Chromium-Binding Ability of Tannin in Water-Extracts from Withered Oak Leaves

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Reaction mixtures containing constant amounts of water-extract from withered oak leaves and various concentrations of potassium chromate were incubated, and each reaction mixture was fractionated by a Sephadex G-25 column. The concentrations of the Cr-tannin obtained in the fractions were determined. Then the Cr-binding ability of the tannin was examined by apparent stability constants and Cr-complexing capacities, following construction of a Scatchard plot which adopts a two-site model. The tannin was shown to have two-classes of Cr-binding sites. Based on the Cr-complexing capacities, 1 mg tannin was shown to bind about 0.5 mg of reduced Cr. Moreover, functional groups contributing to the complexation with Cr were investigated by FTIR and pH titration. The relationship between the Cr-complexing ligans in the two sites and the functional groups in the tannin was also investigated. Finally, Cr ion species which emerged during the reaction between chromate and the tannin were determined by EPR. The spectra showed a sharp signal attributable to Cr(V) and a broad one assignable to Cr(III). Thus, it can be speculated that the tannin most probably forms a final Cr(III)–tannin via Cr(V) species.

Cr(VI) salts are well known to be carcinogens and mutagents and are reduced by various cellular components to form final cross-linked Cr(III) products.[1—5] Soil components such as fulvic and humic acids are equally active in reducing Cr(VI) salts.[6] In the previous study, we reported that water-extracts from withered oak leaves reduced such a toxic Cr(VI) and formed water-soluble complexes (Cr–tannin) with the reduced Cr.[7] Thus, some study on the interaction between Cr(VI) and the tannin is useful to investigate behaviors of biohazards like Cr(VI) in ecological systems. In the present study, we focussed on Cr-binding ability of the tannin to clarify the overall reaction between Cr(VI) and tannin from withered oak leaves. For this purpose, a Scatchard plot[8] for the Cr–tannin was constructed, and apparent stability constants and Cr-complexing capacity in the tannin were calculated by adopting a two-site model.[9,10] Then the relationship between the Cr-complexing capacities in the two sites and the amounts of functional groups was examined using FTIR and pH titration. Moreover, the Cr ion species which emerged during the reaction between chromate and the water-extract were observed by EPR. Those results are described in this report.

Experimental

Preparation of Water-Extract. Withered leaves were collected from oak trees and ground in a high speed grinder (Flitsch, Germany, 15-301). The resulting powder (0.5 g) was mixed with water (50 ml) and heated in a water bath at 100 °C for 1 h. The mixture was centrifuged at low speed (3000 rpm) to obtain a supernatant, which was used as a water-extract for further experiments. Average tannin concentration of the water-extracts was estimated to be 0.5 mg ml^{-1}.

Preparation of Sephadex G-25 Column. Sephadex G-25 (fine) obtained from Pharmacia Biotechnology LKB was equilibrated in water at room temperature for several hours and then decanted from water several times. The Sephadex G-25 was packed into a glass column (1 cm diameter) to make a column of 85 cm length. The column was washed with water (about twice the column volume) before applying samples.

Procedures. Mixtures containing 3-ml of the water-extract, 20 mM of AcOK (pH 6.0) and either 0.35, 0.42, 0.5, 0.6, 1.0, 1.5, 2.0, or 2.5 mM of potassium chromate (M=mol dm^{-3}) in a total volume of 10 ml were incubated at 15 °C for 18 h. A 5-ml volume of each mixture was pipetting into the column prepared as described above. The reaction products were eluted from the column with water at a flow rate of 0.42 ml min^{-1}. Twenty fractions (5 ml of each fraction) were collected, and Cr(VI), total Cr, and tannin in fractions No. 5 to 15 were determined. The concentration of

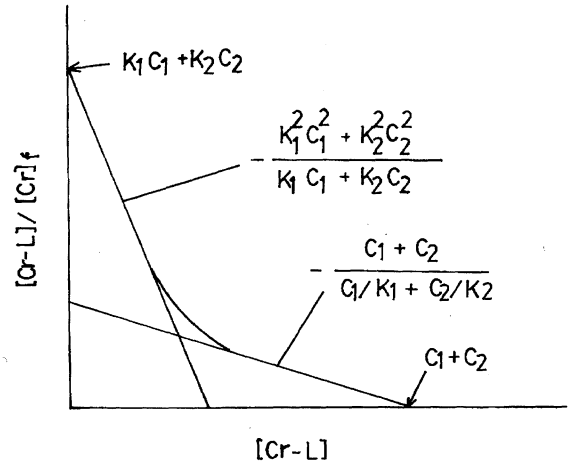

Fig. 1. Scatchard plot.

