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Gel-Based Chemical Cross-Linking Analysis of 20S Proteasome Subunit-Subunit Interactions in Breast Cancer*

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Summary: The ubiquitin-proteasome system plays a pivotal role in breast tumorigenesis by controlling transcription factors, thus promoting cell cycle growth, and degradation of tumor suppressor proteins. However, breast cancer patients have failed to benefit from proteasome inhibitor treatment partially due to proteasome heterogeneity, which is poorly understood in malignant breast neoplasm. Chemical cross-linking is an increasingly important tool for mapping protein three-dimensional structures and protein-protein interactions. In the present study, two cross-linkers, bis (sulfosuccinimidyl) suberate (BS^3) and its water-insoluble analog disuccinimidyl suberate (DSS), were used to map the subunit-subunit interactions in 20S proteasome core particle (CP) from MDA-MB-231 cells. Different types of gel electrophoresis technologies were used. In combination with chemical cross-linking and mass spectrometry, we applied these gel electrophoresis technologies to the study of the noncovalent interactions among 20S proteasome subunits. Firstly, the CP subunit isoforms were profiled. Subsequently, using native/SDS-PAGE, it was observed that 0.5 mmol/L BS^3 was a relatively optimal cross-linking concentration for CP subunit-subunit interaction study. 2-DE analysis of the cross-linked CP revealed that α_1 might pre-interact with α_2, and α_3 might pre-interact with α_4. Moreover, there were different subtypes of $\alpha_1\alpha_2$ and $\alpha_3\alpha_4$ due to proteasome heterogeneity. There was no significant difference in cross-linking pattern for CP subunits between BS^3 and DSS. Taken together, the gel-based characterization in combination with chemical cross-linking could serve as a tool for the study of subunit interactions within a multi-subunit protein complex. The heterogeneity of 20S proteasome subunit observed in breast cancer cells may provide some key information for proteasome inhibition strategy.

Key words: breast cancer; proteasome; chemical cross-linking; protein-protein interaction; three-dimensional gel electrophoresis

The ubiquitin-proteasome system (UPS) is an important regulator of cell growth and apoptosis. This system includes ubiquitin, ubiquitin-activating enzyme E1, ubiquitin conjugating enzyme E2, ubiquitin ligases E3 and proteasome[1]. Mounting evidence has revealed the correlations between a poor prognosis in breast cancer and low levels of $p27^{KIP1}$ possibly due to increased proteasomal degradation[2, 3]. Moreover, the ligand-dependent degradation of α subunit of the estrogen receptor (ESR1), a promising target for luminal subtype of breast cancer, is mediated by different E3 ligases[4]. By contrast, ligand-independent degradation of v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) is mediated by STIP1 homology and U-Box containing protein 1 (STUB1), also known as C terminus of HSC70-interacting protein (CHIP) in collaboration with heat-shock proteins Hsp70 and Hsp90[5]. Therefore, the UPS can be a potential target for breast cancer treatment.

The 26S proteasome, which contains the proteins responsible for proteolysis in a 20S core, is responsible for degradation of ubiquitinated products. The 20S core complex consists of four rings of 28 nonidentical subunits ($\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$) forming a symmetrical barrel-shaped structure[6, 7]. Previous study indicated that 20S proteasomes from tissues or cells included different subtypes[8]. The proteasome activity and biological function may be related to 20S proteasome heterogeneity[9]. And the sensitivity to proteasome inhibition is correlated with the expression levels and composition of proteasome subunits[10]. Though bortezomib, a proteasome inhibitor, has shown great success in the treatment of multiple myeloma[11], it has failed to show a significant clinical effect on breast cancer[12]. It might be attributed to high structural complexity and great heterogeneity of 20S proteasome, which may be related to disease phenotypes and affect the sensitivity of proteasome inhibitors against

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20S proteasome from tissues and cell lines[13, 14].

