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Preparation of Monospecific Polyclonal Antibodies Against *Glechoma hederacea* Agglutinin*

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Abstract *Glechoma hederacea* agglutinin (Gleheda) is a novel glycosylated lectin isolated from the leaves of *G. hederacea*. Like other glycosylated proteins, the detection of Gleheda by immunological methods is often hampered by the cross-reactivity of the polyclonal antibodies with unrelated glycoproteins. Hence a protocol to purify monospecific polyclonal antibodies from a crude antiserum raised against Gleheda was developed. After selective ammonium sulfate precipitation and successive affinity chromatography on columns of Sepharose 4B with immobilized Gleheda and *Robinia pseudoacacia* agglutinin (RPA), respectively, ion-exchange chromatography on a column of Q Fast Flow was used for further purification. The specificity of the antibody fractions from each step was tested by double immunodiffusion assay and analyzed by Western blot. Results revealed that affinity chromatography of the immunoglobulin fraction on the immobilized Gleheda antigen yielded an antibody preparation that still cross-reacted with many proteins in leaf extracts. Depletion of nonspecific cross-reacting antibodies directed against the glycan part of the glycoprotein by affinity chromatography on immobilized RPA removed most but not all nonspecifically reacting antibodies. Only upon further purification by ion exchange chromatography an IgG fraction of monospecific antibodies that reacted exclusively with Gleheda could be obtained and accordingly was suitable for immunodetection studies. This antibody purification procedure promises simplicity and efficiency. In addition, this method does not require expensive facilities.

Key words monospecific polyclonal antibodies, antibody purification, *Glechoma hederacea* agglutinin (Gleheda)

Leaves of ground ivy (*Glechoma hederacea*), which is a typical representative of the plant family Lamiaceae express high concentrations of a lectin called *G. hederacea* agglutinin (Gleheda). Biochemical analyses indicated that Gleheda is a tetrameric glycoprotein consisting of 26 ku and 28 ku subunits, which contain one and two glycan (s), respectively. Molecular cloning and molecular modeling of the deduced sequence demonstrated that Gleheda shares high sequence similarity with the legume lectins and exhibits the same overall fold and three-dimensional structure as the classical legume lectins, indicating that Gleheda belongs to the same lectin family as the legume lectins[1]. Insect feeding bioassays revealed that Gleheda dramatically affects larval growth and reproduction of both caterpillars and beetles [2,3] but exhibits no cytotoxicity towards human and animal cells[2, 4].

The identification of a Lamiaceae lectin with insecticidal activity raises great interest with respect to a study of the insecticidal activity and mode of action of Gleheda as well as possible applications for the generation of transgenic plants with enhanced resistance against some pest insects. To develop highly specific sensitive detection/quantification methods

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based on immunological techniques (like Western blot analysis and immunocytochemistry) a polyclonal antiserum was raised in a rabbit against highly purified Gleheda. However, preliminary experiments indicated that even after partial purification the antiserum still cross-reacted extensively with numerous unrelated proteins, and hence was not suited for a specific detection and/or quantification of the lectin.

This report describes the development of a protocol for the purification of monospecific polyclonal antibodies from a crude antiserum raised against Gleheda. Using a combination of selective ammonium sulfate precipitation, double affinity chromatography on immobilized Gleheda and immobilized *Robinia pseudoacacia* agglutinin (RPA), respectively, and ion-exchange chromatography an antibody fraction was obtained that reacted exclusively with Gleheda and accordingly was suitable for immunodetection studies.

1 Materials and methods

1.1 Preparation of antigens

The protein Gleheda was isolated as described by Wang *et al*[1]. *Robinia pseudoacacia* agglu tinin (RPA) was purified from the bark of the *R. pseudoacacia* tree as described previously[5].

1.2 Immunization of the rabbits and preparation of antiserum

Antibodies against Gleheda were raised in a male New Zealand white rabbit. The rabbit was injected subcutaneously with 1 mg highly purified Gleheda dissolved in 0.2 mol/L NaCl and emulsified in 1 ml of Freund's complete adjuvant to enhance the response to the immunogen. Four booster injections with 1 mg purified protein in 0.2 mol/L NaCl were given with 10-day intervals to obtain a prolonged persistence of the immunogen in tissues and a continuous stimulation of the immune system. Ten days after the final injection, 50 ml blood was collected from an ear marginal vein and kept overnight at room temperature to allow clotting of the blood. The crude antiserum was collected by centrifugation (3 000 *g* for 5 min) and the globulin fraction isolated by three rounds of selective precipitation with ammonium sulfate (40% saturation). After the final precipitation, the proteins were dissolved in 25 ml 0.2 mol/L NaCl. At this stage, the specificity of the antibodies was checked by Western blot analysis of a crude extract from *G. hederacea* leaves.

