

# Journal Pre-proof

A not-glycosylated isoform of  $\gamma$ -conglutin, a hexameric glycoprotein of *Lupinus albus* seed, participates in the oligomerization equilibrium

Giuditta C. Heinzl, Stefano De Benedetti, Nicola Lusignani, Chiara Magni, Alberto Barbiroli, Alessio Scarafoni



PII: S0006-291X(23)00802-1

DOI: <https://doi.org/10.1016/j.bbrc.2023.06.047>

Reference: YBBRC 48798

To appear in: *Biochemical and Biophysical Research Communications*

Received Date: 5 June 2023

Accepted Date: 14 June 2023

Please cite this article as: G.C. Heinzl, S. De Benedetti, N. Lusignani, C. Magni, A. Barbiroli, A. Scarafoni, A not-glycosylated isoform of  $\gamma$ -conglutin, a hexameric glycoprotein of *Lupinus albus* seed, participates in the oligomerization equilibrium, *Biochemical and Biophysical Research Communications* (2023), doi: <https://doi.org/10.1016/j.bbrc.2023.06.047>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2023 Published by Elsevier Inc.

**A not-glycosylated isoform of  $\gamma$ -conglutin, a hexameric glycoprotein of *Lupinus albus* seed, participates in the oligomerization equilibrium**

Giuditta C. Heinzl\*, Stefano De Benedetti, Nicola Lusignani, Chiara Magni, Alberto Barbiroli and Alessio Scarafoni

Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Italy.

\* Corresponding Author

Correspondance to: Giuditta Carlotta Heinzl  
Department of Food, Environmental and Nutritional Sciences,  
Università degli Studi di Milano,  
via G. Celoria 2  
20133 Milano, Italy  
e-mail: giuditta.heinzl@unimi.it  
tel. +39-0250316839

**Abstract**

$\gamma$ -conglutin ( $\gamma$ -C) is a hexameric glycoprotein accumulated in lupin seeds and has long been considered as a storage protein. Recently, it has been investigated for its possible postprandial glycaemic regulating action in human nutrition and for its physiological role in plant defence. The quaternary structure of  $\gamma$ -C results from the assembly of six monomers in reversible pH-dependent association/dissociation equilibrium. Our working hypothesis was that the  $\gamma$ -C hexamer is made up of glycosylated subunits in association with not-glycosylated isoforms, that seem to have 'escaped' the correct glycosylation process in the Golgi. Here we describe the isolation of not-glycosylated  $\gamma$ -C monomers in native condition by two in tandem lectin-based affinity chromatography and the characterization of their oligomerization capacity. We report, for the first time, the observation that a plant multimeric protein may be formed by identical polypeptide chains that have undergone different post-translational modifications. All obtained considered, the results strongly suggest that the not-glycosylated isoform can also take part in the oligomerization equilibrium of the protein.

**Keywords:** plant proteins; legume seed glycoproteins; oligomerization equilibrium; lectins; affinity chromatography.

## 1. Introduction

$\gamma$ -Conglutin ( $\gamma$ -C) is a glycosylated multimeric protein accumulated in lupin seeds, a leguminous plant, and accounts for about 5% of the total seed proteins [1]. It belongs to the class of globulins and its homologues have been detected in many plants including wheat, carrot, cotton, tomato, corn, *Lotus japonicus*, *Medicago truncatula* and *Arabidopsis thaliana* [2].  $\gamma$ -C has long been considered a storage protein (SP), class of proteins that are synthesized and stored in seeds to provide a source of nutrients for the developing embryo during seed germination and defence against pathogens. Recently, its role in plant defense mechanisms has been defined [3]. Moreover, a postprandial glycaemic regulating action exerted by  $\gamma$ -C has been extensively studied for its possible application in human nutrition [4,5].

