

Identification of major rye secalins as coeliac immunoreactive proteins

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Abstract

Six distinct γ - and ω -type secalins, together with two new low molecular mass glycoproteins, have been identified as the major coeliac immunoreactive proteins from a chloroform/methanol soluble extract from rye endosperm. These components were characterized by a combination of reverse-phase high-performance liquid chromatography, immunoblotting using a coeliac serum and microsequencing analysis. This allowed the identification of a group of secalins with different molecular masses according to their N-terminal amino-acid sequence: one ω -type secalin of 40 kDa (ω 1-40); three γ -type secalins, one of 70 kDa (γ -70) and two of 35 kDa (γ -35); as well as two low molecular mass glycoproteins of 15 and 18 kDa, all exhibiting coeliac serum antigenicity. Moreover, four additional rye components, including two low molecular mass proteins, which did not react with coeliac sera, have also been identified. Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of the three main purified coeliac immunogenic secalins, γ -70, γ -35 and ω 1-40, indicated molecular masses of 71 457, 32 240 and 39 117 Da, respectively. The ω 1-40 secalin displays a significant absorption in the visible region which could be related to its peculiar low capacity to bind both coeliac sera antibodies and Coomassie brilliant blue dye.

Keywords: Secalin; Coeliac disease; Immunoblotting; Rye; MALDI-TOF MS

1. Introduction

Cereal storage proteins have been traditionally classified on the basis of their solubility properties [1]. The major group, the alcohol-soluble proteins, are named prolamins reflecting their very high content of proline and glutamine. The prolamins of wheat (gliadins), barley (hordeins), rye (secalins) and oats (avenins) constitute complex mixtures of homologous proteins which can be classified into several groups according to their molecular structure relationship. This classification establishes three groups of prolamins: the sulfur-rich (S-rich) which comprises α - and γ -type, the sulfur-poor which comprises C hordein and ω -type (gliadins, secalins), and the high molecular weight (HMW) prolamins [2]. Prolamins typically possess at least two unrelated domains, one of which contains repeated consensus sequences at the N-terminal region which vary in length, although they all present a preponderance of proline and glutamine residues [3–5].

Based on *in vivo* and *in vitro* studies [6–9] it has been accepted that gliadins and other prolamins from barley and

rye are implicated in coeliac disease (CD), these proteins being responsible for triggering the mucosal damage and malabsorption of carbohydrates, proteins, fats and vitamins characteristic of CD [10,11]. However, the precise mechanism by which these peptides produce the important changes in the intestinal mucosa of coeliac subjects is unknown.

Sera from patients with untreated CD exhibit a high antibody reactivity against the alcohol-soluble protein fractions of wheat, barley, rye and oats. Thus, immunoblotting analysis of the corresponding gliadins, hordeins, secalins and avenins displays a characteristic pattern of circulating antibodies when compared with the reactivity pattern in patients with other intestinal disorders [12–14]. These immunoreactive cereal prolamin components constitute complex mixtures of proteins structurally, chemically and immunologically related which are known to elicit the typical symptoms of CD [11]. However, the precise number of these immunoreactive cereal components has not yet been established. While most of wheat [15–17] and oats [18] coeliac immunoreactive prolamins have been already identified, information concerning rye prolamins still is limited.

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In two recent studies we have identified several oats and wheat prolamins as coeliac immunoreactive proteins and classified them on the basis of their N-terminal amino-acid sequence as α - and γ -type prolamins [17,18]. In the present work, we have extended the study to rye prolamins in order to characterize individual components from rye endosperm with antigenic properties against coeliac sera. This has enabled the identification of distinct γ - and ω -secalins as well as two low molecular mass secalins, all resulting immunoreactive to coeliac sera.

2. Materials and methods

2.1. Reagents

Ultrapure water for high-performance liquid chromatography (HPLC), generated by a Milli-R04 coupled to a Milli-Q water purification system (Millipore, Bedford, MA), was employed in the preparation of all buffers. Acrylamide and *N,N'*-methylene-bis-acrylamide were obtained from Bio-Rad (Richmond, CA). Other reagents not specified were purchased from Merck (Darmstadt, Germany). All reagents and solvents for the sequencer were purchased from Merck, except trimethylamine which was from Applied Biosystems (Warrington, UK).

2.2. Serum samples

Sera from different individuals presenting symptoms compatible with untreated CD and having small bowel biopsies demonstrating subtotal villous atrophy, as well as serum from healthy controls, were used.

2.3. Protein extraction

Rye seeds (*Secale cereal*. cv. Petkus) were used in this study. Rye endosperm was obtained by hand dissection for small samples and by milling for larger samples as described [18]. The ground endosperm was delipidated with ten volumes (1 h, room temperature) of light petroleum and dried under vacuum. The flour was extracted with chloroform/methanol (CM) (2:1, v/v, 3 $\times$ 10 volumes) as described [19], filtered, dried partially in a rotavapor and lyophilized. It was stored dry at -20°C . The CM-extract (300 mg dry weight) was fractionated with 10 ml 0.05 M ammonium bicarbonate (Bi) by stirring overnight at room temperature as described [18]. The pellet (P) was separated from the supernatant (S) by centrifugation at $17\,000 \times g$ for 10 min. The bicarbonate pellet (Bi-P) was washed twice with 10 ml of the same ammonium bicarbonate solution. The pellet (Bi-P) and the resulting supernatant (Bi-S) were lyophilized and resuspended in 5 ml 0.1% trifluoroacetic acid (TFA).