1.3 Immobilization of lectins on Sepharose 4B

Purified Gleheda and RPA were coupled to Sepharose 4B using the divinylsulfone method. Sepharose 4B was poured in a Büchner funnel, washed with 20 volumes of distilled water and equilibrated with a 0.5 mol/L Na_2CO_3 (pH 11). 50 ml of settled gel was suspended in 50 ml of the same carbonate buffer and supplemented with 5 ml divinylsulfone (Aldrich, Bornem, Belgium) to activate the vinyl group of Sepharose 4B. After incubation for 3 h at room temperature with occasional stirring, the suspension was transferred to a Büchner funnel and washed with 250 ml carbonate buffer (pH 11) followed by 1 L distilled water to remove unreacted divinylsulfone. 10 ml aliquots of the activated Sepharose 4B were subsequently suspended in 20 ml of a 0.5 mol/L solution of Na_2CO_3/$NaHCO_3$ (pH 10) containing 50 mg of the protein to immobilize. The mixtures were incubated for approximately 15 h at 37℃ under continuous agitation to allow the proteins to react with the activated Sepharose. After completion of the coupling, the suspension was transferred into single fritted 25 ml columns (International Sorbent Technology Ltd. (IST), Mid Glamorgan, UK) and allowed to settle by draining the coupling medium under gravity. An aliquot of the flow through was collected and its A_{280} was measured to check the coupling efficiency. Under the conditions described here, >98% of the proteins were immobilized to the Sepharose 4B matrix. After settling, the Sepharose 4B gel was washed with 50 ml Na_2CO_3/$NaHCO_3$ (pH 10) buffer and rinsed with 50 ml H_2O. To block the remaining free activated vinyl groups, the matrix was equilibrated with a 0.2 mol/L solution of Tris-HCl (pH 8.7), kept in this buffer for at least 6 h and washed with 50 ml 0.2 mol/L NaCl containing 0.01% Na-azide as an antimicrobial agent. Columns were used immediately or stored at 2℃ until use.

1.4 Affinity chromatography of antiserum on immobilized Gleheda

Affinity chromatography on immobilized Gleheda was done using a single fritted 25 ml column filled with 10 ml of Gleheda-Sepharose 4B. The column was mounted on an IST VacMaster Sample Processing Station. The globulin fraction prepared from the crude antiserum was loaded on the column at a flow rate of approximately 0.5 ml/min. After loading, the column was washed with 0.2 mol/L NaCl until the A_{280} fell below 0.01. Bound antibodies were desorbed with a

solution of 5 mmol/L unbuffered 1,3-propane diamine (pH 11). To prevent inactivation of the antibodies, 1 ml aliquots of the eluate were collected in 0.2 ml 0.4 mol/L phosphate buffer (pH 7.4) containing 1 mol/L NaCl. The presence of anti-Gleheda antibodies in the fractions was checked by immunodiffusion against purified Gleheda. Fractions containing the antibodies were pooled and the pH adjusted to 7.5 with 0.1 mol/L H_3PO_4. The specificity of the antibodies was checked by Western blot analysis of a crude extract from *G. hederacea* leaves.

1.5 Affinity chromatography of antiserum on immobilized RPA

The antibody fraction isolated by affinity chromatography on immobilized Gleheda was brought to a final concentration of 0.2 mol/L NaCl and loaded on a column of RPA-Sepharose 4B (using the same type of column and equipment as described above) at a flow rate of 0.5 ml/ min. After loading, the column was washed with 0.2 mol/L NaCl until the A_{280} fell below 0.01, and eluted as describded in 2.4. The flow through and eluate was collected in fractions of 1 ml. The presence of anti-Gleheda antibodies in the fractions was checked by immunodiffusion against purified Gleheda. Fractions containing antibodies against Gleheda were pooled and dialyzed at 4℃ against 20 mmol/L Tris-HCl (pH 8.7) for 24 h (with 3 changes of buffer). The specificity of the antibodies was checked by Western blot analysis of a crude extract from *G. hederacea* leaves.

