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Proposed formation mechanism, antioxidant activity and MDA-MB-231 cells survival analysis of two glucose–ammonium sulfite caramel colour melanoidins fractions

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ABSTRACT

Ultrafiltration and gel chromatography were used to isolate caramel colour melanoidins, and elemental analysis and pyrolysis-GC/MS was used to analyze the characteristic of glucose–ammonium sulfite caramel colour melanoidins of various molecular weights. Results indicated that furfuran was the main skeleton of glucose–ammonium sulfite caramel colour melanoidins. And 5-hydroxymethyl-2-furaldehyde might be one of the origins of melanoidins skeleton which was formed via aldol condensation. A proposed mechanism on the formation of glucose–ammonium sulfite caramel colour melanoidins might be that bi-furan derivatives were formed after aldol condensation (part A), and sulfite ion could connect with carbon atom of formyl group, and amidolinks might be formed between aldehyde of furfuran and nitrogen atoms (part B). At last, parts A and B polymerized and formed the possible structure of the glucose–ammonium sulfite caramel colour melanoidins. And the formed melanoidins have a significant radical-scavenging activity, and complex effects on MDA-MB-231 cells survival.

1. Introduction

The Maillard reaction takes place during the course of food thermal processing (Carabasa-Giribet & Ibarz-Ribas, 2000; Su, Huang, Yuan, Wang, & Li, 2010; Umemura & Kawai, 2007), and gives rise to the formation of a complex series of compounds called Maillard Reaction Products (MRPs) (Mastrocola & Munari, 2000). In spite of intensive research in the field of melanoidins which have been proved to be the products of the advanced stages of the Maillard reaction, there is not much knowledge about their structural composition that may be generalized (Cammerer & Kroh, 1995; Tressl, Wondrak, Kruger, & Rewicki, 1998; Yaylayan & Kaminsky, 1998).

Recently, there might be two main possible mechanisms on the formation of melanoidin, and the first proposed mechanism was that the carbonyl compound reacts mainly via the Amadori product to form several deoxyosones which are able to react with each other in an aldol-type condensation to form a basic melanoidin skeleton of amino-branched sugar degradation products. For example, the nucleophilic attack of the carbanion in the C3 position of a 3-deoxyhexosulose can take place on the C1 of another molecule of deoxyhexosulose (Cammerer & Kroh, 1995; Hollnagel & Kroh, 2000; Tressl et al., 1998). And Cammerer, Jalyschko and Kroh (2000) have detailed the nitrogen containing melanoidin structure formed from 3-deoxyhexosulose involving amino compounds. The second proposed mechanism on the formation of melanoidins based on the Maillard reaction was that glucosone or 3-deoxyglucosone could form some furan and its derivatives such as carboxylic acid which might polymerize through esterification, oxidation and aldol condensation (Yaylayan & Kaminsky, 1998), which might form some non-nitrogen polymers.

Melanoidins have been studied on their biological in recent years (Faist & Erbersdobler, 2001; Somoza, 2005). Some of researches found melanoidins had a DPPH radical-scavenging activity (Rao, Chawla, Chander, & Sharma, 2011; Ying, Xiong, Wang, Sun, & Liu, 2011). And different melanoidins derived from different carbohydrate and amino compounds always have different DPPH radical-scavenging activity (Chang, Chen, & Tan, 2011; Guan et al., 2010a; Hwang, Kim, Woo, Lee, & Jeong, 2011). Goya, Delgado-Andrade, Rufian-Henares, Bravo, and Morales (2007) reported that a coffee melanoidin has the ability to protect human hepatoma HepG2 cells against an oxidative insult by modulating reduced glutathione concentration, malondialdehyde production and antioxidant enzymes activity. Moreover, melanoidins were also found for antimicrobial activity (Rufian-Henares & Morales, 2006), anticariogenic (Daglia et al., 2002) and antitumoural properties (Wijewickreme & Kitts, 1997). However, structural and bioactive information about caramel colour melanoidins is still unclear even if literatures regarding to melanoidins' structure and bioactivity have been detailed.

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This study aims to investigate the possible structure of glucose–ammonium sulfite caramel colour melanoidins derived from the Maillard reaction. Moreover, the DPPH radical-scavenging and anti-cancer activity on MDA-MB-231 cells was also studied.

2. Materials and methods

2.1. Chemicals

Ammonium sulfite and glucose were purchased from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum and RPMI-1640 media were purchased from Gibco/Invitrogen (Grand Island, NY, USA). Other reagents were chromatographic grade and purchased from Merck (Damstadt, Germany). Human breast cancer MDA-MB-231 cells were obtained from Sun Yat-Sen University (Guangzhou, China).

