Original Article

Conformational study reveals amino acid residues essential for hemagglutinating and anti-proliferative activities of *Clematis montana* lectin

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Introduction

Plant lectins are a class of highly diverse carbohydrate-binding proteins of non-immune origin that contain at least one non-catalytic domain for selective recognition and reversible binding of cells, carbohydrates, and glycoconjugates [1]. Such structural characteristics confer the bioactivities of lectins to agglutinate not only blood cells but also sperm cells and lymphocytes, which were involved in several important physiological and pathological processes, including cancer [2,3].

The *Clematis* genus, which is mainly distributed in subtropical countries, has been used as traditional medicines in India and China since the ancient time [4,5]. In China, *Clematis armandii* and *Clematis montana* called ‘mu tong’ are used to induce urination, reduce fever, stimulate menstrual discharge, and promote lactation [6]. *Clematis montana* lectin (CML) isolated from the air-dried slice of Akebia *Clematis* stems is a mannose-binding protein with two identical subunits. Previous study has established that when the carbohydrate-binding site of CML was blocked by Man-α(1,6)-Man, its apoptosis-inducing activity disappeared [7], indicating that the carbohydrate-binding activity is essential for CML to function as an apoptosis inducer. Therefore, further exploration of the conformational details of CML carbohydrate-binding site will reveal the intricate relationships between structure and bioactivity, and thus benefit the elucidation of the apoptosis-inducing mechanisms of CML.

Fluorescence spectroscopy, a developed technology used to reveal the structural information of indicated proteins [8], shows several advantages in structural research, such as no need to crystallize the protein, easy to apply, less dependent on equipment, and more suitable to monitor dynamic conversion [9]. The fundament of the protein fluorescence spectroscopy is the fluorescent feature of three aromatic amino
acids, namely, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) [10]. Among these three special residues, Trp is the dominant intrinsic fluorophore with a highly rare presence in protein, and thereby the analysis of protein fluorescence spectroscopy is mainly to interpret the Trp spectral data [11]. Notably, Trp fluorescence spectroscopy is extremely sensitive to the solution condition and the micro-environment around it [12]. Therefore, such properties of protein fluorescence spectroscopy can be used to monitor the carbohydrate-binding site of CML at the conformational and dynamic level [13].

In the present study, fluorescence spectra were first used to investigate the conformational changes of CML exposed to chemical modifications, different temperatures, pH, metal ions, and denaturants. Moreover, the relationships among the specific amino acid residues of CML, conformation of carbohydrate-binding site, and CML-induced apoptosis were determined, which provided new insights into the CML antitumor activity based on its structural information.

**Materials and Methods**

**Materials**
The air-dried slice of Akebia *Clematis* stems was purchased in a local traditional Chinese medicine market, Chengdu, China. The purification of CML was performed according to the method described previously [7]. The concentration of CML was determined using the method of Bradford [14].

N-Bromosuccinimide (NBS), 4-nitrobenzenesulfonyl fluoride (NBSF), and diethyl pyrocarbonate (DEPC) were obtained from Acros Organics (Geel, Belgium). 5,5′-Dithiobis (2-nitrobenzoic acid) and 2,3-butanedione were purchased from Merck (Darmstadt, Germany). p-Methylbenzenesulfonyl fluoride (PMSF) was obtained from Sigma (St Louis, USA). Fetal bovine serum (FBS) was purchased from American Type Culture Collection (ATCC, Rockville, USA). PMSF dissolved in isopropanol and CML dissolved in phosphate buffer were added into the phosphate buffer to the final concentration of 5 mM and 1 mg/ml, respectively. Meanwhile, aliquots 1 and 4 were taken as the control and other aliquots were added with 10.0 μl of 10.0 mM NBS (at 5 min interval until the ΔA_{280nm} did not decrease). The number of modified Trp residues in the single CML molecule was obtained with the following equation.

$$N(\text{Trp}) = \frac{1.31 \times \Delta A_{280nm} \times Mr}{5500 \times \rho}$$

where ΔA_{280nm} means the difference of the A value between the native and modified CML, Mr means the molecular mass of CML, and ρ means the concentration of CML in the response system. Afterwards, the residual activity and fluorescence spectrum of each aliquot were determined after removal of excess reagents by dialysis.