1.6 Exchange chromatography of antiserum on Q Fast Flow

The antiserum fraction obtained by successive affinity chromatography on immobilized Gleheda and RPA was applied on a column (1.6 cm ×10 cm, approximately 20 ml bed volume) of Q Fast Flow (Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mmol/L Tris-HCl (pH 8.7). After loading the column was washed with 50 ml of the same buffer and the IgG fraction eluted with 0.1 mol/L NaCl in 20 mmol/L Tris-HCl (pH 8.7). Fractions (1 ml) were collected and the A_{280} determined. The presence of anti-Gleheda antibodies was checked in a double immunodiffusion assay. Fractions containing the antibodies were pooled and specificity of the antibodies checked by Western blot analysis of a crude extract from *G. hederacea* leaves. This final antibody preparation was divided in small portions and stored at −20℃ until use.

1.7 Double immunodiffusion assay

Double immunodiffusion assays were done in small petri dishes (5 cm in diameter) filled with 8 ml PBS containing 1% agarose, 4% (*w*/*v*) polyethylene glycol (*M*r 6 000) and 0.1 mol/L galactose (to prevent aspecific binding of the lectin). Six wells (4 mm in diameter) surrounding one central well were made into the solidified gel and filled with 10 μl samples of proteins or antibodies. Plates were incubated at 37℃ for 15 h. The formation of precipitin lines was checked visually. Alternatively, the plates were washed with 0.2 mol/L NaCl for 24 h to elute the unprecipitated proteins, fixed in a mixture of water/methanol/acetic acid (5∶5∶1, *v*∶*v*∶*v*) and the remaining precipitin lines stained with 0.05% Coomassie Brilliant blue R250 in the same mixture.

1.8 Western blot analysis

Crude extracts were prepared by homogenizing *G. hederacea* leaves in 4 volumes (*w*/*v*) of 20 mmol/L unbuffered 1,3-diaminopropane (pH 11) per g of fresh weight. Homogenates were centrifuged (10 min, 12 000 *g*) and the supernatants used immediately or frozen at −20℃ until use. Extracts and purified proteins were reduced with 2-mercaptoethanol and separated by SDS-PAGE in 15% (*w*/*v*) acrylamide gels[6]. After electrophoresis, gels were electroblotted on an Immobilon P membrane (Millipore, Bedford MA, USA) using a semi dry blotting system from BioRAD (Hercules, CA). To check the blotting efficiency, the gels were fixed and the remaining proteins stained with Coomassie brilliant blue. Prior to the immunodetection the free binding sites on the membrane were blocked with 5% (*w*/*v*) bovine serum albumin in TSB (10 mmol/L Tris, 150 mmol/L NaCl, 0.1% Triton X-100, pH 7.6) for 1 h at room temperature. Then, the membrane was washed with TSB for 5 min and consecutively treated with primary antibody (overnight incubation at room temperature), goat-anti-rabbit antibody (1 h incubation at room temperature) and peroxidase-anti-peroxidase-complex (1 h incubation at room temperature). After each treatment the membrane was washed 3 times with TSB for 5 min. Just before the immunodetection the membrane was washed for 5 min with 0.1 mol/L Tris-HCl (pH 7.6). The peroxidase reaction was carried out in a fresh solution of 0.1 mol/L Tris-HCl (pH 7.6) containing 0.7 mmol/L 3,3'-diaminobenzidine tetra-hydrochloride and 0.01% (*v*/*v*) H_2O_2. The reaction was stopped by washing the membrane in distilled water.

2 Results and discussion

On the day of the fourth injection of the rabbit with Gleheda, a few drops of blood were collected and the presence of anti-Gleheda antibodies checked by a double immunodiffusion assay against purified Gleheda. Since the titer of the antiserum already exceeded 1∶8 (Figure 1a), the rabbit was bled 10 days after the fourth injection and the crude antiserum prepared.

