

Biochemical Characterization of γ -75 k Secalins of Rye

I. Amino Acid Sequences

Claudia Gellrich,¹ Peter Schieberle,¹ and Herbert Wieser^{1,2}

ABSTRACT

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The prolamin fraction of the rye cultivar Danko was reduced with dithioerythritol and separated by reversed-phase HPLC on C₁₈ silica gel. Two major γ -75k secalins, P1 and P2, were collected, purified by rechromatography, derivatized with 4-vinylpyridine, and digested in parallel with α -chymotrypsin, thermolysin, and trypsin. The different enzymatic hydrolyzates were preparatively separated by two-step reversed-phase HPLC on C₁₈ silica gel, and the resulting peptides were characterized by sequence analysis and, in parts, by mass spectrometry. By means of overlapping peptides and by comparison with a known DNA sequence of a γ -75k secalin (gSec2A) derived from a wheat translocation line (2RS. 2BL), 84% of the P1 sequence and 35% of the P2 sequence could be assigned. Heterogeneity at several sequence positions demonstrated that both protein preparations were not pure and contained at least two or three components. The sequence of the C-terminal domain of P1 was almost com-

pletely determined except for one of the 148 residues which could not be identified. The partially determined sequences of P2 were highly homologous with those of P1. The results revealed a close relationship between P1, P2, and gSec2A and a high degree of homology with γ -gliadins of wheat including eight cysteine residues in homologous positions. The partially sequenced N-terminal domain of P1 was similar to that of gSec2A and consisted of repetitive sequences rich in glutamine, proline, and aromatic amino acids. Differences from γ -gliadins were found in the strongly increased number of residues, in the more frequent modifications of the repetitive motifs, and in the presence of a cysteine residue at position 12. The partial amino acid sequence of the N-terminal domain of P2 was in agreement with that of P1, besides a few exceptions in single positions and in the presence of a second cysteine residue.

Previous studies on the storage proteins of rye (secalins) showed that γ -75k secalins are the major protein type in rye flour contributing >30% of total flour proteins and nearly half of the storage proteins (Gellrich et al 2003). In contrast to the storage (gluten) proteins of wheat, information as to the structure of γ -75k secalins are scarcely available. Data on molecular weights differed between 54,000 determined by sedimentation equilibrium ultracentrifugation and 75,000 determined by SDS-PAGE (Shewry et al 1982). N-terminal sequences analyzed up to residue 23 at a maximum (Shewry et al 1982; Rocher et al 1996) and sequences of the C-terminal domain derived from cDNA clone pSc503 (Kreis et al 1985) revealed a high degree of homology with γ -gliadins of wheat. The analysis of amino acid compositions showed some differences to γ -gliadins, notably more Glx and Pro (Shewry et al 1982). According to these results, γ -75k secalins were proposed to have a molecular weight higher than that of γ -gliadins due to the additional repetitive sequences rich in Gln and Pro (Shewry et al 1987). The structural basis for the aggregative behavior of γ -75k secalins remained unclear. Shortly after the experimental work described here was finished, a genomic DNA sequence (clone gSec2A) coding for a γ -75k secalin was published (Murray et al 2001). The clone was isolated from a wheat translocation line carrying the 2RS.2BL chromosome. The predicted amino acid sequence contained 436 residues including a short nonrepetitive N-terminal region (12 residues) followed by a long Gln/Pro-rich repetitive region (272 residues) and a C-terminal domain containing 156 residues. Compared with known partial sequences of γ -75k secalins, the sequence of gSec2A contained Cys instead of Trp in position 12 and several modifications within the C-terminal domain. The same authors resequenced clone pSc503 described by Kreis et al (1985) and identified numerous errors in the original sequence, suggesting that only one residue in position 430 (Glu instead of Val) was different from clone gSec2A. The aim of the present work was to determine the amino acid sequences of γ -75k secalins that are representative of rye flour and to clarify contradictions existing in the literature.

MATERIALS AND METHODS

Extraction of Proteins

Nondefatted flour (5 g) of the German rye cultivar Danko (Gellrich et al 2003) was extracted stepwise three times with 50 mL of 0.4 mol/L NaCl + 0.067 mol/L HKNa PO₄ (pH 7.6) and three times with 50 mL of 60% (v/v) ethanol. Each extraction step was performed by homogenization using an Ultra Turrax homogenizer for 5 min at room temperature (RT 20°C). The suspensions were then centrifuged for 10 min at 11,000 \times g and RT. The ethanolic extracts were combined, dialyzed against 0.1% (v/v) acetic acid, and freeze dried (prolamin fraction).

RP-HPLC of Proteins

The prolamin fraction (100 mg) was dissolved in 10 mL of 60% (v/v) ethanol and reduced with dithioerythritol (1 g) for 10 min at 60°C. RP-HPLC of the filtered solution was performed using an HPLC instrument with a solvent module 125 (Beckman, Munich, Germany) and a Nucleosil 300-5-C₈ column (4.6 \times 240 mm) (Macherey-Nagel, Düren, Germany). Column temperature was 60°C. Elution solvents A and B were 0.1% (v/v) trifluoroacetic acid (TFA) and acetonitrile containing 0.1% TFA, respectively. The applied gradient was stepwise linear with 26% B at the beginning of the run, rising up to 35% after 5 min and to 38% B after 30 min. The flow rate was 1.0 mL/min. The injection volume was 800 μ L, TFA (500 μ L) was injected before and after sample injection (Wieser et al 1998). The eluted proteins were detected by UV absorbance at 210 nm. Effluents from \approx 100 runs were collected as indicated in Fig. 1. The organic solvents were removed in a stream of nitrogen at RT. The proteins were freeze-dried, redissolved in 60% (v/v) ethanol (1.1 g in 1 mL), reduced with dithioerythritol (100 mg), and derivatized with freshly distilled 4-vinylpyridine (Friedman et al 1970). The proteins were then purified by RP-HPLC under the conditions described above but using Nucleosil 300-5-C₁₈ column material (Macherey-Nagel, Düren, Germany).

