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Trypsin inhibitor from starchy endosperm of rye seeds

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Abstract

A method for the preparation of a trypsin inhibitor from starchy endosperm of rye seeds is described. Isolation and purification comprised the following steps: salting out of the water-soluble proteins at pH 4.5 with ammonium sulphate (0.4 saturation), followed by chromatography on CM-Cellulose, Sephadex G-100 and SE Sephadex C-25. The finally purified preparation of inhibitor was found to be homogeneous by both chromatographic and electrophoretic analyses. The preparation inhibited trypsin and chymotrypsin but was inactive against papain, kallikrein and pepsin. The molecular weight of the inhibitor was calculated to be about 10 000.

INTRODUCTION

Since the isolation by Kunitz (1945) of the trypsin inhibitor from soybean seeds, proteins with similar properties have been demonstrated in numerous materials of both vegetal and animal origin (Vogel et al., 1968, Janicki et al., 1970). The common occurrence of proteolytic enzymes inhibitors has prompted investigations for gaining a better knowledge of their physico-chemical and biological properties. In recent years many studies aimed at the elucidation of the influence of these substances on the nutritional value of basic food products of plant origin. Owing to the relation between antiproteolytic activity and the value of fodder produced, based particularly on leguminous plants seeds, these proteins beside antivitamins, haemagglutinins and saponins are considered as factors lowering the nutritional value of plant products (Liener, 1969). Although leguminous seeds are the richest source of trypsin inhibitors, the presence of these compounds has also been demonstrated in other plant species and among them in cereals.

In the seeds of rye antitrypsin activity was also revealed in the endosperm (Polanowski, 1967; Mikola and Kirsi, 1972) and in the embryos (Hochstrasser and Werle, 1969; Mikola and Kirsi, 1972). The former authors isolated from the embryos an inhibitor with molecular weight of about 17 000 and determined its amino acid composition. Three years later Mikola and Kirsi demonstrated that both the embryos and endosperm of rye caryopses contain two inhibitors. One with a molecular weight of about 18 500, probably identical with the inhibitor isolated by Hochstrasser and Werle and another one with a lower molecular weight. In the embryos the inhibitor with molecular weight 18 500 prevails, whereas in the endosperm the quantitative relations of these inhibitors are reversed. The present paper describes the author's own method of inhibitor preparation from the starchy endosperm of rye seeds, a characteristic of this inhibitor is given and some of its properties are described. Moreover, the preservation of the antitrypsin activity of the lyophilized preparation of the inhibitor during storage for 2 years at 2—4°C was checked.

MATERIAL AND METHODS

The investigations were performed on rye caryopses of the variety "Smolickie" from the 1969 and 1970 harvests received from the Institute of Plant Breeding and Acclimatization in Wrocław. The seeds were ground in a Bühler mill; the seed coat and the aleurone layer were separated from the starchy endosperm from which the inhibitor was prepared.

Protein was determined turbidimetrically by the tannin micromethod (Mejbaum-Katzenellenbogen, 1955). Trypsin and chymotrypsin were determined spectrophotometrically at 280 nm in a Carl Zeiss Jena VSU 2P spectrophotometer with the use of the following extinction coefficients: 0.670 for trypsin (Laskowski and Laskowski 1954) and 0.495 for chymotrypsin (Wu and Laskowski, 1955). Antitrypsin activity was measured on casein by the method of Kunitz (1947), on α-N-benzoyl-L-arginine ethyl ester (BAEE) by Brown's method (1960) and α-N-benzoyl-L-arginine p-nitroanilide (BAPA) after Erlanger et al. (1961).

A unit of antitrypsin activity was defined as the amount of inhibitor which inhibited 1 mg of trypsin.

