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Note

Separation of rat liver HSP70 and HSP71 by high-performance liquid chromatography with a hydroxylapatite column

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Heat-shock proteins (HSPs) are induced in response to a broad range of environmental stresses, such as elevated temperature, amino acid analogues, transition metals and metabolic inhibitors (reviewed in refs. 1–3). HSPs with molecular weights of approximately 70 000, one of the major HSPs, are a family of evolutionally conserved proteins encoded by a conserved gene family. In mammalian cells, there seem to be two forms of these proteins, a constitutive form present at appreciable levels under normal conditions and an induced form, the synthesis of which is induced only by stress. These HSPs bind ATP tightly[4] and the property has been exploited to purify the HSPs[5]. Clathrin-uncoating ATPase was identified as a constitutive protein of the HSP70 family[6,7].

We have reported that the elevation of the body temperature of rats induces four HSPs with molecular weights of 70 000, 71 000, 85 000 and 100 000 (HSP70, HSP71, HSP85 and HSP100, respectively) in various tissues, with a concomitant induction of their corresponding mRNAs[8,9]. Among these HSPs of rats, HSP70 is a completely induced protein, whereas HSP71 is a constitutively expressed protein of the HSP70 family that exists abundantly in various tissues of rats. To obtain large amounts of an inducible protein and a constitutive protein of the HSP70 family for a study of their properties, liver tissue of rat may be better than cultured cells as a starting material.

In this paper, we describe a rapid method for the separation of HSP70 and HSP71 from rat liver by high performance liquid chromatography (HPLC) with a hydroxylapatite column.

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EXPERIMENTAL

Materials

A hydroxylapatite column (KB-column, 100 × 7.8 mm I.D.) was obtained from Koken (Tokyo, Japan). DEAE-Sepharose CL-6B and ATP-agarose were obtained from Pharmacia (Uppsala, Sweden) and Sigma (St. Louis, MO, U.S.A.), respectively.

Animals

Male Sprague–Dawley rats weighing 220–240 g were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals and used at 7 weeks of age.

Preparation of HSP70 and HSP71 from rat liver

Whole-body hyperthermia of rats was brought about by elevation of the body temperature of the rats to 42°C for 15 min[8]. Livers (20 g) of rats killed 24 h after the hyperthermia were homogenized in 5 volumes of a buffer containing 20 m*M* Tris–acetate (pH 7.5), 20 m*M* sodium chloride, 0.1 m*M* EDTA and 0.1 m*M* dithiothreitol (buffer A). The homogenates were centrifuged for 10 min at 10 000 *g* at 4°C and the supernatant was further centrifuged for 1 h at 105 000 *g* at 4°C. The second supernatant (80 ml) was applied to a DEAE-Sepharose CL-6B column (60 × 2 cm I.D.) equilibrated with buffer A at 4°C. After the column had been washed with the same buffer, the proteins adsorbed were eluted with a linear gradient of 20–500 m*M* sodium chloride in the same buffer (500 ml). The eluted fractions containing HSP70 and HSP71 (50 ml) were adjusted to a magnesium concentration of 3 m*M* by the addition of 1 *M* magnesium chloride, and were applied to an ATP-agarose column (5 ml) equilibrated with 20 m*M* Tris–acetate (pH 7.5), 20 m*M* sodium chloride, 0.1 m*M* EDTA, 0.1 m*M* dithiothreitol and 3 m*M* magnesium chloride (buffer B) at 4°C. After the column had been washed with buffer B, proteins were eluted with buffer B containing 3 m*M* ATP. The fractions enriched in HSP70 and HSP71 (7.5 ml) were pooled and dialysed against 20 m*M* potassium phosphate buffer (pH 7.6) containing 0.1 m*M* dithiothreitol (buffer C) at 4°C.

High-performance liquid chromatography

Two Altex (Berkeley, CA, U.S.A.) pumps (Model 100) equipped with an Altex Model 420 solvent programmer and a UV-8 spectrophotometer (Tosoh, Tokyo, Japan) with an 8-μl flow cell were used.

The dialysed fractions from the ATP-agarose column were treated by HPLC with a hydroxylapatite column (10 × 0.78 cm I.D.) equilibrated with buffer C. After the column had been washed with the same buffer, the bound proteins were eluted with a linear gradient of 20–200 m*M* potassium phosphate in this buffer in 40 min at a flow-rate of 0.7 ml/min. Fractions containing HSP70 and HSP71 were pooled and dialysed against 20 m*M* potassium phosphate (pH 7.6), 0.15 *M* sodium chloride and 0.1 m*M* dithiothreitol (buffer D) at 4°C, and finally applied to the hydroxylapatite column for HPLC equilibrated with buffer D. The HSP70 and HSP71 were eluted separately with a linear gradient of 20–200 m*M* potassium phosphate in the same buffer in 60 min at a flow-rate of 0.7 ml/min.

One- and two-dimensional gel electrophoreses

For one-dimensional gel analysis, sodium dodecyl sulphate–10% polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli[10]. Two-dimensional gel electrophoresis was performed as reported by O'Farrell[11]. For the first dimension, the isoelectric focusing gel contained 1.6% pH 6–8 ampholites and 0.4% pH 3.5–10 ampholites. For the second dimension, SDS–10% polyacrylamide slab gels were used.

