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SUBUNIT STRUCTURE OF LYSINE SENSITIVE ASPARTATE KINASE FROM SPINACH LEAVES

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Summary

The lysine -sensitive isoenzyme of aspartate kinase was purified to homogeneity from spinach leaves and its subunit composition was studied. The purified preparation had an apparent molecular mass of 280,000 and separated into two subunits- a large subunit with molecular mass of 53000 and smaller subunit with molecular mass of 17,000 by urea treatment and SDS PAGE. The enzyme molecule has subunit composition of 4 large and 4 small subunits. The activity of the large subunit was stimulated more than two fold by the addition of small subunit and the stimulated activity was inhibited by EGTA. This inhibition could be reversed by Ca^{++}. Further characteristics of the smaller subunit such as heat stability, behavior on ion exchange chromatography, elctrophoretic mobility on polyacrylamide gels, amino acid composition and pattern, presence of trimethyl lysine, its ability to activate other calmodulin stimulated enzymes and its calmodulin-like nature in RIA tests suggested that this subunit is identical to calmodulin.

Key Words: Lysine sensitive aspartate kinase, subunit, calmodulin.

Introduction

In earlier communications (1,2) it was shown that aspartate kinase (E.C.2.7.2.4) in spinach leaves occurs as lysine and threonine-sensitive isoenzymes. Studies on the two AK isoenzymes showed that only the threonine-sensitive isoenzyme was activated by Ca^{++} and calmodulin. The lysine-sensitive isoenzyme did not respond to Ca^{++} and calmodulin addition (2). The lysine and threonine-sensitive isoenzymes have now been purified to homogeneity. The studies on threonine-sensitive isoenzyme have shown that it is bifunctional protein having both homoserine dehydrogenase and aspartate kinase activities. Both of these activities are sensitive to threonine and activated by calmodulin (3). Polyclonal antibodies have also been raised against both aspartate kinase isoenzymes (4). The differences

Abbreviations: EGTA, Ethylene glycol bis (B-aminoethylene)N,N,N,N-tetra acetic acid, AK, aspartate kinase.

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between the two isoenzymes with respect to Ca^{++} and calmodulin were intriguing and might be due to the fact that the lysine-sensitive AK isoenzyme binds calmodulin so tightly, that it was not removed by ion exchange chromatography. This implied that calmodulin could be a constituent of this isoenzyme.

In this communication, the purification and properties of the lysine-sensitive isoenzyme of aspartate kinase from spinach leaves are reported. It is shown that lysine-sensitive AK isoenzyme when treated with 4 M urea indeed releases a subunit which is similar to calmodulin in its physiochemical properties. This is a first example of plant enzyme possessing calmodulin-like protein subunit.

Materials and Methods

The early steps in the purification of the lysine-sensitive aspartate kinase isoenzyme were as described earlier (2) and involved purification on DEAE-cellulose and Sephadex G-200 columns. Further purification involved hydroxyapatite, and again Sephadex G-200 chromatography, and finally by FPLC (Pharmacia) using Mono Q column. 50 mM Tris- HCL buffer (pH 7.0) containing 1 mM EDTA was used for eluting the enzyme through different columns. For better results from hydroxyapatite column 1 gm of pre-swollen hydroxyapatite was first equilibrated with buffer and then the enzyme was mixed well with slurry and kept in cold room for 3-4 hours on magnetic stirrer. After that bound enzyme was eluted with different NaCl concentrations. The enzyme from hydroxyapatite was desalted on Sephadex G 200 column and was loaded on a FPLC Mono Q column. The enzyme was eluted with a gradient of 0-0.6 M NaCl in 50 mM Tris buffer (pH 7.5). The active fractions were pooled and dialysed against the same buffer without NaCl to remove salt. The dialysed enzyme was concentrated against glycerol and used for further study.

Protein content was estimated by the procedure of Lowry et al. (5). Aspartate kinase activity was assayed by the hydroxamate method as described by Davies and Miflin (6) and used in our earlier paper (1). One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of one n mol of hydroxamate per minute under standard assay conditions. (The molar extinction coefficient for aspartyl hydroxamate used was 528). Nitrate reductase was assayed by the procedure of Jawali and Sane (7).