Antichymotrypsin activity was determined by the method of Kakade et al. (1970) and antikallikrein activity after Brown (1960). Papain was determined according to Mejbaum-Katzenellenbogen et al. (1966) using casein as the substrate. The papain solution (100 μg/ml) was preincubated before it was added to the substrate for

20 min in 0.05 M EDTA containing 0.01 M cysteine, pH 6.5, Pepsin activity was measured on haemoglobin denaturated with acid in 0.06 N HCl (W i l u s z, 1968).

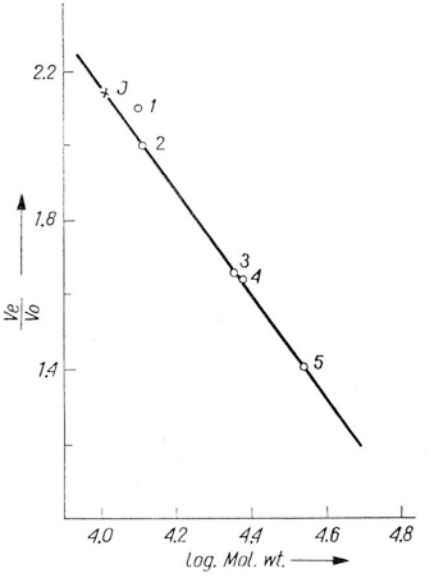

Fig. 1. Determination of molecular weight on Sephadex G-75

On a column of 1 × 80 cm dimensions equilibrated with 0.2 M acetate buffer, pH 4.5, 0.2 ml of 1% protein solution in the same buffer was placed. Fraction of 1 ml were collected at a rate of 30 ml/h.

1 — ribonuclease; *2* — cytochrome C; *3* — chymotrypsin; *4* — trypsin; *5* — pepsin *I* — inhibitor from the starchy endosperm of rye seeds.

The molecular weight of the inhibitor was determined on Sephadex G-75 by extrapolation of the values obtained for standard proteins in the conditions described in the caption to Fig. 1. Tyrosin and tryptophan contents in the inhibitor preparation dried over P_2O_5 were determined spectrophotometrically after B e a v e n and H o l i d a y (1952). Electrophoresis in 15 per cent polyacrylamine gel was run at pH 4.3 according to R e i s f e l d et al. (1962).

R e a g e n t s. Bovine trypsin once crystallized and β-alanin (Koch-Light Lab. Ltd, Colnbrook, Bucks. England); bovine chymotrypsin threefold crystallized (Reanal, Budapest, Hungary); kallikrein (Padutin) (Bayer, Leverkusen, GFR), 10 biological units in one ampoule; white soluble casein; acrylamide and α-N-benzoyl-L-arginine p-nitroanilide (British Drug Houses, Poole, Dorset England); Sephadex G-75, G-100 and SE-C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden); pepsin three times recrystallized (Nutritional Biochemicals Corp., Cleveland, Ohio, USA); carboxymethylcellulose CM 11 (Balston Ltd., Maidstone, Kent, England); N,N′-methylenebisacrylamide and N,N,N′,N′-

-tetramethylethylenediamine (TEMED) (Eastman Kodak N.Y., USA); amide black 10 B (Merck AG, Darmstadt, GFR). The remaining reagents were products of POCH Gliwice.

RESULTS AND DISCUSSION

Inhibitor preparation

The inhibitor was prepared, except where stated otherwise, at room temperature.

1. Extraction: the flour was extracted with distilled water at a 1 : 10 (w/v) ratio with mechanical stirring for 1 h. The mixture was centrifuged for 30 min at 2200 g. The sediment was discarded. Extraction yielded from 1 kg of flour on the average 9.4 g protein with specific activity 0.02 unit/mg.

2. Fractionation with ammonium sulphate. Protein from extract adjusted to pH 4.5 with 2.N HCl in the presence of bromocresol green was salted out with solid ammonium sulphate of 0.4 saturation.

