Trypsin Inhibitor from Rye Endosperm: Purification and Properties

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ABSTRACT

A trypsin inhibitor was isolated from the endosperm of rye seeds by extraction with a 0.1 *M* sodium acetate buffer (pH 4.9), fractionation with ammonium sulfate, heat treatment, ion-exchange chromatography on SP-Sephadex C-50, and gel filtration on Ultrogel AcA 54. The final preparation was homogeneous by electrophoretic analyses. The inhibitor was a protein with a molecular weight of about 12,500 and an isoelectric point of about 9.0. The amino acid composition differed from that of a trypsin inhibitor previously isolated from rye embryo. The action of the endosperm inhibitor on bovine trypsin was linear up to 90% inhibition with benzoyl-*l*-arginine-*p*-nitroanilide as substrate and up to 80% with casein as substrate. The preparation had a weak, nonstoichiometric inhibitor action on bovine chymotrypsin but no activity on porcine elastase. The inhibitor was heat-stable at 100°C, and up to 15% of the original activity could still be extracted from different rye bread types. Immunochemical studies with specific antibodies indicated that the inhibitor may have been modified during the baking process.

Rye seeds have been found to contain four trypsin inhibitors in the embryo (Hochstrasser and Werle 1969) and at least two in the endosperm (Mikola and Kirsi 1972). An inhibitor from the embryo with a reported molecular weight of 17,000 was purified (Hochstrasser and Werle 1969). Another inhibitor with a molecular weight of about 10,000 was isolated from the endosperm (Polanowski 1974), but this inhibitor was not characterized.

Rye is an important crop in many countries (Bushuk 1976). It is used in bread for human consumption and also in animal feeding. Investigation of the possible influence of the trypsin inhibitors in relation to the digestion of proteins in man and animals is therefore important. Preliminary studies indicated that rye seeds contain relatively high amounts of trypsin-inhibiting activity compared to other cereals. The activity in rye seeds was about 50% higher than that in barley seeds and 5–10 times the activity in wheat and oat seeds.

The purpose of the present work is to describe the purification and the chemical and inhibitory properties of the dominant trypsin inhibitor in the endosperm of rye seeds. Because of the possible nutritional significance of the inhibitor in human diets, the inhibitor activities still present in rye bread were determined by immunochemical methods.

MATERIALS AND METHODS

A sample of Danish rye (Secale cereale) was obtained from Schulstad Bread Factory, Glostrup, Denmark.

Trypsin Assay

Benzoyl-l-arginine-p-nitroanilide as Substrate. The activity of trypsin (NOVO, crystalline preparation from bovine pancreas) was determined by the method of Erlanger et al (1961) with some modifications. The substrate was prepared by dissolving 21.8 mg of benzoyl-*l*-arginine-*p*-nitroanilide (l-BAPA) in 1 ml of dimethylsulfoxide and adding prewarmed (37°C) buffer (0.05 *M* tris-HCl, pH 8.2) containing 0.02 *M* $CaCl_2$ to a total volume of 100 ml. This solution was placed in a water bath at 37°C. Then 0.15 ml of enzyme solution (0.05 mg of trypsin per milliliter of 10^{-3} *M* HCl) was mixed with 0.15 ml of 0.1 *M* sodium acetate buffer, pH 4.9, in a small tube at 37°C, and the reaction was started by adding 2.0 ml of substrate solution. The reaction was stopped exactly 10 min later by adding 1.0 ml of 20% acetic acid. The quantity of *p*-nitroaniline liberated by enzyme action was then estimated at 410 nm with a Zeiss PMQ II spectrophotometer.

Casein as Substrate. The assay was done by the method described by Bergmeyer (1974) with some modifications. The reaction was carried out in a thermostatic bath at 37°C. An aliquot (0.15 ml) of enzyme solution (0.1 mg of trypsin per milliliter of 10^{-3} *M* HCl) was mixed with 0.15 ml of 0.1 *M* sodium acetate buffer, pH 4.9, in a small tube. The reaction was started by adding 2.0 ml of substrate solution (0.5% casein in 0.1 *M* phosphate buffer, pH 7.6). The reaction was stopped exactly 10 min later by adding 4.0 ml of 5% trichloroacetic acid. After 30 min the samples were centrifuged, and the absorbance of the supernatants was measured at 280 nm.

