# Application of Ionising Radiation for the Stabilisation of *Trichoderma viride* Cellulases

*Vít PLAČEK[1], Karel VACEK[1], Jan KÁŠ[2], Kateřina DEMNEROVÁ[2], Jarmila ZÍDKOVÁ[2] and Jiří SAJDOK[2]*

*[1]Department of Radiation Applications, Nuclear Research Institute, Řež, Czech Republic; [2]Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, Institute of Chemical Technology Prague, Prague, Czech Republic*

**Abstract**

Plaček V., Vacek K., Káš J., Demnerová K., Zídková J., Sajdok J. (2005): **Application of ionising radiation for the stabilisation of *Trichoderma viride* cellulases**. Czech J. Food Sci., **23**: 111–115.

The solutions of cellulolytic enzymes designated as standards for the cellulase activity assay were exposed in sealed glass ampoules (containing at least 100 Cx-units per ml in 30% w/w glycerol) to gamma radiation within the dose interval of 0–18 kGy. Glycerol was found to be the best enzyme stabiliser, however, the dose for the decontamination had to be increased in comparison with the original solution because glycerol protected also the contaminating microflora. The preparation after such treatment (30% of glycerol, dose 7 kGy) retained about 95% of the initial enzymatic activity without any decrease taking place in the following 6 months. The loss of the side activities did not exceed 10.5% and no bacterial contamination was detected either after 6 months of storage following the irradiation. No difference was found in the immunoreactivity of cellulases or in protein chromatografic (FPLC) pattern between the original and the irradiated enzyme preparations.

**Keywords**: cellulase enzyme; stabilisation; gamma irradiation

The wide application of technical enzymes increased the interest in searching the methods for their stabilisation and microbial decontamination. Many ways of the enzyme stabilisation were proposed using either suitable additives or chemical modifications (e.g. Torchilin & Martinek 1979; Eijsink *et al*. 2004). Among the various approaches leading to microbial decontamination of the enzyme preparations, ionising radiation seems to be very promising because of its simplicity and the possibility to find suitable conditions under which microorganisms are killed and the degree of an enzyme inactivation is low. It is because DNA molecules, which are essential for microbial multiplication, are more sensitive to ionising radiation than other macromolecules including proteins (Kiefer 1981). The lethal effect of ionising radiation on microbial cells is very complex. Apart from the direct incidence, the free radicals formed after the adsorption of

Supported of the Internal Grant Agency of the Ministry of Education, Youth and Sports of the Czech Republic, Project MSM 6046137305.

radiation in water, either in-or outside the cell, play an important role. The final effect of ionising radiation on the biomacromolecules is strongly dependent on the environmental conditions and was recently discussed in the paper of Kempner and Verkman (1988).

The aim of the present paper was to find the most gentle and simple conditions of microbial decontamination of the soluble cellulase preparation by gamma radiation as well as of the simultaneous preservation of its activity.

## MATERIAL AND METHODS

***Chemicals***. Ultrafiltrates of the fermentation broth after cultivation of *Trichoderma viridea*, supplied by Enzyme Plant Kolín (Czech Republic) were used as the original cellulolytic preparations. Galacturonic acid, inulin, apple pectin, and agarose were from Sigma, agar No. 3 from Oxoid, filter paper Whatman No. 1, carboxymethylcellulose from Lovosa, and the other chemicals of analytic grade purity were from Lachema Brno (Czech Republic).

Cx-activity of cellulase preparation was determined using carboxymethylcellulose (CMC) as substrate and estimating the resulting reducing sugars with Somogyi-Nelson method (Somogyi 1950). The Cx-activity unit was defined as the amount of the enzyme releasing 1 mg of reducing sugars during the period of 30 min at pH 5 and 40°C. Very low cellulase activities were measured by plating method with Congo Red (Buriánová *et al*. 1991). FPA (filter paper activity) method was applied according to Montenecourt and Eveleigh (1977). The immunochemical determination of the cellulase concentration was performed using radial immunodifusion technique of Mancini *et al*. (1965).

The side activities were measured in the same manner as Cx-activity except that CMC was replaced with an appropriate substrate (apple pectin, inulin, galacturonic acid and arabinic acid).

The activity of the cellulase solutions was adjusted by dilution either to 100 or 1000 units per ml and various compounds were added as stabilisers. 10 ml of each enzyme sample was sealed in a glass ampoule and then irradiated by gamma rays from cobalt-60 facility at the room temperature. The same dose rate (0.95 kGy/h) was always used. The absorbed doses were checked by alanine dosimetry based on the Fricke dosimeter (Regula & Defner 1989).

The samples of the cellulase preparation were diluted aseptically according to the presumed degree of contamination. 0.1 ml of each sample was applied on the respective cultivation medium (malt agar, complete agar, and nutrient agar) and cultivated either at 28°C (yeasts, fungi) or 37°C (bacteria); the colonies were counted after 2 and 6 days of growth (Collins 1964).

