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# Gelation in protein extracts from cold acclimated and non-acclimated winter rye (*Secale cereale* L. cv Musketeer) ☆

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ABSTRACT

A protein gel is a three-dimensional network consisting of molecular interactions between biopolymers that entrap a significant volume of a continuous liquid phase (water). Molecular interactions in gels occur at junction zones within and between protein molecules through electrostatic forces, hydrogen bonding, hydrophobic associations (van der Waals attractions) and covalent bonding. Gels have the physicochemical properties of both solids and liquids, and are extremely important in the production and stability of a variety of foods, bioproducts and pharmaceuticals. In this study, gelation was induced in phenol extracted protein fractions from non-acclimated (NA) and cold-acclimated (CA) winter rye (*Secale cereale* L. cv Musketeer) leaf tissue after repeated freeze–thaw treatments. Gel formation only occurred at high pH (pH 12.0) and a minimum of 3–4 freeze–thaw cycles were required. The gel was thermally stable and only a specific combination of chemical treatments could disrupt the gel network. SDS–PAGE analysis identified ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) as the major protein component in the gel, although Rubisco itself did not appear to be a factor in gelation. Raman spectroscopy suggested changes in protein secondary structure during freeze–thaw cycles. Overall, the NA and CA gels were similar in composition and structure, with the exception that the CA gel appeared to be amyloidic in nature based on thioflavin T (ThT) fluorescence. Protein gelation, particularly in the apoplast, may confer protection against freeze-induced dehydration and potentially have a commercial application to improve frozen food quality.



## Introduction

Cold-tolerant herbaceous plants, such as winter cereals, can survive subzero temperatures by undergoing a process known as cold acclimation. Cold acclimation is complex and involves growth and development at low, non-freezing temperatures, resulting in numerous biochemical, physiological and molecular changes allowing the plant to acquire freezing tolerance [22,53,59,60]. During this process, numerous proteins accumulate, such as antifreeze proteins (AFPs) and others, which can account for up to 0.9% of the total soluble protein present [59,60]. Many of these proteins are secreted into the apoplast where they are likely to come into contact with the outer surface of the plasma membrane, the primary site of freezing injury [22,39,59]. The plant apoplast is emerging as an important component of plant acclimation, involved in both the perception and transduction of environmental signals [8,26,56,58]. Freezing tolerant plants can adjust their cell wall properties to prevent freezing-induced dehydration and also use the cell wall as physical barrier against ice crystal propagation [8,26,56,58].

Plants survive freezing by forming ice in intercellular spaces [46,47]. The freezing injury that occurs is due primarily to cellular dehydration, caused as intracellular water moves from the cytosol to extracellular ice crystal formation in the apoplast [39,47]. The freezing process is initiated in the apoplast by ice nucleators and as extracellular ice begins to form, growth of the crystal is restricted by several factors, one of which are AFPs [4,19,21,60]. AFPs function by binding to prism faces of ice and modifying the normal growth pattern of the ice crystals. In addition, AFPs depress the

*Abbreviations:* AFP, antifreeze protein; β-ME, β-mercaptoethanol; CA, cold-acclimated; NA, non-acclimated; PEI, polyethyleneimine; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; ThT, thioflavin T.

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freezing temperature of water and are also extremely effective in preventing ice recrystallization [21].

Winter rye (*Secale cereale* L. cv Musketeer) is an over wintering annual plant that can survive temperatures below −30 °C. At least six AFPs with varying levels of antifreeze activity have been identified in apoplastic extracts from cold acclimated winter rye leaves, ranging from 16–35 kDa [21]. These AFPs will form oligomeric complexes which are thought to inhibit ice growth and recrystallization more effectively than the individual polypeptides themselves [21]. Many AFPs are homologous to pathogenesis-related (PR) proteins, including β-1,3-glucanases and chitinases, and can be induced at warm temperatures by other factors such as drought or treatment with pathogenic fungi, salicylic acid, or abscisic acid [21]. However, some of these treatments result in AFPs which lack antifreeze activity. In insects, it is speculated that AFPs can interact with low molecular mass solutes that influence (enhance) their activities, including the organic acid citrate [21].

While proteins with antifreeze activity have been found in many vascular plants since their initial discovery in 1992 [19,61], AFPs are also found in other overwintering organisms, including marine teleost fishes [12]. A type I AFP has been found at high concentrations in the circulatory system and skin of fish (winter flounder) living in subzero seawater, and protects the organism from macromolecular ice growth [12]. While studying AFP mechanism and structure, it was found that high concentrations of this particular AFP in solution formed a translucent gel upon repetitive freeze and thaw cycles [16,17]. Further, these investigators proceeded to demonstrate that type I AFP was converted from a soluble, single α-helical protein into amyloid fibrils in the form of an amyloidotic gel in response to freeze–thaw; conditions appropriate to its physiological function [17,18]. In addition, the authors speculate that this AFP could be used in applications such as cryopreservation, cryosurgery and as a food additive [17]. Therefore, AFPs, at least in fish, appear to be natural protective amyloids [29] and it is interesting to speculate if a similar phenomenon occurs with AFPs in winter rye.

