Research Article

Isolation and Thermal Stabilization of Bacteriocin Nisin Derived from Whey for Antimicrobial Modifications of Polymers

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This work describes novel alternative for extraction of bacteriocin nisin from a whey fermentation media and its stabilization by using polyethylene glycol as matrix with high practical applicability. This product was compared with commercially available nisin product stabilized by sodium chloride and nisin extracted and stabilized by using ammonium sulfate and polysorbate 80. The stability of samples was tested by means of long-term storage at −18, 4, 25, and 55°C to 165 days. The nisin content in the samples was determined by high-performance liquid chromatography and electrophoresis. In addition, effect of whey fortification with lactose on nisin production and antibacterial activity studied against Staphylococcus aureus was tested. Results show that stabilization by polyethylene glycol provides enhanced nisin activity at 55°C after 14 days and long-term stability at 25°C with keeping antibacterial activity.

1. Introduction

Nisin is a water-soluble polycyclic aromatic peptide possessing excellent antibacterial properties against Gram-positive bacterial strains [1]. The bacteriocin that belongs to the group of lantibiotics, in several variants differing in amino acid sequence in their structures, is produced by bacterial strains possessing specific genome. The bacteriocins are designated nisin A, nisin Z, nisin Q, F nisin (Lactococcus lactis), and nisin U (Streptococcus uberis) [2–6]. Recently, nisin H variant produced by Streptococcus hyointestinalis was described by O’Connor et al. [7].

Nisin belongs to the first described bacteriocins and it was recognized as a biological safe food preservative by Food and Agriculture Organization of the United Nations and World Health Organization already in 1968 [8]. It is produced biotechnologically through fermentation of a substrate such as whey or milk by the bacterial strains mentioned above [9].

Since there has been growing scientific and industrial interest in naturally occurring antimicrobial compounds that can be used for effective antimicrobial modifications of polymers, nisin is the ideal modifier showing antimicrobial activity at low concentrations that predetermine its application in food packaging, cosmetics and/or medical devices production. In addition, legislative requirements for introduction of nisin modified polymer system on the market could be significantly lower since it is considered to be a safe compound [8].

Since the nisin stability is limited, utilization of stabilizers is necessary to prolong its activity. Further additives improving its stability are used. The commercially available nisin is usually produced, concentrated, and processed into a powder containing over 90% of the stabilizer salt, especially sodium chloride (NaCl) [10]. Besides chlorides, sulfates [11] and/or surfactants [12] could be used. However, high concentration of the stabilizing salts is limiting for most of the applications dealing with incorporation into polymer systems due to their incompatibility with most of the matrices.

Several works dealing with antimicrobial modification of polymers with nisin have been published. However, most
of the works describe nisin application on plasma activated polymer surface modifier [13] or in matrix of water-soluble polymers [14, 15]. The thermoplastic incorporation into thermoplastic starch matrix has been also reported [16]. On the other hand, nisin incorporation into the cosmetic compositions is rarely described in the relevant literature.

One of the possibilities for the salt-free nisin stabilization is its attachment via both covalent and noncovalent bonds to polymer chains. PEGylation is typical example involving polyethylene glycol (PEG) [17] that is fully biocompatible polymer from the group of polyethers [18]. PEG is commonly used in medicine, pharmaceutics, and cosmetics [19]. Thus combination of nisin as antibacterial agent and PEG as compatible stabilizer can be suitable for most of the applications introduced above.

Despite the fact that the nisin PEGylation has been known, the detailed study describing the PEGylation effect on nisin time-temperature stability and its comparison with the conventional salt stabilization has not been reported.

This work is focused on comparative study of the long-term stability (up to 165 days) and temperature (~4 to 55°C) activity of nisin stabilized by both salt and polymer based compounds. In this study, the nisin was experimentally prepared by fermentation of dairy by-product whey and subsequently stabilized sodium chloride, ammonium sulfate, nonionic surfactant (Tween), and PEG. Its long-term stability under various temperatures was studied by means of high-performance liquid chromatography (HPLC), electrophoresis, and microbiological analysis.

