Effect of *Monascus* sp. as an Adjunct Starter on Physicochemical Properties and Proteolysis in Semi-hard Cheeses during Ripening

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**Abstract**  Two kinds of semi-hard cheeses, with *Monascus purpureus* and without *M. purpureus*, were manufactured, and effects of *M. purpureus* on physicochemical properties and proteolysis were evaluated during 36 days of ripening. Addition of *M. purpureus* changed the microbial survival and showed no significant effect on physicochemical properties of the cheeses, including dry matter and pH. Regardless on the rind or in the core, the indices of proteolysis had no significant difference (p>0.05), whereas there were significant differences of total free amino acid (FAA) and individual FAA between cheeses; this indicated that *M. purpureus* had no significant effect on the primary proteolysis, but affected the content and ratio of individual FAAs during maturation. Electrophoretic analysis showed strong degradation of αs1-casein in the core and on the rind of cheeses, while β-casein was highly degraded on the rind but less in the core. Thus, *Monascus* spp. might have a potential application in the manufacture of cheeses.

**Keywords:** semi-hard cheese, *Monascus purpureus*, proteolysis, ripening

**Introduction**

During cheese ripening, proteolysis is the most complex and, in most varieties, the most important of the three primary biochemical events, since it contributes to textural changes of the cheese matrix due to breakdown of the protein network, decrease in ash through water binding by liberated carboxyl and amino groups, and increase in pH. It also contributes to flavor, and perhaps off-flavor, of cheese directly through the formation of peptides and free amino acids (FAAs) and indirectly through the liberation of substrates (amino acids) for other flavor-generating reactions, as well as by facilitating the release of sapid compounds from the cheese matrix during mastication (1-3).

In most ripened cheeses, the progress of proteolysis can be divided into two phases as follows: primary proteolysis, which serves as the initial hydrolysis of caseins caused by residual coagulant and, to a lesser extent, by plasmin and perhaps cathepsin D and other somatic cell proteinases, resulting in the formation of large- and intermediate-sized peptides, and secondary proteolysis, which is the subsequent degradation of the products of primary proteolysis catalyzed by the coagulant and proteinases from the starter, non-starter, and secondary microflora of the cheese, producing small peptides and FAAs (1,2). Additionally, the general outline of the proteolysis can differ markedly between cheese varieties due to different proteolytic agents occurring in different types of cheeses. Since the extent and pattern of proteolysis in cheese is used as an index of cheese maturity and quality, the extent and pattern of proteolysis for many types of cheeses has been extensively studied, such as Cheddar (4), Camembert (5-7), blue-veined cheese (8,9), and other cheeses (10,11). For surface-ripened cheeses, the secondary cultures, including *Penicillium camemberti* and *Penicillium roqueforti* make a major contribution to proteolysis due to their endopeptidase and exopeptidase activities and are recognized as being as important as the primary cultures (12).

*Monascus* spp. have been widely used in foods and medicines in the Orient for over 1,000 years, especially in China (13). Up to now, *Monascus* spp. are also employed in rice-wine brewing and food fermentation due to the enzymes secreted by them for the breakdown of carbohydrates and proteins (14). Moreover, many results suggest that *Monascus* fermentation products can be effective for reduction of levels of blood cholesterol; lowering of blood pressure; prevention of cancer development; and the management of diabetes, obesity, and Alzheimer’s disease, because they contain numerous active constituents, such as monacolin K, γ-aminobutyric acid (GABA), and dimerumic acid (15,16). Therefore, *Monascus* fermentation products can be used as dietary supplements for medical therapy (16).
now, molds such as *P. camemberti* and *P. roqueforti* have been extensively studied as secondary starters in cheeses. However, there has been little information about *Monascus* spp. as the secondary starter employed in the manufacture of cheese. Recently, *Monascus* sp. was used as the secondary starter for producing the novel *Monascus*-fermented cheese and thus has the potential for producing this new kind of cheese (17).

In this study, semi-hard cheeses inoculated with *P. candidum*, *G. candidum*, and *Monascus* sp. but without *Monascus* sp. as the secondary starter were manufactured and the effects of addition of *Monascus* spp. on the physicochemical properties and proteolysis of semi-hard cheeses during ripening were evaluated.

