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# Radiation-Induced Enhancement of Nitrite Reducing Activity of Cytochrome *c*

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Commercial cytochrome *c* (Cyt *c*) was irradiated with Co-60 $\gamma$-rays in the dose range of up to 3.0 kGy to investigate the enhancement of the nitrite reducing activity of Cyt *c*. The optimum irradiation dose to induce nitrite reducing activity for 30 $\mu$M Cyt *c* solution was 1.0 kGy under an $O_2$ atmosphere. The nitrite reducing activity of Cyt *c* irradiated at this dose was approximately 45-fold that of unirradiated Cyt *c* and ca. 1.2-fold that of nitrite reductase. The irradiation treatment resulted in unfolding of the peptide chain, exposure of the heme group, oxidation of methionine to methionine sulfoxide, dissociation of the sixth ligand (Met), and occurrence of autoxidation in Cyt *c*. Sepharose-immobilized irradiated Cyt *c* had a similar activity to that in solution. The resin retained the activity after five uses even after 1 year of storage. The irradiated Cyt *c* will be able to be used as a substitute for nitrite reductase.

**KEYWORDS: Cytochrome *c*; $\gamma$-irradiation; nitrite reducing activity**

## INTRODUCTION

Nitrite ($NO_2^-$) and nitrate ($NO_3^-$) have been used to improve meat qualities such as color and shelf life for many years (*1*). Nitric oxide (NO) is formed from $NO_2^-$ by reductants in the meat, and it is subsequently complexed with oxidized pigments, mainly metmyoglobin. Nitrosylmetmyoglobin is then converted to nitrosylmyoglobin and, ultimately, to nitrosylhemochrome (*2*). Carcinogenic *N*-nitrosoamines may be produced by the reaction of $NO_2^-$ with secondary amines from meat (*3*).

In our laboratory, the structure/function relationships of cytochrome *c* (Cyt *c*) have been investigated. Cyt *c* is a widely studied soluble hemoprotein, and its heme is coordinated with endogenous ligands, histidine and methionine (*4*).

Yamada et al. reported that heat-treated equine heart Cyt *c* reduces $NO_2^-$ to ammonia ($NH_4^+$) (*5*). This unexpected phenomenon, in which a new catalytic activity appears after heat treatment, is contrary to those observed in well-known biological molecules, which are generally inactivated by heat. Denaturation methods for Cyt *c* to induce its $NO_2^-$ reducing activity (NiR) include the use of organic solvents and supercritical carbon as well as heat treatment (*6*), but $\gamma$-irradiation can also denature proteins. $\gamma$-Rays from Co-60 inactivate *Clostridium botulinum* and *Salmonella* and have been used in food preservation for many years (*7*).

The effect of $\gamma$-irradiation on enzyme activity has been reported on various enzymes such as glucose isomerase from *Streptomyces phaeochromogeus* (*8*), aminopeptidase and endopeptidase from *Musca domestica* (*9*), orinithine decarboxylase from rat (*10*), and thermonuclease (TNase) from *Staphylococcus aureus* (*11*). However, the vast majority of work on the effect of $\gamma$-irradiation on enzyme activity has dealt with enzyme inactivation, and an increase in enzyme activity by irradiation has not yet been reported.

The effect of $\gamma$-irradiation on Cyt *c* has been studied by several investigators. For example, Ambe et al. reported that methionine, histidine, phenylalanine, cystine, serine, and threonine are the most radiation-labile of the amino acids in the irradiated bovine Cyt *c* (*12*). Kondo and co-workers investigated the radiation-induced reduction of Cyt *c* by OH radicals produced by water radiolysis and conformation degradation of Cyt *c* by $\gamma$-irradiation (*13*, *14*). They presumed that the polypeptides of Cyt *c* are unfolded, and the heme and tyrosyl residues in Cyt *c* are exposed by irradiation. However, the radiation-induced enhancement of the NiR of Cyt *c* has not yet been reported. Nitrite reductase does not come into the market, because of low content of the enzyme in plants and microbes and of requirement of a lot of time for the purification. We have tried to prepare a substitute for nitrite reductase by irradiating commercial equine heart Cyt *c* and determining $NO_2^-$ in food and in an aqueous solution.

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In this paper, we report enhancement of NiR of Cyt *c* by $\gamma$-irradiation and the effect of $\gamma$-irradiation on the Cyt *c* structure using UV−visible spectra, circular dichroism (CD) spectra, and amino acid analysis. We also compare irradiated Cyt *c* with heat-treated Cyt *c* for induction of NiR and show the usefulness of immobilized Cyt *c* in applied research.

## MATERIALS AND METHODS

**Preparation of Hemoproteins.** Equine heart Cyt *c* was purchased from Wako Pure Chemical Industries. Bovine blood hemoglobin and equine heart myoglobin were obtained from Sigma Chemical Co. (St. Louis, MO). Cyt *c* was purified by Sephacry S-200 gel filtration column chromatography (1.0 cm × 80 cm) (*15*). The concentration of purified Cyt *c* was determined using pyridine hemochromogen methods (*16*).

**Irradiation.** Cyt *c* solution (30 $\mu$M = 0.375 mg/mL) was saturated with $O_2$ gas by bubbling for 30 min. The solution was irradiated with $\gamma$-rays from Co-60 sources at a dose rate of 5.0 kGy/h. The effect of radicals produced by water radiolysis on Cyt *c* activation was investigated using $5 \times 10^{-2}$ M (This concentration of *tert*-BuOH corresponds to two times the amount of total OH radical produced theoretically by water radiolysis.) *tert*-butanol (BuOH) as an OH radical scavenger (*8*, *17*).

**Measurement of NiR.** The NiR of irradiated Cyt *c* as cited from Blumle and Zumft was utilized (*18*) but with some modifications. The vial containing 0.4 mL of 100 mM sodium phosphate buffer (pH 7.0), 0.4 mL of 10 mM sodium nitrite, 0.5 mL of 3 mM methyl viologen, and 0.4 mL of 30 $\mu$M irradiated Cyt *c* solution was sealed with butyl rubber. After the mixture was preincubated at 37 °C for 5 min, the reaction was started by adding 0.3 mL of 100 mM sodium dithionite dissolved in 50 mM sodium bicarbonate. The reaction was conducted under anaerobic conditions at 37 °C. The reaction mixture was put in a test tube, left to stand for several minutes, and then vigorously shaken until complete decolorization.

