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Constitutive and heat-inducible heat shock element binding activities of heat shock factor in a group of filamentous fungi

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Abstract This study represents the initial characterization of the heat shock factor (HSF) in filamentous fungi. We demonstrate that HSFs from *Beauveria bassiana*, *Metarhizium anisopliae*, *Tolypocladium nivea*, *Paecilomyces farinosus*, and *Verticillium lecanii* bind to the heat shock element (HSE) constitutively (non-shocked), and that heat shock resulted in increased quantities and decreased mobility of HSF-HSE complexes. The monomeric molecular mass of both heat-induced and constitutive HSFs was determined to be 85.8 kDa by UV-crosslinking and the apparent molecular masses of the native HSF-HSE complexes as determined by pore exclusion gradient gel electrophoresis was 260 and 300 kDa, respectively. Proteolytic band clipping assays using trypsin and chymotrypsin revealed an identical partial cleavage profile for constitutive and heat-induced HSF-HSE complexes. Thus, it appears that both constitutive and heat-inducible complexes are formed by trimers composed of the same HSF molecule which undergoes conformational changes during heat shock. The mobility difference between the complexes was not abolished by enzymatic dephosphorylation and deglycosylation, indicating that the reduced mobility of the heat-induced HSF is probably due to a post-translational modification other than phosphorylation or glycosylation. © 1999 Harcourt Publishers Ltd

INTRODUCTION

Cells from diverse species respond to elevated temperatures by transcriptional activation of a specific set of heat shock genes resulting in the rapid synthesis of heat shock proteins (HSPs) (Lindquist and Craig 1988; Morimoto et al. 1994). Besides heat shock, a number of other stress conditions that damage or denature intracellular proteins are capable of inducing this response (Watson 1990; Craig et al. 1994). The HSPs act as molecular chaperones in the renaturation or degradation of damaged proteins, thereby offering protection from the harmful effects of stress (Morimoto et al. 1994). HSPs also function under normal conditions in the synthesis, degradation, and translocation of proteins across membranes (Craig et al. 1994; Frydman and Hartl 1994). It has been suggested that HSPs regulate their own synthesis by feedback inhibition (Craig et al. 1993).

In eukaryotes, the rapid induction of HSP-encoding genes in response to stress is regulated primarily at the level of transcriptional activation through the binding of heat shock factor (HSF) to highly conserved heat shock elements (HSE) in promoters of heat shock genes (Amin et al. 1988; Fernandez et al. 1994). All HSEs contain a simple repeating 5-bp sequence, 5'nGAAn3'; these repeats are contiguous but in alternating arrangements, and the sequence of the HSE is highly conserved over a wide range of organisms (Mager and De Kruijff 1995). Transcriptional activation of heat shock genes is not prevented by blocking *de novo* protein synthesis, indicating that the response involves modification of a pre-existing pool of HSF (Kingston et al. 1987; Sorger et al. 1987; Zimarino and Wu 1987; Karn et al. 1992). The regulation of HSF activity, however, appears to be fundamentally different among different organisms. In higher eukaryotes, activation of HSF involves acquisition of DNA affinity by

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trimerization (Baler et al. 1993; Sarge et al. 1993; Westwood and Wu 1993; Sistonen et al. 1994), but in budding yeasts, HSF molecules exist in the trimeric HSE-binding form at all temperatures. For example, in *Saccharomyces cerevisiae*, a significant level of HSE-binding is detected at normal growth temperatures and no further increase in DNA binding activity is seen as a result of heat shock (Jakobsen and Pelham 1988). In contrast, no such constitutive binding occurs in the fission yeast *Saccharomyces pombe* in which heat shock is required for HSF to bind to HSE (Gallo et al. 1991). As in *S. cerevisiae*, the HSF of the budding yeast *Kluveromyces lactis* when substituted for the endogenous *S. cerevisiae* HSF gene binds to DNA constitutively (Jakobsen and Pelham 1991).

The *S. cerevisiae* HSF gene which codes for a 93.2 kDa protein consisting of 833 amino acids, has been shown to be essential for the viability of the cells at both non-shock and heat shock conditions (Wiederrecht et al. 1988; Sorger and Pelham 1988). The constitutive binding capability of *S. cerevisiae* HSF is in contrast to the heat-inducible binding of HSF from *S. pombe* and higher eukaryotes, and is therefore considered to be a unique exception to the general pattern of heat shock dependent binding of the transcription factor (Mager and De Kruijff 1995; Sorger et al. 1987). It has been suggested that heat shock induced hyperphosphorylation is the modification involved in transcriptional activation of HSF in *S. cerevisiae* as well as in animal cells (Sorger et al, 1987; Larson et al. 1988; Sorger and Pelham 1988; Craig and Gross 1991; Sarge et al. 1993; Xia and Voellmy 1997). In addition, phosphorylation upon heat shock may serve to stimulate the return of the HSF to the inactive form (Hoj and Jakobsen 1994).

The filamentous fungi differ from yeast primarily in cellular organization, filamentous fungi being multicellular. Filamentous fungi have previously been investigated for their response to heat shock and other stress conditions (reviewed in Plesofsky-Vig and Brambl 1985a; 1993). The heat shock response of fungi while similar in many respects to that of other organisms differs in important details, an example being the more transient increase in HSP expression even under maintenance at high temperatures (Plesofsky-Vig and Brambl 1985b). Heat shock protein synthesis has been well documented in *Neurospora crassa* (Kapoor 1986; Plesofsky-Vig and Brambl 1987; Roychowdhury and Kapoor 1990; Kapoor et al. 1995), *Aspergillus niger* (Stephanou and Demopoulos 1986), *Fonsecaea pedrosi* (Laurent-Winter and Ibrahim-Granet 1992), *Physarum polycephalum* (Wright and Tollon 1982), *Ustilago maydis* (Holden et al. 1989), and *Achlya ambisexualis* (Brunt and Silver 1991). Although the presence of consensus sequence motifs resembling HSE have been identified in *N. crassa hsp70* gene (Kapoor et al. 1995), the HSF-HSE interaction, the key component of the heat shock regulatory system, has not been studied in this group of organisms.

