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A novel approach for differentiation of milk fractions and polyvinylpyrrolidone with different molecular weight by patterns derived from cupric chloride crystallization with additives

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Crystallization with additives is based on self-organization, dendritic crystal growth and subsequent pattern formation. The principle is used as an indicator, differentiating food samples according their treatments and origin. The present study focuses on methodological investigations by taking different milk fractions as well as a single polymer as examples. Both kinds of samples alter the patterns derived from cupric chloride crystallization. The changes can be evaluated by image analysis, when a structure related algorithm is applied. The crystallization with additives seems to be an innovative approach following modifications in various sample types. Potential fields of application may be authentication of samples, following processing treatments as well as other modifications in the sample structure during production.

Introduction

It is known that proteins modify crystallization patterns of inorganic materials.[1,2] Recognition of proteins by crystallization patterns has been described by applying microdroplets as the target.[3] Cupric chloride crystallization on pre-conditioned glass plates and under controlled conditions is influenced by several additives from molecules to complex biological systems in an aqueous solution.[4–8] Patterns emerge through a self-organization process.[9] The pattern formation process in this system can be described in two steps, evaporation of water and crystallization.[10] The crystal patterns are evaluated by applying an algorithm resulting in different needle length categories.[11] The images are segmented to binaries, and the binary crystal structure is analysed according to its internodal and terminal branches. Recently, this system was applied to follow different treatments of milk samples using texture image analysis.[12]

To demonstrate how a complex biological system as well as a molecule can change patterns derived from cupric chloride crystallization, we compare different protein concentrates from fresh milk and from polyvinylpyrrolidone (PVP) of two different polymerization levels as additives. We show in this paper that the patterns differ significantly between high and low molecular weight in both, milk and PVP. Low molecular additives give fewer short but more long needles compared to high molecular additives.

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Materials and methods

Reagents and samples

Copper(II) chloride dehydrate was purchased from Merck (Ref. # 1.02733.1000). Polyvinylpyrrolidone (PVP) PVP-700 (M_r 700 000 Da) and PVP-40 (M_r 40 000 Da) were obtained from Fluka (Ref. # 811440). Raw milk was obtained from the experimental station of the Max Rubner-Institut, Kiel, Germany. After skimming, the milk was subjected to microfiltration (ceramic membrane with average pore size 0.1 μm, 50 °C). The permeate of microfiltration was subsequently ultrafiltered (spiral-wound membrane with a nominal cut off of 10 kDa, 50 °C) resulting in a whey protein/casein ratio of >10.[13] Additionally, the proteins of the skim milk were increased by a factor of two by ultrafiltration (cut off 10 kDa, 50 °C). Samples of the whey protein concentrate and the milk protein concentrate were pasteurized (73.5 °C, 20 s) and filled into sterile glass bottles with six replications and sent under cooled conditions (+6 °C) to Kassel University for crystallization as well as analysis. The experiments were carried out in duplicate.

Analysis of milk characteristics

Dry matter, total protein, casein and whey protein were analysed.[14] The acid-soluble whey protein fractions α-lactalbumin (α-LA), β-lactoglobulin (β-LG), bovine serum albumin (BSA), and immunoglobulin G (IgG) were determined.[15,16]

Preparation of chamber solution

The procedure is described for milk samples[12] and for PVP.[7] For the milk experiments two sub samples were prepared as

replicates on three different days resulting in 24–28 single replications in total, when crystallizing in the 43 dishes for one chamber and day. After the glass bottles (500 mL) were shaken gently, 50 mL of the milk was transferred to a 100 mL Erlenmeyer flask and warmed up to room temperature in a water bath (30 min). 2 mL was mixed with 43 mL Milli-Q water (Millipore) and 15 mL 10% $CuCl_2$ solution ($CuCl_2 \cdot 2H_2O$, Merck 1.02733) and shaked at 100 rpm for 30 min (Heidolph Unimax 2010). 6 mL was pipetted into each dish. In order to test how the dry matter content influences the patterns, the protein concentrate was crystallized with the same amount of dry matter as the whey protein concentrate per plate, resulting in 16 additional dishes.

