Changes during storage of lactose hydrolysed extended shelf life (ESL) milk

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The effect of lactose hydrolysis and reduction of carbohydrate content of extended shelf life milk on the proteolysis and the formation of furosine was studied during 8 weeks of storage at 8°C. The reduction of carbohydrate content was carried out by ultrafiltration. The proteolysis was distinct in all test milks as analysed by SDS-PAGE and free tyrosine equivalent content. The proteolysis was weakest in milk with a reduced carbohydrate content and hydrolysed lactose probably due to the inactivation of plasmin during the production process. Proteolytic side activities of lactase were less significant than other proteolytic activities during the storage. Furosine concentrations increased linearly in unhydrolysed, hydrolysed and carbohydrate reduced & hydrolysed milk at 0.19-0.23, 0.34-0.48 and 0.26-0.38 mg/g protein, respectively. The rate of early Maillard reaction during the ESL heat treatment and storage was clearly decreased by the reduction of carbohydrate content in lactose hydrolysed milk. The estimated lysine blockage was nutritionally insignificant in all test milks.

1. Introduction

Proteolysis of milk protein during storage can be a severe problem for the shelf life of UHT-milk (1, 2, 3, 4, 5). When raw milk quality is good and the storage temperature is low, proteolysis is normally not a problem (1). This is the case especially if the shelf life is short, as is the case with pasteurised milks. During recent years ESL milk produced by UHT technology has become popular in many countries (6). Also the consumption of lactose hydrolysed milks and milk drinks has increased (7). The use of lactase enzyme can lead to increased level of proteolysis in UHT-milks (8).

It is also well known that lactose hydrolysis enhances the Maillard reaction during heat-treatment and storage (9, 10, 11). In the beginning of the Maillard reaction lysine reacts with the reducing sugars of milk. As a consequence, the amount of nutritionally available lysine decreases (9, 10).

The aim of this study was to find out the effect of the manufacturing process of ESL lactose hydrolysed milk on proteolysis and lysine blockage during storage at 8°C for 8 weeks. Lysine blockage was followed by furosine content and proteolysis by SDS-PAGE and tyrosine equivalent analyses.

2. Materials and methods

2.1 Milk Samples

Milk used in the test batches was normal fresh milk (Valio Jyväskylä dairy, Finland), which was skimmed and pasteurised (72°C, 15 s) before further processing. Three test skim milks were produced: unhydrolysed milk (UM), hydrolysed milk (HM) and carbohydrate reduced and hydrolysed milk (CRHM). Reduction of the carbohydrate content of milk was performed according to Tossavainen and Sahlstein (12) by ultrafiltration. Ultrafiltration was carried out at 50°C and the UF-retentate was then pasteurised (80°C, 15 s). The ultrafiltration membrane used was GR61PP cut-off value 20 kDa (DSS, Denmark). The remaining lactose was hydrolysed by lactase. The minerals which were lost in the ultrafiltration were returned as milk mineral powder (Valio Ltd., Finland) and the original protein content was restored by diluting water. Lactose hydrolysis was carried out at 5-10°C for 24 h and the lactase (Godo YNL2, Godo Shusei, Japan) dosage was E/S (lactase/lactose) = 2% for both of the test milks hydrolysed.

All the test milks were heat treated in a production scale steam infusion ESL-plant (APV, Denmark). Milk was first heated to 75°C for 22 s by plate heat exchanger and then by steam infusion to 132°C. The heating and holding time was 1 second in all. In steam infusion added water was evaporated in a flash chamber and the temperature was simultaneously lowered to 74°C in 1 s. Next the milk was first homogenised and then cooled to 4°C by plate heat exchanger. Total delay-time at 74°C was 60 s followed by cooling time 30 s. Test milks were filled into 1 l carton packages and stored at 8°C for 8 weeks. The ESL heat treatment of all milks was identical.