2.2. Preparation of caramel colour melanoidins

Glucose–ammonium sulfite solutions were prepared according to the literature with a slight modification (Mudadi, Nkosinathi, & Elizabeth, 1999) to obtain caramel colour solutions. A high-pressure vessel (Parr 4597HP, USA) with a kettle volume of 50 mL was applied to produce caramel colour solutions. In this experiment, 15.0 g glucose and 3.4 g ammonium sulfite were mixed in 15 mL deionized water. The mixture sample was heated at 150 °C for 60 min and then cooled to room temperature by iced water immediately after the thermal reaction.

2.3. Ultrafiltration and isolation of caramel colour melanoidins

Water-soluble caramel colour products were isolated by ultrafiltration according to the method described in the literature (Fenglin et al., 2009). Briefly, after iced water cooling, caramel colour solutions were filtered through a Millex-HN nylon clarification kit of 0.22 μm pore size (Millipore, USA). 200 mL water-soluble caramel colour products diluted solution was subjected to ultrafiltration (FlowMen0015, Xiamen Shida Technology Ltd., Xiamen, China) equipped with 300, 100 and 50 kDa nominal molecular mass cut-off membrane (polysulphone membrane, formed by diaromatic sulphones, Xiamen Shida membrane Technology Ltd.). The 20 mL retentate was topped up to 200 mL with water and washed again. Washing step was repeated at six times. Each fraction was freeze-dried.

A prepared chromatography system (Lisui, China) was used with teflon column (1000 mm × 25 mm i.d.), recorder (Rec. 102), and detector (Uvicord SD, 195 and 405 nm). 0.2 g of melanoidin powder was finely ground and dissolved in 3 mL of distilled water, and then separated using gel chromatography. A 5-mL portion of the deep brown solution was put on a Sephadex column (G 150, GE Healthcare) and eluted with distilled water at a flow rate of 1.2 mL/min. Fractions (10 ml) were collected and analyzed by UV–VIS spectroscopy at 284 and 420 nm. Fractions exhibiting similar spectra were pooled and the solvent was evaporated. The separated polymers was verified by HPLC-Photodiode Array Detector (PAD) system, which consisted of a Ultrahydrogel™ Linear 7.8 mm × 300 mm column (Waters Co., USA), a Waters 600 pump and a Waters 2998 photodiode array detector (Waters Co., USA). The injection volume was 20 μL. The mobile phase was water at a flow rate of 0.5 mL/min. Column temperature was set at 25 °C. Spectral data from all peaks were accumulated in the range 200–800 nm. And the pooled fractions were freeze-dried for further tests.

2.4. Elemental analysis and pyrolysis-GC/MS

A Vario EL III (Elementar, Germany) was used for elemental analysis. And a thermo gravimetric analyzer (PerkinElmer, USA) with a Perkin Elmer Clarus 600 gas chromatograph (PerkinElmer, USA)-mass spectrometer (Perkin Elmer Clarus 600T) (PerkinElmer, USA) was used for the pyrolysis-GC/MS analysis. In all experiments, solid samples of caramel colour (Pa 2.37 mg and Pb 3.61 mg) were introduced inside the quartz tube (0.3 mm thickness) and plugged with quartz wool and inserted inside the coil probe. The pyroprobe was set at 40 °C at a heating rate of 100 °C/min to 900 °C. The pyroprobe interface temperature was set at 210 °C. The samples were introduced under splitless mode (valve was on after 1 min). Capillary direct MS interface temperature was 250 °C, and ion source temperature was 280 °C. The ionization voltage was 70 eV. The mass range analyzed was 45–315. The column was a fused silica Elite-1 (30 m × 0.25 mm ID × 0.25 μm df; Perkin Elmer). The column initial temperature was set at 40 °C for 10 min and then increased to 120 °C at a heating rate of 10 °C/min; immediately the temperature was further increased to 260 °C at a rate of 30 °C/min and kept at 260 °C for 10 min. The structures of the products were identified through library search facility of the HP software.

2.5. Enzymolysis caramel colour melanoidins fractions and HPLC analysis

Trypsin (4 mg) or pepsin (1 mg) was mixed with 100 mL caramel colour melanoidin solution with a concentration of 1%, and then heated at 37 °C for 20, 40 and 60 min. After aqueous thermal treatment, the solution was filtered through a Millex-HN nylon clarification kit of 0.45 μm pore size (Millipore, USA) for analysis by an HPLC-Photodiode Array Detector (PAD) system, which consisted of a Ultrahydrogel™ Linear 7.8 mm × 300 mm column (Waters Co., USA), a Waters 600 pump and a Waters 2998 photodiode array detector (Waters Co., USA). The injection volume was 20 μL. The mobile phase was water at a flow rate of 0.5 mL/min. Column temperature was set at 25 °C. Spectral data from all peaks were accumulated in the range 200–800 nm.

