Bovine Acute Phase Proteins in Milk

Haptoglobin and Serum Amyloid A as Potential Biomarkers for Milk Quality

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Abstract

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The composition and quality of the raw milk is essential for the dairy industry and since only healthy cows produce milk of high quality, it is important with prospering cows. The most important and common disease among dairy cows is mastitis (inflammation of the udder). Mastitis is not only an animal welfare problem, but also results in impaired milk quality, reduced product yield, higher production costs and consequently a higher price for the consumer. The most common way to detect subclinical mastitis is by measuring the somatic cell count (SCC). The SCC is also an important parameter in milk payment systems, highly affecting the price to the producer. Since SCC is influenced by other factors than mastitis, e.g. lactation number, stage of lactation, stress etc., there is a need for new biomarkers for detection of subclinical mastitis as well as for raw milk quality.
The aim of this thesis was to obtain further knowledge about the occurrence of the two major acute phase proteins (APP) in bovine milk, haptoglobin (Hp) and serum amyloid A (SAA), and to evaluate their potential as biomarkers for raw milk quality. For the first time, a method for analysis of Hp using an optical biosensor based on surface plasmon resonance (SPR) technology was developed. The method is fully automated with no need for sample preparation before analysis and each sample requires approximately 8 minutes. The occurrence of Hp and SAA in quarter, cow composite and bulk tank milk samples were investigated and the results showed that APP could be detected in all types of samples. In general, detectable levels of APP in milk were related to high SCC, probably originating from cows with subclinical mastitis. In general, samples containing APP had lower casein content, casein number (casein in relation to total protein) and lactose but also increased whey protein content or increased proteolysis. Hp and SAA were suggested to be useful biomarkers for milk quality, especially the protein quality. To our knowledge, this thesis is the first describing Hp and SAA as potential biomarkers for raw milk quality.

Keywords: bovine, dairy cow, milk quality, acute phase proteins, haptoglobin, serum amyloid A, somatic cell count, mastitis, protein composition, casein content, proteolysis

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Till min syster Christina

Swedish summary

Svensk sammanfattning

För att mejerierna ska kunna förse konsumenterna med säkra, välsmakande produkter av hög kvalitet ställs höga krav på mjölkråvaran. Det är inte ovanligt att dagens kor producerar mellan 50-60 liter mjölk om dagen vilket innebär stora påfrestningar på kon. För att producera mjölk av högsta kvalitet är det därför av stor vikt att de är friska och välmående. Mastit (juverinflammation) är den vanligaste och mest förlustbringande sjukdomen hos mjölkkor. Det är en komplex sjukdom och många faktorer kan ligga bakom, men i de flesta fall är det bakterier som tagit sig in via spenkanalen och orsakat inflammationen. Det finns två former av sjukdomen, klinisk och subklinisk mastit. Vid en klinisk mastit, som årligen drabbar 20 % av korna, uppvisar kon ofta tydliga symptom. De kan exempelvis uppvisa ett rött och svullet juver samt synliga förändringar i mjölken t.ex. blod eller klumpar men även drabbas av ett försämrat allmäntillstånd såsom feber och matvägran. En betydligt vanligare form av mastit är den subkliniska formen. Man har beräknat att 2/3 av de svenska korna har subklinisk mastit någon gång under ett år. Den subkliniska formen kommer i många fall att passera utan att lantbrukaren märker att kon är sjuk och följaktligen hamnar denna mjölk i tanken. Detta på grund av att den subkliniska formen inte uppvisar några synliga förändringar i mjölken eller på kons allmäntillstånd. Oavsett vilken form av mastit kon är drabbad av är mjölkens kvalitet negativt påverkad.

I dag används mjölkens celltal (antalet vita blodkroppar) som en viktig markör för att avgöra om kon har mastit i en juverdel eller inte. Celltalet i tankmjölken ingår även i mjölkbetalningssystemet som ett mått på den allmänna juverhälsan i besättningen och lantbrukaren strävar efter ett så lågt celltal som möjligt för att inte få prisavdrag för den mjölk som levereras. Flertalet faktorer förutom juverhälsan kan dock påverka celltalet, såsom laktationsstadium, laktationsnummer, stress och brunst mm. Celltalet i tankmjölk har därför kritiseras för att vara ett okänsligt och ospecifikt mått på mjölkens kvalitet.

Under många år har man sökt efter alternativa markörer för juverhälsa och mjölkkvalitet men hittills har ingen av de utvärderade parametrarna varit tillräckligt bra för att ersätta eller komplettera celltalet. För ett antal år sedan började forskare intressera sig för de så kallade akutfasproteinerna. Dessa är artspecifika, produceras huvudsakligen i levern och när de detekteras i blod är de en ospecifik markör för skada eller sjukdom. I dag används t.ex. C-reaktivt protein inom humanvården för att avgöra om patienten har en bakteriell eller viral infektion. Hos kor finns det huvudsakligen två akutfasproteiner, haptoglobin (Hp) och serum amyloid A (SAA). I tidigare studier har man undersökt dessa två proteiner i mjölk som tänkbara markörer för mastit, men inte huruvida de skulle kunna vara tänkbara mjölkkvalitetsparametrar.

Syftet med denna avhandling var att undersöka om det finns mätbara halter av Hp och SAA i olika typer av mjölkprov; juverfjärdedels-, samlings- (mjölken från alla

fyra juverdelarna) och tankmjölksprov, samt att studera om det finns något samband mellan förekomsten av dessa proteiner och olika mjölkkvalitetsparametrar.

För att uppnå detta genomfördes fyra olika studier:

I studie I utvecklades en snabb och enkel biosensor metod för att mäta Hp i mjölk. Detta var första gången denna teknik användes för att mäta ett akutfasprotein i mjölk. Denna teknik är helt automatisk och snabb att använda, varje prov tar bara ca 8 minuter att analysera. Dessutom kan man följa alla steg som händer på dataskärmen, vilket är en fördel vid metodutveckling. Tidigare metoder som använts för att mäta Hp och SAA är ofta både tidskrävande och dyra. I studie II analyserades Hp och SAA i olika typer av mjölkprover. Mätbara halter återfanns i såväl juverdels-, samlings- som tankmjölksprov. Detta var första gången någon visade att akutfasproteinerna kunde mätas i tankmjölk. SAA förekom oftare än Hp och även i högre koncentrationer. Generellt sett hade mjölk som innehöll Hp och/eller SAA ett förhöjt celltal.

I studie III och IV samlades samlings- samt tankmjölksprover in och samband mellan olika mjölkkvalitetsparametrar och förekomsten av Hp och SAA studerades. Även här förekom SAA oftare och i högre koncentrationer än Hp i mjölken både i samlings- och tankmjölksprover. Dessa studier visade att Hp och SAA kan ge värdefull information om mjölkens proteinsammansättning, vilket mejerierna har speciellt intresse av. Hp och SAA uppvisade samband med mjölkens innehåll av kaseiner, som är speciellt viktiga vid ost och yoghurttillverkning. Ju mer av de ekonomiskt värdefulla proteinerna, kaseinerna, mjölken innehåller desto mer ost får man ut per kilo mjölk.

Det viktigaste med dessa studier är att det är första gången Hp och SAA relateras till mjölkkvaliteten samt att endast autentiska mjölkprover använts dvs. sådana som normalt skulle ha lämnats till mejeriet. I studie I användes dock ett antal mjölkprover från kor med klinisk mastit för att utvärdera den metod som utvecklades. Akutfasproteinerna Hp och SAA har i tidigare studier visat sig vara intressanta för diagnostik av mastit. Om det vore möjligt att mäta dessa proteiner i automatiska mjölkningssystem skulle det öppna möjligheter för att upptäcka mastit, då mjölken i dag inte inspekteras innan kon mjölkas i detta system. Detta arbete har visat att Hp och SAA även tycks utgöra tänkbara markörer för mjölkkvalitet, speciellt för mjölkens proteinkvalitet. I dag mäts enbart det totala innehållet av protein i mjölken, vilket också är en viktig betalningsgrundande parameter. Det finns idag inte någon enkel teknik för att mäta kaseinerna specifikt i mjölk. Hp och SAA kan i framtiden vara av stor vikt för mejeriindustrin såväl som en kompletterande parameter i mjölkbetalningssystemet.

Contents

Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

Papers I-IV

I. Åkerstedt, M., Björck, L., Persson Waller, K. & Sternesjö, Å. 2006. Biosensor assay for determination of haptoglobin in bovine milk. *Journal of Dairy Research 73*, 299-305.

II. Åkerstedt, M., Persson Waller, K. & Sternesjö, Å. 2007. Haptoglobin and serum amyloid A in relation to the somatic cell count in quarter, cow composite and bulk tank milk samples. *Journal of Dairy Research 74*, 198-203.

III. Åkerstedt, M., Persson Waller, K., Bach Larsen L., Forsbäck, L. & Sternesjö, Å. 2008. Relationship between haptoglobin and serum amyloid A in milk and milk quality. *Accepted for publication in International Dairy Journal*. doi:10.1016/j.idairyj.2008.01.002

IV. Åkerstedt, M., Persson Waller, K. & Sternesjö, Å. 2008. Haptoglobin and serum amyloid A in bulk tank milk in relation to raw milk quality. ***Submitted.***

Paper I, II and III are published by kind permission of the journals concerned.

Maria Åkerstedt's contribution to the papers:

I. Participated in the planning of the experimental work together with the supervisors. Performed all the laboratory work, participated in the evaluation of the results and was responsible for compiling the manuscript.

II. Participated in the planning of the experimental work together with the supervisors. Milked the cows for collection of quarter and cow composite milk samples. Performed all the laboratory work. Main responsibility for the evaluation of results and for writing the manuscript.

III. Planned the work together with supervisors, milked the cows for collection of cow composite milks samples together with one of the co-authors. Performed part of the laboratory work. Main responsibility for the evaluation of results and for writing the manuscript.

IV. Planned the work together with supervisors, participated in the collection of the samples from the dairy. Organised information meetings for people involved and coordinated the handling and analyses of samples. Performed most of the laboratory work. Main responsibility for the evaluation of results and for writing the manuscript.

List of abbreviations

APP	acute phase proteins
BTMSCC	bulk tank milk somatic cell count
CMT	California mastitis test
CRP	C-reactive protein
CV	coefficient of variation
ECM	energy corrected milk
ELISA	enzyme-linked immunosorbent assay
HDL	high density lipoprotein
Hp	haptoglobin
HRP	horse-radish-peroxidase
LOD	limit of detection
MAA	milk amyloid A
M-SAA	mammary derived serum amyloid A
NAGase	N-acetyl-β-D-glucosaminidase
PMN	polymorphonuclear cells
RU	resonance units
SAA	serum amyloid A
SCC	somatic cell count
SD	standard deviation
SDS	sodium dodecyl sulphate
SPR	surface plasmon resonance
TMB	tetramethyl benzidine
UHT	ultra high treatment

Introduction

Dairy production in Sweden

Milk and dairy products are nutritionally important in the diet worldwide. In Sweden 42 % of the delivered milk is sold as liquid milk and fermented products i.e., sour milk, yoghurt, cream and sour cream. The cheese-making industry processes 37 % of the delivered milk and 15 % will be sold as milk powder, 2 % as fodder and the remaining 4 % is used for other purposes (SCB, 2007). Consumption of liquid milk has decreased in Sweden during the last decades; today 111 litres milk are consumed per person and year, compared to 190 litres milk in 1980. The situation for yoghurt and sour milk is the opposite; the consumption of these products has doubled since 1970 (Swedish Dairy Association, 2008).

In Sweden today, there are less than 400,000 dairy cows divided between 8,000 farms i.e., on average 48 cows/herd. It is not unusual that a cow produces between 50–60 kg milk each day during peak lactation. Each cow produces on average 9300 kg energy corrected milk (ECM) per year. The annual figures can be compared with those from 1985 when there were 646,000 dairy cows at 17,200 farms, producing on average 6300 kg 4 % fat corrected milk per cow and year. The numbers of dairy herds and cows have decreased during the last decades but the amount of milk produced per cow has increased during the same time (Figure 1). The total volume of milk produced during the last decade has thus remained almost the same despite the extraordinary change in structure (Swedish Dairy Association, 2008).