Similarly to the classical seed SPs,  $\gamma$ -C is synthesized as a single precursor polypeptide of about 45 kDa that is proteolytically cleaved in juvenile cotyledons into two subunits ( $\alpha$  and  $\beta$ , respectively) of about Mr 32 kDa and 17 kDa [6-8]. The proteolytic cleavage process appears to be incomplete, leaving a certain amount of the protein in an uncut form [9,10]. Additional post-translational processing includes the formation of N-linked glycosylation and six disulfide bridges, one of which links the two subunits [6]. Two genes encoding  $\gamma$ -C have been identified and both codify for protein versions containing a single N-glycosylation consensus motif [1,11]. However, in *Lupinus albus*, only one gene is expressed during seed development [1]. The quaternary structure results from the assembly of six monomers [6] in reversible pH-dependent association/dissociation equilibrium [12]. Storage glycoproteins are a class of proteins that are synthesized and stored in seeds to provide a source of nutrients for the developing embryo during seed germination and defence against pathogens. Legume seeds are rich in storage glycoproteins, which play an essential role in the nutritional quality of these seeds and have emerged as key players in various physiological processes [13,14].  $\gamma$ -C has the typical glycosylation structures that differentiate plant N-glycans from their mammalian counterpart by the absence of sialic acid and the presence of  $\beta$ (1,2)-xylose and  $\alpha$ (1,3)-fucose residues [15]. Glycosylation is a critical post-translational modification (PTM) that can significantly affect the stability, solubility and biological properties of proteins. It can play a role in protein recognition, signalling, trafficking, and localization [16]. For example, the glycosylation pattern of  $\gamma$ -C can affect its folding [17].

Glycosylation of plant proteins has been suggested to play a role in food allergy [18]. Several studies have shown that the removal of the N-linked oligosaccharides can increase protein digestibility and reduce allergenicity. Furthermore, glycosylation could mask the proteolytic cleavage sites leading to incomplete degradation into allergenic peptides [19-21].

The structural features of  $\gamma$ -C glycosidic moiety have been extensively studied by Schiarea et al. [22] and Czubinski et al. [17], who have shown the presence of various isoforms with different glycosidic structures. However, it is still unclear how the various isoforms are distributed in the hexamer and especially whether not-glycosylated monomers are part of the native quaternary structure of the protein. This work describes the isolation of not-glycosylated  $\gamma$ -C monomers and tests for their oligomerization capacity.

## 2. Materials and Methods

### 2.1 Protein purification

Native  $\gamma$ -C was purified from *Lupinus albus* seeds according to Capraro et al. [12] by using a combination of anion and cation exchange chromatography. All the steps were carried out at 4 °C. The homogeneity of the protein preparation was checked by SDS-PAGE. The purified protein was freeze-dried and suspended in the appropriate buffers before use.

## 2.2 Separation of the $\gamma$ -C monomers

For the separation of the monomers from the native assembly two in tandem affinity chromatography have been used. 10 mg of  $\gamma$ -C, dissolved in 5 mL of 50 mM sodium acetate, pH 4.8, containing 500 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 1 mM CaCl<sub>2</sub>, was applied onto *Lotus tetragonolobus* lectin-Sepapore® 4B (Glycomatrix, Dublin, Ohio) column (10 mm x 20 mm), equilibrated with the same buffer. The unbound fraction was immediately loaded onto an AminoLink® Coupling Resin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) column (10 mm x 20 mm) previously coupled with Concanavalin A lectin (Merck, Darmstadt, Germany). Coupling was carried out according to the resin manufacturer's instructions. The protein concentration in the unbound fractions was quantified spectrophotometrically at 280 nm. The unbound fractions were analyzed by RP-HPLC to detect the presence of the glycosylated and the non-glycosylated form (see below) and subjected repeatedly to subsequent tandem chromatography runs until homogeneity in RP-HPLC was reached.

## 2.3 RP-HPLC

RP-HPLC was performed with a SIMMETRY300 C18 (5  $\mu$ m) (4.6 mm x 250 mm) column (Waters, Sesto San Giovanni, Italy) fitted on a chromatographic apparatus (Waters) composed of two 510 HPLC Pumps, a 717plus Autosampler, and a 996 Photodiode Array Detector. Mobile phase flux was 0.8 mL/min, mixing solutions A (TFA 0.1% in water) and B (TFA 0.1% in ACN) as follows: 2 min isocratic 100% solution A, 50 min linear gradient from 37.5% solution B to 75% solution B. Peaks were detected at 220 and 280 nm.

## 2.4 Glycoprotein staining

The qualitative detection of N-glycans was carried out following electrophoretic separation. SDS-PAGE of  $\gamma$ -C and of the purified non glycosylated form was carried out according to Laemmli [23]. The samples were prepared using not-reducing conditions. N-glycans were stained using two different methods. Western blot with Concanavalin A (ConA) detection was carried out to evaluate the presence of mannose residues. Proteins were loaded on a 12% SDS-PAGE and electroblotted onto nitrocellulose [24]. Membranes were washed three times for 5 min with Tris Buffered Saline (TBS), pH 7.4, saturated with 1% fish gelatin in TBS for 1.5 h and incubated with ConA (50  $\mu$ g/mL in TBS, Merck) for 1 h. The membrane was then washed three times as above, incubated with horseradish peroxidase (50  $\mu$ g/mL in TBS, Merck) for 30 min, washed 3 more times and stained with 4-Chloro-1-naphthol (Merck) 0.03% and H<sub>2</sub>O<sub>2</sub> 0.01%. For a more sensitive analysis, the Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit (Thermo Fisher Scientific) was used for fluorescent in-gel detection. Stained blots and gels were digitalized with the use of VersaDoc system (Bio-Rad, Hercules, CA, USA), and analyzed with the ImageJ software [25].