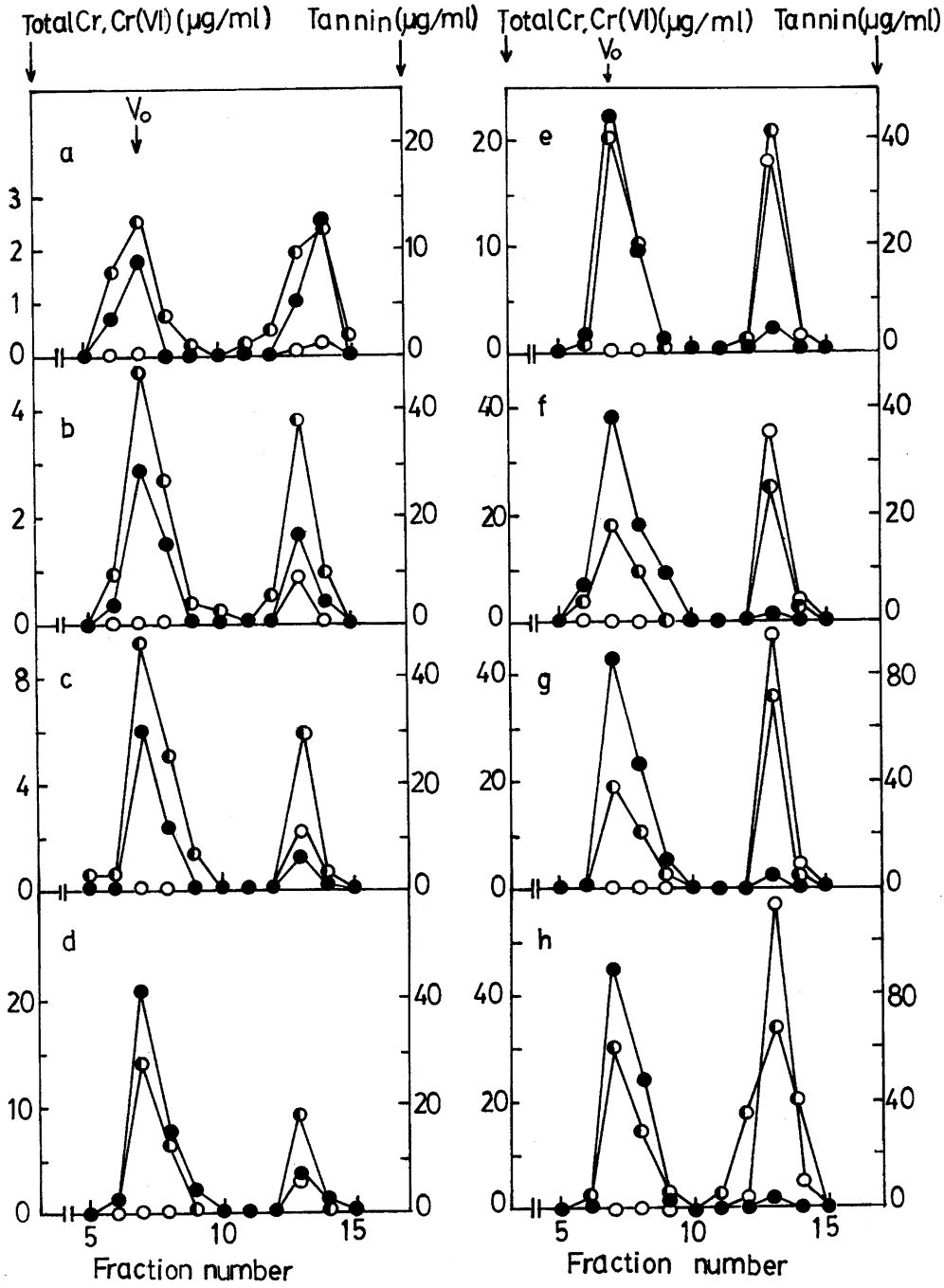

Fig. 2. Sephadex G-25 chromatographies for reaction products from mixtures incubated in the presence of various concentrations of potassium chromate. The concentrations of potassium chromate in panels a—h are 0.35, 0.42, 0.5, 0.6, 1.0, 1.5, 2.0, 2.5 mM, respectively. ●, tannin; ◐, total-Cr; ○, Cr(VI).

