



PURIFICATION OF YEAST GLUCOSE TOLERANCE FACTOR AND ITS EFFECT ON INTRACORPORAL GLUCOSE CONVERSION IN PHYSICAL ACTIVITY

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ABSTRACT

Objective: this paper was aimed to study the separation and purification of yeast glucose tolerance factor (GTF) and its effect on intracorporal glucose conversion of body builders.

Methods: Yeast high-yield GTF was adopted in order to improve the detection sensitivity of chromium; in separation and purification process, the method high performance liquid chromatography- inductively coupled plasma- mass spectrometry/ atomic emission spectrometry (HPLC-ICP-MS/AES) was applied in microanalysis of the distribution of GTF; according to the analysis results, appropriate separation technique was selected for batch separation and purification; by comparing the blood indexes of fed mice, intracorporal glucose conversion of body builders could be deduced.

Results: Chromium/ protein ratio of Peak 1 was much lower than that of Peak 2; Peak1 was micromolecular chromium-binding protein, while Peak 2 was micromolecular chromium-binding protein. N-terminal amino acid sequence analysis results of peak PP1 showed that amino acids in peak PP1 were probably connected with chromium by coordination bonds instead of peptide bonds. In addition, triglyceride and cholesterol level in blood decreased.

Conclusion: Chromium-enriched yeast has an improvement effect on diabetes to some degree.

1. Introduction

Glucose tolerance factor (GTF) is a kind of micromolecular chromium-binding protein (Yuann et al., 2014), also known as a water-soluble complex of chrome with nicotinic acid, glutamate, glycine and cysteine. Due to the physiological functions of GTF, such as adjusting glycometabolism, improving fat metabolism and livestock production performance, GTF has become a research hotspot in the field of drugs, functional food and fodder (Mertz, 1975). As the metabolite of

yeast, GTF can be absorbed by human body at high absorption rate and consumption security (Anderson et al., 1977). Researchers of various countries have made efforts to study the mechanism and functional properties of GTF. In 2012, Sarah et al. (Sarah et al., 2012) applied molecular docking method and speculated that GTF played a role by combining with hxt2p (member of hexose transporter family). In 2014, Qin et al. (Lili et al., 2014) found that homemade high-yield chromium-enriched yeast could prevent the occurrence of type-2 diabetes

in rats and reduce blood glucose level in rats with type 2 diabetes. In 2013, Yang et al. (Yang et al., 2013) analyzed the effect of GTF puerarin functional milk on the metabolism of glucose in the resting cells of beer. In this study, we carried out an experiment on the purification of yeast GTF as well as its effect on intracorporal glucose conversion by taking mice as example, which provided basis for the speculation of the effect on intracorporal glucose conversion of body builders.

2. Materials and methods

2.1. Major instruments and reagents

This study adopted the following instruments and reagents: nucleic acid and protein analyzer, high speed refrigerated centrifuge, N4S ultraviolet visible spectrophotometer, portable glucometer, ammonia water, nitric acid, perchloric acid guarantee reagent (GR), chromium trichloride, glucose, glycosylated hemoglobin determination kit.

2.2. Experimental methods

2.2.1. Cultivation and collection of chromium-enriched thalli cells

Ammonia extracting solution of chromium-enriched yeast was added into a 100 kD ultrafiltration centrifugal tube for 90-min centrifugation at the rate of 2250 r/min, and interception liquid was obtained; then, the centrifugated (100 kD) and filtrated liquid was added into a 50 kD ultrafiltration centrifugal tube for another 90-min centrifugation at the rate of 2250 r/min, and interception liquid was obtained; after that, the centrifugated (50 kD) and filtrated liquid was added into a 10 kD ultrafiltration centrifugal tube for 90-min centrifugation at the rate of 2250 r/min, and interception liquid was obtained; at last, the centrifugated (10 kD) and filtrated liquid was kept at 4 °C as standby.

