin, and α_{s2}-CN *f*(183-207). Most of the WSEs exhibited a bacteriostatic mode of action. The production systems did not affect the inhibitory activity, whereas 6-months-aged cheeses displayed higher inhibition against *L. innocua* than 12 or 18 ripened cheeses. a linear relationship with the peptide concentration was observed.

Introduction

Milk is an optimal source of nutrients and substances effective against infections (McCann et al., 2005). The antibacterial activity of milk is attributed mainly to immunoglobulins, but also the non-immune proteins, lactoferrin, lactoperoxidase and lysozyme exhibit antimicrobial activities (McCann et al., 2005). In addition, several antibacterial peptides encrypted within the sequence of milk proteins can be released by enzymatic proteolysis, for example during cheese ripening or milk processing (Gobbetti et al., 2004). According to literature data, antibacterial peptides can be originated from lactoferrin, α_{s1}-casein (CN), α_{s2}-CN, κ-CN, α-lactalbumin and β-lactoglobulin (López-Expósito and Recio, 2006). The positively charged peptide, corresponding to α_{s2}-CN *f*(150-188), showed inhibitory activity against gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus carnosus*) bacteria (Zucht et al., 1995). Isracidin, deriving from the N-terminal fragment of α_{s1}-CN, is generated by

chymosin and it is able to inhibit the in vitro growth of lactobacilli and other gram-positive bacteria, but only at relatively high concentration (0.1 to 1 mg mL^{-1}) (Lahov et al., 1996). Two reports (Liepke et al., 2001; Malkoski et al., 2001) have shown a direct bacterial growth-inhibition effect of κ-CN-derived peptides whose activity is unrelated to the presence of attached sugar residues in their amino acid sequences.

Many antibacterial peptides have α-helical structures, most of them are cationic and amphipatic, but there are also hydrophobic α-helical peptides which possess antibacterial activity (Epand & Vogel, 1999). In general, the mechanism of action of antimicrobial peptides is not well established. Some antimicrobial peptides have a net positive charge and high content of hydrophobic residues, which allows the interaction with bacterial membranes (Minervini et al., 2003). These peptides are able to kill the target bacteria forming a membrane pore able to destroy the proton gradient of the bacteria. Moreover, it has been demonstrated that some milk-derived antibacterial peptides can reach intracellular compartment and affect the microbial survival by inhibition of its wall and/or nucleic –acid synthesis or by activation of its autolytic enzyme system (Biziulevičius et al., 2005). Other peptides, lacking a net positive charge, can exhibit growth inhibition instead of antibacterial activity (López Expósito & Recio, 2006).

Compared to antibiotics, antimicrobial peptides are able to kill target cells rapidly and, having a broad range of activity, can exert their activity also toward some antibiotic-resistant pathogens in clinics (Gobbetti et al., 2004). Therefore, the presence of these peptides is fundamental because bacterial contamination influences milk safety and organoleptic quality, production and storage of cheese. In particular, outbreaks and sporadic cases of listeriosis have been associated with contamination of different food item and milk among them. *Listeria monocytogenes* is a pathogen of major concern for dairy industry and its survival in many kind of cheeses has been well documented (Nuñez et al., 1997; Rodríguez et al., 2005). Rizzello et al (2005) reported that Pecorino Romano, Canestrato Pugliese, Crescenza and Caprino del Piemonte, characterized by short or medium ripening periods, contained several

antimicrobial peptides able to inhibit the growth of *Listeria* spp. On the contrary, the same authors observed that Parmigiano-Reggiano, Fossa and Gorgonzola water-soluble extracts (WSEs) did not show any antibacterial peptides that justified with the prolonged ripening time that lead to an intense proteolysis.

Asiago d'allevo is a semi-hard cheese made with raw bovine milk and is a typical product of the Veneto region. It is matured for a period ranging from 6 to 18 (and sometimes more) months. Asiago has been designated within the framework of Protected Designation of Origin labelling (PDO, Regulation CEE 1107/1996). The present study considered Asiago d'allevo cheese manufactured with two different milk production systems, *a)* in late spring, cows were housed in a barn and fed a total mixed ration (TMR) and cheese was produced in a dairy plant, *b)* during the summer the cows were sent in alpine grazing and they received a concentrate as supplement and cheese was manufactured *in situ*.

The goal of this study was to evaluate the anti-listerial activity of WSEs of Asiago d'allevo cheeses that differed for the length of ripening and the production system. The main oligopeptides in these extracts were characterized by means of LC/ESI-MS technique.

Materials and methods

Cheesemaking procedure

The cheese samples used in this study were manufactured with raw partially skimmed milk and according to the disciplinary regulation (PDO) both in a dairy plant and in an alpine farmhouse (*in situ*). The morning milk was added to the evening milk, previously left at room temperature in order to settling a large amount of fat, and warmed in the vat at 36 ± 1 °C. The coagulation of milk was induced by the addition of starter bacteria and rennet. In the case of dairy plant, starter culture contained *Streptococcus thermophylus* strain meanwhile in the alpine farmhouse the starter was composed by a mixture of *Streptococcus thermophylus* and *Lactobacillus*

strains. After coagulation the curd was broken into small granules and the temperature raised to 47 ± 2 °C within 18-21 min. The curd was then extracted, placed in moulds and transferred onto a sloping table in order to improve the drainage of whey (3-5 h), facilitated by turning the samples several times. The wheels were then sent to the presalting area where the removal of whey was completed before starting with brining (24% salt concentration at 17 °C and natural humidity for two days). All cheeses were finally ripened in the same storage bay at 11 °C and 80-85% of relative humidity.

