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Short communication

Low-cost purification of nisin from milk whey to a highly active product

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A B S T R A C T

Nisin is a natural peptide used as a preservative in a variety of food products, in which it inhibits mainly Gram-positive bacterial growth, including multidrug-resistant pathogens. However, its application range depends on the cost-effective production and purification of this molecule. Our group has previously produced nisin by *Lactococcus lactis* cultivation in milk whey, which is an industrial residue from dairy production. To our knowledge, no report used milk whey as a culture medium, although several investigators have purified nisin using different techniques. We thus aimed to establish a low-cost purification of nisin obtained by this process. Samples were diluted in ammonium sulphate, applied onto HIC columns (butyl sepharose CL 4B matrix), and eluted with Milli-Q water or PBS. Elution fractions were monitored for protein content and nisin antibacterial activity. Water elution resulted in purification factor values (270, commercial nisin; 775, nisin produced in-house) higher than those obtained with PBS elution. We concluded that purification of nisin does not require precipitation with ammonium sulphate, therefore allowing step/cost reduction. Moreover, purification from milk whey using HIC provides nisin with high activity and low salt content, which can further be applied to a variety of areas.

Keywords: Nisin; Hydrophobic interaction chromatography (HIC); Milk whey; Purification; Lactococcus lactis; Antimicrobial activity

1. Introduction

Certain *Lactococcus lactis* strains produce the antimicrobial peptide nisin as a response to the presence of competitive bacteria, including their spores (Delves-Broughton et al., 1996). Nisin has structure variants due to point mutation that changes its 34 amino acid chain (Field et al., 2008). Regardless of the variant, it can conserve biological products by preventing contamination without toxicity. This property has guaranteed its use in food industry as an established preservative (De Arauz et al., 2009).

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Nisin advantages reached both experimental and commercial frontlines of pharmaceutical, veterinary, and health-care products (De Arauz et al., 2009; Delves-Broughton et al., 1996; Liu et al., 2004; Ukuku and Fett, 2004; Sakamoto et al., 2001; Turner et al., 2004; Aranha et al., 2004; Von Staszewski and Jagus, 2008). The extent of applications requires different levels of product purity, which are achieved by several methods including ion exchange, immunoaffinity chromatography, capillary electrophoresis, ammonium sulfate precipitation, and liquid-liquid and organic solvent extraction (Cheigh et al., 2004; Prioult et al., 2000; Suarez et al., 1997; Yang et al., 1992; Taylor et al., 2007; Jozala et al., 2008; Abts et al., 2011; Espitia et al., 2012).

Hydrophobic interaction chromatography (HIC) is another widespread technique for purification of biomolecules, including nisin (Mahn et al., 2005; Passarinha et al., 2007; Josic et al., 2012). The advantage of HIC for protein purification comes from its ability to separate closely related variants (Zolodz et al., 2010). In addition, columns can be packed with a variety of wide range of media by varying the base matrices and the chemical nature of the ligands, which allows variation in hydrophobicity and consequent protein selectivity (Perkins et al., 1997).

Our research group developed an efficient protocol to produce nisin from *L. lactis*, with whey milk as culture medium (Jozala et al., 2007). This by-product from dairy industry is a low-cost alternative for industrial production, which can provide high yields of this antimicrobial agent. To our knowledge, there are no reports on nisin purification from this medium. In fact, all methods described for nisin purification used complex formulated media, mostly Man, Rogosa, and Sharpe (MRS) medium. The aim of the present work was to purify produced nisin through hydrophobic interaction chromatography (HIC), based on purification profiles of commercial nisin.

2. Materials and methods

2.1. Bacterial strains and media

L. lactis (ATCC 11454) and *Lactobacillus sakei* (ATCC 15521) strains were stored in MRS broth (Difco, Detroit, MI, USA), and supplemented with 40% glycerol at −80 °C (Jozala et al. 2007). *L. sakei*, the nisin-sensitive strain, was grown in MRS broth or agar (Difco, Detroit, MI, USA). *L. lactis*, the nisin-producing strain, was cultivated in milk whey (pH 6.8, kindly provided by a local dairy plant, Brazil).

2.2. Standard nisin

Commercial nisin (2.5%, with sodium chloride and denatured milk solids, Sigma) was used as a standard during purification.

