JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 10049–10054 10049
DOI:10.1021/jf901026m

# A Novel Method for *in Situ* Detection of Hydrolyzable Casein Fragments in a Cheese Matrix by Antibody Phage Display Technique and CLSM

Zhi Duan, Dagmar Adeline Brüggemann, and Henrik Siegumfeldt*

Department of Food Science, Faculty of Life Sciences, The University of Copenhagen, Denmark

A novel method to monitor *in situ* hydrolyzable casein fragments during cheese ripening by using immunofluorescent labeling and confocal laser scanning microscopy (CLSM) was developed. Monoclonal single chain variable fragments of antibody (scFvs) were generated by antibody phage display toward three small synthetic peptides of the $\alpha_{s1}$-casein sequence. These peptides traverse enzymatic cleavage sites of casein during cheese ripening. The specificity of the generated anti-peptide antibodies was determined by ELISA and Western blot. Finally, an immunofluorescent labeling protocol was successfully developed for the detection of scFvs binding to different $\alpha_{s1}$-casein fragments inside a cheese matrix by CLSM. To our knowledge, this is the first demonstrated immunofluorescent labeling method for *in situ* analysis of proteolysis phenomena in the cheese matrix. Additionally, this technique offers a high potential to study *in situ* dynamic spatial changes of target components in complex food systems.

**KEYWORDS: Phage display; scFv; antibody; casein; peptide; CLSM**

## INTRODUCTION

In cheese, casein proteolysis during ripening is one of the major reasons for the development of cheese flavor. Several instrumental methods for the analysis of proteolysis during cheese ripening have been developed, including urea−PAGE, capillary electrophoresis (CE) or RP−HPLC (*1*). More recently immunological methods have been applied in order to detect hydrolyzed casein components. For instance, Senocq et al. (*2*, *3*) and Dupont et al. (*4*) monitored proteolysis of $\beta$-CN with antibodies specifically directed against small peptide fragments traversing the plasmin cleavage sites. Muller-Renaud et al. (*5*−*7*) quantified $\alpha_{s1}$-, $\beta$- and $\kappa$-CN in milk or cheese extracts by using two antibodies which were directed against C- and N-terminals of the proteins. All of these studies were based on conventional hybridoma technology, in which monoclonal antibodies are obtained from animals immunized by a purified antigen (*8*). However, the preparation of antibodies by this hybridoma technology is time-consuming and costly.

Methods for obtaining recombinant antibody fragments were developed in the past few decades. The manipulation of genes encoding antibodies led to the construction of antibody derivates, which retain full antigen binding function. One of these derivates is the scFv, consisting of variable regions of antibody heavy and light chains, connected by a flexible linker (*9*). Functional scFv expressed from a single cDNA sequence can be produced efficiently in bacteria without the need to immunize animals (*10*). Since the pioneering work of Smith (*11*) and further development by McCafferty et al. (*12*), the expression of scFv on the surface of filamentous phage and selection of these phage libraries against target antigen have matured into an extensively used technique to produce recombinant antibodies for research and development of therapeutics (*13*, *14*). Billions of variant scFvs can be displayed in an *in vitro* phage library, where each phage particle in the library carries copies of one single scFv sequence. The production and selection of scFvs on phage surface is much faster compared to the conventional method, and the availability of large and diverse antibody gene repertoires in phage has provided a source of scFvs to almost any antigen (*15*). The antibody phage display technique therefore overcomes some of the limitations of hybridoma technology.

The aim of this study was 2-fold: to detect the specificity of scFvs generated by phage display, and to demonstrate *in situ* analysis of hydrolyzable casein fragments in a cheese matrix, using immunofluorescent labeling. We selected three peptide sequences of $\alpha_{s1}$-CN (F1, F2 and F3) as targets (**Table 1**). These selected casein fractions traverse the cleavage site of enzymes responsible for proteolysis during cheese ripening. As long as the cleavage site is intact, the antibody will bind to the casein. However, after cleavage of the peptide bond by the enzymes, the antibody will no longer be able to recognize the substrate, and as cheese maturation proceeds, there will be a consequent loss in binding sites. Phe23−Phe24, the primary cleavage site of chymosin in $\alpha_{s1}$-CN, is covered by peptide F1. Peptide F2 comprises Leu156−Asp157, which is cleaved at a lower frequency by chymosin. Peptide F3 is situated at the C-terminal sequence, which cannot be cleaved by chymosin.

*Corresponding author: Department of Food Science, Faculty of Life Sciences, University of Copenhagen, 1958 Frederiksberg C, Denmark. Tel: +45 35333286. Fax: +45 35333214. E-mail: hsi@life.ku.dk.