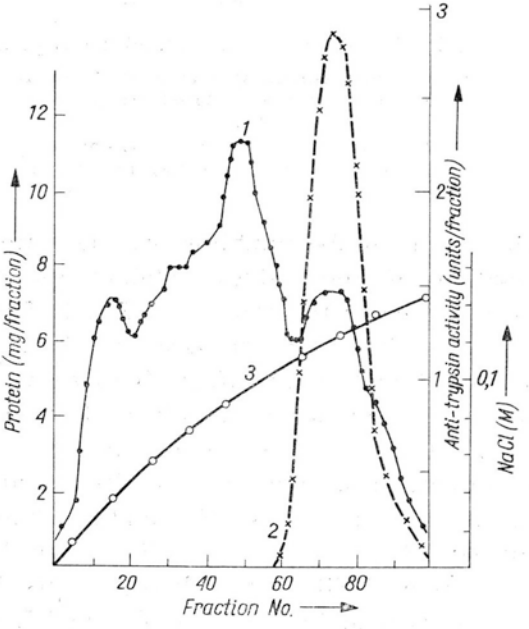

Fig. 2. Chromatography on carboxymethylcellulose

On a 3 × 40 cm column with CM cellulose equilibrated with 0.2 M acetate buffer, pH 4.5 170 ml of solution containing 4.5 g of protein with specific activity 0.04 u./mg were placed. The adsorbed protein was eluted from the column with an increase NaCl concentration in the starting buffer. At an effluent rate of 80 ml/h 14-ml fraction were collected. *1* — protein; *2* — antitrypsin activity determined by Kunitz's method (1947); *3* — NaCl concentration

After 6 h the mixture was centrifuged at 2200 g for 30 min. The protein sediment after washing with ammonium sulphate solution of 0.4 saturation was suspended in water (100 ml/kg flour) and dialyzed at 4°C for 48 h against 30 volumes of the twofold exchanged distilled water, and further for 12 h against 20 volumes of 0.2 M acetate buffer, pH 4.5. After dialysis the insoluble material was removed by centrifugation. The solution contained about one half of the initial amount of protein and almost the entire antitrypsin activity.

3. Chromatography on CM-cellulose. The solution of protein after dialysis was fractionated on a carboxymethylcellulose column equilibrated with 0.2 M acetate buffer, pH 4.5 (Fig. 2). In these conditions about 70 per cent protein containing the entire antitrypsin activity was adsorbed on CM-cellulose. The proteins adsorbed on the carrier, washed with an increasing sodium chloride concentration in the starting buffer separated into 4 fractions. Only the fraction of the fourth peak eluted from the column with 0.11 M NaCl exhibited antitrypsin activity. The protein material of the combined fractions containing the inhibitor was salted out at 0.4 saturation with ammonium sulphate. The salted out protein was suspended in water (5 ml/kg flour) and dialysed at 4°C for 12 h against 0.02 M acetate buffer, pH 4.5. At this stage of preparation 134 mg protein with specific activity 0.325 u./mg were obtained from 1 kg of flour. A 16-fold purification of the inhibitor was achieved in this way with a 23 per cent yield.

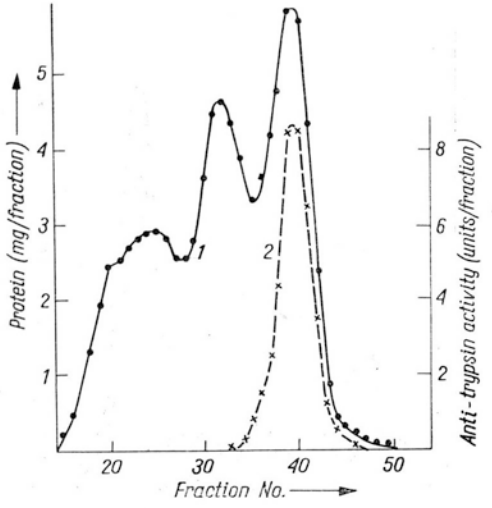

Fig. 3. Chromatography on Sephadex G-100

A 2 × 60 cm columns with Sephadex equilibrated with 0.02 M acetate buffer, pH 4.5. Protein solution (13 ml) containing 43.5 inhibitor units was placed on the column. Protein was eluted from the column with the outset buffer at a rate of 25 ml/h. Three-milliliter fractions were collected.

