[Chem. Pharm. Bull. 26(4)1130—1134(1978)]

UDC 615.355.014.453 : 577.15.07

Microbiological Studies on Drugs and Their Raw Materials. IV.[1] Sterilization of Microbial Contaminants in Enzyme Powder by Gamma Irradiation

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(Received September 3, 1977)

The decimal reduction dose of γ-ray on freeze-dried clean *Escherichia coli* cells and *Bacillus subtilis* spores was about 7×10^4 rad and it was about three times higher than that on *E. coli* cells suspended in saline. *E. coli* cells contained in Takadiastase or trypsin powder showed quite the same susceptibility to γ-irradiation as they were present cleanly. These enzyme activities were not impaired at all even at the dose of 2×10^5 rad. When these enzyme powders containing *E. coli* cells were stored under varied atmospheric relative humidity, the deleterious effect of γ-ray on bacterial cells was highly enhanced in those samples stored under more than 80.5% relative humidities. The additions of excipients, such as glucose and lactose, and of a protectant, L-cysteine, to the bacteria-containing dry enzyme powder did not show any sign of either enhancement or retardation of γ-ray action on enzyme and bacteria. Based on these observations, the utilizability of radiosterilization on biological medicaments is discussed.

Keywords——sterilization; radiosterilization; γ-irradiation; enzyme preparation; Takadiastase; trypsin; *Escherichia coli*; *Bacillus subtilis* spore; γ-ray

Drugs of biological origins are known to be contaminated with microorganisms including toxinogenic fungi and pathogenic bacteria, which cause in some cases the outbreaks of epidemic diseases and vital hazards to patients.[3] Since biological materials are hardly sterilized by simple heating or by chemical treatments, drugs of biological origins, such as enzymes, animal organs and crude drugs, are hitherto left untreated microbiologically. On response to the recent requirement of the reduction of contaminants in these drugs, the sterilization by ionizing radiation may be one of the most desirable methods so far as the biological activities of these drugs are not impaired and undesirable byproducts are not formed. The present experiments are designed to demonstrate the possibility of sterilization of enzyme powders enforced with known numbers of test organisms by γ-irradiation without affecting the enzyme activity.

Materials and Methods

Organisms and Cultivation——*Escherichia coli* W3110 and *Bacillus subtilis* IAM 1144 were cultured with shaking at 27° in nutrient broth. A clean spore sample of *B. subtilis* was obtained as described in the previous paper.[4] Viable cell countings were performed by the capillary tube method of Yanagita[5] for *E. coli* and by the conventional pour plate method for *B. subtilis* and contaminants.

Enzyme Samples and Assay Methods——Enzyme samples employed were as follows: Takadiastase B (for vitamin B_1 assay, Lot No. T60195, Sankyo), trypsin (2000 U/g, Lot No. 201-03272 IEJ8879, Wako

1) Part III: T. Sakai, K. Ageishi, M. Mikage, T. Namba, and T. Yanagita, *J. Gen. Appl. Microbiol.*, **23**, 279 (1977).
2) Location: *a) Gofuku 3190, Toyama*; *b) Nishi-nagae 220, Toyama.*
3) L.O. Kallings, "Contamination in the Manufacture of Pharmaceutical Products," ed. by Secretariat of the European Trade Association, 1973, p. 17.
4) T. Yanagita, T. Miki, T. Sakai, and I. Horikoshi, *Chem. Pharm. Bull.* (Tokyo), **26**, 185 (1978).
5) T. Yanagita, *J. Bacteriol.*, **71**, 381 (1956).

Pure Chem. Ind.), pepsin (1: 3500, Lot No. 16-576 IE17372, W.P.C.I.) and papain (1: 350, Lot No. 164-00172 IE17704, W.P.C.I.). All of these enzyme preparations were the biochemical reagents but not for medicinal use and the latter two were used only for bacteriological examinations. Diastase activity against soluble starch was assayed by the method of Wilstätter and Schudel[6] and trypsin activity against casein (nach Hammersten, Merck, Inc.) by that described by Hagihara[7] using a spectrophotometer (Hitachi-Perkin Elmer 139 UV-VIS).

In all enzyme samples, the activity-enzyme concentration relations were determined preliminarily, and for the enzyme activity assays after γ-irradiation, the concentrations of irradiated preparations in assay systems were adjusted to be equal to that of the control (non-irradiated) one, which was kept at the highest level within a range at which a proportional activity-concentration relation was hold.