Study on protein function often involves the three-dimensional structure of protein and its interaction proteins. Most of these protein play a biological function via protein complexes or protein-protein interactions. Therefore, identifying non-covalent interactions between different proteins will help us to understand the specific molecular mechanisms underlying various biological processes. Chemical cross-linking technology, a new method available in recent years, is used for mapping protein three-dimensional structures and protein-protein interactions[15, 16]. Using high sensitive mass spectrometry, chemical cross-linking technology can be used to investigate conformational structures of proteins and protein-protein assemblies in addition to traditional structural analysis technologies (such as NMR and X-ray diffraction). Unlike traditional technologies, it is suitable for analyzing low-concentration analytes without protein crystallization in special solution, and automating data analysis using software[15].

Previous study found that there were differences in the subunit compositions and subtypes of the 20S proteasomes among different pancreatic cancer cell lines and other tissues[17, 18]. However, the 20S proteaseme heterogeneity and subunit-subunit interactions in breast cancer remain to be elucidated. In the present study, 20S proteasomes were purified from an aggressive human triple negative breast cancer cell line MDA-MB-231 and then cross-linked with two chemical reagents, disuccinimidyl suberate (DSS) and soluble analog-bis (sulfosuccinimidyl) suberate (BS^3), followed by a gel-based proteomics analysis to investigate non-covalent interactions in multi-subunit protein complex.

1 MATERIALS AND METHODS

1.1 Cell Line and Reagents

Human breast cancer cell line MDA-MB-231 was obtained from the Cell Bank of the Chinese Academy of Sciences (China) where they were characterized by mycoplasma detection, DNA-Fingerprinting and cell vitality detection. The MDA-MB-231 cells were cultured in Leibovitz's L-15 medium containing 10% fetal bovine serum. All the cell cultures were maintained at 37°C. Acetonitrile (ACN, HPLC), formic acid, trifluoroacetic acid, BS^3, and DSS were purchased from ThermoFisher (USA). α-cyano-4-hydroxy cinnamic acid (CHCA), bovine thyroglobulin (used as a 670 kD marker protein in non-denaturing PAGE), and silver nitrate were from Sigma-Aldrich (USA). Unmodified and modified sequencing grade trypsins were bought from Roche Diagnositics (Germany). Two-dimensional gel electrophoresis reagents were from GE Healthcare Life Sciences (UK) or Sigma-Aldrich (USA). Other reagents were obtained from Beijing Chemical Works (China).

1.2 Raw Purification of 20S Proteasome by Differential Centrifugation

Raw purification protocol of 20S proteasome was performed by differential centrifugation as described previously[19]. Briefly, 2 g of MDA-MB-231 cells (wet weight, about 0.8×10^9 cells) were lysed in buffer A (20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $MgCl_2$, 100 mmol/L NaCl, 1 mmol/L DTT, 0.2% NP-40 and with 1× Roche protease inhibitor cocktail). Subsequently, the lysates were centrifuged for 30 min at 20 000 × g to remove cellular debris. Differential centrifugation was performed using Beckman Optim Optima XL-80 (Beckman Coulter, USA) with Type 55.2 Ti rotor. Centrifugation at 154 000 × g was performed for 1 h at 4°C to remove ribosome. The ribosome-free supernatant was adjusted to 4.5 mL by the addition of buffer B (50 mmol/L HEPES-NaOH, pH 7.5, 5 mmol/L $MgCl_2$, 100 mmol/L NaCl, and 1 mmol/L DTT), and subjected to consecutive triplicate sedimentations at 198 000 × g for 360 min at 4°C. Glycerol was added to the supernatant at a final concentration of 40%, and the resulting crude 20S core particle (cCP, partially purified) was stored at –20°C.

