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PURIFICATION AND PROPERTIES OF β-CYANO-L-ALANINE SYNTHASE FROM *SPINACIA OLERACEA**

FUMIO IKEGAMI, KYOKO TAKAYAMA, CHIHO TAJIMA and ISAMU MURAKOSHI

Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Chiba 260, Japan

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; β-cyano-L-alanine synthase; cysteine synthase; enzyme purification; amino acid composition; L-cysteine; *O*-acetyl-L-serine; β-cyano-L-alanine; heterocyclic β-substituted alanines.

Abstract—β-Cyano-L-alanine synthase was purified *ca* 6200-fold to homogeneity from the leaves of spinach (*Spinacia oleracea*). The purified enzyme has an apparent M_r of 60 000 and can be dissociated into identical subunits of M_r 30 000. The subunits each contain one molecule of pyridoxal 5′-phosphate. The K_m value is 2.3 mM for L-cysteine and 0.73 mM for cyanide. β-Cyano-L-alanine synthase from *S. oleracea* also catalyses the formation of some *S*-substituted L-cysteines and some heterocyclic β-substituted alanines from L-cysteine or *O*-acetyl-L-serine. The specificity of these additional catalytic activities of the purified enzyme are compared with those of cysteine synthase purified from the same plant, and with those of β-cyano-L-alanine synthase purified from other sources. Some other properties, including the amino acid composition of the purified enzyme, are also described.

INTRODUCTION

β-Cyano-L-alanine (BCA) synthase is widespread in nature. Its metabolic role in plants can be linked to the detoxification of HCN, that arises as a byproduct of ethylene biosynthesis. BCA synthases have been purified from several plant sources [1–5] and also from microorganisms [6–8]. Some BCA synthases purified from plant sources were found to contain one molecule of pyridoxal 5′-phosphate (PLP) per molecule of enzyme, and no subunits; other BCA synthases were found to contain two identical subunits, each subunit containing PLP as a cofactor [5, 8], similar to the cysteine synthases in higher plants [9–15].

In recent years we made comparative studies of some PLP-containing enzymes catalysing the formation of naturally occurring heterocyclic β-substituted alanines [12–17], and we presented evidence that some cysteine synthases from plants, as an additional catalytic activity, can also catalyse the formation of some β-substituted alanines, including BCA, in the presence of *O*-acetyl-L-serine (OAS) and suitable precursors [12–15]. Cysteine synthase purified from spinach (*Spinacia oleracea*) however, could not catalyse the formation of BCA, although high BCA synthase activity was present in extracts of this plant.

In this respect, it seemed of particular interest to extend our studies to BCA synthase which catalyses the reaction between L-cysteine and cyanide to form BCA and hydrogen sulphide in a variety of higher plants, since cyanide serves as a second substrate for the enzyme in the reaction of β-substitution of L-cysteine. Therefore, we have now attempted the purification of BCA synthase from the leaves of *S. oleracea*, which is not a cyanogenic plant, in order to improve our understanding of the biosynthesis of this group of non-protein amino acids.

In this paper we describe the purification and the properties of BCA synthase from *S. oleracea* leaves and its comparison with BCA synthases and cysteine synthases from other sources. Differences between the purified BCA synthase and cysteine synthase present in the same plant are also described.

