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SYNTHETIC CYSTEINE-PEPTIDE AND ITS RELATION WITH HEAVY AND RADIOACTIVE METALS

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The development of radio protective agents with lower toxicity and an extended window of protection has attracted much attention. Glutathione and a new synthesized decapeptide (P10) were used to investigate the protective effect of cysteine peptides. Besides, P10 was characterized by mass spectrometry, atomic force microscopy and circular dichroism spectroscopy. Then, the accumulation and the effect of radioactive mercury on wheat seedlings (*Triticum aestivum L.*) were determined in the course of germination. The radioactive mercury accumulated especially in roots and less in shoots; sugars seem to be the most important in the $^{203}Hg^{2+}$ accumulation. Glutathione diminished the radiotoxic effect of ^{203}Hg as shown by the shoot weight and length of the lots (1.79 g and 310.2 cm in the case of glutathione and ^{203}Hg *versus* 1.56 g and 249.0 cm for the ^{203}Hg treatment). Cystine had also a powerful protective effect, whereas the synthetic decapeptide proved to be harmful in wheat germination experiments.

INTRODUCTION

Toxic mercury exposure can occur from industrial effluents, environmental contamination, occupational exposure, volcanic gasses, and from emissions by coal fired power plants. If excessive exposure is avoided, the human body will efficiently and continuously eliminate mercury and other toxins in the urine, feces, hair, sweat, nails and skin.[1,2] Exposure of cellular peptides to toxic and essential metals is thought to be reflected by the formation of the corresponding metal ion-peptide complexes.[3,4] Many cases related to biological molecules involving proteins and multiprotein systems, in which metals frequently participate, are described.[5] Consequently, a generic metallomics analytical approach is proposed for the identification and quantification of metalloproteins. Therefore, various histidine- or cysteine-containing oligopeptides have been synthesized and metal complexes studied by mass spectrometry (MS), potentiometric, UV-VIS, UV-VIS circular dichroism (CD), and Electron Paramagnetic Resonance (EPR) spectroscopic techniques in solution.[6] The effects of mercury on plants in the presence of thiol-peptides and cystine, the tolerance and the accumulation of mercury (non-radioactive and/or radioactive) in some crops were extensively investigated.[7-11]

A quantitative analysis of protein secondary structure of the mercury-protein complexes showed that the Hg(II) ion coordination was through protein C=O and C-N groups with major Hg-sulfur binding.[12] CD spectra of proteins are sensitive to protein secondary structure. In a previous study[13] we tested the effect of the radioactive and non-radioactive mercury on wheat germination. In our tests, the radioactive solutions were obtained by labeling a non-radioactive solution of $HgCl_2$ with a high-active solution of $^{203}HgCl_2$. During the reviewing process it appeared the question whether the radiotoxicity of $^{203}Hg^{2+}$ is an independent factor or it does represent an additional term to the toxic effect of the heavy metal mercury.

This was the incentive of the present study, in which we tested the behavior of the wheat germination in the as-made $^{203}HgCl_2$ solution at a lower concentration. In this respect, a decapeptide (P10) was synthesized and thoroughly investigated by CD, MS and atomic force microscopy (AFM) techniques in order to understand the relationship between the sequence as well as the secondary

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structure of thiol-peptides and their biological activity. Those results, together with the effect of some cysteine containing peptides are investigated in the present paper. Besides, the thin-layer radio-chromatography was used in order to determine the biochemical components involved in the accumulation of the radioactive mercury.

RESULTS AND DISCUSSION

1. Peptide synthesis

P10 was easily synthesized according to the Fmoc strategy of solid phase peptide synthesis with a high yield and purity (Figure 1).[16] However, further purification by RP-HPLC was necessary to obtain the pure P10 with the expected $[M+H]^+$ at m/z 1379.20. With an error of $\Delta M = 0.28$ mass units and a resolution of 1378.48/0.28 = 4923, the MALDI TOF MS technique proved to be suitable to confirm the primary structure of this oligopeptide.

