Development of Monoclonal Antibodies to the Lantibiotic Nisin A

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Monoclonal antibodies were prepared against nisin A (nisA), an antimicrobial peptide that is used as a food preservative and is produced by strains of *Lactococcus lactis* subsp. *lactis*. NisA antibodies were secreted by hybridomas derived from mice immunized with a nisA−keyhole limpet hemocyanin conjugate. Competitive direct enzyme-linked immunoabsorbent assays (CD-ELISA) were devised whereby free nisA and nisA−horseradish peroxidase competed for antibody binding. The detection limit for nisA in this assay was 10 ng/mL. Antibodies also cross-reacted with nisin Z (nisZ). Mean concentrations of nisA and nisZ required to inhibit 50% antibody binding for the selected clones were 1180 and 3750 ng/mL, respectively. When the nisA CD-ELISA performance was assessed in spiked (250, 1250, and 5000 ng/g) spread cheese, the average recovery was 116.5%, with mean intra- and interassay coefficients of variation of 13.3 and 14.1%, respectively.

Keywords: *Nisin A; monoclonal antibodies; ELISA; lantibiotics; bacteriocins*

INTRODUCTION

Nisin A (nisA); (Figure 1) is a 34-residue peptide lantibiotic with antimicrobial activity that is produced by various strains of *Lactococcus lactis* subsp. *lactis*. Lantibiotics are bacteriocins containing lanthionine residues that form intrachain disulfur rings (Figure 1). NisA and/or nisA-producing food-grade microorganisms have traditionally been used by the dairy industry as food preservatives without any adverse effects observed after ingestion. Because of the safe history of this naturally occurring compound over the years, it was granted GRAS (generally recognized as safe) status in 1988 by the United States Food and Drug Administration (Federal Register, 1988). Today, the use of nisA as a food preservative has been extended to other food industry sectors (bakery, canning, egg), and currently its use is permitted at various levels worldwide.

For many years the most common analytical tool for nisA determination has been the agar diffusion test (ADT) originally described by Trammer and Fowler (1964). This is a bioassay-based method that assesses the inhibitory effect of nisA in a test (indicator) microorganism. The importance of the bioassay is undeniable, but it also has some drawbacks, such as unspecificity and low sensitivity. Thus, the development of alternative methodologies is clearly needed. Immunochemical techniques have proven to be very useful throughout the years in many research fields as routine analytical tools, but not until recently have these techniques been applied to the bacteriocin field. Falahee et al. (1990) described the development of a sandwich-type enzyme-linked immunosorbent assay (ELISA) for nisA in which sheep polyclonal antibodies are used. Recently, our group reported (Suárez et al., 1996) the development of a competitive type of ELISA for nisA with mouse sera and ascites polyclonal antibodies. Both immunoassay compared favorably with the bioassay, are nisin specific, and have detection limits in the parts per billion range. Although the polyclonal antibodies are sensitive and specific, their supply is limited and their characteristics could differ from batch to batch. Now these hurdles of limited supply and varying characteristics can be overcome with hybridoma technology (Köeler and Milstein, 1975), which allows unlimited production of antibodies with defined traits. In this paper we present, for the first time to our knowledge, the production and characterization of nisA monoclonal antibodies (MAb) secreted by stable hybridomas that are derived from mice immunized with a nisA-keyhole limpet hemocyanin conjugate. We also describe the development of competitive direct ELISAs (CD-ELISAs) and their application in foods.

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MATERIALS AND METHODS

Materials. All inorganic chemicals and organic solvents were reagent grade or better. NisA (30 000 units/mg) was from NBS Biologicals (North Mymms, Hartfield, Herts, U.K.), and nisin Z (nisZ) was kindly provided by Dr. Oscar Kuipers (NIZO, Ede, The Netherlands). Ovalbumin (OA; grade III), Tween 20, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS), glutaraldehyde, Freund's adjuvants, pristane, and dimethyl sulfoxide were obtained from Sigma Chemical Company (St. Louis, MO). Keyhole limpet hemocyanin (KLH) and horseradish peroxidase (HRP) were purchased from Pierce Chemical Company (Rockford, IL). RPMI 1640, fetal calf serum, (FCS), 50×HAT (hypoxanthine, aminopterin, thymidine), 50×HT (hypoxanthine, thymidine), L-glutamine, and penicillin/streptomycin (PEST) solutions were from Gibco BRL (Gaithersburg, MD). Poly(ethylene glycol) (MW, 1500; PEG) and BM-Condimed-H1 media were from Boehringer Mannheim Biochemical (Indianapolis, IN). Goat anti-mouse immunoglobulin G Fc fragment was obtained from Cappel Laboratories (Westchester, PA). Tissue culture plasticware and Maxisorp microtiter plates were from Nunc (Roskilde, Denmark). The myeloma cell line P3X63-Ag8.653 was kindly donated by the Instituto Llorente (Madrid, Spain). Mice (BALB/c) were a gift obtained from the in house colonies of the Facultad de Medicina de la Universidad Autonoma, Madrid, Spain.

