EVIDENCE FOR PROTEOLYTIC ACTIVITY OF LACTOBACILLI ISOLATED FROM KEFIR GRAINS

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ABSTRACT
The proteolytic activity of 276 natural isolates from kefir grains was investigated. Evidence for extracellular proteolytic activity was demonstrated for 49 Lactobacillus strains. One strain, Lactobacillus kefir DR22x, was selected as a producer of proteinases. Lactobacillus kefir DR22x strain produces a cell-wall-bound proteinase. The proteinase was removed from the cell envelope by washing the cells with a Ca2+-free buffer. The crude proteinase extract showed the highest activity at pH 7.2 and 37 ºC. The proteolytic activity was shown to be maximal in the late exponential growth. A study using several protease inhibitors suggested that this activity is associated with serine-type proteases. Considering the substrate specificity, the enzyme is similar to the lactococcal P1-type proteinases, since it completely hydrolysed only β-casein, showing very low activity towards α-casein.

Introduction
Kefir is a refreshing fermented milk beverage that has an exotic sour and slightly alcoholic flavor. Kefir has been traditionally consumed for potential health benefits (9). The composition of kefir grains includes subpopulations of lactic acid bacteria (lactobacilli, lactococci, leuconostocs), yeasts and acetic acid bacteria. The microflora of kefir grains has been studied by many workers (10) and several new bacterial species have been described (L. kefir, L. parakefir, L. kefiranofaciens, L. kefiranum). The main function of the microorganisms constituting the grains is the production of lactic acid by fermentation of lactose, as well as the production of ethanol, peroxides, carbon dioxide, acetdehyde, diacetyl and acetoin. However, few of these works studied the proteolytic activity of lactic acid bacteria (LAB) in kefir community (11). The proteinases catalyse the initial steps of the hydrolysis of milk proteins, providing the cell for the amino acids that are essential for LAB growth.

The proteolytic system of lactococci has been a subject of intensive biochemical and genetic research. Their cell-wall-bound proteinases have been divided into two main groups: the P1-type proteinases, which hydrolyse predominantly β-casein, and, the PIII-type proteinases, which degrade α- and κ-caseins in addition to β-casein. The intermediate-type proteinases cleave β-casein in manner similar to that of the P1 type but are also able to hydrolyze αs1-casein (5). In contrast to the lactococcal proteolytic system, a limited information is available for the proteolytic system of lactobacilli. Proteolytic activity of Lactobacillus has been associated with cell-wall-bound proteinases, as in the case of Lactococcus. The protease system most intensively studied is that of Lactobacillus casei (1, 4). The proteinase from Lactobacillus plantarum has similar properties to the L. casei enzyme (3). Cell envelope-associated proteinase, with similar biochemical properties to the lactococcal proteinase PrtP, was also detected in thermophilic lactobacilli. These kinds of proteinases
were found and characterized in various strains of *Lactobacillus helveticus*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii subsp. bulgarius* (2, 10).

The present study was undertaken to investigate the proteolytic activity of lactobacilli isolated from kefir. This information can contribute to the development of starter cultures with predictable characteristics and in order to know better the potential health effects of this bioculture.

**Materials and Methods**

**Kefir grains**
Kefir grains were obtained directly from individual dairies in various parts of the France. These grains (>5 years old) were supported on pasteurized cow’s milk at 22 ºC by daily transfer. Kefir grains were used as a source for bacterial strains.

**Screening of the strains for proteolytic activity**
Kefir grains (10g) were recovered from 24 h fermented mother culture and homogenized (EURO TURAX T25b, Poland) with 90 ml of sterile saline solution 0.9% NaCl. Serial dilutions were performed and aliquots were plated on 10% milk agar for detection and enumeration of proteolytic microflora. The samples were incubated at 28 ºC for 48 h. The aliquots from serial dilutions were plated on MRS agar, supplemented with 5% skim milk (Oxoid, Basingstoke, Hampshire, England) for the selective growth of lactobacilli. MRS plates were incubated under anaerobiosis at 28 ºC for 48 h. The proteolytic strains were detected on the base of clear zones surrounding the colonies.

The selected lactobacilli were purified and tested for proteolytic activity on Calcium caseinate Agar (Merck). Tripsin (Merck, 5 mg/ml) was used as a control. The selected proteolytic strains were stored at −20 ºC in MRS medium containing 50% (v/v) sterile glycerol.

**Identification of isolated lactobacilli**
The isolates were identified by phenotypic criteria (Bergey’s Manual). The identification system API 50CH (BioMerieux, Marcy l’Etoile, France) was applied.

**Determination of the proteolytic activity**
The proteolytic activity was determined according to the method of Kunitz (6). The reaction was stopped by addition of TCA (12%). The samples were centrifuged (12 000 x g, 4 ºC; 5 min) followed by (after 30 minutes) measurement of the supernatant absorbance at λ A_{280}. One Kunitz unit was defined as the quantity of enzyme, which effects an Δ A_{280} of 0.001 under test conditions.

