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Hindring af uønsket proteolyse i langtidsholdbare mejeri-produkter og -ingredienser ved enzyminaktivering

Mejeribrugets
ForskningsFond

NOVEMBER 2015

Final report
for projects funded by MFF

1. Title of Project

Prevention of unwanted proteolysis in long shelf life dairy products and dairy ingredients by inactivation of enzymes.

2. Project manager

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4. Project period

1.2.2012 - 31.3.2015

5. Danish project summary

Projektet omhandler, hvordan et essentielt problem indenfor UHT-behandlede mejeriprodukter, nemlig forekomsten af uønsket proteolyse i forskellige typer af langtidsholdbare flydende eller tørrede produkter og ingredienser kan løses. Ændringerne under lagring og videre anvendelse medfører jævnligt problemer med produkternes funktionelle egenskaber i løbet af holdbarhedsperioden. Årsagerne hertil undersøges i dette projekt gennem karakterisering af de proteolytiske processer og identifikation af de forventede ansvarlige enzymer, for derigennem at kunne foreslå og implementere procestiltag til at forebygge disse uønskede ændringer i produkterne efter deres fremstilling.

Det er vist, at ændring i varmebehandling fra 95ºC i 180 s til 120ºC i 20 s kan forhindre dannelsen af uønskede, uopløselige aggregater i disse drikke. **Vi havde fra starten hypotesen, at en mulig forklaring kunne være, at bovine, bakterielle eller procestilsatte proteaser**

kunne forårsage begrænset proteolyse ved den lavere varmebehandling, som kunne resultere i eksponering af fx hydrofobe områder i proteinerne og medføre aggregering. Dette kunne endvidere føres videre til at foreslå, at sådanne eventuelt ansvarlige proteaser så blev inhiberet/inaktiveret ved den højere varmebehandling, og dermed at kvalitetsforringelsen ikke observeredes ved den temperatur. Dette viste sig imidlertid at være nærmest modsat. I stedet blev der påvist **en øget hydrolyse af både aggregater og opløselige valleproteiner ved den højere varmebehandling, som skyldtes svag syrehydrolyse pga det sure pH kombineret med varmepåvirkningen.** Aggregaterne blev påvist at være beriget med CMP, samt indeholde øvrige valleproteiner også. Da sur hydrolyse især sker C- og N-terminalt til Asp og Glu vil både **deamidering og genetiske varianter påvirke hydrolysegraden** og dermed kvaliteten. Der blev endvidere observeret yderligere molekylære ændringer i valleproteinerne som funktion af varmebehandling, inkl. laktosylering, proteolyse, samt tab af vand. Eftersom aggregaterne er beriget med CMP vil sur hydrolyse af dette molekyle føre til at der ikke dannes aggregater ved høj varmebehanding. Dette skyldes både øget deamidering og øget syrehydrolyse, som virker i synergi. Batchvariationer kan forklares med forskelle i CMP indhold, forskelle i indhold af de genetiske varianter af A og B formerne af CMP.

6. Project aim

The aim of the project is to identify the cause of unwanted protein degradation, which is hypothesized to be caused by enzymes in liquid and powder based UHT products and to come up with solutions on how to implement gentle processing steps to avoid this degradation.

Goals
1. To study the effect of proteolysis on the functionality of UHT (ultra high temperature) treated dairy products.
2. To clarify the cause of proteolysis in these products.
3. To identify which process steps at the dairy may counteract unwanted proteolysis of UHT products.

7. Sub-activities in the entire project period

Fase 1: 1.2.2012-30.09-2013. Karakterisering af enzymatisk betingede ændringer i langtidsholdbare tørrede og flydende mejeriprodukter. Målet er at karakterisere effekten af proteolyse på funktionaliteten af UHT-behandlede mejeriprodukter, ansvarlige enzymer for uønskede funktionelle ændringer (d.v.s. viskositetsændringer, visuelle forandringer, m.m.) i langtidsholdbare tørrede eller flydende produkter, samt generere ”peptidomics-maps” på molekylært niveau, som kan anvendes i studier af mønster genkendelse i fase 2. Peptid fraktionen i udvalgte produkter analyseres vha. 2D geler og LC-MS. Produkternes fysiske egenskaber måles ved reologi og teksturmålinger samt visuelle scoringer.

Fase 2: 1.10.2013-30.05.2014: Sammenholdelse af data vha. multivariat statistik. Hypoteser om proteaser. Modelforsøg/aktivitetsassays til karakterisering af enzymatisk betingede ændringer i langtidsholdbare produkter). Målet er at diagnosticere årsagen til proteolysen i problemprodukter.

Fase 3: 1.6.2014-31.1.2015. Integration af resultater, løsningsforslag og slutpublicering. Målet er at integrere resultaterne fra fase 1 og 2 for derigennem at pege på parametre i produktionen af langtidsholdbare UHT-produkter og derigennem komme med endelige løsningsforslag til, hvordan mere ensartet kvalitet og lang holdbarhed kan opnås. Forslag til - og pilotforsøg - med implementering af resultaterne i praksis. Udarbejdelse af afhandling, samt at fremstille specialproduktioner af udvalgte produkter.

