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Activity, molecular mass and hydrolysis on baker's yeast protein of extracellular proteases from the putative probiotic bacteria *Microbacterium* sp. strain 8L and *Exiguobacterium mexicanum* strain 8N

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Abstract

The bacteria *Microbacterium* sp. 8L and *Exiguobacterium mexicanum* 8N are known to improve the culture of *Artemia franciscana* using baker's yeast as food. Using spectrophotometry, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), substrate-SDS-PAGE and pH-stat *in vitro*-digestibility assays, the activity, molecular mass and hydrolysis on baker's yeast protein of proteases from extracellular polymeric substances (EPS) of the strains 8L and 8N along with the pathogenic strains *Microbacterium* sp. 8R and *Vibrio parahaemolyticus* 588 CECT (Vp) were studied. The EPSs of 8L and 8R showed one activity band, on which the serine inhibitor phenylmethylsulphonyl fluoride (PMSF) had no effect. The EPSs of 8N showed four bands; two were unaffected by PMSF, whereas one was affected, and the other was partially affected. The EPSs of Vp showed two bands, one partially inhibited by PMSF. No inhibitory effects from 1-chloro-3-tosylamido-7-amino-2-heptanone (trypsin inhibitor) were observed in the protease bands of the studied bacteria. The EPSs of 8L and 8N showed a similar degree of hydrolysis (pH-stat). The EPSs of 8L had the lowest Dice index of similarity of yeast protein profiles at 1 h of reaction. We conclude that the strain 8L could benefit *A. franciscana* by providing bacterial proteases for digestion of baker's yeast.

Keywords: *Artemia*, beneficial bacteria, EPS, gnotobiotic

Introduction

Proteases of bacteria associated with the gut lumen of marine crustaceans are believed to play an important role in the nutrition process of their hosts (Donachie & Zdanowski 1998; Ochoa-Solano & Olmos-Soto 2006). The beneficial effects of bacteria in cultures of the brine shrimp of the genus *Artemia* have been explained by their contribution as nutritional supplements, or by competition, where pathogenic bacteria are then excluded (Gorospe, Nakamura, Abe & Higashi 1999; Marques, Dinh, Ioakeimidis, Huys, Swings, Verstraete, Dhont, Sorgeloos & Bossier 2005). The aerobic Gram-positive heterotrophic bacteria *Microbacterium* sp. strain 8L and *Exiguobacterium mexicanum* strain 8N were reported to positively influence the growth, development and survival of *Artemia franciscana* larvae, when both strains (single or mixed) were present in gnotobiotic cultures using baker's yeast *Saccharomyces cerevisae* as food (Orozco-Medina, Maeda-Martínez & López-Cortés 2002). In agnotobiotic (xenic) cultures, the same bacteria supplied either as a single or as a mixed strain also caused significant improvements in the development and survival of *A. franciscana* when fed separately with baker's yeast, corn flour or spirulina (Hipólito-Morales, Maeda-Martínez & Martínez-Díaz 2009).

We hypothesized that some of the mechanisms of the putative probiotic bacteria *Microbacterium* sp. 8L and *E. mexicanum* 8N to produce the beneficial effects on the *A. franciscana* performance are functioning in the intestinal tract by providing additional bacterial

enzymes for digestion of food. Recently, the entrance and the transit in the gut lumen of these bacteria along with baker's yeast were demonstrated in *A. franciscana* larvae. No evidence was recorded that such bacteria adhered or colonized the intestinal epithelium, but they were confined within the chyme in the space limited by the perithrophic membrane (Orozco-Medina, López-Cortés & Maeda-Martínez 2009). The knowledge of extracellular proteases from these bacterial strains is nil. The objectives of our work were to study the activity and the molecular mass of proteases from extracellular polymeric substances (EPSs) of the bacteria *Microbacterium* sp. 8L and *E. mexicanum* 8N, and to determine the degree of hydrolysis (DH%) of their EPSs on proteins of baker's yeast, a common feed used in gnotobiotic and agnotobiotic cultures of *A. franciscana*. For comparative purposes, the pathogenic bacteria *Microbacterium* sp. strain 8R (originally isolated from *A. franciscana* cysts) and *Vibrio parahaemolyticus* (see Orozco-Medina *et al.* 2002) were also included in the analyses. The presence and enzymatic activity of proteases from the EPSs were first studied using spectrophotometry and substrate-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of proteases revealed in zymograms and proteases with trypsin- and serine-like activities were determined through substrate-SDS-PAGE and finally the DH% of the EPSs on baker's yeast protein was studied through pH-stat multi-enzymatic *in vitro* digestibility (pH-stat) assays, whose hydrolysis products were monitored by SDS-PAGE.