**Preparation of the cell wall extract**
Cell wall extract was prepared according to the protocol of Tsakalidou et al. (10). The cells obtained after extraction were designated as treated cells.

**Casein hydrolysis**
Whole cell-suspension (15µl), treated cell-suspension (15µl) or cell wall extract (15µl) were incubated with 15 µl casein solution (α-, β-, total casein, 4 mg/ml, Merck) for 4, 8 and 24 h at 37 ºC. The reaction with cell wall extract was stopped by addition of 60 µl 12% TCA. After 10 min at room temperature the sample was centrifuged (12 000 x g, 5 min, 4 ºC). The sediment was dissolved in 120 µl solubilization buffer, heated for 5 min at 100 ºC, and analyzed by SDS-PAGE. The reaction in the samples with whole and treated cells was stopped by centrifugation (12 000 x g, 5 min, 4 ºC). 30 µl of the supernatant obtained was mixed in a 1:1 ratio with solubilization buffer, heated for 5 min at 100 ºC and analyzed by SDS-PAGE (12.5% acrylamide gel) by the method of Laemmli (7).

**Effect of pH on proteinase activity**
Cell wall extract (1 ml) was incubated with 1 ml of casein solution (4 mg/ml) for 1h at 37 ºC in buffers (2 ml) of various pH values (5.4; 5.7; 6.5; 7.2 and 8). The reaction was stopped by addition of 12% TCA and after 10 min at room temperature the sample was centrifuged and free amino acids and peptides liberated into the supernatant.
TABLE 1

Proteolytic activity of *Lactobacillus* isolates and their identification based on API 50CHL.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Species identification by API 50CHL</th>
<th>Quality of identification</th>
<th>Proteolytic activity (AU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR22x</td>
<td>Lactobacillus kefir</td>
<td>Excellent (98.4%)</td>
<td>530</td>
</tr>
<tr>
<td>DR22r</td>
<td><em>Lactobacillus kefir</em></td>
<td>Very good (92.7%)</td>
<td>290</td>
</tr>
<tr>
<td>GK44eps</td>
<td><em>Lactobacillus delbrueckii</em> subsp. lactis</td>
<td>Very good (94.4%)</td>
<td>340</td>
</tr>
<tr>
<td>GK62N</td>
<td><em>Lactobacillus delbrueckii</em> subsp. lactis</td>
<td>Very good (92.1%)</td>
<td>180</td>
</tr>
<tr>
<td>GK30</td>
<td>Lactobacillus fermentum</td>
<td>Excellent (98.5%)</td>
<td>360</td>
</tr>
<tr>
<td>GK22r</td>
<td><em>Lactobacillus fermentum</em></td>
<td>Very good (90.2%)</td>
<td>189</td>
</tr>
<tr>
<td>GK6</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>Good (85.7%)</td>
<td>380</td>
</tr>
</tbody>
</table>

were determined as described above.

**Effect of temperature on proteinase activity**

Cell wall extract (1 ml) was incubated with 1 ml of casein solution (4 mg/ml) for 1 h in 50 mM phosphate buffer (pH 7.5) containing 15 mM CaCl₂ at various temperatures: 10 °C, 20 °C, 30 °C, 37 °C, 40 °C and 50 °C. The proteolytic activity was determined as described above.

**Proteinase inhibitors**

Cell-wall extract (1 ml) of culture sampled at the late growth phase were incubated at 37 °C for 4 h with β-casein (1 ml) in 2 ml of 50 mM phosphate buffer (pH 6.0). The proteinase inhibitor was added (final concentration of 10 mM). The inhibitors studied were pepstatin A (PEP A), phenylmethylsulfonylfluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) (all reagents from Sigma-Aldrich Chemie). The remaining proteolytic activity was determined as described above.

**Results and Discussion**

**Screening for proteolytic activity**

The analysis of the proteolytic activity of the strains isolated from kefir grains, revealed that proteinase-producing strains (Prt⁺) are often present. The active colonies (*lactobacilli*, lactococci, leuconostoc and yeasts) were shown to be 60% of the isolated population. From 276 *Lactobacillus* spp. strains, 49 were found to produce proteinases. All these isolates were gram-positive, non-motile, non-spore forming rods, arranged singly, in pairs, or occasionally in short chains. The selected strains were catalase and oxidase negative. These properties showed that the isolates belonged to the genus *Lactobacillus*.

To test the proteolytic activity of isolates towards total casein, whole cells from each strain were incubated on Calcium caseinate agar under optimal pH (7.5) and temperature (37 °C). The results showed that all strains hydrolysed casein. However, they differed in the efficiency of casein hydrolysis. The strains DR22x, DR22r, GK6, GK44eps, GK30, GK62, GK22r formed a large clear zone from 5 to 10 mm around the colonies. The strain DR22x produced the largest and clearly visible zone. These results were confirmed by quantitative determination of the proteolytic activity (Table 1).

