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Purification and Characterization of Chromium-Binding Substances from High-Chromium Yeast

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Supporting Information

ABSTRACT: As a highly efficient and easily absorbable source of chromium, the identities of the chromium-binding substances in yeast remain unclear. In this study, a mild extraction procedure involving extraction with ammonia, three-gel filtration, and high-performance liquid chromatography was adopted to obtain two chromium-binding substances from high-chromium yeast. A low-molecular-weight chromium-binding substance was identified, with mass-to-charge ratios (m/z) of 769 and 712, which included glutamic acid, glycine, and cysteine in an approximate ratio of 1:1:1, as well as nicotinic acid and chromium(III). Furthermore, it significantly potentiated (by 51%) the action of insulin to stimulate the conversion of ^{14}C-glucose into lipid in adipocytes. A novel high-molecular-weight chromium-binding substance was also isolated: electrospray ionization tandem mass spectrometry tentatively identified it as HUB1 target protein-1, glyceraldehyde-3-phosphate dehydrogenase, or ribosomal protein L2A(L5A)(rp8)(YL6). This is the first report of a high-molecular-weight chromium-binding substance in yeast and merits further studies.

KEYWORDS: *high-chromium yeast, low-molecular-weight chromium-binding substance, high-molecular-weight chromium-binding substance, glucose tolerance factor*

INTRODUCTION

Yeast glucose tolerance factor (GTF) is a low-molecular-weight substance, comprised of chromium(III); glycine; glutamate, a sulfur-containing amino acid; and nicotinic acid.[1,2] The composition ratio of these components has not been reported. In previous studies, this chromium-rich material from Brewer's yeast was found to be active in stimulating glucose metabolism in fat pad assays and to possess nearly identical properties to GTF from porcine kidney powder.[3−7] Previous studies have supported the idea that supplementation with high-chromium yeast can improve diabetes.[8−11] Chromium may function in vivo as part of a low-molecular-weight chromium-binding substance (LMWCr), to potentiate the action of insulin.[12,13] LMWCr from animals is a naturally occurring oligopeptide of ∼1500 Da, composed of the same amino acids as GTF.[6] Since GTF from yeast is readily absorbed and has been shown to act directly or indirectly to enhance the action of insulin and improve diabetes, it is a potentially important compound that merits further study.

Although purification of GTF from yeast has been carried out for almost 60 years, the reported results of these purification processes have varied. Some of these studies have isolated several chromium-containing substances from yeast, not all of which possessed biological activity;[14,15] conversely, others have obtained compounds with biological activity that contained no chromium.[16,17] It is relevant that these previous GTF isolation procedures involved use of an acid hydrolysis (e.g. 5 M HCl for 18 h) or strong polar resin.[1,14−18] Haylock et al.[14,15] reported the separation of 11 apparently homogeneous, Cr-containing species from brewer's yeast, using Dowex 50W-X12, Dowex 1-X8, and cellulose ion-exchangers, but some of these compounds were biologically inactive. However, the harsh conditions used may have destroyed proteins and peptides that initially may have been associated with chromium.[19,20] In addition, there remain unresolved questions concerning the relationship between GTF and LMWCr: Some researchers have suggested that GTF may, in fact, be an artifact resulting from hydrolysis of porcine LMWCr.[2,5,18]

In this study, we have established a mild purification method for chromium-binding substances from chromium-rich yeast and carried out a determination of the chromium concentration and a bioactivity assay. We used high-chromium yeast, containing organic chromium at a level of 1200 μg/g dry yeast, since ordinary yeast contains organic chromium at only 2−4 μg/g dry yeast. Acid hydrolysis was avoided in the purification procedure, and instead, organic chromium was extracted and purified by aqueous ammonium and three-gel chromatography. High-performance liquid chromatography-inductively coupled plasma atomic emission spectroscopy/mass spectrometry (HPLC-ICP-AES/MS) was used to rapidly and accurately microanalyze the distribution of chromium and protein and ensure that other chromium-binding substances were not missed. In addition, a glucose metabolism assay in adipocytes was used to assess the bioactivities of the substances identified.

MATERIALS AND METHODS

Materials. All chemicals used in this study were of analytic grade, and all aqueous solutions were prepared using double deionized water.

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Sephadex exclusion media (sizes G-75, G-25, and G-15) were obtained from Pharmacia Ltd., UK.

Preparation of High-Chromium Yeast. The strain of yeast used in this work was *Saccharomyces cerevisiae* YS-3, mutated by spaceflight-induced mutagenesis and deposited in the China General Microbiological Culture Collection Center (WDCM550 CGMCC No. 2687). The strain was obtained from the Institute of Agro-Food Science and Technology, Chinese Academy of Agricultural Science, Beijing, China. The organic chromium yield of *S. cerevisiae* YS-3 after spaceflight-induced mutagenesis reached 1200 μg/g dry yeast as compared to 810 μg/g dry yeast from the original strain. After incubation with 400 μg/mL chromium(III) chloride ($CrCl_3$), for 44 h at 28 °C on a rotary shaker (200 rpm), cells were harvested by centrifugation, washed three times with deionized water, and then freeze-dried. The medium used in this study was yeast extract peptone dextrose (YPD) medium (pH 5.6), which contained (per liter) 10 g yeast extract, 10 g peptone, and 20 g glucose.

The high-chromium yeast was a tan powder with a total chromium content of 1512 μg/g dry yeast and an organic chromium content of 1200 μg/g dry yeast, as assayed by atomic absorption spectrometry. The content of organic chromium, which correlates with biological activity, was determined using the ammonia extraction procedure described by Anderson et al.[21] followed by atomic absorption spectrometry using flame atomization. The yeast was found to contain 80% organic chromium.