Material and methods

Extraction of bacterial extracellular-polymeric substances (EPSs)

Pure cultures of *E. mexicanum* strain 8N (DSM 16483^{T}, Mascheroder Weg 1b, Braunschweig, Germany) (López-Cortés, Schumann, Pukall & Stackebrandt 2006), *Microbacterium* sp. strain 8L (DSM 16485^{T}), *Microbacterium* sp. strain 8R and *V. parahaemolyticus* strain 588 CECT (Orozco-Medina *et al.* 2002) were grown in marine broth 2216 (DIFCO, Becton, Dickinson and company Sparks, MD, USA), incubated at 30 °C for 24 h at 100 rpm agitation. The bacterial concentration in the stationary phase of growth was determined by the optical density of the medium at 580 nm (Orozco-Medina *et al.* 2002). Sterile marine broth 2216 was used as control. To obtain the EPSs from each strain and control, the corresponding bacterial culture was agitated for 1 min in a vortex mixer and centrifuged at 6000 *g* for 15 min at 2 °C. The supernatant containing the EPSs was recovered and the remaining bacterial cells in the supernatant were filtered using a 0.2-μm pore-size Whatman cellulose nitrate membrane 7182-004. The EPSs were concentrated 10-fold using a stirred purifier and concentrator of macromolecules in solution (Millipore Ultrafiltrator 8050, Billerica, MA, USA) and a regenerated cellulose membrane (Millipore YM10) of 10-kDa molecular mass cut-off. The concentrated EPS solution was used as an enzymatic extract. All enzymatic extracts were used ice cold and stored at − 56 °C.

Proteolytic activity of the bacterial EPSs on azocasein

The soluble protein in the concentrated EPS solutions was measured by the colorimetric protein assay based on the Bradford (1976) method, microplate-adapted, in four replicates of each bacterial strain culture. Briefly, 20-μL EPS, 30-μL distilled water and 250 μL of the Bradford reagent were poured into a 96-well microplate and the absorbance was recorded at 595 nm. Bovine serum albumin (Sigma A7517, St Louis, MO, USA) at 1 mg mL^{-1} was used as the standard. The total proteolytic activity of the EPSs was estimated using azocasein as substrate (García-Carreño, Dimes & Haard 1993). In brief, 5 μL of the enzyme preparation was incubated in 500-μL 0.5% azocasein as substrate in 50 mM TRIS · HCl buffer at pH 7.5 for 10 min at 25 °C. The reaction was stopped with 500-μL 20% w/v trichloroacetic acid (TCA) solution. Samples were incubated for 10 min at 0 °C and centrifuged at 10 000 *g* for 5 min and the absorbance of the supernatant was recorded at 366 nm. For controls, TCA was added before the substrate. One unit of activity (U) is the absorbance at 366 nm min^{-1} mg protein^{-1} in the crude extract of the EPS (García-Carreño *et al.* 1993).

Substrate-SDS-PAGE of the bacterial EPSs

The profile of proteases present in the bacterial EPSs was revealed twice in 15% substrate-SDS-PAGE with casein as substrate (García-Carreño *et al.* 1993). The electrophoresis was carried out, as described by Laemmli (1970), in a vertical electrophoresis unit (model SE 260, Hoefer, San Francisco, CA, USA) with constant current (15 mA gel^{-1}) at 4 °C using a cold