We are currently investigating the stress response of several genera of deuteromycetous entomopathogenic fungi. Entomopathogenic fungi such as *Beauveria bassiana, Metarhizium anisopliae, Tolypocladium nivea, Verticillium lecanii* and *Paecilomyces farinosus* are used world-wide to control different insect pests (Khachatourians 1996). Since the commercial potential of these fungi as mycoinsecticides is immense (Feng and Khachatourians 1994) it will be important to understand the response of these fungi to environmental stress. In a previous communication, we characterized the heat shock response of the entomopathogenic fungus *Beauveria brongniartii* at the level of HSP synthesis (Xavier and Khachatourians 1996). As part of our ongoing investigations on the biology and molecular aspects of the heat shock response in this group of fungi, we report here the HSF-HSE interactions involved in these fungi.

Our experiments demonstrate both constitutive and heat-inducible sequence specific HSE-binding of HSF from protein extracts of five genera of entomopathogenic fungi. Heat shock resulted in increased quantities and reduced mobility of HSF-HSE complexes. However, the post-translational modification of the heat-inducible HSF-HSE complex is probably not due to phosphorylation or glycosylation. Using UV-crosslinking followed by SDS-PAGE, both the constitutive and heat-inducible *B. bassiana* HSF was estimated to have a similar molecular mass of 85.8 kDa. Determination of the native sizes of the heat-inducible and constitutive HSF-HSE complexes by pore exclusion gradient gel electrophoresis suggests that HSF binds to HSE as a trimer under both non-shock and heat shock conditions. Proteolytic clipping band assays using specific proteases revealed an identical pattern of partial cleavage products for both non-shocked and heat-shocked HSF-HSE complexes further suggesting that both complexes are formed by the same HSF protein.

MATERIALS AND METHODS

Growth and heat-shock conditions

Beauveria bassiana GK 2016, *Metarhizium anisopliae* RS 549, *Tolypocladium nivea* 18768, *Verticillium lecanii* ATCC 46578 and *Paecilomyces farinosus* ATCC 1360 were cultured in 50 ml of yeast extract-peptone-dextrose (YPD) broth at 25°C in 125 ml Erlenmeyer flasks in a New Brunswick rotary shaker (100 r.p.m.) for 5 days. These cultures which consisted primarily of germinated and growing cells from conidiospores called blastospores, were then heat-shocked at 45°C for 1 h in a Haake F-3 water bath as previously described (Xavier and Khachatourians

1996). Duplicate control samples were subjected to a similar treatment except for the incubation temperature which was maintained at 25°C. For experiments with *B. bassiana*, in addition to a 45°C heat shock for 1 h, varying heat shock temperatures (35, 40, 45, and 50°C) were also used. Shift-down experiments involving recovery of heat-shocked *B. bassiana* essentially involved the transfer of heat-shocked (45°C, 1 h) cells to fresh YPD, and samples of recovering cells were withdrawn at varying time intervals of 5, 15, 30, 45, 60 and 120 min. On completion of the treatment, heat-shocked blastospores as well as non-shocked (control) blastospores were cooled on ice immediately and centrifuged at 10 000 g for 15 min at 4°C in a refrigerated centrifuge (Sorvall). The cells were then subjected to protein extraction or stored at –80°C.

Preparation of protein extracts

Total cytoplasmic proteins for gel shift assays were extracted in Buffer C (50 mM Tris-HCl, pH 8.0, 20% glycerol, 0.1 mM EDTA, 2 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml of phenylmethylsulphonyl fluoride) by grinding the cells in an Eppendorf micro-test tube with a motorized pestle (Pellet Pestle Motor, Kontes) using 200–300 μm diameter glass beads (Sigma). For 2-D gel electrophoresis, total cellular protein was extracted from frozen cells in lysis buffer (O'Farrell 1975), containing 9.5 M urea, 2% (w/v) Nonidet P–40, 2% (v/v) ampholines (pH 3–10, Biolyte, BioRad), 5% (v/v) 2-β-mercaptoethanol and 100 μg/ml of phenylmethylsulfonyl fluoride. The extracts were centrifuged in a microfuge at 15 000 g (4°C, 10 min) in order to remove cell debris. Total protein of the supernatant was determined using the dye binding assay of Bradford (Bradford 1976) using bovine serum albumin as a standard. Supernatants were stored in 100 μl aliquots at –80°C for subsequent analyses.

Two-dimensional gel electrophoresis

Proteins were separated using a two-dimensional gel electrophoresis system as previously described (Hochstrasser et al. 1988). The first dimension based on isoelectric focusing (IEF) was run in 15 cm glass tubes with an internal diameter of 0.5 cm and a pH gradient of 4.0 to 8.0 (Biolyte, BioRad). Subsequent to prefocusing at 200 V (15 min), 300 V (30 min) and 400 V (30 min), 25 μg (for silver staining) or 150 μg (for coomassie-blue staining) of total protein was loaded on to tube gels and electrophoresed for a total of 10 000 Vh. In separate tube gels, 2-D molecular weight standards (BioRad) were electrophoresed simultaneously as calibration standards. On completion of electrophoresis, the tube gels were equilibrated in 10 ml of 0.05 Tris-HCl containing 1% (w/v) SDS, 36% (w/v) urea, 30% (v/v) glycerol, and 50 mM dithioethritol for 30 min followed by another equilibration for 30 min in the same buffer with the addition of 4.5% (w/v) iodoacetamide. The second dimension was electrophoresed on a 1 mm thick, homogeneous 10% gel using SDS-PAGE (Laemmli 1970) in a BioRad slab gel unit with constant cooling. Both sets of proteins obtained from heat shocked and control samples were electrophoresed simultaneously using the dual slab gel system. Following electrophoresis, gels were either fixed in 50% ethanol, 10% acetic acid overnight and subsequently silver stained (Bjellqvist et al. 1993) or stained with Coomassie blue R-250. The gels were then digitized and spots quantitatively evaluated based on the relative intensities of the spots by using a video documentation system (IS1000, Canberra Packard). The molecular masses and PI of the observed polypeptides were determined using BioRad 2-D molecular weight standards used either as internal or external standards. Reproducibility of the 2-D gels was consistent for replicate runs from the same sample as well as between samples obtained from separate experiments.

