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SELECTIVE BINDING OF PLANT PROTEINS TO HEAT-SHOCK PROTEIN 70

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Key Word Index—*Phaseolus aureus*; Leguminosae; Mung Bean; Heat shock protein; HSP70; protein binding.

Abstract—Column affinity chromatography using "constitutive HSP70" and "heat-shock HSP70" as affinity ligands has demonstrated that HSP70 binds peptides selectively from [^{35}S]-radiolabelled plant extracts. Eight peptides were bound from native protein extracts and at least 12 further peptides were bound from heat-denatured protein extracts. The total protein bound was about 7.5-fold greater in the case of the denatured extracts compared with that from the native extracts. The "heat-shock-HSP70" columns had a small but significantly greater affinity for proteins than the "constitutive-HSP70" columns did. Affinity chromatography using "native protein" and "denatured protein" as affinity ligands for the binding of radiolabelled HSP70 produced similar results. © 1997 Elsevier Science Ltd

INTRODUCTION

Induction of the enhanced synthesis of heat-shock proteins (HSPs) is ubiquitous in cellular organisms subjected to heat or other stresses [1, 2]. Heat-shock protein 70 (HSP70) is one of the most prominent among these HSPs. It occurs constitutively, where it plays an important role as a chaperonin [3–5] involved in the folding of newly-synthesised peptides, peptide unfolding associated with transport across membranes, the refolding of partially denatured proteins and possibly the detection of temperature change [6, 7]. Its accumulation following stress is believed to be important for the renaturation of damaged proteins and the protection of the organism against the consequences of the stress [5, 8, 9].

All of these functions of HSP70 are dependent upon its ability to bind selectively to partially denatured and even denatured peptides and proteins. The possibility also exists that some non-denatured, functional proteins bind to HSP70 as part of the latter's chaperonin and renaturation functions. HSP70-binding proteins from mammalian sources have been isolated and partially characterised [10–12], but their binding characteristics were described only qualitatively. The present communication describes experiments to assess the degree of specificity associated with the binding of plant proteins to HSP70 and to provide quantitative data on the binding process.

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RESULTS

Two experiments employing affinity chromatography were carried out to measure the binding of proteins to HSP70. In the first experiment, affinity columns were prepared with HSP70 as the affinity ligand and radiolabelled protein extracts were presented to these columns. Two types of column were employed using HSP70 isolated either from non-heat-shocked plants ('constitutive-HSP70' column) or from heat-shocked plants ('heat-shock-HSP70' column). Radiolabelled, native and heat-denatured protein extracts from mung bean hypocotyl were presented to each of the two types of column.

'Constitutive-HSP70' columns bound (retained) 7.5-fold more denatured protein than native protein (Table 1). 'Heat-shock-HSP70' columns similarly bound 7.8-fold more denatured protein than native protein. Much smaller, but statistically significant differences were also noted between the binding powers of the 'constitutive-HSP70' and the 'heat-shock-HSP70' columns; the latter bound about 13% more native protein and about 17% more denatured protein than the former did.

Results from the electrophoretic analysis of the peptides bound by the HSP70 columns are presented in Fig. 1. Eight peptides (or groups of peptides with similar molecular masses) from the native protein extract were bound by both types of column. These had M_r values of 26, 34, 48, 54, 55, 61, 62 and 80 kDa. The 34 kDa peptide(s) was by far the most predominant one in both cases, although it was not a

Table 1. Binding of radiolabelled proteins to HSP70 affinity columns

Constitutive-HSP70 columns		Heat-shock-HSP70 columns	
Native protein bound (dpm)	Denatured protein bound (dpm)	Native protein bound (dpm)	Denatured protein bound (dpm)
31 560 ± 886	236 560 ± 5319	35 567 ± 957	277 517 ± 5993

Each value is the mean ± s.d. from ten replicate experiments.

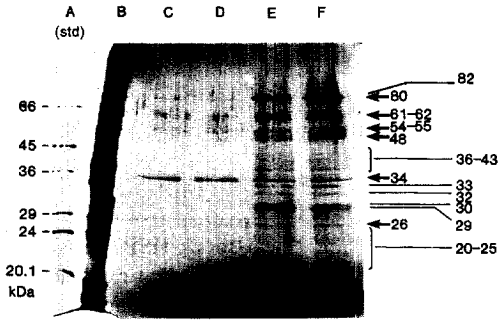

Fig. 1. Electrophoretic analysis of proteins bound to HSP70 columns. Lane A, molecular weight markers; Lane B, total proteins from mung bean; Lane C, native proteins bound to 'constitutive HSP70' column; Lane D, native proteins bound to 'heat-shock HSP70' column; Lane E, denatured proteins bound to 'constitutive HSP70' column; Lane F, denatured proteins bound to 'heat-shock HSP70' column. Lane A is visualised by silver staining. Lanes B–F are visualised by radioautography.

