COMPARISON OF TWO METHODS FOR PURIFICATION OF PLANTARICIN ST31, A BACTERIOCIN PRODUCED BY *LACTOBACILLUS PLANTARUM* ST31

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**ABSTRACT**

Two methods of purification of the plantaricin ST31, a bacteriocin produced by *Lactobacillus plantarum* ST31 are used in this study – the method of ammonium sulfate precipitation, Sep-pack C18 cartridge and reverse-phase HPLC chromatography on C18 Nucleosil column, and the method of direct purification by cation exchange SP Sepharose Fast Flow column Amersham (Pharmacia Biotech). The purity of the products from the two experimental protocols are examined for their molecular weight, aminoacid composition and sequence. Comparison of results show that the plantaricins purified with the two methods are identical. Both methods may be used to purify plantaricin ST31. Comparison of the yield in the purification protocols is 0.8% in the HPLC experimental protocol and 5.9% in the cation-exchange chromatography method.

**Key words:** *Lactobacillus plantarum*, bacteriocin, plantaricin, HPLC, cation-exchange chromatography (IEX)

**INTRODUCTION**

*Lactobacillus plantarum* is important in many food fermentations either as a component of the natural microflora or when used as a starter culture. A number of *L. plantarum* strains produce bacteriocins, many of which have been isolated and partially characterized (2,6,9,10,14,16).

Several methods have been reported for the purification of plantaricins: (a) anion-exchange chromatography (DEAE-Sephadex A-25) and reverse-phase HPLC (9); (b) ammonium sulfate precipitation (80%), cation-exchange chromatography (SP Sepharose fast-flow cation exchange column), hydrophobic interaction chromatography (phenyl-Sepharose CL-4B column) and C2/C18 reverse-phase chromatography (8); (c) ammonium sulfate precipitation (55%), hydrophobic interaction (C8), cation exchange chromatography Mono S cation-exchange column (Pharmacia, Biotech) (6); (d) ammonium sulfate precipitation (40%) and cation exchange – SP-Sepharose (1); (e) ammonium sulfate precipitation (60%), cation-exchange chromatography, hydrophobic interaction chromatography (11); (f) ammonium sulfate precipitation, cation exchange (S- Sepharose), reversed-stationary-phase (octyl-sepharose – CL-4B), stationary-phase C2/C18 chromatography (5,10) and (g) ammonium sulfate precipitation (60%), dialysis, filtration (Amicon, 0.45µm Millipore) and ultrafiltration (4). A rapid and two-step procedure suitable for both small- and large-scale purification of pediocin-like bacteriocins and other cationic peptides have been reported (15). Bacterial cells and anionic compounds passed through the column, with cationic bacteriocins being eluted subsequently with 1 M NaCl (15). The method most commonly used is ammonium sulfate precipitation at different concentrations, followed by HPLC.

We described two methods for the purification of plantaricin ST31: (a) method of precipitation with ammonium sulfate, Sep-pack C18 cartridge and reverse-phase HPLC on a C18 Nucleosil column, and (b) a method of direct purification with SP Sepharose.

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Fast Flow (Pharmacia Biotech). The homogenic nature of plantaricin ST31 purified by the second method was confirmed by reverse-phase HPLC on a C18 Nucleosil column.

MATERIALS AND METHODS

Strains and media
For cultivation of *L. plantarum* ST31, the producer strain and *L. plantarum* LAB 73, the sensitive strain, MRS broth and MRS agar (Merck, Darmstadt, Germany) (3) were used. Incubation was done at 30°C for 24 h. Both strains have been isolated from fermented cereal products (14). The strains were stored at -80°C in MRS broth containing 15% (vol/vol) glycerol. Before use, the strains were cultivated twice for 24 h at 30°C in MRS broth.

Bacteriocin activity assay
Bacteriocin screening was performed by two methods, the agar spot test and the well diffusion method as described by Schillinger and Lücke (12) and Tagg and McGiven (13), respectively. Normally 1.5% agar was used. For overlay 1.0% soft agar was prepared. To eliminate the action of lactic acid on the test organisms, the pH of the supernatants was adjusted to 6.0 with sterile 1N NaOH. The activity was expressed in arbitrary units (AU per ml). One AU was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strain.