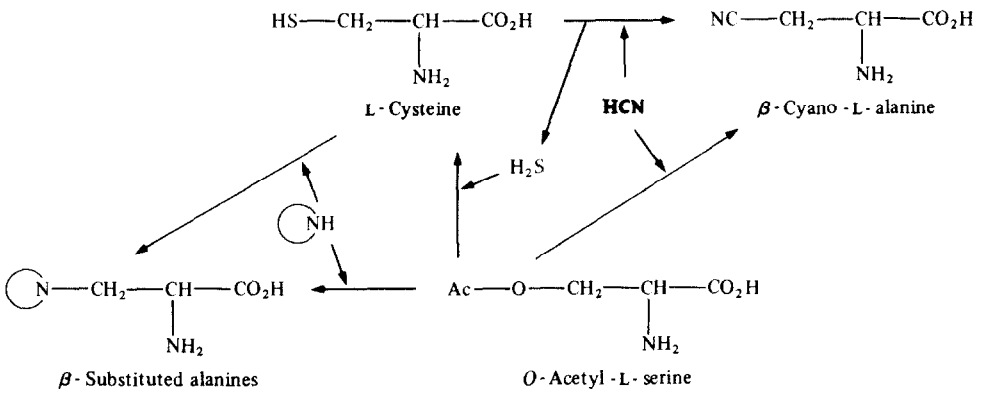
RESULTS

Purification of BCA synthase from spinach leaves

From 8.6 kg of fresh spinach leaves we extracted and purified BCA synthase, mainly using the methods described in previous papers [1, 3, 12–17]. The enzyme was prepared simultaneously with the cysteine synthase activity by a procedure including the preparation of acetonized mitochondria, ammonium sulphate fractionation, ion-exchange chromatography on DEAE-Sephadex A-50, gel filtration on Sephadex G-100 or Ultrogel AcA 44, hydrophobic chromatography on AH-Sepharose 4B and preparative polyacrylamide gel electrophoresis (PAGE) as summarized in Table 1.

The protein demonstrating BCA synthase activity was completely separated from cysteine synthase activity after the first DEAE-Sephadex A-50 column was eluted with a concentration gradient of Tris–HCl buffer. The enzyme activity for BCA synthase was eluted at 230–250 mM and cysteine synthase eluted at 280–300 mM as shown in Fig. 1.

*Parts of this work were reported at the 107th Annual Meeting of the Pharmaceutical Society of Japan at Kyoto, 4 April 1987 (Abstracts, p. 359).



Scheme 1. Biosynthetic pathways for β-cyano-L-alanine, L-cysteine and β-substituted alanines in higher plants.

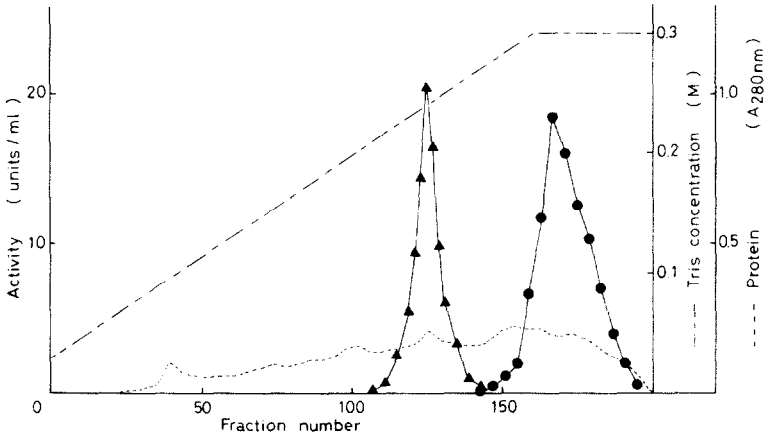


Fig. 1. Elution patterns of β-cyano-L-alanine synthase and cysteine synthase after the first DEAE-Sephadex A-50 column chromatography. β-Cyano-L-alanine synthase activity (▲——▲), cysteine synthase activity (●——●) and protein (A_{280},---) were monitored as shown in the Experimental.

Table 1. Summary of the purification of β-cyano-L-alanine synthase from *Spinacia oleracea*

Purification step	Total activity (units*)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Fold
1. Crude extract†	7030	207000	0.034	100	1
2. Acetonized preparation of mitochondria	4000	12540	0.319	56.9	9.4
3. Ammonium sulphate precipitate‡	3060	2520	1.21	43.5	35.6
4. Sephadex G-100 (peak fractions)	2830	460	6.15	40.3	180
5. 1st DEAE-Sephadex A-50 (230-250 mM)	2080	130	16.0	29.6	470
6. Ultrogel AcA 44 (peak fractions)	1089	33.3	32.7	15.5	962
7. AH-Sepharose 4B (240-275 mM)	571	9.35	59.9	8.1	1760
8. Polyacrylamide-gel electrophoresis	261	1.24	210	3.7	6176
9. 2nd DEAE-Sephadex A-50 (135-145 mM KCl)	235	1.11	212	3.3	6235

*A unit of enzyme activity represents 1 μmol of product formed per min at 30°, in 50 mM Tris–HCl buffer, pH 9.
†Starting from 8.6 kg of the fresh leaves of *Spinacia oleracea*.
‡40–60% saturation and desalted on Sephadex G-25.

