Isolation and Characterization of Ovomucin - a Bioactive Agent of Egg White

JAAKKO HIIDENHOVI

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TURKU, FINLAND – 2015

Food Chemistry and Food Development
Department of Biochemistry
University of Turku, Finland

Supervised by
    Director Eeva-Liisa Ryhänen, Ph.D.
    Green Technology
    Natural Resources Institute Finland (Luke)
    Jokioinen, Finland

    Professor Rainer Huopalahti, Ph.D.
    Department of Biochemistry
    University of Turku
    Turku, Finland

Reviewed by
    Professor Francoise Nau, Ph.D.
    Agrocampus Ouest
    UMR1253 Dairy and Egg Science and Technology
    Rennes, France

    Professor Olli Lassila, MD, Ph.D.
    Department of Biomedicine
    University of Turku
    Turku, Finland

Opponent
    Professor Markku Kulomaa, Ph.D.
    BMT / Molecular Biotechnology
    University of Tampere
    Tampere, Finland

Research director
    Professor Heikki Kallio, Ph.D.
    Department of Biochemistry
    University of Turku
    Turku, Finland

In memory of my parents

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ABSTRACT

The hen's egg is a source of new life. Therefore, it contains many biologically active compounds. In addition to being a very nutritious food and also commonly used in the food industry due to its many techno-functional properties, the egg can serve as a source of compounds used as nutra-, pharma- and cosmeceuticals. One such interesting compound is ovomucin, an egg white protein responsible for the gel-like properties of thick egg white. Previous studies have indicated that ovomucin and ovomucin-derived peptides have several different bioactive properties.

The objectives of the present study were to develop isolation methods for ovomucin, to characterize the structure of ovomucin, to compare various egg fractions as sources of ovomucin, to study the effects of various dissolving methods for ovomucin, and to investigate the bioactive properties of ovomucin and ovomucin-derived peptides.

A simple and rapid method for crude ovomucin separation was developed. By using this method crude ovomucin was isolated within hours, compared to the 1-2 days (including a dialysis step) needed when using several other methods. Structural characterization revealed that ovomucin is composed of two subunits, α- and β-ovomucin, as egg white protein formerly called α1-ovomucin seemed to be ovostatin. However, it might be possible that ovostatin is associated within β- and α-ovomucin. This interaction could even have some effect on the physical nature of various egg white layers.

Although filtration by-product fraction was a very prominent source of both crude and β-ovomucin, process development has reduced its amount so significantly that it has no practical meaning anymore. Thus, the commercial liquid egg white is probably the best option, especially if it generally contains amounts of β-ovomucin as high as were found in these studies. Crude ovomucin was dissolved both by using physical and enzymic methods. Although sonication was the most effective physical method for ovomucin solubilisation, colloid milling seemed to be a very promising alternative. A milk-like, smooth and opaque crude ovomucin suspension was attained by using a colloid mill.

The dissolved ovomucin fractions were further tested for bioactive properties, and it was found that three dissolving methods tested produced moderate antiviral activity against Newcastle disease virus, namely colloid milling, enzymatic hydrolysis and a combination of sonicaton and enzymatic hydrolysis. Moreover, trypsin-digested crude ovomucin was found to have moderate antiviral activity against avian influenza virus: both subtype H5 and H7.

SUOMENKIELINEN ABSTRAKTI

Kananmuna on yksi monikäyttöisimmistä elintarvikkeista. Sen lisäksi, että se on ravintoainekoostumukseltaan rikas elintarvike, sen toiminnallisia ominaisuuksia hyödynnetään elintarviketeollisuudessa laajalti. Muniessaan kana ei kuitenkaan pyri tuottamaan erinomaista ihmisravintoa, vaan sen tarkoituksena on uuden elämän, kananpojan, synnyttäminen. Tämän vuoksi kananmuna sisältää monia biologisesti aktiivisia komponentteja ja on siten potentiaalinen raaka-ainelähde uusille sovelluksille niin elintarvike-, lääke-, kosmetiikka- kuin bioteknologiaa hyödyntävälle teollisuudelle. Yksi tällainen potentiaalinen komponentti on ovomusiiini. Ovomusiini on valkuaisen proteiini, joka ylläpitää sakean valkuaisen geelimäistä rakennetta.

Tämän työn tavoitteena oli kehittää eristysmenetelmiä ovomusiinille, karakterisoida sen rakennetta ja verrata erilaisia valkuaisfraktioita ovomusiinin lähteenä. Tutkimuksessa verrattiin sekä entsymaattisten että erilaisten fysikaalisten menetelmien vaikutusta ovomusiinin liukoisuuteen. Lisäksi tutkittiin ovomusiinin ja siitä lohkottujen peptidien bioaktiivisia ominaisuuksia.

Ovomusiinin eristämiseksi kehitettiin yksinkertainen ja nopea menetelmä, jonka avulla ovomusiini voidaan eristää kananmunasta muutamassa tunnissa, kun monilla muilla menetelmillä siihen kuluu 1-2 päivää. Rakenteellisesti ovomusiini näytti koostuvan vain kahdesta alayksiköstä, α- ja β-ovomusiinista, koska aikaisemmin α1-ovomusiiniksi nimetty proteiini on tutkimusten mukaan ovostatiini. Todennäköisesti ovostatiini on kuitenkin vuorovaikutuksessa ovomusiinin kanssa siten, että sillä on myös vaikutusta kananmunan valkuaisosien rakenteellisiin ominaisuuksiin.

Teollisen munatuotteen, valkuaismassan, valmistuksen yhteydessä muodostuva sivuvirtajae, ns. suodatusfraktio, sisälsi runsaasti ovomusiinia. Valmistusprosessien kehittyessä sivuvirtajakeen määrä on kuitenkin pienentynyt niin merkittävästi, ettei sen hyödyntäminen ovomusiinin lähteenä ole enää käytännöllistä. Käytännön kannalta teollinen valkuaismassa oli potentiaalisin vaihtoehto tutkituista valkuaisfraktioista. Tutkimuksessa verrattiin sekä entsymaattisten että fysikaalisten menetelmien vaikutusta ovomusiinin liukoisuuteen. Fysikaalisista menetelmistä sonikoinnin avulla ovomusiini liukeni parhaiten, mutta myös kolloidimylly on potentiaalinen vaihtoehto. Sen avulla saatiin aikaiseksi maitomainen, opaalinen ja rakenteeltaan miellyttävä ovomusiinisuspensio. Sekä fysikaalisilla että entsymaattisilla menetelmillä voitiin tuottaa ovomusiinipreparaatteja, jotka estivät Newcastlen tautia aiheuttavan viruksen kasvua. Entsymaattisen hydrolyysin avulla tuotettu ovomusiinipreparaatti esti myös lintuinfluenssaa aiheuttavan viruksen kasvua *in vitro*.

LIST OF ABBREVIATIONS

AA	amino acid
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	angiotensin-I converting enzyme
AH	antihemagglutination
AIV	avian influenza virus
BALB/c	Bagg albino (inbred research mouse strain)
BSA	bovine serum albumin
Caco-2	human colon adenocarninoma cells
CV	column volume
Da	Dalton
DH	degree of hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPP4	dipeptyl dipeptidase-4
Gal	galactose
GalNAc	N-acetylgalactosamine
GFC	gel filtration chromatography
GLP-1	glucagon-like peptide-1
GluNAc	N-acetylglucosamine
ELISA	enzyme-linked immunosorbent assay
E/S	enzyme substrate ratio
EW	egg white
FPLC	fast protein liquid chromatography
HI	hemagglutination inhibition
IEP	isoelecric precipitation
IV	influenza virus
LC	liquid chromatography
LDL	low density lipoprotein
LPS	lipopolysaccharide
LYZ	lysozyme
Man	mannose
MBTH	3-methyl-2-benzothiazoline hydrazone hydrochloride
MIC	minimal inhibition concentration
MS	mass spectrometry
MW	molecular weight
NANA	N-acetylneuramic acid = sialic acid
NDV	Newcastle disease virus
OGP	ovomucin glycopeptide
ORAC	oxygen radical absorbance capacity

OVA	ovalbumin
OMUC	ovomucoid
OVMC	ovomucin-C
OVMG	ovomucin-G
OVT	ovotransferrin
PBS	phosphate buffered saline
PITC	phenylthiocarbamate
RP-HPLC	reversed phase-high performance liquid chromatography
RS	relative solubility
RT	room temperature
RV	rotavirus
SAS	statistical analysis system
SR-180	sarcoma-180
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDS-PGGE	sodium dodecyl sulphate-polyacrylamide gradient gel electrophoresis
SCM	sonicated and carboxy-methylated
TNFα	tumour necrosis factor-alpha
UV	ultraviolet
VLDL	very low density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

I.   Hiidenhovi, J.; Aro, H.S.; Kankare, V. Separation of ovomucin subunits by gel filtration: enhanced resolution of subunits by using a dual-column system. *J. Agric. Food Chem.* **1999**, *47*, 1004-1008.

II.   Hiidenhovi, J.; Mäkinen, J.; Huopalahti, R.; Ryhänen, E.-L. Comparison of different egg albumen fractions as sources of ovomucin. *J. Agric. Food Chem.* **2002**, *50*, 2840-2845.

III. Hiidenhovi, J.; Ek-Kommonen, C.; Järvenpää, E.; Huopalahti, R.; Ryhänen, E.-L. Characterization of crude ovomucins obtained from various egg white layers. *Submitted.*

IV.   Hiidenhovi, J.; Hietanen A.; Ek-Kommonen, C.; Järvenpää, E.; Huopalahti, R.; Ryhänen, E.-L. Effects of different dissolving methods on solubility and the bioactive properties of ovomucin. *Submitted.*

1   INTRODUCTION

The hen's egg is a one of the most multipurpose of foods. It is rich in high quality proteins and fats, valuable minerals and trace elements, together with vitamins. In addition, the bioavailability, i.e. the absorption, of these nutritionally important components, is excellent from eggs[1]. Furthermore, eggs are one of a few so-called multifunctional foods. Thus, they are used widely in the food industry e.g. due to their foaming, gelling and emulsification properties.

Structurally, eggs are composed of three parts: the eggshell (including eggshell membranes), the white (or albumen), and the yolk. On average, the yolk comprises 30%, the white 60% and the eggshell 10% of the weight of the egg. Egg white is further divided into four distinct layers: outer thin white, thick white, inner thin white and the chalaziferous layer or inner thick white. The shares of each layer are about 23%, 57%, 17% and 3%, respectively[2]. Proteins are present both in the egg white and the yolk, while virtually all the lipids are in the yolk. Minerals are the major component of the eggshell. More detailed chemical compositions for Finnish eggs are available on the Fineli database (http://www.fineli.fi/). The weight and composition of each part of hen's egg is affected by various factors such as the age of the hen, hybrid and feeding[3].

As a hen is laying an egg, its aim is not to produce food for humans but to give a birth to a new life. Thus the biological roles of both eggshell and egg white are to protect the yolk (and embryo) from mechanical impacts and the penetration of pathogens. In addition, during hatching the egg white serves as a nutriment for the developing embryo[4]. The defence mechanism of the egg white is acting both physically and chemically. The gel-like consistency of egg white attenuates the effect of external shocks and serves as a barrier for microbial movement. Moreover, in fresh eggs both thick egg white layers and chalaza cords are working together to keep the yolk in the middle of the egg[5]. The chemical defence is based mainly on the action of egg white proteins. Many of the proteins have distinctive properties that suggest antimicrobial activity e.g. proteinase inhibition or ability to bind nutrients (vitamins, trace elements) that are essential for microbes[6].The exact number of egg white proteins is not known, but by using mass spec techniques 158 proteins have been identified, and a further 44 proteins were identified tentatively[7]. Naturally, most of these proteins exist in small quantities, and a little over ten proteins comprise about 96% of total egg white proteins[3,8].

One interesting egg white protein is ovomucin, a sulphated glycoprotein, characterised by a high molecular weight and a subunit structure, first

described by Eichholz[9]. In addition to egg white, ovomucin is also found in chalazae [10,11] and in the outer layer of the vitelline membrane[12-15]. Ovomucin is responsible for the gel-like properties of thick egg albumen. Fresh eggs having high quality egg white (expressed as Haugh units) have been reported to contain much more ovomucin in thick white than low quality eggs[16,17]. Egg white thinning, the most important change in egg white during storage, is usually attributed to the degradation of the ovomucin complex[18,19]. In addition, ovomucin and ovomucin-derived peptides have been found to involve different kinds of biological activities. In the review following, the physical and chemical characteristics, isolation methods and various properties of ovomucin will be discussed in more detail.

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2 REVIEW OF THE LITERATURE

2.1 Chemical and structural characterization of ovomucin

2.1.1 Ovomucin subunits

It is generally recognized that ovomucin is comprised of at least two subunits: α-ovomucin and β-ovomucin[20,21]. In addition, Itoh et al.[11] reported that the α-ovomucin, which was fractioned by gel filtration chromatography (GFC), separated into two bands on SDS-PAGE. They named these compounds α1- and α2-ovomucin, and further estimated the molecular weights (MWs) 150, 220 and 400 kDa for α1-, α2- and β-ovomucin, respectively, by using SDS-PAGE. Similar SDS-PAGE patterns for reduced crude ovomucin have been reported by many authors[22-28]. Numerous studies have confirmed the presence of β-ovomucin in the gel region 350-400 kDa, and indicated that the primary area for α-ovomucin seems to be 200-250 kDa in SDS-PAGE[7,11,23,24,28,29-31]. However, the identity of 150 kDa protein is somewhat uncertain.

Shan et al.[26] have further identified the SDS-PAGE bands corresponding to α1- and α2-ovomucin by using tryptic peptide mass analysis, and reported that all the amino acid sequences of peptides obtained from these bands were similar to that of α-ovomucin[32]. In addition, Hayakawa & Sato[33] reported that monomeric α-ovomucin tended to dimerize in dilute salt solutions. Robinson & Monsey[21] have also noted that the reduced α-ovomucin showed multiple sedimentation coefficient values in the phosphate buffer. Similar heterogenous behaviour, e.g. the existence of three compounds, during sedimentation velocity measurements of ovomucin, has also been reported by several authors[34-36].

By contrast, Offengenden et al.[30,31] have reported, that the primary protein detected in 150 kDa band by using mass spectra analysis was found to be ovostatin[37,38]. Similarly, although LC-MS/MS analysis revealed α-ovomucin existence on SDS-PAGE in gel region of 100-250 kDa, and for ovostatin in a gel region of 80-200 kDa, the primary area seems to be 200-250 kDa for α-ovomucin and about 150-180 kDa for ovostatin[7,29]. Moreover, the molecular weight of ovostatin subunit has been estimated to be 162-195 kDa[38-41].

Summarizing, it seems quite likely that, at least in fresh eggs, the main proteins in SDS-PAGE gel regions approximately 350-400, 200-280 and 150-180 kDa are β-ovomucin, α-ovomucin and ovostatin, respectively. However, the existence of smaller α-ovomucins migrating in lower gel regions cannot be totally excluded. As stated by Offengenden et al.[30], degradation of α-ovomucin could happen during egg storage or ovomucin isolation. Very likely the same applies to β-ovomucin.

2.1.1.1 α-Ovomucin – a proteinaceous subunit

The literature review of amino acid and carbohydrate compositions for α-ovomucin obtained from various egg white fractions are shown in **Table 1** and **2**, respectively. As can be seen in **Table 1** α-ovomucin seemed to be rich both in aspartic acid and glutamic acid, thus contributing to the negative charge of ovomucin and suggesting the existence of N-glycans. Moreover, α-ovomucin contains about twice the amount of cysteine as β-ovomucin, and these residues are supposed to enable both the dimerization of α-ovomucin and the polymerization of ovomucin molecules[33].
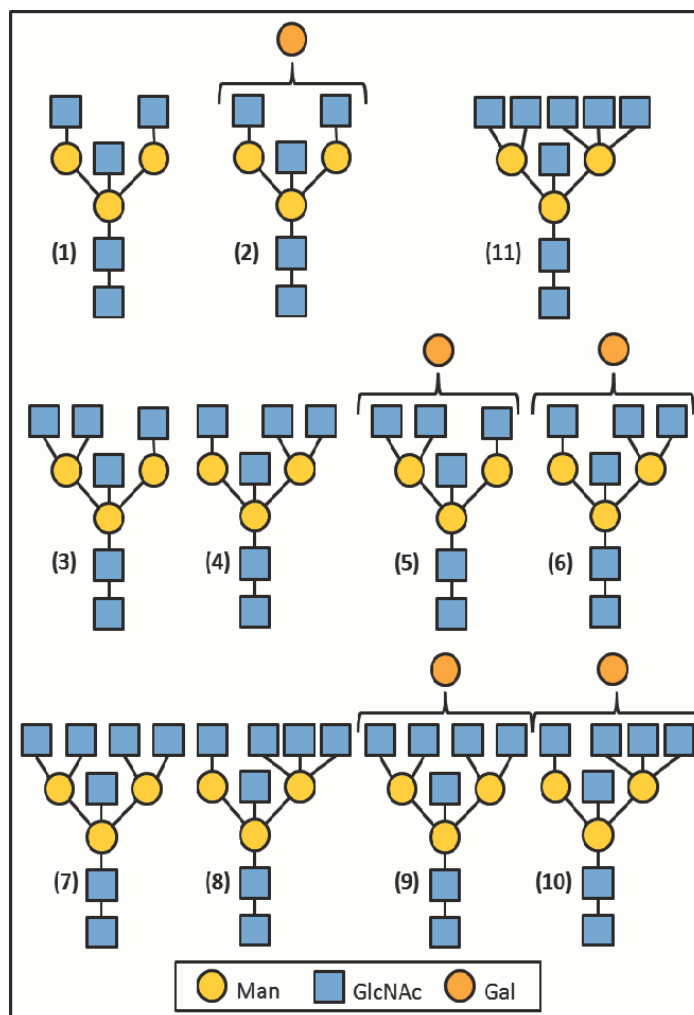
Fig. 1 Possible structures of α-ovomucin N-glycans. These branched chain oligosaccharides are linked to asparagine residues[30,31].

Table 1 Amino acid compositions of α-ovomucin subunits obtained from various egg white fractions.

Egg fraction	Amino acid (mol/100 mol)																		Ref.
	Ala	*Arg*	*Asp*	*Cys*	*Glu*	*Gly*	*His*	*Ile*	*Leu*	*Lys*	*Met*	*Phe*	*Pro*	*Ser*	*Thr*	*Trp*	*Tyr*	*Val*	*Ref.*
Thick egg white	4.8	2.7	9.9	8.7	11.4	6.8	2.0	4.6	6.4	5.5	0.8	4.4	5.4	8.1	7.5	1.3	3.4	6.1	21
	5.1	3.2	10.1	7.0	10.6	7.2	2.2	5.0	6.3	6.5	1.9	4.5	5.8	7.8	7.2	nd	3.8	5.8	11
	4.8	2.3	11.9	5.2	10.9	7.4	2.1	5.2	6.2	6.4	2.2	4.0	5.9	7.7	7.8	nd	3.5	6.6	27
Mean ± SD	**4.9**	**2.7**	**10.6**	**7.0**	**11.0**	**7.1**	**2.1**	**4.9**	**6.3**	**6.1**	**1.6**	**4.3**	**5.7**	**7.9**	**7.5**	**1.3**	**3.6**	**6.2**	
	± 0.2	± 0.5	± 1.1	± 1.8	± 0.4	± 0.3	± 0.1	± 0.3	± 0.1	± 0.6	± 0.7	± 0.3	± 0.3	± 0.2	± 0.3		± 0.2	± 0.4	
Thin egg white	**4.9**	**3.3**	**10.3**	**5.8**	**10.5**	**7.2**	**2.3**	**4.6**	**6.6**	**6.6**	**2.0**	**4.5**	**6.0**	**7.8**	**7.5**	**nd**	**0.7**	**6.4**	11
Gel (Thick)	4.8	2.2	13.9	4.0	10.3	8.2	1.6	4.7	6.2	6.6	2.3	4.5	3.2	8.4	8.0	nd	3.7	7.6	42,43
	4.8	2.6	10.6	7.0	9.4	7.7	2.1	5.6	7.2	5.8	1.7	4.0	5.6	7.8	6.7	nd	3.5	3.6	44
	6.6	6.0	12.8	2.9	9.3	8.1	2.1	4.6	6.9	6.2	1.4	3.9	5.1	8.7	7.7	nd	3.1	4.5	45
Mean ± SD	**5.4**	**3.6**	**12.4**	**4.6**	**9.7**	**8.0**	**1.9**	**5.0**	**6.8**	**6.2**	**1.8**	**4.1**	**4.6**	**8.3**	**7.5**	**nd**	**3.4**	**5.2**	
	± 1.0	± 2.1	± 1.7	± 2.1	± 0.6	± 0.3	± 0.3	± 0.6	± 0.5	± 0.4	± 0.5	± 0.3	± 1.3	± 0.5	±0.7		± 0.3	± 2.1	
Liquid (Thick)	4.9	2.7	10.4	6.7	9.9	7.4	2.1	6.8	6.8	5.6	1.7	3.9	5.4	7.9	6.6	1.3	3.6	6.4	44,46

nd = not determined, SD = standard deviation

Table 2 Carbohydrate composition of α-ovomucin subunits obtained from various egg white fractions.

