PURIFICATION AND SOME PROPERTIES OF A PROTEASE FROM PEA SEEDS, *PISUM SATIVUM* L, S.SP. *ARVENSE* A. AND G.

R. SOEDIGDO AND M. GRUBER

Laboratory of Biochemistry, University of Groningen (The Netherlands)*

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SUMMARY

The purification of a protease from pea seeds (about 350-fold) is described and some properties of the purified enzyme for which the name arvensin is proposed are discussed.

The enzyme acts optimally on casein at pH 8.0; it is inhibited by some salts of heavy metals, but not by iodoacetamide.

It somewhat resembles trypsin. The specific activity of the most pure preparation is about a quarter of that of crystalline trypsin.

INTRODUCTION

During germination seed proteins are broken down into smaller compounds (amino acids and/or peptides) which are then transferred to the growth zones. Proteolytic enzymes from seeds have, however, to our knowledge, not been extensively studied. The degree of purification achieved was low and consequently the resulting preparations were of low activity per unit of protein. The present paper describes the isolation and extensive purification of a protease from pea seeds and some of its properties.

MATERIALS

In all experiments the same batch of non-germinated pea seeds (*Pisum sativum*, s.sp. *arvense* A. and G., race "*aureool*") was used. The seeds were stored in a cool place in the laboratory for more than two years. While the shape, colour and texture showed some changes, the protease content was not appreciably diminished during this period.

The casein (Hammarsten) used was a commercial preparation (Merck; 12.93 % *N* by Kjeldahl method). For use as the substrate, it was always suspended in slightly alkaline water or in buffer solutions, heated at 100° for 30 min with occasional stirring, and then cooled. It was dissolved completely by this treatment. If necessary the solution obtained was adjusted to the appropriate pH. The casein solution could be stored in a refrigerator at 1–4° for at least one week.

Bovine serum albumin was obtained from Poviet Producten N.V., Amsterdam,

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The Netherlands. Its protein content was determined by Kjeldahl analysis, assuming a nitrogen content of 16.0 %.

Hemoglobin was prepared in our laboratory from fresh beef blood by the method of M. L. ANSON[1].

The Soy bean trypsin inhibitor (5 × crystall.; Nutritional Biochemicals Corp.) used was found to be highly active against trypsin[2].

Crystalline trypsin was obtained from the Armour Laboratories (Lot No. S92009, 2910 units/mg).

All reagents used were of analytical grade.

Acetone was purified by treatment with $KMnO_4$ and distillation.

METHODS

Determination of activity

Unbuffered 1 % casein solutions were always used, except where mentioned otherwise.

The proteolytic activity was measured by the spectrophotometric determination of the TCA-soluble fraction after enzyme action in the following way. A mixture of 5 ml of 1 % casein solution of pH 8.0 and 1 ml of a solution of EDTA (0.002 *M* final concentration) adjusted to pH 8.0 was incubated at 38°. After 10 min 2 ml of the enzyme solution were added. 20 min after the addition of the enzyme, 5 ml of an ice-cold 8 % TCA solution were added to the reaction vessel and the mixture well shaken and left at room temperature for 30 min. The precipitated protein was removed by filtration through Whatman No. 3 filter paper and the optical density of the clear filtrate measured in a Zeiss PMQ II spectrophotometer at 276 mμ. Zero time and reaction determinations were performed in duplicate.

The proteolytic activity towards casein was expressed in arbitrary units. One unit was defined as that amount of enzyme, which under the assay conditions used, caused an increase in the optical density of the filtrate of 1.000 at 276 mμ and 1 cm light path.

The specific activity was expressed as units/mg of protein as determined by the ROBINSON–HOGDEN modification of the biuret method[3] or by LOWRY's method[4] when the protein concentration of the solution was lower than 0.5 mg per ml. Bovine serum albumin was always used as a standard.

