Pergamon

PII: S0031-9422(96)00674-7

Phytochemistry, Vol. 44, No. 6, pp. 985–989, 1997

0031-9422/97 $17.00+0.00

ISOLATION OF SIX LOW MOLECULAR WEIGHT HEAT SHOCK PROTEINS AND PARTIAL CHARACTERIZATION OF HEAT SHOCK PROTEIN 29 FROM MUNG BEAN HYPOCOTYL

DAN. H. WU and DAVID L. LAIDMAN*

School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, U.K.

(*Received* 22 *July* 1996)

Key Word Index—*Phaseolus aureus*; mung bean; heat shock protein; HSP29; isotypes; isolation; characterization.

Abstract—Heat shock protein (HSP29) together with five other low-M_r HSPs have been isolated from mung bean hypocotyls by preparative continuous elution SDS–PAGE. Autoradiography and immunochemical analysis of 2D electrophoretograms of the radiolabelled HSP29 revealed that it consists of seven isotypes, two of which are constitutive while the other five are heat-inducible. The pI values of the seven isotypes range from 4.6 to 6.6. A monoclonal antibody raised against the HSP29 isolate reacted with six of the seven isotypes. When HSP29 was subjected to partial proteolysis by V8 *Staphylococcus aureus* protease (EC 3.4.21.19), two fragments of 12 and 17 kDa were identified, neither of which was recognized by the antibody. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Plants, like other cellular organisms, synthesize increased amounts of specific proteins in response to heat or other stresses, while the synthesis of most constitutive proteins is suppressed or even stopped. These stress-induced proteins are commonly called heat-shock proteins (HSPs) [1–3], although many of them are also produced constitutively. A large number of HSPs have been identified (see [4]) with molecular weights ranging from *ca* 14 to 120 kDa. They are classified into five families, i.e. HSP100, HSP90, HSP70, HSP60 and the low molecular weight HSPs (LMW-HSPs) [3]. The LMW-HSPs constitute a gene superfamily, which is more variable than the other HSP families; its members vary both in size and number from species to species [2, 5]. Plants characteristically produce more of these LMW-HSPs than other organisms do, some plants producing more than 30 of them.

The amino acid sequences of many of the LMW-HSPs have been determined through gene sequencing [5]. The various members of the superfamily have two regions of homology, one near the carboxy-terminal end and the other in the central region of the peptide. These sequence analyses have also led to the LMW-HSPs being arranged into four subfamilies according to their sequence homologies.

In contrast to the detailed knowledge about the amino acid sequences of the LMW-HSPs, there is relatively little known about their functions. With a view to facilitating structural and functional studies of one of these proteins (HSP29), we report here its isolation from mung bean hypocotyls and its partial immunochemical analysis. We also report the simultaneous isolation of five other LMW-HSPs from the same source.

RESULTS AND DISCUSSION

Identification and isolation of HSPs

Analytical electrophoresis of extracts from control and heat-shocked radiolabelled hypocotyls revealed the production of 10 HSPs with molecular weight of 20, 21.5, 23, 29, 34, 38, 55, 62, 70 and 85 kDa (Fig. 1). Among them, HSP55 was the most strongly radiolabelled. Six of them, with molecular weights of 20, 21.5, 23, 29, 34 and 38 kDa, fall within the category of LMW-HSPs. This array of HSPs is not unusual compared with those produced by many other species of plants [2]. More surprising is the fact that the mung beans used in the present study accumulated a different set of HSPs compared with the mung beans used by Collins *et al.* [6] in their recent study. Their mung beans produced only one LMW-HSP of 18 kDa compared with the six identified in the present study. Theirs, on the other hand, produced HSPs of 46, 51,

* Author to whom correspondence should be addressed.