Egg fraction	Carbohydrates (% of ovomucin)								
	Hexoses	*Man*	*Gal*	*Hexosamines*	*GlcNAc*	*GalNAc*	*NANA*	*Total*	*Ref.*
Thick egg white	6.4	4.6	1.8	7.3	6.7	0.6	1.0	14.7	21
	4.6	3.4	1.2	5.6	5.3	0.3	1.3	11.5	11
	5.0	3.9	1.1	3.1	3.1	0	1.1	9.2	27
Mean ± SD	**5.3**	**4.0**	**1.4**	**5.3**	**5.0**	**0.5**	**1.1**	**11.8**	
	± 0.9	± 0.6	± 0.4	± 2.1	± 1.8	± 0.2	± 0.2	± 2.8	
Thin egg white	**4.4**	**2.9**	**1.5**	**5.2**	**4.8**	**0.4**	**1.9**	**11.5**	11
Gel (Thick)	6.8	4.1	2.5	6.7	6.7	0	0.8	14.3	42,43
	6.3	2.0	4.3	8.5	8.5	0	0.7	15.5	44
	6.7	4.2	2.5	7.6	7.6	0	1.1	15.4	45
Mean ± SD	**6.6**	**3.4**	**3.1**	**7.6**	**7.6**	**0**	**0.9**	**15.1**	
	± 0.3	± 1.2	± 1.0	± 0.9	± 0.9		± 0.2	± 0.7	
Liquid (Thick)	**6.3**	**1.9**	**4.4**	**7.5**	**7.5**	**0**	**0.3**	**14.1**	44,46

SD = standard deviation

The primary amino acid sequence of α-ovomucin was introduced by Watanabe et al.[32] The polypeptide consists of 2,108 amino acids with a relative molecular mass of 233,553 Da. Thus the estimated size of α-ovomucin could be calculated to be 256–278 kDa, because carbohydrate content of α-ovomucin has been reported to vary from 9.2% to 15.5% (**Table 2**). Analytically, the MW of α-ovomucin has been estimated to be 210–250 kDa or 180–210 kDa by using SDS-PAGE[11,17,23,27,30,31] or analytical ultracentrifugation [21,33], respectively.

α-Ovomucin has been supposed to contain N-glycans as the main carbohydrates are mannose and N-glucosamine (**Table 2**), and it was also reported to contain 24 potential N-glycosylation sites[32]. Offengen et al.[30,31] have studied N-glycolysation of ovomucin in more detail. They found 18 potential N-glycolysation sites in α-ovomucin, of which two were non-glycolysated, and one site was occupied as both glycolysated and non-glycolysated form. The remaining 15 sites were glycolysated. Interestingly, glycopeptide analysis of five glycosylation sites revealed glycan heterogeneity, even up to five glycans *per* site. The identified glycans were found to have a pentasaccharide core of $GlcNAc_2Man_3$ and a bisecting GlcNAc. These glycan forms are also typical of other EW proteins, such as ovalbumin, ovotranferrin and ovomucoid[47], and as glycolysation is known to be tissue- and species-specific[48], it was assumed that N-glycans of α-ovomucin have equal structures as the above-mentioned egg white (EW) proteins. **Figure 1** shows possible structures of α-ovomucin N-glycans as proposed by Offengenden et al[30]. In their studies no terminal sialic acids were reported, although α-ovomucin has been reported to contain a small amount of sialic acid (**Table 2**).

2.1.1.2 β-Ovomucin – a carbohydrate-rich subunit

The results of a corresponding literature review of amino acid and carbohydrate compositions for β-ovomucin obtained from various egg white fractions are shown in **Table 3** and **4**, respectively. The most abundant amino acids are serine and threonine (**Table 3**) thus suggesting the existence of O-glycosidic glycans. **Figure 2** shows the structures of several O-glycans released from ovomucin by β-elimination. Most likely these glycans are from β-ovomucin, as it contains most of the galactose and sialic acid found in ovomucin. The carbohydrates are presented in ovomucin as oligosaccharide moieties consisting of about 2-6 carbohydrate units having both straight and branched chains and also containing terminal sialic acid and ester sulphates. Moreover, two potential N-glycolysation sites have been identified in β-ovomucin[30,31].

To the author's knowledge, the complete amino acid sequence of β-ovomucin has not been revealed officially so far. Watanabe et al.[56] reported already in the early 2000s an amino acid sequence of a β-ovomucin fragment consisting of 827 amino acids and having a molecular weight of 91,836 Da.

Since then, this sequence has been upadated several times, but the status of β-ovomucin sequence is still partial, consisting of 1,185 AAs with a molecular mass of 132,217 Da. However, it should be noted that a website of GenScript (http://www.genscript.com/cgi-bin/orf/refseq.pl?acc=XM_426405) gives a predicted amino acid sequence for β-ovomucin. This sequence consists of 2,677 amino acids, including the above-mentioned partial sequence. The relative molecular mass of β-ovomucin was estimated to be 291,308 Da by using the ProteinCalculator 3.4 program[57]. Furthermore, the estimated size of β-ovomucin could be calculated to be about 630 kDa, if it is assumed the carbohydrate content of β-ovomucin is on average about 54% (**Table 4**). Interestingly, this value is within a range obtained in previous analytical studies, as the molecular weight of the β-ovomucin subunit has been estimated by SDS-PAGE or ultracentrifugation to be between 350 and 720 kDa[7,11,17,23,24,27-31,33,46,58]. Although these results are quite promising, it should be noted that this GenScript-sequence has not been published or loaded into any protein sequence database, such as UniProt (http://www.uniprot.org/). Thus, it has not been peer-reviewed.

2.1.2   Ovomucin complex

Egg white contains two forms of ovomucin: insoluble and soluble ovomucin. Soluble ovomucin is present both in thick and thin albumen, while insoluble ovomucin is found only in thick albumen[59]. In addition, thick white can be divided into gel and liquid fraction by using ultracentrifugation[18]. Insoluble and soluble ovomucin contain different proportions of ovomucin subunits (**Table 5**). Insoluble ovomucin seems to contain about 2.5 times the β-ovomucin compared to that in soluble ovomucin. This abundance is attributed to the higher apparent viscosity of insoluble ovomucin than that of soluble ovomucin[63]. Similarly, the molecular weight of insoluble ovomucin is greater than that of soluble ovomucin. The MW values from 27 to 56×10^6 Da for insoluble ovomucin in 1.1 M KCl (pH 7.9) were estimated using light scattering[64]. For soluble ovomucin, molecular weight values in the range from 5.6 to 8.3×10^6 Da have been reported[35,36,45,65]. The variation of molecular weights in both ovomucin forms is presumably due to differences in both measuring methods and conditions and the heterogeneity of ovomucin

Table 3 Amino acid compositions of β-ovomucin subunits obtained from various egg white fractions.

Egg fraction	Amino acid (mol/100 mol)																		Ref.
	Ala	Arg	Asp	Cys	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val	
Thick egg white	6.4	2.6	7.2	5.4	8.6	4.6	1.7	3.9	6.9	5.1	1.4	3.0	7.4	14.3	13.3	0.8	2.5	4.9	21
	5.0	2.6	5.9	3.0	8.2	4.3	2.1	3.9	7.0	6.1	1.6	3.8	8.7	15.9	14.3	nd	2.6	5.1	11
	7.7	1.6	4.8	1.4	7.9	3.2	1.1	4.1	7.3	4.6	4.4	2.6	9.0	18.9	14.8	nd	1.7	4.9	27
Mean ± SD	**6.4**	**2.3**	**6.0**	**3.3**	**8.2**	**4.0**	**1.6**	**4.0**	**7.1**	**5.3**	**2.5**	**3.1**	**8.4**	**16.4**	**14.1**	**0.8**	**2.3**	**5.0**	
	±1.4	±0.6	±1.2	±2.0	±0.4	±0.7	±0.5	±0.1	±0.2	±0.8	±1.7	±0.6	±0.9	±2.3	±0.8		±0.5	±0.1	
Thin egg white	**5.4**	**2.8**	**5.8**	**3.1**	**7.7**	**4.7**	**1.8**	**3.5**	**6.6**	**5.3**	**1.7**	**3.6**	**9.5**	**15.7**	**15.8**	**nd**	**2.4**	**4.6**	11
Gel (Thick)	6.2	2.4	8.7	2.3	8.1	5.3	1.3	4.2	7.3	5.8	1.8	3.5	4.9	14.5	14.7	nd	2.8	5.5	42,43
	6.0	1.1	2.6	2.5	5.6	2.1	1.1	7.2	7.3	3.0	1.1	2.6	8.9	18.4	20.0	nd	6.1	3.1	44
	6.1	3.8	6.4	2.5	9.6	5.6	2.6	4.0	7.2	6.1	1.8	4.3	5.5	12.8	12.8	nd	2.8	6.2	45
Mean ± SD	**6.1**	**2.4**	**5.9**	**2.4**	**7.8**	**4.3**	**1.7**	**5.1**	**7.3**	**5.0**	**1.6**	**3.5**	**6.4**	**15.2**	**15.8**	**nd**	**3.9**	**4.9**	
	±0.1	±1.4	±3.1	±0.1	±2.0	±1.9	±0.8	±1.8	±0.1	±1.7	±0.4	±0.9	±2.2	±2.9	±3.7		±1.9	±1.6	
Liquid (Thick)	**5.2**	**2.4**	**4.8**	**3.4**	**6.5**	**3.7**	**1.9**	**8.6**	**7.4**	**5.7**	**1.1**	**2.5**	**7.2**	**15.2**	**13.5**	**2.0**	**5.1**	**4.2**	44,46

nd = not determined, SD = standard deviation

Table 4 Carbohydrate compositions of β-ovomucin subunits obtained from various egg white fractions.

Egg fraction	Carbohydrates (% of ovomucin)								Ref.
	Hexoses	Man	Gal	Hexosamines	GlcNAc	GalNAc	NANA	Total	
Thick egg white	23.3	4.1	19.2	19.7	11.0	8.7	13.8	56.8	21
	19.6	0.2	19.4	13.7	7.6	6.1	14.9	48.2	11
	29.9	0.6	29.3	18.6	11.4	7.2	18.2	66.7	27
Mean ± SD	**24.3**	1.6	22.6	**17.3**	10.0	7.3	**15.6**	**57.2**	
	± 5.2	± 2.1	± 5.8	± 3.2	± 2.1	± 1.3	± 2.3	± 9.3	
Thin egg white	**18.0**	0.2	17.8	**15.2**	7.1	8.1	**19.1**	**52.3**	11
Gel (Thick)	18.4	1.5	16.6	18.3	10.4	7.9	11.4	48.1	42,43
	20.1	0.4	19.7	21.6	11.8	9.8	15.3	57.0	44
	18.4	0.4	18.0	19.9	11.3	8.2	12.3	50.6	45
Mean ± SD	**19.0**	0.8	18.1	**19.9**	11.2	8.6	**13.0**	**51.9**	
	± 1.0	± 0.6	± 1.6	± 1.7	± 0.7	± 1.1	± 2.0	± 4.6	
Liquid (Thick)	**19.8**	0.4	19.4	**19.1**	11.1	8.0	**12.6**	**51.5**	44,46

nd = not determined, SD = standard deviation

Fig. 2 Structures of O-glycans of ovomucin. Oligosaccharide moieties consist of 2-6 carbohydrate units with both straight and branched chains that are linked to serine and threonine residues[49-55].

Table 5 Percentage distribution of ovomucin subunits in various ovomucins.

Egg layer/ovomucin form	Percentage distribution (%)		Ref.
	β-ovomucin	α-ovomucin	
Thick /insoluble	27	73	[60]
	34	66	[61]
Thin/soluble	12	88	[60]
Chalazae/insoluble	21	79	[62]
Gel/insoluble	33	67	[46]
Liquid/soluble	13	87	[46]

The literature review of amino acid and carbohydrate compositions of ovomucin complex obtained from various egg white fractions is presented in **Table 6** and **7**, respectively. Surprisingly, the variation of AA compositions among various egg white layers seemed to be quite moderate. Actually, the variation within groups seemed to be about the same magnitude as between the groups. In contrast, the carbohydrate composition of various ovomucins varied considerably: ovomucins obtained from whole, thick and thin egg white contained carbohydrates about 18%, 31% and 19% of ovomucin, respectively (**Table 7**). In addition, the carbohydrate content for gel and liquid fractions was 33% and 16%, respectively. These differences are related to the variation of β-ovomucin content in ovomucin complexes (**Table 5**).

2.2   Isolation and purification of ovomucin

2.2.1   Isoelectric precipitation method

Ovomucin is usually fractionated from egg albumen by using the isoelectric precipitation (IEP) method. Numerous variations of this method are presented in the literature, but usually the IEP method can be said to comprise three steps as follows: precipitation, collection and the washing step. However, in some methods an additional step is added before ovomucin precipitation as lysozyme has been removed by crystallization from egg white[66,77-79]. However, the effectiveness of this procedure seemed to be somewhat questionable, since SDS-PAGE showed that the obtained ovomucin preparations still contained lysozyme as an impurity[81].

In the precipitation step egg albumen is usually diluted with water, followed by acidification to or near to its isoelectric point. Quite often, the pH is adjusted to pH 6.0[18,22,23,60,80,81], but both lower (4.5-5.0)[66,77-80] and higher (7-8)[80,82] pH-values have also been used. Different acids, such as H_2SO_4[18,20,60], HCl[23,79], acetic acid[78,80,82] or lactic acid[22], could be used for acidification. Alternatively, egg white might be diluted with water[11,17,19,21,58,61,83,84] or salt solution[18,76,85] without any pH control.

Table 6 Amino acid composition of ovomucin complex obtained from various egg white fractions.

Egg fraction	Ala	Arg	Asp	Cys	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val	Ref.
												Amino acid (mol/100 mol)							
Whole egg white	5.8	3.5	9.6	6.2	11.0	6.9	2.1	4.6	6.9	5.9	1.9	3.9	5.5	8.2	7.1	nd	4.1	6.6	66
	6.4	3.4	9.4	4.2	11.1	6.0	1.9	5.5	7.7	5.4	2.6	4.4	5.1	8.7	6.3	1.6	3.2	7.5	67
	6.0	3.1	8.7	8.1	10.0	6.9	1.9	4.5	7.1	5.3	0.6	3.5	6.4	9.6	9.0	nd	2.7	6.4	68
	6.2	4.9	12.3	5.9	8.8	7.2	1.7	4.7	7.0	5.6	0.9	4.9	5.1	8.5	7.1	nd	2.9	6.2	22
	3.9	3.7	10.2	6.0	8.1	5.8	2.3	4.9	6.9	6.4	1.3	7.6	5.9	8.4	7.9	nd	4.4	6.4	69,70
	6.4	4.6	12.0	4.7	12.0	7.7	2.5	5.5	8.1	7.4	2.0	3.9	nd	6.8	7.4	nd	3.4	7.8	24,28
Mean ± SD	**5.8** ± 1.0	**3.9** ± 0.7	**10.4** ± 1.5	**5.9** ± 1.4	**10.2** ± 1.5	**6.8** ± 0.7	**2.1** ± 0.3	**5.0** ± 0.4	**7.3** ± 0.5	**6.0** ± 0.8	**1.6** ± 0.8	**4.7** ± 1.5	**5.6** ± 0.6	**8.4** ± 0.9	**7.5** ± 0.9	**1.6**	**3.5** ± 0.7	**6.8** ± 0.7	
Thick egg white	4.7	3.1	9.1	7.2	10.2	5.9	2.3	4.3	6.7	6.3	2.0	3.9	6.4	9.3	8.4	0.8	2.9	6.2	21
	6.1	3.4	11.1	6.7	9.2	8.4	1.9	5.6	6.1	6.5	1.6	5.0	2.2	9.0	6.4	nd	3.3	7.6	34
	4.9	2.9	11.8	7.0	11.5	7.0	2.0	5.5	6.3	6.0	1.8	4.1	4.6	4.8	6.3	1.8	3.6	7.6	71
	5.6	1.8	11.8	8.3	8.7	7.2	1.8	5.2	6.8	4.1	1.9	4.8	5.7	7.1	7.2	1.6	3.7	7.3	71
	4.6	3.2	10.5	7.3	11.5	6.3	2.2	4.7	6.6	6.0	1.6	4.2	6.3	7.8	7.7	nd	3.5	6.1	72
	5.2	2.2	10.4	4.0	9.9	6.8	2.2	4.6	6.5	5.9	2.2	3.7	9.2	9.4	8.5	nd	3.0	6.3	27
Mean ± SD	**5.2** ± 0.6	**2.8** ± 0.6	**10.8** ± 1.0	**6.8** ± 1.5	**10.2** ± 1.2	**6.9** ± 0.9	**2.1** ± 0.2	**5.0** ± 0.5	**6.5** ± 0.3	**5.8** ± 0.9	**1.9** ± 0.2	**4.3** ± 0.5	**5.7** ± 2.3	**7.9** ± 1.8	**7.4** ± 1.0	**1.4** ± 0.5	**3.3** ± 0.3	**6.9** ± 0.7	
Thin egg white	6.6	3.3	9.5	6.3	9.8	8.6	2.2	5.1	7.5	5.4	1.7	5.0	2.3	9.7	8.7	nd	3.4	7.0	34
	7.0	3.6	10.8	6.8	11.1	7.2	1.9	5.3	8.4	6.7	1.7	3.9	4.9	8.0	7.1	nd	3.4	6.0	59
Mean ± SD	**6.8** ± 0.3	**3.5** ± 0.2	**10.2** ± 0.9	**6.6** ± 0.4	**10.5** ± 0.9	**7.9** ± 1.0	**2.1** ± 0.2	**5.2** ± 0.1	**8.0** ± 0.6	**6.1** ± 0.9	**1.7** ± 0.0	**4.5** ± 0.8	**3.6** ± 1.8	**8.9** ± 1.2	**7.9** ± 1.1	**nd**	**3.4** ± 0.0	**6.5** ± 0.7	
Gel (thick)	4.5	2.9	10.0	7.9	10.0	6.4	2.1	4.6	5.8	6.1	1.6	3.9	5.1	7.6	7.5	3.6	3.2	6.3	71
Liquid (thick)	7.3	3.4	10.8	6.0	10.4	7.3	1.8	5.8	8.4	6.6	1.7	4.1	4.8	8.2	7.2	nd	3.5	5.7	59
	4.6	3.4	11.0	7.8	10.8	6.8	2.0	4.6	6.1	5.6	1.6	4.0	5.8	7.6	7.2	1.2	3.4	6.4	71
Mean ± SD	**6.0** ± 1.9	**3.4** ± 0.0	**10.9** ± 0.1	**6.9** ± 1.3	**10.6** ± 0.3	**7.1** ± 0.4	**1.9** ± 0.1	**5.2** ± 0.8	**7.3** ± 1.6	**6.1** ± 0.7	**1.7** ± 0.1	**4.1** ± 0.1	**5.3** ± 0.7	**7.9** ± 0.4	**7.2** ± 0.0	**1.2**	**3.5** ± 0.1	**6.1** ± 0.5	

nd = not determined, SD = standard deviation

Table 7 Carbohydrate composition of ovomucin complex obtained from various egg white fractions.

Egg fraction	Carbohydrates (% of ovomucin)								
	Hexoses	Man	Gal	Hexosamines	GlcNAc	GalNAc	NANA	Total	Ref.
Whole egg white	6.6	2.2	4.4	7.1	4.7	2.4	6.0	19.7	73
	5.3	nd	nd	8.1	6.4	1.7	2.0	15.4	66
	6.5	nd	nd	7.8	6.4	1.4	4.0	18.3	67
Mean ± SD	**6.1**	2.2	4.4	**7.7**	5.8	1.8	**4.0**	**17.8**	
	± 0.7			± 0.5	± 1.0	± 0.5	± 2.0	± 2.2	
Thick egg white	10.7	1.4	9.3	13.9	9.1	4.8	8.7	33.3	74
	9.0	nd	nd	7.5	nd	nd	7.5	24.0	18
	11.3	2.8	8.5	12.0	8.0	4.0	7.4	30.7	21
	11.2	2.7	8.5	12.6	8.6	4.0	7.1	30.9	61
	11.4	nd	nd	12.1	8.0	4.1	7.4	30.9	34
	13.1	3.1	10.0	14.3	11.0	3.3	7.3	34.7	27
Mean ± SD	**11.1**	2.5	9.1	**12.1**	8.9	4.0	**7.6**	**30.8**	
	± 1.3	± 0.8	± 0.7	± 2.4	± 1.2	± 0.5	± 0.6	± 3.7	
Thin egg white	6.7	nd	nd	5.8	nd	nd	3.5	16.0	18
	9.7	nd	nd	10.6	7.7	2.9	6.0	26.3	34
	4.8	nd	nd	7.4	nd	nd	2.8	15.0	59
Mean ± SD	**7.1**	nd	nd	**7.9**	7.7	2.9	**4.1**	**19.1**	
	± 2.5			± 2.4			± 1.7	± 6.3	
Gel (Thick)	12.5	nd	nd	12.8	nd	nd	9.9	35.2	18
	12.3	nd	nd	11.9	nd	nd	9.5	33.7	75
	12.0	3.0	9.0	12.2	8.2	4.0	7.2	31.4	76
Mean ± SD	**12.3**	3.0	9.0	**12.3**	8.2	4.0	**8.9**	**33.4**	
	± 0.3			± 0.5			± 1.5	± 1.9	
Liquid (Thick)	**5.6**	nd	nd	**7.8**	nd	nd	**3.0**	**16.4**	59

nd = not determined, SD = standard deviation

In general, the collection step is carried out by using centrifugation. Ovomucin precipitation has also been collected by settling[64,67,86] or by sieve[23,87]. In the washing step ovomucin precipitate is washed several times with salt solutions, such as 2% KCl, to remove other egg white proteins, and finally with water to wash out salts. Alternatively, salts may be removed by using a dialysis step.[88,89].