1.3 Native PAGE and 2-D IEF/SDS-PAGE (2-DE) of 20S Proteasome

Native-PAGE was based on the approach as described by Elsasser[20]. The crude core particle (CP) samples were separated using 5.5% native-PAGE. Electrophoresis was carried out at 50 V for 1 h, followed by 150 V for 3–4 h at 4°C. After Coomassie staining, the band of CP was excised and destained in a solution containing 50% ACN and 25 mmol/L ammonium bicarbonate, followed by dehydration in ACN. After dehydration, 500–550 μL denaturation solution [7 mol/L urea, 2 mol/L thiourea, 2% 3-(3-(bile amido propyl) dimethylammonio) propanesulfonate (CHAPS), 1.2% Destreak™ and 0.5% IPG buffer (pH 4–7 or 3–10, non-linear)] was added. Then the gel was crushed with a clean tweezers and was placed at room temperature for ≥ 2 h. The mixture was crushed into fine particles and added in IEF. The sample was separated according to two-dimensional electrophoresis: IEF DryStrip ™ pH 3−7 or 3−10 (non-linear, 24 cm) was used as first dimension, then 8% or 10% SDS-PAGE system was taken as second dimension. The CP subunit proteins were visualized on 2-DE map by Coomassie staining or silver nitrate staining.

1.4 Optimized Chemical Cross-Linking and 2-D Native/SDS-PAGE of 20S Proteasome

To optimize the concentration of BS^3 cross-linker, different volumes of freshly prepared 50 mmol/L BS^3 containing 25 mmol/L Hepes-NaOH, pH 7.5, 5 mmol/L $MgCl_2$, 150 mmol/L NaCl were added in cCP solution. The final concentration of BS^3 was 0.1, 0.2, 0.5, 1, 2, 5 and 10 mmol/L, respectively, and placed at 4°C for 2 h. Stop solution (50 mmol/L Tris-HCl, 100 mmol/L-glycine, pH 7.5) was added to terminate the cross-linking reaction.

Subsequently, the cross-linked CP (×CP) was separated in 5.5% native-PAGE. The ×CP band was excised and dehydrated by ACN. After dehydration, 50 μL denaturation solution [250 mmol/L Tris-Cl (pH 6.8), 20% glycerol, 2% SDS, 40 mmol/L DTT and traces of bromophenol blue] was added. The mixture was boiled for 10 min, added with 300 μL water, and then cooled to room temperature for 60 min. After centrifugation, the supernatant was taken and dried to approximately 50 μL with vacuum, and then the mixture was separated by 10% SDS-PAGE.

1.5 Gel Imaging, Mass Spectrometry Analysis and Database Searches

2-DE gel image analysis was performed using the Image-Master 2D Platinum V5.0 software. Spot detection was performed automatically, and spot volume percentages were calculated after the normalization of each 2-D gel. The relative protein quantification was achieved by calculating the average spot volume percentages (vol%) for three experiments.

Stained protein spots were excised and in-gel digestion was performed as described previously. Briefly, 2-DE protein spots were digested with trypsin. Then 1 μL 5% trifluoroacetic acid was added to stop the reaction. Afterwards, 0.7 μL sample was spotted on MTP Anchorchip™ 384/600 target plates. After drying at room temperature, 0.7 μL 0.67 mg/mL CHCA (70% acetonitrile in 0.1% trifluoroacetic acid) was covered on sample. After drying completely, the sample was identified with Autoflex III MALDI-TOF/TOF tandem mass spectrometer (Bruker Daltonics, Germany).

Parameters of Swiss-Prot database were set as follows: relative error of peptide mass fingerprinting (PMF) was 1/10 000, MS/MS error was 1 Da; trypsin maximum leakage cut was 1; fixed modification was homocystine carbamidomethylation; variable modification was methionine oxidation and N-terminal acetylation. In addition, the MS data analysis software MS-Fit (http://prospector.ucsf.edu/prospector/mshome.htm; Protein Prospector, UCSF, USA) was used.

