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Extraction and purification of the lectin found in the tubers of *Eranthis hyemalis* (winter aconite)

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Abstract

Lectin are proteins which play an important role in the defence mechanisms of plants against the attack of microorganisms and insects: this role has provoked particular interest in the fields of biotechnology and agriculture. This paper describes the extraction and purification of the lectin found in tubers of the winter aconite (*Eranthis hyemalis*), with the aim of improving and modernising the existing extraction protocol. The *Eranthis hyemalis* lectin (EHL) is a member of the type-2 Ribosome Inactivating Proteins (RIP) family, proteins which have the ability to inhibit *in vitro* protein synthesis. RIPs have been linked to plant defence by their antiviral, antifungal and insecticidal properties, and some have been found to be potent inhibitors of the Human Immunodeficiency Virus-1 (HIV-1) virus. EHL was purified using affinity column chromatography and ammonium sulphate precipitation; thiourea was used as antioxidant in order to prevent EHL denaturing during the extraction process. The presence of EHL in the extract was verified using a blood agglutination test with rabbit erythrocytes. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was employed to determine the lectin size; EHL was found to be formed of two chains with molecular weights of approximately 31 kDa; the size of the whole protein was estimated as approximately 60 kDa. The concentration of the EHL in the post-column eluent, determined using the Bradford Assay, was 380.1 $\mu g.cm^{-3}$. This improved extraction protocol is the first step which will enable future research on the potential use of EHL in crop protection, by studying its insecticidal, fungicidal and bactericidal properties.

Keywords: Lectin, Ribosome Inactivating Proteins, Affinity chromatography, SDS-PAGE.

1. Introduction

Plant lectins are a class of proteins, non-immune in origin, which show a very specific interaction with carbohydrates. Different lectins bind with specific carbohydrate-containing compounds (e.g. polysaccharides, glycoproteins, and glycolipids), which can be free or bound in cell membranes. Another key characteristic of lectins is their ability to agglutinate erythrocytes, which provides an unambiguous indicator of their presence. Plant lectins have many functions including growth regulation, carbohydrate transport, and plant defence through interaction with microorganisms as well as insect and mammalian predators [1-3]. Additionally, lectins are involved in the detection of nitrogen-fixing bacteria on root surfaces, and the transport of hormones and glycoproteins in plants [3, 4]. These properties make lectins particularly interesting in the context of crop protection, as biological control of pests and diseases. Lectin genes have been inserted into transgenic plants to initiate production of a desired lectin in large quantities, thereby inducing the plant resistance against insect, nematode or mammalian pests [2, 5, 6].

Historically, research on plant lectins has been concentrated in those found in the dry seeds of leguminous plant species. However, lectins have been shown to be present in a variety of plant tissues spanning a wide range of taxonomic groups [1-3]. Investigations into the structure and biochemical properties have identified and classified lectins from a

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variety of plant species including *Canavalia gladiata* (swordbean) [7], *Viscum album coloratum* (mistletoe) [8], *Pisum sativum* (pea) [9], *Arum maculatum* L. [10] and *Eranthis hyemalis* (winter aconite) [11-14].

The lectin on which this study is focused is found in the tubers of winter aconite (*Eranthis hyemalis*), the first member of the Ranunculaceae family known to contain a lectin [11]. The *Eranthis hyemalis* lectin (EHL) makes up around 3% of the whole soluble protein content of the tubers; EHL is specific to N-acetyl-galactoseamine and blood group O eryrthrocytes [11]. EHL has been shown to have pesticidal activity against *Diabrotica undecimpunctata howardii*, a major insect pest of maize crops [12, 14].