EXPERIMENTAL RESULTS

Purification

The purification procedures developed consisted of the following steps:

Preparation of acetone powder: In order to obtain a stable starting material, free from most of the lipids, pigments and other interfering substances, acetone powder was specially prepared.

The seeds were soaked in excess of deionized water for 24 h at 2°, then homogenized in a Waring blendor in a cold room (2°) with 20 volumes of cold acetone (—20°), followed by immediate filtration by suction. The yellow-brownish powder obtained was freed from adhering acetone in a vacuum desiccator. From 1 kg of seeds about 700 g powder was obtained.

Extraction of the enzyme: 700 g of acetone powder were stirred slowly at 2° for 2 h with 3500 ml of a solution of pH 7.0 containing 0.2 *M* NaCl and 0.001 *M* EDTA. The suspension was filtered through a double layer of cheese cloth and the resulting filtrate was centrifuged at 2° for 50 min at 2000 r.p.m. (radius 25 cm) in an International Centrifuge Model PR-2, head No. 276a. The yellow-brownish supernatant (2400 ml) was decanted (A) and the residue discarded.

Isoelectric precipitation: To solution A, 0.05 *M* HCl (*ca.* 130 ml) was added slowly at 2° with careful stirring to avoid foaming to bring the pH to 6.0. The suspension was centrifuged at 2° and the resulting clear supernatant collected. The precipitate (after redissolving in phosphate buffer of pH 7.0) did not show any proteolytic activity and was discarded (B). The supernatant obtained was adjusted to pH 5.0 by adding again 0.05 *M* HCl (*ca.* 330 ml needed). After centrifuging, the resulting precipitate was redissolved in the minimum volume of phosphate buffer of pH 7.0 at 2° and thus solution C (550 ml) was obtained. The phosphate buffer used contained 0.2 *M* NaCl, 0.03 *M* Na_2HPO_4 and 0.02 *M* NaH_2PO_4[5,6]. On further purification of the supernatant by changing the pH, fractions D and E were obtained which exhibited little and no activity respectively.

First fractionation with acetone: To solution C (20 mg protein/ml) cold acetone (—20°) was added until a final concentration of 15 % (v/v).

TABLE I

PURIFICATION OF PROTEASE

700 g acetone powder were used as starting material. For the determinations and a definition of the activity units, see the "Materials" and "Methods" sections.

Fraction	*Total volume (ml)*	*Protein*		*Activity*		*Specific activity*	*Purification*	*Recovery (%)*
		per ml (mg)	*total (mg)*	*per ml (units)*	*total (units)*			
A (crude extract)	2400	12.2	29280	0.153	367.2	0.013	1	100
B (pH 6 precipitate after redissolving)	490	18.0	8820	0	0	0	0	0
C (pH 5 precipitate after redissolving)	550	19.8	10890	0.580	319.0	0.029	2.2	87
D (pH 4 precipitate after redissolving)	350	4.5	1575	0.063	22.1	0.014	1.1	6
E (pH 4 supernatant)	3650	1.6	5840	0	0	0	0	0
F 0–15 % acetone precipitate (after dialysis and redissolving)	465	22.0	10230	0.075	34.9	0.003	0.2	9
G 15 % acetone supernatant (after dialysis)	745	0.48	357	0.323	240.6	0.673	52	66

During this operation, the temperature of the solution was maintained at or somewhat below —2°. The precipitate after centrifuging at —2° was discarded (F) and the clear supernatant was dialyzed against deionized water at 2° for 30 h, and a clear solution G (745 ml) was obtained.

Lyophilization: As the protein concentration of fraction G appeared to be too low to be suitable for further purification with acetone, the solution was lyophilized. This procedure unfortunately caused some loss of activity, but a stable product was obtained, which could be purified further. The lyophilization product is stable for months when stored in well-closed bottles in a deep-freeze cabinet (—20°). It contained about 15–20% of protein.