The detection of $NO_2^-$ was performed according to Ramirez et al. (*19*). For $NO_2^-$ detection, 1.95 mL of water, 1 mL of 1% sulfanilamide, 1 mL of 0.02% *N*-1-naphthylethylenediamine, and 1 mL of water were added to 50 $\mu$L of the reaction mixture. After the reaction mixture was allowed to stand at room temperature for 20 min, its optical density at 540 nm was detemined. One unit of activity was defined as a reduction of 1 $\mu$mol $NO_2^-$ per minute. The value of $k_{cat}$ was calculated from Lineweaver−Burk plots.

The detection of $NH_4^+$ was performed according to Scheiner et al. (*20*). For the detection of $NH_4^+$, 1.9 mL of water, 1 mL of 50 mg% sodium nitroprusside in 10% phenol, 1 mL of 0.06% sodium hypochlorite solution dissolved in 0.1 M disodium hydrogen phosphate−0.25 M sodium hydroxide, and 1 mL of water were added to 100 $\mu$L of the reaction mixture. After the reaction mixture was allowed to stand at room temperature for 60 min, its optical density at 630 nm was determined.

**Spectroscopic Analysis**. The visible spectra of irradiated Cyt *c* were monitored with a HITACHI U3310 spectrophotometer using quartz cuvettes of 1.0 cm path length at 25 °C. CD spectra were recorded at 25 °C in 20 mM sodium phosphate buffer (pH 7.0) with a JASCO J-700 spectropolarimeter in 0.2 cm path length rectangular quartz cuvettes. $\alpha$-Helical content was calculated from the CD absorbance at 222 nm based on the 100% value of $-32 \times 10^{-3}$ deg cm$^2$/dmol (*21*).

**Redox Titration Studies.** Redox titrations were performed under anaerobic conditions, with a continuous stream of argon, in 100 mM sodium phosphate buffer, pH 7.0, at 25 °C (*22*). The potentials were measured with a HORIBA F-13 pH meter equipped with an ORP electrode, and the optical spectra were monitored throughout the titration on a HITACHI U3310 spectrophotometer. The redox mediators were used to stabilize the solution redox potential as described in Yamada et al. (*5*). The redox data were analyzed with a theoretical curve based on the Nernst equation ($n = 1$): $E = E° + (RT/nF)\ \ln([\text{ferric}]/[\text{ferrous}])$.

**Amino Acid Analysis.** Amino acid analysis was based on that described by Kume and Takehisa (*23*). The samples (100 nmol) were hydrolyzed in 6 N KOH for measurement of methionine sulfoxide (*24*) and 6 N HCl in evacuated, sealed tubes at 110 °C for 24 h. For the determination of cysteine (Cys) residue, Cyts *c* were reacted with 2-nitriphenyl sulfenyl chloride for heme cleavage (*25*), which was reacted with iodoacetate (*26*), and Cys was converted after the determination to *S*-carboxymethylcysteine. Tryptophan determination was done according to the hydrolysis with 4 N methanesulfonic acid at 115 °C for 24 h (*27*). Amino acids were analyzed using a HITACHI 655A-11 high-performance liquid chromatograph equipped with a no. 2619F column.

**Table 1.** Assay Components of Nitrite Reducing Activity of Irradiated Cyt *c*[a]

| assay component | concn (mM) | volume (mL) | role |
|---|---|---|---|
| sodium phosphate buffer (pH 7.0) | 100 | 0.4 | to keep the pH of assay |
| irradiated Cyt *c* | 0.03 | 0.4 | sample |
| sodium nitrite | 10 | 0.4 | reaction substrate |
| methyl viologen | 3 | 0.5 | electron carrier |
| sodium dithionite | 100 | 0.3 | reductant |

[a] The total volume of reaction mixture was 2.0 mL.

**Preparation of Sepharose-Immobilized and Acrylamide-Immobilized Cyt *c*.** Sepharose-immobilized and acrylamide-immobilized Cyts *c* were prepared by the methods of Shin and Oshino (*28*). The binding rate of irradiated Cyt *c* to CNBr-sepharose 4B was 99.11%, and the rate of inclusion of irradiated Cyt *c* in acrylamide was 99.86%.

**Protein Analysis.** The degradation of Cyt *c* by $\gamma$-irradiation was checked by 16.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) (*29*). The gel was stained with Coomassie brilliant blue R-250. Protein was determined by the method of Lowry et al. (*30*) with bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

**Enhancement of NiR of Cyt *c* by Irradiation.** The concentration and role of assay components of NiR used in this experiment were showed in **Table 1**. The measurement of NiR of irradiated Cyt *c* was performed using methyl viologen as an electron carrier and sodium dithionite as a reductant according to Blumle and Zumft (*18*). The control mixture contained the sodium phosphate buffer (pH 7.0), sodium nitrite, methyl viologen, sodium dithionite, and sodium phosphate buffer in place of irradiated Cyt *c*. When the methyl viologen as electron carrier was absent in the reaction mixture, the NiR of irradiated Cyt *c* was approximately 1/45 that of irradiated Cyt *c* in the presence of methyl viologen (data not shown). When the sodium dithionite as reductant was absent in the reaction mixture, the irradiated Cyt *c* did not show the NiR at all (data not shown). Therefore, the reaction mixture needs to contain the sodium phosphate buffer (pH 7.0), sodium nitrite, methyl viologen, and sodium dithionite. In this assay method, the irradiated Cyt *c* catalyzed the reduction of $NO_2^-$ to $NH_4^+$, and the overall reaction was a six electron and eight proton reduction.

To investigate the enhancement of the NiR of Cyt *c*, Hb, and Mb, these hemoproteins (30 $\mu$M) were irradiated with Co-60 $\gamma$-rays at 1.0 kGy. The NiR of unirradiated Cyt *c*, Hb, and Mb were nearly the same. In the case of irradiation at 1.0 kGy, only Cyt *c* showed a higher activity (**Figure 1**). In *c* type Cyt, protoheme is covalently bonded via thioether linkage to the two cysteines of the peptide chain, and the axial histidine and Met ligands are coordinated to heme iron as the fifth and sixth ligands, respectively. On the other hand, *b* type hemoproteins such as Hb and Mb have no thioether linkage and bind only the histidine as the fifth ligand.