Gel mobility shift assays

DNA mobility shift assays were performed as previously described (Ovsenek and Heikkila 1990). Binding assays contained 25 μg of fungal protein extract in a final volume of 25 μl. Protein equivalency was previously determined by the Bradford assay and confirmed by analysis on SDS-PAGE gels (Laemmli 1970). The heat shock element oligonucleotide probe used in the binding assay was as described by Ovsenek et al. (Ovsenek et al. 1990) as containing the HSE sequence corresponding to the *Xenopus hsp 70B* gene (5′-GAAATGGAAGCCTCGGGAAACTTCGGGTCGG-3′). Gel shifts were also performed with the consensus HSE oligo (5′-GGGCGTCATAGAATATTCTCGAATTCTGGGTCAGG-3′). Binding reactions were performed in the presence of 1 μg poly (dI-dC), 10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 5% glycerol in a final volume of 25 μl. Subsequent to incubation of the reaction mixture on ice (30 min), the samples were loaded onto 5% non-denaturing polyacrylamide gels containing 6.7 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3.3 mM sodium acetate. Gels were electrophoresed at 150V for 2.5 h, dried on filter paper (1 h, 80°C, with vacuum), and autoradiographed (Kodak X-omat 5 film, –80°C, overnight). Alternatively, samples were run on 5% polyacrylamide gels containing 0.5X TBE (45 mM Tris, 45 mM boric acid, 2 mM EDTA) and autoradiographed.

Competition assays were done with the addition of a 50-fold molar excess (relative to labeled HSE) of cold HSE oligonucleotide into the reaction mixtures. Gel mobility

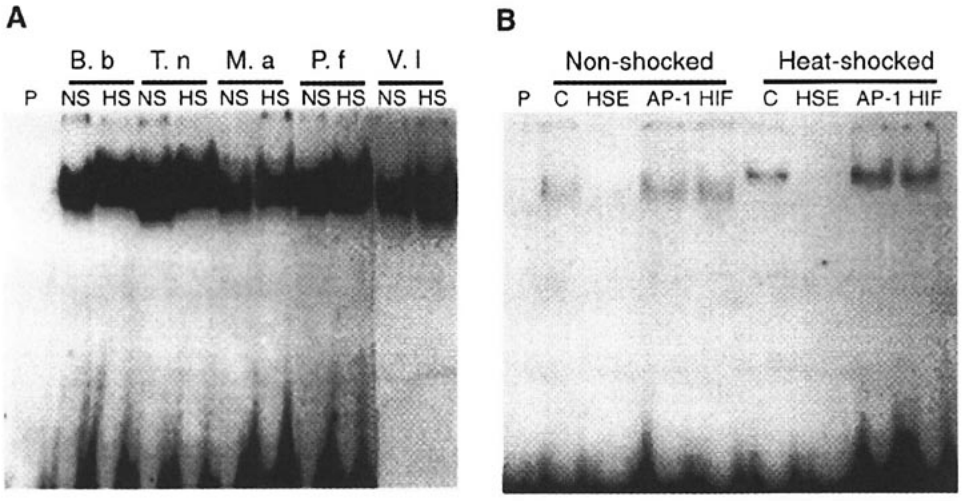

Fig. 1 Constitutive and heat-inducible HSE binding activities of filamentous fungi. (A) Gel mobility shift assays were performed with protein extracts prepared from heat-shocked (HS) and non-shocked (NS) *B. bassiana* (B.b), *M. anisopliae* (M.a), *T. nivea* (T.n), *V. lecanii* (V.l), and *P. farinosus* (P.f). Cellular protein (25 μg) of each fungus was used in binding reactions with a ^{32}P labeled HSE oligonucleotide and the resultant protein-DNA complexes were separated on non-denaturing gels. (B) Sequence-specificity of constitutive and heat-inducible HSE-binding activities of *B. bassiana* as determined by gel mobility shift assays. Competitor DNAs were added at 50-fold molar excess relative to labeled HSE probe. Competitor DNAs were: (1) unlabelled HSE (indicated as HSE), (2) Activator Protein (AP-1) binding site, (3) Hypoxia Induced Factor (HIF) consensus element. Control protein (no competitor added) for both HS and NHS proteins are indicated as C and free probe as P.

shift assays of samples containing a 50-fold molar excess of oligonucleotides containing known binding sites for established transcription factors such as activator protein (AP-1) (5′- CCGGAAAGCATGAGTCAGACAC-3′) (Wang 1993), and hypoxia induced factor (HIF) (5′- GCCC-TACGTGCTGTCTCA-3′) were also performed.

The densities of the HSF-HSE bands were measured by scanning the autoradiographs using an image analyzer software linked to a video documentation system (IS 1000, Canberra Packard)

Dephosphorylation, deglycosylation and proteolytic treatments

Enzymatic dephosphorylation experiments involved incubation of 20 μg of fungal protein at either 37°C or on ice for varying time periods (0, 30, 60 min) with 10 U of calf intestine alkaline phosphatase (Boehringer Mannheim), or with a phosphatase cocktail containing 5 U of calf intestine alkaline phosphatase and 1.0 U each of Type II, III, and IV acid potato acid phosphatase (Sigma).

For deglycosylation experiments, 25 U of PNGase F (*Flavobacterium meningosepticum*, Boehringer Mannheim) was added to 20μg of the fungal protein and the mixture was incubated at 37°C for 60 min. For both of dephosphorylation and deglycosylation experiments, DNA binding assays were performed immediately after treatment with the respective enzymes.

For proteolytic clipping band assays, varying amounts of trypsin and chymotrypsin (Sigma) (0.1, 0.01, 0.001, and 0.0001 U) were added to the DNA-binding reactions prior to electrophoresis as described for gel mobility shift assays. After addition of the respective proteases, the binding reaction was incubated at RT for a further 15 min. Subsequently, the gel mobility shift assay was performed as described earlier.

UV-crosslinking

The apparent molecular mass of the DNA binding fungal protein was estimated by UV-crosslinking using a non-substituted HSE oligonucleotide probe. The volume of DNA binding reactions and the quantity of the protein extract and HSE oligonucleotide probe was scaled up 3-fold. Binding reactions were allowed to proceed for 30 min and protein-DNA complexes were cross-linked by exposing the reaction tube to UV light (254 nm) using a UV transilluminator for a duration of 2 h at a distance of 5 cm. Deoxycholate (0.5 mg/ml) and trichloroacetic acid (12% w/v) were added to cross-linked DNA-protein complexes, and the mixtures were incubated on ice for 15 min. Resultant precipitates containing DNA-protein complexes were collected by centrifugation at 12 000 g for 15 min at 4°C. Precipitates were then dissolved in 50 μl of Laemmli sample buffer (100 mM 2-mercaptoethanol, 60 mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, 0.0005% bromophenol blue) and neutralized by the addition of 5 μl of Tris-HCl (pH 9.0). Samples were boiled for 3 min and subjected to SDS-PAGE on 12% homogenous gels. Protein molecular mass standards (Broad range, BioRad) were run

along with the samples. The gel was then stained with Coomassie blue, destained, dried and autoradiographed (Kodak X-omat 5 film, –80°C, overnight).