**Modification of serine/threonine.** Modification of serine/threonine residues was carried out in phosphate buffer (0.05 M, pH 7.0) according to the method of Kraut et al. [17]. PMSF dissolved in isopropanol and CML dissolved in phosphate buffer were added into the phosphate buffer to the final concentration of 5 mM and 1 mg/ml, respectively. After incubation at 30°C for 30 min, excess reagents were removed by dialysis.

**Modification of Tyr.** Modification of Tyr residues was carried out by NBSF according to the method of Liao et al. [18]. Reaction was carried out in 0.1 M Tris-HCl buffer (pH 8.0).
NBSF was dissolved in isopropyl alcohol before being added to the reaction system. The final concentration of the CML was 1 mg/ml. After incubation at 25 °C for 60 min, the reaction was terminated by addition of 0.1 M HCl to adjust pH value in the range of 5.0–6.0. The hemagglutinating activities were determined after removal of excessive reagents by dialysis.

Modification of arginine, histidine, and sulfydryl. According to the methods described previously [19], arginine and histidine were modified with 2,3-butanedione and DEPC. Modification of sulfydryl was carried out according to the previous method [20,21]. The arginine modification was carried out in the 0.5 M boric acid buffer (pH 8.5). CML and 2,3-butanedione were dissolved in the buffer at the concentration of 1 mg/ml and 100 mM, respectively. The reaction system was bathed at 25 °C for 180 min. For the modification of histidine, CML was dissolved in the NaAc–HAc buffer (0.5 M, pH 6.0) to make a 1 mg/ml solution. CML solution (3 ml) was mixed with cold DEPC ethanol solution (5 ml), and the reaction was carried out at 4 °C for 24 h. The modification of sulfydryl was carried out by addition of 2 μl of 10 mM NEM (at 5 min interval) to 0.1 mg/ml CML, until the absorbance at 300 nm was stable. After removal of excessive reagents by dialysis, the hemagglutinating activities were determined.

Fluorescence spectroscopy

The fluorescence spectrum of all samples was recorded on a spectrofluorometer (Model 4500; Hitachi, Tokyo, Japan). The samples were excited at 295 and 280 nm, and the spectral width of the emission spectrum was 5 nm with scanning speed of 180 nm/min.

Anti-proliferative activity

Cell culture. CML below 0.1% was dissolved in dimethyl sulfoxide which had no detectable effect on cell growth before the experiments. The murine fibrosarcoma L929 cells were cultured in RPMI-1640 medium (Gibco, Gaithersburg, USA) supplemented with 10% FBS, 0.03% L-glutamine (Gibco), 100 U/ml penicillin, and 100 mg/l streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO2. For the experiments, cells were plated 24 h before the treatments to allow adherence.

Cell growth inhibition assay. The L929 cells were dispensed in 96-well flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) at a density of 5 × 10^4 cells/ml. The murine fibrosarcoma L929 cells were pre-incubated in the buffer containing DEPC, PMSF, NBSF, NEM, Diacetyl, or NBS for 30 min, and then treated with the naive CML and group-specific reagents modified CML for 24 h at the concentration of 1 pM. The effects on cell viability/proliferation were measured using MTT assay as previously described [22,23]. Absorbance at 490 nm was measured using a spectrophotometer (Model 3550; Bio-Rad, Hercules, USA). The percentage of cell growth inhibition was calculated as follows:

\[
\text{Inhibition ratio} \% = \frac{A_{490\text{ nm}}(\text{control}) - A_{490\text{ nm}}(\text{sample})}{A_{490\text{ nm}}(\text{control}) - A_{490\text{ nm}}(\text{blank})} \times 100\%
\]

Apoptotic morphological observation. The L929 cells were divided into three groups. The first group was treated as the control, the second group was treated with 1 pM CML, and the third group was treated with the CML previously modified by the NBS. Three groups were cultured at the same condition for 24 h. The cellular morphology was observed using a phase contrast microscope (Leica, Wetzlar, Germany).

