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THE EFFECTS OF GAMMA IRRADIATION ON SOME PROPERTIES OF TWO AEROMONAS PROTEINASES

By

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DAHLE, HANS KOLBEIN and BJARNE UNDERDAL: *The effects of gamma irradiation on some properties of two Aeromonas proteinases.* Acta vet. scand. 1972, *13*, 492—504. — The proteinases A and B of Aeromonas liquefaciens and proteinase B of Aeromonas salmonicida have, in the crude and purified state, been exposed to various doses of γ-irradiation from a Co^{60} source, and the D_{37} values indicate that the proteinase A is the most resistant.

Casein was shown to have a marked protective effect on both proteinases during the irradiation, and while solutions of the purified enzymes were inactivated by doses usually used for food pasteurization, the crude enzymes, or solutions of purified enzymes to which casein was added, required doses usually used for food sterilization before being inactivated. Only minor effects of the environmental pH were observed. The antigenic properties of the enzymes seemed to be qualitatively unchanged in solutions exposed to 150 krad as observed using the casein precipitation inhibition test, and the irradiated proteinases were also inhibited by the naturally occurring proteinase inhibitors in the immune sera. The enzymoserological properties were not influenced by the changes in electrophoretic migration which were demonstrated by the zymogram technique. These proteinases are suitable as models for the examination of the physical properties of food spoiling enzymes and also for taxonomical work.

gamma irradiation; preservation; Aeromonas; proteinases; enzymes; CP-test; zymograms.

Much work has been carried out during the last decades on the applications of ionizing radiation to food and feed preservation (*IAEA* 1963, 1966, 1967, 1968, 1969, 1970). It is known that ionizing radiation inactivates microorganisms, although the D-value varies considerably for the different species. For one of the most resistant spore-forming microorganisms, Clostridium botulinum type A, the D-value is approx. 0.4 Mrad (*Gorseline & Descrosier* 1959), while for strains of some Salmonella species, the D-value is estimated to be approx. 0.02 Mrad (*Underdal & Rossebø* 1972).

Enzymes are, in general, more resistant to radiation than bacteria. Thus, *Rhodes & Meegungwan* (1962) reported that proteolytic and lipolytic enzyme activity of lamb liver could not be completely destroyed by 40 Mrad. One of the obstacles to the successful application of ionizing radiation to food preservation is, therefore, the residual enzyme activity in irradiated foods. On the other hand, the large dose levels which are required for the prevention of food spoilage, tend to cause changes in food quality, and such doses may possibly have an effect on the health of the consumers.

However, the major part of the results, at present available, concerning the effects of ionizing radiation on enzymes refers to work on enzymes of medical and radiological interest. Only a minor part is suitable for application by food technologists.

The present paper deals with gamma irradiation of solutions of the proteinases produced by Aeromonas liquefaciens (A + B) and Aeromonas salmonicida (B) (*Dahle* 1969 b) and analyses of some of their properties after exposure to increasing doses.

MATERIALS AND METHODS

Enzymes

The proteinases were produced by growing Aeromonas liquefaciens (ATCC* 14715) and Aeromonas salmonicida (ATCC* 14174) on semi-solid skim milk agar as described previously (*Dahle* 1971).

The proteinases produced by Ae. liquefaciens were identified as proteinases A and B, while the one produced by Ae. salmonicida was identified as proteinase B by the enzymoserological casein precipitation inhibition test (CPI-test) (*Dahle* 1969 b). The CPI-test is based on the separation of the antiproteinases in immune sera from naturally occurring proteinase inhibitors by paper electrophoresis, followed by the demonstration of inhibition of the casein precipitation reaction (CP-reaction) in the area of the antiproteinases.

Purification of proteinase A and proteinase B was carried out as described (*Dahle* 1971) using a procedure which included precipitation with ammonium sulphate, batchwise treatment with DEAE-cellulose, and gel filtration on Sephadex G-100. The pH in these solutions were 6.8—7.0.

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* American Type Culture Collection, Rockville, Maryland, USA.

Irradiation

The material to be irradiated was transferred, in aliquots of 0.1 ml, to thin-walled 1 ml glass ampoules. The irradiations were carried out at the Institute of Atomic Energy, Kjeller, Norway, using a Co^{60} source. The activity of the source was 20,000 Ci, giving a maximum dose rate of 280 krad per hr. The samples could be placed at different distances from the source, thus giving varying dose rates.

