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AGGREGATION OF CASSAVA LINAMARASE

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ABSTRACT

Cassava linamarase was studied by chromatographic and electrophoretic techniques. Repeated chromatography on Sepharose 4B suggested that linamarase could form large aggregates of 600,000 to 2,000,000 daltons, which did not readily equilibrate with each other. Polyacrylamide electrophoresis under denaturing conditions employing sodium dodecyl sulfate, acid-urea or triton-acid-urea suggested that the linamarase enzyme consisted of a single type of subunit. However, aggregation was also observed by non-denaturing polyacrylamide gel electrophoresis, and the sizes of aggregates were determined as containing 4, 6, 8, 10, 12-14 subunits and larger. Moreover, aggregates of different sizes could also be observed in freshly prepared extracts, so that aggregation might be present in vivo *and play some role in the action of the enzyme. The aggregates did not dissociate in the presence of sodium dodecyl sulfate or β-mercaptoethanol unless heated.*

INTRODUCTION

Cassava is an important calorie source in some 26 tropical countries[1] and is used not only for human consumption but also as an animal feed. This plant contains cyanogenic glycosides, linamarin and lotaustralin[2], which can release the toxic HCN, posing health risks. This hydrolysis is catalyzed by a specific β-glucosidase enzyme, linamarase (EC 3.2.1.21), to yield glucose and an aglycone cyanohydrin, which breaks down spontaneously or enzymatically to yield HCN plus a ketone. Linamarase has been purified from cassava[3-6] and other plants[7-9], and the kinetic properties of these enzymes have been studied in some detail. The enzyme is also produced by some bacteria[10-11] and fungi[12-13]. More recent studies on cassava linamarase have included determination of the nucleotide sequence[14], studies on the location of the enzyme *in vivo*[5,15], and investigation of its catalytic mechanism[16,17]. However, multiple forms of cassava linamarase have been previously been reported[4], so that postranslational events may also be important in linamarase function *in vivo*. In this paper, we report further studies on the heterogeneity of cassava linamarase, especially that resulting from aggregation of subunits.

MATERIALS AND METHODS

Materials

Cassava stem, petiole, leaves and root cortex were obtained from local farms. Linamarin was obtained from BDH Chemicals, Poole, U.K. p-Nitrophenyl-β-D-glycosides and 4-methyl umberriferyl glycosides were from Sigma Chemical Co., St. Louis, MO, USA..

Extraction and Purification

Linamarase was purified from cassava stem, petiole and root cortex using the procedure of Eksittikul and Chulavatnatol[4] with some modifications, with all steps being performed at 4°C. Chopped cassava tissue was homogenized in 0.1 M sodium acetate buffer, pH 5.5, containing 0.1 mM phenylmethylsulphonyl fluoride and 5% (w/w) polyvinylpyrollidone. The extract was filtered through a Miracloth sheet in a Buchner funnel and centrifuged at 12,000 g for 15 min. The supernatant was treated with Dowex 2-X8 for 1 h with stirring, filtered through Miracloth, and centrifuged again as described. The supernatant fluid was brought to 65% saturation with solid ammonium sulfate, and the precipitate was collected by centrifugation at 14,500 g for 20 min, redissolved in 10 mM potassium phosphate buffer, pH 5.5 and dialyzed against the same buffer (2 x 100 vol) overnight. The solution was clarified by centrifuging at 14,5000 g for 20 min and subjected to chromatography on a Sepharose 4B column (2.5 x 80 cm) equilibrated and eluted with 50 mM potassium phosphate buffer, pH 5.5. Fractions of 2.5 ml were collected and analyzed by measurement of absorbance at 280 nm and enzyme activity as described below. Fractions containing linamarase activity were pooled, dialyzed against distilled water and lyophilized.

Repeat chromatography on Sepharose 4B

Occasionally, repeat chromatography of purified cassava linamarase on Sepharose 4B was performed. Tubes corresponding to the high molecular weight and low molecular weight regions of the first Sepharose 4B column were separately taken and separately subjected to a second chromatographic separation on Sepharose. With each pool, tubes were consecutively loaded to the column without pooling, so that the separation on the first column was retained, and improved on the second chromatography step.