To separate the aspartate kinase subunits, the purified enzyme was treated with 4M urea as described by Biswas and Paulus (8) passed through a Sephadex G-200 column. This resulted in the separation of two protein peaks which were used for further studies. The molecular masses were estimated by column chromatography and SDS polyacrylamide gel electrophoresis. Electrophoresis on 10% standard polyacrylamide gels was carried out using 4% stacking gels under the conditions described by Fehrstrom and Moberg (9). Electrophoresis under denaturing conditions was carried out in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulphate as described by Laemmli (10).

For amino acid analysis, 1 mg protein was hydrolyzed with 6N HCL in a sealed tube under vacuum at 104-110° C for 24 h. The sample was evaporated to dryness under reduced pressure. The residue thus obtained was dissolved in sodium citrate buffer (pH 7.2) and analyzed on an LKB-4101 amino acid analyzer after appropriate dilution. RIA was done by Hindustan Lever Reserch Centre, Bombay (India).

Results

Purification of lysine-sensitive isoenzyme:

The details of purification involving a series of chromatographic procedures are given in Table 1. The enzyme was purified to homogeneity as indicated by electrophoresis on native and SDS polyacrylamide gels. The isoenzyme was purified 856- fold with a specific activity of 1711 units/mg protein and 8.8% yield. The purified preparation gave a single band on native polyacrylamide gel and had a molecular mass of about 280,000 as determined on pre-calibrated Sephadex G-200 column (Fig. 1). It was inhibited 80% by 10mM lysine and was insensitive to calcium and calmodulin.

Separation of subunits and subunit structure of isoenzyme:

Electrophoresis of the lysine-sensitive isoenzyme under denaturing conditions (SDS PAGE) revealed the presence of two bands. One of these had a high electrophoretic mobility and corresponded to a molecular mass of

TABLE 1

PURIFICATION OF LYSINE-SENSITIVE ISOENZYME OF ASPARTATE KINASE FROM SPINACH LEAVES

Fraction	Total Protein (mg)	Total Activity Units/min	Specific Activity Units/min	Fold Purification	Yield (%)
I Crude Extract	6498.0	13500	2.1	1.0	100.0
II Amm. Sulfate Fraction	1571.0	12544	7.9	3.8	92.7
III Sephadex G100	1060.0	8737	8.9	4.3	64.7
IV DEAE Cellulose	71.6	5548	78.1	39.0	41.0
V Hydroxyapatite	10.8	5350	500.0	241.5	39.4
VI Sephadex G-200	1.5	1354	920.7	444.7	10.0
VII FPLC Mono Q	0.7	1198	1711.0	856.5	8.8

Enzyme activity assayed by hydroxamate method.