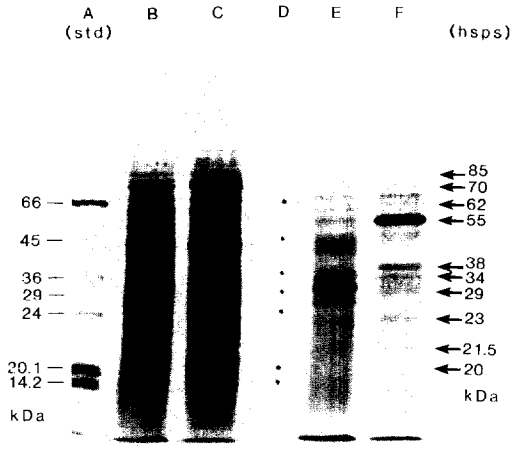

Fig. 1. Identification of radiolabelled HSPs. Lane A, silver-stained molecular weight markers; lane B, Ag-stained crude extract from control tissue; lane C, Ag-stained crude extract from heat-shocked tissue; lane D, pen trace of molecular weight markers from lane A; lane E, autoradiograph from lane B; lane F, autoradiograph from lane C.

73, 79 and 114 kDa, which were not identified in our study. The differences between the two sets of results are presumably reflections of a cultivar difference, since the choice of hypocotyl tissue and the experimental treatments were similar in each case. Collins *et al.* did not record the cultivar that they used.

All of the LMW-HSPs could be separated simultaneously from heat-shocked hypocotyls by continuous-elution SDS–PAGE (SDS–PAGE-CE) using a 13.5% resolving gel (procedure A). The HSPs were located mainly in fractions 33 (HSP20), 49 (HSP21.5), 73 (HSP23), 121 (HSP29), 218 (HSP34) and 266 (HSP38) (Fig. 2). Fractions on either side of these fractions contained smaller amounts of the corresponding HSPs accompanied by other peptides (data not presented). Each of the main HSP fractions contained 1.5–3.0 μg of protein, representing 0.1–0.2% of the total protein submitted to SDS–PAGE-CE. The results indicate that each of the fractions 33, 49, 73, 121, 218 and 266 contained the relevant HSP with relatively little contamination by other peptides.

The SDS–PAGE-CE procedure A was optimized for the separation of HSP29. Generally better separations of the other LMW-HSPs were achieved when procedures B and C were employed. These procedures involved the use of columns with, respectively, 15 and 12% resolving gels. Procedure A gave improved separations of HSP20, HSP21.5 and HSP23, while procedure C was more effective for the separations of HSP34 and HSP39 (data not presented). Analytical SDS–PAGE of the relevant HSP fractions from these procedures gave sharper bands than those from procedure A and there was no evidence for the presence of contaminating peptides. Attempts to further improve the resolution of the LMW-HSPs by using gradient SDS–PAGE-CE did not improve the results beyond those achieved with non-gradient gels.

The present study demonstrates SDS–PAGE-CE as a powerful method for the one-step isolation of plant LMW-HSPs from crude tissue extracts. It produces microgram quantities of the peptides, which run as discrete bands on analytical 1D SDS–PAGE. This does not, of course, exclude the possibility that each isolate contains more than one peptide with the same electrophoretic mobilities, not all of which are HSPs. If such were the case, however, the purifications could undoubtedly be improved by introducing preliminary purifications steps (e.g. ion-exchange chromato-