Cheese samples

The study considered nine samples of Asiago d'allevo cheese produced in a farm located in a mountain area (above 1000 m a.s.l.) the so-called *Altopiano dei Sette Comuni* (Veneto Region, Italy) that reared about 50 lactating Holstein-Friesian cows. Cheeses were manufactured according three periods of the year characterized by two feeding systems: *a)* in May (indoor period), the cows were kept in barn and fed a TMR based on hay and concentrate (maize and soybean); *b)* in July (outdoor period), the cows were carried in alpine site of the farm (1350 m a.s.l. as average) and fed on grazing pasture plus a supplement (maize, barley and soybean) at daily dose of around 4.5 kg of dry matter per cow and *c)* in September similar to *b)* as far as it concerns the rearing system condition. Cheese was made in a dairy plant during the indoor period (May) whereas during the alpine grazing period cheese was manufactured in *malga* that means a cluster of buildings located at high altitude and committed to milking and manufacturing the cheese *in situ*. In each of the three seasonal periods related to the cheese production system (May/barn, July/alpine grazing and September/alpine grazing), three samples were analyzed, respectively was ripened for 6, 12 or 18 months. Sliced samples were storied at -80 °C until the extraction of water-soluble peptides.

Preparation of water soluble extract (WSE)

WSEs were obtained following the procedure described by Kuchroo and Fox (1982) with some modifications. Twenty grams of cheese were resuspended in 40 mL of water and the pH was adjusted to 2.0 using 1 N HCl. The suspension was homogenized for 10 min at room temperature at 20500 rpm using an IKA Ultra-Turrax T25 (Werke GmbH and CO.KG, Germany). Internal standard (5 mL of Phe-Phe, 1mM in MilliQ water, Sigma-Aldrich, St. Louis, MO, USA) for the semiquantification of peptides was added. The homogenate was then heated to 40 °C and held under gentle stirring for 1 h. Centrifugation at 10,000 x *g* for 30 min at 4 °C formed a pellet of insoluble material on the bottom of the tube and a lipid solution in the upper part while the water phase was intermediate. The WSE was recovered and filtered through a glass fibre prefilter (Millipore, Ireland) and then through a 0.45 μm filter (Sartorius Biotech GmbH., Germany). Three extractions with ethyl-ether in order to completely discard the lipophylic component were then carried out. WSEs were ultrafiltered through a 10 kDa cut-off membrane in a stirred ultrafiltration cell module (Millipore, Bedford, MA, USA) and dialyzed for 48 h at 4 °C through a 100 Da cut-off cellulose ester membrane (Spectra/Por® Float-A-Lyzer®, Spectrum®, The Netherlands) to eliminate the salts. The pH of the recovered suspension was adjusted to 7.5 using 10 N NaOH. Finally the permeates were freeze-dried and kept at -20 °C until use. Before the antimicrobial assay, the WSEs were re-hydrated in broth heart infusion (BHI, Fluka, Switzerland) at final dilutions of 5-15-30 and 40 mg mL^{-1} and then filtered through a 0.20 μm cellulose membrane (Sartorius Biotech GmbH., Germany) to be sterilized. The salt concentration was measured by using a chloride analyzer (Sherwood 926, UK). Nisin (Sigma-Aldrich, St. Louis, MO, USA) was used to inhibit the growth of bacteria in the negative control solutions. Nisin stock solution was prepared dissolving the nisin powder in 0.02 M HCl (10000 U mL^{-1}). During the control assays, the final concentration of nisin in solution was 2400 U mL^{-1}

Bacterial strains

To investigate the antimicrobial activity of WSEs, *Listeria innocua* LRGIA 01 and *Listeria monocytogenes* AER 102 were used. *L. innocua* LRGIA 01 was isolated from an industrial plant, while *L. monocytogenes* AER 102 was purchased from Aerial collection (Illkirch, France). Before the investigation, the stock cultures of bacteria were maintained on brain heart infusion (BHI, BioChemika, Buchs, Switzerland) supplemented with 20% glycerol at -80 °C.

The day before the test, the strains were grown in 9 mL BHI for 8 h and then inoculated again in the same broth overnight. Cells in logarithmic phase were diluted by a factor 20 to obtained a final suspension containing approximately 10^8 colony forming units (cfu) mL^{-1}.

Antimicrobial activity assay

The antibacterial activities of the WSEs lyophilisates against the two bacterial strains considered were performed in sterile 96-well microtiter plates. WSEs lyophilisates were added at 4 concentrations (5-15-30 and 40 mg mL^{-1}) in BHI broth. In each well, 270 µL of BHI both with or without (control) resuspended WSE lyophilisate were mixed with 30 µL of bacterial inoculum. The bacterial inocula were prepared by diluting exponentially growing cells in BHI. *Listeria* spp. Cultures without added WSEs lyophilisates were used as positive controls. The negative control wells contained 30 µL of bacterial inoculum and 270 µL of nisin (at a final concentration of 2400 U mL^{-1}) in BHI broth. Growth was monitored hourly over 10-h period (and after 24 h) by measuring the optical density (OD) of the culture at 620 nm using a microplate reader (DTX800, Beckman Coulter, Orange County, CA, USA). The microtiter plates were incubated at 30 °C to allow the bacterial growth. When growth was inhibited by the WSEs lyophilisates, cells of the strains were recovered from microplates, inoculated in a fresh culture broth, and incubated at 30 °C for 24 h to allow the recovery of growth. In all the experiments, three replicates were prepared for each WSE lyophilisate concentration and the mean OD and standard deviation

(SD) were then calculated for each condition. Growth inhibitory activity was expressed as mean percentage of growth inhibition respect to the control without WSE lyophilisate.