2.3. Nisin production

Initially, a frozen aliquot of *L. lactis* (10^8 CFU) was incubated in MRS broth (50 mL; shaker, 100 rpm, 36 h, 30 °C). An aliquot (5 mL) from this pre-culture was transferred to milk whey (50 mL) and incubated (shaker, 100 rpm, 36 h, 30 °C). This culture was sub-cultivated five times, always inoculating aliquots (5 mL) from the previous culture in fresh milk whey (50 mL), and incubated in the same conditions (100 rpm, 36 h, 30 °C). The process was monitored for microorganism contamination (colony morphology and Gram staining) and nisin activity. The last sub-culture was centrifuged (13,200 × *g*, 10 min, and 10 °C), and the supernatant collected and sterile filtered (22-μm pore diameter, Millipore). The sterile solution was called "nisin produced in-house" and stored (4 °C) for further purification.

2.4. Agar diffusion assay for determination of nisin activity

Nisin activity was determined by agar diffusion assay, using *L. sakei* as nisin-sensitive microorganism. Serial dilutions of commercial nisin were utilized to construct the standard curve (10–10^5 Arbitrary Units (AU/mL)), as previously described elsewhere (Arauz et al., 2011). Briefly, *L. sakei* was grown in MRS broth (shaker, 100 rpm, 30 °C, 24 h); an aliquot from this culture was diluted in MRS agar and plated in Petri dishes (10^6 UFC/dish). After agar solidification, 3-mm wells were caved. Samples and standard were independently transferred to wells (50 μL/well) and incubated (4 °C, 12 h; 30 °C, 24 h). After incubation, diameter of growth inhibition zones was determined as the average of four independent measurements. Diameters (mm) determined for commercial nisin were related to the standard curve (AU/mL) (Fig. 1).

2.5. Protein quantification

After elution, the samples were spectrophotometrically monitored (280 nm, Molecular Devices, LLC, USA) for protein content. Samples with nisin activity were quantified by the bicinchoninic acid assay (QuantiPro™ BCA Assay Kit, Sigma) according to the manufacturer's protocol.

2.6. Purification of nisin by hydrophobic interaction chromatography

Commercial nisin was previously diluted in phosphate-buffered saline (0.1 M, pH 7.2, PBS) or Milli-Q water (100 mg/mL, 4 $\log_{10}$ AU/mL), whereas nisin produced in-house (4982 μg/mL of total protein; 4 $\log_{10}$ AU/mL) was not diluted. To guarantee the hydrophobic interaction, ammonium sulphate was added to the samples in sufficient amount to achieve 2 M of final concentration. From these solutions, a sample (3 mL) was loaded onto the column. A column (10-mm diameter, 10-cm length) was packed with butyl sepharose CL 4B matrix (5 mL; GE Healthcare, Uppsala, Sweden). It was equilibrated using 3–4 column volumes (1 mL/min) of ammonium sulphate (2 M) in PBS (first profile) or water (second profile). Diluted samples were sequentially eluted with 3 column volumes of (a) 2 M ammonium sulphate, (b) 1 M ammonium sulphate and (c) solvent (PBS or water).

Elution was performed at a 1 mL/min flow rate and each collected fraction contained 1 mL. Fractions were monitored by absorbance (280 nm), protein content, and nisin activity.

2.7. Electrophoresis

Eluted samples that exhibited nisin activity were analyzed by SDS-PAGE (gradient precast gel 4–20%, Bio-Rad, USA) and compared with non-purified commercial nisin. The gel was stained using the silver stain kit (Bio-Rad, USA). Kaleidoscope polypeptide standard (Bio-Rad, USA) was used as molecular weight standard.

