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G. García-Casado · R. Sánchez-Monge · C. Lopez-Otín
G. Salcedo

Rye chromosome arm 3RS encodes a homodimeric inhibitor of insect α-amylase

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Abstract A new inhibitor of insect α-amylase, designated RDAI-1, has been purified from rye (*Secale cereale* L.) endosperm. RDAI-1 is homologous to wheat homodimeric inhibitors. This homology is supported by their similar N-terminal amino-acid sequences, inhibitory activities towards amylases from *Tenebrio molitor* (Coleoptera) and human saliva, and aggregative properties in gel-filtration chromatography. The gene encoding RDAI-1, *IdhaR1*, is located on the short arm of chromosome 3R, which is homoeologous with wheat chromosome arms 3BS and 3DS, where the genes for homodimeric inhibitors have been previously mapped.

Key words α-amylase inhibitor · Rye
Chromosomal location · Homoeologous genes

Introduction

A single family of inhibitors of animal α-amylases and trypsin accounts for the major fraction of salt-soluble proteins in wheat and barley endosperm. Besides its interest as an abundant protein group in cereal flour, this inhibitor family is receiving considerable attention because of its potential role in plant protection (García-Olmedo et al. 1992), as well as in baker's asthma, the main allergic disease associated with flour manipulation (Sanchez-Monge et al. 1992).

Up to 18 different members of the inhibitor family have been characterized in *Triticum* and *Hordeum* (see García-Olmedo 1992; Carbonero et al. 1993, for reviews). Those active against α-amylases can be classified according to their degree of aggregation into monomeric, homodimeric and heterotetrameric forms. A multigene family dispersed over several chromosomes (homoeology groups 2, 3, 4, 6 and 7) encodes the inhibitor subunits in wheat and barley. The gene dispersion has involved translocations and intrachromosomal duplications, and most of it occurred before the divergence of the barley genome from those genomes included in allohexaploid wheat (Garcia-Olmedo et al. 1992).

In contrast with the extensive knowledge about this protein family in *Triticum* and *Hordeum*, information in rye (*Secale cereale* L.) is very limited at present. Concerning the members active against insect α-amylases, only the N-terminal sequence of one inhibitor, which is closely related to a subunit of the wheat and barley tetrameric inhibitors, has been published (Lyons et al. 1987). No chromosomal assignment of genes encoding rye proteins active towards animal α-amylases has been carried out until now.

We report here the isolation of RDAI-1, the first homodimeric inhibitor characterized from rye, the chromosomal location of the corresponding gene, and its relation with a sub-family of wheat inhibitors.

Materials and methods

Plant material

Flour from *S. cereale* L., INIA c/171-M, hexaploid Triticale (× *Triticosecale* Wittmack) Cachirulo, and *Triticum turgidum* L. cv Enano de Andujar was used in this study. The disomic and ditelosomic rye-wheat addition lines (*S. cereale* cv Imperial × *T. aestivum* cv Chinese Spring) were the gifts of E.R. Sears (University of Missouri, MO., USA) and S.M. Reader (Plant Breeding Institute, Cambridge, UK).

Isolation and characterization of RDAI-1

Crude inhibitor preparations from flour (250 g) were obtained by 0.15 M NaCl extraction and $(NH_4)_2SO_4$ precipitation as previously described (Gomez et al. 1989). These protein preparations were fractionated by gel-filtration on Sephadex G-100 (200 mg of protein loaded; 2.5 cm × 80 cm column; 25 ml/h; 6 ml/fraction) under non-dissociating conditions, using 0.1 M ammonium acetate, pH 6.8, as the elution buffer.

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G. García-Casado · R. Sánchez-Monge · G. Salcedo (✉)
Departamento de Bioquímica, E.T.S. Ingenieros Agrónomos, Ciudad Universitaria, 28040 Madrid, Spain

C. Lopez-Otín
Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain

RDAI-1 was isolated from the appropriate gel-filtration fractions by preparative reverse-phase HPLC (Vydac-C4, 5 mg of protein loaded, 22 mm×250 mm column, particle size 10 µm) using a three-step linear gradient of 10–50% (v/v) acetonitrile in 0.1% trifluoroacetic acid (10–20% in 45 min, 20–35% in 140 min, 35–50% in 100 min; 2 ml/min). WDAI-1 (wheat dimeric α-amylase inhibitor-1) was purified as in Sanchez-Monge et al. (1989).

Protein concentration was quantified by the bicinchoninic-acid assay (Smith et al. 1985).

