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New insight on crystal and spot development in hard and extra-hard cheeses: Association of spots with incomplete aggregation of curd granules

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ABSTRACT

Chemical composition and structure of different types of macroparticles (specks, spots) and microparticles (microcrystals) present in hard and extra-hard cheeses were investigated. Light microscopy revealed that the small hard specks had the structure of crystalline tyrosine, as confirmed by amino acid analysis. Spots showed a complex structure, including several curd granules, cavities, and microcrystals, and were delimited by a dense protein layer. Spots contained less moisture and ash than the adjacent cheese area, and more protein, including significantly higher contents of valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine. Microcrystals were observed by light and electron microscopy and analyzed by confocal micro-Raman. Among others, calcium phosphate crystals appeared to consist of a central star-shaped structure immersed in a matrix of free fatty acids plus leucine and phenylalanine in free form or in small peptides. A hypothetical mechanism for the formation of these structures has been formulated.
Key words: hard and extra hard cheeses, cheese ultrastructure, calcium phosphate crystal, free amino acids, electron microscopy

INTRODUCTION

Hard and extra-hard are attributes used to define cheeses having a firm and brittle body texture (Codex Alimentarius, 1978). Hard and extra-hard cheeses share low moisture content, close structure, and a long ripening period. During ripening, many chemical, biochemical, and microbiological phenomena take place. The biochemical changes are very important for the development of the flavor and texture of these cheeses and are characteristic of the different varieties. Proteolysis is the most relevant among the biochemical phenomena because of its complexity and final effect on the cheese taste. In fact, casein breakdown progressively produces large and medium peptides, followed by small peptides and free amino acids (**FAA**). Because FAA are rather stable, they tend to accumulate with the ripening time and may reach up to 20 to 24% on a cheese protein basis in 10- to 12-mo-old extra-hard cheeses (Masotti et al., 2010). Proteinases and peptidases that catalyze proteolysis in cheese originate from different sources, namely milk, rennet, and starter and nonstarter lactic acid bacteria (**LAB**). The LAB have complex enzyme patterns that release peptides and amino acids from the proteins into the cheese environment to satisfy their own nutritional requirements (Gatti et al., 2014). After vat processing, the loss of water, diffusion of salt, and formation of soluble molecules, such as FAA and lactate, are factors contributing to the increase of solute concentration and concomitant decrease of water activity in cheese throughout the whole ripening period. Beside these main events, minor changes, such as changes in water binding by new carboxylic and amino groups formed upon protein hydrolysis (McSweeney, 2004), contribute to lowering the water activity in cheese. The moisture content and water activity are strongly correlated in cheese throughout ripening (Marcos, 1993).

The increasing solute (salt, ions, FAA) concentration in the cheese water phase may give rise to aggregation and crystallization phenomena that result in the different types of structures in the interior and on the surface of different cheese varieties observed by some authors (Bianchi et al., 1974; Agarwal et al., 2006a; Tansman et al., 2015). Although the earliest studies date back to the 1900s (Babcock et al., 1903; Tuckey et al., 1938), a clear and unambiguous characterization of these structures has not yet been achieved. Moreover, a univocal association between the terminology (e.g., crystals, specks, dots, granules, spots, pearls) and the appearance of these structures is still lacking.

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In long-ripened extra-hard cheeses with a thick, dry rind, these structures develop inside the cheese and become visible when the wheel is cut. Typically, 2 different types of structures visible to the naked eye can be observed these structures are referred to as specks and spots in this article. Specks look bright white and firmer against the cheese matrix, and these are usually smaller than 2 to 3 mm. Consumers (cheese lovers) appreciate the crispness of the specks when they chew them and their contribution to the overall cheese taste. Previous studies about specks reported that they contain clusters of tyrosine, cysteine, other FAA, calcium lactate, and magnesium (Shock et al. 1948) or tyrosine and phenylalanine (Giolitti and Mascherpa, 1970). More recently, Bottazzi et al. (1994) and Tansman et al. (2015) identified the specks in extra-hard cheeses as tyrosine crystals.