8. Project results

Approaches

Observations from the factory records showed that a small change in thermal treatments of WPI drinks (from 95°C for 180s to 120°C for 20s) could prevent the formation of visible aggregates. Thus, four different quality batches of WPI drinks heat-treated at 95°C for 180s or 120°C for 20s were stored for 6 months at 20°C in the dark. The WPI powders were solubilized at 7 % w/v in water, and pH adjusted to pH 3 prior to heat treatments.

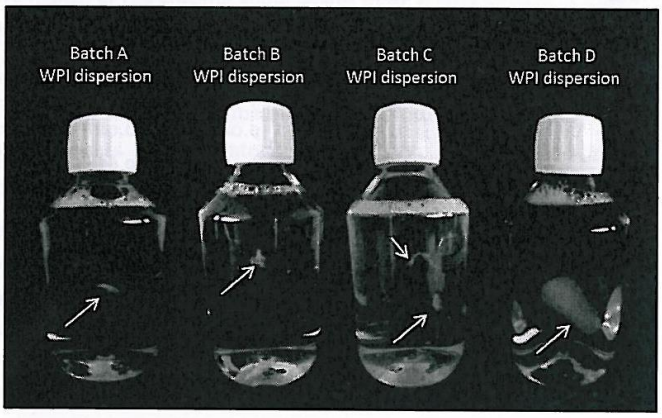

Fig. 1. Picture of the different batches used for the storage experiments. Aggregate ranking is A<B<C<D according to visual scoring, with D having most extensive visible aggregates. All dispersions were heat treated at 95ºC for 180 s, and stored at 20ºC for 3 months.

The developed aggregates and the aqueous phases were analyzed by viscosity and rheology, as well as by 1-D and 2-D electrophoresis (2-DE) and by LC-MS single Q. Selected gel spots were excised and characterized by MALDI MS and an ion trap LC-ESI/MS/MS. Initial investigations furthermore aimed at microscopic investigations of the insoluble aggregates formed during storage, especially in the batches with the biggest problems.

Results

1. *Microbiological investigations and CLSM*

Even though the problem with the insoluble aggregates was not considered to be caused by microorganisms, it was early in the project anyhow tested for presence of microorganisms. The possibility existed that proteolytic enzymes secreted from microorganisms could cause some quality problems, furthermore, presence of microorganisms could cause haze formation, and cause presence of exopolysaccharides. The microbiological analyses were carried out by an external laboratory, ISI food protection (ISI Food Protection, 2013). The results were supplemented with microbiological analyses at Department of Animal Science, Aarhus University, by Ole Højberg.

Both investigations concluded that **viable microorganisms were not present in the liquid drinks,** and therefore not the cause of the problem. By confocal laser scanning microscopy (CLSM) is was examined whether the insoluble aggregates contained exopolysaccharides (EPS), as even though viable microorganisms were concluded not to be the cause of the haze formation some visual similarity exists with EPS. Based on the CLSM images, the ConA probe used for labelling showed no detectable differences in the signal intensity between samples heated at 95ºC for 180 s and the samples heated at 120ºC for 20 s of batch D. Moreover, there were no differences between the clear liquid samples stored for 1 day and samples

stored for 168 days at 20ºC. This indicate that potential EPS in the sample did not develop during storage. The investigations indicated that the ConA labelling was more intense in the insoluble aggregate compared with surrounding liquid of batch D. Therefore, it is a possibility that EPS, when present prior to heat treatment, can be entrapped in the protein network of the insoluble aggregates during storage.

2. Rheological studies

Turbidity (Figure 2) and rheology analysis (obtaining storage and loss moduli G' and G', respectively') of two of the batches of redispersed WPI (A and D) were carried out to obtain measurable quantification and understanding of the changes occurring (reported in Villumsen et al., 2015a) . It was shown that the turbidity indreased markedly in batch D at storage at 20ºC of redispersed WPI heat treated at 95/180, but not of the same sample at 120/20. This clearly shows that the higher heat treatment can prevent the haze formation resulting in increased turbidity.