Pore exclusion electrophoresis

The apparent molecular mass of the native HSF-HSE complexes of *B. bassiana* was estimated using pore exclusion electrophoresis. The DNA binding assay was similar to that used for the gel mobility shift assays. Samples were run on 4 to 25% non-denaturing polyacrylamide gels in 1 X TBE buffer using a Protean II electrophoresis cell (BioRad) with cooling (4°C) at 150 V for 24 h. Native molecular mass markers (LKB Pharmacia) were run alongside the samples. The gel was stained with Coomassie blue, destained, dried and autoradiographed (Kodak X-omat 5 film, –80°C, 1–3d).

RESULTS AND DISCUSSION

The HSE-binding activities in whole cell protein extracts of five entomopathogenic fungi belonging to the genera, *Beauveria, Metarhizium, Tolypocladium, Paecilomyces* and *Verticillium* were examined using gel mobility shift assays. Although the heat shock response has been well documented in several filamentous fungi at the level of protein synthesis, very little is known about the behavior of HSF in this class of organisms. The choice of the heat shock temperature (45°C) used in this study was based on our previous study (Xavier and Khachatourians 1996) that showed maximal induction of heat shock proteins at 45°C in *Beauveria brongniartii.*

Prominent HSE-binding activities were observed in both non-shocked and heat-shocked extracts of fungi from all five genera (Fig. 1A). Although both constitutive and heat-inducible binding of HSF could be detected with all fungi tested, there were overt differences in the mobility of the heat-shocked and control HSF-HSE complexes. In all fungi tested, extracts made from heat-shocked cells yielded complexes whose mobility was clearly diminished relative to those from control extracts In addition to the diminished mobility of HSF-HSE complexes, heat shock also resulted in an increase in the amount of complex formation relative to controls in all fungi except in cultures of *T. nivea* where a slight reduction was observed.

Having established the presence of HSE-binding activities in non-shocked and heat-shocked extracts from each of the five genera tested, the specificity of binding was ascertained by competition experiments. Although the precise identity of the protein as HSF has not been confirmed, we attribute this activity to HSF based on the apparent specificity of the interaction, and its similar binding characteristics with the consensus HSE (data not shown). Among the five genera of fungi reported in this study, *B. bassiana* is the most well studied in terms of biochemical, physiological and genetic characterization. Hence, for competition experiments and further characterization of HSF-HSE interactions, *B. bassiana* was chosen as the model fungus. Both the constitutive and heat-induced HSF-HSE complexes were competed by a 50-fold molar excess of consensus HSE oligonucleotide to DNA-binding reactions (Fig. 1B). Competition experiments with the addition of 50-fold molar excess of oligonucleotides containing binding sites for well established transcription factors such as AP-1 and HIF had no competitive effect on the formation of both constitutive and heat-inducible HSF-HSE complexes (Fig. 1B). These results confirm the DNA-protein complexes of filamentous fungi are sequence-specific HSE-binding activities.

Based on the results obtained with gel shift assays we speculate that the DNA binding domain of the *B. bassiana* HSF shares functional homology with the HSFs from other organisms (*Drosophila, Xenopus,* human, and mouse). To our knowledge, these findings are the first demonstration of HSF-HSE interactions in filamentous fungi. Among fungi, the HSF-HSE interactions have thus far been characterized only in yeast; the constitutive binding of HSF to HSE in the budding yeasts, *S. cerevisiae* and *K. lactis* (Jakobsen and Pelham 1988; 1991); and the inducible binding in the fission yeast, *S. pombe* (Gallo et al. 1991). The major difference in the regulation of DNA-binding activity

Table 1 Characteristics of *B. bassiana* GK 2016 heat shock proteins[a]

SPOT#.	HSP family designation	Mol. mass (kDa)	pI (pH)
1	HSP90	90.2	5.0
2	HSP70	70.5	5.0
3		70.3	5.4
4		70.5	5.5
5		68.3	5.5
6	HSP60	60.2	4.8
7		60.2	5.5
8	HSP50	54.1	4.6
9		54.6	5.0
10		54.6	5.5
11		54.5	6.2
12	HSP40	40.4	5.4
13		43.0	6.7
14	HSP30	35.1	6.1
15		34.4	5.6
16		22.0	5.3

[a]Major representatives of HSPs are identified with their mol. masses, pI and spot numbers as shown in Figure 2.

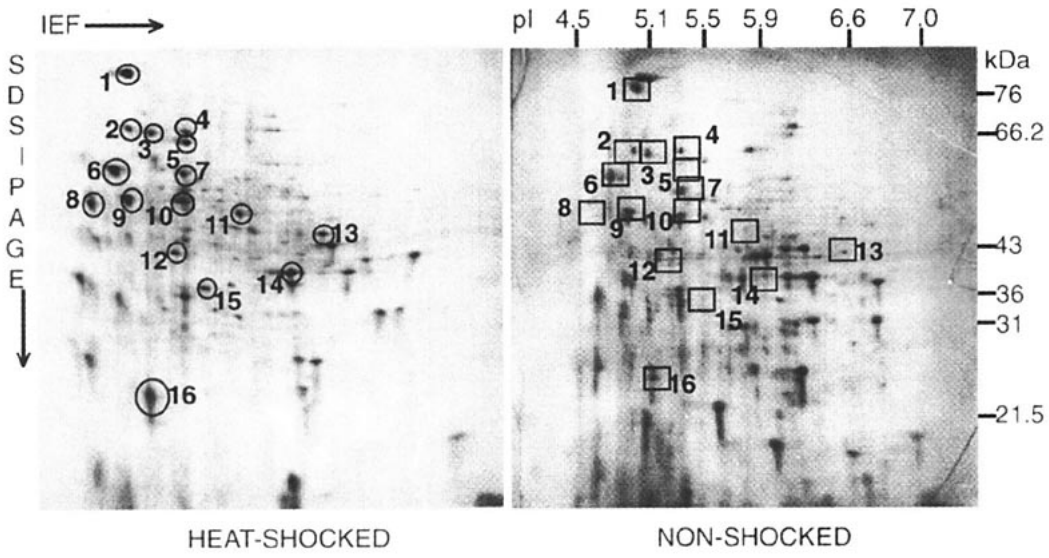

Fig. 2 Heat shock results in increased expression of several polypeptides in *B. bassiana*. Two-dimensional polypeptide map (silver stained gel) of heat-shocked and non-shocked *B. bassiana* GK 2016 total cellular proteins (25 μg). The fungus was grown at 25°C and heat shocked at 45°C for 1 h. Numbered circles in the heat shock gel indicates the heat-induced polypeptides while the corresponding polypeptides in non-shocked gel are shown as numbered boxes.

