6-24-1994

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ABSRACT

Diversity of trypsin inhibitors was studied in 35 spring barley cultivars (*Hordeum vulgare*), 21 samples of *H. spontaneum*, and 3 samples of *H. agriocrithon*. Six variants of trypsin inhibitor spectra were identified by native electrophoresis method followed by specific development of activity. Four variants were found in both cultivated and wild barley, and the other two were revealed only in *H. spontaneum*. Trypsin inhibitor activities (TIA) and soluble protein contents were determined in four cultivars with different variants of trypsin inhibitors. It was shown that TIA differed in the cultivars studied and did not correlate with soluble protein contents.

INTRODUCTION

Trypsin inhibitors found in endosperm of cereals are of great interest for genetics and breeding because they play important role in protection of grain against microorganisms and insect pests [1]. They are also significant for protein utilization by monogastric animals [2]. That is why studying diversity of trypsin inhibitors and variations of their activities in different cultivars is the subject of research programs. Polymorphism of barley trypsin inhibitors was previously described by Salcedo *et al.* [3] and Moralejo *et al.* [4]. They identified three allelic variants of trypsin inhibitors (BTI-CMe1, -CMe2, and -CMe3) and demonstrated that the last two consisted of several components (BTI-CMe2.1, -CMe2.2, -CMe2.3 and BTI-CMe3.1, -CMe3.2 accordingly). TIA of these individual components were shown to be different. This communication describes new variants of endospermal trypsin inhibitors revealed in cultural and wild barley and presents the results of determining total TIA in different barley cultivars with respect to their soluble protein contents.

MATERIALS AND METHODS

Study of 35 *Hordeum vulgare* cultivars (1993 crop) as well as 21 *H. spontaneum* and 3 *H. agriocrithon* samples (1992 crop) was carried out using seeds provided by Dr. A.A. Pomortsev (Russia) and Prof. E. Nevo (Israel). Trypsin inhibitors were extracted from individual embryoless grains by 0.1 M Na-acetate, ph 4.9 (4 v/w) at 4°C during a night and separated by electrophoresis in 6% polyacrylamide gel in tris-Na-EDTA-borate system (ph 8.3) according to [5]. After electrophoresis proteins were transferred from a gel to a gelatine layer of Micrat film for 10 minutes and zones of trypsin inhibitors were developed by the method [6]. The film was dried, put on 1% agarose gel containing 0.05 M tris-HCl, ph 7.8, 0.1 M NaCl, trypsin 250 ng/ml, and incubated with it at 37°C for 60 minutes. Bands of undigested gelatine on the film corresponded to trypsin inhibitors.

Isoelectric focusing in 4% polyacrylamide gel containing 4-9 Servalytes and 9 M urea was performed for determining Isoelectric points (pI) of trypsin inhibitors from cv. Nutans 970 [7]. Proteins were extracted from embryoless ground grains by Na-acetate; supernatant was desalted by gel-filtration on BioGel P6-DG (Bio-Rad) column (12 cm) and concentrated on Minicon B-15 (Bio-Rad) concentrator. The pH gradient of the gel was measured by determining pH values in water extracts from ' cm sections of the gel. Proteins were stained by Coomassie R-250, and bands of trypsin inhibitors were revealed by replication to Micrat film as described before.

Four cultivars with different variants of trypsin inhibitors were chosen for analysis of their TIA and soluble protein contents. Embryoless grains of each cultivar were crushed with Cyclotec 1093 Sample Mill (Tecator). Proteins were extracted with 3 w/v 0.1 M Na-acetate, pH 4.9, at 4°C during a night (three replications for each cultivar), and TIA were determined by method [8] with BAPA (N alpha-benzoyl-DL-arginine-p-nitroanalide HCl, Serva) as substrate. One unit of inhibitor (U) was defined as the amount of inhibitor that could inhibit 1 mg of trypsin (TPCK treated, Serva). Protein contents were determined with Bio-Rad (Breadford) reagent in the same extracts. Bovine serum albumin (Serva) was used as standard for

protein calibration. Systat Version 5.0 was used for statistical calculations of the obtained results. Least significant difference (LSD_{05}) was estimated as described [9].

RESULTS AND DISCUSSION

Study of different samples of cultivated and wild barley by one-dimensional native electrophoresis with subsequent development of trypsin inhibitor bands allowed us to reveal six variants of spectra. Four of them were specific both for cultivated and wild barley, and the other two were found only in *H. spontaneum* (Fig. 1, Table 1). In the studied cultivars the most frequent variant was D, and variants A and C were relatively rare. All three samples of *H. agriocrithon* had the same variant of trypsin inhibitor (Table 1).

