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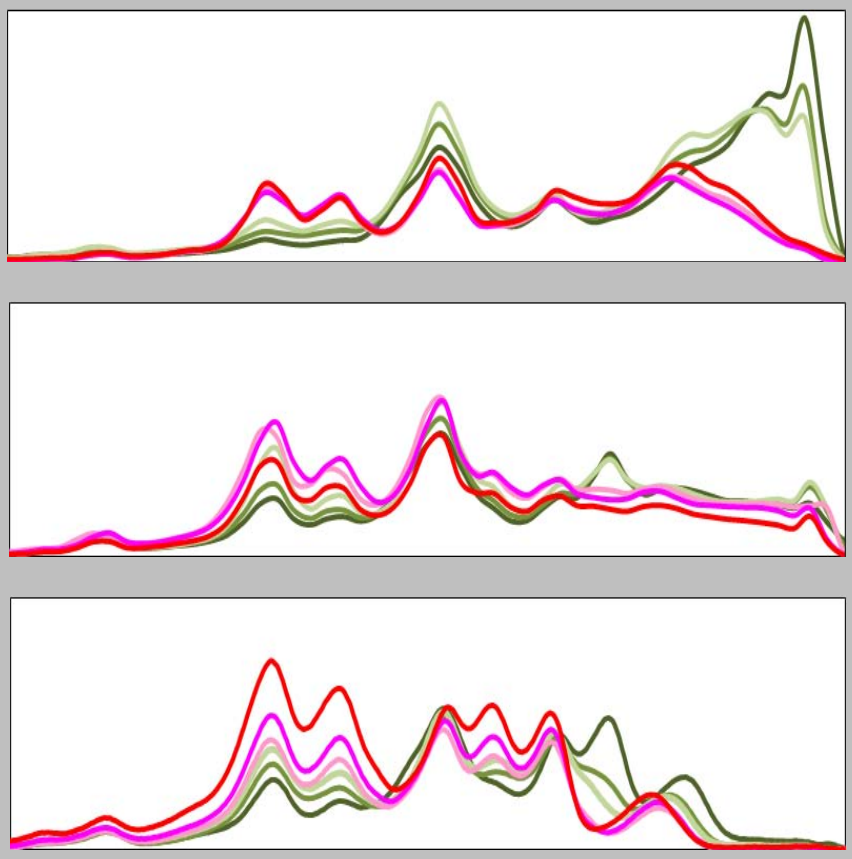

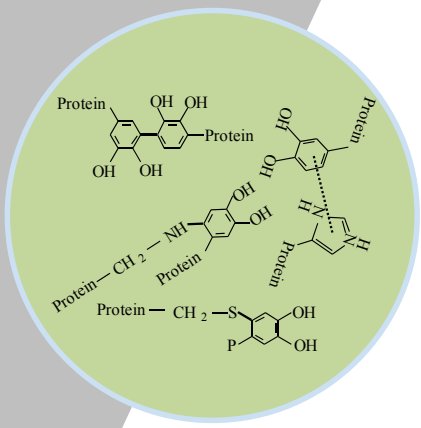

Evanthia Monogioudi

Enzymatic cross-linking of β-casein and its impact on digestibility and allergenicity

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Enzymatic cross-linking of β-casein and its impact on digestibility and allergenicity

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A dissertation for the degree of Doctor of Philosophy to be presented, by permission of the Faculty of Agriculture and Forestry, the University of Helsinki, for public examination and debate in Lecture hall 5 (3003) at the University of Helsinki, Main Building, Fabianinkatu 33, on the 28th of January 2011, at 12 o'clock noon.

ISBN 978-951-38-7421-6 (soft back ed.)
ISSN 1235-0621 (soft back ed.)

ISBN 978-951-38-7422-3 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)

JULKAISIJA – UTGIVARE – PUBLISHER

VTT, Vuorimiehentie 5, PL 1000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 4374

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Edita Prima Oy, Helsinki 2010

Evanthia Monogioudi. Enzymatic cross-linking of β-casein and its impact on digestibility and allergenicity [β-kaseiinin entsymaattinen ristisidonta ja sen merkitys proteiinin digestoituvuuteen ja allergeenisuuteen]. Espoo 2010. VTT Publications 752. 86 p. + app. 66 p.

Keywords tyrosinase, transglutaminase, protein, β-casein, cross-linking, digestibility, allergenicity, SEC-UV/Vis-MALLS, MALDI-TOF MS, 31P-NMR, AFM

Abstract

Protein modification *via* enzymatic cross-linking is an attractive way for altering food structure so as to create products with increased quality and nutritional value. These modifications are expected to affect not only the structure and physico-chemical properties of proteins but also their physiological characteristics, such as digestibility in the GI-tract and allergenicity. Protein cross-linking enzymes such as transglutaminases are currently commercially available, but also other types of cross-linking enzymes are being explored intensively.

In this study, enzymatic cross-linking of β-casein, the most abundant bovine milk protein, was studied. Enzymatic cross-linking reactions were performed by fungal *Trichoderma reesei* tyrosinase (TrTyr) and the performance of the enzyme was compared to that of transglutaminase from *Streptoverticillium mobaraense* (Tgase). Enzymatic cross-linking reactions were followed by different analytical techniques, such as size exclusion chromatography -Ultra violet/Visible – multi angle light scattering (SEC-UV/Vis-MALLS), phosphorus nuclear magnetic resonance spectroscopy (^{31}P-NMR), atomic force (AFM) and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). The research results showed that in both cases cross-linking of β-casein resulted in the formation of high molecular mass (MM ca. 1 350 kg mol^{-1}), disk-shaped nanoparticles when the highest enzyme dosage and longest incubation times were used. According to SEC-UV/Vis-MALLS data, commercial β-casein was cross-linked almost completely when TrTyr and Tgase were used as cross-linking enzymes. In the case of TrTyr, high degree of cross-linking was confirmed by ^{31}P-NMR where it was shown that 91% of the tyrosine side-chains were involved in the cross-linking.

The impact of enzymatic cross-linking of β-casein on *in vitro* digestibility by pepsin was followed by various analytical techniques. The research results

demonstrated that enzymatically cross-linked β-casein was stable under the acidic conditions present in the stomach. Furthermore, it was found that cross-linked β-casein was more resistant to pepsin digestion when compared to that of non modified β-casein. The effects of enzymatic cross-linking of β-casein on allergenicity were also studied by different biochemical test methods. On the basis of the research results, enzymatic cross-linking decreased allergenicity of native β-casein by 14% when cross-linked by TrTyr and by 6% after treatment by Tgase.

It can be concluded that in addition to the basic understanding of the reaction mechanism of TrTyr on protein matrix, the research results obtained in this study can have high impact on various applications like food, cosmetic, medical, textile and packing sectors.

Evanthia Monogioudi. Enzymatic cross-linking of β-casein and its impact on digestibility and allergenicity [β-kaseiinin entsymaattinen ristisidonta ja sen merkitys proteiinin digestoituvuuteen ja allergeenisuuteen]. Espoo 2010. VTT Publications 752. 86 s. + liitt. 66 s.

Avainsanat tyrosinase, transglutaminase, protein, β-casein, cross-linking, digestibility, allergenicity, SEC-UV/Vis-MALLS, MALDI-TOF MS, 31P-NMR, AFM

Tiivistelmä

Proteiinien muokkaus entsymaattisen ristisidonnan avulla on houkutteleva tapa muokata ruoan rakennetta tuotteen laadun ja ravitsemusarvon parantamiseksi. Ristisidonnasta johtuvien muutosten voidaan olettaa parantavan proteiinien rakenteen ja fysikaalis-kemiallisten ominaisuuksien lisäksi myös niiden fysiologisia ominaisuuksia, kuten sulavuutta ruoansulatuskanavassa ja allergeeniutta. Proteiinien ristisidontaan soveltuvia entsyymejä, kuten transglutaminaaseja, on tällä hetkellä kaupallisesti saatavilla, mutta myös muuntyyppisiä ristisidontaan kykeneviä entsyymejä tutkitaan parhaillaan runsaasti.

Tässä väitöskirjassa tutkittiin naudan yleisimmän maitoproteiinin β-kaseiinin entsymaattista ristisidontaa. Entsymaattiset ristisidontareaktiot toteutettiin *Trichoderma reesei* -homeen tyrosinaasilla (TrTyr) ja niitä verrattiin *Streptoverticillium mobaraense* -transglutaminaasin (Tgase) aiheuttamiin reaktioihin. Entsymaattisia ristisidontareaktioita tutkittiin eri analyysimenetelmillä: kokoekskluusiokromatografialla yhdistettynä ultravioletti/näkyvä monikulma-valonsironta ilmaisimeen (SEC-UV/Vis-MALLS), fosfori magneettiresonanssi-spektroskopialla (^{31}P-NMR), atomivoimamikroskopialla (AFM) sekä matriisi-avusteisella laser-desorptio-ionisaatio lentoaikamassaspektrometrialla (MALDI-TOF MS). Tutkimustulokset osoittivat, että kummankin entsyymin tapauksessa β-kaseiinin ristisidonta tuotti levyn muotoisia nanopartikkeleita, joiden molekyylipaino (MP) oli keskimäärin 1 350 kg mol^{-1}, suurta entsyymiannostusta ja pitkää reaktio-aikaa käytettäessä. SEC-UV/Vis-MALLS-tulosten perusteella kaupallinen β-kaseiini pystyttiin lähes kokonaisuudessaan ristisitomaan kummallakin entsyymillä. TrTyr-entsyymin tapauksessa ristisidonnan laajuus varmistui ^{31}P-NMR-analyysissä, jossa selvisi, että 91 % tyrosiini aminohappojen sivu-ketjuista osallistui ristisidosten muodostumiseen.

Proteiinien ristisidonnan vaikutusta ruoansulatukseen mallinnettiin *in vitro* seuraamalla β-kaseiinin pepsiinillä tapahtuvaa pilkkoutumista useilla analyysimenetelmillä. Tutkimustulokset osoittivat, että entsymaattisesti ristisidottu β-kaseiini on stabiili vatsalaukun happamissa olosuhteissa. Lisäksi havaittiin, että ristisidottua β-kaseiinia ei pystytä pilkkomaan pepsiinillä yhtä helposti kuin käsittelemätöntä β-kaseiinia. Entsymaattisen ristisidonnan vaikutusta β-kaseiinin allergeenisuuteen tutkittiin myös erilaisin biokemiallisin testimenetelmin. Tutkimustulosten perusteella entsymaattinen ristisidonta vähensi β-kaseiinin allergeenisuutta 14 %, kun se ristisidottiin TrTyr-entsyymillä ja 6 %, kun se käsiteltiin Tgase-entsyymillä.

Yhteenvetona voidaan todeta, että tutkimustuloksilla on merkitystä paitsi tyrosinaasientsyymin reaktiomekanismitutkimukselle, mutta myös elintarvike-, kosmetiikka-, lääke-, tekstiili- ja pakkausteollisuudelle.

Academic dissertation

Custos

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Preface

The work described in this thesis was carried out at VTT Technical Research Centre of Finland during the years 2007–2010. The research was conducted mainly with the financial support of Marie Curie mobility actions as part of the EU project "Enzymatic tailoring of polymer interactions in food matrix" (MEST-CT-2005-020924). The 4th year of the study was funded by the ABS graduate school (Viikki, Helsinki). In addition, the study presented in Publication II was partially supported by the Academy of Finland ("Enzymatic cross-linking of food proteins: impact of food protein folding on the mode of action of cross-linking enzymes") and Tekes ("Discovery and exploitation of novel lipid modifying enzymes in industrial processes"). COST action 928, titled: "Control and exploitation of enzymes for added-value food products", is acknowledged for the STSM grant for the collaborative study presented in Publication IV.

I would like to thank Prof. Johanna Buchert for giving me the opportunity to participate in this project and for the chance to learn and improvise during all these years. I am grateful to her for the discussions and comments not only while writing this thesis but throughout the study period. I am grateful to my supervisor Docent Maija-Liisa Mattinen for her excellent guidance and endless meetings. I thank her for being supportive and encouraging in good and bad times. I warmly thank all my co-authors for sharing their knowledge. Prof. Harry Gruppen and Dr. Nathalie Creusot are thanked for their help during my visit in the University of Wageningen (Netherlands). Prof. Tanja Cirkovic-Velickovic, Dr. Dragana Stanic and Dr. Natalija Polovic are acknowledged for an excellent collaboration during my participation in the COST 928 short mission (University of Belgrade, Serbia). Moreover, Prof. Tanja Cirkovic-Velickovic is greatly thanked for commenting on this thesis. Prof. Kaisa Poutanen is warmly acknowledged for giving me the opportunity to study the digestibility of proteins and to collaborate with the experienced group of IFR (Norwich, UK).

Prof. Dimitris Argyropoulos is warmly thanked for a most useful discussion on NMR and life in science. Docent Perttu Permi, Dr. Ilari Filpponen, Dr. Michael Lienemann, Dr. Bin Li, MSc Martina Lille and MSc Greta Faccio are warmly thanked for their comments and corrections on the manuscripts. Dr. Raija Lantto is warmly acknowledged for some last minute comments on some parts of the thesis and for being inspiring and motivating every time we met! The ladies in the "big" lab of B-house are thanked for being always smiling and willing to help me. Especially Päivi Matikainen is thanked for showing me the way in the lab and assisting me daily not only in the beginning of this study but whenever I needed it, no matter what the time or how busy she was…

My supervisors at the University of Helsinki, Prof. Marina Heinonen and Prof. Vieno Piironen are warmly acknowledged for helping me to cope with the bureaucratic aspects of this thesis and for taking care of all the issues related to that. Especially Prof. Marina Heinonen is thanked for her useful comments on the thesis and for all her help and support.

Prof. Loredana Mariniello and Dr. Jacob Holm Nielsen are warmly acknowledged for revising the thesis and for their useful comments and corrections.

I would like to thank the rest of the "Marie Curie kids" and office mates, Chiara, Dilek, Hairan, Greta and Jenni for their help and support during all these years. They have been around in easy and difficult times and we have shared our anxieties, fears but also laughter and gossips. I am most grateful. Special thanks go to Greta Faccio. In no language there are the right words to express my gratitude.

Michael Bailey is warmly thanked for his friendship and for making my text… comprehensible! Emilia Miettinen is warmly thanked for not only being a great friend but also for working during the small hours of her busy days on the Finnish version of the abstract! Jenni Rahikainen is specifically acknowledged for some last second corrections of the abstract (only hours before submission!). Prof. Alistair Paterson from Strathclyde University in Glasgow is thanked for being my mentor during my master thesis and for encouraging me to continue my studies in Finland. My friends in Greece, Finland and around the world are warmly thanked for supporting me and having faith in me even though many kilometres have been separating us. If it wasn't for my family (παιδιά και Φούλης), Alistair and Juuso I may had never started the whole journey. I warmly thank them all.

Espoo, December 2010

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp).

List of publications

I Monogioudi, E., Creusot, N., Gruppen, H., Kruus, K., Buchert, J. & Mattinen, M.-L. 2009. Cross-linking of β-casein by *Trichoderma reesei* tyrosinase and *Streptoverticillium mobaraense* transglutaminase followed by SEC–MALLS, *Food Hydrocolloids*, 23: 7, 2008–2015.

II Monogioudi, E., Permi, P., Filpponen, I., Lienemann, M., Li, B., Argyropoulos, D., Buchert, J. & Mattinen, M.-L. Protein Analysis by ^{31}P-NMR Spectroscopy in Ionic Liquid: Quantitative Determination of Enzymatically Created Cross-links, *Journal of Agricultural and Food Chemistry* (In Press).

III Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J. & Mattinen, M.-L. 2011. Effect of enzymatic cross-linking of β-casein on proteolysis by pepsin, *Food Hydrocolloids*, 25: 1, 71–81.

