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PARTIAL PURIFICATION OF NITRILASE FROM CHINESE CABBAGE

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Key Word Index—*Brassica campestris* ssp. *pekinensis*; Brassicaceae; Chinese cabbage; purification; nitrilase; IAA biosynthesis.

Abstract—Nitrilase was purified *ca* 28-fold from Chinese cabbage seedlings. K_m values of 5.2×10^{-4} and 2.6×10^{-3} M were obtained for indoleacetonitrile (IAN) and 3-cyanopyridine (3-CP) as substrates. For hydrolysis of 3-CP, the maximal velocity was 44 times higher than for the natural substrate IAN. The pH optimum is at 7.5. IAA concentrations from 10^{-6} to 10^{-3} M did not inhibit the partially purified enzyme. Nitrilase activity was investigated during development of seedlings grown under continuous light. Roots with hypocotyls exhibited only slightly lower activity than cotyledons based on fresh weight, although their specific activity was *ca* 5 times higher. Etiolated seedlings showed a very similar distribution of nitrilase activity. The significance of the results for IAA biosynthesis is discussed.

INTRODUCTION

In earlier studies of IAA biosynthesis in the Brassicaceae, feeding experiments were performed with radioactive precursors; this work was reviewed by Kutacek and Kefeli [1]. It is generally accepted that IAN is at least one of the natural precursors of IAA in the Brassicaceae. The presence of IAN in hypertrophied Chinese cabbage roots infected with the club root organism *Plasmodiophora brassicae* was demonstrated by Tamura *et al.* [2] and confirmed in our laboratory for leaves and roots of healthy and infected plants (Hilgenberg and Rausch, unpublished results). Whether IAN is formed from glucobrassicin (GLUBR) by myrosinase (EC 3.2.3.1) action [3, 4] or from indoleacetaldoxime (IAOX) by 3-indoleacetaldoxime hydrolyase (EC 4.2.1.29) [5] remains unsettled. The presence of nitrilase activity in intact leaf tissue from several *Brassica oleracea* varieties was first demonstrated by Thimann and Mahadevan [6] in their study of nitrilase (EC 3.5.5.1) from barley but no attempts were made to investigate nitrilase of the Brassicaceae more thoroughly. In the search of possible control points in the biosynthetic pathway of IAA, the nitrilase enzyme is of central interest. It converts IAN directly to IAA and would consequently be involved in both possible pathways. For the demonstration of a possible inhibition by IAA a partial purification of the enzyme was necessary. Furthermore, we searched for changes of nitrilase activity during seedling development and etiolation.

RESULTS

In preliminary experiments we were unable to detect nitrilase activity in crude extracts from different parts of Chinese cabbage plants when IAN was used as substrate. Assuming that enzyme activity was too low to be detected, we tried 3-CP as substrate. Mahadevan and Thimann [7] had shown in their work on enzymatic hydrolysis of different aromatic and aliphatic nitriles that, for the barley enzyme, the V_{max} is *ca* 8-fold higher when 3-CP is used at 9×10^{-3} M. With 3-CP as substrate, nitrilase activity was readily detected in seedlings and different parts of 4-week-old Chinese cabbage plants. The nitrilase enzyme from 5-day-old seedlings was then purified on DEAE-cellulose with prior dialysis of the crude extract. Using a Tris–HCl gradient from 0.125 to 0.5 M (slope: 2mM Cl/ml), we obtained a single sharp peak of activity at 0.185 M Cl. When a less steep gradient from 0.175 to 0.375 M Tris–HCl (slope: 1 mM Cl/ml) was applied, an even better purification (6.6-fold) was obtained with 53% recovery (Fig. 1). For a comparison, nitrilase from barley leaves was subjected to DEAE-cellulose chromatography with a similar gradient. The enzyme eluted as a single peak at the same ionic strength as did the Chinese cabbage enzyme (Fig. 2). Nitrilase activity for crude extracts of different Chinese cabbage materials remained constant during incubation at 35° for only *ca* 30 min and then showed a steady decline. After DEAE-cellulose chromatography, enzyme activity was stabilized and showed no decline for more than 10 hr. With the partially purified enzyme, IAN hydrolysis could now be demonstrated. The pooled active fractions of the columm eluate were taken for the determination of the pH optimum and the K_m for IAN and 3-CP. The enzyme activity showed a typical pH optimum curve with maximum activity at pH 7.5 and half maximum activities at pH 6.7 and 8.7, respectively. Enzyme activity follows Michaelis–Menten kinetics for both substrates. From the Lineweaver–Burk plots, K_m values of 5.2×10^{-4} and 2.6×10^{-3} M were obtained for IAN and 3-CP.

