Antibotulinal Properties of Nisin in Fresh Fish Packaged in an Atmosphere of Carbon Dioxide

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ABSTRACT

The inhibitory effect of nisin on toxin production by Clostridium botulinum type E in inoculated cod, herring, and smoked mackerel fillets, packaged in a 100% carbon dioxide atmosphere, was studied at storage temperatures of 10 and 26°C. Nisin delayed the onset of toxin production in all three fish species. The nisin effect was marked at 10°C and delayed the onset of toxin production by at least 5 d beyond that of the controls. At 26°C the delay was shorter, being one-half day for cod, one day for herring, and two days for smoked mackerel. Nisin did not affect the time to spoilage by nonpathogenic bacteria at either temperatures of storage. It was noted that toxin was formed in untreated cod, mackerel, and herring stored at 26°C and in mackerel stored at 10°C before the samples were judged to be unacceptable to the consumer.

A wealth of evidence has been reported (11,13,16) to indicate that fresh fish may contain Clostridium botulinum type E as part of the natural microflora or as a result of contamination after catching. Under conditions of distribution where fish is chilled on ice and unpackaged, C. botulinum toxin production does not present a problem (11,18). The situation may be different, however, where fish is packaged either under vacuum or in a modified atmosphere in order to expand storage life by reduction of the oxygen level and thereby suppressing the growth of the intrinsic, nonpathogenic spoilage flora. In the case of modified atmosphere, it has been widely shown that gas mixtures rich in carbon dioxide are most effective in inhibiting bacterial spoilage (18), and it is such conditions which may favor the growth of C. botulinum when fish is stored at temperatures above 4°C.

Fears of potential botulism hazards in packaged fish materialized in the 1960's when outbreaks of botulism occurred in the United States of America from the ingestion of hot-smoked, vacuum packaged (VP) fish. Despite much research VP fish which has been reviewed in a number of papers, including those by Baird-Parker (3) and Eyles and Warth (10), in the eyes of some authorities the problem remains unresolved. Furthermore, similar fears have been expressed about the safety of modified atmosphere packaged (MAP) fish (9,14,15).

It has been shown, for a range of fishery products that differences in the rate of toxin production between VP, MAP, and air packed fish are marginal (4,5,6) which leaves the main area of concern to be the relationship between toxicity and the shelf life extension in VP and MAP fish products. It has been found that detection of toxin in white fish packaged in MA and stored at 8 to 12°C either precedes or coincides with the time to sensory rejection (8,18).

The antimicrobial agent nisin, produced by certain strains of Streptococcus lactis, has been reported to be effective in inhibiting the outgrowth of C. botulinum type E spores in broth culture (17). Its use in fish packed in a modified atmosphere (MAP) may delay the onset of toxicity to a point where the product would be spoiled and rejected before consumption.

This study was conducted to investigate the effect of nisin on the growth of C. botulinum type E in fish packaged in a modified atmosphere of 100% carbon dioxide and to evaluate its effect on the botulism hazard from ingestion of MAP fishery products.

MATERIALS AND METHODS

Fish source

Prime quality fish were bought either on the Aberdeen Fish Market or from an Aberdeen processor with emphasis placed on the fact that the fish should be in rigor mortis at the time of purchase. Cod were hand filleted at the Torry Research Station (TRS) then blast frozen and held at -30°C until required for use. Herring were commercially machine filleted and used the same day.

Mackerel were blast frozen whole at TRS and again held at -30°C until required. No fish were stored frozen for more than 2 weeks before being used. For preparation of the hot smoked product the fish were thawed in air, hand filleted, and brined prior to smoking in a Torry kiln for 45 min at 30°C, 30 min at 50°C with a final kiln temperature of 70°C for 40 min. To achieve a projected salt concentration of 1% (percent salt in the aqueous phase of the fish), the fillets were brined in a salt solution containing 52.8 g/L (20 salinometer degrees) for 30 sec then smoked.

Application of nisin

Prior to treatment with nisin, all fillets were trimmed to give a weight of 100 g ± 5 g. The fillets were then placed on 14 cm mesh plastic coated galvanized iron shelves of the type used in smoking kilns and sprayed from above and below using a knapsack pressurized garden sprayer (ASL Airflow Limited, 135 Abbey Road, Aberdeen, Scotland AB9 8DG).
Birmingham, England). Nisin as the commercial concentrate Nisaplin® supplied by Aplin and Barrett Limited (Beaminster, Dorset, England) was used which contains 2.5% active nisin on a carrier of dried milk powder and has an activity of 1 million international units (IU)/g. Fillets were treated with nisin solutions in deionized water containing 8000, 16,000, and 32,000 IU/ml to give target nisin concentrations of 250, 500, and 1000 IU/g of fish, respectively. Higher levels of nisin were used for fish held at 26°C and the lower levels for fish held at 10°C. For control purposes, the required numbers of fillets of each type were sprayed with deionized water. After spraying, the fillets were left to drain for 5 min before transportation to a nearby fish processing plant for packaging.

