

## Differences Between Endospermal and Embryonal Trypsin Inhibitors in Barley, Wheat, and Rye

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Trypsin inhibitors are present in high concentrations (5–10 % of water-soluble protein) both in the embryos and endosperms of grains of barley, wheat, and rye. The inhibitors which occur in the two respective organs are dissimilar to each other, however; in all three species the endospermal and embryonal (main component) inhibitors can be separated by means of gel chromatography on Sephadex G-75. The three endospermal inhibitors possess almost identical elution volumes on Sephadex G-75. The rye inhibitor exhibits partial immunological identity with the inhibitor from barley; the wheat inhibitor does not precipitate antibodies against the barley inhibitor. The single trypsin inhibitor of barley embryos and the main components in wheat and rye embryos possess identical elution volumes on Sephadex G-75 (corresponding to a molecular weight of 18 500). Wheat and rye embryos also contain some minor components which elute at approximately the position of the endospermal inhibitors. The evidence available suggests that these inhibitors also differ from their endospermal counterparts. A single inhibitor, with an unusually high molecular weight (43 500) has been observed in oats grains. All these cereal inhibitors, in contrast to a number of trypsin inhibitors from leguminous seeds, appear to be relatively weak, non-stoichiometric inhibitors of trypsin; in the hydrolysis of benzoyl-DL-arginine *p*-nitroanilide, most of the inhibition effects are linear only as far as 80 % inhibition.

Earlier, we purified a trypsin inhibitor from barley grains.<sup>1</sup> The inhibitor appeared to be a heat-stable, small-molecular protein (mol. wt. 14 400), inhibiting trypsin in a molar ratio 1:1. Subsequently, we have studied the distribution of different proteolytic inhibitors in various tissues of barley.<sup>2</sup> The highest trypsin inhibitor activities were those found present in embryos. However, the active embryo extracts, in contrast to endosperm extracts, did not display immunological reactions with mouse ascites fluids containing antibodies towards the purified inhibitor. The conclusion was drawn that this inhibitor occurs exclusively in the endosperms, and that another (or some other) inhibitor(s) is present in the embryos.

Rather earlier than this, Hochstrasser and Werle had observed the existence of high trypsin inhibitor activities in wheat and rye embryos, and purified an inhibitor from each source.<sup>3</sup> The two inhibitors closely resemble each other; both have a molecular weight of 17 000, both have an amino-terminal serine residue and the sequence -Arg-Ala- at the active centre, and the amino acid compositions are almost identical. The endospermal inhibitor of barley differs greatly from these inhibitors; it is eluted much later from columns of Sephadex G-75 (by reason of smaller molecular size, and reversible adsorption on the dextran gel), and its amino acid composition is different.

As wheat, rye, and barley are relatively close to each other from a taxonomic aspect, the questions arose whether the trypsin inhibitor present in barley embryos resembles the inhibitors purified from the embryos of wheat and rye, and whether the endosperms of the last-mentioned cereals contain inhibitors that are related to the endospermal inhibitor of barley? This paper reports on a series of experiments designed to answer these questions. The trypsin inhibitors of the embryos and endosperms of the three cereals were separated by chromatography on Sephadex G-75, and wheat and rye extracts and inhibitor fractions prepared from the extracts were tested for the presence of antigens corresponding to the endospermal inhibitor of barley. Some comparative data for oats are also presented.

#### MATERIALS AND METHODS

Grain samples were obtained from the following sources: barley (Pirkka, a Finnish 6-row variety), Oy Lahden Polttimo, Lahti; summer wheat (Svenno), winter wheat (Vakka), and oats (Titus) Oy Hankkija, Helsinki; rye (Toivo, a Finnish winter rye), Agricultural Research Centre, Department of Plant Breeding, Jokioinen. All the grains derived from the Finnish harvest of the previous year.

For the separation of embryos and endosperms, the grains of barley and oats were dehusked by sulphuric acid treatment.<sup>3</sup> The incubation times in 50 % (v/v) sulphuric acid at room temperature were 3 h for barley and 5 h for oats. The "naked" grains of all the four cereals were incubated in a shallow layer of water for 1-4 h, and the embryos and endosperms were separated by hand dissection. The separated tissues were dried on filter paper at room temperature, ground in a mortar mill, and defatted by extraction with ten volumes of acetone at -18°.