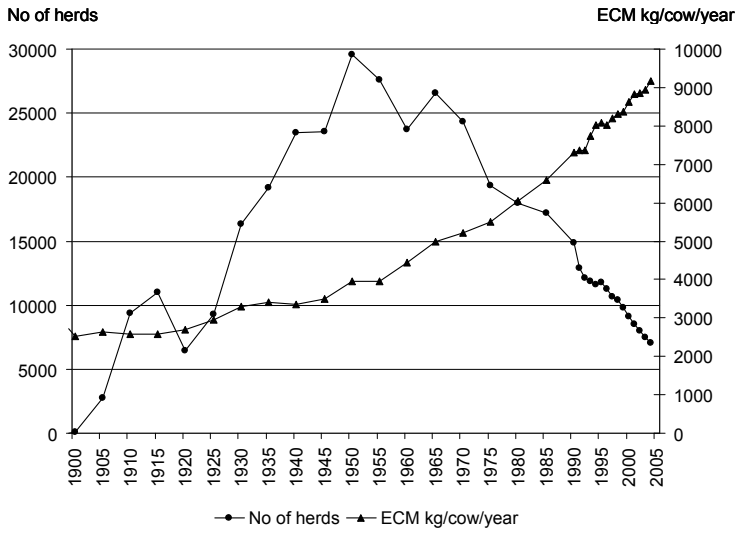

Figure 1. The number of herds and the amount (kg) of energy corrected milk (ECM) produced per cow and year in Sweden between 1900 and 2005 (Swedish Dairy Association, 2008).

For many years dairy cooperatives and consequently milk payment systems encouraged an increase in the volume of milk delivered. The average milk produced from each cow was increased by optimising feeding and breeding strategies, resulting in more diluted milk (Lindmark-Månsson, Fondén & Pettersson, 2003). Today, the payment systems pay more attention to the protein and fat content in the milk than earlier. The dairies require milk of the highest possible compositional and hygienic standards to meet the consumers' demand for safe and high-quality products. Because the dairy cows of today have become such high-producing animals it is extremely important to have healthy and prosperous cows, and only healthy cows will produce raw milk of high quality.

Bovine mastitis

The most important and common disease among dairy cows is mastitis (inflammation in the udder). Mastitis comprises 47 % of all veterinary treated diseases among dairy cows in Sweden (Swedish Dairy Association, 2007) and despite different mastitis control programs it is still the major challenge for the dairy industry (Bradley, 2002). The predominant cause of mastitis is intramammary infection caused by bacteria entering the teat canal and colonising the udder tissue where they can establish and multiply using milk as an optimal substrate (Bramely & Dodd, 1984; Sandholm, Kaartinen & Pyörälä, 1990). Mastitis is not only an animal welfare problem; the farmer, the dairy company as well as the consumer are affected. It is an economic loss for the farmer due to reduced milk production, antibiotic treatment, milk that must be discarded during treatment and withdrawal period, milk price deductions, extra work, and sometimes culling (Bradley, 2002). The dairy industry also faces problems in producing high-quality dairy products. Since the milk composition from cows with mastitis is deteriorated, resulting in reduced product yield, higher production costs, and products with reduced shelf life (Kitchen, 1981; Munro, Grieve & Kitchen, 1984; Le Roux, Laurent & Moussaoui, 2003). This results in higher dairy products prices to the consumers.

Clinical and subclinical mastitis

Mastitis occurs in two different forms; clinical and subclinical mastitis. In Sweden, 20 % of the dairy cows are affected by clinical mastitis annually (Swedish Dairy Association, 2007). This figure is probably an underestimation since only cases treated by a veterinarian are included. Some clinical cases that are not that severe will not be treated with antibiotics and are therefore not included in the statistics. The farmer typically eliminates these cases by massage and frequent milking of the affected quarters. The clinical form of mastitis is recognised by abnormalities of the udder and milk during visual examination. This condition may be characterised by heat, swelling, hardness and pain in the udder as well as clots, flakes or even blood in the milk. Systemical signs like fever and loss of appetite may also be observed. Clinical mastitis may be a severe and painful disease for the animal and can in some cases cause a sudden death. Since clinical mastitis is often easy to detect, these cows are taken out of production and it is not allowed to deliver their milk to the dairy (Harding, 1995; Sandholm *et al.*, 1995).

Subclinical mastitis, on the other hand, is a larger problem for the dairy industry since this condition shows no visible changes in the udder or in the milk. Consequently, many of these cases remain undetected and the milk is therefore delivered to the dairy. It has to be kept in mind that regardless of clinical or subclinical mastitis, the composition of the milk is altered and mastitis pathogens are often present (Harding, 1995). The milk yield is reduced during the inflammation since the epithelial cells producing milk are damaged, and the reduction often becomes permanent throughout the lactation (Hortet & Seegers, 1998; Seegers, Fourichon & Beaudeau, 2003). It is therefore of great importance to detect also the subclinical cases. It is estimated that approximately 2/3 of the cows will be affected by subclinical mastitis during the lactation in Sweden (Swedish Dairy Association, 2006).

Changes in milk composition during mastitis

An inflammation in the udder affects the composition of the milk in several ways. Serum proteins will leak into the milk due to increased permeability between blood and milk. Moreover, epithelial cells are damaged resulting in release of intracellular components to the milk, and finally, the synthesis of milk-specific components produced in the mammary epithelium is reduced (Mattila, Pyörälä & Sandholm, 1986).

The somatic cell count (SCC) increases during mastitis since the somatic cells are part of the defence system that are activated during a microbial infection. The SCC includes lymphocytes, macrophages, polymorphonuclear cells (PMN) and epithelial cells and during inflammation their proportions may change dramatically. In a healthy udder the dominating cells are the macrophages and lymphocytes while during infection the PMN dominate, constituting approximately 90–95 % of the cells (Concha, 1986; Kehrli & Shuster, 1994).

The effect of mastitis on the total fat content in the milk is ambiguous, but regardless if the fat decreases or increases, the composition of the fat will change and thereby deteriorate the quality of the products (Kitchen, 1981; Munro, Grieve & Kitchen, 1984; Sandholm *et al*., 1995). During mastitis the amount of free fatty acids increase, due to increased activity of lipolytic enzymes, which are also active during cold storage of the milk (Ma *et al*., 2000; Santos, Ma & Barbano, 2003). Since some of the lipolytic activity will remain after pasteurization, lipolysis may continue in the product and cause rancid off-flavour, especially in dairy products where the manufacture includes storage of products with long shelflife e.g., butter and ultra-high treatment (UHT) milk (Ma *et al*., 2000).

Most studies are unambiguous about the lactose content, i.e., lactose decreases during mastitis (Linzell & Peaker, 1972; Auldist *et al*., 1995; Holdaway, Holmes & Steffert, 1996; Klei *et al*., 1998; Nielsen *et al*., 2005). Lactose has for many years been evaluated as an indirect marker for mastitis with varying degree of success. It seems to be useful only if applied on quarter level milk and healthy udder quarters as controls (Berglund *et al*., 2007). In bulk tank milk, lactose has never been considered a milk quality parameter.

Total protein is one of the most important milk quality parameters and it is included in raw milk quality programs and is a key factor influencing the milk price to the producer. Different studies on effects of mastitis on total protein show contradictory results. Some studies have not observed any differences in total protein concentration between milk from cows with mastitis and clinically healthy cows (Kitchen, 1981; Munro, Grieve & Kitchen, 1984). The total protein content even increased in quarters with subclinical mastitis (Urech, Puhan & Schällibaum, 1999; Nielsen *et al*., 2005). The reason for this is the change in protein composition of the milk. Most studies have shown that the valuable proteins, the caseins, will decrease while the inflammatory, non-coagulating serum proteins and thus whey proteins will increase during mastitis (Barbano, Rasmussen & Lynch, 1991; Auldist *et al*., 1996). Urech Puhan & Schällibaum (1999) showed that these changes also occur in mild subclinical mastitis.

The lower casein content in milk from cows with mastitis may also be explained by increased proteolysis (Auldist *et al*., 1995; Klei *et al*., 1998; Urech, Puhan & Schällibaum, 1999; Le Roux, Laurent & Moussaoui, 2003; Larsen *et al*., 2004). Proteolysis is a major factor causing inferior quality and stability of milk and dairy products (Mara *et al*., 1998, Barbano, Ma & Santos, 2006; Kelly, O´Flaherty & Fox, 2006). Proteolysis occurs in the udder (Schaar, 1985; Urech, Puhan, Schällibaum, 1999), in the bulk tank on the farm and in the silo at the dairy (Letiner *et al*., 2008) as well as in dairy products since many of the proteases are heat stable and will survive pasteurisation (Santos, Ma & Barbano, 2003; De Noni *et al*., 2007). Plasmin is the most studied proteolytic enzyme in milk, originating from blood, and is in milk normally associated with the casein micelles. Plasminogen is the inactive precursor of plasmin and following several proteolytic cleavages of the precursor, plasmin will become active. Plasmin then binds and hydrolyses casein, resulting in soluble peptides (Bastian & Brown, 1996) which will be lost in the whey fraction during cheese making. Consequently, cheese yield will be reduced presenting a major problem for the dairies. Plasmin is the major proteolytic enzyme in low SCC milk while the importance of other proteases, e.g. cathepsins and elastase originating from PMN (Le Roux, Laurent & Moussaoui, 2003) seems to increase with increasing SCC (Larsen *et al*., 2004). There is evidence that the PMN may ingest caseins as well as fat (Russel, Brooker & Reiter, 1977) but to what extent this will affect the protein composition in the milk has not been evaluated. In addition, exogenous, heat stable proteases originating from psychrotrophs and pathogenic bacteria associated to the infected gland, will also contribute to the degradation of the caseins (Harayani *et al.*, 2003; Haddadi *et al*., 2006). The casein content but also the degree of casein degradation affects the processing properties of the milk and the yield and quality of the dairy products. Properties that may be affected include stability, sensory properties and texture, e.g., in the production of cheese and yoghurt (Lynch, Barbano & Fleming, 1995; Auldist *et al*., 1996; Kelly, O´Flaherty & Fox, 2006).

Mastitis diagnosis

The SCC in milk is the most commonly used parameter in diagnosis of mastitis. SCC is in many countries routinely analysed, using an automated fluoro-opto-electronic cell counting instrument, which is usually limited to central laboratories. Another alternative, mainly used in field, is the California mastitis test (CMT); a cow-side test that will give a rough estimation of the SCC in the milk. SCC together with bacteriological examination is the recommended method for detection and verification of subclinical infectious mastitis (Hillerton, 1999). Bacteriological examination is not an optimal method used in routine, large-scale analysis of mastitis, e.g. in detection of subclinical mastitis. Bacteriological sampling and examination is time consuming, expensive and it takes several days to get a correct diagnosis. One bacterial sampling is often not enough to determine if the quarter is infected or not (Sears *et al*., 1990; Pyörälä & Pyörälä, 1997) and the bacteria count and the SCC will not necessarily peak at the same time (Daley *et al*., 1991).