2. Experimental Part

2.1. Materials. Sweet milk whey was kindly provided by the local dairy research institute (KROMILK, Kromeriz, Czech Republic). Ammonium sulfate ((NH₄)₂SO₄), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium thiosulfate (Na₂S₂O₅), copper(II) sulfate pentahydrate (CuSO₄·5H₂O), potassium sodium tartrate tetrahydrate (KNaC₄H₄O₆·4H₂O), formaldehyde solution, acetic acid, formic acid (85%), and methanol were purchased from PENTA (Prague, Czech Republic). Tween 80, TRIS Pufferan, Tricine Pufferan, tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), mercaptoethanol, bromophenol blue Na-salt, and ammonium persulfate (APS) were supplied from P-Lab (Prague, Czech Republic). Polyethylene glycol (PEG) 2050, lactose, trifluoroacetic acid (TFAA), ammonium bicarbonate (NH₄HCO₃), acrylamide, N,N’-methylenbisacrylamide, Pharmalyte, Coomassie Brilliant Blue R250, glutaraldehyde, molecular weight (MW) and isoelectric focusing (IEF) markers, nisin standard (2.5% (w/v) (NH₄)₂SO₄ and 13% (w/v) (NH₄)PO₄), 48h at 36°C with 0.1 M-NaOH. The inoculated medium was incubated for 48h at 36°C with constant shaking (245 rpm) in laboratory incubator (Incucell 404, BMT a.s., Brno, Czech Republic). The fermentation product was centrifuged (Heraeus Multifuge X1R, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 25 min at 10°C and 15,000 rpm and the supernatant was collected and processed by three different methods. A designation of prepared samples is shown in Table 1.

Method 1: Precipitation with Ammonium Sulfate (AS). A quantity of (NH₄)₂SO₄ (35% (w/v)) was added to the supernatant and continuously shaken at 4°C in conditioned chamber (Climacell 404, BMT a.s., Brno, Czech Republic) for 18 h. Then the mixture was centrifuged for 25 min at 10°C and 15,000 rpm. After that the upper phase was separated and dried using a freeze dryer (ScanVac CoolSafe 110-4 PRO, Lynege, Denmark).

Method 2: Two-Phase Extraction with Tween. The system was prepared by dissolving 1% (w/v) Tween 80 and 20% (w/v) (NH₄)₂SO₄ in the obtained supernatant. Then the mixture was shaken for 30 min and left to stand for 16 hour. After phase separation the upper phase was separated and lyophilized.

Method 3: Two-Phase Extraction with PEG. First the medium pH was adjusted to 5.4–6.5 with 0.1 M-NaOH and 15% (w/v) PEG 2050 and 13% (w/v) (NH₄)₂SO₄ were added. The extraction time was 40 min. After that, the mixture was kept for 1h at 4°C and then the upper phase was separated and lyophilized.

<table>
<thead>
<tr>
<th>Sample designation</th>
<th>Preparation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIS-AS</td>
<td>1</td>
</tr>
<tr>
<td>NIS-Tween</td>
<td>2</td>
</tr>
<tr>
<td>NIS-PEG</td>
<td>3</td>
</tr>
<tr>
<td>NIS-NaCl</td>
<td>Commercially available standard</td>
</tr>
</tbody>
</table>

This work is focused on comparative study of the long-term stability (up to 165 days) and temperature (~4 to 55°C) activity of nisin stabilized by both salt and polymer based compounds. In this study, the nisin was experimentally prepared by fermentation of dairy by-product whey and subsequently stabilized sodium chloride, ammonium sulfate, nonionic surfactant (Tween), and PEG. Its long-term stability under various temperatures was studied by means of high-performance liquid chromatography (HPLC), electrophoresis, and microbiological analysis.

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2.3. Determination of Total Protein Concentration. The total amount of proteins and peptides was evaluated in the liquid phase after fermentation and centrifugation by using the colorimetric method proposed by Bradford [20], with BSA as the standard. The protein contents were determined spectrophotometrically (INFINITE M200Pro NanoQuant, with ELISA reader, Männedorf, Switzerland) at 595 nm.