HPLC/ESI-MS analysis

The WSEs were analysed by HPLC (Alliance Waters 2695 separation module, Waters, Milliford, MA) by using the Jupiter (Phenomenex, Torrance, CA, USA) C18 column, 250 x 4.6 mm. The injection volume was 10 μL. Gradient elution was carried out with a mixture of two solvents (solvent A: 0.2% acetonitrile and 0.1% formic acid in water, solvent B: 0.2% water and 0.1% formic acid in acetonitrile, v/v). Proteins and peptides were eluted as follows: 0-15 min, 100% A; 15-60 min, linear gradient from 100% A to 67 % A; 60-69 min, isocratic 67% A; 69-70 min, from 67% to 60% A; 70-80 min, from 60% to 0% A; 80-85 min, isocratic 0% A. The flow rate was 1 mL min^{-1}. The column was maintained at 30 °C. The column flow was splitted 10:1 before the ESI interface. Detection was performed by a single quadrupole electrospray ionisation mass spectrometer (ESI-MS) according to the following conditions: positive ions, single quadrupole analyzer. capillary voltage 3200 V, cone voltage 30 V, source temperature 100 °C, desolvation temperature 150 °C, cone gas (N_2) 100 L h^{-1}, desolvation gas (N_2) 400 L h^{-1}. Acquisition in total ion mode (100-1900 m z^{-1}), scan time 4 s. Data were acquired by the software MassLynx 4.0. By using a suitable software developed by Sforza et al. (2003), the most abundant peptide were identified according to their molecular weights and their significant fragment ions. For every single peptide, all the possible isobaric sequences contained in the caseins compatible with its molecular weight were first calculated. Among these possible peptides, the software then gave molecular mass of the fragments generated by the loss of the first N- or C-terminal amino acid, allowing to compare them with the fragments actually observed and thus leading to the peptide identification. All identified peptides were semiquantified by comparison with the internal standard Phe-Phe. The oligopeptides

were semiquantitated by measuring the ratio between the peptide area in the extract ion chromatogram (XIC) and the area of the Phe-Phe in its relative XIC.

Statistical analysis

The assumption of normality of residual plot was checked using the Shapiro-Wilks test (PROC UNIVARIATE). Growth inhibitory data were analyzed as repeated measures using a mixed procedure with a CS (compound symmetry) structure. The linear model for both bacterial strains was:

$$Y_{ijklm} = \mu + R_i + M_j + C_{ij} + P_k + T_l + \beta B_{ijk} + \beta A_{ijk} + \varepsilon_{ijklm} \quad (1)$$

where Y is the response for growth inhibition; μ is the overall mean; R_i is the ripening time effect (3 levels: 6, 12 and 18 months), M_j is the cheese production system effect (3 levels: May/barn; July/alpine-grazing; September/alpine-grazing,), C_{ij} is the random effect of each form of cheese nested within R and M, P_k is the WSE lyophilisate concentration effect in BHI broth (4 levels: 5, 15, 30 and 40 mg mL^{-1}), T_l is the time of incubation as repeated effect (4 levels: 7, 8, 9 and 10 h), β is the regression coefficient, B is the $\log_{10}$ cfu mL^{-1} of bacterial cells covariate; A is the residual salt concentration covariate and ε is the error. The degrees of freedom of R effect were used in two orthogonal contrasts: 6 vs. 12+18 and 12 vs. 18 months. When an effect was significant ($P < 0.05$), differences among levels or treatment means were determined using the PDIFF option along with Bonferroni adjust. Considering the fixed effects R_i, M_j, P_k and T_l, none interaction resulted statistically significant, thus they were not introduced in the model. All statistical analyses were carried out by using SAS package (2002).

Results

Antimicrobial activity of water-soluble extracts

The growth of bacterial strains was monitored for 10 h by measuring the optical density at 620 nm (Figure 1). The inhibition was defined as the reduction of the

growth rate of the *Listeria* spp in the Asiago WSEs in comparison to the control experiment (without any WSE).