Kumar, et al, [12] have identified EHL as a type-2 Ribosome Inactivating Protein (RIP), a class of proteins which are highly cytotoxic to eukaryotic organisms [15]. Type-2 RIP lectins provide plants with a defence mechanism against both vertebrate and invertebrate pests, and have also been found to have an inhibitory effect on plant viruses [12]. Other plant species containing RIP type-2 lectins include *Iris hollandica* (Dutch iris) [16] and *V. album coloratum* [8]. Another well know member of this group is the potent cytotoxic agent ricin, extracted from castor beans [12]. RIPs are formed by two polypeptide chains, a cytotoxic A chain and a carbohydrate-binding B chain. Binding of the B chain to a glycoconjugate on a cell surface mediates the entry of the cytotoxic A chain, which inactivates ribosomes. For EHL, initial studies suggest that the size of the A chain is between 28 and 30 kDa, the size of the B chain is 30 to 32 kDa, and the size of the whole lectin is between 55 and 62 kDa [11, 12].

Type-2 RIP lectins have been shown to have an inhibitory effect on viruses [12, 17], which has sparked considerable interest in thee fields of medicine and pharmacology. Various plant lectins have been demonstrated to be useful in the diagnosis and treatment of human diseases such as Human Immunodeficiency Virus-1 (HIV-1); for example, the plant lectin jacalin can effectively block HIV-1 in vitro infection of lymphoid cells [17]. Lectins have also been used as diagnostic and therapeutic tools for cancer treatment [18]. Lectins from species such as mistletoe have a higher affinity to glycoconjugates found on the surface of tumour cells than those present on the surface of healthy cells; this anti-tumoral activity, demonstrated in-vitro, in-vivo and in human case studies, has been applied in cancer treatment in conjunction with conventional radiotherapy and chemotherapy [19, 20]. Lectins have also been studied as potential biomarkers for the early detection of pancreatic cancer, by detecting unique glycosylation patterns of proteins in the patient's serum [21].

Any potential applications of EHL in medicine, pharmacology or pest control rely heavily on the determination of the lectin's structure. Previous research groups were only partially successful, and so far only part of the amino acid sequence of the A chain has been determined [12]. Since purification is a crucial stage when aiming for crystals used for structural analysis of any protein, improvements of the extraction protocol is a first vital step towards obtaining a suitable crystal for such structural analysis. Preliminary work carried out by our research group was successful in isolating EHL [22]. However, the yield obtained was very low, only 37 $\mu g.cm^{-3}$. Moreover, a brown deposit remained in the chromatography column after elution. This residue was attributed to the oxidation of phenols in the tissue by phenoloxidases, a problem often associated with the extraction of proteins and organelles from plant tissues. Phenols are oxidised to quinones which covalently bind to proteins, modifying structure and function; this reaction can be inhibited by the use of a phenoloxidase inhibitor [23]. Thiourea has been proposed as the most appropriate phenoloxidase inhibitor for the extraction of biologically active protein from phenol-rich plant tissues inhibitor [23].

Herein we present the revised extraction and purification of EHL using affinity column chromatography; thiourea was used as an antioxidant in order to prevent the lectin denaturing during the extraction process. The presence of EHL was determined by agglutination assays, using rabbit erythrocytes, since agglutination of red blood cells is a characteristic associated with lectins which provides an unambiguous indicator of lectin presence. Concentration of the extracted EHL was determined using a Bradford assay. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) analysis was used to determine the size of the isolated lectin.

2. Materials and methods

2.1 *Isolation and Purification of EHL*

For the extraction and purification of the lectin from the winter aconite tubers the protocol described by Kumar et al. [12] was modified as follows. Tubers of the winter aconite (*Eranthis hyemalis),* purchased from MAS Seeds Ltd, were cleaned and peeled in an ice-bath before being finely diced. 40 g of the *E. hyemalis* tubers were homogenized in 500ml of 0.2 M sodium chloride solution (pH 5) containing 5 mM thiourea. The homogenized extract was allowed to settle for 30 minutes at 4°C, after which the supernatant was extracted and stored at 4°C. The remaining slurry was thoroughly resuspended in a further 500ml of 0.2 M sodium chloride solution with thiourea and allowed to settle for 30 minutes at 4°C; the supernatant was extracted again, added to the previous supernatant extraction, and stored at 4°C. The combined supernatant fractions were centrifuged at 14000 rpm at 4°C for 20 minutes. The fat layer was removed and discarded and the pH of the extractions was adjusted to 9 using 2M sodium hydroxide before being centrifuged again under the same conditions. The fat layer was again removed and the pH readjusted to pH 9. The extract was then filtered through 3MM Whatman chromatography paper.