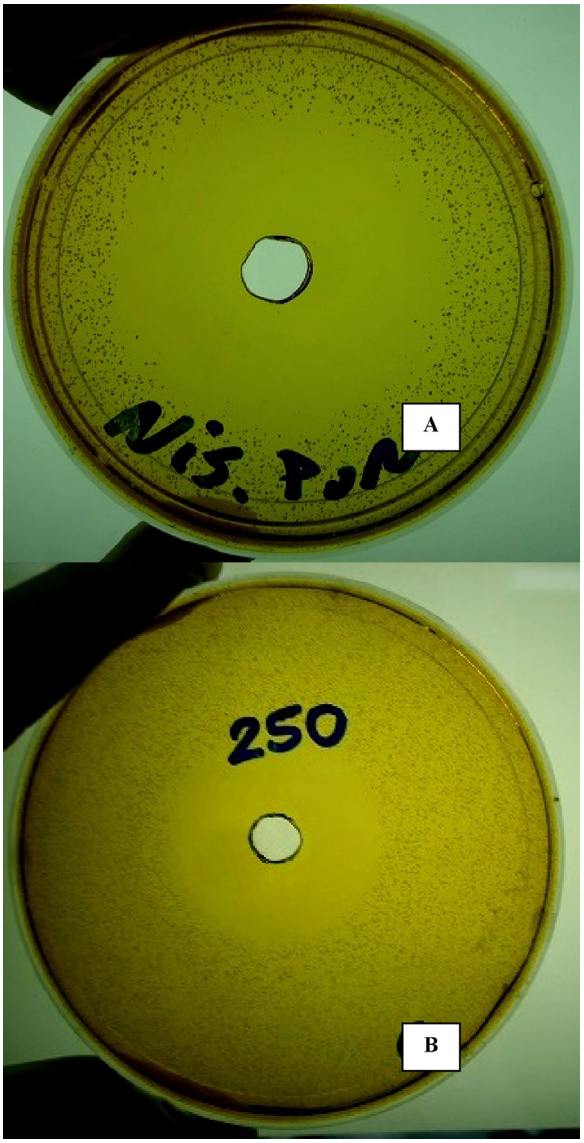

Fig. 1 – Agar diffusion assay of the nisin samples. (A) Commercial nisin activity purified fraction and (B) commercial nisin activity in crude. The diameter of growth inhibition zones was determined as the average of four independent measurements

2.8. *Statistical and mathematical analysis*

Standard curves (log AU × halo diameter) were analyzed by linear regression analysis ($R^2 > 0.9$). Experimental AU values were calculated using the following equation:

$$\text{AU/mL} = 10^{[(0.1423 \times \text{halo diameter}) + 0.1035]} \quad (1)$$

Yield (Y) and purification factor (PF) were calculated using the following equations:

$$\text{PF} = \frac{\text{AU}_{\text{final}}/\text{mg total protein}_{\text{final}}}{\text{AU}_{\text{initial}}/\text{mg total protein}_{\text{initial}}} \quad (2)$$

$$\text{Y} = 100 \times \frac{\text{AU}_{\text{final}} \times \text{V}_{\text{final}}}{\text{AU}_{\text{initial}} \times \text{V}_{\text{initial}}} \quad (3)$$

3. Results and discussion

3.1. *Nisin production/analysis*

We produced nisin from sweet whey, as described above, and labeled it as "nisin produced in-house". As expected, no cross-contamination was detected. Nisin produced in-house (4982 μg/mL total protein; 4 $\log_{10}$ AU/mL) was mixed with ammonium sulphate (1 or 2 M) and tested for antimicrobial activity and protein content after 24 h. Addition of ammonium sulphate did not change the parameters evaluated over time ($p < 0.05$, *T*-test).

Nisin activity is generally measured by the method used in this paper, while protein content determination of peptides varied greatly. Protein content was first measured using the Bradford method, but we did not obtain reproducible results when testing samples with low protein/high nisin content (data not shown). The variability might be due to nisin size (3.4 kDa), which is close to Bradford lower limit (3 kDa), or/and nisin amino acid sequence, which has only five basic groups to react with the dye. On the contrary, BCA has an expanded lower limit (2 kDa) and reacts with peptide bonds at higher temperatures. Use of BCA in our experiments improved nisin detection and allowed reproducible results.

3.2. *Nisin elution profiles*

Commercial nisin was diluted in water and loaded onto a HIC column (Fig. 2A). In the first step, the eluate (2 M ammonium sulphate) contained the highest protein content, although without nisin activity. In the following step, the eluate (1 M ammonium sulphate) contained a low amount of protein. Nisin activity was detected only in the water elution step (Fig. 2A, samples 35–40), in which protein content was lower than in the previous steps. Although elution of commercial nisin with PBS showed a similar profile (Fig. 2B), a decrease was observed in the number of samples with detectable activity (Fig. 2B, samples 36–39).

Regardless of the eluent (PBS or water), nisin was selectively eluted in the step without ammonium sulphate. The second step (elution with 1 M $(NH_4)_2SO_4$) of commercial nisin purification yielded low protein content without nisin activity. Therefore, we decided to suppress this step and perform a 2-step protocol to purify the nisin produced in-house (elution with 2 M $(NH_4)_2SO_4$ and water or PBS).

Elution of nisin produced in-house exhibited the same behavior observed with the commercial one; nisin was selectively eluted in the step without ammonium sulphate. A peak with high protein content was recovered in the first step (2 M ammonium sulphate, with water or PBS), but no nisin activity was found. Most samples collected in the last step were highly active (Fig. 3A and B), and some of them even showed increased activity around 1 log after purification.