## RESULTS AND DISCUSSION

The fundamental characteristics of the original cellulase preparations are given in Table 1. The samples were contaminated with different strains of bacteria (*Bacillus*, *Lactobacillus*, gram negative non-fermentative bacteria).

The preparation No. 1 was diluted in 0.1M acetate buffer, pH 5.0, to the activity of 800, 100, 10, 2 and 1 U/ml. These samples were treated with various doses of ionising radiation and then the cellulase activity was determined. Figure 1 shows that the inactivation was markedly related to the decreasing activity. Simultaneously with the activity assay, the changes in microbial contamination were checked. Table 2 indicates that yests are more sensitive to the ionising radiation than

Table 1. Fundamental characteristics of the cellulase preparations used

| Characteristic | Sample No. 1 | Sample No. 2 |
|---|---|---|
| Cellulase activity – CMC (U/ml) | 800 ± 15 | 3950 ± 20 |
| Dry matter (%) | 7.11 | 9.16 |
| pH | 4.4 | 4.5 |
| Microbial contamination: bacteria cells | $1 \times 10^8$ | $7 \times 10^7$ |
| yeasts | $4.7 \times 10^5$ | $4 \times 10^5$ |
| molds | 0 | 0 |

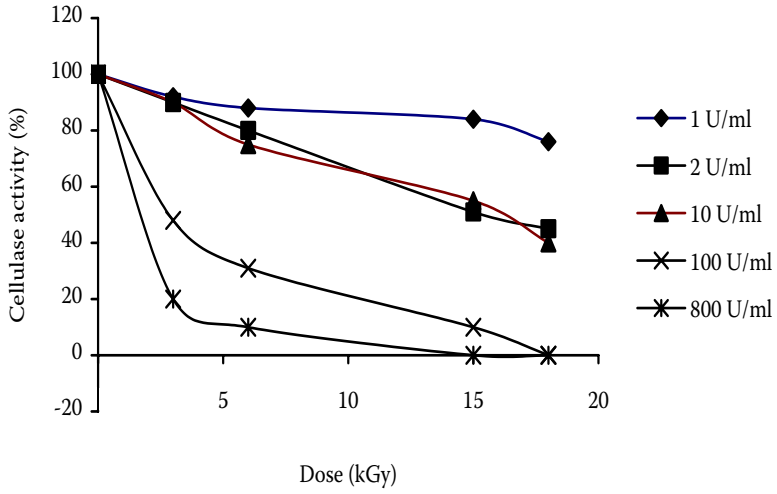


Figure 1. Cellulase activity (%) as a function of dose at various concentrations (units/ml)

bacteria, and that the radiation dose of 6 kGy has a satisfactory pasteurising effect at a relatively high initial contamination level. Using this radiation dose, the loss of enzyme activity in the sample of the highest activity was only 10%. In contrast, in the highly diluted enzyme samples (2 and 1 U/ml) the inactivation of cellulase reached 74% and 92%, respectively. Consequently, some kind of the enzyme stabilisation and also the use of samples with higher enzyme specific activities is needed for the radiation treatment. Some anticipated stabilising agents were tested in the enzyme preparations containing 1000 cellulase U/ml. Besides the optimal radiation dose as determined in the previous experiments (6 kGy), somewhat lower and higher radiation doses were also applied (5 and 7 kGy). The results are given in Table 3 in which the filter paper activity (FPA) is also presented for comparison. The best stabilising effect was achieved by using 30% glycerol. Further increase of the glycerol concentration did not improve the stabilisation effect and, moreover, the high viscosity of the enzyme solution makes the handling diffucult. Cystein, due to its free sulphydryl group, was considered as an efective scavenger of the primary products of radiolysis changing them

Table 2. Effect of radiation dose on the microbial contamination

| Type of microorganism | Cells/ml at radiation dose (kGy) | | | | |
|---|---|---|---|---|---|
| | 0 | 3 | 6 | 15 | 18 |
| Bacteria | $1 \times 10^8$ | $1 \times 10^4$ | 0 | 0 | 0 |
| Yeasts | $4.7 \times 10^5$ | 0 | 0 | 0 | 0 |
| Molds | 0 | 0 | 0 | 0 | 0 |

Table 3. Effect of ionising radiation on cellulase activity in the presence of various stabilisers intended

| Cellulase (1000 U/ml) | Relative cellulase activity at radiation dose 6 k Gy | |
|---|---|---|
| | CMC | FPA |
| Intact | 82 ± 5 | 90 ± 7 |
| + 30% glycerol | 95 ± 5 | 95 ± 7 |
| + 0.2% sodium benzoate | 89 ± 5 | 89 ± 7 |
| + 50% glycerol | 95 ± 5 | 93 ± 7 |

CMC – activity measured by carboxymethylcellulose
FPA – activity measured by filter paper