2.4. Tris-Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (Tris-Tricine-SDS-PAGE). The polyacrylamide (PAA) gel (10% T/3% C) as a uniform separating gel and stock solutions (buffers) for Tris-Tricine-SDS-PAGE were prepared according to Schägger and von Jagow [21]. The protein samples were combined with the buffer solution and the mixture was denatured for 5 min at 80 °C prior to being loaded to the gel. Electrophoresis was carried out with a constant voltage of 190 V by using devices Bluestar (Blirt S.A., Dantsic, Poland) and Mini Vertical Dual (Carl Roth GmbH, Karlsruhe, Germany), power supply Consort EV 202 (Consort bvba, Turnhout, Belgium). Finally, the proteins were fixed and stained with Coomassie Brilliant Blue R250 [22] or with AgNO₃ (when the protein content in a band was less than 0.5 μg).

The assumption was that the band corresponding with molecular weight of about 3.5 kDa according to MW marker contains a mixture of nisin A and nisin Z. After electrophoresis, these bands were excised and extracted. A destaining solution consisted of NH₄HCO₃ and ACN (1:1) and an extraction mixture was composed of 5% formic acid and ACN (1:2). The nisin concentration was then determined according to Bradford [20] and by HPLC. In the case of Bradford method, the calibration curves were constructed by using commercial nisin in the presence of corresponding stabilizing agents (PBS (phosphate buffered saline) for NISAS, Tween 80 for NIS-Tween, and PEG2050 for NIS-PEG samples).

2.5. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). The nisin concentrations were measured using a HPLC Waters 717 plus Autosampler (Waters GmbH, Praha, Czech Republic) equipped with a dual wavelength UV/Vis detector. A XSelect, C18 CS column (5 μm, 4.6 × 250 mm, Waters, Milford, MA, USA) with precolumn Reprosil 100 C18 (5 μm, 50 × 4 mm, Watrex, Prague, Czech Republic) was used. The calibration solutions and samples were prepared by using 0.2 M HCl and filtered through the syringe filter with pore size 0.45 μm. The mobile phase was organized as gradient and composed of 0.05% (v/v) TFAA in HPLC gradient grade ACN (solvent A) and 0.05% (v/v) TFAA in distilled water (solvent B). The gradient consisted of 20% solvent (A) and 80% solvent (B) in 0–5 min, of 80% solvent (A) and 20% solvent (B) in 6–20 min, and of 80% solvent (A) and 20% solvent (B) again in 21–40 min. The injection volume was 25 μl and the flow rate was of 0.6 ml/min. The measurement was performed at wavelengths of 200 and 220 nm (the nisin concentration was calculated from the results of 200 nm).

2.6. Agar Diffusion Testing of Antimicrobial Activity. Nisin activity was determined by agar-well diffusion method, using S. aureus as the indicator microorganism. An aliquot of bacterial culture (10⁶ CFU) was applied with a sterile swab on the surface of the Mueller Hinton agar plate. The test samples were prepared in deionized water so that the starting concentration of nisin corresponded to 25 μg/ml. Afterwards, the solutions were transferred into the bored wells of 8 mm in diameter (250 μl/well) and incubated for 24 h at 35 °C. After incubation, the diameter of inhibition zones was determined as the average of four independent measurements.

2.7. Stability Testing. Prepared samples were assessed for stability during storage for 165 days at −18°C, 4°C, 25°C, and 55°C. The samples were continuously collected and tested by using the Bradford method, RP-HPLC, and agar diffusion assay for determination of nisin activity, as described above. The thermal stability (TS) of the prepared samples was calculated as follows:

\[ TS(\%) = \frac{\text{NIS}_0}{\text{NIS}_t} \cdot 100 \]  

(1)

where NIS₀ is nisin concentration at particular time; NISₜ is starting concentration of nisin.

3. Results and Discussion

3.1. Effect of Lactose Content on Nisin Production. An adequate content of carbon substrate is necessary for optimal production of bacteriocin nisin by lactic acid bacteria. Lactose is one of the possible carbon sources in the by-product, whey, comprising a major part of the fermentation media used in this work. In whey, the lactose content usually varies from 4.5 to 6.0% [23, 24].

The effect of fermentation media fortification by lactose on nisin production has been evaluated in many studies. The optimal nisin production was reported when the medium contained 30 g/l lactose [25, 26]. However, the yield of the reaction depends on many factors such as bacterial strains used, fermentation conditions (i.e., temperature, pH, and time), and composition of the media.