Published on Web 10/15/2009

**Table 1.** Selected Peptides from $\alpha_{s1}$-Casein Sequence

| name | peptide | sequence | traversing cleavage sites by chymosin (pepsin)[a] |
|---|---|---|---|
| F1 | $\alpha_{s1}$-CN f17−31 | NENLLRFFVAPFPEV | 23−24, 24−25, 28−29 |
| F2 | $\alpha_{s1}$-CN f150−163 | FRQFYQLDAYPSGA | 153−154, 156−157, 159−160 |
| F3 | $\alpha_{s1}$-CN f185−199 | PIGSENSEKTTMPLW | no |

[a] Only the reported cleavage sites by chymosin during cheese ripening are listed (*1*). Many other enzymes (e.g., plasmin, cathepsin D, and proteinases/peptidases from LAB) involved in cheese ripening also have cleavage sites on these peptide sequences, which are not shown in the table.

So far, the use of immunodetection has been restricted to the identification of components in aqueous cheese extracts (*2*−*4*), which excludes any spatial or structural information. Cheese as target for antibody detection lacks an innate structural rigidity and must be manipulated to be robust enough to endure liquid solution incubation with antibodies and washing procedures, which are the fundamental steps for immunofluorescent labeling and microscopic analysis *in situ*. We have overcome this problem by applying a simple fixation and embedding method, which stabilizes the fragile structure of the lipid-rich matrix for immunolabeling. This is the first report using the scFv antibodies as probes to *in situ* recognize the spatial distribution of target components inside the cheese matrix with CLSM.

## MATERIALS AND METHODS

**Antibody Phage Libraries and Reagents.** The anti-peptide scFv-phages used in this study were generated by selection of Human Single Fold scFv libraries I + J (Tomlinson I + J) (*16*). This scFv phage library and KM13 helper phage (*17*) were kindly provided by MRC Centre (Cambridge, United Kingdom). All the 96 well microplates were from Nunc A/S (Roskilde, Denmark). The anti-M13/HRP conjugated antibody was from GE Healthcare (Chalfont St. Giles, United Kingdom). The $\alpha_s$-CN had a purity ≥70%. Unless otherwise stated, all commonly used reagents were from Sigma-Aldrich (St. Louis, MO).

**Synthetic Peptides.** Three peptides were selected from $\alpha_{s1}$-CN sequence (**Table 1**) named as F1, F2 and F3. The peptides were synthesized at 80% purity and biotinylated on N-terminals by JPT Peptide Technologies GmbH, Berlin, Germany.

**Generation of Anti-Peptide scFvs by Panning of Phage Libraries.** Tomlinson I and J libraries were used individually in parallel to perform the panning. The biotinylated peptide (20 $\mu$g/mL in PBS, pH 7.2) was incubated 1−1.5 h onto Nunc Streptavidin plates (Nunc A/S, Roskilde, Denmark). After 3 washes by PBS with 1% Tween 20 (1% TPBS), scFv phage library ($10^{12}$ −$10^{13}$ cfu/mL) in 1% TPBS was added and incubated for 2 h. Wells were washed with 1% TPBS by 15 times for first round, 21 times for second and further rounds of panning. The bound phage was eluted by the competitive elution method with 25 $\mu$g/mL biotinylated peptide. 500 $\mu$L of the eluted phage was used to infect 2 mL of exponential phase growing *Escherichia coli* TG1. The subsequent panning procedures were the same as the protocol described previously (*12*). The phage library was subjected to four rounds of panning. The monoclonal scFvs were selected by monoclonal ELISA as previously described (*12*). Briefly, after the last round of panning, individual clones were grown in microtiter wells and phages were produced by addition of KM13 helper phages. Phage was then added to the corresponding wells of a peptide-coated plate and detected by an anti-M13/HRP conjugated antibody.

For each peptide, the monoclonal scFv-phage with the highest affinity was selected for application in this study and named anti-F1, anti-F2 and anti-F3, respectively.

**ELISA.** The protein samples ($\alpha_s$-CN 500 $\mu$g/mL, CN 1 mg/mL, BSA 1 mg/mL, fish gelatin 5 mg/mL) in 0.1 M PBS pH 7.2 were incubated 1−1.5 h with Immo-Amino (Nunc Immobilizer Amino) surface. Rennet treatment of peptides was performed by incubation of 200 $\mu$g/mL biotinylated peptides with 1:3000 diluted CHY-MAX Plus Liquid Coagulant (Chr. Hansen A/S, Hørsholm, Denmark) in PBS (pH 6.5) for 1 h at 37 °C. The nontreated as well as the rennet treated biotinylated peptides (20 $\mu$g/mL) in 0.05% TPBS (Tween 20 in PBS) were incubated 1−1.5 h with Immo-Strep (Nunc Immobilizer Streptavidin) surface. After incubation with scFv-phages for 1 h, ELISA measurements were performed as previously described (*18*), except that 1% TPBS was used for both phage diluting and washing buffers without blocking procedure. A helper phage comprises only the phage particle without scFv and was used as a negative control. Absorbance readings were taken by subtracting the $A_{620}$ from the $A_{450}$ (*18*).