2.4. High-performance liquid chromatography

The Bi-P and Bi-S extracts were fractionated by a reverse-phase high-performance liquid chromatography (RP-HPLC) system using the following equipment: two Waters 510 pumps, a Waters 680 automated gradient controller and a Waters 480 spectrophotometer. Sample injections were performed with a Waters U6K universal injector. Separations by RP-HPLC were carried out on a Nucleosil C₄ silica column (particle size, 5 μm ; pore size 300 Å; 300 $\times$ 8 mm) from Machery Nagel, (Duren, Germany) fitted with a guard column (25 $\times$ 4.6 mm) packed with the same support. The columns were eluted at room temperature with a gradient from 36% to 49% of acetonitrile in 0.1% TFA for 270 min for the Bi-S or from 10% to 25% for 90 min and from 25% to 30% for 95 min for the Bi-P, at a flow of 1 ml/min. The effluent was monitored by absorbance at 230 nm. The protein-containing fractions were lyophilized and resuspended in 0.1% TFA. They were stored at 4°C .

ω 1-40 was analyzed by size-exclusion HPLC in a Smart (Pharmacia, Uppsala, Sweden) HPLC system with a Superdex (Pharmacia) 75 PC 3.2/30 micro column (3.2 $\times$ 30 mm) connected on line with a photodiode array detector. The column was equilibrated and eluted with 0.1% TFA. The column was run at room temperature at a flow rate of 40 $\mu\text{l}/\text{min}$.

2.5. Photodiode array monitoring

A Waters (Milford, MA, USA) 996 photodiode array detector with a dynamic range from the ultraviolet to the visible region (190–800 nm) connected on line with the Smart HPLC System was used. A PowerMate 486/66i personal computer was employed to control the chromatographic system and analyze the resulting chromatograms. Photodiode array spectra were run from 200 to 500 nm in size-exclusion HPLC and analyzed using Millennium (Waters) post-run data programs. The Spectrum Index plot was used to determine absorption spectra automatically at the peak maxima. The Spectrum Review analysis plot was used to provide another view of the spectra at any selected point or to easily discern any similarities or differences. Chromatograms produced by monitoring a separation for absorption at 230 and 330 nm were compared by using the Review Data Window.

2.6. One-dimensional polyacrylamide gel electrophoresis

SDS-PAGE was performed under the same conditions as previously described [17,20]. The separation gel contained 18% (w/v) acrylamide and 0.25% (w/v) bisacrylamide. After electrophoresis the gels were stained overnight in 0.1% (w/v) Coomassie brilliant blue R in

methanol/acetic acid/water (10:10:80) and destained with the same solution in the absence of Coomassie brilliant blue.

2.7. Imidazole-SDS-zinc reverse staining of polyacrylamide gels

The staining procedures were essentially the same as recently described [21,22]. Immediately after electrophoresis gels were immersed and gently shaken in 50–100 ml 0.2 M imidazole containing 0.1% SDS for 5 minutes. Thereafter, the solution was discarded and the gels were immersed in 50–100 ml of 0.3 M zinc sulfate for 15 to 30 seconds until the gel background became deep white, leaving protein as transparent bands. Staining was stopped by discarding the zinc salt solution and rinsing the stained gel with distilled water.

2.8. Electroblothing and sequence analysis of blotted proteins

After one-dimensional SDS-PAGE, proteins were blotted onto Immobilon (Millipore, MA, USA) polyvinylidene difluoride (PVDF) membranes using a Novablot semi-dry apparatus (Pharmacia) in 25 mM Tris-HCl buffer (pH 8.4) containing 0.5 mM dithiothreitol. Blotting was performed at 2 mA/cm² for 3 hours at room temperature. Blotted proteins were detected by briefly staining the PVDF membrane with Coomassie brilliant blue and then rinsed in distilled water. Blotted protein bands were cut out of the membrane and sequenced in a Knauer Modular Protein Sequencer Model 810 equipped on line with a Knauer (Berlin, Germany) PTH-amino-acid analyzer. Phenylthiohydantoin (PTH) amino acids were identified and quantified on a RP-HPLC system based upon a Knauer C-18 column using gradient elution with 95% 13.2 mM sodium acetate, 5% tetrahydrofuran as buffer A and 100% acetonitrile as B.