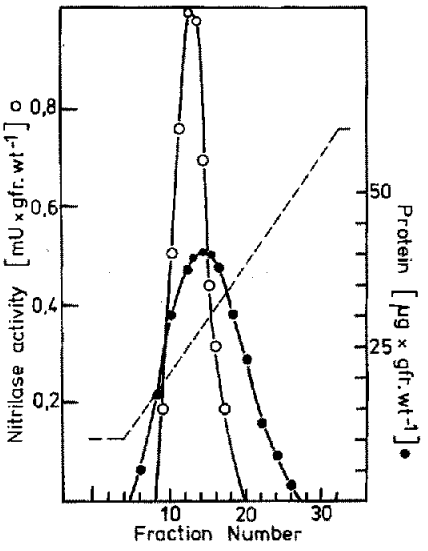

Fig. 1. DEAE-cellulose chromatography of nitrilase from Chinese cabbage. 10 ml of crude extract equivalent to 10 g fr. wt of 5-day-old seedlings were chromatographed with a linear Tris–HCl gradient (0.175–0.375 M, dotted line). Purification was 6.6-fold.

V_{max}, as derived from Eadie–Hofstee plots, was 44 times higher for 3-CP. Attempts were made to further purify the enzyme by gel chromatography on Sephadex G 100. Nitrilase activity eluted in the void volume suggesting that its MW is greater than 100 000. Specific activity was increased 4.2-fold, so that finally purification of *ca* 28-fold was achieved. As nitrilase inhibition by IAA would be of great physiological significance, we tested the effect of IAA on the DEAE-cellulose purified enzyme in several concentrations ranging from 10^{-6} to 10^{-3} M but could not find any significant decrease of activity at pH 7.4.

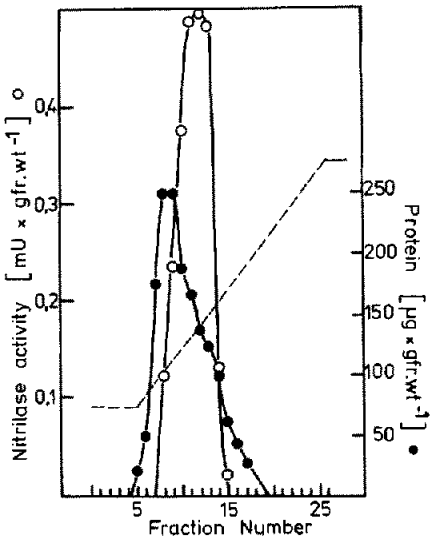

Fig. 2. DEAE-cellulose chromatography of nitrilase from barley. 10 ml of crude extract equivalent to 10 g fr. wt of 10-day-old leaves were chromatographed with a linear Tris–HCl gradient (0.125–0.375 M, dotted line).

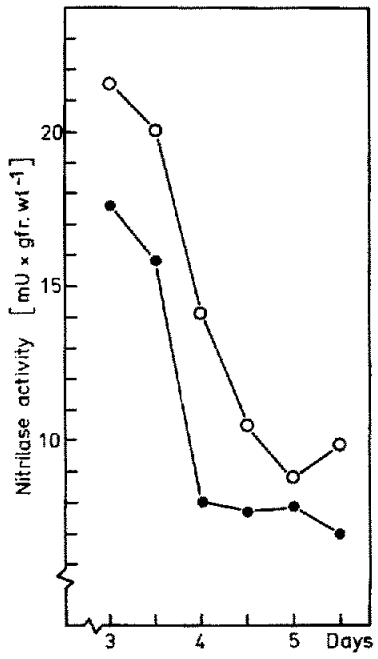

Fig. 3. Nitrilase activity during development of Chinese cabbage seedlings. Seedlings were separated into cotyledons (○—○) and roots with hypocotyls (●—●). 3-CP was used as substrate. Values were arithmetic means of 4 replicates from 1 experiment. Standard deviations were ≤10%. Two independent experiments showed similar results.