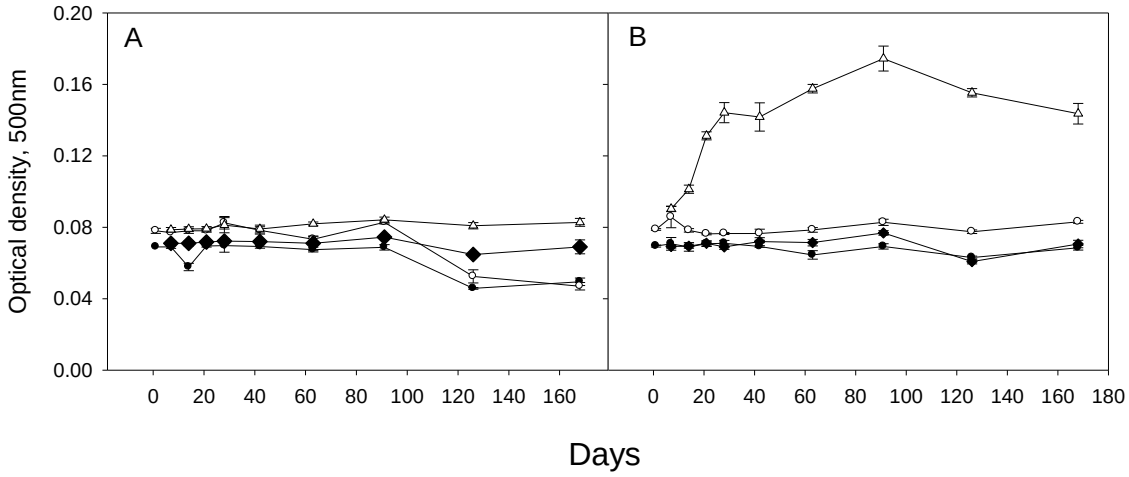

Figure 2: *Time dependent changes in turbidity (optical density at 500nm) in the WPI dispersions subjected to combinations of heat treatments (120 °C/20 s or 95 °C/180s) and storage temperatures (4 °C or 20 °C).* ***A)*** *WPI sample A* ***B)*** *WPI sample D. Black circle (●):heat treatment:120°C/20s, storage T: 4°C. Black diamond (♦): heat treatment 120°C/20s, storage T: 20°C. Open circle(○): heat treatment 95°C/180s, storage T 4°C. Open triangle (∆): heat treatment 95°C/180s, storage T: 20°C. Error bars indicate standard deviations. The samples were homogenized prior to analysis. N=6.*

3. Proteomic investigations of surrounding liquids

Batch A and D were further investigated by compositional analyses by LC-ESI/MS single Q analysis and by 1- and 2D gel electrophoresis (Villumsen et al., 2015b). By these analyses caseinomacropeptide (CMP) was found to be enriched in the visible aggregates compared to the surrounding clear liquid (Villumsen et al., 2015b). Disruption of potential electrostatic interactions between glycosylated and non-glycosylated CMPs were studied by addition of Ca^{++}, acidification and enzymatic treatment by recombinant *Bifidobacterium* sialidase. These different treatment strategies were shown to be able to decrease the time-dependent turbidity developments in acidic WPI dispersions. This suggested that the formation of storage-induced insoluble aggregates may be prevented by the disruption of electrostatic interactions, which was suggested to occur between negative sialic acid residues and positively charged amino acid residues on neighbouring proteins or polypeptides.

2-DE was used to study further the effects of heat-treatment and storage on WPI drinks. The four WPI batches (A, B, C and D) were analysed by 2-DE under reducing conditions. A representative picture of one of the batches, A, is shown in Figure 2. Due to the presence of CMP, which has a pI ranging from 3.15 to 4.15, the samples were focused using IPG strips (pH 3-6). Three distinctive changes of proteins in WPI drinks were resolved on 2-DE gels.

First, changes due to proteolysis appeared as the whey proteins, both α-La and β-Lg, were greatly hydrolysed after storage at both investigated heating methods. Figure 3 reveals the occurrence of two horizontal trains of gel spots below the positions of both intact α-La and β-Lg (region indicated as arrows in 95 and 120ºC samples). Moreover, CMP was hydrolysed in these stored samples, in which a reduction in genetic variant A (verified by MS) spots can be seen (Figure 3).

Secondly, the whey proteins in WPI drinks were deamidated (verified by MS) during storage as evidenced by more acidic spots on 2-DE. The conversion of asparagine (Asn) into asparatic acid (Asp) or glutamine (Gln) into glutamic acid (Glu) introduces additional negative charges of proteins resulting in gel spots of α-La and β-Lg shifted to the left on 2-DE (Figure 3).

Thirdly, minor proteins such as BSA were not found on 2-DE gels of liquid phase of heat-treated samples. Multiple spots varied in pI from 3 to 4.5, Mw about 20 kDa (Box A, Figure 3), as well as those (pI ranged from 4.5 to 5) with Mw approximately 30-35kDa (Box B, Figure 3) were almost vanished in all four batches after heat treatment and storage, as shown for batch A in Figure 2. It could be concluded that thermal processing and storage reduced the amount of intact minor proteins in WPI samples, which could be either due to both cross-linking and/or proteolysis. Interesting bands/spots were excised, trypsin or pepsin digested and analysed by LC-ESI-MS/MS in order to identify the presence of proteins and their modifications on gels, and the MS/MS data are reported in Le et al. (2015).