Trypsin inhibitors were extracted from whole grains and endosperms of barley and wheat by means of water at pH 4.9. A quantity of 10 g of ground tissue was suspended in 20 ml of water, and the suspension was acidified to pH 4.9 with 1 M acetic acid. Extraction was continued for 2 h at 5°, and the extract was separated by centrifugation. For all other tissues, the extraction was effected with 0.146 M sodium acetate buffer of pH 4.9. The extraction ratios were 1 g per 4 ml for grains and endosperms, and 1 g per 20 ml for embryos. The suspensions were agitated during the 2 h extraction at 5°.

For chromatography on Sephadex G-75, the trypsin inhibitors of rye grains and endosperms were purified by adsorption on carboxymethylcellulose in the following way: an amount of 20 ml of extract was dialysed against 0.073 M sodium acetate buffer of pH 4.9. The dialysed solution, clarified by centrifugation, was applied to a 2.5 × 6 cm column of CM-cellulose (Whatman, CM23) equilibrated with the same buffer. After washing with 50 ml of the starting buffer, the inhibitors were eluted with 0.3 M sodium phosphate buffer at pH 6.0 (no further activity was eluted with a corresponding 0.6 M buffer). The inhibitors eluted with the 0.3 M buffer were concentrated by precipitation with ammonium sulphate at 80 % saturation, and dissolved in 10 ml of the pH 4.9 buffer used in the Sephadex runs. The recovery of activity in this purification varied between 82 and 91 %.

The trypsin inhibitors of oats were purified analogously by adsorption on diethyl-aminoethylcellulose. A 2.5 × 6 cm column of DEAE-cellulose (Whatman, DE23) was used

for 40 ml of extract. The starting and eluting buffers were 5 mM Tris-Cl of pH 7.5 and 0.29 M sodium acetate of pH 4.9, respectively. The recovery of activity was 63–72 %.

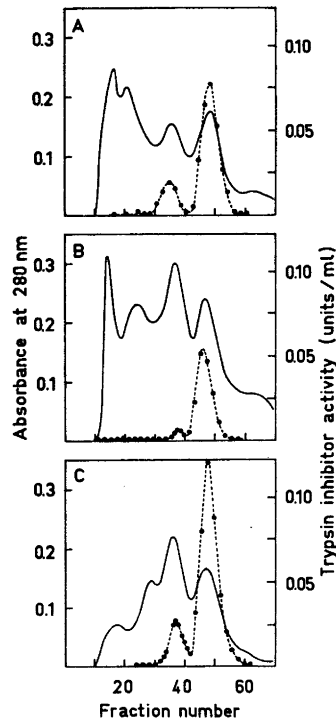
The methods used for trypsin inhibitor assay (based upon the hydrolysis of benzoyl-DL-arginine-*p*-nitroanilide by trypsin), the production of mouse ascites fluids containing antibodies towards barley trypsin inhibitor, and immunodiffusion, have previously been described in detail.<sup>1,2</sup>

Sephadex G-75 (for gel filtration) was obtained from Pharmacia, Uppsala, Sweden. Pure trypsin (from bovine pancreas, 2 × crystallized, Type I) and soybean trypsin inhibitor (3 × crystallized) were products of Sigma Chemical Company, St. Louis, Mo. U.S.A.