In this study, nisin production was examined at various levels of lactose fortification 0–20% (w/v). The results are depicted in Figure 1 as dependence of nisin production effectivity, expressed as concentration determined after 48 hours of fermentation. The nisin production was determined by RP-HPLC technique.

It can be seen that nisin intensity production increases with the rising level of fortification up to 4% of lactose addition. Further lactose addition led to reduction of the nisin biosynthesis due to possible occurrence of competitive processes and elevated osmotic pressure in the fermentation media [27]. Although the lactose concentration of 4% was found to be the best for nisin production, due to technological and economical aspects (high viscosity of the fermentation media at 4 wt.% additional lactose content), the whey fortified with 2 wt.% of lactose was chosen for further nisin experimental preparation.

3.2. Quantitative Analysis of Nisin Content in the Lyophilisates. The lyophilized samples prepared by the methods 1–3 were
It is known that nisin possesses strong antibacterial activity against Gram-positive bacterial strains [15]. There are two

analysed by HPLC and electrophoresis as indicated above. The results presented in Table 2 show that both methods used in this study (HPLC and electrophoresis) bring comparable results. The nisin content determined by the latter method is about 15% higher than in the case of HPLC determination that is caused by the different principles of both techniques. It is caused by the selectivity of both techniques. While HPLC considers only nisin in its A form (due to calibration on commercially available nisin), electrophoresis and subsequent analysis according to Bradford method cover the protein with molecular weight around 3.5 kDa, which includes also nisin Z form [3]. The nisin content in samples stabilized in ammonium sulfate (NIS-AS) and Tween (NIS-Tween) was found to be the same (2.3 mg/g, HPLC). On the other hand, the sample stabilized by using polyethylene glycol (NIS-PEG) had significantly higher nisin content, 5.3 mg/g (determined by HPLC). It was more than 2.3 times higher than in the case of NIS-AS and NIS-Tween.

Since the initial material for nisin extraction was the same for all methods used (i.e., nisin content in the fermentation media was constant), it is clear that extraction step efficiency is the crucial for the nisin content in the resultant samples. The results show that PEG interacts with nisin, which leads to noticeable bacteriocin separation from the fermentation media. The principles of PEG-nisin interactions has been described referring to the ability of formation of protein-polymer conjugates that can display unique combination of protein and synthetic polymer based material properties [28].

3.3. Testing of Thermal Long-Term Stability. It is known that proteins are sensitive to heat treatment, especially in relation to long-term stability and activity [29]. In this study, thermal stability of nisin products was observed during storage (up to 165 days) at four different temperatures representing conditions of production (−18°C, lyophilization), storing (4 and 25°C), and processing (55°C). The nisin content change in the tested samples stored at various temperatures up to 55 days is shown in Figure 2. It can be noticed that the nisin concentration in all powder samples stored at −18°C changed only negligibly. The storage at 4°C caused slight reduction of the nisin content and TS subsequently (9% NIS-NaCl, 33% NIS-AS, 18% NIS-Tween, and 25% NIS-PEG). On the contrary, storage at 25°C led to significant drop in TS values after 55 days. The lowest nisin stability was observed in case of NIS-Tween (87% reduction) followed by NIS-PEG (71%), NIS-NaCl (85%), and NIS-AS (51%). Nevertheless, TS of the samples NIS-NaCl, NIS-AS, and NIS-PEG is relatively high within the first 15 days of the experiment. All samples stored at 55°C underwent steep drop in TS values. In all cases, the reduction of nisin concentration was above 90% after 55 days. TS of NIS-AS was detected up to 11 days due to low nisin stability in the sample leading to unreproducible results of analysis. In addition, it is worth mentioning that despite the TS of NIS-PEG systems is comparable to other investigated samples, the absolute values of nisin concentration are at least 2.3 times higher (see Table 2) (i.e., absolute concentration of nisin is higher).

In Figure 3, TS values of nisin products determined after electrophoresis-Bradford method (A) and by HPLC (B) after 165 days were compared. Stability loss after 165 days of storage at −18°C was about 5–10% for all tested samples and at storage at 4°C it was about 20% for samples except for NIS-AS, where TS was about 40%. Significant changes of stability were observed at storage at 25°C and 55°C, where the highest stability was noticed at NIS-PEG (28% at 25°C and 4% at 55°C) in comparison with other samples including a commercial nisin (NIS-NaCl).