Isolation and Purification. The freeze-dried, high-chromium yeast was extracted with ammonia[21] by shaking at 200 rpm for 3 h at 37 °C with 20 times the volume of 0.1 mol/L ammonia. The supernatants obtained by centrifugation were freeze-dried. The dried material was suspended in distilled water and centrifuged at 8673*g* for 30 min at 4 °C. The supernatant was collected and condensed to the volume of 5 mL using vacuum evaporation at 45 °C. The brownish soluble material obtained was the starting material for isolation of GTF.

All chromatography procedures were carried out in a cold-room maintained at 4 °C. The protein contents of fractions from the column were monitored qualitatively by ultraviolet (UV) absorbance at 280 nm. Simultaneously, the eluates from columns were scanned for UV absorbance at 260 nm, since GTF possesses an UV absorbance maximum at 260 nm.[3,22] The chromium content of the chromatographic effluents was determined by atomic absorption spectrometry using flame atomization.[1,5,14,17,21]

The soluble yeast extract was initially placed onto a Sephadex G75 gel column (1.6 × 80 cm) that had been equilibrated with distilled water. The extract was eluted with distilled water at a flow rate of 0.3 mL/min, and 3.0 mL of each fraction was collected. The chromium content of each tube was determined, and the chromium-containing fractions were pooled and condensed to the volume of 5 mL. During this step, two chromium-rich peaks, peaks 1 and 2, were obtained. Peak 1 was a white liquid, while peak 2 was a yellowish, transparent liquid. Subsequently, reversed-phase HPLC-ICP-AES/MS was used to accurately microanalyze the chromium and protein distributions in peaks 1 and 2. This analysis would form the basis for further purification of GTF.

Next, peak 2 from the Sephadex G75 column was applied to a Sephadex G25 chromatography column (1.6 × 80 cm) that had been equilibrated with 50 mM ammonium acetate buffer (pH 6.0) and was eluted with 50 mM ammonium acetate buffer (pH 6.0). The flow rate was 0.3 mL/min, and 2.2 mL fractions were collected. Fractions containing high concentrations of both chromium and protein were pooled and concentrated by a vacuum concentrator at 40 °C. The resulting yellow clear liquid from the Sephadex G25 column was then loaded onto a Sephadex G15 chromatography column (1.6 × 80 cm) that had been equilibrated with distilled water and eluted with distilled water. The flow rate was 0.3 mL/min, and 2.2 mL fractions were collected. Chromium-containing fractions from this column were pooled, concentrated, and lyophilized for further analysis.

Reverse-Phase HPLC-ICP-AES/MS. *Reverse-Phase HPLC.* Reverse-phase HPLC analysis was performed on a Delta-Pak C18 column (300 Å, 5 μm, 150 × 4.6 mm i.d.) using a Waters HPLC system (Waters Corp.). Analysis was performed at 25 °C with a flow rate of 0.7 L/min using a linear acetonitrile (ACN) gradient: 100% buffer A [5% ACN, 95% water, 0.1% trifluoroacetic acid (TFA)] for 5 min, followed by a linear gradient to 100% buffer B (95% ACN, 5% water, 0.1% TFA) over 40 min. After analysis, the column was equilibrated for 10 min with buffer A. The eluate was monitored at 214 nm, since the increasing amount of acetonitrile used would have resulted in an increase in the baseline absorption level if detection at 200 nm had been chosen.

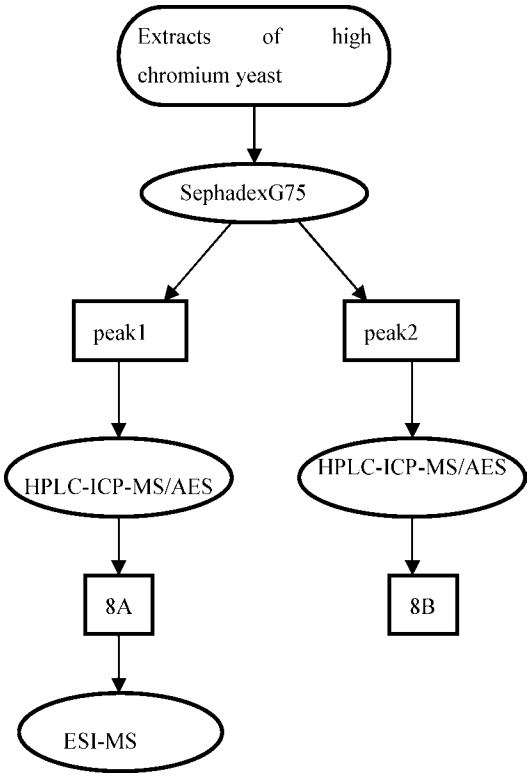

Figure 1. Analysis of the high-molecular-weight chromium-binding substance and the organic chromium distribution using HPLC-ICP-MS/AES and ESI-MS.

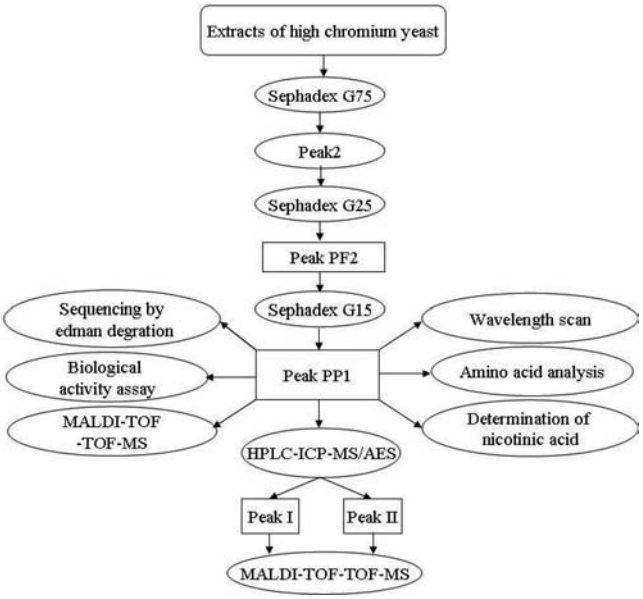

Figure 2. Procedures used for purification and characterization of the low-molecular-weight chromium-binding substance.

