γ-Glutamylpeptidase (Glutaminase) in Germinating Seeds of Chive (*Allium schoenoprasum*)

E. J. MATIKKALA and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

In earlier investigations we have isolated numerous γ-glutamylpeptides from the bulbs and seeds of *Allium*-species.[1] In dormant onion bulb their amount is considerable but they disappear gradually, when the bulb pushes out green leaves. These peptides thus probably function as reserve substances which take part actively in nitrogen metabolism at the beginning of the growth. They are also, after all, derivatives of glutamine which again is both a reserve and an active substance in nitrogen metabolism.

No enzymatic reaction which would explain the decomposition of these peptides in onion bulb has earlier been established conclusively.[2] Weak evidence of hydrolysis and in the presence of pyruvic acid transamination has occasionally been obtained, but not repeatedly. Neither in dormant nor in sprouting onion bulbs and leaves have we found evidence for transpeptidase, though such an enzyme has been observed in plant and aminal tissues. Thompson *et al.*[3] recently demonstrated relatively high transpeptidase activity especially in the seeds and leaves of leguminous plants but confirmed the absence of γ-glutamyltransferase in onion bulbs.

In the seeds of *Allium*-species numerous γ-glutamylpeptides have been found, some of them new, not detected in the bulbs.[1,4] During the germination of the seeds strongly smelling sulphides are formed (dimethyl-S_2, methylpropyl-S_2, dipropyl-S_2, and also acetone and ethanol have been identified by gas chromatography [5]), which demonstrates the enzymatic splitting of different alkyl cysteine sulphoxides by alliinase. In the bulbs the enzymatic reaction takes place only after crushing. γ-Glutamylpeptides were found to disappear in about two weeks in the germinating seeds, so that detectable amounts of enzyme decomposing these peptides could be expected in this material. In preliminary experiments a γ-glutamylpeptidase was observed in the germinating seeds of chive,[6] and the purification and characterization of the enzyme were started.

The purification of the enzyme was performed at 0−3°C, unless otherwise stated. Protein content was determined using the formula: protein content (mg/ml) = $1.55 \times D_{2800} - 0.76 \times D_{2600}$.[7] Determinations of enzyme activity were made in Sörensen's M/15 phosphate buffer pH 7.4, using γ-DL-glutamyl-*p*-nitroaniline (GPNA) as substrate. From this compound which was first used by Orlowski and Meister [8] for estimation of the activity of transpeptidase, *p*-nitroaniline is split off and can be determined spectrophotometrically. The enzymatic reaction was stopped, in our experiments, with abs. ethanol (3.5 ml/1 ml test solution) instead of acetic acid, because the absorption at 4100 Å is greater in the former solvent. The specific activity of proteins = μmole *p*-nitroaniline/mg protein/h.

In preliminary experiments it was found that when chive seeds were germinated for 4.5 days at about 25°C and were then frozen, pulverized at a low temperature, extracted with M/15 phosphate buffer, pH 7.4, dialyzed against tap water, and lyophilized, the dry powder in 0.05 M tris-HCl-buffer, pH 9.0, hydrolyzed GPNA at 37°C. γ-Glutamylisoleucine (isolated from onion bulbs) was also hydrolyzed slightly (formation of isoleucine shown by paper chromatography). In similar experiments with non-germinated seeds, no hydrolysis of GPNA could be found.

It was further found that a powder of germinated seeds could be extracted for 3 min in cold acetone (−10 to −15°C) without loss of enzymatic activity; fat and other substances soluble in acetone could in this way be removed. The extraction was used in all our experiments. Seeds were allowed to germinate and grow for a period, usually, of 6−9 days and were then frozen, pulverized and treated at −10° with a tenfold volume of acetone for 3 min. After filtration and drying *in vacuo* 80 g of the powder extracted with acetone was stirred for 1 h in 1.6 l M/15 phosphate buffer, pH 7.4. The extract (1.4 l) obtained by centrifugation contained 7.74 g protein of specific activity 0.00766 μmole *p*-nitroaniline/mg protein/h.

The extract was saturated with ammonium sulphate, and after 17 h centrifuged. The precipitate was dissolved in M/15 phosphate buffer pH 7.4. The turbid, brown-green solution, 194 ml, contained 6.96 g protein of

specific activity 0.0104. The solution was dialysed for 17 h against M/15 phosphate buffer pH 7.4, and the dialysed solution was diluted to 500 ml with phosphate buffer and centrifuged. The clear supernatant contained 5.18 g protein of specific activity 0.0238.

The proteins were now precipitated from the supernatant by stepwise addition of ammonium sulphate, and fractions from the solutions of 35–65 % and 65–100 % saturation were taken up in phosphate buffer. The solution of the 35–65 % fractions (112 ml) contained 3090 mg protein of specific activity 0.0115 and the solution of the 65–100 % fractions (150 ml) contained 626 mg protein of specific activity 0.0521. Attempts to obtain better fractionation by reprecipitation with ammoniumsulphate were not successful.