Ovomucin has been reported to interact with lysozyme[20,35,43,90-93] as well as with other egg white proteins, lsuch as ovalbumin and ovotransferrin *in vitro*[94]. In addition to ovomucin, lysozyme has also been reported to form complexes with other egg white proteins, such as ovalbumin, ovotransferrin, ovoglobulins and ovomucoid[94,95]. Thus it is not surprising, that in addition to ovomucin, the crude ovomucin precipitate also contains ovotransferrin, ovalbumin, ovomucoid and lysozyme (**Figure 3**). Crude ovomucin without salt washings has been reported to have a purity of about 64%[24-26]. Conventionally co-precipitated proteins were removed with repeated salt washings, although previous studies have shown that extensive KCl washing cause a loss of ovomucin[80,97]. According to Lyndrup[80], only 120 mg of ovomucin was obtained from 100 ml of thick egg white after an extensive washing procedure, while the KCl washings contained altogether about 360 mg of ovomucin. Also, Omana & Wu[25] reported similar results as they extracted ovomucin with 500 mM KCl: the ovomucin yield was about 105 mg compared to 260 mg obtained without salt washings.
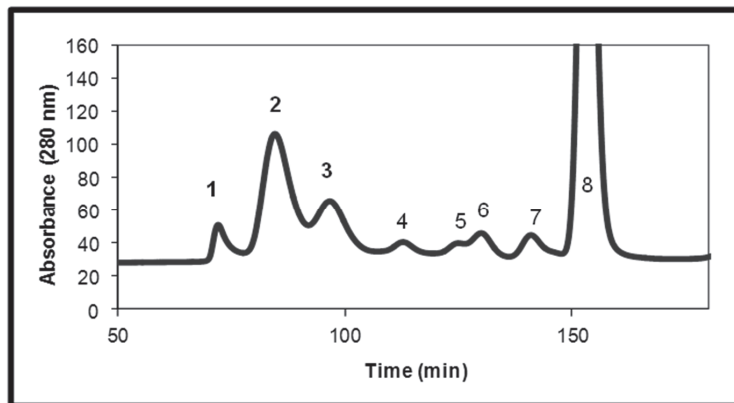
Fig. 3 Elution profile of crude ovomucin obtained by Superose 6 HR gel-filtration chromatography[96]. 1 = β-ovomucin, 2 = α-ovomucin, 3 = ovostatin, 4 = ovotransferrin, 5 = unknown (globulins?), 6 = ovalbumin, 7 = ovomucoid and 8 = lysozyme. Reprinted from Hiidenhovi 2007 with permission. Copyright 2007 Springer Science+Business Media.

Recently, modifications of the IEP method have been proposed, which seemed to overcome this reduction in ovomucin yield[24-26,28]. In this so-called 2-step method, ovomucin was isolated by using two consecutive salt precipitation steps. It has been reported that, by using the 2-step method, it was possible to obtain a high yield (over 400 mg/100g of EW) of ovomucin. However, a closer examination of GFC elution profiles of the ovomucin obtained by 2-step methods indicated that the crude ovomucin precipitate seemed to contain quite a lot of salt as an impurity[24,25,28]. Actually, when the 2-step method was modified by adding a dialysis step, the yield obtained was reduced by half, but the purity was still high, about 90%[88,89]. Shan et al.[98] modified the ovomucin purification method even further; the ovomucin precipitate obtained by the 2-step method was further washed several times with mild (10 mM) NaCl solution followed by a dialysis step. The final purity of ovomucin was calculated to be 96.7%, but no yield was reported.

However, regardless of which method is used, the crude ovomucin precipitate obtained has a limited solubility into conventional aqueous buffer solutions, and can be solubilised completely as subunits with buffers containing dissociating agents such as urea, guanidine hydrochloride or SDS, and reducing agents such as β-mercaptoethanol or dithiotreitol[12,22,34]. In addition, there are several studies in which sonication were used to solubilize ovomucin[11, 33,46,46,99-103]. Alternatively, crude ovomucin can be solubilized by enzymatic hydrolysis to peptides[23,52,81,87,99,100,104-107]. The advantage of the IEP method is that the method itself is very simple, cost-effective and easy to up-scale. **Figure 4** shows ovomucin isolation by using the IEP method on a laboratory scale.
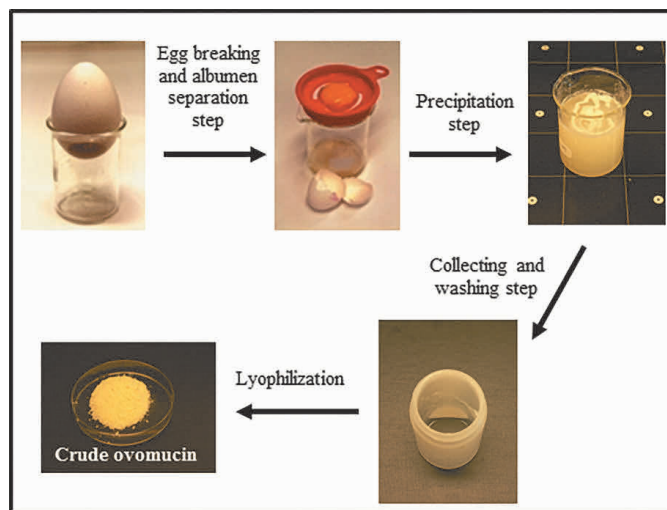

Fig. 4 Schematic diagram of ovomucin isolation by the IEP method.

2.2.2 Chromatographic methods

Alternatively to precipitation methods, whole ovomucin has been isolated from egg albumen by using gel filtration chromatography. The molecular weight of ovomucin is so substantial compared with other egg white proteins that by choosing appropriate gel filtration media, ovomucin will be eluated much before the other egg albumen proteins **(Figure 5)**. Ovomucin has been isolated from egg albumen in a complex form using GFC both without and with detergents as presented in **Table 8**. As can be seen in **Table 8,** much higher flow rates could be used with Sephacryl S-500 due to its rigid structure compared to Sepharose 4B gel. Naturally, a higher flow rate facilitated a timely shorter separation process. Due to this tolerance of high flow rates and high chemical stability, Sephacryl S-500 is well suited for industrial applications[110].
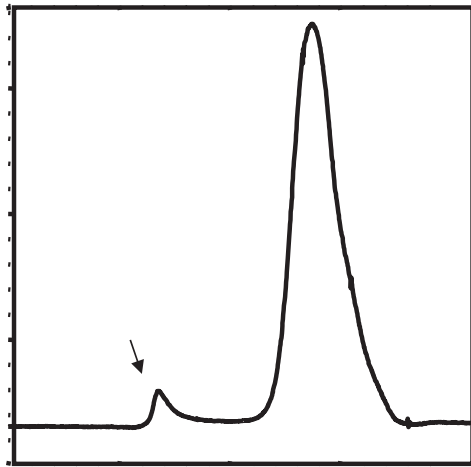
Fig. 5 Elution profile of whole egg white obtained by Sephacryl S-500 HR gel-filtration chromatography[96]. Arrow pointing to ovomucin. Reprinted from Hiidenhovi 2007 with permission. Copyright 2007 Springer Science+Business Media.

One advantage of the GFC method compared with IEP is that the separated ovomucin is usually very pure (free from other egg white proteins) and already in soluble form. The main disadvantage of GFC is a low yield of ovomucin; the reported values are quite modest: 5-10 mg per GFC run[34,69]. Although this is also an upscaling issue, the main reason for the low yield is that samples for GFC are prepared by diluting egg white with a buffer, thus leading to samples containing low amounts of ovomucin. An alternative method to increase the ovomucin yield obtained by GFC has been presented by Hiidenhovi et al.[109] This study is discussed in more detail in Chapter 5.1.2.

Table 8 Examples of applications of GFC for the purification of ovomucin.

Sample	Column*	Injection volume	Sample and elution buffer	Flow rate**	Ref.
Whole EW (50ml) diluted (1+5) with sample buffer c_{sample} = ~17 mg/ml as protein	Sepharose 4B 2.5 x 25 cm CV = 123 ml	10 ml (8.1% of CV)	A) 0.05 M Barbital buffer (pH 8,6) B) 0.85 % Saline buffered to pH 8,0 with ammonium formate	0.83 ml/min (10.2 cm/h)	44
Thick or thin EW diluted (10g+ 90 ml) with sample buffer c_{sample} = ~10 mg/ml as protein	Sepharose 4B 2.3 x 35 cm, CV = 145 ml	10 mL (6.6% of CV)	1% SDS	0.33 ml/min (4.8 cm/h)	68
Liquid (sol) fraction of thick EW	Sepharose 4B 2.5 x 40 cm CV = 196 ml	30 ml (15% of CV)	0.1 M Menzel buffer (pH 9) + 0.4 M NaCl		76
Thick EW (1 + 4) Liquid (sol) fraction of thick EW (1 +4)	Sepharose 4B 3.2 x 28 cm CV = 225 ml	10 ml (4.4% of CV)	0.05 M Barbital buffer (pH 8,6)	0.33 ml/min (2.5 cm/h)	34
EW layers (1g in 50 ml)	Sephacryl S-500 HR 1.6 x 40 cm CV = 80 ml	500 μl (0.6% of CV)	Sample: 0.05 M ImidazoleHCl buffer (pH 7) + 5% SDS Elution: 0.05 M ImidazoleHCl buffer (pH 7) + 0.5% SDS	1 ml/min (30 cm/h)	108
IEP ovomucin from whole egg	Sephacryl S-500 HR 5 x 90 cm CV = 1766 ml	150 ml (8.5% of CV)	Phosphate-buffered saline (pH 7.4)	8 ml/min (24.5 cm/h)	109
2-step IEP ovomucin	Sephacryl S-300 HR	1 ml	0.02 M Tris-HCl buffer (pH 8.4) + 4 M Guanidine-HCl + 0.2 M MgCl$_2$	1 ml/min	26

* CV = column volume. **In addition to a volumetric flow rate (ml/min) a linear flow rate (cm/h) is also presented in parenthesis.

Table 9 Examples of applications of GFC for the purification and analysis of ovomucin subunits.

Sample	Column*	Injection volume	Sample and elution buffer	Flow rate**	Miscellaneous	Ref
Sonicated α- and β-ovomucin from gel fraction of thick EW	Ultrogel ACA 22 1.5 x 24 cm CV = 42 ml		A) 0.1 M Tris-HCl buffer (pH 8) B) + 3 M NaCl C) + 3 M GuHCl D) + 1% SDS		MW (kDa) of subunits: α/ β-ovomucin A) 350 kDa / 510 kDa B) 360 kDa / 500 kDa C) 410 kDa/ - D) 350 kDa /450 kDa	[33]
Sonicated and carboxy-methylated (SCM) α- ja β-ovomucin from gel fraction of thick EW (SCM α- and β-ovomucin)					SCM α / β-ovomucin A) 470 kDa / 510 kDa B) 240 kDa / 500 kDa C) 290 kDa / - D) 210 kDa / 450 kDa	
IEP ovomucin from thick or thin EW	Sepharose 4B 2.6 x 90cm CV = 477 ml	10 ml (2% of CV)	Sample: 0.05 M imidazole-HCl buffer (pH 7) + 5% SDS + 1% β-mercaptoethanol Elution: 0.05 M imidazole-HCl (pH 7) + 0.5% SDS + 0.2% β-mercaptoethanol	0.67 ml/min (7.6 cm/h)	Reduced ovomucin eluated as separate peaks: β-ovomucin and α1 + α2-ovomucin MW(SDS-PAGE) ⇒ β 400 kDa / α1 150 kDa / α2 220 kDa	[11]
(A) Whole egg white (1 + 2) c_{sample} = ~ 6 mg/ml as protein	Superose 6 HR 10/30 1 x 30 cm CV = 24 ml	100 µl (0.4% of CV)	Sample: 0.05 M Tris-HCl buffer (pH 9) + 0.4 M NaCl + 10 mM β-mercaptoethanol Elution: 0.05 M Tris-HCl buffer (pH 9) + 0.2 M NaCl	0.4 ml/min (30.6 cm/h)	Reduced ovomucin eluated as separate peaks	[69,70]
(B) Whole egg white (1 + 2) c_{sample} = ~ 615 mg/ml as protein	Superose 6 prep grade 2.6 x 90 cm CV = 477 ml	10 ml (2% of CV)	Sample: 0.05 M Tris-HCl buffer (pH 9) + 0.4 M NaCl + 10 mM β-mercaptoethanol Elution: 0.05 M Tris-HCl buffer (pH 9) + 0.02 M NaCl	2 ml/min (22.6 cm/h)	Elution profile of preparative GFC was similar to analytical GFC	
IEP ovomucin from thick EW c_{sample} = 10 mg/ml	Sephacryl S-400 HR 2.0 x 75 cm CV = 236 ml	4 ml (1.7% of CV)	Sample: 0.01 M Na-phosphate buffer (pH 7) + 5% SDS + 2% β-mercaptoethanol Elution: 0.01 M Na-phosphate buffer (pH 7) + 0.5% SDS + 0.2% β-mercaptoethanol	0.5 ml/min (9.6 cm/h)	α- and β-ovomucin ⇒ MW (SDS-PAGE) α 220 kDa / β 400 kDa	[78]
IEP ovomucin c_{sample} = 5 mg/ml	Two Superose 6 HR 10/30 columns (1 x 30 cm) connected to series CV = 48 ml	100 µl (0.2% of CV)	Sample: 0.1 M Na-phosphate buffer (pH 7) + 5% SDS + 1% β-mercaptoethanol Elution: 0.1 M Na-phosphate buffer (pH 7) + 0.5% SDS + 0.1% β-mercaptoethanol	0.4 ml/min (30.6 cm/h)	Reduced ovomucin eluated as separate peaks	[111]

* CV = column volume. **In addition to a volumetric flow rate (ml/min) a linear flow rate (cm/h) is also presented in parenthesis.

Table 10 Examples of applications of GFC for both purification and purity analysis of reduced ovomucin eluated as a single peak at column void.

Sample	Column*	Injection volume	Sample and elution buffer	Flow rate**	
Sonicated IEP ovomucin	Sephacryl S-300 HR 2.6 x 70 cm CV = 371 ml	about 4 ml	0.1 M Phosphate buffer (pH 6.6) + 0.2 M NaCl (ref 101)	0.7 ml/min (8.3 cm/h)	101, 102
Void volume fraction obtained by Sephacryl S-300 GFC	Sepharose CL-2B (2 x 40 cm) CV = 126 ml		0.2 M NaCl (ref 102)	0.4 ml /min (7.6 cm/h)	
Pooled ovomucin fractions obtained by Superose 6 prepGFC.	TSK-G3000 SW 0.75 x 30 cm (HPLC) CV = 13 ml		Sample: 0.05 M Tris-HCl buffer (pH 9) + 0.02 M NaCl. Elution: 0.01 M Na-phosphate buffer (pH 7) + 0.2 M NaCl	0.4 ml/min (54.5 cm/h)	69
Whole EW (1 +2) , ref. 11; Thick and thin EW (ref 12 and 13)	Superose 12 HR 10/30 1 x 30 cm CV = 24 ml		Sample: 0.05 M Tris-HCl (pH 9) + 0.4 M NaCl + 10 mM β-mercaptoethanol. Elution: 0.05 M Tris-HCl (pH 9) + 0.1 M NaCl	1 ml/min (47.1 cm/h)	114-116
2-step IEP ovomucin c_{sample} = 5 mg/ml	Superdex HiLoad 200 16/60 prep grade*** 1.6 x 60 cm CV = 121 ml	3 ml (2.5% of CV)	Sample: 0.1 M Na-phosphate buffer (pH 7) + 5% SDS + 1% β-mercaptoethanol. Elution: 0.1 M Na-phosphate buffer (pH 7) + 0.5% SDS + 0.1% β-mercaptoethanol	1 ml	24,25
2-step IEP ovomucin c_{sample} = 5 mg/ml	Superdex HiLoad 200 16/60 prep grade + Superdex HiLoad 75 16/60 prep grade 1.6 x 60 cm +1.6 + 60 cm CV = 242 ml	3 ml (2.5% of CV)	Sample: 0.1 M Na-phosphate buffer (pH 7) + 5% SDS + 1% β-mercaptoethanol. Elution: 0.1 M Na-phosphate buffer (pH 7) + 0.5% SDS + 0.1% β-mercaptoethanol	0.4 ml/min	26

* CV = column volume. **In addition to a volumetric flow rate (ml/min) a linear flow rate (cm/h) is also presented in parenthesis.

*** Poor resolution between reduced ovomucin subunits and ovotransferrin.

In contrast to IEP methods, chromatographic methods also enabled the isolation of ovomucin subunits. Most often ovomucin is separated into α- and β-subunits by using GFC in reducing conditions. **Table 9** shows several examples of GFC applications both for preparative and for analytical scale. Also other chromatographic methods, such as lysozyme-Sepharose 4B affinity chromatography[45] and ion-exchange chromatography after sonication[44,112,113] have been used for subunit isolation.

In addition, GFC methods have been used for both purification and purity analysis of reduced ovomucin in a way that subunits were eluted as a single peak at column void. Examples of such methods are shown in **Table 10.**

2.2.3 Other methods

Garipaldi et al.[82] separated ovomucin from EW by using preparative ultracentrifuge (59,000 × g at 4°C for 16 hrs). The purity of the precipitate obtained was low, about 30%, but nevertheless it contained about 96% of ovomucin that was obtained by using the IEP method. In addition ovomucin was isolated by high-speed centrifugation at high ionic strength[71]. Ovomucin has been separated into α- and β-ovomucin using density gradient ultracentrifugation[21] and density gradient electrophoresis[42].

2.3 Properties of ovomucin

2.3.1 Biological role

Ovomucin is generally recognized as being responsible for the gel-like properties of thick egg albumen, as the most notable difference between thick and thin egg white is the amount of ovomucin. Analytical studies indicated that thick white contained either about two times (1.4-2.3)[19,23,34,59,60,114,115] or at least four times (3.8-6.0)[17,83,117-119] more ovomucin than thin egg white. This variation can be attributed to hen hybrid and/or age, freshness of the eggs as well as different isolation and analysis methods.

Several studies have suggested that the mechanical properties of thick white cannot be explained as a network formed by ovomucin alone, but a better explanation is that the network is formed by the ovomucin-lysozyme complex[83,102,120]. However, the ovomucin preparations used in those studies contained more or less lysozyme as an impurity and this might have effected to these results. Although the occurrence of ovomucin-lysozyme interaction in egg white cannot be ruled out, it might be irrelevant for the gel-forming properties of ovomucin, as it has been noted that ovomucin is able to form a gel individually [31,63,121]. Hayakawa & Sato[63] noted that the apparent viscosity of

soluble ovomucin increased rapidly above 1.5 mg/ml, and at the concentration of 2.0-2.5 mg/ml it formed a gel, whose viscosity was comparable to that of 1.5 mg/ml insoluble ovomucin. Offengenden & Wu[121] showed that at various concentrations (0.5%, 1% and 2%) ovomucin spontaneously formed a gel upon hydration at room temperature. The rheological properties of these gels were similar to structured fluids and other mucins. Similar non-Newtonian behaviour of ovomucin gels has also been reported by Rabouille et al.[101,102].

Actually egg white very much resembles mucus, a complex secretion, which covers the mucosal surfaces of the body, such as gastrointestinal and respiratory tracts, which are exposed to the external environment. Mucus is composed mainly of water (~96%) containing also salts, lipids and proteins, which have a defensive purpose such as lysozyme. The main compound is mucin, a large glycoprotein that is responsible for the viscous and gel-like properties of mucus[122-125]. Without lipids, this description equates extremely well to egg white.

This resemblance is quite expected as ovomucin belongs genetically into mucins. It has been found that ovomucin is encoded by two genes located on a locus on chromosome 5 in chicken. That locus is encoding gel-forming mucins (Muc6, Muc2, Muc5ac and Muc5b) and is quite similar to the corresponding human locus. The most significant difference is the presence of an additional gene in the chicken genome between Muc2 and Muc5a, an ovomucin gene, which encodes α-ovomucin subunit. In addition it has been noted that β-ovomucin is the orthologue of human MUC6[126,127].

Also, the biological role of ovomucin is same as that of mucus and egg white; protection. The gel-like structure of thick egg white induced by ovomucin hinders the movement of micro-organisms through to the albumen and also protects the developing embryo from impacts. In addition, ovomucin is known to contain antimicrobial activity against viruses and bacteria[128-130].

2.3.2   Role in egg white thinning

During storage, the pH of egg white rises from pH 7.6 to pH 9.7 due to loss of carbon dioxide[131,132]. Simultaneously, the structure of thick egg white collapses and changes into liquid form. This phenomenon is called egg white thinning or liquefaction, and is the most important change in egg white during storage. It has been found that one reason for egg white thinning is intrinsic in the white itself, as sterile, separated egg whites thinned during storage[133,134]. Many theories have been suggested to explain egg white thinning, but there seems to be no general consensus among researchers about the causal factors which induce this phenomenon. Such theories have been reviewed, for example, by

Burley & Vadehra[2], Li-Chan & Nakai[8], Vadehra & Nath[135] and Omana & Wu[136]. In what follows, another short review is presented.

2.3.2.1 Enzyme action

In one of the first theories presented for EW thinning it was suggested that ovomucin was degraded by a trypsin-like enzyme[137]. However, this was later refuted by Balls & Hoover[117] who did not find any evidence of active or activatable proteinase in EW. Similar results were also reported by Lineweaver et al.[138,139]. In addition it has been found that the free amino acid content of whole egg remained constant during storage. This was reported to confirm that negligible, if any, proteolytic activity existed in eggs. The slow rise of free amino acids in EW was explained as being caused by the diffusion of amino acids from yolk to EW through vitelline membrane[140].

As the EW contains glycosidase activity, such as α-mannosidase and β-N-acetylglucosaminidase activity[141,142], it has been speculated that the enzymatic degradation some of the glycosidic bonds may cause egg white thinning[91,143]. However, the activities of both enzymes, especially α-mannosidase, are low in EW. In addition, it has been noted that, due to the pH-increase, the β-N-acetylglucosaminidase activity gradually decreases in EW[144-146]. Moreover, it has been reported that the liquefaction of thick egg white did not lead to the release of free hexose or hexosamines[119]. Thus, it is very unlikely that any enzymatic degradation is involved in natural egg white thinning.

2.3.2.2 Reactions of disulphide bonds of ovomucin

Reduction of the disulphide bonds of ovomucin has been proposed to cause EW thinning, as it was found that minor amounts (less than 50 ppm) of reducing agents, such as thioglycol, hydrogen sulphide and sulphur dioxide, caused deteoration of egg white[77,147]. In addition, the alkaline degradation of disulphide bonds by hydroxide ion in alkaline pH was suggested to cause egg white thinning[86]. However, these latter experiments were carried out above pH 11, while natural thinning occurs at lower pH. Therefore, the authors noted that different mechanism might be involved in egg white thinning[86]. Furthermore, a light scattering study[64] of ovomucin demonstrated that alkaline degradation was very slow at pH 7.9. Therefore, it was postulated that a major part of the thinning was caused by disaggregation of gel-like aggregates.