The complete procedure as shown in Table 1 afforded an apparent purification of *ca* 6200-fold for BCA synthase with the specific activity of 212 U/mg protein and the yield of 3.3%, as compared to the total BCA synthase activity of the crude extract.

Properties of the purified BCA synthase

The M_r of the purified enzyme from spinach leaves was estimated by analytical gel filtration using Sephadex G-100 (1.5 × 115 cm) according to the method of ref. [18]. BCA synthase activity was found invariably as a single peak, corresponding to an M_r of 60 000. The purified enzyme was subjected to SDS-PAGE on 12% gels to determine its subunit structure, following the method of ref. [19]. This suggests that BCA synthase from spinach is composed of two identical subunits, with an M_r of 30 000, and that it has been purified to apparent homogeneity.

The purified enzyme had two absorbance peaks at 280 and 410 nm, typical for a PLP-enzyme. The identification of bound PLP in the purified enzyme was demonstrated by direct spectrophotometric measurements [20]. BCA synthase in spinach has one molecule of PLP bound to each subunit like the cysteine synthases and heterocyclic β-substituted alanine synthases from other sources [12–17], although BCA synthases from blue lupine and white lupine have one molecule of PLP per native enzymes [1–4].

The enzyme exhibited a single pH optimum at around pH 9.0–9.5 with Tris–HCl buffer. Lineweaver–Burk plots gave K_m values of 2.3 mM for L-cysteine and 0.73 mM for NaCN. The K_m value for L-cysteine was almost the same value as that determined for BCA synthase from blue lupine [2], while it was higher than that of the enzyme from white lupine [4]. The K_m value for CN^- was also higher than that determined for BCA synthases from both lupine plants [2, 4].

The addition of PLP had an accelerating effect on the activity of this enzyme, the increase being *ca* 30% at a concentration of 1 mM. No requirement for PLP has been reported for BCA synthase from *Chromobacterium violaceum* [8]. The PLP-enzyme inhibitors, hydroxylamine and sodium borohydride at a concentration of 1 mM both caused 10 and 80% inhibition, respectively.

Substrate specificity

Under standard assay conditions, BCA synthase from spinach clearly appears to be specific for L-cysteine as a donor for the amino-substrate. Under identical conditions the activity was 139, 18.4 and 17.3% in the presence of β-chloro-L-alanine, *O*-sulpho-L-serine and L-cystine as compared to L-cysteine at 10 mM, respectively. The purified enzyme showed 1.8% activity in the presence of *O*-acetyl-L-serine. No detectable activity was found with *O*-phospho-L-serine or with L-serine. D-Cysteine or *O*-acetyl-D-serine also did not act as a substrate.

The purified enzyme also showed distinct substrate specificity when a variety of thiol compounds or *N*-heterocyclic compounds were used as an acceptor for the amino-substrates. The relative activities of the purified enzyme with different substrates are shown in Table 2. BCA synthase from spinach could synthesize *S*-substituted L-cysteines from both L-cysteine and OAS when suitable substrates were provided. The purified enzyme could also synthesize β-(pyrazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine with a low activity from both amino-substrates OAS and L-cysteine with the corresponding heterocycles, while this enzyme could not synthesize L-willardiine, L-isowillardiine, L-mimosine or *O*-ureido-L-serine. Thus, the specific activities of the purified BCA synthase towards a variety of substrates are different from those of BCA synthase from other sources [1–8] and cysteine synthase from the same plant [12], and also from those of cysteine synthases from other sources [13–15]. The different thiol or heterocyclic substrates were tested at pH 9.0 with L-cysteine or at pH 8.0 with OAS, the optimal pH for the formation of BCA and L-cysteine, respectively.