Enzyme Assays

Enzyme activity was determined by using linamarin or p-nitrophenyl-β-D-glucoside (pNPG) as substrates. With linamarin[4], the 1 ml reaction mixture containing 0.2 M potassium phosphate buffer, pH 6.8, 1-2 mM linamarin, and 0.05 ml enzyme was incubated for 30 min at 37°C, and the released cyanide was trapped with 0.4 ml of 0.5% picric acid in 2.5% sodium carbonate and quantitated by measurement of A_{515}. With pNPG[18], the 1 ml reaction mixture containing 50 mM tris-HCl, pH 8.0, 1 mM pNPG and 0.1 ml enzyme, was incubated at 40°C for 10 min, and p-nitrophenol released was measured at A_{400} after addition of 2 ml of 1M sodium carbonate.

Electrophoretic analysis under denaturing conditions

Sodium dodecyl sulfate(SDS)-polyacrylamide gels were performed on slab gels (15 cm x 12 cm x 0.1 cm) containing a 7-15% gradient of polyacrylamide, according to the procedure of Laemmli[19], and proteins were stained with Coomassie Blue R-250. Acid-urea gels[20] were performed in 6% polyacrylamide slab gels (15 cm x 13 cm x 0.1 cm) containing 6 M urea, 0.9 N acetic acid, pH 2.7, with buffer vessels containing 0.9 N acetic acid, 1 mM cysteamine. Triton-acid-urea gels[21] were also performed in 6% polacrylamide slab gels (15 cm x 13 cm x 0.1 cm) containing 0.4% triton X-100, 6 M urea, 0.9 N acetic acid, pH 2.7, with buffer vessels containing 0.9 N acetic acid, 1 mM cysteamine. Both types of gel were stained with Coomassie blue R-250.

Electrophoresis under non-denaturing conditions

Non-denaturing polyacrylamide gel electrophoresis was performed 5% polyacrylamide slab gels (15 cm x 12 cm x 0.1 cm) according to the procedure of Cameo and Blaquier[22]. Enzyme activity was detected by staining with 4-methylumberriferyl-β-D-glucoside, and proteins were stained with Coomassie blue R-250. Molecular weight determination of native enzyme by electrophoresis was performed by using the same non-denaturing polyacrylamide gel system, except that different concentrations of polyacrylamide were used (4.5%, 5%, 5.5%, 6%, 7%, 8%, 9% and 10%). The relative mobilities (R_f) of linamarase bands and of standard proteins of known molecular weight were measured at different concentrations of polyacrylamide[23]. Standard proteins used were chick egg albumin (monomer 45,000 daltons), bovine serum albumin (monomer 68,000 daltons and dimer 136,000 daltons), urease (trimer 272,000 daltons and hexamer 545,000 daltons). Graphs were drawn between the 100 [Log (Rf x 100)] on the ordinate against the % gel concentration on the abscissa[23]. The logarithm of the negative slopes so obtained were plotted against the logarithm of the molecular weight, and the molecular weights of the unknown bands were determined from the graph.

Non-denaturing electrophoresis of cassava fresh extracts

Fluid was rapidly obtained from fresh cassava petioles, leaf and stem by inserting samples of tissue into a 10 ml syringe and depressing the plunger to squeeze the tissue and release the fluid. The fluid so obtained was diluted with sample buffer, and applied directly to 5% polyacrylamide gels without prior centrifugation.

RESULTS AND DISCUSSION

Column chromatography of cassava linamarase

Linamarase was extracted from cassava stem, petiole and root cortex as described above. Modifications to the previous procedure[4] included treatment with polyvinyl polypyrollidone and Dowex 2-X8 during extraction to help remove polyphenolic compounds. Moreover, since linamarase was eluted close to the breakthrough peak on Sepharose 6B[4], the larger pore gel Sepharose 4B was used instead and this gave a rather broad peak for linamarase in the size range 600,000-2,000,000 daltons (Figure 1A), in contrast to other plant linamarases,