circulation bath. To determine the presence of trypsin and serine proteases in the extracts, two specific inhibitors were used, 10 mM 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) and 100 mM phenylmethyl sulphonyl fluoride (PMSF) dissolved in isopropyl alcohol. Mixtures of 25 μL of the EPSs and 7.5 μL of the inhibitor, or distilled water for the control, were incubated for 1 h at 25 °C. The mixture was then combined with loading buffer (1:1) before applying it to the gel (García-Carreño *et al.* 1993). After electrophoresis, the gels were immersed in 3% casein solution (Sigma C7080) in 50 mM TRIS · HCl buffer at pH 7.5, placed in an ice bath for 30 min under orbital agitation at 50 rpm and then incubated for 2 h at 25 °C. The gels were then washed and immersed overnight in staining solution containing 40% methanol, 7% acetic acid and 0.5% Coomassie Brilliant Blue R-250. The excess stain was removed with a solution containing 40% methanol and 7% acetic acid. The proteolytic activity of the enzymes was revealed in the zymograms as bands of casein degradation over a blue background, and the molecular mass of the enzymes were estimated using molecular mass markers of proteins (Sigma SDS7) (García-Carreño *et al.* 1993).

pH-stat multienzymatic *in vitro*-digestibility assays

The DH% of baker's yeast protein by the EPSs of the bacteria *Microbacterium* sp. 8L, *E. mexicanum* 8N, *Microbacterium* sp. 8R, *V. parahaemolyticus* 588 CECT and the mixture of *Microbacterium* 8L and *E. mexicanum* 8N (treatments 8L, 8N, 8R, VP and 8L+8N) was evaluated by the pH-stat method (Pedersen & Eggum 1983; Ezquerra, García-Carreño, Civera & Haard 1997; Lemos, Navarrete del Toro, Córdova-Murueta & García-Carreño 2004). The mixture of the strains 8L+8N was used to determine a possible synergistic activity in the hydrolysis of the baker's yeast proteins. The DH% of autoclaved baker's yeast protein by the enzymes present in the EPSs was determined using a 718 Stat Titrino (Metrohom Ion Analysis, Switzerland) with a computer interface (METRODATA MENU COMPUTER PROGRAM 718 STAT TITRINOPC). An appropriate amount of autoclaved baker's yeast dissolved in distilled water to yield 0.08 g of protein was placed in the hydrolysis vessel. The total reaction mass (substrate+water+EPSs) was adjusted to reach 10 g. The pH of this mixture was adjusted to 7.9 with 1 M NaOH and stirred for 1 h to obtain complete solubility of protein and stabilization of the pH. Before starting the reaction, the pH was raised to 8.0 automatically by the pH-stat system by adding 0.1 M NaOH. Four repetitions of each treatment were made. The reaction mixture was maintained at 30 °C for 1 h using a jacketed reaction vessel and a circulating water bath. The software 718 STAT TITRINOPC was used to determine the DH% of protein based on the algorithm described by Adler-Nissen (1982). The DH% of autoclaved baker's yeast without addition of the EPSs was used as a control using the procedure described above.

SDS-PAGE of hydrolysis products

The products of the hydrolysis from the pH-stat essays were monitored for 1 h by SDS-PAGE. Subsamples (25-μL each) taken during the hydrolysis process containing 20–30 μg of protein were combined with 25 μL of loading buffer. The samples were then loaded onto a 15% SDS-PAGE and stained as described previously. The protein bands obtained in the SDS-PAGE were analysed using the GELCOMPAR II™ ver. 4.6 software. The Dice coefficient, a heuristic index that determines similarity of absence–presence data, was used to determine quantitative differences of the number and distribution of protein bands of each treatment revealed in the gels, where a Dice index value of 1 represents equal band profiles. The determination of the Dice index was done using the PAST ver. 1.67 software (http://folk.uio.no/ohammer/past/).

Statistics

The results of bacterial counts, soluble protein content of the EPSs, DH% and Dice index of protein bands in SDS-PAGE were analysed by a nonparametric Kruskal–Wallis one-way analysis of variance of four repetitions using the programme STATISTICA™ ver. 7.0, STATSOFT.