Spots are spherical and paler than the cheese and can be as large as 4 to 5 mm. They appear to be amorphous and firmer with respect to the surrounding cheese matrix, and they become visible after 10 to 12 mo of ripening. Spots can become so numerous and conspicuous that they may influence the visual appeal of the cheese. Spots have been very little studied, and results have been inconsistent (Giolitti and Mascherpa, 1970; Bianchi et al., 1974; Tansman et al., 2015).

Besides specks and spots, extra-hard cheeses contain microscopic crystals, which have mostly been investigated in Cheddar cheese. However, some authors generically referred to crystals, without distinguishing between those that are microscopic and those that are visible to the naked eye (Bottazzi et al., 1982, 1994; Washam et al., 1985; Kaláb et al., 1987).

The aim of the present work was to shed light on the nature and origin of specks, spots, and microscopic crystals in extra-hard cheeses by using a multidisciplinary approach. Based on previous cheese structure studies (Ong et al., 2010; Schrader, 2012; D'Incecco et al., 2015), various microscopy techniques and different dyes (light and fluorescence, confocal, confocal micro-Raman, and transmission electron microscopy) were used in combination with chemical data to achieve an unambiguous characterization of these particles in cheese. Our ultimate goal was to formulate a hypothesis on the origin of these structures as they appear in hard and extra-hard cheeses. This knowledge will contribute useful information to understanding the bioavailability of selected minerals and nutrients in cheese. Furthermore, this knowledge could provide insights into the nature of these structures and might lead to new manufacturing strategies to control the formation of spots in commercial cheeses.

MATERIALS AND METHODS

Cheese Samples and Collection of Specks and Spots

Eleven extra-hard cheeses, ripened for 18 to 20 mo, were kindly provided by 2 dairies producing Grana Padano (7 cheeses) and Parmigiano-Reggiano (4 cheeses), respectively. Specks were harvested from the cheese using a pin, and spots were collected from the cheese with a spatula and then gently brushed to remove the cheese matrix on their surface. Specks and spots were collected separately from individual cheese samples in a sufficient amount (20–22 g) to conduct all the analyses. An equivalent amount of cheese was taken from the portion (0.5-cm thick) immediately surrounding the single spot, as shown in Figure 1, and used as a basis for reference. When necessary, a slice representative of the whole cheese was taken as well. Additional cheese portions were taken as required for microscopy investigations with various techniques. In particular, 20 spots of different size and taken from different cheeses were examined to determine their structural characteristics.

Chemicals

Glutaraldehyde, paraformaldehyde, cacodylate buffer, and osmium tetroxide were purchased from Agar Scientific (Stansted, UK). Toluidine blue, rhodamine, and single amino acids were purchased from Sigma Aldrich (Milan, Italy). Ninhydrin was purchased from Biochrom Ltd. (Cambridge, UK). Water purified with Milli-Q system (Millipore Corp., Bedford, MA) was used.

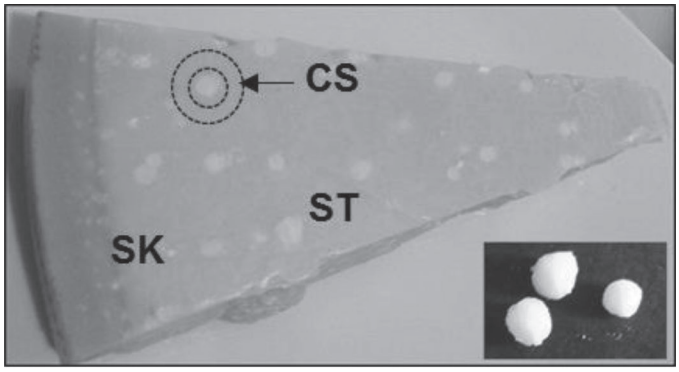

Figure 1. Photograph of extra-hard cheese showing specks (SK) and spots (ST). CS = cheese portion surrounding a spot (in sampling mode). Inset, isolated spots from the cheese. Color version available online.