which have generally have molecular weights of less than 600,000 [7-9]. However, purified linamarase from cassava petiole has been shown to give a single band of 63,000 daltons in SDS-polyacrylamide gel electrophoresis[4] so that the high apparent molecular weight observed in gel filtration under native conditions is therefore probably due to aggregation of subunits. Thus the highest molecular weight regions (Pool 1, tubes 50-62) and lowest molecular weight regions (Pool 4, tubes 88-103) were separately taken and subjected to a second Sepharose 4B chromatography step without prior concentration. For comparison, the elution profile of the repeat chromatography of linamarase from the high and low molecular weight regions are superimposed in Figure 1B, and the A_{280} profile has been omitted for simplicity. On this repeat chromatography (Figure 1 B), linamarase from the high molecular weight region (Pool 1) of the first column was eluted out at an earlier position than linamarase from the low molecular weight region (Pool 4) of the first column. These results indicate that the high and low molecular weight regions of the first column consist of aggregates of different size, which do not readily equilibrate with each other. Moreover gel filtration of linamarase in buffer containing various NaCl concentrations of 0 M, 0.2 M, 0.4 M, and 0.8 M did not show differences in the extent of aggregation (data not shown).

Electrophoretic studies under denaturing conditions

To detect any possible heterogeneity in the subunit composition, purified cassava linamarase was subjected to electrophoresis under various denaturing conditions (Figure 2). In SDS-polyacrylamide gel electrophoresis (Figure 2 A), cassava linamarase purified from petiole (lane 1), stem (lane 2), and root cortex (lane 3) gave single bands of Mr 63,000 as expected, with no evidence of other components. Electrophoretic analysis in 6 M urea, 0.9 N acetic acid gels (Figure 2 B) also showed single bands for linamarase purified from petiole (lane 1), stem (lane 2), and root cortex (lane 3), although some blurring was evident in the latter. Similar results were obtained by triton-acid-urea electrophoresis (Figure 2 C), with petiole (lane 1), stem (lane 2), and root cortex (lane 3) each giving a single band of protein. Thus electrophoretic studies under denaturing conditions did not provide any evidence for the existence of more than one type of subunit.

Electrophoretic studies under non-denaturing conditions

To study the native state of the linamarase molecule, electrophoretic studies were carried out by non-denaturing polyacrylamide gel electrophoresis. The results (Figure 3) showed ladder patterns for both the protein and the activity stains of purified linamarase from petiole (lane 3), stem (lane 4), and root cortex (lane 5). Such ladder patterns suggest the existence of aggregates of different sizes, in agreement with the results of gel filtration.

To determine the molecular weight of the linamarase bands, electrophoresis under non-denaturing conditions was performed at different polyacrylamide concentrations, and graphs were plotted between 100 [Log (R_f x 100)] on the ordinate and % gel concentration on the abscissa . The secondary plots (Figure 4) of the Log (-slope) against Log (molecular weight) were linear (correlation coefficient, r = 0.996). The molecular weights of the linamarase bands were determined from their Log (-slope), with the results shown in Table 1. Assuming a molecular weight of 63,000 daltons for the linamarase subunit, the different linamarase bands correspond to aggregates of 4, 6, 8, 10, and 12-14 subunits. In

TABLE 1. Molecular Weight Determination of Linamarase Bands by non-denaturing polyacrylamide gel electrophoresis at different % gels.

Band number[a]	- Slope[b]	Mol Wt Determined[c]	MW/ 63,000[d]	Number of subunits[e]
1	17.716	272,400	4.32	4
2	20.848	353,800	5.62	6
3	26.118	507,700	8.06	8
4	29.716	624,100	9.91	10
5	35.471	828,400	13.15	12-14

[a] Linamarase bands are numbered starting at the fastest moving major band

[b] Slopes were obtained from the graphs of 100 log [R_f x 100] vs % polyacrylamide for each band

[c] Molecular weight was determined from the graph of standard proteins shown in Figure 4

[d] In calculating the number of subunits, the subunit molecular weight was assumed to be 63,000

[e] Number of subunits calculated to the nearest integer

addition, aggregates of larger size were also detectable, but their sizes were too large for accurate molecular weight determination.