2.6. Determination of DPPH radical-scavenging activity

Ten mg of freeze-dried powder was dissolved in 10 mL deionized water for the determination of DPPH radical-scavenging activity. To 400 μL of caramel colour solutions, 2 mL of 0.12 mmol/L DPPH in ethanol was added. The mixture of caramel colour and DPPH–ethanol solutions were then allowed to stand at room temperature in the dark for 30 min. The absorbance of the mixture was measured at 517 nm using a TU-1901 spectrophotometer (Puxi, China). The control was prepared in the same manner, except that de-ionized water was used instead of caramel colour solutions. DPPH radical-scavenging activity was calculated by the following formula:

$$\text{Radical scavenging activity}\,(\%) = \left(\frac{1 - As}{Ac}\right) \times 100$$

where *As* is the absorbance value of sample. *Ac* is the absorbance value of the control.

2.7. MDA-MB-231 cells culture and survival analysis

Human breast cancer cell line MDA-MB-231 was maintained in a media of MD611 medium with 10% calf serum, and cultivated in 5% CO_2 at 37 °C. The media was changed every other day for 6 days. Cells were harvested by incubation with 0.05% trypsin–EDTA (Invitrogen Corporation, Carlsbad, CA), washed in phosphate-buffered

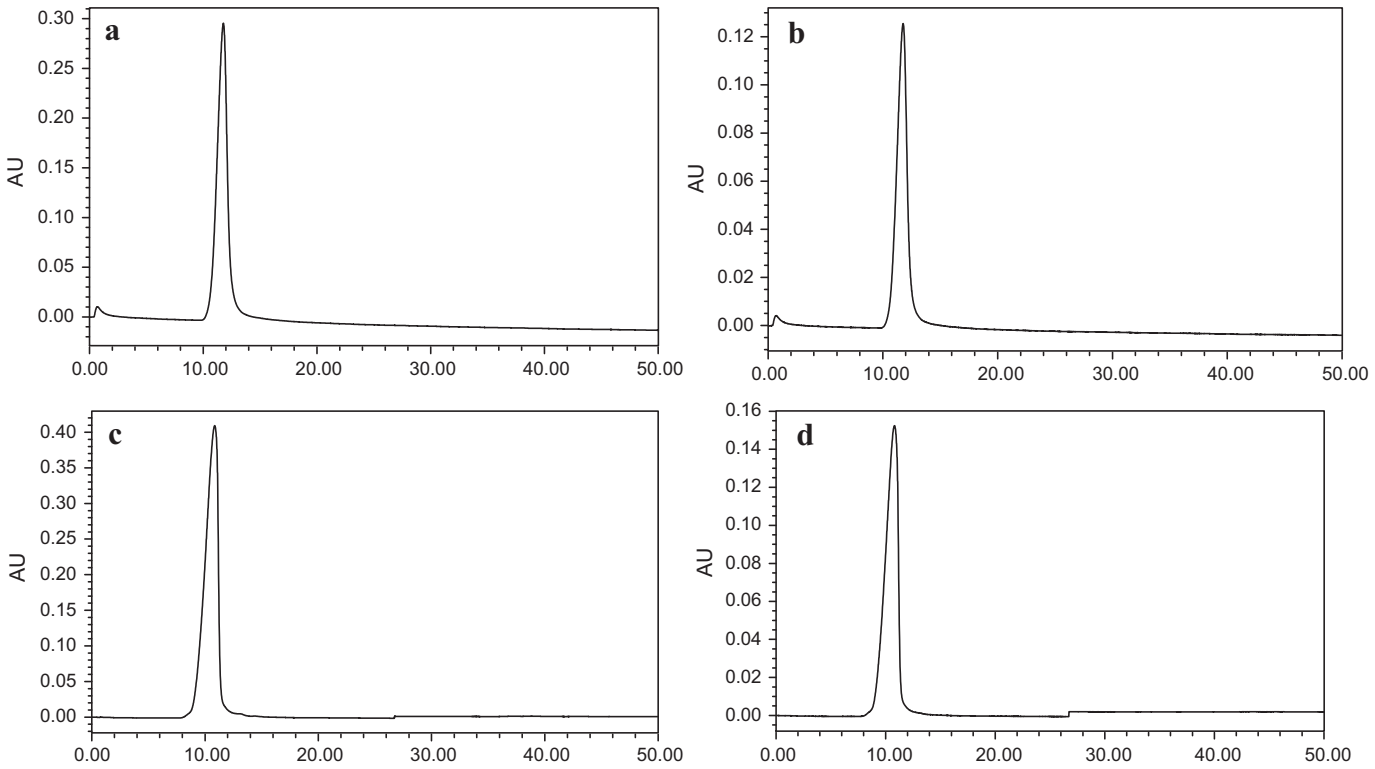

Fig. 1. HPLC-PAD analysis of the isolated glucose–ammonium sulfite caramel colour melanoidins fractions Pa at 284 nm (a) and 420 nm (b); and Pb at 284 nm (c) and 420 nm (d).

saline (PBS). The experiments were repeated twice, and tests were repeated eight times.

The survival analysis of MDA-MB-231 cells was determined by MTT-based colorimetric method (Mosmann, 1983). For glucose–ammonium sulfite caramel colour melanoidins sensitivity assay, MDA-MB-231 cells were plated in 96-wells plates. Cells were cultured for 48 h and then treated with glucose–ammonium sulfite caramel colour melanoidins (concentration, 1 mg/mL) in the media. The HMF solution with various volumes was added to the media containing human breast cancer cell line MDA-MB-231. And the volumes of glucose–ammonium sulfite caramel colour melanoidins and media were shown in Table 1. After 24, 48 and 72 h of incubation, cell survival was analyzed by using the MTT method. 20 μL 0.5% MTT was added to the media to incubate in 5% CO_2 condition at 37 °C for 4 h. After incubation, media was removed and 150 μL DMSO was added to dissolve the crystal fully, and the OD values at 490 nm were determined.