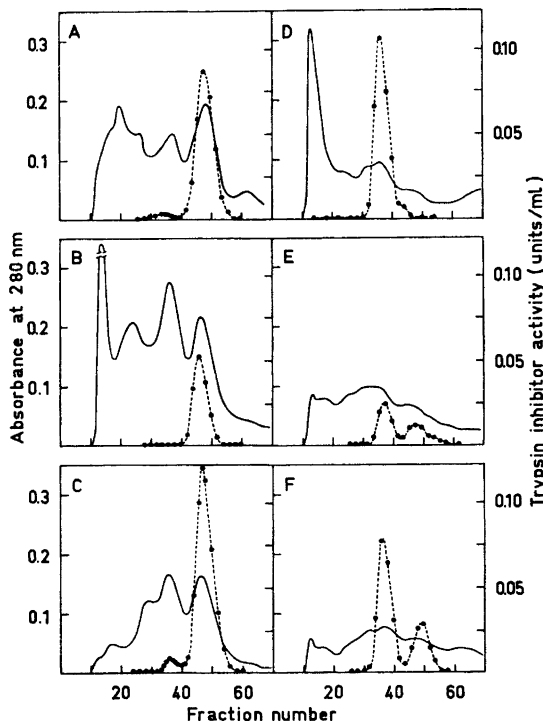
RESULTS

*Inhibitor activities in embryos and endosperms of different cereals.* For determination of the total inhibitor activities, extracts were prepared from the embryos and endosperms of the four cereals by means of the 0.146 M sodium acetate buffer of pH 4.9 (extraction ratio, 1 g/10 ml). The results of the determinations, expressed as inhibitor units per gram of tissue, have been compiled in Table 1. The activities diminish in the order rye, barley, wheat, oats; in all species more inhibitors were observed in the embryos.

*Separation of the inhibitors on Sephadex G-75.* The trypsin inhibitors of whole grains, embryos, and endosperms of barley, wheat, and rye were chromatographed on Sephadex G-75. Most of the runs were made with dialysed extracts. However, the extracts of rye grains and endosperms were too viscous to be chromatographed as such. The inhibitors present in these extracts were



*Fig. 1.* Chromatography of the trypsin inhibitors of whole grains of barley (A), wheat (B), and rye (C) on Sephadex G-75. A 25 × 895 mm column of Sephadex G-75 was equilibrated with sodium acetate buffer of pH 4.9, ionic strength 0.1, and operated at room temperature. The following samples (dialysed extracts or crude protein fractions corresponding to the amounts of tissues indicated) were fed into the column in 9–10 ml of buffer: A, barley grains, 3.4 g; B, wheat grains, 2.8 g; C, rye grains, 3.7 g. The column was eluted with the equilibration buffer at a flow rate of 20 ml/h, and  $E_{280}$  (—) and trypsin inhibitor activity (●---●) were determined from 5.0 ml fractions collected after a prefraction of 100 ml.



*Fig. 2.* Chromatography of the trypsin inhibitors of endosperms and embryos of barley, wheat, and rye on Sephadex G-75. Running conditions and symbols as in Fig. 1. The samples corresponded to:

- |                             |                           |
|-----------------------------|---------------------------|
| A, barley endosperms, 3.7 g | D, barley embryos, 420 mg |
| B, wheat endosperms, 2.8 g  | E, wheat embryos, 480 mg  |
| C, rye endosperms, 3.7 g    | F, rye embryos, 420 mg    |

separated from the viscosity inducing polysaccharides by adsorption on CM-cellulose, and the crude protein fractions eluted from the column with concentrated buffer were used in the Sephadex runs. Typical results for all three species are contained in Figs. 1 and 2.

In whole grains, two trypsin-inhibiting fractions are evident in all three species (Fig. 1A-C). The elution positions of the faster and slower components correspond to the embryonal inhibitors purified by Hochstrasser and Werle,<sup>3</sup> and the endospermal barley inhibitor,<sup>1</sup> respectively. In the endosperms of all species (Fig. 2A-C), the slower component is completely dominant (in barley and rye, minimal activity is eluted at the position of the faster fraction). In wheat, the endospermal inhibitor is eluted slightly earlier than in barley and rye. The difference is more probably attributable to differences in interactions with the dextran gel than to differences in molecular size, as the barley inhibitor is known to display reversible adsorption on Sephadex.<sup>1</sup>

Barley embryos contain only the fast component, which is also dominant in wheat and rye (Fig. 2D-F). The elution volumes are exactly the same. In comparison with a set of standard proteins (ovalbumin, mol.wt. 45 000; soybean trypsin inhibitor, 21 500; cytochrome c, 12 400; glucagon, 3 500) this inhibitor was eluted slightly after soybean trypsin inhibitor, at a position corresponding to a molecular weight of 18 500. This value compares favourably with that arrived at by Hochstrasser and Werle (17 000), when the differences in standard proteins and running pH are taken into consideration. In the elution curves for wheat and rye embryos, smaller, slower fractions are also evident.