Non-denaturing electrophoresis of fresh extracts

To see whether ladder patterns were detectable in fresh extracts, fluid was rapidly obtained by squeezing samples of fresh cassava petioles, leaf and stem through a 10 ml syringe. The fluid so obtained was diluted with sample buffer, and applied directly to 5% polyacrylamide gels without prior centrifugation. The whole process of extraction, from removal of cassava tissues to commencement of electrophoresis took less than 1 h. The results (Figure 5) showed that ladder patterns were visible in fresh extracts of leaf (lane 1), petiole (lane 2) and stem (lane 3), indicating the presence of aggregates even in freshly extracted specimens.

Additional studies on the effect of sodium dodecyl sulfate

To further demonstrate that the aggregates of various sizes were not the result of non-specific interactions or disulfide cross-linkage, additional studies were performed on the effect of the anionic detergent SDS on purified linamarase. Cassava linamarase was found to retain most of its activity (77%) after incubating with 1% SDS for 30 min at room temperature. No further loss was observed when the concentration of SDS was increased (up to 20 % w/v). The electrophoretic patterns of enzyme treated with 1% SDS and mercaptoethanol with and without heat treatment were also studied (Figure 6). When treated with SDS and mercaptoethanol without heating, linamarase still showed ladder patterns with both the protein stain (Figure 6A, lane 2) and the activity stain (Figure 6B, lane 2), indicating that the enzyme still retained activity and that aggregates were present. However, when enzyme treated with SDS and mercaptoethanol was boiled for 2 min, one protein band of 63,000 was present (Figure 6A lane 1) but no activity was detectable (Figure