A preliminary double immunodiffusion test demonstrated that the globulin fraction obtained by ammonium sulfate precipitation yielded clear precipitin lines with pure Gleheda and a crude extract from a plant with a high level of lectin expression but was unreactive towards an extract from a lectin-negative plant (Figure 1b), indicating that the antiserum contains antibodies that were specifically directed against native Gleheda. To test the possible cross-reactivity with other unrelated proteins, a Western blot containing the purified antigen (Gleheda) and crude leaf extracts from a lectin-positive and a lectin-negative plant was developed with the same globulin fraction. As shown in Figure 2a, the antiserum reacted not only with the Gleheda polypeptides but also with several other proteins. According to the

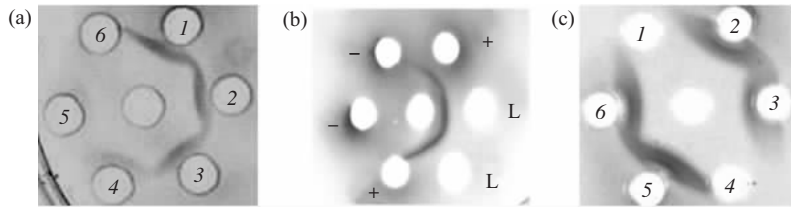

Fig. 1 Double immunodiffusion assays of different antibody preparations

After formation of precipitin lines, soluble proteins were eluted by extensive washing of the gels with 0.2 mol/L NaCl. Afterwards the gel was stained with 0.05% Coomassie Brilliant blue R250. (a) Determination of the titer of the crude antiserum. 10 μl of a 1 g/L solution of Gleheda was applied in the central well. Peripheral wells were filled with 10 μl crude antiserum (well *1*) and 2-, 4-, 8-, 16- and 32-fold diluted antiserum (wells *2*, *3*, *4*, *5* and *6*, respectively). (b) Reaction of the crude antiserum (central well) with pure Gleheda (well L), crude extracts from a lectin-positive (well +) and a lectin-negative (well −) plant. (c) Analysis of the reactivity of different antibody fractions obtained in the different steps of the purification procedure. Gleheda was applied in the central well. The flow-through and bound fractions from the affinity chromatography on immobilized Gleheda were loaded in the wells 1 and 2, respectively. Flow-through and bound fractions from the affinity chromatography on immobilized RPA were applied into wells *3* and *4*, respectively. Wells *5* and *6* were loaded with the fractions eluted with 0.1 mol/L NaCl and 0.5 mol/L NaCl, respectively, from the Q Fast Flow column.

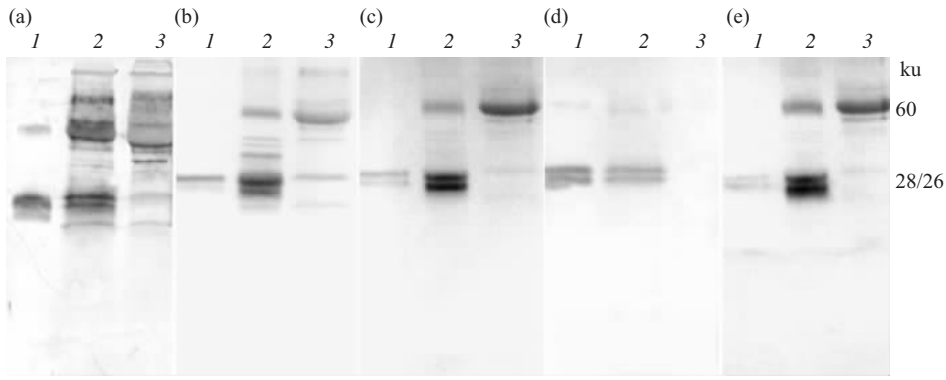

Fig. 2 Analysis of the specificity of different preparations of anti-Gleheda antibodies by Western blot analysis

Lanes *1*, *2* and *3* were loaded with pure Gleheda (0.1 μg), and 10 μl crude extract from leaves of a lectin-positive and a lectin-negative plant, respectively. All samples were reduced with 2% of β-mercaptoethanol. Antibody fractions were used in a dilution of 1∶500. The following antibody fractions were used: (a) Crude antiserum. (b) Fraction bound on Gleheda-Sepharose 4B. (c) Flow-through of the affinity chromatography on RPA-Sepharose 4B. (d) Fraction eluted with 0.1 mol/L NaCl from the Q Fast Flow column. (e) Fraction eluted with 0.5 mol/L NaCl from the Q Fast Flow column. Numbers on the right side of picture (e) show the size of protein marker.

pattern obtained with the lectin-negative extract, the strongest nonspecific reaction occurs with a protein of approximately 60 ku of the extract (Figure 2a). Therefore, a method had to be developed to isolate the monospecific antibodies from the crude globulin fraction.