Table 2. Relative synthetic rates of *S*-substituted L-cysteines and β-substituted alanines from L-cysteine or *O*-acetyl-L-serine by β-cyano-L-alanine synthase purified from *S. oleracea*

Substrate	Amino acid synthesized	Relative velocity of synthesis (%)	
		L-Cysteine	*O*-Acetyl-L-serine
NaCN	β-Cyano-L-alanine	100	1.8
H_2S	L-cysteine	—	0.51
MeSH	*S*-Methyl-L-cysteine	4.49	1.82
$CH_2=CH-CH_2-SH$	*S*-Allyl-L-cysteine	9.72	1.49
$HOOC-CH_2-SH$	*S*-Carboxymethyl-L-cysteine	0.82	0
Pyrazole	β-(Pyrazol-1-yl)-L-alanine	0.44	0.06
3-Amino-1,2,4-triazole	β-(3-Amino-1,2,4-triazol-1-yl)-L-alanine	1.11	0.03
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0.01	0
3,4-Dihydroxypyridine	L-Mimosine	0	0
Uracil	L-Willardiine	0	0
	L-Isowillardiine	0	0
Hydroxyurea	*O*-Ureido-L-serine	0	0

The relative rates of synthesis were compared with that of β-cyano-L-alanine from L-cysteine and NaCN. The reaction conditions are given in the Experimental and are as described before [12–17].

Amino acid composition

The amino acid composition of the purified enzyme is given in Table 3 in comparison with that of cysteine synthase purified from the same plant [12]. The data presented show that BCA synthase from spinach does not contain tryptophan, the same as cysteine synthases from plants [12–15], only BCA synthase purified from blue lupine contains tryptophan [3]. The results obtained here also indicate that BCA synthase from spinach consists of the same number of amino acids (560 residues) as cysteine synthase in the same plant and contains a large amount of glutamic acid (64 residues) and glycine (64 residues). This enzyme however differs in its content of cysteine and methionine residues from the spinach cysteine synthase [12] and other cysteine synthases [14, 15]. The spinach BCA synthase contains 26 residues of methionine, while the number of methionine residues is 2 to 7 for BCA synthase from plants [3, 4] and microorganism [7].

The M_r of BCA synthase, calculated from the amino acid composition, is 59 300, which agrees with the value estimated by gel filtration on Sephadex G-100 (60 000).

DISCUSSION

In the present paper, we deal with the purification and some properties of BCA synthase isolated from spinach, a non-cyanogenic plant. Judging from its profile on SDS-PAGE, it is the first study in which BCA synthase consists of two identical subunits, each containing one molecule of PLP, in higher plants. It appears that BCA synthase differs from cysteine synthase, separating by gradient elutions with Tris–HCl buffer from the DEAE-Sephadex A-50 and AH-Sepharose 4B columns.

Although the kinetic parameters differ considerably, the physicochemical properties of spinach cysteine synthase [12] and BCA synthase purified in this study are remarkably similar. They have the same M_r of 60 000, the same number of amino acid residues, two subunits, contain two molecules of bound PLP per molecule of enzyme, show an absorbance peak at 410 nm due to PLP, while the plant BCA synthases reported before have M_r 50 000–52 000, no subunits, and only one molecule of PLP per molecule of enzyme [1–4]. Interestingly, some cyanide-resistant bacteria also contain BCA synthase activity which is ascribed to a secondary function of cysteine synthase [6, 7]. At least one true BCA synthase, utilizing L-cysteine, occurs in the cyanide-producing bacterium *C. violaceum* [8]. This last enzyme has two identical subunits, each containing one molecule of PLP, analogous to the cysteine synthases from higher plants [9–15] and to the heterocyclic β-substituted alanine synthases [16, 17].

Among the substrates studied so far (Table 2), BCA synthase from spinach could catalyse the formation of *S*-substituted L-cysteines from L-cysteine or OAS and corresponding thiol compounds, and also could synthes-

Table 3. Amino acid compositions of β-cyano-L-alanine synthase and cysteine synthase from *S. oleracea*

Amino acids	Numbers of residues/60 000 g*	
	β-Cyano-L-alanine synthase	Cysteine synthase†
Asp	42	36
Thr	40	28
Ser	44	38
Glu	64	66
Pro	32	32
Gly	64	60
Ala	44	48
Val	42	42
Cys	4	6
Met	26	14
Ile	32	40
Leu	44	46
Tyr	12	14
Phe	16	26
Trp	0	0
Lys	32	42
His	4	4
Arg	18	18
Total	560	560

*Results are expressed as residues/mol and are based on an M_r of 60 000. Values for Thr and Ser are extrapolated to zero-time hydrolysis.