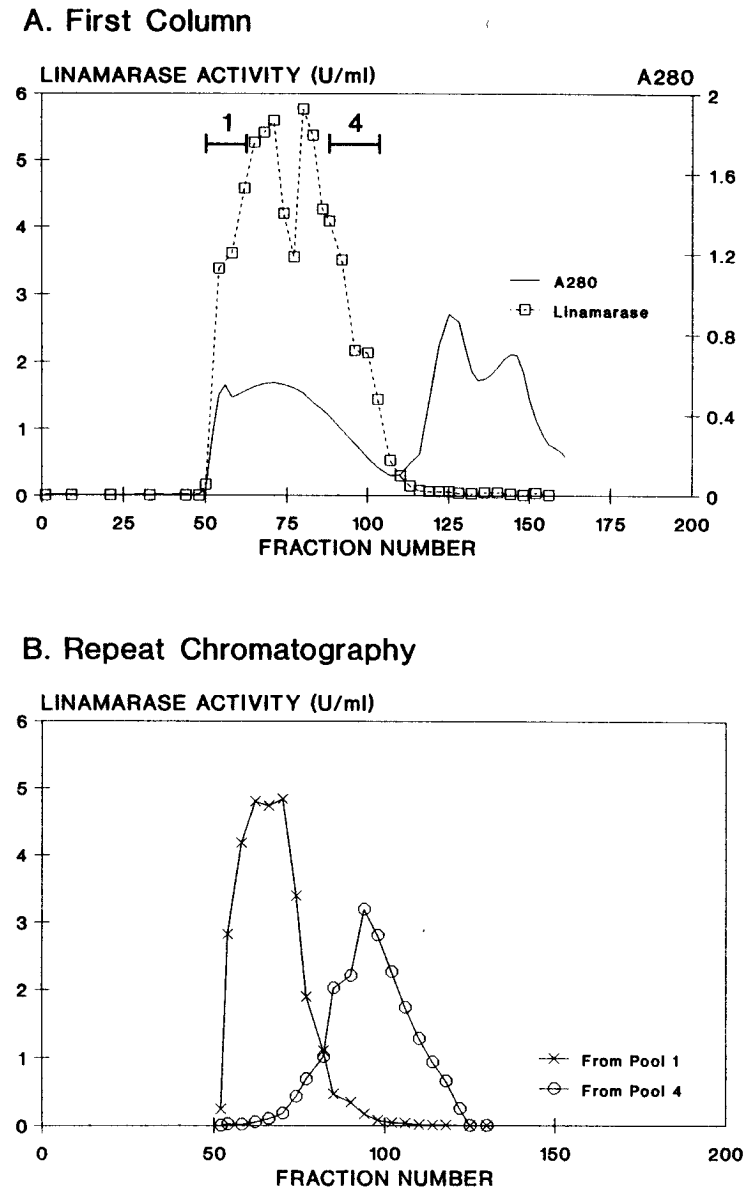

Fig. 1. Sepharose 4B chromatography of petiole enzyme obtained by ammonium sulfate precipitation. *(A) First column:* fractions were monitored for protein by measuring A_{280} (——) and linamarin hydrolyzing activity (❑ - - - ❑). Linamarase activity was found in the MW range 600,000 - 2,000,000, and the high molecular weight region (Pool 1, tubes 50-62) and low molecular weight region (Pool 4, tubes 88-102) were subjected to a repeat chromatography. *(B) Repeat chromatography:* Pool 1 and Pool 4 from the first column were separately chromatographed, but linamarase activities obtained were superimposed for the purposes of comparison. x——x Linamarase derived from Pool 1; O - - - O Linamarase derived from Pool 4.

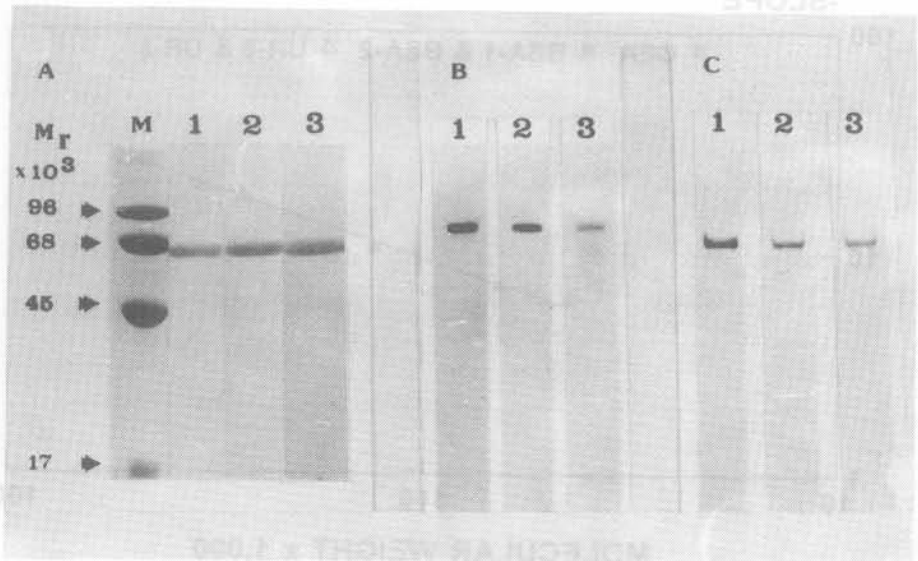

Fig. 2. Electrophoresis of purified cassava linamarase under denaturing conditions in various gel systems, followed by protein staining. *A:* SDS-polyacrylamide gels; *B:* Acid-urea gels; *C:* Triton-acid-urea gels. *M:* Molecular weight markers; *1:* petiole linamarase; *2:* stem linamarase; *3:* root cortex linamarase.

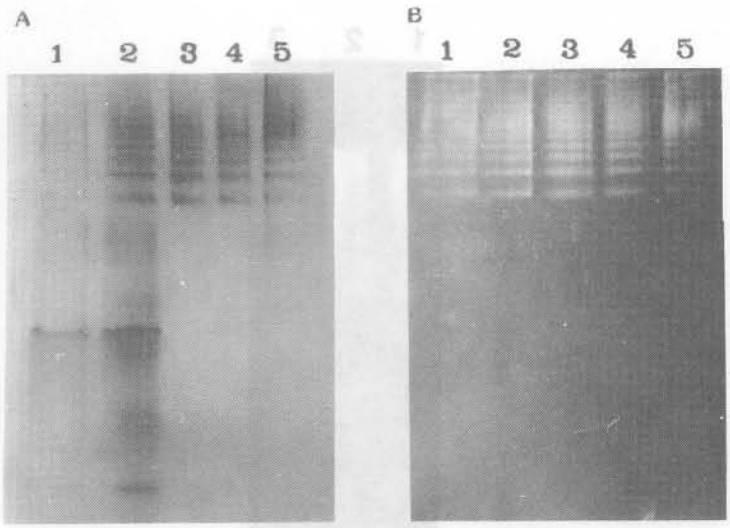

Fig. 3. Non-denaturing polyacrylamide gel electrophoresis on 5% gels. A: Protein stain; B: Activity stain. 1: petiole crude extract; 2: petiole $(NH_4)_2SO_4$ ppte; *3:* petiole Sepharose pool; *4:* stem Sepharose pool; 5: root cortex Sepharose pool.