2 RESULTS

2.1 Characterization of CP Subunit Isoforms in Breast Cancer

Previous findings indicated that 20S proteasomes from tissues or cells included different subtypes. In this study, a native-gel based purification was taken for analysis of intact and active 20S proteasome. After differential centrifugation, raw purified 20S proteasome CP from MDA-MB-231 cells was resolved on 5.5% native-PAGE and CP subunits were further separated by 2-DE (fig. 1). The band with molecule weight (MW) of about 700 kD, which was identified as 20S proteasome by MS analysis, was excised (fig. 1A), followed by 2-DE analysis. As shown in fig. 1B, a total of 36 subunits of subtypes were observed and their corresponding isoelectric point (pI) value range was from 3.0 to 10.0 with MW range of 23–32 kD. Notably, most subunits had two or more different MW or pI subtypes, suggesting that numerous subunit isoforms exist in 20S proteasome.

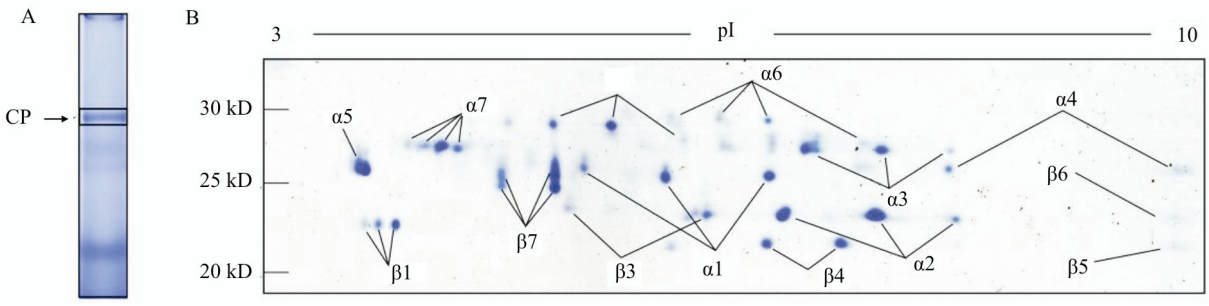

Fig. 1 CP subunits separated with 3-DE, consisting of 5.5% native-PAGE (A) and 2-DE (B)

2.2 Optimization of Chemical Cross-Linking

N-hydroxysuccinimide ester (NHS) is recently a widely used reagent for cross-linking technology. Via a nucleophilic reaction with primary amino group (N-terminal protein ε-amino side chains or lysine) or secondary amino group, it forms a stable amide or imide bond[15, 21]. DSS and its soluble analog—BS^3 belong to the same group of homobifunctional cross-linking agent[21] (fig. 2).

In the present study, using 2-D native/SDS-PAGE technology, we firstly optimized concentrations of BS^3. By using cross-linking, CP first reacted with different concentrations of BS^3 (0, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mmol/L), and the resulting ×CP was separated with 5.5% native-PAGE. Fig. 3A shows that with increasing concentration of BS^3, ×CP electrophoresis migration distance is gradually increased, and with increasing "width" of strips as well. Further SDS-PAGE separation results showed that when $0 < BS^3 < 0.5$ mmol/L, cross-linking reaction of double-subunit (MW 40–60 kD) increased gradually. When 1 mmol/L $< BS^3 <$ 10 mmol/L, cross-linking reaction of multi-subunit (MW > 60 kD) increased. When BS^3 was up to 10 mmol/L, CP covalent was almost completely "bundled" with cross-linking agent. Due to the large molecular weight, CP could not dissociate and aggregate on the edge of SDS-PAGE. Because excessive chemical cross-linking reaction may lead to the changes of CP spatial structure, we chose 0.5 mmol/L as the optimizing concentration of BS^3. Using this BS^3 concentration, CP double-subunit (MW 40–60 kD) has the highest cross-linking.