Approximately 300ml of the raw extract was pumped through a CNBr-activated Sepharose affinity column (GE

Healthcare) coupled with fetuin from fetal calf serum (Sigma Aldrich). The column containing the bound lectin was washed with 150 mM phosphate-buffered saline solution (PBS), pH 7.2, to remove any unbound protein. The column bound EHL was then eluted with 30 ml of 20 mM diaminopropanol (DAP). The presence of lectin in the eluent was determined using a UV path monitor at λ 280 nm (Unicam 5625 UV/VIS Spectrometer). The eluent was neutralised with 9.5ml of 1M Tris-HCl.

2.2. *SDS-PAGE analysis*

Two samples of the post-column eluent were run on 15% Tris-HCl gels in a Mini-PROTEAN 3 electrophoresis module for 40 minutes at 200V. Both samples were incubated at room temperature for 5 minutes. One sample was used to determine the size of the lectin. The other sample was incubated at 95°C before the electrophoresis run, in order to denature EHL and determine the size of the constituent polypeptide chains. The SDS-PAGE gels were stained with Coomassie Brilliant Blue for one hour and de-stained with molecular biology grade water for 24 hours.

2.3. *Agglutination test and Bradford assay*

The presence of lectin in the post-column eluent was confirmed by mixing 0.05 cm^3 of defibrinated rabbit blood with 0.05 cm^3 of column eluent on a welled microscope slide. A control sample was prepared by mixing 0.05 cm^3 of 150 mM PBS with 0.05 cm^3 of defibrinated rabbit blood. Agglutination, assessed under both a high-power and low-power microscope, was also visible to the naked eye.

The concentration of EHL in the column eluent was determined using the pre-programmed Bradford Assay method on a Thermo Scientific BioMate 3 UV-Vis Spectrophotometer. Standard protein solutions where prepared using 0.1, 0.2, 0.3, 0.4 and 0.5 $mg.cm^{-3}$ bovine serum albumin (BSA); absorption readings of these solutions at 595 nm were used to prepare a calibration curve. The absorption reading of a sample of post-column eluent was interpolated in the calibration curve in order to determine the concentration of EHL.

3. Results and discussion

Results suggest that EHL was successfully extracted and purified from the tubers of winter aconite. The sharp peak at λ=280 nm in the UV path monitor indicates the presence of protein in the post column eluent. Since the chromatography column is packed with fetuin, which shows a strong affinity for lectin proteins, any protein detected in the post-column eluent can be assumed to be lectin. The presence of lectin in the post-column eluent is also supported by the results of the agglutination test (see Figure 1). Agglutination of rabbit O-erythrocytes, visible even without the aid of a microscope, was observed in the assay containing the post-column eluent. Agglutination of rabbit O-erythrocytes in the control assay was not observed.

Furthermore, the results from the SDS-PAGE analysis confirm that EHL was successfully extracted from the plant tubers, and corroborate that EHL is a dimeric lectin composed of two polypeptide chains of similar sizes. The size of the unreduced EHL was estimated as approximately 60 kDa. Incubation of the gel at 95°C before analysis produced the breakage of EHL into two chains of approximately 31 kDa in size (see Figure 2). These results are consistent with reported literature values [11, 12].

