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Identification of the three major coeliac immunoreactive proteins and one α-amylase inhibitor from oat endosperm

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Six chloroform/methanol-soluble proteins from oat endosperm (*Avena sativa*) have been isolated and characterized by a purification procedure based on extraction with volatile solvents, followed by reversed-phase high performance liquid chromatography. Three of these proteins, with an assessed molecular weight of 25,000, 27,000 and 32,000 Da, respectively, have been identified by immunoblotting using coeliac sera, as the major coeliac serum IgA-binding components of oat endosperm. The N-terminal amino acid sequence of these proteins indicates that they correspond to α_2, γ_4, and γ_3 avenins, respectively. We have tentatively named them 'coeliac immunoreactive proteins'. Another chloroform/methanol oat component shows weak α-amylase inhibitory activity and exhibits strong homology (60% identity) at the N-terminus with the α-amylase inhibitor from ragi (*Eleusine coracana*).

Coeliac disease; Oat avenins; α-Amylase

1. INTRODUCTION

Cereal proteins are of increasing clinical interest because they are involved in allergic processes [1–3] and intestinal disorders, particularly in coeliac disease [4,5]. Besides, cereal seeds contain protein inhibitors of α-amylases and proteases [6] which play a protective role against endogenous and exogenous hydrolytic activities. Although the proteins involved in coeliac disease and some enzyme inhibitors from wheat, barley and rye have been characterized, nothing is known yet about their presence in oat.

Coeliac disease (gluten-sensitive enteropathy) is characterized by a reaction of hypersensitivity of the small intestine in genetically susceptible individuals after the ingestion of gluten proteins of either flour or meal from wheat, rye, barley and possibly oat [7,8]. Recently some progress on the causative agents has been made, and the alcohol-soluble proteins of the gluten fraction, collectively known as prolamins, and termed hordeins (barley), gliadins (wheat) and secalins (rye) have been reported as being responsible for the toxicity known to cause coeliac disease symptoms [9–11]. However, the implication of oat prolamins (avenins) in coeliac disease is controversial, probably due to the lower proportion (10–20%) of these proteins in the kernel in comparison to that in the other cereal prolamins [12]. Besides, oat prolamins related to coeliac disease have not been identified yet and consequently they could not have been used for clinical or in vitro tests.

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In the present report we describe a simple and reliable chromatographic procedure for the fractionation and isolation of chloroform/methanol-soluble proteins from oat endosperm. This has allowed us to identify three major avenins, which react with coeliac sera and that we have tentatively called oat 'coeliac immunoreactive proteins', as well as one α-amylase inhibitor from oat endosperm. As far as we know, this is the first report identifying prolamins with antigen activity towards IgA from coeliac sera, together with one α-amylase inhibitor from oat endosperm.

2. MATERIALS AND METHODS

2.1. *Extraction and purification*

Soluble proteins from a chloroform/methanol extract of oat endosperm (*Avena sativa*) were obtained as in [13]. The dried residual was extracted with ammonium bicarbonate as also described in [14]. The soluble and insoluble bicarbonate extracts were chromatographed by a reversed-phase high performance liquid chromatography (RP-HPLC) system with a Nucleosil C4 silica column (8 × 250 mm) and eluted with gradients of acetonitrile containing 0.1% trifluoroacetic acid or by a size-exclusion HPLC system with Superose 6 and 12 (10 × 300 mm) columns connected in series, and eluted isocratically with 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min. Mini-slab SDS-PAGE and amino acid analyses were performed as previously described [14].

2.2. *Sequence determination*

The oat proteins were sequenced as described in [15] in a Knauer Modular Liquid Phase Protein Sequencer Model 810 equipped on line with a Knauer PTH-amino acid analyzer.

2.3. *Immunoblotting*

After SDS-PAGE, proteins were blotted to PVDF membranes (Immobilon, Millipore) as basically described in [16]. Blottings were performed at 10 V for 30 min at room temperature, using a Biometra Fast

Blot semi-dry electrophoretic transfer cell. The Immobilon membranes were first incubated for 1 h in Tris-buffered saline (TBS) containing 0.5% bovine serum albumin and 0.5% Triton X-100 and then incubated overnight with a coeliac serum (1:4,000) or normal serum (1:4,000) in the above buffer. The membranes were incubated for 3 h with rabbit anti-human alkaline phosphatase-conjugated Ig-A (1:8,000). For development of the color reaction, the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride.

2.4. *α-Amylase assay*

The activity of α-amylase was determined by using the Bernfeld method [17]. α-Amylase (Type I-A) from porcine pancreas and the wheat α-amylase inhibitor (Type I) were purchased from Sigma Chem. Co. A probable α-amylase inhibitor from barley endosperm (*Hordeum vulgare*) was prepared from a NaCl-soluble protein extract, followed by extraction with ammonium bicarbonate and RP-HPLC as above.