According to Beveridge & Nakai[148], sulfhydryl blocking retained the thinning process, while Sato et al.[85] reported that blocking has no effect on egg white thinning. Similarly, there are inconsistent results when change of sulfhydryl groups in stored eggs has been measured. Heath[149] observed that the concentration of sulfhydryl groups in stored EW increased, in contrast to Kato et al.[150] who noted no change during a 30-day storage. However, Kato et al.[76]

reported that the elution profile of ovomucin obtained by Sepharose 4B for thinned EW after a 20-day storage was similar to that of fresh thick white, in contrast to that of fresh thick white treated with reducing agent. In addition, they found that a definite amount of α-ovomucin remained insoluble even after a 60-day storage, but was readily solubilized by β-mercaptoethanol. Moreover, Miller et al.[36] reported that apparent molecular weight (5.64×10^6 Da), amino acid and carbohydrate composition of native ovomucin were similar to those of ovomucin obtained from stored egg white, while the apparent MWs of reduced ovomucin were 309,500 and 726,200 Da. Thus, these results indicate that non-covalent disaggregation of ovomucin takes place during egg white thinning, without disulphide degradation.

2.3.2.3 Ovomucin-lysozyme interaction

In addition to ovomucin, also lysozyme has been thought to participate in the formation of a gel structure of thick egg white[20,83,86,91,102,151] and the change of ovomucin-lysozyme complex structure was thought to be a cause of egg white thinning. According to Hawthorne[84], the reason for thinning was the breakdown of swollen ovomucin gel due to the formation of insoluble ovomucin-lysozyme complex. However, this was contradicted by Cotterill & Winter[152] who studied ovomucin-lysozyme interaction *in vitro*. They noted that the interaction was highest at pH 7 and decreased as pH increased, suggesting that it is more likely that the dissociation of ovomucin-lysozyme complex might cause thinning. Later, Miller et al.[36] studied ovomucin-lysozyme interaction by using sedimentation equilibrium ultracentrifugation. They noted that the interaction of lysozyme with native ovomucin at pH 6.9 was quite low: 27% and 6% at ionic strength 0.07 and 0.13, respectively. As these experimental conditions were close to natural egg white, Miller et al.[36] suggested that ovomucin-lysozyme interaction is unlikely to be the principal reason for egg white thinning.

2.3.2.4 Changes in the ovomucin subunit structure

Thus, although the ovomucin-lysozyme interaction very likely exists in natural egg white, the changes in the ovomucin itself might be the reason for thinning. As mentioned above (Chapter 2.2) ovomucin consists of two subunits, namely β- and α-ovomucin, and it has been found that during egg white thinning β-ovomucin is gradually dissociated and solubilized from a gel fraction of egg white to a liquid fraction, while a definite amount of α-ovomucin remained in gel fraction even after a 60-day storage[18-21,43,61,76]. Moreover, Kato et al.[75] reported that the release of β-ovomucin was delayed as eggs were kept in CO_2-storage, which has been shown[131] to prevent egg white thinning. Sato et al.[85] suggested that an increase of pH during storage, reduced the ionic strength

induced by other EW proteins and rise of storage temperature seemed to be the main factors that favoured the specific release of β-ovomucin. Later, Kato et al. [150] suggested that the release of O-glycosidically bound carbohydrate units of β-ovomucin due to β-elimination reaction in alkaline conditions might be the reason for the structural deteoration of ovomucin.

Hayakawa et al.[151] examined the effect of β-ovomucin on the solubility of α-ovomucin with or without lysozyme. They noted that α-ovomucin formed an insoluble complex with lysozyme below pH 10, and β-ovomucin inhibited that complex formation in the neutral or slightly alkaline pH region. Relating to these and some other findings of their study, Hayakawa et al.[151] suggested that the gel structure of thick EW ovomucin complex consists of α-ovomucin-lysozyme complex covered by β-ovomucin. Furthermore, they suggested that the disaggregation of β-ovomucin due to alkaline reaction during storage leads to insolubization of the α-ovomucin-lysozyme complex, thus collapsing the gel structure of thick EW.

2.3.2.5 Ovomucin and divalent cations

The involvement of various divalent cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), to EW thinning has also been investigated in several studies. The thinning of thick EW fraction of acidotic hens (feeds containing e.g. 3% NH_4Cl) has been reported to be slower compared to normal eggs. This inhibition was suggested to be related to the maintenance of both pH and divalent cation (Ca^{2+} and Mg^{2+}) content in eggs obtained from acidotic hens[153,154]. In addition, Sauveur[155] has noted that after 20-days storage calcium and magnesium content in EW decreased by 63% and 21%, respectively, in control eggs compared to CO_2-stored eggs. According to Monsey & Robinson[156], thick EW of mature hens (age of 40-65 weeks) contained less magnesium and also thinned more rapidly when compared to the thick EW of young hens. Moreover, Robinson & Monsey[61] reported that addition of small amounts of NaCl or various magnesium salts to thick EW *in vitro* inhibited egg white thinning.

The exact mechanism for how divalent cations affects egg white thinning or ovomucin gelling properties is not known. Although *in vitro*, it has been shown that both Mg^{2+} and Ca^{2+} inhibited the formation of ovomucin-lysozyme complex[91], and that this interaction seemed to have minor importance to gel-like properties of ovomucin, as discussed above. However, it has been reported that calcium has the ability to crosslink human gel-forming mucins into larger aggregates[157,158]. In addition, human mucin aggregates have been found to dissolve by pH increase and by removing calcium[158]. Interestingly, although the apparent viscosity of soluble ovomucin was increased slightly by the addition

of β-ovomucin, in the presence of a small amount of CaCl$_2$ the observed increase was significant[63].

2.3.3 Techno-functional properties

Egg white proteins contain multiple techno-functional properties such as gelling, foaming and emulsification. Therefore, eggs are largely used in the food industry. For example, egg white is one of the most widely used foaming agent for various food applications. Most often, foaming properties are evaluated by measuring the foaming capacity and stability. In addition, thermal coagulation of egg white foams is needed in many food products, especially in bakery products[8,159]. In what follows, the techno-functional properties of ovomucin are briefly reviewed. However, it should be noted that in most of these studies a soluble ovomucin or ovomucin treated in someway (e.g. sonicated or hydrolysed) is used to enhance its solubility. Thus, there is a certain lack of knowledge of the impact of untreated insoluble ovomucin on the techno-functional properties of ovomucin..

Ovomucin has been reported to stabilize egg white foam[160-162]. According to Garipaldi et al.[82] ovomucin-lysozyme complex was heat-denatured during egg white pasteurization causing a longer whipping time due to decrease of mechanical stability of the foam. Johnson & Zabik[163-165] studied the effects of several egg white proteins singly and in combinations on the angel food cake system. They noted that as a single protein ovomucin, ovomucoid and lysozyme had little or no foaming capacity. However, ovomucin association with globulins enhanced foam formation but reduced cake volume. On the contrary, ovomucin-lysozyme interaction depressed foaminess, but produced large cakes. It was suggested that the role of ovomucin is to increase the viscosity of egg white and in this way participates in foam formation. Correspondingly, ovomucin-lysozyme complex was attributed to decrease viscosity, thus reducing foaming capacity.

Kato et al.[164] studied the relationship between the structural and functional properties of various ovomucin types such as soluble, sonicated and reduced ovomucin. In addition to its foaming properties, ovomucin was reported to have excellent emulsifying properties. Both the foaming power and foam stability of each ovomucin type studied were higher compared to that of bovine serum albumin (BSA). At higher protein concentrations (0.5%), the emulsifying activity of sonicated and reduced ovomucins was comparable to that of BSA. Moreover, the emulsion stability of sonicated and reduced ovomucins was much higher compared to that of BSA. The authors suggested that the foaming and emulsifying properties of ovomucins were dependent on viscosity and surface hydrophobicity, respectively[164].

Later, Kato et al.[165] examined the role of negatively charged sialic acid for the functional properties of ovomucin. They found that, even though the removal of terminal sialic acids reduced the viscosity of soluble ovomucin, its foaming properties were increased. According to Kato et al.[165] the explanation for this contradictory phenomenon was the decrease of negative charge which enhanced the interaction of ovomucin molecules at the air-water interface, thus strengthening the foam film. In the case of both sonicated and reduced ovomucins foaming properties were increased after sialic acid removal without viscosity decrease. On the other hand, the emulsifying properties of various ovomucin types were markedly decreased due to sialic acid removal.

More recently, Hammershøj et al.[23] studied the foaming properties of the hydrolyzed ovomucin fractions. The foaming capacity of ovomucin increased along with the degree of hydrolysis (DH), and maximum foam overrun was reached at a DH range of 15 to 40%. Hydrolysis had no significant effect on the foam stability. Hammershøj et al.[23] concluded that limited hydrolysis made it possible to increase the solubility and surface hydrophobicity of ovomucin, which might result in higher foaming capacity and possibly also enhanced foam stability.

Shan et al.[98] suggested that simple pH treatment was an effective way to enhance the functional properties of ovomucin. They reported that the alkali treatment had the greatest effect on for emulsifying properties, while weak acid treatment improved foaming stability.

2.3.4   Bioactive properties

In previous years, ovomucin was studied mainly because of its role in egg white thinning. More recently, however, there has been a growing interest within the food industry in health-promoting, functional foods. Today, proteins are not valuated only by the nutritional value or functional properties, but also by their biological activity. Most of the ovomucin research has lately been focused on its bioactive properties.

2.3.4.1 Antiviral properties

The antihemagglunation (AH) activity of ovomucin against the swine influenza virus was reported already in the late 1940s[65,168,169]. MacDonnell et al.[170] reported that this activity was remarkably resistant, as 0.5-1% solution of ovomucin showed no activity loss e.g. in the following conditions: at pH 7.6 100°C for 30 min, at pH 2 70°C for 60 min and at pH 11 70°C for 60 min. In addition, neither reduction nor chymotrypsin hydrolysis reduced the AH activity of ovomucin. However, both chemical fractionation and lyophilization significantly reduced activity[170].

Tsuge et al.[171] reported that ovomucin also has a hemagglunation inhibition (HI) activity against bovine rotavirus (RV), hen Newcastle disease virus (NDV) and human influenza virus (IV). Ovomucin was found to have the highest HI activity against all three viruses tested, compared to the other main EW proteins. In another study, Tsuge et al.[172] noted that that not only the sialo-oligosaccharide moieties were crucial for antiviral properties of ovomucin. Although the HI activity against NDV needed β-ovomucin (carbohydrate-rich subunit) only, that against RV was implemented its highest by macromolecular ovomucin including both ovomucin subunits. In contrast to earlier findings, both reduction and enzymatic hydrolysis seemed to reduce the HI activity of ovomucin, especially against RV, while against NDV this reduction was not so substantial.

In further studies, the binding activities of various ovomucin fractions to NDV were evaluated by ELISA[78,173,174]. Pronase hydrolysis, heat treatment and pH increase were found to reduce the binding activity of ovomucin to NDV [78,172,173]. These studies also indicated that the terminal sialic acids of β-ovomucin contribute to the binding of ovomucin to NDV, as the binding of desialylated ovomucin to NDV was negligible[173]. In addition, it was demonstrated that the bound-type sialic acid was required for effective hemagglutination inhibition; the amount of free-type needed for 50% inhibition was 1,000 times that of bound-type[173]. Furthermore, a method of preparing Pronase-treated soluble ovomucin fragments having various binding activities to NDV was presented[174].

Recently, Shan et al.[26,175] have reported that the addition of Mg^{2+} enhance HI and virus adhesion activities of ovomucin against NDV. They suggested that minimal amounts of Mg^{2+} (9 μmol/mg of ovomucin) coordinate with the carbohydrate moieties of ovomucin, thus making these functional groups more susceptible to cell signal recognition.

2.3.4.2 Antitumour activity

In preliminary studies, Ohami et al.[176] noted that, by using scanning electron microscopy, the β-ovomucin subunit of ovomucin had a cytotoxic effect on cultured tumour cells (human melanoma cells). In further *in vitro* studies, sarcoma-180 (SR-180) cells were incubated with both α- and β-ovomucin obtained from gel fraction of EW[112]. It was noted that β-subunit supposedly inhibited the cell proliferation rate, in contrast to α-subunit, which predominantly had no effect to it. This cytotoxic effect of β-subunit was found to be both dose- and time-dependent. In addition, scanning and transmission electronmicroscopy demonstrated that β-ovomucin did not damage normal cells (mouse peritoneum macrophage cells), while SR-180 cells were clearly damaged. Moreover, it was shown that β-ovomucin did not induce apoptotic

DNA fragmentation. In the *in vivo* study that follows, the antitumour effect of β-subunit was tested against subcutaneously xenografted SR-180 cells in mice[113]. It was found that the intraperioneal administration of β-ovomucin first suppressed the growth of SR-180 cells and finally cured the tumour. Yokota et al.[113] suggested that the antitumour properties of β-subunit was attributed to activating the immune system, as a vast accumulation of neutrophils, macrophages and lymphocytes were found in the side of the degraded and necrotic tumor tissue area.

It was further demonstrated by using double grafted tumour system that certain highly glycosylated fragments separated from Pronase-treated ovomucin have antitumour properties[177,178]. Double grafted tumours were achieved for the BALB/c mice by simultaneous inoculations of Meth-A fibrosarcoma cells both in the right and left flank. Ovomucin fragments studied were 220 and 120 kDa, a highly glycosylated peptides derived from the β-subunit[177], and 70 kDa, a highly glycosylated peptide derived from α-ovomucin[178]. As the β-subunit derived fragments were injected separately into the right tumour, it was found that both 220 and 120 kDa fragment cured directly and completely the treated tumour and inhibited indirectly and slightly the growth of distant (left) one. In the case of the 70 kDa fragment the direct antitumoural effect was not as potent, as the inhibition of the treated (right) tumour was described as "almost complete", while the indirect effect was moderate. Moreover, treatment with desialylated 120 kDa fragment demonstrated that NANA moieties were not essential for direct antitumour effect, while it seemed to be obligatory for indirect antitumour activity. Further studies indicate, that presumably the 120 kDa fragment inhibit the growth of SR-180 cells due to interaction with SR-180 cells via basic fibroblast growth factor receptor[179].

2.3.4.3 Antiadhesive and antibacterial activity

Ovomucin has been shown to inhibit colonisation of *Helicobacter pylori*, a bacterium which is associated with, for example peptic ulcers and gastric cancer[180]. Furthermore, ovomucin could eliminate *H. pylori* from the stomach in a concentration-dependent manner.

It has been noted that ovomucin glycopeptide (OGP) obtained after Pronase digestion bound exclusively to *E. coli* O157:H7, and sialic acids were necessary for binding[181,182]. As the adhesion of bacteria to host tissues is a preliminary step in the foodborne illness, it was suggested that the OGP has the potential to provide protection against *E. coli* O157:H7 infection. Moreover, in the food hygiene field OGP might serve as a novel probe for *E. coli* O157:H7 detection.

Recently, Shan et al.[183] reported that ovomucin had antibacterial activity against *E. coli* and Salmonella measured by turbidimetry, and the minimal inhibition concentrations (MIC) obtained were 0.05 mg/ml and 0.4 mg/ml, respectively.

2.3.4.4 Immunomodulating activity

Otani & Maenishi[184] reported that ovomucin enhanced the proliferation of mouse spleen lymphocytes, which were induced by lipopolysaccharide (LPS). Therefore, they supposed that ovomucin might be involved in the potential allergenicity to hen eggs. However, recent studies have shown that ovomucin is not very likely an egg allergen[185].

Thus, most likely the immunopotentiator activity of ovomucin is attributed to the bioactive properties of ovomucin, such as antitumour activity, as discussed above. Tanizaki et al.[186] have reported that the glycopeptides of protease-digested ovomucin revealed strong macrophage-stimulating activity, and activated macrophages are known to secrete tumour necrosis factor-alpha (TNFα). The O-linked glycans were identified as active components.

2.3.4.5 Antioxidant activity

Boiled eggs were digested by pepsin and pancreatin, and thus the hydrolysate obtained was fractionated by using ion-exchange chromatograpy and RP-HPLC[187]. Five fractions that contained the most potent antioxidant activities, as measured by ORAC, DPPH and ABTS methods, were further subjected to LC-MS/MS analysis. A total of 63 peptides were identified, of which seven originated from β-ovomucin and four from α- ovomucin. Unfortunately, the antioxidant activities of individual peptides were not determined.

In another study crude ovomucin obtained from whole EW by using the 2-step IEP method was hydrolysed by Protamex, Flavourzyme or Alcalase[188]. The ABTS free radical scavenging activity for Protamex, Flavouzyme and Alcalase hydrolysate was 90.4%, 89.4% and 88.6%, respectively, compared to that of 17.3% for untreated ovomucin. The Protamex hydrolysate was further fractioned by using ultracentrifugal filtration and RP-HPLC. The fraction containing the highest antioxidant activity was subjected to MS analysis, and two peptides derived from α-subunit were identified with great accuracy. Both fragments, LDEPDPL (f686-692) and NIQTDDFRT (f539-549) were synthesized and antioxidant activities were measured to be 51.8 and 24.7% by DPPH assay.

2.3.4.6 Hypocholesterolemic activity

Nagaoka et al.[189] reported that ovomucin inhibits cholesterol absorption in Caco-2 cells *in vitro*. In addition, animal studies with male rats of the Wistar

strain fed the casein a diet showed that diet enriched with ovomucin significantly lowered both the serum total cholesterol and serum LDL + VLDL cholesterol level compared to rats fed the plain casein. Moreover, the total lipid concentration in liver was significantly lower in the ovomucin-enriched group compared to casein-fed rats. It was suggested that the hypocholesterolemic activity of ovomucin was in part due to the direct interaction of ovomucin and cholesterol-mixed micelles thus suppressing cholesterol absorption in jejunum.

2.3.4.7 Dipeptyl dipeptidase-4 (DPP4) inhibitory activity

It has been noted that hydrolysates of several EW proteins, including ovomucin, contained reasonable DPP4 inhibitory activity[190]. DPP4 is an enzyme, which has a substantial role e.g. in a glucose metabolism, as it is responsible for the degradation of glucagon-like peptide-1 (GLP-1). GLP-1 is an incretin hormone having antidiabetic action for example due to its ability to induce satiety and to stimulate insulin secretion and daily gastric emptying. DPP4 inhibitors are supposed to enhance the body's own ability to control blood glucose level, thus being suitable for the treatment of type 2 diabetes[191,192].

2.4.4.8 Antihypertensive activity

Hypertension is one of the risk factors for cardiovascular disease[193]. Angiotensin-I Converting Enzyme (ACE) inhibitors are a group of medicines used for the treatment of hypertension, e.g. for lowering blood pressure[194]. Van Amerongen et al.[195,196] presented an invention related to ACE inhibitory activity of various EW proteins. *In vitro* studies indicated that Alcalase-treated ovomucin had moderate ACE inhibition activity. In addition, it was reported that ovomucin-Alcalase hydrolysate demonstrated a significant blood pressure lowering effect in an *in vivo* test with spontaneously hypertensive rats.

2.4   Summary of the literature review

Ovomucin is a sulphated egg white glycoprotein, characterised by high molecular weight and a subunit structure. In addition to egg white, ovomucin is also found in chalazae and in the outer layer of the vitelline membrane. It is generally recognized that ovomucin consists of at least two subunits: α-ovomucin (protein-rich subunit) and β-ovomucin (carbohydrate-rich subunit).

The primary amino acid sequence of α-ovomucin has been reported to consist of 2,108 amino acids with a relative molecular mass of 233,553 Da. The estimated size of α-ovomucin could be calculated to be 256-278 kDa, as the carbohydrate content of α-ovomucin has been reported to vary from 9.2% to 15.5%. The main carbohydrates of α-ovomucin are mannose and N-

glucosamine, which exist as N-glycan oligosaccharideshaving pentasaccharide core of $GlcNAc_2Man_3$ and a bisecting GlcNAc.

Only a partial primary amino acid sequence of β-ovomucin has been revealed officially, consisting of 1,185 AAs with molecular mass of 132,217 Da. The molecular weight of the β-ovomucin subunit has been estimated by SDS-PAGE or ultracentrifugation to be between 350 and 720 kDa. The carbohydrate content of β-ovomucin is very high: from 48% to 67%. These carbohydrates exist predominantly as O-glycosidically linked oligosaccharide moieties consisting of about 2-6 carbohydrate units having both straight and branched chains also containing terminal sialic acid and ester sulphates. Moreover, two potential N-glycolysation sites have been identified in β-ovomucin.

Egg white contains two forms of ovomucin: insoluble and soluble ovomucin. Soluble ovomucin is presented both in thick and thin albumen, while insoluble ovomucin is found only in thick albumen. Insoluble ovomucin contains about 2.5 times the β-ovomucin compared to that in soluble ovomucin. This abundance is attributed to a higher apparent viscosity of insoluble ovomucin to than that of soluble ovomucin. Similarly, the molecular weight of insoluble ovomucin is greater than that of soluble ovomucin.

Ovomucin is most often isolated from egg albumen by using the isoelectric precipitation method. The crude ovomucin precipitate obtained by using the IEP method is highly insoluble in common buffer solutions, and can be solubilized completely as subunits with the buffers containing dissociating agents and reducing agents or by enzymatic hydrolysis as peptides. The advantages of the IEP method are that the method itself is very simple, cost-effective and easy to up-scale. Alternatively ovomucin has been isolated from EW by using gel filtration chromatography. One advantage of the GFC method compared to IEP is that the separated ovomucin is usually very pure (= free of other egg white proteins) and already in soluble form. The main disadvantage is a low yield of ovomucin.

Ovomucin is responsible for the gel-like properties of thick egg albumen. Although the occurrence of ovomucin-lysozyme interaction in egg white cannot be ruled out, it might be irrelevant for the gel-forming properties of ovomucin, as it has been noted that ovomucin is able to form a gel individually.

Genetically, ovomucin belongs in the mucins. Ovomucin is encoded by two genes located on a locus on chromosome 5 in chicken. That locus is encoding gel-forming mucins and is quite similar to the corresponding human locus. The most significant difference is the presence of an additional gene in the chicken genome, an ovomucin gene, which encodes the α-ovomucin subunit. Moreover, it has been reported that β-ovomucin is the orthologue of human MUC6.