As we were able to detect nitrilase activity in crude extracts with 3-CP as substrate, the enzyme activity was investigated during seedling development of Chinese cabbage. For this purpose the cotyledons of 3- to 5.5-day-old seedlings were removed from the roots and hypocotyls and nitrilase activity was determined separately at 12 hr intervals. The results are shown in Fig. 3. During the growth period from the 3rd to the 6th day, seedlings showed a linear increase in fr. wt. A considerable decrease in nitrilase activity (*ca* 50%) occurred between 3 and 4.5 days for both cotyledons and roots with hypocotyls (for material younger than 3 days, separation of the organs was impossible). Specific activity of nitrilase from roots with hypocotyls was *ca* 5 times higher than from cotyledons, independent of the age of the seedlings (Table 1).

In another experiment we compared 5-day-old seedlings grown under continuous light with etiolated seedlings of the same age. Although the morphology of the etiolated seedlings is drastically changed, as can be seen from the fr. wt ratio of cotyledons/root + hypocotyl, the distribution of nitrilase activity in the separated material was very similar to that obtained for light-grown seedlings (Table 1).

DISCUSSION

The only investigation of a nitrilase enzyme in higher plants that converts IAN to IAA was not done with a member of the Brassicaceae, but with barley [6, 7] although there is still no evidence for IAN as a natural metabolite from that plant species. Two facts point to a similarity of the isolated and partially

Table 1. Nitrilase activity in Chinese cabbage seedlings. (a) Specific activity in cotyledons and root with hypocotyl of seedlings grown under continuous light; (b) distribution of nitrilase activity in seedlings grown either under continuous light or in the dark (5 days)

Day	(a) *Specific activity* ($mU/mg_{Protein}$) Cotyledons	Root+ hypocotyl	Ratio: cotyledons / root+hypocotyl
3.5	4.4	24.7	0.18
4.5	2.2	10.1	0.22
5.5	2.9	15.0	0.19

	(b) *Activity* (mU/g fr. wt) Cotyledons	Root+ hypocotyl	Fr. wt (g) Cotyledons/ root+hypocotyl
Light	12.5	10.6	2.96/2.04
Dark	11.5	10.1	1.34/3.62

purified nitrilase from Chinese cabbage to the barley enzyme. Both enzymes hydrolyse 3-CP more efficiently than IAN [7] and they elute at the same ionic strength (0.185 M Cl^-) from DEAE-cellulose. There seems to be little similarity to microbial nitrilases ([8] and refs. cited therein).

While the physiological significance of the barley enzyme remains unknown, the existence of an IAN-hydrolysing nitrilase in the Brassicaceae has long been postulated for the conversion of IAN to IAA, the last step in IAA biosynthesis. In our work we have shown that nitrilase activity is present in different parts of Chinese cabbage seedlings in varying activities. This is the first system for which both the presence of IAN ([2]; Rausch and Hilgenberg, unpublished results) and nitrilase have been unequivocally demonstrated. Butcher *et al.* [9] have shown that in swede roots (*Brassica napus*) the IAN concentration is *ca* 50 times higher than that of IAA. Therefore we have to investigate if the observed changes of nitrilase activity during seedling development have a physiological significance and if nitrilase is a possible rate-limiting enzyme for IAA biosynthesis.

EXPERIMENTAL

Plant material. After surface sterilization, seeds from *Brassica campestris* ssp. *pekinensis* var. Granat were germinated in Petri dishes on filter paper soaked with 10% Knop's nutrient soln under constant conditions (2 fluorescent lamps Philips TL 55 20 W and TL 32 de Luxe 20 W, 6×10^{-4} W/cm^2; 27°; culture period up to 6 days). Sterile H_2O was added daily to ensure constant humidity. Etiolated seedlings were obtained in the same way but cultured in the dark at 25°. For separate detection of nitrilase activity, cotyledons were removed 2–3 mm from their insertion point. Seeds of *Hordeum vulgare* var. Volgelsang were germinated on moist vermiculite in the greenhouse and leaves were harvested after 10 days.