Results

Activity and molecular mass of bacterial extracellular proteases

The values of enzymatic activity on azocasein of the bacterial EPSs are shown in Table 1. Bacterial concentrations between 4.1×10^{10} and 9.1×10^{14} of bacteria mL^{-1} showed a soluble protein content from 0.94 to 3.08 mg mL^{-1} of EPSs. The EPSs of *E. mexicanum* 8N and *V. parahaemolyticus* 588 CECT had the highest protein contents (2.80 and

Table 1 Concentration of bacteria, protein content of the bacterial extracellular polymeric substances (EPSs), total enzymatic activity on azocasein, and degree of hydrolysis (DH%) of baker's yeast protein of EPSs of the treatments: *Microbacterium* sp. strain 8L, *Exiguobacterium mexicanum* strain 8N, *Microbacterium* sp. strain 8R and *Vibrio parahaemolyticus* strain 588 CECT (VP)

Treatment	Bacteria mL^{-1}	mg protein mL^{-1} of EPSs	Activity (U)*	DH%
8L	4.1×10^{10} (2.2×10^{9})^{a}	1.30 (0.05)^{b}	3.05 (0.06)^{c}	15.4 (1.99)^{b}
8N	3.3×10^{13} (4.7×0^{12})^{c}	2.80 (0.09)^{c}	0.10 (0.09)^{a}	16.5 (2.02)^{b}
8R	6.3×10^{11} (7.4×10^{10})^{b}	0.94 (0.05)^{a}	1.91 (0.07)^{b}	11.2 (0.90)^{a}
VP	9.1×10^{14} (5.5×10^{14})^{d}	3.08 (0.10)^{d}	0.17 (0.03)^{a}	29.3 (4.13)^{c}
8L+8N				27.8 (4.83)^{c}

*One unit of activity (U) is the absorbance at 366 nm min^{-1} mg protein^{-1} in the crude extract of the EPS (García-Carreño *et al.* 1993). Treatment 8L+8N was a mixture (1:1) of the EPSs from *Microbacterium* sp. strain 8L and *E. mexicanum* strain 8N.
Values in parenthesis are standard deviations of $n = 4$. Different superscript letters in a column indicate significant differences between ranks: a < b < c < d at $P < 0.05$ (Kruskal–Wallis test).

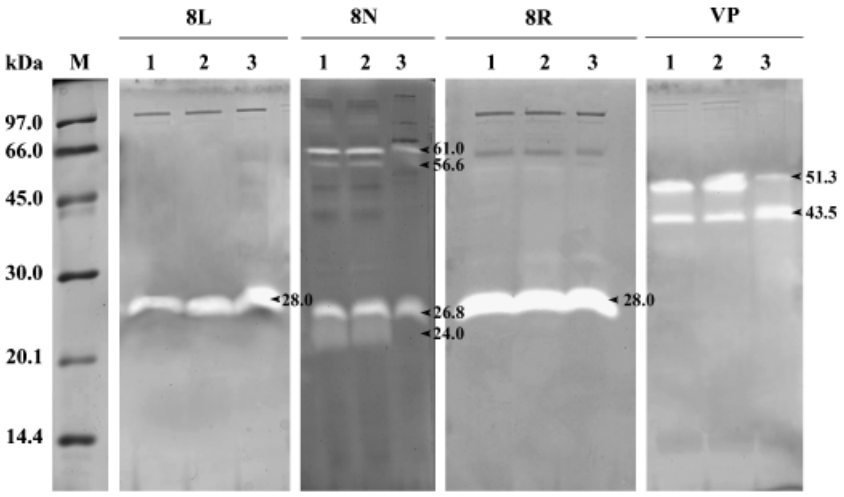

Figure 1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis-casein substrate with enzymatic extracts of extracellular polymeric substances from *Microbacterium* sp. strain 8L (treatment 8L), *Exiguobacterium mexicanum* strain 8N (8N), *Microbacterium* sp. strain 8R (8R) and *Vibrio parahaemolyticus* strain 588 CECT (VP). (1) Without inhibitor, (2) With 1-chloro-3-tosylamido-7-amino-2-heptanone inhibitor for trypsins and (3) With phenylmethylsulphonyl fluoride inhibitor for serine proteases. M, molecular mass markers.

3.08 mg mL^{-1}) at the highest bacterial concentrations ($P < 0.05$), but had the lowest enzymatic activity (0. 10 and 0.17 U) ($P < 0.05$). The EPS from *Microbacterium* sp. 8L showed the highest enzymatic activity (3.05 U) at the lowest bacterial concentration ($P < 0.05$) (Table 1).

