

## A new cysteine protease allergen from *Ambrosia trifida* pollen: proforms and mature forms

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### ABSTRACT

Giant ragweed (*Ambrosia trifida*) pollen is closely associated with respiratory allergy in late summer and autumn, and the prevalence of giant ragweed pollen allergy progressively increases. Compared with short ragweed (*Ambrosia artemisiifolia*), allergenic components from giant ragweed pollen are poorly investigated. To promote component-resolved diagnosis and treatment for giant ragweed pollen allergy, it becomes necessary to identify and characterize unknown allergens from giant ragweed pollen. In the present study, we identified and characterized a new cysteine-protease (CP) allergen from giant ragweed pollen, named as Amb t CP. The cloned Amb t CP gene encoded 387 amino acids. Recombinant Amb t CP (rAmb t CP) and natural Amb t CP (nAmb t CP) were purified by high-affinity Ni<sup>2+</sup> resin and immunoaffinity chromatography respectively. During refolding, purified rAmb t CP could autocatalytically converted to its mature forms displaying a higher enzymatic activity. Moreover, the autocatalytic conversion of proforms to mature forms of nAmb t CP could cause their amount to change in giant ragweed pollen extracts. Then, the allergenicity of Amb t CP was characterized: 23 (33.8%) of 68 Chinese patients with ragweed pollen allergy showed positive IgE binding to nAmb t CP by enzyme-linked immunosorbent assay (ELISA); the result of subsequent ELISA showed that IgE-binding activity of proforms and mature forms of rAmb t CP was different, with positive rate of 39.1% (9/23) and 47.8% (11/23) respectively; Amb t CP showed IgE cross-reactivity with the CP components from short ragweed, *Artemisia annua* and *Artemisia sieversiana* pollen. Our findings will help to promote component-resolved diagnosis and treatment for giant ragweed pollen allergy, standardize allergen products and individualize allergen-specific immunotherapy.

**Abbreviations:** AIT, allergen-specific immunotherapy; IUIS, the International Union of Immunological Societies; CP, cysteine protease; Amb t CP, the cysteine-protease component from giant ragweed pollen; ELISA, enzyme-linked immunosorbent assay; BAT, basophil activation test; *A. annua*, *Artemisia annua*; *A. sieversiana*, *Artemisia sieversiana*; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; rAmb t CP, recombinant Amb t CP; IPTG, isopropyl-β-D-thiogalactoside; DTT, dithiothreitol; pro rAmb t CP, the proform of recombinant Amb t CP; mature rAmb t CP, the mature form of recombinant Amb t CP; nAmb t CP, natural Amb t CP; pro nAmb t CP, the proform of natural Amb t CP; mature nAmb t CP, the mature form of natural Amb t CP; CD, circular dichroism; FITC, fluorescein isothiocyanate; anti-rAmb t CP pAbs, anti-rAmb t CP rabbit polyclonal antibodies; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HRP, horse radish peroxidase; SD, standard deviation; PVDF, polyvinylidene difluoride; ORF, open reading frame.

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## 1. Introduction

As a major source of pollen allergens in late summer and autumn (Pomés and Schulten, 2020), ragweed (*Ambrosia* sp.) pollen is highly associated with allergic diseases and represents a major health problem in multiple countries and regions (D'Amato et al., 2007; Li et al., 2009; Chen et al., 2018). As one of closely related species, the spread of giant ragweed (*Ambrosia trifida*) pollen is consistently enhanced by global warming, and the prevalence of giant ragweed pollen allergy progressively increases (Rasmussen et al., 2017; Dong et al., 2020). In this sense, 12.5% patients with allergic rhinitis in Beijing city (Wang et al., 2006) and 55% patients with allergic rhinitis in Qingyang of Gansu Province (Liu et al., 2013) show a positive skin prick test for giant ragweed pollen extracts, which suggests that allergy to giant ragweed pollen is also a potential health problem in China.

Allergen-specific immunotherapy (AIT) is the only method to alter natural evolution of allergic diseases (Lee et al., 2022), which is based on the administration of disease-causing allergens to induce the immune tolerance (Dorofeeva et al., 2021). To date, the available AIT vaccines are all allergen extracts, and the treatment of allergic diseases can be affected due to the complex components, inconsistent standards and possible exogenous pollutants of allergen extracts (Focke-Tejkl and Valenta, 2012; Dorofeeva et al., 2021). The component-resolved diagnosis and treatment based on single-allergen molecule can effectively overcome these limitations (Valenta et al., 1999; Dorofeeva et al., 2021). Therefore, it is essential to identify and characterize exact allergen molecules to promote component-resolved diagnosis and treatment for giant ragweed pollen allergy.

Up to now, only 3 allergens from giant ragweed pollen have been recorded in the International Union of Immunological Societies (IUIS), including Amb t 5 (Metzler et al., 1992), Amb t 8 (profilin), Amb t 13 (superoxide dismutase). However, as the closely related species of giant ragweed, 12 allergens from short ragweed (*Ambrosia artemisiifolia*) pollen have been recorded by IUIS, suggesting that many allergenic components from giant ragweed pollen remain to be explored.

Proteolytic enzymes were reported as one of allergenic components from pollen (Gaspar et al., 2020). In short ragweed pollen, proteolytic enzymes including trypsin and chymotrypsin-like serine peptidases were identified in 1998, enhancing the inflammatory responses (Bagarozzi and Travis, 1998). Recently, Amb a 11, a cysteine-protease (CP) allergen from short ragweed pollen was identified, with 66% positive IgE-binding activity in American and European patients with ragweed pollen allergy (Bouley et al., 2015; Groeme et al., 2016). Besides Amb a 11, the CP allergens were also reported in mites and fruits as major allergens (Chevigné et al., 2007; Zhang et al., 2007; Chruszcz et al., 2009; Cavic et al., 2014), such as Der f 1 from *Dermatophagoides farinae*, Der p 1 from *Dermatophagoides pteronyssinus* and Act d 1 from *Actinidia deliciosa* (Chapman and Platts-Mills, 1980; Heymann et al., 1986; Pastorello et al., 1998).

These CP allergens belong to the CA1 family (Chevigné et al., 2017; Groeme et al., 2016; Paul et al., 1995; Takai et al., 2002), and they are firstly existed as proproteins and then convert to mature enzymes via an autocatalytic mechanism to remove their N- and C-terminal propeptides (Chevigné et al., 2017; Groeme et al., 2016; Paul et al., 1995; Takai et al., 2002). These terminal propeptides of CP allergens may have an inhibitory effect on the maturation by forming a lid to cover the enzymatic active site (Groeme et al., 2016; Groves et al., 1996). As reported, IgE-binding activity of proforms and mature forms of recombinant Der p 1/recombinant Der f 1 is different because of major conformational IgE-binding epitopes of the mature forms covered by propeptides (Takai et al., 2005), indicating that the autocatalytic maturation of CP allergens is associated with their allergenicity. Moreover, the enzymatic activity of CP allergens can also promote the development of allergic diseases by impairing epithelium, then recruiting immune cells and releasing pro-inflammatory cytokines (Cavic et al., 2014; Chevigné et al., 2017; Ghaemmaghami et al., 2001; Groeme et al., 2016; King et al., 1998). To

date, the CP component from giant ragweed pollen (Amb t CP) is not explored.