Nuclear morphological observation. The cells used for the morphological observation were partly reserved for DAPI staining, and the nuclear morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

All the presented data and results were confirmed in at least three independent experiments. These data were expressed as the mean ± SD. Statistical comparisons were made by Student’s t-test. \( P < 0.05 \) was considered significant.

Results

Endogenous fluorescence spectrometry

CML has been isolated from C. montana Buch.-Ham stem by ion exchange and gel filtration chromatographies on DEAE-Sepharose and Sephacryl S-100 [7]. Our previous study has reported that the purified CML is a homodimer of 11,968.9 Da subunits held together by disulfide linkages. Besides, CML could agglutinate rabbit erythrocytes, and the hemagglutinating activity would be exclusively inhibited by mannan and thyroglobulin [7]. To determine the endogenous fluorescence spectrum of CML, the CML sample was excited at 280 and 295 nm, and the fluorescence intensity was measured at the same time. CML exhibited a fluorescence emission maximum at 338 nm upon excitation at 284 nm (Fig. 1).

Effect of temperature on hemagglutinating activity and fluorescence spectrum of CML

After being treated at different temperatures for 30 min, the activity of CML was evaluated by erythrocyte coagulation testing. As shown in Fig. 2A, CML maintained stable hemagglutinating activities when the temperature ranged...
from 20 to 80°C. However, when the temperature reached 90°C, the activity was drastically decreased by 50% and completely lost at 100°C for 10 min. The maximum fluorescence emission wavelength of CML (Fig. 2B) did not show obvious changes from 40 to 80°C besides enhanced fluorescence intensity compared with native one. However, after treatment at 90 or 100°C for 30 min, the emission peaks increased significantly and red-shifted.

**Effect of pH on hemagglutinating activity and fluorescence spectrum of CML**

When CML was incubated at different pH, its hemagglutinating activities were almost totally maintained in the pH range from 6.0 to 10.0, while a remarkable decrease of activities was observed at the pH below and above this range (Fig. 3A). The effects of pH on the fluorescence emission spectrum of CML were shown in Fig. 3B. There was no obvious change in the emission maximum wavelength of fluorescence spectrum despite of the different fluorescence intensities in testing pH range. The fluorescence intensity maintained high level under the neutral condition, but gradually decreased when the pH changed towards the poles except pH 12.0. On the contrary, CML showed much higher fluorescence intensity at pH 12.0 than at the neutral condition.

**Effect of EDTA and metal ions on hemagglutinating activity and fluorescence spectrum of CML**

We further explored the dependence of CML conformation and activity on ions. The hemagglutinating activity of CML was lost after being treated with EDTA, and a little activity was resumed after removal of EDTA by dialysis. Table 1 shows that the hemagglutinating activities could be totally restored by Ca²⁺ and Mg²⁺ (20.0 mM) and partially restored by Fe³⁺ (62.5%) and Al³⁺ (50%). The effects of metal ions on fluorescence spectrum were plotted in Fig. 4. The relative fluorescence intensity of CML was obviously decreased after treatment with EDTA, while addition of Ca²⁺ and Mg²⁺ into CML solution caused an increase in emission intensity. And, slight red shifts on fluorescence spectrum were observed after being treated with EDTA and metal ions (Fig. 4B).

**Effect of denaturants on hemagglutinating activity and fluorescence spectrum of CML**

Urea, SDS, and GuHCl are widely used as common denaturants to study the structure–function relationships of protein [24]. As shown in Fig. 5, when concentrations of urea were 1.0 and 2.0 M, the hemagglutinating activities of CML were almost unchanged, and CML retained 37% activity when the
concentration up to 4.0 M. Whereas, the activity was completely lost in the concentrations over 6.0 M. In addition, 25% clotting activity was kept when the concentration of GuHCl was 1.0 M and the activity was completely lost when the concentration was 3.0 M. Moreover, the activity of CML gradually decreased with the increased concentrations of SDS. Since hemolysis occurred in the concentrations higher than 1.0 mM, the effects of high concentrations of SDS on hemagglutinating activities were not shown.