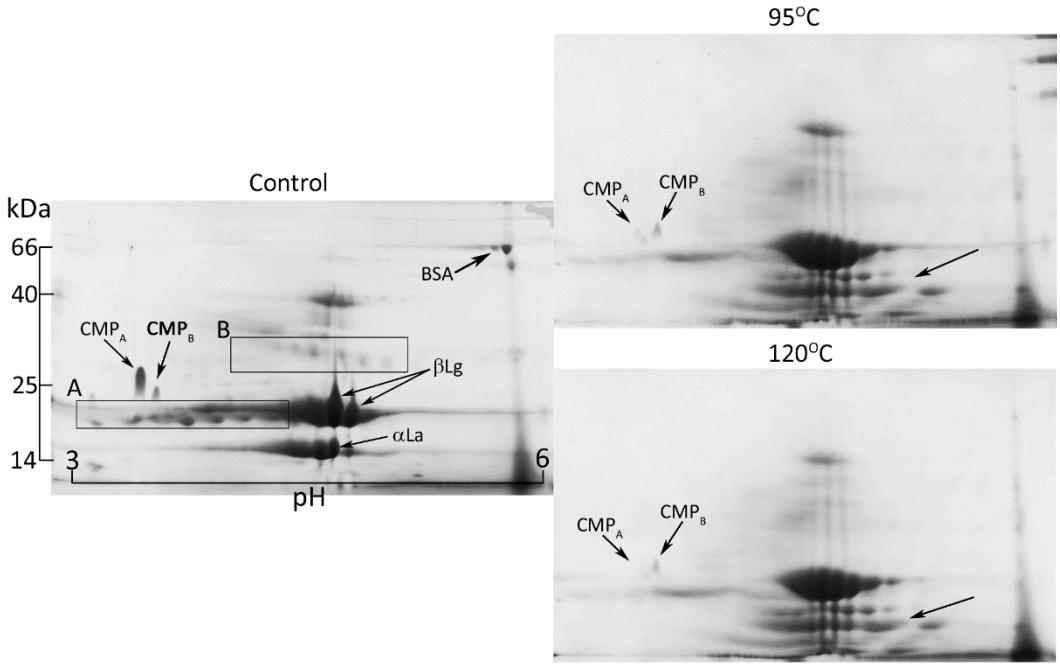

Figure 3. Figure 2. Reducing 2-DE of liquid phase of batch A. The liquid phase of batch A was diluted in lysis buffer, electrophoresed (pH 3-6) and separated on 2-DE. Major changes are boxed: (A) multiple spots with pH ranged from 3 to 4.5, Mw approximately 20 kDa; (B) multiple spots with pH 4.5 to 5, Mw approximately 30-35 kD. Arrows represent noticeable changes in α-La and β-Lg regions in heat-treated samples compared to the control sample.

4. Proteomic analysis of insoluble aggregates

CMP self-assembly as a cause of aggregation varied between batches was proposed by Villumsen *et al*. (2015). Croguennec *et al.* (2014) also suggest the active contribution of CMP into β-Lg forming large aggregates in a model study. Thus, it could be believed that the involvement of other whey proteins (β-Lg, and possibly also α-La) as well in the CMP enriched aggregates.

The protein compositions of WPI aggregates were analysed by 2-DE, LC-MS and MS/MS. Figure 3 shows a comparison between WPI aggregates and the liquid phases from batch D of two different heat treatment conditions (120ºC for 20s and 95ºC for 180s).

