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Short communication

A modified agar medium for the screening of proteolytic activity of starter cultures for meat fermentation purposes

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Abstract

An agar medium, used in the screening of proteolytic activity of dairy-related bacteria, was adapted for assessing the proteolytic capacity of bacteria which were of possible use in meat fermentations. Freeze dried myofibrils, extracted from pork muscle, were incorporated in the medium. The agar plates were inoculated with 20 μl of overnight cultures of different starter strains, and incubated at 30°C for 48 h. After incubation, proteolytic bacteria produced clear zones. Coomassie brilliant blue stain was employed to facilitate the detection of these zones. Proteolytic activity was confirmed in an enzymatic test. © 1997 Elsevier Science Ireland Ltd.

Keywords: Proteolysis; Myofibrils; Meat fermentation; Fermented sausage; Coomassie blue

1. Introduction

In recent years research has focused on the enhancing the ripening of dry fermented sausages, such as salami (DeMasi et al., 1990; Selgas et al., 1993; Johansson et al., 1994). However, an important condition is that sausage characteristics are not affected adversely during the enhanced ripening. It is thought that proteolytic enzymes, both those endogenous to the meat and microbially derived, are important for flavour development during the fermentation process, although their individual roles are not yet clear. Verplaetse et al. (1992) stated that myosin degradation during dry sausage fermentation was mainly due to meat endogenous enzymes, while the production of NPN-compounds was caused by bacterial enzymes. However, the starter culture used in that experiment was not specially chosen for its ability to break down meat protein.

It is common practice, regarding dairy fermentations, to test starter cultures and other bacterial strains found in dairy products for their proteolytic activity using caseinate agar (Atlas, 1993). This facilitates the determination of their possible individual role in flavour development. However, caseinate is a milk protein, and most proteolytic enzymes are substrate specific (Hasegawa et al., 1970; Sikes and Maxcy, 1979). Therefore, screening for proteolytic activity for the purpose of flavour enhancement during meat fermentations should be

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carried out with meat protein, such as myofibrillar protein. For this reason, in the medium described by Atlas (1993), freeze dried sodium caseinate was replaced by freeze dried myofibrils. To facilitate the determination of proteolysis, including partial breakdown of complex proteins, plates were stained with Coomassie brilliant blue. This gives a distinct differentiation between proteins and smaller peptides and amino-acids resulting from proteolysis.

2. Materials and methods

2.1. Extraction of myofibrillar protein

Myofibrils were extracted from lean pork lap muscle, according to a method described by Etlinger et al. (1976), which was slightly modified by Wang (1982). The procedure was as follows: meat was homogenised by use of a blender (Waring, New Hartford, CT, USA), in eight volumes of pyrophosphate buffer (pH 6.8), for two 30 s homogenisations at low speed. After blending, the samples were transferred into 250 ml centrifuge containers and were subjected to several centrifugation and resuspension steps. The samples were centrifuged at 10 000 rpm (relative centrifugal force (RCF) = 15 344 g, JA14 rotor, Beckman J2-21, Beckman Instruments Limited, High Wycombe, UK), for 10 min, unless stated otherwise. The resulting pellet was resuspended in extraction buffer (pH 6.8) and centrifuged, followed by resuspension in extraction buffer and filtering through a double layer of cheesecloth. This filtrate was subsequently centrifuged, the pellet resuspended in extraction buffer, and again centrifuged. The pellet was then washed with Triton 100-X buffer (pH 6.8), and centrifuged for 6 min. The pellet was resuspended in extraction buffer and centrifuged for 10 min, which was repeated twice. Finally, the pellet was lyophilised in a petri-dish and the freeze dried samples were transferred into screw cap containers and stored in a desiccator until required. All buffers were kept at 4°C.

2.2. Preparation of plates

Freeze dried myofibrils were crushed with a mortar and pestle, and subsequently incorporated into the medium, as described by Atlas (1993), using agar (1.5%, Difco Laboratories, Detroit, MI, USA), myofibrils (freeze dried, 1.0%), tryptone (0.5%, Difco), yeast extract (0.25%, Oxoid, Basingstoke, UK), glucose (0.1%, BDH Laboratory Supplies, Poole, UK), $CaCl_2.2H_2O$ (20 ml/l of 1 M solution, BDH) in a 0.015 M trisodium citrate solution (Prolabo, Manchester, UK), pH 6.9±0.1. After autoclaving the medium (121°C, 15 min), plates were poured while swirling the medium regularly and vigorously, to disperse the proteins. Upon solidification of the myofibrillar medium, three wells per plate were made using a sterile Pasteur pipette.