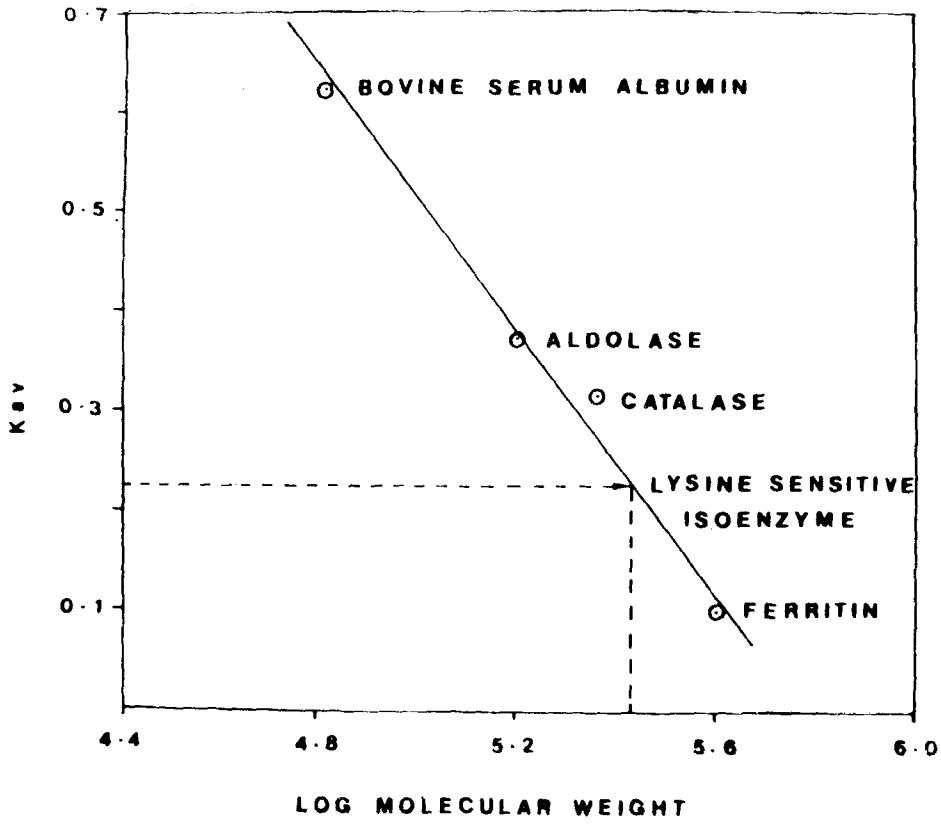

Fig.1. Calibration curve for estimation of the molecular masse using a Sephadex G-200 column. The arrow indicates the log molecular mass of lysine-sensitive isoenzyme. Following standards were used: bovine serum albumin (Mr 66,000), aldolase (Mr 150,000), catalase (Mr 232,000) and ferritin (Mr 440,000).

17,000, whereas the more slowly migrating larger subunit had a molecular mass of 53,000 (Fig. 2). The two types of subunits could be separated by gel filtration on Sephadex G-200 column in the presence of urea. The enzyme renatured into subunits of molecular masses of about 200,000 and 17,000 when eluted with a buffer devoid of urea, but remained separated as subunits in the presence of urea. In view of the molecular mass of about 280,000 of native enzyme, the results suggest a subunit composition of the lysine-sensitive isoenzyme in higher plants of the type $\alpha 4$ ß4.

When the enzyme was treated with 4M urea and then separated with a buffer minus urea on Sephadex G-200 column (pre-calibrated with molecular mass markers), two protein peaks were obtained (Fig.3), one with an apparent molecular mass of 200,000 and another with a molecular mass

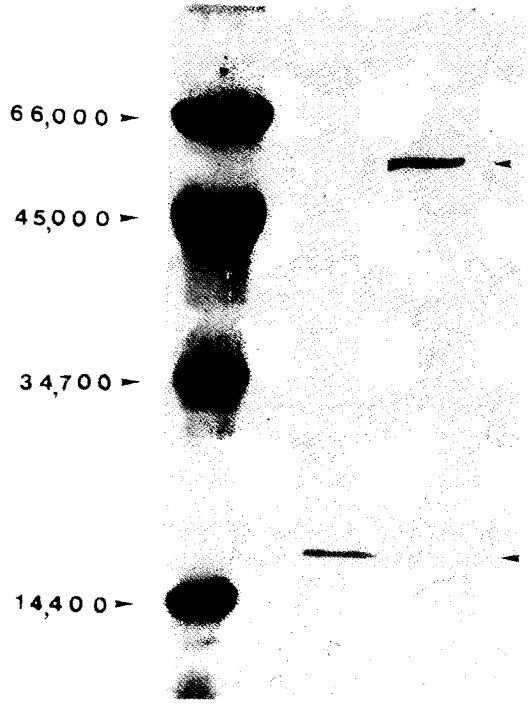

Fig.2. Polyacrylamide gel electrophoresis of lysine- sensitive isoenzyme in the presence of sodium dodecyl sulphate (SDS) on 10% gel. Lane A: Molecular mass markers-bovine serum albumin (Mr 66,000), ovalbumin (Mr 45,000), pepsin (Mr 34,700), egg white lysozyme (14,400) Lanes B and C show two bands in the purified preparation of lysine-sensitive aspartate kinase. The band with low electrophoretic mobility has been designated as LS and the one with high electrophoretic mobility as SS.

of 17,000 (Fig. 4). Low aspartate kinase activity was detected in the first peak whereas the second peak had no activity at all. SDS-PAGE of both the eluted bands separately gave one subunit each (Fig. 2). From the plots of migration distance against logarithm of molecular mass, the molecular mass of the larger subunit was found to be approximately of 53,000 and of the smaller subunit about 17,000 daltons.