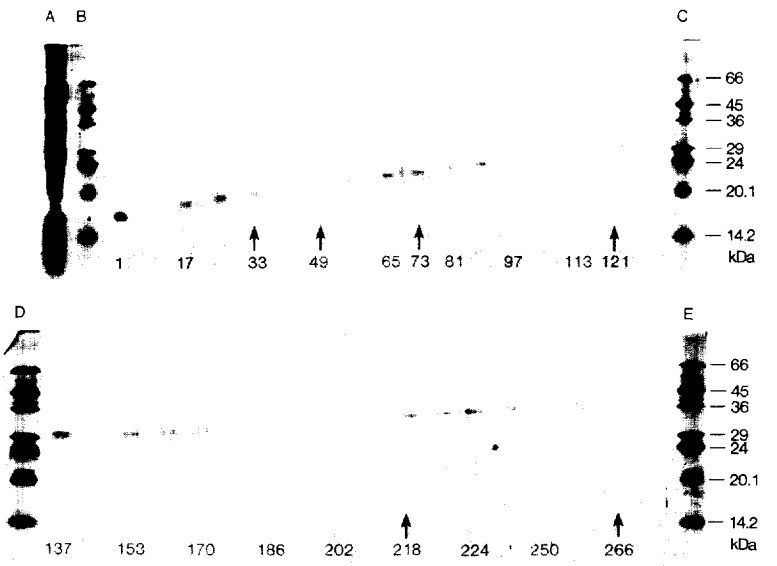

Fig. 2. Separation of LMW-HSPs by SDS–PAGE-CE using procedure A. Lane A, crude extract from heat-shocked tissue; lanes B, C, D and E, molecular weight markers; numbers 1–266, fraction numbers from SDS–PAGE-CE. HSP20 is in fraction 33, HSP21.5 in 49, HSP23 in 73, HSP29 in 121, HSP34 in 218 and HSP38 in 266. All lanes are Ag stained.

graphy) prior to SDS–PAGE-CE. In the case of HSP29 at least, it is clear that the isolate from SDS–PAGE-CE contains only HSP29 isotypes (see below).

Partial characterization of HSP29

A monoclonal antibody raised against HSP29 reacted specifically with the HSP29 band in analytical 1D SDS–PAGE gels run with crude extracts from mung bean hypocotyls (Fig. 3). This allowed us to use the antibody preparation, along with radioautography, for the further study of the HSP29 isolate from SDS–PAGE-CE.

Autoradiograms prepared from 2D SDS–PAGE gels run with the HSP29 isolate revealed the presence of seven radiolabelled peptides (Fig. 4). Five of these peptides with pI values of 4.6, 4.7, 5.6, 6.2 and 6.6 were heat-inducible, while the other two with pI values of 5.9 and 6.4 were constitutive. Six of the seven peptides reacted with the monoclonal antibody (Fig. 5(A)). These had pI values of 4.6, 4.7, 5.6, 5.9, 6.2 and 6.4. The remaining peptide did not react with the antibody, even though it was heat-inducible and therefore a HSP (see Fig. 4). This peptide was also not detected by silver staining (Fig. 5(B)), suggesting that it was present at very low levels. Further, the silver staining showed that the fraction from SDS–PAGE-CE contained only peptides identified as HSPs by radiolabelling. It is concluded that all seven peptides present in the fraction from SDS–PAGE-CE are isotypes of HSP29.

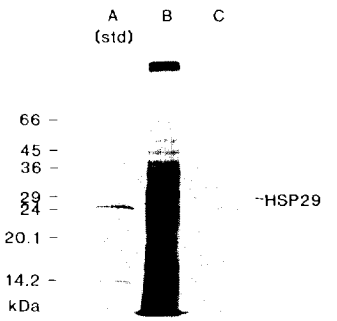

Fig. 3. Immunochemical analysis of HSP29. Lane A, Ag-stained molecular weight markers; lane B, Ag-stained crude extract from heat-shocked tissue; lane C, crude extract from heat-shocked tissue reacted with anti-HSP29 antibody.

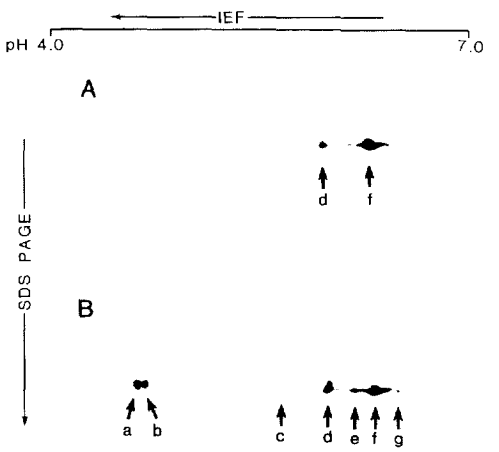

Fig. 4. Autoradiographic analysis of 2D SDS–PAGE gels from purified, radiolabelled HSP29. (A) HSP29 from control tissue; (B) HSP29 from heat-stressed tissue. Isotypes a, b, c, e and g are heat inducible, while isotypes d and f are constitutive.