Cyt *c* (30 $\mu$M) was irradiated with Co-60 $\gamma$-rays in the dose range of 0−3.0 kGy under an $O_2$ atmosphere to investigate the optimum irradiation dose for inducing the NiR of Cyt *c*. From the SDS−PAGE analysis results, the fragmentation and aggregation of Cyt *c* irradiated with doses up to 3.0 kGy were

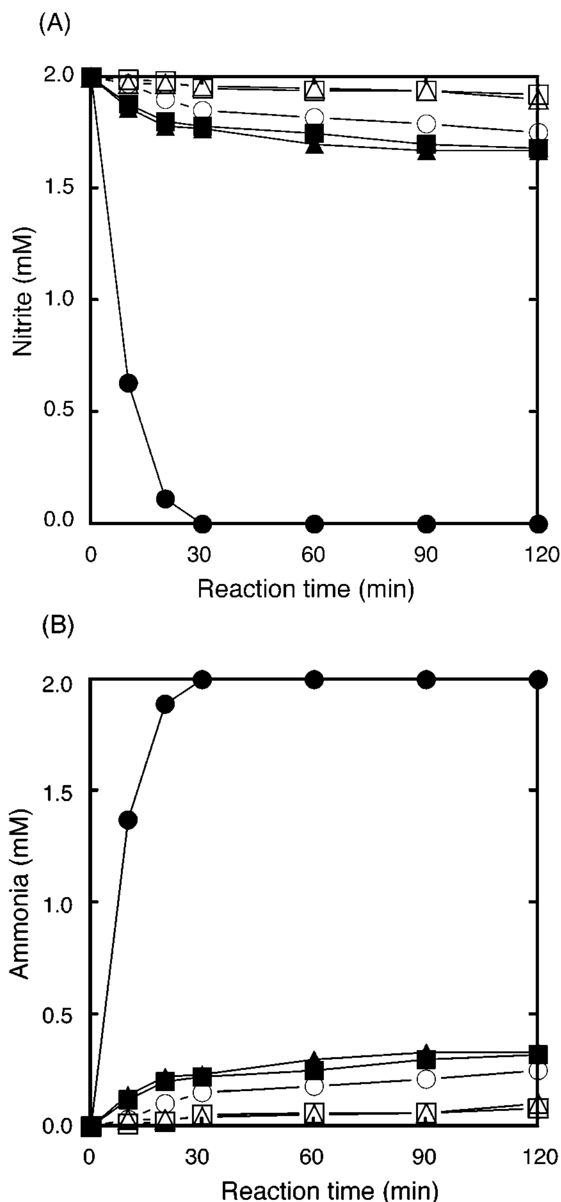


**Figure 1.** Nitrite reduction and ammonia production of irradiated hemoproteins. The hemoproteins were irradiated at 1.0 kGy under an $O_2$ atmosphere. Cyt *c* (○); hemoglobin (△); and myoglobin (□). Closed symbols indicate the corresponding irradiated proteins. (**A**) Nitrite reduction; (**B**) ammonia production.

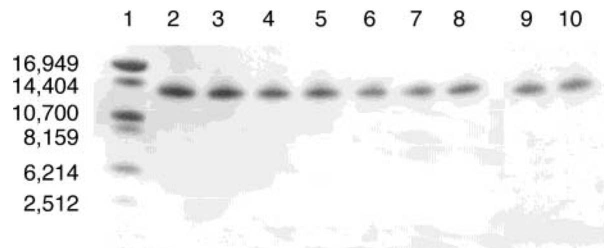


**Figure 2.** SDS–PAGE profile of $\gamma$-irradiated Cyt *c* on a 16.5% acrylamide gel stained with coomassie brilliant blue. Lane 1, molecular weight marker; 2, unirradiated; 3, 0.25 kGy; 4, 0.5 kGy; 5, 0.75 kGy; 6, 1.0 kGy; 7, 1.5 kGy; 8, 2.0 kGy; 9, 2.5 kGy; and 10, 3.0 kGy.

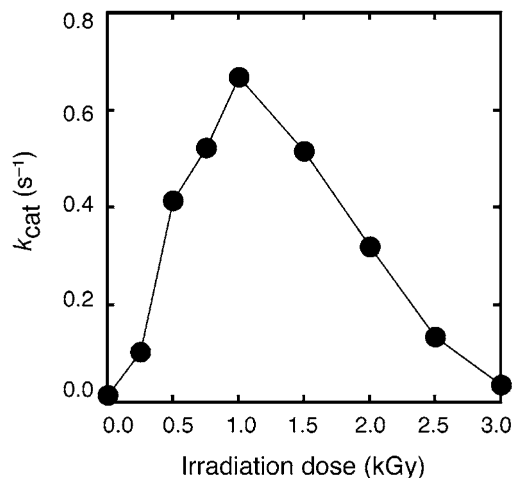


**Figure 3.** Irradiation dose profile of the nitrite reducing activity of Cyt *c*.

not observed (**Figure 2**). Tappel and co-workers investigated the radiation damage of Cyt *c* (0.1% solution) irradiated at doses of 1−500 kGy by SDS−PAGE and amino acid analysis and reported that Cyt *c* aggregation is induced above 10 kGy; 16% of Cyt *c* exposed to 10 kGy and 100% of Cyt *c* exposed to 100 kGy aggregated (*12*, *31*). It was assumed that fragmentation and aggregation of irradiated Cyt *c* were not observed in our study because the Cyt *c* concentration and irradiation dose used were approximately one-third that used by Tappel and co-workers.

To determine the relationship between irradiation dose and Cyt *c* NiR, the NiR of irradiated Cyt *c* was measured, and a plot of $k_{cat}$ value against irradiation dose is shown in **Figure 3**. The NiR of Cyt *c* irradiated at lower doses (0−1.0 kGy) increased with increasing irradiation dose, and the NiR of Cyt *c* irradiated at 1.0 kGy was approximately 45-fold that of unirradiated Cyt *c*. The NiR of Cyt *c* irradiated above 1.0 kGy gradually decreased, and the NiR of Cyt *c* irradiated at 3.0 kGy was similar to that of unirradiated Cyt *c*. Cyt *c* was irradiated at 0.8, 0.9, 0.95, 1.0, 1.05, 1.1, and 1.2 kGy to determine the exact optimum irradiation dose for NiR. It was confirmed that the NiR of Cyt *c* irradiated at 1.0 kGy was maximum. The induction of Cyt *c* NiR by $\gamma$-irradiation has not yet been reported, but the relationship between irradiation dose and the CO affinity of Cyt *c* ($3.01 \times 10^{-5}$ M solution) has already been reported (*14*). In the paper, the CO affinity of Cyt *c* irradiated at lower doses ($0-6.0 \times 10^{18}$ eV/mL (0.93 kGy)) increases monotonically and that of Cyt *c* irradiated at $6.0 \times 10^{18}$ eV/mL is approximately 15-fold that of unirradiated Cyt *c*. However, the affinity of Cyt *c* irradiated above $6.0 \times 10^{18}$ eV/mL gradually decreases, while that of Cyt *c* irradiated at $8.0 \times 10^{18}$ eV/mL (1.24 kGy) is almost the same as that of unirradiated Cyt *c*, and our results agreed with these findings.