Extraction and purification of enzyme. Thoroughly washed 5-day-old seedlings (fr. wt 12 g) of Chinese cabbage were homogenized in a precooled mortar with 6 ml of extraction buffer containing 0.525 M Tris–HCl (pH 7.4), 3 mM EDTA, 15 mM β-mercaptoethanol, and 1.2 g of insoluble PVP by thorough grinding with acid-washed sand. The homogenate was centrifuged at 20 000 g for 1 hr giving *ca* 12 ml of crude extract which was dialysed 3 hr against 1 l. 0.175 M Tris–HCl buffer, pH 7.4 (all buffers used in this study except the extraction buffers contained 1 mM EDTA and 5 mM β-mercaptoethanol) with 2 buffer changes. The dialysed extract was again centrifuged for 30 min at 20 000 g and its conductivity was determined; it was equal to the buffer for dialysis. Dialysed extract (10 ml) was chromatographed on a DEAE-cellulose column, 12×2 cm, pre-equilibrated with 0.175 M Tris–HCl, pH 7.4. The column was washed with 100 ml of equilibration buffer and a linear gradient of 160 ml Tris–HCl, pH 7.4, from 0.175 to 0.375 M was applied. The salt concn of the fractions was determined by conductivity measurements; protein was assessed with the reagent described in ref. [10] (A_{280} values were misleading as some coloured components also bind to DEAE-cellulose). The fraction with the highest nitrilase activity (1.5 ml) was further chromatographed on Sephadex G 100, equilibrated with 0.05 M Tris–HCl, pH 7.4. For comparison, nitrilase from barley (10 g fr. wt of 10-day-old leaves) was chromatographed on DEAE-cellulose by the same method but with different buffers; extraction buffer: 0.375 M Tris–HCl, pH 7.4; buffer for dialysis: 0.125 M Tris–HCl, pH 7.4; gradient; 160 ml Tris–HCl, pH 7.4, from 0.125 to 0.375 M.

Measurement of nitrilase activity. The most active fractions from the DEAE-cellulose eluate were pooled and dialysed for 3 hr against 1 l. of 0.05 M Tris–HCl, pH 7.4, with 2 buffer changes. For determination of the pH optimum, the incubation mixture contained in a total vol. of 1 ml: 0.1 ml enzyme prepn, 0.02 ml 3-CP soln (1 M in EtOH), and 0.88 ml 0.05 M buffer; for pH 5–7.6 K-Pi buffer was prepared for pH 7.4–9.2 Tris–HCl buffer. For obtaining K_m values 0.2 ml enzyme prepn was used for 3-CP and 0.5 ml for IAN; the final substrate concns were from 10^{-3} to 10^{-4} M for IAN and from 10^{-2} to 5×10^{-4} M for 3-CP. The test vols were 1 ml, incubation time was 2 hr for 3-CP and 10 hr for IAN as substrate; temp was at 35°. The reaction was stopped by boiling for 5 min. Precipitated protein was centrifuged off and 0.5 ml aliquots were taken for NH_4^+ determination with GlDH according to ref. [11]. One enzyme unit was defined as 1 μmol NH_4^+/min. Inhibition studies with IAA were carried out at the following concns: 10^{-6}, 10^{-5}, 10^{-4}, 5×10^{-4}, and 10^{-3} M IAA with 3-CP as substrate at 2×10^{-2}, 5×10^{-3}, and 10^{-3} M; pH was at 7.4.

Determination of nitrilase activity in separated material. Crude dialysed extracts were used: the extraction buffer contained 0.1 M Tris–HCl, pH 7.4, 2 mM EDTA and 10 mM β-mercaptoethanol; *x* g fr. wt of the separated and washed plant material was extracted with *x*/10 g insoluble PVP and *x*/2 ml buffer, centrifuged and dialysed against 0.05 M Tris–HCl as outlined above. The test system included in a total vol. of 1 ml: 0.05 ml extract, 0.02 ml 3-CP soln (1 M in EtOH) and 0.93 ml 0.05 M Tris–HCl, pH 7.4. Incubation time was 20 min at 35°. Appropriate controls were either boiled immediately or substrate was omitted and incubation performed as already described. Protein was determined according to the method of ref. [12] after TCA precipitation.

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