IV Stanic, D., Monogioudi, E., Ercili, D., Radosavljevic, J., Atanaskovic-Markovic, M., Vuckovic, O., Lantto, R., Mattinen, M., Buchert, J. & Cirkovic Velickovic, T. 2010. Digestibility and allergenicity assessment of enzymatically crosslinked β-casein, *Molecular Nutrition and Food Research*, 54, 1273–1248.

The author's contribution to the appended publications

I The author planned the work together with Docent Maija-Liisa Mattinen, carried out the experiments and was responsible for the data analysis. Part of the experimental work was performed at University of Wageningen in the Netherlands under the guidance of Prof. Harry Gruppen. The author wrote the paper under the guidance of Doc. Maija-Liisa Mattinen.

II The author planned the work together with Doc. Maija-Liisa Mattinen and carried out most of the experiments at VTT and at the Institute of Biotechnology under the guidance of Doc. Perttu Permi. The experiments concerning the ^{31}P-NMR for single amino acids, small organic molecules and short peptides were performed by Doc. Maija-Liisa Mattinen, Dr. Ilari Filpponen and Dr. Bin Li in North Carolina State University (USA) in collaboration with Prof. Dimitris S. Argyropoulos. The author participated in the interpretation of the data and wrote the paper under the guidance of Doc. Maija-Liisa Mattinen.

III The author planned the work together with Doc. Maija-Liisa Mattinen, carried out all the experiments and was responsible for the interpretation of the data. The author wrote the paper under the guidance of Doc. Maija-Liisa Mattinen.

IV The author carried out the experiments concerning the first part of the paper (cross-linking) in VTT. The experiments concerning the allergenicity studies were performed at the University of Belgrade (Serbia) as part of a STSM COST action 928 under the guidance of Prof. Tanja Cirkovic-Velickovic. The author participated in the writing of the paper under the guidance of Prof. Tanja Cirkovic-Velickovic and Doc. Maija-Liisa Mattinen.

List of symbols

ACN	Acetonitrile
AFM	Atomic force microscopy
[amim]Cl	1-allyl-3-methylimidazolium chloride
C	Cysteine
CBZ	N-carbobenzoxy-L-glutaminylglysine
CHCA	α-Cyano-4-hydroxycinnamic acid
$Cr[acac]_3$	Chromium-(III) acetylacetonate
DH	Degree of hydrolysis
DP	Degree of polymerisation
DTT	Dithiothreitol
E	Glutamic acid
ELISA	Enzyme-linked immunosorbent assay
ESI-FTICR	Electrospray ionisation-Fourier-transform-ion-cyclotron resonance
GI-tract	Gastrointestinal tract
G	Glycine
H	Histidine
IC_{50}	Maximal inhibitory concentration

K	Lysine
L-DOPA	L-Dihydroxyphenylalanine
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight
MALLS	Multi angle light scattering
MM	Molecular mass
MS/MS	Tandem mass spectrometry
P	Proline
^{31}P-NMR	Phosphorus nuclear magnetic resonance spectroscopy
PR [II]	2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane
Q	Glutamine
R_G	Radius of gyration
rms	Root mean square
S	Serine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
T	Threonine
TFA	Trifluroacetic acid
Tgase	*Streptoverticillium mobaraense* transglutaminase
TrTyr	*Trichoderma reesei* tyrosinase
UV/Vis	Ultraviolet/visible spectrophotometry
V	Valine
V_o	Void volume
Y	Tyrosine

1. Introduction

1.1 Protein cross-linking in food applications

Enzymatic cross-linking of proteins is an attractive method for the production of novel structures and functions for food [Buchert et al. 2007, Stangierski et al. 2008], cosmetic [Aimi et al. 2009] and medical [Ashman 2005] applications. The exploitation of cross-linking enzymes in food processing is advantageous over the more traditional chemical and physical methods that have been previously used, since enzymes require mild reaction conditions; they are highly specific and only very small amounts are needed for the reactions [Walsh 2007]. Enzymatic reactions are unlikely to produce toxic by-products [Singh 1991], which is crucial for food applications. Modification of proteins by the use of enzymes can be utilised in a vast range of processes [Creusot & Gruppen 2007, Ercili Cura et al. 2009, Faergemand et al. 1998, Lantto et al. 2006, Lantto et al. 2007a, Steffensen et al. 2008, and Mattinen et al. 2008a].

The commercial exploitation of cross-linking enzymes in food applications started with the discovery of transglutaminase from *Streptoverticillium mobaraense* as reviewed by Yokoyama et al. 2004. Transglutaminase has already been widely exploited in different food applications, such as dairy, fish, meat and baking. By contrast oxidative enzymes, such as tyrosinases, laccases, peroxidases and sulfhydryl oxidases have not yet been industrially exploited as cross-linking agents for food applications. Nevertheless, the research on these types of enzymes is very active and it is anticipated that new applications will be introduced in the future. The technological targets of enzymatic cross-linking in different food product types are summarised in Table 1.

Table 1. Summary of technological targets obtainable with enzyme-aided cross-linking.

Application	Improved technological target	Selected References
Dairy	- gel strength and elasticity of acidified products and cheese - viscosity - water-holding of acidified products - shelf-life - curd yield in cheese making - heat stability	[Yüksel & Erdem 2010, Gauche et al. 2009, De Sa & Bordignon-Luiz, 2010, Ercili-Cura et al. *In Press*, Hiller & Lorenzen 2009, Ozer et al. 2007]
Meat	- shelf-life - gel strength of processed products - ripening time of fermented products - restructuring of low-value cuts & trimmings	[Tseng et al. 2000, Dimitrakopoulou et al. 2005, Lantto et al. 2007a & b]
Baking	- loaf volume - shelf-life - dough handling - baking performance of low-quality gluten - frozen dough bread quality - frozen storage stability	[Gerrard et al. 2000 & 2001, Gerrard 2002, Poza 2002, Wu & Corke 2005, Moore et al. 2006, Selinheimo et al. 2007b, Labat et al. 2000, 2001, Faccio et al. *Submitted*]

1.2 Cross-linking enzymes

Proteins can be cross-linked by transglutaminases (EC 2.3.2.13), tyrosinases (EC 1.14.18.1), laccases (EC 1.10.3.2), peroxidases (EC 1.11.1.1) and sulfhydryl oxidases (EC 1.8.3.3). The reaction mechanisms of these cross-linking enzymes are described below.

1.2.1 Transglutaminases

Transglutaminases are cross-linking enzymes commonly used to modify proteins in food matrices. They belong to the group of acyl-transferases. In proteins, these enzymes form intra- and intermolecular cross-links *via* isopeptide bond formation between glutamine (Q) and lysine (K) side-chains [Folk 1970] as shown in Fig. 1.

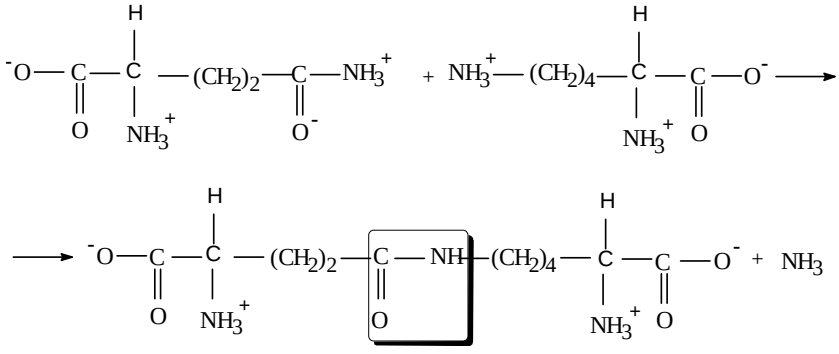


Figure 1. Transglutaminase- catalysed cross-linking between glutamine and lysine side-chains in proteins. The formed isopeptide bond is highlighted with a box.

The first isolated and characterised transglutaminase was the tissue transglutaminase XIIIa. Tissue transglutaminase requires calcium ions for its activity and thus it is mainly exploited nowadays in medical applications [Jones & Messersmith 2007, Hu & Messersmith 2003, Collier & Messersmith 2003]. Microbial transglutaminase from *Streptoverticillium mobaraense* was discovered in 1989 [Ando et al. 1989] and has been investigated in a wide range of food applications [Roos et al. 2003, Mariniello et al. 2007, Tang et al. 2006]. It has wide substrate specificity [Shimba et al. 2002]; it is independent of Ca^{2+} [Yokoyama et al. 2004] and it operates in the pH range of 5–8.

Amino incorporation is also possible *via* the primary amino group of various small organic molecules. The third reaction catalysed by transglutaminases is deamidation. This reaction occurs when amine substrates are not available as acyl acceptors and thus a water molecule is used as such [Folk 1970].

1.2.2 Tyrosinases

Tyrosinases are copper-containing metallo-proteins known for their multiple functions in nature. They play important roles in several biological processes as for example in the pigmentation of hair, skin and eyes [del Marmol & Beermann 1996]. Tyrosinases are responsible for the hydroxylation of monophenols and for the subsequent oxidation of diphenols to diquinones [Lerch 1983 and Robb 1984]. The tyrosinases hitherto isolated and characterised so far are presented in Table 2. The most extensively studied tyrosinase is of mammalian origin. Tyrosinase from *Agaricus bisporus* is commercially available and has been widely investigated in a range of applications [Seo et al. 2003, Selinheimo et al. 2009].

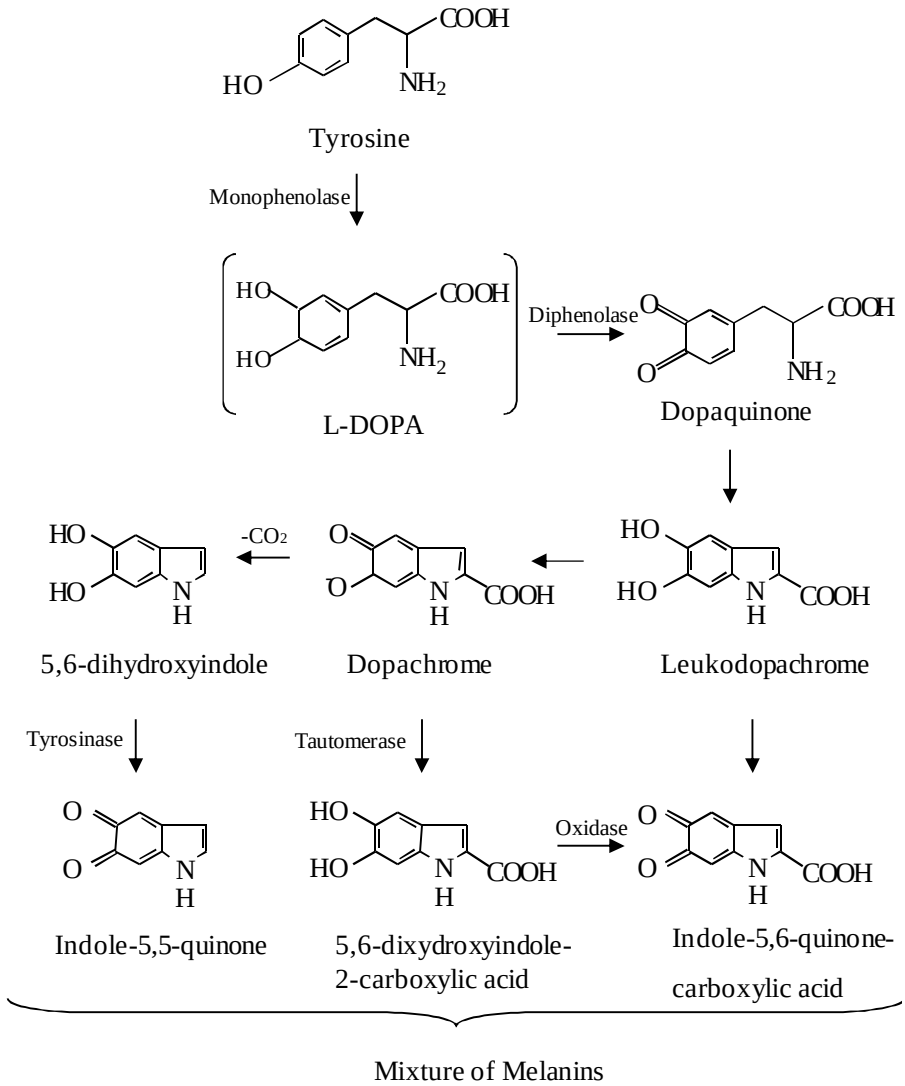
Table 2. Summary of isolated and characterised tyrosinases from different organisms.

		Optimum		
Origin	**Species**	**pH**	**Temp. (°C)**	**References**
Fungal	*Agaricus bisporus*	7	55	[Wichers et al. 2003, Marín-Zamora et al. 2006, Selinheimo et al. 2007a]
	Aspergillus nidulans	7	40	[Bull & Carter 1973, Birse & Clutterbuck 1990]
	Neurospora crassa	5	*	[Lerch 1983, Horowitz et al. 1970]
	Trichoderma reesei	9	30	[Selinheimo et al. 2006]
	Ascovaginospora	*	*	[Abdel-Raheem & Shearer 2002
	Pycnoporus sanguineus	7	60	[Halaouli et al. 2005, Selinheimo et al. 2007a]
	Marinomona smediterranea	*	*	[López-Serrano et al. 2002]
Bacterial	*Streptomyces REN-21*	7	35	[Ito & Oda 2000]
Mammalian	*Human tyrosinase*	7.5	50	[Kong et al. 2000]

* Not known

Generation of cross-links by tyrosinases is a potential alternative to transglutaminases for altering the properties of protein structures. The catalytic mechanism of tyrosinases and the path for the formation of a mixture of melanins are shown in Fig. 2A [Kim & Uyama, 2005].

A.


COOH
HO
NH2
Tyrosine
Monophenolase
HO
HO
COOH
NH2
L-DOPA
Diphenolase
O
O
COOH
NH2
Dopaquinone
HO
HO
N
H
COOH
Leukodopachrome
O
O
N
H
COOH
Dopachrome
-CO2
HO
HO
N
H
5,6-dihydroxyindole
Tyrosinase
O
O
N
H
Indole-5,5-quinone
Tautomerase
HO
HO
N
H
COOH
5,6-dixydroxyindole-
2-carboxylic acid
Oxidase
O
O
N
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COOH
Indole-5,6-quinone-
carboxylic acid
Mixture of Melanins

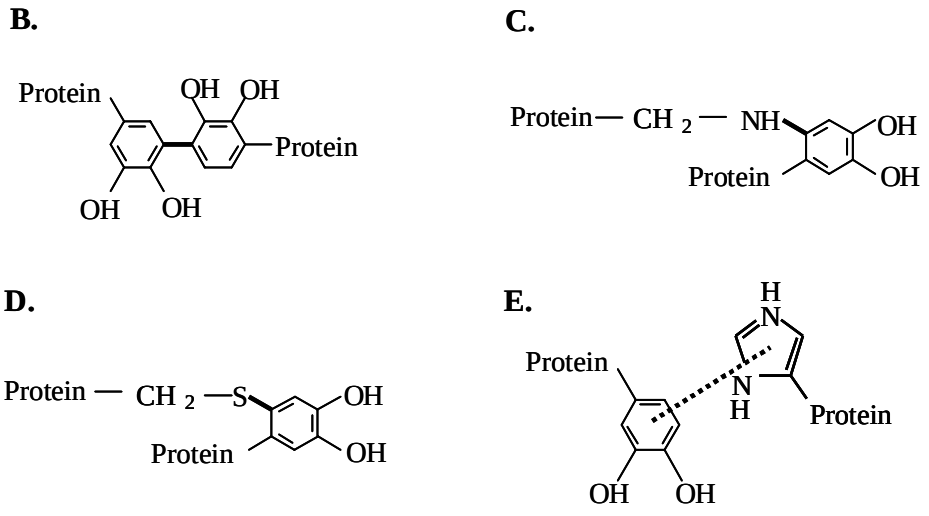

Figure 2. Oxidation of amino acid tyrosine by tyrosinase and possible cross-links formed in proteins. A. Reaction scheme for oxidation of mono- and diphenols and the pathway for the formation of a mixture of melanins. B. Cross-link between two tyrosine side-chains in protein. C. Cross-link between tyrosine side-chain and a primary amino group in protein. D. Cross-link between tyrosine and cysteine side-chains in protein. E. Cross-link between tyrosine and histidine side-chains. In B–D, the position of the cross-link is highlighted with a bold line. In the case of tyrosine-histidine cross-link the exact chemical structure of the cross-link has not been confirmed. However, it has been proposed to be formed between the ε-amine of the histidine side-chain and the aromatic ring of tyrosine side-chain.