These results are principally in agreement with the work published by Rollema et al. [30] who reported chemical stability of nisin A at 20, 37, and 75°C in various buffers at pH range from 2 to 8. The nisin stability after 3 months of storage at 20°C resulted in 50–70% reduction in the bacteriocin content.

3.4. Testing of Nisin Antibacterial Activity in the Lyophilisates. It is known that nisin possesses strong antibacterial activity against Gram-positive bacterial strains [15]. There are two


Table 2: Determination of nisin concentration in prepared lyophilisates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$c_{\text{NIS}}$ [mg/g]</th>
<th>$[\times 10^3 \text{IU/g}]^*$</th>
<th>$c_{\text{NIS}}$ [mg/g]</th>
<th>$[\times 10^3 \text{IU/g}]^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIS-AS</td>
<td>2.3 ± 0.1</td>
<td>94 ± 4</td>
<td>2.6 ± 0.1</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>NIS-Tween</td>
<td>2.3 ± 0.2</td>
<td>93 ± 7</td>
<td>2.7 ± 0.2</td>
<td>109 ± 6</td>
</tr>
<tr>
<td>NIS-PEG</td>
<td>5.3 ± 0.3</td>
<td>210 ± 10</td>
<td>5.9 ± 0.2</td>
<td>234 ± 6</td>
</tr>
</tbody>
</table>

*1 IU of nisin is equivalent to 0.025 μg of pure nisin [8].
Figure 2: Thermal stability of nisin (TS) versus storage time at –18°C (a), 4°C (b), 25°C (c), and 55°C (d). Nisin was determined by HPLC. Data points are average values of three independent analyses. Standard errors were less than 10%. *Commercial product.

Figure 3: Thermal stability (TS) after 165 days of storage at various temperatures determined by electrophoresis-Bradford method (a) and by HPLC (b). Data points are average values of three independent analyses. Standard errors were less than 10%. *Commercial product.
Table 3: Determination of nisin antibacterial activity on the growth of *S. aureus* by agar-well diffusion method. Data points are average values of three independent analyses.

<table>
<thead>
<tr>
<th>Storage temperature</th>
<th>Time [days]</th>
<th>NIS-AS</th>
<th>NIS-Tween</th>
<th>NIS-PEG</th>
<th>NIS-NaCl*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly prepared</td>
<td>0</td>
<td>8.0 ± 0.2</td>
<td>7.4 ± 0.5</td>
<td>5.5 ± 0.5</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td>−18°C</td>
<td>14</td>
<td>8.0 ± 0.2</td>
<td>7.4 ± 0.5</td>
<td>5.5 ± 0.5</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td>4°C</td>
<td>14</td>
<td>8.0 ± 0.2</td>
<td>7.4 ± 0.5</td>
<td>5.5 ± 0.5</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td>25°C</td>
<td>14</td>
<td>2.6 ± 0.5</td>
<td>0.5 ± 0.5</td>
<td>4.5 ± 0.5</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>55°C</td>
<td>165</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

* Commercial product.

... with uncommon unsaturated amino acids (dehydroalanine, dehydrobutyryne) or amino and carboxylic groups of amino acids [17].

4. Conclusions

Novel method for extraction and stabilization of bacteriocin nisin by using polyethylene glycol (PEG) was described in this study. The product NIS-PEG was compared with commercially available nisin product stabilized by NaCl and nisin extracted and stabilized by using (NH₄)₂SO₄ and polysorbate 80 (Tween).

The results reveal that (i) fortification of whey with lactose is optimal at 4 wt.% of added lactose; however, 2 wt.% is convenient due to technological reasons; (ii) quantitative determination of nisin by both analytic techniques used in this work (HPLC, electrophoresis) corresponds to each other; (iii) thermal stability testing of NIS-PEG is comparable with other tested samples; (iv) NIS-PEG product possesses noticeably enhanced long-term stability when stored for 165 days at 25°C; (v) PEG stabilization is more efficient even for samples stored for 14 days at 55°C in comparison with the commercially available product (NIS-NaCl).

The newly introduced method significantly widens applicability possibilities of nisin as antimicrobial modifier for both hydrophobic and hydrophilic polymer systems.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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