Zymograms showed one activity band of 28 kDa for the EPSs of the strains *Microbacterium* sp. 8L and *Microbacterium* sp. 8R. No inhibitory effects were observed when incubated with PMSF (an inhibitor of serine proteases) (Fig. 1). The EPSs from *E. mexicanum* 8N showed protease bands of 24.0-, 26.8-, 56.6- and 61.0 kDa respectively. When incubated with PMSF, the 24.0-kDa band was inhibited, the 56.6-kDa band was partially inhibited and the other two were unaffected (Fig. 1). The EPS from *V. parahaemolyticus* 588 CECT had two activity bands of 43.5- and 51.3 kDa respectively, with the latter partially inhibited by the PMSF (Fig. 1). No inhibitory effects caused by TLCK (an inhibitor of trypsin) were observed in all protease bands of the studied bacteria.

Hydrolysis of baker's yeast protein by the bacterial EPSs

The DH% of the bacterial EPSs on baker's yeast protein is shown in Table 1. The highest values were

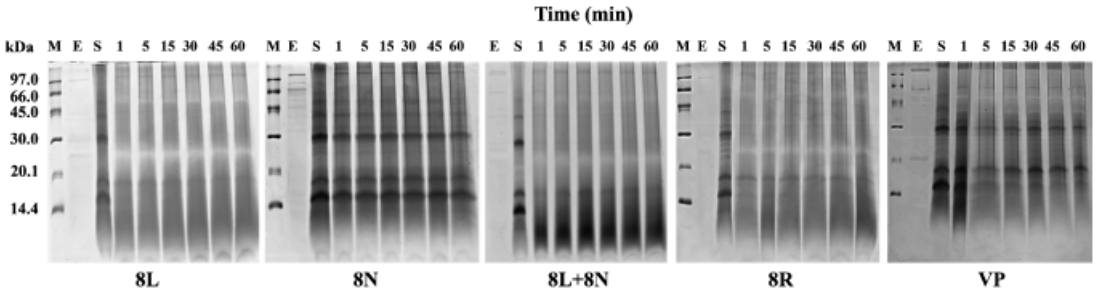

Figure 2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis profiles of baker's yeast protein incubated with enzymatic extracts of extracellular polymeric substances from *Microbacterium* sp. strain 8L (treatment 8L), *Exiguobacterium mexicanum* strain 8N (8N), *Microbacterium* sp. strain 8R (8R), the mix of *Microbacterium* sp. 8L and *E. mexicanum* 8N (8L+8N) and *Vibrio parahaemolyticus* strain 588 CECT (VP) at 1-, 5-, 15-, 30-, 45- and 60 min of hydrolysis *in vitro* by pH-stat titration. M, molecular mass markers; E, extracellular polymeric substances (EPSs); S, Baker's yeast protein substrate.

Table 2 Dice index of similarity of protein profiles of the SDS-PAGE of baker's yeast protein incubated with extracellular polymeric substances (EPSs) from the bacteria *Microbacterium* sp. strain 8L, *Exiguobacterium mexicanum* strain 8N, *Microbacterium* sp. strain 8R, and *Vibrio parahaemolyticus* strain 588 CECT (VP) and a mixture of EPSs from *Microbacterium* sp. 8L and *Exiguobacterium mexicanum* 8N (8L+8N)

8L	8N	8R	VP	8L+8N
0.11 (0.03)[a]	0.8 (0.05)[d]	0.21 (0.04)[b]	0.5 (0.02)[c]	0.14 (0.04)[a,b]

Values in parenthesis are standard deviations of $n = 4$. Different superscript letters indicate significant difference between ranks: a < b < c < d at $P < 0.05$ (Kruskal–Wallis test).
SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

