Purification and enzymatic characterization of three type-1 ribosome inactivating proteins from *Secale cereale* L. seeds

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Seeds of *Secale cereale* L.

INTRODUCTION:

Ribosome-inactivating proteins (RIPs) are enzymes found in several edible plants, exhibiting rRNA N-β-glycosylase activity (EC 3.2.2.22) (1). They remove a specific adenine from the higher rRNA of Sarcin-Ricin Loop (SRL), irreversibly blocking translation. Beyond rRNA, RIPs can also act on different nucleic acid substrates, including DNA and other polynucleotides, reflecting their broader role as polynucleotide:adenosine glycosylases (PNAG) (2). RIPs are classified into two major groups, based on their structural organization. Type-2 RIPs are heterodimeric proteins (~60-kDa) consisting of an active A-chain with rRNA N-β-glycosylase activity and a lectin B-chain, binding cell-surface carbohydrates, thereby enabling cellular uptake. Type-1 RIPs are single-chain proteins (~30-kDa) showing limited ability to enter cells for the absence of B-chain. This structural difference affects their cellular uptake and biological activity (3). In this context, Minami *et al.* reported the presence of a type-1 RIP designed as RPS1 from the seeds of rye (*Secale cereale* L.) (4), an ancient cereal of the Poaceae family, which has been cultivated since the Bronze Age (3000–1200 BC) in Asia Minor (5). However, considering the absence of enzymatic characterization, we decided to further characterized RIPs in rye seeds by reporting the purification and enzymatic characterization of three novel type-1 RIPs from seeds of 'segale del Matese' ('sécena' in the local dialect), a rye landrace, cultivated on the Matese Plateau.

MATERIALS AND METHODS:

The work involved the: (i) purification protocol set-up for the extraction of basic proteins from *S. cereale* by protein acid precipitation and chromatographic steps, exploiting protein charge and molecular weight; (ii) N-β-glycosylase activity assessment by β-fragment release and detection from rabbit, yeast and bacterial ribosomes; (iii) PNAG activity evaluation on salmon sperm DNA and (iv) endonuclease activity on plasmid DNA (6).

RESULTS:

Three protein peaks named Sc1, Sc2 and Sc3 with the ability to inhibit protein synthesis *in vitro*, were eluted from the last purification step by cation exchange chromatography (Fig. 1A). SDS-PAGE analysis of pooled fractions showed a homogeneous protein band with an electrophoretic migration of ~29 kDa with and without reducing agent (Fig 1B). Sc1, Sc2 and Sc3 were obtained with a protein yield of 0.19, 1.9 and 1.5 mg/100 g seeds, respectively. In addition, these proteins are not glycosylated, as they were not stained with the glycoprotein staining kit, differently from quinoin, a glycosylated type-1 RIP from *Chenopodium quinoa* seeds (Fig. 1C-D). The RNA N-glycosylase activity of Sc1, Sc2 and Sc3 was verified by the detection of the diagnostic β-fragment from rabbit and yeast ribosomes after Endo's assay (Fig. 2A-B). However, this activity was not detected on *Escherichia coli* ribosomes (Fig. 2C). Furthermore, like other RIPs, both Sc2 and Sc3 showed PNAG activity on salmon sperm DNA, (Fig. 3A) and all exert endonuclease activity on supercoiled plasmid DNA, by producing the linear form of the plasmid (Fig. 3B).