Nisin was previously purified (produced in MRS) by HIC, in which it was also recovered in an eluent without ammonium sulphate (Gujarathi et al., 2008). However, conditions used in this work are different from the published literature.

3.3. *Purification factor and yield*

Nisin produced in-house showed the highest values for purification factor (PF) 774 for water elution and 384 for PBS elution,

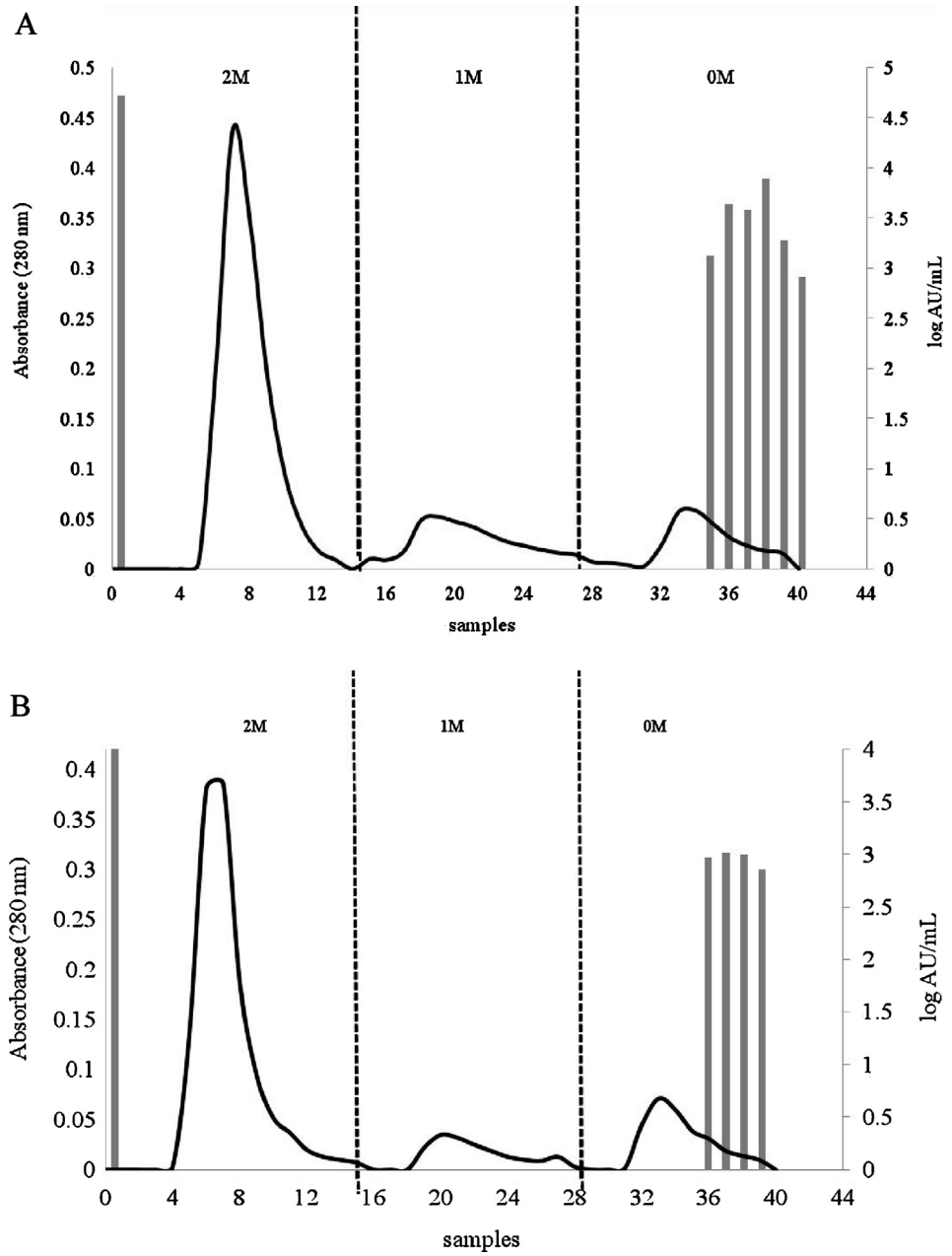

Fig. 2 – Chromatograms of commercial nisin purification by HIC. Black line represents the chromatogram profile recorded at 280 nm (non-bound and bound proteins). Gray solid bars represent nisin activity (Log AU). (A) Commercial nisin, water elution. (2) Commercial nisin, PBS elution.