2. Conformational studies by CD

Circular dichroism spectra in the UV region can be used to monitor changes in the main-chain conformation (Figure 2). CD spectrum of pure decapeptide showed a high degree of ordering (86.9% β-sheet and only 13.1% random coil conformers). The appearance of a positive CD contribution at 220 nm and a negative one at 195 nm are characteristics of the secondary structure of P10 and indicate increased ordering of the main chain. Two other negative contributions were observed at 187 nm and 190 nm, respectively. On adding mercury ions in 1:1 molar ratio, β-sheet populations decreased down to 14.2%, whereas the proportion of random coil conformers became 56.2%. The addition of Ni^{2+} caused a shift of the negative CD band from 195 nm to 194 nm and a drastical increase of its intensity. Besides, the positive maximum of absorption at 220 nm, which is a characteristic of β-sheet structures, was found to be shifted toward 225 nm. The relatively large proportion of β-turn conformers (29.6%) might be related to metal ion binding to the peptide molecule, which results in the formation of metal complexes surrounded by a single molecule of peptide. P10 had also a structural transition associated with the formation of the Ni^{2+} complex, with conversion from β-sheet (86.9%) to an extended β turn-like conformation (39.4%). In addition, the proportion of random coil conformers increased from 13.1% up to 60.6%. We supposed that the Ni^{2+} binding to P10 results in the formation of β-turn structures due to its binding to both SH-group and histidine residues.

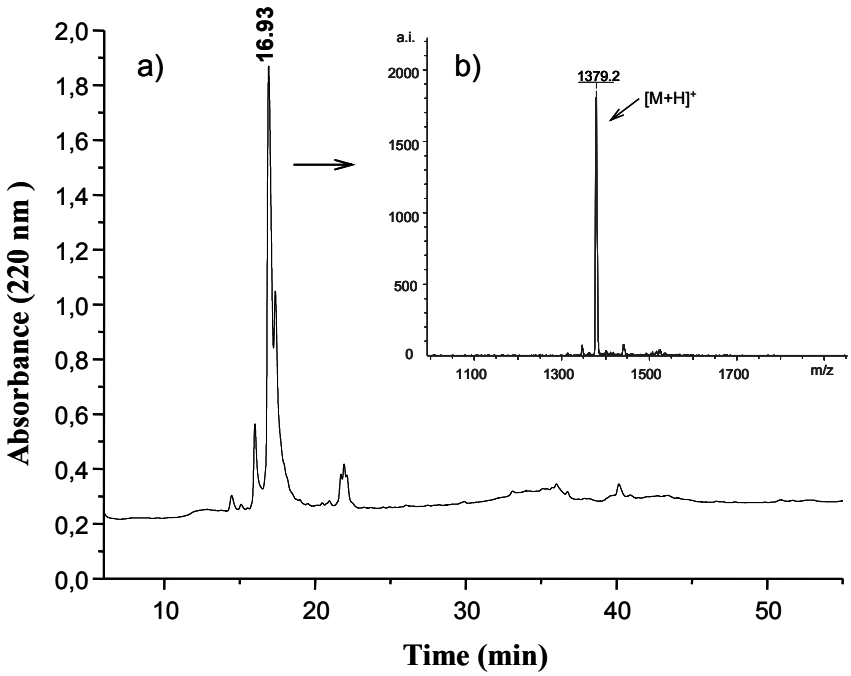

Fig. 1 – RP-HPLC spectrum of the purified P10 (a) as well as its MALDI TOF MS spectrum (b), which show both the molecular weight and the purity of the peptide.

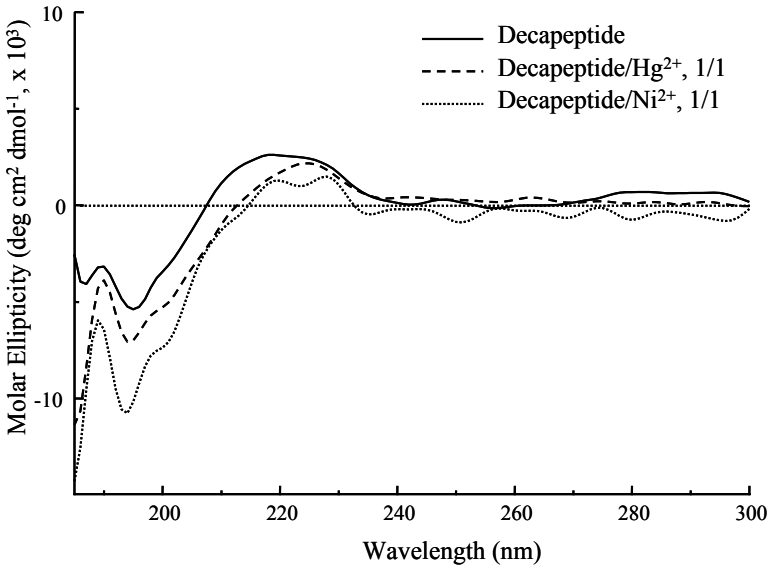

Fig. 2 – Effect of Hg^{2+} and Ni^{2+} ions on the UV CD spectrum of P10.