obtained with the EPS from *V. parahaemolyticus* 588 CECT and with the mix of EPSs from *Microbacterium* sp. 8L and *E. mexicanum* 8N, showing DH% of 29.3% and 27.8% ($P < 0.05$) respectively. The EPSs of *Microbacterium* sp. 8L and *E. mexicanum* 8N showed no differences in the DH% to hydrolyse the autoclaved baker's yeast, with values of 15.4% and 16.5% ($P > 0.05$) respectively.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the products from the pH-stat assays showed hydrolysis of baker's yeast protein from 1 to 5 min of reaction time in all treatments, except for the EPS of *E. mexicanum* 8N that remained almost unaltered (Fig. 2). The reactions containing the EPSs from *Microbacterium* sp. 8L, *Microbacterium* sp. 8R and the mix of EPSs from *Microbacterium* sp. 8L and *E. mexicanum* 8N (8L+8N) showed protein hydrolysis from the first minute of reaction, observed as a reduction in the number and intensity of the protein bands in the gels. The Dice indexes of similarity of protein profiles incubated with the bacterial EPSs are shown in Table 2. The treatment with the EPS of *Microbacterium* sp. 8L had the lowest Dice index of 0.11 at 1 h of reaction in the pH-stat ($P < 0.05$), whereas the EPS of *E. mexicanum* 8N had the lowest ability to digest the baker's yeast protein bands of the gels with a Dice index of 0.8 in the same reaction time ($P < 0.05$). The ability of baker's yeast protein digestion of the treatment with EPSs from *Microbacterium* sp. 8L+*E. mexicanum* 8N had a Dice index of 0.14, which was similar to those from the EPSs of *Microbacterium* sp. 8L and *Microbacterium* sp. 8R ($P > 0.05$). The EPS of the pathogen *V. parahaemolyticus* 588 CECT showed a low ability to hydrolyse the baker's yeast protein bands of the gels, with a Dice index of 0.5 (Table 2).

Discussion

Activity and molecular mass of bacterial extracellular proteases

The protease activity observed in the zymograms (substrate-SDS-PAGE) with EPS extracts of the two strains of *Microbacterium* sp. (8L and 8R) had an activity band of 28.0 kDa that was not a serine- or

trypsin-like protease as shown by the negative influence of the inhibitors TLCK and PMSF (Fig. 1). Extracellular non-serine proteases from the genera *Microbacterium* have been reported by Kanayama and Sakai (2005), who characterized from *Microbacterium liquefasciens* a metalloendopeptidase (EC 3.4.24) of 21 kDa, likely a Zn-dependent protein not inhibited by 20 mM PMSF. Although it degrades gelatin it is not a gelatinase (EC 3.4.24.24, EC 3.4.24.35). Thys and Brandelli (2006) reported from *Microbacterium* sp. strain Kr10, a 42 kDa enzyme with keratinolytic activity and assumed that it was a metalloendopeptidase because it was inhibited by 5 mM EDTA and 1 mM 1, 10-phenanthroline, but not by 5 mM PMSF. Gessesse and Gashe (1997) reported for *Microbacterium* sp. AR-68, isolated from an alkaline-soda lake, the production of a large amount of alkaline proteases, apparently of the serine protease type, because they showed a reduced residual enzymatic activity in the presence of 4% 1 mM PMSF.

A presumptive serine-like protease produced by *E. mexicanum* 8N had a molecular mass of 24 kDa, which is in the range reported for the bacterial serine-alkaline proteases of 15–30 kDa (Rao, Tanksale, Ghatge & Deshpande 1998). This group of proteases is produced by several Gram-positive bacteria (Rao *et al.* 1998; Kumar, Joo, Koo, Paik & Chang 2004) and is active at a high alkaline pH (Rao *et al.* 1998). The other proteases from the strain 8N, with molecular masses of 56.6- and 61.0 kDa respectively, are reported for the first time for the genus *Exiguobacterium*.