3 times higher than PF from commercial nisin it was 268 for water elution and 135 for PBS elution (Table 1).

Differences between nisin formulations might explain the discrepancy between yield values for the commercial and in-house samples: the commercial one contains sodium chloride, whereas nisin produced in-house was not supplemented with salt. Sodium chloride contributes to lipid oxidation (Ladikos and Lougovois, 1990) and might increase

Table 1 – Specific activity, yield and purification factor of nisin after HIC purification.

Samples[a]	Nisin activity (AU/mL)	Protein content (μg/mL)	Specific activity (AU/mg)	Yield (%)	Purification factor (PF)
Commercial nisin					
Initial	51,767.18	10, 000	5.18	100	1
Water elution	3309.97	2.39	1384.92	6.39	267.53
PBS elution	926.57	1.33	697.19	1.79	134.68
Nisin produced in-house					
Initial	3837.16	4982.43	0.77	100	1
Water elution	10,931.35	18.35	595.71	284.88	773.52
PBS elution	5833.14	19.73	295.72	152.02	383.99

[a] Samples were analyzed before (initial) and after purification with water or PBS as the eluent.

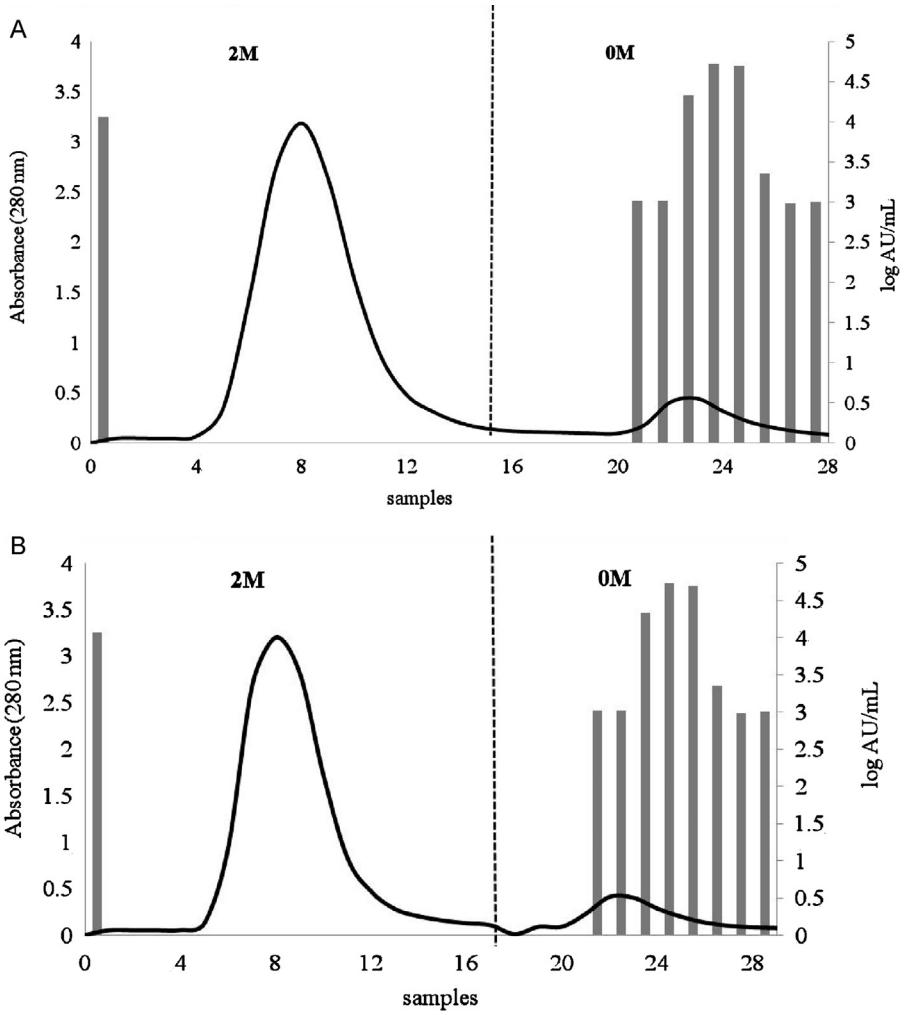

Fig. 3 – Chromatograms of nisin produced in-house purification by HIC. Black line represents the chromatogram profile recorded at 280 nm (non-bound and bound proteins). Gray solid bars represent nisin activity (Log AU). (A) Nisin produced in-house, water elution. (B) Nisin produced in-house, PBS elution.

nisin availability, since nisin-fat clusters were reported to reduce antimicrobial activity (Jung et al., 1992). In addition, this salt prevents formation of insoluble protein aggregates (Costantino et al., 1995; Middelberg, 2002) that may entrap active nisin. Despite sodium chloride advantages in this case, it is likely that the salt interferes with nisin sporicidal activity (Bell and Lacy, 1985) and may reduce HIC efficiency (Roettger et al., 1989). High salt concentration can also induce hyperosmotic stress and kill cells (Burg et al., 2007), which is an undesired outcome for in vitro cytotoxicity tests.