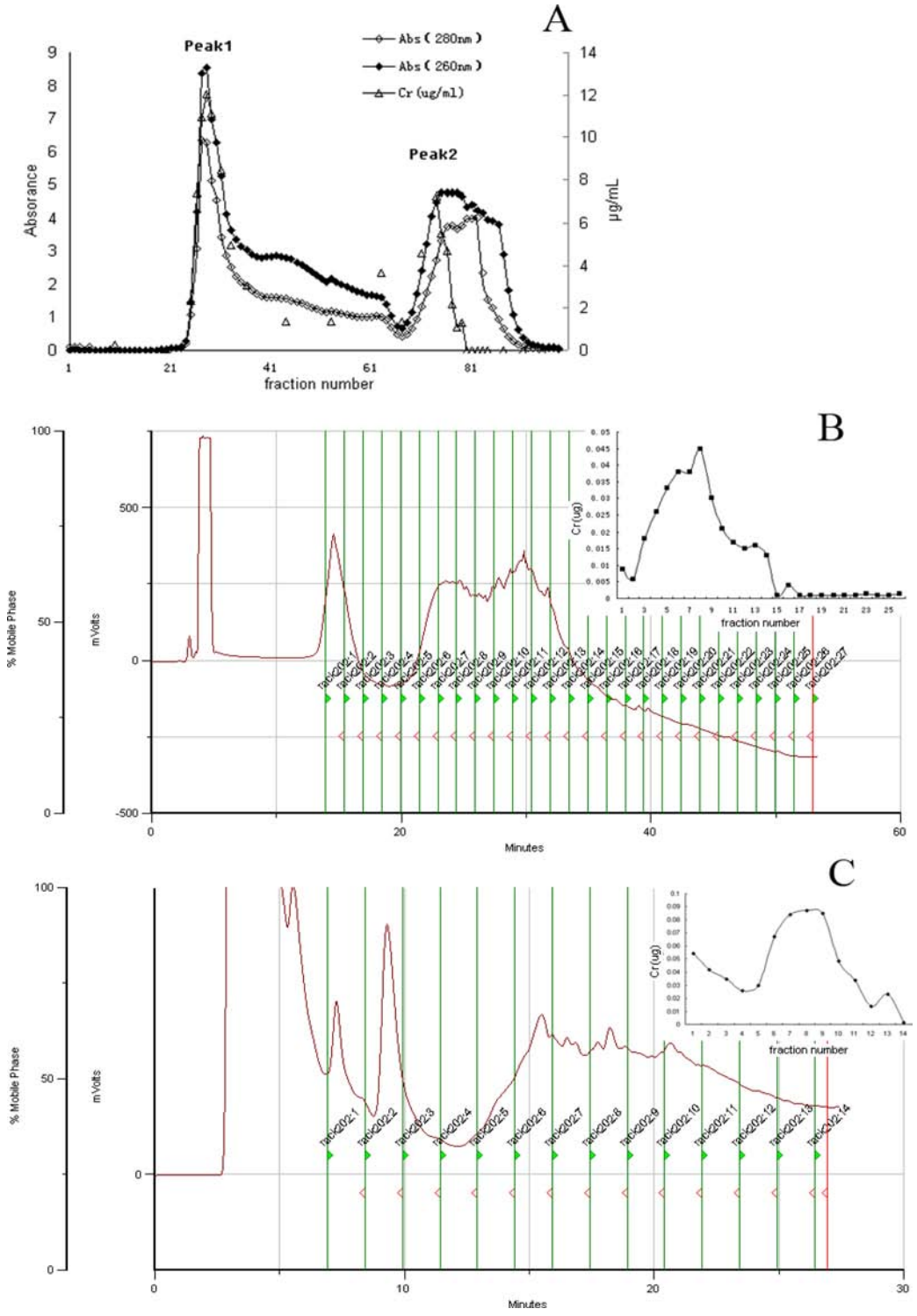

Figure 3. Elution of peaks 1 and 2 from Sephadex G75 and analysis of these peaks using HPLC-ICP-AES/MS. (A) Elution of peaks 1 and 2 from Sephadex G75. (B) HPLC-ICP-AES/MS profile of peak 1. (C) HPLC-ICP-AES/MS profile of peak 2. The freeze-dried ammonia extract was applied to a column (1.6 × 80 cm) equilibrated with distilled water and eluted with distilled water, with 3 mL fractions collected. The chromium and absorbency profiles at 260 and 280 nm are shown in panel A. HPLC of peak 1 is shown in the lower part of panel B. The eluate of peak 1 was collected every 1.5 min, and 27 fractions were collected in total. The green grid in the lower part of panel B marks the collection of each fraction. The chromium in each fraction was determined by ICP-AES/MS, and the chromium profile is shown in the upper part of panel B. HPLC of peak 2 is shown in the lower part of panel C. The eluate of peak 2 was collected every 1.5 min, and 14 fractions were collected in total. The green grid in the lower part of panel C marks the collection of each fraction. The chromium in each fraction was determined by ICP-AES/MS, and the chromium profile is shown in the upper part of panel C.