2.3 Analysis of CP Subunits Chemical Cross-Linking Reaction

Zong *et al* previously reported 2-DE based characterization of post-translational modifications of mammalian 20S proteosome complexes[14]. In this study, 0.5 mmol/L BS^3 was used for cross-linking reaction and ×CP was characterized in 2-DE. As shown in fig. 4A, compared with 2-DE map (fig. 1) of non-cross-linked CP, the majority of subunits separated from monomer CP (lower bands in fig. 3B) migrated to the acidic side, which means that pI became smaller. The reason may be that most subunits reacted as class I cross-linking. Among them, acidic side migration was observed obviously by

subunit α_2, β_3, and β_4. Experimental results also showed that some proteins present "transverse" smearing or partial dispersion, which might lead to decreased protein amount per spot and thus some proteins could not be successfully identified. Fig. 4B shows MS/MS spectrum in which α_2 peptide reacted as class I cross-linking (^{71}LNLYELK $^{(\times)}$EGR^{80}, where the ^{77}K was cross-linked with BS^3).

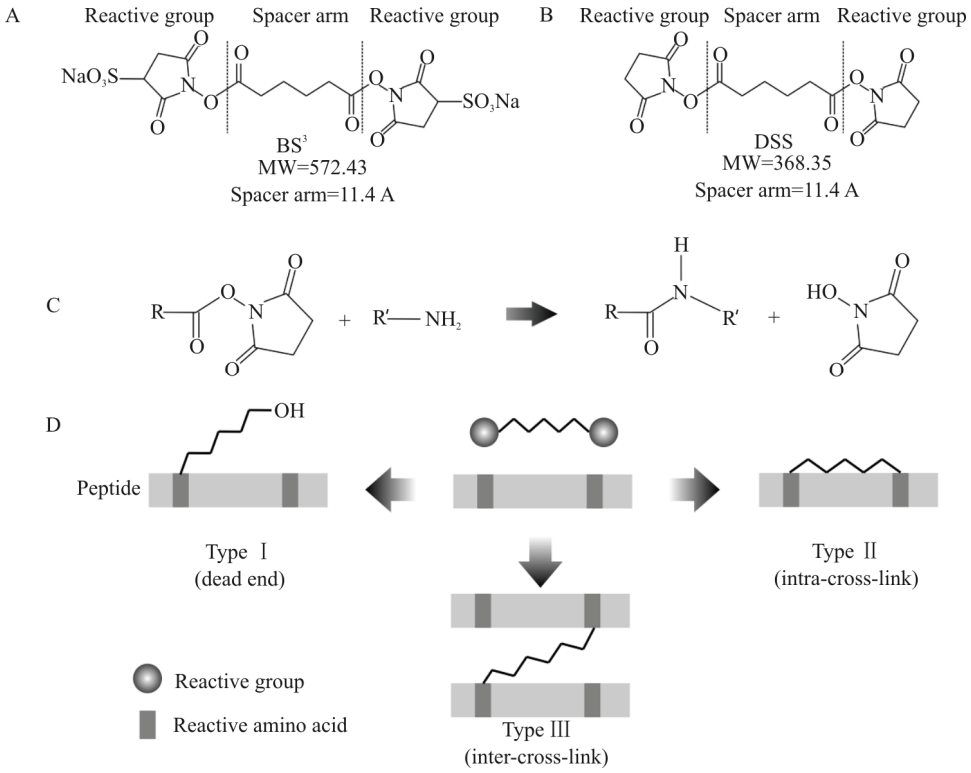

Fig. 2 BS^3 (A), DSS (B), NHS reaction (C) and three types of cross-linking reactions (D)