As may be seen from Fig. 1, three variants of trypsin inhibitors (C, D, and E) are close to each other by their electrophoretic mobilities (patterns 1-3 and 7-9), the fourth one (A) is considerably different (patterns 4-5), and the remaining two have intermediate mobilities (patterns 6, 10). It should be noted that it was difficult to determine correspondence of our variants of trypsin inhibitors revealed by one-dimensional electrophoresis with the known trypsin inhibitors classified by Moralejo [4] based on the results of two-dimensional electrophoresis. These difficulties were connected with differences in sets of varieties studied and with resolution capabilities of the methods used. So we preliminarily designated our variants as A-E according to their electrophoretic mobilities (Fig. 1). One variant of *H. spontaneum* (Fig. 1, pattern 10) consisted of two components. However, because the component with higher activity against trypsin had the same mobility as B-variant, we designated it as B'. A-variant of trypsin inhibitor seems to be interesting because of its extremely fast mobility in alkaline electrophoresis. So we further characterized it by isoelectric focusing.

The results of IEF presented in Fig. 2 demonstrated that A-variant of trypsin inhibitor (cv. Nutans 970) consisted of four components with pI 6.2, 6.1, 5.8, and 5.6 as well as two minor components with pI 5.9 and 5.7, thus, all the components of this variant have acid pI.

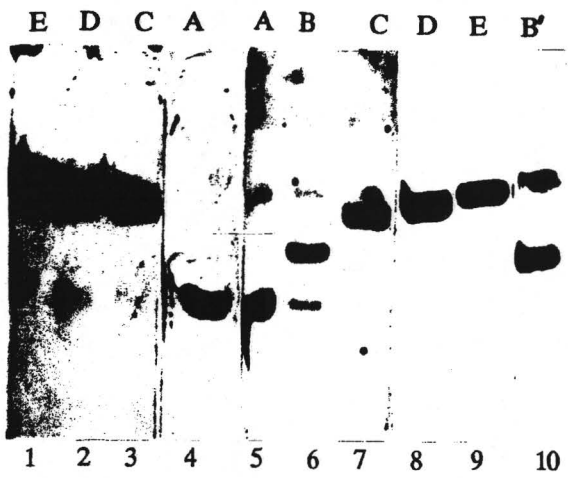

Fig. 1. Diversity of trypsin inhibitors in cultural (1-4) and wild (5-10) barley.

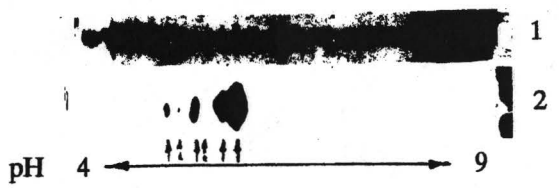

Fig. 2. Bands of proteins (1) and trypsin inhibitors (2) of cv. Nutans 970 revealed by isoelectric focusing.

Table 2. Trypsin inhibitor activities and soluble protein contents in four barley cultivars.

Variety	TIA (U/g of flour)	Soluble protein Content (mg/g of flour)
Pirkka	0.94	9.97
Kashticky	0.83	9.97
Nutans 558	0.37	10.07
Nutans 970	0.83	11.23
LSD_{05}	0.14	2.75

The presence of trypsin inhibitors with acid pI in endosperm of barley cultivars Emir and Proctor was previously demonstrated by Bruhn and Djurtoft [10]. However, in these cultivars trypsin inhibitors with pI 5.8-6.2 were only additional for the main trypsin inhibitor with alkaline pI and are characterized by weak trypsin inhibitors activity (1/3 of the total TIA). In cv. Nutans 970 bands of trypsin inhibitors with alkaline pI were absent. Our preliminary studies allows us to assume that additional bands of trypsin inhibitors with acid pI increase or appear in cvs. Emir and Proctor during storage or accelerated aging of barley grains (Ladogina, unpublished). However, further investigations are needed for clarifying the nature of these changes in trypsin inhibitor spectra.

The next aim of our work was to study TIA and soluble protein contents in four barley cultivars (Pirkka, Kashticky, Nutans 558, and Nutans 970) with different variants of trypsin inhibitors (E, D, C, and A variants respectively). Data of the analysis presented in Table 2 demonstrate that variations in protein contents were not significant in all cultivars studied ($F = 1.0114$, P 0.436). However, variations in TIA were highly significant ($F = 65.19$, P 0.000).

Within the restricted set of the examined barley cultivars we could not reveal correlations between soluble protein contents and TIA. Data, similar to ours, were obtained by Tanner and Reinbergs [11] for wheat and rye: in their study TIA was independent of the level of soluble and total protein content.

In our study cv. Nutans 558 differed from the other cultivars by extremely low TIA (approximately two-fold). The set of the studied cultivars is insufficient for analysis of reasons of such decrease of TIA in cv. Nutans 558, and additional investigations are needed. However, it may be possible to use this cultivar for obtaining barley forms with low TIA.

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