EXPERIMENTAL MYCOLOGY **17**,362-367 (1993)

BRIEF NOTE

Purification and Partial Amino Acid Sequence of the Major 70,000-Dalton Heat Shock Protein in *Neurospora crassa*

FRANCO FRACELLA, SAADAT MOHSENZADEH, AND LUDGER RENSING

Biology Department, University of Bremen, Leobenerstrasse/NW2, D-28359 Bremen 33, *Germany*

Accepted for publication May 18, 1993

FRACELLA, F., MOHSENZADEH, S., AND RENSING, L. 1993. Purification and partial amino acid sequence of the major 70,000-Dalton heat shock protein in *Neurospora crassa. Experimental Mycology,* 17, 362-367. The major heat shock protein of 70 kDa (hsp70) from heat-shocked mycelial extracts of *Neurospora crassa* was purified to near homogeneity employing DEAE anion-exchange chromatography followed by affinity chromatography on ATP-agarose. The isolated hsp70 migrates as a single band on one-dimensional sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), with a molecular mass of ~69 kDa. On two-dimensional gels it is resolved into two polypeptides with isoelectric points in the acidic range of ~pH 5.2. The first 53 amino terminal amino acids of the major protein were sequenced and compared with hsp70 of other species. The amino acids aspartic acid, arginine, and phenylalanine occur at positions 27, 28, and 44 (from the methionine terminus) in contrast to the main consensus sequence. These three differing amino acids are shared by yeast, and, in addition, the first two by *Arabidopsis,* petunia, and maize. © 1993 Academic Press. Inc.

INDEX DESCRIPTORS: Heat shock protein; hsp70; *Neurospora crassa;* protein isolation; amino acid sequence.

Heat shock (or stress) proteins (hsps) are produced by both prokaryotic and eukaryotic cells under normal and stress conditions. The hsps belong to the most conserved proteins in phylogeny. Among the hs-genes in eukaryotic cells, a multigene family exists that encodes several related 70-kDa stress proteins (the hsp70 family), differing in their intracellular location, function, and regulation. This family includes constitutive (or cognate), stressinducible, and glucose-regulated proteins. Members of the hsp70 family playa major role in the folding, unfolding, and translocation of polypeptides as well as in the assembly and disassembly of oligomeric protein complexes. All these processes involve ATP binding and hydrolysis (for reviews see Hightower and Nover, 1991; Lindquist and Craig, 1988; Nover, 1991). The heat shock response of the filamentous fungus *Neurospora crassa* is characterized by the enhanced synthesis of about 6 to **11** hsps (Plesofsky-Vig and Brambl, 1985; Kapoor and Lewis, 1987; Zoeger *et al.,* 1992). The hsp70 family of *N. crassa* consists of at least seven proteins resolved in twodimensional gels (Kapoor and Lewis, 1987; Zoeger *et al.,* 1992). We isolated the major hsp70 of *N. crassa* and determined the amino acid sequence of the amino terminal portion in order to determine sequence homologies to other species.

N. crassa (bd A strain, FGSC No. 1858) was cultured in Vogel's minimal medium (Vogel, 1956) supplemented with 1 m*M* $CaCl_2$ and 2% sucrose under continuous shaking in constant light at 28°C for about 24 h after inoculation with 1.2 x 109 conidia per liter. For heat shock treatment, the culture flasks were kept at 45°C for 60 min. The *N. crassa* hsp70 is most highly expressed after 60 min of heat exposure (Plesofsky-Vig and Brambl, 1985).

Growing mycelia were labeled with [^{35}S]methionine (Amersham, 40 yCi/ml me

0147-5975/93 $5.00

dium) for the last 50 min during heat exposure. Control cultures were labeled at the same time and harvested in parallel with the treated cultures. After washing and harvesting, cells were homogenized and the amount of TCA-precipitable radioactive proteins was determined in a scintillation counter (Mans and Novelli, 1961).