SDS-PAGE was carried out according to Laemmli (1970) and two-dimensional electrophoresis (isoelectrofocusing, IEF×starch-gel electrophoresis, SGE) as in Gomez et al. (1989).

The N-terminal amino-acid sequence was determined by standard methods using an Applied Biosystems 477A gas-phase sequenator.

Inhibition of *Tenebrio molitor* (Coleoptera) and human salivary α-amylases was tested by the method of Bernfeld (1955), with the modifications previously described (Gutierrez et al. 1990, 1993). According to the pH optima of both enzymes, the assays were carried out at pH 5.4 (20 mM sodium acetate, 100 mM NaCl, 0.1 mM $CaCl_2$) for *T. molitor* and at pH 6.9 (20 mM potassium phosphate, 67 mM NaCl, 0.1 mM $CaCl_2$) for the human enzyme. All tests were performed using approximately one unit of α-amylase, defined as the amount of enzyme required to produce the reducing equivalents of 1 µmol of maltose under our experimental conditions.

Chromosomal location of the RDAI-1 gene

Individual kernels (approximately 35 mg) of the disomic and ditelosomic rye-wheat addition lines were delipidated with petroleum ether (2 ×, 1:10 w/v, 1 h, boiling point 50–70 °C), and the residues extracted with 70% ethanol (2 x, 1:10 w/v, 1 h). The extracts were analyzed by two-dimensional electrophoresis (IEF, pH 5–8×SGE, pH 3.2).

Results and discussion

Isolation of inhibitor RDAI-1

The crude inhibitor preparation from rye flour was fractionated by gel-filtration on Sephadex G-100 under non-dissociating conditions, as previously described for a similar protein preparation from wheat (Gomez et al. 1989; Gutierrez et al. 1990). In contrast with the results reported both in *T. turgidum* and *T. aestivum*, a poorly-resolved profile was obtained in rye (data not shown). However, the major components of the *S. cereale* inhibitor preparation appeared chiefly in a chromatographic peak with elution volumes that partially correspond to those of both dimeric and monomeric inhibitors from wheat endosperm. The main component of this gel-filtration fraction (Fig. 1a) was isolated by preparative reverse-phase HPLC (Fig. 1b). The purified protein behaved as a single band of around 13 kDa in SDS-PAGE (Fig. 1c), and as a single spot in two-dimensional electrophoresis (IEF×SGE; data not shown).

RDAI-1 is homologous to wheat homodimeric inhibitors

The first 23 N-terminal amino acids of RDAI-1 were determined (Fig. 2). No heterogeneity was found in any of the residues sequenced. By comparison with sequences of the α-amylase/trypsin inhibitor family previously reported, the results obtained clearly indicated that the rye inhibitor is closer to WDAI-1 (syn. 0.53), WDAI-2 (syn. 0.19) and WDAI-3 (Maeda et al. 1985; Sanchez-Monge et al. 1989) than to any other member of the protein family. Therefore, RDAI-1 can be included in the subfamily of these wheat homodimeric inhibitors. Interestingly, at least in the determined N-terminal sequence, the differences between the wheat components are of the same order as those between them and RDAI-1 (Fig. 2), which suggests a similar degree of intra- and inter-specific divergence within this inhibitor subfamily.

The homology with the wheat homodimeric inhibitors was further supported when the inhibitory properties of RDAI-1 were analyzed. The isolated protein was active towards amylases from the coleopteran *T. molitor* and from human saliva, showing inhibition profiles like those of WDAI-1 (Fig. 3). These data corroborate, and extend to a member of an additional species, our previous proposal that there are no significant differences in inhibitory properties within the subfamily of dimeric inhibitors (Sanchez-Monge et al. 1989).