Corresponding fractions have been observed by Hochstrasser and Werle. The slower peak of wheat embryos is clearly asymmetrical (Fig. 2E) (the same result was obtained several times), indicating the presence of more than one inhibitor. Obviously, only one of these can be identical with the endospermal inhibitor. In rye, the slower peak is symmetrical. However, the following section will show it did not give the immunological reaction displayed by the endospermal inhibitor of rye. It is thus possible that even the smaller-molecular trypsin inhibitors of wheat and rye embryos are dissimilar to the corresponding endospermal inhibitors.

The chromatograms illustrated in Figs. 1A and 2A,D were obtained with barley which had been dehusked with 50 % sulphuric acid. For confirmation that this treatment does not affect the trypsin inhibitors, the run indicated in Fig. 1A was repeated, using barley which had not been dehusked. Changes were apparent in the  $E_{280}$ -curve, but the activity curve was identical with that in Fig. 1A. It seems that the inhibitors are not modified either by the presence of husks, or by the dehusking procedure.

Emerging rootlets and coleoptiles are the only vegetative tissues of barley which contain trypsin inhibitor activity.<sup>2</sup> For determination of the kind of inhibitor responsible for the activity, the inhibitors of freeze-dried rootlets of green malt (Pirkka, 9 days germination) were chromatographed on Sephadex G-75. The extracts (pH 4.9 buffer, 4 ml/g) were too viscous for direct chro-

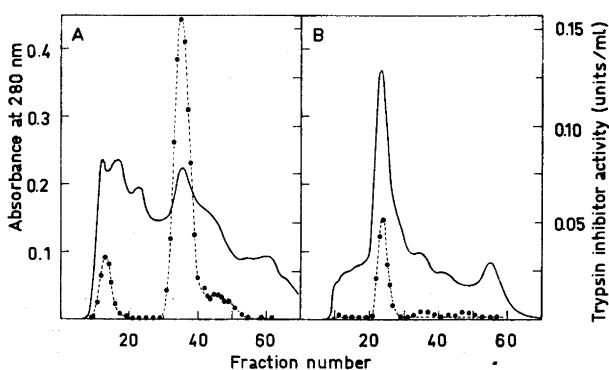


Fig. 3. Chromatography of the trypsin inhibitors of barley rootlets (A) and oats grains (B) on Sephadex G-75. Running conditions and symbols as in Fig. 1. Feeding: A, inhibitor fraction purified by adsorption on CM-cellulose and corresponding to 8.5 g of barley rootlets; B, inhibitor fraction purified by adsorption of DEAE-cellulose and corresponding to 11.3 g of de-husked oats grains.

Table 1. Trypsin inhibitor activities of endosperms and embryos of different cereals,

	Inhibitor activity (Units/g dry weight)	
	Endosperm	Embryo
Rye (Toivo)	2.9	10.0
Oats (Titus)	0.3	1.1
Barley (Pirkka)	1.5	9.1
Wheat (Vakka)	0.4	2.8

matography, but the inhibitors could be purified by adsorption on CM-cellulose by application of the method used for rye extracts. A representative run is illustrated in Fig. 3A. The major fraction is exactly at the position of the embryonal inhibitor; in addition, two small fractions are evident.

The trypsin inhibitor activities of oats extracts were lower than those of the other species (Table 1). Moreover, aqueous extracts of oats have a high degree of viscosity. Attempts to concentrate and purify the inhibitors by CM-cellulose treatment proved unsuccessful owing to the incomplete adsorption of inhibitor activity. The inhibitors were consequently concentrated by adsorption, followed by elution from DEAE-cellulose (see Materials and Methods). A Sephadex G-75 run of a crude protein fraction eluted from DEAE-cellulose is illustrated in Fig. 3B. The single activity peak is eluted much earlier than either of the barley inhibitors. The elution volume, which only