Cr–tannin ([Cr–tannin]) was determined by measuring the total Cr in each fraction containing tannin. If Cr(VI) was contained in the fraction, the Cr(VI) was subtracted from the total Cr to estimate the actual amount of reduced Cr with the tannin.

Calculations. To estimate apparent stability constants and Cr-complexing capacities, a Scatchard plot was constructed. The equilibrium of chromium and the tannin can be written as follows:

$$\mathrm{Cr} + \mathrm{Li} \rightleftharpoons \mathrm{Cr\text{–}Li}$$

where Cr is total Cr(VI) added and Li is the binding site of the tannin.

If it is assumed that there are m sites in Cr–tannin formation, which are independent of each other, the mass balance can be described by Eqs. 1, 2, and 3.

$$[\mathrm{Cr}]_t = [\mathrm{Cr}]_f + \sum^{m}[\mathrm{Cr\text{–}Li}] \quad (1)$$

$$C_i = [\mathrm{Li}] + [\mathrm{Cr\text{–}Li}] \quad (2)$$

$$K_i = [\mathrm{Cr\text{–}Li}]/[\mathrm{Cr}]_f[\mathrm{Li}] \quad (3)$$

where $[\mathrm{Cr}]_t$ is total Cr concentration, $[\mathrm{Cr}]_f$ is the concentration of free Cr ions, K_i and C_i show the apparent stability constants

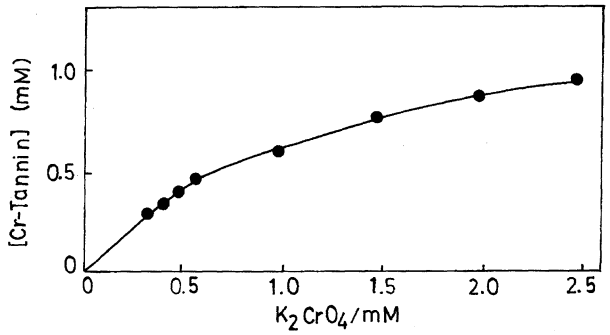

Fig. 3. $[Cr]_t$ vs. [Cr–tannin] plot. A 5 ml sample solution containing 0.35—2.5 mM Cr(VI) was used.

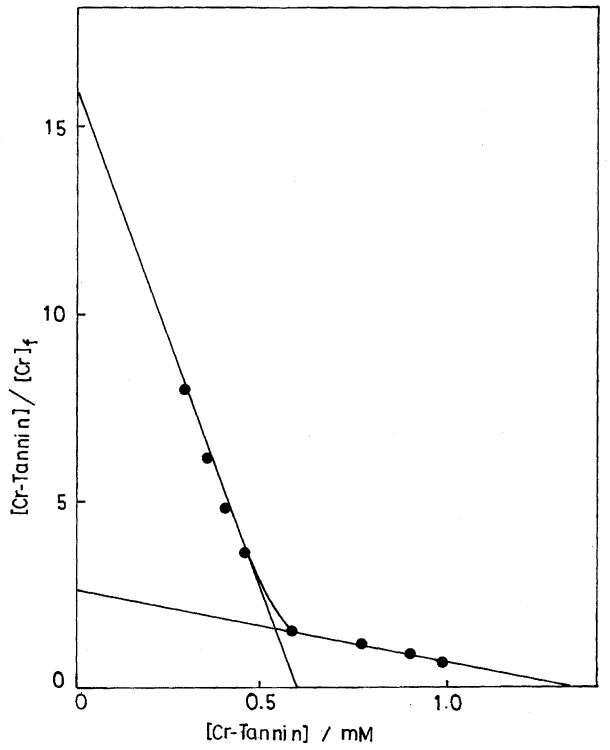

Fig. 4. Scatchard plot for Cr–tannin complex adopting two-site model.

and Cr-complexing capacities of each site, respectively. Upon rearrangement of these equations, the expression becomes

$$[Cr–Li] = (K_i[Cr]_f Ci)/(1 + K_i[Cr]_f). \quad (4)$$

If it is assumed that there are two independent Cr-binding sites, we can get from Eq. 4,

$$[Cr–Li]/[Cr]_f = K_1C_1/(1 + K_1[Cr]_f) + K_2C_2/(1 + K_2[Cr]_f). \quad (5)$$

The Scatchard plot is the lower projected curve, as shown in Fig. 1. The two lines can be extrapolated for this curve, the their slopes and intercepts can be related to the apparent stability constants and Cr-complexing capacities. The regression analysis was used to divide the result into the two lines.

