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Studies on Growth and Toxin Production of C. Botulinum
Type E on Cod Homogenate Treated with A combination of
Spices, Sodium Chloride and γ -Radiation

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ABSTRACT

Cod homogenates inoculated with spores of C. botulinum type E strain Erimo at 10^2 and 10^4 /g were treated with 1% and 2% sodium chloride, 0.25% each of mustard, garlic and turmeric and 0.3 Mrad γ -radiation either in single or combination treatments. The growth and toxin production of type E spores in the inoculated homogenates were followed at incubation temperatures of 30°, 10° and 5°C for 7, 28 and 56 days respectively. Growth and gas formation were noted in all the samples but type E toxin could not be detected. The reason for the absence of toxin in both the untreated and treated homogenates could not be ascertained. Inadequate detection method, unfavourable growth conditions in the homogenate and weak toxigenicity of the strain employed have been advanced as probable factors that contributed to the negative results on the toxin assay.

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INTRODUCTION

C. botulinum, a dangerous foodborne sporeformer, is the most resistant of all the intoxicating bacteria that are encountered in the radiation preservation of fish and fishery products. Any preservation method used must ensure complete safety of the preserved product from this deadly organism. To achieve such a high degree of safety from C. botulinum requires very high doses of radiation which cause damage to the organoleptic qualities of fish. For this reason, it is essential to find a preservation method which will completely eliminate the danger of botulism and at the same time retain the desirable palatable qualities of the preserved product.

There are some ways by which the dose requirement for effective inactivation of C. botulinum spores present in food can be significantly reduced. One way to achieve this is by the use of chemical additives and spices (1). A combination of mustard and γ -radiation was found to inhibit the toxin production of C. botulinum inoculated in meat. Corriander seed and nutmeg in combination with γ -radiation also exerted an additive inhibitory effect on botulinum toxin production. The advantage of using spices are that they are a popular and traditional food additive and their use in food does not present health hazard problem. Spices add characteristic flavour to foods and enhance their natural flavour. Spices are primarily used as flavouring agents and their antimicrobial role, if any, is a secondary consideration.

This study was initiated to investigate the effect of γ -radiation combined with spices and NaCl on the growth and toxin production of C. botulinum type E inoculated in cod homogenate and incubated at 30°, 10° and 5°C for various periods of time. Fewer reports on similar work are available and practically very little study has been carried out on inoculated packs using fish homogenate.

The objectives of this study were to determine:

- i) Whether the combination treatments of γ -radiation, spices and NaCl could minimize the risk of botulism by affecting the growth and toxin production of the organism in fishery products.
- ii) Whether the combination treatments of fishery products could create more favourable conditions for the growth and toxin production of C. botulinum, and
- iii) Whether there was a margin of safety period between the times of toxin production and termination of shelf life of fishery products treated with the combination treatments.

MATERIALS AND METHODS

Organism: C. botulinum type E strain Erimo which was isolated from a causative food involved in human botulism was used for inoculation of cod homogenate. The strain was tested for toxin production in artificial medium before inoculation. The toxicity of this strain was compared with that of another type E strain, Iwanai, also an isolate from human botulism.

Spore preparation: Spores of Erimo strain were produced at 30°C in trypticase-peptone-glucose medium (2). The foreculture was prepared by incubating heat shocked (65°C for 13 min) spores at 30°C for 30 h. This culture was again heat-shocked at 65°C for 13 min and 1 ml was transferred to the sporulation medium which incubated for 28-30 h during which a good sporulation occurred. After harvesting, the spores were cleaned by centrifugation and by the lysozyme-trypsin enzyme treatment (3). Spores freed of vegetative debris, were resuspended in distilled water to give a 10^8 /ml suspension which was stored at 4°C until used for inoculation. The suspension was standardized by a replicated deep tube technique using pork infusion agar.

Preparation of fish homogenate: Fresh cod flesh was macerated with equal quantity of water in a homogenizer. The homogenate was put in screwcapped plastic bottles (ca. 10 g) at the rate of 5 g in each. Spores at 10^2 and 10^4 per g were injected into the homogenate by a sterile syringe. The inoculated homogenate was stored at wet-ice temperature until irradiated.

Spices and NaCl: The spices used were finely powdered commercial mustard, garlic and turmeric. Spices at 0.25% and NaCl at 1% and 2% concentrations were added to fish before homogenization. These were used without sterilization.