Using only SCC in diagnosis of subclinical mastitis has been criticised for not being sensitive and specific enough since SCC is influenced by many different factors. Except the inflammatory status of the udder quarter, there are many other factors affecting SCC, e.g. lactation number, stage of lactation, milk production, stress, season, breed and parity (Harmon, 1994; Schepers *et al*., 1997; Jayarao *et al*., 2004). Threshold values for SCC in diagnosis of mastitis have been discussed for many years in quarter and cow composite milk (Mattila, Pyörälä & Sandholm, 1986; Holdaway, Holmes & Steffert, 1996). Values suggested differ between studies since they are from different countries, use different breeds, and different types of milk samples. The SCC also shows a day-to-day variation making it more difficult to determine a cut-off value (Mattila, Pyörälä & Sandholm, 1986). Decades ago, in the 1970s, the threshold SCC for a healthy quarter was 500,000 cells mL^{-1} and today it is suggested to be below 100,000 cells mL^{-1} (Hamann, 2003; Le Roux, Laurent & Moussaoui, 2003; Pyörälä, 2003). SCC does not always correlate with udder health status as defined by repeated bacteriological sampling (Schepers *et al*., 1997). Different mastitis pathogens have different pathogenicity and consequently the host response may differ (Brolund, 1985; Harmon, 1994; Schepers *et al*., 1997; Coulon *et al*., 2002; Djabri *et al*., 2002; Middleton *et al*., 2004). Middleton *et al*. (2004) concluded that the sensitivities of both CMT and SCC were too low for use as reliable markers to identify infected quarters in herds with high bulk tank milk somatic cell count (BTMSCC).

Despite more than 30 years of research in mastitis diagnostics there are few alternatives to SCC in practical use for identification of cows with subclinical mastitis. Much effort has been invested to find alternative biomarkers to replace or complement SCC, e.g. antitrypsin, serum albumin, electrical conductivity, lactose and N-acetyl-β-D-glucosaminidase (NAGase) activity but with limited success (Mattila, Pyörälä & Sandholm, 1986; Berning & Shook, 1992; Nielen *et al*., 1995; Biggadike *et al*., 2002; Pyörälä 2003). New indicators for inflammation are needed for a more sensitive and specific identification of infected animals (Pyörälä, 2003; Leitner *et al*., 2006).

It is important to detect the subclinical cases of mastitis to prevent that bacteria may spread between cows in the herd. Together with repeated bacteriological examination, SCC is still a valuable tool to distinguish between healthy and infected udder quarters, even though this routine is not practical. Another important aspect for detection of mastitis is the increasing number of automated milking systems. Since these systems lack visual inspection of the milk appearance as in common traditional systems there is a need for on-line analysis of new biomarkers indicating udder health disturbances (Eckersall *et al*., 2001; Grönlund *et al*., 2003; Pyörälä, 2003; Eckersall, 2004; Hiss *et al*., 2007).

Somatic cell count as marker for milk quality

Today BTMSCC is an important parameter in the milk payment system highly affecting the price to the producer. There is, however, no unambiguous scientific evidence at what SCC level the bulk tank milk composition is negatively affected. Still, the pressure on milk producers to reduce the BTMSCC below today's standard has increased. According to EC regulation 853 (2004) the BTMSCC should not exceed 400,000 cells mL^{-1} during three consecutive months. In the Scandinavian countries many dairy cooperatives pay premium if the BTMSCC is below 200,000 cells mL^{-1}. When using BTMSCC as a marker for the udder health status of the herd as well as a milk quality parameter one should be aware that the SCC will be influenced by the number of cows in the herd that are infected by a mastitis pathogens, herd size as well as the proportion of mastitic milk compared to milk from healthy udders (Emanuelson & Funke, 1991; Leitner *et al*., 2008). Therefore, bulk tank milk with the same BTMSCC may differ with in milk quality.

There are studies indicating that SCC is not a reliable marker for proteolysis in quarter milk samples (Le Roux, Colin & Laurent, 1995; Urech, Puhan, Schällibaum, 1999). Larsen *et al.* (2004) showed that milk from quarters adjacent to an infected quarter have increased proteolysis even though the SCC is not elevated in these quarters. Some recent published studies have also reported that SCC alone is not a trustworthy marker for raw milk quality intended for cheese production in quarter (Leitner *et al*., 2006), as well as cow composite, bulk tank and dairy silos samples (Leitner *et al*., 2008). Saeman *et al.* (1988) demonstrated that the proteolytic activity remained higher than during preinfection even though the SCC has returned to normal levels after experimentally-induced mastitis.

In conclusion, using SCC as marker for the protein quality of the raw milk is doubtful. Since the protein quality is of great importance for the dairy industry, a reliable biomarker for protein composition of the raw milk is needed.

Acute phase response and acute phase proteins

During tissue injury, e.g., infection, surgical or other trauma or burns, the acute phase response will be activated, resulting in a number of systemic and metabolic changes. These changes will help the individual to survive during the period after injury through destruction of the infectious agent, removal of damaged tissue and repair of the affected organ. Tissue injury might be of a fatal or minor character,

and the acute phase response will respond in proportion to the damage (Kushner, 1982). In Figure 2, the acute phase response is described. The body will respond to tissue injury through different defence mechanisms, the innate (non-adaptive) and adaptive immune response will be activated. The most important difference between these mechanisms is that the adaptive immune response is highly specific and has a memory remembering the infectious agent. On the other hand, the innate immune response will respond in the same manner regardless of invading pathogen. Among the innate mechanisms, the activation of monocytes/macrophages is important. These cells release protein hormones, i.e. cytokines. These will have local effects on adjacent cells but also systemic effects on other organs that can be reached through the blood stream. In this way, the cytokines will affect the hepatocytes to produce and release the acute phase proteins (APP) (Roitt, Brostoff & Male, 2000). In addition, there is extra hepatic production of APP in different tissues. APP are thus a part of the acute phase response, which is a non-specific reaction in animals due to tissue damage. The acute phase response will radically change the plasma protein concentrations; up regulate the production of some APP (positive APP) and down regulate the production of others (negative APP). The production of APP differs between species, i.e. they are species specific, and the positive APP are also divided into minor, moderate and major APP, depending on their increase in concentration during stimuli (Eckersall, 2000; Petersen, Nielsen & Heegard, 2004).

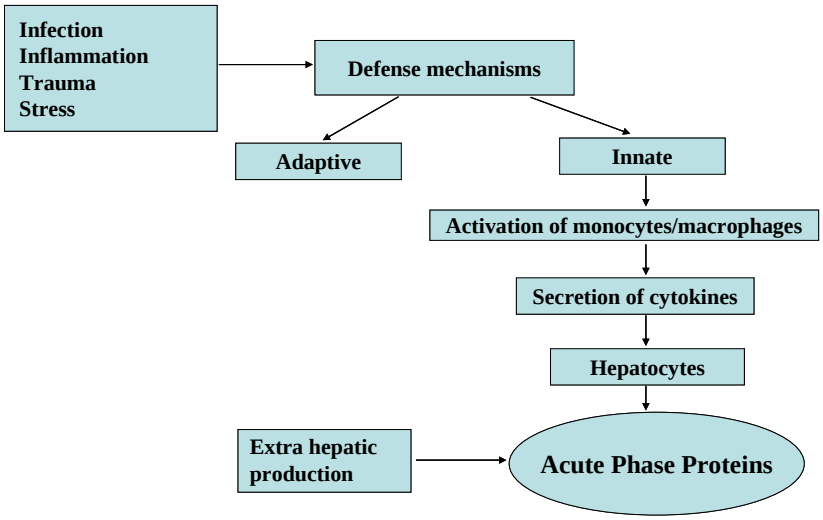

Figure 2. Schematic illustration showing the acute phase response and the resulting production and release of acute phase proteins (APP) by hepatic and extra hepatic cells.

In human medicine C-reactive protein (CRP), the major human APP, has become an important diagnostic marker to distinguish between bacterial and viral infection (Jaye & Waites, 1997). CRP is also a major APP in dogs and pigs, increasing 100 to 1000 fold in plasma concentrations. During the last decade the diagnostic potential of APP has been evaluated in veterinary medicine due to their low or undetectable concentrations in healthy animals, the dramatic increase in concentration during inflammation as well as rapid decrease after recovery (Gruys, Obwolo & Toussaint, 1994; Eckersall, 2000; Murata, Shimada & Yoshioka, 2004; Petersen, Nielsen & Heegard, 2004).

Even though elevated serum concentrations of APP are generally regarded as non-specific markers of inflammation, they are considered to be valuable in diagnosis in veterinary medicine (Gruys, Obwolo & Toussaint, 1994; Murata, Shimada & Yoshioka, 2004; Petersen, Nielsen & Heegard, 2004). APP has also been suggested as marker for differentiation between acute and chronic inflammation in cows (Alsemgeest *et al*., 1994; Horadagoda *et al*., 1999). This is important since acute inflammation processes are often reversible, while in chronic inflammations prognosis is poor. In some studies increased level of APP in serum has been observed when calves were exposed to physical stress (Alsemgeest *et al*., 1995b, c). Others studies have looked at the APP response in serum during different kind of infections in cows (Hirvonen, Pyörälä & Jousimies-Somer, 1996; Heegard *et al*., 2000; Jacobsen *et al*., 2004). APP has also been suggested as a biomarker of animal health at slaughter (Gruys *et al*., 1993; Saini *et al*., 1998). The latter application is especially important for human food safety reasons.

The major bovine acute phase proteins

In cattle there are two major acute phase proteins, i.e. haptoglobin (Hp) and serum amyloid A (SAA) which both increase during tissue injury and disease (Eckersall & Conner, 1988; Alsemgeest *et al*., 1994; Gruys, Obwolo & Toussaint, 1994). SAA is also a major APP in human and other domestic animal species while Hp is only a moderate APP in man and pig (Murata, Shimada & Yoshioka, 2004).

Haptoglobin

Hp is a highly glycosylated protein and the name haptoglobin is derived from its ability to form a stable complex (haptein = to bind) with haemoglobin (Javid & Liang, 1973). Hp consists of a light (α) chain and a heavy (β) chain and the latter will bind haemoglobin in one of the strongest known binding in nature. Human Hp has three different phenotypes, Hp 1-1, Hp 2-1 and Hp 2-2, with the molecular masses of 100, 220 and 400 kDa, respectively (Putnam, 1975). Most other mammals only have the Hp 1-1 phenotype, except for ruminants, who have an Hp form most similar to human Hp 2-2 (Eckersall & Conner, 1990). Hp 1-1 is a homo-dimer in which two Hp molecules are linked together by a disulphide bond between the two α-chains (Wejman *et al*., 1984). There is also a longer variant of the α-chain in humans, probably originating from an unequal crossover of two alleles. Disulphide bonds will link the α-chains, humans homozygous for the long α-chains will have a multimeric Hp phenotype (Hp 2-2) while humans

heterozygous will have both Hp dimers and multimers named Hp 2-1 (Valette *et al*., 1981; Kristiansen *et al*., 2001). The subunit organisation of the different phenotypes of Hp and examples of how haptoglobin-haemoglobin complexes might be organised are presented in Figure 3.

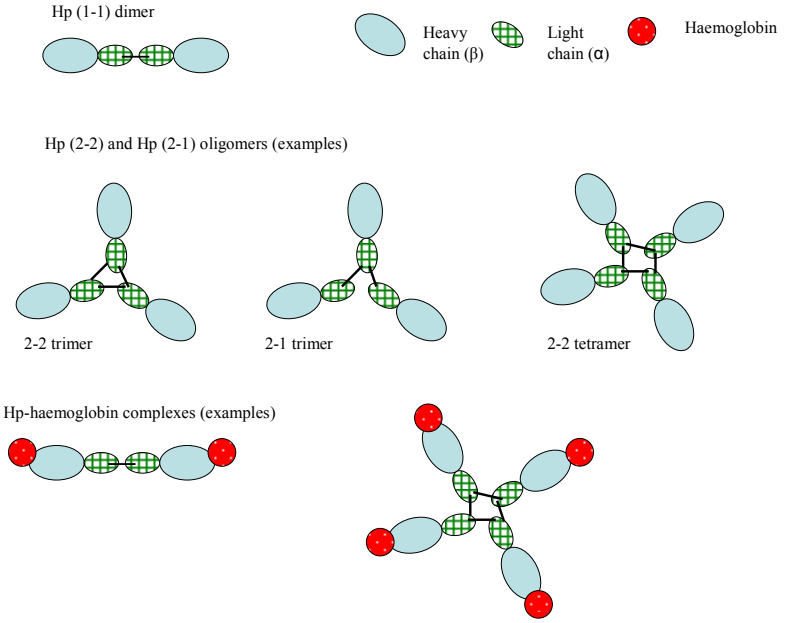

Figure 3. The subunit organisation of the different human phenotypes of haptoglobin (Hp), 1-1, 2-1 and 2-2, and examples of how haptoglobin-haemoglobin complexes might be organised. The illustration is modified from Kristiansen *et al*. (2001), with kind permission from the authors.