Trypsin Inhibitor Assay

In the trypsin assay, varying quantities of inhibitor were included in the 0.15 ml of sodium acetate buffer. One inhibitor unit was defined as the amount of inhibitor that could inhibit 1 mg of trypsin in the assay with l-BAPA as substrate. In routine assays, l-BAPA was always used as substrate. The inhibitor activity was calculated from inhibitor concentrations that inhibited the trypsin activity in the range of 20–70%.

Chymotrypsin Assay

The activity of chymotrypsin (NOVO, crystalline preparation from bovine pancreas) was determined by the method of Erlanger et al (1966) using glutaryl-*l*-phenylalanine-*p*-nitroanilide as substrate or by the method described by Bergmeyer (1974) using casein as substrate.

Elastase Assay

The activity of elastase (Boehringer, suspension from porcine pancreas) was determined with casein as substrate using the same method as described for trypsin.

Extraction of Rye Bread

Extracts of eight different types of rye bread (2.5 g per 10 ml of 0.1 *M* sodium acetate buffer, pH 4.9) were made by homogenizing the bread in the buffer with an Ultra Turrax, type TP 18/2, for 1 min. The homogenization was performed in small tubes placed in an ice bath. After homogenization, extraction was done with vigorous magnetic stirring for 1 hr at 4°C. After centrifugation, the suspensions were analyzed for inhibitor activity.

Chromatography of a Concentrated Rye Extract

To a rye extract (10 g of rye extracted by 30 ml of 0.1 *M* sodium acetate buffer, pH 4.9), ammonium sulfate was added to 45% saturation. The precipitate was redissolved in 15 ml of extraction buffer and the irreversible precipitate removed by centrifugation. A 1.5-ml aliquot (which corresponds to 1.0 g of rye) was fed to a 0.9 ×60-cm column of Sephadex G-75 equilibrated with 0.1 *M* sodium acetate buffer, pH 4.9, and operated at 4°C (flow rate: 5 ml/hr). Fractions of 1.5 ml were collected.

Preparation of Inhibitor

Fine ground rye was suspended in 0.1 *M* sodium acetate buffer, pH 4.9 (0.5 kg per 1.5 L). The suspension was allowed to stand overnight at 4°C, and then the extract was isolated by

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centrifugation (13,000 *g* for 15 min). Most of the trypsin-inhibiting activity was precipitated by addition of solid ammonium sulfate to give 30% saturation, followed by stirring for 1 hr at 4°C. The precipitate containing the activity was collected by centrifugation and suspended in 200 ml of 0.1 *M* sodium acetate buffer, pH 4.9. The suspension was heated for 5 min in a boiling water bath, and after cooling in an ice bath the precipitate was removed by centrifugation. The clear supernatant from the centrifugation was dialyzed overnight against 5 L of 0.05 *M* sodium acetate buffer, pH 5.5, and then passed through a 2.5 × 25-cm column of SP-Sephadex C-50 (Pharmacia) equilibrated with the same buffer. Finally the activity was eluted with a linear salt concentration gradient. The active fraction from the preceding step was collected and concentrated by ultrafiltration to a volume of 20 ml (Amicon ultrafiltrator, model 202, diaflo PM-10 membrane). The concentrated sample was chromatographed at 4° C on a 2.5 × 90-cm column of Ultrogel, AcA 54 (LKB) equilibrated with 0.1 *M* sodium acetate buffer, pH 4.9 (flow rate: 25 ml/hr). Fractions of 9.8 ml were collected and active top fractions were stored in the buffer at −20° C.

Preparation of Specific Immunoglobulins

One milligram of the purified rye trypsin inhibitor was dissolved in 2.5 ml of a 0.05 *M* phosphate buffer, pH 7.5, and polymerized at room temperature by adding 20 μl of a 25% glutardialdehyde solution, as suggested by Bollum (1975). After a few minutes the solution had become turbid, and the reaction was stopped by adding 0.25 ml of a 1% sodium borohydride solution. The polymerized product to be used as antigen was dialyzed overnight against a 0.9% NaCl solution. Two rabbits were injected initially with a mixture of 0.25 ml of antigen and 0.25 ml of Freund's complete adjuvant. Each week for the following three weeks, injections with 0.25 ml of antigen and 0.25 ml of incomplete adjuvant were given. One week after the last injection, blood samples (40 ml) were collected from the rabbits and tested. They contained potent and specific immunoglobulins against rye trypsin inhibitor. After four months of further immunization (boosting), the immunoglobulins were recovered, concentrated, and purified as described by Harboe and Ingild (1973).