Analytical Methods. Tannin in each fraction was determined by the method using iron(II) tartrate and ethyl gallate as a standard.[11] Cr(VI) was spectrophotometrically determined using 1,5-diphenylcarbonohydrazide.[12] Total Cr was determined by atomic absorption spectrometry (Hitachi, 180-30) with an air-acetylene flame at 357.9 nm. Carboxyl group in the water-extract was determined using the method with sulfuric acid–carbazole.[13] FTIR spectra of the water-extracts were measured on a Perkin–Elmer 1600 Series spectrometer in a matrix of KBr, following evaporation by rotary evaporator. Cr ion species were determined with a Varian Century-Series EPR spectrometer operating at X-band with a 100-kHz magnetic field modulation. The conditions used to obtain the frozen spectra (at 77 K) were field 3430±250 gauss, microwave frequency 9.43 GHz, receiver gain 12500, microwave power 10

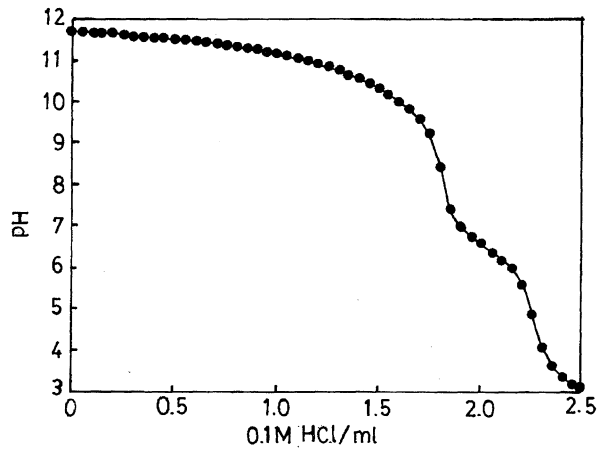

Fig. 5. pH titration curve for water-extract from withered oak leaves. A 30 ml sample solution containing 3.5 mg tannin was used.

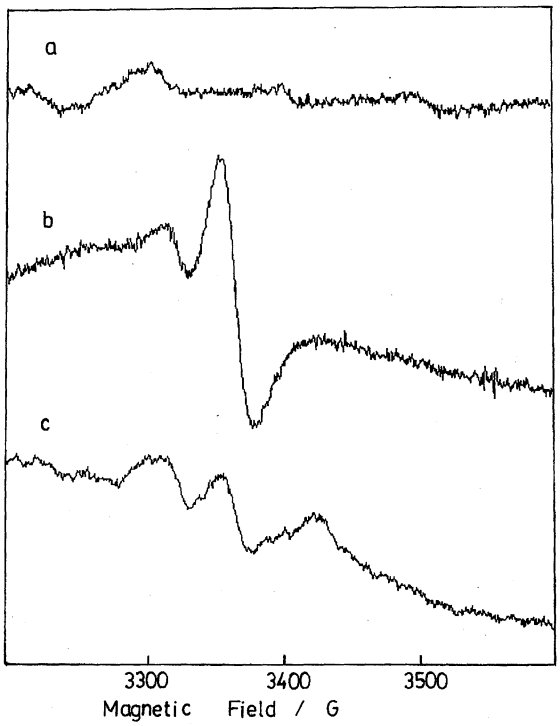

Fig. 6. EPR spectra of reaction mixtures measured at 77 K. Water-extract was prepared by boiling 5 g ground withered oak leaves with 50 ml water for 1 h. Reaction mixtures containing the water-extract and chromate (10 mM) in a volume ratio of 1 : 1 were incubated at room temperature for 4 h (b) and 18 h (c). Panel (a) is a spectrum of the water-extract.

mW, and time constant 0.25 s.