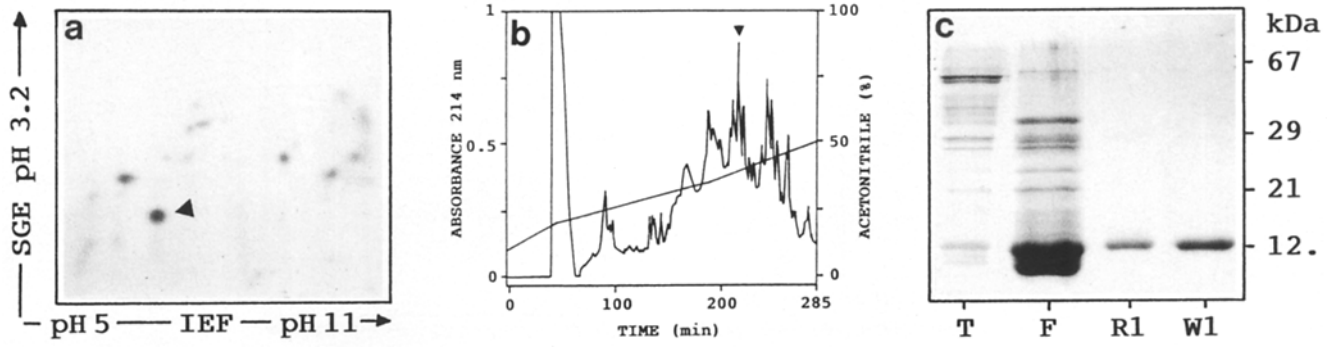


Fig. 1a Two-dimensional electrophoretic separation (IEF×SGE) of the gel-filtration fraction from the rye inhibitor preparation (400 µg of protein was loaded) used for further purification steps. **b** Reverse-phase HPLC profile of the gel-filtration fraction shown in **a**. The position of RDAI-1 is indicated by an *arrowhead* in both **a** and **b**. **c** SDS-PAGE of the following samples: crude inhibitor preparation from rye (*T*; 25 µg); gel-filtration fraction shown in **a** (*F*; 12 µg); purified RDAI-1 (*R1*; 2 µg) and the wheat homodimeric inhibitor WDAI-1 (*W1*; 2 µg). Molecular masses (kDa) of reference proteins appear on the right side of the figure

PROTEIN	N-TERMINAL SEQUENCE			IDENTICAL RESIDUES			
	1	10	20	R1	W1	W2	W3
RDAI-1	SGPWMCYPGQAFQVPALPNCRPV			-	21	21	20
WDAI-1 (syn.0.53)			G L		-	22	20
WDAI-2 (syn.0.19)			A L			-	19
WDAI-3		Y K	G				-

Fig. 2 Alignment of the N-terminal sequence of RDAI-1 with those of the wheat homodimeric inhibitors WDAI-1, -2 (Maeda et al. 1985) and -3 (Sanchez-Monge et al. 1989). Only the differences in the sequences of wheat inhibitors with respect to that of RDAI-1 are represented

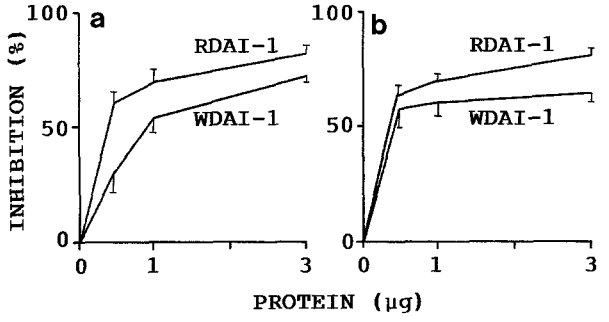


Fig. 3 a, b Inhibitory activities of RDAI-1 towards *Tenebrio molitor* (**a**) and human salivary (**b**) α-amylases. WDAI-1 was included for comparison. Values are means±SE (*vertical bars*) of four replicates within a single sample

RDAI-1 is associated in Triticale with the dimeric inhibitor fraction

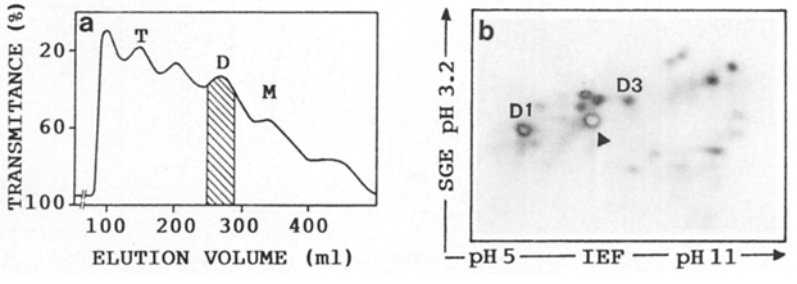
To confirm the dimeric nature of RDAI-1, the crude inhibitor preparation from Triticale Cachirulo (genomes AABBRR), was fractionated by gel-filtration under non-dissociating conditions. In this way, the different types of wheat inhibitors encoded by genomes A and B could be used as proper internal markers to study the aggregative properties of rye proteins. An elution profile with prominent well-separated peaks, and with elution volumes similar to those of wheat tetrameric, dimeric and monomeric inhibitors, was obtained (Fig. 4 a). When gel-filtration fractions corresponding to the three inhibitor types were analyzed by two-dimensional electrophoresis (IEF×SGE), RDAI-1 was detected only in the fraction which included the wheat homodimeric inhibitors WDAI-1 and -3 (Fig. 4 b). Consequently, the rye inhibitor can be classified in the homodimeric category.