Second fractionation with acetone: This was carried out with only part of the powder obtained by freeze-drying. A solution of the lyophilized product was prepared in deionized water with a concentration of 4.5 mg of protein/ml (I). Cold acetone (—20°) was added to give a final concentration of 30 % (v/v) in the same manner and the mixture was centrifuged as described before. The inactive precipitate was discarded (II). More cold acetone was added to the supernatant to give a concentration of 50 % (v/v). The precipitate obtained after centrifugation showed only slight activity after dialyzing (III). Again cold acetone was added to the supernatant to a final concentration of 75 % (v/v). After centrifuging the suspension both precipitate and supernatant were dialyzed for 36 h at 2° (fractions IV and V respectively).

TABLE II

FURTHER PURIFICATION OF PROTEASE

As the starting material part of the lyophilized fraction G (see Table I) containing 54 mg protein, was used. The values between brackets in the columns "Purification" and "Recovery" are expressed relative to the crude extract (Fraction A) and thus show total purification and recovery respectively.

Fraction	*Total volume (ml)*	*Protein*		*Activity*		*Specific activity*	*Purification*	*Recovery (%)*
		per ml (mg)	*total (mg)*	*per ml (units)*	*total (units)*			
I redissolved lyophilized fraction G	12	4.5	54.0	2.740	32.9	0.61	1 (47)	100 (60)
II precip. 0–30 % acetone, dialyzed;								
supernatant	14	0	0	0	0	0	0	0
precipitate	13	1.7	22.1	0	0	0	0	0
III precip. 30–50 % acetone, dialyzed;								
supernatant	9	0.5	4.5	0.045	0.4	0.09	0.1 (5)	1
precipitate	5	1.1	5.5	0.225	1.1	0.21	0.3 (14)	3
IV precip. 50–75 % acetone, dialyzed	11	0.35	3.9	1.540	16.9	4.40	7.2 (338)	51 (31)
V 50–75 % acetone supernat.; dialyzed	73	traces	traces	0	0	0	0	0

Fraction IV showed the highest activity of all the fractions. The purification was about 7- and 350-fold relative to fraction I and the crude extract A respectively.

The results of the various steps are described in Table I and II.

Properties

Effect of variation in enzyme concentration on protein breakdown: The breakdown of casein by partial purified pea seeds protease (fraction G) of varying concentration is illustrated in Fig. 1. The casein concentration was maintained constant. A linear relationship was obtained. This was not the case, however, when the crude extract A was used, probably owing to the simultaneously occurring splitting of the seed proteins by the enzyme.

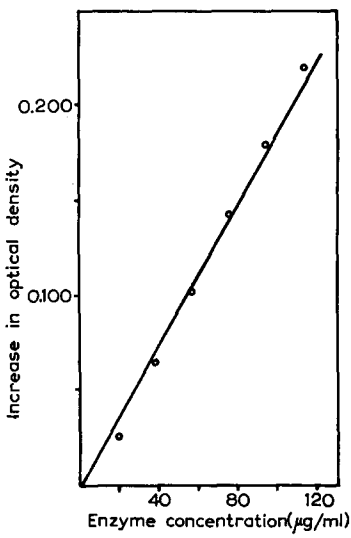

Fig. 1. Effect of enzyme concentration on casein breakdown. Activity determinations were carried out as described in "Methods" section, except without the addition of EDTA. The enzyme concentration was expressed in μg enzyme protein per ml final concentration.

Effect of pH: The pH dependence of the enzymic activity is shown in Fig. 2. The pH optimum appears to be at pH 8.0 and the curve is relatively symmetrical. Measurements below pH 6 were not possible as the enzyme and the substrate precipitated.

Effect of temperature on reaction rate: The rates of protease activity at 20°, 30° and 40° respectively were compared as is shown in Fig. 3.

Substrates: Besides casein, hemoglobin and serum albumin were broken down by this enzyme. In the case of hemoglobin, the enzyme acted upon the native as well as on the heat-denatured form, while with serum albumin only the denatured protein was split to a measurable degree. Another native protein, soy bean trypsin inhibitor, was also attacked by the enzyme (see following section).