2.3. Strains, inoculation and incubation

The following micro-organisms were tested for their proteolytic activity: *Micrococcus varians* (Nestec, Lausanne, Switzerland), *Lactobacillus pentosus* (Rudolf Müller, Pohlheim, Germany), and two *Staphylococcus xylosus* strains, strain A (Nestec), and B (CHR Hansen, Hørsholm, Denmark). With the exception of *S. xylosus* A, in initial studies all these strains proved to be positive on sodium caseinate agar (results not shown). The staphylococci and micrococcus strains were grown overnight in a broth consisting of tryptone (1%, Difco), yeast extract (0.5%, Oxoid), sodium chloride (0.5%, Prolabo) and glucose (0.01%, BDH), pH 7.2. The lactobacillus strain was grown in MRS broth (Oxoid). All strains were incubated at 30°C, for 24 h. The myofibrillar agar plates were inoculated in triplicate, with 20 μl of whole suspension per well, and incubated at 30°C, for 48 h. Each strain was evaluated in two independent tests.

2.4. Staining of plates

After 48 h, the complete agar layers were removed from the petri-dishes, and were stained for 5 min in a solution containing 0.05% (w/v) Coomassie brilliant blue R-250 (BDH), 50% (v/v) methanol, and acetic acid (9.2% (v/v), BDH), in distilled water. Subsequently, they were destained overnight using 25% (v/v) ethanol and 5% (v/v) acetic acid in distilled water.

2.5. Spectrophotometric evaluation of enzymatic protein breakdown

An enzyme test was performed on all strains to confirm proteolytic activity. The proteolytic activity

in a solution containing freeze dried pork myofibrils was measured over a period of 6 h. The test procedure is described as follows: culture supernatant (max. 100 μl) was added to 0.5 ml of solution I (5% of 1 M Tris–HCl solution (Sigma, St. Louis, Missouri, USA) pH 7.5, 10 ml 4% Na-azide (Sigma), 0.074% (w/v) $CaCl_2.2H_2O$ and 0.8% (w/v) freeze dried pork myofibrils, in distilled H_2O, shaken at 37°C, and stored at 4°C) in an Eppendorf tube, and incubated at 37°C in a shaking waterbath. The enzymatic reaction was stopped by the addition of 0.5 ml of solution II (1.634% (w/v) trichloroacetic acid (BDH), 1.804% (w/v) sodium acetate (BDH), 1.886% (v/v) acetic acid, in distilled H_2O, stored at 4°C). The samples were left at room temperature for at least 30 min, after which they were centrifuged for 15 min at 14 000 rpm (RCF = 15 996 g, F-45-18-11 rotor, Eppendorf centrifuge 5415C, Engelsdorf, Germany). The absorption of the supernatant was measured in a Beckman DU 640 spectrophotometer (Fullerton, California, USA) at 275 nm (UV), in a quartz cuvette. A negative control (reagent blank) was also included.

3. Results and discussion

Micrococcus varians, *Staphylococcus xylosus* B, and *Lactobacillus pentosus* showed a positive reaction, indicated by a clear zone surrounding the wells (see Fig. 1), with diameters of 22±0.0 mm, 18±2.8 mm and 13±1.4 mm, respectively. No clear zone could be observed for *S. xylosus* A, indicating that proteolytic activity had not occurred. The repeated tests gave comparable results. The staining with Coomassie brilliant blue facilitated the determination of whether or not proteolysis had taken place. Fairly clear, unstained zones became visible after staining and subsequent destaining, despite the fact that the myofibrils were not fully dissolved and some strains were apparently not able to break down these rather complex proteins fully, which was visible as some dark stained granules.