Also the biological role of ovomucin is considered to be the same as that of egg white; protection. The gel-like structure of thick egg white induced by ovomucin hinders the movement of micro-orgasms through to the albumen and also protects the developing embryo from impacts. In addition, ovomucin contains antimicrobial activity. Egg white thinning, the most important change in egg white during storage, is usually attributed to the degradation of the ovomucin complex. It has been reported that, during egg white thinning, β-ovomucin is gradually dissociated and solubilized from the gel fraction of egg white to liquid fraction.

Ovomucin has also been found to contain some techno-functional properties such as foaming and emulsification. In addition, ovomucin and ovomucin-derived peptides have been reported to possess multiple bioactive properties. Ovomucin, therefore, seems to be a highly potential source of bioactive ingredients for novel functional foods or pharmaceuticals.

3   AIMS OF THE STUDY

The objectives of the present study were:
- − to develop isolation methods for ovomucin (I-III)
- − to characterize ovomucin structure (I-III)
- − to compare various egg fractions as sources of ovomucin (II, III)
- − to study the effects of various dissolving methods for ovomucin (IV)
- − to investigate the bioactive properties of ovomucin and ovomucin-derived peptides (III, IV)

4   MATERIALS AND METHODS

4.1   Egg samples

The shell eggs used in the studies (**I-III**) were obtained from the local hen house (MTT Agrifood Research Finland, Animal Feeding Section. As of 1 January 2015, MTT Agrifood Research Finland is part of Natural Resources Institute Finland [Luke]) or from a local store (**III**). The liquid egg white (**II, IV**) and the filtration by-product fraction (**II**) were obtained as a gift from Muna Foods Oy (former Munakunta, Piispanristi, Finland). Fraction **II** was obtained from an egg processing plant as e.g. chalazae were filtered off after egg breaking and yolk separation prior to further EW processing.
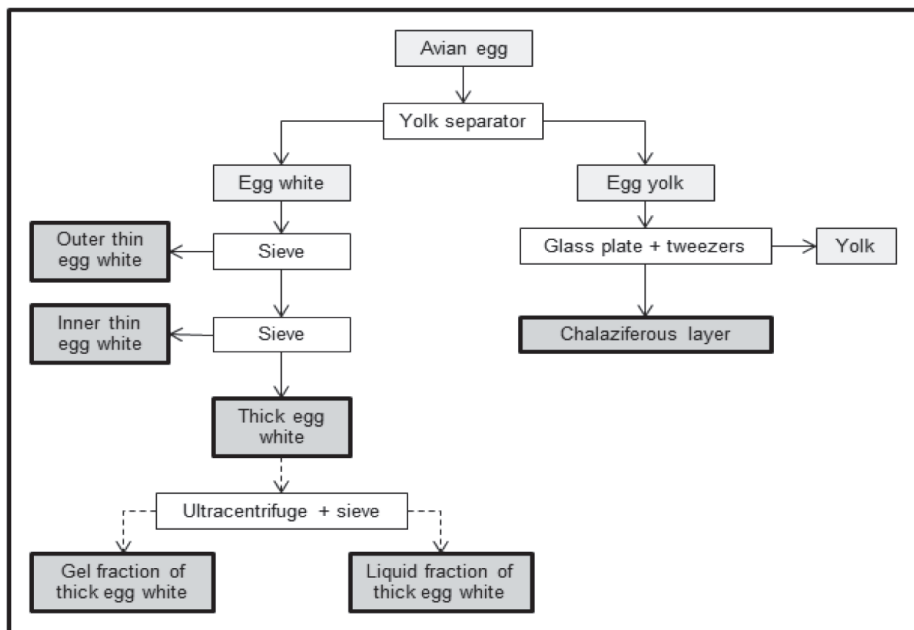
Fig. 6 Fractionation of egg white layers. Figure is obtained from Publication **III**.

4.2   Separation of egg white fractions

The whole egg white fraction (**II, III**) was obtained by separating the albumen and yolk with a household yolk separator. Whole egg whites (chalaza cords removed by using tweezers) were pooled and homogenized with a household mixer at low speed so as to avoid foaming. Thick egg white fraction (**I, II**) was separated by using a sieve (having a square aperture of 2.0 mm) according to

the modified method of Holst & Almquist[197] and Brooks & Hale[120]. The separation of EW layers (**III**) was accomplished as shown in **Figure 6**.

4.3   Isolation of ovomucin

4.3.1   Isoelectric precipitation method (I–IV)

Crude ovomucin was isolated by using the isoelectric precipitation method described in Nakamura et al.[198] with a few modifications. Briefly, homogenized albumen was diluted with three volumes of Milli-Q water, stirred for 10 **(I)** or 30 **(II)** minutes by using a magnetic stirrer and then adjusted to pH 6 with 1 N HCl. The albumen dispersion was stirred for an additional 30 min (**III**) and then centrifuged (10,000 × g, 10 min [**I, II**] or 20,000 × g, 20 min [**III**] at room temperature) to precipitate the crude ovomucin. The crude ovomucin precipitate was washed twice (**I-III**) with Milli-Q water by centrifugation and then freeze-dried.

Crude ovomucin for dissolving tests **(IV)** was prepared by using a combination of methods developed by Nakamura et al.[198] and by Donovan et al.[67] Liquid egg white was diluted with three volumes of de-ionized water, stirred for 30 minutes and then adjusted to pH 6 with 2 N HCl. After overnight settling, supernatant was siphoned off and the remaining suspension was collected and centrifuged (10,000 × g, 20 min, RT) to precipitate crude ovomucin. The crude ovomucin precipitate was washed twice with water by centrifugation and then freeze-dried.

4.3.2   Gel filtration chromatography (GFC) method (I, III)

4.3.2.1 Preparative Sephacryl S-400 GFC with reducing agent (**I**)

Gel filtration was performed with fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences, Uppsala, Sweden) equipped with two Sephacyl S-400 HR columns (2.6 × 95 cm and 1.6 × 90 cm) connected in series. Ovomucin was dissolved in 150 mM imidazole-HCl buffer (pH 7.0) containing 5% SDS and 1% β-mercaptoethanol.

4.3.2.2 Preparative Superose 6 GFC with reducing agent (**III**)

Preparative GFC was performed using an Äkta explorer system (GE Healthcare Life Sciences, Uppsala, Sweden) equipped with Superose 6 prep grade XK 26/100 column (2.6 × 95 cm). Lyophilized IEP ovomucin was dissolved in 100 mM of sodium phosphate buffer (pH 7.0) containing 5% SDS and 1% ß-mercaptoethanol.

4.3.2.3 Preparative Superose 6 GFC without reducing agent (**III**)

Preparative GFC was performed using an Äkta explorer system (GE Healthcare Life Sciences, Uppsala, Sweden) equipped with Superose 6 prep grade XK 26/100 column (2.6 × 95 cm). IEP ovomucin precipitate (without lyophilisation) was dissolved in 100 mM of sodium phosphate buffer (pH 7.0) containing 5% SDS.

4.4   Characterization of ovomucin

4.4.1   Analytical GFC (II-IV)

Subunit profile and amounts of co-precipitated egg white proteins in crude ovomucin samples were determined with high-performance liquid chromatography (HPLC) equipped with two Superose 6 HR 10/30 gel filtration columns in series. Lyophilized IEP ovomucin was dissolved in 100 mM of sodium phosphate buffer (pH 7.0) containing 5% SDS and 1% ß-mercaptoethanol.

Standard curves (peak area *vs*. mg of protein) were created for each co-precipitated egg white protein (ovotransferrin, ovalbumin, ovomucoid and lysozyme) by using commercial protein preparations. Ovalbumin (A-5503, purity 98%), ovotransferrin (C-0755, purity 80%), ovomucoid (Fluka 93621, purity 80%), and lysozyme (L-6876, purity 90%), were purchased from Sigma (St. Louis, MO, USA). The purity announced by the manufacturer for each protein was taken into account when generating standard curves. In Study **II** different ovomucoid was used (T-2011, purchased from Sigma, St. Louis, MO, USA). The purity was analysed by using GFC and found to be 99%.

4.4.2   SDS-PAGE (I-III)

SDS-PAGE was performed by PhastSystem using PhastGel Gradient 4-15-minigels and PhastGel SDS Buffer Strips (GE Healthcare Life Sciences, Uppsala, Sweden). The Silver Stain SDS-PAGE Standards, High Range -kit (Bio-Rad Laboratories AB, Hercules, CA, USA) was used for molecular weight (MW) estimations.

4.4.3   Amino acid analysis (I)

Prior to amino acid analysis, the lyophilized crude ovomucin samples were freed from SDS by the ion-pair extraction method of Koningberg & Henderson[199]. Amino acids were analysed as phenylthiocarbamate (PITC) derivates by using HPLC[200].

4.4.4 Carbohydrate analysis (II)

Total hexose content was determined by a resorcinol sulphuric acid micromethod[201]. Ovomucin (2 mg) was hydrolyzed with 2 M HCl for 3 hr at 95°C. Galactose was used as a standard.

Total hexosamine was estimated by the 3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH) method using N-acetylglucosamine as a standard[202]. Each sample (2 mg) was hydrolyzed with 2 M HCl for 3 hr at 95°C.

Sialic acid (N-acetylneuraminic acid, NANA) was determined by using the periodate-resorcinol method[203]. Ovomucin (2 mg) was hydrolyzed with 0.01 M HCl for 1 hr at 90°C. N-Acetylneuraminic acid was used as a standard. It is known that free sialic acids are destroyed during the hydrolysis step. In Study **II** this loss was measured to be 12%, which was then corrected to reported values.

Standard curves (peak area vs. µg of carbohydrate) were created for each carbohydrate by using commercial preparations. Galactose (48259, purity ≥ 99.5%), N-acetylglucosamine (01140, purity ≥ 99.0%) and sialic acid (01398, purity ≥ 99.0%) were purchased from Fluka (Buchs, Switzerland).

4.5 Enzymatic hydrolysis of ovomucin (III, IV)

Crude ovomucin dispersion was hydrolysed by trypsin (enzyme-substrate ratio [w/w] 1/100) at 45°C for 3 hr. The enzymatic reaction was terminated by heating (100°C/6 min). In all tests phosphate buffered saline (PBS, pH 7.4) and ovomucin concentration 0.2% (2 mg/ml) was used. After hydrolysis, the ovomucin sample was centrifuged (20,000 × g, 20 min) and aliquots of supernatant were taken for further analysis.

4.6 Characterization of ovomucin hydrolysates (III, IV)

Degree of hydrolysis (DH) was determined (**III**, **IV**) spectrophotometrically by measuring the number of α-amino groups released during hydrolysis following reaction with o-phthaldialdehyde by the method of Church et al.[204] as modified by Frister et al.[205].

Relative solubility (RS) was calculated (**III**, **IV**) by using the following formula:

$$RS = \frac{\text{protein content (mg) of hydrolysed crude ovomucin sample}}{\text{weight (mg) of crude ovomucin in sample}} \times 100$$

Protein content of hydrolysed ovomucin sample was measured by using Bio-Rad protein DC assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA).

NANA content of hydrolysed ovomucin sample was measured (**III**, **IV**) as described in Chapter 4.4.4.

Molecular weight estimations **(III)** of ovomucin hydrolysates were determined with high-performance liquid chromatography (HPLC) equipped with Superdex 200 HR 10/30 column (GE Healthcare Life Sciences, Uppsala, Sweden). The following standard proteins (Gel Filtration Standard, 151-1901, Bio-Rad, Hercules, CA, USA; MW in parentheses) were used in calibrating the Superdex 200 HR column for preparing standard curves for molecular weight estimation: thyroglobulin (670,000 Da), bovine gamma globulin (158,000 Da), chicken ovalbumin (44,000 Da), equine myoglobin (17,000 Da) and vitamin B-12 (1,350 Da). Molecular mass distribution was calculated by dividing the total chromatogram area into three ranges of molecular mass (> 100 kDa, 100 – 10 kDa, < 10 kDa) and expressed as a percentage of total area.

Hemagglutination inhibition (HI) activity of ovomucin preparations was determined (**III, IV**) by using conventional method[206]. Briefly, various concentrations of PBS diluted ovomucin samples were mixed with a constant amount of virus suspension in microtitre plates. The mixture was incubated at 22°C for one hour where after 1% chicken red blood cells were added. The plates were further incubated at 4°C and read after 30-40 min. HI activity was expressed as the minimal inhibition concentration (MIC) that was the highest dilution (µg/ml) of a sample protein that completely inhibited virus hemagglutination.

4.7   Solubilisation of ovomucin (IV)

In all the tests phosphate buffered saline (PBS, pH 7.4) and ovomucin concentration 0.2% (2 mg/ml) were used. After each dissolving test the ovomucin sample was centrifuged (20,000 × g, 20 min) and aliquots of supernatant were taken for further analysis.

Conventional stirring. Ovomucin suspended in PBS was stirred (3 hr, RT) using a magnetic stirrer.

Sonication. Ovomucin was dispersed in PBS and sonicated for 6 × 1 min or 12 × 1 min at 80W (low) using LABSONIC 2000 Ultrasonic-Homogenizer

(B.Braun, Melsungen, Germany). During sonication, samples were kept in an ice-bath.

Colloid milling. Ovomucin suspension was treated (16,000 RPM) for 1 min by using IKA magicLAB equipped colloid mill MK module (IKA WERKE Gmbh & Co., Staufen, Germany).

*Enzymatic hydrolysi*s**. Described above (Chapter 4.5).

Sonication + enzymatic hydrolysis. After 6 min sonication the ovomucin sample was hydrolysed by trypsin as described above.

Colloid milling + enzymatic hydrolysis. Ovomucin suspended in PBS was treated both by colloid milling and by enzymatic hydrolysis in series. Both treatments were conducted as described above.

4.8   Solubilisation rate of ovomucin (IV)

Relative solubility (RS) was calculated by using the following formula:

$$\text{RS} = \frac{\text{protein content (mg) of dissolved crude ovomucin in supernatant}}{(\text{weight (mg) of crude ovomucin in sample } \times 0.884)} \times 100$$

Protein content of dissolved ovomucin sample was measured by using Bio-Rad protein DC assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Factor 0.884 is a protein content of crude ovomucin analysed by the Kjeldahl method.

Ovomucin peak area (Area-%) was calculated by using the following formula:

$$Area - \% = \frac{Area(t)}{Area(0)} \times 100$$

where Area(t) is the total peak area of dissolved ovomucin subunits after treatment, and Area(0) is the total peak area of crude ovomucin subunits sample (2 mg/ml) before treatment. Subunit profile and areas of ovomucin subunits were determined by using Superose 6 GFC method described by Hiidenhovi et al.[II].

NANA content of ovomucin sample was measured (**III**, **IV**) as in Chapter 4.4.4.

4.9 Yield determination of various ovomucin aggregates

In this dissertation, the ovomucin precipitation obtained by IEP method is referred to as **crude ovomucin**. The protein composition of crude ovomucin was determined by GFC. Combination of the first three proteins (peaks 1-3, **Figure 8**) is called **ovomucin-G,** while the combination of β-ovomucin and α-ovomucin (peaks 1-2, **Figure 8**) is called **ovomucin-C**. The yields for each ovomucin form were determined as described below.

In addition it should be noted that the percentage amount of ovomucin-G (section 4.9.3) is the same as purity-% of crude ovomucin, a terminology used in paper **I**.

4.9.1 Crude ovomucin (II–IV)

The freeze-dried ovomucin preparations were weighed, and these obtained weights were used to calculate the crude ovomucin contents of the albumen fractions in 100 g of albumen and as a percentage of total protein. The total protein content of the EW fractions was analysed by the Kjeldahl method using a conversion factor of 6.25.

The amount of crude ovomucin (mg/100 g) was calculated by the following formula:

$$W(crude) = \frac{W1}{W2} \times 100$$

where W1 is the weight (mg) of the freeze-dried crude ovomucin precipitate, W2 is the weight (g) of the egg white used for crude ovomucin preparation

4.9.2 Ovomucin-G (II–IV)

Amount of ovomucin-G (mg/100 g) was calculated by following formula:

$$W(OVMG) = \frac{W(crude) \times OVMG\%}{100}$$

where W(crude) was the weight (mg) of freeze-dried crude ovomucin per 100 g of EW. The percentage amount of ovomucin-G (OVMG%) was obtained from gel filtration data by subtracting the amounts of co-precipitating proteins (obtained by using standard curves) from the amount of crude ovomucin.

4.9.3 Ovomucin-C (III, IV)

During the GFC analysis, a UV-detector (A_{280}) measured only the protein concentration of eluated compounds. Because proteins eluted in the first three peaks are known to be glycoproteins, the peak areas of peaks 1, 2 and 3 were divided by 0.4, 0.85 and 0.88 respectively so as to take into account the amount of carbohydrate in each protein, as β-ovomucin, α-ovomucin and ovostatin were supposed to contain 60%, 15% and 12% of carbohydrate, respectively[41,96].

The amount of ovomucin–C (mg/100 g) was calculated by following formula:

$$W(OVMC) = \left(\frac{A1 + A2}{A1 + A2 + A3}\right) \times W(OVMG)$$

where A1, A2 and A3 were corrected peak areas.

4.10 Statistical analysis (III, IV)

The obtained data were subjected to analysis of variance using the general linear model procedure of SAS (SAS version 9.3, SAS Institute, Cary, NC, USA), and significant differences were determined using Duncan's multiple range test ($p < 0.05$).

5   RESULTS AND DISCUSSION

5.1   Isolation of ovomucin by using modified IEP method (I–III)

Figure 7 shows a schematic diagram of three IEP methods. Crude ovomucin was separated in studies **I-III** by using a modified method (A) as described in Materials and Methods. The salt washing steps which are generally applied in IEP methods were omitted from this procedure for two reasons: firstly, to maximize the yield of ovomucin, and secondly, to keep the ovomucin separation procedure as simple as possible. Consequently, just two water washing steps were included in this purification protocol to remove only the major contaminants, thus leading to an ovomucin precipitate containing various amounts of other co-precipitated egg albumen proteins. Study **II** the purity of whole EW crude ovomucin was about 64% and the obtained ovomucin-G yield 277 mg/100 g of whole EW. In addition, for thick EW crude ovomucin the corresponding values were 343 mg and ~65%.

As a conventional method (B) the method described by Nakamura et al.[198] is given here as an example (**Figure 7**). It has been reported in previous publications that, in order to achieve efficient removal of other co-precipitated albumen proteins, the ovomucin precipitate should be washed several times and/or kept in contact with water and KCl washing solutions for 24 hr per washing step[80,83]. Depending on the harshness of the washing procedure, one acquired purer ovomucin but simultaneously the yield was lower. Only 120 mg/100 ml of thick EW ovomucin, corresponding to about 25% of the ovomucin present in thick albumen, was obtained after vigorous washing procedure[80].

The third method (C), 2-step method, first introduced by Omana & Wu[24] was reported to produce large quantities of ovomucin (over 400 mg/100 g of EW) with high purity. **Figure 7**C shows the original protocol, by which the ovomucin isolation took over two days. However, a method with reduced settling time from overnight to 2 h showed an almost equal yield and purity compared to original protocol[24,28]. Thus, crude ovomucin isolation was possible to complete within one day by using 2-step method. However, the salt removal, e.g. by dialysis, will take an additional day. Moreover, when the 2-step method was modified by adding a dialysis step, ovomucin-G yield has been reported to decrease to 208 mg[88] or 214 mg/100 g of whole EW[89]. In the first case, the purity of crude ovomucin was about 89%.
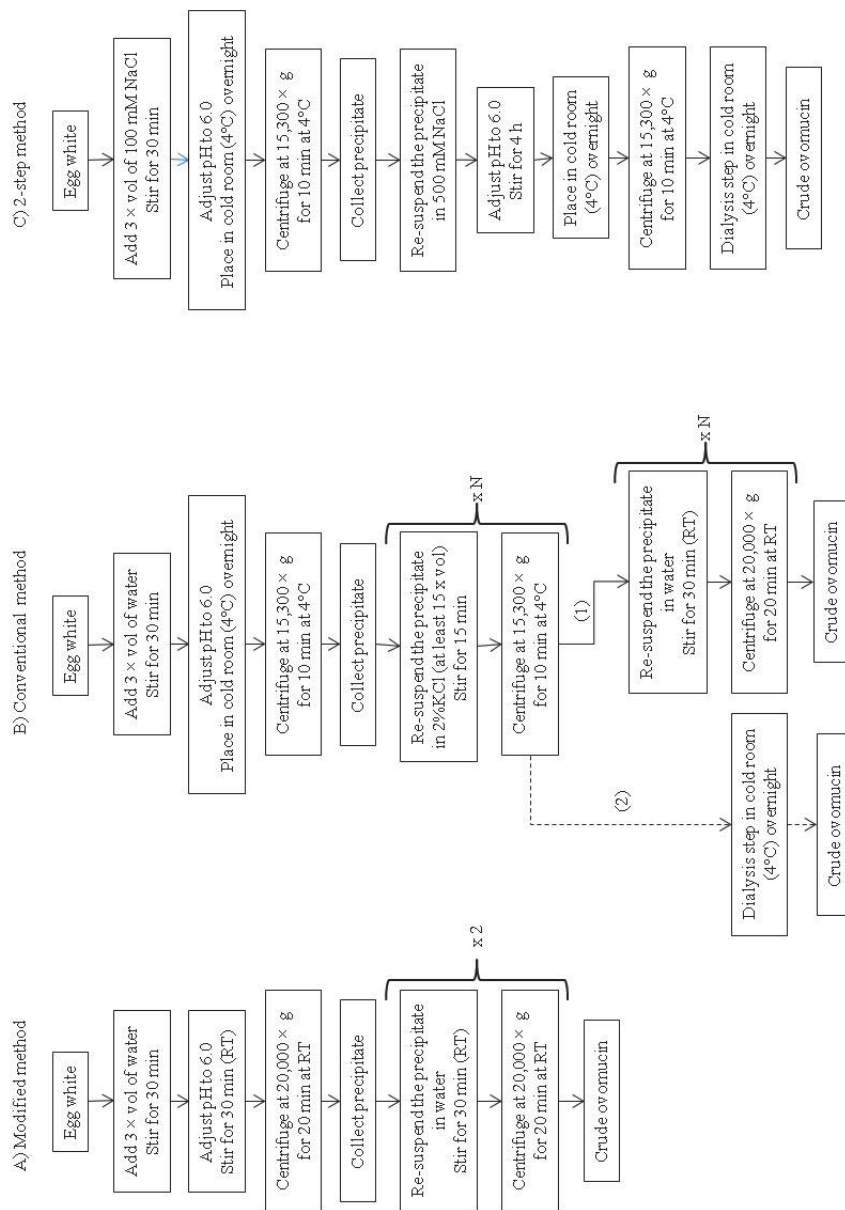
Fig. 7 Schematic protocols of various isoelectric precipitation methods for crude ovomucin isolation.

