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Response to Comment on Purification and Characterization of Chromium-Binding Substance from High-Chromium Yeast

Glucose tolerance factor (GTF), natural chromium complexes, from yeast was first reported in 1973.[1] Chromium was proposed to be its acitve component, which played an important role in maintaining proper carbohydrate and lipid metabolism. Up to now, the identification and structure of GTF are still not clear. Previous GTF isolation procedures involved the use of either acid hydrolysis (e.g., 5 M hydrochloric acid for 18 h) or a strong polar resin.[2−5] Some researchers have suggested that GTF may be an artifact resulting from hydrolysis of a porcine low-molecular-weight chromium-binding substance.[6,7] Many experiments have shown that high-chromium yeast could help to enhance the action of insulin and improve diabetes in animals.[8−11] The pancreas of the diabetic mice supplemented with high-dose, high-chromium yeast (1000 μg/kg·day) showed a slight decrease in the number and bulk of islet cells compared with low-dose, high-chromium yeast group (250, 500 μg/kg·day) and a control group (supplemented with normal yeast, chromium <0.1 μg/g dry yeast).[10] Therefore, the existence of the chromium-binding substance in yeast before hydrolysis is worth exploring. (It may be different from the original definition or the original composition proposed. GTF is named after its original function, which is active in stimulating glucose metabolism in fat pad assays. It also shows nearly identical properties to GTF from porcine kidney powder.)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) spectrum of PP1 (Figure 6 in ref 12) showed two MS signals at m/z 712 and 769, respectively. The reverse-phase high-performance liquid chromatography (HPLC) of PP1 showed two peaks (peaks I and II).[12] The content of chromium in these two peaks was determined by inductively coupled plasma atomic emission spectroscopy/mass spectrometry (ICP-AES/MS). The identifications of molecules in these two peaks were determined by MALDI-TOF-TOF-MS-MS. The content of chromium in peak I was 9 μg, which came off with the solvent and had no MS signals detected. However, chromium was partially retained in peak II through separation (3 μg), which rendered two MS signals at m/z 712 and 769, respectively. The MALDI-TOF-MS spectrum of PP1 was in accordance with the MALDI-TOF-MS spectrum of peak II (both gave MS signals at m/z 712 and 769). Hence, the condition of reverse-phase HPLC of PP1 (Figure 6 in ref 12) could result in some chromium shield from PP1 during separation in advance. Chromium in peak I could not be ionized during MALDI-TOF-TOF-MS-MS and, hence, no signal was observed for peak I. Other than the reverse-phase HPLC of PP1 (Figure 6 in ref 12), HPLC of PP1 in Edman degradation was also obtained.[12] An unknown compound (just one peak) remained unchanged throughout the whole experiment (Figure 2 in the Supporting Information for ref 12). It was a pity that the peak was not collected to be tested furthermore. Recently, a modified preparative HPLC was carried out, and only one peak was accquired, which contained chromium (Figure 1). The acetonitrile concentration range for elutions was narrowed from 20 to 22% during the whole elution. Further analysis on its constructure and function is underway.

As to Dr. Vincent's conjecture about the low concentration of 35% amino acids, PP1 not only contained glu, gly, and cys but also contained chromium and nicotinic acid,[12] which were determined by ICP-AES/MS analysis and microbiological analysis, respectively. Furthermore, 100% hydrolysis could not be achieved in the test,, which could influence the result. Except for 17 common amino acids, no other amino acids were analyzed.