**Preparation of Cheese Samples.** To prepare fresh cheese curd, commercial milk (1.5% fat) was purchased from a local supermarket and precultured overnight at 30 °C with a combination of several different *Lactococcus lactis* (similar to DL culture). Subsequently, milk was inoculated with 1% preculture and incubated at 30 °C for 1 h. $CaCl_2$ was added to a final concentration as 0.1 mg/L. After adding 1:3000 CHY-MAX Plus Liquid Coagulant (Chr. Hansen A/S, Hørsholm, Denmark), the milk was put into the container at 37 °C for around 1 h. The separated curd (coagulated part) was cut into small pieces and stirred for 30 min. Finally, the curd was filtered by a tea/coffee towel to remove most of the whey, pressed in a container and left overnight at room temperature.

Commercial Danbo 45+ (fat 25%) and Cheddar (fat 33%) cheeses were purchased from a local supermarket.

**SDS−PAGE and Western blotting.** Aqueous cheese extracts were prepared by grinding 10 g of cheese sample with 40 mL of distilled water in a stomacher bag with filter (BagPage, Breveté, France and Etranger) by Seward Stomacher 400 Blender at room temperature for 5 min. The supernatant was filtered through a 0.45 $\mu$m acetate filter (Millipore, Billerica, MA). The aqueous cheese extracts were stored at −18 °C until used. Rennet treated $\alpha_s$-CN sample was obtained by incubation of 5 mg/mL $\alpha_s$-CN with 1:3000 diluted CHY-MAX Plus Liquid Coagulant in PBS (pH 6.5) for 1 h at 37 °C.

The samples were treated with NuPAGE LDS Sample Buffer and Reducing Agent (Invitrogen) according to the manufacturer's instructions. SDS−PAGE was carried out using NuPAGE 10% Bis-tris gels (Invitrogen) and NuPAGE MES running buffer. Molecular weight marker was Precision Plus Protein Standards (Bio-Rad). Proteins were transferred immediately from the gel onto a 0.45 $\mu$m nitrocellulose membrane (Bio-Rad) and run at a constant 30 V for 1 h by XCell Blot Module (Invitrogen). Membranes were blocked overnight in 2% BSA and 2% fish gelatin in PBS at 4 °C. After incubation with scFv-phage diluted as $10^{11}$ phage/mL in 1% Tween 20, 2% BSA and 2% fish gelatin in PBS, immunodetection was performed with anti-M13/HRP conjugate. Blots were developed using a TMB Liquid Substrate System (Sigma-Aldrich, St. Louis, MO) for 1 min.

**Confocal Laser Scanning Microscopy.** For microscopy, small slices were cut from blocks of cheese. After fixation in 4% paraformaldehyde in PBS, the samples were labeled with Alexa Fluor 350 carboxylic acid, succinimidyl ester (Molecular Probes, Invitrogen, Eugene, OR) for protein visualization, and with Bodipy (Molecular Probes, Invitrogen, Eugene, OR) for lipid visualization. The labeled blocks were subsequently incubated in 3% porcine gelatin in PBS. A second fixation in 4% paraformaldehyde−PBS was introduced before the final immunolabeling procedure was performed (*19*). After incubation with anti-peptide scFv-phages, a mouse anti-M13 phage antibody (GE Healthcare, Chalfont St. Giles, United Kingdom) was added and incubated with the fluorescently labeled Alexa Fluor 647 donkey antimouse antibody (Molecular Probes, Invitrogen, Eugene, OR). The washing buffer was 1% TPBS, and blocking buffer comprised 2% BSA, 2% gelatin in PBS. In order to measure the nonspecific labeling from phage particles, a sample incubated with KM13 helper phage instead of scFv-phage was used as a negative control.

The CSLM work was carried out using a Leica SP2 confocal scanning laser microscope (Leica Microsystems GmbH, Wetzlar, Germany) using $\lambda_{ex}$ 365, $\lambda_{em}$ 375 to 450 nm (for protein matrix); $\lambda_{ex}$ 488, $\lambda_{em}$ 498 to 540 (for lipids); and $\lambda_{ex}$ 633 nm, $\lambda_{em}$ 643 to 700 nm (for scFv-phages). To avoid false positives in collecting fluorescence emission from adjacent fluorophore channels, a sequential confocal imaging technique was used. The resulting images from each channel were false colored. The presented images are maximal projections of a series of *XY* plane images and overlays of the three recorded channels.

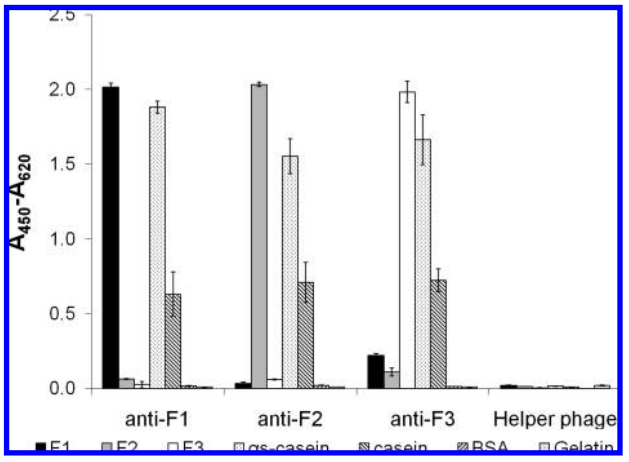


**Figure 1.** ELISA of anti-peptide scFv-phages against different peptides and proteins. Biotinylated peptides were coupled on an Immo-Strep surface. All proteins were immobilized on an Immo-Amino surface. Helper phage (phage particle without scFv) was used as control. Each error bar represents the standard deviation of 4 replicates.