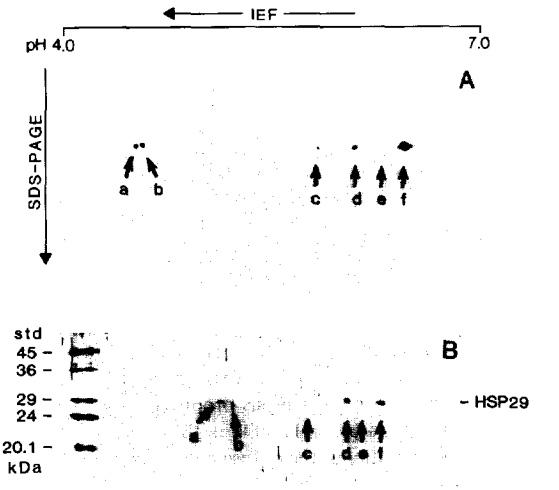

Fig. 5. Immunochemical analysis of 2D SDS–PAGE gels from purified HSP29. (A) Reacted with anti-HSP29 antibody; (B) Ag-stained. All the isotypes except isotype g reacted with the antibody. Isotype g was also not detected by Ag staining. Ag staining detects only HSP29 isotypes and not other peptides.

The existence of several isotypes of HSP29 is similar to the situation described earlier for HSP70 [7] and it raises the possibility that other HSPs may also be polytypic. The full significance of this is not known. It has been suggested that some HSPs confer protection and promote recovery from abiotic stress by virtue of their ability to bind partially denatured proteins and to protect them from heat-induced aggregation [8–11]. Perhaps a range of HSP29 isotypes with different surface characteristics (different pI values) allows their protect and repair functions to be carried out on a wider range of client proteins than would be possible with a single HSP. Also, some of the different isotypes might be chemically modified (phosphorylated) derivatives of a parent HSP involved in the renaturation process.

When the purified HSP29 was subjected to limited hydrolysis by *Staphylococcus aureus* protease (endo Glu-C; EC 3.4.21.19), it was cleaved into two fragments of *ca* 12 and 17 kDa (Fig. 6). Neither of the fragments was recognized by the monoclonal antibody (data not presented). This finding indicates that the epitope that is recognized by the antibody involves a region or regions of sequence homology which is common to six of the seven isotypes of HSP29. Cleav-

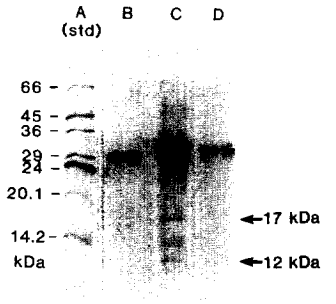

Fig. 6. Limited proteolysis of HSP29. Lane A, molecular weight markers; lane B, HSP29 before proteolysis; lane C, HSP29 plus *S. aureus* protease after proteolysis; lane D, *S. aureus* protease. The hydrolysis products are marked with arrows. The band at *ca* 11 kDa is the bromophenol blue marker. The identity of the diffuse band at *ca* 14 kDa is not known.

age in the central region of the peptide by the *S. aureus* protease destroys the epitope. These characteristics make the antibody an ideal tool for further studies of the structure and function of HSP29.

EXPERIMENTAL

Plant material. Mung beans (*Phaseolus aureus* cv. Roxborough) were soaked for 8 hr at 25° in distilled H_2O and germinated for 48 hr on moist filter paper (Whatman No. 1) contained in a glass bowl and incubated in the dark at 25°. Seedlings were harvested and sterilized for 10 min in dilute NaOCl (0.2% available Cl). A 2-cm hypocotyl segment was cut from below the plumular hook of each seedling and used as experimental material.