The very high proportion of β-sheet conformers under the solid state was explained by the ionic bonds between the carboxyl group at the C-terminal chain end and the NH_3^+ groups at the N-terminal one (Figure 3). In addition, many other hydrogen bonds are possible between OH groups of tyrosine and the electron-rich nitrogen atom in the histidine moiety. Therefore, an anti-parallel orientation of the peptide molecules is favored. In aqueous solutions, all these highly hydrophilic groups are surrounded by water molecules, resulting in the rapid increase in the proportion of unordered conformers.

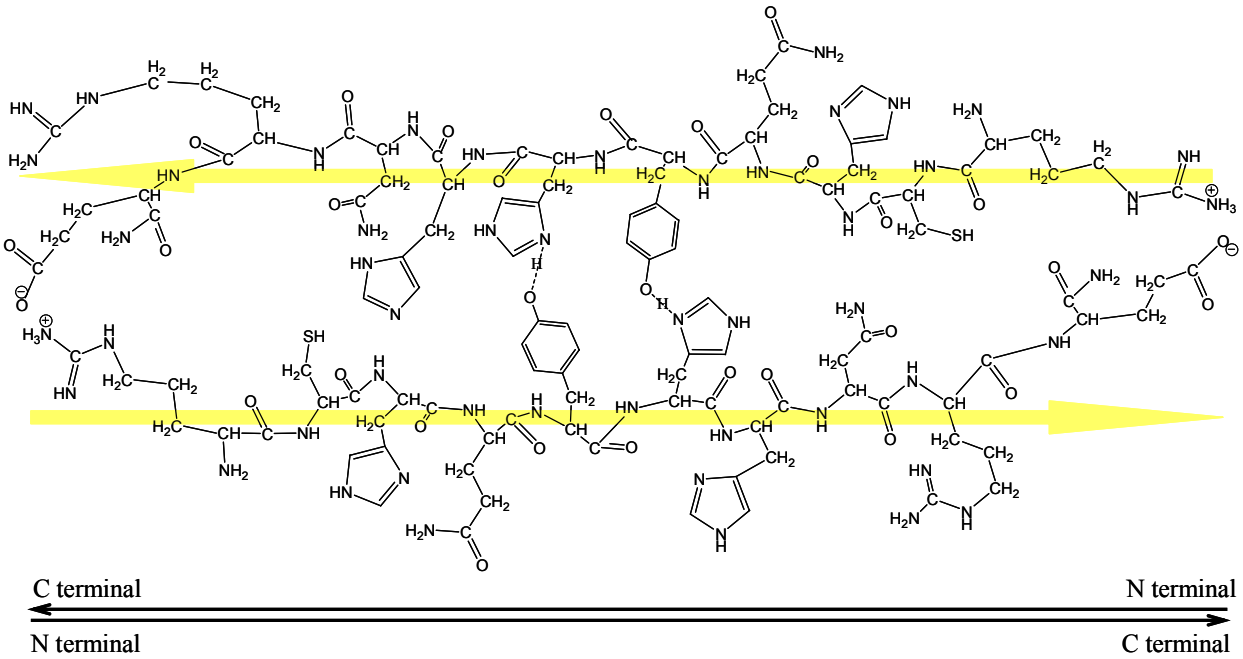

Fig. 3 – The formation of β-sheet conformers of P10 under solid state.
The top chain is left side C-terminal; the bottom one is with C-terminal to the right.

Mercury binding to P10 was favored by SH group as shown previously in the case of glutathione.[13] However, the carboxyl groups may also bind mercury ions although these ions had a great tendency to form -S-Hg-S- bridges between two peptide molecules. CD data were concordant to all these structures, showing that β-sheet structures turned to β-turn and unordered ones. The

high toxicity of P10 and its mercury complex could therefore be correlated to a conformational shift and not to the radioactivity of mercury. To confirm the hypothesis that β-structures are responsible for the toxicity, further research is necessary in which experiments with this peptide and heavy and radioactive metals should be done.

3. AFM studies

Atomic force microscopy produced faithful high-resolution images of peptide films in the aqueous environment and showed the stark contrast between the morphological properties of P10 under various environments (Figure 4). The bidimensional micrographs of pure P10 proved to be very simple and gave little information on the peptide film layered on the glass plate. On the contrary, the tridimensional micrographs showed complex nanostructures characteristic to β-sheet rich proteins. These ones display numerous small sharp microhills on the protein surface.

