Phytochemistry, 1969, Vol. 8, pp. 827 to 830. Pergamon Press. Printed in England

PARTIAL PURIFICATION AND CHARACTERIZATION OF AN AMIDOHYDROLASE FROM SOYBEAN

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(*Received* 29 *November* 1968)

Abstract—A soluble enzyme with amidohydrolase activity was isolated from the roots of soybean seedlings and purified 45-fold by ammonium sulfate fractionation, gel filtration and cellulose ion-exchange chromatography. The enzyme hydrolyzed primarily N^{α}-benzoyl-DL-arginine *p*-nitroanilide and to a slight extent the *p*-nitroanilides of glycine and L-leucine, but it did not hydrolyze casein, Azocoll and the *p*-nitroanilides of L-lysine, L-cystine, succinyl-L-phenylalanine and glutaryl-L-phenylalanine. The apparent K_m for N^{α}-benzoyl-DL-arginine *p*-nitroanilide was $5{\cdot}78 \times 10^{-5}$ M. The effects of temperature, pH, metal ions and sulfhydryl reagents on the enzyme were also investigated.

INTRODUCTION

AMIDOHYDROLASE activity detected by the chromogenic substrate N^{α}-benzoyl-DL-arginine *p*-nitroanilide is known to be present in germinated barley (*Hordeum vulgare*) and wheat (*Triticum aestivum* L.). Suolinna *et al.*[1] have reported that an enzyme hydrolyzing this substrate was extracted from barley flour. Burger and Siegelman[2] have investigated the location of this activity in the barley kernel and Burger *et al.*[3] have partially purified an enzyme with similar activity from germinated barley. Prentice *et al.*[4] studied the distribution of this activity in barley and wheat kernels and found that the activity increased with time during germination.

A recent survey in our laboratory showed that the N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrolase activity is quite widespread in the roots and shoots of many crop plant seedlings such as soybean (*Glycine max* Merril), pea (*Pisum sativum* L.), corn (*Zea mays* L.), sunflower (*Helianthus annuus* L.), cotton (*Gossypium hirsutum* L.) and bean (*Phaseolus vulgaris* L.)

This paper describes the partial purification and characterization of an amidohydrolase extracted from the roots of soybean seedlings, using N^{α}-benzoyl-DL-arginine *p*-nitroanilide as the primary substrate.

RESULTS AND DISCUSSION

Purification

The results of a typical purification experiment are presented in Table 1. These data indicate a 45-fold purification of the enzyme with 63 per cent recovery. The purification was followed by the standard N^{α}-benzoyl-DL-arginine *p*-nitroanilide assay according to the method of Erlanger *et al.*[5]

* Published with the approval of the Director, North Dakota Agricultural Experiment Station, Journal article No. 162.

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Fraction III was chromatographed on a DEAE cellulose column. The first peak of the chromatogram contained Fraction IV or the partially purified enzyme of this study. This was tested for substrate specificity and the results are reported in Table 2. The low relative activities for glycine *p*-nitroanilide and L-leucine *p*-nitroanilide seemed to indicate either residual impurities in the preparation or a somewhat broader specificity. The second peak, following closely, hydrolyzed glycine *p*-nitroanilide to a greater extent than N^{α}-benzoyl-DL-arginine *p*-nitroanilide. The character of the second peak is presently being investigated. Several inactive peaks were also observed.

TABLE 1. PURIFICATION OF SOYBEAN ROOT AMIDOHYDROLASE

Fraction	Total volume (ml)	Total protein (mg)	Total milli-units* (mU)	Specific activity (mU/mg protein)	Recovery (%)	Purification (fold)
I (crude)	162	239	1633·0	6·8	100	1
II ($(NH_4)_2SO_4$)	176	146·1	1474·4	10·0	90·3	1·5
III (Sephadex)	27	44·3	1419·2	32·0	86·9	4·7
IV (DEAE)	31	3·4	1026·3	301·9	62·8	44·4

* 1 milli-unit (mU) = 1 millimicromole substrate per min.