2.9. Immunoblotting

After one-dimensional SDS-PAGE, proteins were electrophoretically transferred as described above, this time employing a transfer buffer which contained 25 mM Tris and 192 mM glycine (pH 8.3). The blot was carried out on duplicate gels at the same time. After blotting, the PVDF membranes were incubated in 20 ml of a buffer composed of 20 mM Tris, 500 mM NaCl (pH 7.5) containing 0.5% bovine serum albumin (Sigma, St. Louis, MO, USA) and 0.5% Triton X-100 (Sigma) for 1 h. The two membranes were then incubated overnight with a coeliac serum (1/4000) or normal serum (1/4000) in the above buffer. The membranes were incubated for three hours with alkaline phosphatase-conjugated rabbit anti-human IgA (Dakopats, The Netherlands) (1/8000). Finally, the membranes were stained for 20 min with 5-bromo-4-chloro-3-

indolyl phosphate *p*-toluidine salt and *p*-Nitro blue tetrazolium chloride. The reaction was stopped by rinsing the membrane briefly with distilled water. All incubations were performed at room temperature.

2.10. Sample preparation and MALDI-TOF MS

Nine $\mu\text{g}/\mu\text{l}$ of total protein extract or purified proteins were suspended in 80% aqueous ethanol solution and centrifuged for 2 min. Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) was used as a matrix for laser desorption and ionization of proteins. One μl of the supernatant was mixed with 50 μl of a saturated solution of sinapinic acid in 30% aqueous acetonitrile and 0.1% trifluoroacetic acid. One μl of the mixture was then deposited onto a stainless steel probe tip and dried at room temperature. The sample was measured on a Bruker Reflex mass spectrometer (Bremen, Germany) equipped with an ion source with visualization optics and Himas detector. All spectra were recorded in the linear positive mode at 35 kV acceleration. About 100 spectra of single laser shots were accumulated. For the calibration of the instrument horse heart cytochrome *c* (12 360 Da) and BSA (66 430 Da) were used.

2.11. Carbohydrate staining of blotted proteins on PVDF membrane

A highly sensitive staining technique based on Problott, a PVDF-type membrane for screening glycoproteins using biotinylated lectins and peroxidase-conjugated avidin, was employed as described [23].

3. Results

3.1. Fractionation of secalins from the CM-protein extract from rye endosperm

Fig. 1, the center part, shows the Coomassie brilliant blue stained SDS-PAGE pattern of the chloroform/methanol protein extract from rye endosperm. The CM-extract contains a large number of different proteins including prolamins (secalins), CM-proteins, and albumins. The gel shows the presence of three main components, with estimated molecular masses of 70, 35 and 40 kDa respectively, along with some low molecular mass components of about 10 to 20 kDa.

Fig. 1, left, displays an immunoreactive pattern obtained after blotting in which differences among the intensities of the immunoreactive bands are observed depending on the coeliac sera employed. Under these electrophoretic separation conditions, the immunoreactivity and the Coomassie brilliant blue staining pattern of the CM-extract indicate a main group of immunoreactive bands in addition to a minor group of non immunoreactive bands (Fig. 1).

In order to separate and identify both immunoreactive

and non immunoreactive components, the rye CM-extract was subjected to a procedure combining an ammonium bicarbonate extraction, RP-HPLC and SDS-PAGE, followed by immunoblotting with a coeliac serum and microsequencing analysis. The CM-extract was first fractionated using a bicarbonate solution into two more simple mixtures, Bi-S and Bi-P (Fig. 1, center). The main protein components of about 35 to 70 kDa were distributed in both the soluble and insoluble mixtures, while low molecular mass components were only in the soluble mixture (Fig. 1, center).

The resulting supernatant Bi-S and pellet Bi-P fractions were further chromatographed by RP-HPLC in a Nucleosil-C₄ column, using separation times ranging from three to four hours. 14 peaks in both Bi-S and Bi-P distributed along the full length of the chromatogram were obtained (Fig. 2, top). Amino-acid composition of all these fractions has allowed to identify prolamins components on the basis of their glutamic/glutamine and proline content. On fractions S 9–14 and P 1–14, the Glx and Pro content ranges from 35 to 47% and 14 to 36% respectively, which is characteristic of prolamins. On the other hand, the Glx and Pro content on fractions S 1–2 are 11% and 13% respectively, while on fractions S 3–8 they are 27–31% and 11–18%.

The resolution of the SDS-PAGE analysis of these peaks allowed a well-resolved separation of proteins, most

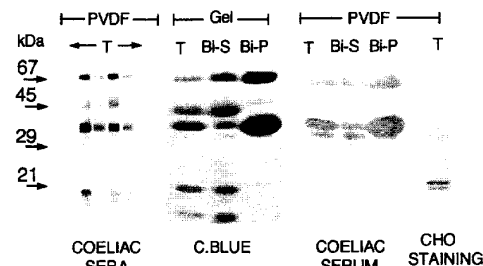


Fig. 1. SDS-PAGE and immunoblotting analysis from the total CM extract from rye endosperm. Left: immunoblotting analysis of the total CM extract (T) against four different sera from patients with CD. Center: SDS-polyacrylamide gel stained with Coomassie brilliant blue and the corresponding immunoblotting onto PVDF membrane, incubated with a coeliac serum and visualized by developing with anti-human IgA labeled with alkaline phosphatase. The control using normal sera was negative (data not shown). Right: carbohydrate detection onto PVDF of the total CM extract. The glycoproteins IgA and human protein HC used as positive controls for the carbohydrate staining have not been included. CHO: carbohydrate.

of which yielded a unique N-terminal sequence, thus permitting their chemical identification.