Eckersall & Conner (1990) purified Hp from bovine plasma and found that the quarternary structure of the protein is a tetramer, consisting of two α-chains (~16 kDa) and two β-chains (~40 kDa). It was shown that Hp existed as large polymers with a molecular mass >1000 kDa. The β-chain is highly conserved between species and one reason for this might be that this chain is responsible for the strong binding to haemoglobin (Lustbader *et al*., 1983).

The biological functions of Hp have not been completely elucidated but like other APP they have a protective role in limiting the damage caused by the infection or inflammation as well as enhancing repair and recovery (Petersen, Nielsen & Heegard, 2004). However, one very important function that is well understood is that Hp by binding haemoglobin prevents losses of iron via urine after haemolysis, thereby protecting the kidney from being damaged by free haemoglobin. It is of great importance to remove free haemoglobin released from erythrocytes because of the iron-containing haems that have oxidative and toxic properties (Putnam, 1975). The Hp-haemoglobin complex is recognised via a specific cell surface receptor located on macrophages and once bound the complex will rapidly be

removed from circulation (Kristiansen *et al*., 2001). It has also been reported that Hp inhibits bacteria dependent on heam iron for growth (Eaton *et al*., 1982). For many years researchers believed that Hp in milk only originated from Hp production by the hepatocytes, but recent research has shown that Hp mRNA is expressed in the mammary gland (Hiss *et al*., 2004).

Serum amyloid A

SAA is a non-glycosylated apolipoprotein and its molecular weight varies between 11 and 14 kDa depending on species (Westermark *et al*., 1986; Alsemgeest *et al*., 1995a). Most of the protein is transported with the high density lipoprotein (HDL) fraction in the blood (Husebekk *et al*., 1988; Malle, Steinmetz & Raynes, 1993). The SAA family consists of several apolipoproteins expressed to different extents acute phase SAA and consecutive SAA (Uhlar & Whitehead, 1999). The acute phase SAA can increase in concentration 500- to 1000-fold upon stimulation, while consecutive SAA increase only slightly during inflammation, indicating that it is not a major APP (Malle, Steinmetz & Raynes, 1993). Like Hp, the biological function of SAA is not fully understood but it is known that SAA is involved in lipid transport/metabolism (Malle, Steinmetz & Raynes, 1993; Uhlar & Whitehead, 1999). Recent research has also demonstrated that SAA binds Gram-negative bacteria (Hari-Dass *et al*., 2005; Larson *et al*., 2005), possibly to facilitate the uptake by macrophages and neutrophiles (Larson *et al*., 2005).

SAA is also produced extrahepatically, e.g. by the mammary gland epithelial cells and a mammary-associated form of SAA has been identified in milk (McDonald *et al*., 2001; Larson *et al*., 2005; Jacobsen *et al*., 2005; Eckersall *et al*., 2006). Different names are used for SAA occurring in milk, which might be confusing. Whereas McDonald *et al*. (2001) and Larson *et al*. (2005) refer to mammary-associated serum amyloid A as M-SAA 3, O'Mahony *et al*. (2006) use MAA (milk amyloid A) for SAA measured in milk regardless if it is hepatically- or locally-produced. According to the Nomenclature Committee of the International Society of Amyloidosis, extrahepatically-produced SAA should be referred to as SAA3 (Sipe, 1999). Many studies only use SAA (Grönlund *et al*., 2003; Lehtolainen, Røntved & Pyörälä, 2004; Grönlund, Hallén Sandgren & Persson Waller, 2005) referring to SAA that has been measured in milk. The SAA ELISA used in most of these studies detects SAA regardless of whether it is hepatically- or mammary-produced since these two forms share 83 % amino acids identity (McDonald *et al*., 2001).

Haptoglobin and serum amyloid A as markers for mastitis

Recently there has been an increased interest in the potential of APP in milk as markers for mastitis (Eckersall *et al*., 2001; Grönlund *et al*., 2003; Pedersen *et al*., 2003; Pyörälä 2003; Lehtolainen, Røntved & Pyörälä, 2004; Grönlund, Hallén Sandgren & Persson Waller, 2005; Jacobsen *et al*., 2005; Eckersall *et al*., 2006; O'Mahony *et al*., 2006; Hiss *et al*., 2007; Kováč, Popelková & Tkáčiková, 2007). Hp has been suggested to discriminate between minor and major mastitis pathogens (Hiss *et al*., 2007) and also increase in concentration in milk along with

increasing severity of the signs (Pyörälä *et al*., 2006). Eckersall *et al.* (2001) and Hiss *et al.* (2007) evaluated the sensitivity and specificity for Hp as biomarker to distinguish between healthy quarters and quarters with mastitis. Hp showed sensitivity between 85 to 86 % and specificity between 92 to 100 %. SAA was also investigated, with the sensitivity and specificity of 93 and 100 %, respectively (Eckersall *et al.*, 2001).

Several studies have investigated the correlation between APP in serum and milk. Most studies have found that there is a correlation between Hp in serum and milk (Eckersall *et al*., 2001; Nielsen *et al*., 2004; Kováč, Popelková & Tkáčiková, 2007) while no correlation was found between SAA in serum and milk (Eckersall *et al*., 2001; Lehtolainen, Røntved & Pyörälä, 2004; Nielsen *et al*., 2004; O'Mahony *et al*., 2006). Several studies have demonstrated that SAA will increase faster in milk than in serum (Lehtolainen, Røntved & Pyörälä, 2004; Pedersen *et al*., 2004; Jacobsen *et al*., 2005; Eckersall *et al*., 2006). Since the APP appeared later in serum, it is most likely that they originate from local production in the mammary gland. Jacobsen *et al*. (2005) showed that the isoforms of SAA produced hepatically as well as locally are present in milk during experimentally-induced mastitis. Interestingly, locally-produced isoforms were detected in the milk 6 to12 h post-inoculation of the pathogen, compared to serum concentrations which began to increase 12 to 24 h post-inoculation. It was also demonstrated that the locally-produced SAA isoforms could not be detected in plasma, while the hepatically-produced isoforms appeared in milk, probably due to increased permeability.

Some studies have also investigated the correlation between APP and SCC in quarter and cow composite milk samples. Positive correlation was found between Hp and SCC (Nielsen *et al*., 2004; Grönlund, Hallén Sandgren & Persson Waller, 2005; Hiss *et al*., 2007; Kováč, Popelková & Tkáčiková, 2007) as well as between SAA and SCC (Nielsen *et al*., 2004; Grönlund, Hallén Sandgren & Persson Waller, 2005; O'Mahony *et al*., 2006; Kováč, Popelková & Tkáčiková, 2007). There is also a study that concluded that SAA will increase faster in milk than SCC during experimentally-induced mastitis (Pedersen *et al*., 2004).

Hp and SAA are interesting as biomarker for mastitis. The local mammary production of APP in the udder might indicate that these proteins have a special function in the udder's defence against invading microorganisms. Both Hp and SAA have antibacterial functions through binding free haem and bound bacteria facilitating fagocytosis, consequently they are interesting as early biomarkers for mastitis. Since mastitis is one of the most common causes for deterioration of the raw milk quality, it is important to study if APP may be used as biomarkers for milk quality. In addition, there are only few studies describing levels of APP in milk from clinically healthy cows delivering milk to the dairy. No studies have investigated if it is possible to detect APP in bovine bulk tank milk. To our knowledge there are no earlier studies focusing on raw milk quality parameters in relation to APP either.

Since the APP are very interesting in serum as well as biomarkers for mastitis detection in milk, rapid, sensitive assays determine APP are a key factor. The tests available in veterinary medicine pose a problem in contrast to those in human medicine where the major human APP, CRP, is frequently analysed using a quick test only requiring a couple of minutes. To determine bovine APP in serum and milk it is most common with different enzyme-linked immunosorbent assays (ELISA). These are often cumbersome and time consuming when used for large-scale analysis.

BIAcore technology

BIAcore technology (GE Healthcare, Uppsala, Sweden) is the world-leading manufacturer of SPR (surface plasmon resonance) biosensors, and the instruments are appreciated both for method development and in routine analysis. This technique was first described by Jönsson *et al.* (1991) and the use of SPR biosensors has rapidly increased over the years. During the last decade several applications of SPR biosensor technology for quantitative analysis of components and residues in foods, e.g. milk have been described (Baxter *et al*., 2001; Gillis *et al*., 2002; Indyk *et al*., 2002; Samsonova *et al*., 2002; Nygren, Sternesjö & Björck, 2003; Dupont, Rolet-Repecaud & Muller-Renaud, 2004; Gustavsson *et al*., 2004; Haasnoot *et al*., 2004). The instrument uses an optical phenomenon, SPR, to study interactions on the sensor surface in real time.

The instrument is composed of three major parts; the sensor surface, the integrated flow system and the optical system. The sensor chip consists of a gold-coated glass slide covered with a coupling matrix, e.g. carboxymethylated dextran. The ligand is covalently immobilised to the sensor surface, the most commonly used immobilisation method being the amine coupling procedure where the ligand is coupled to the carboxylated dextran through primary amine groups. There is a constant flow of buffer over the sensor surface before and after injection of the sample. Finally, the optical system registers changes in the optical phenomenon when interaction takes place, and converts the event to a signal. When the analyte of interest binds to the immobilised ligand on the sensor surface, this will result in an increased response signal due to increased mass on the sensor surface. The change in mass is indicated as a change in resonance units (RU). The analyte is then dissociated from the ligand by injection of an appropriate regeneration solution and the sensor surface can be reused for hundreds of injections, depending on the nature and stability of the ligand. Since this technique is based on real time measurements, all steps can be observed on the computer screen, which is a great advantage especially during method development (BIAtechnology Handbook, 1994).

Concentration analysis using Biacore

Concentration analysis is one type of application that the biosensor may be used for, and there are different approaches when designing a concentration assay. The most straightforward way is to develop a direct assay, requiring that the analyte has a molecular weight of 100 to 200 Da to give rise to a measurable signal. In this case a binding molecule for example an antibody is coupled to the sensor surface and the analyte is injected over the sensor surface (BIAtechnology Handbook, 1994). A sensorgram describing a direct concentration assay is described in Figure 4.

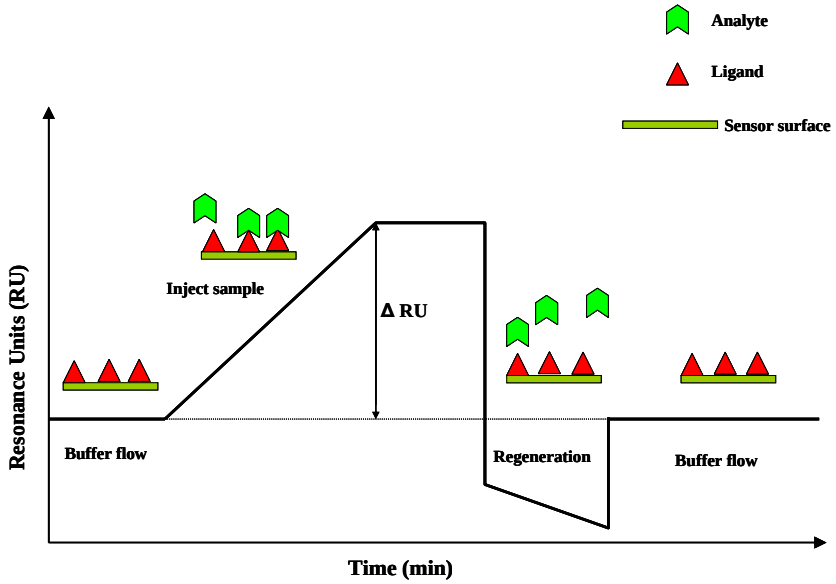

Figure 4. Sensorgram describing direct concentration analysis by surface plasmon resonance (SPR) technology. At first, only buffer flows across the sensor surface with the covalently-coupled ligand. When the sample containing the analyte is injected, interaction between free analyte and ligand on the sensor surface takes place. This result in a mass increase on the surface, giving rise to an increasing signal measured as resonance units (RU). Finally, a regeneration solution is injected to dissociate the analyte from the ligand, and the surface is ready for injection of a new sample.