As can be seen in **Figure 7**, modified IEP method (A) was much simpler compared both to conventional (B) and to the 2-step (C) method. In addition to simplicity, it was also faster, as the ovomucin separation was accomplished within hours, compared to 1–2 days (including the dialysis step) needed when using two other methods. Also, the crude ovomucin yields obtained by using method (A) were somewhat higher.

The main disadvantage was the lower purity of the crude ovomucin obtained. A preliminary study (Hiidenhovi et al.[207], unpublished data), in which an additional salt washing step was included in method (A), has been conducted so as to overcome this problem. After the collection step, the crude ovomucin obtained was re-suspended in the concentrated (500 mM) salt solution, followed by the washing step as usual. The main impurities in the reference crude ovomucin were ovalbumin and lysozyme (**Figure 8A**). Each salt solution treatment reduced especially the amount of lysozyme in order, $CaCl_2 >$ NaCl ~KCl (**Figure 8** and **Table 11**). Due to this decrease in co-precipitated proteins, the purity-% of crude ovomucin (= a percentage of ovomucin-G) increased from 61% to 84% (**Table 11**).

Table 11 Effect of salt on both crude ovomucin and ovomucin-G yield.

Procedure	Crude ovomucin		Ovomucin-G		
	mg/100 g of egg white	% of total protein	mg/100 g of egg white	% of total protein	Ovomucin-G (%) (purity-%)
Reference	525 ± 0^a	4.5 ± 0.0^a	322 ± 0^a	2.8 ± 0.0^a	61.4 ± 1.8^b
NaCl	350 ± 35^b	3.0 ± 0.3^b	274 ± 27^{ab}	2.4 ± 0.2^{ab}	78.5 ± 4.1^a
KCl	325 ± 36^b	2.8 ± 0.2^b	259 ± 29^b	2.2 ± 0.2^b	79.9 ± 1.1^a
$CaCl_2$	212 ± 18^c	1.8 ± 0.2^c	178 ± 15^c	1.5 ± 0.1^c	84.0 ± 5.1^a

Given values are mean values and standard deviation of duplicate analyses. Dissimilar letters in the same column were significantly different ($p < 0.05$).

It should be noted that, instead of a dialysis step for salt removal, salt-treated crude ovomucin precipitate was washed just twice with Milli-Q water by centrifugation and then freeze-dried. Unfortunately, due to the low amount of salt-treated crude ovomucin precipitates obtained, the ash content analysis to ensure the efficiency of salt removal could not be carried out. Therefore, a factor, which took account of the moisture and ash content in ovomucin precipitates, was estimated. These factors were 0.94, 0.94 and 0.90 for NaCl, KCl and $CaCl_2$ treated ovomucin, respectively. Thus, especially in the case of $CaCl_2$ a more efficient salt removal step might be convenient. Nevertheless, the yield of ovomucin-G varied from 178 mg to 274 mg after a salt treatment (**Table 11).** Hence, by using the IEP method (A) including additional salt

washing step, it was possible to produce comparable amounts of ovomucin with reasonable purity.
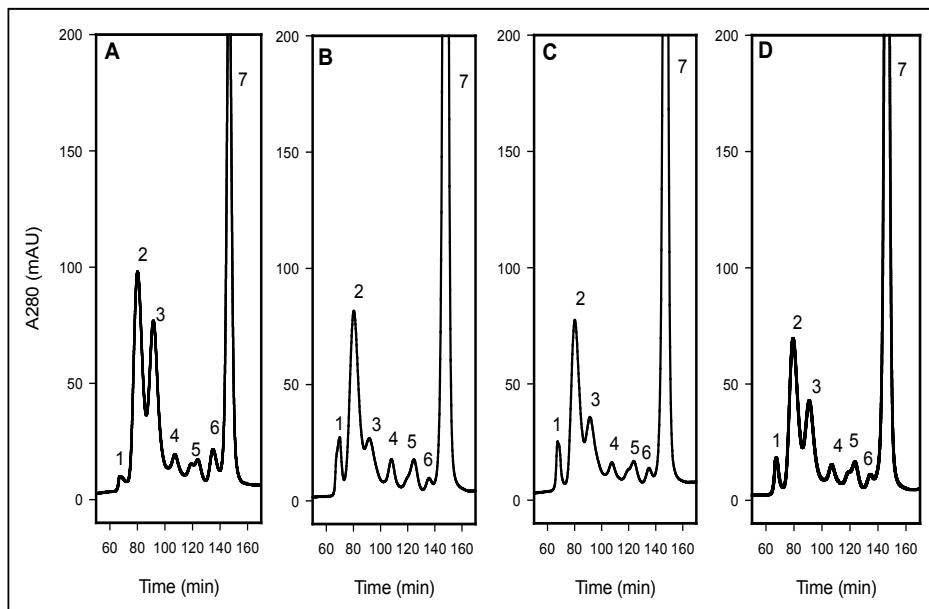

Fig. 8 Superose 6 HR Gel filtration chromatogram of crude ovomucin prepared by the IEP method without and with the salt treatment step. Ovomucin isolation procedure: A = reference, B = 500 mM NaCl, C = 500mM KCl and D = 500 mM CaCl$_2$. Peak numbering: 1 = β-ovomucin, 2 = α-ovomucin, 3 = ovostatin, 4 = ovotransferrin, 5 = ovalbumin, 6 = ovomucoid and 7 = lysozyme. Analysis conditions are described in Materials and Methods.

As mentioned in Chapter 2.2, the low yield of ovomucin is the main disadvantage in the use of GFC for ovomucin isolation. An alternative protocol to increase the ovomucin yield obtained by GFC has been presented by Hiidenhovi et al.[109], in which the modified IEP method (A) was used as a pre-purification step and the crude ovomucin obtained was further dissolved into a PBS buffer and centrifuged. The insoluble materials were collected, and further analysed by using Superose 6 HR GFC. Analysis showed that this precipitatecontained (insoluble) ovomucin with a purity of 99%. The supernatant, containing dissolved ovomucin, was further concentrated by using membrane filtration with a 100 kDa cut-off. Besides increasing the ovomucin concentration, this step also reduced the amounts of co-precipitated EW proteins having MW's less than 100 kDa. An aliquot of concentrated soluble ovomucin sample was loaded onto a preparative Sephacryl S-500 HR column. The proteins in the ovomucin sample were eluted in two peaks: A containing
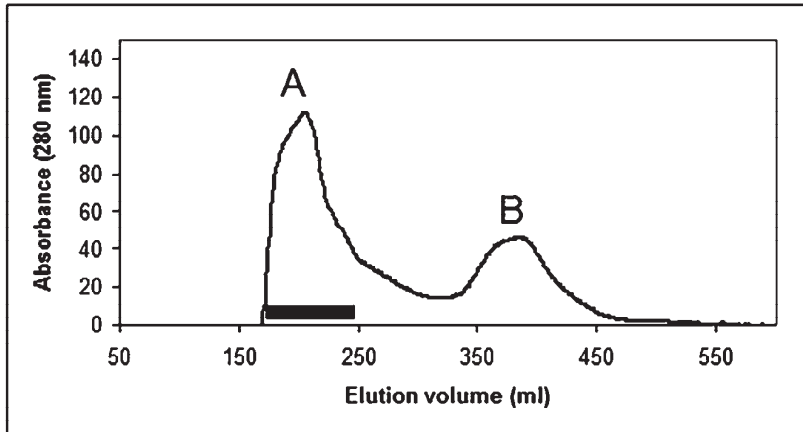
Fig. 9 Elution profile of membrane filtration concentrated ovomucin sample obtained by preparative Sephacryl S-500 HR GFC. Peak A = ovomucin, and peak B = other egg white proteins. Thick line = collected fractions. Reprinted from Hiidenhovi et al. 2003[109] with permission.
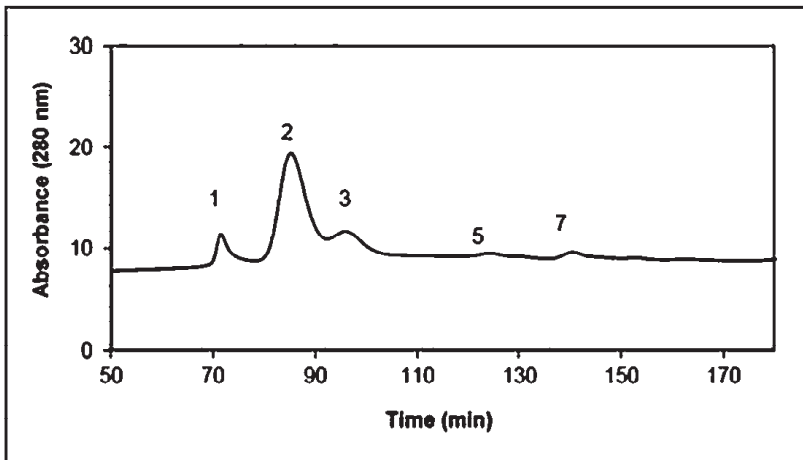

Fig. 10 Elution profile of peak A obtained by Superose 6 HR 10/30 GFC. Peak 1 = β-ovomucin, peak 2 = α-ovomucin, peak 3 = ovostatin, peak 5 = unknown (globulins?), and peak 7 = ovomucoid. Reprinted from Hiidenhovi et al. 2003[109] with permission.

ovomucin and B containing other EW proteins (**Figure 9**). An aliquot of the pooled fractions corresponding to peak A was dialyzed against water, lyophilized, and weighed. Thus, it could be estimated that about 110 mg of the soluble ovomucin was purified in one GFC run. Moreover, analytical Superose

6 HR GFC revealed that the soluble ovomucin fraction appeared to be almost free of other EW proteins, and its purity was calculated to be 97% (**Figure 10**). The obtained ovomucin yield by using the IEP method (A) + GFC combination was remarkably higher compared to previously reported values, which were 5-10 mg *per* GFC run[34,69].

5.2   Characterization of ovomucin

5.2.1   Subunit structure of ovomucin (I-III)

In the studies (**I**-**III**) ovomucin was isolated by using the IEP method, and the ovomucin precipitate obtained was referred to as crude ovomucin. The protein composition of the crude ovomucin was evaluated either by using preparative Sephacryl S-400 HR dual-column GFC (**I**) or by analytical Superose 6 HR dual-column GFC (**II, III**).
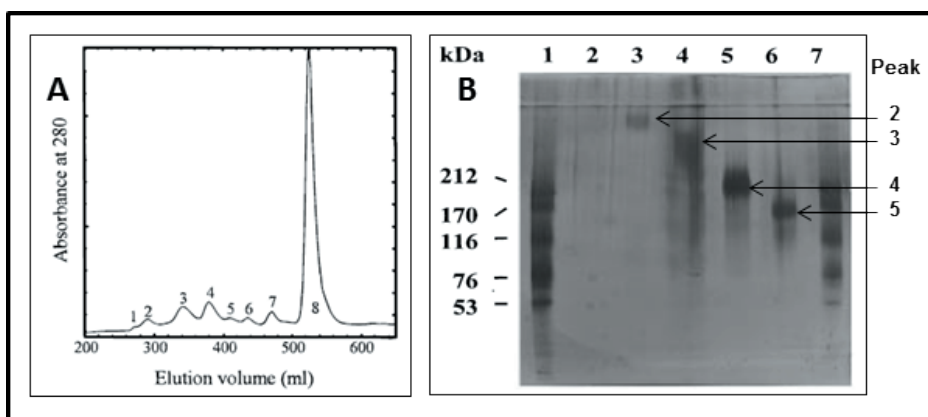
Fig. 11 Elution profile of crude ovomucin obtained by Sephacryl S-400 HR GFC (A) and SDS-PAGE of pooled fractions from Sephacryl S-400 HR GFC. Analysis conditions are presented in Materials and Methods, and peak and lane numbering in Publication **I**. Reprinted and adapted with permission from Hiidenhovi et al. 1999. Copyright 1999 American Chemical Society.

Sephacryl S-400 HR GFC separated crude ovomucin into eight peaks (**Figure 11A**). According to SDS-PAGE performed with EW protein standards, peak 6, 7 and 8 were identified as ovalbumin, ovotransferrin and lysozyme, respectively. Peak 1 could not be identified properly, and was suggested to be an unknown high MW ovomucin aggregate. In Study **I**, the identification of peaks 2-5 was based both on amino acid composition analysis and on MW

estimation by SDS-PAGE (**Figure 11B**). Suggested identifications of peaks 2-5 are shown in **Table 12**. It should be noted that the SDS-PAGE results have also been re-calculated by using the same protocol as used in Study **III**.
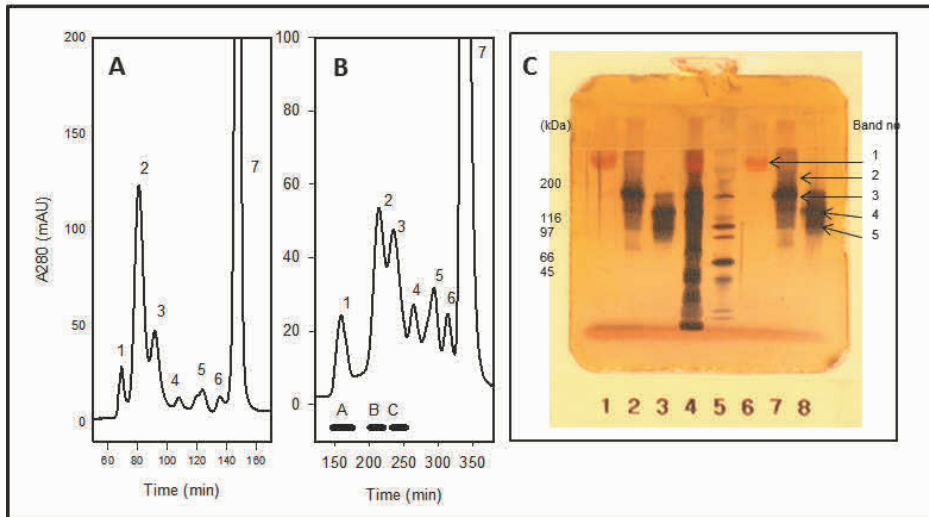

Fig. 12 Elution profile of crude ovomucin obtained by analytical (A) and by preparative (B) Superose 6 HR GFC with SDS-PAGE (C) of pooled ovomucin fractions purified by preparative GFC. Analysis conditions are presented in Materials and Methods, and peak and lane numbering in Publication **III**. Figure is obtained and adapted from Publication **III**.

Due to long elution time (over 24 hr) of preparative Sephacryl 400 HR GFC, an analytical Superose 6 HR GFC method was developed in Study **II** that enabled crude ovomucin analysis in 3 hours. Superose 6 HR GFC separated crude ovomucin into seven peaks (**Figure 12A**). According to GFC performed with EW protein standards, peaks 4-7 were identified as ovotransferrin, ovalbumin, ovomucoid and lysozyme, respectively. In Study **III**, the MWs of the main proteins eluting as peaks 1-3 were estimated by using SDS-PAGE. In addition, the use of SDS-PAGE enabled the more precise examination of the purity of the peaks. In order to ensure sufficient material for the studies, a preparative Superose 6 GFC was used (**Figure 12B**). Fractions were collected and pooled from peaks 1-3, and further analysed by SDS-PAGE (**Figure 12C**). Suggested identifications are shown in **Table 13**.

Table 12 Suggested identifications on proteins in peaks 2-5 obtained by Sephacryl S-400 HR GFC including the corresponding band) appeared in SDS-PAGE with estimated molecular weights (MW).

Peak number (GFC)	MW (kDa) (SDS-PGGE)*	MW (kDa) (SDS-PAGE)**	Suggested protein identification
2	610	460	β-ovomucin
3	350	320	α-ovomucin
4	210	210	α-ovomucin
5	160	160	ovostatin

*determined by SDS-PGGE technique presented by Lambin et al.[208] **re-estimated by the protocol used in Study **III**.

Table 13 Suggested identifications on proteins in peaks 1-3 obtained by Superose 6 HR GFC including the corresponding band(s) appeared in SDS-PAGE with estimated molecular weights (MW).The main protein of each peak is shown in bold.

Peak number * (GFC)	Band number (SDS-PAGE)	MW (kDa)	Suggested protein identification
1	**1**	**370**	**β-ovomucin**
2	2	290	α-ovomucin
2	**3**	**220**	**α-ovomucin**
3	**4**	**150**	**ovostatin**
3	5	120	α/β-ovomucin

In Study **II**, peaks 1, 2 and 3 obtained by Superose 6 HR GFC were called β-, α2- and α1-ovomucin, respectively, according to nomenclature presented by Itoh et al.[11] However, the results shown both in **Table 12** and **13** indicate that, at least with fresh eggs, the main proteins in SDS-PAGE gel regions approximately 350-400, 200-280 and 150-180 kDa are β-ovomucin, α-ovomucin and ovostatin, respectively. In addition, both studies (**I and III**) suggested an existence of another α-subunit in gel region around 300 kDa. The MW difference between the proteins in peaks 3 and 4 (**Table 12**) and in bands 2 and 3 (**Table 13**) might be caused by a different degree of glycosylation between these two proteins. On the other hand, some previous studies indicate self-association of α-ovomucin in dilute salt solutions[21,33].

Besides ovostatin (150 kDa band), peak 3 contained also another protein with an estimated MW of 120 kDa (band 5, **Figure 12C**). Also Hammershøj et al.[23] have reported a protein with the same order of magnitude (122 kDa) in SDS-PAGE pattern of crude ovomucin, and suggested that it could be α1-ovomucin. Furthermore, Hayakawa & Sato[33] reported that the sonicated α-ovomucin treated with mercaptoethanol appeared as a slight diffuse band in the region around 100 kDa in SDS-PAGE (Weber-Osborn protocol). However, it

has been reported that proteolytic degradation of reduced and alkylated β-ovomucin indicated the presence of 112-120 kDa fragment.[27,58,174] Although quite likely, this 120 kDa-fragment was orientated from ovomucin, and more specific analytical methods, such as MS, are needed to precise identification.

Because in previous studies ovomucin yields have been calculated for ovomucins consisting of both two and three subunits, the following nomenclature is used in this study: the combination of the first three proteins (peaks 1-3, **Figure 12A**, corresponding β-ovomucin, α-ovomucin and ovostatin/ovomucin) is called ovomucin gel (ovomucin-G), while the combination of β-ovomucin and α-ovomucin (peaks 1-2, **Figure 12A**) is called ovomucin complex (ovomucin-C) to facilitate comparison of previous data with present results (**Table 14-19**).

5.2.2   Subunit structure of ovomucin-G (III)

Heterogeneous behaviour, e.g. the existence of three compounds, has been noted during sedimentation velocity measurements of ovomucin[31,59,60]. In Study **III**, the composition of crude ovomucin was studied more closely. Crude ovomucin was dissolved into a buffer that contained SDS but no reducing agents, and introduced into a preparative Superose 6 HR column. Elution was also performed without reducing agents. It was noted that a high molecular weight protein was eluted as a single peak well ahead of other EW proteins (**Figure 13**). Pooled fractions, collected from the middle of this peak and further reduced by using β-mercaptoethanol, were subjected to SDS-PAGE analysis, which separated it into three bands. The MWs of these bands were estimated to be 370, 220 and 160 kDa, which corresponded to β-ovomucin, α-ovomucin and ovostatin, respectively, as discussed above (Chapter 5.2.1). Thus, it seems quite likely that ovostatin is somehow associated with β- and α-ovomucin. This possible interaction might even have some effect on the physical nature of various EW layers, as the amount of ovostatin varied from 30 to 370 mg per 100 g of EW (**Table 19**). This interaction might be accomplished by electrostatic forces or by hydrogen bonds, as after the severe washing procedure, only β-ovomucin and α-ovomucin were detected in SDS-PAGE[27]. Clearly, the role of ovostatin in the ovomucin gel forming process might be worth investigating more closely.
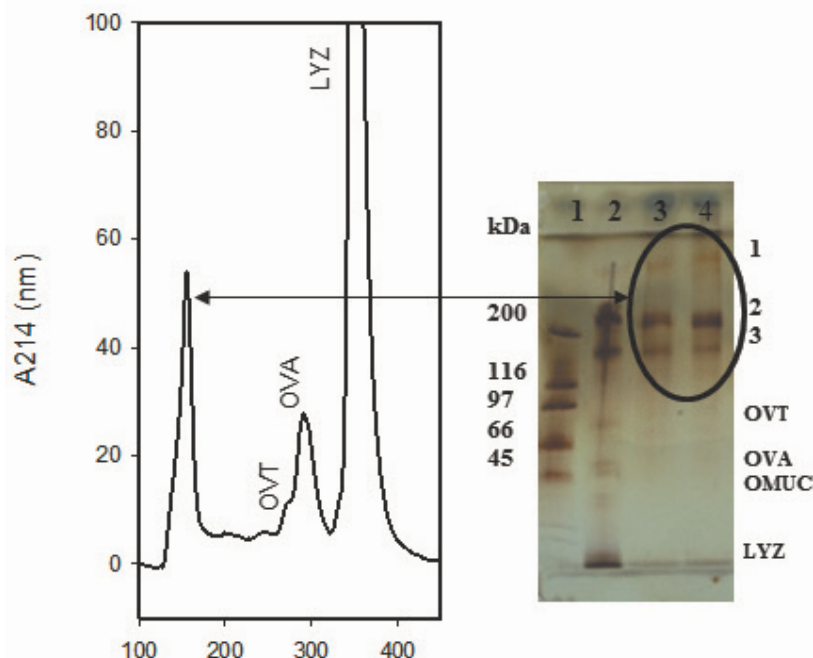
Fig. 13 Elution profile of crude ovomucin obtained by GFC without reducing agents (A) and SDS-PAGE of reduced ovomucin samples (B). Lane 1 = molecular weight markers, lane 2 = injected crude ovomucin sample, lane 3 = ovomucin fraction (1+1 with SDS-PAGE sample buffer), lane 4 = ovomucin fraction (reduced in elution buffer). OVT = ovotransferrin, OVA = ovalbumin, OMUC = ovomucoid and LYZ = lysozyme. Figure is obtained from Publication **III**.