3.2. Immunoblotting with coeliac serum

In order to identify the rye coeliac immunoreactive components, we chose a high titer coeliac serum capable

Table 1
NH₂-terminal amino-acid sequence of the blotted rye proteins from the Bi-S and Bi-P extracts

Protein	kDa	N-terminal amino acid sequence	Refs
<i>γ</i> 70- <i>Secalin</i>		N M Q V N P S G Q V Q W P Q Q Q P F P Q P Q Q	(3, 11)
^a S 10–12	<i>γ</i> 70	N M Q V N P S G Q V Q W P Q Q Q P F P Q P Q Q	
^a P 4–12	<i>γ</i> 70	N M Q V N P S G Q V Q P P Q Q Q P F P Q P Q Q	
<i>γ</i> 40- <i>Secalin</i>		N M Q V G P S G Q V E W P Q Q Q P L P Q	(3, 11)
S 14	<i>γ</i> 35	N M Q V G P S G	
P 9–12	<i>γ</i> 35	N M Q V G P S G Q V E W P Q Q Q P L P Q	
P 13–14	<i>γ</i> 35	N M Q V G P S G Q V S W P Q Q Q P L P Q P Q Q X V Y Q X P	
<i>ω</i> 1- <i>Secalin</i>		R Q L N P S E Q E L Q S P Q Q P V P G E Q S Y P	(24)
P 1–2	<i>ω</i> 1-40	R Q L N P S E Q E L Q S P Q Q P V P G E Q S Y P	
S 3	<i>Rye-15</i>	L D T T X S Q G Y G Q X Q L Q Q Q M N Y T X A A F L Q Q X X T P	
S 4–8	<i>Rye-18</i>	L D T T X S Q G Y G Q X Q L Q Q	
S 1–2	<i>Rye-10</i>	T G P Y E Y A G N	(18)
S 1–2	<i>Rye-12</i>	S V G G Q X V P G L A M P K N P L - G A R E T V	(25, 26)
S 9–11	<i>Rye-45</i>	T S I Y Q X Y E G	
S 13	<i>Rye-40</i>	I V L G Q S Q	

Unidentified positions are indicated with an X. Single letter designations for amino acids are used.

^a P and S indicate proteins from the ammonium bicarbonate pellet and supernatant respectively. The assessed molecular weights of *γ*3 and *ω*-secalins are indicated with italic numbers. Coeliac immunoreactive proteins are indicated in dashed boxes. Amino acid differences are indicated in square boxes.

of yielding the strongest reactivity with the different protein components. Fig. 2, bottom, shows the immunoblotting employing this serum against the different frac-

tions obtained by RP-HPLC from both Bi-S and Bi-P. The results indicate that the highest immunoreactivity is associated with the two main components of about 35 and 70