Enzymatic Hydrolysis

In preliminary experiments the following enzymes were tested: α -chymotrypsin (Merck no. 2307), TLCK-chymotrypsin (Sigma no. C-3142), trypsin (Merck no. 24579), thermolysin (Boehringer no. 161586), and pepsin (Sigma no. P-6807). The ratios of enzyme

¹ Deutsche Forschungsanstalt für Lebensmittelchemie und Kurt-Hess Institut für Mehl- und Eiweißforschung, Lichtenbergstr. 4, D-85748 Garching.

² Corresponding author. E-mail: h.wieser@Lrz.tum.de

to substrate (w/w 1/10, 1/25, 1/50, 1/100) and of substrate to buffer volume (w/v 1/1,000, 1/2,000), and the incubation time (8 hr, 16 hr) were varied for optimization. The degree of hydrolysis was monitored by RP-HPLC of aliquotes. In preparative assays, 0.64–1.60 mg of protein (P1, P2) was digested with TLCK-chymotrypsin, thermolysin and trypsin (ratio 1/25). The buffers were 0.1 mol/L Tris-HCl pH 7.8 (chymotrypsin, trypsin) and 0.1 mol/L Tris-HCl pH 7.8 + 2 mmol/L CaCl₂ (thermolysin) using a ratio of 1:1,000 (chymotrypsin) and 1:2,000 (trypsin, thermolysin). The temperature was 37°C, and the incubation times 6 hr (chymotrypsin, trypsin) and 16 hr (thermolysin). The reactions were stopped by adjusting to pH 3.0 with 1 mol/L of HCl. The digests were filtered, and the filtrates were used for RP-HPLC.

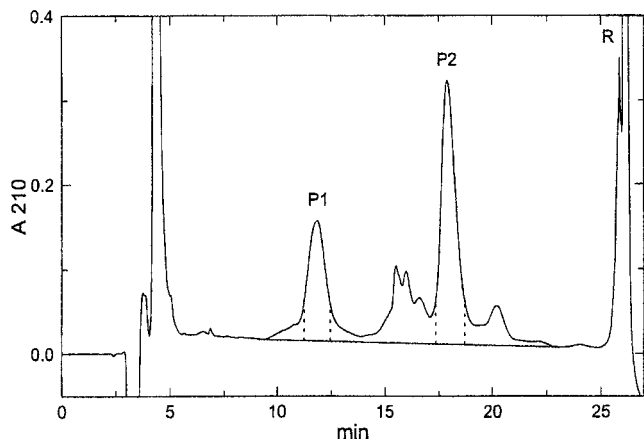


Fig. 1. Preparative RP-HPLC of the prolamin fraction from Danko rye flour on C₈ silica gel.

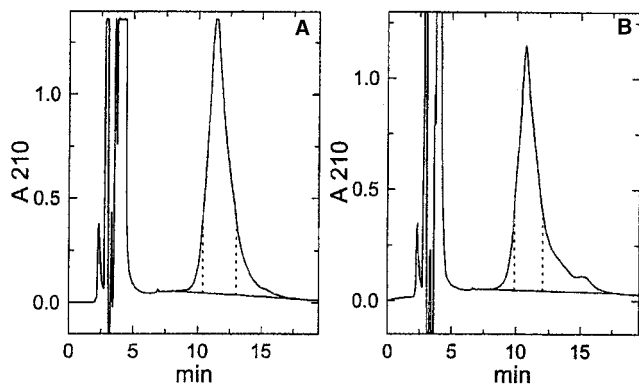


Fig. 2. Preparative rechromatography of γ -75k secalins P1 (A) and P2 (B) on C₁₈ silica gel.

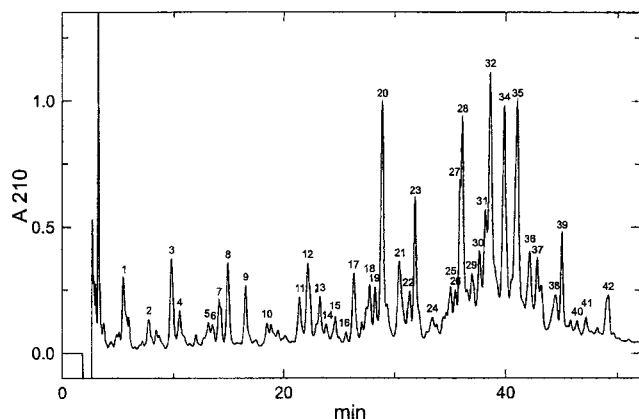


Fig. 3. Preparative RP-HPLC of the chymotryptic digest of P1 on C₁₈ silica gel (peptides P1/Ch 1-42).