In a search for a synthetic substrate, benzoyl-argininamide (0.05 *M*), glycyl-phenylalaninamide (0.05 *M*) and glycyl-glycine (0.05 *M*) were tried, but were not split within 2 h by the most purified enzyme preparation in high concentration (400 μg of enzyme protein/ml of test solution).

Inhibitors and activators: In the following experiments casein solutions were used

as the substrate. Salts of heavy metals, notably $CuCl_2$, $HgCl_2$, $FeCl_2$ and $MnCl_2$ inactivated the enzyme, but $FeCl_3$ had no inhibitory effect. The inactivation by $CuCl_2$ could be abolished by addition of EDTA. However, this substance could not restore activity after inactivation by $FeCl_2$ and $HgCl_2$.

Iodoacetamide (IAA) a well-known inhibitor of SH-enzymes, had no significant effect, even when the enzyme was pre-incubated with the inhibitor (3×10^{-2} *M* IAA)

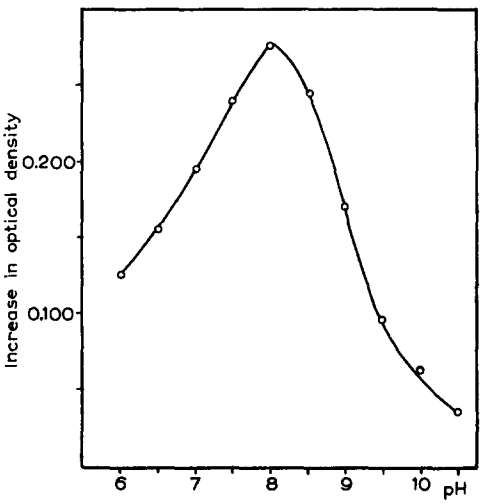

Fig. 2. Effect of pH on casein breakdown. The activity was determined in 1 % unbuffered casein solutions the pH of which was adjusted with *N* HCl or *N* NaOH. The pH was measured by a glass electrode (Radiometer pH meter 24) and remained constant within 0.1 pH units during the enzymic digestion. Blank determinations were carried out at all pH values. A solution of the lyophilized product was used as the enzyme solution (final concentration 63 μg enzyme protein/ml). Activity determinations were carried out without the addition of EDTA.

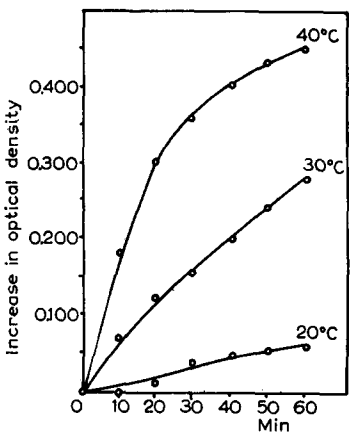

Fig. 3. Effect of temperature on reaction rate. For activity determinations see the "Methods" section. A solution of the lyophilized fraction G was used as the enzyme source (final concentration 63 μg enzyme protein/ml).

at 25° for 30 min before being added to the substrate. On the other hand L-cysteine had a remarkable inhibitory effect, which increased with increasing cysteine concentration.

As the enzyme showed some resemblance to trypsin, and some beans contain inhibitors of tryptic proteolysis, the effect of crystalline soy bean trypsin inhibitor on the enzyme was investigated. The inhibitor which appeared to be highly active against trypsin, did not only show no inhibition of the protease, but even somewhat activated it when present in 0.01 % final concentration (twice the amount of enzyme protein present). On further examination it was found that soy bean trypsin inhibitor was split by the enzyme to form TCA-soluble, u.v.-absorbing substances more quickly than casein under the experimental conditions used. It should be noted that this inhibitor is quite stable towards denaturation under these circumstances.