Mycelia were harvested by filtration, broken with a precooled mortar and pestle after adding acid-washed seas and and buffer A (20 m*M* Tris-HCl, pH 7.5, 20 mM NaCl, 10 m*M* $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride), and then suspended in buffer A and centrifuged for 10 min at 4°C and 48000*g*. The supernatant was filtered through Schleicher & Schuell ME 25 and again centrifuged at 48000*g* and 4°C for 1 h. The hsp70 was purified by the method of Welch and Feramisco (1985) with a few modifications. The resulting supernatant was directly passed through a 60-ml DEAE column (Fractogel TSK DEAE 650 M, Merck). The fraction eluted from the column between 0.08 and 0.14 *M* NaCl in buffer A was loaded on a 1-ml A TP-agarose (A2767, Sigma) column. After washing with buffer A and B {buffer A + 0.6 *M* NaCl), bound protein was eluted with buffer C (buffer A + 10 mM Na2ATP). The ATPeluate was found to contain virtually homogeneous hsp70.

Protein concentration was determined according to Neuhoff *et al.* (1979). SDSPAGE was performed on 10% acrylamide0.3% bisacrylamide gels (Laemmli, 1970). Two-dimensional gel electrophoresis was conducted essentially as described by O'Farrell (1975) using isoelectric focusing in tube gels with a mixture of 1 % ampholines (pH 2-11, Serva) and 4% ampholines (pH 5-7, Serva) in the first dimension. The second dimension of gel electrophoresis was carried out as described above. Gels were stained by Coomassie blue. Fluorography was carried out by permeating the gels with dimethyl sulfoxide and 2,5 diphenyloxazole (Bonner and Laskey, 1974). The gels were vacuum dried and exposed to Kodak XAR-5 film at -80°C for various times. For electrophoretic blotting, the proteins separated in one-dimensional gel electrophoresis were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Trans Blot Cell (Bio-Rad) (Towbin *et al.,* 1979). The N-terminal amino acid sequence of hsp70 blotted through PVDF membrane was analyzed by automated Edman degradation in a dualphase protein sequencer (Knauer, Berlin, Model 816 E). The sequence obtained was compared to the sequences of other proteins in the EMBL data bank.

Using the method of Welch and Fera

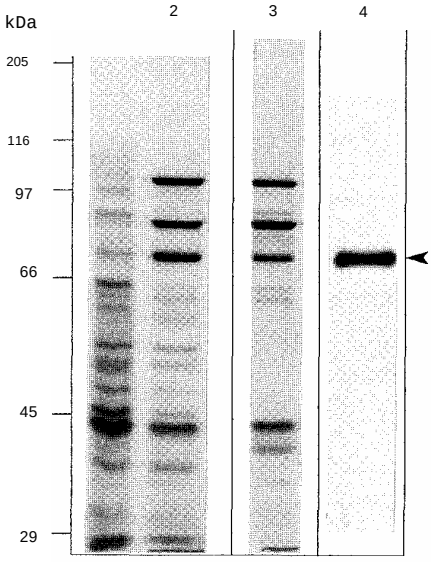

FIG. 1. Fluorograph of the 10% SDS-PAGE profile of the cytoplasmic fraction of a heat-shocked *N. crassa* lysate at successive steps of purification. Lane 1, crude lysate from a control mycelium (10^4 cpm in 18 yg protein); lane 2, crude lysate from heat-shocked mycelium (10^4 cpm in 21 yg protein); lane 3, fraction from DEAE column from heat-shocked mycelium eluted between 0.08 and 0.14 *M* NaCl containing the majority of hsp70 (10^4 cpm in 5 yg protein); lane 4, fraction from ATP-agarose from heat-shocked mycelium (2×10^4 cpm in 46 yg protein). hsp70 is indicated by an arrowhead.

