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Protein deamidase from germinating wheat grains

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A new enzyme catalyzing the deamidation of seed storage proteins was found in germinating wheat grains and was partially purified. It also acts on egg lysozyme, horse hemoglobin and reduced RNAse, glutamine and Gly-L-Gln-L-Tyr. No activity was observed when using ovalbumin, serum albumin, RNAse, insulin, asparagine and an asparagine-containing peptide. Only glutaminyl residues appear to be deamidated by this enzyme. It differs from transglutaminase and proved to be a true protein deamidase.

Protein deamidase; Germinating seed; Wheat

1. INTRODUCTION

The degradation of storage proteins during seed germination is a complex multistage process [1]. Deamidation preceding their complete proteolytic breakdown was observed in germinating seeds of several leguminous species [2–4] and is especially intensive in germinating wheat grains [5]. Up to 35% of amido groups of gluten – the complex of wheat grains protein – is deamidated during the first 3 days of germination [5]. It may be inferred from this that the process of deamidation of storage protein in germinating seeds is of a general nature. The high rate of deamidation is indicative of its enzymatic character.

The objectives of the present work were the detection, partial purification and characterization of the deamidating enzyme from germinating wheat grains. The latter were chosen as the enzyme source due to the highest content of amidated amino acids in their storage proteins, which is suggestive of a higher activity of the deamidating enzyme.

2. MATERIALS AND METHODS

2.1. *Substrates tested*

The following proteins were isolated using the methods described in the references: isoelectrically precipitated soybean globulins [6], soybean 11 S and 7 S proteins [7], kidney bean 7 S protein [8], kidney bean hemagglutinin [9] and gluten [10]. Gly-L-Gln-L-Tyr was a gift from Prof. V.M. Stepanov. Other substrates were obtained from commercial sources: ovalbumin, insulin, lysozyme and bovine serum albumin from Serva (Germany), RNAse from Pharmacia (Sweden), horse hemoglobin, L-glutamine, L-asparagine and DL-Ala-DL-Asn from Reanal (Hungary).

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2.2. *Determination of the deamidase activity*

The incubation mixture consisted of 0.2 ml of substrate solution (3 mg/ml of protein or 0.5 mg/ml of amino acid or peptide) in phosphate–citrate buffer, pH 6.8 and ionic strenght 0.067, and 0.4 ml of enzyme solution. In the control experiments the substrate solution was substituted for 0.2 ml of the buffer. After 30 min incubation at 30°C the reaction was stopped by the addition of 0.5 ml of 4% solution of $Na_2B_4O_7 \cdot 10H_2O$ containing enough NaOH to bring up the pH of the incubation mixture to 10. NH_3 was distilled isothermally into 1 M H_2SO_4 and determined by a variant of the Nessler's method [11]. All the determinations were performed at least in triplicate except in the chromatographic fractions where they were made in duplicate.

Soybean globulin was used as a working substrate during purification and study of the properties of the enzyme. Its natural substrate, gluten, is less suitable for the determination of the activity in vitro because as a result of clumps of gluten at the beginning of incubation, the reaction proceeds slowly and the reproducibility is poor. The enzyme activity was expressed in mU (nmol of NH_3 released per min).

2.3. *Other determinations*

The content of the amide N in the substrate prior to and after incubation with enzyme was determined by measuring NH_3 released by hydrolysis with 2 N HCl [12]. Transglutaminase activity was tested using NH_2OH and protein (soybean 11 S protein or kidney bean 7 S protein) as substrates [13]. Protein determination was carried out by the dye-binding method of Bradford [14] using bovine serum albumin for calibration.

2.4. *Enzyme purification*

Wheat grains (Odesskaya 51 variety) were soaked in distilled water for 6 h, germinated in the dark at 25°C during the specified time (the time of soaking included) and then homogenized with water in a ratio of 3:5 (weight of dry seeds/water volume). After centrifugation a mixture of dry NaH_2PO_4 and K_2HPO_4 in the molar ratio of 83:17 was added to the supernatant to up to 3.7 M concentration. The pH of the solution was in the range of 6.1–6.4. The precipitate was discarded and the supernatant was dialyzed against water, concentrated by ultrafiltration, then introduced into a column of DEAE-cellulose (Whatman DE-32) equilibrated with phosphate buffer, pH 6.8 ionic strength 0.067, and eluted with the same buffer. The most active fractions were combined and concentrated by ultrafiltration where necessary.