### Materials and Methods

#### Cheese manufacture and sampling

Two types of semi-hard cheeses, experimental cheeses (HB cheese) and control cheese (B cheese), were repeated twice on 2 consecutive days, and were manufactured from whole cow milk. The B cheeses were manufactured without any *Monascus* sp. strains but with the commercial lactic starters; however, the HB cheeses were manufactured with the *Monascus* sp. strain plus the commercial lactic starters added in the B cheeses. Whole milk (250 L) was refrigerated, carried at 4°C to the pilot plant, pasteurized (75°C for 15 s) in a 400 L/H pasteurizer (SPX Corporation, Charlotte, NC, USA), and cooled to 30°C. The milk was then divided into two vats of 100 L each. The lactic acid starter containing *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Streptococcus salivarius* subsp. *thermophilus* (1.5 g/100 L, CHOOZIT™ RA 21; Danisco, Sassenage, France) was inoculated into each tank. At the same time, the *M. purpureus* BD-M-4 starter (1.7×10⁶ CFU/mL, CGMCC No.9712), stored in China General Microbiological Culture Collection Center, Beijing, China, was inoculated into the tank for experimental cheeses. Then, the red yeast rice powder (0.2 g/L, Jiajie®; Shanghai Jiajie Natural Food Pigment Co., Ltd., Shanghai, China) was added into the tank for experimental cheeses, and the milk was gently stirred for 5 min in order to evenly mix in the red yeast rice powder. After 80-110 min, as pH of the milk became 6.3, rennet (1 g/100 L; Marzyme®; Streptococcus thermophilus subsp. thermophilus; Thermolactobacillus ■; Danisco) was added. The progress of coagulation lasted about 25 min, and the curd was cut into cubes (1 cm³) after 40 min of hardening. The mixture of curd and whey was left standing for 10 min in the vat, and the whey was drained for 40 min at room temperature. Then, the curd was loaded into plastic molds, with a diameter of 105 mm and a height of 30 mm, producing cheeses weighing 220±20 g. The cheeses were pressed for 4-8 h and pickled for 1 h in sterile (30 min at 121°C) supersaturated brine at 144°C after the end of molding. Then, the cheeses were immersed into the solution containing *G. candidum* (6×10⁸ spores per milliliter; CHOOZIT™ GEO 17 LYO 2D; Danisco) and *P. candidum* (1×10⁶ spores per milliliter; CHOOZIT™ PC 12 LYO 20D; Danisco) for 1 h. After 1 h of drying to eliminate excess water, the cheeses were transferred to ripening chambers for 4 days under a controlled condition (25°C and 90% relative humidity [RH]) and then for 17 days at 14°C and 90% RH. On day 5, the cheeses were turned. On day 21, they were wrapped and ripened at 4°C until day 36. At each ripening period (1, 8, 15, 22, 29, and 36 days), two cheese samples were taken randomly from the rind (5 mm thick all over the cheese surface) and the core (10 mm around the center of the cheese sample) for analysis.

#### Physicochemical analysis of cheeses

For each part of the cheeses (the rind and the core of cheese mass), dry matter (DM), pH, and the content of nitrogen compounds in the soluble fractions were determined. DM was analyzed in triplicate by drying 3±0.3-g cheese samples at 150°C in an MB 45 moisture analyzer (Ohaus International Trading [Shanghai] Co., Ltd., Shanghai, China) until constant weight. Ground cheeses (2±0.1 g) were mixed with 10-mL deionized water; then, the mixture was stirred for 10 min, and its pH was measured using a Delta 320 pH meter (Mettler-Toledo Ltd., Shanghai, China).

#### Nitrogen fractions and FAA measurements

Proteolysis was assessed based on determination of total nitrogen (TN), pH 4.6 acid-soluble nitrogen (ASN), 12% trichloroacetic acid (TCA)-soluble nitrogen (NPN), and 5% phosphotungstic acid-soluble nitrogen (PTASN), according to the method presented by Leclercq-Perlat et al. (18). Proteolytic indexes, including the ratios of ASN/TN, NPN/TN, and PTASN/TN, were used as cheese-ripening indicators.

Individual FAAs were determined in duplicate from PTASN filtrates. An aliquot of PTASN filtrate was filtered on a 0.22-μm pore diameter membrane after its pH was adjusted to 1.7-2.2 by adding 10 N NaOH solution. Then, its individual FAAs were analyzed on a high-speed amino acid analyzer (L-8900; Hitachi, Tokyo, Japan) using the method recommended by De Freitas et al. (19) with modification. All analysis was carried out in duplicate.

#### Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed using the gradient gel system (12% w/v) by Laemmli (20), as described by Zhang and Zhao (21), with some modifications. Two grams of pH 4.6-insoluble nitrogen fraction separated from pH 4.6-SN was washed three times using 6 mL of 1 mol/L sodium acetate buffer (pH 4.6). The residual fat was eliminated by washing with 4 mL of diethyl ether two times. The residue (~0.2 mg) was mixed with 2 mL Tris-buffer (pH 6.8) containing glycercin (25% w/v), β-mercaptoethanol (5% v/v), SDS (2% w/v), and bromophenol blue (0.1% w/v). Then, the solution was diluted twenty times using Tris-buffer (0.06 mol/L, pH 6.8), mentioned above, in order to obtain the solution with protein concentration ranging from 5 to 10 mg/mL. After boiling and centrifuging at 10,000 ×g for 10 min, the supernatant was separated for SDS-PAGE analysis. The gradient gel was 1.5 mm thick, consisting of a 2-cm stacking gel and 10-cm running gel. A 10-μL aliquot of the supernatant...