Preparation of Immunoconjugates and Immunization. NisA was conjugated by the glutaraldehyde method (Avrameas and Terninck, 1969; Briand et al., 1985) to keyhole limpet hemocyanine (nisA−KLH; 1/2, w/w) for use as immunogen and to horseradish peroxidase (nisA−HRP; 1/5, w/w) by the periodate method (Nakane and Kawoi, 1974) for use in the CD-ELISA.

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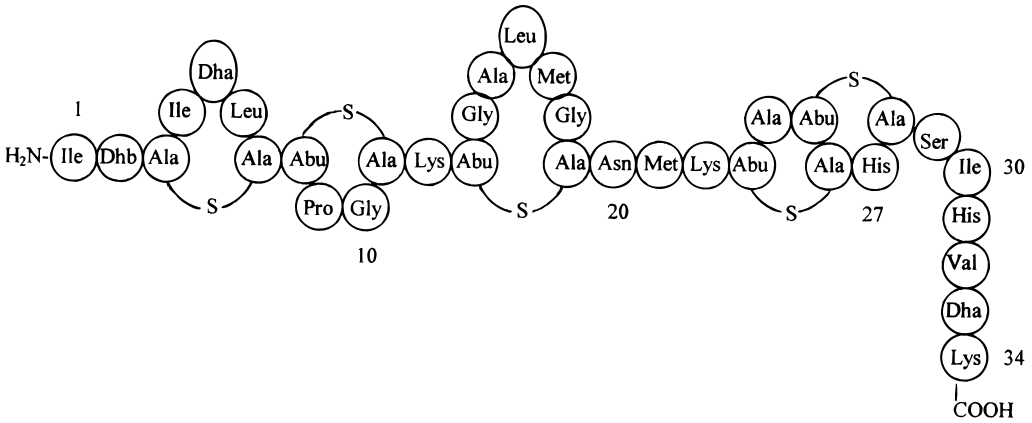

Figure 1. Nisin A structure. Nisin Z has an Asn residue at position 27 instead of the His. Dha, dehydrolalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine.

Female BALB/c mice (6−8 weeks of age) were subcutaneously immunized at 2-week intervals with 25 μg of nisA−KLH. The initial injection consisted of 0.3 mL of conjugate in a 1:1 ratio of saline and Freund's complete adjuvant. The second dose consisted of 0.3 mL of conjugate in saline and Freund's incomplete adjuvant (1:1), and the subsequent doses were presented in saline (0.2 mL). Ether-anesthetized mice were bled from the retrobulbar plexus, and serum was obtained after overnight incubation of blood at 4 °C and centrifugation at 1000*g* for 15 min. Four days prior to the fusion, a final dose of immunogen was given to the mouse whose antiserum showed optimal characteristics.

Immunoassays. A CD-ELISA (type I CD-ELISA) was used to screen for nisA-specific antibodies in mouse sera during immunization. Plates were coated overnight by air drying at 40 °C with 125 μL of nisA antisera (appropriately diluted in coating buffer: 0.1 M sodium carbonate−bicarbonate buffer, pH 9.6). Plates were washed four times with 300 μL of washing solution [0.02% (v/v) Tween 20 in 0.01 M phosphate buffered (pH 7.2−7.4) saline (PBS)]. Wells were blocked for 30 min at 37 °C with 300 μL of 1% ovalbumin (grade III) (w/v) in PBS (OA-PBS) and then washed four times. Next, to each well was added consecutively 50 μL of nisA standard (or sample) and 50 μL of nisA-HRP (2 μg/mL in OA-PBS). Plates were incubated for 1 h at 37 °C and washed eight times, and bound peroxidase was determined with ABTS substrate as described elsewhere (Harlow and Lane, 1988). Absorbance was read at 405 nm with a Labsystems iEMS reader with a built-in software package for data analysis (Labsystems, Helsinki, Finland).