ICP-AES/MS. ICP-MS analysis (DRC-e, Perkin-Elmer Inc.) used a radio frequency (rf) power of 1.15 kW, a cooling gas flow rate of 15 L/min, an atomizer flow rate of 0.7 L/min, and an auxiliary gas flow rate of 1.0 L/min. For ICP-AES (Vista-MPX, Varian),

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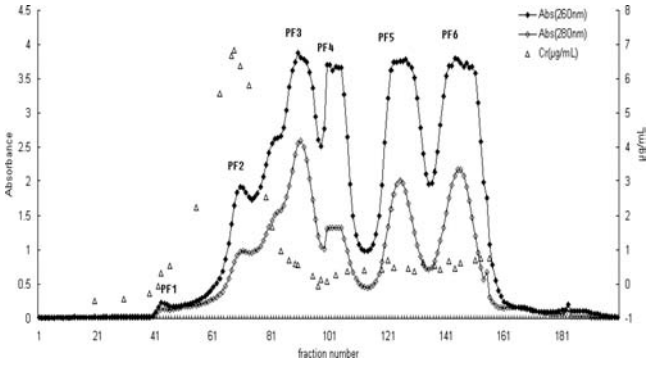

Figure 4. Elution profile of peak 2 from Sephadex G25. Freeze-dried peak 2 was applied to a Sephadex G25 column (1.6 × 80 cm) equilibrated with 50 mM ammonium acetate buffer (pH 6.0) and eluted with the same buffer, with 2.2 mL fractions collected. The chromium and absorbency profiles at 260 and 280 nm are shown. Samples with an absorbance >2 were diluted to allow their determination within the normal range of the instrument. The absorbance of the sample was dilution factor × absorbance of the dilution.

conditions were as above and observations were made at a height of 12 mm.

Analysis of Amino Acid Composition. Samples were hydrolyzed under vacuum in 6 M HCl at 110 °C for 22 h, and analysis was then carried out using an 8500A amino acid composition analyzer (Hitachi) employing standard techniques.

Microbiological Determination of Nicotinic Acid. The test organism used was *Lactobacillus arabinosus* 17−5 (ATCC No. 8014) and the method employed was based on the observation that *L. arabinosus* 17−5 requires nicotinic acid for growth.[23] The method used was a modification of that described previously for the assay of nicotinic acid.[24] Using a basal medium complete in all respects except for nicotinic acid, the growth responses of the organism were compared quantitatively between standard and unknown solutions. The acid produced by the organism was measured to determine the extent of the growth and thereby provide a measure of the amount of nicotinic acid in the test solution. Incubation was carried out at 37 °C for 3 days, and the acid produced was titrated to neutrality with 0.1 N NaOH.

Wavelength Scanning. Light absorption spectra (λ = 190−600 nm) were measured using a dual-wavelength recording spectrophotometer (UV-3010, Hitachi) with deionized water as the solvent.[18]

ESI-MS and MALDI-TOF-TOF-MS. Electrospray ionization tandem mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight time-of-flight mass spectrometry (MALDI-TOF-TOF-MS) were performed by the Beijing Proteome Research Center. The material referred to as 8A from peak 1, fractionated by reversed-phase HPLC, was analyzed by ESI-MS. peaks PP1, I, II were analyzed by MALDI-TOF-TOF-MS.

Determination of ^{14}C-Glucose Metabolism in Rat Adipocytes. The biological activities of the fraction PP1 obtained from the Sephadex G15 chromatography column was assayed for its biological activity by measurement of ^{14}C-glucose metabolism in rat adipocytes. Insulin was used as an internal standard. Rat epididymal fat pads were digested with collagenase in 10 mL of Krebs Ringer's/HEPES−bovine serum albumin (KRH-BSA) medium containing 0.1 mg/mL dextrose. The digestion was carried out at 37 °C in a water-bath shaker at 100 rpm for 25−30 min. The resulting adipocytes were then filtered through a layer of nylon cloth and collected in a 15 mL centrifuge tube. The adipocytes were washed five times with Krebs Ringer's buffer and then centrifuged in a clinical centrifuge (500*g*, 1 min) to remove the collagenase.

Isolated adipocytes were incubated for 1 h in KRH-BSA with U-^{14}C-glucose (5 mmol/L, specific activity 0.89 × 10^6 dpm/μmol), $NaHCO_3$ (10 mmol/L), palmitic acid (0.25 mmol/L), and adenosine deaminase (800 U/L), together with either insulin (60 pmol/L) or PP1 (10 μg/mL). After a 1 h incubation, the medium was separated from the adipocytes by gentle centrifugation (1000*g*, 1 min) and collected for subsequent analysis. Lipids were extracted from the adipocytes with 2-propanol/heptane/H_2SO_4 (1 mol/L) in the ratio 40:10:1. Glucose metabolism was measured from the incorporation of U-^{14}C-glucose into total lipids. Triplicate measurements were made for each variable in each experiment. All values were corrected for zero-time incubation.

Statistical Analysis. Data are presented as means ± standard errors (SE). A pooled Student's *t* test was used to evaluate the differences in the mean concentrations of U-^{14}C-glucose converted into lipid between the basal and other groups. $p < 0.05$ was considered to be indicative of statistically significant differences.

RESULTS AND DISSUSSION

Many studies have utilized GTF bioactivity tests, such as yeast bioassays and glucose metabolism tests in adipocytes or hepatocytes, during the purification process to help identify the target.[4,5,14−17,21,22,25−29] Of these bioactivity tests, yeast bioassays have been found to lack a high degree of accuracy, resulting in either false positive results for chromium-containing compounds or positive results for substances that did not contain chromium.[6,14,15,17,25] To ensure the purification of only chromium-binding substances in our study, both the measurements of organic chromium content and a fat pad assay (for glucose metabolism) were adopted.