misco (1985) we isolated nearly pure hsp70 from *N. crassa* cytoplasm. On Coomassie blue stained one-dimensional SDS-P AGE, isolated hsp70 migrates as a single band with a molecular mass of ~69 kDa (not shown). Also on fluorographs, reflecting an isolation routine with [^{35}S]methioninelabeled proteins, no contaminating proteins were detectable (Fig. 1). On fluorographs of two-dimensional gels it was resolved into two polypeptides with isoelectric points in the acidic range of ~pH 5.2 (Fig. 2c). To examine the entire cytoplasmic hsp70 family crude nontreated and heat-shocked extracts were passed through a ATP-agarose column only. The fluorograph of a twodimensional gel of heat-shocked extract shows seven polypeptides with a molecular mass in the range of 70 kDa (Fig. 2b). Four members of these cytoplasmic hsp70 are constitutively synthesized in moderate amounts in unstressed cells (Fig. 2a). The other three polypeptides appeared only in the heat-shocked extract (Fig. 2b). These induced hsp70 members showed isoelectric points in a slightly more basic range (~pH 5.5) than the constitutive hsp70 members. Alternatively, some of these spots could be interpreted as differently phosphorylated isoforms of one protein.

The fluorographs shown in Fig. 2 suggest that there are only a very few proteins present other than members of the hsp70 family. Coomassie blue staining of the gels of which the fluorographs in Figs. 2a and 2b were taken revealed some other proteins of different molecular weight (not shown). These proteins were eliminated when the whole purification procedure according to Welch and Feramisco (1985) was applied

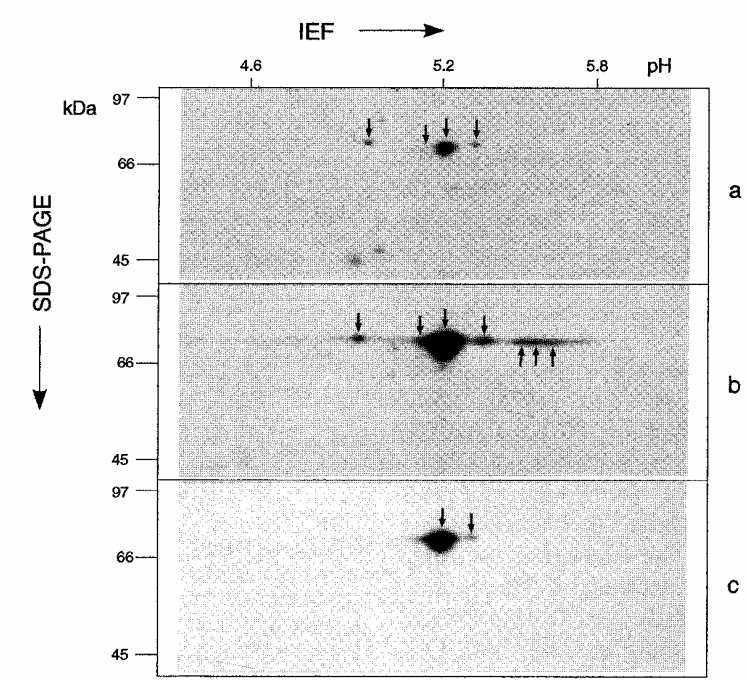

FIG. 2. (a, b) Fluorographs of two-dimensional electrophoresis of a control extract (a, 3 x 10^4 cpm in 11 yg protein) and a heat-shocked extract (b, 1.2 x 10^5 cpm in 36 yg protein) containing the whole set of cytoplasmic hsps passed through ATP-agarose columns only. (c) Fluorograph of twodimensional electrophoresis of the purified hsp70 (3 x 10^4 cpm in 42 yg protein). hsp70 spots are identified by arrows.

(Fig. 2c). The total amount of cytoplasmic proteins from crude heat-shocked extracts that binds to A TP-agarose was approximately 40--60% higher than that from control extracts, mainly due to the increased synthesis of the hsp70s.