2.8. Statistical analysis

All experiments were carried out at least triplicates. Means and standard deviations of the data were calculated for each treatment.

Table 1
The volume of adding HMF solution to media containing human breast cancer cell line MDA-MB-231.

No.	HMF solution volume, μL	Media volume, μL
1	1	99
2	2	98
3	4	96
4	8	92
5	16	84

Analysis of variance (ANOVA) was carried out to determine any significant differences ($P < 0.05$) among the applied treatments by the SPSS package (SPSS 10.0 for windows).

3. Results and discussion

3.1. Structure analysis of caramel colour melanoidins

As shown in Fig. 1, two fractions (i.e. Pa and Pb) of the glucose–ammonium sulfite caramel colour melanoidins were isolated. Elemental analysis showed that Pa and Pb have the empirical formula $C_{408}H_{682}O_{199}N_{42}S_{19}$ and $C_{401}H_{611}O_{222}N_{37}S_{16}$, respectively. Pyrolysis-GC/MS showed that furfuran and sulfur dioxide were the main fractions of the glucose–ammonium sulfite caramel colour melanoidins after GC/MS analysis. Other chemical groups such as nitrogen heterocyclic were also found (Table 2). Possible explanation should be that bi-furan derivatives (part A) were formed after aldol condensation between two 5-hydroxymethyl-2-furaldehyde (HMF) moleculars. And sulfite ion could connect with carbon atom of formyl group of HMF by a nucleophilic addition and formed part B. At last, part A and B could polymerize and form the main skeleton of the glucose–ammonium sulfite caramel colour melanoidins. Moreover, side chain might contain pyrazine, 5-methyl-pyrimidine and furfural (Fig. 2).

As shown in Fig. 3a–d, both trypsin and pepsin could degrade Pa and Pb. A main degraded compound was detected as HMF after comparing the spectrum with standard HMF in early research (Guan, Zhu, Yu, Xu, & Shi, 2011b). This result indicated that the side chain of Pa contains amidolinks. And a proposed mechanism might be that the aldehyde group of HMF might connect with nitrogen atoms of pyrazine or pyrimidine, and formed amidolink. And plau-