1

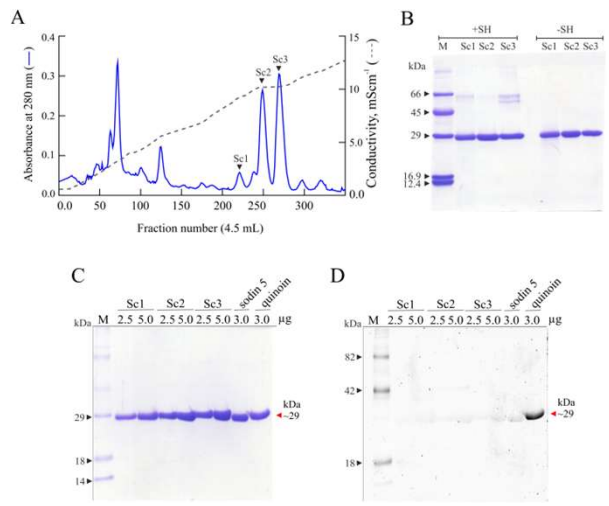

Fig. 1. (**A**) Elution profile from cation-exchange chromatography using CM-Sepharose resin, showing three peaks named Sc1, Sc2, and Sc3 from *S. cereale* seeds. (**B**) SDS-PAGE analysis in a 12% polyacrylamide gel of Sc1, Sc2 and Sc3 pooled fractions (3.0 μg) with (+SH) and without (-SH) reducing agent. Lane M, molecular weight markers. (**C**) 12% SDS-PAGE analysis of Sc1, Sc2, and Sc3 under reducing conditions, followed by (**D**) in-gel glycoprotein detection using the Pro-Q Emerald 300 glycoprotein staining kit. Stained proteins were visualized by UV trans-illumination. Quinoin (3.0 μg), glycosylated type-1 RIP from *C. quinoa* seeds used as a positive control. Sodin 5 (3.0 μg), non-glycosylated type-1 RIP from *Salsola soda* seeds used as a negative control. M, CandyCane™ glycoprotein molecular weight standards.

2

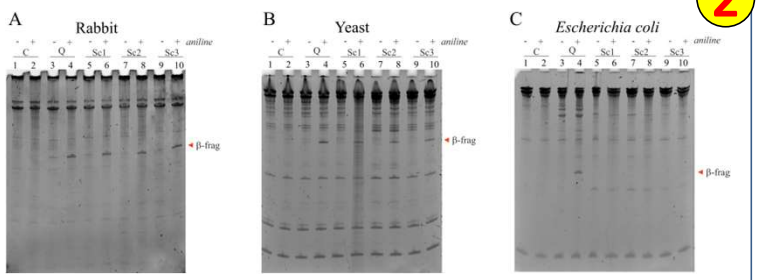

Fig. 2. rRNA N-β-glycosylase activity of Sc1, Sc2 and Sc3 (3.0 μg) evaluated by Endo's assay on (**A**) rabbit reticulocyte lysate, (**B**) yeast S30 supernatants and (**C**) *E. coli* ribosomes. Quinoin (3.0 μg), type-1 RIP from *C. quinoa* seeds, used as a positive control. The release of the characteristic β-fragment (β-frag), hallmark of N-β-glycosylase activity was indicated by the red arrows.

3

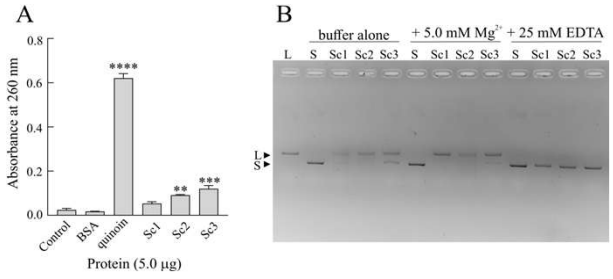

Fig. 3. (**A**) PNAG activity of BSA (negative control), quinoin (positive control) and Sc1, Sc2 and Sc3. Proteins (5.0 μg) were assayed on salmon sperm DNA. Data were analysed by one-way ANOVA with Dunnet's post hoc test (****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$).
(**B**) Endonuclease activity of Sc1, Sc2 and Sc3 on pUC18 plasmid DNA under different conditions (buffer alone, 5.0 mM Mg^{2+} and 25 mM EDTA), analyzed by 0.8% agarose gel electrophoresis and stained with PAGE GelRed (Biotium).

CONCLUSIONS:

Three different type-1 RIPs named Sc1, Sc2 and Sc3 have been isolated from *S. cereale* seeds. All enzymes are able to release the β-fragment following incubation with eukaryotic ribosomes. Moreover, both Sc2 and Sc3 display additional PNAG activity, while all are able to convert the supercoiled plasmid into the relaxed form due to their nuclease activity. Overall, this research aims to revisit the presence of type-1 RIPs in *S. cereale*, in light of their possible use as antiviral, antifungal or antipathogenic tools in agriculture.

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Meeting del Gruppo Proteine della Società Italiana di Biochimica (SIB) "Proteine 2026"

Structural and functional characterization of proteins and their modulation.

DATES: 20-22 Maggio 2026

CONGRESS VENUE: Fisciano Campus | University of Salerno