Irradiation: Samples were given 0.3 Mrad dose with a Co-60 source at a dose rate of 1 Mrad per hour. Irradiation was done at 0°C. The dose used was slightly less than $2D_{10}$ value of the Erimo strain in distilled water medium which was estimated by irradiating the organism in the 0-1 Mrad dose range at intervals of 0.2 Mrad.

Incubation of inoculated packs: After irradiation the samples were incubated at 30°C, 10°C for 6, 28 and 56 days respectively.

Toxin assay: The samples stored at 30°C were assayed every day for 6 days, those at 10°C on 6, 9, 12, 15, 18, 21, 24 and 28 days and the samples incubated at 5°C were analysed at 2 weeks intervals up to 8 weeks. At each designated assay period, duplicate samples per variable were assayed for odour, gas formation, pH and the presence of type E toxin. The contents of two bottles mixed, diluted with equal amount of water and centrifuged. The supernatant was injected intraperitoneally into two mice in both untrypsinized and trypsinized states at 0.25 ml/mouse. In instances where mouse deaths were noted the nature of toxin was varified by specific antitoxin protection test, heat shock treatment of the toxic supernatant at 80°C for 20 minutes and by injection of chloramphenicol and oxy-tetracyclin to mouse to prevent nonspecific deaths. The antibiotic injection was given according to the method described by Segner and Schmidt (4).

Storage life: The maximum storage life of the samples was judged on the basis of odour of the raw homogenate.

RESULTS AND DISCUSSION

The Erimo strain which was used for inoculating the packs was found to produce a weakly toxic culture compared with the Iwanai strain in brain heart infusion broth (results not shown). Irradiation of Erimo spores in distilled water medium yielded a D_{10} value of 0.155 Mrad (Fig. I). The dose used in irradiating the homogenate came to twice the D_{10} value of the organism.

Nonspecific mouse death occurred in all the nonirradiated samples including the uninoculated homogenate which was more pronounced at 30°C than at 10°C and 5°C incubation. All samples formed gas; the nonirradiated reference samples after the termination of the maximum storage life and the combination method treated ones before the termination of storage life. Gas formation was very insignificant in the samples treated with garlic and radiation. At 5°C incubation gas production was indistinct and irregular. The estimation of shelf life of these samples was made difficult for this reason and also due to the masking off of spoilage odour by the spices.

None of the inoculated packs under study was found to contain type E toxin at any temperature or period of incubation (Table I). Trypsinization of the homogenate filtrate also did not show detectable toxin in any sample. This finding is in disagreement with those of other investigators carrying out inoculated pack study. Ando *et al.* (5) reported toxin production in irradiated (0.1 and 0.3 Mrad) flat fish homogenates inoculated with Iwanai spores at 10^3 , 10^4 /g at 10°C and 20°C incubations. They also reported type E toxicity in irradiated and inoculated fish fillets stored at 10°C (6). Segner and Schmidt (7) found type E toxin in haddock fillets irradiated at 0.1 and 0.2 Mrad. Slavin and Ronsivalli (8) detected toxicity in irradiated fillets inoculated with *C. botulinum* type E strain Beluga. None of these investigators worked with the combination methods, so little is known about their effect on the growth and toxin production of *C. botulinum* type E in inoculated fillet or homogenate.

The reason for our failure to detect toxicity in the inoculated combination or reference treatments could not be known. A combination of factors, such as low levels of toxin produced, unfavourable conditions of the medium, inadequacy of the method of detection employed, weak toxigenicity of the botulinal strain used *etc.* might be acting in disfavour of toxin production or appearance of toxicity in the inoculated homogenates.

Detection of low levels of toxin is complicated by the fact that the toxin is found intracellularly and released primarily by vegetative cell lysis (7). If the cell lysis process is hampered or slowed down under unfavourable growth condition toxicity may not appear in the medium unless some means is used to extract the toxin from the cells or cause them to lyse.

Alternatively, the physical and chemical conditions of the inoculated homogenate might adversely affect the growth and toxin production. Fish homogenate does not provide a completely anaerobic environment which is essential for the growth and toxin production of

C. botulinum. Air is trapped inside the spongy homogenate which cannot easily diffuse out on to the empty space of the air-tight pack. As a result the homogenate does not become sufficiently reduced. A properly reduced medium is necessary for good growth and toxin production (9). Outgrowth is markedly affected by the oxidation-reduction potential (Eh) of the medium, although this is again dependent on other factors such as pH. All these conditions mentioned above might affect the homogenate in a way to inhibit either the production or the appearance of toxin in the homogenate.