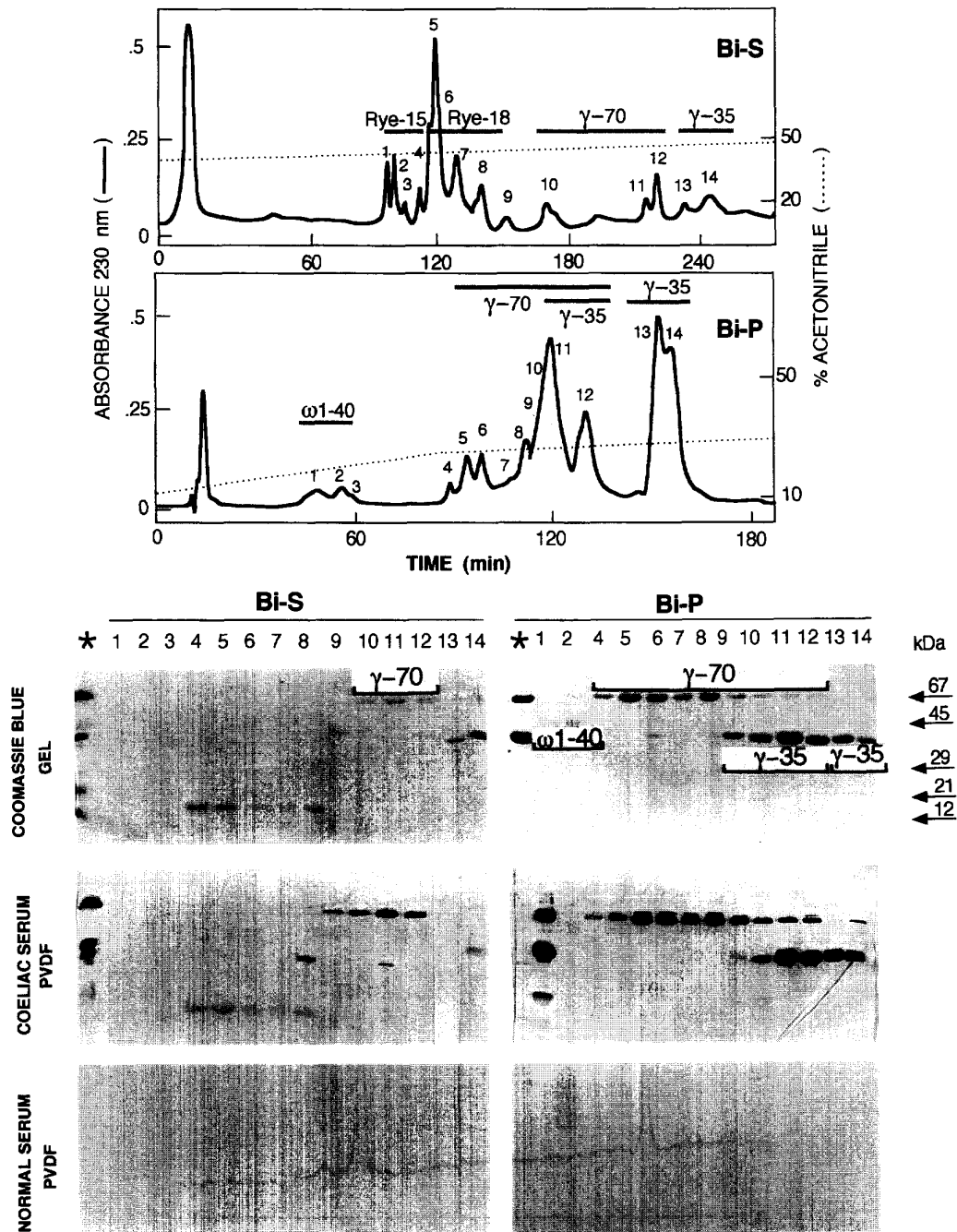


Fig. 2. Fractionation by RP-HPLC of the ammonium bicarbonate soluble, Bi-S, and insoluble, Bi-P, fractions from rye endosperm. Top: the extracts were resuspended in 0.1% TFA and injected into a Nucleosil C-4 column. Bottom: SDS-PAGE stained with Coomassie brilliant blue of fractions of the chromatograms and the corresponding immunoblotting developed with a coeliac or normal serum. Serum dilutions for a coeliac patient and a healthy blood donor were 1/4000 and for anti IgA 1/8000. Numbers indicated above the gels correspond to the peak of the chromatogram. Arrows show the mobility of the standard proteins. The lanes marked with an asterisk correspond to the total bicarbonate soluble and insoluble extract. Identified components are indicated by Greek letters.

kDa, while only a faint immunoreactivity is associated with a component of about 40 kDa in the Bi-P and two low molecular mass components present in the Bi-S (Fig. 2, bottom, center). On the contrary, normal serum at the same concentration failed to react with these components (Fig. 2, bottom). The non immunoreactive rye components were localized in a few minor bands in fractions 1–2, 9–11 and 13 in the Bi-S which did not react with coeliac serum.

3.3. Chemical classification of the coeliac immunoreactive rye prolamins by N-terminal microsequencing

In order to characterize and chemically classify these coeliac immunoreactive rye components, proteins bands associated with a strong or faint immunoreactivity were subjected to microsequencing analysis.

The N-terminal amino-acid sequence of bands S 10–12 and P 4–12, of 70 kDa, and S 14, P 9–12 and P 13–14, of 35 kDa, fully coincides with that of a typical γ -type secalin [3,11] (Table 1). On the other hand, band P 1–2 of 40 kDa fully coincides with the N-terminal amino-acid sequence of ω 1-secalins [24] (Table 1). The three immunoreactive components have been called γ -70, γ -35

and ω 1-40, respectively. Moreover, the sequence of bands S 3 and S 4–8, corresponding to the two low molecular mass immunoreactive components of 15 and 18 kDa, did not match any described secalin or typical wheat, barley, rye and oats prolamins. These two components have been tentatively called Rye-15 and Rye-18, according to their molecular masses.

Several bands in S 1–2, S 9–11 and S 13, which were not antigenic against coeliac serum, were also sequenced (Table 1). The two bands in S 9–11 and S 13 of 40 and 45 kDa, respectively, were not homologous with known cereal proteins; they have been designated Rye-40 and Rye-45. Two bands in S 1–2, of 10 and 12 kDa, have been called Rye-10 and Rye-12, respectively. The former has some homology with oats-C [18], and the latter with oats-A [18] and wheat, barley [25] and rye [26] CM α -amylase inhibitors.