The radiation-absorbed dose was determined by placing dosimeters together with the different samples. In the lower dose ranges (5—50 krad) Fricke dosimeters (*Fricke & Hart* 1966) were used, while doses above 50 krad were determined by using perspex dosimeters, Red-400 (*Thomassen* 1970).

During the irradiations the temperature was kept at 0—4°C. Non-irradiated controls were treated in the same way and kept at the same temperatures.

Enzyme assay

The concentrations of proteinases (CP-titre) were determined by the casein precipitation method of *Sandvik* (1962), as modified by *Dahle* (1969 a). A hot solution containing 1.40 % Bacto-agar* 0140-01, 1.00 % sodium caseinate** (added as 4 % solution of pH 6.2), 0.01 % merthiolate and 0.004 M-$MgCl_2$ in distilled water, was poured into a glass tray to a depth of 2 mm and allowed to solidify. Aliquots of 25 µl from serial 2-fold dilutions of the proteinase solution were transferred to wells of 7 mm diameter in the sodium caseinate-containing agar before incubation at 37°C for 16 hrs. The estimation of diffusion units was based on the diameters of the precipitation zones which occurred (*Dahle* 1969 a). For extremely concentrated proteinase solutions, preliminary dilutions were performed before preparing the serial 2-fold dilutions.

Proteolytic activity was determined by a modified *Kunitz* (1947) procedure. The digestion of 1 ml 2 % casein solution (Hammarsten quality)*** with 1 ml enzyme solution was performed by incubating the mixtures for 20 min. at 37°C, after which the addition of 3 ml 5 % trichloracetic acid terminated the

* Difco Laboratories Inc., Detroit, Michigan, USA.
** Eastman Kodak, Rochester, N.Y., USA.
*** Merck, Damstadt, Germany.

enzymatic activity and precipitated the undigested proteins. The absorbance at 280 nm was measured for the supernatants after centrifugation. As solvent for the proteinases, 0.2 M Tris-HCl buffer, pH 8.1 was used.

Electrophoretic and antigenic properties of the proteinases after irradiation

Zymograms of the proteinases were prepared as described by *Dahle* (1970) using 1 % agar* in 0.05 M phosphate buffer, pH 6.2, and using the same buffer as running buffer.

Serological identification of the proteinases was carried out by the CPI-test (*Dahle* 1969 b).

Table 1. Irradiation of crude proteinase solutions of Aeromonas liquefaciens and Aeromonas salmonicida using a Co^{60} source.

Proteinase solution*	% CP-activity remaining after exposing the enzyme solutions to the following doses (krad)									
	0	10	50	100	250	500	750	1000	2500	5000
Ae. liquefaciens (A and B)	100	100	85	60	25	15	10	5	1	0
Ae. salmonicida (B)	100	85	80	55	20	10	5	1	0	0

* The proteinase solutions are indicated by the organism by which the proteinases are produced (30,000 diffusion units equal 100 % CP-activity).

RESULTS

The crude proteinase solutions of high activity obtained by centrifugation of the semi-solid skim milk cultures of Ae. liquefaciens (proteinases A and B) and Ae. salmonicida (proteinase B) were exposed to increasing doses of γ-irradiation. Table 1 shows that these crude enzyme solutions retained some activity even when exposed to doses above 1 Mrad. It is also seen that the Ae. liquefaciens solution requires exposure to the highest doses before being completely inactivated, although both solutions contain approximately the same amount of diffusion units per ml.

In order to compare the proteinase A and the proteinase B of these aeromonads, solutions of the purified proteinases were prepared, containing approx. 50,000 diffusion units per ml (CP-

*Special Noble-agar 0142-01; Difco Laboratories Inc.

method) or 0.1 unit per ml based on the method of *Kunitz* (1947). Serial 2-fold dilutions from these solutions were exposed to increasing doses of γ-irradiation. Figs. 1 a and 1 b show logarithmic plots of the CP-activities as a function of the dose. The horizontal dotted line marks the D_{37} values for the individual dilutions. The D_{37} values for the two proteinases, as a function of the dilution are presented in Fig. 2. The slope of the line representing the proteinase A solutions is greater than that of the line for the proteinase B solutions.

The stabilizing effect of the substrate (casein of Hammarsten quality) is shown in the histograms in Figs. 3 a and 3 b. The activities of the purified enzyme solutions are reduced to one fourth of the original activities by a dose of 100 krad, while the activities of the enzyme-substrate mixtures are only reduced by 30—50 % by the same dose. The proteinase A was the most resistant of the two proteinases.