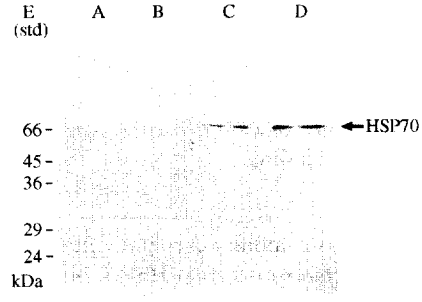

Fig. 2. Electrophoretic analysis of HSP70 bound to protein columns. Lane A, 'constitutive' HSP70 bound to 'native protein' column; Lane B, 'heat-shock' HSP70 bound to 'native protein' column; Lane C, 'constitutive' HSP70 bound to 'denatured protein' column; Lane D, 'heat-shock' HSP70 bound to 'denatured protein' column. All lanes are visualised by radioautography.

major peptide in the original crude protein extracts presented to the columns. In the case of the denatured protein extracts, at least 12 further peptides, in addition to the above eight, were bound to HSP70 columns. These additional peptides had M_r values 20, 21, 23, 25, 29, 30, 31, 33, 36, 40, 43 and 82 kDa. The peptides corresponding to 29, 30, 34, 80 and 82 kDa were quantitatively the most important of these peptides. There were no obvious differences between the two types of HSP70 columns regarding the profiles of the peptides that they bound.

In the second experiment, the affinity columns were constituted with proteins as the affinity ligand and radioactive HSP70 was presented to the columns. Two types of column were again employed, one prepared from native protein extracts ('native-protein' column) and one from heat-denatured protein ('denatured-protein' column). Radiolabelled 'constitutive' and 'heat-shock' HSP70 preparations were presented to each of the two types of column. The results from this experiment are presented in Table 2 and Fig. 2. They confirm and extend those from the first experiment. The 'native-protein' columns bound somewhat more (16%) 'heat-shock' HSP70 than 'constitutive' HSP70 (Table 2). Similarly, the 'denatured-protein' columns bound 26% more 'heat-shock' HSP70 than 'constitutive' HSP70. Also, 'denatured-protein' columns bound more HSP70 than 'native-protein' columns did. In the case of 'constitutive' HSP70, the binding was 4.3-fold greater, while it was 4.7-fold greater in the case of 'heat-shock' HSP70.

Electrophoresis of the material retained by the protein columns (Fig. 2) failed to demonstrate any HSP70 binding by 'native' protein columns, although this had taken place (cf. Table 2). On the other hand, both 'constitutive' and 'heat-shock' HSP70 were clearly bound by 'denatured-protein' columns. This binding was specific, as no other peptide bands were observed in the electrophoretograms. The failure to demonstrate binding in the case of the 'native-protein' columns was probably due to the small amount of

Table 2. Binding of radiolabelled HSP70 to protein affinity columns

Native-protein columns		Denatured-protein columns	
Constitutive HSP70 bound (dpm)	Heat-shock HSP70 bound (dpm)	Constitutive HSP70 bound (dpm)	Heat-shock HSP70 bound (dpm)
1258 ± 53	1462 ± 81	5412 ± 219	6825 ± 244

Each value is the mean ± s.d. from three replicate experiments.

radioactivity present in the relevant fractions from these columns.

DISCUSSION

Our results are concordant with those from other studies which have demonstrated the existence of HSP70-binding proteins using *in vivo* pulse-chase radiolabelling and co-immuno-precipitation techniques [10, 12, 13]. They also confirm and extend results from *in vitro* affinity chromatography experiments on mammalian proteins [11, 14]. In particular, our experiments show that heat-denatured proteins are more strongly bound to HSP70 than native proteins are, supporting the view that HSP70 is involved in the repair/renaturation of stress-damaged proteins [8, 9]. The observed binding of native proteins supports the view that HSP70 is also involved in routine procedures not associated with stress [15]. In this case, however, we cannot be emphatic that all of the peptides in our native extracts had remained entirely in their native conformation; on the contrary, some would expectedly have been partly denatured during the extraction process. Our finding that 'heat-shock' HSP70 has a greater affinity for proteins than 'constitutive' HSP70 does, also has biological implications. Although this difference was relatively small, it could have considerable *in vivo* significance, because HSP70 levels can be many times greater in stressed than in unstressed cells [16]. This affinity difference is presumably a reflection of the different isotype spectrum of the HSP70 produced as a result of heat stress compared with that of constitutive HSP70. Our data also show that HSP70 expresses its affinity towards a surprisingly small number of peptides from among the relatively large number presented to it. Moreover, this is true of heat-denatured as well as native proteins.