For some analyses, e.g. low molecular weight analytes, or very large molecules and particles such as viruses, it is not always possible to develop a direct assay. The alternative is then to use an inhibition assay, where the analyte of interest is coupled to the sensor surface and the sample is mixed with a known amount of a binding molecule, e.g. an antibody, before injection. When the sample is injected, the binding molecule will bind to the immobilised analyte on the sensor surface unless the sample contains free analyte inhibiting the interaction on the surface. A sensorgram describing an indirect concentration assay is described in Figure 5.

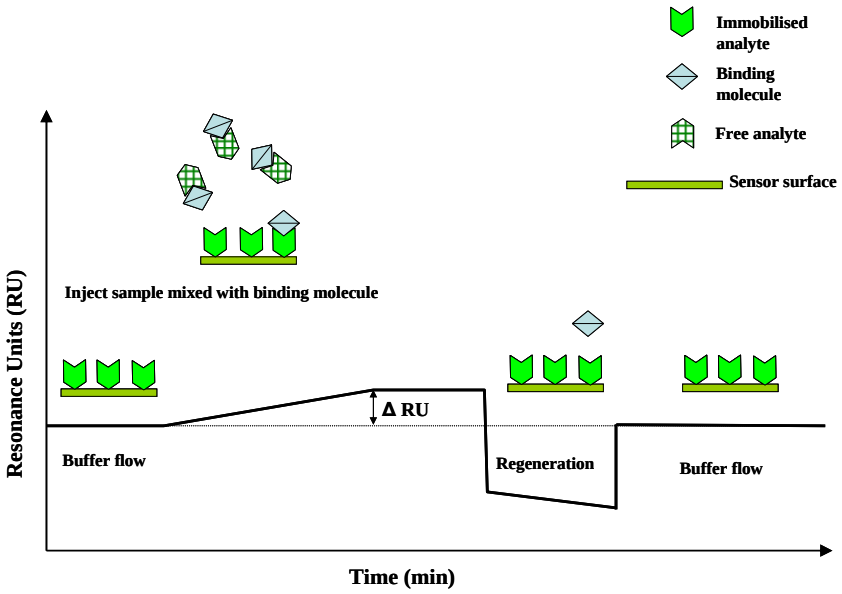

Figure 5. Sensorgram describing the different steps in an indirect concentration analysis by surface plasmon resonance (SPR) technology. At first, only buffer flows across the sensor surface with the covalently bound analyte. When the sample containing the analyte is mixed with binding molecule before injection, interaction between the free analyte and binding molecule takes place. This inhibits the interaction between the added binding molecule and analyte immobilised on the surface. Consequently, less mass increase occurs on the sensor surface, giving rise to a smaller signal measured as resonance units (RU). Finally, a regeneration solution is injected to dissociate any binding molecule from the analyte bound to the surface, and the surface is ready for injection of a new sample.

As mentioned earlier APP in serum and milk are most commonly determined using ELISA. Comparing ELISA with the biosensor technology the former is often cumbersome and time consuming when used for large-scale analysis. Advantages with the biosensor assay include that it is fully automated and rapid (each sample requiring 5–10 min), analysis is in real time, detection is label free and there is no sample preparation before analysis i.e., whole milk samples could be analysed directly. No earlier studies have evaluated the possibility to use an optical SPR biosensor to analyse APP in milk. To quote the title of a paper written by Eckersall (2004), “The time is right for acute phase proteins assays.”

Aims

The overall aim of this thesis was to obtain further knowledge about the occurrence of the two major APP, Hp and SAA, in bovine milk and their potential as biomarkers for raw milk quality.

The specific aims of the present work were to:

• develop and validate a rapid, fully automated biosensor assay to determine Hp in milk (paper I).

• screen for Hp and SAA in quarter, cow composite, and bulk tank milk samples and investigate if detectable levels of Hp and SAA were related to SCC (paper II).

• investigate if cow composite milk samples with detectable levels of Hp and SAA differed with respect to milk composition in comparison with samples without detectable levels of Hp and/or SAA (paper III).

• evaluate if bulk tank milk samples with detectable levels of Hp and SAA differed with respect to milk composition and coagulating properties in comparison with samples without detectable levels of Hp or SAA (paper IV).

Materials and methods

This section summarises the materials and methods applied in the studies of the thesis. For more detailed descriptions, see papers I-IV.

Animals

The quarter and cow composite milk samples used in **papers I-III** were of the two main Swedish dairy breeds, Swedish Red and Swedish Holstein. The cows were fed according to Swedish recommendations (Spörndly, 2003). The samples were from clinically healthy cows, i.e. cows without systemic symptoms or clinical signs of disease or abnormalities in the udder or in the milk, when observed by visual examination and palpation of the udder. Except for the samples used in **paper I**, where the quarter milk samples originated from cows with clinical mastitis.

In **paper II**, 165 cows were included. They were in lactation number 1–9 (median 1), lactation week 5–62 (median 36) and produced 12–51 kg milk per day (median 24). The average cow composite SCC was 187,100 cells mL^{-1} (median 70,000).

In **paper III**, 89 cows were included. They were in lactation number 1–5 (median 2), lactation week 2–63 (median 26) and produced 4–59 kg milk per day (median 31). The average cow composite SCC was 386,800 cells mL^{-1} (median 83,000).

Sampling of quarter, cow composite and bulk tank milk

In **paper I,** quarter milk samples (n=28) from clinical cases of mastitis were obtained from the National Veterinary Institute, Uppsala, Sweden.

In **paper I** (n=43), **paper II** (n=165), and **paper III** (n=89) cow composite milk samples from two University experimental dairy farms and from one private dairy farm in the region of Uppsala, Sweden were collected. In **papers II and III** all cows in the herds delivering milk at the sampling occasion were included in the study and cows were sampled only once.

In **paper II,** quarter and cow composite milk samples were collected with a special milking machine intended for quarter milking, constructed and provided by DeLaval International AB (Tumba, Sweden) with monovac, pulsation ratio 70/30 and system vacuum 42 kPa. This milking machine collects the total milk volume from each quarter in a separate vessel for the whole milking, and after milking is completed, milk from the four vessels are commingled and a representative cow composite milk sample was taken. This milking machine was also used in **paper III** for collecting representative cow composite milk samples.

Bulk tank milk samples (n=96) used in **paper II** were provided from the Swedish central milking grading laboratory, Eurofins Steins Laboratory (Jönköping, Sweden) and the average BTMSCC was 269,000 cells mL^{-1} (median 259,000). In

paper IV, the bulk tank milk samples (n=91) were collected at Milko Dairy Cooperative (Grådö Dairy Plant, Hedemora, Sweden) and the average BTMSCC for these samples was 195,000 cells mL^{-1} (median 146,000).

Milk analyses

Milk gross composition

SCC was measured on fresh milk samples by electronic fluorescence-based cell counting (Fossomatic 5000, A/S N. Foss Electric, Hillerød, Denmark). Lactose, fat and total protein were measured in fresh milk by mid-infrared spectroscopy (Fourier Transform Instrument, FT 120, Foss Electric). The casein content was determined by an indirect method whereby protein in the whey fraction was determined by mid-infrared spectroscopy after rennet coagulation of the caseins. The proportion of casein was then calculated from the proportion of whey and total protein content.

Proteolysis

Proteolysis was determined after thawing of defatted frozen (-70°C) milk samples according to the fluorescamine method (Wiking *et al*., 2002). In short, milk proteins are precipitated with trichloroacetic acid. After centrifugation, and peptides present as a result of proteolytic enzymes in the milk sample, will appear in the supernatant. These are coupled to a reagent, fluorescamine, which after reaction with amino terminals will fluoresce. The fluorescence (excitation 390 nm, emission 480 nm) was measured by a Luminescence spectrometer (Perkin-Elmer, LS 50 B, Norwalk, CT, USA). The extent of proteolysis was expressed as equivalence (mM) leucine, using a standard curve constructed by analysis of leucine diluted in 0.01mM HCl.

Coagulating properties

Coagulating properties were determined in fresh milk samples using a Bohlin VOR Rheometer (Malvern Instruments Nordic AB, Uppsala, Sweden) according to Hallén *et al*., (2007), with a minor modification. The rennet used was Chymax Plus, strength 200 International Milk Clotting Units per gram (Christian Hansen A/S, DK-2970, Hørsholm, Denmark) instead of pure chymosin. In short, milk samples (12 mL) treated with bronopol, were incubated for 60 min in a water bath (30°C) before 20 µL Chymax solution was added to the milk sample. The temperature was kept constant and each measurement lasted for 30 min. The coagulation time was measured, i.e. the time (s) elapsed from Chymax addition until a weak coagulum corresponding to 5 Pa was formed. In addition, curd firmness (Pa) was measured 25 min after Chymax addition.

Determination of serum amyloid A

SAA was determined after thawing of the frozen (-70°C) whole milk samples, using a commercial ELISA (PhaseTM Serum Amyloid A Assay, Tridelta Development Ltd, Wicklow, Ireland). Diluted samples were added to the wells coated with a monoclonal antibody specific for SAA, and biotinylated anti-SAA

monoclonal antibodies were added. SAA present in the sample will be captured by the primary antibodies and labelled with the secondary, conjugated antibodies. Streptavidin horse-radish-peroxidase (HRP) conjugate and finally tetramethyl benzidine (TMB) substrate solution were added to the wells. The colour intensity obtained was proportional to the concentration of SAA in the sample. The milk samples were initially diluted 1:50, and samples with an optical density above the range of the standard curve were further diluted and re-analysed. Optical densities were read on an automatic plate reader (Model ELx 800; Bio-Tek Inc, CA. USA) at 450 nm with a reference at 630 nm. The detection limit (LOD) of the ELISA was 0.3 mg L^{-1} according to the manufacturer.

Determination of haptoglobin using a biosensor assay

Hp was determined after thawing of the frozen (-70^{o}C) whole milk samples using an indirect optical biosensor method presented in Figure 5. The method is based on the strong interaction between Hp and haemoglobin. The milk sample is mixed with bovine haemoglobin before injection over a sensor surface with covalently bound Hp. When there is no, or small amounts of Hp present in the sample, haemoglobin will bind to immobilised Hp on the sensor surface. When Hp is present in the sample, it will form a complex with added haemoglobin, inhibiting haemoglobin binding to the surface. Thus, the biosensor response is inversely proportional to the amount of Hp in the sample.

In **paper I,** the human form of Hp was used for immobilisation on the sensor surface and construction of standards (Biogenesis, Poole, UK). When initiating **paper II**, human Hp 2-2 had become commercially available (Sigma St Louis, MO 63178, USA) and was used in this study. In **papers III and IV,** purified bovine Hp was available (Life Diagnostic, Clarkston, GA, USA) and therefore used in the studies. Standard curves using the different proteins in combination with bovine haemoglobin were used, but no differences in the performance of the assays could be detected between human and bovine forms of Hp (unpublished results). Bovine haemoglobin was used in all studies **(papers I–IV)**.