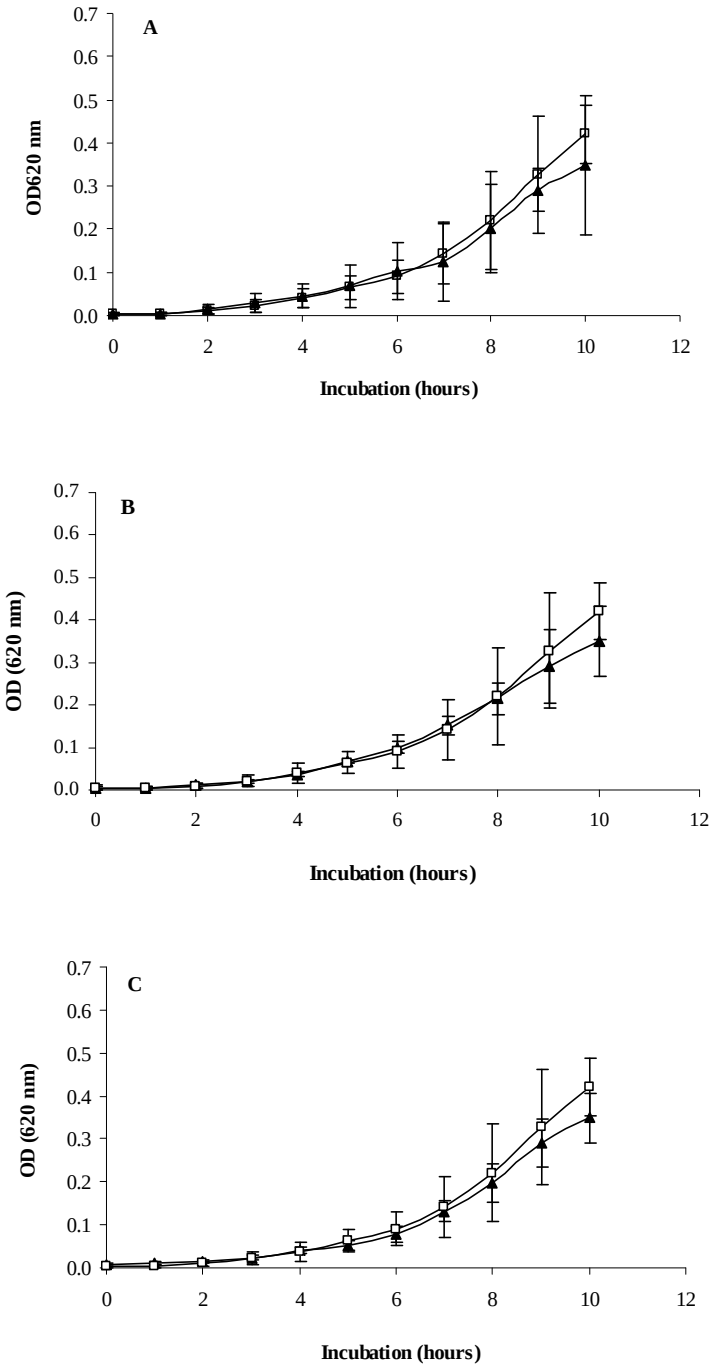

Figure 1 Effect of WSEs at 30 mg ml^{-1} and ripened for 6 (panel A), 12 (panel B) and 18 (panel C) months on the OD_{620} of *L. innocua* LRGIA 01 using the 96-well plate assay. □, *L. innocua* incubated alone; ▲, *L. innocua* incubated with WSEs. Samples were prepared in triplicates and data shown are the mean value (± SD) of the OD measurements

Regarding to *L. innocua* LRGIA01 assay, the significance of all fixed effects and the covariates considered in this study were reported in Table 1. Lyophilisates of 6-months aged cheeses displayed a slightly higher ($P < 0.05$) inhibition (26.4%) than 12- and 18-months-aged cheeses (20.1% and 18.1%, respectively) as shown in Figure 2. The different cheese production system did not affect the inhibitory response, whereas the WSE lyophilisates concentration in BHI broth significantly affected the growth inhibition ($P < 0.001$). In fact, the increasing in WSE lyophilisates concentration corresponded to an increase of inhibitory potency. The lowest WSEs lyophilisates concentration (5 mg mL^{-1}) was not active against *L. innocua* (i.e., below 3% of growth inhibition) while at 40 mg mL^{-1} of the WSEs the highest inhibitory potency (50%) was recorded (Figure 3a). The log_{10}cfu mL^{-1} covariates significantly ($P < 0.05$) affected the bacterial growth, while the effect of residual salt content of lyophilisates tended to be also significant ($P < 0.10$). As expected, the starting concentration of bacterial cells showed a negative (β=-5.2) relationships with the inhibition growth whereas the residual salt concentration (11±4.2 mg mL) was positively (β=0.6) correlated with the inhibition. The time of incubation did not affect the inhibitory activity of WSEs against *L. innocua.* Furthermore, for each experimental ripening period, a similar trend of inhibition along the hours was observed (Figure 4). Since the *L. innocua* growth occurred after 24 h (data not tabulated), it may be hypothesized a bacteriostatic mode of action of the WSEs peptides.

Table 1 *P*-values of the fixed effects and covariates on the growth inhibition of *L innocua* LRGIA 01 and *L. monocytogenes* AER 102

	Listeria innocua LRGIA 01		*Listeria monocytogenes* AER 102	
Fixed effect	*P*	β^a/*P*	*P*	β^a/*P*
Ripening time	0.11	-	0.43	-
Milk system	0.51	-	0.23	-
Peptide concentration	≤ 0.001	-	0.03	-
Time of incubation	0.27	-	0.12	
Log_{10}CFU mL^{-1} (covariate)	-	-5.2 / 0.02	-	0.08 / 2.7
Salt concentration (covariate)	-	0.6 /0.06	-	0.44 / 0.16

[a]β is the coefficient of regression of the covariates log_{10}CFU ml^{-1} and salt concentration; none interaction resulted statistically significant