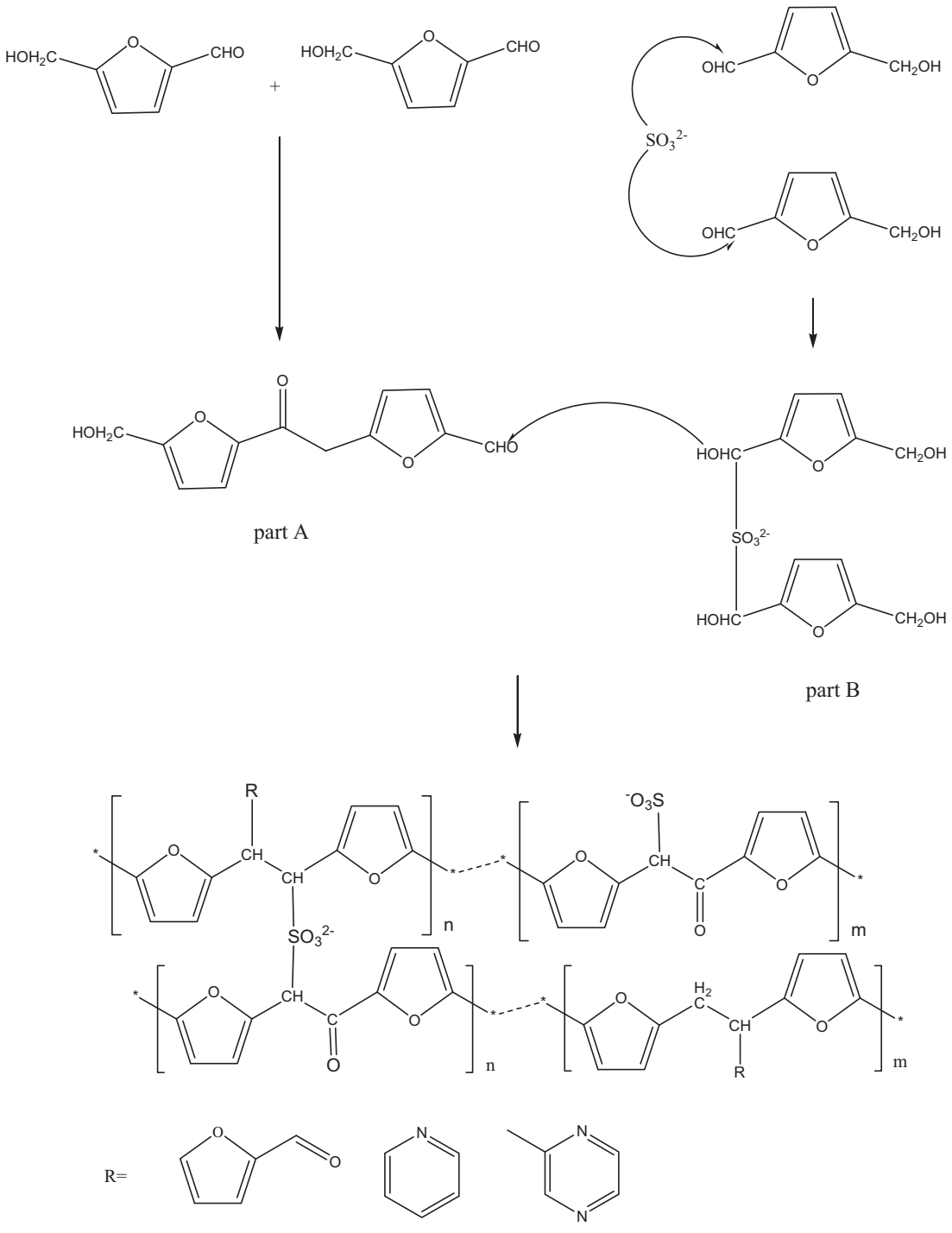

Fig. 2. Proposed structure and mechanism of formation of the glucose–ammonium sulfite caramel colour melanoidins.

sible mechanisms were proposed for the formation of polymers Pa and Pb based on spectroscopic data, and shown in Fig. 3e.

3.2. Changes in DPPH radical-scavenging activity

The scavenging activity of caramel colour melanoidins Pa and Pb on DPPH radical was depicted in Fig. 4a. Results showed that DPPH radical-scavenging activity ratio of Pb has a higher antioxidant activity, about 1.02 times higher than Pa, which means that glucose–ammonium sulfite caramel colour melanoidins may play a role in protection against cell oxidative damage. Proposed explanation, shown in Fig. 4b, should be that the single electron of DPPH could connect with the lone pair electrons of caramel colour melanoidins molecule, which leads to a decrease of A_{517}. And Pb might contain more single electron than Pa. On the other hand, caramel colour melanoidins Pa and Pb might react with free radicals and repair free-radical-induced damage through electron-transfer reactions, which is a main mechanism on antioxidant activity (Goya et al., 2007). This result was in accordance with that of Lertittikul, Benjakul, and Tanaka (2007), who found that the scavenging activity of DPPH frequently increased as the Maillard reaction was extended in the porcine plasma protein–glucose system. This result