Fig. 4 a Gel-filtration on Sephadex G-100 of the crude inhibitor preparation from Triticale, Cachirulo. Peaks corresponding to wheat tetrameric (*T*), dimeric (*D*) and monomeric (*M*) inhibitors are indicated. **b** Two-dimensional electrophoretic separation of the gel-filtration fraction (200 µg of protein were loaded) designated as *D* in **a**. The position of RDAI-1 (*arrowhead*) and the wheat homodimeric inhibitors WDAI-1 (*D1*) and -3 (*D3*) are marked

Idha R1, the gene for RDAI-1, is located in chromosome arm 3RS

The chromosomal assignment of the gene encoding RDAI-1 was carried out by two-dimensional electrophoretic analysis of 70% ethanol extracts from the disomic and ditelosomic rye-wheat addition lines. It has been previously shown that cereal proteins included in the inhibitor family under study are readily extractable with this solvent, and that the corresponding two-dimensional protein maps are simpler and show less overlapping than those obtained using 0.15 M NaCl (Sanchez-Monge et al. 1986). RDAI-1 was present only in the addition lines 3R and 3RS (Fig. 5). Identity of the rye inhibitor spot in the two-dimensional electrophoretic maps of cv Imperial and INIA c/171-M, as well as in those of the addition lines mentioned above, was confirmed by co-electrophoresis of the corresponding ethanol extracts and the purified inhibitor. It can be concluded that *Idha R1*, the gene for RDAI-1, is located in the short arm of chromosome 3R, which is the rye homoeologue to the wheat chromosome arms 3BS and 3DS, where the genes encoding the corresponding homodimeric inhibitors have been assigned (Sanchez-Monge et al. 1986, 1989). An unidentified component (marked with an asterik in Fig. 5 d), that was not detected in Chinese Spring wheat, Imperial rye, or the others addition lines analyzed, was also present in both the 3R and 3RS addition lines.

The location of *Idha R1* further extended not only the relationship between RDAI-1 and the wheat inhibitors, but also represents additional evidence on the homoeology of the group-3 chromosomes of *Triticum* and *Secale* (Devos and Gale 1993).



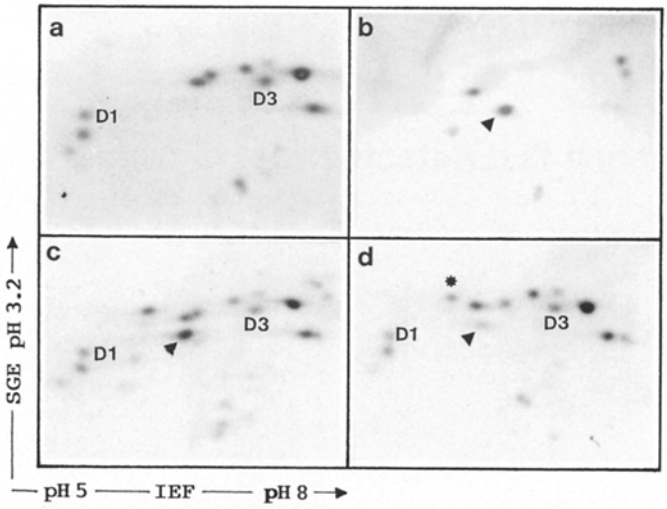


Fig. 5a–d Two-dimensional electrophoretic separations of 70% ethanol extracts from individual endosperms of the following genetic stocks: *T. aestivum* cv Chinese Spring (**a**), *S. cereale* cv Imperial (**b**), a mixture of Chinese Spring and Imperial extracts (**c**) and Imperial/Chinese Spring ditelosomic addition line 3RS (**d**). The position of RDAI-1 is indicated by *arrowheads*. The wheat homodimeric inhibitors WDAI-1 (*D1*) and -3 (*D3*) are also marked. The asterisk marks a spot present in the addition line that is not detected in either Chinese Spring or Imperial extracts

No homologous component to RDAI-1 has been found in barley. A dimeric protein active against *T. molitor* amylase is present in *Hordeum vulgare* endosperm, but its inhibitory specificity, amino-acid sequence, and the chromosomal location of its corresponding gene (chromosome 6H) are all very different to those of the rye and wheat homodimeric inhibitors (Lazaro et al. 1988; Mena et al. 1992). The characterization of new members of the inhibitor family in rye, together with the data reported here, will help to explain the evolution of the multigene family encoding this protein group in the *Triticeae*.

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