J. P. Roozen

Department of Food Science,
Agricultural University, Wageningen

Inactivation of peroxidase, pectinesterase and alkaline phosphatase in polymers as a model for irradiation of dried foodstuffs

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Abstract

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Aqueous solutions containing an enzyme (peroxidase, alkaline phosphatase, pectinesterase) and a polymer (carboxymethylcellulose, soluble starch, polyvinylpyrrolidone, sodium pectate) were freeze-dried. The systems thus obtained were incubated over saturated salt solutions at various levels of water activity. They were then irradiated by an electron generator. The storage and radiation sensitivity of the enzymes depended on the polymer used in the model, the enzyme concentration, the humidity and the pH.

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The author graduated as Doctor of Agricultural Sciences, Agricultural University, Wageningen, the Netherlands, on a thesis with the same title and contents.

1 Introduction and literature

Dried foodstuffs Since ancient times foodstuffs have been dried to preserve them from spoilage which can be microbiological, chemical or enzymatic. The shelf life of dried products depends on their water content. Acker (1962) has written a detailed review on enzymatic spoilage in relation to the water activity of food. Most enzymes do not react at moisture contents below the inflection point of the sorption isotherm (Acker & Lück, 1958). However, lipids may be oxidized at such low moisture contents (Purr, 1970). Even if there is no problem of oxidation it is often difficult to achieve or maintain the low moisture content. In practice therefore it is still necessary to blanch dried and frozen vegetables to prevent enzymic deterioration. Replacement of this heat treatment by irradiation could improve some of the existing products; then even vegetables that wither on heating could be stored in a frozen or dried state.

Irradiation of macromolecules The most important effects of ionizing radiation on macromolecules are degradation and crosslinking. The disruption of secondary structures have so far only been described in proteins and deoxyribonucleic acid (Bacq & Alexander, 1966). Chapiro (1964) discussed some aspects of the radiation chemistry of polymers. In irradiated solid polymers, degradation and crosslinking can largely be ascribed to reactions by free radicals. When polymers are irradiated in solution, these reactions are influenced by free radicals from the solvent. Thus in the solid state, variations in water content have been shown to cause changes in the radiosensitivity of deoxyribonucleic acid (Lett & Alexander, 1961), carboxymethylcellulose (Munzel & Schenkel, 1964), starch (Reuschl & Guilbot, 1966), pectin (Wahba & Massey, 1966), and polyvinylpyrrolidone (Alexander & Charlesby, 1957).

Radiosensitivity of enzymes From a survey of literature, Marples & Glew (1958) concluded that enzymes in food are extremely radio-resistant. A great measure of protection for the enzymes is provided by the extraneous material in food. The irradiation energy taken up by foodstuffs is probably distributed between the enzyme and the other material present.

A decrease in radiosensitivity of enzymes, when enzyme-polymer complexes are formed, has been found for

1. chelated complexes such as carboxypeptidase A-cysteine (Fultz, 1968), catalase-glycerol (Lohmann et al., 1964) and catalase-hydrogen cyanide (Sutton, 1956),

2. enzyme-substrate complexes such as deoxyribonuclease I-deoxyribonucleic acid (Okada, 1957), polyphenol oxidase-tyrosin and actomyosin-adenosine triphosphate (Arnaud & Coelho, 1969),
3. covalent-bound complexes such as papain-poly-p-aminophenylalanine (Giovannozzi-Sermanni & Di Marco, 1965) and
4. unidentified complexes such as trypsin-agar (Holladay et al., 1966).

In biological material all the various complexes are likely, because of the diversity of the material. For intercellular yeast catalase, activity increases by irradiation of cell suspensions (Aronson et al., 1956). This phenomenon is accompanied by a significant lowering of the energy of activation for the catalase-hydrogen peroxide reaction and a considerable increase in the sensitivity of the enzyme to inactivation by heat. The explanation of the authors is, that ionization can lead to the dissociation of a ribonucleic acid-catalase complex and the release of the enzyme from the interface. A combined irradiation and heat treatment might therefore inactivate catalase in yeast cells very effectively.

Defining purpose of study Loss of enzyme activity by storage and irradiation of foodstuffs was simulated in systems of enzymes and polymers. For ease, the polymers used were water soluble. The enzymes were selected from different groups for their importance in food technology, availability in pure form and for easy assay (Table 1).