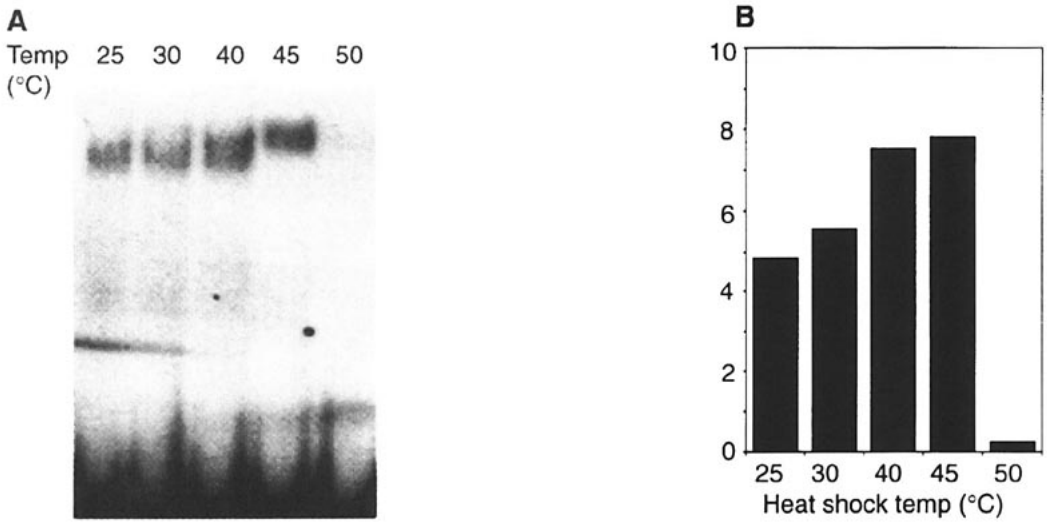

Fig. 3 The level of HSF induction in *B. bassiana* is dependent on the severity of heat shock. Temperature profile of heat-induced HSF-HSE binding activity of *B. bassiana*. (A) Protein extracts (25 μg) prepared from non-shocked (25°C) *B. bassiana*, and cells heat-shocked at different temperatures (35, 40, 45, and 50°C for 1 h) were analyzed by gel mobility shift assay. The heat shock temperatures are indicated above each lane. (B) Relative DNA binding ability of HSF obtained by densitometric quantitation of the HSF-HSE complexes shown in (A).

among lower and higher eukaryotes is the constitutive and heat-shock inducible binding of HSF to HSE. In higher eukaryotes, a heat-inducible binding activity is seen, with a 10–20 fold increase in the binding activity over basal levels after exposure to heat shock, while no binding can be detected under non shock conditions. In some yeast species (*S. cerevisiae* and *K. lactis*), unlike higher eukaryotes, a significant level of HSF binding activity can be detected in vitro at non-shock temperatures (Kingston et al. 1987; Sorger et al. 1987), the only exception reported being *S. pombe* in which HSF-binding to HSE is heat shock dependent (Gallo et al. 1991). The yeast HSF is, therefore, considered unique in its ability to bind DNA under non-shock conditions (Sorger et al. 1987). Results of the gel mobility shift assays in this study demonstrate that this unique aspect of the DNA-binding property of yeast is also