†This result was recalculated based on ref. [12].

The numbers of residues of amino acids were calculated based on the results of analyses after 24, 48 and 72 hr acid hydrolysis of native enzyme. Means of duplicate analyses are given. Determination of tryptophan was made by alkaline hydrolysis and methanesulphonate hydrolysis [12, 28].

ize heterocyclic β-substituted alanines such as β-(pyrazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine in low activities. Our data presented here indicate that BCA synthase also has the potential to catalyse analogous reactions with appropriate sulphydryl or imino analogues like cysteine synthases previously described [12–15].

In recent years, pathways for the formation of BCA have been studied in many plants and several species of microorganisms [1–8]. The results obtained in this paper and other results mentioned previously [1, 12–15] confirm previous suggestions [21, 22] that BCA synthase can play a role in the detoxification of endogenous molecules such as cyanide or hydrogen sulphide. Although spinach is a non-cyanogenic plant, it could be that BCA is the first intermediate in the process of cyanide assimilation in higher plants [22]. Cyanide is formed naturally from the ethylene pathway in higher plants and is detoxified by BCA synthase [23, 24]. Since BCA synthase appears to be located mainly in the mitochondrion [1, 23], BCA synthase has been postulated to have a role in protecting mitochondrial metabolism from the possible toxic effects of cyanide, arising from the biosynthesis of ethylene which takes place in the mitochondria. Besides its potential role in cyanide detoxification, this enzyme also catalyses the formation of heterocyclic β-substituted alanines and *S*-substituted L-cysteines from L-cysteine or OAS. Moreover, some cysteine synthase isoenzymes also catalyse the formation of BCA from OAS (not L-cysteine) and cyanide [13–15]. This group of enzymes, BCA synthase and cysteine synthase, thus can play a similar role in the detoxification of endogenous- or eventually exogenous-toxic molecules such as cyanide, hydrogen sulphide, triazole or pyrazole, thereby forming secondary metabolites like the β-substituted alanines, which may have a role as allelochemicals.

BCA synthase from spinach and cysteine synthase from the same plant seem to have the same chain length: 560 amino acid residues per enzyme or 280 residues per subunit (Table 3). The amino acid compositions of the two purified enzymes also show some similarities, like the absence of tryptophan, and the same number of proline, histidine and arginine residues. On the other hand BCA synthase contains more methionine and more hydroxy amino acids (Ser + Thr) but less phenylalanine and lysine than cysteine synthase. When a mathematical method is used [25] to compare amino acid compositions, it is suggested that BCA synthase from spinach seems to be related to cysteine synthase from the same plant [12] and also to cysteine synthases from other plant sources [14, 15], except the isoenzyme B from pea seedlings [14]. This relatedness does not exist with the BCA synthases from blue lupine [3], white lupine [4] or from *Enterobacter* sp. 10-1 [7]. These latter enzymes also differ in the presence of tryptophan and in their physicochemical properties.

From the above it seems possible that the two spinach enzymes BCA synthase and cysteine synthase may have arisen by duplication of an ancestral gene. Further study of this group of PLP-dependent BCA synthase and cysteine synthase enzymes, including the amino acid sequencing, may give information on the phylogenetic relationship of these plants, and on the evolution of non-protein amino acids. Such a group of enzymes with similar properties but with varying substrate specificities may become important for the further development of protein engineering.

EXPERIMENTAL

Materials. Spinacia oleracea L. cv Parade was grown in the medicinal plant gardens of our University. Leaves were collected and then cooled for 1 hr at 0–4° before enzyme extraction. Sephadex G-25, G-100, DEAE-Sephadex A-50 and AH-Sepharose 4B were purchased from Pharmacia. Ultrogel AcA 44 was obtained from LKB. All other chemicals used were of the highest commercial grade available.