As the present enzymes are active over wide pH-ranges (*Dahle* 1971) it was also of interest to investigate the effect of the environmental pH on the inactivation by γ-irradiation. Fig. 4 shows the CP-activities as functions of the irradiation doses for enzyme-substrate solutions adjusted to pH 8.5, 7.0 and 5.6 using Tris-HCl buffer, phosphate buffer and acetate buffer, respectively. Only minor differences were observed.

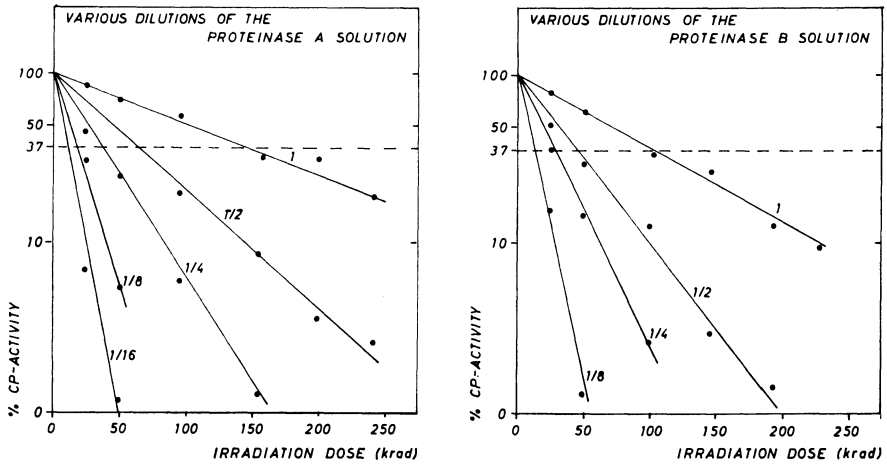

Figure 1. The logarithm of the CP-activities as a function of the irradiation dose for dilutions of the proteinase A solution (a) and the proteinase B solution (b).

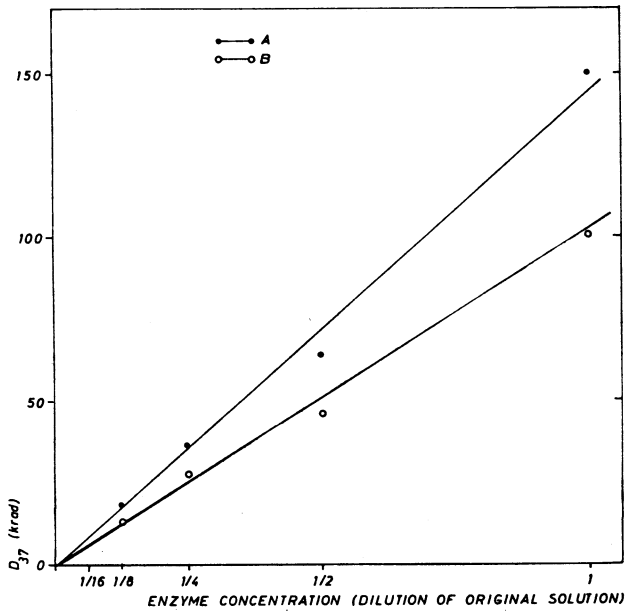

Figure 2. The D_{37} values from Fig. 1 as a function of the dilution of the proteinase solutions of proteinase A and proteinase B.

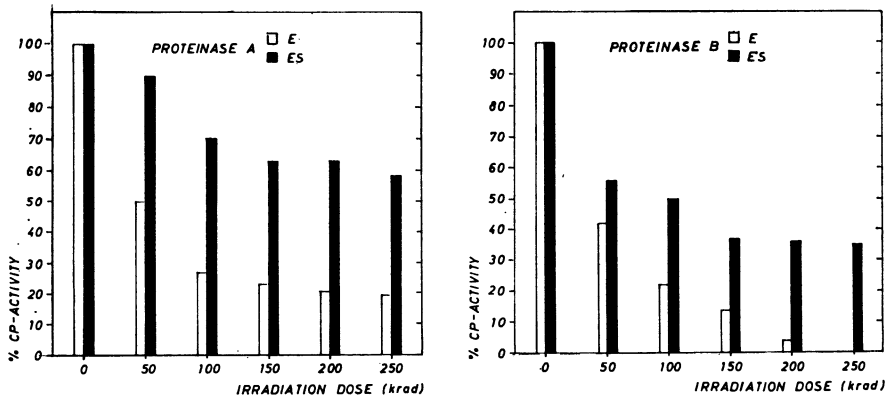

Figure 3. Histograms of proteinase solutions and proteinase-substrate solutions of proteinase A and proteinase B exposed to increasing dose levels. The original proteinase solutions were mixed with one part distilled water (E) or one part 2 % casein (Hammarsten quality) solution (ES) before being irradiated. The activities were determined by the casein precipitating method.