Crystallization

Two chambers[10,12] were used for every run in parallel (no. B and C). Experiments were performed for a medium evaporation time of 13 h at 30 °C and with 53% relative humidity at the start above the plates. For this experiment a total of 208 single plates were crystallized and scanned for image analysis. For PVP 36 single plates were used, 18 for each PVP sample. Each scanned single pattern was connected to the documented information (temperature, humidity, evaporation and crystallization process) when pattern analysis was applied. The dishes were placed circular on the crystallization unit as described previously.[7,10] All dishes received the same conditions in terms of relative humidity and temperature.[7,10] The two parameters were controlled in the outer chamber as constant.[7,10] The crystallization is not induced or further controlled.[10]

Pattern evaluation and data analysis

Pattern evaluation was performed on each single image and data analysis was performed on each experiment.[11,12] Boxplots and Principal Component Analysis (PCA) were performed in "R-cran" (http://www.R-project.org) and were plotted against the different region of interests (ROIs) of the scanned image.[6,10] ROIs are circular parts of the image around the geometric center and are calculated for 20–90% of the total image. This also reflects the crystallization process from the center to the periphery of the dish. *F*- and *p*-values are calculated by means of a linear-mixed effects mode.[17] Also *F*- and log(*p*)-values were plotted against different ROIs from 20 to 90%.

Results and discussion

Milk characteristics

According to the filtration treatments, the composition of the different milk protein fractions is significantly different (Table 1). The content of α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulin was determined after isoelectric precipitation at pH 4.6 of casein and denatured whey protein.

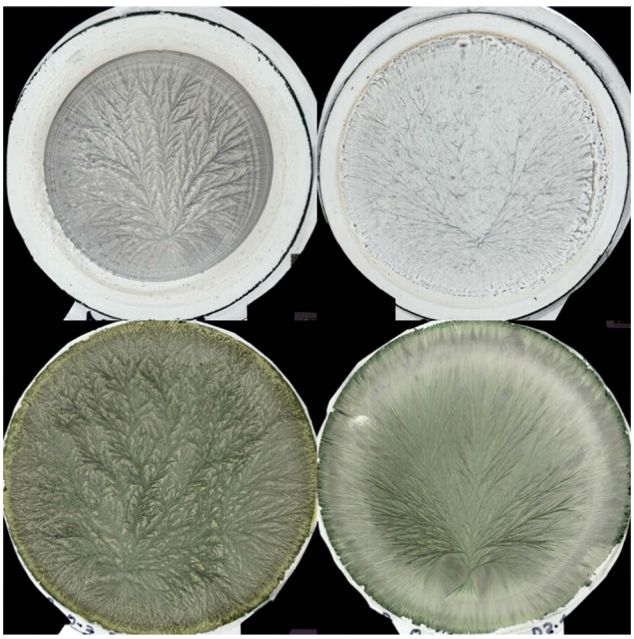

Fig. 1 Patterns from cupric chloride crystallization with above PVP-700 (left) and PVP-40 (right) and below milk protein concentrate (left) and whey protein concentrate (right) as additives.

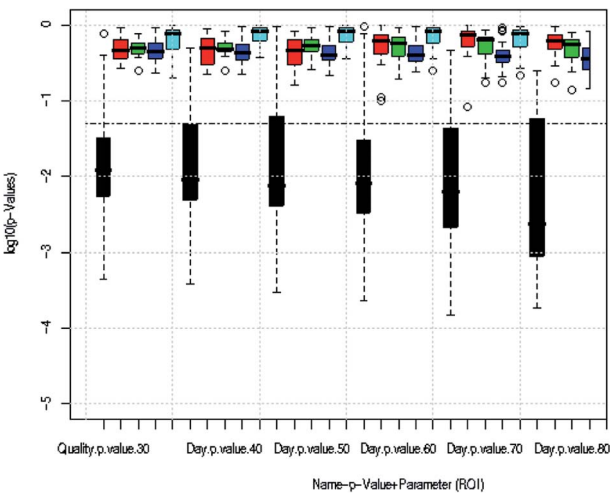

Fig. 2 *p*-value for the difference between the protein concentrate and the whey protein concentrate from skim milk as additives in cupric chloride crystallization patterns *versus* circular parts of the pattern (ROI) (black: quality; red: chamber; green: day; dark blue: quality day; light blue: chamber day).