Preparations of Freeze-Dried Bacteria and Enzyme Samples Containing Test Organisms——The washed cell suspension of *E. coli* cultured with shaking for 48 hr at 27° and the clean spore suspension of *B. subtilis* were subjected to freeze-drying using an apparatus (Ulvac, Japan Vacuum Eng. Co) to obtain dry clean cell powders. For the preparation of dry enzyme powders containing test bacteria homogeneously, washed cells of *E. coli* or clean spores of *B. subtilis* were suspended in enzyme solutions in an ice bath and subjected to freeze-drying. Dried samples were powdered finely and stored in a desiccator at 4°.

γ-Irradiation and Dose Assay——γ-Irradiation apparatus employed was a ^{60}Co-encapsulated Toshiba RCR 20-1 (3000 Ci on May, 1974). Samples were contained in glass tubes (48 × 6 mm diameter with plastic caps) and those tubes were placed on a plastic rack arranging them radially and horizontally so as to be irradiated equally as far as possible. Irradiation was performed at a constant distance of 30 cm from a ^{60}Co source within a irradiation field of 12 × 12 cm^2. The dose of irradiation was varied by changing the duration of irradiation. The absorbed dose or dose rate was determined by Fricke's ferrous-ferric chemical dosimetry.[8] The dose rate under the present condition was estimated to be 3.45×10^4 rad/hr at room temperature in glass tubes.

Results

Bacterial Numbers in Enzyme Preparations

As a preliminary experiment, the viable cell numbers of contaminating bacteria in commercial enzyme preparations were counted. As shown in Table 1, rather high counts were observed in some of these preparations. From the results, the number of test bacteria to be enforced in enzyme preparations in the following experiments were chosen at least two orders of magnitude higher than the number of contaminants, viz., 10^8—10^{10} cells/g. Assuming that the contaminants and test bacteria are equally sensitive to γ-irradiation, the

Table I. Viable Cell Numbers of Contaminants Contained in Commercial Enzyme Preparations

Enzymes	Viable cell number (cells/g)
Takadiastase	4.0×10^6
Trypsin	1.0×10^3
Papain	2.3×10^8
Pepsin	3.2×10^5

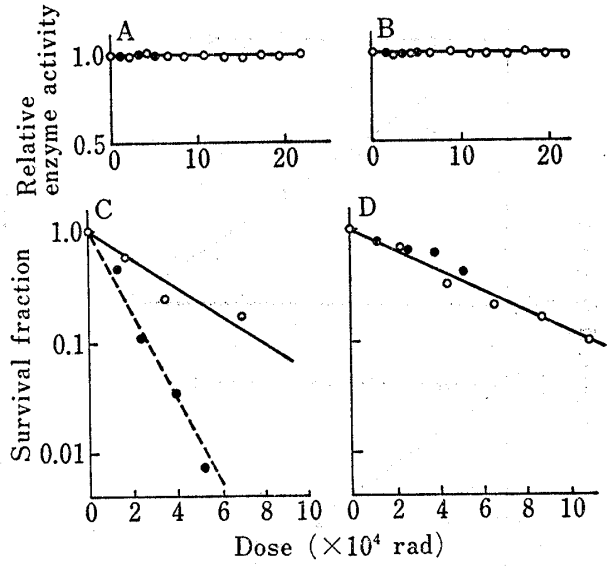

Fig. 1. Effects of γ-Irradiation on Enzyme Activities and Bacterial Viability

A. Takadiastase activity. B. trypsin activity. C. *E. coli* cell viability. D. B. *subtilis* spore viability. ○: dry samples, ●: enzymes dissolved in water or cells suspended in saline.

6) R. Willstätter and G. Schudel, *Ber.*, **51**, 23 (1918).
7) B. Hagihara, "Methods in Enzymology," (in Japanese), Vol. 2, ed. by S. Akabori, Asakura, Pub., Tokyo, 1956, p. 238.
8) N. Miller and J. Wilkinson, *Trans. Faraday Soc.*, **50**, 690 (1954).

presence of contaminants in these preparations enforced with such a high count of test bacteria may be accounted for negligible.

Effect of γ-Irradiation on Dry and Dissolved Enzyme Preparations and on Dry and Suspended Bacterial Cells

Using dry powders and solutions of Takadiastase (6.25 mg/ml) and trypsin (50 mg/ml), different doses of γ-ray were irradiated. As seen in Fig. 1A and B, the activities of these enzymes were not impaired over the wide range of dose so far examined.

Similar experiments were also performed using dry clean cell powders and saline suspensions of *E. coli* cells and *B. subtilis* spores. Results shown in Fig. 1 C and D, representing the relationship between logarithm of survival fraction *versus* γ-ray dose, clearly indicate that in *E. coli* cells the doses for decimal reduction in viability were 7×10^4 and 2.5×10^4 rad for dry and suspended cells, respectively. Thus, dry cells were about three times more resistant than suspended cells. By contrast, in *B. subtilis* spores the decimal reduction dose for either dry or suspended spores did not differ so widely showing about 11×10^4 rad. It is interesting to note that the sensitivities to γ-irradiation of dry *E. coli* cells and *B. subtilis* spores were nearly the same.