1 — protein; *2* — antitrypsin activity determined by Kunitz's method

4. Chromatography on Sephadex G-100. For further purification of the inhibitor Sephadex G-100 equilibrated with 0.02 M acetate buffer, pH 4.5, was used. By fractionation (Fig. 3) three well separated protein peaks were obtained. The entire antitrypsin activity was localized in the fraction of the peak eluted latest from the column. The proteins of the combined fractions containing the inhibitor were salted out at 0.4 saturation with solid ammonium sulphate. The protein sediment was suspended in a small volume of water and dialysed at 4°C for 12 h against distilled water, and for the next 12 h against 0.2 M acetate buffer, pH 4.5. The inhibitor preparation thus obtained showed an activity of 1.43 u./mg protein.

5. Chromatography on SE Sephadex C-25. The inhibitor prepared in the preceding step was purified additionally on a SE Sephadex C-25 column equilibrated with 0.2 M acetate buffer, pH 4.5 (Fig. 4). The adsorbed protein was eluted with a rising sodium chloride concentration in the outset buffer. In the elution profile a symmetric protein peak was obtained practically including the entire antitrypsin activity. The proteins of this fraction were salted out as previously, and after 48-h dialysis at 4° against four times exchanged distilled water, lyophilized.

By the method of isolation above described 18 mg of 110 times purified inhibitor as compared to the initial activity were obtained from 1 kg of flour in a 21 per cent yield.

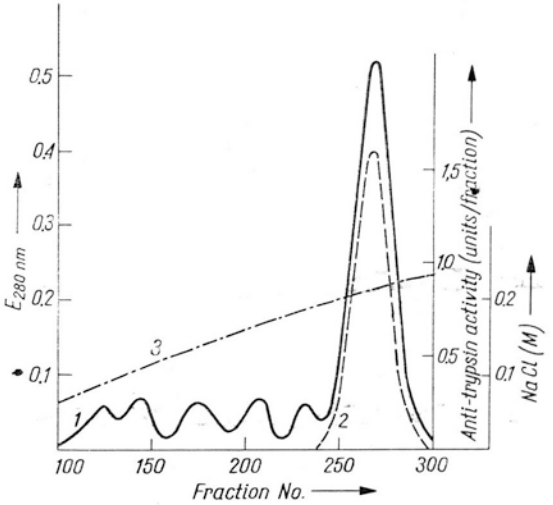

Fig. 4. Chromatography on SE Sephadex G-25

A 1.8 × 50 cm column with SE Sephadex equilibrated with 0.2 M acetate buffer, pH 4.5. Protein was eluted by a continuous NaCl gradient in the same buffer at a rate of 50 ml/h. Three-milliliter fractions were collected.

1 — absorption at 280 nm, *2* — antitrypsin activity, *3* — NaCl concentration

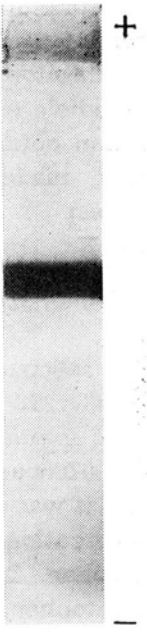

Photo 1. Discelectrophoresis of the inhibitor on 15% polyacrylamide, gel at pH 4.3

Protein (50 μg) was separated at a current intensity of 5 mA per tube. The gel was stained for protein with amide black. The excess of the stain was removed with a 7% acetic acid solution and after decoloration photographs were taken according to the method described by Oliver and Chalkley (1971)

The preparation proved to be homogeneous in electrophoresis in 15 per cent polyacrylamide gel, pH 4.3 (Photo 1).

The particular steps of purification are shown in Table 1.