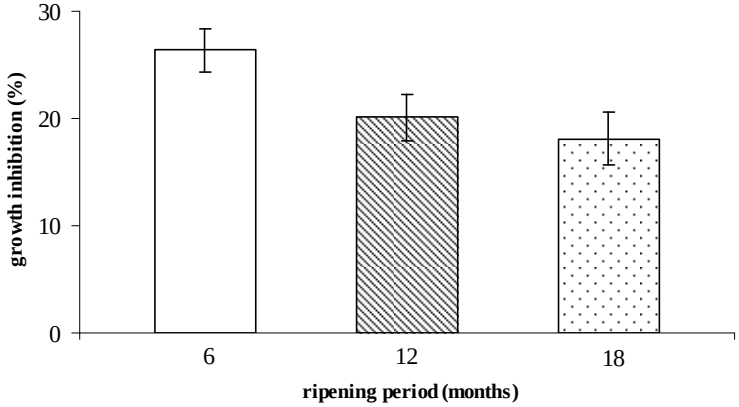

Figure 2 Percentage of growth inhibition of WSEs against *L. innocua* LRGIA 01 during the ripening period (orthogonal contrast: 6 vs. 12+18: $P < 0.05$; 12 vs. 18: P=0.56)

The statistical results for *L. monocytogenes* inhibition are presented in Table 1. The inhibition against this pathogenic strain was not affected by the effects considered in this work, except for the WSE lyophilisate concentration ($P < 0.05$). In opposition to *L. innocua*, it seems that a concentrations of WSEs lyophilisates in BHI broth above 15 mg mL^{-1} did not cause any significant improvement in the inhibitory activity (Figure 3b).

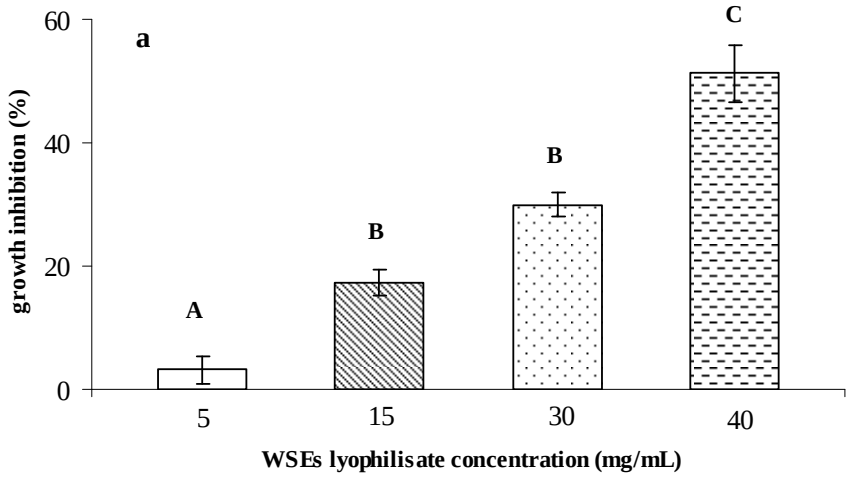

Figure 3a Relationship between peptide concentration, expressed as mg per mL, and percentage of growth inhibition (%) against *L. innocua* LRGIA 01. Data with different letter differ at P-value <0.01

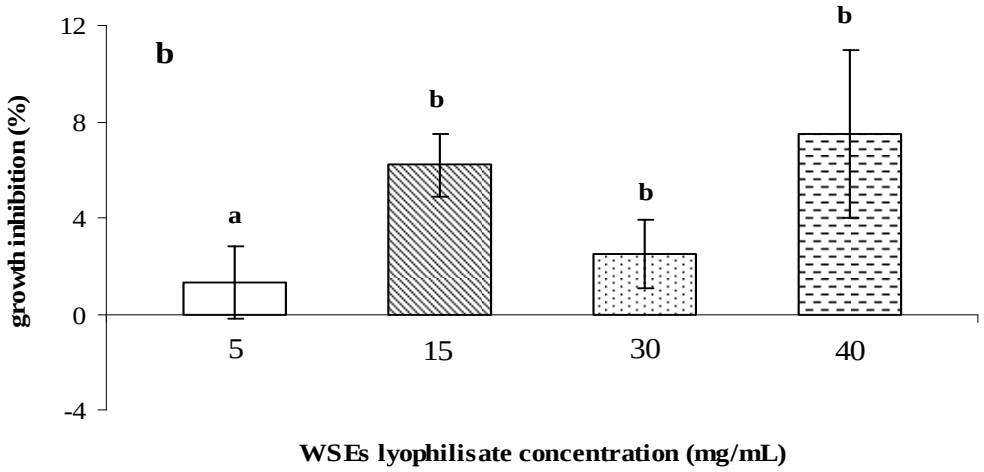

Figure 3b Relationship between peptide concentration, expressed as mg per mL, and percentage of growth inhibition (%) against *L. monocytogenes* AER 102. Data with different letter differ at *P*-value <0.05