Isolation and Purification of Chromium-Binding Substances. Purification and analysis of a high-molecular-weight chromium-binding substance from high-chromium yeast are shown in Figure 1, while those of GTF, utilizing three-gel filtration chromatography, are shown in Figure 2.

The concentration of GTF in normal yeast is very low, increasing the chance that a purification procedure may fail to isolate some of the compounds that are present in the yeast.[4,16] Under certain conditions, yeast has the ability to accumulate trace elements and form organic bonds with these, at concentrations several times higher than the normal level.[30] Providing yeast with supplemental chromium in a more concentrated form can produce high-chromium yeast, containing chromium at a concentration of 1200 μg/g dry yeast. Extraction of high-chromium yeast with aqueous ammonia is able to transfer chromium-binding proteins into the water phase, and such extracts have been shown to contain 75% of the chromium initially present in the yeast biomass.[21] In contrast, ultrasonic disruption, which has often been used in the extraction of bioactive substances, results in only 10% of the chromium initially present in the yeast biomass being retained in the extracts.[19,31] For this reason, we chose to adopt extraction with aqueous ammonia as the first step in our isolation procedure.

Extracts from high-chromium yeast were chromatographed on a Sephadex G75 gel, which was then eluted with distilled water. The elution profiles from the Sephadex G75 column are presented in Figure 3A. Two definite absorption peaks, 1 and 2, evident at both 280 and 260 nm, which coincided with the chromium peak, were present in tubes 21−34 and tubes 70−81. On the basis of the fractionation range (3000−80 000 Da) of Sephadex G75 and the location of the two peaks in the elution profile, the proteins in peak 1 were high-molecular-weight proteins (>80 000 Da), while those in peak 2 were low-molecular-weight proteins (<3000 Da).

HPLC-ICP-MS/AES is a useful analytical method employed in metallomics for the separation of an unknown metalloprotein and rapid determination microelement in food; advantages include high efficiency, a low detection threshold for

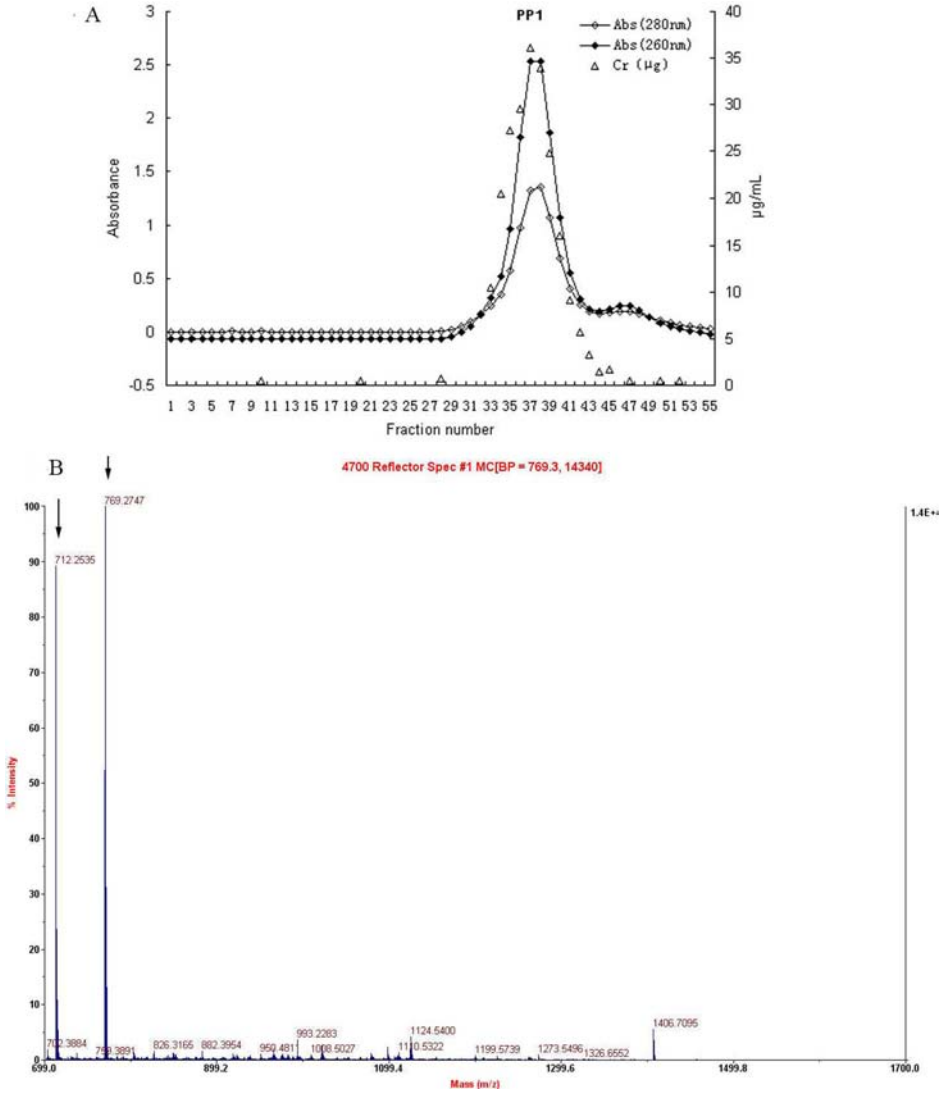

Figure 5. Elution profile for peak PP1 from Sephadex G15 column and its MALDI-TOF-MS spectrum. (A) Elution profile for peak PP1, using a Sephadex G15 column. (B) MALDI-TOF-MS spectrum of peak PP1. Freeze-dried peak PF2 was applied to a Sephadex G15 column (1.6 × 80 cm) equilibrated with distilled water and eluted with distilled water, with 2.2 mL fractions collected. The chromium and absorbency profiles at 260 and 280 nm are shown in panel A. Two molecular ions (m/z 712 and 769) are observed in the MALDI-TOF-MS spectrum of PP1.