RP-HPLC of Peptides

The separation of the enzymatic digests was performed as follows: column: Hypersil C₁₈ (5 μ m, 12 nm) 4.6 \times 240 mm (Thermoquest, Runcorn, UK); temperature: 60°C; elution solvents: (A) 0.01 mol/L of triethylammonium formate, pH 3.5 (TEAF) and (B) TEAF/acetonitrile (60/40, v/v); gradient: stepwise linear, 0 min 25% B, 55 min 80% B (chymotryptic digest) or 0 min 10% B, 60 min 80% B (tryptic and thermolytic digest); flow rate: 1.0 mL/min; injection volume: 50 μ L (analytical) and 250–700 μ L (preparative); detection: UV absorbance simultaneously at 210 and 254 nm. The effluents were collected corresponding to the different peptide peaks and freeze-dried. Rechromatography of the peptides was performed under the conditions described above but with pH 6.0 of the TEAF solvent and with solvent gradients optimized for each peptide which were finally freeze-dried.

Analytical Methods

Amino acid analysis, the determination of amino acid sequences, SDS-PAGE, and MALDI-TOF mass spectrometry were performed under the conditions described previously (Gellrich et al 2003). Peptide masses were determined by nanospray mass spectrometry using an LCQ mass spectrometer (Finnigan, Bremen, Germany).

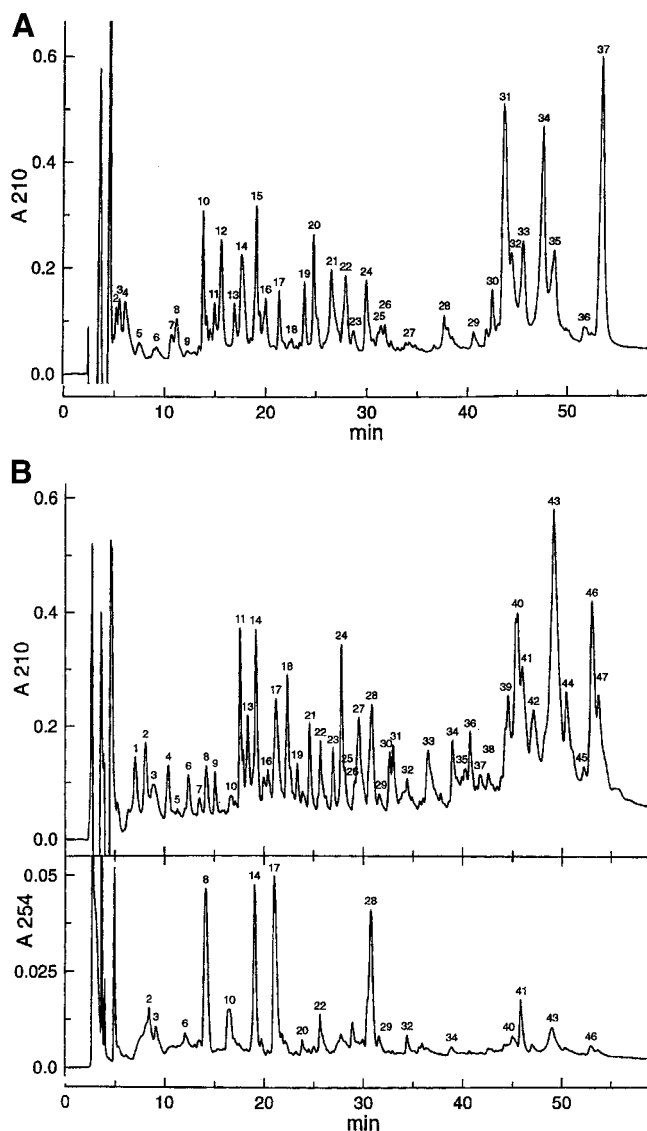


Fig. 4. Preparative RP-HPLC of the thermolytic digest of P1 (A) and P2 (B) on C₁₈ silica gel (peptides P1/Th 1-37 and P2/Th 1-47).

RESULTS AND DISCUSSION

Isolation and Fragmentation of γ -75k Secalins

Previous studies on the secalins of two rye flours (cvs. Danko and Halo) had demonstrated that the major portion of γ -75k secalins occurred in the alcohol-soluble prolamins fractions (Gellrich et al 2003). γ -75k Secalins were isolated by extraction of rye flour Danko with 60% ethanol after albumins and globulins were removed by extraction with a salt solution. The proteins of the prolamins fraction were reduced and then separated by preparative RP-HPLC on C_8 silica gel optimized for the separation of γ -75k secalins. Two major γ -75k secalins, P1 and P2, were collected (Fig. 1), derivatized with 4-vinylpyridine, and purified by rechromatography on C_{18} silica gel (Fig. 2). SDS-PAGE of the isolated proteins (not shown) revealed that P1 corresponded to the upper band, and P2 to the lower band of the γ -75k secalin region

(Gellrich et al 2003). The amino acid compositions of P1 and P2 agreed with those of the raw fractions Pr/e and Pr/f isolated previously (Gellrich et al 2003). MALDI-TOF mass spectrometry indicated a single mass for P1 (52,200) and three different masses for P2 (50,600/51,500/52,500). According to these results the amino acid sequence of P1, which appeared pure, was intended to be determined as extensively as possible. P2, which was heterogenous, should be at least characterized by amino acid sequences containing Cys residues to get basic information for the determination of the disulfide structure.

For conventional sequence analysis, the fragmentation of proteins (by enzymes) is necessary. At first, different enzymes were tested, and the conditions for the enzymatic digestion were optimized by preliminary experiments. P1 and P2 were then preparatively hydrolyzed in parallel assays with α -chymotrypsin (only P1), thermolysin and trypsin.