According to Fig. 2A, tyrosinases catalyse the oxidation of the phenolic ring of a monophenol i.e. tyrosine to di-quinones *via* 3, 4-dihydroxy-L-phenylalanine (L-DOPA) intermediate. The formed di-quinones can act as precursors for the formation of a mixture of melanins in proteins [Sánchez-Ferrer et al. 1995, Rescigno et al. 2002]. They can also react further chemically with different amino acid side-chains, such as sulfhydryls, amines, amides, indoles as well as other tyrosine side-chains, leading to the formation of inter- and intramolecular cross-links as presented in Fig. 2B–E [Ito et al. 1984, Mattinen et al. 2008b, Burzio & Waite 2000, Hellman et al. *In Press*].

It has been shown that tyrosinase from the filamentous fungus *Trichoderma reesei* can polymerise short tyrosine peptides such as GYG and EGVYVHPV as well as α- and β-caseins [Mattinen et al. 2008b, Selinheimo et al. 2007a]. The polymerisation was proposed to occur *via* the formation of covalent chemical bonds between the aromatic side-chains of tyrosine residues and amino groups

of lysine, sulfhydryl groups of cysteine or other tyrosine side-chains [Ito et al. 1984, Bittner 2006, Burzio & Waite 2000]. Hellmann et al. [In press], recently showed, using $DRKSH_3$ model protein as tyrosinase substrate, that histidine residues may also participate in the cross-linking reactions with tyrosine side-chains in proteins [Hellman et al. *In Press*].

Not all the possible cross-links in proteins catalysed by tyrosinases have yet been fully characterised. The reactions depend on the accessibility of the target amino acids in the protein as well as on the reaction conditions, such as pH, temperature, buffer and phenolic modifiers. Depending on the reaction conditions, coloured products of varying shades from pink to black are typically formed [Ito et al. 1984, Bittner 2006, Sánchez-Ferrer et al. 1995, Raper 1928], indicating the formation of different types of chemical bonds. The colour formation may limit the use of tyrosinases in certain food applications.

1.2.3 Other cross-linking enzymes

In addition to tyrosinases and transglutaminases, proteins can be cross-linked by laccase, peroxidase and sulfhydryl oxidase. Laccases are multi-copper enzymes with broad substrate specificity. They catalyse the oxidation of phenolic compounds leading to the formation of free radicals, while the molecular oxygen participating in the reaction is reduced to water [Thurston 1994, Yaropolov et al. 1994]. The optimum pH and temperature of laccases vary considerably depending on their source [Kiiskinen et al. 2002, Martins et al. 2002]. Proteins are reported to be rather poor substrates for laccases [Lantto et al. 2005, Færgemand et al. 1998]. However, addition of small phenolic compounds such as ferulic and caffeic acid appears to facilitate the cross-linking process [Selinheimo et al. 2008, Ercili Cura et al. 2009, Mattinen et al. 2008a].

Peroxidases belong to the group of oxidoreductases that use H_2O_2 as an electron acceptor, and are able to modify peptides containing tyrosine [Malencik & Anderson 1996, Steffensen et al. 2008]. The optimum pH and temperature as well as the exact substrate specificity of these enzymes depend on their origin [Kawaoka et al. 2003]. The biological role of plant peroxidases in nature is related to the synthesis and degradation of the cell wall of lignin [Tai & Zhong 002], to the catabolism of auxin [Hinman & Lang 1965] as well as to the defensive responses to the treatment of wounds [Espelie et al. 1986].

Sulfhydryl oxidases are another type of enzymes catalyzing the formation of *de novo* disulfide bonds from free thiol groups present in small molecules such

as glutathione, to cysteine residues present in proteins [Thorpe et al. 2002, Thorpe & Coppock 2007]. The cross-linking ability of sulfhydryl oxidases has not hitherto been adequately described.

1.3 Milk proteins

Milk is a common source of proteins for children and adults. It contains two major groups of proteins i.e. caseins (ca. 80%) and whey proteins (ca. 14%) [Fox & McSweeney 1998]. Bovine milk consists of four caseins, α_{s1}, α_{s2}, β and κ. The degradation product of β-casein, γ-casein is sometimes also referred to as a milk protein [Wong et al. 1996]. Caseins are random coil proteins with no secondary or tertiary structure. Caseins precipitate at pH ~4.6 and below 6 °C they form aggregates that can be sedimented by centrifugation. They are very stable in heat treatment, a characteristic which has proved to be advantageous in the production of heat-sterilised milk products. Caseins have high proline content, which explains to a great extent the lack of well defined tertiary structure [Fox 2003]. Another common characteristic of caseins is that they are all phosphorylated, albeit to different degrees. The phosphate groups are esterified to serine residues [Hipp et al. 1952]. They are significant for the functional properties of caseins as they define their molecular charge and metal binding sites. Caseins have high surface hydrophobicity due to the lack of secondary and tertiary structure, meaning that their hydrophobic residues are completely exposed. However, caseins are not uniformly hydrophobic proteins but they also have hydrophilic sites, giving them an amphiphilic structure [Fox 2003]. This feature gives caseins high foaming and emulsifying abilities, rendering them a good choice for many applications [Dickinson 2003]. Additionally, caseins are susceptible to proteolytic enzymes.

β-casein is the most abundant milk protein [Swaisgood 2003]. It is a random coil protein with MM ~24 kg mol^{-1} depending on the genetic variant. It has been shown that β-casein is a monomeric protein at low temperatures with a Stokes radius of 3.7 nm and a radius of gyration of 4.6 nm [Schmidt & Payens 1972]. In a more recent study, it was reported that at temperatures below 15 °C in protein concentration of 1 mg ml^{-1}, β-casein is in a molecular state, with an average diameter of 7–8 nm. However, when the temperature is increased above 35 °C micellization of the protein occurs and the micelle diameter increases to 20–25 nm [Dauphas et al. 2005]. Micellization can be understood through the amphiphilic nature of β-casein [Nagarajan & Ruckenstein 1979]. The amino acid sequence of

β-casein consists of 209 amino acids, of which 4 are tyrosine (Y), 11 lysine (K), 20 glutamine (Q) and 5 histidine (H) residues as can be seen in Fig. 3, in which the full amino acid sequence of β-casein is shown. Thus, β-casein is an excellent target protein for enzymatic modifications by tyrosinases and transglutaminases.

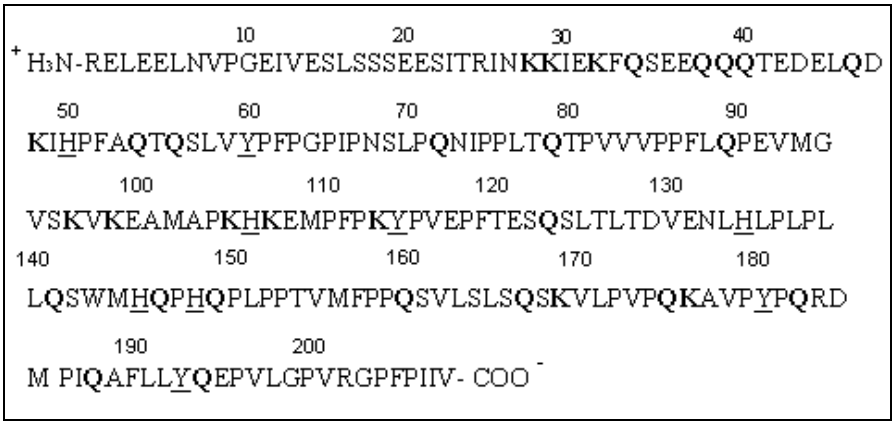

Figure 3. Amino acid sequence of β-casein. The reactive amino acid residues for tyrosinase are underlined and for transglutaminase bolded.

The other 20% of milk proteins are whey or serum proteins. They are remaining in the supernatant of the milk after precipitation at pH 4.6. Whey proteins are composed of β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins [Fox 2003]. Of these proteins the most abundant and important are β-lactoglobulin corresponding to ~50% of the whey proteins and ~12% of all milk proteins, as well as α-lactalbumin corresponding to about 20% of the whey proteins and 3.5% of total milk proteins. These globular proteins are more water soluble than caseins and are subject to heat denaturation. Bovine β-lactoglobulin is resistant to hydrolysis by proteolytic enzymes in the stomach and thus some of the protein may remain intact after digestion [Schmidt & van Markwijk 1993]. Heat denaturation of β-lactoglobulin at temperatures above 80 °C causes irreversible structural changes. These changes make β-lactoglobulin more prone to digestion by pepsin at the low pH of the stomach [Reddy et al. 1988, Kitabatake & Kinekawa 1998]. Native whey proteins have good emulsifying, gelling and whipping properties. These properties depend not only on their amino acid composition but also on the processes occurring during milk treatments as reviewed by Singh and Havea [Singh & Havea 2003].

1.4 Enzymatic cross-linking of milk proteins

The 3D structure of a substrate protein to be modified by cross-linking enzymes plays an important role in the success of the cross-linking reactions. In their native state, globular proteins, such as β-lactoglobulin, are usually unfavourable substrates for cross-linking enzymes [Mattinen et al. 2008b, Mattinen et al. 2006 and Sharma et al. 2001]. However, after denaturation of the protein, i.e. after opening the protein folding and reduction of disulphide bridges e.g. by chemical (acidic or alkaline) or by other physical treatments (high pressure, heat), the extent of cross-linking may increase due to exposure of the reactive amino acid side-chains [Færgemand & Qvist 1999, Eissa & Khan 2006, Eissa et al. 2004, Eissa & Khan 2005, Thalmann & Lötzbeyer 2002, Færgemand et al. 1998]. In the case of random coil proteins having no 3D structure, as for example in the case of β-casein, enzymatic cross-linking is possible. Færgemand and Qvist and Lorenzen and colleagues reported that caseins in general are good substrates for transglutaminase [Færgemand & Qvist 1999, Lorenzen et al. 1998]. Naturally, in addition to the structure of the protein, the number of target amino acid side-chains is a key factor affecting the extent of enzymatic cross-linking. In Table 3 some cases where β-casein has been used as a model protein for enzymatic cross-linking reactions are summarised.

Table 3. Summary of β-casein cross-linking studies.

Enzyme	**Result**	**References**
Streptoverticillium mobaraense transglutaminase	Extensively cross-linked	[O'Connell & de Kruif 2003, Hinz et al. 2007, Liu & Damodaran 1999]
Tissue transglutaminase	Large polymers	[Kaartinen et al. 1997]
Trichoderma reesei tyrosinase	Large polymers	[Mattinen et al. 2008b]
Agaricus bisporus tyrosinase	No cross-linking occurred	[Mattinen et al. 2008b]
Horseradish peroxidase	Mixture of low and high molecular mass polymers	[Boeriu et al. 2004]

1.5 Methods for analysing protein cross-linking

Enzymatic cross-linking of proteins has been studied by a number of analytical methods as shown in Table 4. The MM of the cross-linked proteins can be determined by different analytical techniques such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC) connected to a UV/Vis detector or UV/Vis-MALLS detector amongst others. In the case of Tgase cross-linking reactions, the rate of cross-linking can be monitored indirectly through the measurement of ammonia, which is released as a co-product [de Jong & Koppelman 2002, Tang et al. 2006]. It is often necessary to exploit more than one method to analyse the cross-linking reactions.

Table 4. Summary of methods used for analysing enzymatic protein cross-linking.

Parameters to be measured	Purpose	Limitations	References
SDS-PAGE	Separation and MM determination	Max MM: 200 kg mol^{-1}	[de Jong & Koppelman 2002]
SEC-UV/Vis	Separation and MM determination	Resolution limitations	[de Jong & Koppelman 2002]
SEC-MALLS	Separation and MM determination	Light scattering proportional to MM×Concentration	[Tang et al. 2008, Ye 2006]
HPLC	Separation and MM determination	Resolution limitations	[Macierzanka et al. *In Press*]
MALDI-TOF MS	MM determination	Max MM: 150 kg mol^{-1}	[de Jong & Koppelman 2002]
ESI-FTICR-MS/MS	Separation and MM determination	MM limitations	[Sinz 2006]

In the following sections, the basic principles, advantages and limitations of the techniques used in analysing the enzymatic cross-linking of proteins are described.

1.5.1 SDS-PAGE

SDS-PAGE is commonly used for separating proteins according to their electrophoretic mobility [Laemmli 1970]. Prior to analysis, proteins are treated with reducing agents, such as SDS, in order to open their three dimensional structure. SDS-PAGE has been used as a standard method to monitor enzymatic cross-linking [Mattinen et al. 2008 a & b, Ercili-Cura et al. 2009, Lantto et al.

2007a & b]. However, it has some limitations. SDS may not denature all proteins effectively; thus they may retain their shape and structure, affecting movement of the molecules through the gel. Binding of the SDS is affected by the charge, hydrophobicity as well as by post-translational modifications of the protein [Marshall & Inglis 1986]. The maximum separation limit of the gel is another limiting factor. Usually the upper limit of a gel does not exceed 200 kg mol^{-1}. Therefore, extensively cross-linked proteins cannot be monitored.

1.5.2 Size Exclusion Chromatography

MM of cross-linked proteins can also be monitored by SEC. In this method, analytes pass through a column packed with stable and inert porous spherical particles, known as *"stationary phase"*. The separation takes place according to the hydrodynamic volume of the molecules [Sun et al. 2004]. Large analyte molecules cannot penetrate this porous surface and elute first from the column, whereas smaller molecules pass through the pores and elute last. Molecules with a size below or above the column limits elute as one broad peak in the Void Volume (V_o). SEC can be used for defining the MM of proteins and peptides when the column has been calibrated with proteins of known molecular masses [Wen et al. 1996]. However, MM determined by this method may vary significantly from the theoretical mass [Xie et al. 2002] calculated on the basis of the amino acid sequence of the peptide or protein, as the elution of molecules in SEC depends on a number of parameters such as shape, hydrodynamic volume and interaction with the column matrix in addition to absolute molecular mass [Oliva et al. 2001]. When the molecular masses of the proteins in question are large and no known molecular mass standards are available, an external detector i.e. MALLS can be linked to determine the exact molecular masses.

1.5.3 Multi Angle Light Scattering

Light scattering methods such as MALLS offer accurate measurements of the MM without the need for external calibration [Fox & McSweeney 1998, Burdalo et al. 2000, Fredheim et al. 2002, Wang & Lucey 2003]. When studying very large polymers, such as cross-linked proteins, external calibration can be a serious problem due to the lack of appropriate protein standards as described above [White 1997]. Determination of the MM of small polymers having MM < 10 kg mol^{-1} is not very straightforward with MALLS, because small proteins

have poor light scattering properties [Carlshaf & Jönsson 1991]. The success of the method depends additionally on the accurate determination of the refractive index increment with molecular concentration (dn/dc) [Ye 2006]. The basic principle of the technique is shown in Fig. 4. The scattered light from the centre of a sensor cell is measured by a number of photocells, set at different fixed angles to the incident light. Generally, the more photocells the device contains the more data points can be collected, resulting in more accurate results.