A second type of CD-ELISA (type II CD-ELISA) was devised to assess the production of nisA-specific antibodies in the hybridoma supernatants. This type II assay was further used to evaluate the specificity and sensitivity of the antibodies secreted by the stabilized cell lines and to determine the recoveries in spiked samples. Plates were coated overnight by air drying at 40 °C with 125 μL/well of goat anti-mouse immunoglobulin G Fc fragment specific (8 μg/mL in coating buffer). After washing and blocking, 50 μL of anti-nisA antibody were added per well, and plates were incubated for 30 min at 37 °C. Following five more washes, to each well was added consecutively 50 μL of nisA standard (or analogue or sample) and 50 μL of nisA−HRP. The assay was then completed as just described for the type I CD-ELISA.

To identify cultures containing anti-nisA antibodies in fusion and cloning wells, after washing and blocking, 40-μL aliquots of each hybridoma supernatant were added into sets of four wells (of plates coated with anti-mouse immunoglobulin G Fc fragment specific) and incubated at 37 °C for 30 min. After washing five times, 50-μL aliquots of PBS were added as nisA-free blanks to each of two wells, and 50-μL aliquots of nisA (5 μg/μL in PBS) were added to the two other wells; then, to each of these four wells, 50 μL of nisA−HRP was added consecutively. The assay was then completed as already described.

Hybridoma Production. Spleen cells from a hyperimmunized mouse were fused with P3X63-Ag8.653 myeloma cells with PEG as fusogen reagent (Galfre and Milstein, 1981). Fused cells were resuspended in complete medium [RPMI 1640 (supplemented with 1% glutamine and 100 U/mL of PEST solution) containing 15% FCS and 10% BM-Condimed-H1 media], then distributed into wells (125 μL/well) of microculture plates and incubated at 37 °C in a humid atmosphere of 5−7% CO_2. After 24 h, 125 μL of HAT medium (complete medium with HAT solution) were added per well and incubated for 48 h. Next, half of the supernatant from each well was removed and replaced with an equal volume of fresh HAT medium. This operation was repeated every 2−3 days for a period of 2 weeks, after which HAT media was eliminated by gradual replacement with HT medium (the same composition of HAT medium but with HT solution instead). Supernatants of hybridoma cultures were tested for the presence of specific antibody of type II CD-ELISA as described before. Hybrid lines secreting the most sensitive antibodies were cloned by limiting dilution at 0.5−1 cell/well (Goding, 1980). Clones yielding optimal antibody activity were then isotyped (Isotyping system ISO-2; Sigma Chemical Company, St. Louis, MO), and finally stored in FCS:dimethyl sulfoxide (9:1) under liquid nitrogen. Mass production of nisA monoclonal antibodies were done either by culture expansion of the selected clones or in ascites fluid of pristane primed mice injected with 1×10^7 cells of a specific clone (Harlow and Lane; 1988). Antibodies were purified and concentrated from cell-free culture supernatants or ascites fluid by precipitation with 50% saturated ammonium sulfate (Hebert et al., 1973).

Microorganisms. The bacteriocin-producing lactic acid bacteria used to test antibody cross-reactivity are described in Table 2. Microorganisms were propagated overnight in MRS broth (Oxoid Ltd., Basingstoke, U.K.) at 37 °C. Next, $\sim 1 \times 10^5$ CFU of a fresh culture was inoculated in 5 mL of MRS broth and incubated for 16 h at 37 °C. Culture supernatants were obtained by centrifugation at 12 000 *g* for 10 min at 4 °C. The pH of the supernatants was adjusted to 6.1 with 1 N NaOH, next they were filtered through 0.22-μm pore size filters (Millipore Corp., Bedford, MA), and finally they were stored at −20 °C until required.

Spiking and Extraction of Cheese Samples. A Kraft Philadelphia spread cheese (free of nisin) was used in the recovery studies. The extraction procedure was done as described by Fowler et al. (1975). NisA (125 μL) was added to 2.5 g ± 0.1 g of cheese dispersed in four volumes (w/v) of 0.02 M HCl, to give final concentrations of 250, 1250, and 5000 ng/g. Next, the pH was adjusted to 2.0 and the samples were heated for 5 min at 98 °C in a water bath. After cooling at room temperature, the volume of the samples was adjusted to 12.5 mL with 0.02 M HCl, and the samples were centrifuged at 4000*g* for 20 min at 4 °C. After 30 min at 4 °C, the supernatants were collected and filtered through Whatman filter paper (No. 1), and their pHs were adjusted to 6.5 with 5 N NaOH. NisA standard curves were prepared in nisA-free cheese extracts (NFCE) obtained as described.