2.2.2. Sephadex G-25 gel filtration chromatography

A certain amount of Sephadex G-25 dry glue was added to the ammonia acetate buffer (50 mM, pH 6.0) at 90 °C for 90-min intumescence (Neudachina et al., 2014); when the intumescence was over, the mixture was stirred mildly so as to remove floating broken particles; then, a certain amount of buffer was added to adjust the concentration to 73%. Before packing, ultrasonic wave was applied to remove bubbles for 30 min (Skilbeck et al., 2014). Then, ammonia acetate buffer (50 mM, pH 6.0) was used as the eluent; the flow rate was 0.5 mL/min, and the temperature was set at 0 ~ 4 °C; 2.6mL of eluent was collected for each tube. The eluent was traced and checked at 250nm and 270 nm; flame atomic absorption method was applied to detect metal content. At last, liquid in target tube was collected and merged for standby application after freeze drying processing.

2.2.3. Ultraviolet full wavelength scanning

N4S ultraviolet (UV) visible spectrophotometer was used to scan the absorbance of samples within wavelength coverage of 200 ~ 600 nm; blank control liquid and target samples were put into the quartz cuvette which was placed in a UV spectrophotometer for baseline correction, with deionized water as the blank control.

2.2.4. Determination of protein content

Referring to Bradford method, zymoprotein content was determined, with bovine serum albumin as a standard protein.

2.2.5. Analysis of amino acid composition

A certain amount of sample was weighed and placed at the bottom of a hydrolysis test tube, and 3 ml of muriatic acid (5.7 mol / L) was added. The sample was placed in an ultrasonic tank for oscillation and degassing; then, it was put in a blast burner and the pipe orifice was sealed; then, it was placed in an oven for 22-hour hydrolysis at 110 ± 1 °C, and proteins or peptides were hydrolyzed into free

amino acids (Lan et al., 2010). After cooling, the test tube was cut open, and the hydrolysate was filtrated into a 5 ml volumetric flask; test tube and filter paper were flushed with deionized water, and the hydrolysate was diluted to the constant volume. Then, 5 ml of filtrate was placed in an evaporation pan and dried by water bath. Residue was dissolved with 4.5 ml of deionized water and evaporated to dryness, which was repeated for 3 times. Precisely, 5 ml of citrate buffer solution (pH 2.2) was added to dissolve the extractive; 1.5 ml of sample was centrifuged at a high speed refrigerated centrifuge; with an injector, 50 μ l of supernatant was drawn and stored in the spiral pipe for computer analysis. According to the concentration and retention time of amino acid criterion liquid, concentrations of amino acid in sample solution were determined.

2.2.6. Sequence analysis of protein/polypeptide-N terminal with Edman degradation method

Edman degradation method was used for sequence analysis of protein/ polypeptide-N terminal, aiming to analyze N terminal amino acid sequence of protein / polypeptide with free N terminus. It was applied to sequencing amino acids in a peptide. The amino-terminal residue was labeled and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues (Edman *et al.*, 1950).

2.2.7. Determination of blood biochemical indicators

After being fed, the mice were on fasting; then, orbital blood was drawn and then centrifugated at the rate of 2250 r/min for 25 min, thus serum was separated; then, blood glucose level, total cholesterol and triglyceride were determined.

3. Results

3.1. Microanalysis on distribution of metal chromium and protein in Peak 1 and Peak 2 (from Sephadex G-75 gel chromatography) by HPLC-ICP- MS/AES

Sephadex G-75 was used to separate chromium-enriched yeast ammonia organic chromium extract, and two peaks (Peak 1 and Peak 2) that contained organic chromium were obtained. Peak 1 was a protein with large molecular weight (greater than 10 kD), while Peak 2 was a protein with smaller molecular weight (approximately less than 3000 D). This study adopted the method HPLC-ICP-MS/AES to analyze distribution of protein and chromium in the two groups of peaks, which indicated research direction for the subsequent experiment.

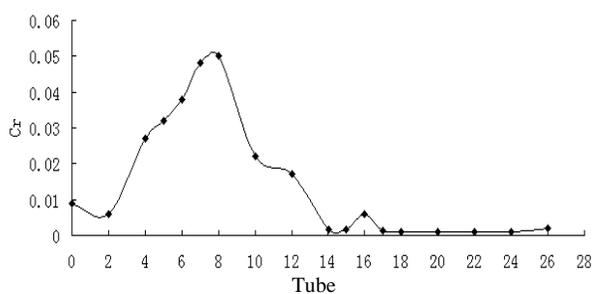


Figure 1. Distribution of organic chromium in high performance liquid chromatography (HPLC) of Peak 1