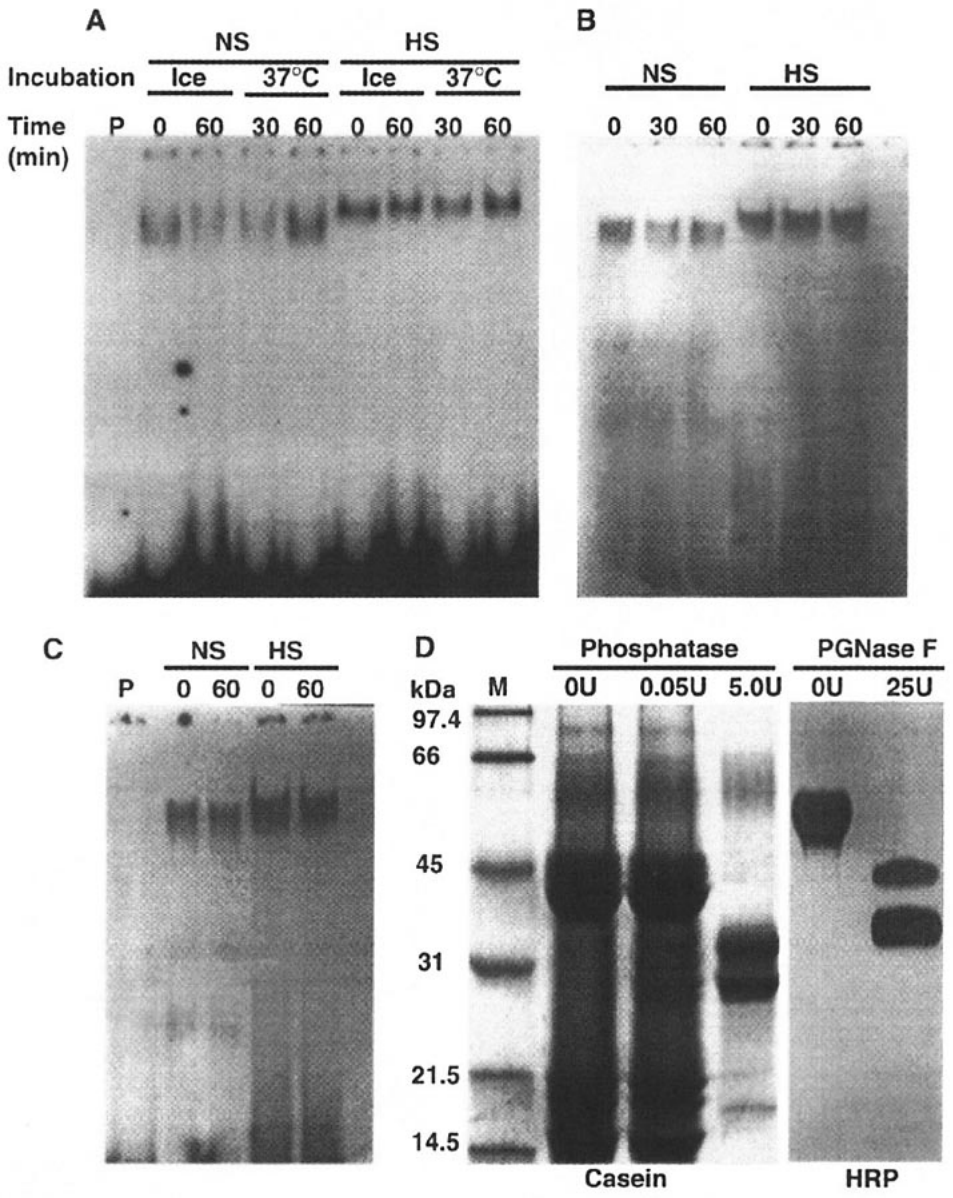

Fig. 4 Effect of phosphatase and glycosidase treatments on HSF-HSE complexes in heat-shocked (HS) and non-shocked (NS) blastospores of *B. bassiana*. Phosphorylation or glycosylation is not responsible for the diminished mobility or increased levels of the heat-induced HSF-HSE complex. Extracts were incubated with calf intestinal alkaline phosphate (A), phosphatase cocktail (B) and the glycosidase PGNase F (C). Protein extracts were incubated with respective enzymes for varying periods of time at 0°C or 37°C prior to performing the DNA binding reaction and gel mobility shift assay. (D) Dephosphorylation and deglycosylation of 20 μg of control proteins α casein (casein), and horse radish peroxidase (HRP), respectively, as observed by SDS-PAGE.

seen with filamentous fungi belonging to 5 different genera, *Beauveria, Metarhizium, Tolypocladium, Paecilomyces,* and *Verticillium*. The constitutive binding property of HSF in these fungi although similar to yeast differs with respect to stress induced increase in HSF binding activity. This observation could be attributed to increased synthesis of HSF, or post- translational modification leading to increased HSE-binding affinity after heat shock.

Apart from budding yeasts, constitutive binding attributed to HSF2 activity has been shown in mouse and human cells undergoing differentiation and development and is not induced by stress (Sistonen et al. 1992; Mezger et al. 1994; Murphy et al. 1994; Sarge et al. 1994; Sistonen et al. 1994; Goodson et al. 1995). Similarly, a distinct, developmentally regulated constitutive HSE-binding activity in stages I, II and testes tissue of *Xenopus* has

been reported (Gordon et al. 1997). It is not known if the constitutive binding activity reported in this study is a result of differentiation and development of these fungi. Results of heat shock experiments showed an increase in the HSF-HSE complex formation relative to controls (Fig. 1A). This increase in heat-inducible HSE-binding activity of HSF is not seen in yeast but is similar to that of higher eukaryotes in which this activity is heat shock dependent. Interestingly, experiments involving a mutant strain of *S. cerevisiae* with a temperature-sensitive defect in HSF show that HSF is necessary for the high constitutive expression of several heat shock genes under optimal growth conditions, and also that it is essential for the viability at normal and heat shock temperatures (Sorger and Pelham 1988; Wiederrecht et al. 1988). Thus, constitutive binding of HSF serves an important function not necessarily related to protection from heat shock. Based on the constitutive HSF-binding observed in this study, we suggest that high levels of HSF activity could be equally as important for the growth and survival of filamentous fungi. Indeed relatively high constitutive expression of heat shock proteins has been demonstrated in *B. brongniartii* (Xavier and Khachatourians 1996) and several other major entomopathogenic fungi including *B. bassiana, M. anisopliae, T. nivea, V. lecanii* and *P. farinosus* (Xavier and Khachatourians, unpublished data).

Since there has been no previous characterization of the stress response in *B. bassiana,* it was important to demonstate that a heat shock resulted in the synthesis of HSPs in vivo. In *B. bassiana,* increased expression of at least 16 polypeptides with a concomitant decrease in several others was detected by 2-D PAGE (Fig. 2) following a heat shock of 45°C. Thus, the overexpression of HSPs at 45°C could be correlated to the changes occurring in HSF. The pI and molecular mass of each of the heat shock-induced polypeptides are shown in Table 1. Similar results were observed in at least five repeats of this experiment. Due to the apparent increase in mobility of HSF-HSE complexes we were next interested in determining the effect of a temperature gradient on HSE-binding activity. Protein extracts from cells heat-shocked at various temperatures were analyzed by gel mobility shift assay (Fig. 3A). The mobility of HSF-HSE complexes was reduced after a 30°C heat shock, and a decrease in mobility of HSF-HSE complexes was observed at the 45°C heat shock temperature. The change in mobility was also accompanied by an increase in the quantity of HSF-HSE complex relative to the control (Fig. 3B). The amount of HSE-binding activity was barely visible after heat shock at temperatures of 50°C, but this was probably due to the greatly reduced survival of *B. bassiana* at this temperature as observed in parallel viability assays (data not shown). The change in the mobility and quantity of HSF-HSE complexes at increased temperatures indicates that the level of HSF induction in *B. bassiana* is dependent on the severity of heat shock. This feature of the heat shock response is seen in higher eukaryotes (Lis and Wu 1992) but an increase in the quantity of HSF-HSE complex is not seen in yeast. Therefore, it is likely that the temperature-dependent modulation of HSF binding plays an important regulatory role in the control of heat shock gene transcription in *B. bassiana.*

The diminished mobility of the heat-induced HSF-HSE complexes observed in heat-shocked extracts (Fig. 1–3) indicated that heat shock brought about post-translational modifications of HSF. Several studies in various organisms have implied that phosphorylation plays a role in the regulation of HSF. The mobility difference observed in *S. cerevisiae* HSF-HSE complexes has been shown to be significantly reduced by treatments of crude extracts with phosphatase thus attributing the heat shock-induced reduction in gel mobility to hyperphosphorylation (Sorger et al. 1987; Sorger and Pelham 1988). Similar effects have been observed with the *Tetrahymena pyriformis* HSF-HSE complex (Avides et al. 1990). We, therefore, postulated that the mobility changes observed in extracts of heat shocked filamentous fungi could be caused by hyperphosphorylation of HSF. In order to test this, we treated the protein extracts from non-shocked and heat-shocked cells with calf intestine alkaline phosphatase (CIAP) prior to performing DNA-binding assays. Phosphatase treatment had no effect on the mobility of both constitutive and heat-shocked HSF. Repeated experiments with greater amounts of CIAP (up to 10 U) and with treatment periods of 1 h at RT had no effect on the mobility of either the constitutive or heat-induced HSF-HSE complexes (Fig. 4A). Similar results were obtained in phosphatase experiments performed with several types of phosphatase cocktails, including CIAP and Types II, III, and IV acid phosphatases (Fig. 4B).