Production studies
Tween-free MRS medium prepared from basal ingredients was sterilized by autoclaving (15 min, 120°C) and was aseptically transferred to a bioreactor connected to an automatic pH and temperature controller (Set 2M; SGI, Toulouse, France). The medium was inoculated with 2% (vol/vol) of an overnight grown culture of *L. plantarum* ST31. The pH was maintained at 6.0 with 6M NaOH. The temperature was controled at 30°C, and agitation was set at 100 rpm. Samples were taken at different time intervals for determination of optical density at 600 nm and antimicrobial activity were determined at each step of the purification process.

Mass spectrometry
Active peptide fractions collected from HPLC was subjected to electrospray mass spectrometry (ESMS). ESMS was done on a VG Bio-Q quadrupole with a mass range of 4000 Da (Bio-Tech, Manchester, UK) in the positive mode. The protein was dissolved in H₂O/CH₃CN (50/50, v/v) with 1% acid at a concentration of about 5 pmol/µl (by volume); 10-µl aliquots were introduced into the ion-source at a flow rate of 4 µl/min. Scanning was usually performed from m/z = 500 to m/z = 1,500 in 10 s with the resolution adjusted so that the peak at m/z = 998 from horse heart myoglobin was 1.5 – 2 wide on the base. Calibration was performed by using the multiply charged ions produced by separate introduction of horse heart myoglobin (16,950.4 Da) (7).

RESULTS AND DISCUSSION

Purification by precipitation with ammonium sulfate, Sep-pack C₁₈ cartridge and reverse-phase HPLC on a C₁₈ Nucleosil column. Since the maximum activity was found in the culture
medium at the beginning of the stationary phase, the bacteriocin was isolated from 24-h-old cultures in MRS medium at pH 6.0. The bacteriocin was pre-purified by ammonium sulfate precipitation. The recovery was approximately 40%. The precipitate was subjected to filtration on a Sep-Pack C18 cartridge. The active fraction was eluted with 40% iso-propanol. At this stage of purification, the recovery was 4% and the specific activity increased approximately 1.9x10³-fold.

Separation of the active fraction on a HPLC C18 reverse-phase column yielded an active peak which eluted at 38.08 min. (data not shown). At this stage the purification factor reached 1.2x10⁴-fold and the recovery was 0.8% (Table 1). The active fraction was purified by subsequent reverse-phase chromatography and its amino acid sequence determined. The peptide consists of 20 amino acids with a total mass of 2,755.63 Da, as estimated by mass spectrometry. The sequence was determined to be: Lys-Arg-Lys-Lys-His-Arg-Xaa-Gly-Val-Tyr-Asn-Asn-Gly-Met-Pro-Thr-Gly-Met-Tyr-Arg.

A second active peak, which eluted at 36.62 min, was also recorded (data not shown). The protein recovery was identical to that recorded for that eluted at 38.08 min. The molecular mass of the peptide was 2,771.58 Da. Analysis of the amino acid sequence revealed primary structure similar to that of the 2,755.63 Da peptide, but with oxygenated methionine.

**Purification with a cation exchange SP Sepharose Fast Flow column (Amersham, Pharmacia Biotech).**

For the purification, an active supernatant from the same fermentation flask of the previous experiment was used. The active supernatant was subjected to cation-exchange chromatography (SP Sepharose, Amersham, Pharmacia Biotech). The active fraction was eluted with 100% buffer B - 1M NaCl after 20 min from the beginning of the gradient (data not shown). At this stage of purification, the recovery was 5.94% and the specific activity increased approximately 1.1x10²-fold (Table 2). Further separation by HPLC on a C18 reversed-phase column, yielded an active peak which eluted at 63.71 min. (data not shown). The active fraction was purified by reverse-phase chromatography and its amino acid sequence determined. Mass spectrometry of the active peptide corresponded to that recorded for plantaricin ST31, as purified by the previously described method. The amino acid composition was also identical to previously described.