The purpose of the study was to look for a set of conditions which would increase the radiosensitivity of enzymes in foodstuffs. Augenstine (1962), in his excellent review, said: 'The radiosensitivity of a given enzyme is affected not only by temperature, pH, gases and the assay method employed, but more importantly by the nature of the system in which they are irradiated. Thus, analysis of the mechanisms of enzyme inactivation in heterogeneous systems, which more nearly simulate cellular conditions, is complicated by the effects of moisture content, the presence of other solutes and the possibility that the enzyme is adsorbed at an interface.'

Table 1. Data on the enzymes.

Enzyme	Molecular weight	Prostetic group	Bound metal	Supplier	Assay
POD	40.000[1]	+	Fe	Worthington	spectrophotometer
AP	100.000[1]	—	Zn	Boehringer	spectrophotometer
PE	26.000[2]	—	—	Worthington	titrimeter

1. Dixon & Webb, 1965
2. Delincée & Radola, 1970

2 Definitions and abbreviations

Before irradiation the samples were freeze-dried and stored for three weeks. The residual enzyme activities were determined and plotted as a function of water activity. The curves obtained were a measure for the *storage stability* of the enzyme in question. The enzyme activity immediately before irradiation is called *the blank*.

The enzyme activities of the irradiated samples were expressed as a percentage of the blank and plotted as a function of the dose. The inactivation-dose relationship is exponential for many enzymes (Augenstine, 1962), but linear for papain (Sanner & Pihl, 1963) and neither exponential nor linear for α-amylase (Gorin et al., 1969). In my systems exponential inactivation was observed so that the D_{37} value could be used as an index for the radiosensitivity. The D_{37} *value* is the dose that leaves 37% of the original enzyme activity.

After storage of the solutions of the irradiated systems for a week in the refrigerator the D_{37} value was determined again and called the D'_{37} *value*. Then the *after-effect* could be defined as:

$$\frac{D_{37} - D'_{37}}{D_{37}} \times 100\%$$

In a general kinetic analysis of enzyme inactivation in solution by ionizing radiation, Sanner & Pihl (1963) defined the *intrinsic radiosensitivity* as the probability that a radical which becomes inactivated by the enzyme will concurrently inactivate the enzyme. For papain the authors worked out a graphic method which was used for processing the data of this study. The reciprocal of the slope of the curve D_{37} = f (enzyme concentration), is a measure of intrinsic radiosensitivity. At water activities higher than 45% the samples contain 'free water', in which radicals can be generated by ionizing radiations. These samples can therefore be considered as *concentrated solutions*, and an intrinsic radiosensitivity can be calculated. For this purpose the enzyme concentration was taken as the reciprocal of the water content (% H_2O) of the system.

CMC : carboxymethylcellulose
NaP : sodium pectate
PVP : polyvinylpyrrolidone
sS : soluble starch
AP : alkaline phosphatase
PE : pectinesterase
POD : peroxidase

3 Methods

According to Table 2 an enzyme solution was added to a 2% polymer solution, after which the mixture was freeze-dried. Precautions were taken to avoid changes in pH of the enzyme-polymer systems during the handling period (Roozen & Pilnik, 1971a). The freeze-dried material was ground, divided between 42 polyethene bags, incubated for one week in a desiccator and then for a fortnight over saturated salt solutions. The bags were then sealed and the samples were irradiated up to 6.10^6 rad by a vertical beam Van de Graaf electron generator. Blue cellophane was used as a dosimeter.

To determine the enzyme activities, the samples were dissolved in distilled water and then stored overnight in a refrigerator. The assays used were standard methods (Roozen & Pilnik, 1970, 1971c, 1971d) which were adapted to process 50 assays per day.

For the various water activities the D_{37} values of the enzyme-polymer systems were calculated with the help of linear regression analysis (ln % residual enzyme activity versus dose). The D_{37} values were plotted against the water activities but results with a correlation coefficient less than 0.9 were omitted. The intrinsic radiosensitivities were then calculated from linear regression analysis of the D_{37} values versus the reciprocals of the water contents.

As control the pH values of the solutions of the irradiated systems were also measured. The solutions of the irradiated systems were stored for a week in the refrigerator after which the D'_{37} values were determined and the after-effects calculated.