For the sequence analysis the purified hsp70 was first electrophoresed by SDSPAGE and then the separated protein transferred to PVDF membrane and stained with Coomassie blue. A strip of the membrane containing the hsp70 was excised and submitted directly to the sequencer. The amino terminal amino acid turned out to be alanine. The first 53 N-terminalamino acids of the protein were compared to the corresponding region of other known hsp70s and the consensus hsp70 (Nover, 1991). The *N. crassa* amino acid sequence alignment is presented in Fig. 3. The sequence deduced from the *Drosophila melanogaster* gene coding for the 71-kDa heat shock cognate protein (Perkins *et al.,* 1990) appears to exhibit the closest homology with the *N. crassa* hsp70. However, even though *Saccharomyces cerevisiae* hsp70s (SSA1, SSA2) have less sequence homology to *N. crassa* hsp70 than *D. melanogaster,* there are three amino acids (aspartic acid, arginine, and phenylalanine at positions 27, 28, and 44 from the methionine terminus) in identical positions to those of *N. crassa* which differ from the consensus hsp70. Interestingly, amino acids 27 and 28 also correlate with the amino acids of *Arabidopsis,* petunia, and maize. In comparison, the amino acid sequence of the small hsps revealed also a relatively higher degree of homology between *N. crassa* and yeast as well as several plant species whereas

```
                aa   Da      com   hom
aa Nr.                              1        10         20         30         40         50         60
Nc-hsp70                    p               ------A... .........? ....REDR.. .......... ...F....-. ..........
Consensus hsp70                        87%  MXXXXKGPAV GIDLGTTYSC VGVFQHGKVE IIANDQGNRT TPSYVAFTXD TERLIGDAAK
01 Dm-hsc71   651  71059   na      87%  .S---.A... .......... .......... .......... ........-. ..........
02 Dm-hsp70   641  70258   na      75%  .------..I .......... ...Y...... .N.Y...... ........-. S...N.EP..
03 Hs-hsc70   646  70898   na      85%  .S---..... .......... .......... .......... ........-. ..........
04 Hs-hsp70   640  69868   na      85%  .A---.AA.. .......... .......... .......... ........-. ..........
05 Mm-hsc70   646  70837   na      85%  .S---..... .......... .......... .......... ........-. ..........
06 Mm-hsp70   633  69740   na      82%  .SAR--...I .......... .......... .......... ........-. ..........
07 At-hsp70   120  13209   f,na    78%  .SGKGE...I .......... ...W..DR.. .......... ........-. S.........
08 Ph-hsp70   651  71226   na      78%  .AGKGE...I .......... ...W..DR.. .......... .....G..-. ..........
09 Zm-hsp70   645  70602   na      80%  .A-KSE...I .......... ..LW..DR.. .......... ........-. ..........
10 Sc-SSA1    642  69767   na      85%  .S-----K.. .......... .AH.ANDR.D .......... ...F....-. ..........
11 Sc-SSA2    639  69470   na      83%  .S-----K.. .......... .AH.SNDR.D .......... ...F.G..-. ..........
12 Sc-SSA4    642  69651   na      85%  .S-----K.. .......... .AH.ANDR.. .......... ........-. ..........
13 Ec-dnaK    638  69115   na      48%  .-----.KII .......N.. .AIMDGTTPR VLE.AE.D.. ...II.Y.Q. G.T.V.QP.K
Consensus hsp70                        87%  MXXXXKGPAV GIDLGTTYSC VGVFQHGKVE IIANDQGNRT TPSYVAFTXD TERLIGDAAK
Nc-hsp70                    p               ------A... .........? ....REDR.. .......... ...F....-. ..........
aa Nr.                              1        10         20         30         40         50         60
```