Effect of γ-Irradiation on Dry Enzyme Preparations Containing Test Organisms

In the case of Takadiastase (Fig. 2A), the enzyme activity was not impaired even at the dose of more than 10^5 rad. The susceptibility of dry *E. coli* cells to γ-ray was almost the same (decimal reduction dose being 7×10^4 rad) as that observed on a clean *E. coli* cell powder. Quite a similar results were also obtained on trypsin preparations (Fig. 2B). From these observations the presence of enzyme proteins covering *E. coli* cells does not seem to protect from or enhance the deleterious effect of γ-ray.

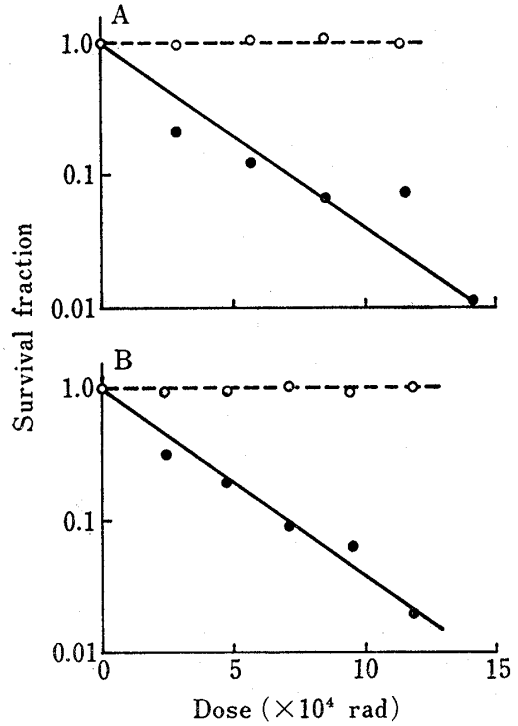

Fig. 2. Effects of γ-Irradiation on Freeze-dried Enzyme Preparation Containing *E. coli* Cells

A. Takadiastase preparation. B. trypsin preparation. --○--: enzyme activity, —●—: *E. coli* cell viability.

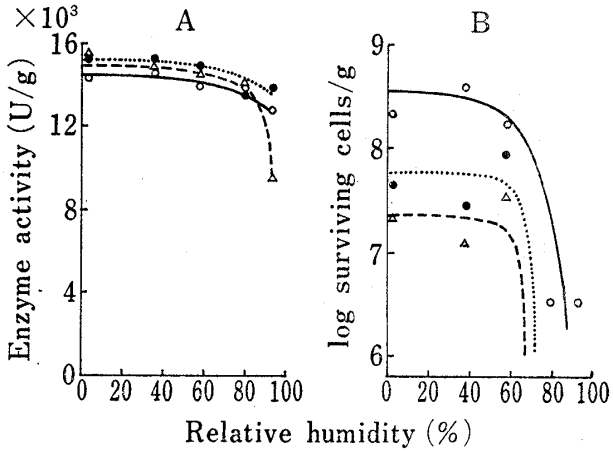

Fig. 3. Effects of γ-Irradiation on Enzyme Preparation Containing *E. coli* Cells stored under Different Atmospheric Relative Humidities

A. Takadiastase activity. B. *E. coli* cell viability. —○—: control (non-irradiated), ···●···: irradiated at the dose of 7.4×10^4 rad, --△--: irradiated at the dose of 10.5×10^4 rad.

Effect of Moisture on the Susceptibility to γ-Ray of Enzyme Powders Containing Test Organisms

Takadiastase powder containing *E. coli* cells were stored overnight at 23° in desiccators containing varied concentrations of sulfuric acid to keep the respective atmospheric relative humidities.[9] These enzyme preparations were irradiated at the doses of 7.0×10^4 and 10.5×10^4 rad leaving control ones unirradiated. Results are shown in Fig. 3.

Enzyme activities of control preparations were reduced slightly in samples stored under higher humidities (Fig. 3A). Irradiation of 10.5×10^4 rad on samples stored under 93.9% relative humidity resulted in a marked decrease in activity. As seen in Fig. 3B, though the data seems to be rather fluctuating under higher humidities probably because of an increased lability of cells, viable cell counts of *E. coli* in control samples were also affected largely by the storage under more than 80.5% relative humidity. Under less than 58.3%, the susceptibility of *E. coli* cells to γ-ray seemed to be not affected (*cf.* Fig. 1 or 2), whilst under more than 80.5% it was largely enhanced: these samples were almost completely sterile under such conditions.