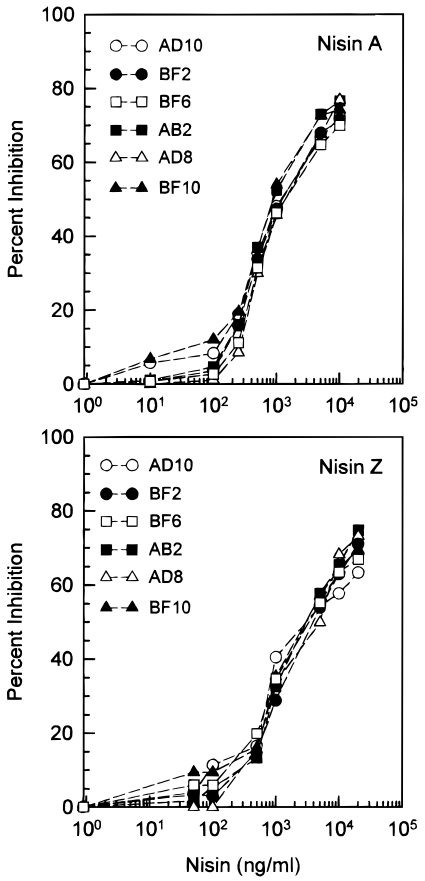

Figure 2. Specificity of nisA MAbs derived from different clones as determined by CD-ELISA. Data for each nisin variant represents the average value of triplicate determinations in a single microtiter plate.

RESULTS AND DISCUSSION

Mouse Immunization and Hybridoma Production. After the third immunization with the nisA-KLH conjugate, all the animals ($n = 4$) showed the presence of specific nisA antibodies, with serum titer values ranging from 12 800 to 25 600 [serum titer assessed by indirect ELISA (Suárez et al., 1996) was arbitrarily designated as the maximum dilution that yielded at least twice the absorbance of the same dilution of nonimmune control serum]. Binding inhibition values (average and range) of nisA-HRP to nisA antibodies when 0.1-, 1-, and 10-μg/mL concentrations of free nisA were assayed were 13% (9−16), 41% (28−57), and 72% (58−83), respectively. Spleen cells from the animal with the best antiserum were fused with P3X63-Ag8.653 myeloma cells at a 7.5:1 ratio. From 480 seeded wells, only one produced anti-nisA antibodies as determined by type II CD-ELISA. Upon cloning, six clones (AD10, BF2, BF6, AB2, AD8, and BF10) were selected for further characterization.

Characterization of Monoclonal Antibodies. The isotype of all six clones immunoglobulins was IgG_1. Sensitivity and specificity of the monoclonal antibodies was assessed with pure nisins (Figure 2; Table 1). The detection limit of the type II CD-ELISA for the best clones was 10 ng/mL, with response curves ranging from 10−50 to 5000 ng/mL. The concentration of free nisin required to inhibit antibody binding by 50% ranged from 900 to 1450 ng/mL (mean, 1180 ng/mL) for nisA, and from 3000 to 5100 ng/mL (mean, 3750 ng/mL) for nisZ. Mean cross-reactivity for nisZ was 33% (Table 1), which was lower than the 65% value reported when using polyclonal antibodies (Suárez et al., 1996). Cross-reactivity toward the naturally found nisin variant nisZ was expected because it only differs from nisA in a single amino acid at position 27 (Figure 1; Mulders et al., 1991). However, considering the very high homology between both nisin variants, the result was relatively low. Thus, low or no cross-reactivity could be expected with other type A lantibiotics like subtilin, epidermin, pep5, etc. (Shal et al., 1995) based on their sequence differences, although this needs further confirmation. This nisZ cross-reactivity results indicate that the epitope of these nisA MAb includes the histidine residue at position 27 in ring E. The specificity of the CD-ELISA with nisA MAb (pool of different clones) was also studied in supernatants of bacteriocin-producer lactic acid bacteria (Table 2). The immunoassay positively reacted with the supernatants of nisA- and nisZ-producer strains. Slight differences in reactivity detected among strains was most likely due to the variable amounts of bacteriocin produced by each microorganism. The immunoassay did not detect activity in the supernatants of the plasmid-free non-nisin-producing strain nor in the supernatants of other non-lantibiotic bacteriocin-producer microorganisms.