**Discussion.** Comparison of the two methods revealed that separation by HPLC is more expensive and tedious. Compared to the HPLC method, the utilisation of cation-exchange chromatography is less expensive, and more rapid. Furthermore, half of the plantaricin ST31 purified by HPLC is converted into an oxygenated form. This phenomenon reduced the yield of plantaricin ST31 by 50%.

**Table 1. Purification of plantaricin ST31 by precipitation with ammonium sulfate, Sep-pack C18 cartridge and reverse-phase HPLC on a C18 Nucleosil column.**

<table>
<thead>
<tr>
<th>Sample (ml)</th>
<th>Total activity (AU)</th>
<th>Protein (mg)</th>
<th>Specific activity (AU/mg)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant / 200</td>
<td>6.4 x 10⁵</td>
<td>1810</td>
<td>3.5 x 10²</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation / 20</td>
<td>2.5 x 10⁵</td>
<td>10.6</td>
<td>2.4 x 10⁴</td>
<td>40</td>
<td>6.9 x 10³</td>
</tr>
<tr>
<td>SepPack 40% isopropanol / 2</td>
<td>2.5 x 10⁴</td>
<td>0.038</td>
<td>6.6 x 10⁵</td>
<td>4</td>
<td>1.9 x 10⁵</td>
</tr>
<tr>
<td>HPLC C18 / 0.2</td>
<td>5.1 x 10⁴</td>
<td>0.001</td>
<td>5.1 x 10⁶</td>
<td>0.8</td>
<td>1.2 x 10⁴</td>
</tr>
</tbody>
</table>

**Table 2. Purification of plantaricin ST31 by SP Sepharose Fast Flow (Pharmacia Biotech) Chromatography.**

<table>
<thead>
<tr>
<th>Sample (ml)</th>
<th>Total activity (AU)</th>
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<td>100</td>
<td>1</td>
</tr>
<tr>
<td>SP Sepharose Fast Flow column / 30</td>
<td>3.8 x 10⁴</td>
<td>0.96</td>
<td>3.9 x 10⁵</td>
<td>5.94</td>
<td>1.1 x 10²</td>
</tr>
</tbody>
</table>

The differences in the results of the purification of plantaricin ST31 and the yield in the first and second experimental protocols (0.8% by HPLC and 5.94% by cation-exchange chromatography) might be explained by the longer HPLC experimental procedure and the different treatments of the supernatant.

Precipitation with ammonium sulfate was not required prior to cation-exchange, as reported by other authors (1,5,8,10,11). The molecular mass obtained for plantaricin ST31 by using the two methods was identical when the oxygenated form of methionine is taken into account (MM: 2771.58 +/-0.11 and MM: 2763.32 +/-0.11, respectively).

From these results, we can conclude that *Lactobacillus plantarum* ST31 produces one form of plantaricin ST31. The existence of two active forms of plantaricin ST31 (with oxygenated and non-oxygenated at methionine) suggests methionine is not present in the part of the molecule responsible for the bacteriocin activity.

**ACKNOWLEDGMENTS**

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RESUMO

Comparação de dois métodos de purificação da plantaricina ST31, a bacteriocina produzida por Lactobacillus plantarum ST31

Dois métodos de purificação de plantaricina ST31, uma bacteriocina produzida por Lactobacillus plantarum ST31 foram usados neste estudo - o método de precipitação pelo sulfato de amônia usando cartucho Sep-pack C18 para a filtração e HPLC de fase reversa em coluna de C18 Nucleosil, e o método de purificação direta por troca catiônica SP Sepharose “Fast Flow column Amersham” (Pharmacia Biotech). A pureza dos produtos obtidos pelos dois protocolos foi examinada através da determinação dos pesos moleculares, composição e sequência dos aminoácidos. A comparação destes resultados revelou que, em termos da pureza dos produtos, não havia diferenças entre os dois métodos de purificação podendo-se, portanto, utilizar qualquer um dos protocolos de purificação testados. No entanto, o rendimento da purificação pelo método da troca catiônica foi de 5.9% enquanto o do método HPLC foi de 0.8%.

Palavras-chave: Lactobacillus plantarum, bacteriocina, plantaricina, HPLC, cromatografia por troca catiônica (IEX)

REFERENCES