Table 4. Microbial contamination of cellulase preparations in dependence on radiation dose

| Cellulase | Bacteria counts (ml) after radiation dose (kGy) | | | | |
|---|---|---|---|---|---|
| | 0 | 3 | 5 | 6 | 7 |
| Intact | $7 \times 10^7$ | $1.3 \times 10^4$ | 0 | 0 | 0 |
| + 30% glycerol | $7 \times 10^7$ | $2 \times 10^5$ | 200 | 0 | 0 |
| + 50% glycerol | $7 \times 10^7$ | $5 \times 10^5$ | 1000 | 250 | 0 |
| + 30% glycerol and 0.02% sodium benzoate | $7 \times 10^7$ | $2 \times 10^5$ | 500 | 40 | 0 |

Table 5. Changes of the side enzyme activities of cellulase preparation in intact and stabilised (30% of glycerol) forms after ionising radiation (6 kGy)

| Enzyme | Original enzyme activity* (U/ml) | Enzyme activity (%) after radiation | |
|---|---|---|---|
| | | intact enzyme | protected with 30% glycerol |
| Amylase | 87 ± 7 | 87 ± 5 | 91 ± 5 |
| Pentosanase | 24 ± 7 | 85 ± 5 | 90 ± 5 |
| Inulinase | 114 ± 8 | 86 ± 5 | 96 ± 5 |
| Polygalacturonidase | 556 ± 8 | 92 ± 5 | 95 ± 5 |
| Arabinase | 142 ± 8 | 85 ± 5 | 90 ± 5 |
| Pectinase | 365 ± 7 | 88 ± 5 | 92 ± 5 |

*The activity of the individual enzymes is expressed in mg of reducing sugars released from the appropriate substrate at pH 5 and 40°C during the radiation

to less reactive compounds. Unfortunately, this anticipated effect was not confirmed.

The microbial contamination (Table 2) and the changes in the levels of side activities (Table 5) of the irratiated cellulase preparations stabilised with glycerol were also tested. Table 4 demonstrates that the increase of the glycerol concentration to 50% had another unfavourable effect, i.e. the protection of bacteria against ionising radiation, and that the 30% glycerol concentration represents an acceptable compromise between the stabilisation of the enzyme and bacteria protection. Table 5 shows that also the other enzymes present are protected against radiation. Although certain small diferences between the radiation sensitivities of the individual enzymes were found, the suggested method of cellulase stabilisation against the inactivating effect of ionising radiation can be proposed as a general approach to the protection of other enzymes. No changes existing in the immunochemical properties of the intact and irradiated cellulase, as well as the same chromatografic properties on Mono Q column of FPLC (Pharmacia, Uppsala), show that the ionising radiation of 6 kGy is an acceptable procedure for the stabilisation of enzymes in solution when 30 w/w of glycerol is added.

## References

Buriánová T., Kopečný J., Sajdok J., Káš J. (1991): Assay of very low cellulolytic activity in fodder supplemented with enzyme preparation. Animal Feed Science and Technology, **33**: 41–48.

Collins C.H. (1964): Microbiological Methods Butterworths. London: 86–109.

Eijsink V.G.H., Gaseidnes S., Synstad B., van den Burg B. (2004): Rational engineering of enzyme stability. Journal of Biotechnology, **113**: 105–120.

Kempner E.S., Verkman A.S. (1988): Direct effects of ionizing-radiation unique to macromolecules. Radiation Physics and Chemistry, **32**: 341–347.

Kiefer J. (1981): Biologische Strahlenwirkung. Springer Verlag, Berlin: 301–303.

Mancini G., Carbonare A.O., Heremaus J.F. (1965): Immunochemical quantitations of antigens by singel radial immundiffusion. Immunochemistry, **2**: 235–254.

Montenecourt B.S., Eveleigh D.E. (1977): Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. Applied and Environmental Microbiology, **34**: 777–782.

Regula D.F., Defner U. (1989): Dose estimation by ESR spectroscopy at a fatal radiation accident. International Journal of Radiation Applications and Instrumentation. Part A. Applied Radiation and Isotopes, **40**: 1039–1043.

Reese E.T., Mandels M. (1980): Stability of the cellulase of *Trichoderma reesei* under use conditions. Biotechnology and Bioengineering, **22**: 323–335.

Somogyi M. (1950): Studies of arteriovenous differences in blood sugar. Biological Chemistry, **186**: 513–526.

Torchilin V.P., Martinek K. (1979): Enzyme stabilization without cariers. Enzyme and Microbial Technology, **1**: 74–82.

Received for publication February 25, 2005
Accepted after corrections May 2, 2005

*Corresponding author:*

Doc. Ing. Jiří Sajdok, CSc., Vysoká škola chemicko-technologická, Fakulta potravinářské a biochemické technologie, Ústav biochemie a mikrobiologie, Technická 5, 166 28 Praha 6, Česká republika
tel.: + 420 220 445 102, fax: + 420 220 445 167, e-mail: sajdokj@vscht.cz