NisA competition curves in PBS and in a nisA-free cheese extract (NFCE) obtained by simultaneous incu-

Table 1. Reactivity of Nisin A Monoclonal Antibodies Produced by Different Clones As Determined by CD-ELISA

	amount required for 50% inhibition (ng/mL)[a]	
clone	nisin A	nisin Z
AD10	1100 (100)[b]	3300 (33)
BF2	1300 (100)	4300 (30)
BF6	1450 (100)	3500 (41)
AB2	940 (100)	3200 (29)
AD8	1400 (100)	5100 (27)
BF10	900 (100)	3000 (38)

[a] Data from Figure 1. [b] Cross-reactivity defined as (ng/mL) of nisin A required for 50% inhibition)/(ng/mL of analogue required for 50% inhibition) × 100.

Table 2. Reactivity of Nisin A Monoclonal Antibodies against Culture Supernatants of Lactic Acid Bacteria As Determined by CD-ELISA

microorganism[a]	source[b]	cross-reactivity[c]
Lactococcus lactis BB24 (nisin A)	our collection	100
Lactococcus lactis G18 (nisin A)	our collection	84
Lactococcus lactis 5876 (nisin A)	IFR	85
Lactococcus lactis ATCC 11454 (nisin A)	ATCC	74
Lactococcus lactis CNRZ 150 (nisin Z)	INRA	95
Lactococcus lactis MG1614 (no producer)	IFR	nr
Lactobacillus sake 148 (lactocin S)	our collection	nr
Pediococcus acidilactici 347 (pediocin PA-1)	our collection	nr
Pediococcus acidilactici L50 (pediocin L50)	our collection	nr

[a] Bacteriocin produced in parentheses. [b] INRA, Station de Reserches Laitières (Jouy-en-Josas, France); IFR, Institute of Food Research, Norwich Laboratory (Norwich, U.K.); ATCC, American Type Culture Collection (Rockville, MD). [c] Cross-reactivity defined as [(antibody binding inhibition produced by a culture supernatant/antibody binding inhibition produced by supernatant of *Lactococcus lactis* BB24) × 100]; nr, no reactivity.

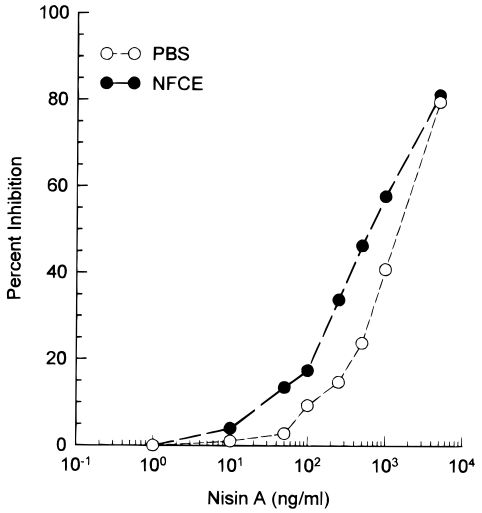

Figure 3. CD-ELISA standard curve of nisA in PBS, and in a nisin-free cheese extract (NFCE). Each data point represents the average value of triplicate determinations in a single microtiter plate. Absorbance values at 100% for PBS and NFCE were 2.0 and 1.9, respectively.

Table 3. Nisin A Recovery from Spiked Spread Cheese As Determined by CD-ELISA

nisin A added (ng/g)	sample[a]	recovery[b,c] ng/g	recovery[b,c] %	CV,[d,e]
250	A	266 ± 43	106	16.2
250	B	341 ± 39	137	11.5
250	C	370 ± 113	148	30.4
1250	A	1274 ± 43	102	3.4
1250	B	1697 ± 36	136	2.2
1250	C	1528 ± 178	122	11.6
5000	A	4294 ± 548	86	12.8
5000	B	6096 ± 1166	122	19.1
5000	C	4331 ± 565	87	13.0

[a] Triplicates of each sample spiked separately were analyzed after extraction on different days (A−C). [b] Mean recoveries for samples containing 250, 1250, and 5000 ng/g of nisA. [c] Interassay coefficients of variation ($n = 3$) for 250, 1250, and 5000 ng/g were 13.5, 11.6, and 17.1%, respectively; mean interassay CV was 14.1%. [d] Intraassay coefficient of variation; mean intraassay CV was 13.3%.

bation of nisA and nisA−HRP over anti-nisA MAb AD10 are shown in Figure 3. Response range for the curves in PBS and NFCE were 50−5000 and 10−5000 ng/mL, respectively. The NFCE curve pattern resembled that in PBS, indicating that interferences from matrix and extraction procedure were minimal. Thus, samples could be analyzed by ELISA, by direct comparison to standard in a nisin-free matrix.