In the first attempt to increase the specificity, the antibodies that reacted with Gleheda were selectively isolated from the globulin fraction by affinity chromatography on immobilized Gleheda. This approach yielded a relatively large quantity of anti-Gleheda antibodies (Figure 3a), which reacted very well with Gleheda in a double immunodiffusion assay (Figure 1c, well 2). However when this affinity-purified fraction was used in a Western blot analysis, it still exhibited an extensive cross-reactivity with many unrelated polypeptides other than the lectin subunits (Figure 2b). Similar to the Western blot pattern obtained with the crude antiserum (Figure 2a), the Western blot pattern with the affinity-purified antibody fraction (Figure 2b) clearly showed cross-reactivity especially with a polypeptide of approximately 60 ku in crude extract from leaves of a lectin-positive and a lectin-negative plant. It appears, therefore, that affinity chromatography on immobilized Gleheda yields a fraction that is highly rich in anti-Gleheda but also in antibodies that cross-react with unrelated proteins and especially with a polypeptide of approximately 60 ku.

The most plausible explanation for the strong cross-reactivity is the presence of a subset of antibodies that is not directed against the polypeptide chains but against the N-glycan structures of the *G. hederacea* lectin. It is well-known, indeed, that serological cross-reactivity between unrelated plant proteins often relies on the presence of structurally identical N-glycans. Many plant proteins contain N-glycans with a xylose residue β-1,2 linked to the core mannose residue and a fucose residue α-1,3 linked to the asparagine-linked GlcNAc-residue. These structures are common in plant N-glycans but do not occur in N-glycans from mammalian origin, and hence are very potent antigens[7, 8]. As described in the paper of Wang *et al* in 2003[1], the 26 and 28 ku subunits of Gleheda contain one and two N-glycans, respectively. Though the structure of these N-glycans on Gleheda is not known, one can reasonably expect on the analogy of the N-glycans present on the subunits of the related lectins from the Lamiaceae species *Clerodendron trichotomum* [9] and *Salvia sclarea* [10] that Gleheda contains β-1,2 linked xylose, and α-1,3 linked fucose residues. Accordingly, there is a reasonable chance that the strong cross-reactivity of the affinity-purified anti-Gleheda antibody fraction is due to the presence of antibodies that are specifically directed against the N-glycans. If so, there is a possibility to remove the cross-reacting antibodies by affinity chromatography on an immobilized plant glycoprotein as has been demonstrated for the preparation of monospecific antibodies against a glycosylated type 2 ribosome-inactivating protein from elderberry (*Sambucus nigra*) bark [11]. In this particular case, the antiserum was depleted from N-glycan binding antibodies by specific retention on a column of immobilized *Robinia pseudoacacia* agglutinin (RPA), which is a glycosylated lectin belonging to the family of legume lectins[6].

Since RPA does not bind rabbit immunoglobulins through protein-carbohydrate-interactions, the technique developed for the elderberry type 2 ribosome-inactivating protein could in principle also be applied for the purification of anti-Gleheda antibodies for removal of those antibodies that cross-react with N-glycans. In addition, affinity chromatography on immobilized RPA could also increase the specificity of the antiserum by selective removal of the antibodies with the highest affinity for the polypeptide chain of the distantly related legume lectin (thus eliminating antibodies reacting with epitopes common to legume lectins). Therefore, the antibody fraction isolated by affinity chromatography on immobilized Gleheda was loaded on a column of immobilized RPA. As shown in Figure 3b, most of the proteins were not retained indicating that they did not react with the N-glycans of the immobilized RPA. Only a small fraction bound to the column and could be eluted by increasing the pH.

Double immunodiffusion tests revealed that only the antibodies present in the flow through reacted with purified Gleheda (Figure 1c, well *3*). However, a subsequent specificity assay by Western blot analysis demonstrated that despite an obvious increase in specificity (as compared to that of the affinity-purified antibody fraction obtained after Gleheda-Sepharose 4B) the flow through fraction still exhibited a strong cross-reactivity especially with a 60 ku polypeptide in the crude extracts of leaves (Figure 2c). Obviously, the depletion of cross-reacting glycan-recognizing

antibodies on an RPA-affinity column increases the specificity of the antibody preparation but does not yield monospecific anti-Gleheda antibodies.