Although the large subunit alone had some aspartate kinase activity, this activity could be stimulated over two-fold by the addition of small subunit (Table 2). The stimulated activity was inhibited by 5 mM EGTA, but the inhibition could be reversed by the addition of calcium. The stimulation of activity by small subunit that had been subjected to heat treatment at

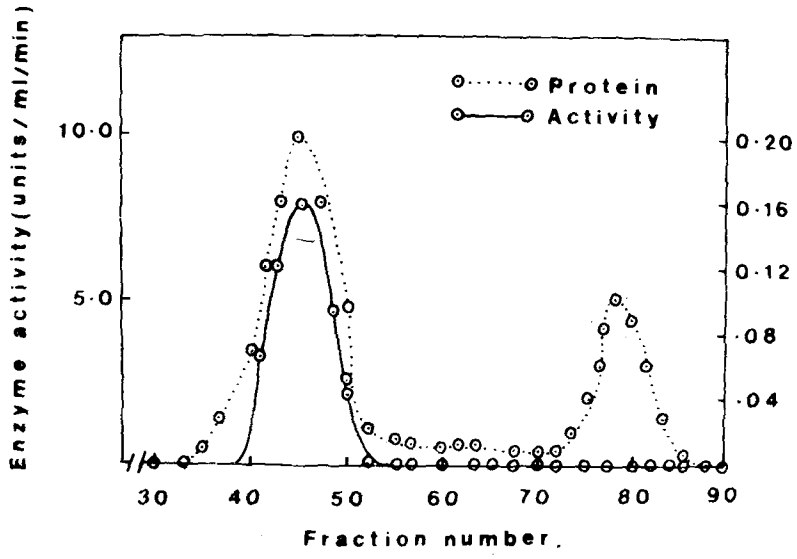

Fig.3. Protein and activity profile of the lysine-sensitive isoenzyme of aspartate kinase when passed through a column of Sephadex G-200 after treating it with 4 M urea. Only the larger peak showed some enzyme activity.

80° C for 3 min (Table 2) could be mimicked by calmodulin, provided that Ca^{++} was also added.

In order to test whether the small subunit may be identical to calmodulin, the activation of the threonine-sensitive isoenzyme of aspartate kinase and of nitrate reductase by the small subunit was tested. Both these enzymes were previously shown by us to be activated by calmodulin (2,3,11). The results presented in Table 3 show that both the threonine sensitive aspartate kinase isoenzyme and nitrate reductase could be stimulated 2-3 fold by the small subunit, with calmodulin from bovine brain producing a similar degree of stimulation.

Amino acid composition and spectral properties of the small subunit:

The amino acid composition of the purified small subunit of aspartokinase is given in Table 4. The results show a high content of acidic amino acids, viz. glutamate and aspartate,similar to calmodulin. Indeed, the overall, amino acid composition of the subunit compares fairly well with the calmodulin in bovine brain by Watterson et al. (12). The amino acid analyzer results showed a peak in the position expected for trimethyl lysine both in calmodulin and small subunit of aspartokinase (Fig. 5) besides the standard amino acids.