In the present study, we identified and characterized Amb t CP from giant ragweed pollen. We confirmed the autocatalytic maturation of Amb t CP by enzymatic activity test, mass spectrum and immunoblot. We also studied the allergenic characteristics of Amb t CP by enzyme-linked immunosorbent assay (ELISA), immunoblot, basophil activation test (BAT) and inhibition immunoblot. Our findings will help to promote component-resolved diagnosis and treatment for giant ragweed pollen allergy, standardize allergen products and individualize AIT.

## 2. Methods and materials

### 2.1. Serum samples

Patients' sera selected for the study showed positive IgE binding to *Ambrosia* pollen extracts (code: W1, ragweed pollen-specific IgEs > 0.35 kU/L), which was detected by Phadia 1000 Immunoassay Analyzers (Phadia, Uppsala, Sweden) (Table S1). Additionally, almost all patients' sera included in the study showed positive IgE binding to giant ragweed, *Artemisia annua* (*A. annua*) and *Artemisia sieversiana* (*A. sieversiana*) pollen extracts by ELISA (Fig. S1). The negative controls included in the study consisted of 13 non-allergic individuals, with their total IgEs < 60 kU/L (Table S2) (Fokkens et al., 1992; Al-Mughales, 2016). We also confirmed that these non-allergic individuals showed negative IgE binding to giant ragweed pollen extracts by ELISA (Fig. S2). The study was conducted under the guidance of the Helsinki Declaration and approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

### 2.2. Preparation of pollen extracts

Pollen including giant ragweed, short ragweed, *A. annua* and *A. sieversiana* was purchased from Beijing Macro. Union Pharmaceutical Limited Corporation (Beijing, China). Total proteins were extracted from pollen mixed with PBS (1:5 w/v) by end-over-end mixing at 4 °C overnight, which was protected by 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sangon, Shanghai, China). After centrifugation, the supernatant of pollen extracts was pooled and filtered through a 0.22 µm membrane, then stored at − 80 °C until use.

### 2.3. Cloning of Amb t CP gene

A potential CP sequence from giant ragweed pollen was selected via comparing splicing sequences identified by next-generation sequencing with CP allergen sequences recorded by IUIS. The next-generation sequencing of giant ragweed pollen was performed by Allwegene technology corporation (Beijing, China). The total RNA of giant ragweed pollen was extracted using TaKaRa MiniBEST plant RNA extraction kit (Takara, Dalian, China) according to manufacturer's instructions. The Amb t CP gene was amplified by polymerase chain reaction (PCR) using the following primers: the forward 5'-TCCAACCATCAAGATCATAATG-3' and the reverse 5'-GATTGCACCTCAAACCTCTCTA-3'. The PCR program was run as follows: 98 °C 5 s, 30 cycles of 98 °C 10 s, 52 °C 30 s and 72 °C 1 min, followed by 72 °C 10 min. The amplified Amb t CP gene was cloned to the T vector (Vazyme, Nanjing, China). Positive clones were selected by means of the kanamycin resistance. The nucleic acid sequence of Amb t CP gene was confirmed by DNA sequencing, which was carried out in Genscript biotech corporation (Nanjing, China).

### 2.4. Expression, purification and characterization of recombinant Amb t CP (rAmb t CP)

After sequencing, the Amb t CP gene was cloned to the pET-28a plasmid between Nco I and Xho I sites by a one-step-clone kit (Vazyme, Nanjing, China) to construct the Amb t CP-recombinant

plasmid. Then, the Amb t CP-recombinant plasmid was transferred into *E. coli* BL21 (DE3) (Vazyme, Nanjing, China). The isopropyl- $\beta$ -D-thiogalactoside (IPTG) (Takara, Dalian, China) at final concentration of 1 mM was added to induce the expression of rAmb t CP. rAmb t CP was purified from the pellet of cell lysates after ultrasonication under the denaturing condition by the high-affinity  $\text{Ni}^{2+}$  NTA resin (Genscript, Nanjing, China). The refolding of rAmb t CP was conducted according to Groeme's study (Groeme et al., 2016). Briefly, rAmb t CP with the addition of dithiothreitol (DTT) (Sangon, Shanghai, China) at final concentration of 10 mM was incubated at 37 °C for 1 h. After the urea-gradient dialysis, rAmb t CP was diluted in refolding buffer (50 mM Tris-HCl, 0.5 M NaCl, 5 mM ethylene diamine tetraacetic acid, 1 mM glutathione, 0.1 mM oxidized glutathione, 0.5 M arginine, 10% glycerol, pH 8.5) (Sangon, Shanghai, China). Finally, rAmb t CP was dialyzed against PBS.

A new protein appeared after refolding, which was identified as the mature form of recombinant Amb t CP (mature rAmb t CP) according to subsequent analysis. The HiTrap Q HP column (Uppsala, Sweden) was used to isolate the proform of recombinant Amb t CP (pro rAmb t CP) and mature rAmb t CP by a linear gradient elution with 20 mM Tris containing 1 M NaCl (pH 8.5) using the ÄKTA go protein purification system (GE Healthcare, Uppsala, Sweden). The circular dichroism (CD) analysis (pro rAmb t CP: 0.1 mg/mL; mature rAmb t CP: 0.05 mg/mL) was performed by Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK) at 200–250 nm wavelength range. The data of CD were analyzed using the K2D3 program (Louis-Jeune et al., 2012). Subsequently, their enzymatic activity was detected using Fluoro™ protease assay kit (Sangon, Shanghai, China) according to manufacturer's instructions. Briefly, 100  $\mu\text{L}$  of fluorescein isothiocyanate (FITC)-labeled casein as the general protease substrate, 100  $\mu\text{L}$  of 1  $\times$  Fluoro™ assay buffer and 5  $\mu\text{L}$  of protein samples, including pro rAmb t CP, mature rAmb t CP and natural Amb t CP (nAmb t CP) were added and mixed in the plate, which was incubated for 60 min at room temperature in dark. The buffer was used as the negative control. The plate was read by the Cytation 5 fluorometer (BioTek, Vermont, USA).

## 2.5. Purification and characterization of nAmb t CP

nAmb t CP was purified by immunoaffinity chromatography coupling with anti-rAmb t CP rabbit polyclonal antibodies (anti-rAmb t CP pAbs). Firstly, anti-rAmb t CP pAbs were produced by immunizing a New Zealand White rabbit according to Calabozo's study (Calabozo et al., 2001), and detailed steps were same to those of the reported study (Zhu et al., 2021). Subsequently, anti-rAmb t CP pAbs were purified by HiTrap rProtein A HP column connected to the ÄKTA go protein purification system (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions. Purified anti-rAmb t CP pAbs were coupled to CNBr-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden). The giant ragweed pollen extracts in PBS were incubated with the immunoaffinity gels with continuous addition of 1 mM PMSF (Sangon, Shanghai, China) at 4 °C overnight. After washed with PBS, nAmb t CP immunosorbent on the gels was eluted with 100 mM glycine-HCl buffer (pH 2.7). The eluted fractions were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Purified nAmb t CP was firstly characterized by mass spectrum. The nAmb t CP excised from the SDS-PAGE gel was digested with trypsin, which was detected by Easy-nanoLC 1200 coupled with a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA) at Nanjing heyelan biotechnology Co. Ltd. The result of mass spectrum was analyzed by pNovo with default settings (Chi et al., 2010). Subsequently, whether purified nAmb t CP was glycosylated was detected by Pro-Q® emerald 300 glycoprotein gel and blot stain kit (Thermo Fisher Scientific, Massachusetts, USA) according to manufacturer's instructions. Briefly, the CandyCane™ molecular weight standards, purified nAmb t CP, pro- and mature rAmb t CP were firstly separated by SDS-PAGE. Then, the gel was fixed in 100 mL of fixing solution

containing 50% methanol and 5% acetic acid, which was further oxidized in 25 mL of periodic acid solution. After washed, the gel was stained in 25 mL of Pro-Q® Emerald staining solution at room temperature for 2 h in dark. Finally, the stained gel was visualized by ChemiDoc XRS+ System (BioRad, California, USA).