Table 1 Dry matter and milk constituents of two different milk fractions after filtration

	DM (%)	Total protein (%)	Casein (%)	Whey protein (%)	α-La (mg per 100 mL)	β-LG (mg per 100 mL)	BSA (mg per 100 mL)	IgG (mg per 100 mL)
Protein concentrate	12.26	6.46	5.22	1.08	188.8	914.4	62.1	71.7
Whey protein concentrate	9.62	3.97	0.33	3.48	646.0	3656.9	63.0	140.7

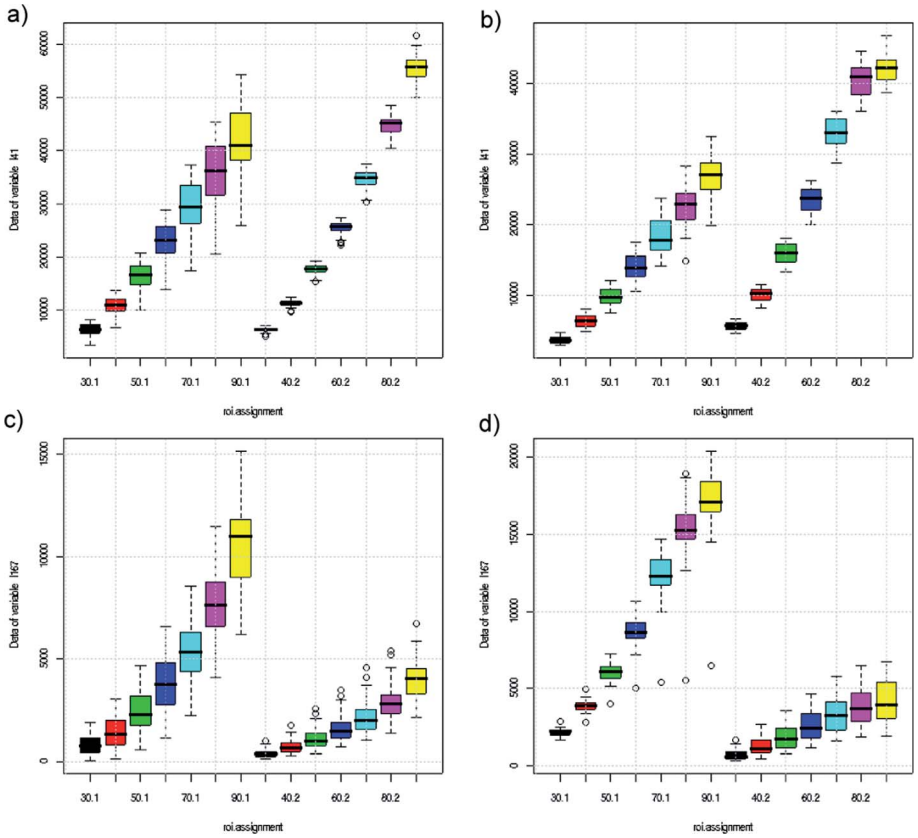

Fig. 3 Boxplots for two different length categories (above for L41 and below for L167) from the milk protein concentrate and the whey protein concentrate (a and c) and PVP 700 and PVP-40 (b and d) *versus* circular parts of the patterns (ROI). Colors represent different ROIs.

Crystallization patterns with additives

The two different protein concentrates gave different crystallization patterns (Fig. 1a below). Visually the protein concentrate gives ramifications from the center to the periphery with lots of side branches, whereas the whey protein concentrate shows longer and unbranched needle stems. When patterns derived from PVP-700 and PVP-40 are compared visually (Fig. 1b above), the high molecular PVP gave ramifications with many side branches, whereas PVP-40 shows much fewer side branches as well as longer, unbranched needles. When patterns from milk and PVP are compared, the PVP patterns are just filling about 80% of the area of milk patterns.

When the structure algorithm is applied to the patterns from the milk protein concentrate with and without reduced dry matter content as well as from the whey protein concentrate, PCA shows three groups (data not shown). Although the reduction in dry matter alters the patterns derived from the milk protein concentrate, it is still different from those derived from the whey protein concentrate. The difference between the patterns according to the calculated L-categories from the two different milk fractions is significant for each single ROI, independent from day and chamber (Fig. 2). This was similar in the repetition of the experiment on another date.

Based on the single structure variable lengths of the needles (L categories), the milk protein concentrate gives more of the shorter and fewer of the longer needles compared to the whey protein concentrate (Fig. 3a and c). This is also found when comparing PVP-700 and PVP-40. PVP-700 gave more of the shorter and fewer of the longer needles compared to PVP-40 (Fig. 3b and d).