Analytical Methods

Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed as described by Jensen et al (1980). Molecular weights were calculated using ovalbumin (mol wt 45,000), carboanhydrase (mol wt 30,000), and cytochrome C (mol wt 12,400) as standard proteins.

Isoelectric focusing was made in 2% Ampholine, pH 3–10 (LKB), in 74 × 2.7-mm acrylamide gel rods. Experimental conditions have previously been described in detail (Bruhn and Djurtoft 1977).

Samples for amino acid analyses were hydrolyzed in 6 *N* HCl at 110° C in evacuated sealed tubes for 24 or 72 hr and analyzed with a Durrum amino acid analyzer. The samples were hydrolyzed in the presence of phenol to protect tyrosine. Cysteine was determined after oxidation to cysteic acid by hydrogen peroxide. Tryptophan was estimated in the intact protein by the colorimetric method of Beaven and Holiday (1952) as modified by Wetlaufer (1962). The amount of free sulfhydryl groups was assayed according to the procedure of Ellman (1959).

Pure inhibitor samples in 0.1 *M* sodium acetate buffer, pH 4.9, were heat-treated in a series of small test tubes placed in a boiling water bath for periods of 5–30 min.

Immunoelectrophoresis experiments were performed essentially as described by Axelsen et al (1973).

RESULTS AND DISCUSSION

Gel filtration of a redissolved 45% ammonium sulfate precipitate from a rye extract results in the separation of the trypsin-inhibitng substances in several active peaks (Fig. 1). According to Mikola and Kirsi (1972) the slowest—and also the most dominant—peak corresponds to a trypsin inhibitor from the endosperm. Purification and study of this endosperm inhibitor is the subject of the present study.

Purification procedures for trypsin inhibitors very often include an initial heat treatment of the extract in order to remove heat-labile material. A high content of polysaccharides in the rye extract causes complete gelification when the extract is heated, ruling out heat treatment at this point in the preparation procedure. Initial studies by gel filtration experiments on the extract showed, however, that nearly all the endospermal trypsin inhibitor precipitated at 30% ammonium sulfate concentration. Heat treatment of the suspended 30% ammonium sulfate precipitate did not cause any decrease in the trypsin inhibitor activity and resulted in a significant enrichment of inhibitor proteins by irreversible precipitation.

A pure preparation of the endosperm rye trypsin inhibitor was obtained from a rye extract by ammonium sulfate fractionation, heat treatment, cation exchange chromatography, and gel filtration, as described above. The results are summarized in Table I. The major loss in activity (from 319 to 188 inhibitor units) occurred during ammonium sulfate precipitation, which (up to a concentration of 30%) leaves the embryo trypsin inhibitors in solution. This was demonstrated by gel filtration on the suspended 30% ammonium sulfate precipitate. Only one peak appeared, corresponding to the slowest-moving (most active) peak in Fig. 1. (This result is not shown.)

In the gel filtration step (Fig. 2), the rye endosperm trypsin inhibitor adsorbed markedly to the gel material. This resulted in an elution volume for the active peak a little higher than the bed volume and indicated a molecular weight below 5,000. However, by

TABLE I
Purification of Rye Trypsin Inhibitor

Fraction	Volume (ml)	Activity (U[a])	Protein[b] (mg)	Specific Activity (U/mg of protein)	Recovery (%)
Extract	1,092	319	11,200	0.028	100
Ammonium sulphate precipitate, 30%, resuspended and heat treated	220	188	1,620	0.11	59
After dialysis	225	146	1,470	0.10	46
SP-Sephadex peak	56	110	100	1.1	34
AcA 54 peak	39	98	52	1.9	31

[a] Inhibitor unit, defined as the amount necessary to inhibit 1 mg of trypsin.
[b] Protein was estimated by the method of Lowry et al (1951).