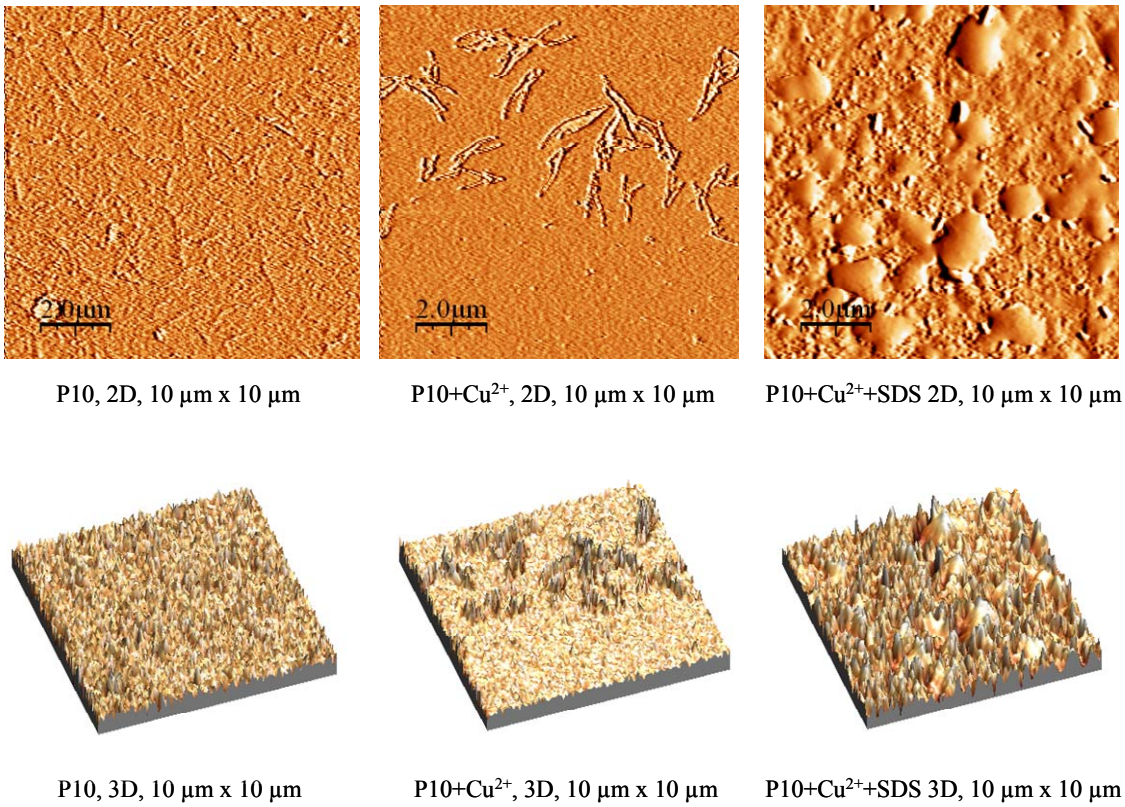

Fig. 4 – Bi- and tridimensional AFM images of pure P10, Cu^{2+}-P10 complex in water and in the presence of sodium dodecylsulfate (SDS).

On adding metal ions such as copper ones, the image drastically changes to display large nanostructures that are characteristic to non-covalent polymer forms. In the presence of sodium dodecylsulfate (SDS), all the polymer complexes turn to monomolecular complexes with metals, as shown by the corresponding change of AFM images. Therefore, our results showed that the self-assembled nanostructures of the P10 molecules in aqueous solution were significantly influenced by metal ions, although they are related to the size and the sequence of these peptides. Some parameters were calculated to quantify the differences between the three images of P10. Thus, root mean square roughness for the image of pure P10 was 0.148 nm and the average height 0.347 nm, whereas the same parameters of copper-decapeptide complex were 0.322 nm and 0.233 nm, respectively. Maximum height of copper-peptide nanostructures was 1.916 nm. On adding SDS, a quite different image was obtained, which was characterized by a root mean square roughness of 0.298 nm, an average height of 0.753 nm, and a maximum height of 1.446 nm, respectively. The images of P10 suggested its tendency of aggregation to form oligomers, which can be highly toxic to the living cells. AFM

images of P10 supported and extended the features of self-aggregates observed by CD.