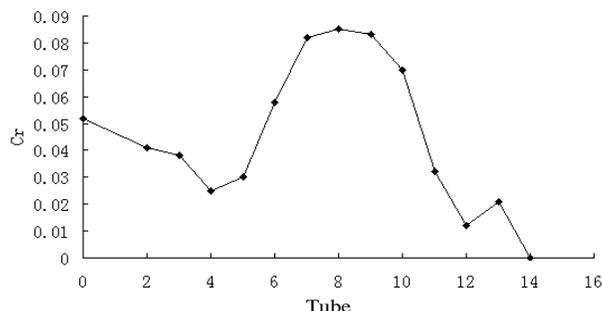


Figure 2. Distribution of organic chromium in HPLC of Peak 2

Figure 1 shows the distribution of organic chromium in HPLC of Peak 1. In Peak 1, protein was spread effectively, and the whole process of elution and separation lasted about

45 min. According to the distribution map, there was a peak value of chromium content in Peak 1, which was 0.05 μg ; content of chromium was the highest in tube 6, 7, 8. Figure 2 shows the distribution of organic chromium in HPLC of Peak 2. It can be

observed that there was a peak value of chromium content, which was 0.087 μg ; the content of chromium was the highest in tube 7, 8, 9.

Table 1. Distribution of chromium and protein in ultra-filtration (100kD, 50kD, 10KD)

	Chromium (μg)	Protein content (mg)	Chromium/protein ($\mu\text{g}/\text{mg}\cdot\text{pro}$)
Ammonia extracting solution	975.12	19.45	51.23
$\geq 100\text{kD}$	523.19	14.23	38.22
100~50kD	67.16	2.51	4.79
50~10KD	121.22	2.29	55.79
$\leq 10\text{KD}$	141.62	0.50	339.13

3.2. Ultrafiltration centrifugation

Membranes of different cut-off molecular weights were used for protein fractionation in organic chromium ammonia extracting solution. The distribution of protein and chromium is shown in table 1. In view of the absolute chromium content in cut-off products, chromium content and protein content (accounting for almost three quarters of the total protein) were the highest in the membrane cut-off components when membrane molecular weight was greater than 100 kD, while chromium content was the lowest when membrane molecular weight was 100 ~ 50 kD, and protein content was the lowest when membrane molecular weight was less than 10 kD.

3.3. Sephadex G-25 gel filtration chromatography

After ultra-filtration through the membrane (with the molecular weight of 100 kD, 50 kD and 10 kD) for 3 times, the ammonia extraction liquid was given Sephadex G-25 filtration chromatography. Ammonium acetate buffer (pH 6.0, 55 mM) was used as the eluent (Jitka et al., 2006), the flow rate was 0.25 mL/min; 2.6 mL was collected in each tube. During elution, the detection wavelengths were A280

and A260. From the experiment, it can be observed that the detection wavelength showed there were 6 protein peaks in the whole separation process, and the peaks overlapped. According to the distribution of protein peaks, the collected tubes were selected for metal content detection, and the results are shown in Figure 3. Through Sephadex G-25 gel chromatography, large quantities of impure proteins were eliminated.

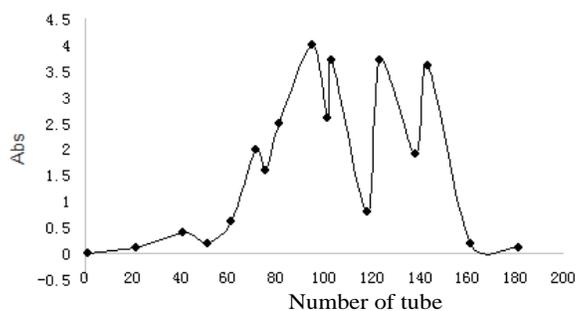


Figure 3. Distribution of metal chromium in Sephadex G-25 gel filtration chromatography

3.4. Sephadex G-25 gel desalination chromatography

Figure 4 shows the distribution map of organic chromium. According to the experimental process and figure 4, it can be

seen that there was only one protein peak in the elution process, while distribution of chromium also formed a peak, and protein peak coincided with chromium peak, with symmetrical distribution of peak curves. Through ultrafiltration with three cutoff membranes (100 kD, 50 kD, 10 kD) and combination of two gel chromatographic separation, we obtained the only protein peak (PP1) that contained chromium and had symmetrical distribution of peak curves.