The effects of urea, GuHCl, and SDS concentrations on the fluorescence spectrum of CML were shown in Fig. 6. Endogenous fluorescence spectrum of CML presented two stages of changes with different concentrations of urea. The fluorescence intensity of CML increased but the peak position unchanged at low concentrations of urea (1.0–4.0 M), while the fluorescence intensity declined at higher concentrations of urea (4.0–8.0 M) (Fig. 6B). Figure 6 shows the effects of different concentrations of GuHCl on the fluorescence intensity of CML. The fluorescence intensity was gradually stronger with the increasing concentrations of GuHCl and reached a maximum at 2.0 M. Thereafter, the fluorescence intensity decreased with the increasing concentrations of GuHCl, and meanwhile the $\lambda_{\text{max}}$ slightly red-shifted [25] (Fig. 6A). The effects of different concentrations of SDS on the fluorescence spectra showed that the fluorescence intensity of CML increased, but the maximum emission wavelength slightly blue-shifted with the addition of 0.1–1.0 mM SDS. On the contrary, the fluorescence intensity began to decline with increasing concentrations of SDS (Fig. 6C).

### Chemical modification on CML
The effects of chemical modification of amino acid residues on the hemagglutinating activity of CML were shown in Table 2. The CML activity was gradually reduced with the addition of NBS, and completely lost in 30 µl of 10 mM NBS (data not shown). According to the previous method [16], $\approx$ 4 (4.4) Trp residues were modified under non-denaturing condition and $\approx$ 6 (6.1) Trp residues were modified after addition of urea. The effects of Trp residues modification on the hemagglutinating activity were shown in Fig. 7A. It was found that modification of one Trp residue did not affect the hemagglutinating activity, modification of two Trp residues resulted in 60% loss of activity, and modification of three Trp residues completely destroyed the hemagglutinating activity. Changes of fluorescence spectrum upon modification of Trp residues were shown in Fig. 7B. The fluorescence intensity was gradually weakened after addition of the modifier and the maximum emission peak constantly blue-shifted at 295 nm.

The hemagglutinating activities of CML retained only 20% and 50% after modification of arginine and sulfydryl, respectively (Table 2). Modifications of Tyr, histidine, and serine/threonine did not cause any changes in the hemagglutinating activity of CML (Table 2). After modifying the residues by DEPC, PMSF, NBSF, NEM, and Diacetyl,
fluorescence intensity of the spectrum excited at 295 nm severely declined with blue shift at the peak position (Fig. 8).

The correlation between chemical modification of CML and its anti-proliferative activity

Previous report proved that the CML could inhibit the growth of murine fibrosarcoma L929 cells by inducing apoptosis [7]. To assess the effects of chemical modification on the anti-tumor activity of CML, we pre-incubated CML in the buffer containing DEPC, PMSF, NBSF, NEM, Diacetyl, or NBS at 37°C for 30 min, respectively. After the murine fibrosarcoma L929 cells were treated with above-mentioned CML for 24 h, cell inhibitory ratio was measured by the MTT assay. As shown in Fig. 9A and Table 3, NBS modified the Trp and nearly completely inhibited the CML anti-proliferative activity, while Diacetyl and NEM caused 76.99% and 49.64% loss of the anti-proliferative activity of CML. As for the other amino acid modifiers, no inhibiting effects were observed after the treatment. The DAPI was used to specially stain the DNA to observe the nuclear morphological changes. The L929 cell treated with CML exhibited round and smaller cellular profile and the DAPI staining showed the condensed chromatin and fragmented nucleus. On the contrary, the CML modified by NBS did not exhibit such cellular and nuclear morphological alterations (Fig. 9B).