While extracting protein from cold acclimated winter rye leaves for proteomic analyses, we made an interesting observation. After several freeze–thaw cycles, the protein extracts became more viscous and the formation of a gel was observed. This was similar to the translucent gel which formed upon freezing and thawing in winter flounder type I AFP [17].

The global protein ingredient (gelling/viscosity modifying agents) market is currently dominated by animal-derived proteins such as casein, gelatin, ovalbumin and whey. With increasing consumer fears towards animal-based proteins (i.e. prion disease), coupled with changing dietary restrictions and preferences due to religious or moral beliefs, the food and pharmaceutical industries are searching for plant-based protein alternatives [42].

In this study we examine protein extracts from non-acclimated and cold acclimated winter rye leaves and characterize the conditions required for this gel formation, as well as a biochemical analysis of the gelling and non-gelling components of the extracts to determine their composition and rheological properties. By successfully identifying and characterizing the compounds and conditions responsible for protein gelation in plant extracts, it is hoped that this information will advance commercial applications for use a gelling/viscosity modifying agent in foods, bioproducts and pharmaceuticals.

## Materials and methods

### *Plant material and growth conditions*

Seeds of winter rye (*Secale cereale* L. cv Musketeer) were sown in 7-cm plastic pots containing coarse vermiculite at a density of 7–10 seeds per pot. Plants were grown using controlled environment growth chambers (Controlled Environments Ltd., Winnipeg, MB, Canada). Fluorescent tubes (Cool White, 160 W, F72T12/CW/VHO, Sylvania) provided a photosynthetic photon flux density (PPFD) of 250 μmol photons $m^{-2}$ $s^{-1}$ of photosynthetically active radiation (PAR) over a 16 h day length with temperatures of 20/20 °C (day/night). Non-acclimated (NA) plants were grown under these conditions for 21 days. For cold acclimated (CA) material, plants grown under NA conditions for 7 days were shifted to a chamber set for 5/4 °C (day/night) temperatures with all other conditions constant for an additional 49 days. All plants were watered with a nutrient solution as required [34]. All fully expanded and healthy leaves were harvested for subsequent analyses.

### *Protein extraction*

Total leaf protein was extracted from NA and CA tissue using two protocols as described in detail by Isaacson et al. [30]. The first involved a trichloroacetic acid (TCA)-acetone precipitation. Subsequent experiments utilizing this procedure proved unsuccessful and it was abandoned. The second protocol made use of a phenol extraction coupled with ammonium acetate–methanol precipitation [27,30] and this was adopted for all future experiments unless otherwise indicated.

Leaf tissue (1 g) was frozen with liquid nitrogen and ground into a fine powder with a pre-chilled mortar and pestle using 5 mL of ice cold tissue extraction buffer (0.1 M Tris [pH 8.8], 10 mM EDTA, 0.9 M sucrose, 0.4% [v/v] β-mercaptoethanol (β-ME) added immediately before use) for 2 min. The homogenate was transferred to a 50 mL plastic tube and mixed in a 1:1 ratio (5 mL) of phenol saturated with Tris–EDTA, pH 8.0 (Omni Pur®; EMD Chemicals, Gibbstown, NJ, USA). The mixture was agitated for 30 min at 4 °C followed by centrifugation at 6000*g* for 15 min. Two milliliter of the phenol phase (top phase) was removed and placed in a 15 mL plastic tube. This solution was mixed with 5 volumes of ice-cold 0.1 M ammonium acetate in methanol and incubated overnight at −20 °C to allow for protein precipitation. The precipitate was collected by centrifuging at 6000*g* for 15 min at 4 °C. Two volumes (based on the volume [5 mL] of the last collected phenolic phase) of ice-cold 0.1 M ammonium acetate in methanol were added to wash the pellet and remove phenol, lipids and pigments. The sample was centrifuged at 6000*g* for 10 min in ice-cold 80% (v/v) acetone to remove the ammonium acetate. The resultant protein pellet was gently air dried in a fume hood at room temperature (22 °C) for 30 min. The pellet was stored at −80 °C for future analysis or suspended in appropriate buffer as described below.

Protein concentrations were determined using the Pierce® BCA protein assay (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions with BSA (Fraction V; EMD Chemicals) as the standard [57].

### *Induction of gel formation*

Protein pellets were suspended in 400–800 μL of either 50 mM Tris or 50 mM borate buffer (depending on experiment) by sonication (VirSonic 600 Ultrasonic Cell Disrupter, VirTis Company, Gardiner, NY, USA) using a microtip probe for two times at 10 s each (intensity 0.5) and once or twice for 10 s (intensity 1.0) until the protein pellet was fully dissolved. Gel formation was induced by subjecting the protein extracts to multiple freeze–thaw cycles in a −20 or −80 °C freezer (Frigidaire, model FFU14M5H W Mississauga, ON, Canada; Revco Technologies, model ULT2586, Asheville, NC, USA, respectively). The freezing treatment was a minimum of 3 h or up to 4 days followed by thawing on the bench at room