Packaging of fish

Treated fillets were packed under semicommercial conditions at Clipper Seafoods Ltd, Aberdeen, using a Multivac RV700 system (Multivac UK Ltd, Swindon, England). The packaging used comprised a lower laminate of 300 microns uPVC (unplasticised polyvinyl chloride) and 100 microns low density polythene with an antifog coating. Laminates were supplied by D.R.G. Flexible Packaging, Bristol, England. The ratio of gas to fish was 6:1 and the initial composition of the gas in the packs was established to be entirely carbon dioxide by gas analysis using a Gow-Mac instrument. (Gow-Mac Instrument Co. (UK) Ltd, Gow-Mac House, PQ Box G13, 6 Livingston Circus, Gillingham ME7 2NH, Kent). Packs were checked for gas composition and seal integrity during and after each production run.

On return to TRS a dab of Arbosil 1081 silicone sealer (Aldshead, Ratcliffe and Co Ltd, Belper, Derby) was applied to the top surface of all packages that were to be inoculated, to provide an inoculation septum. This sealer cures sufficiently fast to allow inoculation of the packs within 1 h of application.

Inoculation of the fish with C. botulinum

A spore cocktail was prepared by the method described by Cann et al. (4) which comprised an equal mixture of five strains of C. botulinum type E with a viable count of 1 x 10⁵/ml. The strains that were used were Hazen, strain 35396 (N.C.I.B. 4207); Beluga (N.C.I.B. 4248); Tenno (N.C.I.B. 4207); Minnesota (F.T. 52); and Birmingham (U. 25301).

For toxicity studies, all packages were inoculated with 1 ml of the C. botulinum spore suspension to give a concentration of 1 x 10⁴ to 1 viable spores in the fish. The suspension of spores was introduced into the package by injection through the septum using a hypodermic needle and syringe. Fish were inoculated by spraying the suspension over the surface of each fillet.

Temperature of storage

In the first experiment of the series using cod, storage temperatures of 8 and 26°C were chosen. However, due to the poor toxin development at the lower temperature subsequent experiments were conducted at 10 and 26°C. The former figure was chosen to simulate mild temperature abuse during commercial handling and storage and the latter, gross temperature abuse which might take place during the height of summer either commercially or more probably in the home.

Sampling

Five replicates of test packs and controls were sampled for analysis at regular intervals according to the expected toxigenicity of the fish product under test in relation to the days prior to development of toxin in an inoculated control, cooked meat broth held under identical conditions of storage.

Bacteriological analysis

Fish products were homogenized and assayed for toxin production using the mouse bioassay test as described by Cann et al. (7) with the trypsinized extract prepared from each fillet being inoculated into duplicate mice. The cooked meat broths were assayed similarly. Toxicity was recorded only when typical signs of botulism were observed followed by death of the mice and confirmation of type E using monovalent antisera. Nonspecific deaths were not a problem as they were eliminated by the trypsinization techniques. The concentration of nisin in the fish before incubation was determined by the plate diffusion method as described by Tramer and Fowler (19).

Sensory analysis

The organoleptic properties of raw odor, cooked odor, and cooked flavor of control uninoculated packaged fish taken from the same batch and held under identical conditions of storage were assessed using a trained taste panel of 4-6 judges. Duplicate packs of fish were examined at each sampling point, steamed in casserole dishes for 20 min, and assessed using the Torry objective score sheets for cod, herring, and mackerel. Herring were rejected at a score of 3.0 on a 7 point scale. Cod and mackerel were rejected at a score of 4.5 on a 10 point scale (5).

RESULTS

Nisin levels

The actual levels of nisin achieved within each batch of fillets are shown in Tables 2, 3, and 4.

The average salt in the water phase of smoked mackerel fillets throughout the storage period was determined to be 1.5% (1).

The sensory assessment of uninoculated fillets stored at 26°C gave a shelf life of around 2 d for cod, 4 d for mackerel, and 1 d for herring as judged by cooked odor and cooked flavor (Table 1). However, when judged on appearance the cod fillets were deemed unacceptable after storage for 1 d because of excessive drip which contained a milky white curd. Nisin treated fish spoiled at the same rate as the control fillets as judged by cooked odor and flavor, indicating that nisin has no inhibitory effect on the normal spoilage organisms of fish packed under modified atmosphere. The sensory assessment of uninoculated fillets stored at 10°C gave a shelf life of 1 d for herring, 7 1/2 d for cod as judged by cooked odor, and greater than 12 d for mackerel as judged by cooked odor (Table 1). There was no noticeable effect by nisin on the organoleptic property of the fish. Toxin did not develop in any of the inoculated fish over the storage period of 16 d.

