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Thermostability of peptidases secreted by microorganisms associated with raw milk

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ABSTRACT

Peptidases of psychrotolerant microorganisms are known to be thermostable and withstand the thermal processing of milk products. The thermostability of peptidases from 231 recently isolated raw milk microorganisms was evaluated after cultivation in milk medium at 6 °C. Nearly all peptidases secreted showed residual peptidase activity of at least 5% after heat treatments at 70 and 95 °C for 5 min. Peptidases of three isolates from three novel *Pseudomonas* species were produced in a bioreactor at conditions simulating the storage of raw milk (6 °C), then purified and characterised biochemically. The protective effect of milk protein and influence of autoproteolysis were shown in extensive studies. The peptidases withstood ultra-high temperature treatment with residual activities of 45.8–58.9% and are able to cause destabilisation of milk products during storage; this was demonstrated for the peptidases from one of the novel *Pseudomonas* isolates in pilot-scale production of milk treated at ultra-high temperatures.

1. Introduction

The microbiota of raw milk is very diverse due to its numerous possibilities for contamination and its high nutrient content and includes a large pool of unknown species (Quigley et al., 2013; Von Neubeck et al., 2015a). The refrigeration of raw milk selects for psychrotolerant microorganisms, such as *Pseudomonas* (Fricker, Skånseng, Rudi, Stessl, & Ehling-Schulz, 2011; Lafarge et al., 2004; Rasolofo, St-Gelais, LaPointe, & Roy, 2010). Von Neubeck et al. (2015a) investigated the biodiversity of raw milk and detected 33 *Pseudomonas* species, which included 18 potentially novel species. Psychrotolerant microorganisms, especially pseudomonads, are known to produce thermostable peptidases during growth and have been studied for decades (Adams, Barach, & Speck, 1975; Baglinière et al., 2013; Griffiths, Phillips, & Muir, 1981; Owusu, Makhzoum, & Knapp, 1991; Sørhaug & Stepaniak, 1997; Wiedmann, Weilmeier, Dineen, Ralyea, & Boor, 2000).

Peptidases secreted by pseudomonads are aprX/aprA-gene-encoded and belong to the M10B subfamily (serralysin, EC 3.4.24.40) of the clan MA of metallopeptidases (Rawlings, Waller, Barrett, & Bateman, 2014). Studies dealing with the thermostability of *Pseudomonas* peptidases have shown their ability to withstand heat treatments even in the UHT region (135–150 °C) and that the enzymes are able to destabilise milk products by causing bitterness or gelation (Adams et al., 1975; Baglinière et al., 2013; Barach & Adams, 1977; Baur et al., 2015b; Datta & Deeth, 2003; Mu, Du, & Bai, 2009; Schokker & van Boekel, 1997a). The inactivation kinetics of *Pseudomonas* peptidases do not usually conform to the Arrhenius equation over a wide temperature range, because the rate of heat inactivation at moderate temperatures (40–60 °C) is disproportionally high compared with temperatures in the UHT region (Owusu & Doble, 1994). This phenomenon is

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often called low-temperature inactivation in the literature (Adams et al., 1975; Barach, Adams, & Speck, 1978; Kroll & Klostermeyer, 1984; Schokker & van Boekel, 1998b).

The inactivation of enzymes can be generally described by the three-state model of Lumry and Eyring (1954). In a first step, the native protein molecule is reversibly transformed into an unfolded, inactive form. Native and unfolded molecules are equal at the denaturation temperature T_d, this means that 50% of the molecules are folded and 50% are unfolded. At temperatures above T_d, the unfolded molecules are inactivated in a second irreversible step by, for example, the reshuffling of disulphide bonds, destruction of amino acid residues, or aggregation or formation of incorrect structures (Ahern & Klibanov, 1988). An additional irreversible type of inactivation is possible for peptidases. At temperatures in the range of T_d, unfolded molecules can be hydrolysed by native peptidases (Ahern & Klibanov, 1988). Autoproteolysis can, therefore, be a possible explanation for the low temperature inactivation of peptidases from *Pseudomonas* species.

The aim of this study was the investigation into the thermostability of peptidases from 231 raw milk isolates to show their relevance as spoilage organisms of raw milk. The cultivation was carried out in milk medium at 6 °C to simulate the conditions of raw milk storage. Furthermore, the peptidases of three *Pseudomonas* isolates belonging to three potentially novel species were produced, purified and partially characterised. The thermostability of the purified peptidases was investigated extensively in synthetic milk ultrafiltrate (SMUF) and milk to show the potential protective effect of the milk protein.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma Aldrich (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany) or Applichem (Darmstadt, Germany). Sodium caseinate (Na–CN) was obtained from Bayerische Milchindustrie eG (Landshut, Germany). Extended shelf life (ESL) milk (3.8% fat, Schwarzwaldmilch GmbH, Freiburg, Germany) and ultra-high temperature (UHT) treated milk (entrahmte H-Milch, 0.1% fat, homebrand, EDEKA supermarket, Germany) were purchased from a local supermarket.