2.2 Analytical determinations

The raw milk batches received in the dairy and used for test milks were sampled prior to processing. The test milks were sampled before and after hydrolysis and immediately after the ESL-treatment and later every 7 to 10 d for pH, furosine, reactive lysine, carbohydrates, free tyrosine equivalent and α-amino-nitrogen. pH was measured with Mettler Delta 320 pH meter (Mettler-Toledo Ltd, Halstead, UK). Reactive lysine was analysed spectrophotometrically by o-phthaldialdehyde method according to Vigo et al, (13) in 6 replicates. The lysine blockage was estimated according to the Finot principle (10, 9). Lactose, glucose and galactose were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection. The analysis was based on the method of De Slegte (14). Furosine was analysed according to IDF (15) and α-amino-nitrogen according to Lieske and Konrad (16) in 4 replicates. Free tyrosine equivalent was analysed as described by Matsubara (17) in 8 replicates. Samples from test milks for protein (18), fat (19), ash (20) and total solids (21) analysis

were taken after ESL heat-treatment. Samples for microbiological analysis were taken from raw milks and from test milks before ESL heat treatment and after 3, 5 and 8 weeks of storage. Standard plate count (22) was analysed from the samples before ESL heat treatment and raw milk. *Bacillus cereus* (23) and psychrotrophs (24) were analysed from the samples before ESL heat treatment.

SDS-PAGE analysis was carried out according to Laemmli (25) by using ready made 18% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, USA). The amount of protein added to each sample well was 10 µg. Protein bands were stained with Coomassie G-250 (GelCode Blue Stain Reagent, Pierce, USA) and compared with molecular weight markers (Prestained SDS-PAGE standards, broad range, Bio-Rad, USA). The samples for SDS-analysis were taken immediately after heat treatment and at the end of the storage period.

3. Results

3.1 Test milks

The microbiological quality of the raw milk and the test milks before the ESL heat treatment was good (Table 1). Standard plate count of the raw milk batches used for test milks was higher than in the test milks before ESL-heat treatment because the raw milk was pasteurised before further processing. During the storage period no microbes were found in the samples.

Table 1: Microbiological quality of raw milk batches and test milks (cfu/g)

	Raw milk	Before ESL-heat treatment		
	Standard plate count	Standard plate count	*Bacillus cereus*	Psychro-trophic
UM	24000	3600	0	0
HM	9000	3200	45	0
CRHM	29000	3100	20	0
Symbols: UM unhydrolysed milk, HM hydrolysed milk, CRHM carbohydrate reduced and hydrolysed milk.				

Composition of the packed test milks are presented in Table 2. UM and CRHM milks were slightly diluted with water during the production process which can be seen as a slightly lower protein content. Otherwise their compositions are similar except for their different carbohydrate content and composition. In lactose hydrolysed milks galactose and glucose contents did not change over the storage period (data not shown), but galactose content was always slightly lower than glucose content. This is likely to be related to the formation of galactooligosaccharides during the lactose hydrolysis (26). Similar results have been found earlier (27).

3.2 Changes in pH

There were no significant changes in pH during the storage in any of the test milks (Fig. 1) and no difference between the test milks were noticed.
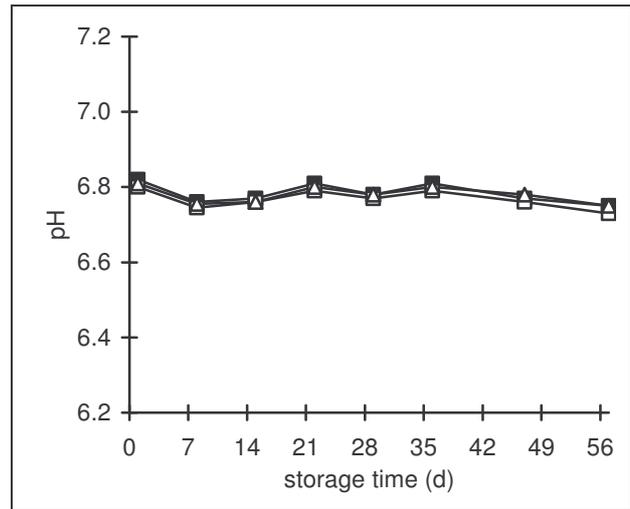

Fig. 1: pH of the test milks during the storage at 8 °C. Symbols: ■ unhydrolysed milk, □ hydrolysed milk, △ carbohydrate reduced and hydrolysed milk.