## 2.5 SEC-MALS

Analysis of the oligomeric structures of  $\gamma$ -C and of the purified non glycosylated form was performed by using the Size-Exclusion Chromatography (SEC) combined with Multi-Angle Light Scattering (MALS). Detection was performed in a HPLC system composed by a Waters 515 HPLC Pump, a Waters 2487 Dual Absorbance detector (Waters), a Wyatt Dawn Heleos MALS and a Wyatt Optila T-REX differential refractive index detector (Wyatt Technology, Santa Barbara, Ca).

200  $\mu$ L of 0.2 mg/mL samples dissolved in 50 mM sodium acetate, pH 4.8, were run on a Superose 12 10/300 GL column (GE Healthcare, Milan, Italy). The mobile phase was always 50 mM Tris-HCl, pH 7.5 containing 100 mM NaCl, and the flow rate 0.5 mL/min. Molar masses were calculated by means

of the Astra V software vs. 5.3.4.20 (Wyatt), using a dn/dc value of 0.185 and 0.188 for the not-glycosylated and the glycosylated protein, respectively.

### 3. Results and discussion

The initial working hypothesis was that the  $\gamma$ -C hexamer is made up of both glycosylated and not-glycosylated monomers, and the first step of the present work aimed to detecting the coexisting nature of the two isoforms in *L. albus* seeds. When separated in RP-HPLC, *L. albus*  $\gamma$ -C purified to homogeneity show the presence of two peak (Fig. 1). The possibility that heterogeneity could be due to the presence of several gene products is excluded because it is well established that only one is accumulated in *L. albus* seeds [1,6]. By specific glycosylation staining procedures, we have been able to assign the peaks at 9 and 12 minutes to the not-glycosylated (NG) and glycosylated isoforms, respectively (Fig. 1). This result confirms the hypothesis that not-glycosylated monomers are present in  $\gamma$ -C extracted under native conditions from the seeds [12]. The amount of the not-glycosylated monomers is around 5% (Table1).

$\gamma$ -C from *L. albus* seeds (as well as that from *L. angustifolius* seeds) is characterized by different N-glycoforms which contain N-acetyl-glucosamine, mannose, fucose and xylose in different amounts [17,22]. Interestingly, up to twenty different N-glycans have been detected in  $\gamma$ -C and may be grouped as containing or not fucosyl residues. All N-glycans contain mannosyl residues [17].

In order to perform the separation of the isoforms of  $\gamma$ -C under native conditions we have adopted a combination of affinity chromatography using two different immobilized lectins. *Lotus tetragonolobus* lectin is capable of binding fucose residues [26], whereas *Canavalia ensiformis* lectin (ConA) has an affinity for terminal  $\alpha$ -D-mannosyl and  $\alpha$ -D-glucosyl residues. To preserve the integrity and functionality of the two lectins, and in accordance with the supplier's indications, all separations have been carried out at pH 4.8. At these conditions  $\gamma$ -C dissociate as monomer for the majority but some dimers and a small amount of hexamers are also still present [12]. The dissociation equilibrium of  $\gamma$ -C has been exploited to isolate increasing amount of not-glycosylated protein (Table 1, Fig. 2). Thus, after five serial repetitions of affinity chromatography steps the homogeneity has been reached (Fig. 2A). At each step, all the not retained fractions were collected and directly analyzed through RP-HPLC as described above to evaluate the amounts of the two isoforms and the enrichment levels. The presence of residual N-glycans was estimated by fluorescent staining and, in all cases, the effective absence of any glycan moieties was confirmed after the last run (Fig. 2B, red line). Horseradish peroxidase (HP) was used as positive control samples of glycosylation.

It is worth noting that on the last chromatogram (Fig. 2A) at the beginning (five minutes) of the run, some proteins did not interact with the column. This fraction accounts for about 31% of the total peak area (Table 1) and likely contains aggregates, that have not been further investigated.