FIG. 3. Partial amino acid sequence of the amino terminus of the *N. crassa* hsp70 compared with corresponding regions of other hsp70s. The amino acid residues are indicated by the one-letter code. For comparison of sequences I to 13 with the hypothetical consensus hsp70 sequence (Nover, 1991) and the partial *N. crassa* hsp70 sequence (53 amino acids), identical amino acids are indicated by dots, missing amino acids by hyphens, and unknown amino acids or either one of the 20 amino acids by X. The? in the *N. crass a* hsp70 sequence indicates a possible cystein at this position. In a sequencing routine cystein fails to produce a peak and is therefore not directly detectable. aa, total amino acids; Da, Dalton; com, comment (f, fragment; na, sequence from nucleic acid; p, sequence from protein); horn, percentage homology with *N. crassa* hsp70. 01 Dm-hsc71, *D. melanogaster* (Perkins *et al.,* 1990); 02 Dm-hsp70, *D. melanogaster* (Ingolia *et al.,* 1980; Karch *et al.,* 1981; Torok and Karch, 1980); 03 Hs-hsc70, Human (Dworniczak and Mirault, 1987); 04 Hs-hsp70, Human (Hunt and Morimoto, 1985); 05 Mm-hsc70, Mouse (Giebel *et al.,* 1988); 06 Mm-hsp70, Mouse (Zakeri *et al.,* 1988); 07 At-hsp70, *Arabidopsis thaliana* (Wu *et al.,* 1988); 08 Ph-hsp70 *Petunia hybrida* (Winter *et al.,* 1988); 09 Zm-hsp70, Maize (Rochester *et al.,* 1986); 10 Sc-SSAl, S. *cerevisiae* (Slater and Craig, 1989); 11 Sc-SSA2, S. *cerevisiae* (Slater and Craig, 1989); 12 Sc-SSA4, S. *cerevisiae* (Boorstein and Craig, 1990); 13 Ec-dnaK, *E. coli* (Ohki *et al.,* 1986; Slater and Craig, 1989).

Drosophila and other animal species showed less identical amino acids (Piesofsky-Vig *et al.*, 1992; de Jong *et al.*, 1993).

ACKNOWLEDGMENTS

We are grateful to Dr. F. Reimann for help in determining the protein sequence and Dr. M. Vicker for critically reading the manuscript.

REFERENCES

BONNER, W. M., AND LASKEY, R. A. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochern.* 46: 83-88.

BOORSTEIN, W. R., AND CRAIG, E. A. 1990. Structure and regulation of the SSA4 hsp70 gene of *Saccharomyces cerevisiae. J. Bioi. Chern.* 265: 1891218921.

DE JONG, W. W., LEU NISSEN, J. A. M., AND VOORTER, C. E. M. 1993. Evolution of the a-crystallin/small heat-shock protein family. *Mol. Bioi. Evol.* 10: 103-126.

DWORNICZAK, B. P., AND MIRAULT, M.-E. 1987. Structure and expression of a human gene coding for a 71 kd heat shock "cognate" protein. *Nucleic Acids Res.* 15: 5181-5197.

GIEBEL, L. B., DWORNICZAK, B. P., AND BAUTZ, E. K. F. 1988. Developmental regulation of a constitutively expressed mouse mRNA encoding a 72kDa heat shock-like protein. *Dev. Bioi.* 125: 200207.

HIGHTOWER, L., AND NOVER, L. 1991. *Heat Shock and Development.* Springer-Verlag, Berlin.

HUNT, C., AND MORIMOTO, R.I. 1985. Conserved features of eukaryotic hsp70 genes revealed by comparison with nucleotide sequence of human hsp70. *Proc. Natl. Acad. Sci. USA* 82: 6455-6459.

INGOLIA,T. D.,CRAIG,E. A.,ANDMcCARTHy,B. J. 1980. Sequence of three copies of the gene for the major *Drosophila* heat shock induced protein and their flanking regions. *Cell* 21: 669-679.

KAPOOR, M., AND LEWIS, J. 1987. Alteration of the protein synthesis pattern in *Neurospora crassa* cells by hyperthermal and oxydative stress. *Can. J. Microbiol.* 33: 162-168.

KARCH, R., TOROK, I., AND TISSIERES, A. 1981. Extensive regions of homology in front of two hsp70 heat shock variant genes in *Drosophila melanogaster. J. Mol. Bioi.* 148: 219-230.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

LINDQUIST, S., AND CRAIG, E. A. 1988. The heat shock proteins. *Annu. Rev. Genet.* 22: 631-677.