The influence of some buffers were also examined when used as solvents for casein. Casein dissolved in phosphate–borate–citrate buffer of pH 8.0[7] was split less than an unbuffered casein solution. The most suitable buffer was a 0.05 *M* veronal–HCl buffer[8]. Casein solutions in this buffer were digested more quickly than the unbuffered

TABLE III

EFFECT OF SOME SUBSTANCES ON THE PROTEASE ACTIVITY TOWARDS CASEIN

For experimental conditions, see "Materials" and "Methods" sections. The phosphate–borate–citrate buffer contained 0.0100 *M* H_3PO_4, 0.0114 *M* H_3BO_3, 0.0067 *M* citric acid, 0.0690 *M* NaOH and 0.0280 *M* HCl.

Added substances and their final concentrations	*Buffer of substrate*	*pH*	*Purity of the enzyme preparation used*	*Enzyme protein concentration per ml of reaction mixture (μg)*	*Relative activity (without addition = 100)*
10^{-4} *M* $CuCl_2$	unbuffered	6.5	58-fold	60	60
$2 \cdot 10^{-4}$ *M* $CuCl_2$	unbuffered	6.5	58-fold	60	32
10^{-3} *M* $CuCl_2$	unbuffered	6.5	58-fold	60	0
10^{-3} *M* $HgCl_2$	unbuffered	7.0	58-fold	60	0
10^{-3} *M* $FeCl_2$	0.05 *M* veronal	7.0	300-fold	70	18
10^{-3} *M* $FeCl_3$	0.05 *M* veronal	7.0	300-fold	50	100
10^{-3} *M* $MnCl_2$	0.05 *M* veronal	7.0	300-fold	75	64
10^{-2} *M* $MnCl_2$	0.05 *M* veronal	7.0	300-fold	75	0
$3 \cdot 10^{-2}$ *M* IAA (pre-incubated)	unbuffered	7.0	58-fold	75	93
10^{-2} *M* L-Cysteine	0.05 *M* veronal	7.0	300-fold	50	80
$2 \cdot 10^{-2}$ *M* L-Cysteine	0.05 *M* veronal	7.0	300-fold	50	10
$4 \cdot 10^{-2}$ *M* L-Cysteine	phosphate–borate–citrate	6.5	58-fold	100	82
$8 \cdot 10^{-2}$ *M* L-Cysteine	phosphate–borate–citrate	6.5	58-fold	100	33
Soy bean trypsin inhibitor (0.01 %)	0.05 *M* veronal	8.0	300-fold	50	170
$2 \cdot 10^{-3}$ *M* EDTA	unbuffered	8.0	58-fold	100	135
$4 \cdot 10^{-3}$ *M* EDTA	unbuffered	8.0	58-fold	100	140
$2 \cdot 10^{-3}$ *M* EDTA	phosphate–borate–citrate	8.0	58-fold	100	100
$2 \cdot 10^{-3}$ *M* EDTA	0.05 *M* veronal	8.0	58-fold	100	100

TABLE IV

INFLUENCE OF BUFFERS ON PROTEASE ACTIVITY TOWARDS CASEIN

For explanation, see Table III.

Buffer used	*pH*	*Purity of the enzyme preparation used*	*Enzyme protein concentration per ml of the reaction mixture (μg)*	*Relative activity (without addition = 100)*
Phosphate–borate–citrate	8.0	58-fold	100	72
0.05 *M* veronal	8.0	58-fold	100	210

substrates. Moreover these solutions appeared to be stable for more than 2 weeks if stored at about 2°.

Tables III and IV show the effects of some inhibitors or activators.

Milk-clotting effect: The lyophilized fraction G (90 μg of enzyme protein/ml test solution) incubated at 38° in the presence of 20 % skimmed milk powder in Tris-buffer (0.05 *M*) of pH 7.5 did not show a noticeable milk-clotting effect within 3 h.

Stability: In general the solution of this enzyme in deionized water at neutral pH could be kept in the frozen state (—20°) for more than two months with only little loss of activity. However, frequent thawing and freezing caused denaturation.