4. The biological experiments

The number of seedlings in a given lot were not influenced by the treatment with 10^{-4} M solutions mercury(II) chloride (Table 1). The addition of glutathione or cystine to the both forms of mercury resulted in increasing the number of germinated seeds.

The slight stimulatory effect of radioactive mercury could be related to the non-specific stress against which the living organism reacts in order to adapt. However, the decapeptide used here proved to have an inhibitory effect on wheat germination, which was independent of the mercury treatment.

Table 1

The effect of a 10^{-4} M solution of radioactive mercury on wheat germination, in the presence or absence of glutathione and P10 (10^{-3} M; six independent experiments; thirty three seeds in a lot)

Treatment	Plantlets in the lot	Total height of the shoots (nm)	Weight of the stems and leaves (g)	Root weight of the lot (g)
H_2O (Blank)	31± 3	197.9 ± 19.5	1.38 ± 0.17	1.17 ± 0.12
Hg^{2+}	29 ± 4	183.0 ± 22.1	1.26 ± 0.19	1.18 ± 0.14
$^{203}Hg^{2+}$	31 ± 3	194.9 ± 24.4	1.30 ± 0.16	1.29 ± 0.16
$^{203}Hg^{2+}$ + Decapeptide	29 ± 3	93.4 ± 10.7	1.07 ± 0.14	1.21 ± 0.15
$^{203}Hg^{2+}$ + Glutathione	31 ± 3	199.7 ± 26.1	1.32 ± 0.23	1.35 ± 0.17
D; s_x	4.9; 1.2	21.4; 4.7	0.14; 0.04	0.22; 0.05

The non-radioactive mercury moderately decreased the percentage of vigorous plantlets, whereas the radioactive form drastically reduced the percent of plantlets up to 78%. Glutathione treatment resulted in a low increase in the number of plantlets grown up on a radioactive solution. The highest number of plantlets was counted in the cystine-treated lots, where only 2% of the seeds did not germinate and only 1% of them germinated without growing.

All of the treatments decreased the total height of the plantlets in a lot as compared to the control (402.4 nm). However, the radioactive mercury displayed the most harmful effect on the seeds; it reduced significantly both the weight and the length of the resulted plantlets as compared to the blank and the non-radioactive mercury, respectively. Glutathione reduced the radiotoxic effect of ^{203}Hg as shown from the shoot weight and length (1.79 g and 310.2 nm in the case of glutathione and ^{203}Hg *versus* 1.56 g and 249.0 nm for the ^{203}Hg treatment). The weight of the roots also increased on adding glutathione to the radioactive solution (1.54 g *versus* 1.21 g). Surprisingly, cystine had a more powerful protective effect as compared to glutathione.

5. Radioactivity in plants

The present experiment re-confirms the root tropism of radiomercury.[13] The not-extracted ^{203}Hg was associated with some other biochemical fractions presented in plantlets, but not separated by the method we used (*e.g.*, proteins, inorganic compounds, etc.).

$^{203}Hg^{2+}$ was accumulated especially in roots and less in the leaves and stems (Table 2). The decapeptide provoked a substantial increase in the radioactivity of the roots due to the retention of $^{203}Hg^{2+}$ at this level. However, a 26% increase in the radioactive content of the roots cannot be the cause for the observed toxicity. Although the decapeptide retained the Hg^{2+} in roots, the experimental data suggested that the decapeptide might be toxic by itself. Moreover, the decapeptide was associated with a less amount of radioactive mercury in shoots. The low concentration of $^{203}Hg^{2+}$ in the treatment solutions was unable to generate toxic effects. We presumed that the decapeptide entered the seeds and then the roots and accumulated in the latter ones. It bound the Hg(II) ions except for a small part of them, which migrated towards the shoots.

On the other hand, glutathione protected roots from entering the $^{203}Hg^{2+}$, till once entered they displayed a similar distribution pattern between roots and leaves to that of the treatment with mercury only (22% of the total mercury was found in shoots *vs.* 26% in the absence of glutathione). A ten times increase in the treatment concentration of $^{203}HgCl_2$ resulted in only two times enhancement of the radioactivity in roots. A protection mechanism against mercury that hinders the

accumulation of high amounts of heavy metal ions could be suspected in wheat. Cystine had no protective action against mercury entering, which accumulated both in roots and in leaves. Although cystine cannot react with $^{203}Hg^{2+}$ due to its disulfide bridge (as glutathione and decapeptide do), it protected tissues from the radiation damage probably by capturing the new generated radicals. Thus, a 37% increase in the root radioactivity had no effect on root growth. The experiments with cysteine containing compounds showed that they could have a protective effect against radiotoxicity if they are tolerated by the mercury-contaminated organisms; in the present case, the decapeptide was less tolerated.