## RESULTS AND DISCUSSION

**Identification of Specificity of scFvs by ELISA.** An ELISA was performed to identify the specificity of the anti-peptide scFvs (**Figure 1**). A high specificity of the anti-peptide monoclonal scFv-phages was demonstrated. All anti-peptide monoclonal scFv-phages showed high affinity with the corresponding peptides, high binding ability with $\alpha_s$-CN and CN, and no cross-reaction with the negative control proteins, BSA and gelatin. The control (helper phage) showed that the detected binding was caused by scFvs and not by phage particles. Both anti-F1 and anti-F2 scFvs possessed a good specificity and had a very low reaction with other nontarget peptides. Anti-F3 scFv showed some limited cross-reaction with the peptides F1 and F2.

A further ELISA quantification of anti-peptide scFv-phages binding before and after rennet treatment shows that anti-F1 and anti-F2 primarily binds to intact peptides (**Figure 2**), whereas anti-F3 retains its binding capacity, even after rennet treatment of F3. This demonstrated that the anti-peptide scFv-phages could clearly differentiate between intact and rennet treated peptides. However, some absorbance remained after the rennet treatment, which may be due to an incomplete cleavage of the peptides by the rennet, or by a residual binding of scFvs to the cleaved peptides. The saturated absorbance values between **Figure 1** and **Figure 2** are different for the same antibody−antigen reactions, because there is a batch-to-batch variation of ELISA, which is caused by different TMB substrate stocks used.

**Further Identification of Specificity of scFvs by Western Blot.** A more detailed analysis of the binding pattern was achieved by SDS−PAGE and Western blot (**Figure 3**). Protein profiles of all samples investigated were visualized by Coomassie Blue G-250 staining of gels. The binding of scFv-phage was detected by an anti-M13/HRP conjugate and visualized with a TMB Liquid Substrate System. The commercially obtained $\alpha_s$-CN (lane 1) was separated into several bands by SDS−PAGE due to impurity of the sample. The rennet treated $\alpha_s$-CN (lane 2) contained several more bands than untreated $\alpha_s$-CN, which are the hydrolyzed products by rennet. The different caseins were separated by SDS−PAGE into the mixture of bands between 20 to 30 kDa in the skim milk solution (lane 3). SDS−PAGE also revealed a large amount of different hydrolyzed casein products (below 30 kDa) in the cheese aqueous extracts (lanes 4 and 5).

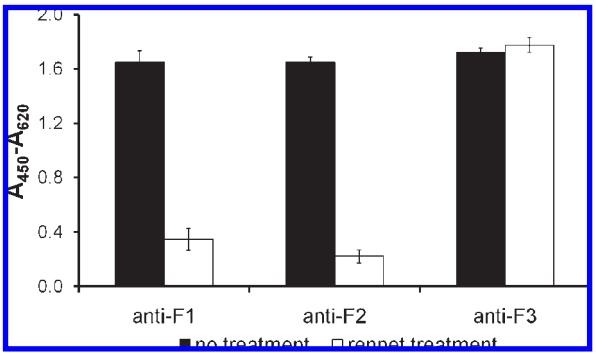


**Figure 2.** Specificity of anti-peptide scFv-phages toward rennet treated and nontreated corresponding target peptides quantified by ELISA. Error bar represents the standard deviation of 4 replicates.

Comparing all samples, the anti-F2 and anti-F3 scFv-phages bound to similar bands, while the anti-F1 showed a different binding profile. By the interpretation of their binding patterns, it was possible to identify the separated bands from the SDS−PAGE. This would not have been possible without high specific antibodies. One clear and strong band at around 30 kDa, which appeared on protein profiles of all samples and was detectable by all scFv-phages, was consequently identified as $\alpha_s$-CN (mixture of $\alpha_{s1}$-CN and $\alpha_{s2}$-CN). $\beta$-CN was tentatively identified as an impurity in $\alpha_s$-CN. The fragment $\alpha_{s1}$-CN f41−149 was identified in rennet treated $\alpha_s$-CN. This is because no scFv-phages bind to it, and no any other rennet cleavage sites have been reported on $\alpha_{s1}$-CN between Ser41 and Leu149 under the performed hydrolysis conditions (*20*).

In order to facilitate the description of the other resulting bands, these bands were grouped into three areas (A, B and C) according to their molecular weight. Bands at area A observed in all samples corresponded to high molecular weight proteins. In general, they were detected by all scFv-phages, especially in the cheese samples. These bands could be caused by incomplete dissociation of casein micelles in milk (*21*). Band B, around 25 kDa, is identified as $\alpha_{s1}$-CN f24−199 (MW 20.2 kDa), because Phe23−Phe24 is the main cleavage site on $\alpha_{s1}$-CN by rennet (*20*), and only anti-F2 and anti-F3 have high binding on these bands. The two scFv-phages also revealed the bands in area C. These bands should include several C-terminal $\alpha_{s1}$-CN fragments, e.g. f99−199, f120−199, f104−199, f110−199, which are always found in cheese extracts (*1*).