In the later experiments the enzyme purification was simplified. The water extraction and subsequent salting-out were replaced by direct homogenization with 3.7 M phosphate.

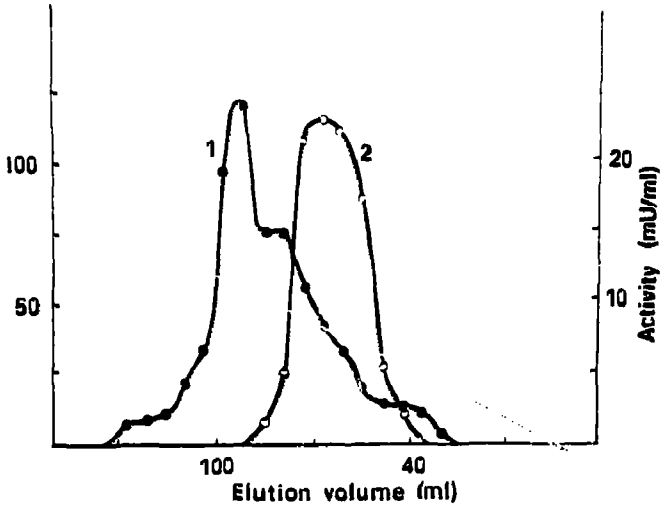

Fig. 1. Chromatography of protein deamidase on DEAE–cellulose. (1) Protein concentration. (2) Enzyme activity. Column 1.5 × 60 cm; elution with phosphate buffer, pH 6.8, ionic strength 0.067; elution rate 20 ml/h; fraction volume 4 ml.

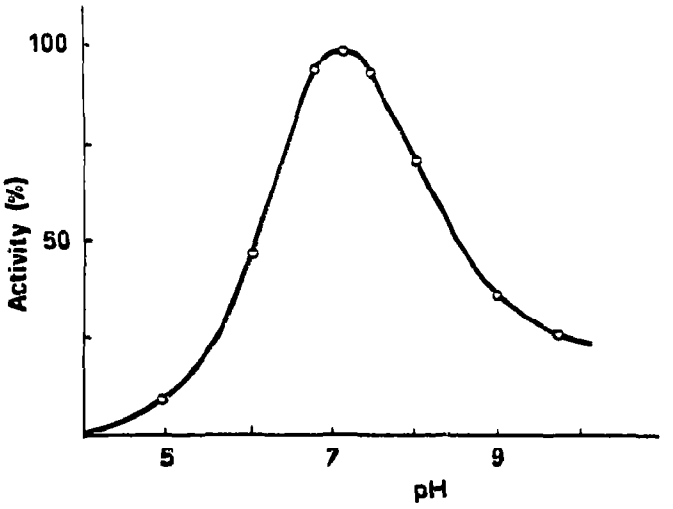

Fig. 3. Variation of protein deamidase activity with pH. Values were normalized relative to the highest observed activity (equal to 100).

3. RESULTS AND DISCUSSION

The formation of NH_3 was detected under the action of water extracts obtained from 24-h germinated grains on soybean globulins: it increased markedly during further germination and was about 4 times higher after 72 h. At this time, however, proteolytic enzymes acting on reserve proteins are very active [15]. The peptides and free amides formed may be deamidated at a higher rate by the enzyme studied and, possibly, by other enzymes acting on amides and amide-containing peptides. In further studies, therefore, we used only the extracts from grains germinated for 30–32 h.

The results of a representative purification experiment are shown in Table I. Most of the proteins are salted out, while only about 20% of the initial activity is precipitated, under the conditions used. During the chromatography of the supernatant on DEAE–cellulose most of the protein was eluted with the void volume. The enzyme was retarded on the column and partially separated from the protein peak (Fig. 1). In several experiments the separation was complete. Preparations purified 250–500-fold with specific activities of 1,300–2,500 mU/mg were obtained and used in the further work.

Direct extraction with phosphate resulted in preparations close to those obtained by water extraction and subsequent salting out.

Under the conditions of incubation used the rate of reaction was constant during the first 30 min (Fig. 2). The pH optimum was about 7 (Fig. 3).