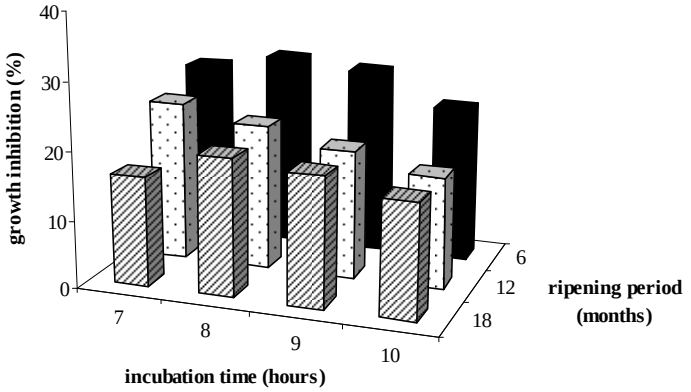

Figure 4 Percentage of inhibition during the hours of incubation (T) according to the ripening period (R) against *L. innocua* LRGIA 01 (*P*-value of the interaction between T and R was not statistically significant)

Identification of peptides

Using the HPLC/ESI-MS analysis, the main peptides present in WSEs of Asiago d'allevo cheese were identified. Among the identified peptides, some peptides already known in literature as having bioactivity and others deriving from the cleavage of the fragment α_{s1}-CN *f*(1-23) with none detected bioactivity yet are reported in Table 2. The known antimicrobial peptides observed in this work are α_{s1}-CN *f*(1-23), α_{s2}-CN *f*(183-207) and β-CN *f*(193-209). Their sequences contain basic amino acid residues such as lysine, histidine and arginine. Figure 5 shows the

average relative content of three antimicrobial peptides (peptides XIC area/Phe-Phe XIC area) in relation with the cheeses ripening duration. In the case of *L. innocua*, the concentration of these antimicrobial peptides did not explain the inhibitory activity related to the cheese ageing. For two antimicrobial peptides, β-CN *f*(193-209) and α_{s2}-CN *f*(183-207), the higher concentration was detected both in 12- and 18-months-aged cheeses.

Table 2 A brief list of water-soluble peptides isolated from 10 kDa WSEs of Asiago d'allevo cheeses with demonstrated bioactivity or derived from the antibacterial peptide α_{s1}-CN *f*(1-23)

MW[a] (Da)	Identification	Amino acid sequence[b]	Bioactivity	Reference
489	α_{s1}-CN *f*(27-30)	PFPE	ACE-inhibitory	Ong et al., 2007
1052	α_{s1}-CN f(24-32)	FVAPFPEVF	ACE-inhibitory	Ong et al., 2007
1881	β-CN *f*(193-209)	YQEPVLGPV RGPFPII	Immunomodulatory Antimicrobial, ACE-inhibitory	Yamamoto et al., 1994
2764	α_{s1}-CN *f*(1-23)	RPKHPIKHQGLP QEVLNENLLRF	Antimicrobial, immunomodulatory	Hill et al., 1974
3116	α_{s2}-CN *f*(183-207)	VYQHQKAMKPWI QPKTKV IPYVRYL	Antimicrobial	Recio & Visser, 1999
487	α_{s1}-CN *f*(18-21)	ENLL	unknown	-
1380	α_{s1}-CN *f*(2-13)	PKHPIKHQGLPQ	unknown	-

[a] MW: molecular weight; [b] The one-letter amino acid codes was used

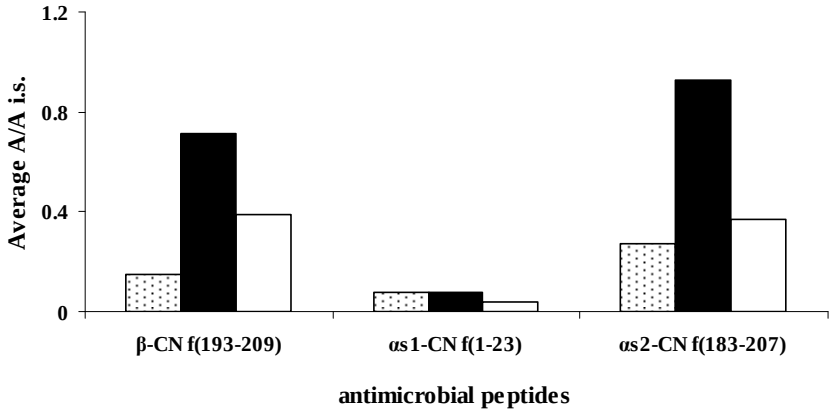

Figure 5 Average relative amounts of antimicrobial peptides (peptide XIC area/Phe-Phe XIC area) in cheeses ripened for 6 (▒), 12 (■) and 18 months (□)

Discussion

The water-soluble fractions (WSEs) of cheese is known to contain a wide amount of proteolytic products ranging from small proteins, to peptides and amino acids and nitrogen compounds derived from the previous ones, such as amines, urea and ammonia. This soluble fraction of cheese is often rich in peptides with functional properties such as antithrombotic, antihypertensive, immunostimulating and opioid (Korhonen & Pihlanto, 2006). As explained in the introduction, peptides derived from the milk lactoferrin and α_{s1}- and α_{s2}-CN have been shown to exhibit growth inhibition toward a wide range of gram-positive and gram-negative bacteria (Lahov et al., 1996; McCann et al., 2005; Zucht et al., 1995)