Both protein fractions were dialysed overnight against running tap-water at about 4°C and the suspensions were lyophilized. The residues were extracted with 0.01 M tris-HCl-buffer, pH 7.5, and the insoluble material was removed by centrifugation. The extract of the 65–100 % fractions (130 ml) contained 370 mg protein of specific activity 0.155. The extract was fractionated on a DEAE-cellulose column (3.04 × 37.9 cm), equilibrated with 0.01 M tris-HCl-buffer, pH 7.5. The proteins were washed on the column with 40 ml of buffer, after which gradient elution was started, using 1 litre 0.01 M and 1 litre 0.5 M tris-HCl-buffers pH 7.5. Fractions, each of 20 ml, were collected. The activity was found in two groups of fractions. Fractions 7–23 (enzyme preparation A) contained 36.0 mg protein and fractions 39–47 (enzyme preparation B) 43.6 mg (fraction 45 was lost). Protein was precipitated from both solutions by saturating with ammonium sulphate, and the precipitates were dissolved in M/15 phosphate buffer pH 7.4 and dialysed against the same buffer for 17 h. After dialysis the volume of the enzyme A solution was 70.5 ml and the specific activity of the proteins was 0.158. The purification was thus 21-fold. The volume of the enzyme B solution was 36.5 ml (a small amount was lost). The specific activity of the proteins was 1.21, and thus the purification of the enzyme was about 158-fold. The greatest specific activity of the proteins obtained in our purification experiments was 1.44 μmole *p*-nitroaniline/mg protein/h. The purification of the enzyme was in this case more than 200-fold but cannot be exactly calculated because fractions of two different experiments were used for purification.

The tris-HCl-buffer extract of the 50–65 % fractions also was fractionated by gradient elution from a DEAE-cellulose column (3.04 × 35 cm), using the same solutions as before. The highest specific activity of the proteins obtained was only 0.455 (59-fold purification). The stages in the purification of γ-glutamylpeptidase can be seen in Table 1.

The purity of the enzyme preparations was studied electrophoretically. An enzyme solution, containing proteins of specific activity 1.36, was lyophilized, the dry residue was dialysed against distilled water for 69 h, and the protein solution was again lyophilised. Electrophoretic runs were performed with the dry powder using Romero's[9] thin-film-starch-gel method and buffer solutions as described by Poulik,[10] (170 V, 5 mA). Staining with amido black showed three protein zones, the fastest having moved about 9.2 cm towards the anode, the next about 7.7 cm and the slowest, a diffuse zone, about 5.5 cm. The lastmentioned contained γ-glutamylpeptidase. Thus the enzyme preparation still contained more than one protein. This explains why one of our purified preparations had α-peptidase effect and the other not.

Regarding the properties of the enzyme preparation isolated, should be mentioned that a γ-glutamyltranspeptidase effect could not be found in any preparation. This was revealed in systems which contained either γ-glutamyl-isoleucine isolated from onion, L-methionine, enzyme preparation (specific activity 1.4), and phosphate buffer pH 7.4 (also tris-HCl-buffer pH 9.0) or glutathione (GSH), methionine, enzyme preparation (specific activity 1.4) and phosphate buffer pH 7.4 and 7.8. The γ-glutamyl peptides were decomposed only hydrolytically. The formation of glutamic acid and isoleucine in the former system, and glutamic acid and cysteinylglycine but not glycine in the latter system could be demonstrated by paper chromatography. In neither system could γ-glutamylmethionine be shown (reference compound γ-glutamyl-methionine isolated from onion).

The pH optimum of the enzyme preparation B was 8.0 in 0.1 M KH_2PO_4–0.1 M glycine/NaOH-buffer. At pH 5.5 the activity was still nearly 50 % of the maximum and at pH 10 about 25 %. The optimum of both preparations B and A in 0.05 M tris-phosphate/NaOH-buffer was pH 8.0–8.3. In M/15 phosphate buffer the optimum was not below pH 8.0.

The activity was highest in phosphate buffer, and nearly the same in phosphate buffer (0.05 M) after the addition of Na-

Table 1. Purification of γ-glutamyl peptidase.

Stage of purification	Protein mg	Specific activity μmole *p*-nitroaniline/ mg prot./h.
Centrifugation of the extract	7740	0.00766
I $(NH_4)_2SO_4$-precipitation	6960	0.0104
Dialysis and centrifugation	5180	0.0238
II $(NH_4)_2SO_4$-precipitation		
35 % saturation	576	0.00153
65 % »	3090	0.00115
100 % »	626	0.0521
Further treatment of the 65 – 100 % fraction		
Dialysis against tap-water, lyophilization and extraction with 0.01 M tris-HCl-buffer pH 7.5	370	0.155
DEAE-cellulose,		
Fraction A	36.0	0.158
Fraction B	43.6	1.21
Further treatment of the 35 – 65 % fraction,		
50 % $(NH_4)_2SO_4$-precipitation	956 mg	0.00342
100 % » »	2160	0.0108
Further treatment of the precipitate (fraction 50 – 65 %) obtained from the 100 % solution		
Dialysis against tap-water, lyophilization and extraction with 0.01 M tris-HCl-buffer pH 7.5	695	0.0406
DEAE-cellulose		
Fraction A	52.0	0.0627
Fraction B_2	15.3	0.455
Fraction B_3	56.0	0.109