The separation limit of the performed SDS−PAGE (10 kDa) prevents the discrimination of the binding profiles of anti-F2 and anti-F3. Anti-F3 presumably binds to much smaller fragments containing the C-terminal. The results of Western blot show that the three scFv-phages have high specificity toward their target peptide sequences, and scFv-phages can recognize the intact or hydrolyzed fractions of $\alpha_{s1}$-CN which contain these target peptide sequences.

Under the conditions used for SDS−PAGE, $\alpha_s$-CN, a mixture of $\alpha_{s1}$-CN (MW 22.9 kDa) and $\alpha_{s2}$-CN (MW 24.3 kDa), could not be separated completely. This is because the two proteins have similar molecular weight. The same observation was also described by Gaiaschi et al. (*22*). However, the indicated molecular weight of the casein or casein fragments from the molecular weight marker in the gel was inconsistent with the reported molecular weight. For instance, $\alpha_s$-CN showed a molecular weight around 30 kDa. This may be because the loaded samples were treated with denaturing and reducing agents, where the molecular weight marker was untreated, and in our experiments, the prestained marker Precision Plus Protein Standards (Bio-Rad) could therefore

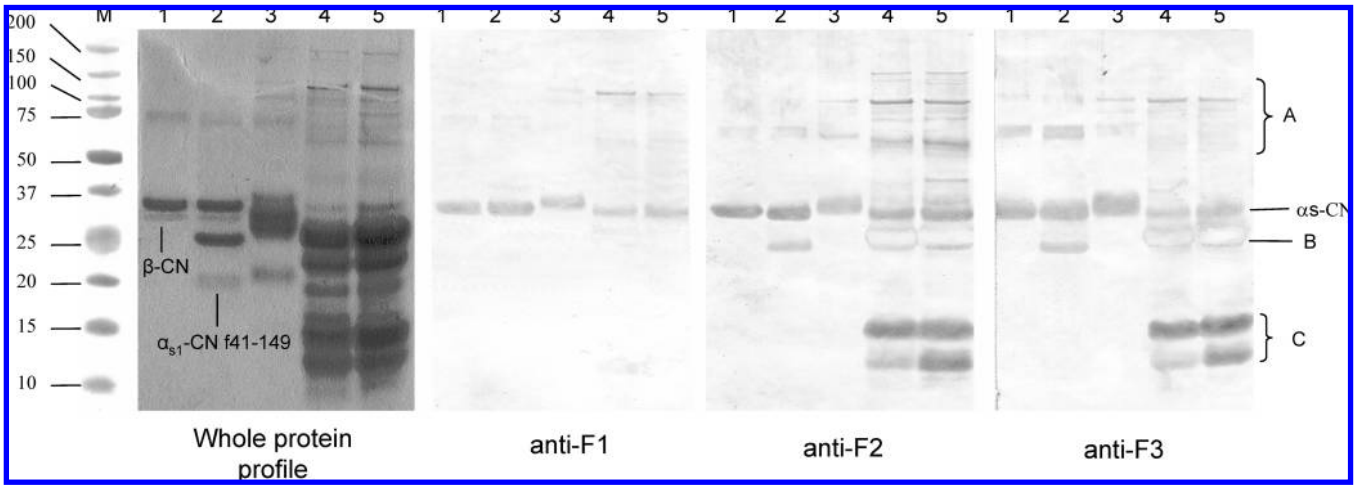


**Figure 3.** Specificity of anti-peptide scFv-phages toward protein fragments from ripened Danbo and Cheddar cheeses is revealed by Western blot. The gel was stained by Coomassie brilliant blue G-250 to reveal the whole protein profile. TMB substrate was used to visualize the binding of scFv-phages (anti-F1, anti-F2, anti-F3) against protein samples transferred onto nitrocellulose membranes. M, molecular weight mark; lane 1, αs-CN; lane 2, rennet treated αs-CN; lane 3, skim milk solution; lane 4, Danbo cheese aqueous extracts; lane 5, Cheddar cheese aqueous extracts. The bands at areas A−C are revealed by scFv-phages.

not predict the precise molecular weight of treated samples. The use of the prestained marker is necessary as an indicator for Western blot.

***In Situ* Analysis of Cheese by scFv-Phage.** Paraformaldehyde fixation after embedding with gelatin preserves the structure of the cheese matrix after immunolabeling procedures and allows for the investigation of the cheese matrix with CLSM. The structure of the cheeses showed networks of protein as a continuous matrix in which lipids were trapped as discrete globules, and empty holes (air or water) inside cheese were also clear (**Figure 4**). Both Danbo and Cheddar cheeses showed many small protein conjugation units connected together to form the whole protein matrix, but laboratory-made fresh cheese had a different protein structure. This may be because the milk used for fresh cheese was common commercial milk, which had been homogenized.