Discussion

Plant lectin has become researchers’ focus because of its anti-tumor activity to diverse types of cancer cells [25,26]. Notably, several plant lectins have been shown to induce apoptotic cancer cell death [27,28], but the mechanism is still not clear. In our previous study, we reported the extraordinary anti-tumor activity of CML and determined its N-terminal amino acid sequence as DNVKYSGQVKNTGSA [7]. BLAST results showed that it does not have any homology with all other reported lectins (data not shown). The lack of complete CML sequence makes homology modeling impossible. In the present study, we explored the role of specific amino acid residues in the conformation of CML to understand the intricate relationships between the carbohydrate-binding site structure and the anti-tumor function of CML.

Previous study has proved that only Trp can be excited at 295 nm, while both Trp and Tyr can be excited at 280 nm [29]. However, the undetectable fluorescence peaks of Tyr residues in the fluorescence spectrum of CML may indicate the energy transfer from Tyr to Trp in the emission [30]. The
emission peaks showed a blue shift of 10 nm at 280 and 295 nm compared with the emission peak of free Trp (348 nm) [10], indicating the presence of Trp in a relatively hydrophobic region [31–33]. This hydrophobic region might be the hydrophobic core of the lectin and the Trp residues buried in this region are crucial for the protein stability [34].

CML retained nearly 100% of its hemagglutinating activity at 80°C (Fig. 2A), in contrast to other lectins that are unstable even at 50°C [35,36]. As for the fluorescence spectrum (Fig. 2B), the red-shifted emission peak suggested that Trp residues were located at a less hydrophobic environment, and the increasing fluorescence intensity indicated that heat induces Trp residues to expose to the surface of the molecule. Given such reorientations of Trp residues, the high temperature probably destructed the conformation of CML [31]. CML is more stable under basic pH than acidic pH (retaining only 12.5% hemagglutinating activity at pH 2, and 62.5% at pH 12), which is similar to the lectin from rhizome of *Setcreasea purpurea* [37]. The extremely alkaline condition caused elevated emission peak (Fig. 3B), indicating that the Trp residues exposure might be due to the alteration of conformation caused by abnormal molecular surface charge [38]. Therefore, CML exhibited decreased hemagglutinating activity with the conformational changes of active center in extremely hot and alkaline environment.

It is well known that metal ions are required for maintaining the spatial conformation and bioactivities of certain proteins [39,40]. The completely lost activity of EDTA-treated CML (Table 1) suggested that ions are essential for CML activity like other lectins [41]. Some studies have revealed that ions can interact with some amino acid residues to maintain the structure of active site [42,43]. Compared with CML in the native condition, the dropping fluorescence intensity, along with slight red shift in the EDTA-treated group, (Fig. 4B) suggested that the conformation of CML was altered by deionization. Moreover, the distinct effects of ions with different valencies on EDTA-treated CML (Fig. 4B and

<table>
<thead>
<tr>
<th>Modified amino acid residues</th>
<th>Modification reagents</th>
<th>Hemagglutinating activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>NBS</td>
<td>0</td>
</tr>
<tr>
<td>Arg</td>
<td>Diacetyl</td>
<td>20</td>
</tr>
<tr>
<td>Tyr</td>
<td>NBSF</td>
<td>100</td>
</tr>
<tr>
<td>Ser and Thr</td>
<td>PMSF</td>
<td>100</td>
</tr>
<tr>
<td>–SH</td>
<td>NEM</td>
<td>50</td>
</tr>
<tr>
<td>His</td>
<td>DEPC</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 6. Effects of different denaturants on the fluorescence spectrum of CML. (A) The final GuHCl concentrations were 0.0, 0.15, 1.0, 2.0, 3.0, and 6.0 M for colored curves, respectively. (B) The final urea concentrations were 0.0, 1.0, 2.0, 4.0, 6.0, and 8.0 M for colored curves, respectively. (C) The final SDS concentrations were 0.0, 0.10, 0.50, 1.0, 5.0, and 10 mM for colored curves, respectively, excited at 295 nm.
suggested that the bivalent metal cations are the specific ions to maintain the conformation of active site [44]. In other words, bivalent metal cations are essential for the hemagglutinating activities of CML because of their specific interaction with residues which retains the spatial conformation of CML [40,45].