The toxin producing ability of Erimo strain under the experimental conditions might also contribute to the negative results on toxin assay. In artificial medium Erimo strain was found to be three-fifth as toxic as Iwanai strain. The toxin production of Erimo strain in brain heart infusion broth was found to be inhibited by incorporation of 1% NaCl and 0.25% each of mustard, garlic or turmeric while that of Iwanai strain was not affected (Table II). A test was then run to compare the toxin production of these two strains of type E in nonirradiated and irradiated cod homogenate. While Erimo strain gave a negative result Iwanai strain produced toxin in the irradiated (0.3 Mrad) homogenate incubated at 30°C for 4 days (Table II) but the nonirradiated sample remained nontoxic till the termination of the experiment (7 days). This finding agree with those of Ando et al. (5), Slavin et al. (8) and others (10) who reported earlier production of botulinal toxin in radurized fish homogenate or fillet. From this finding it appears that the weak toxigenicity of Erimo strain coupled with the unfavourable growth conditions in the cod homogenate might explain the reason for the nonappearance of toxicity. Since no toxin was detected the effect of the combination treatment of spices, NaCl and γ -radiation on toxin production could not be evaluated.

Spices, particularly garlic, had an effect on lengthening the shelf life and improving the colour of the homogenate, but did not affect gas formation and probably the growth of the organism.

From the results obtained and discussed above it may not be entirely out of substance to make the assumption that many of type E botulinal strains found toxic when grown in artificial media may not be toxic in foods. In artificial media all conditions of growth are carefully met, one or more of which a food medium may lack. This needs confirmation by further investigative and screening studies with as many C. botulinum types and strains as possible using a variety of foods and food products as substrates.

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Table 1. Toxin assay results on inoculated cod homogenates treated with combination and single treatments of spices¹ NaCl and γ -radiation

Treatment	Shelf life in days ² at incub. temps. of			Fraction of samples toxic ³
	30°	10°	5°	
1% NaCl + mustard + 0.3 Mrad	2	15	36	0/2
2% NaCl + " + "	2	18	30	0/2
1% NaCl + garlic + 0.3 Mrad	2	21	42	0/2
2% NaCl + " + "	3	24	48	0/2
1% NaCl + turmeric + 0.3 Mrad	2	15	36	0/2
2% NaCl + " + "	2	18	38	0/2
Mustard + 0.3 Mrad	3	20	42	0/2
Garlic + 0.3 Mrad	2	18	35	0/2
Turmeric + 0.3 Mrad	2	16	30	0/2
1% NaCl + 0.3 Mrad	2	16	35	0/2
2% NaCl + 0.3 Mrad	3	20	40	0/2
Uninoculated homogenate	1	7	22	0/2
Inoculated homogenate	1	7	22	0/2
1% NaCl	1	10	26	0/2
2% NaCl	2	15	30	0/2
Mustard	1	8	24	0/2
Garlic	1	10	27	0/2
Turmeric	1	8	24	0/2
1% NaCl + mustard	1	11	28	0/2
1% NaCl + garlic	1	15	30	0/2
1% NaCl + turmeric	1	12	28	0/2
2% NaCl + mustard	2	18	30	0/2
2% NaCl + garlic	2	20	35	0/2
2% NaCl + turmeric	2	18	32	0/2

¹ The concentration of spices used was 0.25% in powder forms.

² Maximum storage life in days of each treatment at 30°, 10° and 5°C incubations for 6, 28 and 56 days respectively.

³ Number of samples containing type E toxin per number of samples per treatment assayed at each designated period.

Table 2. Effect of NaCl, spices and γ -radiation on the relative toxicities of Erimo and Iwanai strains of C. botulinum type E incubated at 30°C under different conditions

Treatment	Incubation		Fraction of samples toxic ¹	
	Medium	Time (days)	Erimo	Iwanai ²
Untreated	BHI broth	5	3/3	3/3
1% NaCl	"	5	3/3	3/3
0.25% mustard	"	5	2/3	3/3
0.25% garlic	"	5	3/3	3/3
1% NaCl + 0.25% mustard	"	5	0/3	3/3
1% NaCl + 0.25% garlic	"	5	0/3	3/3
0.0 Mrad	Fish homogenate	7	0/3	0/3
0.3 Mrad	"	7	0/3	3/3

¹ Number of samples containing type E toxin per number of samples assayed.

² Toxin was detected on the 4th day of incubation.

Fig. 1. Dose-survival curve of Erimo strain of C. botulinum spores at 0°C.