**Change in Structure of Irradiated Cyt *c*.** To examine the secondary structural change of Cyt *c* due to $\gamma$-irradiation, the far-UV CD spectra of irradiated Cyt *c* were measured. In the far-UV CD spectra, the absorbances at 222 and 216 nm showed $\alpha$-helices and $\beta$-sheets, respectively (*32*), but equine heart Cyt *c* has no $\beta$-sheets (*33*). Therefore, the secondary structural change of Cyt *c* by $\gamma$-irradiation was investigated using absorbance at 222 nm. The CD spectra of irradiated Cyt *c* showed a lower mean residue ellipticity of 222 nm with increasing irradiation dose, and this peak of Cyt *c* irradiated at 3.0 kGy disappeared (**Figure 4**). To investigate the changes of CD spectra of irradiated Cyt *c*, the CD spectra of irradiated Cyt *c* were compared with that of Cyt *c* treated with urea and heated. The CD spectra of Cyt *c* treated with urea (9.5 M) as a denaturant and heated at 100 °C for 30 min showed a lower mean residue ellipticity of 222 nm than that of the native Cyt

**Table 2.** Changes in Amino Acid Content of Irradiated Cyt *c*[a]

| | amino acid content (residues/molecule) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | unirradiated | 0.25 | 0.5 | 0.75 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 (kGy) | *tert*-BuOH[f] |
| aspartic acid | 8.0 | 8.0 | 7.7 | 7.7 | 7.5 | 7.5 | 7.2 | 6.8 | 6.6 | 8.0 |
| methionine sulfoxide[b] | 0.0 | 0.0 | 0.5 | 0.7 | 1.1 | 1.4 | 1.4 | 1.5 | 1.5 | 0.0 |
| threonine | 10.0 | 9.9 | 9.5 | 9.2 | 9.0 | 9.0 | 8.9 | 8.5 | 8.0 | 10.1 |
| serine | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| glutamic acid | 12.0 | 12.0 | 12.0 | 11.9 | 11.8 | 11.6 | 11.7 | 11.8 | 11.6 | 11.8 |
| proline | 4.0 | 3.8 | 3.7 | 3.7 | 3.4 | 3.3 | 3.5 | 3.3 | 3.5 | 3.9 |
| glycine | 12.0 | 11.9 | 11.7 | 11.3 | 10.9 | 10.8 | 10.7 | 10.3 | 10.2 | 11.9 |
| alanine | 6.0 | 5.8 | 5.6 | 4.8 | 4.0 | 4.0 | 4.0 | 3.9 | 3.8 | 6.0 |
| cysteine[c] | 2.0 | 1.9 | 1.9 | 1.7 | 1.6 | 1.7 | 1.7 | 1.6 | 1.6 | 1.9 |
| valine | 3.0 | 2.9 | 3.0 | 2.9 | 2.9 | 2.3 | 2.2 | 2.1 | 2.2 | 3.0 |
| methionine | 2.0 | 1.8 | 1.2 | 1.0 | 0.5 | 0.4 | 0.4 | 0.3 | 0.3 | 2.1 |
| DOPA | 0.0 | 1.0 | 1.8 | 2.4 | 2.8 | 2.6 | 2.6 | 2.3 | 2.3 | 0.0 |
| isoleucine | 6.0 | 6.0 | 6.0 | 5.9 | 6.2 | 6.2 | 6.1 | 5.9 | 5.9 | 6.0 |
| leucine | 6.0 | 5.9 | 5.9 | 5.8 | 5.8 | 5.7 | 5.5 | 5.5 | 5.3 | 6.0 |
| tyrosine | 4.0 | 3.3 | 2.1 | 1.9 | 1.2 | 1.2 | 1.2 | 1.0 | 0.9 | 4.0 |
| phenylalaine | 4.0 | 3.9 | 3.8 | 3.7 | 3.5 | 3.4 | 3.4 | 3.3 | 3.4 | 4.0 |
| tryptophan[d] | 1.0 | 0.9 | 0.8 | 0.7 | 0.7 | 0.7 | 0.7 | 0.6 | 0.6 | 1.0 |
| $NH_3$ | 1.2 | 1.3 | 1.3 | 1.3 | 1.7 | 1.7 | 1.8 | 1.9 | 2.1 | 1.3 |
| lysine | 19.0 | 19.1 | 18.9 | 18.7 | 18.3 | 18.3 | 18.1 | 18.0 | 16.8 | 18.9 |
| histidine | 3.0 | 2.9 | 2.9 | 2.9 | 2.8 | 2.7 | 2.5 | 2.2 | 2.2 | 2.8 |
| arginine | 2.0 | 2.0 | 2.0 | 1.9 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 2.0 |
| total[e] | 104.0 | 103.0 | 101.0 | 98.4 | 95.8 | 94.6 | 93.6 | 90.7 | 88.5 | 103.3 |

[a] Cyt *c* (100 nmol) was hydrolyzed in 6 N HCl, and its amino acids were analyzed using a HITACHI 655A-11 high-performance liquid chromatograph equipped with a no. 2619F column. [b] According to ref *24.* [c] According to ref *26.* [d] According to ref *27.* [e] Excluding $NH_3$. [f] Cyt *c* containing *tert*-BuOH was irradiated at 1.0 kGy.

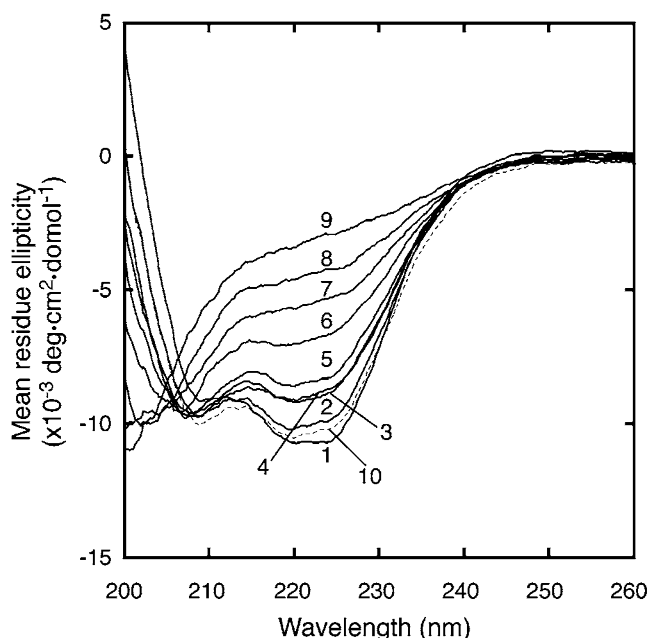


**Figure 4.** CD spectra of $\gamma$-irradiated Cyt *c*. The spectra were recorded in 10 mM sodium phosphate (pH 7.0) at 25 °C. Lane 1, unirradiated; 2, 0.25 kGy; 3, 0.5 kGy; 4, 0.75 kGy; 5, 1.0 kGy; 6, 1.5 kGy; 7, 2.0 kGy; 8, 2.5 kGy; 9, 3.0 kGy; and 10, Cyt *c* containing *tert*-BuOH irradiated at 1.0 kGy.