In **papers I–III,** the LOD of the Hp assay was 1 mg L^{-1} while after different modifications the LOD could be reduced to 0.3 mg L^{-1} in **paper IV**. There are different ways to increase the sensitivity of the biosensor assay, e.g. by increasing the contact time between sample and surface and decreasing the concentration of added binding molecule, i.e. haemoglobin. In the original method the contact time was 60 sec and the haemoglobin concentration 2.5 mg L^{-1}, whereas in the modified assay it was 75 seconds and 1.5 $L^{-1,}$ respectively. In the final version of the assay the Hp surface was prepared using a solution of 25 mg L^{-1} instead of 500 mg L^{-1} Hp in 0.01 M acetate buffer and the activation of the sensor surface was reduced to 3 min compared to 7 min in the original method. Two extra standard points were added (0.6 and 0.3 mg L^{-1}) to the standard curve, and an extra reconditioning step of 50 mM glycine pH 9.5 was included after the regeneration step. The substantial reduction of the amount of protein used during immobilisation and the extra reconditioning step after regeneration contributed to a more robust assay and a sensor surface that lasted much longer. It was also

necessary to increase the concentration of sodium dodecyl sulphate (SDS) from 2 mM to 2.5 mM in the solution used for regeneration, especially when bovine Hp was used.

Determination of haptoglobin by ELISA

An ELISA for Hp in milk (PhaseTM Bovine Haptoglobin Enzyme Immunoassay, Tridelta Development Ltd, Bray, Co. Wicklow, Ireland) was used to evaluate the performance of the biosensor assay. In the ELISA, the wells are coated with haemoglobin that will bind free haptoglobin in the sample. An HRP-labelled anti-haptoglobin monoclonal antibody is added, which will bind to the captured Hp. Finally, a TMB substrate solution is added, and the colour intensity obtained is proportional to the concentration of Hp in the sample. The assay was performed according to the manufacturer's instructions and the LOD of the ELISA was 0.3 mg L^{-1}.

Statistics

The statistical analysis in **papers I–IV** was performed by using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA) or Minitab (Minitab Inc. version 14, 2003, State College, PA, USA). In **papers II–IV** descriptive statistics for the data included was presented. A p-value ≤ 0.05 was considered significant in all studies.

Wilcoxon signed rank test was used in **paper I** to study the agreement between the results from the developed biosensor Hp assay and the commercially ELISA for Hp in milk. Chi-square was used to evaluate relationships between Hp, SAA and SCC in **paper II**. SCC was then categorised in 5 groups, and categorisation of samples with respect to Hp and SAA was based on the LOD for the assay used, i.e. detectable versus undetectable concentrations of the APP in the milk sample. Parametric t-test was used to compare samples with and without detectable levels of APP regarding different milk quality parameters in **papers III and IV**. SCC and curd firmness were logarithmically transformed to obtain normally distributed data.

Results

Biosensor assay to determine haptoglobin in milk (Paper I)

An affinity sensor assay based on the interaction between Hp and haemoglobin was developed using SPR biosensor technology. The LOD of the developed assay was determined to 1.1 mg L^{-1}. The intra-assay repeatability (CV, coefficient of variation) ranged between 3.6 and 6.8 % for bulk tank milk spiked with human Hp (4.0 and 12 mg L^{-1}) and authentic cow composite milk samples containing Hp (4.1 and 7.2 mg L^{-1}). For the same samples the inter-assay repeatability ranged between 4.5 and 8.6%. Results from precisions studies are presented in Table 1.

Table 1. Precision of the haptoglobin (Hp) biosensor assay, intra- and inter-assay repeatability determined by analysis of bulk tank milk samples spiked with human Hp at two different concentrations (4.0 and 12.0 mg L^{-1}) and two cow composite milk samples containing 4.1 and 7.2 mg L^{-1} of bovine Hp according to ELISA.

	Bulk tank milk spiked with human Hp		Real cow composite milk samples containing Hp	
	4.0 mg L^{-1}	12.0 mg L^{-1}	4.1 mg L^{-1}	7.2 mg L^{-1}
Intra-assay (n=10)				
Mean value (mg L^{-1})	4.8	12.6	4.1	6.4
Standard deviation (CV %)	0.2 (3.6)	0.7 (5.5)	0.2 (4.3)	0.4 (6.8)
Inter-assay (n=3)				
Mean value (mg L^{-1})	4.5	11.8	4.1	6.5
Standard deviation (CV %)	0.3 (5.9)	1.0 (8.6)	0.2 (5.9)	0.3 (4.5)

Haptoglobin and serum amyloid A in different milk samples (Paper II)

Quarter (n=103), cow composite (n=165) and bulk tank milk samples (n=96) were analysed for Hp, SAA and SCC. The study concluded that it was possible to find detectable levels of Hp and SAA in milk samples from clinically healthy quarter and cow composite milk samples but also in bulk tank milk samples. Detectable levels of APP were associated with high SCC in all types of milk samples except for Hp in bulk tank milk. Moreover, there was a relationship between Hp and SAA, i.e. samples with detectable levels of Hp also contained SAA and vice versa. This relationship was significant at quarter and cow composite level, but not in bulk tank milk samples.

In this study we also demonstrated that SAA was present more often and also at higher concentrations than Hp. Results for samples containing detectable levels of Hp and SAA are presented in Table 2 and 3, respectively.

Table 2. Number of samples, range and median for haptoglobin (Hp) concentration in quarter, cow composite and bulk tank milk samples containing detectable levels of Hp (≥ 1 mg L^{-1}). Median somatic cell count (SCC) for the samples containing detectable levels of Hp is also presented.

Milk sample	No of samples containing Hp	Range Hp (mg L^{-1})	Median Hp (mg L^{-1})	Median SCC ($\times 10^3$ cells mL^{-1})
quarter	9/103 (9%)	1.3-18.5	5.3	2,375
cow composite	28/165 (17%)	1.0-15.0	1.5	280
bulk tank	39/96 (41%)	1.0-3.8	1.3	285

Table 3. Number of samples, range and median for serum amyloid A (SAA) in quarter, cow composite and bulk tank milk samples containing detectable levels of SAA (≥ 0.3 mg L^{-1}). Median somatic cell count (SCC) for the samples containing detectable levels of SAA is also presented.

Milk sample	No of samples containing SAA	Range SAA (mg L^{-1})	Median SAA (mg L^{-1})	Median SCC ($\times 10^3$ cells mL^{-1})
quarter	54/103 (52%)	0.5-54.8	2.5	161
cow composite	82/165 (50%)	0.4-106.2	2.2	127
bulk tank	79/96 (82%)	0.4-6.7	1.3	281

Twenty-six cows were included when sampling the quarter milk samples, one udder quarter on one cow was dried-off. Representative quarter milk samples as well as a representative cow composite milk sample were collected. In 7 out of these 26 cows, all quarter and the belonging cow composite milk samples contained no detectable levels of Hp or SAA. Consequently, 19 of the cows contained detectable levels of APP. Only 9 % of the quarters contained detectable concentrations of Hp (Table 2), but these quarters had extremely elevated SCC even though no abnormalities were observed in the milk. As much as 52 % of the quarter milk samples contained detectable levels of SAA (Table 3). When analysing the quarter and the associated cow composite milk samples, results showed that occasionally, detectable levels of SAA were found without effects on

SCC. In some cows, SAA levels in several quarters resulted in a cow composite milk sample with detectable SAA levels although the same pattern could not be seen for SCC in these cows. In Table 4, results for six of the cows are presented to illustrate detectable levels of Hp and SAA in comparison to SCC.

Table 4. Illustration of the variation in milk haptoglobin (Hp) and serum amyloid A (SAA) concentrations between udder quarters within cow, and the presence in cow composite milk in relation to the somatic cell count (SCC). The table shows results from 6 out of 19 cows with milk containing Hp and/or SAA. RF = right fore, RR = right rear, LF = left fore, LR = left rear quarter.

cow id	udder quarter	SCC x10^3 (cells mL^{-1})	Hp (mg L^{-1})	SAA (mg L^{-1})
861	RF	1914	7.2	11.2
	RR	57	<	<
	LF	1618	2.7	29.3
	LR	36	<	0.5
	composite	104	<	0.7
891	RF	38	<	1.9
	RR	27	<	1.4
	LF	319	<	6.9
	LR	37	<	1.4
	composite	62	<	2.0
896	RF	51	<	5.7
	RR	106	<	8.6
	LF	51	<	6.9
	LR	181	<	11.9
	composite	99	<	7.8
936	RF	30	<	2.0
	RR	19	<	1.8
	LF	302	<	2.5
	LR	17	<	1.6
	composite	46	<	1.5
992	RF	46	<	1.1
	RR	383	1.3	7.1
	LF	27	<	1.1
	LR	52	<	1.4
	composite	124	<	3.2
1171	RF	19	<	2.5
	RR	2522	5.3	46.6
	LF	918	<	12.7
	LR	178	<	6.0
	composite	887	1.0	9.8

< = below LOD for the Hp (1 mg L^{-1}) and SAA (0.3 mg L^{-1}) assay, respectively.

Haptoglobin and serum amyloid A in cow composite milk samples in relation to raw milk quality (Paper III)

Paper III demonstrated that the presence of the major bovine APP, Hp and SAA, in cow composite milk samples (n=89) was related to important changes in milk quality parameters. Cow composite milk samples with detectable levels of Hp had lower total protein, lower casein content and higher SCC compared to samples without detectable levels of Hp. Samples with detectable levels of SAA had lower casein number (casein in relation to total protein), lower lactose content and higher SCC compared to samples without detectable levels of SAA. Table 5 gives a summary of the results presented in **paper III**.

Table 5. Milk quality parameters for cow composite milk samples, with (+) and without (-) detectable levels of haptoglobin (Hp) (≥ 1 mg L^{-1}) and serum amyloid A (SAA) (≥ 0.3 mg L^{-1}) were investigated. Differences between the groups were evaluated by parametric t-test and were considered significant if $p \leq 0.05$.

Parameter	Hp+ (n=28)	Hp- (n=61)	p-value (Hp+/Hp-)	SAA+ (n=46)	SAA- (n=43)	p-value (SAA+/SAA-)
Total protein (%)	3.51	3.71	0.02	3.63	3.67	NS
Casein (%)	2.58	2.72	0.03	2.65	2.71	NS
Casein number	0.73	0.73	NS	0.73	0.74	0.03
Whey protein (%)	0.93	0.99	NS	0.99	0.96	NS
Proteolysis (eq leu)	1.15	1.12	NS	1.13	1.13	NS
Fat (%)	4.66	5.06	NS	4.94	4.93	NS
Lactose (%)	4.51	4.49	NS	4.44	4.55	0.01
Log SCC (cells mL^{-1})	5.27	4.79	<0.001	5.29	4.57	<0.001

NS=not significant
eq leu= equivalent mM leucine

Since SCC is an indirect marker for milk quality, relationships between SCC and the different parameters were also investigated using the same statistical method as for the APP. Cow composite milk samples with elevated SCC (>83,000 cells mL^{-1}) had decreased lactose content and increased whey protein concentrations compared with samples with low SCC (<83,000 cells mL^{-1}).

Haptoglobin and serum amyloid A in bulk tank milk samples in relation to raw milk quality (Paper IV)

Paper IV demonstrated that the presence of Hp and SAA in bulk tank milk samples (n=91) was related to important changes in milk quality parameters. Bulk tank milk samples with detectable levels of Hp showed lower casein content, lower casein number, increased proteolysis, lower lactose content and higher SCC compared to samples without detectable levels of Hp. Milk samples with detectable levels of SAA had lower casein number, increased whey protein content, lower lactose content and increased SCC compared to samples without detectable levels of SAA. Table 6 gives a summary of the results presented in **paper IV**.

Table 6. Milk quality parameters for bulk tank milk samples, with (+) and without (-) detectable levels of haptoglobin (Hp) (≥ 0.3 mg L^{-1}) and serum amyloid A (SAA) (≥ 0.3 mg L^{-1}) were investigated. Differences between the groups were evaluated by parametric t-test and were considered significant if $p \leq 0.05$.