TABLE 2. SUBSTRATE SPECIFICITY OF SOYBEAN ROOT AMIDOHYDROLASE

Substrate	Relative rate of hydrolysis
N^{α}-Benzoyl-DL-arginine *p*-nitroanilide	100
Glycine *p*-nitroanilide	9
L-Leucine *p*-nitroanilide	4

The reaction mixture for *p*-nitroanilide derivatives contained 28 μg of Fraction IV (DEAE) enzyme, 0·5 μmole of the nitroanilide substrate, 150 μmoles of phosphate buffer, pH 8·2, plus distilled water in a total of 3 ml. The reaction was run for 5 min and the change in absorbance at 410 nm was determined. The enzyme was also tested for proteolytic activity with 1·0 per cent casein using the standard protease assay and with Azocoll measuring the rate of dye release at 580 nm for 180 min at 35°.

Enzyme Properties

The substrate specificity study of Fraction IV showed (Table 2) that the enzyme predominantly hydrolyzed the amide bond of N^{α}-benzoyl-DL-arginine *p*-nitroanilide, and displayed only low activities for glycine *p*-nitroanilide and L-leucine *p*-nitroanilide. The *p*-nitroanilides of L-lysine, succinyl-L-phenylalanine and glutaryl-L-phenylalanine and the bis-*p*-nitroanilide of L-cystine were not hydrolyzed and there was no visible proteinase activity on casein or Azocoll.

Table 3 shows the results of enzyme activation and inhibition studies. Some thiol inhibitors were only moderately effective, but *N*-ethylmaleimide strongly inhibited the amidohydrolase activity. Dithiothreitol and EDTA produced a slight stimulatory effect. In addition,

iodoacetate (0·5 mM), phenylmethanesulfonyl fluoride (0·25 mM), 2-mercaptoethanol (0·5 mM), L-cysteine (0·5 mM), soybean trypsin inhibitor (0·5 mg/ml), $FeSO_4$ (0·5 mM), KCl (0·5 mM) and $CaCl_2$ (0·5 mM) were tested under the same conditions, but none of these caused activation or inhibition.

TABLE 3. INHIBITION AND ACTIVATION OF SOYBEAN ROOT AMIDOHYDROLASE

Inhibitor or activator	Concentration (mM)	Per cent change
o-Iodobenzoate	0·25	−8
p-Chloromercuribenzoate	0·5	−16
N-Ethylmaleimide	0·5	−81
Dithiothreitol	0·5	+11
EDTA	0·5	+6
$FeCl_3$	0·5	+7
$MnCl_2$	0·5	+14
$CoCl_2$	0·5	+10
$CuSO_4$	0·5	−60
$ZnCl_2$	0·5	+8
$Al(NO_3)_3$	0·5	+8
$HgCl_2$	0·5	−98

The complete system contained 57 μg Fraction IV (DEAE) enzyme, 0·5 μmole N^{α}-benzoyl-DL-arginine *p*-nitroanilide, 100 μmoles of phosphate buffer, pH 8·2, and inhibitor or activator, plus distilled water in a total of 3 ml. The inhibitors or activators were incubated for 15 min at 25° with the enzyme, buffer, and distilled water before the addition of the substrate N^{α}-benzoyl-DL-arginine *p*-nitroanilide. The reaction was then run for 5 min at 25° and the change in absorbance was determined at 410 nm.

The pH optimum for N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrolysis was found to be at 8·0. The temperature optimum for this activity under the standard assay conditions was 50°; the heat stability of the enzyme was low and the N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrolase activity declined rapidly above 45° after 5 min exposure, with total inactivation at 65°. The double reciprocal plot of initial velocities at several concentrations of N^{α}-benzoyl-DL-arginine *p*-nitroanilide gave a straight line. The kinetic data were processed on an IBM 1620 computer by use of the Fortran program of Cleland.[6,7] The apparent K_m for N^{α}-benzoyl-DL-arginine *p*-nitroanilide was calculated as $5{\cdot}78 \times 10^{-5}$ M.

