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One-Step Purification of Nisin A by Immunoaffinity Chromatography

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The lantibiotic nisin A was purified to homogeneity by a single-step immunoaffinity chromatography method. An immunoadsorption matrix was developed by direct binding of anti-nisin A monoclonal antibodies to *N*-hydroxysuccinimide-activated Sepharose. The purification procedure was rapid and reproducible and rendered much higher final yields of nisin than any other described method.

Nisin is a ribosomally synthesized and posttranslationally modified antimicrobial peptide produced by certain *Lactococcus lactis* subsp. *lactis* strains that has practical applications in the food industry (6, 8, 18). This fact, together with its potential in human and animal therapy, has stimulated considerable research in the development of analytical techniques for its detection, quantification, and purification. Two natural variants of nisin, nisin A and nisin Z, have been found among lactococcal strains; the two mature peptides differ in only one amino acid residue, His^{27} in nisin A and Asn^{27} in nisin Z (15, 20). Pure nisin preparations are obtained by various procedures (2–5, 9, 10, 12, 13, 15, 21) that usually involve multiple steps, making them laborious, time consuming, inefficient, and/or barely reproducible.

We recently described the generation of anti-nisin A specific monoclonal antibodies and the development of nisin immunoassays for the detection and quantification of this bacteriocin in bacterial cultures and in food products (19). This communication reports the use of a monoclonal antibody produced by AD10 hybridoma cell culture for the immunopurification of nisin A. The purification procedure developed is efficient, specific, and reproducible and can be easily scaled up.

Monoclonal antibodies of the immunoglobulin G1 isotype produced by hybridoma cell line AD10 were purified and concentrated from cell-free supernatants or ascitic fluid by precipitation with 50% saturated ammonium sulfate (19). Anti-nisin A monoclonal antibodies were coupled to a 1-ml HiTrap *N*-hydroxysuccinimide-activated column from Pharmacia (Uppsala, Sweden) as recommended by the manufacturer. Briefly, after washing of the column with ice-cold 1 mM HCl, 10 mg of antibody dissolved in 1 ml of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was injected into the column. After 1 h at room temperature, the remaining active groups were deactivated and the nonspecifically bound ligand was removed by washing alternately with alkaline buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and acidic buffer B (0.1 M acetate, 0.5 M NaCl, pH 4) three times. After the second buffer A wash, the column remained at room temperature for 1 h. Finally, the column was washed with 0.01 M phosphate-buffered saline (PBS; pH 7.4) and stored in 0.01% (wt/vol) thimerosal in PBS at 4°C until required. Coupling efficiency was determined by measuring protein concentration at 280 nm. Previously, *N*-hydroxysuccinimide groups released during the coupling reaction were removed by gel filtration in PD-10 desalting columns (Pharmacia). Approximately 8.3 mg of the antibodies was successfully bound to the column.

The performance of the immunocolumn was initially assessed by passing 1-ml aliquots of pure nisin A (30,000 U mg^{-1}; NBS Biologicals, Hartfield, United Kingdom) solutions in PBS (0, 1,000, and 10,000 ng ml^{-1}). After application of each sample, the column was washed with 3 ml of PBS and nisin was eluted with 4 ml of glycine-hydrochloride buffer (0.05 M glycine-HCl, 0.5 M NaCl, pH 2.7). All of the steps were carried out at room temperature. An agar diffusion test (18) showed that all of the antimicrobial activity was present in the elution fractions. *Pediococcus acidilactici* 347 was used as the indicator strain.