Since  $\gamma$ -C has been shown to undergo pH-dependent quaternary organization between a monomeric unit at acidic pH and oligomeric assembly at neutral values [12], SEC was carried out to assess the capability of the not-glycosylated monomers to acquire the multimeric status. The not-glycosylated protein showed a general aggregative behavior very close to the glycosylated one (Fig. 3). The MW of the peaks, as calculated by SEC-MALS, are in good accordance with those previously estimated [12].

It should be noted that, in our case, the proteins were dissolved at pH 4.8 but the column was equilibrated at pH 7.5. In other words, sample was injected as monomer into the column, and the switch of the equilibrium toward the polymeric form (dimer and hexamer) is triggered by the solvent change occurring in the separation. This may explain possible little discrepancies in the calculated MWs due to kinetics limitations on achieving equilibrium.

Overall, these results suggest that the absence of the glycosylation does not hinder, or prevent, aggregation. In turn, also in the absence in the glycosylation, the monomer may reach the correct

folding and therefore undergoes the other PTMs apart from glycosylation. Finally, it is interesting to note that the ability of NG to form aggregates also justifies its presence in the native hexamer, in association with the glycosylated monomers.

On the other hand, sample NG shows the presence of a high MW fraction in the excluded volume (15 min in Fig. 3). Since the separation was carried out in native condition, it can be assumed that some aggregates are formed, confirming what was hypothesized following the RP-HPLC experiment (Fig. 2A). Whether this aggregation is due to an aggregation equilibrium shift or to a lower conformational stability of the non-glycosylated form compared to the glycosylated one will be the subject of future studies.

We report, for the first time, the observation that a plant multimeric protein may be formed by identical polypeptide chains that have undergone different PTMs. Some monomers seem to have completely 'escaped' the process in the Golgi during the PTM process.

Therefore, all considered, the results obtained strongly suggest that the not-glycosylated protein can also take part in the oligomerization equilibrium of the protein.

### Authors' contribution

G.C.H. and A.S. developed the theoretical framework. G.C.H., S.D.B., N.L., C.M. and A.B. performed the experiments. G.C.H. and A.S. wrote the manuscript. S.D.B. prepared the figures. All authors participated in the final revision and agreed to the submitted version of the manuscript.

### Funding

This research received no funds. G.C.H. Ph.D. program in Nutritional Sciences was funded by the University of Milan.

### Declaration of competing interest

The authors declare that they have no competing interests or personal relationships that could have appeared to influence the work reported in this paper.

### References

- [1] A. Scarafoni, A.D. Cataldo, T.D. Vassilevskaia, E.P. Bekman, C. Rodrigues-Pousada, F. Ceciliani, M. Duranti, Cloning, sequencing and expression in the seeds and radicles of two *Lupinus albus* conglutinin Q genes, *Biochimica et Biophysica Acta*. (2001). [https://doi.org/10.1016/S0167-4781\(01\)00225-1](https://doi.org/10.1016/S0167-4781(01)00225-1)
- [2] H. Hirano, Basic 7S globulin in plants, *Journal of Proteomics*. 240 (2021) 104209. <https://doi.org/10.1016/j.jprot.2021.104209>.
- [3] S.D. Benedetti, E. Galanti, J. Capraro, C. Magni, A. Scarafoni, *Lupinus albus*  $\gamma$ -Conglutinin, a Protein Structurally Related to GH12 Xyloglucan-Specific Endo-Glucanase Inhibitor Proteins (XEGIPs), Shows Inhibitory Activity against GH2  $\beta$ -Mannosidase, *IJMS*. 21 (2020) 7305. <https://doi.org/10.3390/ijms21197305>.
- [4] T.J. Guzmán, M. Düfer, M. Wiedemann, R. Olguín-Alor, G. Soldevila, C.M. Gurrola-Díaz, *Lupin*  $\gamma$ -conglutinin protects against cell death induced by oxidative stress and lipotoxicity, but transiently inhibits in vitro insulin secretion by increasing KATP channel currents, *International Journal of Biological Macromolecules*. 187 (2021) 76–90. <https://doi.org/10.1016/j.ijbiomac.2021.07.088>.