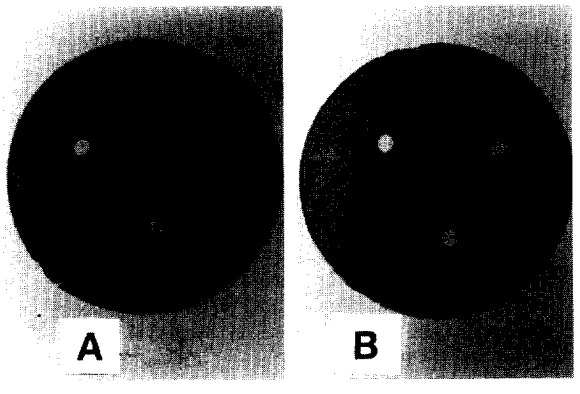

Fig. 1. Proteolytic activity by *Lactobacillus pentosus* (A), and no proteolytic activity by *Staphylococcus xylosus* A (B) on the agar medium containing freeze dried pork myofibrils.

As shown in Fig. 2, in the enzyme test *S. xylosus* B, *M. varians* and *L. pentosus* gave an increase in absorption over time, indicating proteolytic activity, whereas both *S. xylosus* A and the negative control showed no increase, i.e., no proteolytic activity. The difference in the level of absorption between the negative control and the negative strain may be explained by the presence of some culture medium, which contributed to the level of absorption.

Earlier research performed by Klement et al. (1974) and by García de Fernando and Fox (1991) showed that during the fermentation of meat the amount of non-protein-nitrogen (NPN) from myofibrillar origin is much higher than from sarcoplasmic origin. This indicates that it is favourable to use myofibrillar protein in a screening method for the detection of proteolytic activity of bacterial strains intended for meat fermentation purposes. Moreover, because the amounts of myofibrillar and sarcoplasmic protein were approximately 60% and 30% of total muscle protein, respectively, this may imply that the ability to break down myofibrillar protein rather than sarcoplasmic protein will be important regarding the development of fermentation characteristics. Therefore, strains thus selected for their enhanced proteolytic activity on meat proteins may be of greater importance than has been thought to date.

The amount of pork and beef meat used for the production of fermented sausages can vary a lot, from 20% up to 70% pork, and from 0% to 50% beef, depending on the type of sausage made (Astiasaran et al., 1990; Díaz et al., 1993; Montel et al., 1993; Johansson et al., 1994; Næs et al., 1995). In this research pork myofibrils were used, but the test could also be performed using beef myofibrils.

The slope of the absorption values set against time, measured for the different strains in the enzyme test, was linearly related to the zone diameter obtained with the agar medium. A linear regression

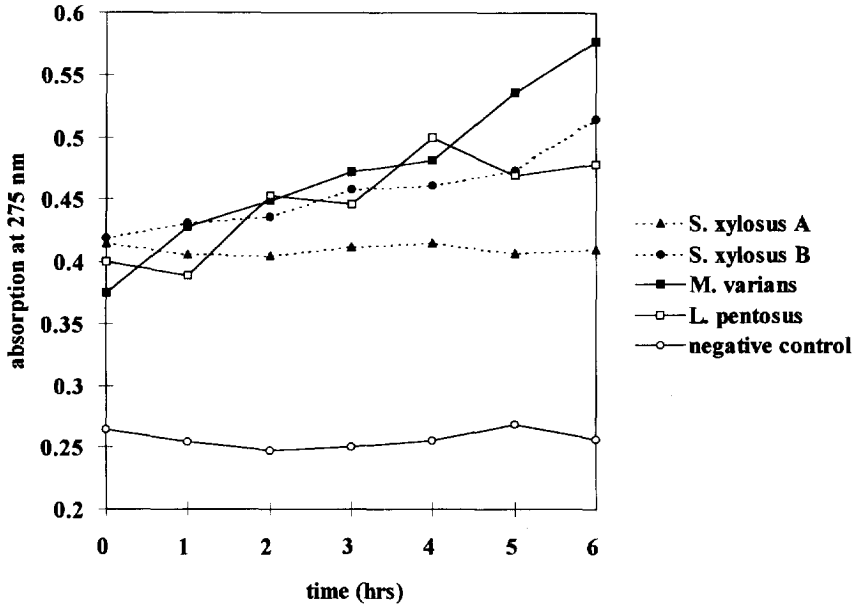

Fig. 2. Proteolytic activity of different starter strains in an enzyme test using freeze dried pork myofibrils.

analysis revealed a correlation coefficient of 0.89. This indicates that this method can be used as a semi-quantitative method for the screening of proteolytic activity of starter strains.

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