Mikola and Kirsi (1972) demonstrated by extraction of the endosperm of rye seeds with acetate buffer, pH 4.9, and further adsorption of the extract proteins on CM cellulose and chromatography

Table 1

Preparation of inhibitor from starchy endosperm of rye

Step of preparation	Protein mg/kg flour	Activity u./kg flour	Specific activity u./mg protein	Yield, %	Degree of purification
Extraction	9400,0	188,0	0.020	100	1,0
Fractionation $(NH_4)_2SO_4$ and dialysis	4500,0	180,0	0.040	96	2,0
Chromatography on CM-cellulose	134,0	43,5	0,325	23	16,2
Chromatography on Sephadex G-100	28,8	41,2	1,430	22	71,5
Chromatography on Sephadex C-25	18,0	39,6	2,200	21	110,0

on Sepahadex-75, the presence of two trypsin inhibitors differing in molecular weight. The inhibitor isolated from starchy rye endosperm in the present study corresponds probably to the inhibitor of lower molecular weight of Mikola and Kirsi from the whole endosperm. Additional chromatography, however, of the preparation obtained after molecular filtration on a SE Sephadex C-25 column made possible a higher degree of purification (from 71.5 to 110 times).

Physico-chemical properties

An aqueous solution of the inhibitor gives in UV a spectrum typical for protein with minimum at 250 and maximum at 278 nm. The absorption ratio 280/260 is 2.0 and $E^{1\%}_{278}$ is 8.61. The extinction coefficients of the inhibitor in 0.1 N NaOH are K_{280} — 1.0705 and K_{294} — 1.0706. With the use of Heaven and Holiday's equation it was calculated from these values that 1 g of the inhibitor preparation contains 0.3523×10^{-3} M tyrosin and 0.0995×10^{-3} M tryptophan. In the inhibitor molecule the molar ratio of tyrosin to tryptophan is 3.54:1. The inhibitor is an alkaline protein, it does not dialyse through cellophane membranes, it is precipitated by 5 per cent trichloroacetic acid and does not contain sugars. Heating of the inhibitor at 80°C for 30 min. in water solution as well as in 0.01 N HCl does not causes its inactivation. The thermolability of the antitrypsin activity of aqueous extracts from rye flour demonstrated in the previous paper (Polanowski, 1967) was probably due to coprecipitation of the inhibitor with other proteins after heating. Storage of the lyophilized inhibitor preparation at 2—4°C for two years did not produce any noticeable changes in its activity.

Antiproteolytic properties

The influence of the inhibitor on trypsin activity towards casein is shown in Fig. 5. It results from the diagram that 1 μg of trypsin is inhibited by 0.45 μg of the inhibitor preparation. The inhibitor does not require preincubation for forming an inactive complex with trypsin. If we assume that the inhibitor forms stoichiometric complexes with trypsin, it may be calculated that its molecular weight is about 10 600, this being in agreement with the value obtained after molecular filtration.

Esterase and amidase activity of 1 μg trypsin is inhibited by 0.5 μg of the inhibitor preparation (Fig. 6). The inhibitor also inhibits chymo-

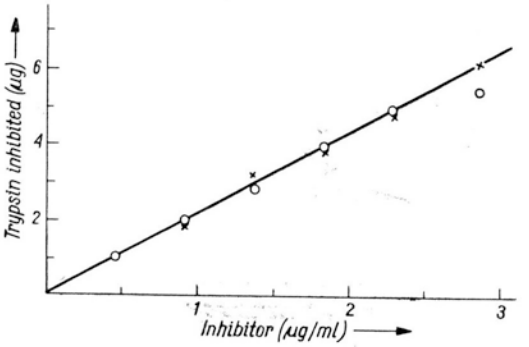

Fig. 5. Influence of inhibitor on trypsin activity

Activity determined by the method of Kunitz, incubation mixture contained in 1 ml: 5 mg casein, increasing inhibitor amounts and 5.6 (O-O), or 11.2 μg of trypsin (×-×)