The strain DR22x was heterofermentative and according to biochemical and physiological characteristics obtained by API 50 CH system, showed 98.4% similarity to the *Lactobacillus kefir*. This is the first report of proteolytic activity of *L. kefir*.

The strain DR22x exhibited a great increase of the activity in the late exponential growth, corresponding to 530 Ku units (Fig. 1). According to the literature (8) the proteinases of *L. curvatus* and *L. homolochii* showed maximum activity in the early exponential phase. The strain DR22x differed in this property, which may be due to his origin – kefir grains presented too complex media.
Fig. 1. Cell concentration (1) and proteolytic activity (2) measured along the growth curve of *Lactobacillus kefir* DR22x.

Fig. 2. The action of cell-wall-extract (A) on total casein (lane 1) after 4h (lane 2), 8 h (lane 3), and 24 h (lane 4); action of cell-wall-extract (A) on α-casein after 8 h (lane 5) and 24 h (lane 6); action of cell-wall-extract on β-casein after 8 h (lane 7) and 24h (lane 8). The action of whole cells (B) on total casein (lane 1) after 4h (lane 2), 8 h (lane 3), and 24 h (lane 4); action of whole cells (B) on α-casein after 8 h (lane 5) and 24 h (lane 6); action of whole cells (B) on β-casein after 8 h (lane 7) and 24h (lane 8). The action of treated cells (C) on total casein (lane 1) after 4h (lane 2), 8 h (lane 3), and 24 h (lane 4); action of treated cells (C) on α-casein after 8 h (lane 5) and 24h (lane 6); action of treated cells (C) on β-casein after 8 h (lane 7) and 24h (lane 8).

In order to investigate the location of the proteolytic enzymes of *L. kefir* DR22x the release of proteinases from the cell was performed by the method of Tsakalidou. The ability of *L. kefir* DR22x to hydrolyze α-, β- and total casein was tested after induction of the proteinases in MSR supplemented with 5% milk. As shown in Fig. 2 the crude proteinase extract obtained by washing the cells in a Ca²⁺- free buffer, predominantly hydrolyzed β-casein, α-casein and the total casein were weakly hydrolyzed. The same results were obtained when the whole and treated cells were
tested on α-, β- and total casein. As it expected the proteolytic activity of the whole cells was much higher than that of the cell wall extract. The lowest activity was detected when treated cells were used. The results were confirmed by a photometric determination of the free amino groups (data not shown). These results suggested that L. kefir DR22x had cell-wall-bound proteinases with an extracellular location.

**Determination of optimal pH and temperature**

To determine the pH optimum for casein hydrolysis cell wall extract of the strain L. kefir DR22x was incubated at various pH values using β-casein as a substrate. The optimal pH (at 37 °C) appeared to be 7.2 (Fig. 3). In a parallel experiment, a determination of the temperature optimum (at pH 7.2) for strain DR22x was done. The results showed that an optimal digestion of β-casein was achieved at 37 °C (Fig. 4). The final determination of caseinolytic activity was carried out at optimal pH and temperature.

**Effect of proteinase inhibitors**

This work aimed to elucidate the nature of the enzymes responsible for the proteolytic activity present in the cell wall extract. Table 2 shows the remaining proteolytic activity obtained when protease inhibitors were used. EDTA inhibited 27% of the proteolytic activity of the both – cell extract and whole cells. When Pepstatin A was used (as an acidic proteases inhibitor) the remaining proteolytic activity was 72% for
TABLE 2

<table>
<thead>
<tr>
<th>L. kefir DR22x</th>
<th>Without inhibitor</th>
<th>EDTA</th>
<th>PMFS</th>
<th>PEP A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>100</td>
<td>73</td>
<td>5</td>
<td>72</td>
</tr>
<tr>
<td>Whole cells</td>
<td>100</td>
<td>73</td>
<td>8</td>
<td>75</td>
</tr>
</tbody>
</table>

Effect of protease inhibitors on the proteolytic activity of *Lactobacillus kefir* DR22x (results expressed as % of remaining proteolytic activity)

the cell extract and 75% for the whole cells. PMFS (inhibitor of serine proteases) strongly inhibited the crude proteinase (95%) and whole cells (92%). It can be concluded that several proteases determined the proteolytic activity of the strain investigated. The results from this study suggested that the strain DR22x produced proteolytic complex in which serine-type proteases were the main component.

This paper presents an initial attempt to clarify the proteolytic systems of one *Lactobacillus* species, frequently present in kefir grains, namely *L. kefir* DR22x. This strain produced at least one cell-wall-bound proteinase, which resembled to the lactococcal P1-type proteases. The enzyme exhibited similar biochemical properties to those reported previously for lactococcal and lactobacillus enzymes. Further genetic information is necessary to improve our knowledge of its functional properties.

REFERENCES