3.4. MALDI-TOF mass spectrometric analysis of total soluble prolamins and γ -35, ω 1-40 and γ -70 rye secalins

To determine accurate molecular masses of these coeliac immunoreactive secalins, the total rye CM-extract was analyzed by means of MALDI-TOF MS. Fig. 3 shows

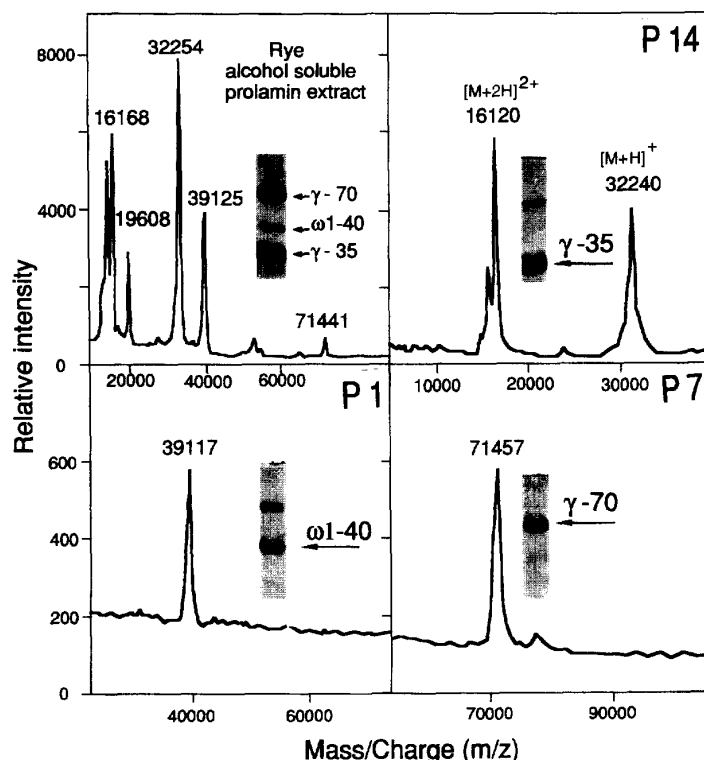


Fig. 3. Mass spectra of the total alcohol soluble prolamins extract. The protonated molecular mass $[M + H]^+$ values determined for the purified γ -35, ω 1-40 and γ -70 were 32240, 39117 and 71457 Da, respectively. The slightly different masses obtained for the same components, 32254, 39125 and 71441, from the total soluble extract are due to the resolving power of the mass spectrometer used. SDS-PAGE patterns of the total soluble extract and fractions P14, P1 and P7 from Bi-P are inserted.

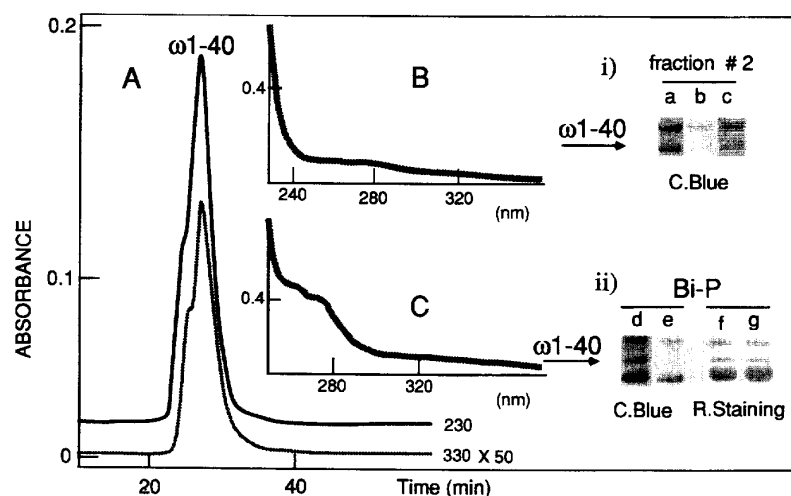


Fig. 4. Analytical fractionation by size-exclusion HPLC of the purified ω 1-40 secalin. Column: Superdex 75 PC 3.2/30 equilibrated in 0.1% TFA; flow-rate 40 μ l/min. (A) The chromatograms display the light absorption of the effluents at 230 and 330 nm. (B) and (C) are the absorption spectra from 230 to 360 nm and from 242 to 400 nm respectively, recorded by the photodiode array detector from the peak maximum. SDS-PAGE of (i) fraction 2 (Fig. 1) staining with Coomassie brilliant blue (a) for six hours, (b) for 24 hours and (c) restained and destained for six hours; (ii) Bi-P extract stained with Coomassie brilliant blue and destained (d) for six hours, (e) for 24 hours. (f) is the Bi-P extract stained by reverse staining and (g) is obtained by reverse staining of (e).

mass signals at 16 168, 19 608, 32 254, 39 125 and 71 441 Da which are likely to correspond to Rye-15, Rye-18, γ -35, ω 1-40 and γ -70, respectively. The molecular masses of γ -35, ω 1-40 and γ -70 were confirmed by MALDI-TOF MS analysis of fractions P14, P1 and P7 (Fig. 2), which yielded masses of 32 240, 39 117 and 71 457 Da, respectively (Fig. 3).