## 2.6. IgE-binding activity of Amb t CP in Chinese patients with ragweed pollen allergy

ELISA, immunoblot and BAT were used to characterize the allergenicity of Amb t CP in Chinese patients with ragweed pollen allergy. For ELISA, purified nAmb t CP in PBS (0.5  $\mu\text{g}/\text{well}$ ) was coated to microtiter plates (Corning Costar, Massachusetts, USA) at 4 °C overnight. These unspecific sites were blocked with 1% bovine serum albumin (BSA) in PBS-0.1% Tween 20 at 37 °C for 2 h. Sera of ragweed pollen-allergic patients and non-allergic individuals functioned as the first antibody (1:10 diluted in PBS-0.1% Tween 20), which were added to the plate and incubated at 37 °C for 2 h. Goat against human anti-IgEs linked by horse radish peroxidase (HRP) (1:2500 diluted in PBS-0.1% Tween 20) (KPL, Maryland, USA) functioned as the second antibody to detect IgEs binding to nAmb t CP. The reaction was visualized by 100  $\mu\text{L}$  of 3, 3', 5, 5'-tetramethylbenzidine substrate (Beyotime, Shanghai, China) at room temperature for 30 min in dark, and stopped by 50  $\mu\text{L}$  of 2 M  $\text{H}_2\text{SO}_4$ . The absorbance values were read at 450 nm by the Multiskan GO (Thermo Fisher Scientific, Massachusetts, USA). The cut-off values were presented as mean (negative controls) + 3  $\times$  standard deviation (SD) (negative controls).

For immunoblot, 10  $\mu\text{g}$  nAmb t CP after SDS-PAGE was transferred to the 0.22  $\mu\text{m}$  polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Massachusetts, USA). The membrane was blocked by 5% skimmed milk in PBS-0.1% Tween 20 at room temperature for 2 h. Then, the membrane was cut into strips, which were incubated with 13 sera of ragweed pollen-allergic patients and 2 sera of non-allergic individuals (1:10 diluted in PBS-0.1% Tween 20) at 4 °C overnight. Goat against human anti-IgEs linked by HRP (1:5000 diluted in PBS-0.1% Tween 20) (KPL, Maryland, USA) as the second antibody were used to detect IgEs binding to nAmb t CP. The IgE-binding strips were visualized by Immobilon™ western HRP substrate luminol reagent (Merck Millipore, Massachusetts, USA) using ChemiDoc XRS+ System (BioRad, California, USA).

The allergenicity of Amb t CP was further assessed by BAT. 100  $\mu\text{L}$  of fresh whole blood from the patient with ragweed pollen allergy and non-allergic individual was treated with 2 mL of red blood cell lysis buffer (Femac, Nanjing, China) at room temperature for 12 min. Pelleted cells were washed with PBS-0.1% fetal bovine serum and Tris buffer (25 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.6). The isolated leukocytes were resuspended in 100  $\mu\text{L}$  of Tris buffer containing 0.6 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  (pH 7.6). Then, basophils with the addition of anti-IgEs, giant ragweed pollen extracts, nAmb t CP, pro rAmb t CP and mature rAmb t CP at final concentration of 10  $\mu\text{g}/\text{mL}$  were incubated at 37 °C for 30 min. 900  $\mu\text{L}$  of precooled PBS containing 2.5 mM ethylene diamine tetraacetic acid ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  free) was used to stop the reaction. After centrifugation, pelleted cells were resuspended in staining buffer (0.05% BSA, 2 mM ethylene diamine tetraacetic acid in PBS buffer) with FITC-conjugated anti-CD63 antibodies (Biolegend, California, USA) and phycoerythrin-conjugated anti-CCR3 antibodies (Biolegend, California, USA), which were stained for 20 min on ice. Basophil expressions of CCR3 and CD63 were evaluated using FACS Canto Plus and analyzed by the build-in Diva software (BD Bioscience, California, USA).

## 2.7. Pro- and mature rAmb t CP differently reacted to IgEs from sera of Chinese allergic patients to ragweed pollen

The reactivity of pro- and mature rAmb t CP to IgEs from sera of Chinese patients with ragweed pollen allergy was further investigated by ELISA. Detailed steps of ELISA were same to the above described in

**Section 2.6.** Twenty-three sera of ragweed pollen-allergic patients, with positive IgE binding to nAmb t CP, were included. Three sera of non-allergic individuals were used as negative controls. The cut-off values of pro- or mature rAmb t CP were presented as mean (negative controls of pro- or mature rAmb t CP) + 3 × SD (negative controls of pro- or mature rAmb t CP).

### 2.8. nAmb t CP was autocatalytically converted from pro- to mature forms in giant ragweed pollen extracts

Considering the autocatalytic conversion of rAmb t CP during refolding and different allergenicity of pro- and mature rAmb t CP, we intended to investigate whether the proform of nAmb t CP (pro nAmb t CP) autocatalytically converted to the mature form of nAmb t CP (mature nAmb t CP), further changing the amount of pro- and mature nAmb t CP in giant ragweed pollen extracts. Total proteins were extracted from giant ragweed pollen, and detailed steps were described in Section 2.2. Giant ragweed pollen extracts were divided into 1.5 mL tubes (1 mL each), and each was differently treated (storage for 3, 5, 10, 14, 22 days at 4 °C or −20 °C). Finally, the amount of pro- and mature nAmb t CP in giant ragweed pollen extracts was determined by immunoblot using anti-rAmb t CP pAbs. The detailed steps of immunoblot were same to the above described in Section 2.6.

### 2.9. IgE cross-reactivity of Amb t CP between *Artemisia* and *Ambrosia* pollen

Most patients included in the study were sensitized to *Ambrosia* and *Artemisia* pollen according to their clinical characterizations. IgE cross-reactivity between *Ambrosia* and *Artemisia* pollen was largely reported (Asero et al., 2006; Grewling et al., 2018). Therefore, we intended to investigate IgE cross-reactivity of the CP components from *Ambrosia* and *Artemisia* pollen. Considering *A. annua*, *A. sieversiana* as abundant species in China (Tang et al., 2015; Guan et al., 2019) and short ragweed as the closely related specie of giant ragweed, short ragweed, *A. annua* and *A. sieversiana* pollen extracts were included in the study. The detailed steps about preparation for pollen extracts were described in Section 2.2.