Isolation and Characterization of Peptides

The different enzymatic hydrolyzates of P1 and P2 were separated by RP-HPLC on C_{18} silica gel. The effluents were monitored simultaneously by UV absorbance at 210 and 254 nm. The absorbance at 254 nm allowed the specific detection of pyridyl-ethylated Cys residues. The peptide patterns measured at 210 nm are shown in Figs. 3–5, an example for the patterns measured at 254 nm is given in Fig. 4B. According to the amino acid compositions of P1 and P2 (Gellrich et al 2003) and the specificity of the enzymes, the digestion with α -chymotrypsin and thermolysin resulted in various peptide distribution within the overall range of elution (peptides P1/Ch 1-42, Fig. 3; peptides P1/Th 1-37 and P2/Th 1-47, Fig. 4). By contrast, the chromatograms of the tryptic digests showed only few dominant peaks during the first half of the elution and a broad peak near the end (peptides P1/Tr 1-38

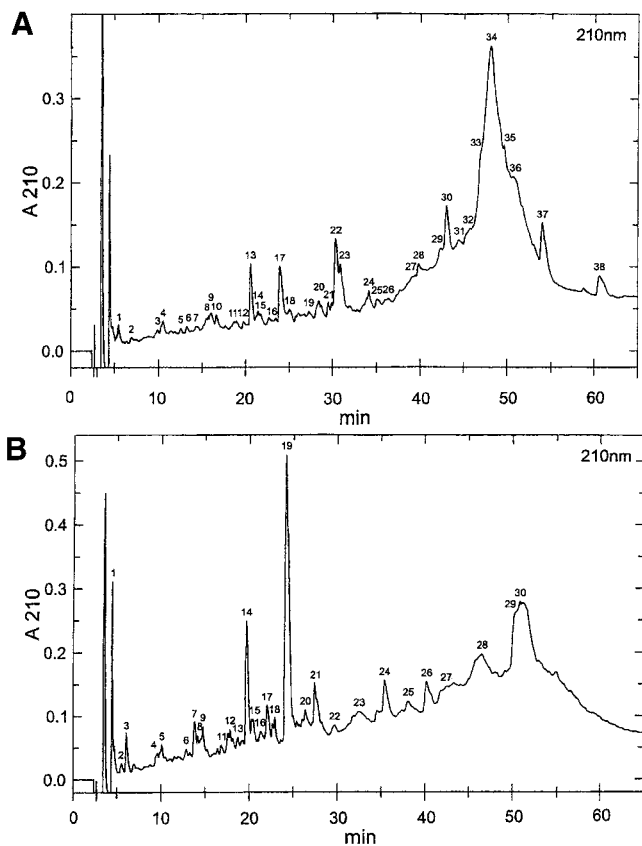


Fig. 5. Preparative RP-HPLC of the tryptic digest of P1 (A) and P2 (B) on C_{18} silica gel (peptides P1/Tr 1-38 and P2/Tr 1-30).

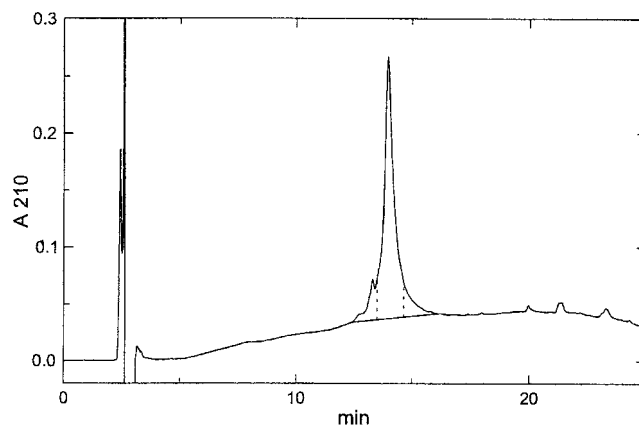


Fig. 7. Rechromatography of peptide fraction P1/Tr 33 + 34 on C_{18} silica gel.

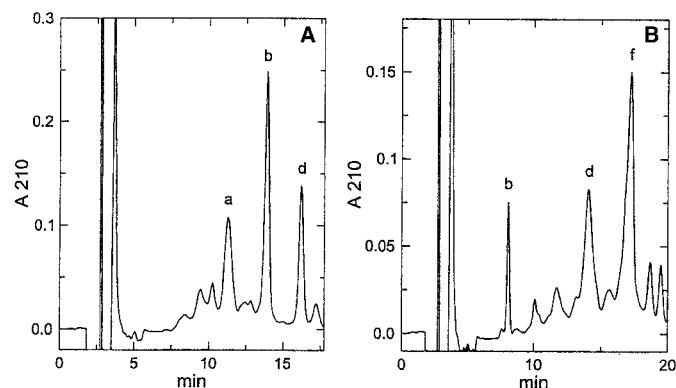


Fig. 6. Rechromatography of peptide fractions P1/Ch 32 (A) and P1/Ch 34 (B) on C_{18} silica gel.