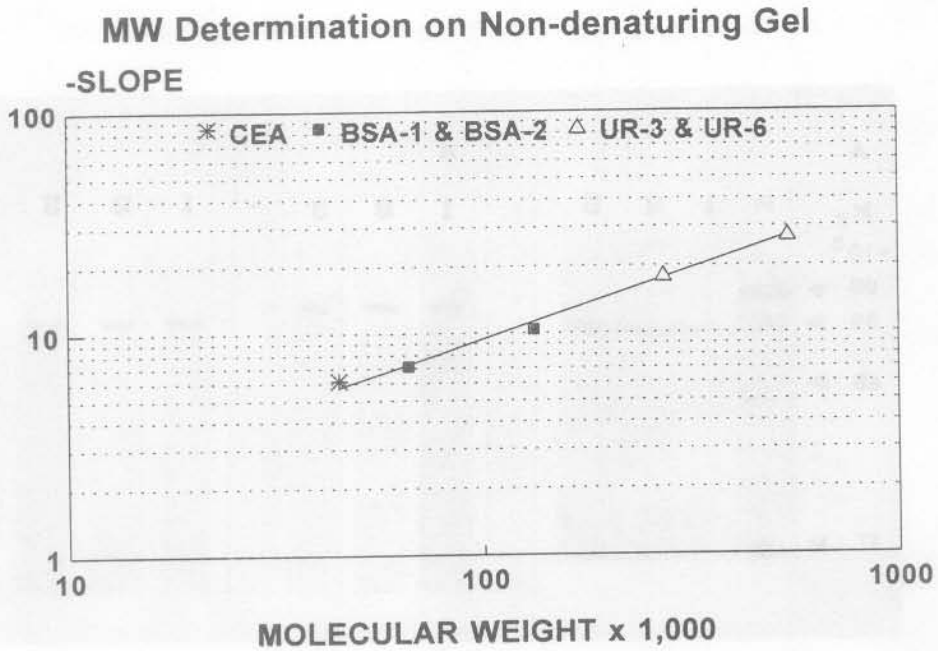

Fig. 4. Molecular weight determination of linamarase bands by electrophoresis under non-denaturing conditions at various % gel concentrations. Negative slopes from the primary plot of 100 Log (R_f x 100) vs % gel concentration were plotted against molecular weight in a double logarithmic manner. MW markers were: chick egg albumin (CEA); bovine serum albumin monomer (BSA-1) & dimer (BSA-2); urease trimer (UR-3) & hexamer (UR-6). Vertical dashed lines indicate molecular weights determined for linamarase bands.

Fig.5. Non-denaturing polyacrylamide gel electrophoresis of fresh crude extracts obtained from cassava tissues, after staining for β-glucosidase activity. *1:* petiole extract; *2:* leaf extract; *3:* stem extract.

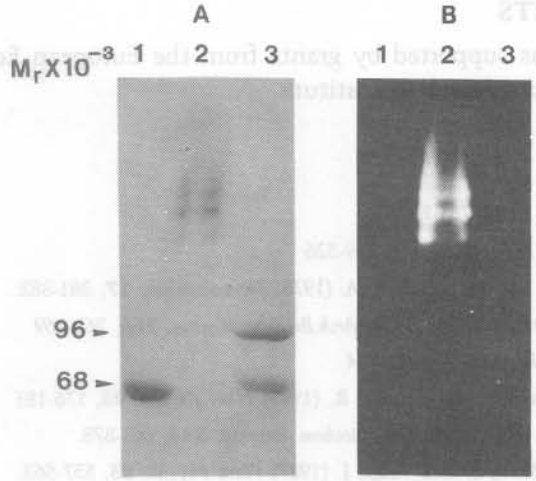

Fig. 6. SDS-polyacrylamide electrophoresis of purified cassava petiole linamarase after different treatments. *A:* Protein stain; *B:* Activity stain. *1:* treated with 1% SDS and 1% mercaptoethanol, and boiled for 2 min; *2:* treated with 1% SDS and 1% mercaptoethanol, but not heated; 3: molecular weight markers, 68,000 and 94,000 daltons.