Activity assays. The enzyme preparations obtained were dissolved in 50 mM Tris–HCl buffer, pH 9. Substrate concns were 10 mM for both L-cysteine and NaCN. Incubation was at 30° for 10 min; the total reaction vol. was 0.6 ml, utilizing up to 0.2 ml of enzyme (corresponding to 0.4–500 μg of protein). Reactions were terminated by the addition of 0.4 ml of 95% EtOH and the formation of BCA was measured spectrophotometrically at 640 nm using a ninhydrin reagent after subjecting the reaction solns to TLC in pyridine–*n*–BuOH–H_2O (1:1:1) as described previously [13]. The formation of L-cysteine was also measured spectrophotometrically according to the method of ref. [26] as described before [12–15]. The unit of enzyme activity used in this paper is equivalent to 1 μmol of BCA or L-cysteine produced per min. Protein was determined by the method of ref. [27].

Purification of BCA synthase from S. oleracea *leaves.* All operations were carried out at 0–4°. BCA synthase was prepared from 8.6 kg of fresh leaves. Each 100 g of the leaves was repeatedly homogenized in 0.1 M Tris–HCl buffer, pH 9, containing 0.35 M sucrose, 10 mM 2-mercaptoethanol and 0.5 mM EDTA. The homogenate obtained was centrifuged at 15000 *g* for 20 min, and the supernatant was discarded and then the ppt. was completely suspended in a minimum vol. of 50 mM Tris–HCl buffer, pH 8.5, containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA (buffer A). To the suspension obtained, 5 vol. of chilled acetone (−20°) was added and homogenized for 30 sec in a Waring blendor, followed by centrifugation at 5000 *g* for 10 min. Acetonized mitochondria was prepared according to a partially modified method of refs [1, 3]. After discarding the acetone, buffer A was added for the solubilization of the enzyme and then homogenized for 10 min in the same buffer. After standing for 2 hr, the ppt. which had formed was removed by centrifugation at 15000 *g* for 20 min. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation, and a 40–60% saturated $(NH_4)_2SO_4$ fraction was resuspended in buffer A. The resulting soln was applied to a column (8 × 25 cm) of Sephadex G-25 (fine) pre-equilibrated with buffer A. The protein fraction was concentrated by $(NH_4)_2SO_4$ pptn and then applied to a column (4.6 × 90 cm) of Sephadex G-100 pre-equilibrated with the same buffer. The active fractions were pooled and then applied to the first DEAE-Sephadex A-50 column (4.6 × 6 cm) pre-equilibrated with buffer A and the enzymes eluted with a linear gradient of Tris (50–300 mM) in the same buffer. BCA synthase activity was eluted at 230–250 mM and cysteine synthase activity was eluted at 280–300 mM Tris–HCl buffer, respectively, and the first active fractions (230–250 mM Tris fractions) were concentrated by $(NH_4)_2SO_4$ pptn. The active fraction was applied to a column (3 × 100 cm) of Ultrogel AcA 44 pre-equilibrated with buffer A. The eluates were collected and active fractions were concentrated by Immersible CX-10 (Millipore). The resulting soln was then applied to a column (1.2 × 8.5 cm) of AH-Sepharose 4B pre-equilibrated with buffer A and the enzyme eluted with a linear gradient of Tris (50–500 mM) in the same buffer. BCA synthase was eluted 240–275 mM and concentrated by Immersible CX-10. The resulting soln was subjected to prep. PAGE on 7.5% gel at pH 8.3 (Tris–glycine buffer). BCA synthase fraction obtained from gel slices was finally applied to a column (1.2 × 2 cm) of

DEAE-Sephadex A-50 pre-equilibrated in buffer A. The highly purified enzyme fraction (135–145 mM KCl fractions) was a pale yellow soln and was stable for at least one month at 0°. This enzyme preparation in 50 mM Tris–HCl buffer, pH 9 was used in all further expts.

Properties of BCA synthase were studied by the methods of ref. [16].

Identification of heterocyclic β-substituted alanines and S-substituted L-cysteines as reaction products was performed as described previously [12].

Determination of amino acid composition was also performed as described previously [12]. This was also achieved by the method of ref. [28].

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