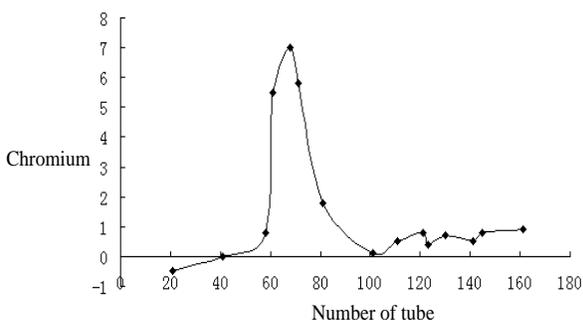


Figure 4. Distribution map of chromium in the process of separation and purification with Sephadex G-25

3.5. Analysis on separation purity of PP1 with combined method HPLC-ICP-MS/AES

Based on protein molecules of different sizes, gel filtration chromatography was applied to achieve the purpose of purification by fractionation; however, it was not suitable for the fractionation of protein components that had similar molecular weights. In the separation and purification of YS-3 yeast ammonia extract, fractionation was performed according to molecular size of protein (Liu et al., 2011). Although we obtained the only protein peak that contained chromium and had symmetrical distribution of peak curves, its purity could not be determined accurately. Therefore, we made further analysis on peak PP1 with the aid of HPLC-ICP-AES/MS method (Tabb et al., 2004); thus the specific purity could be analyzed; on the other hand, by HPLC, PP1 was given high efficiency separation so as to test the separation prospect (Dębski et al., 2004).

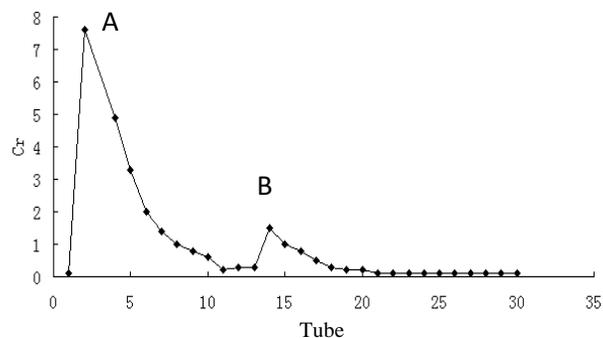
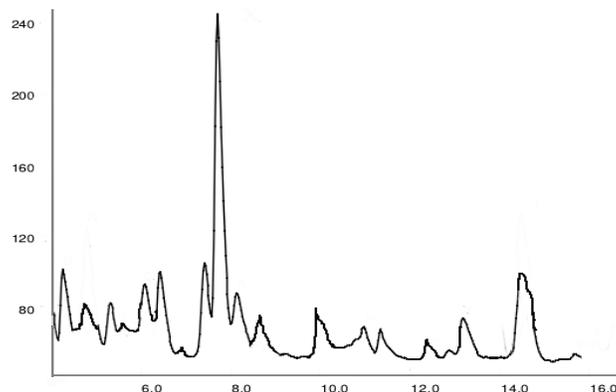


Figure 5. Distribution map of organic chromium in high performance liquid phase of PP1

Figure 5 shows the distribution of organic chromium of PP1. In tube 3 and tube 14, there were two chromium peaks (peak A and peak B). As shown in the figure, chromium content of peak A was significantly higher than that of peak B, while UV detection signal of peak A was weaker than that of peak B. Peak B showed symmetrical peak shape, while chromium distribution of peak A was asymmetrical.

3.6. Sequence analysis of protein / polypeptide-N terminal

The four graphs of Figure 6 show the liquid phase diagram of residue 1, residue 2, residue 3 and residue 4 (of amino acids). It can be seen that each peak shape and height did not change with hydrolysis, which indicated that this kind of substance seemed to remain unchanged without being cleaved.