*c*, and the difference indicated that the α-helices of Cyt *c* were unfolded (*5*, *34*). From these, we presumed that the α-helices of Cyt *c* were unfolded by $\gamma$-irradiation as well as by other denaturation methods.

Amino acid analysis was performed to investigate the amino acid changes in Cyt *c* due to $\gamma$-irradiation. The contents of the 17 kinds of amino acids of Cyt *c* decreased with increasing irradiation dose, and the total residual amino acid (%) of Cyt *c* irradiated at 3.0 kGy was approximately 85% of that of unirradiated Cyt *c* (**Table 2**). Among the amino acids in **Table 2**, phenylalanine (Phe), tyrosine (Tyr), and methionine (Met) contents tended to decrease at irradiation doses above 0.25 kGy, and the residual Phe, Tyr, and Met contents were approximately 85, 23, and 13%, respectively, of those of unirradiated sample. Cyt *c* (0.1% solution) was irradiated in the dose range of 1−500 kGy to investigate the effect on the contents of 17 amino acids. It was found that cystine, Met, histidine, Phe, Tyr, and threonine are most susceptible to radiation damage up to 10 kGy. On the other hand, aspartic acid, glutamic acid, lysine, and alanine were among the most stable (*31*). These results were similar to those of hemoproteins such as Hb and Mb (*12*, *35*).

The Phe and Tyr contents of Cyt *c* gradually decreased above 0.25 kGy and leveled off at a constant value at 1.0 kGy, and the residual Phe and Tyr contents were 47 and 16%, respectively, of those of unirradiated Cyt *c*. On the other hand, 3,4-dihydroxyphenylalanine (dopa) was formed above 0.25 kGy. Dopa formation was 2.8 residues at 1.0 kGy. The Phe and Tyr contents decreased to 0.5 and 2.8 residues/molecule, respectively, as compared with those of unirradiated Cyt *c*, and dopa formation rates were about the same even with the decrease in Phe and Tyr contents (**Figure 5**). It was considered that dopa was formed from the oxidation of Phe to Tyr or the direct oxidation of Tyr. Dopa is formed via two pathways: Phe converts first to Tyr, and the Tyr formed further converts to dopa upon OH radical production by water radiolysis, as shown by Garrison (*36*). Hydroxyl radicals react with aromatic and heterocyclic amino acids, and they were found to be most efficient in inactivating enzymes. Kume et al. (*8*) reported that the irradiation of glucose isomerase (79 $\mu$g/mL) from *S. phaeochromogens* with up to 0.15 kGy, and OH radicals are effective in inactivating glucose isomerase. The activity of this enzyme irradiated at 0.15 kGy was one-half that of the unirradiated one. Another study shows the irradiation of lysozyme (7 $\mu$M) from *Micrococcus lysodeikticus* below 0.05 kGy, and the reaction of OH radicals with tryptophan 108 in lysozyme, causing its activity to become one-fifth that of the unirradiated sample (*37*). Equine heart Cyt *c* has five α-helices, and in this Cyt *c*, Phe10 and Tyr67, 74, and 97 compose helices I, III, IV, and V, respectively (*38*, PDB code: 1HRC). The

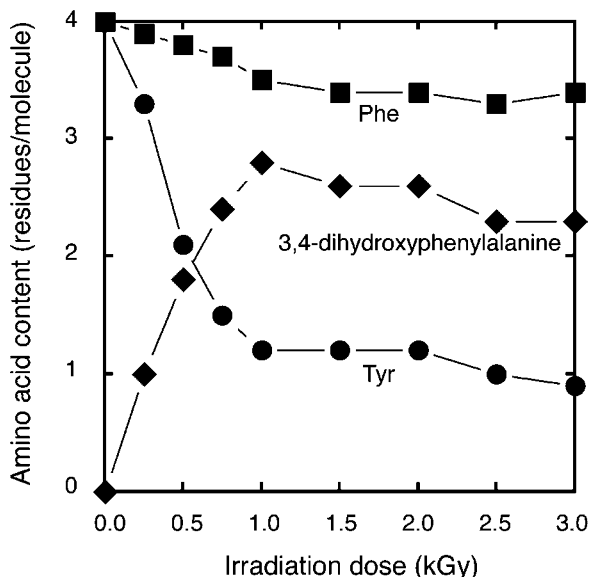


**Figure 5.** Effect of irradiation dose on Tyr, Phe, and 3,4-dihydroxyphenylalanine content of Cyt *c*. Amino acids were hydrolyzed in 6 N HCl.

values of Phe and Tyr were 1.66 and 0.74, respectively, in conformational preferences of amino acids for the helix by Williams and co-workers (*39*). Phe has a tendency to compose the α-helix as compared with Tyr. From the paper, it was considered that Tyr has a tendency to compose the α-helix as compared with dopa. To investigate the effect of OH radicals produced by water radiolysis on Phe, Tyr, and α-helix contents, Cyt *c* containing *tert*-BuOH ($5 \times 10^{-2}$ M) as an OH radical scavenger was irradiated. In Cyt *c* irradiated under this condition, the Phe and Tyr contents were both four residues/molecule, and these results were similar to those of the unirradiated one as determined by amino acid analysis (**Table 1**). The α-helical content (33.2%) of irradiated Cyt *c* containing *tert*-BuOH was almost the same as that of the unirradiated sample (34.3%) as determined by CD spectra (**Figure 4**). On the other hand, the results for the irradiated Cyt *c* containing *tert*-BuOH were different from those for the irradiated Cyt *c* without *tert*-BuOH (Phe content, 3.5 residues/molecule; Tyr content, 1.2 residues/molecule; and α-helical content, 26.2%) (**Table 1** and **Figure 4**). The $k_{cat}$ value of irradiated Cyt *c* containing *tert*-BuOH was $15.6 \times 10^{-3}$ $s^{-1}$. This value was consistent with that of the unirradiated Cyt *c* ($k_{cat}$: $13.3 \times 10^{-3}$ $s^{-1}$) and approximately 1/40 of that of the irradiated Cyt *c* without *tert*-BuOH ($k_{cat}$: 0.669 $s^{-1}$). From these results, it was concluded that the Cyt *c* NiR was induced because the helices of Cyt *c* were unfolded by the conversion of Phe to Tyr and dopa.