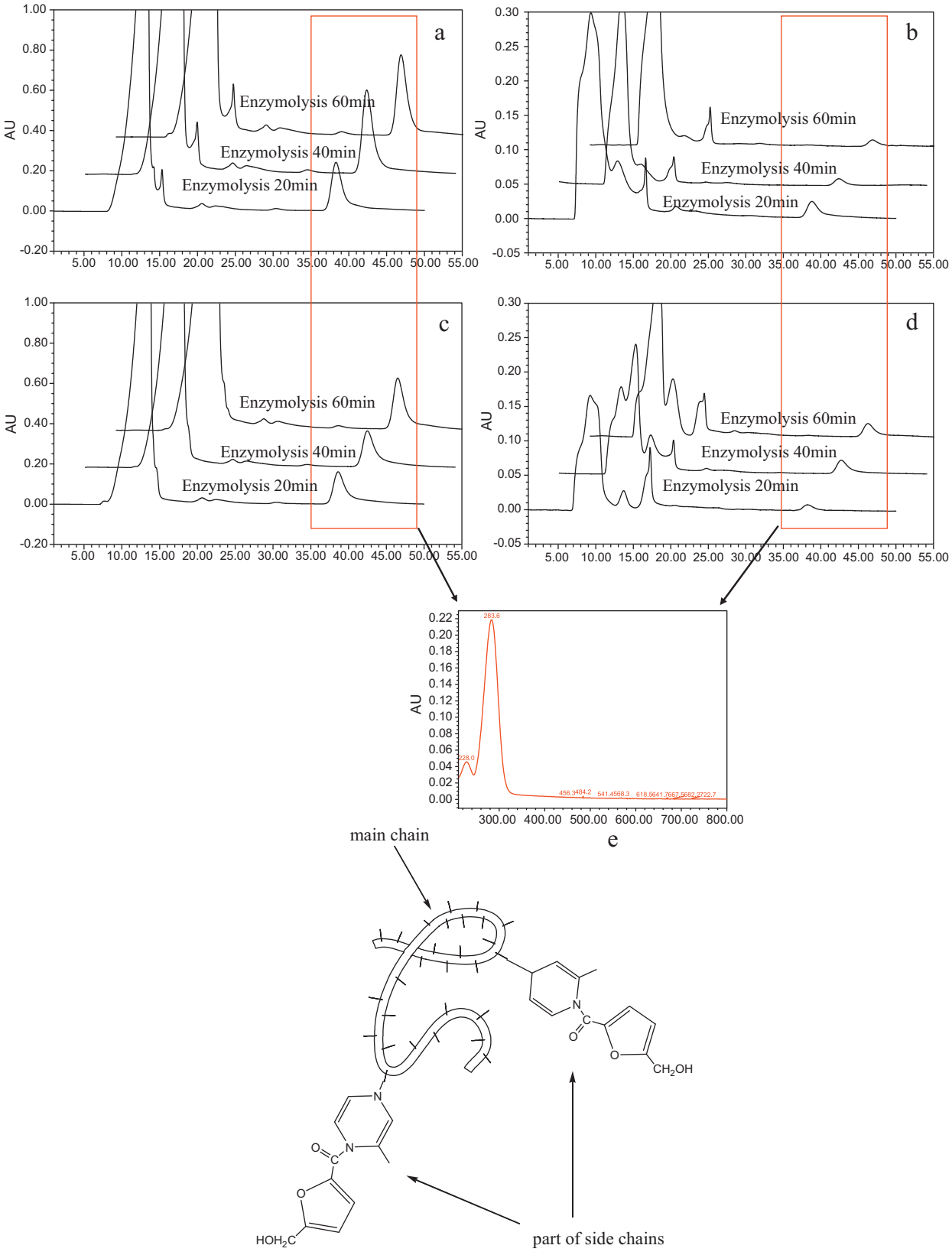

Fig. 3. HPLC analysis the glucose–ammonium sulfite caramel colour melanoidins; (a) added pepsin to Pa; (b) added pepsin to Pb; (c) added trypsin to Pa; (d) added trypsin to Pb; and (e) The formation of the side chain of glucose–ammonium sulfite caramel colour melanoidins.