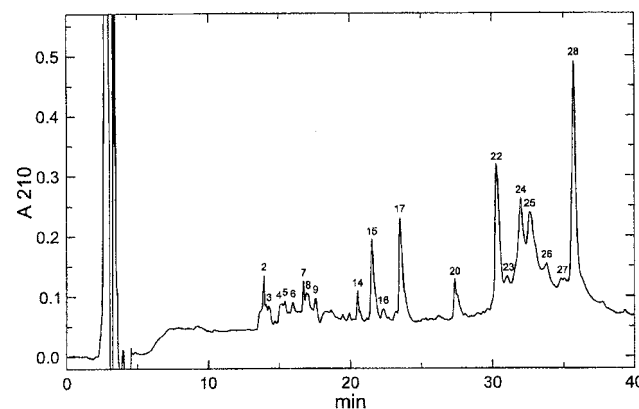


Fig. 8. Preparative RP-HPLC of the thermolytic digest of peptide fraction P1/Tr 33 + 34 on C_{18} silica gel (peptides P1/Tr 33+34/Th 2-28).

and P2/Tr 1-30, Fig. 5). The effluents corresponding to single peaks were collected and freeze-dried. Most peptide fractions obtained were rechromatographed on the same column under changed conditions; thereby numerous pure peptides could be isolated. Two examples of rechromatography are shown in Fig. 6. The rechromatographed peptide fraction P1/Tr 33 + 34 from the tryptic digest of P1 (Fig. 7) was analyzed by means of mass spectrometry. The result ($M_r \approx 36,000$) indicated that the fraction corresponded to the intact N-terminal domain of P1. The fraction was digested with thermolysin, and the resulting smaller peptides were separated by RP-HPLC on C_{18} -silica gel (peptides P1/Tr 33+34/Th 2-28, Fig. 8) and freeze-dried.

The amino acid sequences of all isolated peptide fractions were analyzed by automatic Edman degradation. The sequences of short peptides could be completely determined, those of longer peptides only in parts up to 27 residues at a maximum. (Tables I–VI). Most fractions appeared pure showing unique sequences. Few fractions revealed either modifications of single residues (P1/Th 32a, P1/Th 36a, P1/Tr 28a, P1/Tr 33 + 34/Th 4, P2/Tr 20d) or they contained two or three peptides with completely different sequences (P1/Ch 9a, P1/Tr 23, P1/Tr 24, P2/Tr 19d, P2/Tr 23a). In the latter case, the single sequences were designated by comparison with the known sequences of clone gSec2A.

Amino Acid Sequences of P1 and P2

By means of overlapping peptides and clone gSec2A, the partial sequences of the peptides could be assigned to the total sequences of P1 and P2 (Table VII). The sequences of the C-terminal domain (residues 289–436) of P1 were completely determined except one residue in position 349. Within the N-terminal domain (residues 1–288) 77% of the residues could be assigned according to the sequences of clone gSec2A. The longest gap was situated between positions 67 and 105; these sequences consisted almost exclusively of Gln, Pro, Phe, and Tyr and were not cleaved into smaller peptides by the applied enzymes. Summarizing both domains of P1, 19 positions revealed two different residues indicating that the protein was not pure and consisted of at least two components.

TABLE I
Amino Acid Sequences of Chymotryptic Peptides
from γ -75k Secalin P1

Peptide P1/Ch no. ^a	Sequence ^b	Position ^c	Mass ^d
3a	QQCCQQL	328-335	1187.5
7a	XQIPQQL..	336-342	-
7b	NMQVNPSSGQVQCPQ..	1-14	-
8b	AS I ETGIVGH	424-436*	982.3
9a	XLFPQT	167-172	-
9a	XXXXXXXXPQVTSQPQQPFPAQPP..	174-197	-
11b	QQCSVAL	303-310	949.4
12a	VHQPPQSPQQQP..	271-284	2067.2
13c, 19b	XXFPQQPQQPFPQ..	107-120/123-136	-
17b	QCSTIQAPF	418-426	1098.5
18b	AQVQGI IQPQQL	382-393	1321.6
18e	IFPQSECQV	318-326	-
20a	QQFPQQPQQSFPQQPQQP..	123-141*	-
20b	QNLPTMCNVY	405-414	1285.5
21b	SKIFPQSECQVM	316-327	1500.5
22a	SQQPQQPFPQP..	219-230*	-
23c	QQFPQQPQQPFPQXXQXV..	123-141	-
25b	SQQPQQPFPQP..	219-232*	-
27a, 28d	SQQPYPQEPQQLFPQSQPQ..	204-224	-
28a	QSIQPPQFPFPQX..	245-261	-
28e	QSQPQQPQVTSQPQQP..	174-191	-
31b	SQQPFPQPPQSSPQQ..	37-54*	-
32a, 32b	QSPQPPQPPFPQSSPQQ..	131-157*	-
32d	QQFPQQPQQPFPQVVPQSSQ..	123-147*	-
34d, 34f	QQFPQQPQQSSPQQ..	15-29*	-
35b, 36c	NMQVNPSSGQVQCPQQPFPQ..	1-20	-
37c	XQFPQQPQQPFPQ..	107-121/123-137	-
39b	XSIQPPQPPFPQPP..	245-261	-

^a See Fig. 3.

^b One-letter codes for amino acids; X, not identified; .., incomplete sequence; bold letters indicate differences to clone gSec2A.

^c Corresponding to sequence of clone gSec2A; *, modified.

^d Determined by mass spectrometry.

The sequences of P1 and gSec2A were highly homologous and did not match only in positions 152 (Thr/Gln), 155 (Pro/Gln), 156 (Gln/Ser), and 286 (Ile/Ser). As mentioned before, the characterization of P2 was focused on Cys containing peptides. Therefore,