Previous studies on extracellular proteases of *V. parahaemolyticus* have shown these enzymes to be chymotrypsin-like and collagenases, generally studied for their pathogenic properties (Ishihara, Kawanishi, Watanabe, Tomochika, Miyoshi & Shinoda 2002; Lee, Cheng, Yu & Pan 2002; Aguirre-Guzmán, Mejía-Ruíz & Ascencio 2004; Miyoshi, Nitanda, Fujii, Kawahara, Li, Maehara, Ramamurthy, Takeda & Shinoda 2008). The extracellular proteases observed in this work from the EPS of *V. parahaemolyticus* 588 CECT had molecular masses of 43.5- and 51.3 kDa respectively, the latter a probable serine-like protease because of its partial inhibition with PMSF (Fig. 1). Their molecular masses are similar to the enzymes already reported for *V. parahaemolyticus* (Ishihara *et al.* 2002; Lee *et al.* 2002). An extracellular monomeric protein of 43 kDa designated as protease A from *V. parahaemolyticus* No. 93 was inhibited by PMSF (at concentrations of 1- and 10 mM respecively) and by soybean trypsin inhibitor (0.1 mg mL^{-1}) (Lee *et al.* 2002). Although protease A was believed to be a serine protease, it was inhibited by the metal chelator 1, 10 phenanthroline at 20 mM, but its activity was not restored by addition of divalent metal ions such Ca^{2+} and Zn^{2+}, as shown by metalloproteases (Lee *et al.* 2002). An extracellular protease of 50 kDa measured by SDS-PAGE (designated as VPP1) was obtained from the pathogenic strain *V. parahaemolyticus* KX-V137 (Ishihara *et al.* 2002). The purified protease VPP1 was a calcium-dependent serine protease because it was inhibited by the calcium chelators EDTA and EGTA (1 mM) and also by the serine protease inhibitors chymostatin and PMSF (Ishihara *et al.* 2002). Miyoshi *et al.* (2008) recorded the same protease VPP1 in the strain *V. parahaemolyticus* V98-401 and also noted that it is a chymotrypsin-like protease based on results of inhibition on chymostatin. However, Miyoshi *et al.* (2008) suggested that the protease A of 43 kDa isolated by Lee *et al.* (2002) and the VPP1 protease of 50 kDa reported by Ishihara *et al.* (2002) were the same, based on the comparison of N-terminal amino acid sequences and the responses to the inhibitors.

Hydrolysis of baker's yeast protein by the bacterial EPSs

The DH% of baker's yeast protein by the EPSs from all the bacterial strains tested was followed in SDS-PAGE (Fig. 2) during 1 h of reaction. The enzymes present in the EPSs had different specificities to the substrate and the protein bands were hydrolysed at different levels. The EPSs of *Microbacterium* sp. 8L and *E. mexicanum* 8N had no difference in their DH% to hydrolyse the autoclaved baker's yeast (Table 1); however the EPSs of these bacterial strains showed significant differences in their proteolytic activity on azocaseine (Table 1) and in their Dice index (Table 2). The pH-stat method detects changes in the pH. These changes are expected to be caused by the increase of protons (H^+) liberated during the hydrolysis of the peptide bonds and are compensated for by the addition of NaOH. The volume of alkali added is used to calculate the DH% (Pedersen & Eggum 1983). Thus, in the reaction mixture (substrate+enzymes) in the treatment *E. mexicanum* 8N, it is probable that besides the proteolysis, other reactions such as the hydrolysis by glycosylases increased the acidity of the reaction solution. It is known that *E. mexicanum* 8N can hydrolyse *N*-acetyl glucosamine (López-Cortés *et al.* 2006), which is present as a structural compound of

the cell wall of the baker's yeast *Saccharomyces cerevisiae* (Aguilar-Uscanda & François 2003).

Other species of the same genera analysed in this work have also been related to the function of the intestinal tract of marine animals. Ringo, Sperstad, Kraugerud and Krogdahl (2008) reported *Microbacterium oxidans* OUZ46 and *Exiguobacterium artemiae* 9AN associated with the gut lumen of the marine fish *Salmo salar*. *Microbacterium oxidans* OUZ46 was recovered when the fish were fed with feed containing purified soy-nonstarch polysaccharides. In comparison, *E. artemiae* 9AN was recovered from fish fed with a fish-meal feed containing a negligible level of nonstarch polysaccharides. Nishino and Nakayama (2006) reported bacteria of the genus *Microbacterium* as part of a bacterial consortium useful to produce free amino acids. Donachie and Zdanowski (1998) reported that heterotrophic bacteria isolated from the gut lumen of the marine crustacean *Euphasia superba* had significantly more activity of lipase (C14), trypsin-like substances and acid phosphatase than the same bacteria in seawater, as determined by the Api Zym system™ (bioMérieux, MO, USA). According to these authors, their results support the concept that the bacterial metabolic activity participates in the digestion of dietary components of *E. superba*. We conclude that the results of our study on the SDS-PAGE of the hydrolysis products (Dice index) support the hypothesis that at least the putative probiotic *Microbacterium* sp. 8L could benefit *A. franciscana* by providing additional bacterial proteases for digestion of baker's yeast protein.

Acknowledgments

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