MATERIALS AND METHODS

Extraction and Purification Procedure

Soybean (*Glycine max* Merril, var. Hawkeye) seedlings were harvested, after 7 days' germination between moist paper towels, without addition of nutrient under controlled conditions in a growth chamber. All steps of the extraction and purification procedure were carried out at 4° in a cold room. To each 20 g fresh weight of excised roots 80 ml of 0·05 M phosphate buffer, pH 8·2, was added and the mixture was homogenized at high speed in a

[6] W. W. CLELAND, *Nature* **198**, 463 (1963).

[7] W. W. CLELAND, in *Advances in Enzymology* (edited by F. F. NORD), Vol. 29, p. 1, Interscience Publishers, New York (1967).

Waring blendor for 90 sec. The homogenate was squeezed through cheesecloth and the filtrate centrifuged in a Sorvall SS-3 centrifuge at 40,000 × *g* for 20 min. The pellet was discarded and the supernatant (Fraction I) was brought to 30% saturation with $(NH_4)_2SO_4$. The precipitate was then removed by centrifuging at 40,000 × *g* for 20 min and the pellet was discarded. The supernatant (Fraction II) was adjusted to 60% $(NH_4)_2SO_4$ saturation and the precipitate was collected by centrifuging at 40,000 × *g* for 20 min. The supernatant was discarded, and the active pellet was resuspended in 2·0 ml 0·05 M phosphate buffer, pH 8·2, and applied to a 2·0 × 22 cm column of bead form Sephadex G-75.

The Sephadex had been previously equilibrated with 0·05 M phosphate buffer, pH 8·2. The column was eluted with 0·05 M phosphate buffer, pH 8·2, under low pressure. The first peak of the chromatogram contained the enzymatic activity (Fraction III). Fraction III was lyophilized, redissolved in 2–3 ml 0·05 M phosphate buffer, pH 8·2, and applied to a 2·5 × 30 cm column of 0·87 meq/g DEAE cellulose equilibrated with 0·05 M phosphate buffer, pH 8·2. The column was eluted with a linear gradient of 0–0·75 M NaCl in 0·05 M phosphate buffer, pH 8·2, at a rate of 0·5 ml/min. The first peak corresponded to 0·25–0·30 M NaCl and contained the N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrolase activity (Fraction IV).

Assay Methods

Protein was determined by the method of Lowry *et al.*[8] The distribution of protein in column eluates was monitored by recording the absorbance at 254 nm.

The extent of the hydrolysis of N^{α}-benzoyl-DL-arginine *p*-nitroanilide, glycine-*p*-nitroanilide, L-leucine *p*-nitroanilide, L-lysine *p*-nitroanilide, L-cystine *bis*(*p*-nitroanilide), succinyl-L-phenylalanine *p*-nitroanilide, and glutaryl-L-phenylalanine *p*-nitroanilide was measured by determining the increase of absorbance at 410 nm corresponding to *p*-nitroaniline formation.[5] The standard reaction mixture contained 0·5 μmole of the substrate *p*-nitroanilide, 150 μmoles of phosphate buffer, pH 8·2, enzyme, and distilled water in a total of 3·0 ml, at 25°. The reaction was initiated by adding the enzyme and the change in absorbance at 410 nm was determined after 5 min.

The proteolytic activity on casein was tested according to the method of Kunitz,[9] by measuring the absorbance at 280 nm of the trichloroacetic acid-soluble extract of the casein digest incubated at 25° for 3 hr. The activity on the protein-dye conjugate, Azocoll,[10] was assayed by incubating a mixture of the enzyme and 10 mg Azocoll in a total of 3·0 ml of 0·05 M phosphate buffer, pH 8·2, at 25° for 3 hr and measuring the absorbance of the dye released from the complex at 580 nm.

Specific activities are given as milli-units (mU) per mg protein.[11] The mU is defined as the amount of enzyme required for the hydrolysis of 1 millimicromole of substrate per minute under the conditions of the assay.

Substrates and resins used were high quality, commercial samples.

Acknowledgements—This research was supported in part by the U.S. Department of Agriculture, Agricultural Research Service, Grant No. 12-14-100-9170 (34). The authors wish to thank Michael Koszalka for his technical assistance during the course of this work.

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