5.3   Eggs as source of ovomucin (II, III)

Two studies (**II** and **III**) were conducted in which various egg fractions were investigated as a source of ovomucin to find the most relevant fraction for value-added utilization of ovomucin. In Study **II**, both shell egg and industrial EW fractions were compared. Whole EW was used as a reference, and thick EW was chosen because many studies linked to ovomucin's bioactive properties have been carried out with ovomucin isolated from thick EW. Similarly, two industrial EW fractions were used: liquid EW, a pasteurized EW product, and a filtration by-product fraction. The latter fraction was obtained from the egg processing plant as e.g. chalazae were filtered off after egg breaking and yolk separation prior to further EW processing.

In Study **III**, EW was fractioned into four basic EW layers, outer thin white, thick white, inner thin white, and chalaziferous layer (including chalazae). In

addition, the thick EW layer was further divided into a gel and a liquid fraction. Thus, altogether six "natural" fractions were compared.

It should be noted that, in order to make the results of these studies more comparable, the GFC results of Study **II** were re-integrated. In addition, in this dissertation the by-product results are presented as a daily base in contrast to the original paper (**II**), where they were presented as a united one. This separation also enabled the demonstration of possible differences between consecutive days.

5.3.1 Protein composition of crude ovomucin

Protein compositions of crude ovomucins obtained in Study **II** are shown in **Table 14**. The highest percentage amount of ovomucin-G was found to be in the liquid EW (about 71%), while in the other fractions it was practically equal but lower (63-65%). Naturally, this variation was linked to other co-precipitated egg white proteins. The main impurities in the crude ovomucins were ovalbumin and lysozyme, which was consistent with previous studies[23-25]. However, there was not such a considerable variation in percentage amount of lysozyme between different EW fractions, as was noted in Study **III** (**Table 15**). Interestingly, there was no variation between protein compositions of by-product ovomucins between two consecutive days. However, a longer follow-up time is needed for precise conclusions.

Protein compositions of crude ovomucins obtained from various EW layers are presented in **Table 15**. To our knowledge, this was the first time as the protein profiles of crude ovomucins obtained from these six egg white layers were presented simultaneously, facilitating the comparison of ovomucins obtained from different egg white layers. As expected, the percentage amount of ovomucin-G (OVMG%) varied among the EW layers. Interestingly, OVMG% was highest in outer thin EW crude ovomucin. Moreover, OVMG% of liquid thick EW was higher than in the gel fraction and equal into the chalaziferous fraction. Thus, the share of ovomucin-G in various crude ovomucins was highest in most liquid egg white fractions. In Study **III**, especially the amount of lysozyme varied considerably between EW layers. For example, thick white crude ovomucin contained about 2.5 times the amount of lysozyme compared to outer thin white crude ovomucin. It has been reported that lysozyme interaction with β-ovomucin is more extensive than with α-ovomucin[90]. However, this possible interaction did not solely explain the difference noticed, since, even though gel fraction contained about twice the amount of β-ovomucin compared to liquid fraction, the amount of lysozyme was quite the same in both fractions (**Table 15** and **19**). Similar behaviour also existed

among EW fractions in Study **II**, e.g. whole EW *vs*. filtration by-product (day 2) fraction (**Table 14** and **16**).

Ovomucin–lysozyme interaction is mainly considered to be electrostatic, although some hydrophobic and hydrogen interactions also exists[36,82,90,93,94]. The protein-protein interactions are known to be influenced by pH, salt type and salt concentration[209]. Thus, the action of various cations might also cause this observed difference. Actually, Robinson[91] has reported that Ca^{2+} and Mg^{2+} inhibited ovomucin-lysozyme interaction. Therefore, in the future, it might be advantageous also to measure the concentrations of several cations, especially divalent ones, in the four basic EW layers.

5.3.2   Various complex forms of ovomucin

The amounts of different ovomucin aggregates, namely crude ovomucin, ovomucin-G and ovomucin-C, are shown in **Table 16** and **17**. In Study **II** the amounts obtained for thick EW crude ovomucin, ovomucin-G, and ovomucin-C were about 530, 340 and 310 mg, while in Study **III** the corresponding values were about 570, 330 and 280 mg/100 g of EW (**Table 16** and **17**), respectively. In addition, when crude ovomucin, ovomucin-G and ovomucin-C was presented as a percentage of total protein corresponding values were for Study **II** 5.3%, 3.4% and 3.1%, and in Study **III** 5.7%, 3.3% and 2.8%, respectively. These results were surprisingly similar, as samples were obtained from different eggs, and moreover, there was a gap of many years between analyses. Thus, the IEP method (A) seems to be quite reliable and repeatable.

In Study **II**, both shell eggs and industrial EW fractions were studied. Despite the complex form of ovomucin, industrial fractions contained more ovomucin than shell egg fractions (**Table 16**). Three EW fractions containing most of the ovomucin were: filtration by-product (day 2) > liquid EW > filtration by-product (day 1). Thus, for now in the case of filtration by-product there was also statistically significant difference between the dates of collection.

The results of Study **III** are shown in **Table 17**. There were large differences in the amounts of various ovomucin complex forms among the EW layers, but more or less the order was about the same despite the ovomucin form. The three most ovomucin-rich fractions were: the chalaziferous layer > the gel fraction of the thick EW layer > the thick EW layer. Actually both the chalaziferous layer and the gel fraction contained more ovomucin than filtration by-product (day 2) fraction.

Table 14 Protein compositions of crude ovomucins obtained from different white fractions by using IEP method.

Egg white fraction	Ovomucin-G (%)	Ovotransferrin (%)	Ovalbumin (%)	Ovomucoid (%)	Lysozyme (%)
whole egg white	64.4 ± 2.0^b	1.4 ± 0.3^{bc}	6.5 ± 0.1^a	3.5 ± 0.7^b	24.2 ± 0.8^{ab}
thick egg white	64.5 ± 1.8^b	1.2 ± 0.0^c	6.2 ± 0.3^a	1.8 ± 0.3^c	26.3 ± 1.3^a
liquid egg white	70.6 ± 0.6^a	1.4 ± 0.0^{bc}	4.3 ± 0.1^a	3.6 ± 0.0^b	20.1 ± 0.7^c
filtration by-product, day 1	62.9 ± 0.7^b	2.2 ± 0.4^{ab}	5.6 ± 0.5^a	4.8 ± 1.1^{ab}	24.7 ± 1.2^{ab}
filtration by-product, day 2	63.0 ± 0.5^b	2.7 ± 0.5^a	6.9 ± 2.1^a	6.3 ± 0.1^a	21.3 ± 2.2^{bc}

Given values are mean values and standard deviation of triplicate analyses. Dissimilar letters in the same column were significantly different ($p < 0.05$).

Table 15 Protein compositions of crude ovomucins obtained from different egg white layers by using IEP method.

Egg white layer	Ovomucin-G (%)	Ovotransferrin (%)	Ovalbumin (%)	Ovomucoid (%)	Lysozyme (%)
outer thin white	73.4 ± 0.2^a	2.0 ± 0.0^d	4.9 ± 0.2^d	7.2 ± 0.1^a	12.5 ± 0.3^e
thick white	57.2 ± 0.4^d	3.2 ± 0.3^b	7.1 ± 0.3^b	2.4 ± 0.2^c	30.1 ± 0.1^a
inner thin white	65.5 ± 2.1^c	1.9 ± 0.2^d	5.5 ± 0.8^{cd}	2.8 ± 0.3^c	24.2 ± 0.9^b
chalaziferous layer	67.0 ± 1.4^{bc}	2.5 ± 0.3^c	6.4 ± 0.6^{bc}	1.1 ± 0.4^d	22.9 ± 0.9^c
gel fraction of thick white	58.2 ± 2.0^d	5.0 ± 0.4^a	12.3 ± 1.3^a	2.6 ± 0.3^c	21.8 ± 1.0^c
liquid fraction of thick white	68.8 ± 0.8^b	1.9 ± 0.2^d	5.5 ± 0.4^{cd}	4.6 ± 1.0^b	19.1 ± 0.4^d

Given values are mean values and standard deviation of triplicate analyses. Dissimilar letters in the same column were significantly different ($p < 0.05$).

Table 16 Amounts of various ovomucin forms obtained from different egg fractions using IEP method.

Egg white layer	Amount of crude ovomucin		Amount of ovomucin-G		Amount of ovomucin-C	
	mg/100 g of egg layer	% of total protein	mg/100 g of egg layer	% of total protein	mg/100 g of egg layer	% of total protein
whole egg white	430 ± 5[d]	4.3 ± 0.1[d]	277 ± 3[d]	2.7 ± 0.0[d]	229 ± 1[e]	2.3 ± 0.01[e]
thick egg white	531 ± 10[c]	5.3 ± 0.1[c]	343 ± 6[d]	3.4 ± 0.1[c]	312 ± 0.1[d]	3.1 ± 0.0[d]
liquid egg white	714 ± 2[b]	6.9 ± 0.02[b]	504 ± 1[b]	4.8 ± 0.1[b]	424 ± 1[b]	4.1 ± 0.01[b]
filtration by-product, day 1*	688 ± 20[b]	6.9 ± 0.2[b]	433 ± 13[c]	4.4 ± 0.1[c]	363 ± 0.4[c]	3.6 ± 0.04[c]
filtration by-product, day 2*	953 ± 34[a]	8.7 ± 0.3[a]	600 ± 21[a]	5.5 ± 0.2[a]	492 ± 1[a]	4.5 ± 0.01[a]

Given values are mean values and standard deviation of triplicate analyses (*n = 6). Dissimilar letters in the same column were significantly different (p < 0.05).

Table 17 Amounts of various ovomucin forms obtained from different egg white layers by using IEP method.

Egg white layer	Amount of crude ovomucin		Amount of ovomucin-G		Amount of ovomucin-C	
	mg/100 g of egg layer	% of total protein	mg/100 g of egg layer	% of total protein	mg/100 g of egg layer	% of total protein
outer thin egg white	280 ± 20[de]	2.8 ± 0.2[de]	206 ± 1[f]	2.0 ± 0.01[f]	125 ± 1[e]	1.2 ± 0.01[e]
thick egg white	573 ± 31[c]	5.7 ± 0.3[c]	328 ± 3[c]	3.3 ± 0.02[c]	276 ± 4[c]	2.8 ± 0.04[c]
inner thin egg white	367 ± 104[d]	3.7 ± 1.0[d]	240 ± 2[d]	2.4 ± 0.08[d]	186 ± 6[d]	1.9 ± 0.06[d]
chalaziferous layer	1,833 ± 58[a]	15.3 ± 0.5[a]	1,229 ± 26[a]	10.3 ± 0.22[a]	858 ± 11[a]	7.2 ± 0.09[a]
gel fraction of thick white	1,111 ± 19[b]	11.1 ± 0.2[b]	647 ± 22[b]	6.5 ± 0.22[b]	554 ± 15[b]	5.6 ± 0.15[b]
liquid fraction of thick white	250 ± 43[e]	2.7 ± 0.5[e]	172 ± 2[e]	1.8 ± 0.02[e]	142 ± 2[f]	1.5 ± 0.02[e]

Given values are mean values and standard deviation of triplicate analyses. Dissimilar letters in the same column were significantly different (p < 0.05).

Table 18 Protein distribution of various ovomucin forms obtained from different egg fractions using IEP method.

Egg white fraction	Protein distribution of ovomucin-G			Amounts of subunit proteins			Protein distribution of ovomucin-C	
	β-ovomucin (%)	α-ovomucin (%)	ovostatin/ovomucin (%)	β-ovomucin (mg/100 g of EW)	α-ovomucin (mg/100 g of EW)	ovostatin/ovomucin (mg/100 g of EW)	β-ovomucin (%)	α-ovomucin (%)
whole egg white	13 ± 0.1[c]	69 ± 0.2[a]	18 ± 0.4[a]	37 ± 0.3[d]	192 ± 0.7[c]	48 ± 1.0[d]	16 ± 0.1[c]	84 ± 0.1[a]
thick egg white	18 ± 0.1[c]	73 ± 0.1[a]	9 ± 0.03[c]	62 ± 0.2[d]	249 ± 0.3[b]	32 ± 0.1[e]	20 ± 0.1[c]	80 ± 0.1[a]
liquid egg white	24 ± 0.3[b]	60 ± 0.1[b]	16 ± 0.3[b]	120 ± 2.0[c]	304 ± 0.3[a]	80 ± 1.3[b]	28 ± 0.3[b]	72 ± 0.3[b]
filtration by-product, day1	35 ± 1.0[a]	49 ± 0.9[c]	16 ± 0.1[ab]	153 ± 4.1[b]	210 ± 3.8[c]	70 ± 0.4[c]	42 ± 1.1[a]	58 ± 1.1[c]
filtration by-product, day2	31 ± 1.1[a]	51 ± 1.2[c]	18 ± 0.1[a]	185 ± 6.6[a]	307 ± 7.5[a]	109 ± 0.8[a]	38 ± 1.4[a]	62 ± 1.4[b]

Given values are mean values and standard deviation of triplicate analyses. Dissimilar letters in the same column were significantly different (p < 0.05).

Table 19 Protein distribution of various ovomucin forms obtained from different egg white layers by using IEP method.

Egg white layer	Protein distribution of ovomucin-G			Amounts of subunit proteins			Protein distribution of ovomucin-C	
	β-ovomucin (%)	α-ovomucin (%)	ovostatin/ovomucin (%)	β-ovomucin (mg/100 g of EW)	α-ovomucin (mg/100 g of EW)	ovostatin/ovomucin (mg/100 g of EW)	β-ovomucin (%)	α-ovomucin (%)
outer thin egg white	4 ± 0.6[e]	57 ± 0.2[e]	39 ± 0.4[a]	9 ± 1.3[e]	116 ± 0.2[e]	81 ± 0.6[b]	7 ± 1.0[e]	93 ± 1.0[a]
thick egg white	22 ± 1.3[b]	62 ± 0.7[b]	16 ± 0.7[e]	73 ± 4.5[b]	203 ± 2.5[c]	52 ± 2.1[c]	26 ± 1.3[b]	74 ± 1.3[d]
inner thin egg white	19 ± 0.3[c]	58 ± 0.6[d]	23 ± 0.3[c]	46 ± 2.0[c]	140 ± 3.9[d]	54 ± 2.2[d]	25 ± 0.5[b]	75 ± 0.5[d]
chalaziferous layer	13 ± 1.1[d]	57 ± 0.3[e]	30 ± 1.1[b]	162 ± 11.7[a]	695 ± 12.2[a]	371 ± 20.7[a]	19 ± 1.3[c]	81 ± 1.3[c]
gel fraction of thick white	25 ± 1.1[a]	60 ± 1.2[c]	14 ± 0.7[f]	163 ± 6.1[a]	391 ± 15.5[b]	93 ± 7.7[b]	29 ± 1.3[a]	71 ± 1.3[e]
liquid fraction of thick white	13 ± 0.8[d]	70 ± 1.2[a]	17 ± 0.4[d]	22 ± 1.3[d]	121 ± 2.7[d]	30 ± 0.6[d]	15 ± 1.0[d]	85 ± 1.0[b]

Given values are mean values and standard deviation of triplicate analyses. Dissimilar letters in the same column were significantly different (p < 0.05)..

5.3.3   Ovomucin subunits

The percentage distribution of ovomucin subunits both for ovomucin-G and for ovomucin-C is shown in **Table 18** and **19**, in addition the amount (mg/100 g of EW fraction) of each subunit protein in various egg white layers. Respectively, **Table 3** summarizes the previous data of the relative content of α– and β-ovomucins in ovomucin complexes obtained from various EW layers. These values agreed quite well with results obtained in Study **III** for subunit distribution of ovomucin-C (**Table 19**). Thus ovomucin-C equates well with earlier models for ovomucin structure, which was thought to consisting of two subunit, namely α-ovomucin and β-ovomucin[20,21,33,42-46]. On that basis, it is reasonable to assume that the subunit protein distribution presented for various ovomucin-G forms (**Table 18** and **19**) is also equally plausible.

One aim of these studies (**II, III**) was to compare different egg white fractions as sources of ovomucin. Especially, the amount of β-ovomucin was very interesting, as many of the bioactive properties of ovomucin are linked to β-ovomucin. In both studies (**II, III**), the same three fractions that contained most of ovomucin also contained the largest amounts of β-ovomucin. In the study **II** the order was: filtration by-product (day 2) > filtration by-product (day 1) > liquid EW, and in Study **III** it was: gel fraction of thick EW layer ~ chalaziferous layer > thick EW layer.

Although filtration by-product fraction seemed to be a very prominent source of β-ovomucin, process development has reduced its amount so significantly that it has no practical meaning any more. Similarly, the amount of the chalaziferous layer is so low that it has only academical interest. The production of the gel fraction of thick EW on a larger scale is possible, but in doing this one should find some use for the thin EW fractions. Thus, the commercial liquid EW fraction seemed to be the best option, especially if it generally contains as high an amounts of β-ovomucin as was found in Study **II**.

5.4   Dissolving of ovomucin (IV)

5.4.1   Dissolving of ovomucin by using physical methods

The effects of various physical methods for crude ovomucin solubilisation are demonstrated in **Table 20** and **Figure 14**. The lowest solubility was achieved after conventional stirring (3 hours) regardless of the method used for measuring. However, RS-value for stirring seemed to be too high (about 49%), without statistically significant difference between stirring and colloid milling. As can be seen in **Figure 13A**, during conventional stirring mainly the co-

precipitated EW proteins are dissolved into PBS. Both Area-% and NANA values obtained for the conventional stirring fraction were lower compared to other crude ovomucins (**Table 20**), indicating a lower amount of solubilized ovomucin. In all other cases, the difference was statistically significant between stirring and other dissolving methods. Thus, it was quite obvious that, if the solubility measuring method of crude ovomucin is based only on protein determination, it will produce results that are too high in some cases. Similar results have been reported by Hammershøj et al.[23]. They noted that solubility of "unwashed" ovomucin, which contained co-precipitated EW proteins as impurities, into deionised water was about 30% compared to 10% obtained for "washed" ovomucin.

Table 20 The effect of various physical methods to crude ovomucin solubility.

Treatment	RS (%)	Area-% (%)	NANA (µg/ml)
Stirring (3 hr)	49.1 ± 1.8^c	19.2 ± 1.5^d	5.6 ± 0.6^c
Colloid milling (1 min)	49.6 ± 3.6^c	31.1 ± 1.3^c	16.1 ± 1.0^b
Sonication (6 min)	60.8 ± 3.6^b	47.1 ± 1.5^b	19.1 ± 0.7^b
Sonication (12 min)	79.5 ± 2.4^a	68.0 ± 12.1^a	26.9 ± 3.0^a

Given values are mean values and standard deviation of triplicate analyses. Dissimilar letters in the same column were significantly different ($p < 0.05$).

Colloid milling is commonly used to produce dispersion and to homogenise mixtures. In Study **IV**, a milk-like, smooth and opaque crude ovomucin suspension was attained by using IKA colloid mill. This "high-speed mixing" favoured ovomucin dissolution into PBS compared to conventional stirring (**Table 20** and **Figure 13A** and **13B**). Thus, much more ovomucin was dissolved in just 1 min by using colloid milling compared to 3 hours of stirring.

A higher solubilisation rate was achieved by using both sonication treatments compared to colloid milling. However, it should be noted that the treatment times used for sonication were higher, 6 and 12 minutes, compared to 1 minute of colloid milling. Nevertheless, in Study **IV** the highest solubilisation rate by using various physical treatments was obtained with 12-minutes sonication. The longer sonication time clearly enhanced solubilisation of crude ovomucin into PBS. Thus, sonication seemed to be a time-dependent phenomenon that was in accordance with a previous study[44], in which the MW of ovomucin was found to decrease in the course of time.
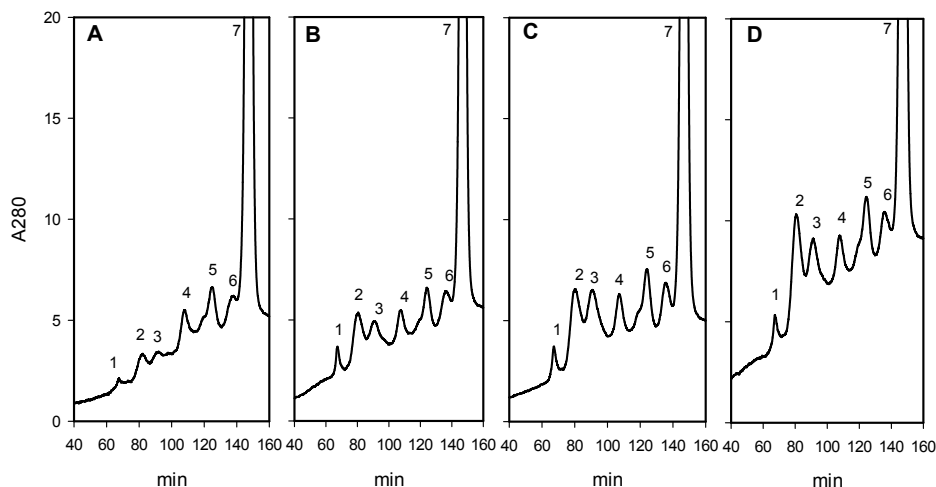
Fig. 14 Elution profiles of degraded ovomucins obtained by Superose 6 HR gel-filtration chromatography**.** A = conventional stirring, B = colloid milling, C = sonication (6 min), D = sonication (12 min). Peak numbering: 1 = β-ovomucin, 2 = α1-ovomucin, 3 = ovostatin, 4 = ovotransferrin, 5 = ovalbumin, 6 = ovomucoid and 7 = lysozyme. Figure is obtained from Publication **IV**.