Next, we evaluated the performance of the immunoaffinity matrix with the culture supernatant of a nisin A-producing lactococcal strain. *L. lactis* BB24 (18) was grown in MRS broth (Oxoid Ltd., Basingstoke, United Kingdom) at 32°C for 16 h. Culture supernatants were obtained by centrifugation at 12,000 × *g* for 10 min at 4°C, adjusted to pH 7.4 with 1 N NaOH, and filtered through 0.22-μm-pore-size filters (Millipore Corp., Bedford, Mass.). Ten milliliters of supernatant was pushed through the column with a P-1 peristaltic pump (Pharmacia) at a flow rate of 0.5 ml min^{-1}. After washing of the column with 25 ml of PBS, the nisin bound to the column was eluted with 20 ml of glycine-hydrochloride buffer. Flowthrough, washing, and elution fractions were collected in 1-ml aliquots, and their A_{280}, A_{254}, and A_{220} were measured. Nisin activity was detected by a microtiter plate assay system (14). One bacteriocin unit was defined as the reciprocal of the highest dilution exhibiting 50% growth inhibition of the indicator microorganism *P. acidilactici* 347 (18). In addition, a competitive direct enzyme-linked immunosorbent assay (CD-ELISA) (19) was used to quantify nisin A after adjustment of the pH of the samples to 7.4 ± 0.2 with 1 N NaOH. Although some nisin was detected in the flowthrough fraction (25.7%) and the first washing fraction (3%), most of the bacteriocin (72.7%) was retained and recovered in the elution step. The purification process resulted in a 10-fold increase in specific activity (Table 1). Maximum activity and quantity of eluted nisin, as determined by the microtiter assay and the CD-ELISA, respectively, were coincident with an absorbance peak at 220 nm (Fig. 1).

The purity of nisin A obtained from the culture supernatant of *L. lactis* BB24 was assessed by using a PepRPC HR 5/5 C_2/C_{18} reverse-phase chromatography column adapted to a fast protein liquid chromatography system (Pharmacia). After the column was equilibrated with aqueous 0.1% trifluoroacetic

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TABLE 1. Purification of nisin A from a culture supernatant of *L. lactis* BB24

Fraction	Vol (ml)	Total A_{220}[a]	Total activity[b]	Sp act[c]	Relative sp act (fold)	Total nisin[d] (ng)	Yield (%)
Sample (supernatant)	10	12.64	13,716	1,085	1	46,365	100
Flowthrough	10	12.37	597	48.26	0.044	11,936	25.74
Washing	25	5.49	555	101.13	0.093	4,189	9.03
Elution	20	0.73	8,122	11,187	10.31	33,690	72.70

[a] Total A_{220} is A_{220} times the volume in milliliters.
[b] Total activity (in bacteriocin units) was determined by a microtiter plate assay.
[c] Specific activity is total activity divided by the total A_{220}.
[d] Total nisin was determined by a CD-ELISA. Each value is the sum of the average values of the different fractions determined in triplicate in a single microtiter plate.

acid, a sample with approximately 34,000 ng of nisin, as estimated by a CD-ELISA, was applied to the column. The bacteriocin was eluted with a linear gradient ranging from 10 to 60% 2-propanol containing 0.1% trifluoroacetic acid at a flow rate of 1 ml min^{-1}. A sample containing approximately the same concentration of pure commercial nisin A in glycine-hydrochloride buffer was passed through the column as a control. A major absorbance peak at about 36% 2-propanol was observed for both the sample and the control. The peaks were identical in size. The non-nisin A active peaks seemed to be the result of the background absorbance generated by the glycine-hydrochloride buffer (Fig. 2). Furthermore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of commercial purified nisin A to homogeneity and purified nisin A by immunoaffinity chromatography in a PhastSystem electrophoresis unit showed that the electrophoretic patterns of the molecules were similar (results not shown).

We have previously developed a nisin A purification protocol that involves ammonium sulfate precipitation followed by cation-exchange, hydrophobic interaction, and reverse-phase chromatography (18). This protocol is useful for studies of structure-function relationships and biochemical research but is inefficient when significant amounts of the bacteriocin are needed for food and clinical studies and applications (11). Moreover, such an approach is complex, laborious, and time consuming and the recovery is only 3.5% of that obtained with the immunoaffinity chromatography method reported here and the yield is approximately 30-fold lower (results not shown). Similar disadvantages have been reported for other nisin purification schemes previously described (7, 16).