TABLE II
Amino acid Sequences of Thermolytic Peptides
from γ -75k Secalin P1

Peptide P1/Th no. ^a	Sequence ^b	Position ^c	Mass ^d
2a	IETG	429-432*	428.1
3b	VVHA	350-353	424.3
4b	PAS	426-428	-
5a	LVS	310-312	-
7a	LQC	342-344	-
9ab	VMQCCQ	325-334	-
10a	VHQPPQSPQQQP..	271-285	-
11b	IQAP	422-425	-
12b	LQCS	302-306	-
13a	SSPQSQPYPQEPQ	200-214	1765.6 ^e
14c	LNPCKN	294-299	792.4
14d	VYVQRCS	413-420*	1086.5
15a	AQFPQSSPQSQPYPQ..	194-211	-
15b	VYVPRQ	413-418	760.4
16c	LIMQREOR	354-363	1230.6
16d	VYVPR	413-417	632.4
17a, 18a	LAQIPQ	335-341	796.3
19a	LQFPQPPQ..	122-130/106-114	-
20b	VTSQPQQPFPQ	183-193	1255.5
21b	LFPQTQSSPQQP..	168-180	-
21c	LNPCKN	294-300	-
22a	IFPQSECQ	318-325	-
22b	VYVPRQCS	413-421	1156.5
24a	SFPQPPQPPVPPQ..	132-144*	-
25a	VTSQPQQPFPQAPFPQ..	183-200	-
26ab, 29a	VQCPQQPFP..	10-19	-
28a, 31a	LQFPQPPQPPFPQPPQXV..	122-141*	-
30c	LVLQNLPT	402-409	896.3
31b, 33b	LVLQNLPTMCN	402-412	-
	Q		
32a, 36a	SQP PQQPYPQPPFPQXQ..	48-66*	-
	P		
33a	SQPQQPFPQSPQPPFPQ..	24-44*	-
34a, 35a	VQCPQQPFPQXQSPQPPQ..	10-30*	-
36c, 37a	LFPQSPQPPFPQPPQXQ..	215-233*	-

^a See Fig. 4.

^b One-letter codes for amino acids; X, not identified; .., incomplete sequence; bold letters indicate differences to clone gSec2A.

^c Corresponding to sequence of clone gSec2A; *, modified.

^d Determined by mass spectrometry.

^e K-adduct.

TABLE III
Amino Acid Sequences of Tryptic Peptides
from γ -75k Secalin P1

Peptide P1/Tr no. ^a	Sequence ^b	Position ^c	Mass ^d
4ab	AIIMQREOR	353-361	-
8a	QQCCQQL	328-335	1202.4
8b-d, 9abd	SQLEVV	394-400	829.5
13a	AS I ETGIVGH	427-436*	982.4
17	SLQQQLNPCK	289-298	1262.6
18b	NLPTMCNV..	406-413	-
20a, 31a	NMQVNPSSGQVQCPQ..	1-15	-
20b, 22b	QCSTIQAPF	418-426	1098.5
23	IFPQSECQVM	318-327	-
23	AQIPQQLQCAAIH	336-348	-
24	IFPQSECQVM	318-327	-
24	NLPTMCNVY	406-414	-
25a	EGVQILLPQSHK	362-373*	-
27a, 32b	NMQVNPSSGQVQCPQQPFPQ..	1-20	-
	V		
28a	EGVQILLVQ HQHVGGALAVQV..	362-385*	-
	S		
29a	NMQVNPSSGQVQCPQQPFP..	1-18	-
30, 37b	SLVLQNLPTMCNVY	401-414	1699.4
31b	SLVLQNLPTMCNVY..	401-414	-
34a, 35b	NMQVNPSSGQVQCPQQPFP..	1-19	-
34b	NVLLQCSVALV	299-311	-
38c	NVLLQCSVALVSSVR	299-315*	1916.9

^a See Fig. 5.

^b One-letter codes for amino acids; X, not identified; .., incomplete sequence; bold letters indicate differences to clone gSec2A.

^c Corresponding to sequence of clone gSec2A; *, modified.

^d Determined by mass spectrometry.

only ≈35% of the total sequences was determined, the larger part located in the C-terminal domain. P2 was highly homologous with P1, and differences occurred only in the positions 197 (Ile/Pro), 201 (Cys/Ser), and 430 (Val/Glu). As already indicated by mass spectrometry, the preparation of P2 was not pure, thus showing heterogeneity in several position. Differences to gSec2A were found in the positions 197 (Pro/Ile) and 201 (Ser/Cys).

In summary, P1 and P2, two major γ -75k secalins of the rye flour Danko, are closely related to the γ -75k secalin gSec2A occurring in a wheat translocation line. This demonstrates that the amino acid sequence of gSec2A is representative for the γ -75k secalin type of rye. The corrections of the sequences of clone pSc503 (Kreis et al 1985) done by Murray et al (2001) were, in fact, justified. Moreover, the presence of a Cys residue in position 12 of the N-terminal domain was confirmed by sequencing P1 and P2. The presence of Trp at this position postulated by Shewry et al (1982) and Rocher et al (1996) is either untypical for γ -75k secalins or the consequence of an error in sequencing due to the fact that underivatized Cys is difficult to detect by sequence analysis. The comparison of γ -75k

secalins with γ -gliadins of wheat (Bartels et al 1986; Rafalski et al 1986) revealed a high degree of homology (≈80%) in the C-terminal domains. The differences are caused by point mutations and, in a few cases, by an insertion of Gln residues between the positions 361 and 362 of clone gSec2A. Remarkably, the C-terminal domain of both γ -75k secalins and γ -gliadins contain eight Cys residues in homologous positions, and it is likely that γ -75k secalins form the same intramolecular disulfide bonds as γ -gliadins (Müller and Wieser 1997).