Results and Discussion

In the previous study, reaction mixtures containing water-extract from withered oak leaves and potassium chromate (2 mM) in a volume ratio of 1 : 1 were incubated at room temperature for various periods. We found that formation of a water-soluble Cr–tannin complex (Cr–tannin) reached almost a maximum after incubation for 18 h. To carry out quantitative analysis on the formation, reaction mixtures containing constant amounts of the water-extract (3 ml), 20 mM potassium acetate (pH 6.0) and various concentrations of potassium chromate (0.35, 0.42, 0.5, 0.6, 1.0, 1.5, 2.0, and 2.5 mM) in a total volume of 10 ml were incubated at 15 °C for 18 h. A 5 ml volume of each mixture was separately applied to the Sephadex G-25 column and fractionated the products. Twenty fractions (5 ml) were collected, and total Cr, Cr(VI), and tannin in each fraction were determined. Figure 2 shows the elution patterns of the total Cr, Cr(VI), and tannin from each mixture. Panels a, b, c, d, e, f, g, and h in Fig. 2 correspond to 0.35, 0.42, 0.5, 0.6, 1.0, 1.5, 2.0, and 2.5 mM potassium chromate-containing mixtures, respectively. A significant part of the elution patterns of the total Cr and tannin were found to overlap each other in all the panels, indicating formation of the Cr–tannin in all the mixtures. As shown on the vertical axis of the panels, the amounts of Cr incorporated into the Cr–tannin increased as the concentration of potassium chromate increased. Moreover, the mixtures containing 0.35, 0.42, 0.5, and 0.6 mM potassium chromate gave two types of Cr–tannin complexes, one having a higher molecular weight of over 2000 and the other lower molecular weight of about 500. The rate of the large complex increased as the concentrations of potassium chromate increased. In fact, the mixtures containing 1.0, 1.5, 2.0, and 2.5 mM potassium chromate gave only the large complex. Figure 3 shows the relationship between concentrations of exogenously added potassium chromate and concentrations of the Cr–tannin formed. When the concentrations of potassium chromate added were 0.35, 0.42, 0.5, 0.6, 1.0, 1.5, 2.0, and 2.5 mM, the concentrations of Cr–tannin formed were estimated to be 0.311, 0.361, 0.431, 0.472, 0.598, 0.784, 0.907, and 1.0 mM, respectively. The tannin effectively coordinated chromium, and the concentration of total Cr(VI) added vs. concentrations of Cr–tannin plot was linear in the range of 0 to 0.5 mM. However, the plot became the upper projected curve at the concentrations of over 0.6 mM. These results suggest that the tannin has at least two chromium binding sites. Thus, we constructed a Scatchard plot of Cr–tannin complex. It was found to be lower projected curve (Fig. 4) and fitted a two-site model. The curve was divided into two linear sections based on regression analysis. The apparent stability constants (K_1 and K_2) and chromium-complexing capacities (C_1 and C_2) were then estimated from these slopes and intercepts, using equations described in the Experimental section. We use the term "strong binding site" for the site with higher stability constant K_1 and lower Cr-complexing capacity C_1 and "weak binding site" for the site with lower stability constant K_2 and higher Cr-complexing capacity C_2. The logarithm of the apparent stability constants ($\log K_1$ and $\log K_2$) and Cr-complexing capacities in the tannin are as follows: $\log K_1$=4.44, C_1=0.53 mM, $\log K_2$=3.1, C_2=0.8 mM. Based on the data from C_1 and C_2, the total concentration of Cr-binding ligand is estimated to be 1.33 mM. The amount of tannin in the original mixtures (10 ml total volume) is about 1.5 mg. Thus, it can be calculated that 1.0 mg tannin approximately bound 0.5 mg of reduced Cr.