All three denaturants (GuHCl, urea, and SDS) can denaturate CML by destroying the secondary structure of the protein and the unfolded CML loses its activity [46]. Intriguingly, treatment with these three denaturants led to a two-stage transition of the fluorescence spectrum (Fig. 6). It has been shown that native protein is first transformed into a native-denatured mixed state, and then totally denatured to the unfolded state [47], which might explain why the two-stage change occurs.

The red shift and blue shift observed in the fluorescence spectrum (Fig. 6) revealed the effects of denaturant on CML’s Trp residues in the following aspects: (i) the increasing concentrations of urea did not affect the hydrophobic microenvironment of Trp residues, but its presence disturbed the microenvironment around the residues; (ii) the higher the concentrations of GuHCl, the stronger the disturbance to the microenvironment; and (iii) the low concentrations of SDS provided a more hydrophobic environment to Trp, and the high SDS concentrations might totally unfold the CML and affect the hydrophobic environment [33].

Some amino acid residues located at the active site may facilitate the active center to form unique and substrate-accessible structure and interact with the specific group of the substrates [48,49], and thereby can be considered as the specific group to exert or maintain the protein activity. To reveal the roles of specific amino acid residues in the active site, groundbreaking work has been done in the chemical modification of amino acid residues [50,51]. In addition, molecular biology methods have been universally used to construct mutants and alter the amino acid at particular site [52]. Compared with the molecular approaches, chemical modification is easy to operate, less time-consuming to perform, and less disturbing to the environment of amino acid residues. Chemical modification has been used to resolve the structural information and predict the secondary structure of several proteins [53]. Furthermore, in combination with hemagglutination assay, the roles of specific residues in the lectin active site have been successfully studied with the modification of amino acid residues [31,54].

As the study on the CML biochemical properties went further, we found that Trp may play an important role in the molecular conformation and bioactivity of CML. According to the crystal structure of the Polygonatum cyrtonema lectin

| Table 1 |
Figure 9. Effects of chemical modifiers on CML-induced cell death and morphological changes  

(A) Effects of six different chemical modifiers on lectin-induced cell death. The murine fibrosarcoma L929 cells were pre-incubated in the buffer containing DEPC, PMSF, NBSF, NEM, Diacetyl, or NBS for 30 min, and then treated with the CML (1 pM) for 24 h. The cell inhibitory ratios were measured by the MTT assay ($n = 3$, the mean $\pm$ SD). *$P < 0.05$, vs. CML group.  

(B) Morphological observation of L929 cells. The morphological changes were observed after the cells were incubated with medium, 1 pM CML or 1 pM CML pre-incubated with NBS for 24 h under a phase contrast microscope (1, medium; 3, 1 pM CML; 5, CML pre-incubated with NBS; magnification ×200) or under a fluorescence microscope by DAPI staining (2, medium; 4, 1 pM CML; 6, CML pre-incubated with NBS; magnification ×200).
Table 3. Effects of modification of amino acids on anti-tumor activity of CML

<table>
<thead>
<tr>
<th>Modified amino acid residues</th>
<th>Modification reagents</th>
<th>Inhibitory ratio (%)a</th>
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</thead>
<tbody>
<tr>
<td>–</td>
<td>Native</td>
<td>56.93 ± 3.18</td>
</tr>
<tr>
<td>Trp</td>
<td>NBS</td>
<td>1.61 ± 0.53</td>
</tr>
<tr>
<td>Arg</td>
<td>Diacetyl</td>
<td>13.10 ± 1.15</td>
</tr>
<tr>
<td>Tyr</td>
<td>NBSF</td>
<td>54.50 ± 2.41</td>
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<tr>
<td>Ser and Thr</td>
<td>PMSF</td>
<td>54.87 ± 2.57</td>
</tr>
<tr>
<td>–SH</td>
<td>NEM</td>
<td>28.67 ± 1.15</td>
</tr>
<tr>
<td>His</td>
<td>DEPC</td>
<td>56.03 ± 2.46</td>
</tr>
</tbody>
</table>

aData represent mean values of inhibitory ratio (%) from three independent experiments (the mean ± SD).