Toxicity studies on fish stored at 26°C indicated that in cod, fillets became toxic after storage for 1 1/2 d. The degree of toxicity was proportional to the concentration of nisin present, with all replicates of the control fish being toxic (Table 2). After storage for 2 1/2 d, the inhibitory effect of nisin was lost when all replicates of both control
and treated fish became toxic. In mackerel, toxin development in the control fish occurred after 2 d storage (Table 3). This was delayed in the treated fish to 2 1/2 d for the lower and 4 d for the higher levels of nisin that were used. Throughout the 5 d storage period, toxicity was not detected in all five replicates of the nisin treated fish, whereas all the control fillets became toxic after a further 2 1/2 d storage. In herring, toxin was present in three of the 5 replicates of control fish at the first analysis after 18 h storage while all replicates were toxic after a further 6 h storage (Table 4). Fish treated with nisin became toxic after 40 h of storage. A quantitative effect of nisin was apparent as toxicity did not develop in all replicates treated with the stronger solution until 64 h of storage.

Toxicity studies at 10°C indicated that in cod, toxin occurred in one of the inoculated control packs after storage for 2 d (Table 2). No further toxin was detected until 10 d of storage when all sampled replicates of the control fish became toxic. Subsequently during the storage period of

### TABLE 1. Time to spoilage, in days, of fish packaged in 100% carbon dioxide, as judged by cooked odor, raw odor, and cooked flavor.

<table>
<thead>
<tr>
<th>Product</th>
<th>Sensory analysis</th>
<th>Temperature of storage (°C)</th>
<th>8°C</th>
<th>10°C</th>
<th>26°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod</td>
<td></td>
<td></td>
<td>10</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Smoked *mackerel</td>
<td></td>
<td></td>
<td>14</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Herring</td>
<td></td>
<td></td>
<td>7-9</td>
<td>71/2</td>
<td>2</td>
</tr>
</tbody>
</table>

*Due to the risk of botulism and scombrotulin poisoning of taste panelists, the cooked flavor analysis of the smoked mackerel was not conducted as it is traditionally eaten without further cooking.

14 d toxicity was more erratic. None of the nisin treated fish developed toxin over the test period. In mackerel, one pack of the control fish was found to be toxic at the first sampling point after 7 d storage (Table 3). From 7-17 d toxicity was variable but toxicity of all replicates was not observed. The nisin treated fish developed toxin after 13 and 14 d storage in fish treated to give 173 and 409

### TABLE 3. Toxin production by C. botulinum type E in hou-smoked mackerel sprayed with varying concentrations of nisin (IU/g) and packaged in a modified atmosphere of 100% carbon dioxide.

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>Temperature of storage (°C)</th>
<th>Nil</th>
<th>409</th>
<th>600</th>
<th>Nil</th>
<th>173</th>
<th>409</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>1 1/2</td>
<td>0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>1 1/2</td>
<td>0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

### TABLE 2. Toxin production by C. botulinum type E in cod sprayed with varying concentrations of nisin (IU/g) and packaged in a modified atmosphere of 100% carbon dioxide.

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>Temperature of storage (°C)</th>
<th>Nil</th>
<th>675</th>
<th>637</th>
<th>Nil</th>
<th>333</th>
<th>675</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>1 1/2</td>
<td>0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2 1/2</td>
<td>0/10 0/10 0/10 N.T. N.T. N.T.</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>**N.T. 0/10 0/10 N.T. N.T. N.T.</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>N.T. N.T. N.T. 1/10 0/10 0/10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>N.T. N.T. N.T. 0/10 0/10 0/10</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>N.T. N.T. N.T. 0/10 0/10 0/10</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>N.T. N.T. N.T. 0/10 0/10 0/10</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>N.T. N.T. N.T. 10/10 0/10 0/10</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>N.T. N.T. N.T. 5/10 0/10 0/10</td>
<td>11</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>N.T. N.T. N.T. 9/10 0/10 0/10</td>
<td>12</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>N.T. N.T. N.T. 8/10 0/10 0/10</td>
<td>13</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>N.T. N.T. N.T. 4/10 0/10 0/10</td>
<td>14</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

* Superscript = number of deaths of mice from botulism.

Subscript = number of inoculated mice.

**N.T. = not tested.
nisin IU/g, respectively, and then remained toxic, albeit at a low level to the end of the experiment. Toxin was detected in some packs of herring after 7 d storage, and this situation continued until 15 d when all replicates were found to be toxic (Table 4). Toxin did not develop in the nisin treated fish until after 11 d storage, and toxicity of all replicates was not observed up to 15 d.