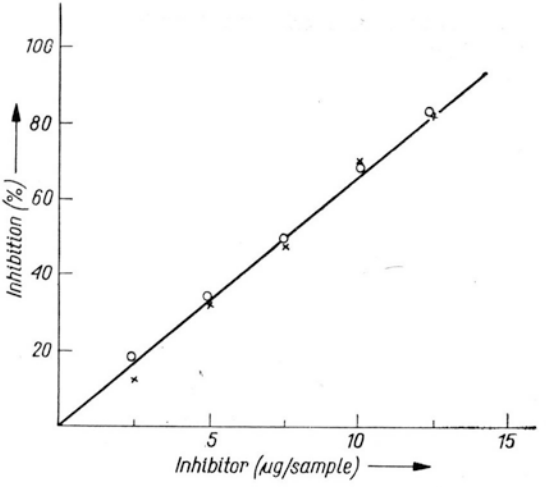

Fig. 6. Influence of various inhibitor concentration on esterase and amidase activity of trypsin

Esterase activity was determined after Brown (1960). Incubation mixture consisted of 2 ml 0.01 M BAEE solution in 0.05 M veronal buffer, pH 7.8, 0.02 M $CaCl_2$ and 0.5 ml of solution containing 30 μg trypsin and increasing amounts of the inhibitor in the buffer. Amidase activity was determined after Erlanger at al. (1961) on BAPA in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$. The incubation mixture (2 ml) contained 30 μg trypsin and increasing amounts of the inhibitor. The amount of p-nitroanilin formed was determined spectrophotometrically at 410 nm.

O-O — esterase activity; ×-× — amidase activity

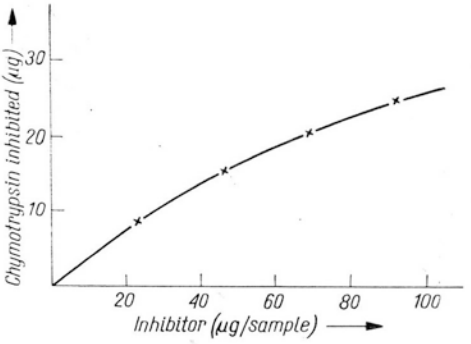

Fig. 7. Influence of inhibitor on chymotrypsin activity

Incubation mixture (4 ml) contained 20 mg casein in 0.1 M borate buffer, pH 7.6, increasing amounts of the inhibitor and 44 μg of chymotrypsin

trypsin activity. This inhibition is of nonlinear character (Fig. 7). The antitrypsin activity of the inhibitor is several times higher than that against chymotrypsin. The inhibitor does not, however, inhibit papain, kallikrein and pepsin.

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Inhibitor trypsyny z części bielmowej endospermy ziarniaków żyta

Streszczenie

W wyniku frakcjonowania wyciągów wodnych z części bielmowej ziarniaków żyta przy pomocy siarczanu amonu, chromatografii na CM-celulozie, sefadeksie G-100, i SE sefadeksie C-25, otrzymano preparat inhibitora trypsyny 110 razy oczyszczony w stosunku do aktywności wyjściowej z wydajnością 21%. Preparat hamuje trypsynę i chymotrypsynę, nie hamuje natomiast papainy, kalikreiny i pepsyny. Ogrzewanie inhibitora w 80° przez 30 minut zarówno w roztworze wodnym jak i w 0,01 N HCl nie powoduje jego inaktywacji. Preparat okazał się homogenny w elektroforezie w żelu poliakrylamidowym w pH 4,3. Inhibitor jest białkiem o c. cz. około 10 000. Stosunek absorpcji 280/260 wodnego roztworu inhibitora wynosi 2,0 a $E^{1\%}_{278}$ — 8,61. Stosunek molowy tyrozyny do tryptofanu wynosi 3,54 : 1.