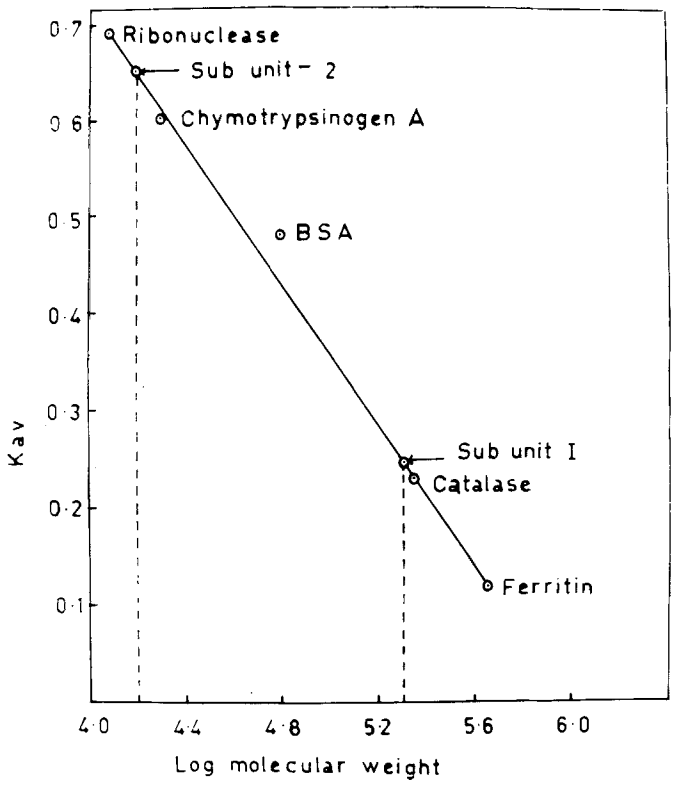

Fig.4. Calibration curve for estimating the molecular mass of the aspartate kinase subunits by elution from a Sephadex G-200 column. One of the proteins had an apparent molecular mass of 200,000 (subunit 1) and the other had molecular mass of about 17,000. Following molecular mass markers were used : ribonuclease (Mr 13,7000), chymotrypsiniogen (Mr 25,000), bovine serum albumin (Mr 66,000), catalase (Mr 232,000) and ferritin (Mr 440,000).

Radioimmunoassay of the small subunit of the lysine-sensitive asprtokinase isoenzyme was carried out by Hindustan Lever Research Centre at Bombay and showed that the small subunit cross reacted with antibodies against calmodulin (data not shown).

Discussion

The results presented in this communication demonstrate that lysine-sensitive isoenzyme has a molecular mass of about 280,000 and consists of two dissimilar subunits with molecular masses of 53,000 and 17,000. This

TABLE 2

EFFECT OF SMALL SUBUNIT, CALMODULIN, EGTA AND Ca^{++} ON THE ACTIVITY OF LARGE SUBUNIT OF ASPARTATE KINASE

Additions	Enzyme Activity (units/ml/min)
Large subunit (LS) 80 μg	33.0
LS + Small subunit (SS) 150 μg	72.7
LS + SS 150 μg + EGTA 5 mM	50.7
LS + SS + EGTA + Ca^{++}	70.3
LS + SS (Heated at 80°C)	67.6
LS + Calmodulin (authentic) 150 μg	25.0
LS + Calmodulin + Ca^{++} 250 μM	68.3

One unit enzyme produced 1 nmol of hydroxamate per ml/min at 30°C in the presence of 50 mM aspartate.

TABLE 3

ACTIVATION OF THREONINE-SENSITIVE ISOENZYME AND NITRATE REDUCTASE ACTIVITY BY SMALL SUBUNIT OF LYSINE-SENSITIVE ISOENZYME FROM SPINACH LEAVES

Additions	Enzyme Activity
Threonine-sensitive aspartokinase	100[a]
Threonine-sensitive + Small Subunit (150 μg)	353[a]
Threonine-sensitive + Calmodulin (125 μg)	340[a]
Enzyme (NR)	100[b]
Enzyme (NR) + Small Subunit (75 μg)	281[b]

a: Aspartate kinase acitivity 100 = 14.9 units/mg/ml/min.

b: Nitrate reductase activity 100 = 8.7 μg nitrite/mg/protein

Bovine brain calmodulin (Sigma) was added to the extent of 125 μg per ml of reaction mixture. Small subunit was added to the extent of 150 μg per ml of reaction mixture in case of aspartate kinase and 25 μg per ml of reaction mixture in case of nitrate reductase.

TABLE 4

COMPARISON OF THE AMINO ACID COMPOSITION OF THE SMALL SUBUNIT (SS) OF LYSINE-SENSITIVE ISOENZYME OF ASPARTATE KINASE AND OF CALMODULINS FROM VARIOUS SOURCES

Amino Acid	(SS) Subunit	Spinach[a] Calmodulin	Bovine Brain Calmodulin[b]
Aspartic Acid	14.0	11.6	11.5
Threonine	7.0	6.2	8.1
Serine	4.0	2.7	2.7
Glutamic Acid	14.0	13.7	14.2
Proline	1.8	1.4	1.4
Glycine	11.4	6.8	7.4
Alanine	9.1	7.5	7.4
Half Cystine	0.3	0.7	0
Leucine	-[c]	7.5	6.1
Valine	4.4	5.5	4.7
Isoleucine	-[c]	4.8	5.4
Methionine	3.2	5.5	6.1
Tyrosine	1.3	0.7	1.4
Phenylalanine	4.9	6.2	5.4
Histidine	1.2	0.7	1.0
Arginine	3.9	3.4	4.1
Tryptophan	0[d]	0	0