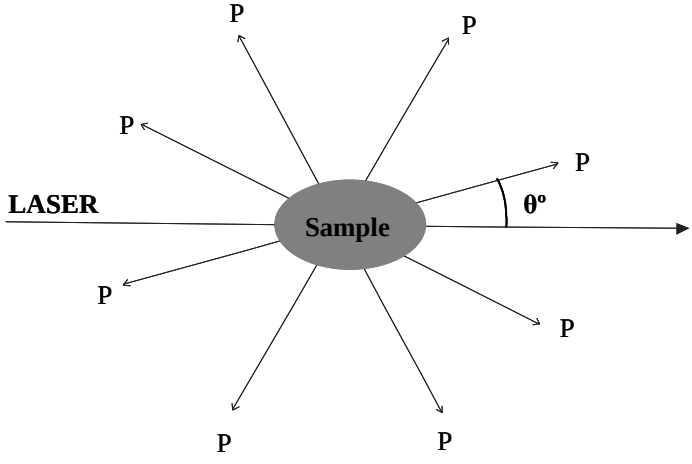

Figure 4. Graphic representation of the basic principle of a light scattering detector. The laser passes through the sample and is scattered at different angles (θ˚) which are detected by a number of photocells (P).

In MALLS the calculation of the average molecular mass of the molecule can be obtained from equation [Hoffmann et al. 1997] (1):

$$MM = \frac{R(\theta, c)}{P(\theta) \times \left[Kc - 2A_2 c \times R(\theta, c)\right]} \quad (1)$$

where,

MM = average molecular mass,

R (θ,c) = light scattered per unit,

c = concentration (g ml^{-1}),

$P(\theta)$ = function that describes the dependence of the scattered light on the angles,

A_2 = second virial coefficient in the virial expansion of the osmotic pressure and which can be neglected in cases of low analyte concentrations and

$$K = \frac{4\pi^2 n_o^2 (dn/dc)^2}{\lambda_o^2 N_A} \quad (2)$$

where,

n_o = refractive index of the solvent,

dn/dc = specific refractive index increment of the dissolved molecules at the same wavelength as that of the measurement,

λ_o = vacuum wavelength of the incident light and

N_A = Avogadro's number.

The MM range that can be monitored by a MALLS detector extends from 10^3 to 10^9 g mol^{-1} and the *rms* radii from 10 to 500 nm. The errors of the method are below 0.5% for the MM determination and below 3% for the radius of gyration (R_G) [Wyatt Technology Corporation 2005].

1.5.4 Matrix Assisted Laser Desorption / Ionisation – Time of Flight Mass Spectroscopy

MALDI-TOF MS is a technique that can be used to ionise short peptides and relatively small proteins. The upper mass limit of a TOF mass analyser is indefinite; however, the highest mass hitherto detected is a dimeric form of IgG, i.e. 300 kg mol^{-1} [Rutzinger et al. 2004]. In practice, the lowest mass limit is about 800 g mol^{-1}, due to the signals arising from the matrix used for the ionisation which interfere with the detection of small molecules. This can be overcome by the use of complementary techniques such as LDI (laser desorption/ionisation) without the use of a matrix [Hillenkamp & Peter-Katalinić 2007]. This soft-ionisation technique does not cause major fragmentation of the analytes and thus molecular ions of analytes can be detected even within mixtures [Liland et al. 2009]. The method is very sensitive and even attomoles

of analytes have been detected with the most advanced mass spectrometers [Hillenkamp & Peter-Katalinić 2007].

Sample preparation is a crucial step for the success of measurement by MALDI-TOF MS. Samples are mixed with a matrix having high laser light absorbing ability. α-Cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA) are typically used for peptides and proteins, respectively. Thereafter, they are applied onto a MALDI target and left to dry into a crystalline deposit. Due to the application of the laser, positive and negative ions will form. The exact ionisation mechanism is not completely understood, although it is known that the generated ions enter the mass analyser where they are separated according to their mass and charge. Thus, the different ions move towards the detector at different velocities. The velocity of each ion is inversely proportional to the square root of its mass-to-charge ratio (m/z).

After ionisation, every ion has a kinetic energy (E_k), proportional to its charge (z), its elementary charge (e) and the potential difference U applied between the sample support and the nearby electrode as shown in Fig. 5 and described by equation (3):

$$E_k = z \times e \times U \tag{3}$$

Ions having the same kinetic energy will have different velocities (v) which depend on their masses (m). Thus:

$$E_k = ½ \times m \times v^2 \text{ or } v = \sqrt{2E_k / m} = \sqrt{2zeU / m} \tag{4}$$

Hence, the arrival time for an ion to the detector can be calculated by the following equation:

$$t = L\sqrt{m / 2zeU} \tag{5}$$

where,

L = the length of the flight tube.

The mass to charge ratio for a specific ion depends on the length of the tube and the flying time accordingly:

$$m/z = 2eU\left(t/L\right)^2 \tag{6}$$

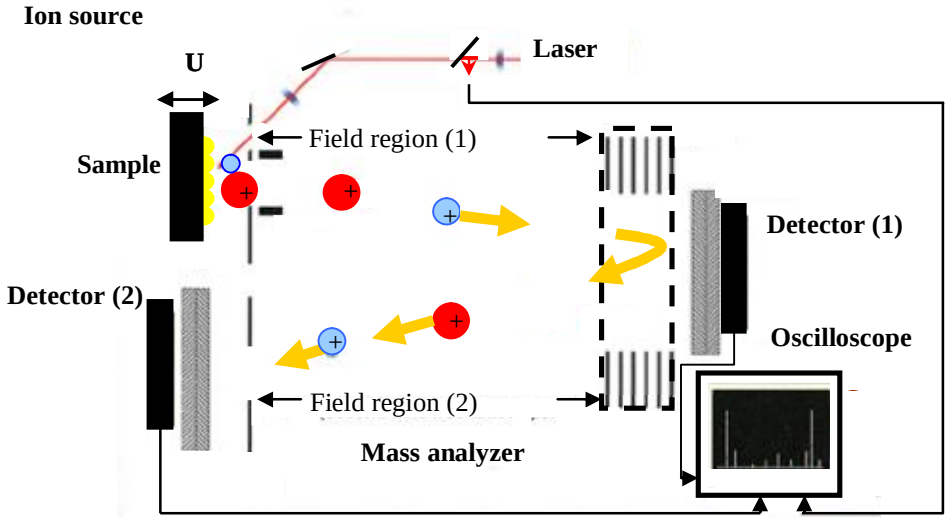

Figure 5. Basic principle of linear/reflectron mode MALDI-TOF mass spectrometer (Modified from [Hillenkamp & Peter-Katalinić 2007]).

Depending on the sample, e.g. peptides or proteins, the linear or reflector mode can be used. Linear mode is usually used for large proteins (MM > 10 kg mol^{-1}). In this mode, the ions travel along a linear path to the detector (1). The reflector mode is usually chosen when analysing peptides or small proteins (MM < 10 kg mol^{-1}). An ion mirror (deflector) is used to reflect ions having slightly different velocities to the detector (2), which improves the resolution of the spectrum [Hillenkamp & Peter-Katalinić 2007].

1.5.5 ^{31}Phosphorus-Nuclear Magnetic Resonance spectroscopy

NMR spectroscopy is one of the most important techniques for structural characterisation of proteins in solution. In addition, high resolution NMR has been used to study the kinetics of enzymatic reactions [Brindle 1988, Briggs et al. 1985, Seymour et al. 1983] and to characterise polymerised reaction products [Douglass 1980]. The most commonly used nuclei in NMR spectroscopy of biomolecules are ^{1}H, ^{13}C and ^{15}N having nuclear spin quantum numbers equal to ½. ^{31}P is also an attractive nucleus for NMR spectroscopy because various functional groups present in e.g. proteins may be modified by specific phosphorus chemicals.

The principle of NMR spectroscopy is based on the phenomenon that nuclei with non-zero spin quantum number have a magnetic dipole moment. Due to this

property, when an external magnetic field is applied to a molecule along the z-axis, nuclei with spin are quantised and orientated in parallel or anti-parallel to the applied magnetic field depending on the strength of the magnetic field applied [Cavanagh et al. 1996]. In the equilibrium an overall macroscopic magnetisation, i.e. "*net magnetisation*", is formed along the z-axis. When a radiofrequency pulse is applied to the sample, the equilibrium state of the net magnetisation is disturbed (Fig. 6A) and is turned from the z-axis, towards the x, y-plane (Fig. 6B). After that, the net magnetisation starts to precess around the z-axis returning to the equilibrium state. When the process is finished, the net magnetisation aligns again along the z-axis (Fig. 6C).

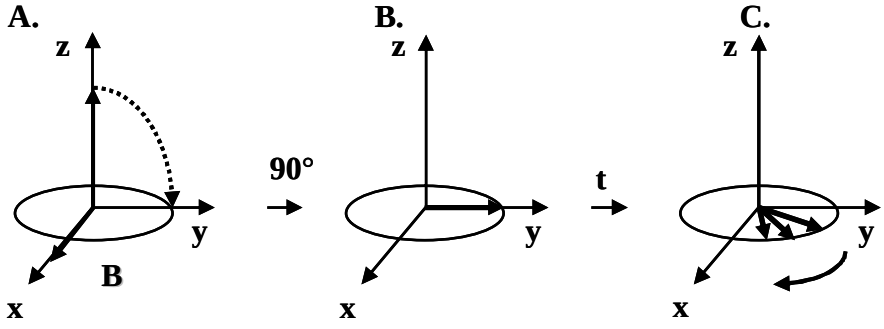

Figure 6. Application of external magnetic field which results in net magnetisation in the transversal x,y plane. After the pulse, the magnetisation returns to equilibrium.

The signal measured during the relaxation of the net magnetisation is called free induction decay (FID). The relaxation process can be divided into lattice-spin relaxation, which accounts for the return of the pulse signal to the equilibrium state, and spin-spin relaxation, which is responsible for the decay of the transverse magnetisation in the x, y plane. When the FID is Fourier transformed, the time-dependent magnetic signal is turned into a frequency-dependent signal i.e. a 1D NMR spectrum as shown in Fig. 7 [Cavanagh et al. 1996].

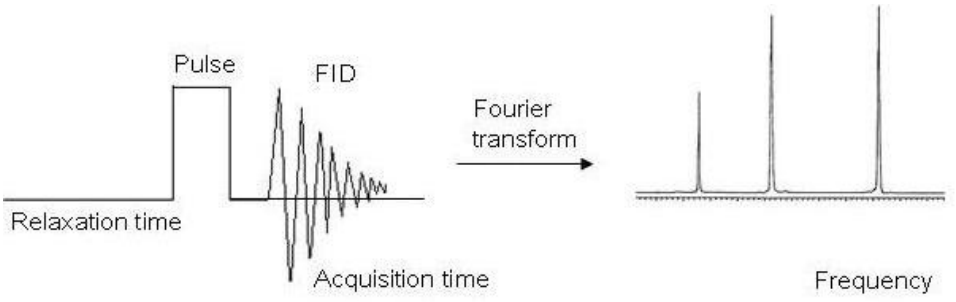

Figure 7. Schematic representation of a one-pulse NMR experiment. The FID recorded during the acquisition time (A) is transformed to the 1D NMR spectrum (B) *via* Fourier transformation.

The detected frequency (chemical shift) obtained for a specific nucleus depends on the chemical surrounding of the nucleus itself, thus enabling the discrimination signals between identical nuclei in the molecule having somewhat different chemical environments. The radio frequency pulse covers a wide range of chemical shifts, but in some cases e.g. when detecting ^{31}P nuclei the spin-lattice relaxation times may be rather long [Braun et al. 1990]. This problem may be overcome e.g. by the addition of an auxiliary relaxation agent such as chromium-(III) acetylacetonate ($Cr(acac)_3$) [Braun et al. 1990].

Protein NMR experiments are often performed in organic solvents [Serebryakova et al. 2009] or buffered water solutions [Guilloteau et al. 1992]. However, recently ionic liquids have been exploited for the solubilisation of various biomolecules [King et al. 2009, Mattinen et al. 2009, Granström et al. 2008, Swatloski et al. 2002, Junghans et al. 2006]. Ionic liquids are molten salts, consisting of positive and negative ions; they are characterised by weak interactions that depend on the cation and charge-delocalised anion that they consist of [Armand et al. 2009]. Due to their non-volatile, non-flammable and thermally stable nature they have been proposed as alternatives to organic solvents [Huddleston et al. 2001]. Ionic liquids have been widely used in analysing various wood components [Ren et al. 2003, Swatloski et al. 2002, Turner et al. 2004, Wu et al. 2004, Xie et al. 2007, Zhang et al. 2005, Granata & Argyropoulos 1995 and Mattinen et al. 2009], but they have also been exploited in the solubilisation of protein materials such as silk fibres [Gupta et al. 2007, Phillips et al. 2004 and Phillips et al. 2005]. Their use in protein applications has been limited to molecules having relatively small molecular masses [Fujita et al. 2006, Baker et al. 2004]. Much research has recently been done for improving

the properties of ionic liquids and extending their use in different applications [Armand et al. 2009]. The dissolution mechanism of different ionic liquids is not yet fully understood, but it is believed that they are responsible for the breaking down of hydrogen bonds between the polymeric chains in woods [Swatloski et al. 2002]. By contrast, in the case of proteins it has been reported that the 3D structures of proteins may be stabilised in ionic liquids [Fujita et al. 2006, Baker et al. 2004 and Fujita et al. 2005].

1.5.6 Atomic Force Microscopy

AFM is a scanning probe microscopy technique. In comparison to other microscopy techniques e.g. electron microscopy, the AFM samples must be firmly attached to a mica surface, but no surface coating or staining is required [Colton et al. 1998]. Originally the method was developed for imaging the topography of various surfaces; nowadays AFM is also used e.g. for measuring intra- and intermolecular interactions as well as for determining mechanical, chemical and biochemical properties of sample surfaces [Carl et al. 2001, Oberhauser et al. 1998, Rief et al. 1997].

In AFM technique, a tiny tip (~ 10 nm) is attached to the free end of a cantilever, which touches the surface of the sample during the measurement. This creates forces causing bending of the cantilever and subsequent deflection of a laser beam, measured by a photosensitive detector. In AFM both horizontal and vertical deflection of the cantilever can be measured with nanometre accuracy. In addition, AFM can be operated in different modes, the most common ones being contact and non-contact modes. However, in cases of soft samples, while the first mode would easily cause destruction of the surface, the second, even though non-destructive, would yield poor resolution. Thus, a combination of the positive properties of both modes would be most appropriate when soft samples such as proteins are to be analysed. This combination of the techniques could occur if the probe is oscillated in such a way that there is only intermittent contact between probe and sample during each oscillation period. This method is known as the “tapping method” [Colton et al. 1998].

1.6 Impact of enzymatic cross-linking on physiological properties of β-casein

Enzyme-aided protein cross-linking can also affect the physiological properties of food proteins.

1.6.1 Digestibility

Understanding the impact of protein cross-linking on protein digestion is very important as the rate of proteolysis and the subsequent amino acid absorption have a major effect on protein anabolism in the body [Boirie et al. 1997]. Digestibility of proteins is known to be altered through heating [Genovese & Lajolo 1996, Duodu et al. 2003] and/or through naturally occurring Maillard reactions [Friedman 1999, Fayle et al. 2000]. Enzymatic cross-linking affects protein structure and therefore it is assumed that it may also affect the breakdown of food products in the GI-tract by proteolytic enzymes.

During digestion, food components in the alimentary tract are broken down by numerous digestive enzymes. Digestion takes place in the oral cavity, the stomach, the duodenum and the jejunum [Kong & Singh 2008]. A number of proteases are responsible for the breaking down of protein material in humans. Amongst them, the most important ones include three endopeptidases, pepsin (EC 3.4.23.1), trypsin (EC 3.4.23.4) and chymotrypsin (EC 3.4.23.2). Proteins pass through the stomach, where they may remain for several hours depending on the food structure and composition. Pepsin-catalysed proteolysis occurs in the stomach. Afterwards, the cleaved proteins i.e. large peptides move to the upper small intestine (i.e. duodenum and jejenum), where they are further cleaved by pancreatic enzymes [Schmelzer et al. 2007]. In a final step, the polypeptides and oligopeptides appearing after pancreatic digestion are further broken down into amino acids in the intestinal lumen by other peptidases. A schematic representation of the process is shown in Fig. 8.