The presence of the CP components in *Artemisia* and *Ambrosia* pollen extracts was confirmed by immunoblot using anti-rAmb t CP pAbs. For inhibition immunoblot, 10 µg purified nAmb t CP after electrophoresis was firstly transferred to the PVDF membrane (Merck Millipore, Massachusetts, USA). The membrane was blocked using 5% skimmed milk in PBS-0.1% Tween 20 and then cut into strips. Pooled sera of 5 ragweed pollen-allergic patients functioned as the first antibody (1:10 diluted in PBS-0.1% Tween 20). The positive control was pooled sera of ragweed pollen-allergic patients without treatments. The treatment groups were pooled sera of ragweed pollen-allergic patients preincubated with giant ragweed, short ragweed, *A. annua* and *A. sieversiana* pollen extracts at final concentration of 10 µg/mL. The negative control was pooled sera of non-allergic individuals. Then, these strips with differently treated sera were incubated at 4 °C overnight, and subsequently incubated with goat against human anti-IgEs linked by HRP (1:5000 diluted in PBS-0.1% Tween 20) (KPL, Maryland, USA). The IgE-binding strips were visualized by Immobilon™ Western HRP substrate luminol reagent (Merck Millipore, Massachusetts, USA) using Tanon 5200 multi-imaging system (Tanon, Shanghai, China). Additionally, the inhibition immunoblot assay was performed by using the allergen extracts (giant ragweed, short ragweed, *A. annua* and *A. sieversiana* pollen) with pooled sera of ragweed pollen-allergic patients which were preincubated with nAmb t CP at final concentration of 10 µg/mL. The detailed steps were same to the above.

### 2.10. Statistical analysis

GraphPad Prism 9.00 was used for statistical analysis. Unpaired *t* test was used to compare whether IgE-binding activity of pro rAmb t CP was

different from that of mature rAmb t CP to same individual sera of patients with ragweed pollen allergy. The data were presented as mean ± SD (*n* = 3). *p*-values less than 0.05 were considered as significantly different. \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001.

## 3. Results

### 3.1. Cloning of Amb t CP gene

The Amb t CP gene amplified by PCR was resolved on 1% agarose gel, which sited at approximately 1200 bp (Fig. 1A, the arrow). According to the result of sequencing, the Amb t CP gene with an 1164-bp open reading frame (ORF) encoded 387 amino acids, and its N-terminal signal peptides, N-terminal and C-terminal propeptides were respectively marked in blue, orange and purple according to Amb a 11 (Fig. 1B). For multiple alignments with homologous CP allergens from other species, the identity of amino acid sequences of Amb t CP and Amb a 11, Der p 1, Der f 1, Act d 1 and Eur m 1 was 95.34% 19.56%, 27.39%, 34.51%, 24.87% respectively (Fig. 2).

### 3.2. Expression, purification and characterization of rAmb t CP

Purified rAmb t CP (approximately 43 kDa, pro rAmb t CP) was resolved on SDS-PAGE gel, and a new protein band (approximately 38 kDa, mature rAmb t CP) appeared after refolding (Fig. 3A). Two proteins isolated by anion-exchange chromatography were resolved on SDS-PAGE gel (Fig. 3B), and isolated proteins were characterized. According to the result of CD, the secondary structure contents assessed by the K2D3 program were α-helices (70.55%) and β-sheets (2.86%) in pro rAmb t CP, and α-helices (76.83%) and β-sheets (1.34%) in mature rAmb t CP (Fig. 3C). The enzymatic activity of mature rAmb t CP was higher than that of pro rAmb t CP (Fig. 3D). Additionally, pro- and mature rAmb t CP showed positive reactions to anti-rAmb t CP pAbs (Fig. 4B).

### 3.3. Purification and characterization of nAmb t CP

Purified nAmb t CP by immunoaffinity chromatography coupling with anti-rAmb t CP pAbs was resolved on SDS-PAGE gel, which displayed the enzymatic activity (Figs. 3D, 4A). Purified nAmb t CP was also characterized by mass spectrum (Fig. S3). The coverage of amino acid sequences identified by mass spectrum was up to 34.6%, and the amino acid (N<sub>127</sub>) of nAmb t CP was glycosylated (Fig. 1B). Purified nAmb t CP (two protein bands, approximately 35 and 40 kDa) showed positive reactions to anti-rAmb t CP pAbs, which corresponded to those of giant ragweed pollen extracts (Fig. 4B). For the glycosylation analysis, two protein bands of purified nAmb t CP were highlighted, which suggested that they were glycosylated (Fig. 4C).

### 3.4. IgE-binding activity of Amb t CP in Chinese patients with ragweed pollen allergy

According to the result of ELISA, IgE-binding activity of nAmb t CP in 68 Chinese patients with ragweed pollen allergy was 33.8% (23/68) (Fig. 5A). We also found that ragweed (W1) pollen-specific IgEs and IgE reactivity to Amb t CP showed a positive correlation (Fig. S4). Subsequently, 13 sera (OD values over 1.2 × cut-off values) of ragweed pollen-allergic patients were included, and the result of immunoblot showed that IgE-binding activity of the approximately 40 kDa protein (pro nAmb t CP) and the approximately 35 kDa protein (mature nAmb t CP) was 76.9% (10/13) and 15.4% (2/13) respectively (Fig. 5B). Representative flow cytometry plots of basophils from the ragweed pollen-allergic patient and non-allergic individual stimulated with anti-IgEs, giant ragweed pollen extracts, nAmb t CP, pro rAmb t CP, mature rAmb t CP and the buffer were showed in Fig. S5. According to the result of BAT, anti-IgEs and the buffer as the positive and negative control, could activate 39.0%, 9.8% basophils from the patient with ragweed

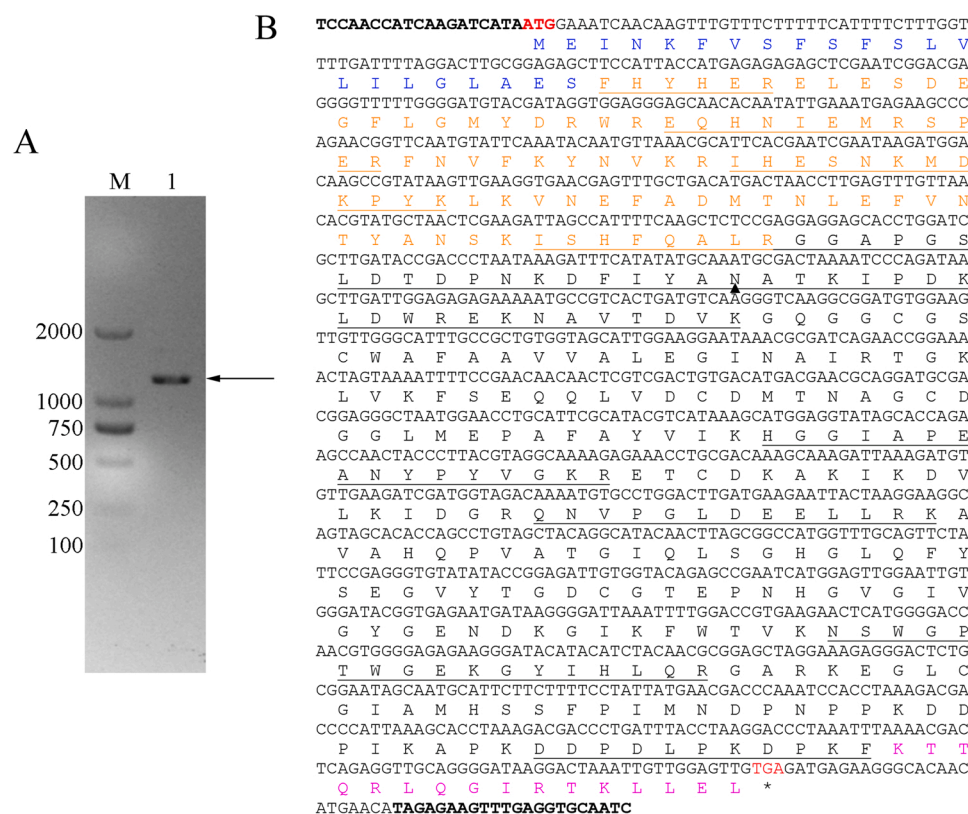


**Fig. 1.** Cloning of Amb t CP gene. A The PCR amplification of Amb t CP gene. Lane M - DNA marker, Lane 1 - PCR amplified fragment of Amb t CP gene (the arrow). B The nucleic acid sequence of Amb t CP gene. The Amb t CP gene with an 1164-bp ORF encoded 387 amino acids. The N-terminal signal peptides, N-terminal and C-terminal propeptides of Amb t CP protein were marked in blue, orange and purple respectively according to Amb a 11. The initiator and terminator of Amb t CP gene were marked in red. Bold nucleic acid sequences represented the primers. The underlined sequences represented amino acid sequences of Amb t CP protein identified by mass spectrum. The N-glycosylation site (N<sub>127</sub>) of Amb t CP protein was marked by a triangle.