Since dephosphorylation experiments did not alter the mobility of the HSF-HSE complexes we next tested the potential effects of glycosylation, which is known to regulate activity of several transcription factors (Berk 1989). Treatment of protein extracts with the glycosidase PGNase F did not affect the mobility of constitutive and heat-induced HSF-HSE complexes (Fig. 4C). To confirm the activity of the enzymes used in these assays we performed control dephosphorylation and deglycosylation experiments using the phosphoprotein α casein and the glycoprotein horse radish peroxidase (HRP) as positive controls. Subsequent to enzymatic treatments, samples were run on SDS-PAGE gels and stained with coomassie blue. Results showed that the respective enzyme treatments were effective in dephosphorylating and deglycosylating the respective control proteins as seen by increased mobility of enzyme treated bands relative to controls (Fig. 4D).

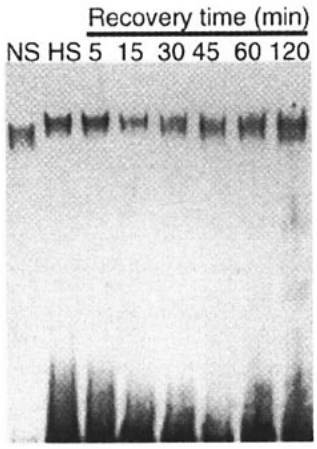

Fig. 5 A progressive increase in the mobility of the HSF complex during recovery. The effect of recovery from heat shock on HSE-binding activity of protein preparations from blastospores of *B. bassiana* was studied. Cells were heat-shocked (HS) at 45°C for 1 h and subsequently transferred to control (NS) temperature (25°C). Samples were withdrawn at periodic intervals of recovery times (5, 15, 30, 45, 60, and 120 min) and the HSF-HSE complex analyzed by gel mobility shift assays. Times of recovery are indicated above the panel.

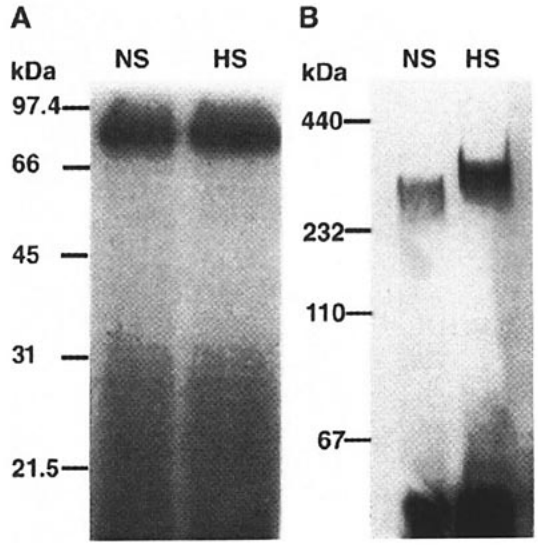

Fig. 6 *B. bassiana* HSF exists as a trimer under both non shock and heat shock conditions. The native and denatured molecular masses of constitutive and heat-inducible HSF-HSE complexes was determined. (A) Molecular mass of denatured HSF-HSE complexes as observed by SDS-PAGE analysis of UV cross linked HSF-HSE complexes of heat-shocked (HS) and non-shocked (NS) blastospores of *B. bassiana*. Protein extracts were UV cross-linked to a ^{32}P labeled HSE oligonucleotide and resolved on a 12% homogenous SDS-PAGE gel. The molecular masses of the HSFs were determined by comparison with coomassie blue stained broad range molecular standards (indicated on left). (B) Estimation of the molecular mass of native HSF-HSE complexes using pore exclusion gradient gel electrophoresis. The HSF-HSE complexes from heat-shocked (HS) and non-shocked (NS) *B. bassiana* were separated on a 4–25% non-denaturing pore gradient polyacrylamide gel for 24 h at 150V with cooling at 4°C. Native molecular mass standards are indicated on the left side.

Hyperphosphorylation of HSF in heat-shocked cells has been correlated with increased ability to promote transcription in all eukaryotes studied so far (Larson et al. 1988; Sarge et al. 1993). In *S. cerevisiae*, it has been suggested that hyperphosphorylation of HSF upon heat shock triggers transcriptional activation through the induction of a conformational change, unmasking transcriptional activator domains (Sorger and Pelham 1988). Later evidence, however, suggests that hyperphosphorylation in yeast serves as a regulatory mechanism to deactivate HSF, and may not be involved in activation (Hoj and Jakobsen 1994). The overall conclusion of the experiments shown here is that neither phosphorylation nor glycosylation was responsible for the diminished mobility or increased levels of the heat-induced HSF-HSE complex. Thus, the absence of phosphorylation mediated transcriptional activation of the HSF may be an exception in this group of filamentous fungi. It must be noted here that the mobility differences between heat shocked and non-shocked HSF of *S. cerevisiae* could not be totally removed by dephosphorylation (Sorger and Pelham 1988). The suggestion of the authors that this reflects the inability of the added phosphatases to remove all phosphates is further substantiated by the results of this study which suggest the possibility of additional modifications or conformational changes of the heat-induced HSF besides phosphorylation. Interestingly, hyperphosphorylation of chick HSF1 could not be detected after heat shock (Nakai et al. 1995). We are not able to conclude from our experiments whether the lack of mobility changes observed in phosphatase treatments was due to absence of HSF phosphorylation, or inability to remove phosphate groups.