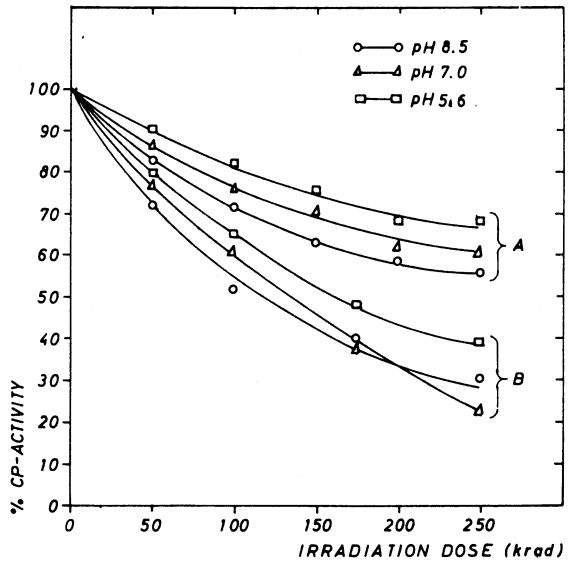

Figure 4. The effect of pH on the irradiation of proteinase A and proteinase B.

In the present study, proteinase solutions which were irradiated with 150 krad and still retained CP-activity were used for developing the electrophoretic patterns of antisera aginst the two Aeromonas proteinases (CPI-test). Fig. 5 shows that the antigenic properties are still intact after the irradiation, and that the proteinases are also inhibited by the naturally occurring proteinase inhibitors in the immune sera. In addition it was of interest to compare the electrophoretic properties of the enzymes. Zymograms of the crude proteinase solutions were, therefore, prepared before and after irradiation with 100 krad and are shown in Fig. 6.

While both the CP-method and the method of Kunitz were used for the proteinase determinations on the original solutions, only the CP-method was suitable for corresponding determinations on the irradiated material, due to the greater sensitivity of the method (*Fossum* 1970).

DISCUSSION

The radiation sensitivity of enzymes is usually expressed by their G-value, i.e. the number of enzyme molecules inactivated per 100 eV absorbed. The G-value can be conveniently calculated

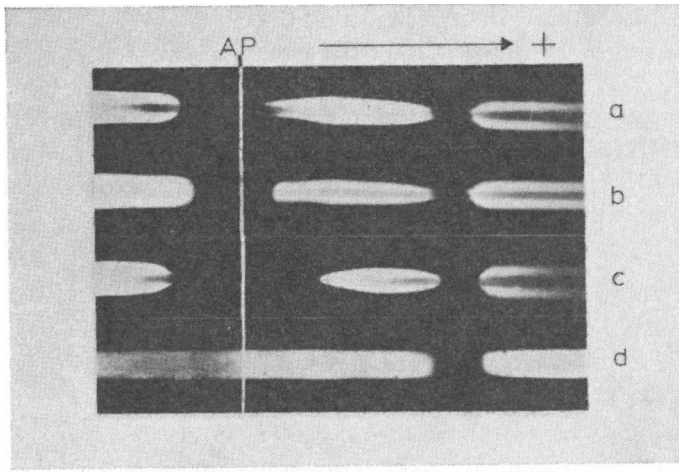

Figure 5. Electrophoretic patterns of antisera against the proteinase A (a and b) and proteinase B (c and d) transferred to sodium caseinate agar. Development was performed with the original solutions of proteinase A (a), the same solution after irradiation with 150 krad (b), the proteinase B solution after irradiation with 150 krad (c), and in the control (d) the development was performed with a heterologous type of enzyme which was not irradiated. The specific antibodies are localized in the area of the line of application (AP). The naturally occurring serum inhibitors can be seen to the right of this line. The electrophoresis was carried out in 0.05 M phosphate buffer, pH 6.2, for 18 hrs. at 120 v.