pollen allergy respectively (Fig. 5C). With the addition of giant ragweed pollen extracts, nAmb t CP, pro rAmb t CP and mature rAmb t CP at final concentration of 10 µg/mL, their average proportion (%) of CD63-positive out of CCR3-positive cells was 48.6%, 67.3%, 55.6% and 28.6% respectively (Fig. 5C).

### 3.5. Pro- and mature rAmb t CP differently reacted to IgEs from sera of Chinese allergic patients to ragweed pollen

ELISA was also carried out to determine whether IgE-binding activity of pro- and mature Amb t CP were different. The result suggested that IgE-binding activity of pro- and mature rAmb t CP was 39.1% (9/23) and 47.8% (11/23) respectively (Fig. 6). Moreover, serum samples (number 2, 5 and 19) showed positive IgE binding to mature rAmb t CP but negative IgE binding to pro rAmb t CP, whereas number 12 was contrary to number 2, 5 and 19 (Fig. 6). Among sera showing positive IgE binding to pro- and (or) mature rAmb t CP, 9 individual sera (2, 3, 4, 6, 7, 8, 17, 20, 21) showed significantly different IgE-binding activity to pro- and mature rAmb t CP ( $p < 0.05$ ) (Fig. S6, Fig. 6).

### 3.6. nAmb t CP was autocatalytically converted from pro- to mature forms in giant ragweed pollen extracts

The result showed that pro nAmb t CP gradually converted to mature nAmb t CP at 4 °C over time, which indicated that the amount of pro- and mature nAmb t CP in giant ragweed pollen extracts changed (Fig. S7). When giant ragweed pollen extracts were stored at 4 °C for 22 days, pro nAmb t CP completely converted to mature nAmb t CP, whereas the autocatalytic conversion of pro- to mature nAmb t CP was not obvious when giant ragweed pollen extracts were stored at -20 °C (Fig. S7).

### 3.7. IgE cross-reactivity of Amb t CP between *Artemisia* and *Ambrosia* pollen

The existent components of *A. annua*, *A. sieversiana* pollen extracts showed positive reactions to anti-rAmb t CP pAbs by immunoblot, which migrated between 35 and 40 kDa (Fig. 7A). IgEs binding to nAmb t CP was inhibited in different degree, which was exerted by preincubating pooled sera of ragweed pollen-allergic patients with giant ragweed, short ragweed, *A. annua* and *A. sieversiana* pollen extracts at final concentration of 10 µg/mL by inhibition immunoblot (Fig. 7B, Fig. S8). Compared with other treated groups, IgEs binding to Amb t CP was almost completely inhibited by preincubating pooled sera of ragweed pollen-allergic patients with giant ragweed pollen extracts (Fig. 7B, Fig. S8). Also, IgEs binding to the CP components (approximately 35–40 kDa) from giant ragweed, short ragweed, *A. annua* and *A. sieversiana* pollen could be inhibited by preincubating pooled sera of ragweed pollen-allergic patients with nAmb t CP at final concentration of 10 µg/mL (Fig. S9).

## 4. Discussion

Under global warming, the spread of giant ragweed is consistently expanding, which further causes that allergy to giant ragweed pollen becomes a potential problem of human health (Rasmussen et al., 2017; Dong et al., 2020). However, the exact allergenic molecules from giant ragweed pollen are poorly investigated. Therefore, the identification and characterization of unknown allergens from giant ragweed pollen is essential, which will help to promote component-resolved diagnosis and treatment for giant ragweed pollen allergy.

In this study, the Amb t CP gene with an 1164-bp ORF encoding 387 amino acids was cloned. The amino acid sequence of Amb t CP shared 95.34% identity with that of Amb a 11 (Bouley et al., 2015). Compared with other homologous CP allergens such as Der p 1, Der f 1, Act d 1 and Eur m 1, the sequence identity was 19%–35% (Chevigné et al., 2007;