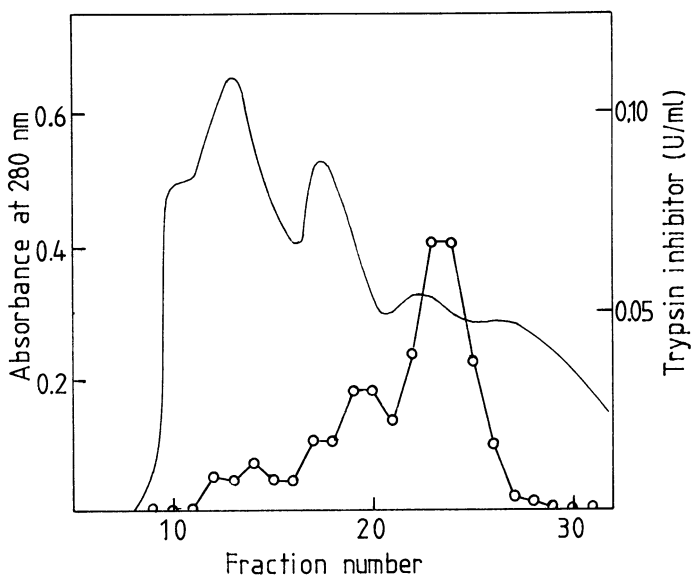

Fig. 1. Chromatography of a concentrated rye extract on Sephadex G-75. —— = Absorbance at 280 nm, o——o = trypsin inhibitor activity.

SDS-gel electrophoresis, one zone, with a molecular weight of about 12,000, was found (Fig. 3). Isoelectric focusing also gave only one zone. The location of the zone corresponds to a pI of about 9.0 (estimations higher than 8.5 are not accurate in the procedure used).

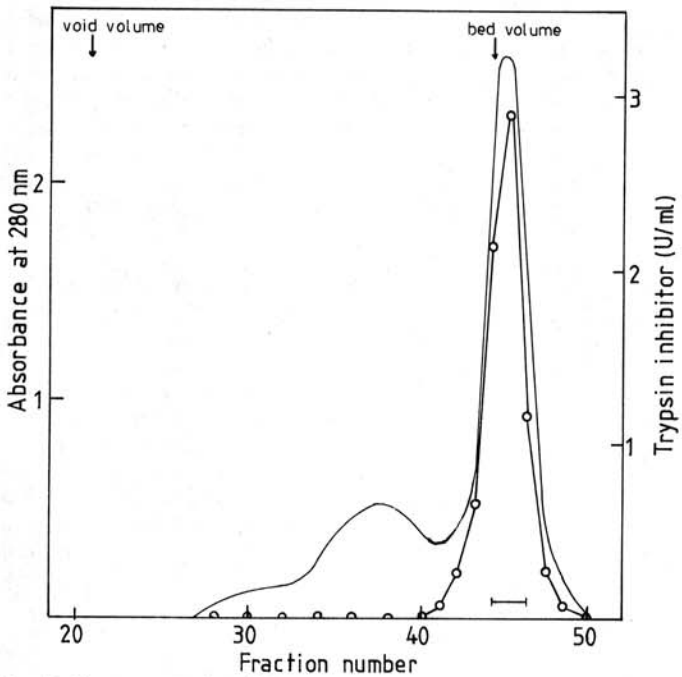

Fig. 2. Chromatography of the partly purified rye trypsin inhibitor on Ultrogel AcA 54. —— = Absorbance at 280 nm, o——o = trypsin inhibitor activity. Active top fractions used for further studies are indicated by a bar at the base line.

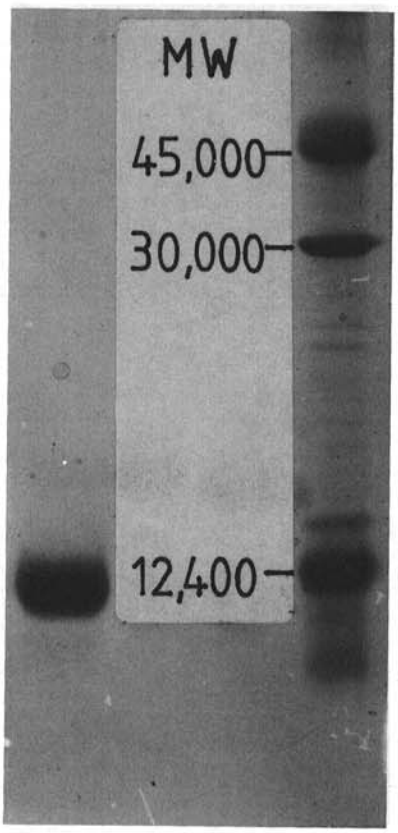

Fig. 3. Left, sodium dodecyl sulfate-gel electrophoresis of the purified rye endosperm trypsin inhibitor. Right, molecular weight standards: upper, ovalbumin; intermediate, carboanhydrase; lower, cytochrome C.