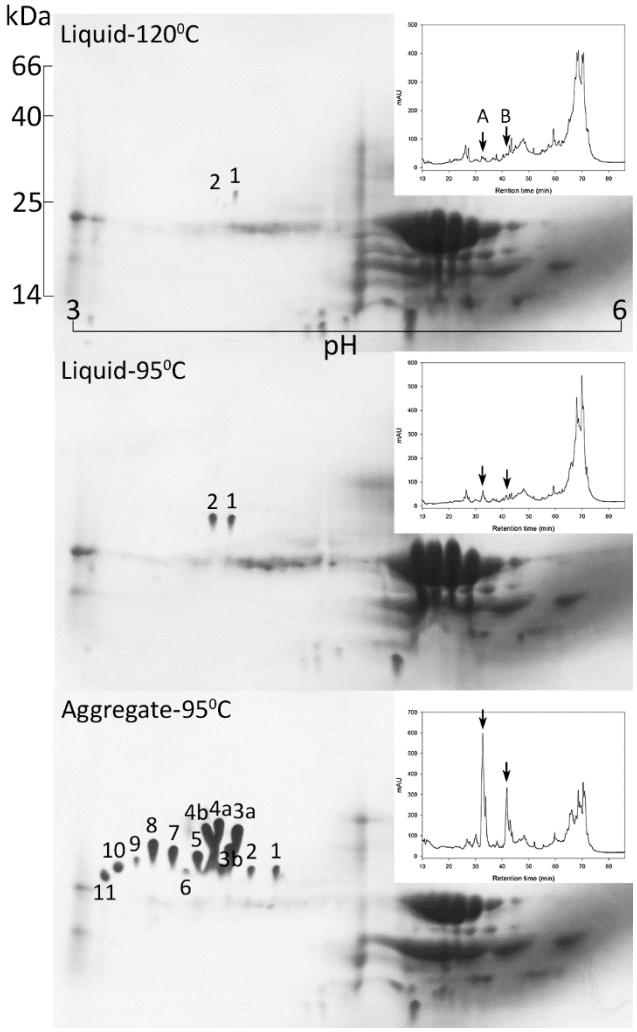

Figure 4. Reducing 2-DE and LC-MS results of aqueous and aggregate phases of batch D heat-treated at 120°C for 20s and 95°C for 180s stored at 20ºC for 12 months. Different glycosylated and phosphorylated forms of CMP_A and CMP_B were labelled from 1 to 11. Arrows show changes in mono-phosphorylated CMP_A (A) and CMP_B (B) between the liquid phases and the aggregates in LC chromatogram.

Figure 4 shows that different forms of CMP (labelled as 1, 2, 3a, 3b, 4a, 4b, 5, 6, 7, 8, 9, 10, and 11) were accumulated in the storage induced aggregates. Only mono-phosphorylated

CMP of both genetic variants A and B were detected in surrounding liquids by LC-MS and 2-DE. This could be because heat-treatment and storage causes protein degradation leading to a decrease in concentration (below the limit of detection by 2-DE and LC-MS) of other glycosylated forms of CMP. From the pI values, the LC-MS and MS/MS results, phosphorylated and glycosylated forms of CMP_A and CMP_B were detected. The singly and doubly phosphorylated forms of CMP_A and CMP_B were the most dominant forms in which CMP_A was more susceptible to hydrolysis (Figure 4). The reason for that is due to the presence of Asp in the replacement to Ala in CMP_A, which provides an extra site for proteolytic cleavage by weak acid hydrolysis. In all WPI batches (A, B, C and D), 2-DE and LC-MS results showed that CMP in 120ºC heat-treated products was more prone to degradation during storage (results not shown). This leads to a reduction in the concentration of intact CMP.

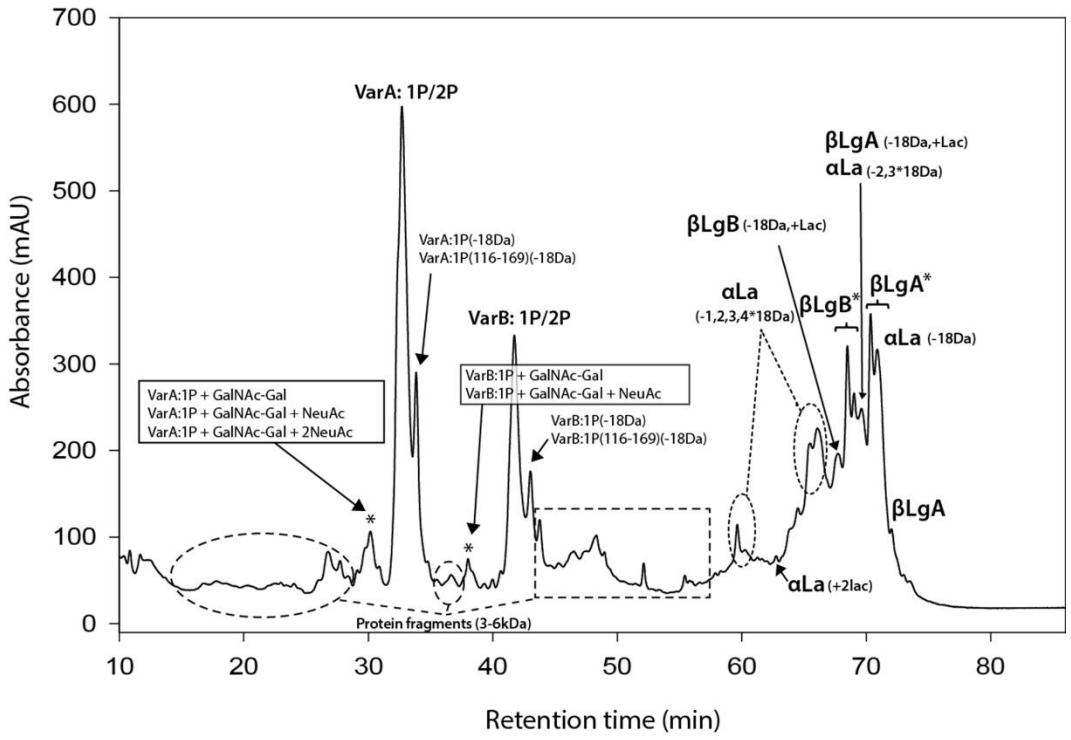

*Figure 5. LC-MS analysis of isolated aggregates from batch D. The aggregates were separated from the surrounding liquid of batch D, reduced by DTE and analysed by LC-ESI-MS. All post-translational modifications of α-La, β-Lg and CMP are labelled as P (phosphorylation), Lac (lactosylation), * (glycosylation), GalNAc (N-acetylgalactosamine), NeuAc (sialic acid), α-La* (deamidated forms of α-La), β-Lg* (deamidated forms of β-Lg), and 116-169 (CMP fragment from 116 to 169).*