3.3 Hydrolysis of protein during storage

Proteolysis was analysed by three different methods: α-amino-N/total nitrogen, tyrosine equivalent and SDS-PAGE. There were no changes in the α-amino-N/total-nitrogen content in the test milks during storage and the test milks can not be distinguished (data not shown). In this case the α-amino-N/total-nitrogen method would not to be sensitive enough for the analysis of proteolysis.

Figure 2 shows that the amount of free tyrosine equivalent clearly increased during the follow-up time in all three test milks. The free tyrosine equivalent content was highest in HM and lowest in CRHM. The production technology might offer an explanation for the difference. In the production of CRHM the milk was ultrafiltrated during which part of the NPN-fraction was lost into the permeate. During the first two weeks of storage no proteolysis was observed but later proteolysis proceeded linearly in all test milks. There were also slight differences in the slopes of the fitted lines or in the rate of proteolysis between the test milks. Proteolysis was weakest in CRHM and strongest in HM.

The results from SDS-PAGE analysis are shown in Fig. 3. It can be seen that proteolysis has proceeded in all test milks over 56 days. The formation of γ-casein was significant in each case which indicates the presence of plasmin, the major indigenous protease of milk (28, 29). However, the size and intensity of colour of α_{s2}- and β-casein bands decreased less in CRHM than in UM and HM. Proteolytic activity seems to have been weaker in CRHM than in UM and LM, where proteolysis was at the same level. The analysis of tyrosine equivalent content supports these results.

3.4 Formation of furosine and lysine blockage

There were no significant differences in the amount of reactive lysine between the test milks during storage (Fig. 4). However, there was a slightly increasing

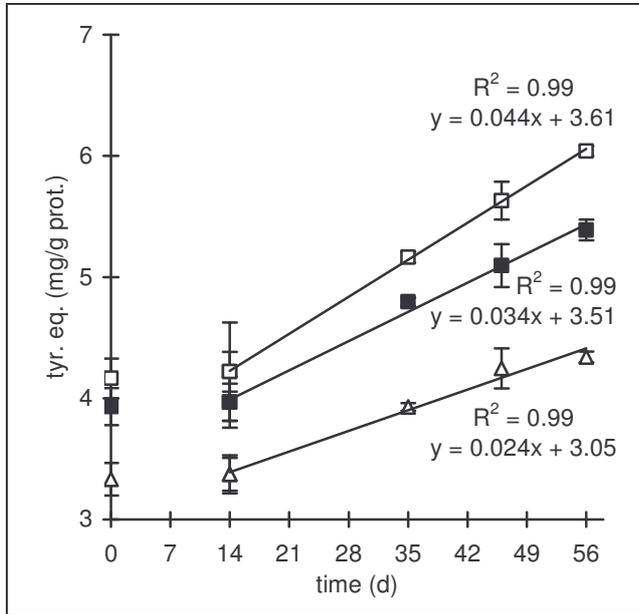| Table 2: Chemical composition of the test milks (%) - Symbols as in Table 1							
	Protein	Fat	Ash	Total solids	Lactose	Glucose	Galactose
UM	3.1	0.04	0.7	7.9	3.8	<0.1	<0.1
HM	3.1	0.05	0.7	7.9	< 0.1	2.0	2.0
CRHM	3.6	0.06	0.7	7.4	< 0.1	1.5	1.5

Fig. 2: Effect of storage time on free tyrosine equivalent in test milks. Symbols: ■ unhydrolysed milk, □ hydrolysed milk, △ carbohydrate reduced and hydrolysed milk.