To investigate possible functional groups for Cr-binding sites, the water-extract was analyzed by FTIR. The analysis showed a broad band centered around 3300 cm^{-1} with a weak shoulder around 2900 cm^{-1}, indicating the presence of a phenolic-hydroxyl group and a carboxylic group. The former group was expected by observation of bands around 1500—1600 cm^{-1} attributable to the aromatic carbon–carbon stretching vibration. The bands around 1750, 1400, and 1250 cm^{-1} were attributed to stretching vibration of C=O, bending vibration of O–H, and stretching vibration of C–O, respectively. These are all typical bands found in carboxylic acid, supporting the presence of carboxyl groups. The presence of carboxyl groups in the water-extract was also shown by analysis of each fraction eluted from Sephadex G-25 column. The elution profile of the carboxyl group closely resembled that of tannin (data not shown). Moreover, in the previous study we have found gallic acid residues in acid-hydrolysates obtained from the water-extract under a condition with 5% sulfuric acid at 100 °C for 6 h. Functional groups such as hydroxyl groups in sugars are known to be responsible for ability in chromate reduction.[14] Furthermore, the reducing ability is shown to be stronger in the carboxyl group-containing sugars (e.g. uronic acid) than in carboxyl-free sugars. It was, therefore, speculated that the ability in the chromate redution was not only due to reducing ability of the compound but also to the complexing nature of the compound. Thus, it is plausible that both the phenolic-hydroxyl groups and the carboxyl groups in the water-extract are responsible for the Cr-complexing capacities.

To investigate the relationship between the functional groups and Cr-complexing capacities in the two sites, a pH titration experiment was carried out. In this study, 3.5 mg of evaporated water-extract was dissolved in 30 ml water and then adjusted to pH=11.69 by adding 1.0 M potassium hydroxide. The mixture was titrated with 0.1 M hydrochloric acid, and the titration curve shown in Fig. 5 was obtained. Two equivalence points were found, at the volumes of 1.8 and 2.25 ml HCl. A clear lag attributable to one functional group was observed at a position between the first and the second equivalence points. However, it was obscure if there was another functional group before the first equivalence point. The concentration of hydroxyl ion in the mixture was calculated to be 0.0049 M before titration, since the pH was 11.69. If only KOH consumed the HCl added, 1.47 ml HCl was necessary to achieve the first equivalence point. Thus, the extra 0.33 ml HCl was most likely due to consumption by some acidic functional group in the water-extract. Therefore, the water-extract can be speculated to contain two acidic

functional groups: One is a weak group and the other is a strong group. The pK_a values for each functional group were estimated to be (a) 11.2 and (b) 6.45 by reading the pH values at the volumes of HCl corresponding to half of the first-equivalence point and the average of the first plus second equivalence points, respectively. The weak functional group in (a) probably corresponds to a phenolic hydroxyl group, and the strong functional group in (b) probably corresponds to a carboxyl group. The amount of the weak functional group was estimated to be 0.033 mequiv HCl, since the extra 0.33 ml HCl was consumed. The amount of the strong functional group was estimated to be 0.015 mequiv HCl, since the lag started at the volumes of 1.95 ml HCl and ended at 2.1 ml HCl. The percentages of the weak and strong functional groups were calculated to be 69 and 31%, respectively. On the other hand, the Cr-complexing capacities in weak and strong sites were estimated to be 0.8 and 0.53 mM. The percentages of the Cr-complexing ligands in the weak and strong sites were therefore calculated to be 60 and 40%, respectively. Thus, amounts of the weak and strong functional groups do not correspond to the concentrations of Cr-complexing ligands in each sites. Consequently, the difference in the two-classes of sites may not be due to only the type of functional groups. Rather, the difference of geometry including the functional groups might be due to the two sites. Viewed collectively, our findings suggest that the strong binding site at lower concentrations of potassium chromate (0.35—0.60 mM) chelated Cr to form a small Cr–tannin at first, followed by formation of the large complex. Association of the small Cr–tannin complexes probably occurred. This result is in agreement with the result that the apparent molecular weight of humic acids increased under the presence of di- and trivalent metals.[15] As the concentration of chromate increases (over 1.0 mM), the more numerous weak sites predominate and bind further Cr to the sites to form the final large-complex.

Finally, Cr ion species which emerged during the reaction between chromate and the tannin were investigated by EPR. In this experiment, water-extract was prepared by boiling of 5 g ground withered oak leaves in 50 ml of water for 1 h. Then we incubated the water-extract with potassium chromate (10 mM) at room temperature in a molar ratio of 1 : 1. As shown in Fig. 6-a, the water-extract frozen at 77 K showed no significant EPR signal. The reaction mixture incubated for 4 h showed a sharp signal (g=1.984) attributable to Cr(V) and a broad signal assignable to Cr(III) species (Fig. 6-b). The ratio of the Cr(V) in the total Cr decreased after 18 h incubation (Fig. 6-c). Taken together, the tannin most likely reduced Cr(VI) in chromate to produce a final Cr(III)–tannin through Cr(V) species as intermediates.

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