Radiolabelling and extraction of proteins. Batches of 4 sterilized hypocotyl segments were placed upright into glass vials (8 mm i.d.) containing 1.5 ml 1 mM HEPES–KOH buffer (pH 8.5) with 15 μCi L-[^{35}S]methionine (1 300 μCi mmol^{-1}) and incubated for 2 hr at either 25°C (control) or 42°C (heat-shock). After incubation, segments were washed several times in 1 mM HEPES–KOH buffer containing 0.1 mM non-radioactive L-methionine and blotted dry. They were then homogenized for 1 min at 4° in 20 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM Na EDTA, 0.1 mM β-mercaptoethanol, 2 mM phenylmethanesulphonyl fluoride, 1 mM benzamidine and 5 mM 6-amino-*n*-hexanoic acid, using a top-drive homogenizer. The vol. of the extraction medium was 1 ml g^{-1} tissue. Triton X-100 (1%, v/v) was added during the last 10 sec of homogenization. The homogenate was centrifuged for 20 min at 12 000 *g* and 4° and the supernatant was filtered through glass fibre (Whatman CF/C). The filtrate (protein extract) was stored at −20° for not more than 2 days before further processing.

Analytical electrophoresis and isoelectric focusing. 1D SDS–PAGE was conducted at pH 8.3 using a 3% stacking gel and an 11% resolving gel, each with 2.67% cross-linker. Protein samples were applied in loading buffer (12.5 mM Tris–HCl, pH 6.8, 2% glycerol, 0.4% SDS and 5% β-mercaptoethanol). After SDS–PAGE, proteins were transferred at pH 8.3 from the gels to nitrocellulose membranes where they were stained with $AgNO_3$ and labelled proteins were detected by radioautography.

For 2D SDS–PAGE, sepn in the first dimension was by isoelectric focusing using micro-glass tubes (1 × 8 mm) containing 3.8% gel with 5% cross-linker, 5.5 M urea, 2% NP-40 detergent and 4% ampholytes (pH 3–10). The developed gel was expressed from the tube and washed for 20 min in 0.25 M Tris–HCl buffer, (pH 6.8) containing 10% glycerol, 2% SDS and 2% β-mercaptoethanol. It was then submitted to SDS–PAGE (see above) in the second dimension.

Purification of LMW-HSPs. Crude extracts containing non-radiolabelled proteins were mixed (1:4) with loading buffer, containing 12.5 mM Tris–HCl buffer (pH 6.8), 2% glycerol, 0.4% SDS, 5% β-mercaptoethanol and bromophenol blue marker, and applied (8 mg protein in 2 ml) to the separating column (28 mm i.d.) of an SDS–PAGE-CE apparatus (Prep Cell Model 491, BioRad Labs, Hemel Hempstead, U.K.). Three procedures were used as follows.

In procedure A, a 13.5% non-gradient resolving gel (65 mm long) and a 3% stacking gel (25 mm long), both with 2.67% cross-linker, were employed. For the separating stage, the applied current was 60 mA, the eluting fluid flow rate was 10 ml hr^{-1} and the cooling fluid flow rate was 120 ml min^{-1}. Both eluting and cooling fluids consisted of 25 mM Tris–glycine buffer, (pH 8.3) and 0.1% SDS. When the marker reached the bottom of the column, the elution stage was begun. For this, the current was changed to 50 mA and the eluting and cooling fluid rates were adjusted to 60 ml hr^{-1} and 150 ml min^{-1}, respectively. After a further 4 hr and until completion, the current was changed to 70 mA and the eluting fluid rate was adjusted to 75 ml hr^{-1}. Fr. collection (1.5 ml fr.) was started 2.5 hr after the current was changed to 50 mA. Total running time was *ca* 9 hr.

In procedure B, the resolving column consisted of a 15% non-gradient gel. For the separating stage, the current was 50 mA, the eluting fluid flow rate was 10 ml hr^{-1} and the cooling fluid flow rate was 120 ml hr^{-1}. At the beginning of the elution stage, the elution fluid rate was increased to 90 ml hr^{-1}. Other conditions were the same as for procedure A.

In, procedure C, the resolving column consisted of a 12% non-gradient gel. Other conditions were the same as for procedure B.