- [5] G.C. Heinzl, M. Tretola, S. De Benedetti, P. Silacci, A. Scarafoni, Lupinus albus  $\gamma$ -Conglutin: New Findings about Its Action at the Intestinal Barrier and a Critical Analysis of the State of the Art on Its Postprandial Glycaemic Regulating Activity, *Nutrients*. 14 (2022) 3666. <https://doi.org/10.3390/nu14173666>.
- [6] J. Czubinski, J. Barciszewski, M. Gilski, K. Szpotkowski, J. Debski, E. Lampart-Szczapa, M. Jaskolski, Structure of  $\gamma$ -conglutin: insight into the quaternary structure of 7S basic globulins from legumes, *Acta Crystallogr D Biol Crystallogr*. 71 (2015) 224–238. <https://doi.org/10.1107/S1399004714025073>.
- [7] E.D. Johnson, J. Knight, K.R. Gayler, Biosynthesis and processing of legumin-like storage proteins in *Lupinus angustifolius* (lupin), *Biochemical Journal*. 232 (1985) 673–679. <https://doi.org/10.1042/bj2320673>.
- [8] M. Duranti, A. Consonni, C. Magni, F. Sessa, A. Scarafoni, The major proteins of lupin seed: Characterisation and molecular properties for use as functional and nutraceutical ingredients, *Trends in Food Science & Technology*. 19 (2008) 624–633. <https://doi.org/10.1016/j.tifs.2008.07.002>.
- [9] M. Duranti, A. Scarafoni, C. Gius, A. Negri, F. Faoro, Heat-induced synthesis and tunicamycin-sensitive secretion of the putative storage glycoprotein conglutin gamma from mature lupin seeds, *Eur J Biochem*. 222 (1994) 387–393. <https://doi.org/10.1111/j.1432-1033.1994.tb18877.x>.
- [10] J. Czubinski, M. Montowska, E. Fornal, Post-translational cleavage pattern of *Lupinus angustifolius*  $\gamma$ -conglutin:  $\gamma$ -Conglutin post-translational cleavage pattern, *J. Sci. Food Agric*. 98 (2018) 5212–5219. <https://doi.org/10.1002/jsfa.9057>.
- [11] R.C. Foley, L.-L. Gao, A. Spriggs, L.Y. Soo, D.E. Goggin, P.M. Smith, C.A. Atkins, K.B. Singh, Identification and characterisation of seed storage protein transcripts from *Lupinus angustifolius*, *BMC Plant Biol*. 11 (2011) 59. <https://doi.org/10.1186/1471-2229-11-59>.
- [12] J. Capraro, P. Spotti, C. Magni, A. Scarafoni, M. Duranti, Spectroscopic studies on the pH-dependent structural dynamics of  $\gamma$ -conglutin, the blood glucose-lowering protein of lupin seeds, *International Journal of Biological Macromolecules*. 47 (2010) 502–507. <https://doi.org/10.1016/j.ijbiomac.2010.07.005>.
- [13] A. Ceriotti, M. Duranti, R. Bollini, Effects of N-glycosylation on the folding and structure of plant proteins, (1998).
- [14] Y. Nagashima, A. von Schaewen, H. Koiwa, Function of N-glycosylation in plants, *Plant Science*. 274 (2018) 70–79. <https://doi.org/10.1016/j.plantsci.2018.05.007>.
- [15] P. Lerouge, M. Cabanes-Macheteau, C. Rayon, A.-C. Fischette-Lainé, V. Gomord, L. Faye, N-Glycoprotein biosynthesis in plants: recent developments and future trends, in: J. Soll (Ed.), *Protein Trafficking in Plant Cells*, Springer Netherlands, Dordrecht, 1998: pp. 31–48. [https://doi.org/10.1007/978-94-011-5298-3\\_2](https://doi.org/10.1007/978-94-011-5298-3_2).
- [16] A. Ghazaryan, K. Landfester, V. Mailänder, Protein deglycosylation can drastically affect the cellular uptake, *Nanoscale*. 11 (2019) 10727–10737. <https://doi.org/10.1039/C8NR08305C>.
- [17] J. Czubinski, E. Lattová, Z. Zdráhal, R. Strasser, Characteristics of N -Glycosylation and Its Impact on the Molecular Behavior of *Lupinus angustifolius*  $\gamma$ -Conglutin, *J. Agric. Food Chem*. 71 (2023) 7359–7369. <https://doi.org/10.1021/acs.jafc.3c00727>.