Parameter	Hp+ (n=19)	Hp- (n=72)	p-value (Hp+/Hp-)	SAA+ (n=68)	SAA- (n=23)	p-value (SAA+/SAA-)
Total protein (%)	3.49	3.55	NS	3.55	3.49	NS
Casein (%)	2.53	2.59	0.02	2.59	2.56	NS
Casein number	0.725	0.731	<0.001	0.728	0.734	<0.001
Whey protein (%)	0.91	0.91	NS	0.92	0.88	<0.01
Proteolysis (eq leu)	1.16	1.10	0.04	1.12	1.08	NS
Fat (%)	4.35	4.49	NS	4.48	4.50	NS
Lactose (%)	4.55	4.62	0.05	4.58	4.69	<0.001
Log SCC (cells mL^{-1})	5.53	5.10	<0.001	5.26	4.99	<0.001
Coagulation time (s)	110	123	NS	122	113	NS
Log curd firmness (Pa)	2.55	2.52	NS	2.52	2.53	NS

NS=not significant
eq leu= equivalent mM leucine

Relationships between SCC and the different raw milk quality parameters were also investigated using the same statistical method as for the APP. Bulk tank milk samples with elevated SCC (>195,000 cells mL^{-1}) had a lower casein number than samples with a lower SCC (<195,000 cells mL^{-1}).

General discussion

Biosensor assay development

When initiating the work to develop a biosensor assay to measure Hp in milk, an antibody based biosensor assay was one option. It would then be necessary to have pure bovine Hp for immunisation as well as a standard, but at that time bovine Hp was not commercially available. Therefore, an attempt to purify Hp from pooled serum samples from cows with clinical mastitis and newly calved cows was accomplished. The purification succeeded but problems came up after storage at -70^{o}C, probably due to formation of large aggregates of the protein. The work to produce our own reagents was not continued.

Since the binding of Hp to haemoglobin is not species specific (Makinen, Milstien & Kon, 1972; Valette *et al*., 1981), a biosensor assay based on the strong interaction between Hp and haemoglobin was another alternative. A direct assay, with haemoglobin coupled to the surface was our first choice of assay design since this is the most straightforward way when developing an assay. For unknown reasons we did not succeed with this approach. One possible explanation might be sterical hindrance at the surface between the two proteins involved. Hp is a macromolecular protein and its molecular mass has been determined to 1,000-2,000 kDa (Eckersall & Conner, 1990; Morimatsu *et al*., 1991) to be compared with haemoglobin, having a molecular mass of approximately 60 kDa. It was never investigated if sterical hindrance was the cause to the problem during development of a direct assay, instead an inhibition assay was developed. The developed biosensor assay is an indirect assay, with Hp covalently coupled to the sensor surface. Before injection, the milk sample is mixed with a known amount of haemoglobin.

To validate the developed biosensor assay another method to determine Hp in milk was needed. When this work started, only one commercial ELISA was available for determination of Hp in milk (PhaseTM Bovine Haptoglobin Enzyme Immunoassay, Tridelta Development Ltd, Bray, Co. Wicklow, Ireland), and this assay was used for validation of the developed biosensor assay. When the validation of the biosensor assay was initiated, samples from cows with clinical mastitis were collected for comparison of the biosensor method with the commercial ELISA. The reason for choosing these milk samples was that we wanted samples with elevated Hp concentrations to compare the performance of biosensor assay with the ELISA. Milk from cows with severe clinical mastitis has a substantially different composition compared with milk from clinically healthy cows, and it is possible that these samples were contaminated by blood, even though this was never observed by visual examination. One drawback with the biosensor assay is that even the slightest amount of haemoglobin from blood may disturb the analysis. This will result in two different possible and probable situations that may occur separately as well as in combination with each other. Contaminating haemoglobin will bind Hp in the sample and consequently the inhibitory effect on added haemoglobin will decrease. Moreover, contaminating

haemoglobin will bind to immobilised Hp on the surface which will result in an increased relative response, masking Hp in the sample. In both cases the biosensor assay will underestimate the level of Hp. To what extent these situations occur are difficult to estimate, but it is important to be aware of the problem with contaminating haemoglobin. With the exception of the quarter milk samples used for assay development, milk from cows with clinical mastitis was never included in the subsequent studies **(papers II-IV)**, since there is no need for a method confirming these cases of mastitis. To be confident that the Biacore assay delivered correct results many samples in **papers II and III** were also analysed with the ELISA. The results obtained by the biosensor assay agreed well with the results from the ELISA. In conclusion, the developed biosensor was validated in an unsuitable way using milk samples from cows with clinical mastitis. The following studies **(papers II and III)** demonstrated that the biosensor assay agreed well with the ELISA in contrast to what was originally reported in **paper I**.

There were no earlier studies reporting analysis of whole milk samples in the BIAcore instrument, and since milk fat might clog the thin tubes it was uncertain if the biosensor would manage injection of whole milk samples. In **paper I** the milk samples were therefore defatted before analysis, but in **papers II–IV** whole milk samples were analysed with success. To verify that Hp was not associated with the fat a number of samples containing detectable levels of Hp were analysed both as whole milk and defatted milk in the ELISA (results not shown). As expected, the same result was observed in the defatted and whole milk samples.

The commercial ELISA used for assay validation and for verification of results obtained by the biosensor is not available today. Consequently there is no commercial assay to determine Hp in milk. There is thus a need for new methods analysing APP in serum and milk samples. The developed method has potential for Hp screening in milk samples and is simple, robust and fully automated.

The importance of representative milk samples

In **papers II and III** a specially designed milking machine was used to collect quarter and cow composite milk samples. Since there are no investigations about variations in concentration of Hp and SAA during milking it was important to use representative milk samples, i.e. samples reflecting the average concentrations of components analysed throughout the entire milking.

The milk composition varies during milking, especially concerning the milk fat (Urech, Puhan & Schällibaum, 1999). The milk fat may vary between 1–10 % during milking, while residual milk may contain as much as 20 % fat (Harding, 1995). Since SAA is an apolipoprotein (Westermark *et al*., 1986; Alsemgeest *et al*., 1995a) it is likely to be associated with the fat in the milk. To investigate if SAA was affected when using defatted milk samples, a number of milk samples were analysed as both whole milk and defatted milk. The results showed a clear decrease in SAA concentration when the samples were defatted, indicating that some of the SAA in milk was associated with the fat (results not shown). In several studies milk samples were defatted before analysis of SAA (Jacobsen *et*

al., 2005; Lindmark-Månsson *et al*., 2006). Moreover, quarter and cow composite milk samples were not collected in a representative manner in previous studies where often only some mL was sampled after discarding the first jets of milk (Nielsen *et al*., 2004; O'Mahony *et al*., 2006). Since SAA seems to be associated with the fat and since fat varies during milking, representative samples are important when comparing studies and observed concentrations of the protein.

In some studies (Grönlund, Hallén Sandgren & Persson Waller, 2005; Kováč *et al*., 2007) cow composite milk samples were constructed by pooling equal volumes of milk from the four different quarters. This will not reflect the differences in milk volume between quarters and may thus affect the observed level of APP in the cow composite milk sample. To illustrate this, Table 4 (presented in the result section) illustrates representative milk samples from each quarter as well as a cow composite milk sample. The first cow in the table, cow number 861, has two quarters with SCC 1,914,000 and 1,618,000 cells mL^{-1}, respectively. The SAA concentrations in these quarters were 11.2 and 29.3 mg L^{-1}, respectively. Interestingly, the SCC in the real cow composite milk sample was 104,000 cells mL^{-1} and would not draw attention. If we had used a pooled milk sample, with equal volumes from each quarter, the SCC in the cow composite milk sample would instead exceed 900,000 cells mL^{-1} and the SAA concentration would be 10 mg L^{-1}. This example clearly illustrates the effect of the milk volume produced in each quarter on the concentrations of parameters analysed.

When studying bulk tank milk composition and coagulating properties we used field samples from herds delivering milk to the dairy. Previous studies in this area used a small number of animals and only categorised them according to the SCC (Barbano, Rasmussen & Lynch, 1991; Rogers & Mitchell, 1994; Mazal *et al*., 2007). Other studies have constructed batches of milk by pooling milk with extreme SCC and average SCC to obtain milk with high and low SCC. Since milk with extreme SCC often originates from cows with clinical mastitis, the composition of the milk is substantially deviant from milk originating from clinically healthy cows regarding serum components e.g. enzymes. Consequently, this kind of constructed milk samples is not representative for a real bulk tank milk sample, where milk from the whole herd is commingled.

Occurrence of haptoglobin and serum amyloid A in milk

In our studies, we have observed that SAA was present more often and also in higher concentrations than Hp **(papers II–IV)** in agreement with Kováč, Popelková & Tkáčiková (2007). One explanation might be that the LOD of the SAA assay was lower than that of the initial Hp assay. Since many of the samples were not only analysed by the biosensor assay but also with the Hp ELISA, the possibility that differences in LOD between the SAA and Hp assays would explain this observation was therefore unlikely.

In studies on APP it is often assumed that milk samples from clinically healthy cows or quarters contain no detectable levels of Hp or SAA. In some of these studies, milk from the healthy/control quarters have, however, contained

detectable levels of SAA but not Hp (Grönlund *et al*., 2003; Nielsen *et al*., 2004; Pedersen *et al*., 2004). O'Mahony *et al.* (2006) also noticed that SAA was detected in quarters with low SCC, but it was unclear whether these quarters were located in udders having one quarter with much higher concentration of SAA in the milk. The authors called this a "spill-over" effect and suggested that it originated from hepatically-produced SAA or that it was actually subclinically inflamed quarters not responding with elevated SCC. It is thus important to remember that different studies define a healthy quarter differently.

Another explanation for SAA being frequently observed in milk samples might be communication between quarters. It has been assumed that each quarter constitutes its own milk producing system, and that there is no connection between adjacent quarters. However, during recent years studies have reported that infection in one quarter will affect the adjacent quarters (Larsen *et al*., 2004; Bansal *et al*., 2005; Merle, Schröder & Hamann, 2007). Many of the cows in **paper II** had elevated SCC in only one quarter whereas SAA was often present also in neighbouring quarters. Larsen *et al*. (2004) demonstrated that casein degradation occurred in quarters adjacent to the infected quarter without affecting the SCC in these quarters. Considering the results in **papers III and IV**, where we suggested that APP might indicate the protein quality, it is possible that the protein quality in all quarters were affected if the cow had an udder infection. It is therefore likely that some of the cows in **paper II** had subclinical mastitis in one of the quarters, possibly affecting the raw milk quality in adjacent quarters without showing an elevated SCC.

In other recently published studies, different biological cut-off values to indicate an inflammatory response in the udder have been suggested. Berry, Hillerton & Torgerson (2006) used 0.8 mg L^{-1} for SAA in cow composite milk. O'Mahony *et al*. (2006) used the same cut-off value, although using quarter milk samples. Grönlund *et al.* (2003) suggested >1.4 mg L^{-1} as a cut-off value indicating abnormal levels of SAA in quarter milk samples. Jacobsen *et al*. (2005) reported that the SAA concentration in quarter milk samples ranged between 0.0–1.3 mg L^{-1} prior to inoculation of the pathogen. In a study by Grönlund, Hallén Sandgren & Persson Waller (2005) cows with chronic subclinical mastitis were studied and >0.9 mg L^{-1} was suggested to indicate abnormal udder quarters. The results from these studies are in agreement with the concentrations found in our studies analysing clinically healthy cows **(papers II and III)** and all studies mentioned above have used the same ELISA for determination of SAA in milk.