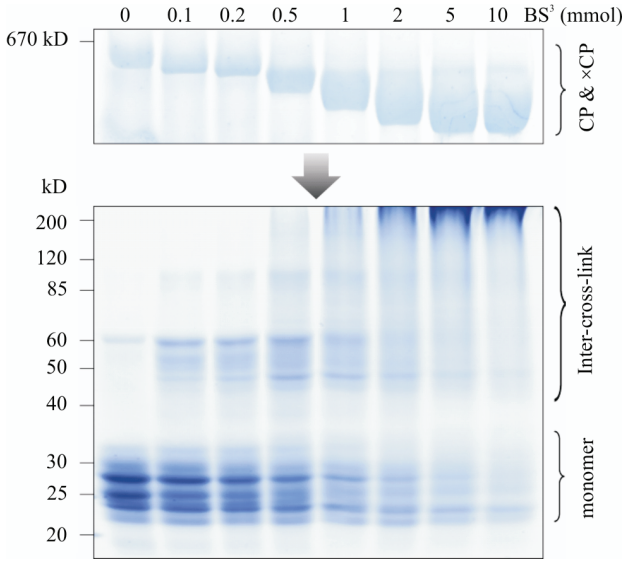

Fig. 3 BS^3 concentration optimization using 2-D native/SDS-PAGE, consisting of 5.5% native-PAGE (A) and 10% SDS-PAGE (B)

Then we investigated class III subunit cross-linking (MW 40–60 kD) with mass spectrometry. Due to the low protein gel point within this molecular weight, we used silver nitrate staining. Fig. 5A and table 1 indicate that there are interactions between α_1 and α_2, as well as α_3 and α_4 using PMF and MS/MS. It also indicates that the interaction between α_3 and α_4 was presented as two different types (fig. 5A). This may be due to the multiple cross-linking sites or linkage pattern, resulting in significantly different 2-DE behavior in term of pI and molecular weight. Alternatively, it is due to the presence of different types of CP (i.e. proteasome heterogeneity). The different types of CP may be derived from not only the post-translational modifications of subunits, but also different forms of non-covalent interactions between the subunits.

In term of characteristic, DSS chemical reaction is very similar to BS^3, but it has a strong hydrophobicity. In general, a small molecule with a strong hydrophobic region is easier to access the hydrophobic interaction region between protein subunits. CP-linking reaction *in vitro* showed there was no significant difference by hydrophobic DSS in cross-linking reaction (fig. 5B). This result might suggest that interactions between certain CP subunits are electrostatic interaction and/or hydrogen based.