- [18] M. Bardor, Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha(1,3)-fucose and core xylose, *Glycobiology*. 13 (2003) 427–434. <https://doi.org/10.1093/glycob/cwg024>.
- [19] S. Dam, M. Thaysen-Andersen, E. Stenkjær, A. Lorentzen, P. Roepstorff, N.H. Packer, J. Stougaard, Combined N-Glycome and N-Glycoproteome Analysis of the *Lotus japonicus* Seed Globulin Fraction Shows Conservation of Protein Structure and Glycosylation in Legumes, *J. Proteome Res.* 12 (2013) 3383–3392. <https://doi.org/10.1021/pr400224s>.
- [20] H. Kaulfürst-Soboll, S. Rips, H. Koiwa, H. Kajiura, K. Fujiyama, A. von Schaewen, Reduced Immunogenicity of *Arabidopsis hgl1* Mutant N-Glycans Caused by Altered Accessibility of Xylose and core Fucose Epitopes, *Journal of Biological Chemistry*. 286 (2011) 22955–22964. <https://doi.org/10.1074/jbc.M110.196097>.
- [21] K. Matsuo, U. Kagaya, N. Itchoda, N. Tabayashi, T. Matsumura, Deletion of plant-specific sugar residues in plant N-glycans by repression of GDP-d-mannose 4,6-dehydratase and  $\beta$ -1,2-xylosyltransferase genes, *Journal of Bioscience and Bioengineering*. 118 (2014) 448–454. <https://doi.org/10.1016/j.jbiosc.2014.04.005>.
- [22] S. Schiarea, L. Arnoldi, R. Fanelli, E. De Combarieu, C. Chiabrando, In-Depth Glycoproteomic Characterization of  $\gamma$ -Conglutin by High-Resolution Accurate Mass Spectrometry, *PLoS ONE*. 8 (2013) e73906. <https://doi.org/10.1371/journal.pone.0073906>.
- [23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680–685.
- [24] J. Capraro, A. Clemente, L.A. Rubio, C. Magni, A. Scarafoni, M. Duranti, Assessment of the lupin seed glucose-lowering protein intestinal absorption by using in vitro and ex vivo models, *Food Chemistry*. 125 (2011) 1279–1283. <https://doi.org/10.1016/j.foodchem.2010.10.073>.
- [25] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, *Nat Methods*. 9 (2012) 671–675. <https://doi.org/10.1038/nmeth.2089>.
- [26] C.J. Thomas, A. Surolia, Mode of Molecular Recognition of l-Fucose by Fucose-Binding Legume Lectins, *Biochemical and Biophysical Research Communications*. 268 (2000) 262–267. <https://doi.org/10.1006/bbrc.2000.2110>.

## Figure legends

**Figure 1.** Separation of seed-extracted  $\gamma$ -C by RP-HPLC. The inset represents the Western blot analysis with Concanavalin A (ConA) detection. NG refers to ConA-negative fraction, whereas  $\gamma$ -C indicated the positive reacting polypeptide. See text for experimental details.

**Figure 2.** Panel A: RP-HPLC chromatograms of unbound fractions collected after serial steps (indicated by numbers from 1<sup>st</sup> to 5<sup>th</sup>) of tandem affinity chromatography using fucose-binding and mannose-binding lectins. T0 indicates control  $\gamma$ -C as in Fig. 1. Panel B: SDS-PAGE with in-gel glycoprotein fluorescent staining. NG: not retained fraction;  $\gamma$ -C: intact  $\gamma$ -C protein; STI: soybean trypsin inhibitor; HP: horseradish peroxidase. The two latter were negative and positive control samples of glycosylation. See text for experimental details.

**Figure 3.** SEC-MALS of NG (blue line) and pure  $\gamma$ -C (red line) at 0.2 mg/mL in sodium acetate buffer, pH 4.8, and run on Superose 12 10/300 GL column equilibrated 50 mM Tris-HCl, 100 mM NaCl, pH 7.5 as mobile phase. The MW, as calculated by SEC-MALS, are indicated by the letters above each peak and are 104000 kDa (D: dimer); 258100 kDa (H: hexamer); >400000 (A: aggregates).

**Table 1.**

Area distribution of the peaks separated by RP-HPLC of Fig. 2A.

Round indicates the number of repetitions of subsequent tandem chromatography runs until homogeneity was reached. Figures are the means of two different experimental settings.

n.d.: not detectable

Round	NG $\gamma$ -C (%)	$\gamma$ -C (%)	Aggregates (%)
0	5.82	93.93	0.26
1	14.87	84.64	0.49
2	25.58	72.42	2.01
3	52.98	44.99	2.03
4	85.95	11.18	2.87
5	68.94	n.d.	31.06