3. RESULTS AND DISCUSSION

In order to isolate and to identify oat proteins implicated in coeliac disease, the chloroform/methanol protein extract from oat endosperm was fractionated with ammonium bicarbonate and the resulting soluble and insoluble fractions were analyzed by SDS-PAGE and immunoblotted using coeliac and normal sera (Fig. 1). Strong reaction with coeliac serum was associated to the bicarbonate insoluble proteins, and no reaction was found in the bicarbonate soluble fraction (Fig. 1, center). This coeliac serum was tested against the corresponding bicarbonate insoluble proteins from wheat, barley and rye, and gave similar intensity bands to the oat fraction (data not shown). Similar results were also obtained with a total of ten coeliac sera (data not shown).

To identify these oat-reactive proteins, the bicarbonate insoluble fraction was chromatographed by size-exclusion HPLC. As shown in Fig. 2A, only peak 1′ was homogeneous when analyzed by SDS-PAGE. Peaks 1 and 2 were rechromatographed by RP-HPLC while peak 3 was rechromatographed by the same size-exclusion system as above, and a total of eleven pure proteins showing a single band on SDS-PAGE were obtained (Fig. 2).

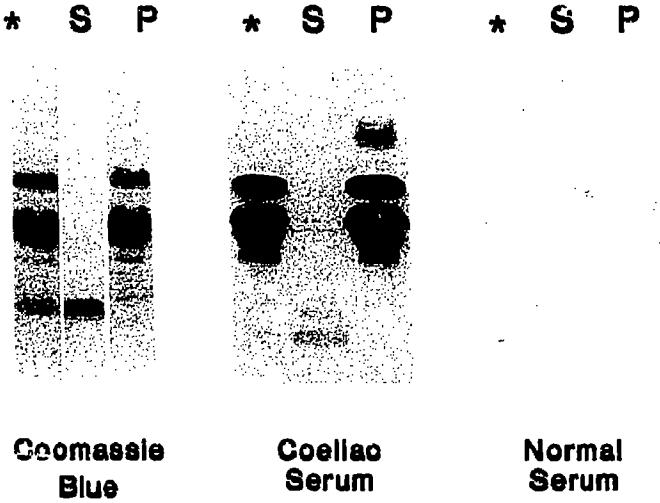

Fig. 1. SDS-PAGE and immunoblotting analysis of the supernatant (S) and pellet (P) bicarbonate fractions from the total CM extract (*) from oat endosperm. Left: SDS-acrylamide gels stained with Coomassie blue, Center and Right are the corresponding immunoblotting onto PVDF membranes, incubated with coeliac and normal serum, respectively, and visualized by developing with anti-human IgA labeled with alkaline phosphatase.

The amino acid composition of all purified components reveals a high glutamic/glutamine (40%) and proline (10%) content which is the characteristic composition of oat prolamins [18]. Based on amino acid composition (data not shown), electrophoretic mobilities in SDS-PAGE, and sequence data, we have tentatively classified the eleven pure components (1a, 1b, 1c, 1′, 2a, 2b, 3a, 3b, 3c, 3d and 3e), in three different groups, called 1, 2 and 3 with respective molecular weights of 27,000, 32,000 and 25,000 Da (Fig. 2). The NH_2-terminal sequence of proteins 1, 2 and 3 fully coincided with the γ_4, γ_3 and α_2 avenins reported from oat [18] (Fig. 3) and do not exhibit significant sequence homology with other known prolamins from oat [19], as well as from wheat, barley and rye [18]. It is of interest that proteins 1, 2 and 3 migrate as several peaks on the same reversed-phase chromatogram (Fig. 2B–D). In addition, protein 1 and 1′ which present the same amino acid composition, N-terminal amino acid sequence and electrophoretic mobility in SDS-PAGE in the presence or in the absence of 2-mercaptoethanol, migrate as two peaks in a size-exclusion column (Fig. 2A). This chromatographic behavior, could be due to denatured and native forms of avenins, as well as glutamine desaminations as it has been recently reported for other cereal proteins isolated by the same purification procedure [14].

Nevertheless, the possibility that some of these isolated avenins, could represent heterogeneous components [18] due to (i) multigen families exhibiting a few amino acid substitutions in the two distinct tanden repeat consensus sequences [19], (ii) gene duplications, or (iii) carbohydrate heterogeneity as described for gliadins [20], have yet to be investigated. In fact, proteins grouped as 1, 2 and 3 could simply be eleven isolated genetic variants of avenins with minor variations in the amino acid sequence and consequently migrating differently in the RP-HPLC (Figs. 2 and 3).