Hiss *et al.* (2007) studied Hp as a marker for subclinical mastitis and suggested that a cut-off value between 2.2–2.7 mg L^{-1} could discriminate between quarters with subclinical mastitis and healthy quarters. Grönlund *et al.* (2003) suggested that >4.3 mg L^{-1} in quarter milk samples indicated abnormal levels of Hp in milk in a study on experimentally induced acute and chronic *Staphylococcus aureus* mastitis. For chronic subclinical mastitis, Grönlund, Hallén Sandgren & Persson Waller (2005) suggested that values >LOD of the assay (0.3 mg L^{-1}) indicated abnormal udder quarters. In our studies, samples containing detectable levels of Hp also contained SAA and elevated SCC **(papers II–IV)**. It therefore seems

unlikely that Hp should have a biological cut-off level as high as several mg L^{-1}. It is possible that differences in analytical method may have contributed to the variation in results.

Since the number of studies on milk APP levels of clinically healthy cows is small, it is difficult to suggest appropriate cut-off values for Hp and SAA. When discussing cut-off values it is also important to distinguish between concentrations in quarter, cow composite and bulk tank milk samples, since cow composite and bulk tank milk samples will be diluted resulting in decreased APP levels.

Haptoglobin and serum amyloid A as potential biomarkers for mastitis

In **paper II**, when analysing quarter and cow composite milk samples, SAA was occasionally detected in the cow composite milk samples due to elevated SAA in one or more quarters, although the cow composite milk SCC was not affected. **Paper III** also demonstrated that it was possible to find SAA in cow composite milk samples even though the SCC in the sample was low. When analysing SCC in cow composite milk samples, it is quite common that a subclinical mastitis in one quarter is masked due to the dilution effect. Berglund *et al.* (2004) showed that among cow composite milk samples with SCC <100,000 cells mL^{-1}, 10 % of the quarters had a California Mastitis Test (CMT) score ≥ 3, and 50 % of those quarters were bacteriologically positive. Given our results for SAA, it might be speculated that SAA may be a better tool to use in cow composite milk samples when there is a low-grade inflammation/infection in one quarter. Therefore it would be interesting to study if APP can be used as biomarkers for mastitis at cow composite level to be used on farms, for example in automated milking systems.

It is likely that Hp and SAA are present in milk during different stages of the inflammatory process and also respond differently to different udder pathogens. Pyörälä *et al.* (2006) studied Hp and SAA in milk in relation to different pathogens and found remarkable differences in concentrations dependent on the causing pathogen. This is in agreement with Hiss *et al.* (2007) who studied Hp in subclinial cases of mastitis. Both studies demonstrated that *Escherichia coli* caused a substantial increase in Hp whereas coagulase-negative staphylococci only caused a minor increase. Pyörälä *et al.* (2006) also found that *Arcanobacterium pyogenes* increased the Hp concentration remarkably, while the SAA concentration was only slightly increased. In a study by Åkerstedt *et al.* (unpublished results), the individual udder quarters of 10 clinically healthy cows were milked twice a day during 21 consecutive days and bacteriological sampling was performed from all udder quarters every morning. One of the cows had a naturally occurring subclinical infection caused by *Enterobacter* spp, which resulted in SAA concentrations sometimes exceeding 300 mg L^{-1} in the infected udder quarter, without any clinical signs in the cow or in the milk.

In a study by Nielsen *et al.* (2004) the authors suggested that SAA was present in milk only when bacteria were present. In another study by Eckersall *et al.* (2006) the authors demonstrated that during subclinical mastitis the increase of SAA

mRNA was greater than the increase of Hp mRNA in mammary tissue. On the contrary, in hepatic tissue the Hp mRNA was greater than the SAA mRNA. Given these data, a hypothesis why we found SAA more frequently in the milk than Hp might be production of locally-produced SAA as soon as bacteria are present in the milk. Presence of bacteria in milk does not always give rise to an infection or may induce subclinical infections that are cured before the infection is detected. Therefore, locally-produced SAA might be detected more frequently in milk. Hp and hepatically-produced SAA might be detected in milk if the bacteria become established in the mammary tissue, with a consequent change in milk composition. Moreover, the production of Hp and SAA seems to differ, which may indicate that they could be used as potential biomarkers for different stages of the inflammation. It is of great importance that research on APP in relation to subclinical mastitis and different udder pathogens continues as it would give further information about the biological functions of APP in the mammary gland. Such knowledge is also necessary to elucidate if the APP levels in milk give information about the severity of the inflammation.

Haptoglobin and serum amyloid A as potential biomarkers for raw milk quality

In **papers III and IV**, results show that the casein number and the casein content as well as lactose content was often much lower when APP was present in the milk sample. Reduced casein and lactose content can be the result of several factors. Udder health disturbances often result in increased permeability between blood and milk, damaged epithelial cells resulting in decreased synthesis of proteins and increased proteolysis (Mattila, Pyörälä & Sandholm, 1986; Le Roux, Laurent & Moussaoui, 2003). Increased permeability will often result in elevated whey protein content since serum proteins like immunoglobulins and serum albumin will leak into the milk. The reduced ability of epithelial cells to synthesise will also result in a decrease of components produced in these cells, e.g. lactose, caseins and the whey proteins β-lactoglobulin and α-lactalbumin (Sandholm *et al*., 1995).

Cow composite milk samples with detectable levels of Hp showed significantly lower casein and total protein content **(paper III)**. The most possible explanation was reduced synthesis of the milk proteins produced by the epithelial cells. Another explanation could be increased proteolysis, but this was not observed in this study. A possible explanation why proteolysis could not be observed is that the fluorescamine method used was not sensitive enough to detect small differences in proteolysis between samples with and without detectable levels of Hp (Harayani *et al*., 2003). On the other hand, increased proteolysis and lower lactose was found in bulk tank milk samples with detectable levels of Hp **(paper IV)**. Reduced lactose content is often described due to decreased synthesis in the epithelial cells, but Silanikove *et al*. (2000) demonstrated that reduced lactose might be associated to increased proteolysis. The authors demonstrated that when casein is degraded, the release of specific peptides might have a regulatory effect on lactose secretion. Bulk milk samples with detectable levels of Hp also contained significantly lower casein content and casein number **(paper IV)**. A

possible explanation to the fact that increased proteolysis was observed in bulk tank milk samples is that this milk consists of commingled milk from different milkings. Consequently, milk from subclinically infected glands will be commingled with milk from healthy glands in the tank. During storage proteolysis may continue for a longer time in the tank compared to the situation for the cow composite milk samples **(paper III)**, which were collected and frozen immediately after milking.

Cow composite milk samples with detectable levels of SAA had lower casein number and lower lactose content **(paper III)** and bulk tank milk samples containing detectable levels of SAA had lower casein number, increased whey protein content and lower lactose content **(paper IV)**. Since detectable levels of SAA in bulk tank milk samples was related to increased whey protein content and lower lactose, the reduced casein number is in this case most probably affected by the influx of whey proteins from blood. It is both possible and probable that decreased synthesis, proteolysis and influx of components from the blood occur simultaneously but in these studies it was not possible to evaluate to what extent.

The protein composition of the milk is greatly influencing the yield, shelf-life and overall quality of many dairy products. Despite the fact that casein is considered the most valuable milk protein for the dairies, casein content is not included in milk quality programmes. It would be more relevant to determine casein instead of total protein level in the milk. Today, the experience from routine analysis of casein in raw milk is limited, although methods based on mid-infrared spectroscopy are available. Earlier the milk was collected every day in Sweden but due to the decreasing number of dairy herds and dairies, milk has been collected every second day since the 1960s. Since the number of dairy herds is decreasing, the distance between herds increases, especially in northern Sweden. Collecting the milk every third day would result in reduced transportation costs with a positive effect on the environment. If the milk would be collected every third day, the protein quality of the milk would be even more critical for the dairies, since proteolysis is one major factor determining the shelf-life of the products. Consequently, problems with subclinical mastitis and its affect on milk quality will then be even more critical.

Final remarks

Since mastitis is one major cause for raw milk deterioration and consequently constitutes a large problem for the dairies, early detection of subclinical mastitis is of great importance. SCC is still the golden standard used for diagnosis of subclinical mastitis and it is an important parameter in milk payment systems. Since SCC is influenced by various factors and a suitable threshold value defining the udder health status is difficult to determine, new, more specific markers are needed. The APP have not been extensively studied in milk, but since they are locally produced, speculations regarding different biological functions make APP even more interesting as early markers for mastitis and consequently impaired raw milk quality.

In general, there is a lack of simple, reliable methods to determine APP in milk and serum for animals. The most common way to measure APP in milk is by often different cumbersome in-house ELISA. Consequently, simple and rapid techniques analysing APP in milk as well as serum are needed to continue the evaluation of their potential as biomarkers in diagnostics as well as for raw milk quality grading purposes. **Paper I** describes the first APP assay using a biosensor technique to detect Hp in real time. In addition, the numbers of automated milking systems increase and there is a need for new online methods detecting mastitis. If it would be possible to use APP as markers for subclinical mastitis at cow composite level, and if easier methods could be developed to detect APP in milk, new possibilities for their implementation in automatic milking systems would occur.

Another important aspect of this work was that we applied APP research in the field of product quality, i.e. raw milk quality. To our knowledge **paper II** was the first to describe presence of APP in bovine bulk tank milk, but **papers III and IV** were the first studies published on APP in milk in relation to the composition and processing properties of the raw milk. If APP could be used to determine milk quality they would have a great potential in raw milk quality grading and for milk payment purposes. SCC has been reported to be a doubtful marker for raw milk quality, especially in relation to the protein quality of the milk. Our studies indicate that APP could be potential biomarkers for the protein composition of the milk, which is of great importance for the dairy industry. Therefore, it is essential for the dairy industry as well as for the veterinarians and farmers to continue research related to APP as early markers for subclinical mastitis and raw milk quality.

Main conclusions

The results presented in this thesis give insight into the occurrence of the major acute phase proteins in bovine milk, Hp and SAA, in relation to milk quality and udder health. The main outcomes from the studies are:

- A rapid and fully automated biosensor assay to determine Hp in milk was developed (LOD 0.3 mg L^{-1}).
- Hp and SAA could be found in quarter, cow composite and bulk tank milk samples.
- SAA was detected more often and also at higher concentrations than Hp in quarter, cow composite and bulk tank milk samples.
- In general, detectable levels of Hp and SAA were related with elevated SCC in all types of samples.
- Cow composite milk samples with detectable levels of Hp had lower total protein and casein content and higher SCC than samples without detectable levels of Hp.
- Cow composite milk samples with detectable levels of SAA had lower casein number, lower lactose content and higher SCC than samples without detectable levels of SAA.
- Bulk tank milk samples with detectable levels of Hp had lower casein content and casein number, increased proteolysis, lower lactose content and higher SCC than samples without detectable levels of Hp.
- Bulk tank milk samples with detectable levels of SAA had lower casein number, increased whey protein content, lower lactose content and higher SCC than samples without detectable levels of SAA.
- Hp and SAA are suggested to be useful biomarkers for the quality of the raw milk, especially regarding the protein composition.

Suggestions for future research

In general, since there is a need for new diagnostic markers for mastitis as well as biomarkers for raw milk quality and processing properties, more studies on APP in quarter, cow composite and bulk tank milk samples are needed.

Some suggestion for future research:

- Investigations on the possibility to use an acute phase protein index, combining positive and negative APP for a more accurate diagnosis of subclinical mastitis and a more specific and sensitive biomarker for raw milk quality.

- Research related to relevant cut off values for APP in milk. If appropriate, cut off levels indicating udder health disturbances (mainly in quarter and cow composite milk samples) and changes in milk quality (mainly in bulk tank milk samples).

- Investigations to find out if and how the APP varies during milking. This is especially important if APP analysis would be incorporated into automated milking system.

- Studies on the effect of different udder pathogens on APP concentrations and milk quality.

- Studies to find out if APP in milk are less influenced by physiological factors, such as oestrus, stage of lactation, lactation number, around calving and drying off, than SCC.

- Development of simple, rapid and sensitive assays to determine APP in milk, and assays that can distinguish between hepatically and locally produced APP.

- Studies to define biological functions of locally produced APP and evaluation of the diagnostic relevance of these proteins.

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