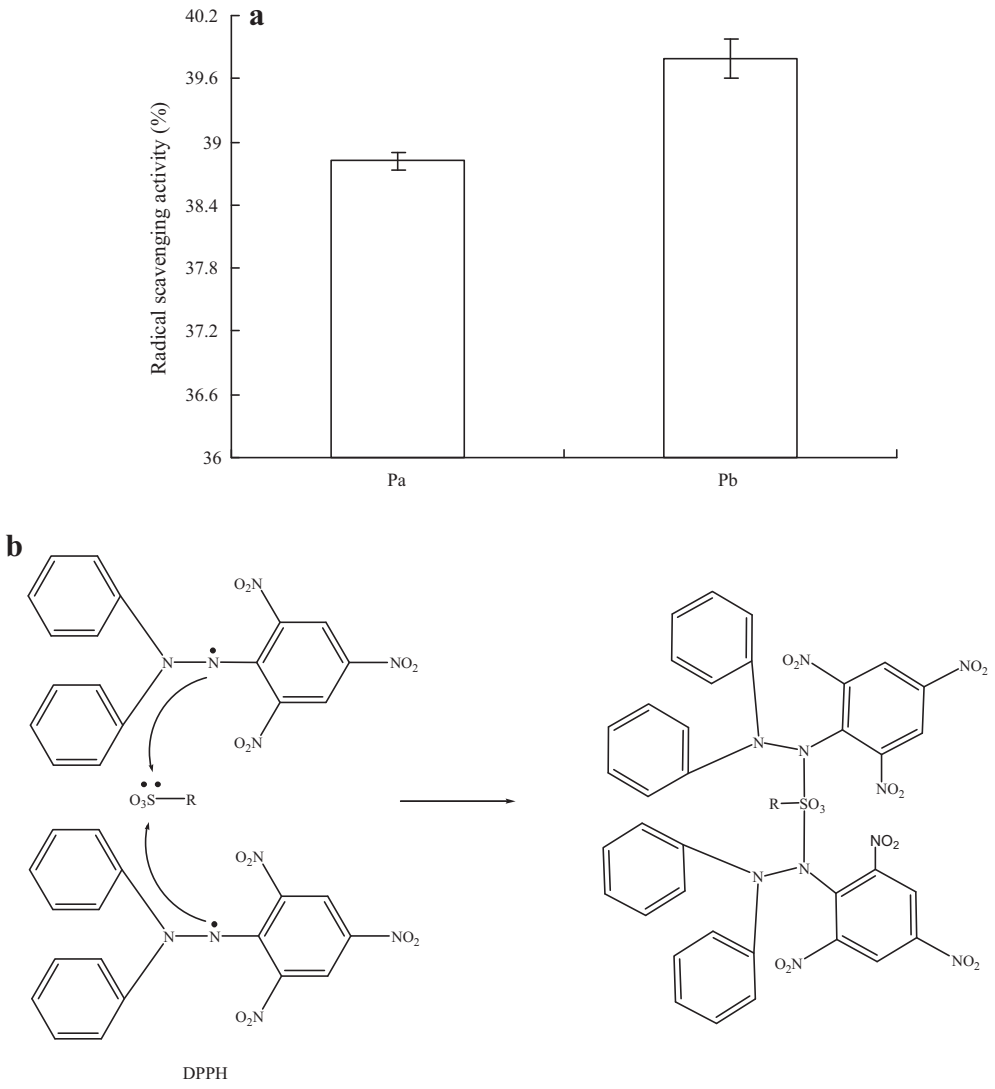

Fig. 4. DPPH radical-scavenging activity of of Pa and Pb (a); and proposed mechanism on DPPH radical-scavenging activity using glucose–ammonium sulfite caramel colour melanoidins, R-SO_2 means glucose–ammonium sulfite caramel colour melanoidins (b).

Table 2
Pyrolysis products of the glucose–ammonium sulfite caramel colour melanoidins in Pa and Pb.

Retention time (min)	Compounds	Structural formula
6.126	Sulfur dioxide	
7.924	Furan, 2,5-dimethyl-	
11.011	Pyrazine	
13.022	Pyrimidine, 5-methyl-	
14.468	Furfural	

was also in accordance with that of Guan et al. (2010a), who found that the Maillard reaction products have a significant scavenging activity of DPPH.