The cheese matrix was successfully labeled by scFv-phages, as seen by the many small dots scattered inside the protein matrix. All cheeses had a relatively small amount of $\alpha_{s1}$-CN peptide fragments containing the F1 sequence compared to the C-terminal F3 sequence. The fragments of both sequences within the protein matrix were distributed homogeneously. The samples incubated with KM13 helper phage have been used as a negative control (only the images from Danbo and Cheddar are shown). No detectable signal from the negative control proved that all the observed small dots must be binding from scFvs, and not nonspecific phage particle binding.

A stratified labeling procedure, including several gentle fixation steps, allowed for the first time the *in situ* immunolabeling of cheese. Using porcine gelatin as an embedding medium for cheese has many advantages over classical embedding media, e.g. paraffin or resin (*23*, *24*), as no dehydration and defatting is required. Furthermore, the gelatin proteins do not interfere with the antigenicity of the $a_{s1}$-CN peptide fragments. An additional advantage is that lipid behavior and the protein network can be studied together. Less fat and smaller lipid globules were found in the laboratory-made fresh cheese than in the Danbo and Cheddar cheeses. Lipid globules were particularly large in the Cheddar cheese, which is due to the different production procedures. The amount of lipid globules represented in the images reflects the overall fat content, as the laboratory-made cheese was produced from semiskimmed milk (1.5%), and the Danbo and Cheddar from full fat milk.

The bound scFv-phages were revealed as many small dots scattered inside the cheese protein matrix. As each small dot had an observed diameter of 1−2 $\mu$m, it is thought to represent an individual scFv-phage, because the length of a standard M13 phage with scFv fragment is around 0.9 $\mu$m (*25*). Due to this length, many secondary antiphage antibodies, and subsequently many fluorophores carrying tertiary antibodies, can bind to it, resulting in a strong amplification of the signal, which makes it possible to visualize the binding of a single scFv. A few larger dots (diameter $> 5\,\mu$m) were observed, which may be due to an uneven distribution of the $\alpha_{s1}$-CN peptide fragments, or more likely an accumulation of phages.

The scFv-phages could penetrate 10 $\mu$m into the cheese matrix as determined by optical sectioning with the microscope (results not shown), which is in accordance with detection depth of antibodies in most animal tissues. It indicates that the penetration ability of scFv-phage into cheese matrix is only marginally influenced by its large size. This might be due to the relatively loose structure of cheeses.

82% proteins of bovine milk are CN, in which 37% are $\alpha_{s1}$-CN (*26*), and only 10% intact $\alpha_{s1}$-CN was left in the 2 month ripened cheese (*27*, *28*). Therefore, there should be approximately 3% ($=82\%\times37\%\times10\%$) total proteins as intact $\alpha_{s1}$-CN are still retained in the normal commercial ripened cheese (e.g., Danbo and Cheddar in this study). The cheese fragments that are labeled by anti-F1 probably represent the intact $\alpha_{s1}$-CN, because the bond Phe23−Phe24 (included in the F1 sequence) is the most active cleavage site by chymosin and is cleaved fast during the earlier stage of cheese ripening. It is more difficult to predict how many intact C-terminal fragments (F3 sequence) are left in a ripened cheese, because a plethora of peptide fragments containing the C-terminal sequence has been identified except intact $\alpha_{s1}$-CN (*1*). But we can conclude that there must be more intact F3 sequence than intact F1 sequence in a ripened cheese. The obtained images demonstrate that, in all cheeses, the binding of anti-F3 is much more abundant than that of anti-F1 (**Figure 4**). This is consistent with the proteolysis procedure during cheese ripening.

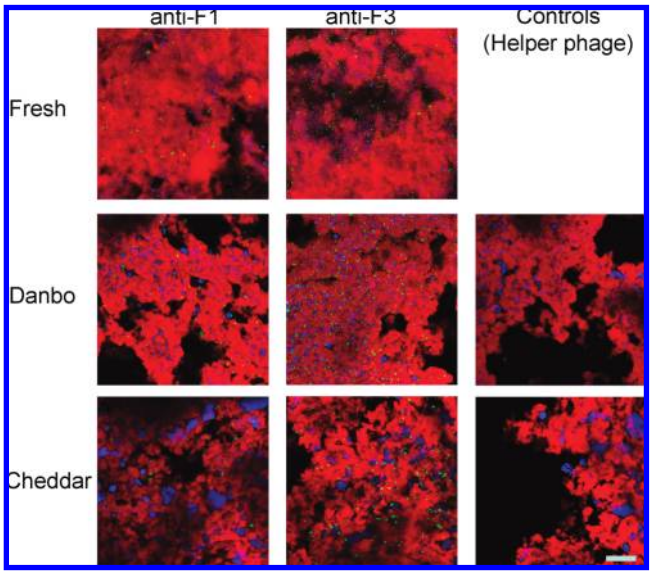


**Figure 4.** Immunofluorescent labeling of real cheese samples. Maximum projection of stacks of CLSM images was performed on three types of cheeses (laboratory-made fresh, Danbo and Cheddar). Red color represents the protein network stained by Alexa Fluor 350. Blue color represents lipids stained by Bodipy. Green color represents scFv-phages stained by Alexa Fluor 647 donkey antimouse antibody. Bar = 30 $\mu$m. The KM13 helper phage which comprises only phage particle without scFv was used as a negative control (only the images from Danbo and Cheddar are shown).