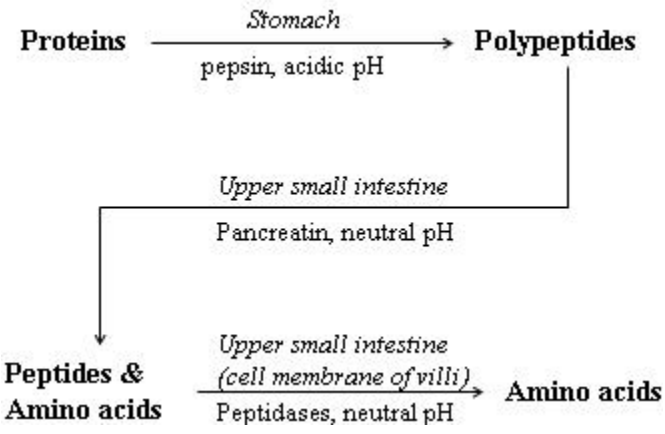

Figure 8. Schematic representation of protein digestion [Guyton & Hall, 1996].

The effect of protein cross-linking by microbial transglutaminase on *in vitro* digestibility has been studied with a variety of protein substrates such as soy [Tang et al. 2006] and sodium caseinate [Hiller & Lorenzen 2009]. Generally, cross-linking is reported to decrease digestibility. A summary of digestibility studies of enzymatically cross-linked proteins is shown in Table 5. The Tgase cross-linked casein has also been fed to rats. The differences in the food intake and overall health of rats were not significant when cross-linked casein was compared to that of native casein, indicating good digestion and retained nutritional value of the cross-linked protein. Mariniello et al. reported that cross-linking of phaseolin by microbial Tgase resulted in products which were resistant to both pepsin and trypsin. The resistance of cross-linked phaseolin to proteolysis could be applied in a wide range of applications from drug delivery systems to the production of phaseolin cultures resistant to insect attacks [Mariniello et al. 2007].

Table 5. Effect of enzymatic cross-linking on digestibility.

Cross-linking enzymes	Origin	Substrates	Impact	Reference
Laccase	*Myceliophthera thermophila*	Sodium Caseinate	Decreased	[Hiller, Lorenzen 2009]
	Myceliophthera thermophila	Whey protein isolate	Decreased	[Hiller, Lorenzen 2009]
	Trametes versicolor	β-lactoglobulin	Increased	[Stanic et al. 2009]
Lactoperoxidase	Bovine Milk	Whey protein isolate	Increased	[Hiller, Lorenzen 2009]
	Bovine Milk	Sodium Caseinate	No change	[Hiller, Lorenzen 2009]
Glucose oxidase	*Aspergillus niger*	Whey protein isolate	Increased	[Hiller, Lorenzen 2009]
	Aspergillus niger	Sodium Caseinate	Increased	[Hiller, Lorenzen 2009]
Transglutaminase	*Streptoverticillium mobaraense*	Phaseolin *(Phaseolus vulgaris)*	Decreased	[Mariniello et al. 2007]
	Streptoverticillium mobaraense	Soy protein isolate	Decreased	[Tang et al. 2006]
	Streptoverticillium mobaraense	Soybean proteins	Increased	[Volken de Souza et al. 2009]
	Streptoverticillium mobaraense	Vicilin – rich kidney protein	Increased	[Tang et al. 2008]
	Streptoverticillium mobaraense	Sodium Caseinate	Decreased	[Roos et al. 2003, Hiller & Lorenzen 2009]
	Streptoverticillium mobaraense	Whey protein isolate	Decreased	[Hiller & Lorenzen 2009]
	Streptoverticillium mobaraense	Chicken meat	Decreased	[Volken de Souza et al. 2009]

1.6.2 Allergenicity

Food allergies constitute a serious health concern. As much as 4–6% of children and 1–3% of adults in developed countries suffer from some sort of food allergy [Mills et al. 2007, Sicherer & Sampson 2006]. The foods and thus the proteins

incriminated in these allergies are many and their number is constantly increasing as novel food products are being produced. Milk, egg and peanut proteins are particularly responsible for allergic reactions caused in children and adults [Sicherer & Sampson 2006]. It is assumed that food allergens are very robust and that they can survive the harsh proteolytic conditions of the stomach [Mills et al. 2004]. Although there is much evidence to show that resistance to proteolysis is an indicator of increased allergenicity [Astwood et al. 1996, FAO/WHO 2001], recent studies have been contradictory [Fu 2002, Fu et al. 2002]. Hence, protein allergenicity appears to depend not only on the protein itself, but also on the processing [Mills & Mackie 2008], the presence of various surfactants such as phospholipids [Moreno et al. 2005], the gastric emptying rate and intestinal transit.

Cross-linking enzymes are also anticipated to have an impact on protein allergenicity. The allergenic impact when using microbial transglutaminase as a cross-linking enzyme has been extensively studied on a broad range of proteins, from soy [Babiker et al. 1998] to peanuts [Clare et al. 2008]. In most cases indications of reduced allergenicity have been observed *in vitro*, although opposite effects have also been reported (Table 6). For both soy and peanut proteins, allergenicity was decreased after cross-linking by transglutaminase [Babiker et al. 1998, Clare et al. 2008, Clare et al. 2007]. On the other hand, treatment of raw peanuts by peroxidase, even though it did not significantly affect cross-linking, caused a decrease in the levels of the major allergens [Chung et al. 2004].

Table 6. Summary of allergenicity studies of cross-linked proteins.

Cross-linking enzymes	Origin	Substrates	Impact	Reference
Transglutaminase				
(-/+ DTT)	*Streptoverticillium mobaraense*	Peanut proteins	Retained	[Clare et al. 2007]
(+ cysteine)	*Streptoverticillium mobaraense*	β-lactoglobulin	Decreased	[Villas-Boas et al. 2010]
	Streptoverticillium mobaraense	Kefir	Decreased	[Wróblewska et al. 2009]
	Streptoverticillium mobaraense	Soy	Decreased	[Babiker et al. 1998]
	Streptoverticillium mobaraense	Soft & hard flour	Decreased	[Watanabe et al. 1994]
	Tissue	Peptic fraction of ω-5 gliadin	Increased	[Palosuo et al. 2003]
Polyphenol oxidase (-/+ caffeic acid)	Mushroom	Peanut allergens	Decreased	[Chung et al. 2004]
Peroxidase	Horseradish	Roasted peanut allergens	Decreased	[Chung et al. 2005]

The effect of enzymatic cross-linking on the allergenic properties of different food proteins is an interesting research field due to its importance for human health and well-being worldwide. It is evident that enzymatic cross-linking may significantly affect the allergenicity of protein-based food products [Chung et al. 2004, Palosuo et al. 2003].

1.7 Techniques for analysing protein allergenicity

1.7.1 Immunoblotting

Immunoblotting, also known as Western Blot is an analytical technique for detecting proteins which can bind to specific antibodies [Towbin et al. 1979]. In immunoblotting, antibodies are used for specific recognition of a chosen antigen from a solution containing various proteins and other components. The method relies on separation of the chosen antigen on the basis of its molecular mass and

antibody-antigen recognition specificity [Towbin et al. 1979]. In the first step of immunoblotting, the gel electrophoresis for separation of proteins is similar to that explained in Section 1.5.1. After separation, the proteins are transferred to a membrane, such as nitrocellulose, and they are probed by antibodies specific to the protein in question [Towbin et al. 1979, Renart et al. 1979].

1.7.2 CAP inhibition of specific IgE binding

Development of allergy symptoms is a result of complex interactions between allergens, antibodies and parts of the human immune system [Prescott et al. 2001]. Through sensitisation to an allergen, specific IgE antibodies appear in human plasma or serum. Presence IgE antibodies can be detected by following their concentration in the serum or plasma [Sasai et al. 1996, Yunginger et al. 2000]. The measurement is commonly performed using an *in vitro* test. The allergenic protein to be tested is immobilised covalently onto cellulose solid phase such as ImmunoCap^{TM}, where it can interact with the IgE present in the serum or plasma taken from a patient [Johansson & Yman 1998]. Non-specific IgE is washed away and enzyme-labelled antibodies against IgE are added to the sample. After incubation, any unbound enzyme anti-IgE is washed away and a developing agent is added to the serum or plasma. The concentration of the IgE antibodies is analysed by fluorescence measurement [Fontaine et al. 2007]. A high concentration indicates sensitization to the allergen being tested. In addition to the identification and measurement of allergens, the method can be exploited to predict the risk of developing an allergy in the future [Sasai et al. 1996].

1.7.3 ELISA inhibition of specific IgE binding

Enzyme-linked immunosorbent assay (ELISA) is a method used to detect the presence of an antibody or an antigen in a sample [Engvall & Perlmann 1971]. There are different types of ELISAs, such as direct or indirect, competitive or non-competitive. The inhibition or competitive-indirect type of ELISA is used to compare allergenic potencies of two or more allergens and it is based on inhibition of the reaction taking place between a specific enzyme-labelled antibody and an immobilised antigen. The immobilised antigen may be the same as, or different from the allergen tested [Tremblay et al. 2003]. The results are expressed quantitatively by calculating the maximal inhibitory concentration (IC_{50}) through a logistics equation [Raab 1983].

1.7.4 Basophil activation assay

Basophil activation assay is a robust and reliable assay for *in vitro* measurements of allergies [Boumiza et al. 2005]. In this assay, the activation of basophils by allergens through the increase of the antigen CD63 at the surface of a cell is measured. The measurement takes place through flow cytometry [Sainte-Laudy et al. 1994, Sabbah & Sainte-Laudy 1996]. Flow cytometry is a valuable tool for the analysis of many different cell types and can be used to identify specific populations of cells, even when they are present in low numbers [Boumiza et al. 2005]. In addition to CD63, the antigen CD203c is also used as a marker for the activation of basophils. CD203c catalyses the cleavage of a number of molecules [Bollen et al. 2000]. Because of its specificity it has been proposed as a new tool for allergy diagnosis [Platz et al. 2001, Hauswirth et al. 2002, Bühring et al. 2004, Kahlert et al. 2003]. Comparison of the two antigens, CD63 and CD203c with regard to basophil activation has revealed that the latter is more sensitive [Boumiza et al. 2003]. However, as the expression of the two antigens depends on different stimulatory pathways [Hennersdorf et al. 2005], it was suggested that both markers could be used for increased sensitivity [Bühring et al. 2004].

2. Aims of the work

The overall aim of this study was to investigate the enzymatic cross-linking of a milk protein by tyrosinase from *Trichoderma reesei* and to compare the results to cross-linking by transglutaminase from *Streptoverticillium mobaraense*. β-casein, the most abundant protein in bovine milk, was used as a model protein and several analytical methods were applied.

The specific aims of the study were:

1. To examine the extent of cross-linking of β-casein by *Trichoderma reesei* tyrosinase on a molecular and chemical bond level in comparison to *Streptoverticillium mobaraense* transglutaminase (Publications I & II).
2. To investigate the effects of enzymatic cross-linking of β-casein on digestion by the proteolytic enzyme pepsin (Publications III & IV).
3. To assess the allergenicity of enzymatically cross-linked β-casein under physiological conditions (Publication IV).

3. Materials and methods

The materials and methods used in this study are also described in detail in the corresponding publications I–IV. Enzymatic cross-linking conditions are described in the publications. In the following sections some general information about the materials and methods used is summarised.

3.1 Chemicals

Protein model compounds are summarised in Table 7. Single amino acids, peptides and chromatographically purified β-casein were used as model compounds for the different techniques. β-casein enzymatically cross-linked by TrTyr and by Tgase was used as a substrate for the digestibility and allergenicity studies.

Table 7. Substrates used in the study.

Substrates	MM ($g\ mol^{-1}$)	Supplied by	Used in publication
4-hydrobenzoic acid	138.1	Fluka	II
2-hydroxyethanoic acid	76.1	Fluka	II
Y	181.2	Fluka	I, II
S	105.1	Fluka	II
E	147.1	Fluka	II
GYG	295.3	Bachem	I, II
EGVYVHPV	899.0	AnaSpec	II
β-casein	24 000	Sigma-Aldrich	I–IV
Purified β-casein	24 000	Sigma-Aldrich (purified at VTT)	II

3.2 Enzymes

TrTyr was produced and purified at VTT [Selinheimo et al. 2006]. In addition, commercial transglutaminase from Ajinomoto and further purified at VTT was used in all cross-linking experiments.

Digestibility of the cross-linked β-casein was investigated using two proteolytic enzymes, pepsin (Sigma-Aldrich) and pancreatin (Sigma-Aldrich).

3.3 Enzymatic reaction conditions

3.3.1 Cross-linking experiments

The reaction conditions used for cross-linking of β-casein by TrTyr and Tgase are summarised in Table 8. The pH (7 or 8) was chosen so as to ensure the solubility of all chemicals used in the experiments. Both enzymes are active in these pH conditions [Selinheimo et al. 2006, Ando et al. 1989]. A temperature of 40 °C was chosen as an average optimum for both enzymes [Selinheimo et al. 2006, Ando et al. 1989]. Substrates were dissolved in 150 mM NaCl except in the case of the ^{31}P-NMR and AFM experiments, when the chemicals were dissolved in distilled water because salts would interfere with the measurements. Vials were kept open so as to ensure oxygen availability during enzymatic cross-linking by TrTyr.

Table 8. Enzymatic reaction conditions used for the cross-linking experiments.

Enzyme	Enzyme dosage (nkat g^{-1})	Reaction time (h)	Concentration of β-casein (mg ml^{-1})	pH	Used in publication
TrTyr	10, 100, 1 000	2, 6, 24	1	7	I–III
	1 000	24	1.7	8	IV
Tgase	10, 100, 1 000	2, 6, 24	1	7	I–III
	100, 1 000	24	1.7	8	IV

3.3.2 Digestion experiments

Digestion of native and enzymatically cross-linked β-casein was performed in two different cases. In the first case the effect of cross-linking of β-casein by TrTyr and Tgase on digestibility was investigated using pepsin at acidic pH, mimicking gastric conditions. Relatively low pepsin dosage was used so as to ensure that differences in the proteolysis efficiency could be followed.

Digestibility was also studied in Publication IV. In this study, biphasic digestibility of native and cross-linked β-casein was followed under the physiological conditions occurring in the human GI-tract. Additionally, partial gastric digestion of non cross-linked and enzymatically cross-linked β-casein was followed for 15 min prior to CAP inhibition assay. Temperature was set at 37 °C in all incubations. A summary of the digestion reaction conditions is presented in Table 9.

Table 9. Reaction conditions for the digestion experiments.

Enzyme	**Concentration (mg ml^{-1})**	**Reaction time (h)**	**pH**	**Used in Publication**
Pepsin	0.01	1–20	7	II
	6.8	0.25, 1	8	IV
Pancreatin	40	1	8	IV

3.4 Analytical techniques

Different analytical methods were employed during this study. The methods and selection criteria are shown in Table 10.

Table10. Methods used in the Publications I–IV.