An overview of protein compositions and major changes in WPI storage-induced aggregates is shown in Figure 5. The isolated aggregates analysed were composed of a mixture of major proteins, α-La, β-Lg, CMP and small protein fragments. Phosphorylated and glycosylated forms of CMP_A and CMP_B were accumulated in the storage-induced aggregates. Glycosylated CMP in the aggregates consisted of various glycans, such as N-acetylgalactosamine (GalNAc), galactose (Gal) and sialic acid (NeuAc) residues (Le et al., 2015). The chromatogram also demonstrates that the major proteins were chemically modified during processing and storage. The changes observed include proteolysis, deamidation, dehydration and lactosylation. α-La and β-Lg of WPI were largely deamidated during storage as an evidence of gel spots shifted to the more acidic region (Figure 3) as well as peaks in the LC-MS analysis split-up and slightly shifted to the more hydrophilic regions on the LC chromatogram (Figure 5). Interestingly, Figure 5 shows that dehydrated forms of both α-La, β-Lg and CMP were present in the

aggregates. The losses of water resulted in a mass reduction of 18 Da, and noticeably up to four water molecules were observed to be lost in α-La (Le et al., 2015)

5. Weak acid hydrolysis

Degree of proteolysis was analysed by the fluorescamine assays. The level of N- terminals, expressed as leucine equivalents, were quantified relatively. For all the analysed samples (4 WPI batches A-D x 2 heat treatments), protein hydrolysis continued over time throughout 163 days of storage (Figure 6). For the WPI liquid phases of the four batched A-D, the level of hydrolysis was significantly different between those solutions subjected to heat treatment at 95°C for 180 s and those subjected to heat treatment at 120°C for 20 s ($P < 0.001$). The leucine equivalent values clearly demonstrated that higher levels of N-terminals were produced after heat treatment at 120°C for 20s than after 95°C for 180s for the same WPI batch solution. It is worth noting that the initial N-terminal values correlated positively with ranking of the WPI batches on the product qualities regarding the amount of aggregates (A<B<C<D).

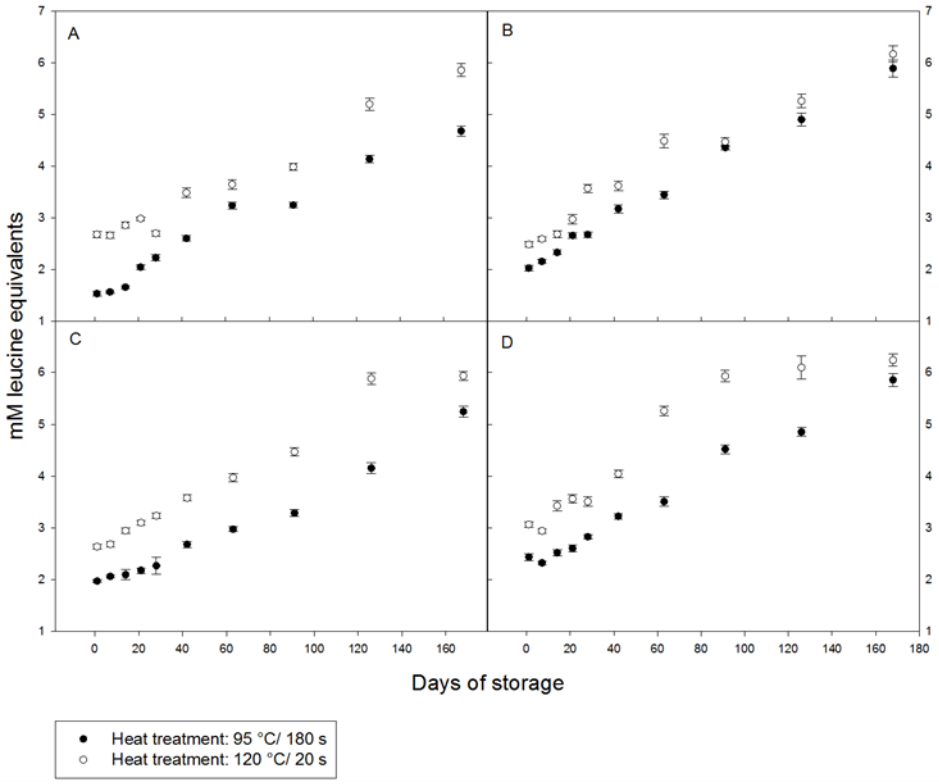

Figure 6. Free N-terminal measurements of four WPI batches by fluorescamine assay: A, B, C and D, with D representing the batch with the highest level of aggregate formation after heat treatment at 95/180. (●) Samples subjected to heat treatment of 95 °C for 180 s. (○) samples subjected to heat treatment of 120 °C for 20 s. Error bars indicate standard deviations (n=3).