With regard to Dr. Vincent's conjecture about nicotinic acid in PP1, the content of nicotinic acid in PP1 was determined through microbiological assays but not by the ultraviolet absorption of PP1 at 262 nm. The determination showed that the concentration of nicotinic acid in normal yeast was 0.045%, and that in PP1 was 3.8% (0.308 mmol/L).[12] In that paper,[12] a low-molecular-weight chromium-binding substance was mentioned as PP1 in the result of PP1 (MALDI-TOF-TOF-MS-MS analysis of peak PP1). Two chromium-binding substances were purified from high-chromium yeast. 8A purified from peak 1 was named as a high-molecular-weight chromium-binding substance because of its larger molecular weight. To distinguish these two chromium-binding substances in this paper, low-molecular-weight chromium-binding substance was mentioned as GTF. Originally, low-molecular-weight chromium-binding substance (LMWCr, also called chromodulin) was purified from the liver, kidney, and other organs of mammals (rabbit, bovine, chicken, etc.) and the compositions of amino acids of LMWCr from different sources were different.[13−16] During the purification, an in vitro chromium loading procedure was utilized requiring the addition of 3.4 mmol of chromium as dichromate per 2 L of homogenate, to guarantee the material contained its full complement of chromium. LMWCr is maintained in liver cells as apoprotein. It was difficult to sequence the LMWCr by Edman degradation and MALDI-TOF-MS, because the bound Cr suppresses the signals or post-translational modifications.[13] A combination of X-ray absorption and electron paramagnetic resonance spectroscopies was used to analyze the structure of LMWCr and synthetic Cr complex.[16] According to E:G:C:D:4:2:2:2 of LMWCr from bovine liver, Dinakarpandian made a genomic search of the nonredundant database for all possible decapeptides of the reported composition. Three exact matches were found, EDGEECDCGE, DGEECDCGEE, and CEGGCEEDDE.[17] The low-molecular-weight chromium-binding substance described in the present study included glutamic acid, glycine, and cysteine in an approximate ratio of 1:1:1, as well as nicotinic acid and chromium(III). Due to the lack of sufficient sample, the ratio of chromium, amino acids, and nicotinic acid in

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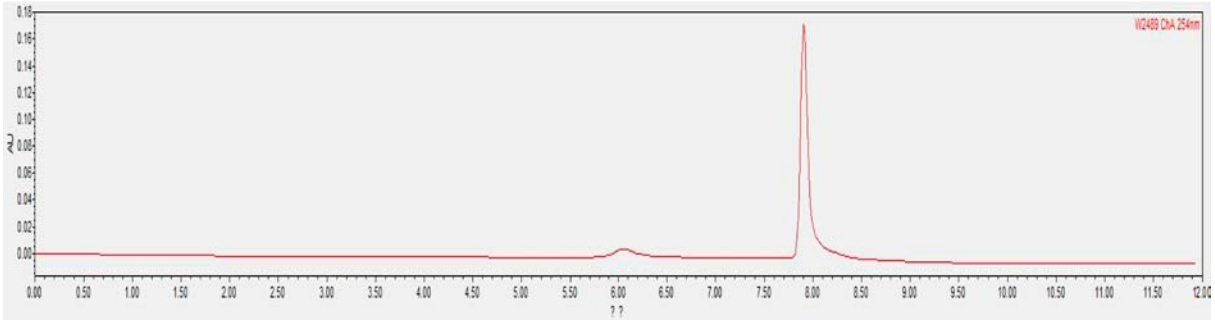

Figure 1. Preparative HPLC of peak PP1. Reverse-phase HPLC analysis was performed on an XBridge BEH 130 Prep C18 column (10 μm, 250 × 10 mm i.d.; injection = 0.5 mL;) using a Waters HPLC system (Waters Corp. USA). Analysis was performed at 25 °C with a flow rate of 1.5 mL/min. Buffer A: water/TFA = 1000:1. Buffer B: ACN/TFA = 1000:0.8. The ACN concentration range for elutions was narrowed from 20 to 22% during the whole elution. The eluate was monitored at 254 nm.

stoichiometric amounts was not calculated. The batch purification of GTF from yeast is being carried out to provide sufficient sample (precise ratio of chromium, amino acids, and nicotinic acid) for further experiments such as genomic search, structural identification, and biological activity.

The rat epididymal fat pads used in the experiment were derived from male Sprague−Dawley rats (180−220 g) and were provided by Professor Jin Wen from Institute of Medicinal Plant Development, Chinese Academy of Medical Science, Beijing, China.

It cannot be denied that the identification of GTF is still a mystery since its first report.[1] Some studies have isolated several chromium-containing substances from yeast, not all of which possessed biological activity;[2,3] conversely, others have obtained compounds that contained no chromium with biological activity.[4,5] The variation of results in such studies might be caused by the harsh conditions (Dowex 50W-X12, Dowex 1-X8) used in the purification process, which could destroy proteins and peptides that initially associated with chromium.[2,3,18,19] A mild purification method for chromium-binding substances was established to get the intact material (chromium-binding substance) from chromium-rich yeast. By avoiding acid hydrolysis in the purification procedure, organic chromium was extracted and purified by aqueous ammonium and three-gel chromatography. The primary limitation of this purification was the low treatment capacity. Therefore, further isolates from PP1 could not be obtained through HPLC for other structure tests. A pilot purification is underway, and further experiments are planned to test its function, structure, and formation when sufficient quantities of purified preparations are achieved.

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