The concentration of EHL in the post-column eluent was measured using the Bradford assay. From the absorbance of the lectin eluent and those of the BSA standards, the concentration of EHL was determined as 380.1 $\mu g.cm^{-3}$. Preliminary work carried out by our research group [22], although successful in isolating EHL, produced a yield of only 37 $\mu g.cm^{-3}$. The low yield was attributed to protein oxidation during the extraction process, evidence of which was a brown deposit remaining in the chromatography column after elution. The use of thiourea as an antioxidant eliminated the presence of this brown residue in the affinity column. This suggests that the residue was, as suspected, the result of oxidisation processes which resulted in the denaturing of the lectin protein. Beside the use of thiourea as antioxidant, additional measures taken in order to maintain the integrity of the EHL included ensuring that the sample was constantly maintained at approximately 4°C. These precautions could explain the significant improvement observed in the concentration of EHL in the post-column eluent.

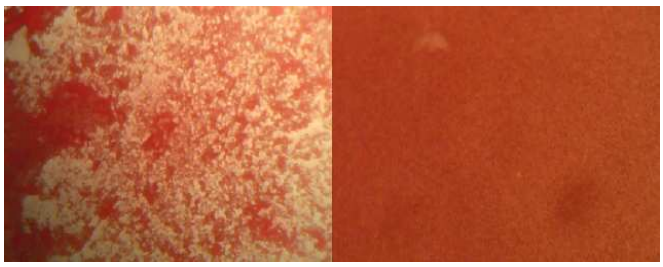

Figure 1. Blood smear (left) exhibits agglutination in the presence of the extracted EHL. Blood smear (right) exhibits even distribution of erythrocytes in the presence of the phosphate buffered saline (PBS) control; no agglutination is present (magnification x100).

Variations from the original method published by Kumar et al [12] include the use of PBS as buffer solution. Moreover, the original paper used two columns for the separation, one containing asialofetuin immobilised on agarose, followed by a Sepharose Q Fast flow anion exchange column. These have now been replaced with a single column step, using Fetuin bound to a Sepharose 4B column. Furthermore, tetraborate (used in the original paper) has been replaced by DAP, as the latter has been found to be more effective in eluting the lectin from the column, hereby reducing the volume of desorbant needed.

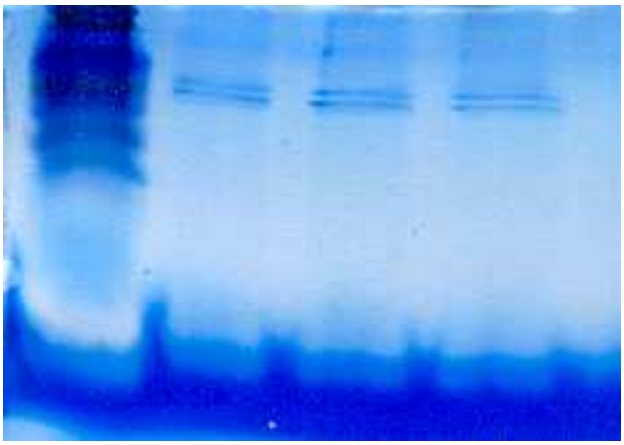

Figure 2. SDS-PAGE results for the gel incubated at 95°C before the electrophoresis run, in order to denature EHL. The gel shows the standard Kaleidoscopic marker (left hand side column) and EHL post-column eluent (three columns on the right). All EHL bands show two strands at approximately 31 kDa, corresponding to the reduced lectin.

4. Conclusion

The lectin protein found in tubers of winter aconite (*Eranthis hyemalis*) has been successfully extracted and characterised. EHL was purified using affinity column chromatography and ammonium sulphate precipitation; thiourea was used as an antioxidant in order to prevent the lectin denaturing during the extraction process. The success of the extraction protocol was verified using a blood agglutination test with rabbit erythrocytes. Using SDS-PAGE analysis, the size of the unreduced EHL was established as approximately 60 kDa; furthermore, EHL was determined to be formed of two non-identical chains with molecular weights of approximately 31 kDa. The concentration of the EHL in the post-column eluent, determined using the Bradford Assay, was 380.1 $\mu g.cm^{-3}$. This is an order of magnitude improvement over our previous work, and enables us to undertake in-depth study of effect of the lectin on various plant pathogens and pests, and thus to determine its potential crop protection applications.

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