MANS,R. J.,ANDNoVELLI,G. P.1961.Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Arch. Biochern. Biophys.* 94: 48-53.

NEUHOFF, V., PHILIPP, K., ZIMMER, H. G., AND MESECKE, S. 1979. A simple, versatile, sensitive and volume-independent method for quantitative protein determination which is independent of other external influences. *Hoppe-Seyler's* Z. *Physiol. Chern.* 360: 1657-1670.

NOVER, L. 1991. *Heat Shock Response.* CRC Press, Boca Raton.

O'FARRELL, P. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Bioi. Chern.* 259: 4007-4021.

OHKI, M., TAMURA, F., NISHIMURA, S., AND USHIDA, H. 1986. Nucleotide sequence of the *Escherichia coli* dnaJ gene and purification of the gene product. *J. Bioi. Chern.* 261: 1778-1781.

PERKINS, L. A., DOCTOR, J. S., ZHANG, K., STINSON, L., PERRIMON, N., AND CRAIG, E. A. 1990. Molecular and developmental characterization of the heat shock cognate-4 gene of *Drosophila melanogaster. Mol. Cell. Bioi.* 10: 3232-3238.

PLESOFSKy-VIG, N., AND BRAMBL, R. 1985. The heat shock response of *Neurospora crassa:* Protein synthesis and induced thermotolerance. *J. Bacteriol.* 162: 1083-1091.

PLESOFSKy-VIG, N., VIG, J., AND BRAMBL, R. 1992. Phylogeny of the a-crystallin-related heat-shock proteins. *J. Mol. Evol.* 35: 537-545.

ROCHESTER, D. E., WINER, J. A., AND SHAH, D. M. 1986. The structure and expression of maize genes encoding the major heat shock protein, hsp70. *EMBO J.* 5: 451-458.

SLATER, M. R., AND CRAIG, E. A. 1989. The SSAI and SSA2 genes of yeast *Saccharomyces cerevisiae. Nucleic Acids Res.* 17: 805-806.

TOROK, I., AND KARCH, R. 1980. Nucleotide sequence of heat shock activated genes in *Drosophila melanogaster.* I. Sequences in the regions of the 5' and 3' ends of the hsp70 gene in the hybrid plasmid 56H8. *Nucleic Acids Res.* 8: 3105-3123.

TOWBIN, H., STAEHELIN, T. M., AND GORDON, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350-4354.

VOGEL, H. J. 1956. A convenient growth medium for *Neurospora* (medium M). *Microb. Genet. Bull.* 13: 42-43.

WELCH,W. J.,ANDFERAMISCO,J. R.1985.Rapidpurification of mammalian 70,000-Dalton stress proteins: Affinity of the proteins for nucleotides. *Mol. Cell. Bioi.* 5: 1229-1237.

WINTER, J., WRIGHT, R., DUCK, N., GASSER, C.,

FRALEY, R., AND SHAH, D. M. 1988. The inhibition of petunia hsp70 mRNA processing during CdClz stress. *Mol. Gen. Genet.* 211: 315-319.

Wu, C., CASPAR, H. T., BROWSE, J., LINDQUIST, S., AND SOMERVILLE, C. 1988. Characterization of an hsp70 cognate gene family in *Arabidopsis. Plant Physiol.* 88: 731-740.

ZAKERI,Z. F., WOLGEMUTH, D. J., AND HUNT, C. R. 1988. Identification and sequence analysis of a new member of the mouse hsp70 gene family and characterization of its unique cellular and developmental pattern of expression in the male germ line. *Mol. Cell. Bioi.* 8: 2925-2932.

ZOEGER, D., SCHOLLE, C., SCHRODER-LoRENZ, A., TECHEL, D., AND RENSING, L. 1992. Some starvation-induced proteins in *Neurospora crassa* are related to glucose-regulated proteins. *Exp. Mycol.* 16: 138-145.