Table 2

Distribution of radioactive mercury in the stems and roots of seven-day seedlings in the presence or absence of glutathione, cysteine-containing decapeptide, and cystine (six independent measurements)

Experiment	Treatment	Activity (Bq)		Specific activity (Bq/g)	
		Roots	Shoots	Roots	Shoots
No. 1	$^{203}HgCl_2$, 10^{-4} M	10892±1281	3938±633	8422±943	3007±520
	$^{203}HgCl_2$, 10^{-4} M + Decapeptide	13023±1416	2091±309	10621±1175	1954±290
	$^{203}HgCl_2$, 10^{-4} M + Glutathione	7105±765	2089±313	5293±567	1496±229
No. 2	$^{203}HgCl_2$, 10^{-3} M	20937±2008	8643±947	16585±1651	4991±561
	$^{203}HgCl_2$, 10^{-3} M + Glutathione	16182±2007	4762±507	10847±1354	2764±294
	$^{203}HgCl_2$, 10^{-3} M + Cystine	28859±2409	8715±789	14503±1244	4502±440

EXPERIMENTAL

1. Materials

Most of the reagents, biological material, and instruments were used as previously.[13,14] In addition, sampling procedure, treatment solutions, germination tests, and statistics were similar to those described in.[13,15] NovaSyn TGR resin was purchased from Novabiochem. Protected amino acids for peptide synthesis were provided by GL Biochem (Shanghai, China). All solvents for peptide synthesis were commercially analytical grade and were redistilled before use. L-cystine was purchased from Merck.

2. Methods

The decapeptide with the sequence NH_2-RCHQYHHNRE-$CONH_2$ was prepared manually by the classic Fmoc methodology using the TGR resin, as previously shown.[16] A BIO-RAD Model 2700Elite RP-HPLC system (Bio-Rad Laboratories GmbH, Germany), using a Spherisorb (ODS) C_{18} reversed-phase analytic column, was used for the purification of the decapeptide.

The MALDI-TOF mass spectrometer (Finnigan MAT LASERMAT 2000GTI) equipped with a nitrogen UV laser (λ_{max} = 337 nm) was used for all measurements. An external calibration was carried out using the average masses of single-proton ion signals of some known peptides.

CD spectra were recorded using a Spectropolarimeter (Labor und Datentechnik GmbH, Germany) equipped with a thermal circulator accessory and a spectrometer software (Dr. Jörg Nerkamp). The measurements were carried out at 25.0 ± 0.2 °C.

Images were taken at room temperature (22 ˚C) on a SPM Solver PRO-M AFM (NT-MTD Co. Zelenograd, Moscow, Russia) in tapping mode. This mode was used in order to reduce sample surface distortion due to tip and sample interactions. All images were acquired using a High Resolution Noncontact "Golden" Silicon NSG10/Au/50 cantilever with Au conductive coating. Image analysis was done with scanning probe microscopy software, WSxM 4.0 Develop 10.0 (Nanotec Electronica S.L.).

In order to obtain additional information about the mechanism of the accumulation process, a thin-layer method based on radiochromatography was used.[17,18] Briefly, 50 seeds were germinated in the 10^{-4} M solution of $^{203}HgCl_2$ as described above. After 7 days of germination, the plants were collected, washed, dried and mechanically separated in roots, seeds and stems/leaves. Samples of 1 g of such materials were extracted with 50 mL of acetone for 5 days. Then, 0.01 mL of such extract was used in further radiochromatographic experiments (five replications).

CONCLUSIONS

The solid phase synthesis of peptides may offer the best opportunities to create new cysteine-containing biopolymers with high metal binding capacity. The radioactive mercury showed an inhibitory effect on the wheat germination, and accumulated in roots and less in shoots. Thiol-peptides such as glutathione reduce the radiotoxic effect of ^{203}Hg, increasing the weight and length of the resulted seedlings. Cystine has a more powerful protective effect, whereas the synthetic decapeptide proved to be harmful in wheat germination experiments. However, some thiol-peptides, such as the decapeptide investigated here may have a complex effect on the interaction between living cells and heavy or radioactive metals.

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