Table 2. Enzyme concentrations of the systems (in mg enzyme per gram dry polymer).

Polymers	Enzymes			
	POD	PE	AP	
			I	II
CMC	1	4	0.05	1
sS	1	4	0.05	1
PVP	1	4	0.05	1
NaP	–	4	–	–

4 Results

In addition to the results given in Table 3 some other aspects of the enzyme-polymer systems were studied.

Peroxidase systems At 25°C and the characteristic pH values of the polymers, big differences in the storage stability of POD were observed (Roozen & Pilnik, 1970). In the CMC systems POD had highest storage stability at the extremes of the water activities used. This enzyme was very unstable in the PVP systems and lost all its activity at high water activities. In the sS systems the activity of POD could be regenerated by water after incubation at the lower water activities.

The D_{37} values of the POD-polymer systems were calculated with the help of linear regression analysis. The extrapolated curves, found for the sS systems did not intersect at the blank point on the activity coordinate, whereas the curves of the CMC and PVP systems cut the activity coordinate in the blank point.

Pectinesterase systems For this enzyme the same methods and polymers were used as for POD. NaP-PE systems were also studied. In these systems one can expect enzyme-product-complexes (Jansen, 1960) that provide information about

Table 3. Results, obtained with enzyme-polymer systems, that illustrate influence of water activity on storage stability and radioresistance of enzymes.

System	Storage stability: residual activity curve[1]	Radioresistance: D_{37}-curve[2]	Intrinsic radio-sensitivity ratio	After-effect
POD-CMC	minimum	maximum	3	≈ 40%
POD-sS	horizontal	descending	4	≈ 50%
POD-PVP	minimum	maximum	2	none
PE-CMC	descending	maximum	2	none
PE-sS	descending	maximum	2	none
PE-PVP	horizontal	ascending	–	none
PE-NaP	horizontal	maximum	1	none
AP-CMC	horizontal	maximum	3	≈ 25%
AP-sS	horizontal	maximum	5	≈ 30%
AP-PVP	horizontal	ascending	–4	none

1. % enzyme activity versus increasing water activity (shape of curve)
2. D_{37}-versus increasing water activity (shape of curve)

the radiosensitivity of enzymes in such complexes. The storage stability of PE depended little on the water activity of the systems studied. It is rather the nature of the polymer which determines the stability of the enzyme.

The ln activity versus dose curves of all the PE systems studied cut the activity axis in the blank point. As mentioned before the forming of enzyme-polymer complexes has been proved in the NaP-PE systems (Jansen, 1960). Both facts indicate that in all systems the active PE molecules are complexed with polymer molecules.

In solutions of the sS systems, the pH decreased as a function of the radiation dose. Over the whole range the change of the pH was 0.2 units per 10^6 rad.

Alkaline phosphatase systems For this enzyme two different AP concentrations were chosen (Table 2) to obtain information about the influence of the concentration on the storage and irradiation behaviour of enzymes. The storage stability of AP was the same in the high and low concentration systems and was not strongly influenced by the water activity. The storage stability depends mainly on the nature of the polymers.

The dose versus ln activity curves of all the AP systems studied can be extrapolated to the blank point on the activity axis. In AP systems, all the active enzyme molecules seemed to be complexed with the polymers. The calculated D_{37} values were plotted as a function of their water activities. The plots of both enzyme concentrations were similar to the corresponding plots of the PE systems. A 20-fold increase of the enzyme concentration increased the radioresistance (D_{37}) of these systems by a quarter.

PVP systems showed no after-effects. For the sS and CMC systems the after-effect depended on the enzyme concentration: it was about 25% for the low concentration systems and ranged from 5 tot 15% for the high concentration systems.

As in the sS-PE systems the pH of the sS-AP systems was decreased by the action of ionizing radiation. Here the decrease of the pH was 0.07 units per 10^6 rad.

5 Discussion and conclusions

Radioresistance of the enzymes The radioresistance of an enzyme in an enzyme-polymer system was calculated from a linear regression analysis of the ln % residual enzyme activities versus irradiation doses. The fact that the extrapolated curves of the sS-POD systems did not intersect at the blank point on the activity axis, whereas the curves of all the other enzyme-polymer systems cut the activity axis in the blank point can be explained as follows. In the sS-POD systems there are both bound and free enzyme molecules. The latter are much more radiosensitive than the bound ones, so that there is a discontinuity in the curve ln % residual enzyme activity versus irradiation dose. In the other systems this discontinuity does not occur, because the active enzyme molecules are only present in a bound form.