However, the total amount of the labeled peptide fragments is smaller than the theoretical value (e.g., there perhaps should be 3% of F1 sequence in total cheese proteins). We think this is mainly caused by masking of peptide epitopes inside the cheese structure, because the fixing step randomly conjugates the cheese proteins to generate a new protein network, during which the target peptide epitopes could be embedded inside a three-dimensional protein network and difficult to be reached by scFv-phages. So all the dots in **Figure 4** should reveal the location of peptide fragments from $\alpha_{s1}$-CN, but probably not all target sequences were revealed by scFv-phages.

This is the first time that specific antibodies recognizing casein protein fragments were generated using antibody phage display, and that these phage-antibodies were used to visualize the spatial distribution of the target casein fragments inside the cheese matrix by CLSM. Three peptide sequences were labeled to show the hydrolysis of $\alpha_{s1}$-CN during cheese ripening. This study demonstrates an innovative method for *in situ* analysis of proteolysis phenomena inside the cheese matrix, but more studies are necessary to follow a cheese ripening process, where a progressive reduction of binding sites for antibodies (particularly F1 and F2) is expected during the cheese maturation. However, previous unknown information about spatial distribution of target components in cheese has been revealed by this method, and it is expected that the techniques developed in this study will be ideal for evaluating the influence of processing parameters and starter cultures on the final cheese quality. Future improvements would only require specific antibodies against various targets (e.g., other enzyme cleavage sites), and for this purpose, antibody phage display is a reliable and fast-output resource.

## ABBREVIATIONS USED

scFv, single chain variable fragment of antibody; F1, F2 and F3, synthetic peptides from $\alpha_{s1}$-casein sequence; PBS, phosphate-buffered saline; TPBS, Tween 20 in PBS; Immo-Amino, Nunc Immobilizer Amino surface; Immo-Strep, Nunc Immobilizer Streptavidin surface; CN, casein; BSA, bovine serum albumin; HRP, horseradish peroxidase; MW, molecular weight; CLSM, confocal laser scanning microscopy.

## LITERATURE CITED

(1) Upadhyay, V. K.; McSweeney, P. L. H.; Magboul, A. A. A.; Fox, P. F. Proteolysis in cheese during ripening. In *Cheese: Chemistry, Physics and Microbiology*, 3rd ed.; Fox, P. F., McSweeney, P. L. H., Cogan, T. M., Guinee, T. P., Eds.; Elsevier Academic Press: 2004; pp 391−433.

(2) Senocq, D.; Dupont, D.; Rolet-Repecaud, O.; Faurie, F.; Levieux, D. Antipeptide antibodies recognizing plasmin sensitive sites in bovine beta-casein sequence. *J. Agric. Food Chem.* **2001**, 49 (3), 1571–1577.

(3) Senocq, D.; Dupont, D.; Rolet-Repecaud, O.; Levieux, D. ELISA for monitoring the cleavage of beta-casein at site Lys(28)-Lys(29) by plasmin during Comte cheese ripening. *J. Dairy Res.* **2002**, 69 (3), 491–500.

(4) Dupont, D.; Rolet-Repecaud, O.; Senocq, D. A new approach to monitoring proteolysis phenomena using antibodies specifically directed against the enzyme cleavage site on its substrate. *Anal. Biochem.* **2003**, 317 (2), 240–246.

(5) Muller-Renaud, S.; Dupont, D.; Dulieu, P. Quantification of kappa-casein in milk by an optical immunosensor. *Food Agric. Immunol.* **2003**, 15 (3−4), 265–277.

(6) Muller-Renaud, S. P.; Dupont, D.; Dulieu, P. Quantification of beta-casein in milk and cheese using an optical immunosensor. *J. Agric. Food Chem.* **2004**, 52 (4), 659–664.

(7) Muller-Renaud, S.; Dupont, D.; Dulieu, P. Development of a biosensor immunoassay for the quantification of alpha(S1)-casein in milk. *J. Dairy Res.* **2005**, 72 (1), 57–64.

(8) Kohler, G.; Milstein, C. Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity. *Nature* **1975**, 256 (5517), 495–497.

(9) Bird, R. E.; Hardman, K. D.; Jacobson, J. W.; Johnson, S.; Kaufman, B. M.; Lee, S. M.; Lee, T.; Pope, S. H.; Riordan, G. S.; Whitlow, M. Single-Chain Antigen-Binding Proteins. *Science* **1988**, 242 (4877), 423–426.