3.3. Effects of caramel colour melanoidins on MDA-MB-231 cells

In this study, the effects of Pa and Pb on the growth of MDA-MB-231 cells were investigated by MTT assay. As shown in Fig. 5, the inhibitory effects on the growth of MDA-MB-231 cells increased significantly ($P < 0.05$) with the increase of addition volume of Pa for 72 h, and Pb for 48 and 72 h incubation. However, an interesting phenomenon was found that accelerated effect on the growth of MDA-MB-231 cells was found after 24 h incubation. Frankly, the mechanism of accelerating growth of MDA-MB-231 cells has not been clear. But the mechanism of inhibiting MDA-MB-231 cells by Pa for 72 h, and Pb for 48 and 72 h incubation might be explained as follows. Some of the antioxidative compounds may play a role in protection against cell oxidative damage, and inhibit the cancer cells growth (Goya et al., 2007). Moreover, some of the genotoxic and redox responses to MRPs by cells may reflect prooxidant rather than antioxidant activity (Goya et al., 2007). Therefore, the higher anticancer activity of Pa for 72 h, and Pb for 48 and 72 h incubation might be attributed to its higher scavenging activity on

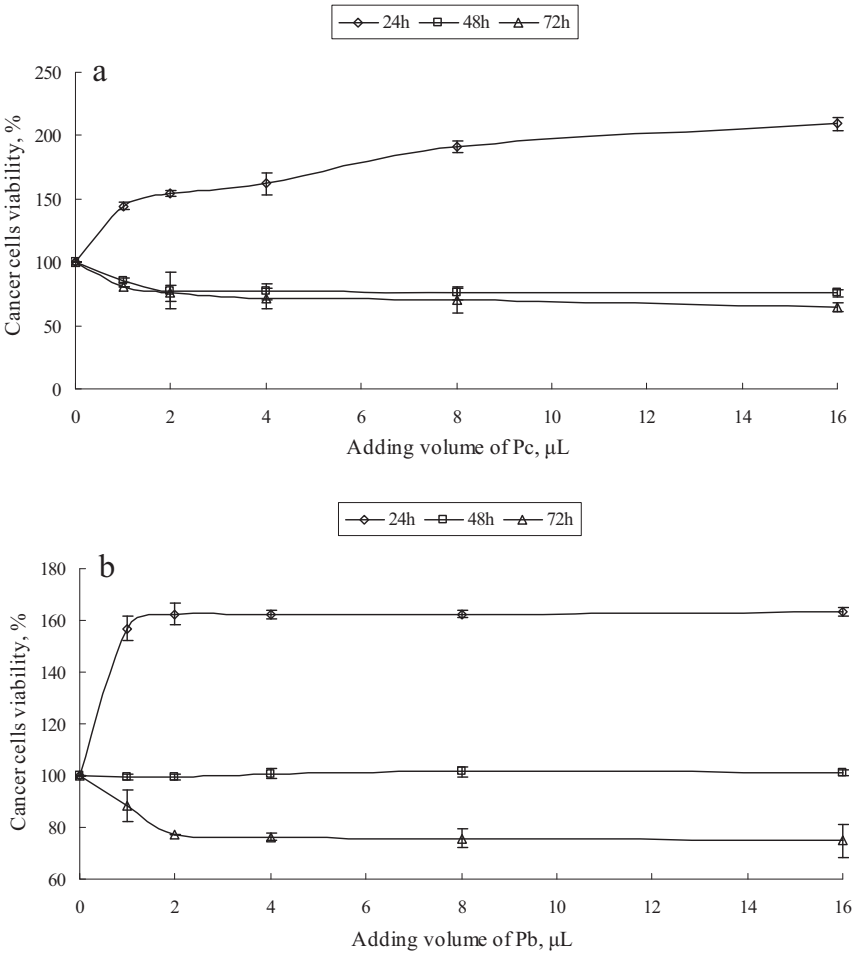

Fig. 5. Effects of Pa (a) and Pb (b) on MDA-MB-231 cells survival.

free radicals. However, the phenomenon, i.e. accelerating effect on the growth of MDA-MB-231 cells as adding Pa and Pb for 24 h incubation, cannot be explained recently, and should be further detailed.

4. Conclusion

(1) Two polymers, i.e. Pa and Pb, could be isolated using ultrafiltration and gel chromatography, and both of them might be formed by furfuran as the main skeleton, which might be formed by the aldol condensation between HMF molecules. The side link of glucose–ammonium sulfite caramel colour melanoidins might contain amidolinks connecting between aldehyde of furfuran and nitrogen atoms. However, the proposed mechanism should be confirmed in further study.
(2) Glucose–ammonium sulfite caramel colour melanoidins have a significant DPPH radical-scavenging activity. And the radical-scavenging mechanism still needs to be clearly understood, even if a proposed mechanism has been stated in this paper.
(3) Glucose–ammonium sulfite caramel colour melanoidins could inhibit the growth of MDA-MB-231 cells after relative long time incubation. The mechanism might be that higher scavenging activity on free radicals leads to an inhibition on the growth of cancer cells. However, the reason on accelerating the growth of MDA-MB-231 cells after adding glucose–ammonium sulfite caramel colour melanoidins for a short time incubation still need further research.

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