The content of the amide N in the protein substrate decreased during incubation. When 7.2 μg of ammonia N were formed the decrease of amide N was found to

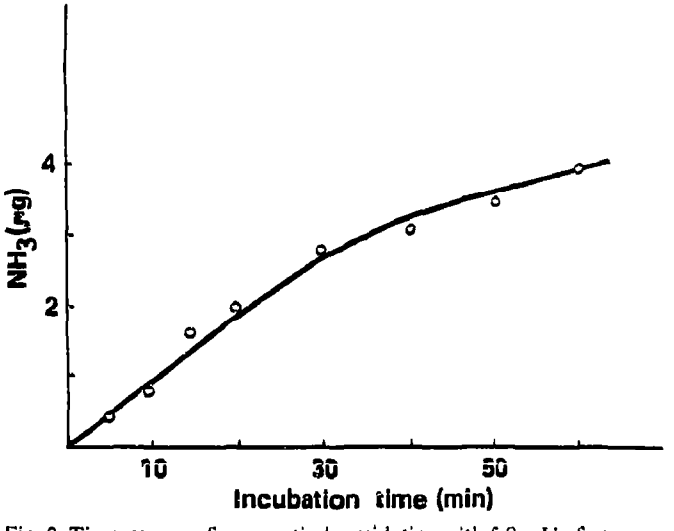

Fig. 2. Time-course of enzymatic deamidation with 5.9 mU of enzyme and soybean globulin as substrate.

Table I

Summary of protein deamidase purification

Step	Protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification (-fold)
Water extraction*	220	1,065	5	100	1
Salting out and dialysis	3.6	738	205	70	41
Concentration	3.2	410	128	39	26
DEAE–cellulose chromatography	0.16	318	1,990	30	400

*Water extract was derived from 40 g of dry grains.

Table II

Action of protein deamidase on some substrates

Substrate	Relative activity* (%)
Soybean globulin	100
Soybean 11 S protein	47 ± 5
Squash 11 S protein	67 ± 4
Soybean 7 S protein	90 ± 6
Kidney bean 7 S protein	90 ± 5
Kidney bean hemagglutinin	35 ± 3
Wheat gluten	48 ± 28
Horse hemoglobin	9 ± 1
Lysozyme	13 ± 2
Reduced RNAse	55 ± 2
Glutamine	25 ± 2
Gly-Gln-Tyr	75 ± 5

*Values normalized relative to the activity with soybean globulin as substrate.

Table III

Action of some effectors on the activity of protein deamidase

Effector	Activity (%)
Control	100
$CaCl_2$ (0.01 M)	39 ± 4
JCH_2COO^- (5×10^{-4} M)	96 ± 4
2-Mercaptoethanol (0.01 M)	83 ± 10

be 6.6 μg, thus confirming the deamidating action of the enzyme studied.

The action of the enzyme on several other potential substrates is shown in Table II. It deamidated all the seed storage proteins tested. From the other native proteins tested only lysozyme and hemoglobin showed a marked deamidation. The activity with egg and serum albumins, insulin and RNAse as substrates was within the error of determination. The latter was deamidated after the reduction of its disulfide bonds. Glutamine and glutamine-containing peptides were also deamidated while asparagine and asparagine-containing peptides were not affected by the enzyme. In all probability, in proteins, too, only glutaminyl residues will be deamidated. In the tripeptide both amino and carboxyl groups of the glutaminyl residue are protected. The rate of its deamidation is markedly higher than that of the free glutamine. This is consistent with the specificity of the enzyme acting on proteins.

The difference in sensitivity of protein substrates to the action of the deamidating enzyme is evidently determined by the content of amidated amino acid residues (glutaminyl residues if the enzyme specificity suggested is true) and their availability. It is quite natural that the highest deamidation is observed when seed storage proteins are used as substrates. They are rich in glutaminyl residues and are obviously the natural substrates of corresponding deamidating enzymes.

Splitting off the amide groups from a protein may be caused not only by the action of deamidase, but also by a transglutaminase [16]. However, the deamidating enzyme studied does not catalyze the reaction of hydroxamate formation characteristic of transglutaminase. In addition, Ca^{2+} is an obligatory factor for transglutaminase activity [13], but it inhibits the activity of the enzyme studied (Table III). In contrast to transglutaminase [13], SH-reagents have no significant effect on its activity. Thus, it may be concluded that the enzyme found is a true deamidase.

The enzymes deamidating free asparagine and glutamine are well known. Two enzymes deamidating glutamine peptides were described [17] of which one was found to cause some deamidation of heat-denatured soybean seed protein [18]. However, to the best of our knowledge the existence of a deamidase acting on native proteins was not proven up till now.

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