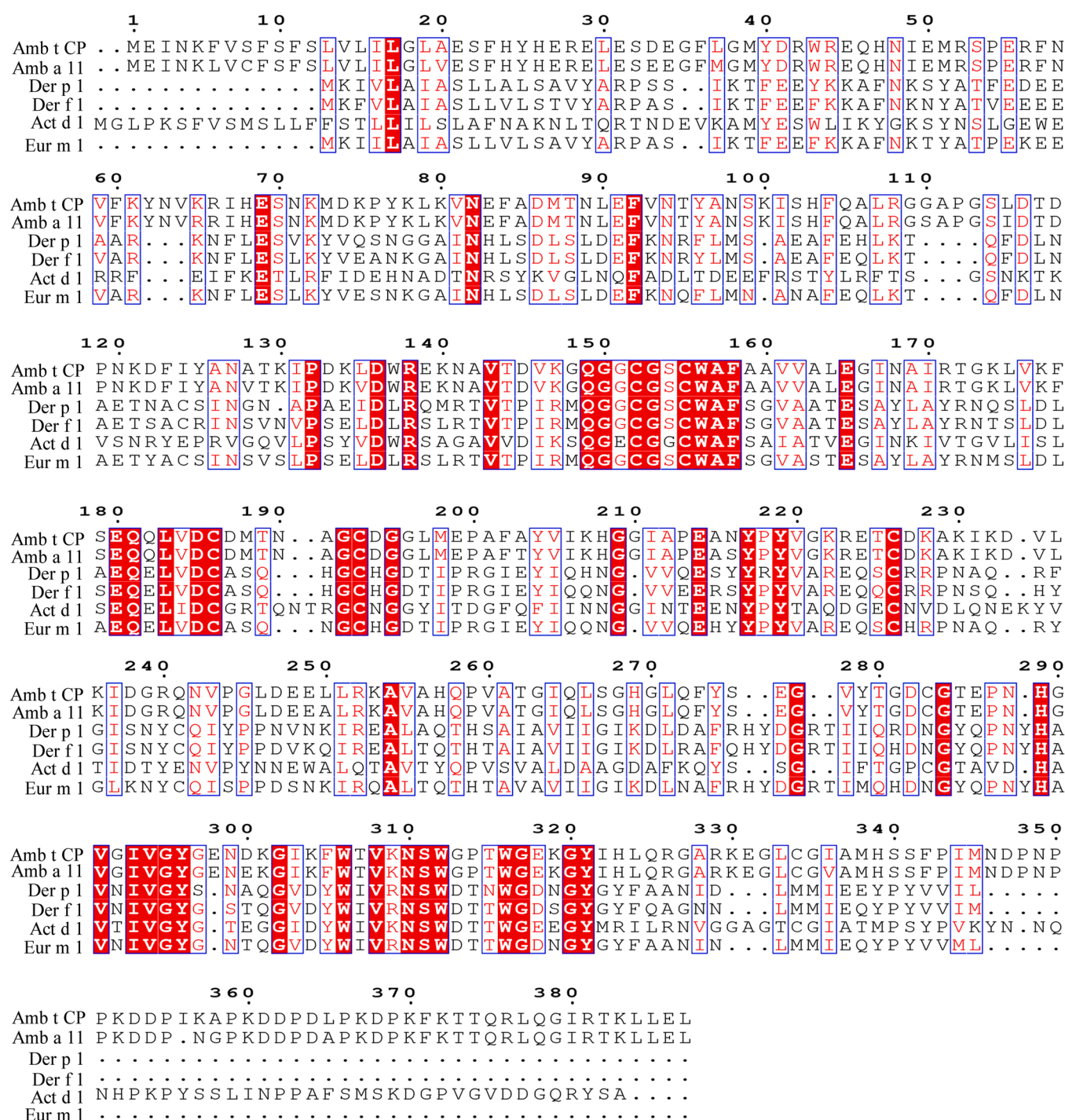


**Fig. 2.** Multiple alignments with homologous CP allergens from other species. The amino acid identity of Amb t CP and Amb a 11, Der p 1, Der f 1, Act d 1 and Eur m 1 was 95.34%, 19.56%, 27.39%, 34.51%, 24.87% respectively. The blue and red boxes represented that the amino acid identity of these CP allergens was over 70% and 100% respectively. The accession numbers of Amb a 11, Der p 1, Der f 1, Act d 1, Eur m 1 were AHA56102.1, AAB60215.1, BAC53948.1, CAA34486.1, AAC82351.1 respectively.

Zhang et al., 2007; Chruszcz et al., 2009; Cavic et al., 2014). It was reported that the structures of mature domains of these CP allergens, such as Amb a 11, Der p 1 and papain, which belonged to the CA1 family, were very similar (Bouley et al., 2015). It possibly indicated that these CP allergens had a relatively conserved three-dimensional structure.

According to our results, a new protein band below purified rAmb t CP appeared after its refolding, which showed higher enzymatic activity and positive reactions to anti-rAmb t CP pAbs. The secondary structures of purified rAmb t CP and the new appearing protein were similar.

Considering the autocatalytic maturation of CP allergens belonging to the CA1 family (Groeme et al., 2016; Paul et al., 1995; Takai et al., 2002), we speculated that the new appearing protein after refolding was possibly mature rAmb t CP. Moreover, purified nAmb t CP (two protein bands, approximately 35 and 40 kDa) showed positive reactions to anti-rAmb t CP pAbs, which corresponded to those of giant ragweed pollen extracts. Purified nAmb t CP was also identified by mass spectrum. Additionally, the protein (approximately 40 kDa) gradually decreased, and the other (approximately 35 kDa) gradually increased in

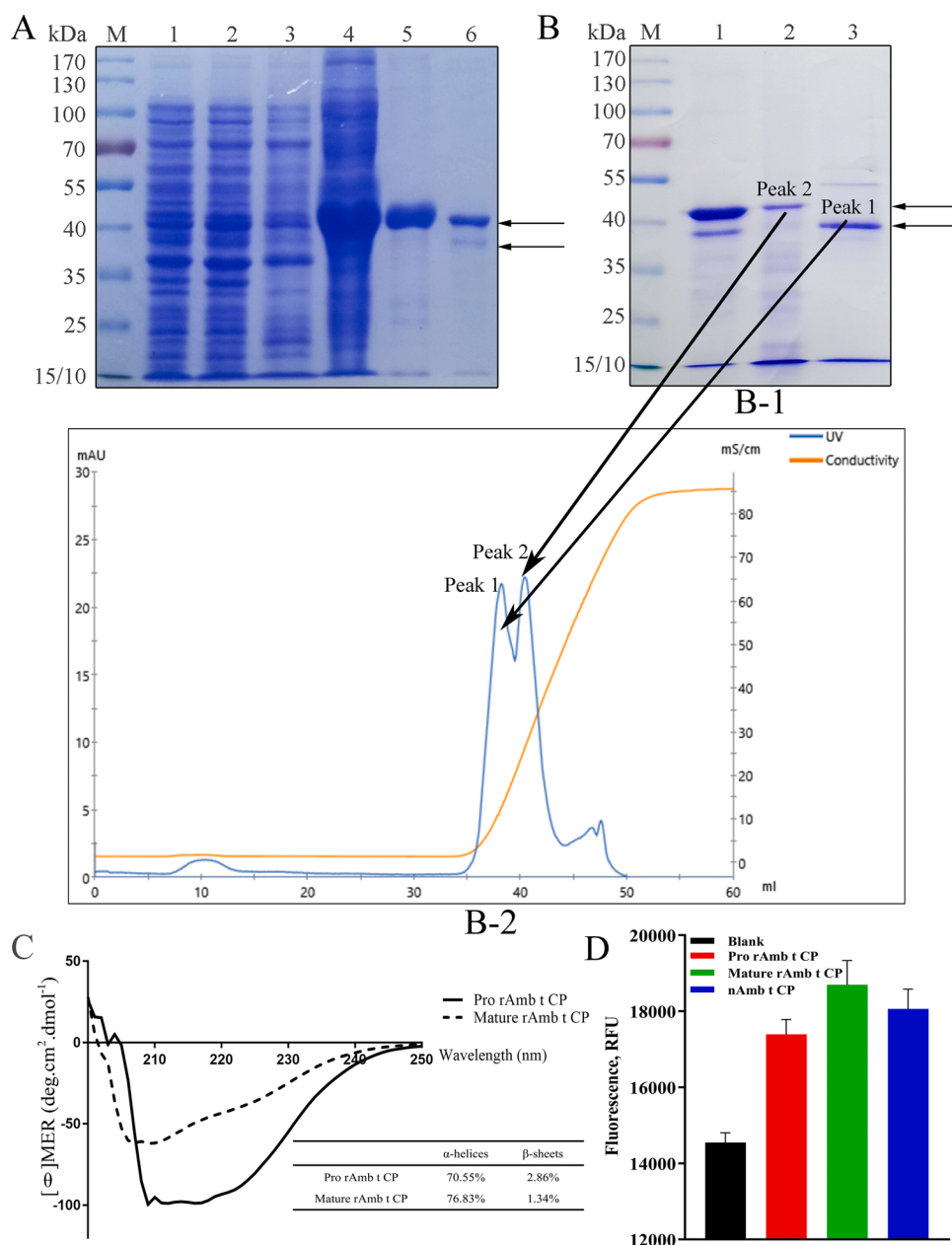


**Fig. 3.** Expression, purification and characterization of rAmb t CP. **A** rAmb t CP purification. Lane M - protein marker; Lane 1, 2 - lysates of whole cells induced with 0 mM, 1 mM IPTG respectively; Lane 3 - the supernatant of cell lysates after ultrasonication; Lane 4 - the pellet of cell lysates after ultrasonication; Lane 5 - purified rAmb t CP; Lane 6 - purified rAmb t CP after refolding (pro- and mature rAmb t CP were marked by arrows). **B** Pro- and mature rAmb t CP. Lane M - protein marker, Lane 1 - purified rAmb t CP after refolding, Lane 2 - pro rAmb t CP, Lane 3 - mature rAmb t CP; Peak 1 - mature rAmb t CP, Peak 2 - pro rAmb t CP. **C** CD analysis. According to the result of CD,  $\alpha$ -helices dominated in the secondary structures of pro- and mature rAmb t CP. **D** Enzymatic activity. The enzymatic activity of pro rAmb t CP was lower than that of its mature form, and the enzymatic activity of mature rAmb t CP was higher than that of nAmb t CP including pro- and mature nAmb t CP.