The two milk fractions represent two different protein compositions which are different in their chemical as well as physical characteristics. They alter the crystallization patterns in different ways. The PVP samples have different molecular weights and also alter the patterns differently. The direction in which the four samples change the patterns seems comparable. The protein concentrate as well as the PVP with high molecular weight results in more shorter and fewer longer needles compared to the complementary samples. One field of future application of the cupric chloride crystallization with additives seems therefore to be the indication of changes in systems due to molecular weight.

Conclusions

Although the cupric chloride crystallization with additives may not replace existing methods, it seems to be an innovative approach to follow changes in complex and simple systems with focus on changes in molecular weight of the constituents. Potential fields of application may be authentication of samples, following processing treatments as well as other modifications in the sample structure during production.

Sample characteristics which are expressed during the self-organized crystallization process may be related to physical processes. The approach seems not to be able to detect single constituents or residues.

References

1 R. D. Deegan, O. Bakajin, T. F. Dupont, G. Huber, S. R. Nagel and T. A. Witten, *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.*, 2000, **62**, 756–765.
2 T. L. Yakhno, V. G. Yakhno, A. G. Sanin, O. A. Sanina and A. S. Pelyushenko, *Tech. Phys.*, 2004, **49**, 1055–1063.
3 V. N. Morozov, N. N. Vsevolodov, A. Elliott and C. Bailey, *Anal. Chem.*, 2006, **78**, 258–264.
4 J. P. Gallinet and B. Gauthier-Manuel, *J. Colloid Interface Sci.*, 1991, **148**, 155–159.
5 T. Shibata, S. Matsumotoa, M. Kogureb, T. Iguchic, A. Tanakad, T. Naganoa and T. Ogawae, *J. Cryst. Growth*, 2000, **219**, 423–433.
6 M. Szulc, J. Kahl, N. Busscher, G. Mergardt, P. Doesburg and A. Ploeger, *Comput. Electron. Agr.*, 2010, **74**, 218–222.
7 N. Busscher, J. Kahl, J.-O. Andersen, M. Huber, G. Mergardt, P. Doesburg, M. Paulsen and A. Ploeger, *Biol. Agric. Hortic.*, 2010, **27**, 1–23.
8 K. Seidel, J. Kahl, F. Paoletti, I. Birlouez, N. Busscher, U. Kretzschmar, M. Särkkä-Tirkkonen, R. Seljasen, F. Sinesio, T. Torp and I. Baiamonte, *J. Food Sci. Technol.*, 2013, DOI: 10.1007/s13197-013-1109-5.
9 M. O. Kokornaczyk, G. Dinelli and L. Betti, *Naturwissenschaften*, 2013, **100**, 111–115.
10 N. Busscher, J. Kahl, P. Doesburg, G. Mergardt and A. Ploeger, *J. Colloid Interface Sci.*, 2010a, **344**, 556–562.
11 P. Doesburg and A. F. M. Nierop, *Comput. Electron. Agr.*, 2013, **90**, 63–67.
12 J. Kahl, N. Busscher, P. Doesburg, G. Mergardt, M. Huber and A. Ploeger, *Eur. Food Res. Technol.*, 2009, **229**, 175–178.
13 W. Hoffmann, N. Johannsen and D. Ströbel, *Kiel. Milchwirtsch. Forschungsber.*, 2006, **58**, 41–51.
14 VDLUFA, *Methodenbuch Band VI: Chemische, Physikalische und Mikrobiologische Untersuchungsverfahren für Milch, Milchprodukte und Molkereihilfsstoffe*, VDLUFA-Verlag, Bonn, Germany, 2003.
15 German Institute for Standardization, *DIN 10473: Determination of acid-soluble β-lactoglobulin in heated milk*, Beuth-Verlag, Berlin, Germany, 1997.
16 I. Clawin-Rädecker, C. Kiesner and E. Schlimme, *Kiel. Milchwirtsch. Forschungsber.*, 2000, **52**, 323–334.
17 A. Meelursarn, PhD thesis, University of Witzenhausen, 2007, http://kobra.bibliothek.uni-kassel.de/handle/urn:nbn:de:hebis34-2007041017616.