Equine heart Cyt *c* has two Met residues (Met 65 and 80), and Met 80 is the sixth heme axial ligand (*40*). The Met content of Cyt *c* gradually decreased as irradiation dose increased from 0.5 kGy, and the residual Met content was approximately 15% of that of the unirradiated sample at 3.0 kGy. Methionine sulfoxide (Met═O) formed with decreasing Met content, and the Mets were approximately oxidized to Met═O at 3.0 kGy (**Figure 6A**). Met (0.05 M) was irradiated, and oxidation reactions on the sulfur atom led to the synthesis of Met═O, which on further oxidation formed methionine sulfone at $6.72 \times 10^{20}$ eV/mL (108 kGy), which in turn formed homocysteic acid at $7.68 \times 10^{20}$ eV/mL (123 kGy) (*41*). In our study, it was assumed that methionine sulfone and homocysteic acid were not formed, because the protein concentration and irradiation dose used were approximately one-half and 1/40, respectively, of the dose used in previous studies. It is evident that Met═O

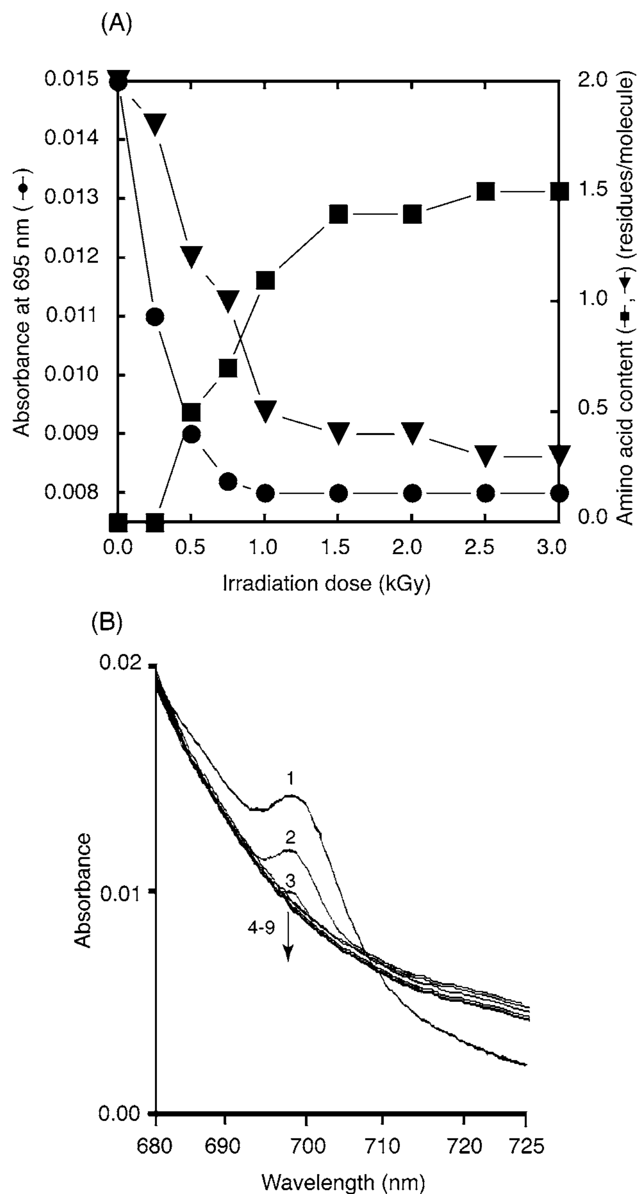


**Figure 6.** Effect of irradiation dose on Met content of Cyt *c*. (**A**) Decomposition of Met (▼), formation of methionine sulfoxide (■), and plots of absorbance at 695 nm of oxidized forms of Cyt *c* (●). (**B**) Absorption spectra at 695 nm of oxidized forms of γ-irradiated Cyt *c*. Line 1, unirradiated; 2, 0.25 kGy; 3, 0.5 kGy; 4, 0.75 kGy; 5, 1.0 kGy; 6, 1.5 kGy; 7, 2.0 kGy; 8, 2.5 kGy; and 9, 3.0 kGy.

formation from Met occurred in the presence of OH radical produced by water radiolysis, because the oxidation from Met to Met═O was not observed in the irradiated Cyt *c* containing *tert*-BuOH (**Figure 1**).

In the visible spectra, a peak at 695 nm indicated the characteristics of the coordination between methionine sulfur and heme iron in the oxidized forms of Cyt *c*. The disappearance of this peak showed that the methionine ligand dissociated from the heme of Cyt *c* (*42*). The absorbance at 695 nm for the oxidized form of irradiated Cyt *c* gradually decreased with increasing irradiation doses above 0.25 kGy and completely disappeared at 1.0 kGy (**Figure 6B**). The peak at 695 nm of the equine heart Cyt *c* treated at 100 °C for 30 min and *Hydrogenobacter thermophilus* Cyt $c_{552}$ treated at 85 °C for 15 min completely disappeared, and the methionine ligand dissociated from the heme iron of both Cyts *c* (*5*, *43*). The peak at 695 nm of Cyt *c* (400 μM) reacted with chloramine-T (804 μM)