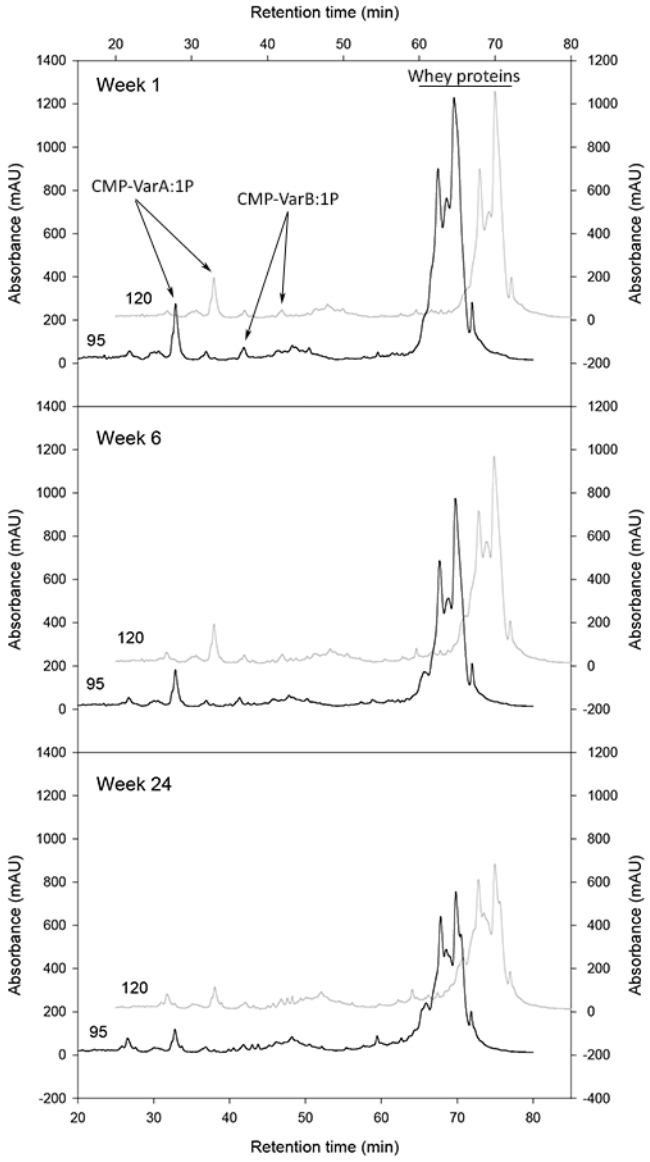

Figure 7. LC-MS analysis of batch D liquid. WPI drinks were heat-treated at 95ºC for 180s (black line) and 120ºC for 20s (grey line), and stored for 1, 6 and 24 weeks. The samples were reduced by DTE and analysed by LC-ESI-MS. Whey proteins and variants A and B of CMP with one phosphate attachment are presented in the chromatogram.

The protein profile of batch D drinks (liquids) were obtained by LC-MS. Figure 7 shows mono-phosphorylated CMP_A and CMP_B as well as whey proteins. Protein degradation was evident already after 1 week of storage at both heat-treated conditions. The amount of intact CMP and whey proteins decreased rapidly over storage time in which higher proteolysis occurred in WPI liquids heat-treated at 120ºC for 20s.This confirms that CMP were fragmented more in 120^0C heat-treated batches than 95^0C's. Thus, the concentration of intact CMP in 120^0C heat-treated batches could be lower than in 95^0C's.

Table 1 represents the list of hydrolysed peptides from CMP of the batch A (Le et al., 2015). The cleavage of CMP mainly occurs at N- and C-terminals of Asp (D) or Glu (E) (Table 1). These amino acids act as nucleophiles attacking the carbon in the carbonyl groups of the protein backbone, resulting in the formation of intermediate five or six membered ring structures. This leads to the cleavage at the N- or C-terminus of Asp or Glu at respectively. There are 38 peptides shown in Table 1, in which most of the peptides were generated from the cleavage of Asp or Glu at both terminals.