Antibody reactivity against these purified oat avenins was investigated by immunoblotting, with a serum from a patient with coeliac disease (Fig. 2), as well as with ten different coeliac sera (data not shown). As can be seen, they all reacted strongly with coeliac serum in contrast to the negative reaction obtained with a normal serum, used as control (Fig. 2). According to these results the reactivity of oat avenins γ_4, γ_3 and α_2 against coeliac sera appears quite clear in the present study, despite uncertainties in the literature as to the specificity of oat avenins in coeliac disease [10]. As far as we know, this

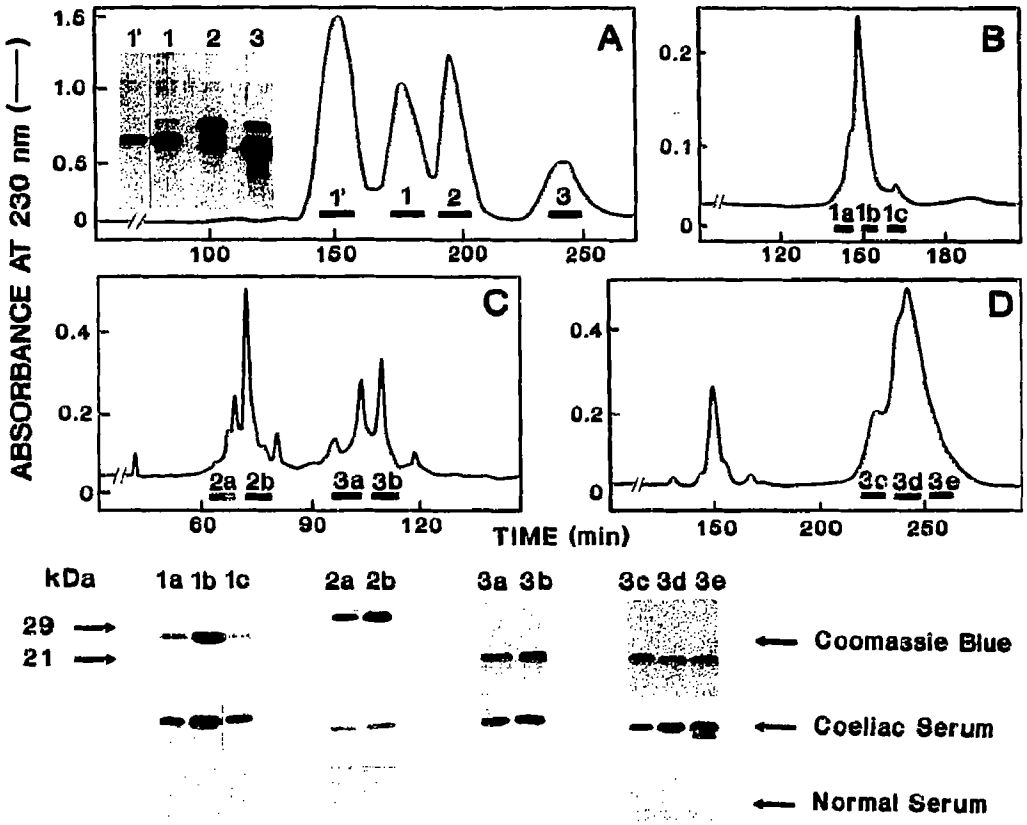

Fig. 2. Fractionation of the bicarbonate insoluble fraction from oat endosperm. (A) The bicarbonate-insoluble extract was resuspended in 0.1% TFA and injected into two connected in series Superose 6 + Superose 12 columns and eluted with 0.1% TFA. SDS-PAGE stained with Coomassie blue of the fractions indicated by bars is shown as an insert. (B) and (C) repurification of peaks 1 and 2 by RP-HPLC on a Nucleosil C4 column and eluted with a linear gradient of acetonitrile from 36 to 50% containing 0.1% TFA. (D) Purification of peak 3 by the same size-exclusion Superose columns as (A). SDS-PAGE stained with Coomassie blue of the fractions indicated by bars, in B, C and D and the corresponding immunoblotting developed with coeliac serum or normal serum are shown at the bottom. The mobilities of the standard proteins are also indicated.

is the first report on the identification of oat prolamins with immunoreactive properties with coeliac sera. We have tentatively designated them as oat 'coeliac immunoreactive proteins' (CIP) and we have called the three isolated reactive oat prolamins 1, 2 and 3 as Oat CPI-1, CPI-2 and CPI-3, respectively.