PBS when giant ragweed pollen extracts were stored at 4 °C over time. Collectively, both rAmb t CP and nAmb t CP had proforms and mature forms. The autocatalytic conversion was the important feature of Amb t CP as other CP proteins.

It was widely accepted that the autocatalytic conversion of these CP proteins was triggered by an acidic pH (Yasuhara et al., 2001; Huet et al., 2006; Roy et al., 2012; Bouley et al., 2015; Cheign   et al., 2017). However, some studies reported that other conditions also triggered the autocatalytic conversion of pro- to mature CP. The C-terminal extension of ervatamin-C, the CP protein from *Ervatamia coronaria*, was removed at higher pH of 8.0, which depended on the electrostatic interaction of N/C-terminal propeptides in acidic/basic pH and the amino acid characteristic of propeptides (Dutta et al., 2011). The CP protein from *Entamoeba histolytica* converted to its mature form when it was incubated in 10 mM DTT (Hellberg et al., 2002; Quintas-Granados et al., 2009). Moreover, a study about actinidin, the CP protein from Kiwifruit, pointed that the autocatalytic maturation possibly varied (Paul et al., 1995). As reported, the in vitro maturation of the proform of

recombinant Amb a 11 was completely abolished when the cysteine (cys-155) was mutated to a serine, which was a prerequisite for the enzymatic activity of the mature form of recombinant Amb a 11 (Groeme et al., 2016). In other words, the enzymatic activity of mature forms was critical for the self-activation maturation. We found that the autocatalytic conversion of pro- to mature rAmb t CP during refolding could be triggered with the enzymatic activity of rAmb t CP increasing. Additionally, giant ragweed pollen extracts contained pro- and mature nAmb t CP, and pro nAmb t CP could autocatalytically convert to mature nAmb t CP. So, we speculated that the enzymatic activity of Amb t CP was critical for its self-activation maturation. However, the detailed autocatalytic mechanism about Amb t CP remains to be further investigated.

The allergenicity of Amb t CP in Chinese patients with ragweed pollen allergy was characterized. Firstly, IgE-binding activity of Amb t CP in Chinese patients with ragweed pollen allergy was analyzed by ELISA and BAT. The result of ELISA suggested that Amb t CP had relatively high IgE-binding activity. Moreover, Amb t CP at final

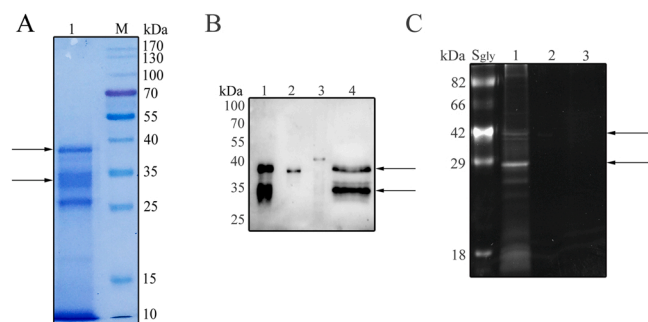


**Fig. 4.** Purification and characterization of nAmb t CP. A nAmb t CP purification. Lane 1 - purified nAmb t CP (pro- and mature nAmb t CP were marked by arrows); Lane M - protein marker. B Immunoblot of Amb t CP. Lane 1 - purified nAmb t CP; Lane 2 - mature rAmb t CP; Lane 3 - pro rAmb t CP; Lane 4 - giant ragweed pollen extracts. C Glycosylation analysis. Lane Sgly - Candy-Cane™ molecular weight standards, Lane 1 - purified nAmb t CP, Lane 2 - pro rAmb t CP, Lane 3 - mature rAmb t CP. These highlighted bands meant that they were glycosylated. Glycosylated pro- and mature nAmb t CP were marked by arrows.

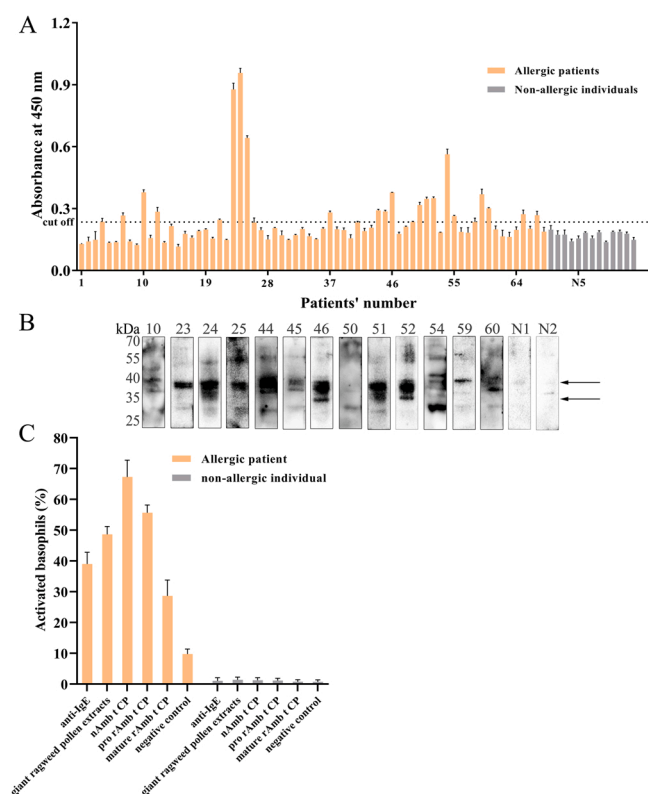


**Fig. 5.** IgE-binding activity of Amb t CP in Chinese patients with ragweed pollen allergy. A ELISA. The OD values of nAmb t CP reactions to IgEs from sera of patients with ragweed pollen allergy over cut-off values were considered as the positive, otherwise the negative. The cut-off values were 0.235. The data were presented as mean  $\pm$  SD ( $n = 3$ ). B Immunoblot. The reactivity of purified nAmb t CP to IgEs was further analyzed in 13 sera of patients with ragweed pollen allergy (OD values over  $1.2 \times$  cut-off values; The above numbers corresponded to those of Fig. 5A). Pro- and mature nAmb t CP were marked by arrows. C BAT. The vertical axis represented their average proportion of CD63-positive out of CCR3-positive cells. The data were presented as mean  $\pm$  SD ( $n = 3$ ).

concentration of 10  $\mu$ g/mL, had a strong ability to activate basophils from the patient with ragweed pollen allergy, suggesting that Amb t CP had the potential to trigger allergic symptoms.