We believe that HIC purification concentrated the active peptide in commercial samples, resulting in high values for PF. A selection to obtain the most purified fractions can significantly reduce their yield (Ward and Swiatek, 2009), as occurred in this purification. On the contrary, nisin produced in-house has demonstrated better activity than commercial nisin. In this case, purification probably separated impurities that were interacting with nisin in the crude extract. The increase in activity resulted in high values for PF and yield.

Regarding the eluent effect, the values for yield and PF in all preparations eluted with water were higher than those eluted with PBS (Table 1). This might be explained by the pH factor: nisin is more active in acidic solutions, whereas PBS maintains a neutral pH. As a consequence, the antimicrobial activity decreases, which reflects on the yield/PF values.

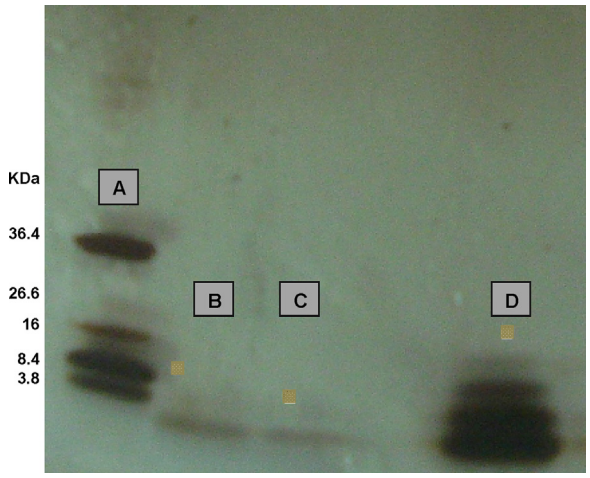

Fig. 4 – SDS-PAGE (gradient gel 4–20%, silver stain). SDS-PAGE (gradient precast gel 4–20%, Bio-Rad, USA). The gel was stained using the silver stain kit (Bio-Rad, USA). Kaleidoscope polypeptide standard (Bio-Rad, USA) was used as molecular weight standard. At 150 V, 200 mA for 90 min. (A) Molecular weight standard; (B) purified form of commercial nisin; (C) purified fraction of nisin produced in-house; (D) commercial nisin in crude.

Researchers Gujarathi et al. (2008), who purified MRS-derived nisin using HIC, obtained 50% recovery and a PF of 10.87. As stated above, most conditions used by this group were different from ours: their protocol involved several purification steps; the sample was previously concentrated by ammonium sulphate precipitation; the column was packed with phenyl Sepharose instead of butyl Sepharose, among other details (Gujarathi et al., 2008). To our knowledge, there are no articles describing purification of whey milk-derived nisin, thus precluding any comparisons.

3.4. *Electrophoresis*

Purified and crude samples were subjected to SDS-PAGE (4–20%) and revealed with silver (Fig. 4). The gels showed the "smiley effect", probably due to high salt content in some samples. Purified samples showed no bands but nisin, in agreement with nisin activity (high) and protein content (low) assays. As expected, commercial nisin (not purified, Fig. 3B) showed multiple bands and contained high protein content.

4. Conclusion

The present study established a low-cost purification of milk whey-derived nisin by HIC. We obtained a high degree purification of nisin without protein precipitation, which allows step/cost reduction. It was possible to recover nisin with water or PBS, which allows more possible applications of nisin. Water was the best eluent, with an increase of up to 775-fold in the antimicrobial activity. As per the outcomes of this work, milk whey probably contains factors, as remains of precipitated proteins, derived from the nisin production process, that decrease its activity, although high activity values were obtained in the crude extract. These factors were easily removed by HIC purification, evidenced by an even higher antimicrobial activity.

We conclude that production of nisin using milk whey as culture medium and HIC in the downstream process is cost-effective and results in a highly purified nisin product.

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