2.2. Isolation and identification of microorganisms

The microorganisms investigated in this study were isolated from raw milk and identified by Von Neubeck et al. (2015a). In addition, some microorganisms isolated from semi-finished milk products (von Neubeck, unpublished data) have also been analysed; semi-finished products are products that can be used in further processes, such as skim milk concentrate or milk protein concentrate. The identification was done by 16S rRNA or 26S rRNA gene sequencing for bacteria and yeasts, respectively (Von Neubeck et al., 2015a). The gene for the sigma 70 subunit (*rpoD*) of the RNA polymerase was used for the identification of *Pseudomonas* isolates, because the 16S rRNA gene is not discriminatory enough (Von Neubeck et al., 2015a). Three isolates (WS 4992, WS 4993 and WS 4672) belonging to three potentially novel *Pseudomonas* species have been investigated in more detail in this study. They were designated as *Pseudomonas* sp. nov. 1 (WS 4992), *Pseudomonas* sp. nov. 7 (WS 4993) and *Pseudomonas* sp. nov. 16 (WS 4672) in the study of Von Neubeck et al. (2015a). The isolate *Pseudomonas* sp. nov. 7 (WS 4993) is the type strain of the novel species *Pseudomonas weihenstephanensis* (Von Neubeck et al., 2015b). The peptidase gene sequences were determined by whole-genome sequencing, as described by Baur et al. (2015b) and deposited at GenBank under the accession numbers JYLN00000000 (WS 4672), JYLO00000000 (WS 4992) and JYLF00000000 (WS 4993).

2.3. Investigation of the thermostability of the peptidases from raw milk isolates

2.3.1. Culture conditions

Cryopreserved isolates were revived in 5 mL plate count (PC) medium (5.0 g L^{-1} peptone, 2.5 g L^{-1} yeast extract, 1.0 g L^{-1} glucose) for 24 h at 30 °C on a platform shaker (120 rpm) (TR-250, Infors AG, Bottmingen, Switzerland). Each isolate was then cultivated in duplicate in test tubes containing 7 mL heat-sterilised 10% (v/v) ESL milk. The test tubes were inoculated with 100 µL of the revived culture and incubated for 8 d at 6 °C and for 2 d at 30 °C on a platform shaker (120 rpm) (TR-250, Infors AG, Bottmingen, Switzerland). Peptidase activity was determined in the supernatant after centrifugation at 8000 × *g* for 15 min at 4 °C (5804 R, Eppendorf AG, Hamburg, Germany) with azocasein as a substrate (see Section 2.5.1). All cultivations were performed in duplicate. The mean ± deviation was calculated with Excel.

2.3.2. Heat treatment of the extracellular peptidases in the supernatant

The thermostability of the extracellular peptidases in the supernatant was investigated by heat treatment at 70 and 95 °C for 5 min. Before the different heat treatments were realised, the samples were diluted to a defined peptidase activity of 6.6 ΔA h^{-1} mL^{-1} using the standard assay buffer (50 mM 3-(N-morpholino)propansulfonic acid (MOPS), pH 6.7, 1 mM $CaCl_2$) and the standard assay conditions with azocasein as a substrate (see Section 2.5.1). If the activity was lower than 6.6 ΔA h^{-1} mL^{-1}, the heat treatment was done directly with the untreated sample. The heat treatment was carried out in 500 µL scale using 500 µL safe-lock tubes and two ThermoMixers (Eppendorf AG, Hamburg, Germany). The samples, which were heated to 70 °C, were preheated in a ThermoMixer adjusted to 95 °C for 30 s to reduce the period of heating. Thereafter, the samples were transferred to a ThermoMixer adjusted to 70 °C and treated with heat for 5 min. The samples, which were heated to 95 °C, were heated directly in a ThermoMixer adjusted to 95 °C for a total time span of 7.5 min. After the heat treatment, the samples were cooled immediately in liquid nitrogen (N_2). Residual peptidase activity was determined using azocasein as a substrate (see Section 2.5.1).

2.4. Peptidases from three novel Pseudomonas species

2.4.1. Peptidase production

Isolates of three novel *Pseudomonas* species, namely *Pseudomonas* sp. nov. 1 (WS 4992), *Pseudomonas* sp. nov. 7 (WS 4993) and *Pseudomonas* sp. nov. 16 (WS 4672), were cultivated at 750 mL scale for peptidase production. Precultures were grown overnight in test tubes in PC medium at 30 °C on a platform shaker (120 rpm). Enzyme production was carried out in a stirred-tank reactor (Multifors, Infors, Bottmingen, Switzerland) in 750 mL UHT milk. The bioreactor was inoculated with 7.5 mL preculture and fermentation was carried out at a stirring speed of 150–500 rpm, 0.5 vvm air gassing ($pO_2 > 30\%$), at 6 °C and pH 6.7 ± 0.5, (controlled with 2 M NaOH and 0.67 M H_3PO_4). Samples were taken every 2 d during the fermentation to analyse growth and peptidase activity. Growth was monitored by plating 10 µL of various dilutions on PC agar. Peptidase activity was analysed in the supernatant after centrifugation at 8000 × *g* and 4 °C for 15 min using azocasein as a substrate (see Section 2.5.1). The cultivation was terminated when the peptidase activity no longer increased. The supernatant was