The N-terminal domains of γ -75k secalins and γ -gliadins are characterized by repetitive sequences rich in Gln, Pro, and aromatic amino acids (Phe, Tyr). The major difference comprises the number of residues, the sequences of the repetitive motifs, and the presence of a Cys residue. The N-terminal domain of γ -75k secalins contains ≈280 residues and that of γ -gliadins ≈110–150 residues. The repetitive sequences of γ -75k secalins have been divided into two regions (Murray et al 2001). One region comprises 15 units each consisting of eight residues (e.g., PQQPFPQQ) that have been proposed to be similar to corresponding units of ω -secalins and C-hordeins. The second region contains 17 units each consisting of 7–10 residues (XQQPQQPFPQ) similar to those observed in γ -gliadins. Modification of these sequences are more frequent in γ -75k secalins compared with γ -gliadins.

In contrast to monomeric γ -gliadins gSec2a, P1 and P2 have a Cys residue in the N-terminal domain which is likely to form intermolecular disulfide bonds and therefore could be responsible for the fact that γ -75k secalins do not occur in a monomeric form. In P2, a second Cys residue has been detected in the N-terminal domain (peptide P2/Th 22a, Table V). According to the results of mass spectrometry and sequence analysis, the P2 preparation consisted of more than one protein. Therefore, it cannot be decided whether each component contains one or two Cys residues in the N-terminal domain.

Both γ -75k secalins P1 and P2 were isolated from the prolamins fraction. Previous studies demonstrated that γ -75k secalins from the glutelin fractions agreed with P1 and P2 according to the mobility

TABLE IV
Amino Acid Sequences of Thermolytic Peptides
from Peptide Fraction P1/Tr 33 + 34

Peptide P1/Tr 33+34/Th no. ^a	Sequence ^b	Position ^c
2	VHQPQQPSPQQQP . .	271-284
4	L ^Q P ^A SP	274-279*
7	AQPPQQSSPQSQQPY . .	194-208
8	AQPPQQSSPQ . .	194-203
14	VTSQPQQPF	183-191
15	SXPQTQQSXPQ . .	239-249*
17	VTSQPQQPFPQAQP . .	183-196
20	LQQFPQQPQQSFPQQ . .	122-136*
22	LQQFPQQPQQPFPQQPQQXV . .	122-141
24	VNPSGVQXPQQQPF . .	4-18
28	LFPQSQQPQQPFPQQPQQ . .	215-234

^a See Fig. 8.

^b One-letter codes for amino acids; X, not identified; . . , incomplete sequence; bold letters indicate differences to clone gSec2A.

^c Corresponding to sequence of clone gSec2A; *, modified.

TABLE V
Amino Acid Sequences of Thermolytic Peptides
from γ -75k Secalin P2

Peptide P2/Th no. ^a	Sequence ^b	Position ^c	Mass ^d
3a	IVGH	433-436	424.2
3b	MCN	410-412	-
8a	LQC	342-344	-
10ab	VMQQCCQQ . .	326-334	-
14a	NMQVNPSGQVQCPQQ . .	1-15	-
14b	LQQCSF	302-307	779.4
17a, 26a	LNPCKN	294-299	-
17b	VA Q ^Q SH ^Q QH	367-375*	-
22a	AQ ^P I ^Q Q ^S CP ^Q S ^Q P ^Q Y ^Q E . .	194-211*	-
26b	VYVPRQC	413-419	968.4
28a	IFPQSECQ	318-325	1055.3
28b	VYVPRQCS	413-420	1055.8
32	VQCPQQPFPQPQ	10-22	-
41	LVLQNLPTMCN	402-411	-

^a See Fig. 4.

^b One-letter codes for amino acids; X, not identified; . . , incomplete sequence; bold letters indicate differences to clone gSec2A.

^c Corresponding to sequence of clone gSec2A; *, modified.

^d Determined by mass spectrometry.

TABLE VI
Amino Acid Sequences of Tryptic Peptides
from γ -75k Secalin P2

Peptide P2/Tr no. ^a	Sequence ^b	Position ^c	Mass ^d
1d	VPR	415-417	-
5a	AIIMQQEQR	353-361	-
7b	QQCCQQ . .	328-334	-
8a	SQLEVV	394-400	829.5
12b	IFPQSECQVMQ . .	318-329	-
12d	SLVLQ	401-405	558.2
14b, 15b	SLQQQLNPCK	289-298	1262.4
15b, 20d	NLPTMCNVY	406-414	-
17b	NMQVNPSGQVQCPQQ . .	1-16	-
18d	EC STIQA . .	418-424*	-
19d	ASIVTGVGH	427-436	952.2
19d	IFPQSECQVM	318-327	1285.4
19d	QCSTIQA . .	418-424	-
20d	IFPQSECQVM ^Q QQCCQ . .	318-333	-
21e	EGVQILLPQSHK ^E	362-373*	1347.8
23a	NMQVNPSGQVQCPQQP . .	1-18	-
23a	SQPQQ . .	185-189	-
24c	SLVLQNLPTMCNVY	401-414	-
26d	NVLLQQCS ^P VAL	299-310	1388.6

^a See Fig. 5.

^b One-letter codes for amino acids; X, not identified; . . , incomplete sequence; bold letters indicate differences to clone gSec2A.

^c Corresponding to sequence of clone gSec2A; *, modified.

^d Determined by mass spectrometry.