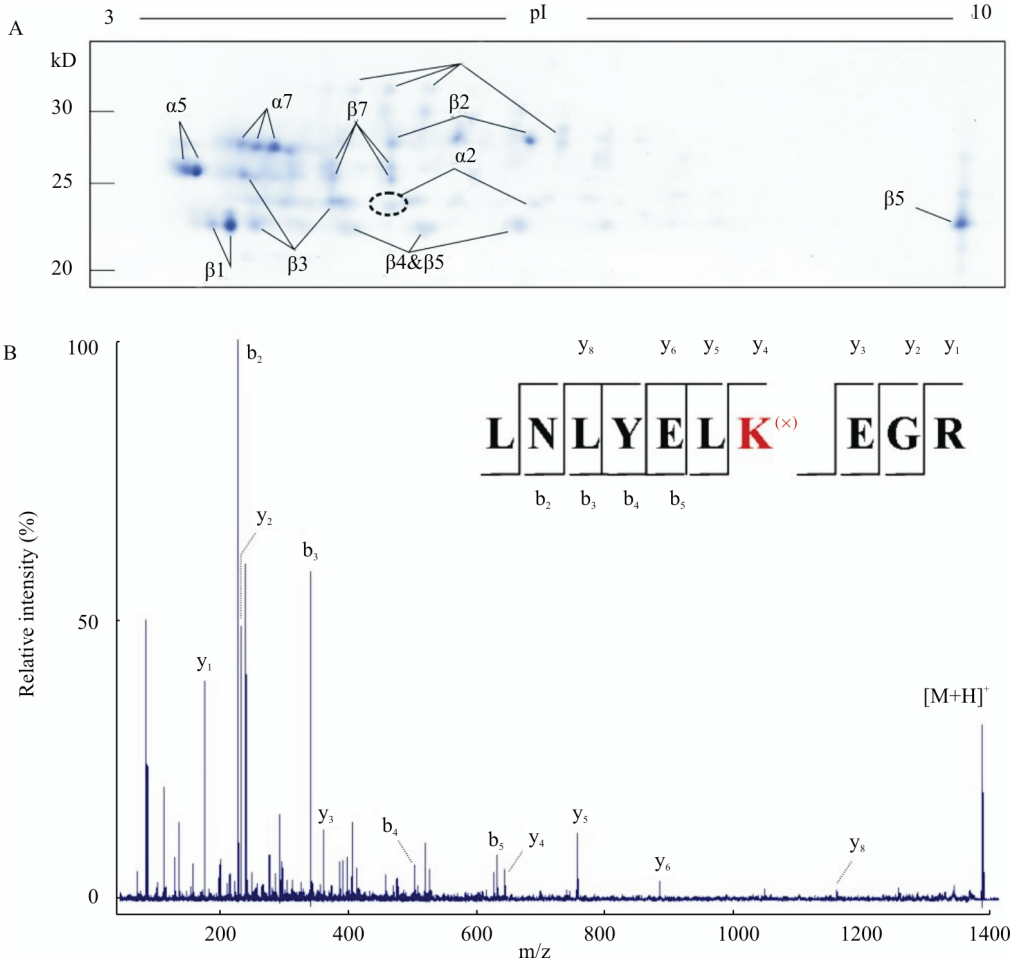

Fig. 4 Separation of subunits from cross-linked monomer CP by 2-DE (A) and MS/MS spectrum of α_2 tryptic peptide ^{71}LNLYELK$^{(\times)}$EGR^{80} (B)
Circle: α_2 isoforms modified by BS^3

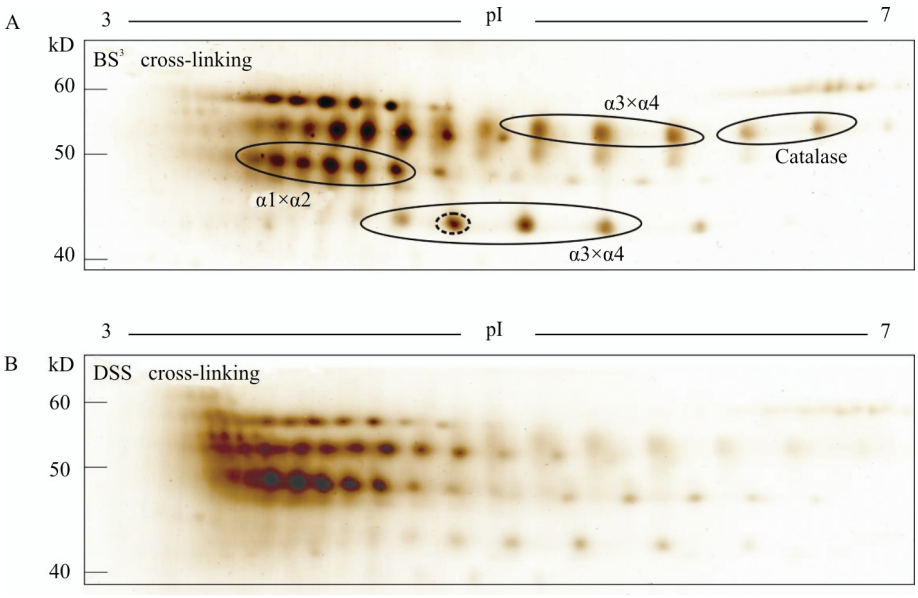

Fig. 5 Silver-stained 2-DE maps obtained from CP cross-linked with BS^3 (A) and DSS (B)

Table 1 Identification of cross-linked subunits of 20S proteasome as shown in fig. 5A

Cross-link	Subunit	Swiss-Prot	PMF coverage (%)	Peptide sequences identified by MS/MS
$\alpha_1 \times \alpha_2$	α_1	P60900	59	ARYEAANWK HITIFSPEGR AINQGGLTSVAVR ILTEAEIDAHLVALAERD
	α_2	P25787	32	SILYDER
$\alpha_3 \times \alpha_4$ (40 to 50 kD)	α_3	P25789	26	TTIFSPEGR LSAEKVEIATLTR LLDEVFFSEK QKEVEQLIK
	α_4	O14818	60	LTVEDPVTVEYITR
$\alpha_3 \times \alpha_4$ (50 to 60 kD)	α_3	P25789	26	TTIFSPEGR LSAEKVEIATLTR
	α_4	O14818	60	LTVEDPVTVEYITR
Catalase		P04040	54	GPLLVQDVVFTDEMAHFDR GAGAFGYFEVTHDITK LSQEDPDYGIR LFAYPDTHR