The results of the amino acid analyses of the purified inhibitor (Table II) were expressed as residues per two residues of lysine. The total number of residues calculated from the nearest integer was 114, and the formula weight was 12,570, which was in good agreement with the value found by SDS-gel electrophoresis. Search for free sulfhydryl groups in the inhibitor gave a negative result, indicating that the inhibitor does not contain cystein. The analytical finding of eight half-cystine groups in the hydrolysate (Table II) is interpreted as indicating four disulfide bridges in the intact molecule.

The amino acid composition of the endosperm trypsin inhibitor (Table II) differs greatly from that of the embryo trypsin inhibitor isolated by Hochstrasser and Werle (1969). The amino acid composition of the inhibitor isolated by Polanowski (1974) was not

TABLE II
Amino Acid Composition of Rye Trypsin Inhibitors

	Trypsin Inhibitor from		
	Endosperm		
Amino Acid	Residues per mole[a]	Nearest Integer	Embryo[b]
Aspartic acid	6.7	7	15
Threonine	6.9	7	11
Serine	4.2	4	17
Glutamic acid	11.3	11	12
Proline	10.8	11	20
Glycine	10.2	10	7
Alanine	8.1	8	12
Half-cystine	8.2[c]	8	24
Valine	8.8	9	7
Methionine	3.5	4	2
Isoleucine	3.5	4	5
Leucine	10.4	10	2
Tyrosine	4.7	5	3
Phenylalanine	1.9	2	5
Histidine	2.8	3	1
Lysine	2	2	10
Arginine	7.4	7	15
Tryptophan[d]	2.0	2	...
Total		114	168
Molecular weight		12,570	18,282

[a]The composition was calculated by extrapolation of or from the average values estimated on samples after 24 and 72 hr of hydrolysis.
[b]Values from Hochstrasser and Werle (1969).
[c]Determined as cysteic acid.
[d]Method of Beaven and Holiday (1952), as modified by Wetlaufer (1962).

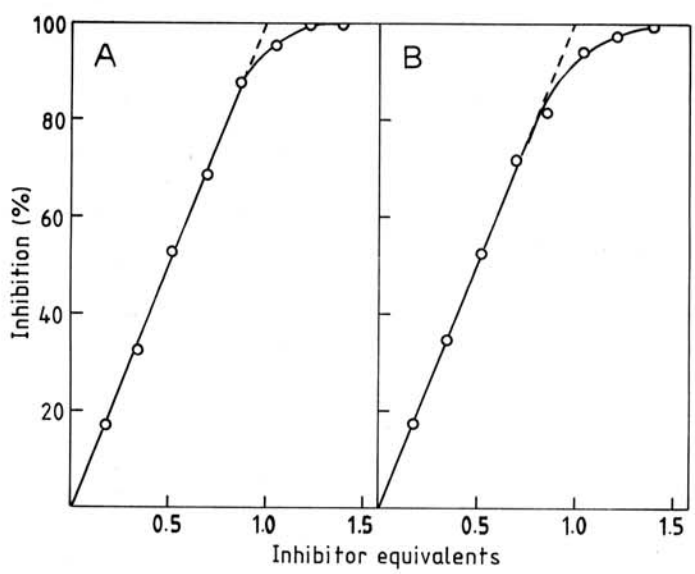

Fig. 4. Inhibition of trypsin by rye trypsin inhibitor. Hydrolysis of benzoyl-*l*-arginine-*p*-nitroanilide at pH 8.2 (**A**) and of casein at pH 7.6 (**B**) by bovine trypsin.

reported.

The effects of the purified inhibitor on the hydrolysis of l-BAPA and casein by pure bovine trypsin are shown in Fig. 4A and B, respectively. The inhibitory action of trypsin with l-BAPA as substrate is seen to be both linear and stoichiometric up to 90% inhibition. The same is the case with casein as substrate, but only up to about 80%. One inhibitor equivalent is defined as double the amount of inhibitor necessary for 50% inhibition in the trypsin assay.

The preparation also inhibited bovine chymotrypsin, although this inhibition was weak and nonstoichiometric (Fig. 5). Preincubation of the inhibitor with trypsin (up to double the amount of inhibitor) did not reduce the inhibition of chymotrypsin. The active sites against trypsin and chymotrypsin must therefore be located at different positions in the molecule. No inhibitory effect was observed on elastase, even when incubation was performed using 5 moles of inhibitor per mole of elastase.