[a]Spinach calmodulin from Lucas et al. (1988) Pl. Physiol. 75, 5788-5795.
[b]From the amino acid sequence of the bovine brain protein, Watterson (1980) J. Biol. Chem. 225, 962-975.
[c]Not estimated.
[d]Estimated from ultraviolet spectrum.

suggests that lysine-sensitive isoenzyme consists of 4 large subunits and 4 small subunits. A schematic representation of the proposed structure of the enzyme and its dissociation into subunits is presented in Fig. 6. The occurrence of two subunits of the lysine-sensitive isoenzyme with different molecular masses and properties has already been reported in bacteria (*Bacillus polymyxa)* by Biswas et al. (13). Biswas and Paulus (8) and Paulus (14) where the subunits have molecular masses of 43,000 and 16,000.

An important finding that emerges from our investigation is that the 17 KDa subunit of lysine-sensitive aspartokinase seems to be similar or

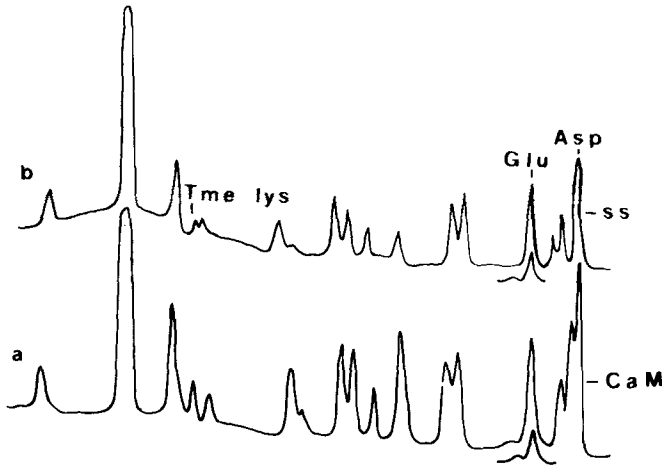

Fig.5. Amino acid profile of acid hydrolysates of calmodulin (bovine brain) and small subunit of lysine-sensitive aspartate kinase.

identical to the Ca^{++} dependent modulator calmodulin, by a variety of criteria. These include its heat stability, molecular mass, amino acid composition, cross-reaction with calmodulin on radioimmuno assay and ability to activate other calmodulin-activated enzymes, e.g., threonine-sensitive aspartokinase isoenzyme and nitrate reductase.

The interaction between the lysine-sensitive aspartokinase isoenzyme and the calmodulin-like subunit appears to be rather strong, as they were not separated during the normal course of purification and could be dissociated only under denaturing conditions. This observation and the fact that the activity of threonine-sensitive isoenzyme of aspartate kinase is regulated by calmodulin, suggests that the lysine-sensitive aspartokinase isoenzyme may be more ancient in the evolutionary scale and that the tightly bound aspartokinase subunit may have evolved into less strongly bound calmodulin-like polypepetide which ultimately assumed the role of regulatory protein.

A variety of cellular processes in plants, such as cytoplasmic streaming, cell division, and exocystosis (Poovaiah and Reddy, 18), and plant enzymes such as ATPases and protein kinases, have been reported to be under Ca++ -calmodulin regulation (15-21). The occurrence of calmodulin as a subunit of an enzyme has also been reported in some animal enzymes such as myosin light chain kinase of smooth muscle (22), and skeletal muscle phosphorylase kinase (23,24). However, the lysine-sensitive