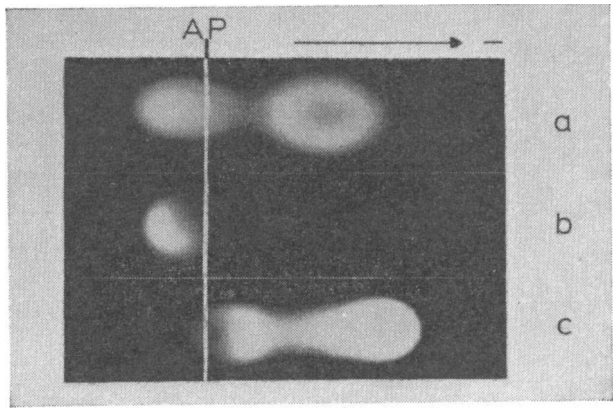

Figure 6. Zymograms in agar gel of crude proteinase solutions obtained from Aeromonas liquefaciens cultures (a) and Aeromonas salmonicida cultures (b) after irradiation with 100 krad, as compared with a solution from Ae. liquefaciens culture (c) which was not irradiated. The Ae. liquefaciens solution contains both the proteinase A (fast moving) and the proteinase B (slow moving) while the Ae. salmonicida solution contains the proteinase B (slow moving) only.

from exponential dose-inactivation curves on the basis of the D_{37} dose (*Sanner & Pihl* 1969), and it can be estimated both for enzymes in the dry state and enzymes in solution, if the concentrations of the enzymes are known. In the present study the concentrations of the proteinases in the crude and purified enzyme solutions are known, in terms of diffusion units (CP-method), but not as weight/volume concentrations. This limits the possibilities for calculating the G-values, but does not, however, reduce the value of studying radiation sensitivity in microbial proteinase systems. Thus, Figs. 1 and 2 show how the D_{37} doses are related to various dilutions of the proteinase solutions, in which the concentrations of proteolytic enzymes are easily determined by the CP-method. Table 1 also shows that the crude proteinase solutions are very resistant to γ-irradiation, in that more than one Mrad is required for complete inactivation.

Figs. 1 a and 1 b also show that when the CP-activity is plotted as a function of the irradiation dose, the curves representing each dilution are of exponential type. This may be due to the rate of interaction of the water radicals produced ($OH^{\cdot}$ and $H^{\cdot}$) with the active and inactivated enzyme molecules being the same. Furthermore, the D_{37} doses are shown to decrease regularly with increasing dilution, and Fig. 2 demonstrates clearly that the D_{37} doses for the dilutions of proteinase A (molecular weight 22,100 (*Dahle* 1971) are larger than the D_{37} doses for the proteinase B (molecular weight 43,600). The probability that the energy quanta and the induced radicals could collide with molecules increases with increasing molecular weight, and consequently the D_{37} dose decreases correspondingly. The figure also shows that the D_{37} dose increases linearly with the enzyme concentration, and this is in accordance with results published by other authors (*Vas* 1969).

In food technology, the problems of food spoiling enzymes are closely related to the composition of the food, and to which extent the food is suitable as substrate for the enzyme in question. An important question is, therefore, to which extent substrates protect enzymes by binding to the active site. Generally the enzymes may be inactivated (1) by destruction of the active sites, (2) by a general denaturation, or (3) by a combination of these two effects. Fig. 3 shows that both of the Aeromonas proteinases are protected by the casein solution in all the dilutions examined, and this favours the theory of destruction of the active site by

the irradiation. These observations are also in accordance with the results given in Table 1, where the crude proteinase solutions are shown to require the very high doses of one Mrad before being inactivated. The proteinase activities in the crude solutions were of the order of 30,000 diffusion units per ml as compared with 50,000 diffusion units per ml in the purified enzyme solutions inactivated at the much lower dose of 350 krad (Fig. 3). However, experiences regarding substrate protection of other enzymes against irradiation vary somewhat (*Pihl & Sanner* 1963). In the present study, proteinase A was shown to be the more resistant of the two enzymes. Proteinase A has previously been shown to possess the greatest temperature stability (*Dahle* 1971), and the present observation is another criterium of the differences in physical properties of the two proteinases. The difference in molecular weights should be kept into mind in this connection.