metal, and a high separation speed.[32−34] In this study, HPLC-ICP-MS/AES was used to analyze the distributions of organic chromium and protein. The results from reversed-phase HPLC-ICP-AES/MS analysis of chromium-rich peaks 1 and 2 are presented in Figure 3B,C. As shown in Figure 3B, there was a single chromium peak in accordance with the absorption peak in the elution profile of peak 1, reaching 0.045 μg. The chromium-containing fraction in tube 8, named 8A, was analyzed by ESI-MS; as shown in Figure 3C, a single chromium peak was evident, in accordance with the absorption peak at 214 nm in the elution profile of peak 2 reaching 0.087 μg.

The material referred to as 8A from peak 1, fractionated by reversed-phase HPLC, was analyzed by ESI-MS, and the series of fragment ions obtained in the collision was used as the basis for protein identification, undertaken by searching the yeast protein database (www.ncbi.nlm.nih.gov) using SEQUEST software. The high-molecular-weight chromium-binding protein may be homologous to ubiquitin-1 (HUB1) target protein-1, glyceraldehyde-3-phosphate dehydrogenase, or ribosomal protein L2A(L5A)(rp8)(YL6) [Table 1 (results of ESI-MS) in the Supporting Information]. To our knowledge, this is the first report of the purification and tentative identification of a high-molecular-weight chromium-binding protein. However, the results should be regarded as preliminary, and further detailed studies will be required for confirmation of the identity.

Purification of a high-molecular-weight chromium-binding protein from yeast has, to date, not been reported. A possible reason for this may have been the use of assays of biological activity alone in previous purification procedures, rather than determination of organic chromium content.[4−6,14−18,25,26] If so, this would imply that the high-molecular-weight chromium-binding protein discovered in this study does not possess the same bioactivity as that of the low-molecular-weight chromium-binding protein. The bioactivity of the high-molecular-weight compound and the mechanism by which chromium and protein combine require further investigation.

Although there was a single chromium peak in both peaks 1 and 2, the chromium content of peak 2 was higher than that of peak 1. Furthermore, the proteins in peak 2 were of low

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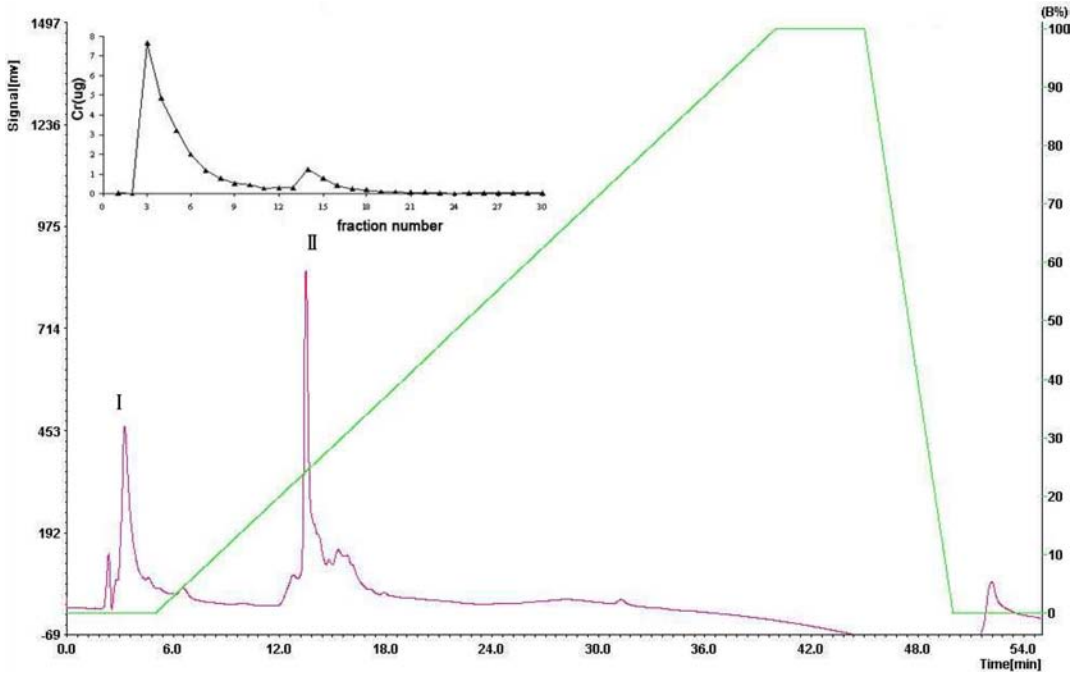

Figure 6. HPLC chromatography of peak PP1 and the organic chromium distribution. HPLC chromatograph of peak PP1 is shown in the lower part of the figure. During HPLC of peak PP1, the eluate was collected every 1 min, and a total of 30 fractions were collected. The chromium in each fraction was determined by ICP-AES/MS, and the chromium profile is shown in the upper part of the figure.