Finally, the applicability and performance of a CD-ELISA with AD10 anti-nisA MAb was assessed in extracts of spiked spread cheese (Table 3). Recoveries estimated by direct comparison with a nisA standard curve in NFCE in samples spiked with nisA at 250, 1250, and 5000 ng/g were 130, 120, and 98%, respectively. The mean recovery was 116%, with mean intra- and interassay coefficients of variation of 13.3 and 14.1%, respectively. Possible sources of variation besides the immunoassay itself are sample spiking and assay extraction efficiency. The recovery values reported here, as well as the intra- and interassay coefficients of variation are slightly higher than those reported by Falahee et al. (1990) when using the sheep polyclonal-based sandwich ELISA, but similar to those described for other analytes using competitive ELISAs (Azcona-Olivera et al., 1990, 1992).

Summary. We report here the production of a highly specific nisA MAb and the development of a sensitive competitive ELISA and it application in foods. Approved limits for nisA in different foods varies from country to country (some, do not even require limits) and, in most instances, is in the parts per million range (from 2−100 mg/kg food). The MAb-based ELISA described in this work should be useful for the rapid estimation of nisA in different food products because the detection limit of the assay is 10 ng/mL. The recovery results of the spiking studies in the 0.25−5-μg/g range support the usefulness of the MAb-based ELISA, which will be sufficient for the estimation of nisA in foods at the parts per million level. Other potential applications of these nisA MAb include their use (1) for rapid identification and isolation of nisin-producer strains, (2) as a tool for regulation and mechanistic studies, and (3) for immunopurification purposes.

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LITERATURE CITED

Avrameas, S.; Ternynck, T. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry* **1969**, *6*, 53−66.

Azcona-Olivera, J. I.; Abouzied, M. M.; Pestka, J. J. Detection of zearelone by tandem immunoaffinity-enzyme linked immunosorbent assay and its application to milk. *J. Food Prot.* **1990**, *53*, 577−580.

Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. Production of monoclonal antibodies to the mycotoxins fumonisins B_1, B_2 and B_3. *J. Agric. Food Chem.* **1992**, *40*, 531−534.

Briand, J. P.; Muller, S.; Van Regenmortel, M. H. V. Synthetic peptides as antigens: pitfalls on conjugation methods. *J. Immunol. Methods* **1985**, *78*, 59−69.

Falahee, M. B.; Adams, M. R.; Dale, J. W.; Morris, B. A. An Enzyme immunoassay for nisin. *Int. J. Food Sci. Technol.* **1990**, *25*, 590−595.

Federal Register. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. *Fed. Regist.* **1988**, *54*, 11247−11251.

Fowler, G. G.; Jarvis, B.; Tramer, J. The assay of nisin in foods. *Soc. Appl. Bacteriol. Tech. Ser.* **1975**, *8*, 91−105.

Galfre, G.; Milstein, C. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* **1981**, *73*, 3−46.

Goding, J. W. Antibody production by hybridoma. *J. Immunol. Methods* **1980**, *39*, 285−308.

Harlow, E.; Lane, D. In *Antibodies. A Laboratory Manual*; Cold Spring Harbor Laboratory: New York, 1988.

Hebert, G. A.; Pelham, P. L.; Pittman, B. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse and goat antisera. *Appl. Microbiol.* **1973**, *25*, 26−36.

Köeler, G.; Milstein, C. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* **1975**, *256*, 495−497.

Mulders, J. W. M.; Boerrigter, I. J.; Rollema, H. S.; Siezen, R. J.; de Vos, W. M. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *Eur. J. Biochem.* **1991**, *201*, 581−584.

Nakane, P. K.; Kawaoi, A. Peroxidase-labeled antibody: a new method of conjugation. *J. Histochem. Cytochem.* **1974**, *22*, 1084−1091.

Shal, H. G.; Jack, R. W.; Bierbaum, G. Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. *Eur. J. Biochem*. **1995**, *230*, 827−853.

Suárez, A. M.; Rodríguez, J. M.; Hernández, P. E.; Azcona-Olivera, J. I. Generation of polyclonal antibodies against nisin: immunization strategies and immunoassays development. *Appl. Environ. Microbiol*. **1996**, *62*, 2177−2121.

Tramer, J.; Fowler, J. J. Estimation of nisin in foods. *J. Sci. Food Agric*. **1964**, *15*, 522−528.

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