if heat stable proteases of bacterial origin are present due to poor raw milk quality (4, 30, 31). In addition, industrial enzymes often contain heat stable proteolytic side activities. TOSSAVAINEN and KALLIOINEN (8) found that proteolysis can be stronger in lactose hydrolysed than in unhydrolysed UHT-milk. However, it is obvious
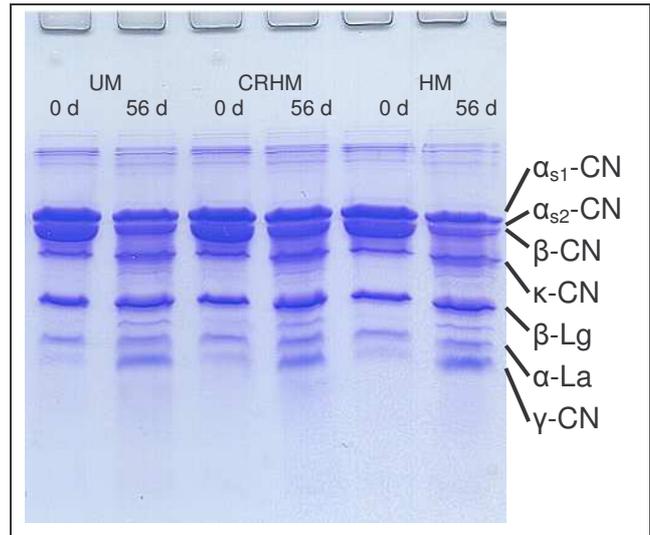

Fig. 3: SDS-PAGE analysis of test milks stored at 8°C after storage of 0 and 56 days. Symbols as in Table 1.

trend observed in all test milks. It is well known that proteolysis causes an error by increasing the result when reactive lysine is analysed by o-phthaldialdehyde-method.

Figure 5 shows the distinct differences in furosine content during storage. In all test milks the amount of furosine increased linearly. The slope of the fitted lines are for HM and CRHM, 0.0026 and 0.0021, respectively, while the slope is clearly smaller for test milk UM at 0.0006. This means that the formation of furosine is approximately 4 times faster in lactose hydrolysed milks than in normal milk and 20 % lower in CRHM than in HM.

The estimated lysine blockage varied from 0.8 to 1.7% after 56 days' storage (Table 3). Lysine blockage was practically insignificant in all test milks during the storage time.
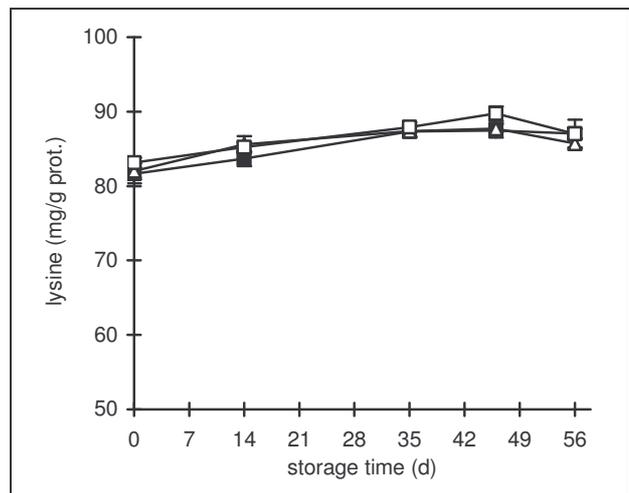

Fig. 4: Amount of reactive lysine in test milks during the storage of 56 days. Symbols: ■ unhydrolysed milk, □ hydrolysed milk, △ carbohydrate reduced and hydrolysed milk.

Table 3: Lysine blockage estimated from the furosine content during the storage time							
	Storage time (day)						
	0	7	14	21	28	35	46	56
UM	0.7	0.7	0.7	0.8	0.8	0.8	0.8	0.8
HM	1.2	1.3	1.3	1.4	1.5	1.5	1.6	1.7
CRHM	1.0	1.0	1.1	1.1	1.2	1.2	1.3	1.4

4. Discussion

The shelf life of UHT-milks is often restricted by proteolysis caused by indigenous proteases of which the most important is plasmin. The problem is more severe