Table 1. Analysis of the Amino Acid Composition of Peak PP1

amino acid	mmol	%
Asp	0.1390	1.85
Glu	1.0809	15.9
Gly	0.9441	7.09
Cys	0.8423	10.2

molecular weight, showing greater similarity to previous reports of GTF.[25,35,36] Therefore, our further work focused on the purification of peak 2. A summary of the purification and analysis of the low-molecular-weight chromium-binding substance is shown in Figure 2. The pooled peak 2 fraction from the Sephadex G75 column was condensed by freeze-drying and applied to a Sephadex-G25 column. As shown in Figure 4, there were six absorption peaks at both 260 and 280 nm; only peak PF2 contained chromium. The pooled peak PF2 (fractions 61−71) was then condensed to the minimum volume and chromatographed on a Sephadex G15 column. Figure 5A shows that a symmetrical, single, sharp peak, peak PP1 (fractions 33−41), appeared during the elution that contained Cr. The pooled peak PP1 was concentrated and lyophilized for further analysis.

MALDI-TOF-TOF-MS Analysis of Peak PP1. There were only two molecular ions evident in the negative mode of the MALDI-TOF-mass spectrum of PP1, at m/z 712 and 769 (Figure 5B), indicating that the purity of PP1 was very high. Comparison of the MALDI-TOF-TOF mass spectra of m/z 712 and 769 revealed many similarities, such as the ions at m/z 496, 286, 175, 144, 129, 119, 87, and 70 [Figure 1 (MALDI-TOF-TOF-MS spectrum for m/z 712 and 769) in the Supporting Information]. This implied a certain correlation between m/z 712 and 769 and indicated that the two ions had similar structure. The difference between m/z 712 and 769 was 57, a small value that may represent the loss of an amino acid functional group. According to these results, the fragment ion with m/z 712 may be formed from decomposition of the fragment ion with m/z 769, although further work is required to confirm whether this is indeed the case. Nonetheless, we have established that the low-molecular-weight chromium-binding substance (PP1) purified from yeast has an m/z of 712−769.

Peak PP1 was further analyzed by reversed-phase HPLC-ICP-AES/MS. As shown in Figure 6 (lower part), two sharp peaks were observed, with peak I being lower than peak II. Furthermore, peak I appeared before gradient elution, indicating that the compounds in peak I were those that could not be adsorbed onto the C18 column. There was only one peak during gradient elution, which was peak II. The distribution of organic chromium in the HPLC chromatograph of peak PP1 is shown in the upper part of Figure 6. There were two chromium peaks that were in accordance with peaks I and II, and the chromium content of peak I was clearly higher than that of peak II. MALDI-TOF-TOF-MS was used to analyze peaks I and II, the figures of which are not included in this paper; compared with the MALDI-TOF-TOF-MS spectrum of blank, no mass spectroscopic signal was detected for peak I, whereas two species at m/z 712 and 769 were detected in the mass spectrum of peak II. On the basis of the results of HPLC-ICP-AES/MS of peak PP1 (Figure 6) and MALDI-TOF-MS spectrum of peaks I and II, peak I may have been the chromium that could not be adsorbed onto C18, shielded from the low-molecular-weight chromium-binding substance and hence appeared before gradient elution, while peak II may have been the low-molecular-weight chromium-binding substance that had partially retained chromium through separation.

Analysis of Amino Acid Composition and Nicotinic Acid Level of PP1. Many studies showed that the predominant components were glutamic acid, glycine, cysteine, and aspartic acid, but the ratio of these amino acids from different sources (mammalian liver and yeast) was inconsistent.[1,22,27−29]

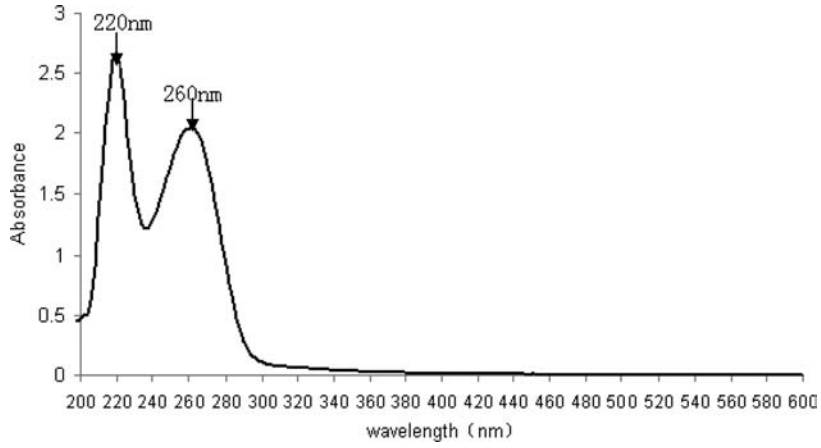

Figure 7. UV wavelength scanning of peak PP1. Light absorption spectra (λ = 600−190 nm) were measured using a dual-wavelength recording spectrophotometer (UV-3010, Hitachi) with deionized water as the solvent.

The ratios of the components of GTF have not been reported yet.[2] LMWCr from bovine colostrum contained aspartic acid, glutamic acid, glycine, and cysteine in a ration of 5:4:2:1.[27] LMWCr from rabbit liver contained glutamic acid, glycine, cysteine, and aspartic acid in a ration of 6:5:3:3.[22] The amino acid composition of PP1 is shown in Table 1. The concentration of glutamic acid, glycine, and cysteine is 6−8 times higher than that of aspartic acid. The main components were glutamic acid, glycine, and cysteine in an approximate ratio of 1:1:1. Given the molecular weight from MS in the 700s, the material appears to have an amino acid composition of 2:2:2 Glu:Gly:Cys.