Antibody production. General procedures followed the protocols described in ref. [12]. Balb/c mice were immunized (×4 times) with purified HSP29 using complete and incomplete Freund's adjuvants. After sacrifice, their spleens were removed and the spleen cells were fused with a myeloma cell line (X63-Ag8-653) using PEG (M_r = 1 500) according to the method of ref. [13] as modified in ref. [14]. Hybridoma cells were screened for antibody production by Western

analysis using nitrocellulose strips containing purified HSP29 (see below). For this, the IgG antibody fr. from hybridoma cultures was purified using a Protein A column (Econo-pac, BioRad Labs).

Western analysis. Proteins from electrophoresis gels were transferred to nitrocellulose membranes and air dried for 1 hr. Remaining binding sites in the membranes were blocked with 1% (w/v) fat-free milk powder in TBST (25 mM Tris–HCl buffer, pH 7.2, containing 0.9% NaCl and 0.1% Tween-80). The membranes were then incubated for 1 hr in 25 mM TBST containing either purified HSP29 antibody (for screening of HSP29 in gels) or hybridoma culture supernatant (for detection of HSP antibody in the supernatant). The membranes were then incubated with anti-mouse IgG-peroxidase conjugate in TBST (Sigma) and finally with a solution containing 0.05% 4-chloro-1-naphthol and 0.003% H_2O_2 in 20% MeOH. After each incubation, the membranes were washed ($\times 3$) with TBST).

Limited proteolysis of HSP29. A 1D SDS–PAGE gel (pH 6.8) was prep with a 3% stacking gel and 13% resolving gel, each with 2.67% cross-linker. Purified HSP29 (5 μg) in loading buffer was loaded into the first well, HSP29 (5 μg) plus V8 *S. aureus* protease (5 μg; Sigma) was loaded into the 2nd well, and the protease (5 μg) was loaded into the 3rd well. Power was applied until the samples reached the stacking gel, when the power was switched off for 30 min. Electrophoresis was then completed as normal. The completed gel was dried and stained with $AgNO_3$.

Acknowledgement—We are indebted to the Overseas Development Administration for financial support to D.H.W.

REFERENCES

1. Schlesinger, M. J., Ashburner, M. and Tissieres, A., *Heat Shock: From Bacteria to Man.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982.
2. Nover, L., *Heat Shock Response.* CRC Press, Boca Raton, FL, 1990.
3. Vierling, E., *Annual Reviews of Plant Physiology and Plant Molecular Biology*, 1991, **42**, 579.
4. Nover, L. and Scharf, K. D., in *Heat Shock Response*, ed. L. Nover. CRC Press, Boca Raton, FL, 1990, p. 41.
5. Waters, E. R., Lee, G. J. and Vierling, E., *Journal of Experimental Botany*, 1996, **47**, 325.
6. Collins, G. G., Nie, X. L. and Salveit, M. E., *Journal of Experimental Botany*, 1995, **46**, 795.
7. Wilkinson, M. C., Wheatly, P. A., Smith, C. J. and Laidman, D. L., *Phytochemistry*, 1990, **29**, 3073.
8. Jinn, T.-L., Yeh, Y.-C., Chen, Y.-M. and Lin, C.-Y., *Plant Cell Physiology*, 1989, **30**, 463.
9. Jinn, T.-L., Chen, Y.-M. and Lin, C.-Y., *Plant Physiology*, 1995, **108**, 693.
10. Jacob, U., Gaestel, M., Engel, K. and Buchner, J., *Journal of Biological Chemistry*, 1993, **268**, 1517.
11. Lee, G. J., Pokala, N. and Vierling, E., *Journal of Biological Chemistry*, 1995, **270**, 10432.
12. Liddle, J. E. and Cryer, A., *A Practical Guide to Monoclonal Antibodies.* John Wiley, Chichester, U.K., 1991.
13. Galfre, G. and Milstein, C., *Methods in Enzymology*, 1981, **74**, 3.
14. Goding, J. W., *Monoclonal Antibodies: Principles and Practice*, 2nd edn. Academic Press, London, 1986.