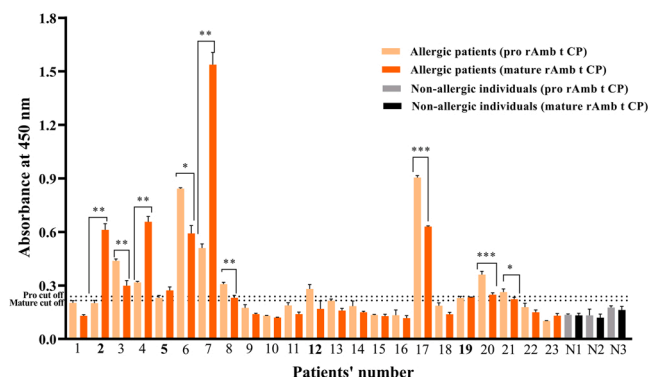


**Fig. 6.** Pro- and mature rAmb t CP differently reacted to IgEs from sera of Chinese allergic patients to ragweed pollen. Bold serum numbers meant that they had positive IgE binding activity to either pro rAmb t CP or mature rAmb t CP. Pro cut off and mature cut off represented the cut-off values of pro- and mature rAmb t CP respectively (pro cut-off values = 0.238, mature cut-off values = 0.216). The individual sera of patients with ragweed pollen allergy had significantly different IgE-binding activity to pro- and mature rAmb t CP ( $p < 0.05$ ), which was marked by “\*”. The 7 sera (number 3, 6, 7, 8, 17, 20, 21 in Fig. 6) corresponded to these sera (number 10, 23, 24, 25, 54, 59, 60 in Fig. 5B). The data were presented as mean  $\pm$  SD ( $n = 3$ ).

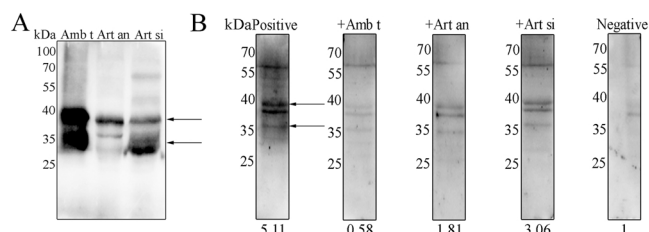


**Fig. 7.** IgE cross-reactivity of Amb t CP between *Artemisia* and *Ambrosia* pollen. A The CP components of giant ragweed, *A. annua* and *A. sieversiana* pollen. Amb t - giant ragweed pollen extracts, Art an - *A. annua* pollen extracts, Art si - *A. sieversiana* pollen extracts. The arrows represented the CP components from giant ragweed, *A. annua*, *A. sieversiana* pollen. B IgE cross-reactivity between *Artemisia* and *Ambrosia* pollen. Positive - pooled sera of ragweed pollen-allergic patients without treatments; +Amb t, +Art an, +Art si - pooled sera of ragweed pollen-allergic patients preincubated with giant ragweed, *A. annua*, *A. sieversiana* pollen extracts respectively; Negative - pooled sera of non-allergic individuals. The arrows represented pro- and mature nAmb t CP. Numbers below the immunoblot strips represented the signal ratio between the Positive, +Amb t, +Art an, +Art si and Negative blot by imageJ.

Secondly, IgE-binding activity of pro- and mature Amb t CP was different. According to the result of immunoblot, pro nAmb t CP showed more positive results than mature nAmb t CP. Considering the possible disruption of conformational IgE-binding epitopes during SDS-PAGE process, IgE-binding activity of pro- and mature Amb t CP was also determined by ELISA. The result showed that the reactivity of pro- and mature rAmb t CP to IgEs from 23 sera of Chinese patients with ragweed pollen allergy was 39.1% (9/23) and 47.8% (11/23) respectively. Among sera showing positive IgE binding to pro- and (or) mature rAmb t CP, 9 individual sera (2, 3, 4, 6, 7, 8, 17, 20, 21) showed significantly different IgE-binding activity to pro- and mature rAmb t CP ( $p < 0.05$ ). However, among some patients with ragweed pollen allergy, pro Amb t CP showed higher IgE-binding activity than mature Amb t CP. It was possibly explained that N, C-terminal propeptides of pro Amb t CP formed IgE-binding epitopes and (or) IgE-binding epitopes of pro Amb t CP were more accessible. A similar result that proforms showed higher IgE-binding activity than or equivalent IgE-binding activity to mature forms was also observed in Der f1/Der p1 allergens (Takai et al., 2005). Compared the allergenicity conducted by ELISA and immunoblot, their

results were inconsistent. Possible reasons were as follows: 1. Conformational IgE-binding epitopes of Amb t CP were destroyed during SDS-PAGE process. 2. The conversion of pro- to mature Amb t CP involved in the cleavage of propeptides, which possibly promoted the conformational change and further changed IgE-binding epitopes of Amb t CP. 3. The ratio of linear and conformational IgE-binding epitopes was possibly changed. The inconsistent result of immunoblot and ELISA possibly indicated that conformational IgE-binding epitopes were critical for mature Amb t CP mediating IgE-binding responses. To sum up, different IgE-binding activity of pro- and mature Amb t CP might result from their IgE-binding epitope change during the autocatalytic maturation.

Thirdly, the Amb t CP glycosylation had no effect on its IgE-binding activity. Equivalent inhibitory effect was reported when comparing natural Der f 1/natural Der p 1 with recombinant Der f 1/recombinant Der p 1 with or without N-glycosylation (Takai et al., 2005). Also, similar IgE-binding activity of recombinant Amb a 11 without glycosylation and its natural form was observed (Bouley et al., 2015). Considering the high sequence identity and same N-glycosylation site with Amb a 11, the glycosylation of Amb t CP may not be associated with its allergenicity. However, the effects of Amb t CP glycosylation on its allergenicity still need to be further clarified.

Finally, the inhibition immunoblot result showed that IgEs binding to Amb t CP was almost completely inhibited by giant ragweed pollen extracts, whereas IgEs binding to Amb t CP was partially inhibited by short ragweed, *A. annua* and *A. sieversiana* pollen extracts. The result indicated that Amb t CP and the CP components from short ragweed, *A. annua* and *A. sieversiana* pollen shared IgE cross-reactivity, and Amb t CP possibly played a primary role in the CP components from giant ragweed, short ragweed, *A. annua* and *A. sieversiana* pollen mediating IgE-binding responses. IgEs binding to the CP components (approximately 35–40 kDa) from giant ragweed, short ragweed, *A. annua* and *A. sieversiana* pollen could be inhibited by nAmb t CP, which further suggested that Amb t CP and the CP components from short ragweed, *A. annua* and *A. sieversiana* pollen shared IgE cross-reactivity. IgE cross-reactivity between short ragweed and giant ragweed pollen was largely described (Leiferman et al., 1976; Christensen et al., 2015), which suggested that short ragweed single-specie immunotherapy was sufficient for patients with diverse ragweed pollen allergy. However, the study by Asero et al. suggested that short and giant ragweed pollen were not allergenically equivalent, which indicated that the diagnosis and treatment for patients with ragweed pollen allergy needed to be individualized (Asero et al., 2005).

In summary, a new allergen from giant ragweed pollen, Amb t CP, was identified and characterized, with relatively high IgE-binding activity in Chinese patients with ragweed pollen allergy, which would help to promote component-resolved diagnosis and treatment for giant ragweed pollen allergy.

#### CCRediT authorship contribution statement

**Ji-Fu Wei:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Ying Zhu:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Jin-Lyu Sun:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Xiao-Jing Ling:** Writing – original draft, Writing – review & editing, Methodology, Software, Validation, Visualization. **Yong-Shi Yang:** Writing – review & editing, Methodology, Software, Validation, Visualization. **Zhi-Qiang Xu:** Writing – review & editing, Methodology, Software, Validation, Visualization. **Yan-Jun Zhou:** Methodology, Software, Validation, Visualization. **Ye Wang:** Methodology, Software, Validation, Visualization. All authors contributed to drafting or editing of parts of the manuscript.

#### Conflict of interest

The authors declare that they have no relevant conflicts of interest.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.molimm.2022.05.003](https://doi.org/10.1016/j.molimm.2022.05.003).

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