Differently form most of the data reported in the literature, in this experimental work the WSEs were not fractionated by liquid chromatography and complete WSEs were used. In this study, their effectiveness on *Listeria* spp. inhibition seems to be related to Asiago ripening period: 6-months-aged samples had slightly higher inhibitory potency than 12- and 18-months-aged cheeses. Even if in the absence of similar studies, these results may indicate that antibacterial peptides once liberated by proteolytic enzymes, after one year of ripening were degraded to inactive fragments due to further extensive proteolysis (Meisel, 1997). Actually, 18-months-aged Parmigiano Reggiano extracts did not contain fractions that showed antibacterial activity; on the other side, 6-months-aged Canestrato Pugliese displayed a moderate growth inhibition toward several bacterial strains (Rizzello et al., 2005). Moreover, cheeses characterized by a soluble nitrogen / total nitrogen (SN / TN) ratio ranging from 12 to 24% could contain noticeable amounts of bioactive peptides (Rizzello et al., 2005). Our samples ripened up to 6 months were characterized by the lowest SN/TN ratio (21.4%) as compared to 12 and 18 months-aged cheeses (26.1% and 27.6% respectively), which reflects a significant ($P < 0.05$) lower level of proteolysis in the shorter stage of ripening (unpublished data).

The different cheese production systems seemed not to influence the generation of antimicrobial peptides. Probably the combined effect of starter lactic acid bacteria

proteases and the action of chymosin combined with the mild heating (45 °C) treatment were the main responsible of the peptide system, while the contribution of autochthonous bacteria that characterized the environment of the specific cheese production system is less effective.

During the exposure of *L. innocua* to WSEs, a moderate inhibition was revealed up to 10 h of incubation, but, after 24 h, a marked bacteria growth was detected in all cases. Indeed, the growth of cells resuspended in fresh culture broth confirmed that WSEs lyophilisates were not able to kill the bacteria. Therefore, the slight bacteriostatic activity and the lack of bactericidal activity could be due to the complex mixture of the Asiago peptides extracted in this assay. The antimicrobial effect became more significant as the amount of peptide concentration increased. The 40 mg mL^{-1} concentration of WSEs had a greater efficacy than lowest concentration (5 mg mL^{-1}) against this bacterial strain. This result could suggest a linear dose-response curve, but the biochemical action of antimicrobial peptides of Asiago d'allevo cheese remains still unclear. Salt is generally considered a preserving and an antimicrobial agent (Bidlas & Lambert, 2008). The residual salt in WSEs was reduced through dialysis in order to reduce as possible its potential antibacterial effect. However, it is impossible to eliminate all sal therefore, the residual salt concentration may have a significant effect on inhibition and, as expected, an increasing salt content corresponded to a lower growth.

This preliminary study showed that WSEs moleculars did not affect the growth of *L. monocytogenes* because the highest value of inhibition was around 12% was not significant. Only the peptide concentration had a significanteffect on growth, highlighting that a low WSEs lyophilisates concentration was sufficient to inhibit bacteria growth, even if slightly. We may hypothesize the presence of a threshold already efficient at the middle concentration of 15-30 mg mL^{-1}. In the comparison with the results observed in *L. innocua*, the minor inhibition may suggest an uncertain bacteriostatic activity of peptides against *L. monocytogenes*.

All Asiago d'allevo samples contained the peptide α_{s1}-CN *f*(1-23), called isracidin, corresponding to the primary site of cleavage of α_{s1}-CN by chymosin during cheese ripening, it also known to inhibit the in vitro growth of lactobacilli and of a variety of gram-positive bacteria but only at relatively high concentrations (0.1 to 1 mg mL^{-1}, López-Expósito & Recio, 2006). During Asiago late ripening, further breakdown of isracidin into smaller fragments could be expected leading to a decrease of antibacterial activity. Probably enzymes of both starter and not starter bacteria could be the responsible of these cleavages.

Other antimicrobial peptides, such as cationic α_{s2}-CN *f*(183-207) and β-CN *f*(193-209) have been identified in Asiago WSEs. These positively charged peptides are believed to make contact with the negatively charged phospholipids bilayer and form channels into the microbial membrane (Rizzello et al., 2005). Although it has been recognized that positive charge is crucial to antibacterial activity, increased positive charge alone does not guarantee increased activity (Epand & Vogel, 1999). Positioning of positive charge within the sequence of peptides has been demonstrated to modulate activity, but also hydrophobic residues have an (indirect) effect on activities influencing structure or structural stability of bacterial membrane (Epand & Vogel, 1999).

As shown in Figure 5, two peptides having a known antimicrobial activity (Hill et al., 1974; Recio & Visser, 1999; Yamamoto et al., 1994) than the fragment α_{s1}-CN *f*(1-23) were found in higher concentration in 12-months-aged Asiago cheeses. This evidence is in apparent contrast with the significant effect of ripening time that displayed the highest inhibitory activity against *L. innocua* in the 6-months-aged cheeses. Thus we may suppose that other peptides found in Asiago WSEs could contribute to the inhibitory potency.

Conclusions

This study demonstrated that Asiago d'allevo cheese contained antimicrobial peptides which were able to moderately inhibit mainly *L. innocua* LRGIA 01 rather than *L. monocytogenes* AER 102. Moreover 6-months-aged cheese had higher *L. innocua* inhibitory potency than the other two aging period thesis and a linear relationship with the lyophilisates cncentration in culture broth was observed. The Asiago WSEs had no bactericidal action, they were able to slightly inhibit the bacteria growth for the first ten hours, but after 24 h this inhibition disappeared. The action of chymosin and lactic acid bacteria proteases rathen than the effect of cheese production system may be predominant for the generation of antimicrobial fragments during Asiago ripening. In this study, the entire WSEs lyophilisates were tested against bacterial strains, therefore, further investigations will be carried out in order to assess the antimicrobial activity of purified fractions achievable by RP-HPLC separation.