The solution of the enzyme at neutral pH was stable for about one week at 2–4°. It quickly lost activity at pH values below 4 and above 10.

The enzyme was rather sensitive towards heat. Incubation of the purified enzyme solution at 38° for 20 min in the absence of substrate, caused an activity loss of about 60 %. Addition of protein substrate stabilized the enzyme towards increase in temperature. This effect will be investigated further.

Storage of the enzyme solution in the presence of toluene caused a considerable loss of activity.

DISCUSSION

The experiments have shown that it is possible to purify a protease from seeds about 350-fold, compared to the crude extract, by the repeated use of isoelectric precipitation and acetone fractionation. As the crude extract contains only a quarter of the total seed protein, the protease described forms about 0.05 % of the total protein or less. Its spatial distribution in the seeds has not yet been investigated, but will be considered in further studies.

Some aspects of the purification deserve comment.

It appeared necessary to use acetone powder as the starting material. Acetone apparently removes substances, probably of lipid nature, which interfere with the purification. It also dissolves part of the colouring material which can interfere with spectrophotometric measurements.

The lyophilization step, though time-consuming, was also necessary in order to obtain a stable product.

It is of advantage to carry out the second acetone fractionation in the stepwise manner described. Direct fractionation by adding acetone in one step until 50 % (v/v) yielded products with appreciably lower specific activities. This phenomenon is still unexplained; it might be due to protein–protein interaction.

A combination of the procedure described with ammonium sulfate fractionation did not give satisfactory results. Fractionation of the crude extract with ammonium sulfate yielded most of the enzyme activity in the 40 % saturation precipitate, but caused heavy losses of activity.

Calcium gel adsorption methods have also proved unsuccessful up-to-now.

Further purification of the enzyme will be attempted by ion-exchange chromatography. We have no unambiguous indications about the state of purity of the preparation obtained. Paper electrophoresis did not appear to be suitable.

For an explanation of the curious effects of some metals further experiments will be necessary. We therefore prefer to leave a discussion of them to a later publication.

Preliminary qualitative experiments have shown that the products of the enzymic splitting of casein are mainly or only peptides. This provides evidence that the enzyme is of the endopeptidase type and not *e.g.* an aminopeptidase. These experiments are being continued to obtain more quantitative information.

A comparison of the properties of this enzyme with the more purified plant proteases described, shows that it is different from papain in many respects. It does not possess reactive SH-groups necessary for activity, does not clot milk, is inhibited by cysteine, etc. On the other hand, it quite resembles trypsin in most of its properties and also in its pH dependence*. In order to get some rough idea of its activity in the

* See NOTE ADDED IN PROOF.

purified state it was compared to a preparation of crystalline trypsin. It appeared that per mg of protein this enzyme is about a quarter as active as crystalline trypsin on casein. As the preparation is certainly not yet pure, this fact shows that this plant protease has an activity of the same order of magnitude as the most powerful enzymes purified from animal sources.

In view of the fact, that an enzyme of this type has, to our knowledge, not yet been described, we propose the name arvensin for it. We believe that this enzyme is quite different from that demonstrated in green peas by MERGENTIME *et al.*[9,10] which has a pH optimum of 5.5 acting on casein and is present in the supernatant at pH 4.0. The proteolytic enzyme from yellow pea seeds described by DANIELSSON[10] has a pH optimum between 6.3 and 7.0 depending upon the substrate. Whether it resembles arvensin cannot be decided as other properties have not been described.

It is not yet known how the proteolytic activity in germinating seeds is regulated. According to some workers[10,11] the proteolytic activity increases during germination while according to others it remains constant[12]. It is clear, however, that the enzyme investigated by us, is present in ungerminated seeds. Studies of its level in germinating seeds will be carried out.

NOTE ADDED IN PROOF

The enzyme is not inhibited by diisopropylphosphofluoridate (10^{-3} *M*), even after preincubation for 20 min at 30°.

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