(PCL), three Trp residues were shown to partially maintain the PCL conformation through strong van der Waals interactions [55]. There are also some studies supporting that Trp is crucial to maintain the conformation of mannose-binding lectin [56]. Trp residues are also involved in the carbohydrate-binding site. The crystallized lectin EW29 from the earthworm *Lumbricus terrestris* identified Trp161 as an important residue to form the polar contacts and van der Waals interactions with carbohydrates [57]. With the help of the computational studies, Trp was demonstrated to form hydrogen bonds with the sugar and create a more suitable binding phase for the sugar [58]. Therefore, we hypothesized that the Trp residues may participate in the carbohydrate-binding site and keep the bioactive conformation of CML.

To test our hypothesis, we performed chemical modification of CML with NBS, a specific modification reagent to destroy the beta-indolyl of Trp under particular condition [59]. We found that NBS caused CML hemagglutination activity loss in a dose-dependent manner, and CML was completely inactive at high dose of NBS. Further investigation suggested that there were six Trp residues in CML, four of which were exposed to the external environment, and other two hided in the molecular hydrophobic core. The unaltered hemagglutinating activity after the modification of one Trp residue and the 60% and 100% activity loss following the modification of one more and two more Trp residues (Fig. 7A) indicated that the external Trp residues might cooperatively maintain the carbohydrate-binding activity. The relationship between Trp residues and CML conformation was clarified by the fluorescence spectrum of CML modified by increasing concentrations of NBS. With more NBS, weaker fluorescence intensity was detected at the emission peak along with the blue shift at 295 nm excitation (Fig. 7B). It is rational to conclude that Trp residues exposed to the external and the relatively hydrophilic environment are essential to maintain the CML conformation, and each of four Trp residues contributes unequally.

After Arg and sulfhydryl were modified with Diacetyl and NEM, the activity retained only 20% and 50%, respectively (Table 2), suggesting that these two residues were partially required for the hemagglutination activity and probably maintained the conformation of active center indirectly. However, compared with the spectrum of NBS-modified CML, the fluorescence spectra of other modified CML exhibited declined fluorescence intensity, and the blue shift like NBS-modified CML’s spectrum was detected (Fig. 8). The fact that 20% and 80% activity retained after the Arg and –SH modification of CML suggested that Arg and sulfhydryl may participate in the formation of sugar-binding site conformation, but not indispensable.

CML can induce apoptosis in murine fibrosarcoma L929 cells through caspases-dependent pathway [7]. The activation of apoptosis requires interaction between CML molecule and cell surface receptors [60], the prerequisite of which is the accessible conformation of CML for the cell surface receptor. The Trp-modified CML nearly completely lost its anti-proliferating activity (Table 3), and was unable to induce the L929 cell apoptosis as the native CML (Fig. 9B). A previous report has proposed that the apoptosis of L929 cell induced by CML can be inhibited by specific carbohydrates [7]. Therefore, we conclude that the apoptosis-inducing activity of CML is probably maintained by the specific carbohydrate-binding site conformation being kept by Trp residues. In addition, the partial loss of anti-proliferation activity and the dropping fluorescence intensity with the blue or red shift after the Arg and –SH modification suggested that Arg and sulfhydryl may not be so important in the apoptosis-inducible conformation of CML.

In summary, we used chemical modification and fluorescence spectroscopy to identify the amino acid residues that are crucial for CML’s bioactive conformation. The obtained information is valuable to understand the mechanism of CML-induced apoptosis and its anti-tumor activity. Further investigations are still needed to elucidate the detailed interaction between the CML’s carbohydrate-binding site and the saccharide chain of cell surface receptors. A complete elaboration of the structural basis of CML’s apoptosis-inducing activity will help to develop CML as a potential anti-cancer reagent.

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