Table 21 Percentage protein distribution of ovomucin-G.

Egg white layer	Protein distribution of ovomucin-G		
	β-ovomucin (%)	α-ovomucin (%)	ovostatin (%)
Stirring (3 hr)	17.6 ± 3.0^b	48.8 ± 5.9^a	33.6 ± 4.4^a
Colloid milling (1 min)*	23.2 ± 1.2^a	47.6 ± 3.3^a	29.3 ± 3.3^b
Sonication (6 min)	18.0 ± 3.0^b	45.8 ± 2.4^a	36.2 ± 2.4^a
Sonication (12 min)	16.7 ± 3.7^b	50.4 ± 3.2^a	32.9 ± 1.7^{ab}
Reference ovomucin **	17.9 ± 0.7^b	49.2 ± 0.7^a	32.9 ± 0.3^{ab}

Given values are mean values and standard deviation of six analyses (*n = 5, **n = 3). Dissimilar letters in the same column were significantly different ($p < 0.05$).

The effect of dissolving methods investigated on the percentage distribution of ovomucin-G subunits is shown in **Table 21**. The subunit distribution of ovomucin–G after sonication (both 6 and 12 minutes) and conventional stirring were quite similar, and resembled that of reference crude ovomucin, as no statistical difference between these treatments were noted. In contrast, colloid milling seemed to enhance β-ovomucin dissolution into PBS.

5.4.2 Dissolving of ovomucin by using enzymatic hydrolysis

Enzymatic hydrolysis is commonly used to enhance ovomucin solubility into conventional buffers[23,52,81,87,104-107]. In Study **IV**, trypsin was used both alone and in combination with some physical dissolving methods. In each case, DH values obtained were quite low due to a low E/S-ratio (1/100) (**Table 22**). In addition, hydrolysis temperature was a little higher (45°C) than normally used (37-40°C) for trypsin. In comparison, a DH value of 11% was reached already after 2 hours of trypsin hydrolysis when E/S=1/25 and 40°C was used[87]. Nevertheless, trypsin treatment seemed to enhance the ovomucin solubilisation attained by physical methods. For example, after 3 hours of enzymatic hydrolysis the NANA content was 20.7 µg/ml, compared to 5.6 µg/ml obtained by 3 hours of stirring (**Table 22**). Similarly, enzymatic hydrolysis enhanced ovomucin solubilisation both after 6 min of sonication and after colloid milling (**Table 21** and **22**). Actually, the NANA-content 28.8 µg/ml after a combination method of sonication *plus* hydrolysis was the highest value attained in Study **IV**.

Table 22 The effect of enzymatic hydrolysis to crude ovomucin solubility.

Treatment	DH (%)	RS (%)	NANA (µg/ml)
Stirring (3 hr)	nd	49.1 ± 1.8^c	5.6 ± 0.6^d
Enzymatic hydrolysis (3 hr)	3.7 ± 0.1^b	62.9 ± 8.4^b	20.7 ± 1.2^c
Sonication (6 min) + enzymatic hydrolysis*	5.5 ± 0.8^a	76.1 ± 2.1^a	28.8 ± 1.4^a
Colloid milling + enzymatic hydrolysis	3.0 ± 0.3^b	61.6 ± 4.0^b	25.8 ± 1.2^b

Given values are mean values and standard deviation of triplicate analyses. *RS and NANA (n = 2). nd = not determined. Dissimilar letters in the same column were significantly different ($p < 0.05$).

In another study (Hiidenhovi et al.[87]), 10 different enzymes were compared for their efficiency to hydrolyse ovomucin. Additionally, the effect of different enzyme and substrate concentrations on enzymatic hydrolysis of ovomucin was studied in more detail using three enzymes (Pronase, Proteinase K, and Trypsin). All 10 enzymes had a significant effect on ovomucin solubility. Pronase and Proteinase K were the most efficient of all 10 enzymes, whereas Trypsin and Pepsin were the most efficient among the food-grade enzymes. Enzyme concentration was found to affect the composition of the obtained ovomucin hydrolysates. Interestingly, with Pronase, we found considerable variation in the amount of peptides having a MW > 100 kDa when the enzyme concentration was changed from 0.1 to 2%, even though the RS of both hydrolysates was in the same range. The RS of hydrolyzed ovomucin decreased as the substrate concentration increased from 5 to 20 mg/ml (E/S = 1%),

whereas it had virtually no effect on DH values. Thus, it was possible to produce ovomucin hydrolysates with very different peptide compositions, not only by using different enzymes but also by changing the hydrolysis conditions. These results can be used to produce ovomucin hydrolysates having different biological activities.

5.5   Antiviral properties of ovomucin

5.5.1   Antiviral properties of crude ovomucins obtained from various EW layers (III)

In Study **III**, the crude ovomucins obtained from various EW layers were treated by trypsin to obtain antiviral peptides. Under rather mild hydrolysis conditions, E/S = 1% and hydrolysis time of 3 hrs, the degradation of various crude ovomucins was rather limited. The DH was about 4-6% for all the other egg fractions, except for liquid fraction, for which it was little over 10% (**Table 23**). The relative solubility was also quite low: the variation being from 45% to 65%. The highest RS values were found to be in crude ovomucins, in which β-ovomucin content was least, namely outer thin and liquid fraction of thick EW. About 40-65% of dissolved peptides were found to still have MW over 100 kDa indicating the presence of ovomucin fragments in hydrolysates.

Only three hydrolysed ovomucin fractions were found to contain HI activity against NDV, namely thick EW, inner thin EW and gel fraction of thick EW (**Table 23**). Interestingly Tsuge et al.[172] have reported that the MIC value for trypsin-treated thick white ovomucin was equally 46 µg/ml. However, it should be noted that the observed MIC values cannot be compared straightforwardly, because the virus concentration used by Tsuge et al.[172] was not given. Moreover, both thick EW and gel fraction ovomucin showed HI activity against avian influenza virus (AIV) subtype H5. In addition, gel fraction ovomucin inhibited also AIV subtype H7. All three ovomucin hydrolysates having HI activity also contained the highest amount of NANA (**Table 23**) the difference being statistically significant. Thus, quite likely NANA residues of the β-ovomucin have similar ability to bind also to AIV as to NDV[173,210].

Table 23 Characterization of crude ovomucin hydrolysates.

Egg white layer	RS (%)	DH (%)	NANA (μg/mL)	Molecular weight distribution (kDa)			MIC (μg/ml)		
				>100	100-10	<10	NDV	AIV5	AIV7
outer thin egg white	65.9 ± 4.6^a	4.8 ± 0.10^c	22.7 ± 1.2^c	51.0 ± 4.4^{bc}	19.4 ± 0.8^a	29.6 ± 3.7^b	n.i.	n.i.	n.i.
thick egg white	44.6 ± 3.7^c	5.9 ± 0.06^{bc}	47.2 ± 5.2^a	50.4 ± 3.3^{bc}	18.5 ± 1.0^a	31.1 ± 3.1^b	46 ± 14	36 ± 13	111 ± 9
inner thin egg white	52.1 ± 1.8^{bc}	5.7 ± 0.04^{bc}	53.0 ± 2.5^a	40.2 ± 7.5^d	21.5 ± 1.6^a	38.3 ± 6.0^a	65 ± 2	n.i.	n.i.
chalaziferous layer	57.6 ± 9.8^{ab}	6.3 ± 0.30^b	31.6 ± 2.6^b	45.3 ± 1.2^{cd}	15.1 ± 2.1^b	39.6 ± 3.2^a	n.i.	n.i.	n.i.
gel fraction of thick white	52.0 ± 7.1^{bc}	5.9 ± 0.05^{bc}	51.9 ± 3.9^a	55.5 ± 4.1^b	19.0 ± 1.6^a	25.5 ± 2.7^{bc}	83 ± 23	140 ± 7	n.i.
liquid fraction of thick white	64.6 ± 5.4^a	10.2 ± 0.03^a	33.3 ± 2.8^b	65.8 ± 4.4^a	13.6 ± 2.1^b	20.6 ± 2.3^c	n.i.	n.i.	n.i.

Given values are mean values and standard deviation of triplicate analyses. Dissimilar letters in the same column were significantly different ($p < 0.05$). MIC = minimal inhibition concentration, NDV= Newcastle disease virus, AIV5 = avian influenza virus, subtype H5, AIV7 = avian influenza virus, subtype H7, n.i. = no inhibition

However, the low NANA content in chalaziferous layer hydrolysate, as well as the absence of HI activity, was unexpected, as the crude ovomucin isolated from chalaziferous layer contained the highest amount of β-ovomucin (about 160 mg/100 g of EW, **Table 19**). The mild hydrolysis conditions used in Study **III** were probably inadequate to degrade the chalaza layer ovomucin properly. Most likely, this inadequacy was caused by the structural difference of the chalaza cords (tight bundle of strands) compared to other EW layers (viscous liquids or gels). Moreover, the hydrolysed ovomucin samples were kept frozen for several months, and that might also have a negative effect on HI activity.

5.5.2 Effects of various dissolving methods on antiviral properties of ovomucin (IV)

Table 24 Hemagglutination inhibition activity of different ovomucin samples.

Treatment	MIC (µg/ml)		
	NDV	AIV5	AIV7
Stirring (3 hr)	n.i.	n.i.	n.i.
Sonication (6 min)	n.i.	n.i.	n.i.
Sonication (12 min)	n.i.	n.i.	n.i.
Colloid milling (1 min)	122 ± 9	n.i.	n.i.
Enzymatic hydrolysis (3 hr)	154 ± 21	77 ± 10	77 ± 10
Sonication (6 min) + enzymatic hydrolysis*	186 ± 5	n.i.	n.i.
Colloid milling + enzymatic hydrolysis	n.i.	n.i.	n.i.

Given values are mean values and standard deviation of triplicate analyses. MIC = minimal inhibition concentration, NDV= Newcastle disease virus, AIV5 = avian influenza virus, subtype H5, AIV7 = avian influenza virus, subtype H7, n.i. = no inhibition *n = 2.

The solubilized ovomucin fractions obtained in Study **IV** were further tested for HI activity. As can be seen in **Table 24,** only three dissolving protocols tested produced moderate antiviral activity against NDV, namely colloid milling, enzymatic hydrolysis and a combination of sonication and enzymatic hydrolysis. Moreover, trypsin-digested crude ovomucin was found to have antiviral activity (MIC being approx. 77 µg/ml) against avian influenza virus, both subtype H5 and subtype H7 (**Table 24**).

It has been shown that the NANA residue in the β-ovomucin contributed greatly to the binding of ovomucin to various viruses, such as NDV and AIV[173,177,210]. Therefore, it was not surprising that HI activity was not found after conventional stirring, due to the minimal solubility of ovomucin achieved by this method. Instead, the lack of HI activity after both sonication treatments was strange. Especially in the case of 12-minute sonication, as the sonicated ovomucin contained about twice the amount of NANA found in the colloid

milling sample, the latter fraction was still the active one, having HI activity against NDV (**Table 21** and **24**). Due the large amount of bound-NANA in 12 minute-sonicated ovomucin fraction, the reason for the lack of HI activity might be some kind of, as yet unknown, structural modification during sonication. In addition to NANA residue, also the amino acid sequence seems to be important for interaction with both viruses and for inhibition the propagation of both NDV and AIV[211-213].

Colloid milling was the only physical treatment that induced HI activity alone. Strangely, this HI activity was lost after enzymatic hydrolysis, while the other enzymatically hydrolysed fractions were found to have HI activity (**Table 24**). Unfortunately, these above-mentioned deviations might also be caused by some kind of problems, e.g. in sample solubility, when frozen samples were reconstituted into HI analysis. Another cause might be prolonged storage of IEP ovomucin in the refrigerator before lyophilisation.

Table 25 Effect of various enzymes and hydrolysis time on crude ovomucin solubility and HI activity.

Enzyme	RS (%)	DH (%)	MIC µg/ml)		
			NDV	AIV5	AIV7
Alcalase (3h)	73 ± 3^b	5.3 ± 0.6^{bc}	45 ± 2.0^e	22 ± 1.0^c	22 ± 1.0^d
Alcalase (24h)	72 ± 5^b	5.7 ± 0.04^{bc}	176 ± 11.2^a	44 ± 2.8^a	88 ± 5.6^b
Esperase (3h)	36 ± 0^e	2.2 ± 0.3^d	117 ± 0.0^b	22 ± 0.0^c	88 ± 0.0^b
Esperase (24h)	46 ± 1^d	2.9 ± 0.1^d	56 ± 0.8^d	28 ± 0.4^b	112 ± 1.7^a
Neutrase (3h)	58 ± 3^c	4.5 ± 0.6^c	71 ± 3.9^c	18 ± 1.0^d	$35 \pm 2,0^c$
Neutrase (24h)	75 ± 1^b	5.9 ± 0.6^b	46 ± 0.7^{de}	23 ± 0.3^c	23 ± 0.3^d
Protamex (3h)	85 ± 2^a	6.0 ± 0.5^b	52 ± 1.1^{de}	13 ± 0.3^e	13 ± 0.3^e
Protamex (24h)	75 ± 2^d	8.6 ± 0.9^a	46 ± 1.1^{de}	11 ± 0.3^e	11 ± 0.3^e

Given values are mean values and standard deviation of duplicate analyses. Dissimilar letters in the same column were significantly different ($p < 0.05$).

The latter cause might be the most probable explanation, as another experiment (**IV**) was conducted, in which the effect of enzymatic hydrolysis on HI activity was investigated by using several enzymes. The EW and the crude IEP ovomucin before and after hydrolysis were frozen as before, but the storage time was much shorter compared the one used in dissolving test (**IV**). Moreover, the E/S ratio was a little higher (2%) compared to dissolving test, but without any prominent effect to DH or RS. As shown in **Table 25**, much higher HI activities were obtained this time. The effect of longer hydrolysis time (24 hours) varied among enzymes used, but generally there was no need for longer hydrolysis time meaning lower process costs.

Both Newcastle disease and avian influenza are pathogenic, thus causing substantial loss to the poultry industry. In addition, there is still a great concern that highly pathogenic, avian influenza virus (H5N1 or bird flu virus), with a mortality rate over 60% mutate so as to adapt to humans[210,214]. The controlling of AIV infection in poultry is crucial to prevent its outbreak among humans[215]. Thus, any possible compound that has the ability to inhibit propagation of AIV (or NDV), such as ovomucin, is worthy of further investigation.

6   SUMMARY AND CONCLUSION

A simple and rapid IEP method for crude ovomucin isolated was developed. By using this method, ovomucin separation was accomplished within hours, compared to the 1-2 days (including the dialysis step) needed when using several other methods. The main disadvantage was the low purity (about 60-65%) of obtained crude ovomucin. However, by using the IEP method including the additional salt washing step, it was possible to produce comparable amounts of ovomucin with reasonable purity

Structural characterization revealed that ovomucin is composed of two subunits, α- and β-ovomucin, as egg white protein formerly called α1-ovomucin seemed to be ovostatin. However, ovostatin tends to elute with both α- and β-ovomucin as a single peak in non-reducing GFC. Therefore, it might be quite possible that ovostatin is associated within β- and α-ovomucin. This interaction could even have some effect on the physical nature of various EW layers, as the ovostatin content varied a great deal, up to ten times, between different egg white layers. Thus, in future, the role of ovostatin in the ovomucin gel-forming process might be worth to investigating more closely. In addition, there was some evidence of self-association of α-ovomucin.

Various EW fractions were compared as a source of ovomucin. Although filtration by-product fraction was a very prominent source of both crude and β-ovomucin, process development has reduced its production so significantly that it has no practical meaning any more. Similarly, while the chalaziferous layer is an ovomucin-rich fraction, the actual amount of this EW layer is so low that it has only academic interest. The isolation of the gel fraction of thick EW on a larger scale is possible, but simultaneously one should also find some use for thin EW fractions. Thus, the commercial liquid egg white is possible the best option, especially if it generally contains as large an amounts of β-ovomucin as was found in these studies.

Crude ovomucin was dissolved both by using physical and enzymic methods. Of the physical methods, sonication was the most effective in dissolving ovomucin, but colloid milling seemed to be a very promising alternative. A milk-like, smooth and opaque crude ovomucin suspension was attained by using colloid mill. In addition, this method is easily up-scaled.

The dissolved ovomucin fractions were further tested for HI activity, and three dissolving protocols tested produced antiviral activity against NDV, namely colloid milling, enzymatic hydrolysis and a combination of sonication and enzymatic hydrolysis. Interestingly, trypsin-digested crude ovomucin was found to have antiviral activity against avian influenza virus: both subtype H5 and subtype H7.

ACKNOWLEDGEMENTS

Forssa, April 2015

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APPENDIX: ORIGINAL PUBLICATIONS

I.   Reprinted from *Journal of Agricultural and Food Chemistry*, 1999, 47, 1004-1008, with permission from American Chemical Society.

II.   Reprinted from *Journal of Agricultural and Food Chemistry*, 2002, 50, 2840-2845, with permission from American Chemical Society.

III. Submitted

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IV.   Submitted

DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic herring flesh lipids. (Organic chemistry).

2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.

3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.

4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.

5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.

6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.

7. **PÄIVI LAAKSO (1992)** Triacylglycerols – approaching the molecular composition of natural mixtures.

8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.

9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.

10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: food use potential.

11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.

12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: estimation of optimal *Lactobacillus* GG concentrations.

13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.

14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.

15. **SUSANNA EEROLA (1997)** Biologically active amines: analytics, occurrence and formation in dry sausages.

16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.

17. **TUULA VESA (1997)** Symptoms of lactose intolerance: influence of milk composition, gastric emptying, and irritable bowel syndrome.

18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.

19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.

20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.

21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.

22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole – compounds associated with boar taint problem. (Biotechnology).

23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: studies on diagnosis, prevalence and nutritional management.

24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.

25. **BAORU YANG (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.

26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.

27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.

28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.

29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria x ananassa* Duch.) fruit.

30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota – a target for treatment in infant atopic eczema?

31. **TARJA ARO (2003)** Chemical composition of Baltic herring: effects of processing and storage on fatty acids, mineral elements and volatile compounds.

32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.

33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.

34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.

35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.

36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.

37. **FANDI FAWAZ ALI IBRAHIM (2006)** Lactic acid bacteria: an approach for heavy metal detoxification.

38. **JUKKA-PEKKA SUOMELA (2006)** Effects of dietary fat oxidation products and flavonols on lipoprotein oxidation.

39. **SAMPO LAHTINEN (2007)** New insights into the viability of probiotic bacteria.

40. **SASKA TUOMASJUKKA (2007)** Strategies for reducing postprandial triacylglycerolemia.

41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: studies on polydextrose, lactose and cocoa mass.

42. **RENATA ADAMI (2007)** Micronization of pharmaceuticals and food ingredients using supercritical fluid techniques.

43. **TEEMU HALTTUNEN (2008)** Removal of cadmium, lead and arsenic from water by lactic acid bacteria.

44. **SUSANNA ROKKA (2008)** Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections.

45. **ANU LÄHTEENMÄKI-UUTELA (2009)** Foodstuffs and medicines as legal categories in the EU and China. Functional foods as a borderline case. (Law).

46. **TARJA SUOMALAINEN (2009)** Characterizing *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 as a new probiotic combination: basic properties of JS and pilot *in vivo* assessment of the combination.

47. **HEIDI LESKINEN (2010)** Positional distribution of fatty acids in plant triacylglycerols: contributing factors and chromatographic/mass spectrometric analysis.

48. **TERHI POHJANHEIMO (2010)** Sensory and non-sensory factors behind the liking and choice of healthy food products.

49. **RIIKKA JÄRVINEN (2010)** Cuticular and suberin polymers of edible plants – analysis by gas chromatographic-mass spectrometric and solid state spectroscopic methods.

50. **HENNA-MARIA LEHTONEN (2010)** Berry polyphenol absorption and the effect of northern berries on metabolism, ectopic fat accumulation, and associated diseases.

51. **PASI KANKAANPÄÄ (2010)** Interactions between polyunsaturated fatty acids and probiotics.

52. **PETRA LARMO (2011)** The health effects of sea buckthorn berries and oil.

53. **HENNA RÖYTIÖ (2011)** Identifying and characterizing new ingredients *in vitro* for prebiotic and synbiotic use.

54. **RITVA REPO-CARRASCO-VALENCIA (2011)** Andean indigenous food crops: nutritional value and bioactive compounds.

55. **OSKAR LAAKSONEN (2011)** Astringent food compounds and their interactions with taste properties.

56. **ŁUKASZ MARCIN GRZEŚKOWIAK (2012)** Gut microbiota in early infancy: effect of environment, diet and probiotics.

57. **PENGZHAN LIU (2012)** Composition of hawthorn (*Crataegus* spp.) fruits and leaves and emblic leafflower (*Phyllanthus emblica*) fruits.

58. **HEIKKI ARO (2012)** Fractionation of hen egg and oat lipids with supercritical fluids. Chemical and functional properties of fractions.

59. **SOILI ALANNE (2012)** An infant with food allergy and eczema in the family – the mental and economic burden of caring.

60. **MARKO TARVAINEN (2013)** Analysis of lipid oxidation during digestion by liquid chromatography-mass spectrometric and nuclear magnetic resonance spectroscopic techniques.

61. **JIE ZHENG (2013)** Sugars, acids and phenolic compounds in currants and sea buckthorn in relation to the effects of environmental factors.

62. **SARI MÄKINEN (2014)** Production, isolation and characterization of bioactive peptides with antihypertensive properties from potato and rapeseed proteins.

63. **MIKA KAIMAINEN (2014)** Stability of natural colorants of plant origin.

64. **LOTTA NYLUND (2015)** Early life intestinal microbiota in health and in atopic eczema.

65. **JAAKKO HIIDENHOVI (2015)** Isolation and characterization of ovomucin – a bioactive agent of egg white.