Similar observations were also carried out employing a trypsin preparation. However, we could not be able to obtain reproducible results probably because of the unstable progress of the digestion of *E. coli* cells by trypsin under higher humidities during storage.

Effect of Additives Contained in Enzyme Preparation on Deleterious Action of γ-Ray

In the medicinal processing of enzyme preparations, excipients are usually added. Therefore, the effect of the additions of sugars, such as glucose and lactose, to an enzyme preparation on the γ-irradiation action were examined. Dry Takadiastase powder containing *E. coli* cells was mixed thoroughly with the same amount of anhydrous glucose or lactose and stored overnight under 58.3% relative humidity. In Table II are listed data of relative activity of the enzyme and of survival ratio of *E. coli* cells after γ-irradiation at different doses. Results showed that the effect of γ-irradiation on neither enzyme activity nor on survival ratio was affected by the presence of these sugars.

Table II. Effect of γ-Irradiation on Relative Activity of Takadiastase and Survival Ratio of *E. coli* Cells in Enzyme Preparations containing Chemical Substances

Subjects assayed	Substances added	γ-Ray dose ($\times10^4$ rad)		
		6.8	8.5	10.2
Takadiastase	None	1.00	1.03	1.02
	Glucose	1.10	—	1.00
	Lactose	0.95	—	1.04
	L-Cysteine	1.02	1.03	0.98
E. coli cells	None	0.06	0.04	0.03
	Glucose	0.08	—	0.02
	Lactose	0.07	—	0.03
	L-Cysteine	0.07	0.04	0.01

Enzyme powders containing chemical substances were stored under 58.3% relative humidity. Activity or survival ratio of non-irradiated control was taken as unity.

Since L-cysteine is known to have a protective activity on living cells against ionizing radiations, the effect of this amino acid was also examined. In this experiment, Takadiastase was dissolved in washed cell suspension of *E. coli* and L-cysteine hydrochloride was added at the concentration of 11 mM and the solution was subjected to freeze-drying. Thus the contact

9) C.D. Hodgman, R.C. Weast, and C.W. Wallace (ed.), "Handbook of Chemistry and Physics," 35th Ed., Chem. Rubber Pub., Cleveland, 1953, p. 2310.

between *E. coli* cells and cysteine was expected to be highly intimate in the dry preparation. As understood from the results of γ-irradiations shown in Table II, the protecting effect of cysteine on *E. coli* cell viability could not be demonstrated at all and no effect was also observed on enzyme activity.

Discussion

In most studies on the radiation effect on microorganisms, wet or suspended cells are employed as test organisms. Our present investigation revealed that the dry cell sample was almost three times more resistant to γ-irradiation than suspended cells. The fact is also noticeable that the dry *E. coli* cells are as resistant as spores of *B. subtilis* to γ-irradiation. From studies on the effect of moisture on the radiation activity, we found that no appreciable effect was exhibited when the irradiated preparations were stored under less than 58.3% relative humidities. Since biological materials such as enzymes are usually stored under dryness, enhancement of sterilization effect of γ-irradiation by humidity could not be expected in practice.

It should be stressed that the radiosterilization can be performed at room temperature and can reduce the number of either vegetative cells or spores of bacteria by two orders of magnitude at the dose as low as 1.5×10^5 rad, at which enzyme activity was not impaired at all. Ueno *et al.*[10] reported that even at the dose of 5 Mrad, protease activity reduced only about 10% and bacterial number reduced from 10^7 to 10^2 cells/g. Since radiosterilized proteinaceous foodstuffs are known to be edible, radiosterilized enzymes and some of the other biological medicaments may be used for the medical use.

It was already reported that the crude and purified enzyme preparations did not differ in their susceptibility to γ-irradiation.[10] We also found that the additions of excipients, such as glucose and lactose, resulted in neither enhancement nor retardation of γ-ray activity. Even the addition of L-cysteine showed any sign of protection of living bacteria from the γ-ray action, probably because of the dry state of the cells. These observations seem to make it simple to design the radiosterilization practice. Since the inclination of the straight line relationship between survival fraction and radiation dose was quite reproducible under a certain condition, if we know the number of contaminants in a preparation, we can easily estimate the proper dose of γ-irradiation to reduce the number of contaminants to a known level.

Acknowledgement The present work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.

10) T. Ueno, F. Yoshizako, A. Nishimura, and T. Kotaka, *Ann. Rep. Rad. Center, Osaka*, **16**, 62 (1975).