The apparent lack of an association between phosphorylation or glycosylation with changes in the behavior of HSF-HSE complexes led us to speculate that distinct HSF proteins formed the constitutive and heat inducible HSE-binding activities. Earlier experiments with *B. bassiana* (Xavier and Khachatourians, unpublished data) have shown that protein synthesis was not blocked by cycloheximide at concentrations as high as 1000 μg ml^{-1}. Thus the inherent resistance of *B. bassiana* towards cycloheximide precluded the use of protein synthesis inhibitors to test whether the heat inducible isoform was synthesized *de novo*. Therefore, as a preliminary exploration of the possibility that distinct proteins formed the constitutive and heat-induced HSF-HSE binding activities, we investigated changes in the pattern of HSF-HSE binding activity in heat-shocked cells following a temperature shift down. Cells were subjected to a 45°C heat shock and then allowed to recover at control temperature, and the HSF-HSE binding activity of recovering cells was

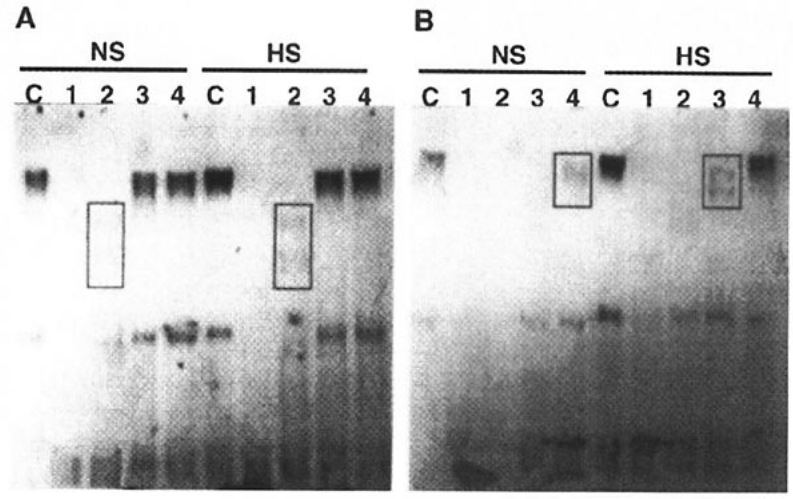

Fig. 7 The same HSF binds to HSE in both unstressed and heat-shocked *B. bassiana* cells. Proteolytic clipping band shift assays of HSF-HSE complexes from heat-shocked (HS) and non-shocked (NS) blastospores of *B. bassiana*. Proteolytic clipping band assays were done by the addition of varying amounts of trypsin (A) and chymotrypsin (B) to completed DNA-binding reactions and further incubated at RT for 15 min. Samples were analyzed by gel mobility shift assays. Lanes 1–4 indicate 0.1, 0.01, 0.001, and 0.0001 enzyme units, respectively. Controls (no enzyme) of both NS and HS samples are indicated as C and identical cleavage products are boxed.

monitored over time. The surmise of this experiment was that if the constitutive and heat-induced HSF complexes were formed by different proteins, the appearance of two distinct HSF-HSE complexes could be detected during recovery: a declining level of heat-induced complex, and an increasing level of constitutive complex. Although a progressive increase in the mobility of the HSF complex was observed during recovery (Fig. 5) only a single band was detected in each sample, and distinct formation of different complexes was not observed. The relative mobility of heat induced HSF-HSE complex returned to control levels within 2 h of recovery indicating a return to normal physiological conditions and a reversion of HSF to the constitutive conformation (Fig. 5). Gel mobility shift assays using alternate buffer systems such as TBE or high ionic Tris-glycine also did not result in the resolution of distinct HSF-HSE complexes (data not shown).

Since differences in the molecular masses of the constitutive and heat-induced HSF complexes would indicate the existence of different proteins, we next determined the size of these complexes using UV-crosslinking. Results showed that the molecular mass of both heat-induced and constitutive HSF was 85.8 kDa (Fig. 6A). The molecular mass of *B. bassiana* HSF is well within the range of other known HSFs such as *S. cerevisiae* (93.2 kDa) (Wiederrecht et al. 1988); *X. laevis* (88 kDa) (Ovsenek and Heikkila 1990); *Schistosoma mansoni* (60 and 80 kDa) (Levy-Holtzman and Schechter 1996); and *T. pyriformis* (70 kDa) (Avides et al. 1990). Since only one distinct band with a molecular mass of 85.8 kDa was detected on SDS-PAGE gels, it is highly probable that identical homomeric protein complexes comprise both constitutive and heat-induced bandshifts detected in this group of filamentous fungi.

We were next interested in determining the size of the native HSF-HSE complex. This was done using pore exclusion gradient polyacrylamide gel electrophoresis. Results showed that the native apparent molecular mass of the heat-induced and constitutive HSF-HSE complexes was approximately 260 and 300 kDa, respectively (Fig. 6B). The difference in the molecular masses of heat-induced and constitutive HSF complexes was consistent with the relative migration patterns observed with gel mobility shift assays. A comparison of the size of the denatured form (85.8 kDa) with the native sizes of HSFs from non-shocked and heat-shocked cells indicates that *B. bassiana* HSF exists as a trimer under both non-shock and heat shock conditions. This finding is consistent with the results obtained with *S. cerevisiae* wherein HSF exists as a trimer in solution and when bound to DNA (Sorger and Nelson 1989). The similarity in monomeric molecular mass of both HSFs as determined by UV-crosslinking, and the results of phosphatase and glycosidase experiments suggest that the reduced mobility of heat-induced HSF in gel shift assays and native gels was due to a conformational change in the native shape of the heat-induced HSF. Presumably, SDS-PAGE eliminated the difference in the conformational changes in the native globular protein structures of both constitutive and heat-inducible HSFs.

We further examined the identity and relatedness of the HSF molecules from non-shocked and heat-shocked cells using proteolytic clipping band assays. In these

assays, various dilutions of trypsin and chymotrypsin were added to reaction mixtures upon completion of DNA-binding reactions. This treatment results in the production of partial cleavage products that become more completely cleaved with higher concentrations of protease. The electrophoretic 'fingerprint' pattern of partial and complete cleavage products of constitutive and heat inducible HSFs were compared (Fig. 7). The cleavage products of both heat-induced and constitutive HSFs were identical with treatment of trypsin (Fig. 7A) and chymotrypsin (Fig. 7B). We, therefore, suggest that the same HSF binds to HSE in both unstressed and heat-shocked cells, although further confirmation is required. Based on the results of this study, we hypothesize that a single HSF which undergoes conformational changes other than phosphorylation or glycosylation during heat shock, controls constitutive and inducible expression of HSP genes of the filamentous fungi studied here. Further studies including the cloning of HSF gene from filamentous fungi will be required to determine the mechanisms by which filamentous fungi respond to stress and regulate the activity of HSF.

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