EXPERIMENTAL

Plant material. Mung beans (*Phaseolus aureus* Cv. Roxborough) were soaked for 12 hr at 25° in distilled H_2O and germinated for 48 hr wrapped in moist filter paper (Whatman no. 1) contained in a glass container and incubated in the dark at 25°. The seedlings were harvested and sterilised for 10 min in dil. NaOCl (0.2% available chlorine). A 2 cm hypocotyl segment was cut from below the plumular hook of each seedling and used as the experimental material.

Radiolabelling and extraction of proteins. For radiolabelling, batches of four sterilised hypocotyl segments were placed upright into glass vials (8 mm i.d.) containing 1.5 ml 1 mM HEPES-KOH buffer, pH 8.0, with 100 μCi L-[^{35}S]methionine (1300 μCi mmol^{-1}) and incubated for 2 hr at either 25° (control) or 42° (heat-shock). After incubation, the segments were washed several times in 1 mM HEPES-KOH buffer containing 0.1 mM non-radioactive L-methionine and blotted dry.

Radiolabelled and unlabelled batches of hypocotyl segments were homogenised 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 phenylmethane sulphonyl fluoride, 1 mM benzamidine and 5 mM 6-amino-*n*-hexanoic acid, using a top-drive homogeniser. The vol. of the extraction medium was 1 ml g^{-1} tissue. Triton X-100 (1%) was added during the last 10 s of homogenisation. The homogenate was centrifuged for 20 min at 12000 *g* and 4° and the supernatant was filtered through glass fibre (Whatman CF/C). Protein was then pptd from the supernatant by addition of Me_2CO at −30° and resolubilised in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl. This soln was stored at −20° for not more than 2 days before further processing.

Heat-shock protein 70 was isolated from the protein extracts using the procedure described by Wilkinson *et al.* [17]. Where total protein extracts were to be denatured, this was done by heating for 2 min at 100°. Protein concns were determined using the BioRad reagent (BioRad Laboratories Ltd., Hemel Hempstead, U.K.).

Affinity chromatography. Affinity chromatography columns were prepd according to the standard procedures [18]. Four different stationary phases were prepd. The support in each case was Sepharose 4B (Pharmacia LKB Biotechnology, Sweden), while the affinity ligand was either 'constitutive' HSP70, 'heat-shock' HSP70, 'native' protein or 'denatured' protein (see Results section). For the coupling of the ligand to the support, 1 g of CNBr-activated Sepharose 4B was reacted with either 30 mg HSP70 or 100 mg protein. The supernatants from the completed reactions were analysed for their protein content to ensure that equal coupling had taken place in the two HSP70 preparations and in the two protein preparations. The prepd stationary phases were packed into glass columns (0.8 mm × 50 mm).

Before use, the columns were equilibrated with TEB (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol, 2 mM phenlmethane sulphonyl fluoride, 1 mM benzamidine and 5 mM 6-amino-*n*-hexanoic acid). In the case of the HSP70 columns, radiolabelled protein (200 mg, 7.4×10^3 Bq mg^{-1}) was then applied in TEB (5 ml) and the column was washed with TEB (50 ml) to remove unbound protein. Bound proteins were then eluted, first with 20 mM Tris–HCl, pH 7.4, containing 1 M NaCl (10 ml) and then with 0.2 M glycine–HCl buffer, pH 2.8, until no more radioactivity was eluted. The two eluates were combined, dialysed against TEB and concd by freeze drying. In the case of the protein columns, the same procedure was followed with the following differences:- radiolabelled HSP70 (0.5 mg, 16×10^3 Bq mg^{-1}) was applied in TEB (5 ml) and the first elution was carried out with 20 mM Tris-HCl, pH 7.4, containing 1 M NaCl and 10 mM ATP. The dialysed, reduced frs from the different columns were adjusted

to equal vols with distilled H_2O before analysis by liquid scintillation spectrometry and electrophoresis.

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