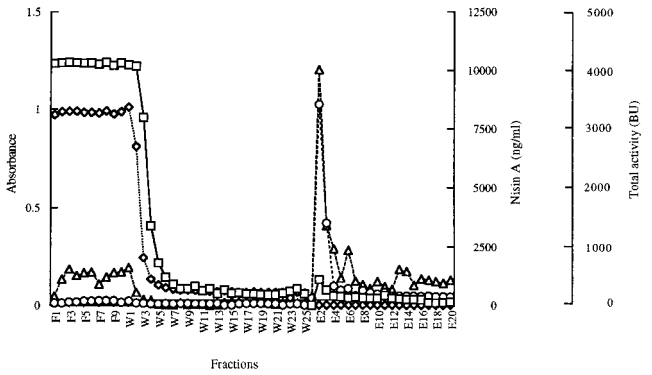

FIG. 1. Immunoaffinity chromatography purification of nisin A from a culture supernatant of *L. lactis* BB24. Flowthrough (F), washing (W), and elution (E) fractions are shown. Absorbance was measured at 220 (□) and 280 (◇) nm. Total activity (○) was determined by the microtiter plate assay. Nisin A concentration (△) was determined by a CD-ELISA. BU, bacteriocin units.

To our knowledge, this is the first immunologically based method for purification of nisin. The one-step purification of nisin A by immunoaffinity chromatography is clearly easier to perform (involving only one chromatography step), quicker (requiring only a few hours), less expensive (sophisticated equipment is not needed), and more productive (the initial sample is 100 times smaller and the final yield is 30-fold higher) than any purification technique previously described for this bacteriocin. Although immunoaffinity techniques have been used for many years to purify proteins, these methods have not found an application in the bacteriocin field, mainly because it is difficult to develop antibodies against such antimicrobial peptides. The generation of antibodies against bacteriocins of lactic acid bacteria is a main research target because it permits their detection and quantification in food substrates, as well as their purification in a single step through similar strategies. The specific monoclonal antibodies against nisin A have provided a method that is able to purify this bacteriocin even from a complex medium such as MRS broth without unspecific adsorption. The procedure is simple, rapid, highly specific, reproducible, and inexpensive and permits the purification of nisin to homogeneity in a single step. These characteristics will surely allow the development of a scaled-up method by which to obtain pure nisin A in the large quantities required for food and clinical trials and applications. Interestingly, production of highly purified nisin preparations and enhancement by chela-

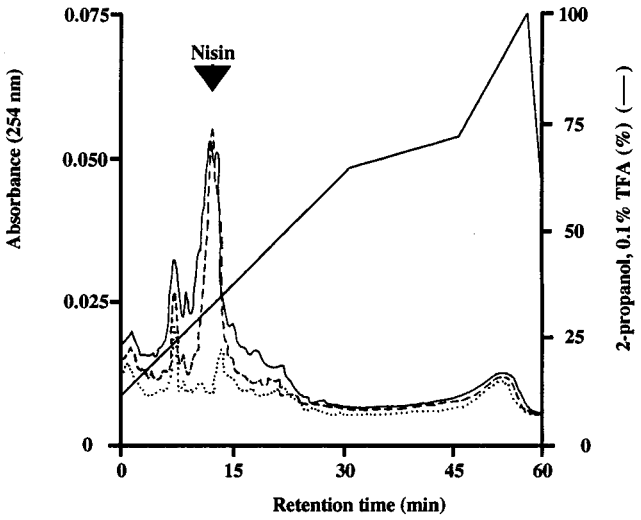

FIG. 2. Reverse-phase chromatography of eluted fractions from the immunoaffinity chromatography step (——), pure nisin A (– – –), and glycine-hydrochloride buffer (· · · ·). Fractions were collected and assayed for bacteriocin activity. TFA, trifluoroacetic acid.

tors have led to interest in the use of nisin for human ulcer therapy, gingival inflammation, topical skin infections, mastitis control in cattle, and the treatment of multiple-drug-resistant systemic infections (6).

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