Table 1. Identification of peptides originated from CMP present in the liquid phase of batch D. PTMed peptides are labeled as p (phosphorylation), d (deamidation), (d) (potential sites for deamidation).

m/z	Mass	Residues	Sequence
572.00	1141.99	106 - 115	MAIPPKKN**d**QD
458.96	915.91	116 - 123	KTEIPTIN**d**
510.76	1019.50	116 - 124	KTEIPTIN**d**T
601.17	1200.33	116 - 126	KTEIPTINTIA
673.29	1344.57	116 - 128	KTEIPTINTIASG
777.77	1553.53	116 - 129	KTEIPTINTIAS**p**GE
837.12	1672.22	116 - 131	KTEIPTIN**d**TIASGEPT
880.70	1759.39	116 - 132	KTEIPTINTIASGEPTS
863.80	2588.37	116 - 140	KTEIPTINTIASGEPTSTPTTEAVE
1054.69	3161.06	116 - 146	KTEIPTINTIASGEPTSTPTTEAVESTVATL
1097.71	3290.10	116 - 147	KTEIPTINTIASGEPTSTPTTEAVESTVATLE
1136.16	3405.46	116 - 148	KTEIPTIN**(d)**TIASGEPTSTPTTEAVESTVATLED
837.13	2508.37	124 - 148	TIASGEPTSTPTTEAVESTVATLED
803.48	2407.41	125 - 148	IASGEPTSTPTTEAVESTVATLED
1112.30	2222.59	127 - 148	SGEPTSTPTTEAVESTVATLED
1040.24	2078.47	129 - 148	EPTSTPTTEAVESTVATLED
859.34	1716.665	130 - 146	PTSTPTIEAVESTVATL
918.23	1834.44	130 - 147	PTSTPTTEAVESTVATLE
650.90	1949.69	130 - 148	PTSTPTTEAVESTVATLED
833.23	1664.45	133 - 148	TPTTEAVESTVATLED
568.10	1134.18	138 - 148	AVESTVATLED
418.40	834.78	141 - 148	STVATLED
696.69	1391.37	149 - 160	S**p**PEVIESPPEIN**d**
746.76	1491.50	149 - 161	S**p**PEVIESPPEINT
960.34	1918.67	149 - 165	SpPEVIESPPEINTVQVT
703.60	2107.79	149 - 167	SpPEVIESPPEIN**(d)**TVQ**(d)**VTST
1100.41	2198.80	149 - 169	SPEVIESPPEIN**(d)**TVQ**(d)**VTSTAV
761.44	2281.31	149 - 169	S**p**PEVIESPPEIN**d**TVQVTSTAV
564.59	1127.17	151 - 160	EVIESPPEIN**d**
614.68	1227.34	151 - 161	EVIESPPEINT
922.37	1842.72	151 - 167	EVIESPPEINTVQVTST
1007.80	2013.58	151 - 169	EVIESPPEINTVQVTSTAV
943.31	1884.60	152 - 169	VIESPPEINTVQVTSTAV
772.31	1542.61	155 - 169	SPPEINTVQVTSTAV
729.13	1456.24	156 - 169	PPEINTVQVTSTAV
567.15	1132.28	159 - 169	INTVQVTSTAV
453.46	904.91	161 - 169	TVQVTSTAV
403.36	804.70	162 - 169	VQ**(d)**VTSTAV

In conclusion, different proteomics methods were used to characterize protein compositions and chemical modifications occurring in visually storage-induced aggregates and liquid phases of WPI beverages. Weak acid hydrolysis of CMP, and other whey proteins, was found as a main cause of the differences in the development of storage-induced aggregates between low- and high-heat treated WPI drinks. Deamidation could be a boost-factor for the hydrolysis reaction or an increase in negative charges preventing intermolecular aggregation. The concentration of intact CMP is likely to have an effect on the aggregation process of whey proteins or on the size of the aggregates. Moreover, the genetic variants of CMP also contributed to more peptide hydrolysis; thus accounting for batch-to-batch variation. Other chemical reactions such as lactosylation and protein cross-linking might have an impact on WPI aggregation. Apart from applying high-temperature

heat treatment to promote weak acid hydrolysis to break down the CMP and aggregates, another approach to avoid unwanted aggregate formation at lower heat treatments could be the production of WPI with a minimised CMP content or selective removal of the least degradable variant B.

Other activities

Afslutnings- og opsamlingsmøde planlagt til 4. dec. 2015 på AU.

Deltagelse i the 9.th Nizo dairy conference on Milk protein functionality, Papendal, The Netherlands, 30. Sep.-2. Oct. 2015.

Møde hos AFI, oktober, 2014, samt dec. 2014.

Deltagelse i 11. International Symposium for Milk Genomics and Human Health. Aarhus, Denmark, Oct. 6.-8. 2014.

Deltagelse IMGC workshop, Tools and possibilities for optimized milk, Aarhus, Denmark, Oct. 9.-10. 2014

9. Deviations

9.1 Scientific

Projektet er blevet færdigarbejdet i 2015, og den seneste artikel submitted okt 2015.

9.2 Financial Ingen. Projektet er støttet gennem Mejerirationaliseringsfonden.

9.3 Timetable Ingen i forhold til tidligere slutrapport

10. Plans within the next six months

[Short presentation of each sub-activity]
The project is finished. Publicationwise, there is expected to be some editing on the paper just submitted to IDJ. Furthermore, an end-paper in Danish for Mælkeritidende is going to be written in Nov 2015 and submitted.

11. Communication and knowledge sharing about the project

[Publications, oral presentations etc.]

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