Method	**Purpose**	**Used in Publication**
UV/Vis spectrophotometry	Quantification of cross-linked β-casein, colour formation	I
SDS-PAGE	Monitoring early stages of cross-linking, pH stability, digestibility	I, III, IV
SEC-UV/Vis chromatography	Purification of β-casein, digestibility	II
SEC-UV/Vis-MALLS	MM determination	I
MALDI-TOF mass spectrometry	Analysis of peptides after digestion and MM determination of purified β-casein	II, III
^{31}P-NMR spectroscopy	Determination of reactive groups in β-casein after cross-linking by TrTyr	II
AFM	Morphology of cross-linked material	II
pH-stat	Monitoring hydrolysis and calculating the DH	III
Immunoblotting	Probing of antigenic peptides after partial digestion	IV
CAP inhibition	Monitoring the presence and levels of specific IgE antibodies in serum	IV
ELISA inhibition	Monitoring specific IgE binding of cross-linked β-casein	IV
Basophil activation assay	Analysis of the ability of milk allergens to activate degranulation of basophils	IV

4. Results and discussion

4.1 Structural characterisation of tyrosinase cross-linked β-casein (Publications I & II)

The chemical cross-linking reactions initiated by tyrosinase are very complex (Fig. 2) and depend on the chemical structure of the substrate and the reaction conditions. The random coil β-casein contains four tyrosine residues and several free amino groups that could be cross-linked by TrTyr in different combinations, making it an excellent choice as substrate protein for these studies. As β-casein does not contain any cysteine residues, the possibility of tyrosine-cysteine cross-linking is excluded.

SDS-PAGE was used for studying the extent of cross-linking of β-casein by TrTyr. When high enzyme dosages and long incubation times were used, the enzymatically cross-linked β-caseins were so large that they could not penetrate the SDS-PAGE gel, indicating that their MM was above 200 kg mol^{-1} which is the limit of the gel (Publication I, Fig. 2A). Thus, only the early stages of polymerisation could be followed by SDS-PAGE.

In order to study the enzymatically cross-linked β-caseins with molecular mass above 200 kg mol^{-1}, SEC-UV/Vis-MALLS was employed. From the numerical data extracted from SEC-UV/Vis-MALLS, it became clear that the end point of the TrTyr cross-linking reaction was obtained when the highest enzyme dosage of 1 000 nkat g^{-1} was used, regardless of the incubation time (2, 6 or 24 h). The chromatograms at the end point of the enzymatic cross-linking when using TrTyr are shown in Fig. 9. The small variation in molecular mass (MM), radius of gyration (R_G) and degree of polymerisation (DP) values are attributed to the possible different pairings of the reactive amino acid side-chains. In all cases, the reaction products were monodisperse. Use of tenfold lower enzyme dosage i.e. 100 nkat g^{-1} led to the formation of intermediate-sized

products with a DP an order of magnitude lower than when using the highest enzyme dosage. When β-casein was cross-linked using the lowest enzyme dosage (10 nkat g^{-1}), no polymerisation was observed (Publication I).

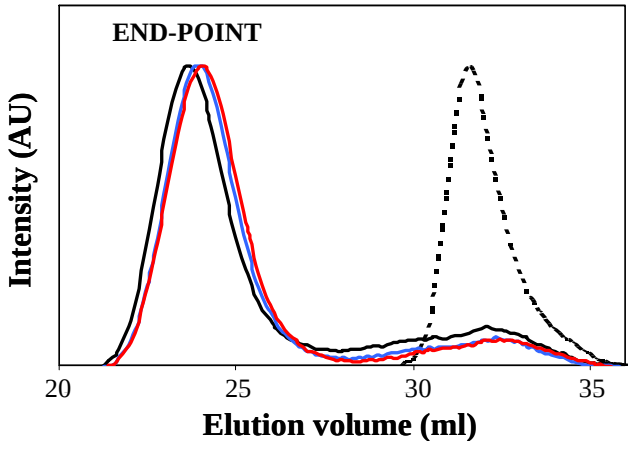

Figure 9. Size-exclusion chromatographs of β-casein polymerised by TrTyr at the end point of the cross-linking reactions. Untreated β-casein (reference) is shown with a black dotted line. Chromatograms were measured from the polymerised β-caseins after 2 (black), 6 (blue) and 24 h (red) incubation times and are shown with solid lines. At the end point of the cross-linking reactions, the average MM, R_G and DP values were 1 700 ± 7, 1 300 ± 5, 1 500 ± 6 kg mol^{-1}; 52 ± 2.8, 45 ± 2.9, 46 ± 2.9 nm; 71, 54, 63 respectively.

The extent of cross-linking of β-casein by TrTyr was also studied by ^{31}P-NMR spectroscopy in ionic liquids (Publication II). Thus, cross-linked β-casein was solubilised in 1-allyl-3-methylimidazolium chloride i.e. [amim]Cl, a solvent system commonly used for wood components. The extent of cross-linking was monitored by ^{31}P-NMR using 10, 100 and 1 000 nkat g^{-1} enzyme dosages and 24 h incubation time as shown in Fig. 10. The method allows quantification of aliphatic, phenolic and carboxylic acid hydroxyl groups in the reaction products [Spyros et al. 2000, Jiang et al. 1995]. When the lowest enzyme dosage was used for the cross-linking of β-casein, 85% of the phenolic hydroxyls remained intact for further derivatisation by the 2-chloro-4,4,5,5-tetramethyl-1,3,2 dioxaphospholane i.e. PR [II] reagent, indicating a relatively low extent of cross-linking and hence confirming the SEC-UV/Vis-MALLS results (Publication I, Fig. 3 & Table 1). In the case of a tenfold higher enzyme dosage, 47% of the reactive phenolic hydroxyl groups remained un-cross-linked, while in the case of the highest enzyme dosage only 9% of the reactive phenolic hydroxyl groups were not

involved in the cross-linking reactions and thus were available for reacting with the PR [II] reagent.

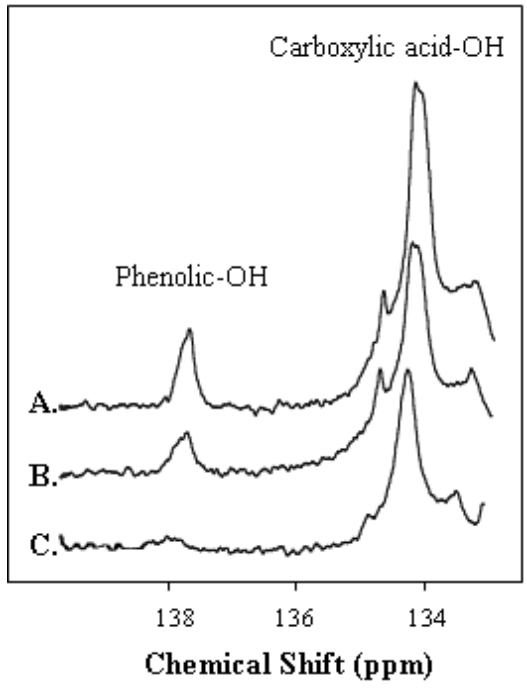

Figure 10. Selected area of the ^{31}P-NMR spectra measured from chromatographically purified β-casein cross-linked by TrTyr using enzyme dosages of (A) 10 nkat g^{-1}, (B) 100 nkat g^{-1} and (C) 1 000 nkat g^{-1} and 24 h incubation time. The assignments of the various hydroxyl groups are shown above the corresponding peaks.

Cross-linking of β-casein by TrTyr resulted in the formation of disk-shaped, nanoscaled particles as visualized by AFM (Publication II). The average diameter of these particles was around 19 nm when the lowest enzyme dosage (10 nkat g^{-1}) was used. The diameter was approximately two times larger than that of the non cross-linked β-casein particles. Unexpectedly, the use of a tenfold higher enzyme dosage (100 nkat g^{-1}) resulted in somewhat smaller particles, ca. 15 nm. This result was in good agreement with the SEC-UV/Vis-MALLS (Publication I) and ^{31}P-NMR (Publication II) data, where under the selected reaction conditions only intermediately cross-linked products were formed. When the highest enzyme dosage of 1 000 nkat g^{-1} was used, the average size of the disk-like shaped particles was around 80 nm, although larger particles of approximately 340 nm most probably aggregates, were also detected (Fig. 11).

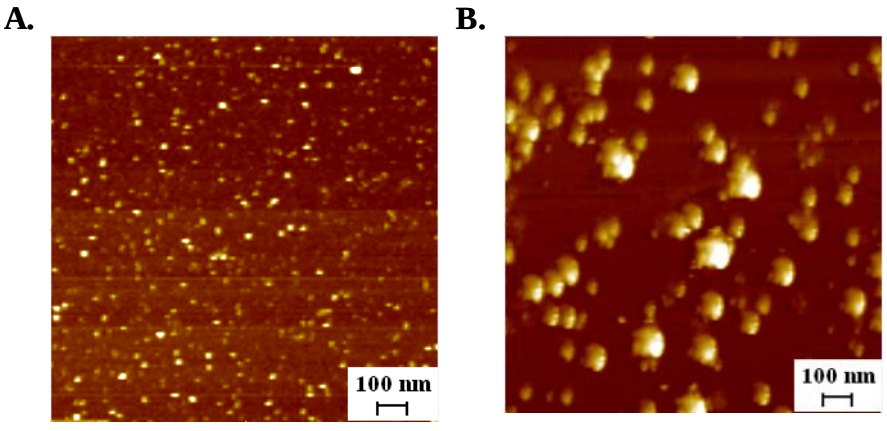

Figure 11. Morphology of enzymatically cross-linked β-casein as analysed by AFM. A. Selected, magnified area of the mica surface of β-casein (reference). B. Selected, magnified area of the mica surface of β-casein cross-linked by TrTyr (enzyme dosage: 1 000 nkat g^{-1} and incubation time: 24 h). Chromatographically purified β-casein was used for the experiments.

4.1.1 Colour formation

As previously reported [Bittner 2006, Sánchez-Ferrer et al. 1995, Hellman et al. *In Press*], tyrosinase-catalysed oxidation products were coloured and the intensity of the colour varied from purple *via* yellow to very dark brown when using tyrosine, GYG peptide and β-casein as substrates for TrTyr as shown in Fig. 12. Colour formation is a very complex reaction, which in the case of proteins is often attributed to the presence of free cysteine or tyrosine residues oxidised by tyrosinase [Sánchez-Ferrer et al. 1995, Ito et al. 1984, Raper 1928]. However, because β-casein does not contain any cysteine residues, the detected colours were derived from the covalent bonds formed after enzymatic oxidation of tyrosine side-chains coupled with each other or amine and histidine residues as shown in Fig. 2. These bonds could be formed between the aromatic rings of tyrosine residues [Roberts et al. 1972], as well as between the aromatic rings and free amino groups [de Oliveira et al. 2006] caused by the non-enzymatic chemical reactions of highly reactive di-quinones produced by TrTyr as shown in Fig. 2, page 19 [Ito et al. 1984, Raper 1928].

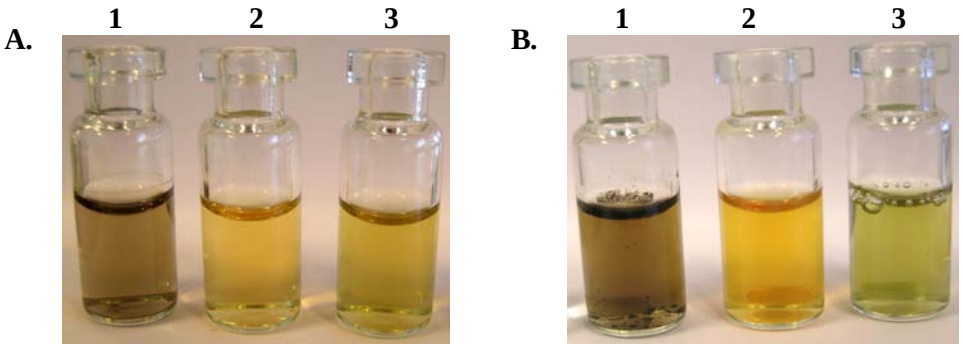

Figure 12. Colour formation after treatment of (**1**) tyrosine amino acid, (**2**) GYG peptide and (**3**) β-casein protein using the highest TrTyr dosage and overnight incubation time. In (**A**) the reactions were performed at pH 6.5 and in (**B**) at pH 8.0.

Colour formation was also monitored on-line by UV/Vis spectrophotometry. However, due to the complexity of the measured spectra (Publication I, Fig. 5) the different bands detected from the UV/Vis spectra could not be unambiguously interpreted on the level of molecular structure.

The pH stability of β-casein cross-linked by TrTyr was verified at low pH (Publication III, Fig. 2). However, the stability of the colour and thus the covalent structure of the enzymatically cross-linked β-casein, an important parameter for the exploitation of the enzyme in various applications, remain to be studied.

4.2 Structural characterisation of transglutaminase cross-linked β-casein (Publications I & II)

When β-casein was treated by Tgase, highly cross-linked β-casein was produced already when using 100 nkat g^{-1} enzyme dosage and 6 h reaction time, whereas when the highest enzyme dosage (1 000 nkat g^{-1}) was used the MM was slightly lower (Fig. 13) This was attributed to the large number of reactive side-chains of β-casein. The amino acid sequence of β-casein has 11 K- and 20 Q- residues. Thus, it is clear that a number of different pairings of these reactive amino acid side-chains may occur, leading to slightly different cross-linked reaction products. Use of the lowest enzyme dosage (10 nkat g^{-1}) did not result in the formation of fully cross-linked polymers; even when the longest reaction time was used only intermediately sized cross-linked products were formed.

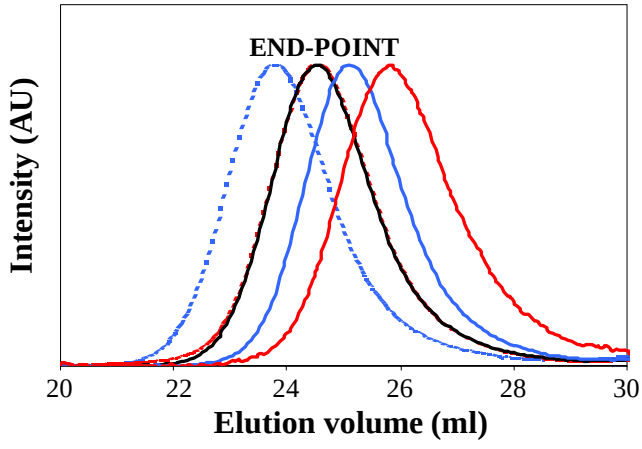

Figure 13. Size-exclusion chromatographs of β-casein polymerised by Tgase at the end point of the cross-linking reactions. Chromatographs measured from the polymerised β-caseins using 100 nkat g^{-1} (dotted lines) and 1 000 nkat g^{-1} (solid lines) Tgase dosages, after 2 (black), 6 (blue) and 24 h (red) reaction times. At the end point of the cross-linking reactions, the average MM, R_G and DP values were 1 600 ± 06, 1 100 ± 04, 850 ± 08, 1 200 ± 08, 1 100 ± 04 kg mol^{-1}; 50 ± 2.7, 35 ± 3.1, 50 ± 2.9, 45 ± 5.0, 38 ± 3.3 nm; 66, 46, 35, 50 and 46 respectively.

The ^{31}P-NMR spectra of β-casein cross-linked by 10, 100 and 1 000 nkat g^{-1} of Tgase showed no effect of the enzyme on the intensities of the aliphatic, phenolic and carboxylic acid hydroxyl groups as expected, because these functional groups do not participate in the cross-linking reactions induced by Tgase.

When β-casein was cross-linked by Tgase, the average diameter of the disk-shaped particles obtained after using the lowest enzyme dosage of 10 nkat g^{-1} and the shortest reaction time (2 h) was approximately 22 nm, as analysed by AFM. The average diameter almost doubled (39 nm) when a tenfold higher enzyme dosage was used (data not shown). Interestingly, the highest enzyme dosage of 1 000 nkat g^{-1} Tgase led to the formation of smaller particles with an average diameter of 20 nm. Moreover, large aggregates of approximately 360 nm were also detected (Fig. 14B). Based on these results it becomes apparent that the protein network formed after extensive cross-linking of β-casein by Tgase results in tightly packed protein nanoparticles, also indicating intramolecular cross-linking.