**DISCUSSION**

In the present study the time taken for fish to become toxic when stored at the gross abuse temperature of 26°C compared well with earlier studies of fish packaged in atmospheres rich in CO₂. Findings of toxicity in 2 d at 26°C (16) have been reported for cod in comparison with the 1 1/2 d reported here. Similarly, Cann, et al. (5) reported the onset of toxicity in herring and smoked mackerel after 1-2 and 2-4 d at 20°C, respectively, in comparison with the present finding of 1 and 2 d, the shorter times to toxicity in the latter study reflecting the higher storage temperature. The rate of organoleptic spoilage in fish stored at 26°C was surprisingly slow and probably reflected mesophilic bacterial spoilage rather than the psychrophilic spoilage typically found in chilled fish (12).

A large number of studies has been conducted on the botulinogenic properties of packaged fish stored at the moderate abuse temperature of 10°C. These have been summarized in a number of reviews, the most recent of which are those by Hobbs (11) on fishery products in general and by Statham (18) specifically on modified atmosphere packaged fish.

The comparative times to the onset of toxicity and spoilage obtained in this study agree well with the earlier studies for similar species of fish, although strict comparisons are difficult because of differences in experimental design and the criteria used for sensory rejection. For instance, in this work the cut-off point for rejection was taken when off-flavors first became apparent. If lower quality levels were to be used, the degree to which fish became toxic before becoming spoiled would be more marked.

The inhibitory effect of nisin on toxin production in packaged fish, although not complete, did have a significant delaying effect in all three species of fish examined. In cod, the effect was marginal at 26°C even with the highest level of nisin used, when 1337 IU/g only delayed the complete toxicity of all replicate fillets by half a day. Comparison of the shelf life of the fillets from the viewpoint of acceptability and safety shows that fish stored at the high temperature of 26°C became toxic while still considered to be edible using the Torry criteria of acceptability. At 10°C, if the transient development of toxin in one replicate pack of untreated fish after 6 d is ignored, the safe shelf life of the control fish was 9 d, 2 1/2 d after rejection by sensory analysis. The addition of nisin at levels of 333 and 675 IU/g further extended the safe shelf life to at least 14 d.

The storage data for toxin production in smoked mackerel indicated that at the higher abuse temperature of 26°C nisin at levels of 409 and 600 IU/g extended the safe shelf life by 1/2 d and 1 1/2 d, respectively. Control fillets and those treated with the lower level of nisin both became toxic before rejection by sensory analysis. At 10°C, the control fish became toxic after 7 d storage, some 5 d before sensory rejection of parallel uninoculated samples, whereas fish treated with nisin at levels of 173 IU/g and 409 IU/g had safe shelf lives of 12 and 13 d, respectively, exhibiting an extension of safe shelf life to a point beyond sensory rejection.

In assessment of any treatment for extension of wholesomeness of herring, the perishable and highly botulinogenic properties of this species must be taken into account, particularly when the studies are conducted near the optimum growth temperature for *C. botulinum* type E. At 26°C, a significant extension of the safe shelf life was observed, with control fish becoming toxic 6 h before sensory rejection and treated fish at both dosage levels some 16 h later. The data obtained for fillets stored at 10°C showed a considerable inhibitory effect by nisin which extended the safe shelf life by 4-5 d. However, fillets stored at this temperature were found to be unacceptable by sensory analysis after 2 d storage.

These studies indicate that the treatment of fresh fish packed in a modified atmosphere of carbon dioxide with nisin can significantly delay the onset of toxin production by *C. botulinum* type E. It may be argued that in commercial practice spores of *C. botulinum* may be present deep in the flesh. However, the natural incidence of this organism is in the intestines and to a lesser extent the gills and rarely on the skin of fish (13). Contamination, therefore, can be expected to occur largely on the surface of fish fillets. It is inevitable, however, that spores will penetrate the flesh of badly handled fish but so should nisin by the same route. Furthermore, toxin production in deeply inoculated fish has been shown to occur at much the same rate as surface inoculated, packaged fish (4,5,6). Although the use of this antimicrobial agent could not be advocated to replace good manufacturing practice (GMP), its use could offer a degree of protection against temperature abuse which inevitably occurs during commercial handling and processing, and particularly during and after retail purchase (2). Nisin, in reducing the risk of onset to toxicity before rejection by the consumer because of spoilage, affords added protection to MAP fish products while having no effect on their normal pattern of spoilage. Furthermore, as nisin is not used medically and is widely used internationally in cheese manufacture, its use in packaged fish should not suffer the common objections attendant on the introduction of a new food preservative.

**REFERENCES**


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