The heat stability at 100° C of the pure inhibitor preparation was examined. The inhibitory activity decreased about 5% during the first 5 min but did not decrease further during the next 25 min.

Next to wheat, rye is the most important cereal for bread making (Bushuk 1976). Rye breads made in Denmark contain about 60% rye and are normally baked at 170° C in the oven, but inside the bread, the temperature does not exceed 97° C.[1] Therefore, we can expect to find some of the inhibitor activity remaining in the bread. In the extracts of eight different rye bread types, the inhibitory activity was about 5–15% of the amount present in untreated rye grains.

Specific immunoglobulins against the purified trypsin inhibitor were prepared as described above. Rocket immunoelectrophoresis of the purified inhibitor conducted at pH 8.6 showed only one precipitate that was moving towards the cathode (Fig. 6). This is in agreement with the high isoelectric point of the inhibitor. The figure also shows that with extracts from rye and rye bread, some components are also moving towards the anode. This may be explained by impurities in the preparation used for immunization, but it may also be caused either by the presence of other compounds with the same immunological determinants as the purified inhibitor or by the tendency of the inhibitor to bind strongly to components that run in the "normal" anodic direction.

Tandem crossed immunoelectrophoresis on extract of rye bread and pure rye endosperm trypsin inhibitor (Fig. 7) confirmed the identity of this inhibitor and the trypsin-inhibiting activity in rye bread. However, separate crossed immunoelectrophoresis under identical conditons (Fig. 8) indicated that the inhibitor had to some degree been modified during the baking process, resulting in a lower mobility.

Our extraction procedure at pH 4.9 gave the maximum amount of inhibitor extracted from the bread. More experiments closely simulating the passage of food through the alimentary tract have to be performed to finally evaluate the importance of rye trypsin inhibitors in human nutrition. However, in animal nutrition, rye

[1] Personal communication, T. Jönsson, Schulstad Bread Factory, Glostrup, Denmark.

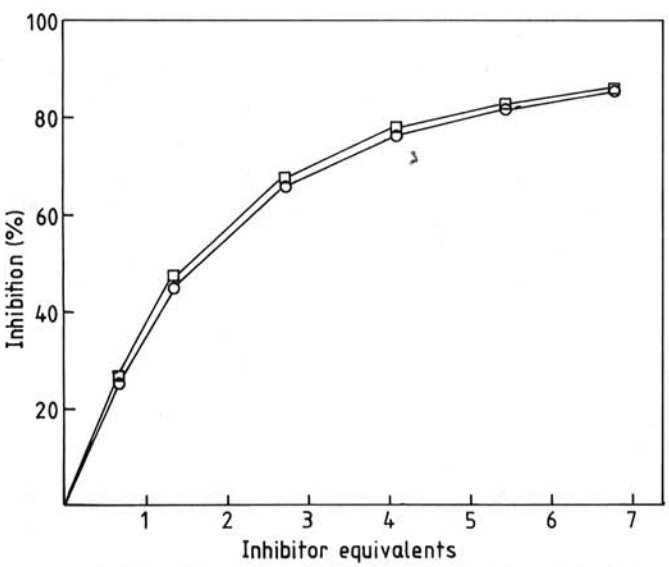

Fig. 5. Inhibition of chymotrypsin in rye trypsin inhibitor. Hydrolysis of glutaryl-*l*-phenylalanine-*p*-nitroanilide at pH 7.6 (-o-o-) and of casein, also at pH 7.6 (□-□).