Fig. 4 indicates that the proteinases are more sensitive to γ-irradiation near their pH-optima (7.9 for A and 9.0 for B) (*Dahle* 1971) than at lower pH-values, although no large differences in the activities remaining were observed. The pH-effects are probably more distinct for enzymes with narrower pH-ranges. Thus, for cathepsin C *Giovannozzi-Sermanni et al.* (1969) have reported distinct differences in activity when the enzyme was irradiated at various pH-values. Corresponding effects are also obtained in irradiation experiments on Clostridium botulinum toxins (*Skulberg* 1964).

The antigenic properties of macromolecules are of particular interest when studying biochemical degradation reactions, because the determinant groups on the molecules have to be intact if positive serological reactions are to be obtained. The fact that the antigenic properties seem to be intact for the proteinase solutions exposed to moderate irradiation (Fig. 5) is, therefore, of particular interest for the identification of the enzymes and also for the value of the CPI-test for such purposes. It should be noted that the CPI-test combines examinations of both the determinant groups on the molecule and the active site of the enzyme. However, in the present study no attention was paid to differentiating between these sites, although the results demonstrate that both functions were intact in solutions retaining enzyme activity after irradiation. As far as the authors are aware similar results have not yet been published. It is also interesting that

the electrophoretic properties changed for both the proteinases (Fig. 6) after exposure to 100 krad, although the typical serological properties of the enzymes were still intact. It seems possible that certain charged groups may be removed from the molecules by the irradiation, as the proteinase B (slow moving) no longer possessed its original cathodic migration, and proteinase A (fast moving) was also somewhat retarded. However, this did not seem to influence their antigenic properties by the method used. Similar observations have previously been described by *Dahle & Sandvik* (1971) for proteinases of certain strains of Vibrio comma which were stored for some time.

The presented results indicate that although the purified proteinases can be inactivated by the γ-irradiation doses usually used for food pasteurization (0.2—0.4 Mrad), the crude enzyme solutions and the substrate-protected enzyme solutions, which both resemble practical situations in food technology, require doses nearer the level usually called sterilization doses (4—5 Mrad). The question of possible damage by such doses to the quality of food is therefore very important for the public health. Thiamine in fish fillets, for example, is partially destroyed by 0.3 Mrad, while 50 % of the Vitamin A content is still intact after irradiation with 3.0 Mrad (*Mameesh et al.* 1964).

The enzymoserological relationship between the proteinases produced by Ae. liquefaciens and Ae. salmonicida makes the proteinases an interesting model for further experimentation. The organisms are widely distributed in nature, in fish and fish products, and Ae. liquefaciens is also common in poultry and egg environments (*Breed et al.* 1955). Thus, the proteinases are associated with food spoilage problems, and as such their physical properties are of particular interest. On the other hand, the proteinases are excellently suited as models for enzymoserological investigations, and thereby also of interest for taxonomical work.

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SAMMENDRAG

Virkninger av gammabestråling på noen av egenskapene til to Aeromonas proteinaser.

Proteinasene A og B produsert av Aeromonas liquefaciens og proteinase B produsert av Aeromonas salmonicida ble bestrålt i renset og urenset tilstand med varierende doser av gamma-stråler fra en Co^{60} kilde. D_{37}-verdiene indikerer at proteinasen A er den mest resistente av dem. Det ble vist at kasein har en betydelig beskyttende virkning på begge proteinasene under bestråling. Mens løsninger av de rensede enzymene ble inaktivert av doser som vanligvis anvendes ved pasteurisering av mat, trengtes det doser som brukes for sterilisering av mat for å inaktivere løsninger av rensede enzymer som var tilsatt kasein, og likedan var det for løsninger av de urensede enzymer. Omgivelsenes pH-verdier hadde liten virkning på inaktiveringen av enzymene. De antigene egenskapene til enzymene syntes ikke å forandres kvalitativt ved bestråling av løsninger opp til 150 krad, noe som ble vist ved hjelp av kaseinpresipitasjonsinhibisjonstesten. De bestrålte proteinasene ble også hemmet av de naturlig forekommende proteinaseinhibitorene i immunseraene. Noen forandringer i den elektroforetiske vandring, som kunne påvises ved hjelp av zymogramteknikken for proteinaser, syntes ikke å ha innvirkning på de enzymoserologiske egenskapene. De anvendte proteinasene var vellegnet som modell for undersøkelse over fysikalske egenskaper hos enzymer som ofte finnes i forbindelse med bedervelse av mat, og også for taksonomiske arbeider.

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