The separated proteins were stained with Coomassie brilliant blue. Molecular weight markers included phosphorylase b (92 500), bovine serum albumin (69 000), α-amylase (55 000), ovalbumin (45 000) and carbonic anhydrase (29 000).

Peptide mapping

Peptide mapping was performed as described by Cleveland *et al.*[12]. To 20 μl of each purified HSP at a concentration of 100 μg/ml, 5 μl of 0.25 *M* sodium hydrogencarbonate and 2.5 μl of *Staphylococcus aureus* V8 protease at various concentrations from 40 to 300 μg/ml were added, and proteolytic digestion was carried out at 30°C for 30 min. The reaction was stopped by the sample being boiled for 2 min, and the digest was separated by SDS-PAGE and detected by silver staining.

RESULTS AND DISCUSSION

Procedures have been developed for the purification of proteins of the HSP70 family from different cells and animals[5,13–15]. The proteins of the HSP70 family are purified rapidly by a two-step procedure involving DE-52 cellulose column chromatography followed by ATP-agarose affinity chromatography[5]; the latter is based on the tight binding of these proteins to ATP. However, the inducible protein and constitutive protein of the HSP70 family have similar physical properties, so these proteins are not separated by column chromatography on hydroxylapatite, DE-52 cellulose, DE-53 cellulose, DEAE-Sepharose, Sephacryl S-300, Bio-Gel P-300 or phenyl-Sepharose[13–15]. Isoelectric points of the inducible protein and constitutive protein of the HSP70 family from rats are 5.2 and 5.0, respectively, under non-denaturing conditions, and 5.8 and 5.6, respectively, under denaturing conditions[14]. By making use of the p*I* differences between these proteins, they can be separated from each other by preparative isoelectric focusing[14].

We established a method for purifying the HSP70 and HSP71 from the livers of rats that had been subjected to whole-body hyperthermia. These proteins were first recovered in the 150–180 m*M* sodium chloride eluate from DEAE-Sepharose Cl-6B column chromatography. The eluate was next chromatographied on an ATP-agarose column and an affinity-purified fraction contained mainly HSP70 and HSP71 with a main contaminating protein with a molecular weight of 54 000 and minor contaminating proteins with molecular weights of 45 000, 92 000 and 30 000. HSP70 and HSP71 were further purified, with removal of the contaminating proteins, by HPLC on a hydroxylapatite column (Fig. 1). These proteins were recovered in the 80–100 m*M* phosphate eluate. The second peak of A_{280}, which eluted at 110–130 m*M* phosphate, contained little protein but did contain ATP. Under the chromatographic conditions used, HSP70 seemed to be eluted slightly faster than HSP71 in the first peak, so we attempted to separate the two proteins more completely by adjustment of the chromatographic conditions.