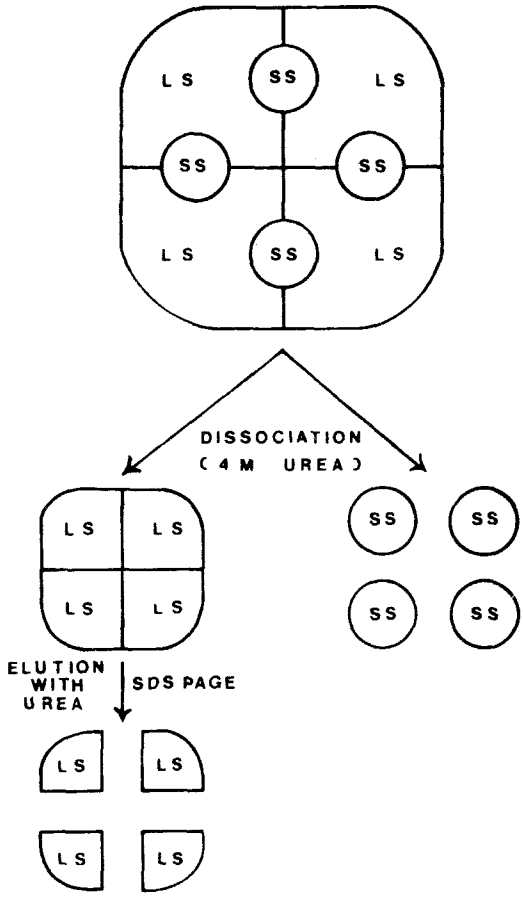

Fig.6. Proposed subunit structure of lysine-sensitive isoenzyme of aspartate kinase.

aspartate kinase may be the first example of a plant enzyme with calmodulin as a subunit.

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References

1. Sane, P.V., Kochhar, S., Kumar, N. and Kochhar, V.K. (1984) FEBS Lett. **195**, 238-242.
2. Kochhar, S., Kochhar, V.K. and Sane, P.V. (1986) Biochem. Biophys. Acta **880**, 220-226

3. Pavagi, S., Kochhar, S., Kochhar,V.K. and Sane, P.V. (1995) Biochem. Mol. Biol. International 36, 649-658.
4. Kochhar, S., Kochhar, V.K. and Sane, P.V. (1993) Ind.J. Biochem.Biophy. **30**, 199-203.
5. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randell, R.J. (1951) J. Biol. Chem. **193,** 265-275.
6. Davies, H.M. and Miflin, B.J. (1977) Sci. Lett. **9,** 329-333.
7. Jawali, N. and Sane, P.V. (1984) Phytochem. **23,** 225-228.
8. Biswas, C. and Paulus, H. (1973) J. Biol. Chem. **248,** 2894-2900.
9. Fehrstrom, H. and Moberg, U. (1977) LKB Application Note No 306, pp. 12-15.
10. Laemmili, U.K. (1970) Nature **227,** 680-689.
11. Sane, P.V., Kumar, N., Baijal, M., Singh, K.K.and Kochhar, V.K. (1987) Phytochem. **26,** 1289-1291.
12. Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980). J. Biol. Chem. **225,** 962-975.
13. Biswas,C., Grym, E. and Paulus, H. (1970) J. Biol. Chem. **245,** 4900-4906.
14. Paulus, H. (1984) J. Niol. Sci. **6,** 403-418.
15. Dieter, P. and Marme, P. (1980) Proc. Natl. Acad. Sci. USA **77,** 7311-7314.
16. Veluthambi, K. and Poovaiah, B.W. (1984) Science **223,** 167-169.
17. Veluthambi, K. and Poovaiah, B.W. (1986) Plant Physiol. **1,** 836.
18. Poovaiah, B.W. and Reddy, A.S.N. (1987) Crit. Rev. Plant Sci., CRC Press **6,** 47-103.
19. Hepler, P.K. and Wayne, R.D. (1985) Ann. Rev. Plant Physiol. **36,** 397-439.
20. Muto, S. (1992) Rev. Cytology **142,** 305-345.
21. Roberts, D.M. and Harmon, A.C. (1992) Ann. Rev.. Plant. Physiol. Mol. Biol. **43,** 375-414.
22. Drabowska. R., Aromatoril, O.D., Sherry, J.M.F. and Hartstone, D.J. (1977) Biochem. Biophy. Res. Comm. **78,** 1263-1272.
23. Yagi, K., Yazawa, M., Kakuichi, S., Oshimo, M. and Veushi, K. (1978) J. Biol. Chem. **253,** 1338-1340)
24. Cohen, P., Burchell, A., Foulkes, J.G., Cohen, P.T.W., Vanaman, T.C. and Naim, A.C. (1978) FEBS Lett. **92,** 287-293.