3.5. Photodiode array analysis of ω 1-40 secalin

During this study, it was noted that the ω 1-40 secalin band exhibits an unusual purple colour after staining with Coomassie brilliant blue. The intensity of this colour progressively diminishes until it disappears during gel destaining as seen in Fig. 4. Fig. 4, right, shows in more detail the colour intensity evolution of ω 1-40 present both on fraction 2 (Fig. 4, right, top) on the Bi-P chromatogram (Fig. 4, right, bottom), and on the unfractionated Bi-P extract, after staining with Coomassie brilliant blue followed by destaining for a short period of time (6 hours, lanes a and d) or a long period of time (24 hours, lanes b and e). Although the protein was apparently destained, it still remained on the gel as revealed after further restaining the gel with Coomassie brilliant blue (Fig. 4, lane c) or imidazole-Zn-SDS.

To look for attached ligands which could explain the behaviour with Coomassie brilliant blue, purified ω 1-40 was analyzed by micro size-exclusion HPLC using photodiode array detection. When monitored at two different wavelengths, the absorbance at 230 nm fully coincided with that at 330 nm (Fig. 4, Part A). Absorption spectra at the peak maxima displayed significant absorption in the

300–400 nm range (Fig. 4, Parts B and C), thus suggesting the presence of chromophores in the visible region.

3.6. Carbohydrate staining

Since glycoproteins stain faintly with Coomassie brilliant blue, we also investigated whether this peculiar staining of ω 1-40 was due to carbohydrates. Thus, rye prolamins were blotted to PVDF and stained by a specific carbohydrate stain. Fig. 1, right, shows that only the two low molecular mass components stained positively for carbohydrate. These components were identified by amino-acid sequencing as the coeliac immunoreactive proteins Rye-15 and Rye-18.

4. Discussion

Rye prolamin immunoblotting shows an antibody pattern with coeliac sera, consisting of six to eight main and minor immunoreactive bands ranging from 10 to 70 kDa whose relative intensity varies with the coeliac sera employed (Fig. 1, left). Although the immunoreactivity and the Coomassie brilliant blue staining pattern are very similar, indicating that all protein bands are apparently immunoreactive, or suggesting a non-specific reaction, the use of a better resolution technique like RP-HPLC + SDS-PAGE two dimensional combination (Fig. 2, bottom), has allowed to distinguish between immunoreactive and non-immunoreactive proteins. The separation provided by this procedure allowed microsequence for chemical characterization of a large number of individual bands.

N-terminal sequence analyses of approx. 15 identified immunoreactive bands allow classification of at least six γ - and ω 1-type secalins. Proteins with identical N-terminal sequence and molecular mass but showing in different peaks in the chromatogram have been tentatively grouped under a single name and called γ -70, γ -35 and ω 1-40. Their sequences fully coincide with those deduced by amino acid and nucleotide sequence analyses of ω 1- and γ -type secalins [3,11,24]. In addition, two apparently new coeliac immunoreactive low molecular mass components, Rye-15 and Rye-18, with N-termini atypical secalin, are also described. They could not be chemically classified, since they did not exhibit significant sequence homology with other known prolamins from wheat, barley, rye or oats.

Some of the identified secalins displaying identical N-terminal sequence appear to migrate with different retention time upon RP-HPLC (Fig. 2, top). As an example, γ -70 appears in peaks 10–12 and 4–12 in the Bi-S and Bi-P, respectively, while γ -35 appears in the Bi-P in peaks 9–12 and as a unique protein in peaks 13–14. Thus, protein bands exhibiting identical N-terminal sequences and molecular weight are considered and called as a unique molecule.

These coeliac immunoreactive secalins with the same N-terminal amino-acid sequence and molecular weight (two γ -70, three γ -35 and Rye-15 and Rye-18) could constitute heterogeneous components closely related representing homologous groups of proteins resulting from conservative, non-lethal mutations among duplicated genes. Whether RP-HPLC peaks correspond to a unique polypeptide sequence existing in multiple forms, or they are due to chemical modifications under chromatographic acidic conditions (e.g., deamidation) as also described for coeliac immunoreactive wheat and oats [17,18], or to the effect of temperature [27], needs further insight.

γ -70 and γ -35 secalins have highest binding capacities

against coeliac sera (Figs. 1 and 2); on the contrary, ω 1-40 secalin, another main component, has a much lower antigenic capacity (Figs. 1 and 2).

Both Rye-15 and Rye-18, the newly described immunoreactive secalins, seem to be glycosylated (Fig. 3). To our knowledge, this is the first report describing glycoproteins in rye endosperm. Nevertheless, the precise carbohydrate attachment site(s) of glycosylation, the elucidation of the carbohydrate moiety nature, and the possibility that carbohydrates have a biological role in Rye-15 and Rye-18 require further research.