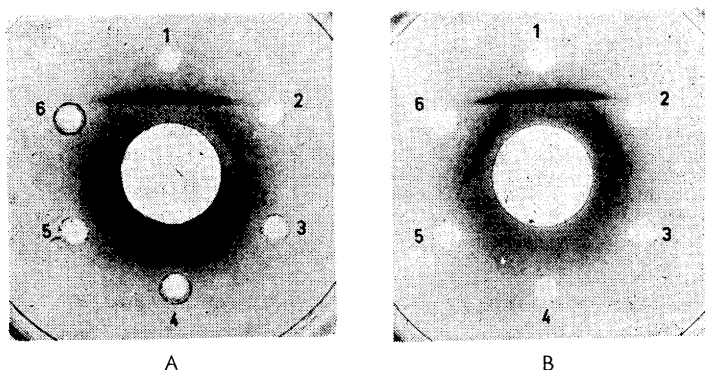
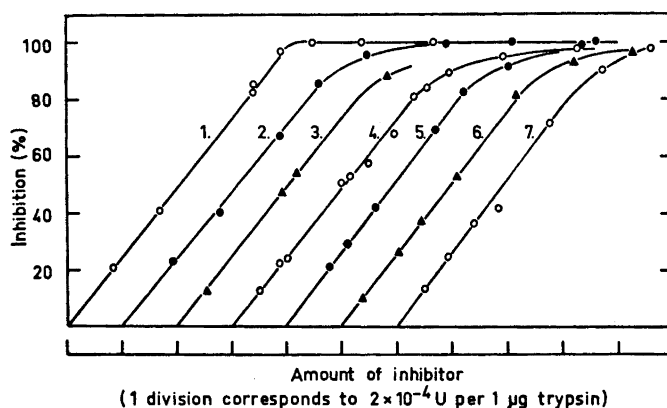


Fig. 4. Comparison by immunodiffusion of various cereal trypsin inhibitors with the endospermal inhibitor of barley. The central wells were filled with undiluted ascites fluid from a mouse immunized with purified barley trypsin inhibitor; approximately equal amounts of trypsin inhibitor activity were pipetted into the outer wells as follows: A. Reactions of tissue extracts: endosperm extracts of barley (1), rye (2), and wheat (6), and embryo extracts of rye (3), barley (4), and wheat (5). B. Reactions of different rye inhibitors separated on Sephadex G-75: 5 µg of purified barley inhibitor (1), inhibitor from rye endosperms (2 and 6), faster component from rye embryos (3), slower component from rye embryos (4 and 5).

slightly exceeds that of ovalbumin, corresponds to a molecular weight of 43 500.

*Search for antigens corresponding to barley trypsin inhibitor in other cereals.* For examination of the expected homology between the endospermal trypsin inhibitors of barley, wheat, and rye, immunodiffusion experiments were made. Extracts were prepared from the separated tissues, using the pH 4.9 buffer (4 ml/g for endosperms, 10 ml/g for embryos). In diffusion against mouse ascites fluids containing antibodies towards the purified barley inhibitor, only rye endosperms gave a precipitate line. A typical plate is reproduced in Fig. 4A. Corresponding experiments were made with inhibitor fractions obtained from the Sephadex runs. The tubes in an activity peak were combined, the inhibitors were precipitated with ammonium sulphate (80 % saturation), the precipitate was dissolved in a small volume of pH 4.9 buffer, and excess salt was removed by dialysis against the same buffer. In immunodiffusions, the slow fractions from barley and rye endosperms were the only ones to precipitate (Fig. 4B). Neither of the inhibitor fractions from rye embryos gave a precipitate in diffusions repeated a number of times with different ascites fluids. This result indicates the probability that even the slower inhibitor fraction of rye embryos differs from the endospermal inhibitor.

In the immunodiffusion plates illustrated in Fig. 4, the precipitation line of the barley trypsin inhibitor does not fuse completely, but extends beyond the line attributable to the rye inhibitor (or the antigen present in rye). This indicates the existence of only partial immunological identity between the two inhibitors (only a part of the antibodies produced in response to the barley inhibitor reacts with the rye antigen). Apparently rye trypsin inhibitor possesses some but not all of the immunological determinants of the barley inhibitor.



*Fig. 5.* Inhibition of trypsin by different cereal inhibitors. Hydrolysis of benzoyl-DL-arginine-*p*-nitroanilide at pH 8.2 by 28  $\mu$ g of pure trypsin in a reaction volume of 3.0 ml; the curves refer to the following inhibitors, most of which were partially purified by chromatography on Sephadex G-75: 1. Soybean trypsin inhibitor, pure. 2. Endospermal inhibitor of rye. 3. Endospermal inhibitor of wheat. 4. Embryonal inhibitor of barley. 5. Dialysed extract of rye embryos. 6. Embryonal inhibitor of wheat. 7. Inhibitor from oats grains.