(10) Huston, J. S.; Levinson, D.; Mudgetthunter, M.; Tai, M. S.; Novotny, J.; Margolies, M. N.; Ridge, R. J.; Bruccoleri, R. E.; Haber, E.; Crea, R.; Oppermann, H. Protein Engineering of Antibody-Binding Sites - Recovery of Specific Activity in An Anti-Digoxin Single-Chain Fv Analog Produced in Escherichia-Coli. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, 85 (16), 5879–5883.

(11) Smith, G. P. Filamentous Fusion Phage - Novel Expression Vectors That Display Cloned Antigens on the Virion Surface. *Science* **1985**, 228 (4705), 1315–1317.

(12) Mccafferty, J.; Griffiths, A. D.; Winter, G.; Chiswell, D. J. Phage Antibodies - Filamentous Phage Displaying Antibody Variable Domains. *Nature* **1990**, 348 (6301), 552–554.

(13) Watkins, N. A.; Ouwehand, W. H. Introduction to antibody engineering and phage display. *Vox Sang.* **2000**, 78 (2), 72–79.

(14) Kretzschmar, T.; von Ruden, T. Antibody discovery: phage display. *Curr. Opin. Biotechnol.* **2002**, 13 (6), 598–602.

(15) Griffiths, A. D.; Williams, S. C.; Hartley, O.; Tomlinson, I. M.; Waterhouse, P.; Crosby, W. L.; Kontermann, R. E.; Jones, P. T.; Low, N. M.; Allison, T. J.; Prospero, T. D.; Hoogenboom, H. R.; Nissim, A.; Cox, J. P. L.; Harrison, J. L.; Zaccolo, M.; Gherardi, E.; Winter, G. Isolation of High-Affinity Human-Antibodies Directly from Large Synthetic Repertoires. *EMBO J.* **1994**, 13 (14), 3245–3260.

(16) Duan, Z. Interactions between macro-molecules and starter cultures in the cheese curd using antibody phage display technique and bioimaging. In *European Congress of Chemical Engineering - 6 Book of Abstracts* (*Electronic version*); Gani, R., Dam-Johansen, K., Eds.; **2007**.

(17) Kristensen, P.; Winter, G. Proteolytic selection for protein folding using filamentous bacteriophages. *Folding Des.* **1998**, 3 (5), 321–328.

(18) Harrison, J. L.; Williams, S. C.; Winter, G.; Nissim, A. *Screening of phage antibody libraries*; Academic Press Inc.: San Diego, 1996.
(19) Bruggemann, D. A.; Risbo, J.; Pierzynowski, S. G.; Harrison, A. P. Muscle Contraction and Force: the Importance of an Ancillary Network, Nutrient Supply and Waste Removal. *Int. J. Mol. Sci.* **2008**, 9, 1472–1488.
(20) McSweeney, P. L. H.; Olson, N. F.; Fox, P. F.; Healy, A.; Hojrup, P. Proteolytic Specificity of Chymosin on Bovine Alpha-S1-Casein. *J. Dairy Res.* **1993**, 60 (3), 401–412.
(21) Creamer, L. K. Some Aspects of Casein Micelle Structure. *ACS Symp. Ser.* **1991**, 454, 148–163.
(22) Gaiaschi, A.; Beretta, B.; Poiesi, C.; Conti, A.; Giuffrida, M. G.; Galli, C. L.; Restani, P. Proteolysis of alpha(s)-casein as a marker of Grana Padano cheese ripening. *J. Dairy Sci.* **2000**, 83 (12), 2733–2739.
(23) Ercolini, D.; Hill, P. J.; Dodd, C. E. R. Development of a fluorescence in situ hybridization method for cheese using a 16S rRNA probe. *J. Microbiol. Methods* **2003**, 52 (2), 267–271.
(24) Awad, R. A.; Abdel-Hamid, L. B.; El-Shabrawy, S. A.; Singh, R. K. Texture and Microstructure of Block Type Processed Cheese with Formulated Emulsifying Salt Mixtures. *Lebensm.-Wiss. -Technol.* **2002**, 35 (1), 54–61.
(25) Model, P.; Russel, M. Filamentous phage. In *The Bacteriophages*, 2nd ed.; Calendar, R., Ed.; Oxford University Press: New York, 2006; pp 147−160.
(26) Fox, P. F.; McSweeney, P. L. H. Chapter 4 Milk Proteins. In *Dairy Chemistry and Biochemistry*; Fox, P. F.; McSweeney, P. L. H., Eds.; Springer: 1998; pp 146−238.
(27) Christensen, T. M. I. E.; Kristiansen, K. R.; Werner, H. Casein Hydrolysis in Cheeses Manufactured Traditionally and by Ultrafiltration Technique. *Milchwissenschaft* **1991**, 46 (5), 279–283.
(28) Albillos, S. M.; Busto, M. D.; Perez-Mateos, M.; Ortega, N. Chemometrical analysis of capillary electrophoresis casein fractions for predicting ripening times of milk mixture cheese. *J. Agric. Food Chem.* **2005**, 53 (15), 6094–6099.

**Received March 27, 2009. Revised manuscript received September 21, 2009. Accepted September 23, 2009. This work was supported by research grants from Danisco A/S and LMC (Centre for Advanced Food Studies).**