TABLE VII
Alignment of Amino Acid Sequences of γ -75k Secalins P1 and P2
from Rye Flour Danko with Predicted Amino Acid Sequences Derived
from cDNA gSec2A (Murray et al 2001) and pSc503 (Kreis et al 1985)^a

	10	20	29	39
gSec2A	NMQVNPSSGQV	QCPQQQFFPQ	PQQSSP .QQP	QQPFPQQSQQ
pSc503	-----	-----	-----	-----
P1	NMQVNPSSGQV	QCPQQQFFPQ	PQQSSP .QQP	QQPFPQQSQQ
			Q Q P Q	E
P2	NMQVNPSSGQV	QCPQQQFFPQ	PQ-----	-----
	48	58	68	78
gSec2A	.PFPQQPQQS	SPQPQQPYYPQ	QFPFPQQPQP	YPQQPQQPFP
pSc503	-----	-----	-----	-----
P1	.PFPQQPQQS	SPQPQQPYYPQ	QFPFP-Q-Q--	-----
	Q	Q F		
P2	-----	-----	-----	-----
	88	98	108	118
gSec2A	QQPQQPYYPQ	PQQQFPQQPQ	QPVPQQPLQQ	FPQQPQQPFP
pSc503	-----	-----	-----	-----
P1	-----	-----	-----LQQ	FPQQPQQPFP
P2	-----	-----	-----	-----
	128	138	147	157
gSec2A	QQPLQQFPQQ	PQQPFPQQPQ	QPV .PQQSOO	FPFQTQQPQQ
pSc503	-----	-----	-----	-----
P1	QQPLQQFPQQ	PQQPFPQQPQ	QPV .PQQSQQ	FPF-QQQQS-
		S	QFV	
P2	-----	-----	-----	-----
	167	177	187	197
gSec2A	PFPQPQQPQQ	LFPQTQQSSP	QQPQQVTSQP	QQPFPQAQPP
pSc503	-----	-----	-----	-----
P1	-----	LFPQTQQSSP	QQPQQVTSQP	QQPFPQAQPP
P2	-----	-----	-----SQP	QQ---AQPI
	207	217	227	237
gSec2A	QQSSPQSQQP	YPQEPQQLFP	QSQQPQQPFP	QPQQPQQPFP
pSc503	-----	-----	-----	-----
P1	QQSSPQSQQP	YPQEPQQLFP	QSQQPQQPFP	QPQQPQQ---
				QP Q
P2	QQSCPQSQQP	YPQE-----	-----	-----
	247	257	267	277
gSec2A	QPQPQTQQSI	PQPQQPFPQP	QQPFPQSQEQ	FPQVHQPPQP
pSc503	-----PQQLF	PLPQQPFPQP	QQPFPQSQEQ	FPQVHQPPQP
P1	-S-PQTQQSI	PQPQQPFPQP	QQPF-----	---VHQPPQP
				L A
P2	-----	-----	-----	-----
	287	297	307	317
gSec2A	SPQQQQPSIQ	LSLQQQLNPC	KNVLLQQCSP	VALVSSLRSK
pSc503	SPQQQQPSIQ	LSLQQQLNPC	KNVLLQQCSP	VALVSSLRSK
P1	SPQQQQPS--	-SLQQQLNPC	KNVLLQQCSP	VALVSSVRSK
P2	-----	-SLQQQLNPC	KNVLLQQCSP	VAL-----
	327	337	347	357
gSec2A	IFPQSECQVM	QQCCQQLAQ	IPQQLQCAAI	HSVVHAIIMQ
pSc503	IFPQSECQVM	QQCCQQLAQ	IPHHLQCAAI	HSVVHAIIMQ
P1	IFPQSECQVM	QQCCQQLAQ	IPQQLQCAAI	H-VVHAIIMQ
P2	IFPQSECQVM	QQCCQQLAQ	----LQC---	----AIIMQ
		E		
	367	377	387	397
gSec2A	QEQRREGVQIL	LPQSHQQHVG	QGALAQQVQGI	IQQQLSQLE
pSc503	QEQRREGVQIL	LPQSHQQHVG	QGALAQQVQGI	IQQQLSQFN
P1	QEQRREGVQIL	LPQSHQQHVG	QGALAQQVQGI	IQQQLSQLE
		V V K		
P2	QEQRREGVQIL	LPQSHQQH--	-----	-----SQLE
		V AQ K		
	406	416	426	436
gSec2A	VVRSL .VLQN	LPTMCNVYVP	RQCSTIQAPF	ASIVTGVVGH
pSc503	VGIIVLQMLQN	LPTMCNVYVP	RQCPSRRRLH	HAM .SLVCGH
P1	VVRSL .VLQN	LPTMCNVYVP	RQCSTIQAPF	ASIVTGVVGH
P2	VVRSL .VLQN	LPTMCNVYVP	RQCSTIQA--	ASIVTGVVGH
				E

^a One-letter code for amino acids; bold letters indicate modifications of the gSec2A sequence; -, not determined; ., spaces introduced to maximize alignment; numbers indicate positions within the gSec2A sequence.

on SDS-PAGE, retention time, and elution profile of RP-HPLC, and N-terminal sequences (Gellrich et al 2003). Nevertheless, the results presented here leave open the possibility that γ -75k secalins characterized by small but crucial differences may still be found in the glutelin fraction.

CONCLUSIONS

Sequence analysis of two major γ -75k-secalins of rye flour Danko have revealed a high degree of accordance with sequences derived from a cDNA clone of a wheat translocation line coding a γ -75k secalin. The C-terminal domain of γ -75k-secalins is homologous with that of γ -gliadins and includes eight cysteine residues in analogous positions. The N-terminal domain of γ -75k-secalins, consisting mainly of repetitive sequences rich in Gln, Pro, Phe, and Tyr, is related to that of γ -gliadins but has more than twofold numbers of residues. In contrast to the monomeric γ -gliadins, γ -75k-secalins possess at least one Cys residue in the N-terminal domain, and this residue is likely to be responsible for the aggregative nature of γ -75k secalins.

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