In a final attempt to obtain monospecific antibodies, the preparation obtained by successive affinity chromatography on immobilized Gleheda and immobilized RPA was further purified by ion-exchange chromatography on a column of Q Fast Flow. After binding of the antibodies in Tris buffer the IgG fraction was eluted with 0.1 mol/L NaCl and the remaining proteins with 0.5 mol/L NaCl. Both the large IgG fraction in the 1st peak and the small fraction in the 2nd peak (Figure 3c) gave a positive reaction upon double immunodiffusion against Gleheda (Figure 1c, well *5* and *6*). However, Western blot analysis revealed that the IgG present in the first peak reacted exclusively with the Gleheda polypeptides (Figure 2d) whereas the antibodies present in the second peak exhibited a strong cross-reactivity especially with the 60 ku protein in the crude extract of leaves(Figure 2e).

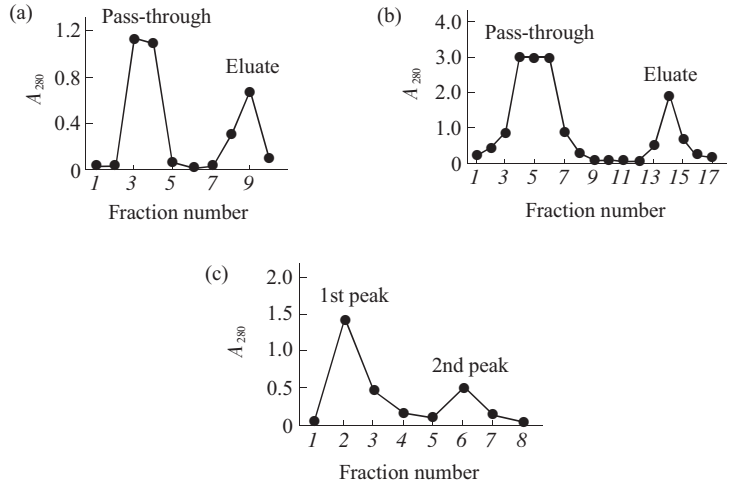

Fig. 3 Overview of the chromatography steps included in the purification protocol of monospecific anti-Gleheda antibodies

(a) Affinity chromatography on Gleheda-Sepharose 4B. (b) Affinity chromatography on RPA-Sepharose 4B. (c) Anion-exchange chromatography on Q Fast Flow.

3 Conclusion

Our objective was to develop a highly specific polyclonal antibody against Gleheda for application in immunological studies. Using a combination of ammonium sulfate precipitation, double affinity chromatography and a ion-exchange chromatography, a preparation of monospecific polyclonal antibodies that reacted exclusively with Gleheda could be prepared from a crude antiserum. Since the final preparation exhibited no cross-reactivity towards any unrelated proteins in a crude extract it is also suitable for immunodetection and immunolocalization studies. The present antibody purification protocol is not only simple, but is also specific and cost effective, does not require expensive facilities. Hence it may be applicable to the preparation monospecific polyclonal antibodies against other glycosylated plant proteins.

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抗欧亚活血丹凝集素特异性多克隆抗体的制备 *

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摘要 欧亚活血丹外源凝集素(Gleheda)是分离自欧亚活血丹 (*Glechoma hederacea*) 叶片中的一种糖基化植物新蛋白. 如同其他糖基化蛋白，通过免疫学方法探测 Gleheda 的过程中通常受到一些不相干糖蛋白的妨碍，为此制定了抗 Gleheda 特异性多克隆抗体的纯化方案. 免疫血清蛋白经硫酸铵选择性沉淀后，分别以 Gleheda 和刺槐外源凝集蛋白 (RPA) 结合在 Sepharose 4B 作为亲和配体，采用亲和层析法连续纯化 2 次，然后进一步采用离子交换层析 Q Fast Flow 提纯. 经每一步骤提纯得到的抗体组分对 Gleheda 的特异性，均同时采用双向免疫扩散检验和 Western blot 分析. 结果表明，以 Gleheda 为配体，亲和纯化制备得到的抗体组分对叶片粗提物中的许多植物 (糖) 蛋白仍然表现交叉反应. 为除去由植物糖蛋白中的聚糖所引起这些非特异性交叉反应抗体，接着以 RPA 为配体再次进行亲和纯化，Western blot 分析显示，抗体的特异性得到提高但并非除去了所有非特异性交叉反应的抗体. 最后进一步采用离子交换层析制备得到仅抗 Gleheda 蛋白的特异性抗体组分，此抗体组分适用于免疫探测研究. 该抗体纯化制备程序简易而高效，而且不需要昂贵的设备.

关键词 特异性多克隆抗体，抗体制备，欧亚活血丹外源凝集素 (Gleheda)
学科分类号 Q5-3

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