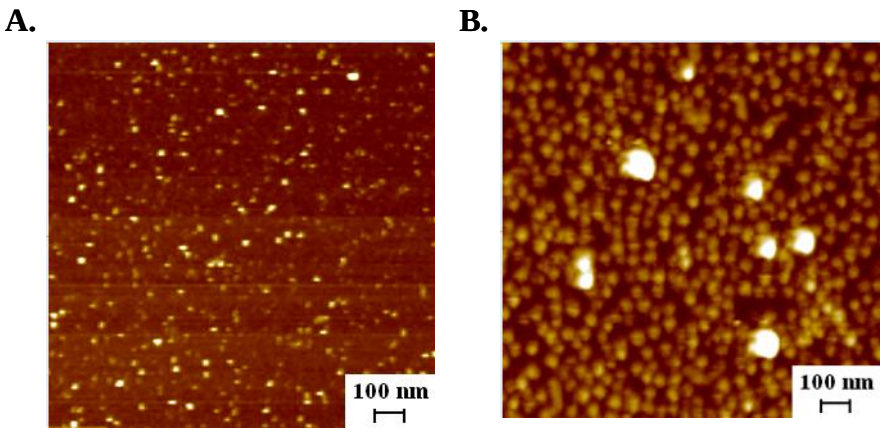

Figure 14. Morphology of enzymatically cross-linked β-casein as analysed by AFM. **A.** Selected, magnified area of the surface of chromatographically purified β-casein. **B.** Selected, magnified area of the surface of β-casein cross-linked by Tgase (enzyme dosage: 1 000 nkat g^{-1} and incubation time: 2 h). Chromatographically purified β-casein was used for the experiments.

4.3 Comparison of the cross-linking enzymes

According to SEC-UV/Vis-MALLS, polymerisation of β-casein by TrTyr and Tgase occurred to the same degree (ca. 90%) when the optimum reaction conditions of enzyme dosage and incubation time for each enzyme were used. Commercial β-casein that was used as a substrate contains globular whey proteins as impurities (Publication I, Fig. 2), and thus 100% polymerisation could not be obtained. Neither TrTyr nor Tgase can polymerise globular proteins [Raper 1928, Gauche, et al. 2008]. It is characteristic that for TrTyr cross-linking the highest enzyme dosage was required for obtaining fully polymerised product. The incubation time (2, 6, and 24 h) did not affect significantly the degree of polymerisation. In the case of Tgase, medium enzyme dosage (100 nkat g^{-1}) and shorter reaction time (6 h) were sufficient to obtain the same size of cross-linked product. However, it is important to note at this stage that a comparison based strictly on the enzyme dosage, incubation time and final MM is not adequate for drawing conclusions on the performance of the enzymes in food matrix, as *Trichoderma reesei* tyrosinase and *Streptoverticillium mobaraense* transglutaminase have completely different reaction mechanisms.

The R_G obtained by AFM were smaller for all cross-linked samples in comparison to the radii of gyration obtained by SEC-UV/Vis-MALLS. The

reason for this apparent inconsistency lies in the fact that the samples were dried on the mica surface prior to analysis by AFM. The protein particles probably shrunk due to the drying, causing a decrease in their radii. By contrast, analysis by the SEC-UV/Vis-MALLS was performed in solution and therefore the samples did not shrink during the measurement. Martin et al. (2006) reported the same phenomenon when analysing casein micelles by Scanning Electron Microscopy (SEM). Morphological analysis of enzymatically cross-linked β-casein showed that independently of the type of enzyme used, the cross-linked polymers were of a disk-like shape. On the basis of the experimental data presented in this study, it was shown that when β-casein was cross-linked by Tgase, large polymers were formed. After extended incubation time more cross-links were probably formed, resulting in a tight spherical nanostructure rather than a polymer of higher MM. By contrast, in the case of treatment by TrTyr, as β-casein has less reactive sites for the enzyme (four tyrosine and five histidine amino acid residues) and thus a looser protein network is formed. This is visualised by the larger diameter of the nanoparticles.

Cross-linking of protein material by TrTyr results in colourful polymerised products, whereas treatment of tyrosine, GYG peptide and β-casein by Tgase results in colourless products. The colours observed depended on the incubation time as well as on the pH of the solution.

4.4 Effect of enzymatic cross-linking on digestibility by pepsin (Publication III)

A number of studies, as summarised in Table 5, p. 36, have been made regarding the digestibility of various enzymatically cross-linked protein materials. In most cases, *Streptoverticillium mobaraense* transglutaminase was used as cross-linking enzyme but laccase and peroxidase have also been employed. The study presented in Publication III is the first one in which the digestibility of β-casein cross-linked by tyrosinase (from *Trichoderma reesei*) and transglutaminase (from *Streptoverticillium mobaraense*) was studied on a molecular level by various analytical techniques, such as SEC-UV/Vis and MALDI-TOF MS. Prior to studying the effect of digestibility by the proteolytic enzyme pepsin on enzymatically cross-linked β-casein, it was ensured that enzymatically cross-linked β-casein was stable under the acidic conditions present during proteolysis (Publication III, Fig. 2).

The digestibility of native β-casein as well as of fully enzymatically cross-linked β-casein was monitored by SEC-UV/Vis, MALDI-TOF MS and pH-stat (Publication III). The SEC-UV/Vis profiles of native and enzymatically cross-linked β-casein digested by pepsin for 1, 3, 6 and 20 h are shown in Fig. 15. The chromatograms were divided into four areas for clarity and for facilitating interpretation of the data. On the basis of the results it was clear that proteolysis by pepsin starts immediately after pepsin addition. After 1 hour of incubation time, significant changes were already detected. The non cross-linked β-casein was already broken down into smaller particles ($16 < MM < 10\ kg\ mol^{-1}$), whereas the enzymatically cross-linked β-casein was not digested to the same extent. Thus protein particles ($MM > 16\ kg\ mol^{-1}$) eluted at V_o, i.e. in area I (Fig. 15A). After 20 h of digestion, enzymatically cross-linked β-casein presented protein particles larger than $16\ kg\ mol^{-1}$ that could not be digested by pepsin (Fig. 15D).

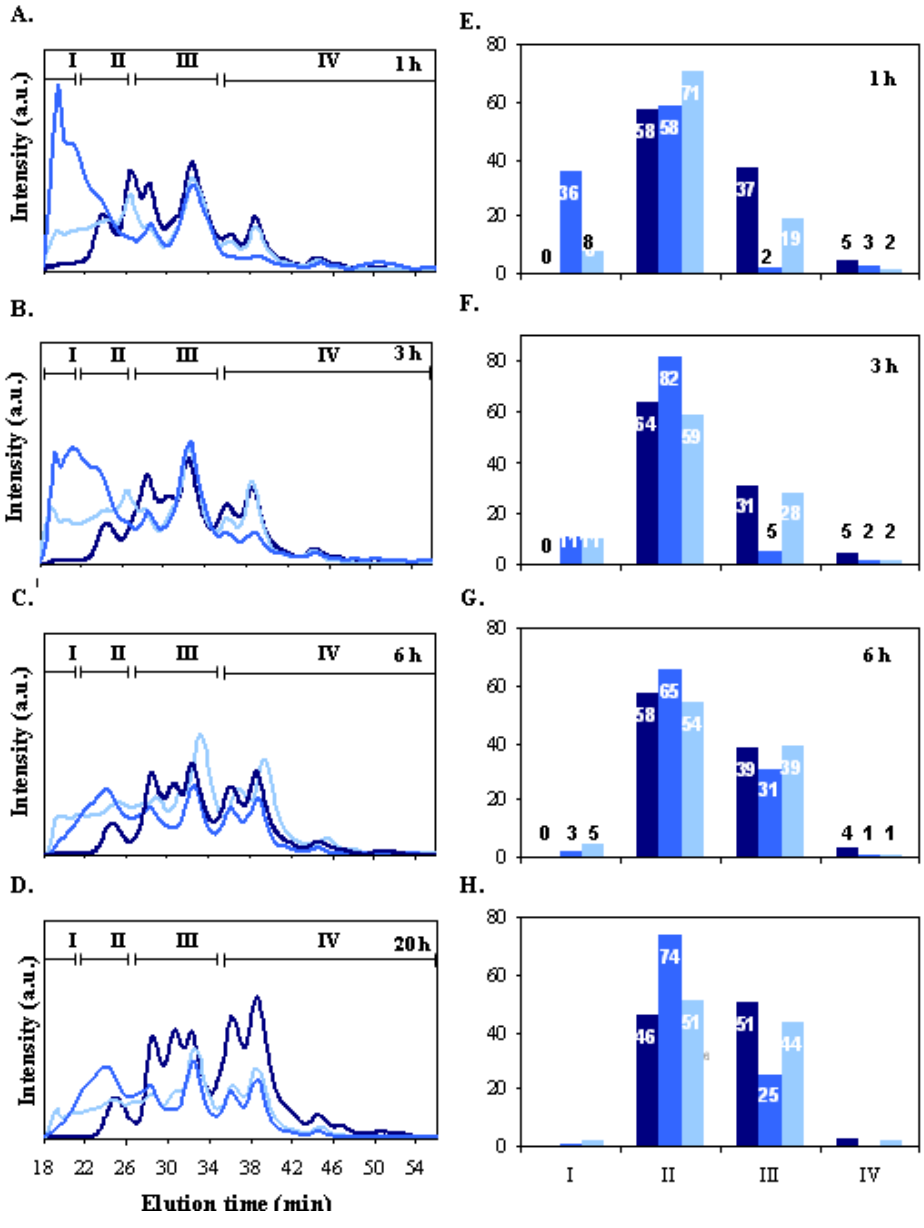

Figure 15. Size-exclusion chromatograms of enzymatically cross-linked β-casein digested by pepsin after different digestion incubation times (**A–D**) [dark blue: β-casein (reference), blue: TrTyr (enzyme dosage: 1 000 nkat g^{-1}, incubation time: 24 h, light blue: Tgase (enzyme dosage: 1 000 nkat g^{-1}, incubation time: 24 h] and quantitative data from the digested protein material (**E–H**). I. High molecular mass proteins eluting at Vo; II. medium sized proteins, (MM: 16–10 kg mol^{-1}); III. peptides (MM: 10–4 kg mol^{-1}); IV. single amino acids, tri- and dipeptides (MM ≤ 4 kg mol^{-1}).

In the case of non cross-linked β-casein, the amount of large protein particles ($16 < MM < 10$ kg mol^{-1}) remained relatively constant throughout the digestion process (Fig. 15E-H), whereas the amount of shorter peptides ($10 < MM < 4$ kg mol^{-1}) increased from ~32% to ~51% after 20 h digestion. The amount of single amino acids remained constant at around 5%. When β-casein was cross-linked by TrTyr, large protein particles eluting in V_o steadily decreased, although even after 20 h digestion, a small amount of ~1% was detected (Fig. 15H). As expected, the amount of medium sized proteins ($16 < MM < 10$ kg mol^{-1}) showed a small decrease (around 10%) as the amount of larger proteins ($MM > 16$ kg mol^{-1}) decreased. The peptides ($10 < MM < 4$ kg mol^{-1}) increased from 1 to 20 h digestion time as was expected. Similar profiles were observed in the digestion process of β-casein cross-linked by Tgase. The pepsin-catalysed digestion appeared to have proceeded faster in the Tgase cross-linked β-casein as compared to TrTyr cross-linked β-casein. Thus, the amount of medium sized proteins decreased from 82% (1 h) to 51% while the amount of peptides ($10 < MM < 4$ kg mol^{-1}) increased from 22% to 44%. Single amino acids, tri- and dipeptides did not change (Fig. 15E–H).

In addition to SEC-UV/Vis chromatography, short peptides and amino acids (area IV: $MM \leq 4$ kg mol^{-1}) were analysed by MALDI-TOF MS. A number of differences depending on (1) the type of enzyme used for the cross-linking, (2) the enzyme dosage and (3) the digestion time, were observed in the peptide patterns. In general some of the peptides derived from the enzymatically cross-linked β-casein, whether by TrTyr or by Tgase, disappeared compared to the products of the reference sample. At the same time, some new peaks could be also detected from the spectra. However, it was not always possible to understand the reasons for these disappearances/appearances as not all the peptide sequences have been identified. Moreover, not all of the disappearing peptides of known sequences had tyrosine amine and/or histidine residues that could explain their absence after treatment by TrTyr.

The identified peptides [Schmelzer et al. 2004] disappearing after cross-linking of β-casein by TrTyr and Tgase are shown in Table 11. The amino acid sequence of β-casein is shown in Fig. 3, page 23.

Table 11. List of peptides disappearing after TrTyr and Tgase treatment.

	Possible cross-linked amino acids			
	TrTyr		Tgase	
Peptides	**Y**	**H**	**Q**	**K**
VYPFPGPIPNSLPQNIPPLTQT	1	0	2	0
PVVVPPFLQPEVM	0	0	1	0
GVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLT	1	1	-	-
TDVENLHLPLPLLQS	0	1	-	-
QSWMHQPHQPLPPTVMFPPQSVLS	0	2	4	0
SLSQSKVLPVPQKAVPYPQRDM PIQAF	-	-	4	2

- These peptides did not disappear after peptic digestion of the enzymatically cross-linked β-casein.

Although gastric digestion of native β-casein has been described in several studies [Schmelzer et al. 2007, Schmelzer et al. 2004, Qi et al. 2007, Fountoulakis & Lahm 1998, Otte et al. 2007, Kitazawa et al. 2007], digestibility of cross-linked β-casein by TrTyr and Tgase has not previously been analysed at this level. It could be concluded that when bovine β-casein was cross-linked by TrTyr or by Tgase, the digestibility was retarded in comparison to that of the non cross-linked β-casein. This difference in the digestibility between cross-linked and non cross-linked β-casein can be understood by the covalently compact nature of the enzymatically cross-linked protein in comparison to the flexible random coil nature of β-casein [Smiddy et al. 2006].

In summary, digestion of cross-linked β-casein by TrTyr and Tgase leads to the formation of protein fragments and peptides that vary to some extent depending on the cross-linking conditions (Publication III, Table 1). In the case of β-casein cross-linked by TrTyr, the digestion was complete after a reaction time of 20 h, even though retarded in comparison to the control. By contrast to TrTyr, enzymatic modification by Tgase led to more resistant protein particles (MM > 20 kg mol^{-1}) that remained in the reaction mixture even after 20 h of reaction time.

4.5 Effect of enzymatic cross-linking on allergenicity of β-casein (Publication IV)

Commercial β-casein was cross-linked by four enzymes, TrTyr and AbTyr, Tgase and *Trametes hirsuta* laccase (ThL). Cross-linking of β-casein was performed

in slightly higher substrate concentration (1.7 mg ml^{-1}) and pH 8.0 than in the previous cross-linking experiments. These changes did not cause significant differences in the extent of cross-linking by TrTyr and Tgase (Publication IV, Fig. 1A). Allergenicity was measured *in vitro* by different analytical techniques. The effect of cross-linking of β-casein by AbTyr and by ThL on allergenicity was most interesting, as allergenicity was decreased to a great extent in comparison to the non cross-linked β-casein. However, only tyrosinase from *Trichoderma reesei* and transglutaminase from *Streptoverticillium mobaraense* are examined in this thesis.

In CAP inhibition assay using sera of patients allergic to cow's milk, the binding of casein specific IgE was reduced by 14% in the case of TrTyr and only by 6% in the case of Tgase in comparison to that of the native β-casein. Allergenicity was also tested for partially digested cross-linked caseins (15 min proteolysis by pepsin) and after biphasic digestion (1 h proteolysis by pepsin and subsequently 1 h by pancreatin). The binding potential of IgE for the fragments occurring after partial digestion by pepsin increased by 10% and 17% for TrTyr and Tgase, respectively. However, after the biphasic digestion the binding potential decreased almost to the levels of the non cross-linked β-casein. Finally, the ability of the allergens to activate the degranulation of the basophils was tested in seven patients. None of the cross-linked caseins showed significant reduction in the ability to activate basophils of the allergic patients. It is important to note that non-atopic subjects did not react to the enzymatically cross-linked material and also that enzymatic cross-linking of β-casein did not enhance allergic effects in non-atopic subjects. As discussed in publication IV, it can be concluded that enzymatic cross-linking of β-casein by TrTyr decreases allergenicity of the β-casein more than Tgase.