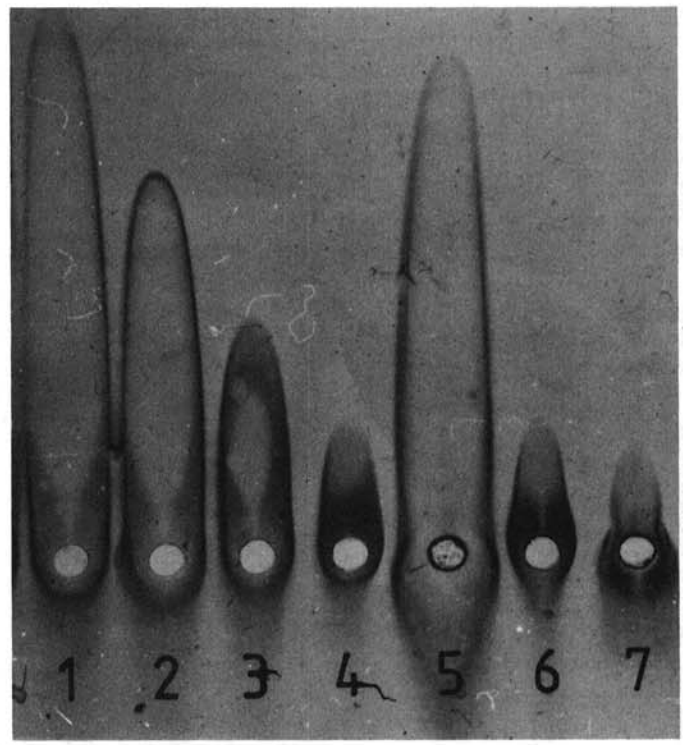

Fig. 6. Rocket immunoelectrophoresis performed at pH 8.6. The following samples (5 μl) were applied: **1–4,** Dilutions of purified rye trypsin inhibitor preparation corresponding to 0.16, 0.12, 0.08, and 0.04 U/ml, respectively; **5,** extract of rye undiluted; **6,** extract of rye diluted five times with buffer; **7,** extract of rye bread (1 g/3 ml 0.1 *M* sodium acetate, pH 4.9).

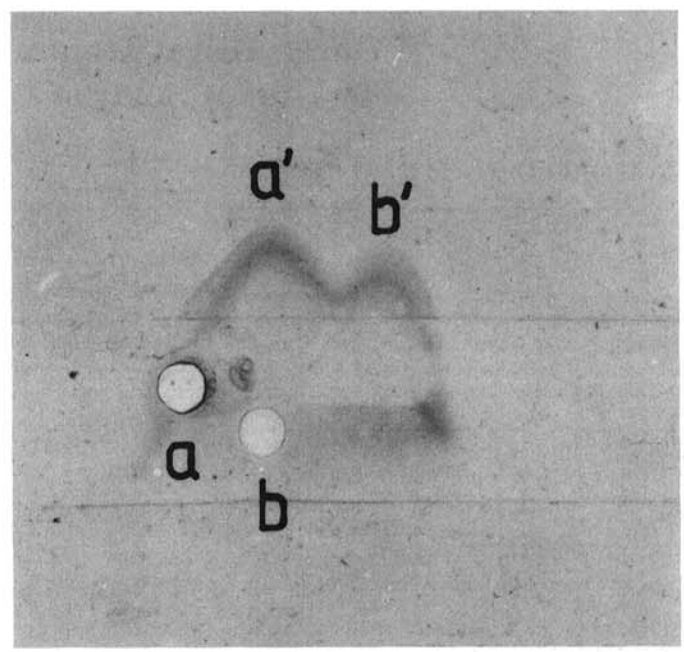

Fig. 7. Tandem crossed immunoelectrophoresis of extract of rye bread and purified rye endosperm trypsin inhibitor. **a** and **b,** application well for the rye bread extract and inhibitor, respectively. **a'** and **b'**, precipitate peaks from a and b, respectively.

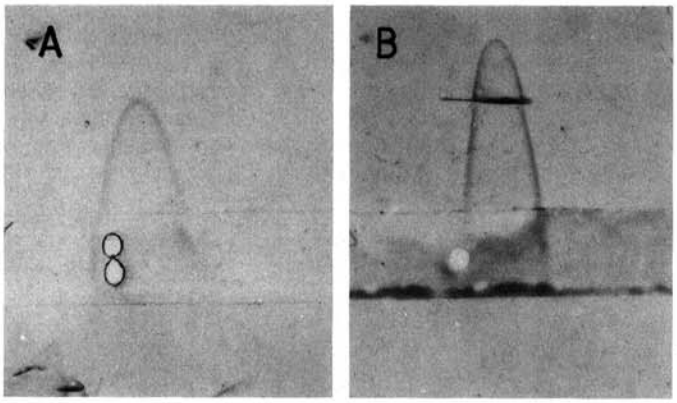

Fig. 8. Crossed immunoelectrophoresis of extract of rye bread (**A**) and of the purified rye endosperm trypsin inhibitor (**B**).

seeds are consumed without any pretreatment, and in this case the rather high content of inhibitor may really cause a lowered protein digestion. Animal feeding experiments using purified inhibitor preparations may also give more definite answers to these questions.

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