In the systems used in this study it was found that the enzymes are least radioresistant at high water activities ('free water') except for the PVP systems of PE and AP. An explanation could be that water radicals are more reactive to the enzymes than to the polymers, but for the exceptions the water radicals are more reactive to PVP. The radioresistance of AP in its systems was increased in the higher enzyme concentration systems. This protective behaviour could be caused by a higher concentration of impurities including inactivated enzyme molecules. Therefore environmental factors strongly determine the radioresistance of an enzyme.

Intrinsic radiosensitivity As shown in Table 3 the intrinsic radiosensitivities of the enzymes studied in the CMC and sS systems are in the following order:

$$PE < POD < AP$$

The molecular weights of these enzymes have the same sequence. Although there were not sufficient data for establishing a clear relationship, a regression analysis was carried out for intrinsic radiosensitivity as a function of the molecular weight (Roozen & Pilnik, 1972). The intrinsic radiosensitivities of PE in the NaP systems and of the enzymes in the PVP systems are out of this order. These deviations could be caused by the forming of an enzyme-product complex (PE-NaP) and by the high reactivity of hydrated electrons with PVP (Hart, 1964). In the experiments of Okada (1957) the protective effect of proteins seemed to be a function of their respective molecular weights. His results imply that the number of reactive sites on the protein molecule increase with the size of the molecule. In analogy, in aqueous systems the intrinsic radiosensitivity of an enzyme is partly determined by its molecular weight (Roozen & Pilnik, 1972).

After-effects For some time after the actual irradiation of aqueous solutions of deoxyribonucleic acid with X-rays there is a slow decrease of viscosity which is called an after-effect. Daniels et al. (1953) considered this after-effect to be due to a slow hydrolysis of labile substances which were formed during the irradiation process. I found similar after-effects with the enzyme activity of dissolved enzyme-polymer systems. It is therefore possible that unstable enzyme molecules are formed during irradiation and that these molecules will be inactivated more quickly than the original ones.

Summary

The effect of ionizing radiation on the inactivation of enzymes was studied as a function of their moisture contents. For this purpose the following enzyme polymer solutions were freeze-dried: POD-CMC, POD-sS, POD-PVP, PE-CMC, PE-sS, PE-PVP, PE-NaP, AP-CMC, AP-sS and AP-PVP. The freeze-dried material was ground and then divided between 42 polyethene bags. The samples were incubated for one week in a desiccator and then for a fortnight over saturated salt solutions in order to obtain samples with the desired moisture content. These samples were then irradiated, dissolved and assayed for enzyme activity.

During the treatment before irradiation the stability of POD depends on temperature, moisture content, pH and the nature of the polymers. The same factors influence the radiosensitivity of POD. An after-effect of the irradiation was detected in the solutions of the CMC-POD and the sS-POD systems, but no after-effect was found in the solutions of POD-PVP systems.

Incubated over silica gel the PE systems lost minimum enzyme activity. In all systems the enzyme was least radiosensitive at 40% relative humidity except in the PVP system in which the enzyme decreased in radiosensitivity with increasing relative humidity.

In all the systems the radiosensitivity of AP had the same magnitude but over 40% relative humidity the sensitivity decreased in the PVP systems and increased in the CMC and sS systems. An increase in enzyme concentration decreased the radiosensitivity of AP. There was no after-effect in the solutions of the AP-PVP systems, while the after-effects were more pronounced in the solution of the CMC and sS systems with the lower enzyme concentrations.

At neutral pH, the intrinsic raidosensitivities of the studied enzymes in the CMC and sS systems were in the proportion of:

$$PE < POD < AP$$

The molecular weights of these enzymes have the same sequence. Therefore a regression analysis was carried out from the intrinsic radiosensitivity as a function of the molecular weight. The intrinsic radiosensitivities of PE in the NaP systems and of the enzymes in the PVP systems were out of the order. These deviations could be caused by the formation of an enzyme-product complex (PE-NaP) and by the high reactivity of hydrated electrons with PVP.

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