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GENERAL CONCLUSIONS

The potential health benefits of milk protein-derived peptides have been a subject of growing interest in the context of health promoting functional foods. In fact, health-conscious consumers have made functional food the leading trend in the food industry; the functional dairy products are the leader product sector because many milk proteins possess specific biological properties that make these components potential ingredient of health-promoting foods. These peptides are inactive within the sequence of the parent protein molecule and may be released by hydrolysis of proteolytic enzymes, by proteolytic starter and not starter cultures and by gastrointestinal digestion. Once liberated, these peptides may be potential modulators of various regulatory processes in the living system. At present, a variety of naturally formed bioactive peptides have been found in fermented dairy products, such as yoghurt, sour milk and cheese. There are already a few commercial dairy products supplemented with milk protein-derived bioactive peptides whose health benefits have been documented in clinical human studies.

Also in cheeses having a protected denomination of origin there is an interest in isolate these peptides and evaluate their bioactivity because their presence could increase the added value of cheeses. In this thesis, the choice fell upon oligopeptides because they have never been studied in Asiago cheese. Every chapter shows that also Asiago d'allevo cheese is a natural source of oligopeptides.

These oligopeptides are useful for discriminate cheeses according to milk production system, cheese making procedure and length of ripening. The principal component analysis is the better statistical solution to grouped cheese according to many variabiles related to the peptides identified in all samples under investigation. Several peptides originating from α_{s1}-casein and β-casein were identified and an increasing content was observed as ageing proceeds. The results of this research confirmed that multivariate statistical analysis based on oligopeptidic profiles was an objective and powerful approach for evaluating proteolysis in cheese and discriminate cheeses mainly according to ripening age. Peptide pattern also allowed to differentiate the samples on account of the use of starter. Moreover, the peptide

pattern was found different in relation to the cow feeding and the environmental condition effects but only considering the longer maturing period. Finally, oligopeptides may be considered useful molecular markers as a tool of authenticity of PDO Asiago in relation to both ageing and the use of starter, however the generation of oligopeptides is affected by several proteolytic proceccess which could reduce the efficacy of this methodology.

In this thesis, a potential angiotensin converting enzyme (ACE) inhibitory activity was investigated: WSE of Asiago cheeses ripened for 6, 12 and 18 months were considered. The presence of moderate ACE inhibitory activity in the 6 months-old WSEs and with low molecular mass (< 3 kDa) was observed. This latter evidence is in agreement with the results of several studies, which have found that the majority of peptides are of low molecular mass corresponding to the relatively short chain peptides. As ripening proceeded, the proteolysis led to a degradation of bioactive peptides and consequently the exceed proteolysis may determine a loss of antihypertensive peptides. The fragments α_{s1}-CN *f*(1-13), *f*(24-30), β-CN *f*(47-52) and *f*(193-209) having an antihypertensive activity have been identified and they may be responsible for the detected activity. The gastrointestinal condition used in this trial was not efficient to produce more antihypertensive peptides. Therefore the use of other gastrointestinal enzymes may be useful to highlight the generation of new peptides upon simulated digestion and to detect a resistance of these peptides against hydrolysis. Howerver, the physiological function of these peptide needs to be also established *in vivo* through trials with spontaneously hypertensive rats.

The last experimental contribution had been focused on the potential antimicrobial activity of WSEs lyophilisates. 6-months aged Asiago d'allevo cheeses contained antimicrobial peptides which were able to moderately inhibit mainly *L. innocua* LRGIA 01 rather than *L. monocytogenes* AER102. Moreover it was observed a a linear relationship between the inhibiton and the WSEs lyophilisate concentration. Using LC/MS-ESI technique, a few antibacterial peptides such as α_{S1}-CN *f*(1-23), α_{S2}-CN *f*(183-207) and β-CN *f*(193-209) have been identified. Unfortunately a weak inhibition

was detected against *L. monocytogenes* AER 102. It could be interesting to assess the inhibitory activity of our WSEs using other *L. monocytogenes* strains.

Moreover Asiago WSEs had no bactericidal action, in fact they were able to slightly inhibit the bacteria growth for the first 10 h, but after 24 h this inhibition disappeared. In this study, the effect of cheesemaking procedure conducted in farm and dairy plant was not significant. Therefore, the ripening effect was the main responsible for the generation of antimicrobial peptides; in this regard, the contribution of starter lactic bacteria enzymes was essential. However, in a further investigation (data not reported) a major inhibiton was detected in a farm which did not use the starter culture in Asiago cheesemaking procedure. This evidence may suggest that the non starter lactic acid bacteria, originating from environment and milk, probably contribute to release peptides with potential antibacterial activity.

Summing up this thesis, as regard Asiago d'allevo cheese it seems that the better ripening period may be inferior to one year, characterized by a medium index of proteolysis, both for biofunctional point of view and for the decreasing of the cost of storage.

Concluding, both producers and consumers are interested in the nutritional values of the dairy products. In particular, Asiago cheese is an important resource of oligopeptides that may exert a potential biological function. The LC/MS is an useful methodology to gain more information on the proteolytic process in cheese and to identify peptides produced throughout the ripening. The valorisation of Asiago produced in mountain area in this side will increase its high value also as a functional food.

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