Some immunodiffusions were also made with extracts of oats embryos and endosperms. No precipitates were observed.

*Strength of trypsin inhibition by the different inhibitors.* In contrast to a number of inhibitors from leguminous seeds, the endospermal trypsin inhibitor of barley is not a strong, stoichiometric inhibitor to trypsin.<sup>1</sup> In the hydrolysis of both casein and benzoyl-L-arginine-*p*-nitroanilide, the inhibition effects were linear up to only 65 % inhibition. For comparison of the cereal inhibitors in this respect, inhibition curves for the hydrolysis of benzoyl-L-arginine-*p*-nitroanilide were constructed for the different inhibitors. Inhibitor fractions from the Sephadex runs (concentrated as described in the preceding section) were employed as inhibitor solutions in most experiments; a dialysed extract was used in experiments concerned with rye embryos. The results, collected in Fig. 5, indicate that the endospermal and embryonal trypsin inhibitors of barley, wheat, and rye, as well as the oats inhibitor, are all relatively weak, non-stoichiometric inhibitors, most of the inhibition effects being linear up to about 80 % inhibition.

#### DISCUSSION

The gel filtration data presented above conclusively demonstrate that the trypsin inhibitor present in barley embryos resembles the inhibitors purified by Hochstrasser and Werle from the embryos of wheat and rye, and that the endosperms of the two species last-mentioned each contain an inhibitor resembling the endospermal inhibitor of barley. The elution diagrams of whole grain extracts further indicate that the endospermal inhibitor is the dominant component in whole grains of all three cereals.

The trypsin inhibitor of rye endosperms exhibits partial immunological identity with the inhibitor purified from barley; consequently, the two proteins are most probably homologous. In view of the close taxonomical relationships between the three species, it seems probable that the endospermal inhibitor of wheat also belongs to the same series of homologous proteins. In complete analogy, the embryonal inhibitors of wheat and rye are certainly homologous,<sup>3</sup> and similar localization and molecular size also suggest homology for the corresponding barley inhibitor.

The differences between oats and the other species are quite pronounced; the trypsin inhibitor content of both embryos and endosperms is less than those in the other species, a single inhibitor is evident in Sephadex G-75 runs of whole grain extracts, and its molecular weight is much higher than those of other cereal inhibitors. This inhibitor, by virtue of the smaller size of the embryo, is at least mainly of endospermal origin. Indeed, the results do not exclude the possible existence of another inhibitor in oats embryos.

Maize, not included in this study, appears to represent a third type of cereal in regard to trypsin inhibitors. Hochstrasser *et al.*<sup>4,5</sup> have purified an inhibitor from this source, and stated that this inhibitor is the only one occurring in whole grains of maize. The purified inhibitor has a molecular weight of 21 200, and appears to be a trimer of three identical subunits, bonded together non-covalently. The inhibitor possesses the sequence -Arg-Leu- at the



active centre, and its amino acid composition differs widely from those of the inhibitors purified from wheat, rye, and barley.

Earlier, we estimated that, in whole grains of barley, trypsin inhibitors account for 4.5 % of water-soluble protein.<sup>1</sup> To derive corresponding percentages for the separated tissues, calculation was made of their inhibitor content (in mg/g) from the activities presented in Table 1 (for rootlets, a value of 1.8 U/g was determined separately), and from specific activities 2.4 U/mg for the endospermal inhibitor<sup>1</sup> and 2.0 for the embryonal inhibitor. Water-soluble proteins were extracted at pH 4.9, and assayed after dialysis by the Biuret-reaction; the values obtained were 7 mg/g for endosperms, 53 mg/g for embryos, and 17 mg/g for rootlets. Based on these figures the trypsin inhibitors accounted for 9, 9, and 5 % of the water-soluble proteins in endosperms, embryos, and rootlets, respectively. Consequently, whatever their physiological significance may be, trypsin inhibitors represent quantitatively important protein fractions, both in the tissues of the resting grain and in emerging rootlets of barley.

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