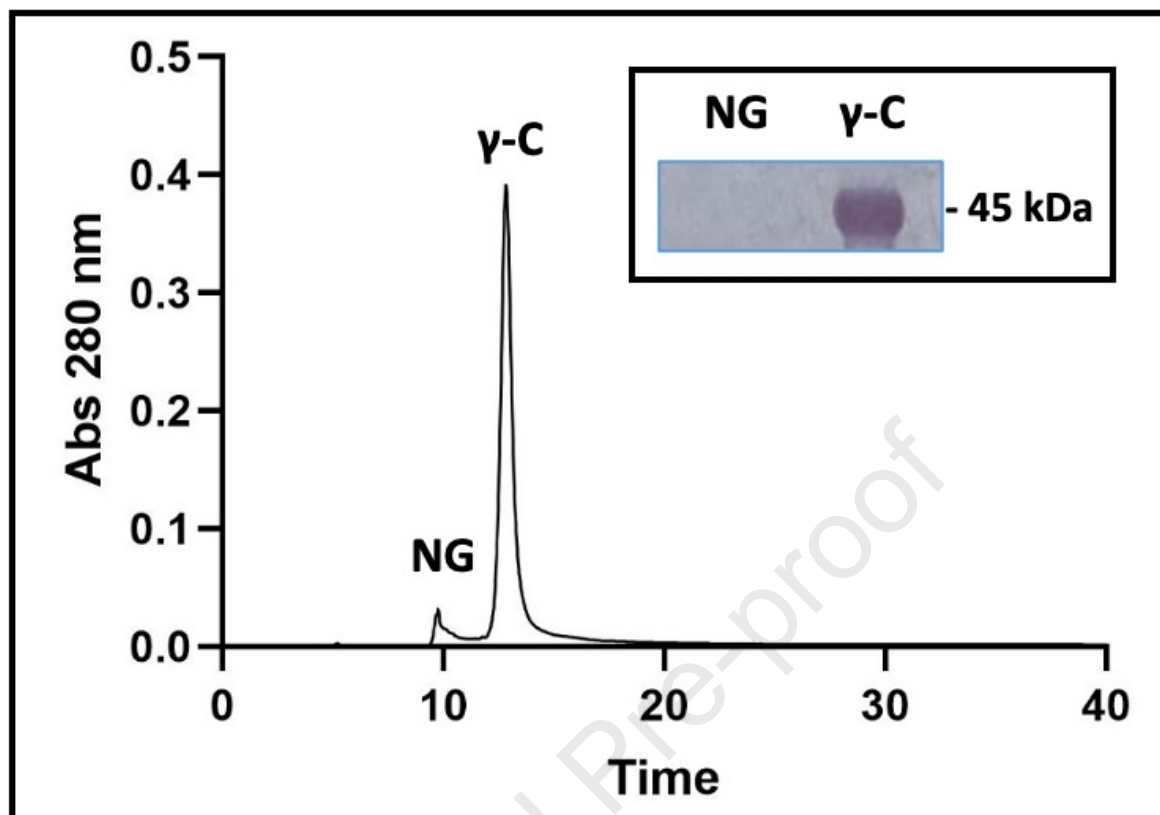


Figure 1

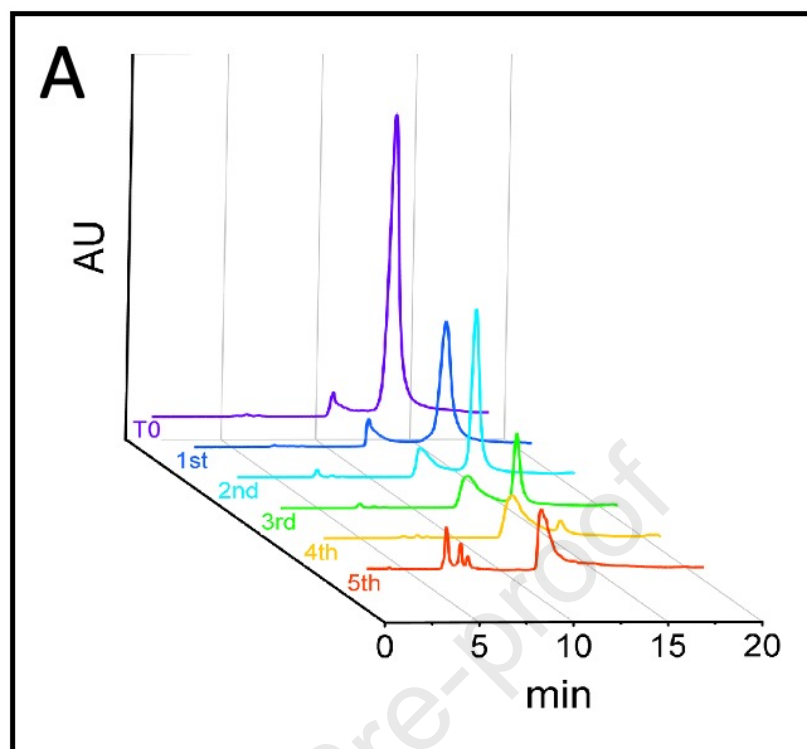
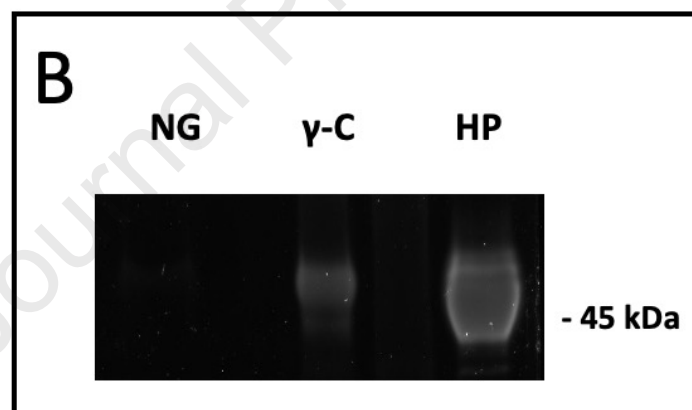