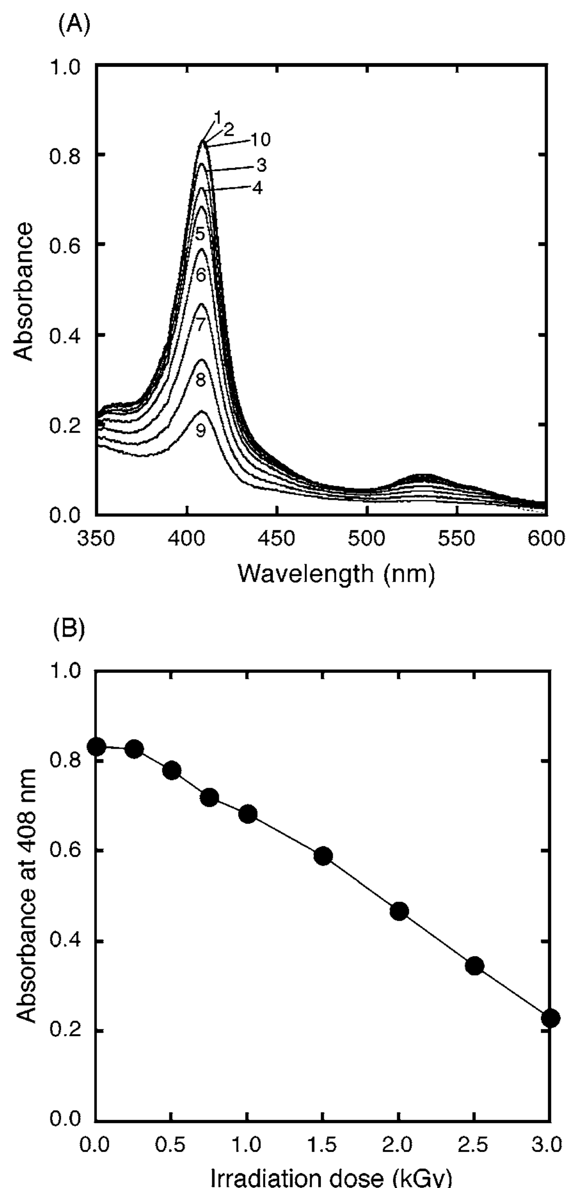


**Figure 7.** UV/visible absorption spectra of oxidized forms of $\gamma$-irradiated Cyt *c* (**A**). Lines 1, unirradiated; 2, 0.25 kGy; 3, 0.5 kGy; 4, 0.75 kGy; 5, 1.0 kGy; 6, 1.5 kGy; 7, 2.0 kGy; 8, 2.5 kGy; 9, 3.0 kGy; and 10, Cyt *c* containing *tert*-BuOH irradiated at 1.0 kGy, and plots of absorbance at 408 nm of oxidized forms of Cyt *c* (**B**).

with modifications of Met to Met=O, and the methionine ligand dissociated from the heme iron of Cyt *c* due to oxidation of Met to Met=O (*44*). From these papers, it has become apparent that the Met 80 axial ligand dissociated from the heme iron due to oxidation of Met to Met=O upon $\gamma$-irradiation. It is conceivable that the NiR of Cyt *c* irradiated at 1.0 kGy is maximum in this study, because the Met ligand completely dissociated from the heme iron of Cyt *c* irradiated at 1.0 kGy.

A study has shown that the Soret absorbance at 408 nm of Cyt *c* (0.2 mg/mL) decreases upon treatment with hydrogen peroxide ($1.5 \times 10^{-2}$ M), and OH radicals produced by hydrogen peroxide attack the α-methene bridge of the porphyrin ring, leading to the opening of the heme porphyrin ring (*45*). To investigate heme destruction, the UV/visible spectra, and the Soret absorbance at 408 nm of oxidized Cyt *c* gradually decreased as irradiation dose increased above 0.25 kGy, and this peak of Cyt *c* irradiated at 3.0 kGy was approximately 20% as compared with that of the unirradiated Cyt *c* (**Figure 7**). The Soret absorbance at 408 nm of Cyt *c* containing $5 \times 10^{-2}$ M *tert*-BuOH as an OH radical scavenger did not change as compared with unirradiated Cyt *c*. This means that the heme destruction of Cyt *c* occurred due to OH radicals produced by water radiolysis.

**Comparison of Irradiated Cyt *c* with Heat-Treated Cyt *c*.** The NiR of Cyt *c* irradiated at 1.0 kGy was measured under anaerobic conditions, and it was observed that the $NO_2^-$ was completely reduced to $NH_4^+$. This reaction was the same as that of the ferredoxin−nitrite reductase from *Chlorella fusca*, *Porphyra yezoensis* (*46*), and spinach (*47*) and Cyt *c* treated at 100 °C for 30 min (*5*).

The $k_{cat}$ values of irradiated and unirradiated Cyt *c* were 0.669 and $13.3 \times 10^{-3}$, respectively, and the activity of irradiated Cyt *c* was approximately 45-fold that of unirradiated Cyt *c*. The NiR of irradiated Cyt *c* was approximately 10-fold that of Cyt *c* treated at 100 °C for 30 min ($k_{cat}$value was $71.3 \times 10^{-3}$) (*5*), and this activity was similar to that of the nitrite reductase from *Escherichia coli* ($k_{cat}$ value was 0.570) (*48*). From these results, it is apparent that the irradiated Cyt *c* shows a high NiR.

The irradiated Cyt *c* had an optimum pH of 7.0 (**Figure 8A**), which is close to those of the nitrite reductase from *Porphyra yezoensis* and *Chlorella fusca* (pH 7.5) (*45*, *46*, *49*) and is the same as that of Cyt *c* treated at 100 °C for 30 min (pH 7.0) (*5*).

To examine the autoxidation of irradiated Cyt *c*, visible spectra were monitored after the addition of dithionite to irradiated Cyt *c*. The absorption maxima at 550 (α peak) and 520 ($\beta$ peak) nm descreased with time and completely disappeared at 90 min, the absorbance at 412 (Soret) nm shifted to 408 nm with time, and the peak showed a change from the reduced form to the oxidized form at 90 min (**Figure 8B**). This spectral change suggested that irradiated Cyt *c* is autoxidizable, and this result is similar to those of the Cyt *c* treated at 100 °C for 30 min (*5*) and the M80A Cyt *c* mutant (the sixth ligand (M80) was replaced by alanine), which do not have six coordination (*50*).

It has been suggested that the redox potential of Cyt *c* resulted in one of an index of denaturation, and the potential was decreased with denaturing (*51*). For example, the redox potential of Cyt *c* was 260 mV (*40*, *52*), but the potential of Cyt *c* treated at 100 °C for 30 min was 0 mV (*5*). Ferri et al. reported that the potential of Cyt *c* treated with guanidine hydrochloride (6 M) as a denaturant was −10 mV (*53*). The degree and mechanism of denaturation of Cyt *c* by $\gamma$-irradiation will naturally differ from that of Cyt *c* by heat and detergent treatment. The redox potential of irradiated Cyt *c* was −77 mV (data not shown). As shown in amino acids analysis (**Table 2**) and CD spectra (**Figure 4**) of preceding section, the helices of irradiated Cyt *c* were unfolded by the conversion of Phe to Tyr and dopa. As shown in amino acids analysis (**Table 2**) and visible spectra (**Figure 6B**) of preceding section, the Met 80 axial ligand dissociated from the heme iron due to oxidation of Met to Met=O by $\gamma$-irradiation. From these results, we assumed that the redox potential of Cyt *c* would be decreased with exposure of the heme in Cyt *c* to solvent by irradiation.