3 DISCUSSION

20S proteasome CP is an active core of proteasome, and MW is about 700 kD. It consists of 14 different subunits, α_1–α_7 and β_1–β_7 (MW 20–30 kDa), respectively, and forms a barrel structure. Previous study indicated that 20S proteosomes from tissues or cells included different subtypes[8]. These subtypes have different physical and chemical properties such as MW, pI and protein degradation function. This phenomenon is described as proteasome heterogeneity[13, 22]. In the present study, our 2-DE map (fig. 1) showed that each subunit has more than two spots, and each spot is one isoform of the subunit. When all the subunit isoforms are restructured to an intact 20S proteasome, there may be more than billions of kinds of 20S proteasome theoretically. Biochemical studies on proteasome are hampered by the limitations of the current purification protocols such as affinity chromatography and biochemical approaches due to a heterogeneous population of proteasome[23, 24]. The molecular basis of protein heterogeneity is that various components of proteasome subunits have different splicing in mRNA or proteins, post-translational modification or chemical modification *in vitro*[25].

NHS cross-linking reagent involves in three different cross-linking reactions: (a) Class I: NHS reaction group reacts with proteins/peptides, another group is hydrolyzed to form a hydroxyl group; (b) Class II: two groups react with the same proteins/peptides; (c) Class III: two groups react with two different proteins/peptides (fig. 2C and 2D). Until now, there has been no standard to optimize concentrations of cross-linking agent for protein-protein interactions. In this study, we examined chemical cross-linking reactions in CP subunits by using non-linear concentration gradient of BS^3. As shown in fig. 3A, with increasing dose of BS^3, the MW of CP complex became greater and greater. The increasing of electrophoresis mobility is mainly because there is no reaction between a NHS group of BS^3 and residues (mainly lysine) of side chains, and NHS reactive groups hydrolyze to form hydroxyl. In alkalic, non-denaturing electrophoresis conditions, the negative charge of protein increases significantly and leads to an increase of electrophoresis mobility. Further SDS-PAGE analysis of ×CP revealed that with increasing concentration of BS^3, the amount of protein decreased when subunits (MW 20–30 kD) reacted as class I/II chemical cross-linking. The amount of protein increased when subunits (MW > 40 kD) reacted as class III chemical cross-linking (fig. 3B).

2-DE analysis of the non-cross-linked monomer CP (MW 20–30 kD) showed that the majority of subunits migrated to the acidic side, which means that pI became smaller. The reason may be that most subunits were reacted as class I cross-linking. In contrast, 2-DE analysis of the ×CP revealed that α_1 might pre-interact with α_2, and α_3 might pre-interact with α_4. Moreover, there were different subtypes of $\alpha_1\alpha_2$ and $\alpha_3\alpha_4$ due to proteasome heterogeneity. Our findings indicated the presence of multiple isoforms for all the subunits and showed a high degree of heterogeneity of the CP, which may be related to cancer cell property, proteasome activities and inhibitor sensitivities.

In this study, we used gel electrophoresis technology and proteomics technology to investigate the interactions between CP subunits. The results showed that the gel-based characterization in combination with chemical cross-linking could serve as a tool for the study of subunit interactions within a multi-subunit protein complex. Moreover, the 20S proteasome subunit heterogeneity observed in this study may provide some key information for proteasome inhibition strategy in future cancer treatment. It should be noted that a different protein complex may require different chemical cross-linking conditions. Further studies could use isotopically labeled cross-linking agent, in order to measure interaction sites between subunits.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organizations or corporations or individuals that can inappropriately influence this work.

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