Analysis by MALDI-TOF MS of alcohol-soluble rye prolamins or purified secalins (Fig. 3) permits more precise molecular masses to be obtained for the identified coeliac immunoreactive secalins than can be determined by SDS-PAGE (Figs. 1 and 3): 71 441 Da for γ -70, 39 172 for ω 1-40, 32 240 for γ -35, 19 608 for Rye-18 and 16 168 for Rye-15. The mass of ω 1-40 is only slightly lower than the values of 39 426 and 39 324 from two genomic clones [24]. These small differences could be ascribed to deletion of amino acids or to deamidation during sample manipulation.

According to our results, ω 1-40 secalin has several exceptional properties: (i) in contrast to other secalins, ω 1-40 displays an unusual purple colour on acrylamide gel after staining with Coomassie brilliant blue; (ii) this colour nearly disappears upon prolonged storage in destaining solution, although the protein remains in the gel; (iii) ω 1-40 displays a significant absorption in the visible region; and (iv) ω 1-40 appears to have lower antibody binding capacity than the other coeliac immunoreactive secalins. This peculiar behaviour seems to be associated to ω -type prolamins, as also observed in ω -gliadins [28], which also have low antibody binding capacity in comparison to α -, β - and γ -gliadins [28]. This phenomenon could be ascribed to the Coomassie brilliant blue metachromasia effect, as also described in secalin fractions [29,30] and

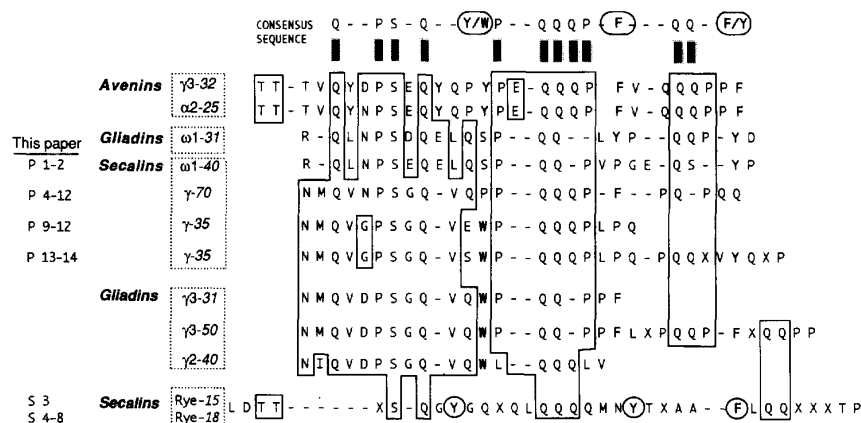


Fig. 5. Amino-acid sequence homology between coeliac immunoreactive secalins, gliadins, and avenins. Common sequences are indicated in boxes. Gaps are included to achieve maximal homology. Conserved residues are in bold and indicated also by shaded boxes. Aromatic amino acids are in circles.

tentatively attributed to the high proline content of these proteins. Nevertheless, some other factors (e.g., chromophore attached ligands, like those of ω 1-40) must be responsible for this metachromatic effect, as most proline-rich cereal proteins do not display it.

N-terminal sequence analyses indicate that the immunoreactive γ -70, γ -35 and ω 1-40 secalins display strong homology with coeliac immunoreactive prolamins described in oats and wheat [3,11,18,31] (Fig. 5) and are completely different from the non-immunoreactive rye components (Table 1). The two immunoreactive glycoproteins, Rye-15 and Rye-18 seem to be non true prolamins, and could be albumins or globulins since they shown less homology than γ -70, γ -35 and ω 1-40 secalins (Fig. 5) as well as a lower Glx and Pro content.

Moreover, immunoreactive prolamins are structurally unrelated with the non-reactive components, which have some homology with oats, wheat, barley and rye CM-components [18,19,25,26]. No homologies were found for Rye-45 or Rye-40.

N-terminal region of the immunoreactive rye, oats and wheat prolamins, shows three main groups of conserved sequences (Fig. 5). One comprises the first 10–11 amino-acid residues; two other groups, comprising 4–6 and 4 residues, respectively, display a high proline and glutamine content, including the QQQP peptide sequence toxic in CD. These three groups share 11 conserved amino-acid residues, including glutamine, proline and serine, and are separated by aromatic amino acids at equivalent sequence positions. Conserved amino-acid residues permit to deduce a peptide consensus sequence (Fig. 5, top) once more indicating a strong homology among these cereal components.

The presence of the QQQP toxic peptide in these immunoreactive secalins suggests a possible relationship between immunoreactivity and toxicity; nonetheless, the identification of other toxic sequences in γ -70, γ -35 and ω 1-40 will be required in order to elucidate their toxic implication in CD, as described for coeliac active peptides from gliadins [32–35].

The existence of common structural characteristics among these related coeliac immunoreactive secalins, gliadins and avenins could explain their antigenic similarities. Thus, coeliac sera raising circulating antibodies against gliadins [12–14], which in turn display antibody reactivity against hordeins and secalins, have been described [36]. On the other hand, it has been suggested that coeliac patients may raise circulating antibodies against distinct gliadin epitopes positioned in the N-terminal region, what could explain that other prolamins, structurally related to gliadins, show antigenic similarities [37].

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