In the case of β-casein, it has been shown that 50% of patients with bovine milk allergy are allergic to this protein [Docena et al. 1996, Shek et al. 2005]. Hence, β-casein represents a serious health risk for children and adults. Chatchatee and colleagues as well as Cerecedo et al. reported a number of regions in the β-casein sequence that were recognised by allergic patients and are thus considered to be IgE-binding epitopes. The epitopes distinguishing between tolerant and non-tolerant patients, were: AA 25-50, 52-74, 55-70, 83-92, 108-121, 135-144, 154-173 and 186-208 [Chatchatee et al. 2001, Cerecedo et al. 2008]. In a comparison of their findings to the results presented in Publications III and IV, it was noted that some of these peptides were missing from the MALDI-TOF MS spectrum after pepsin digestion of the enzymatically

cross-linked β-casein (Table 11, page 60). Thus, the 14% decrease of allergenicity of β-casein when cross-linked by TrTyr could be attributed to the disappearance of some of these peptides such as those with amino acid sequences 59-80; 81-93 and 94-128 (See Fig. 3 for the amino acid sequence of β-casein). At the same time, the remaining peptides such as 185-207, which have also been reported to be allergenic, explain why allergenicity was not completely eliminated by TrTyr treatment. Similar conclusions can of course be drawn when using Tgase as a cross-linking enzyme.

5. Conclusions and future outlook

In this study, β-casein was cross-linked by a fungal tyrosinase from *Trichoderma reesei* and by transglutaminase from *Streptoverticillium mobaraense*. Both enzymes cross-linked β-casein to the same extent according to SEC-UV/Vis-MALLS data. With the highest TrTyr dosage used (1 000 nkat g^{-1}) and the longest reaction time (24 h), 91% of all tyrosine residues of β-casein participated in the cross-linking reactions according to ^{31}P-NMR experiment. Pepsin proteolysis of β-casein cross-linked by TrTyr was retarded in comparison to the proteolysis of native β-casein. However, the enzymatically cross-linked protein was eventually broken down into small peptides and amino acids after 20 h of incubation time. These small digested peptides were rather similar to those of native β-casein, as evidenced by MALDI-TOF MS. Despite the similarities in the peptide profiles of both samples, some peptides were not detected after the digestion of the sample cross-linked by TrTyr, indicating their possible participation in the cross-linking. Allergenicity of β-casein cross-linked by TrTyr was slightly decreased in comparison to the native β-casein. The colour of the cross-linked products after treatment of β-casein by TrTyr varied depending on the cross-linking conditions such as pH and enzyme dosage.

In the case of the reference enzyme, Tgase, high molecular mass products (~ 1 200 kg mol^{-1}) were also formed after enzyme treatment. A tenfold smaller enzyme dosage (100 nkat g^{-1}) was adequate for the formation of similar sized products, as in the case of TrTyr treatment. The protein network after cross-linking by Tgase was rather tight as evidenced by AFM, and disk-shaped particles of very small diameter were formed. The digestibility of β-casein cross-linked by Tgase was retarded in comparison both to the control and to the β-casein cross-linked by TrTyr. Mariniello et al. reported that when phaseolin from *Phaseolus vulgaris* was cross-linked by Tgase, the introduced isopeptide bonds contributed to polymers with increased resistance to proteolysis

[Mariniello et al. 2007]. In this case, the digested peptides also showed some similarities but some differences in comparison to the native and cross-linked β-casein, as evidenced by the MALDI-TOF MS spectra. Interestingly, allergenicity of β-casein cross-linked by Tgase was less mitigated than in the case of TrTyr. Furthermore, the products formed after enzymatic cross-linking by Tgase were not coloured. A comparative summary of how tyrosinase from *Trichoderma reesei* affects cross-linking of β-casein in comparison to transglutaminase from *Streptoverticillium mobaraense* at the end point of the cross-linking and digestion reactions is shown in Table 12. The results obtained from these studies further confirm TrTyr as a cross-linking agent for protein modification, despite the drawback of colour formation.

Table 12. Summary of the properties of fully cross-linked β-casein.

	Cross-linking enzyme	
Properties	**TrTyr**	**Tgase**
Molecular mass	~ 1 500 kg mol^{-1}	~ 1 170 kg mol^{-1}
Dispersity	Monodisperse	Monodisperse & aggregates
Cross-linking	Covalent	Covalent
Average Size	80 nm	20 nm
Shape	Disk-shaped	Disk-shaped
pH – Stability	Yes	Yes
Colour	Yes	No
Digestibility	Retarded >	Retarded >>
Allergenicity	Decreased >>	Decreased >

For the purpose of these studies a number of advanced analytical techniques were employed. The methods used for studying the extent of cross-linking of β-casein by TrTyr and Tgase were complementary to each other and all possible information was extracted. Although widely used for studying the extent of enzymatic cross-linking of proteins, SDS-PAGE has major limitations particularly with regard to estimation of the molecular mass of the products formed. When the formed polymers have molecular mass above 200 kg mol^{-1}, this method is not applicable because they cannot penetrate the gel.

For this reason, SEC-UV/Vis connected to a MALLS detector was used. The use of this detector was necessary and advantageous as it could monitor large

polymers without the use of molecular mass standards and calibration. Moreover, as the strength of the light scattering signal is proportional to the molecular mass of the molecule and to its concentration, detection of the formed polymers after enzymatic cross-linking was possible even though low concentrations were used. The ^{31}P-NMR method was useful in determining in a quantitative manner the number of tyrosine side-chains cross-linked after treatment by tyrosinase from *Trichoderma reesei*. However, using this 1D method it was not possible to determine the position of the formed covalent bonds.

When studying the impact of cross-linking of β-casein on digestibility, SEC-UV/Vis could not provide detailed information due to the low resolution, although it did reveal differences in the obtained peptide profiles. Analysis of peptides with a molecular mass from 1 to 4 kg mol^{-1} was performed by MALDI-TOF MS. Above these molecular masses (i.e. > 4 kg mol^{-1}) none of the above-mentioned methods were able to give any supplementary information. Use of a pH-stat for monitoring the rate of proteolysis proved to be inadequate for complex samples such as enzymatically cross-linked β-caseins.

Further studies should focus on understanding the exact chemical bonds formed after cross-linking and the exact reaction mechanism of tyrosinase. This knowledge would facilitate understanding the origin of the colour formed after treatment of proteins by TrTyr, as this colour may hinder the exploitation of this enzyme in food applications. However, melanin formation is not necessarily a negative phenomenon. Schweitzer and colleagues recently showed that structures able to protect bone marrow from the ionising radiation applied during radio-immunotherapy of tumours were formed by the polymerisation of melanin precursors on the surface of silica nanoparticles [Schweitzer et al. *In Press*].

The impact of cross-linking of β-casein by TrTyr and Tgase on biphasic digestion should also be further studied. As shown in Publication IV, even the largest cross-linked products formed after cross-linking by 1 000 nkat g^{-1} Tgase were eventually digested after treatment with pancreatin. It is therefore assumed that enzymatically cross-linked β-casein after biphasic digestion is broken down into small particles at the size level of short peptides and single amino acids. It would thus be interesting to study the structure of these small particles and how they together with the delayed presence of bigger particles in the GI-tract, would affect food intake, satiety and human health in general.

The nano-scaled material produced after enzymatic cross-linking could be used in different non-food applications, such as in drug delivery systems (DDS). The small size of the enzymatically cross-linked polymers allows improved bio-

distribution of drugs in the human circulation system and improved solubility [Maham et al. 2009]. Other applications of these small but pH-stable particles could include coatings and adhesives [Huppertz & de Kruif 2008]. Song and colleagues demonstrated that hydrogels formed from the enzymatic cross-linking of commercial casein powder by Tgase prolonged the release of Vitamin B12 [Song et al. 2010].

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Series title, number and report code of publication

VTT Publications 752
VTT-PUBS-752

Author(s)
Evanthia Monogioudi

Title

Enzymatic cross-linking of β-casein and its impact on digestibility and allergenicity

Abstract

Protein modification *via* enzymatic cross-linking is an attractive way for altering food structure so as to create products with increased quality and nutritional value. These modifications are expected to affect not only the structure and physico-chemical properties of proteins but also their physiological characteristics, such as digestibility in the GI-tract and allergenicity. Protein cross-linking enzymes such as transglutaminases are currently commercially available, but also other types of cross-linking enzymes are being explored intensively.

In this study, enzymatic cross-linking of β-casein, the most abundant bovine milk protein, was studied. Enzymatic cross-linking reactions were performed by fungal *Trichoderma reesei* tyrosinase (TrTyr) and the performance of the enzyme was compared to that of transglutaminase from *Streptoverticillium mobaraense* (Tgase). Enzymatic cross-linking reactions were followed by different analytical techniques, such as size exclusion chromatography -Ultra violet/Visible – multi angle light scattering (SEC-UV/Vis-MALLS), phosphorus nuclear magnetic resonance spectroscopy (^{31}P-NMR), atomic force (AFM) and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). The research results showed that in both cases cross-linking of β-casein resulted in the formation of high molecular mass (MM ca. 1350 kg mol^{-1}), disk-shaped nanoparticles when the highest enzyme dosage and longest incubation times were used. According to SEC-UV/Vis-MALLS data, commercial β-casein was cross-linked almost completely when TrTyr and Tgase were used as cross-linking enzymes. In the case of TrTyr, high degree of cross-linking was confirmed by ^{31}P-NMR where it was shown that 91% of the tyrosine side-chains were involved in the cross-linking.

The impact of enzymatic cross-linking of β-casein on *in vitro* digestibility by pepsin was followed by various analytical techniques. The research results demonstrated that enzymatically cross-linked β-casein was stable under the acidic conditions present in the stomach. Furthermore, it was found that cross-linked β-casein was more resistant to pepsin digestion when compared to that of non modified β-casein. The effects of enzymatic cross-linking of β-casein on allergenicity were also studied by different biochemical test methods. On the basis of the research results, enzymatic cross-linking decreased allergenicity of native β-casein by 14% when cross-linked by TrTyr and by 6% after treatment by Tgase.

It can be concluded that in addition to the basic understanding of the reaction mechanism of TrTyr on protein matrix, the research results obtained in this study can have high impact on various applications like food, cosmetic, medical, textile and packing sectors.

ISBN
978-951-38-7421-6 (soft back ed.)
978-951-38-7422-3 (URL: http://www.vtt.fi/publications/index.jsp)

Series title and ISSN	Project number
VTT Publications 1235-0621 (soft back ed.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)	

Date	Language	Pages
December 2010	English, Finnish abstr.	86 p. + app. 66 p.

Name of project	Commissioned by

Keywords	Publisher
Tyrosinase, transglutaminase, protein, β-casein, cross-linking, digestibility, allergenicity, SEC-UV/Vis-MALLS, MALDI-TOF MS, 31P-NMR, AFM	VTT Technical Research Centre of Finland P.O. Box 1000, FI-02044 VTT, Finland Phone internat. +358 20 722 4520 Fax +358 20 722 4374

Julkaisun sarja, numero ja raporttikoodi

VTT Publications 752
VTT-PUBS-752

Tekijä(t)
Evanthia Monogioudi

Nimeke

β-kaseiinin entsymaattinen ristisidonta ja sen merkitys proteiinin digestoituvuuteen ja allergeenisuuteen

Tiivistelmä

Proteiinien muokkaus entsymaattisen ristisidonnan avulla on houkutteleva tapa muokata ruoan rakennetta tuotteen laadun ja ravitsemusarvon parantamiseksi. Ristisidonnasta johtuvien muutosten voidaan olettaa parantavan proteiinien rakenteen ja fysikaalis-kemiallisten ominaisuuksien lisäksi myös niiden fysiologisia ominaisuuksia, kuten sulavuutta ruoansulatuskanavassa ja allergeeniutta. Proteiinien ristisidontaan soveltuvia entsyymejä, kuten transglutaminaaseja, on tällä hetkellä kaupallisesti saatavilla, mutta myös muuntyyppisiä ristisidontaan kykeneviä entsyymejä tutkitaan parhaillaan runsaasti.

Tässä väitöskirjassa tutkittiin naudan yleisimmän maitoproteiinin β-kaseiinin entsymaattista ristisidontaa. Entsymaattiset ristisidontareaktiot toteutettiin *Trichoderma reesei* -homeen tyrosinaasilla (TrTyr) ja niitä verrattiin *Streptoverticillium mobaraense* -transglutaminaasin (Tgase) aiheuttamiin reaktioihin. Entsymaattisia ristisidontareaktioita tutkittiin eri analyysimenetelmillä: kokoekskluusiokromatografialla yhdistettynä ultravioletti/näkyvä monikulma-valonsironta ilmaisimeen (SEC-UV/Vis-MALLS), fosfori magneettiresonanssispektroskopialla (^{31}P-NMR), atomivoimamikroskopialla (AFM) sekä matriisiavusteisella laser-desorptio-ionisaatio lentoaikamassaspektrometrialla (MALDI-TOF MS). Tutkimustulokset osoittivat, että kummankin entsyymin tapauksessa β-kaseiinin ristisidonta tuotti levyn muotoisia nanopartikkeleita, joiden molekyylipaino (MP) oli keskimäärin 1 350 kg mol^{-1}, suurta entsyymiannostusta ja pitkää reaktioaikaa käytettäessä. SEC-UV/Vis-MALLS- tulosten perusteella kaupallinen β-kaseiini pystyttiin lähes kokonaisuudessaan ristisitomaan kummallakin entsyymillä. TrTyr-entsyymin tapauksessa ristisidonnan laajuus varmistui ^{31}P-NMR-analyysissä, jossa selvisi, että 91 % tyrosiini aminohappojen sivuketjuista osallistui ristisidosten muodostumiseen.

Proteiinien ristisidonnan vaikutusta ruoansulatukseen mallinnettiin *in vitro* seuraamalla β-kaseiinin pepsiinillä tapahtuvaa pilkkoutumista useilla analyysimenetelmillä. Tutkimustulokset osoittivat, että entsymaattisesti ristisidottu β-kaseiini on stabiili vatsalaukun happamissa olosuhteissa. Lisäksi havaittiin, että ristisidottua β-kaseiinia ei pystytä pilkkomaan pepsiinillä yhtä helposti kuin käsittelemätöntä β-kaseiinia. Entsymaattisen ristisidonnan vaikutusta β-kaseiinin allergeenisuuteen tutkittiin myös erilaisin biokemiallisin testimenetelmin. Tutkimustulosten perusteella entsymaattinen ristisidonta vähensi β-kaseiinin allergeenisuutta 14 %, kun se ristisidottiin TrTyr-entsyymillä ja 6 %, kun se käsiteltiin Tgase-entsyymillä.

Yhteenvetona voidaan todeta, että tutkimustuloksilla on merkitystä paitsi tyrosinaasientsyymin reaktiomekanismitutkimukselle, mutta myös elintarvike-, kosmetiikka-, lääke-, tekstiili- ja pakkausteollisuudelle.

ISBN
978-951-38-7421-6 (nid.)
978-951-38-7422-3 (URL: http://www.vtt.fi/publications/index.jsp)

Avainnimeke ja ISSN	Projektinumero
VTT Publications 1235-0621 (nid.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)	

Julkaisuaika	Kieli	Sivuja
Joulukuu 2010	Englanti, suom. tiiv.	86 s. + liitt. 66 s.

Projektin nimi	Toimeksiantaja(t)

Avainsanat	Julkaisija
Tyrosinase, transglutaminase, protein, β-casein, cross-linking, digestibility, allergenicity, SEC-UV/Vis-MALLS, MALDI-TOF MS, 31P-NMR, AFM	VTT PL 1000, 02044 VTT Puh. 020 722 4520 Faksi 020 722 4374

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ISSN 1235-0621 (soft back ed.)
ISBN 978-951-38-7422-3 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)