The content of nicotinic acid in yeast and PP1 was determined though microbiological assays. The concentration of nicotinic acid in normal yeast was 0.045%, and that in PP1 was 3.8% (0.308 mmol/L). The effort to sequence the N-terminus of PP1 by Edman degradation failed. During Edman degradation it was observed that a signal intensity of an unknown compound remained unchanged and it appeared not to be degraded by stepwise acid cleavage from the N-terminus [Figure 2 (N-terminal amino acid analysis of PP1) in the Supporting Information]. Edman degradation proceeds from the N-terminus of the protein and will not work if the N-terminal amino acid has been chemically modified or concealed within the body of the protein.[37] Some researchers proposed that the structure of GTF contains liganded glutamic acid, glycine, cysteine, and nicotinic acid,[1,38] which was supported by the failure of the Edman degration of PP1 to some degree.

Ultraviolet Spectra of PP1. In previous studies, the absorption spectrum of GTF ranged only from 220 to 340 nm.[1,21,28,31] In this study, the UV absorption spectrum of the low-molecular-weight chromium-binding substance was extended, ranging from 190 to 600 nm. There were two peaks (λ = 220 and 260 nm) in the UV spectrum of PP1 (Figure 7). The peak of the absorption spectrum in the UV region (λ = 260 nm) observed in this study was in agreement with that previously reported for GTF by Mertz (λ = 262 nm)[1,35] and Yamamoto et al. (λ = 260 nm).[22,27] Vlatka et al. found that the presence of nicotinic acid in the yeast complex was responsible for the peak at 260 nm.[30] Except the peak (λ = 260 nm), a concave downward tendency in the UV region (λ = 220 nm) was also observed in the study of Yamamoto et al.[27]

Effect of PP1 on the Metabolism of ^{14}C-Glucose to Lipid (Triglycerides and Fatty Acids). Bioactivity tests for GTF include the yeast bioassay and the glucose metabolism test in adipocytes or hepatocytes.[4,5,14−17,21,22,25−29] Since the yeast bioassay is less accurate,[6,14,15,17,25] we decided to study the effects of PP1 on glucose metabolism in vitro, using rat adipocytes. In preliminary experiments, a range of PP1 concentrations was tested (0.1−100 μg/mL). The lowest reliably active concentration was found to be 10 μg/mL, and this was chosen for subsequent investigations. Insulin (60 pmol/L) enhanced the conversion of glucose to lipid by 278% (Figure 9, $p < 0.05$ vs basal). As shown in Figure 8, purified PP1 alone (10 μg/mL) was without effect on the conversion of glucose to lipid (a nonsignificant apparent increase of 7%; $p > 0.05$ vs basal). The combination of PP1 and insulin significantly enhanced the conversion of glucose to lipid in the adipocytes ($p < 0.05$), such that PP1 potentiated the effect of insulin by 51%.

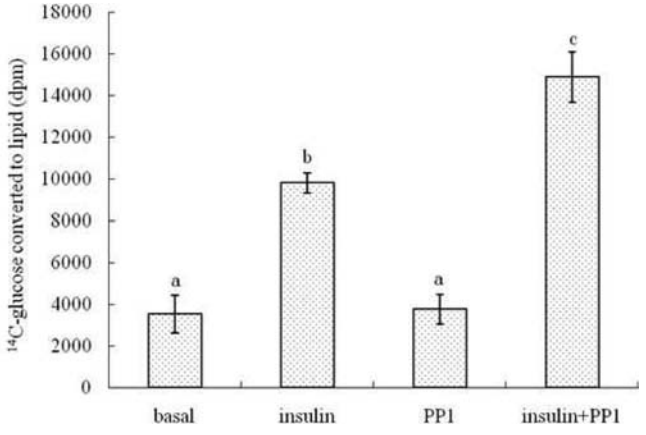

Figure 8. Effect of PP1, insulin, and their combination on conversion of glucose into lipid in rat adipocytes. Preliminary experiments showed that the lowest reliably active concentration of PP1 was 10 μg/mL. Adipocytes isolated from rat fat pads were incubated with insulin (60 pmol/L), PP1 (10 μg/mL), or a combination of both. The data are presented as the means ± SE of 10 independent experiments. Compared with basal, bars with different letters mean significant statistics difference, $p < 0.05$. PP1 alone was without effect on the conversion of glucose to lipid, but in the presence of insulin, PP1 enhanced the rate of glucose conversion by 51%.

Some researchers have proposed that GTF is an artifact produced by acid hydrolysis of a low-molecular-weight chromium-binding substance.[2,6,18] In this paper, GTF was isolated from high-chromium yeast using a mild purification

method not involving acid hydrolysis or strong polar resins. Our analysis showed that PP1 contained cysteine, glycine, and glutamic acid in the ratio 1:1:1, as well as nicotinic acid and chromium(III), and was able to stimulate glucose metabolism, which had an m/z of 712−769. The physical characteristics and biological activity of PP1 compare favorably with those of GTF and LMWCr from mammalian liver reported previously.[22,27,28,39] The other compound isolated was a high-molecular-weight chromium-binding protein that was tentatively identified as HUB1 target protein-1, glyceraldehyde-3-phosphate dehydrogenase, or ribosomal protein L2A(L5A)(rp8)(YL6). To our knowledge, this is the first identification of a high-molecular-weight chromium-binding protein, and further detailed studies are required to determine its function.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table 1, results of ESI-MS; Figure 1, MALDI-TOF-TOF-MS spectrum for m/z 712 (A) and m/z 769 (B); Figure 2, N-terminal amino acid analysis of PP1. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

GTF, glucose tolerance factor; ESI-MS, electrospray ionization tandem mass spectrometry; LMWCr, low-molecular-weight chromium-binding substance; MALDI-TOF-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight time-of-flight mass spectrometry; HPLC-ICP-MS/AES, high-performance liquid chromatography-inductively coupled plasma atomic emission spectroscopy/mass spectrometry; m/z, mass-to-charge ratios.

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