Figure 2



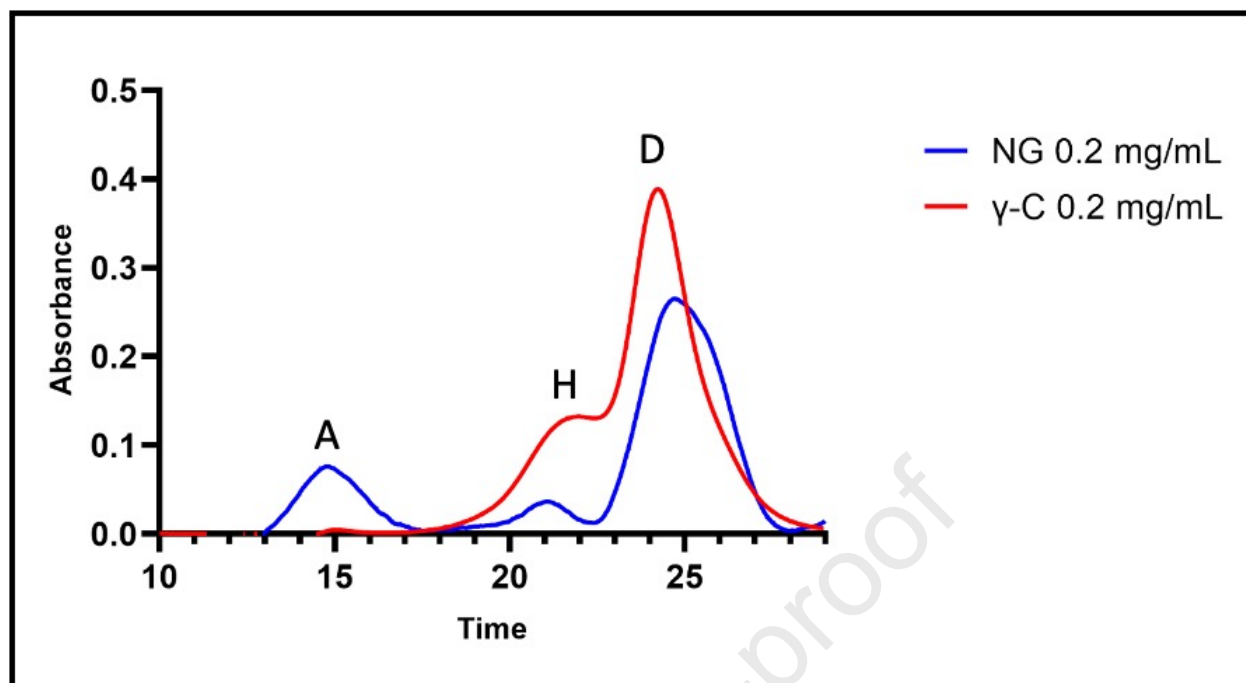


Figure 3

- A not-glycosylated  $\gamma$ -C monomers from the native protein has been isolated for the first time
- Native  $\gamma$ -C is a hexamer made up by both glycosylated and not-glycosylated monomers
- The seed glycoprotein is formed by identical polypeptide chains that have undergone different PTMs
- the not-glycosylated polypeptide is able to take part in the oligomerization equilibrium of  $\gamma$ -C

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

--