6B, lane 1), indicating that dissociation into monomers had occurred with loss of activity. Since the unheated sample (Figure 6, lane 2) contained mercaptoethanol, aggregation is unlikely to be the result of disulfide bonding. Moreover, extensive attempts using various dissociating agents to obtain monomers or dimers with enzymatic activity have, so far, not been successful, suggesting aggregation may be a necessary requirement for activity.

CONCLUSIONS

Extensive information is now available on the structure[14], mechanism of action[16,17], and localization[5,15] of cassava linamarase. However, the physiological action of an enzyme necessarily depends on its native form at its site of action. In the present experiments, we have further studied the heterogeneity of cassava linamarase by chromatographic and electrophoretic studies. Electrophoretic studies under various denaturing conditions showed no evidence for the existence of more than one type of subunit, despite the fact that these techniques were selected to employ different principles in separation, namely molecular weight in SDS-polyacrylamide gel electrophoresis, content of basic amino acids in acid-urea gel electrophoresis, and content of hydrophobic amino acids in triton-acid-urea gel electrophoresis. Chromatographic studies on Sepharose 4B suggest that linamarase subunits combine to form large aggregates. This was further confirmed by the ladder pattern found in non-denaturing polyacrylamide gel electrophoresis, and the sizes of these aggregates were further determined to consist of 4, 6, 8, 10, 12-14 subunits and larger. Such aggregates do not appear to result from disulfide bonding or ionic interaction. The fact that aggregates of various sizes were also found in fresh extracts suggests that they may be present *in vivo* and may play some role in the physiological action of the enzyme.

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บทคัดย่อ

เอ็นไซม์ลินามาเรสจากมันสำปะหลังได้รับการศึกษาโดยวิธีโครมาโตกราฟี และอีเล็คทรอโฟริซิส เมื่อผ่านเอ็นไซม์เข้าไปในคอลัมน์เซฟาโรส 4 บี ซ้ำสองครั้ง พบว่าเอ็นไซม์ลินามาเรสรวมตัวกันเป็นกลุ่มหลายขนาดตั้งแต่ 600,000 ถึง 2,000,000 ดาลตัน โดยที่กลุ่มเหล่านี้ไม่เปลี่ยนกลับไปมา การวิเคราะห์ด้วยวิธีอีเล็คทรอโฟริซิส ภายใต้สภาวะที่ทำให้เสียสภาพด้วยโซเดียมโดเดซิล-ซัลเฟต กรด-ยูเรีย หรือไตรตอน-กรด-ยูเรีย ชี้ให้เห็นว่าเอ็นไซม์ลินามาเรสประกอบด้วยหน่วยย่อยเพียงชนิดเดียว การจับกันเป็นกลุ่มๆ จะตรวจพบได้ด้วยวิธีอีเล็คทรอโฟริซิสที่ใช้โพลีอะคริลาไมด์ที่แยกขนาดได้ในสภาวะที่ไม่ทำให้เสียสภาพ ขนาดของกลุ่มต่างๆ ที่สามารถตรวจวัดได้ประกอบด้วยกลุ่ม 4, 6, 8, 10, 12-14 หน่วยย่อยและกลุ่มที่ใหญ่กว่า นอกจากนี้กลุ่มขนาดต่างๆ เหล่านี้ตรวจพบได้ในสารสกัดสดจากใบมันสำปะหลัง แสดงว่ากลุ่มขนาดต่างๆ มีอยู่ในสภาพธรรมชาติ และอาจมีบทบาทในการทำงานของเอ็นไซม์กลุ่มต่างๆ ไม่แยกตัวเป็นหน่วยย่อยเมื่อเติมโซเดียมโดเดซิลซัลเฟต หรือ เบตา-เมอร์เคปโตเอธานอล นอกจากจะให้ความร้อนด้วย