carbonate (0.05 M). Na-borate (0.05 M) in phosphate buffer (0.05 M) inhibits strongly γ-glutamylpeptidase. Thompson *et al.*[3] have noticed the same with γ-glutamyltranspeptidase, but this enzyme is inhibited also by carbonate and the pH optimum is in the range 9 – 10.

No metal salt (0.001 M) investigated was observed to have a clear activating or inhibiting effect, except $HgCl_2$ which caused a complete inhibition. Sodium cyanide and the SH-group inhibitors, iodoacetate and *p*-chloromercury benzoate, did not clearly inhibit the enzyme. Glutamine inhibited the reaction vigorously in all experiments. The inhibition was probably competitive, because the preparation hydrolyses glutamine strongly. Asparagine was not hydrolysed.

The results show that the enzyme responsible for the splitting of γ-glutamylpeptides in germinating seeds of chive hydrolyses the γ-glutamyl bond also in glutamine. The absence of γ-glutamyltranspeptidase in the germinating seeds is somewhat surprising because this enzyme has been shown to occur in many plants, especially in legume tissues, and to be responsible for dipeptide biosynthesis in these plants.[3] Animal tissues also contain active transpeptidase but there are striking differences in the properties of the plant and animal enzymes. The glutaminases of pig kidney[11] and *Allium* have some similarities (*e.g.* pH optimum) but more differences (*e.g.* inhibitors). Kidney preparations seem to have both transpeptidase and glutaminase activity.[12]

The separation of glutaminase on a DEAE-cellulose column into two components (enzymes A and B) may have occurred during the fractionation on ion exchangers.[13] The pH-optimum of the preparations A and B was the same in tris-phosphate buffer. Because of the small amount of the preparation A it was not compared further with the preparation B. In a deepfreeze box both dry powders and frozen solutions of the enzyme preparations retain their activity for months.

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γ-Glutamylpeptidase in Sprouting Onion Bulbs

E. J. MATIKKALA and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Our observations on the function of glutaminase in the germinating seeds of chive [1] have prompted further study on the decomposition of γ-glutamylpeptides in onion bulbs. In these experiments in which γ-DL-glutamyl-*p*-nitroaniline (GPNA) was used as substrate, we have at last been able to show that an enzyme which splits the γ-glutamyl bond hydrolytically is to be found in sprouting onion bulbs. The enzyme seems to be taken up with difficulty by M/15 phosphate buffer pH 7.4, whereas it is obtained from the germinating seeds with this buffer. Hydrolysis is best achieved using crushed onion bulbs at the sprouting stage as the enzyme preparation. An onion (71.5 g) which had been kept in moistened sand for a month and which had green leaves of about 1 cm length (the sprouting was very slow in winter), was frozen, ground finely and dialyzed in M/15 phosphate buffer pH 7.4 for 22 h against the same phosphate buffer. Dialysis was then continued for four hours against M/15 phosphate buffer pH 7.8, after which the outer solution was renewed and dialysis continued a further 16.5 h. 1 ml of dialyzed suspension and the same volume of M/15 phosphate-buffer pH 7.8 were used in the experiment. The amount of GPNA in the test solution was 0.8 mg. The test solutions were kept at 37°C for 3 h, after which 7 ml absolute ethanol was added to each of them. The solution could not be clarified by centrifugation or filtration. The composition of the test solutions and optical densities (4100 Å) are presented in Table 1 (P = phosphate buffer).

The results showed that onion crush had a γ-glutamyl peptidase effect. Because of the turbidity of the solution the experiment could not yet be regarded as satisfactory. Therefore another experiment was performed with the same dialyzed onion crush, which had been stored at −20° for 2 days. In each experiment there were 3 ml of onion crush and 3 ml of M/15 phosphate buffer pH 7.8. The amount of GPNA in the test solution was again 2.4 mg. After 3 h at 37°C the test solutions were centrifuged, without prior precipitation of proteins with ethanol. When the solutions were turbid, they were kept overnight at about −20°, after which centrifugation gave clear solutions which were then filtered through paper. The results are shown in Table 2. On the basis of this experiment it has been definitely proved that active γ-glutamyl-peptidase is found in sprouting onion.

The same crushed onion was used in a further experiment, which was similar to that shown in Table 1 except that the turbid solutions, centrifuged after adding ethanol, were kept overnight at −20°C, after which they were centrifuged and filtered through paper. Clear solutions were then obtained. Also determinations made with heated crushed onion were included in the experiment. The results are shown in Table 3. This experiment confirmed the results given in Table 1.