$y = 0.0026x + 0.3411$
$R^2 = 0.99$

$y = 0.0021x + 0.2593$
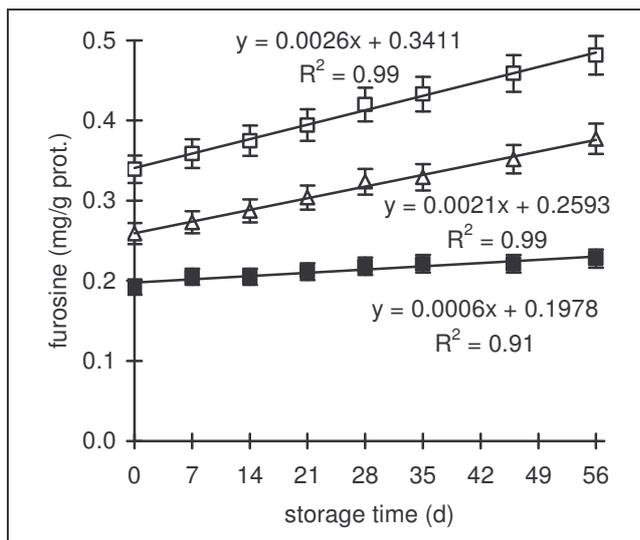$R^2 = 0.99$

$y = 0.0006x + 0.1978$
$R^2 = 0.91$

Fig. 5: Furosine content in test milks. Symbols: ■ unhydrolysed milk, □ hydrolysed milk, △ carbohydrate reduced and hydrolysed milk.

that proteolysis of milk proteins does not normally deteriorate the quality of ESL-milk due to the low storage temperature and quite short storage time.

In this study we found that in all ESL test milks proteolysis was distinct regardless of the low storage temperature (8 °C) and the fairly short storage time of 8 weeks. We also noticed that natural proteolytic activity is a more important factor than the proteolytic side activities of lactase in ESL-milks stored at 8 °C. Plasmin seems to be less active in milk with reduced carbohydrate content than in normal or lactose hydrolysed milk. A possible explanation may be the heat load in the ultrafiltration process-step. Ultrafiltration was carried out at 50 °C and UF-retentate was pasteurised at 80 °C for 15 seconds. It has been reported that plasmin activity can be affected by preheat treatment in the production of UHT-milk (32, 1). It is proposed that denatured β-lactoglobulin interacts with plasmin and plasminogen which reduced plasmin activity in milk (33) and also that denatured whey proteins are bound to the surface of the casein micelles which hinders proteolysis caused by plasmin (34).

Plasmin, plasminogen (precursor of plasmin) and plasminogen activators are associated with the casein micelles (29) in milk. The molecular mass of plasmin and plasminogen is about 88 kDa (29) and Lu and Nielsen (35) approximated molecular mass of plasminogen activators at 27 – 93 kDa. The cut-off value of used ultrafiltration membranes was 20 kDa. Then it follows that most of the potential proteolytic activity of the plasmin system in CRHM was not lost into the milk-permeate but that all the components needed for the fully active plasmin were present in the test milk.

The furosine content of the ESL-milks during storage varied from 0.2 to 0.5 mg/g protein (6-15 mg/kg) which is clearly higher than typically found in pasteurised milks but much lower than in UHT-milk (27, 8). During ESL heat treatment and storage the reaction kinetics of Maillard reaction depended on the carbohydrate content in both hydrolysed test milks. The furosine content of CRHM was 76% of HM and the carbohydrate content was 75%, respectively, after the

ESL heat treatment. During storage the formation of furosine was 20% slower in CRHM than in HM. The concentration of monosaccharides seemed to be a restrictive reactant of the Maillard reaction in hydrolysed milks. However, the rate of the Maillard reaction was very slow in the test milks during storage, and hence, also the estimated lysine blockage was insignificant in all test milks from a nutritional point of view.

5. Conclusion

Proteolysis in ESL-milks was clearly measurable regardless of chilled storage. It was weaker in milk with a reduced carbohydrate content probably due to the more considerable heat load during the production process, which is likely to reduce plasmin activity. Meanwhile the proteolytic side activities of lactase were not significant during storage. Early stages of the Maillard reaction proceeded faster in lactose hydrolysed milks than in unhydrolysed milk. The reduction of carbohydrate i.e. monosaccharide content of lactose hydrolysed milk to 3.0% reduced approximately in same ratio the rate of Maillard reaction during ESL heat treatment and storage. However, due to the chilled storage, the reaction rate is rather slow and lysine blockage is insignificant. The process for reduction of carbohydrate content combined with ESL seems a good choice for the manufacture of a stable lactose hydrolysed product like lactose free milk.

6. References

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