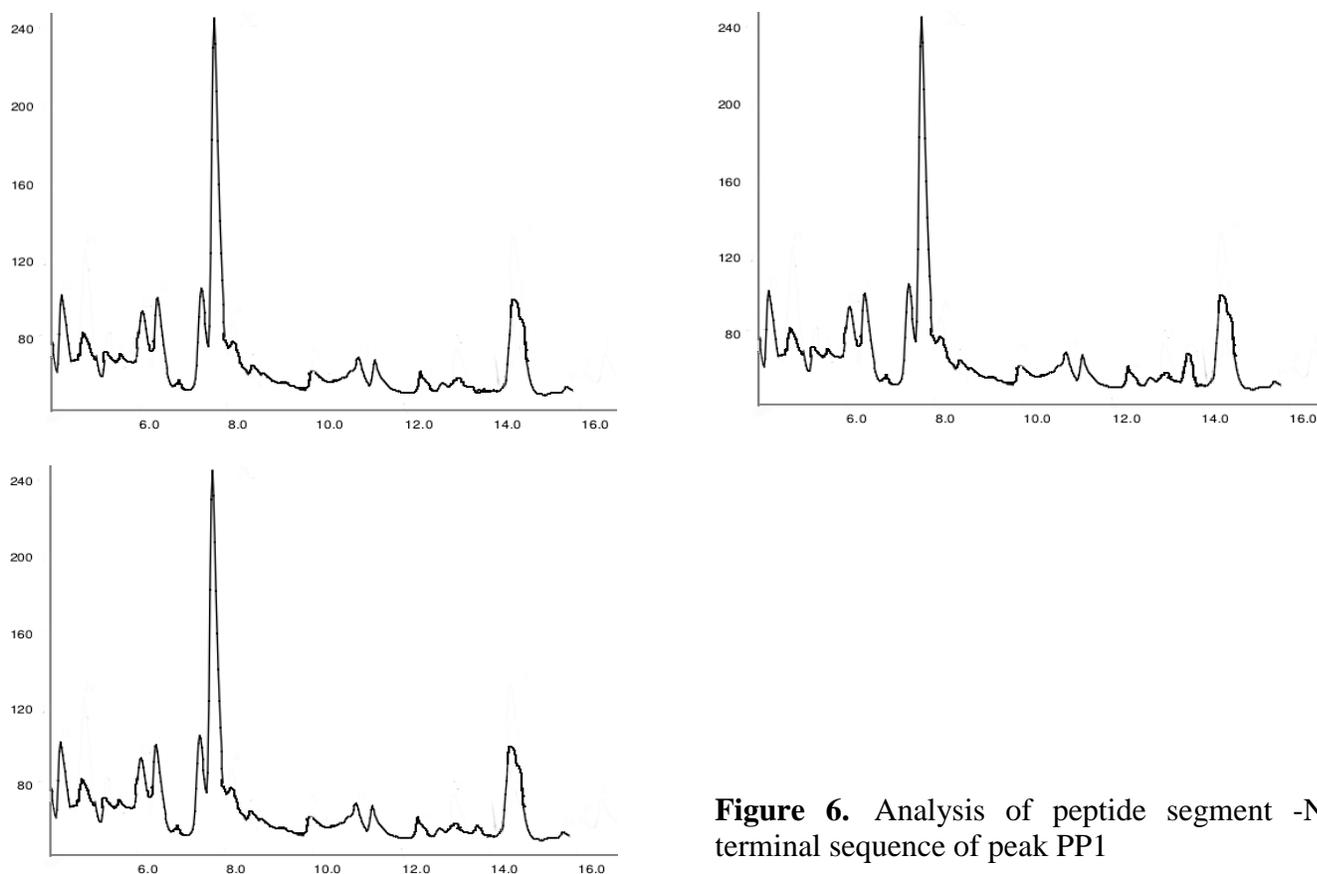


Figure 6. Analysis of peptide segment -N terminal sequence of peak PP1

3.7. Blood lipid level

Table 2. Blood lipid level in each treatment group after 15 weeks

	Normal	Model	Control	Low	Middle	High
Triglyceride	471.8±95.7	301.6±55.9	550.9±71.9	413.9±34.9	246.1±31.9	401.2±33.1
Total cholesterol	204.1±14.9	231.5±13.1	184.9±11.6	161.5±16.2	167.6±12.1	225.9±10.9

Table 2 shows blood lipid level in each group (normal group, model group, positive control group and low/ middle/ high dose chromium-enriched yeast groups) after 15-week medication. In comparison with control group, triglyceride in middle dose chromium-enriched yeast group decreased significantly ($p < 0.05$) and was lower than that in normal group; triglycerides in low dose group, high dose group and positive control group were higher than that of control group.