Reversed-phase high performance liquid chromatography of the bicarbonate soluble fraction is shown in Fig. 4. Six components showing a single band around 10,000 to 12,000 Da were isolated. The amino acid composition of these proteins indicates that they do not belong to the prolamin class (data not shown) and they do not show any reaction to coeliac serum (data not shown). With the analytical criteria mentioned above the six proteins (A1, A2, B1, B2, C1 and C2) were also found to consist of only three different groups, with respective molecular weights of 12,500, 11,000 and 10,000 Da (Fig. 4) and we have designated them as protein Oat-A, Oat-B and Oat-C, respectively. The N-terminal sequence of Oat-A and Oat-C does not have any clear homology with other cereal components, but Oat-B shows a significant homology (60–88% of identity) with the α-amylase inhibitor from ragi [6], as well as with the two probable α-amylase inhibitors from rice [21] and barley [22] (Fig. 3).

In order to characterize the possible function of Oat-B, α-amylase assays were performed with the porcine pancreatic α-amylase. Oat-B showed a weak inhibition of this enzyme of porcine pancreas (32%) in comparison with the 70% inhibitory effect of the wheat α-amylase

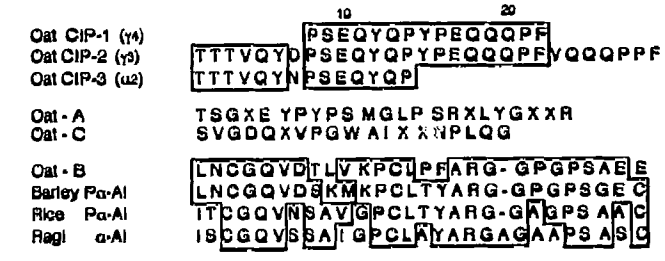

Fig. 3. N-terminal amino acid sequence of the isolated oat proteins. Common sequences are indicated in boxes. Oat-CIP-1, 2 and 3 correspond to the following known components: γ_4, γ_3 and α_2 avenins, respectively. Ragi α-amylase inhibitor (α-AI) [6] and the probable rice and barley α-amylase inhibitors (Pα-AI) [21,22] have been included to be compared with the above avenins. Unidentified positions are indicated by X. Gaps are included to achieve maximum homology.

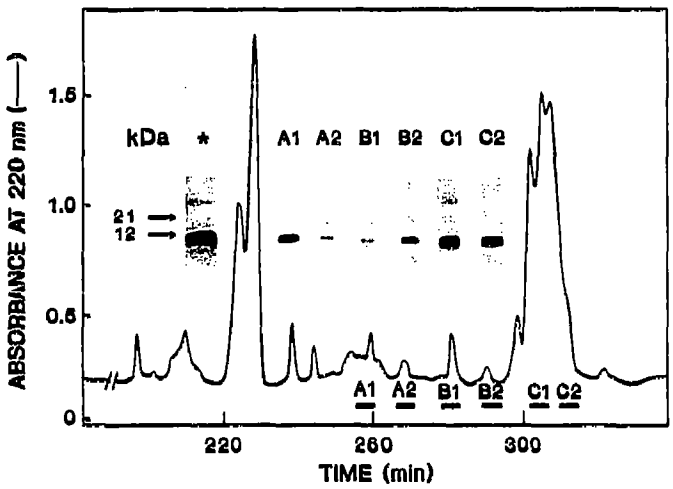

Fig. 4. Fractionation by RP-HPLC of the bicarbonate soluble fraction from oat endosperm. The bicarbonate soluble extract in 0.1% TFA was injected into a Nucleosil C4 column and eluted with a linear gradient of acetonitrile from 0 to 50% containing 0.1% TFA. SDS-PAGE of the fractions indicated by bars are inserted. The lane marked with an asterisk corresponds to the total soluble extract. The mobilities of the standard proteins are also indicated.

inhibitor. No inhibitory effect was obtained with the probable α-amylase inhibitor from barley used as control (data not shown), which does not inhibit α-amylase activity [22]. This finding establishes for the first time the existence of proteins from oat endosperm with α-amylase inhibitory activity. The existence in Oat-B of another inhibitory effect against an entirely unrelated enzyme Oat-B, as already described for other bifunctional cereal proteins (α-amylase/protease inhibitor) [21,22], has yet to be investigated.

In summary, according to the present results, six chloroform/methanol proteins from oat endosperm, including three major coeliac immunoreactive proteins (previously described as α_2, γ_3 and γ_4 avenins), and a new α-amylase inhibitor (Oat-B), have been identified and characterized. The characterization of these oat antigens could contribute to the understanding of some immunological aspects of coeliac disease and also to determine their possible harmful effects in clinical tests and in vitro tissue culture [23].

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