**Immobilized Irradiated Cyt *c* and Its NiR, Stability, and Reuse.** Generally, proteins and enzymes are unstable at high temperatures and their recovery after a reaction is difficult. We tried to improve the reusability and stability of irradiated Cyt *c* and thus prepared irradiated Cyt *c* immobilized in CNBr-sepharose or acrylamide. The NiR of these immobilized irradiated Cyt *c* were then measured. The NiR of CNBr-sepharose-immobilized irradiated Cyt *c* was double that of acrylamide-immobilized irradiated Cyt *c*, and CNBr-sepharose-

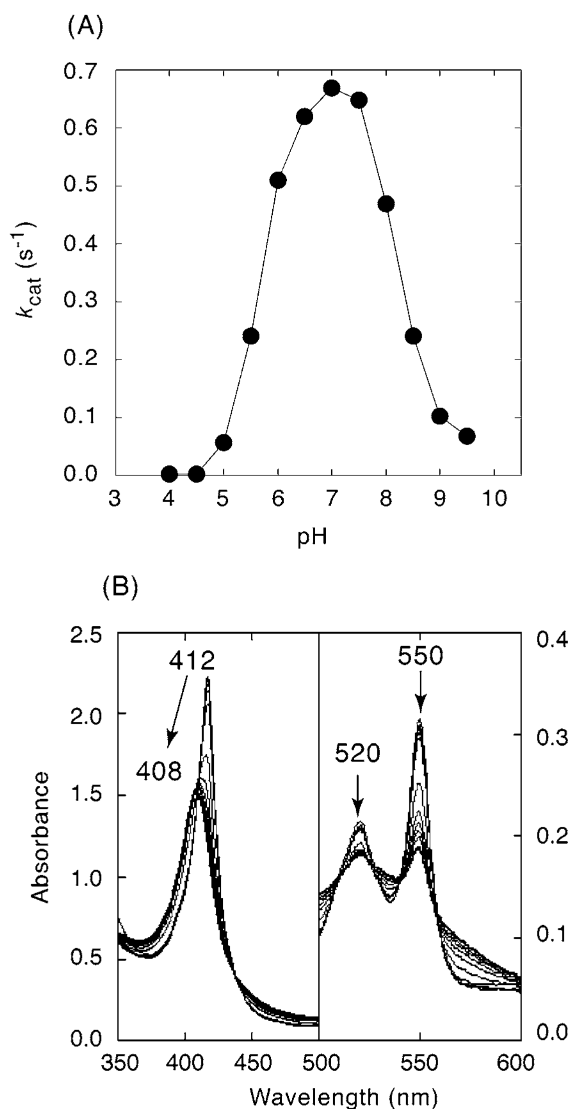


**Figure 8.** Physicochemical properties of irradiated Cyt *c*. (**A**) pH profile of the nitrite reduction by irradiated Cyt *c*. Each pH was maintained with sodium citrate (pH 4–5), sodium phosphate (pH 6–7), Tris-chloride (pH 8), and glycine–NaOH (pH 9–9.5). (**B**) Spectral change of irradiated Cyt *c* due to autoxidation.

immobilized irradiated Cyt *c* had nearly the same activity as the soluble protein (**Figure 9**). The heme in CNBr-sepharose-immobilized irradiated Cyt *c* was exposed to a solvent; however, in the case of the acrylamide-immobilized irradiated Cyt *c*, the irradiated Cyt *c* is surrounded by a matrix. It was presumed that the NiR of CNBr-sepharose-immobilized irradiated Cyt *c* is higher than that of acrylamide-immobilized irradiated Cyt *c*. The NiR of irradiated Cyt *c* immobilized in CNBr-sepharose was approximately 10 times that of the heat-treated Cyt *c* immobilized in CNBr-sepharose. The residual activities of CNBr-sepharose and acrylamide-immobilized irradiated Cyts *c* were 99.2 and 99.3%, respectively, even after storage at 4 °C for 1 year (**Table 3**). The CNBr-sepharose-immobilized irradiated Cyt *c* after the fifth use had the same NiR as that of fresh samples (**Table 4**).

In conclusion, irradiated Cyt *c* showed almost the same NiR as nitrite reductase. Irradiated Cyt *c* demonstrated improved stability, with long-term storage achieved by immobilization. Irradiated Cyt *c* may thus be a new molecule for simple applications such as the determination of $NO_2^-$ in food and in an aqueous solution and will be able to be used as a substitute for nitrite reductase.

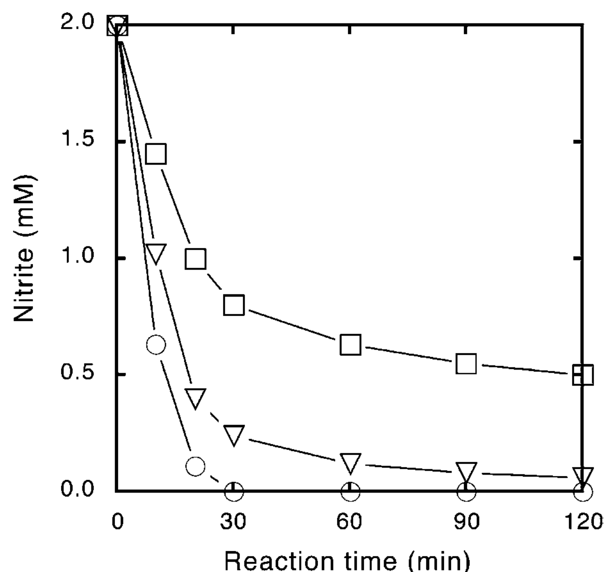


**Figure 9.** Nitrite reducing activity of immobilized Cyt *c*. Free Cyt *c* (○); sepharose-immobilized (▽); acrylamide-immobilized (□).

**Table 3.** Effect of Storage Time on the Residual Nitrite Reducing Activity of Immobilized Cyt *c*

| | residual activity (%) | |
|---|---|---|
| storage duration at 4 °C | CNBr-sepharose | acrylamide |
| none | 100 | 100 |
| 1 week | 99.9 | 99.8 |
| 1 month | 99.6 | 99.5 |
| 1 year | 99.2 | 99.3 |

**Table 4.** Effect of Reuse on the Residual Nitrite Reducing Activity of Immobilized Cyt *c*

| | residual activity (%) | |
|---|---|---|
| reuse | CNBr-sepharose | acrylamide |
| 1st | 100 | 100 |
| 2nd | 99.8 | 84.9 |
| 3rd | 99.5 | 72.3 |
| 5th | 99.3 | 51.2 |

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