In comparison with the model group, total cholesterol level decreased significantly in low and middle dose groups ($p < 0.01$) as well as positive drug control group ($p < 0.05$), while no

change of total cholesterol level was found in high dose group. The decrease of triglyceride and cholesterol level in blood indicated that chromium-enriched yeast had improvement effect on blood lipid level of diabetes to some degree.

3.8. Overall discussion

According to previous researches, GTF was reported to be low-molecular-weight chromium-enriched protein (peptide) (Koch et al., 2011). However, it has never been reported that macromolecular chromium-binding protein could be separated from chromium-enriched yeast. It was because previous studies adopted

cytoactive test to detect GTF in separation liquid, while this study detected the distribution of organic chromium in separation liquid; on the other hand, it was also related to the adoption of different separation methods were applied.

As an emerging method of metal proteomics, HPLC-ICP-MS/AES combines chromatographic separation ability with qualitative function of inductively coupled plasma mass spectrometry so as to realize the quantitative and qualitative analysis of complex mixtures, with fast separation speed, high efficiency and low metal detection threshold (Troccoli et al., 2004). In addition, the pretreatment process of samples can be simplified and sample analysis is more convenient.

According to chromium content determination results, it was found that chromium content was much higher in Peak 2 than in Peak 1, and Peak 2 was small molecular weight component, which was basically consistent with the previous report that GTF was micromolecular peptide. Based on the previous research reports and the analysis results of Peak 1 and Peak 2, subsequent purification work will focus on the separation of components; in addition, the tube with the highest chromium content in Peak 1 will be analyzed to explore the physicochemical properties of chromium-binding molecular protein.

GTF is a kind of compound of chromium and protein; therefore, single evaluation based on chromium content or protein content is one-sided. This study adopted the ratio of chromium and protein as a reference index. In reference to previous studies, separation range of Sephadex G-75 was 80 ~ 3 kD, in this study, the filtrate that was fractionated through 100 kD, 50 kd and 10 kD membrane was used for subsequent purification.

The binding of metal and protein is different from that of amino acids in proteins. Amino acids in proteins are bonded by peptide bonds (Beili et al., 2010), while metals bond with proteins by coordination. Each metal ion

has certain coordination configuration; as long as the spatial arrangement of configuration atoms meets the basic requirements of the configuration, stable complexes can be formed. Metal ions bind to protein molecules through coordination with endogenous ligands and exogenous ligands (such as water molecules, porphyrin ring and small organic molecules), thus metal active sites are formed, which affects protein structure. Based on the experimental results, it was confirmed that peak PP1 was chromium-binding small molecular peptide; through sequence analysis of protein/polypeptide-N terminal, it was found that some substance could not be dissociated to produce amino acids, suggesting that the amino acid in the substance is linked with chromium by coordination bonds instead of peptide bonds; therefore, amino acid analysis based on N-terminal dissociation could not be achieved.

In general, fasting glucose level reflects the amount of hepatic glucose output, without reflecting the sensitivity of peripheral tissues to insulin, which is due to the decrease in fasting insulin level and increase in glucagon level. Therefore, we speculated that the absence of Fyn in liver could influence gluconeogenesis under fasting conditions, since hepatic glucose output failed to balance the high clearance rate of glucose. Insulin resistance leads to the increase of intracellular hydrolysis in adipose cells, thus more fatty acids are released into blood vessels, which promotes liver to form more low-density cholesterol and triglyceride. If insulin sensitivity is increased, the opposite reaction will happen in blood lipid.

4. Conclusions

Through the experimental study, we found that chromium/ protein ratio of Peak 1 was far less than that of Peak 2; Peak 1 was chromium-binding macromolecular protein, while Peak 2 was chromium-binding micromolecular protein. According to the UV wavelength scanning on peak PP1 and amino acid composition analysis, it was found that peak PP1 was a chromium-binding small peptide consisting of aspartic acid, glutamic acid, glycine and ysteine. We

assumed that chromium-enriched yeast thalli had an improvement effect on diabetes to some degree. However, due to the limitations of various conditions in the experiment, there are still some defects in this study, based on which we will make some improvement in the subsequent researches.

5. Acknowledgement

Participated project: name of project (strategy research on mass sports development in construction of new-type urbanization) project number (14008) title (not concluded)

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