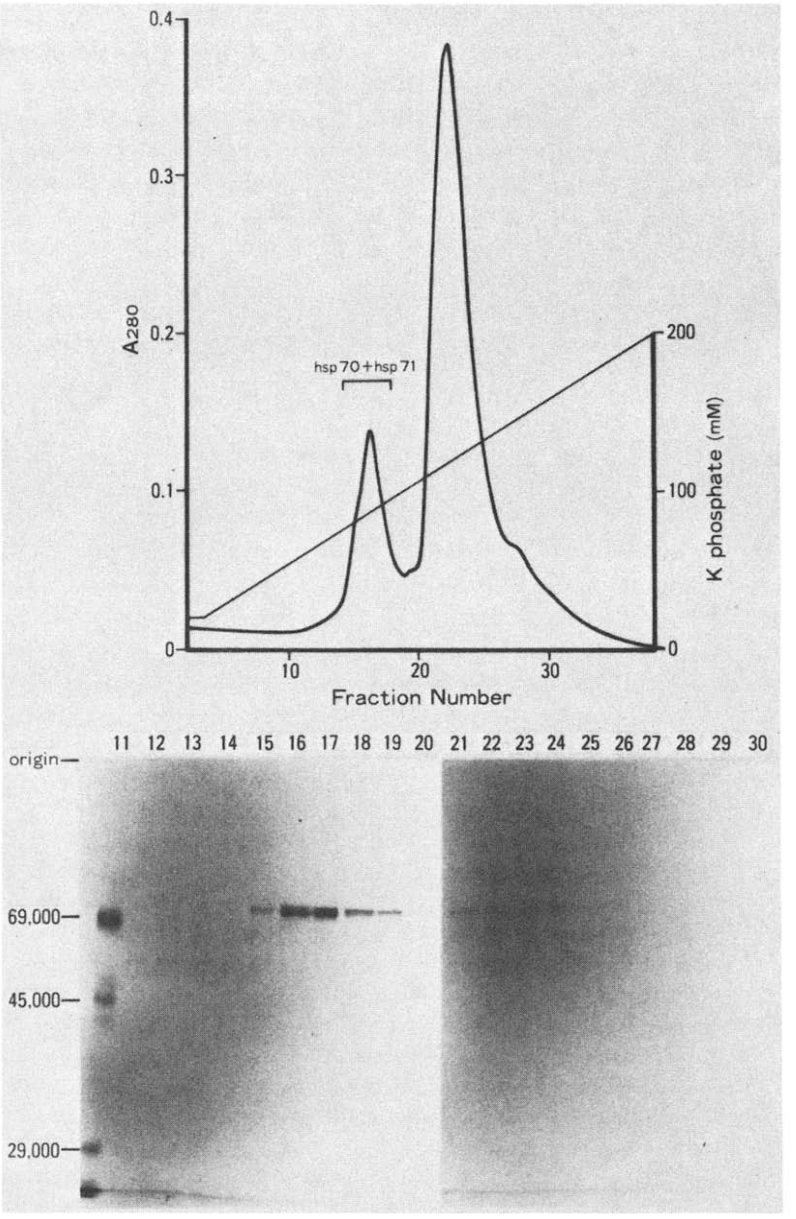

Fig. 1. Purification of HSP70 and HSP71 by HPLC with a hydroxylapatite column. Fractions containing HSP70 and HSP71 eluted from an ATP-agarose column were pooled and dialysed against buffer C. The solution was applied to a hydroxylapatite column equilibrated with buffer C. An elution profile of the column developed with a linear gradient of 20–200 m*M* potassium phosphate in buffer C is shown in the upper portion. In the lower portion is shown Coomassie blue staining of SDS–10% polyacrylamide gels loaded with 20 μl of each fraction. In this and subsequent figures, molecular weight markers are indicated on the left.

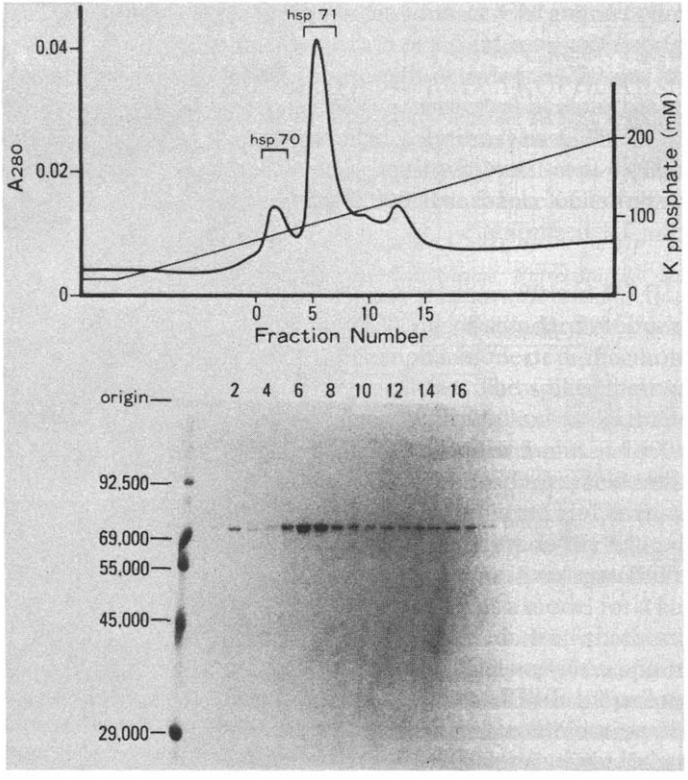

Fig. 2. Separation of HSP70 and HSP71 with a hydroxylapatite column in the presence of 0.15 *M* sodium chloride. Fractions containing HSP70 and HSP71 that eluted from the first hydroxylapatite column were dialysed against buffer D that contained 0.15 *M* sodium chloride and applied to a hydroxylapatite column equilibrated with buffer D. An elution profile of the column developed with a linear gradient of 20–200 m*M* potassium phosphate in buffer D is shown in the upper portion. In the lower portion is shown Coomassie blue staining of a SDS-10% polyacrylamide gel loaded with 20 μl of each fraction. The relative proportions of HSP70 and HSP71 are different in Fig. 1 and 2, because results obtained from different series of experiments are presented in each.

Fig. 2 shows an elution profile of HSP70 and HSP71 on rechromatography on a hydroxylapatite column with a potassium phosphate gradient in the presence of 0.15 *M* sodium chloride. Under these conditions, these proteins were separated from each other. HSP70 was eluted at 80 m*M* phosphate and HSP71 was eluted mainly at 90–110 m*M* phosphate, with tailing peaks eluting at 110–130 m*M* phosphate. HSP71 that eluted at the main peak and that which eluted at the tailing peaks were not different according to analyses by two-dimensional gel electrophoresis and by peptide mapping. Fractions containing HSP70 and HSP71, shown in Fig. 2, were pooled as purified HSP70 and HSP71.

When the purified proteins were analysed by two-dimensional gel electrophoresis, HSP70 and HSP71 gave nearly single spots on the gels (Fig. 3). When HSP70 and HSP71 were mixed with liver extract of heat-shocked rats, these proteins migrated

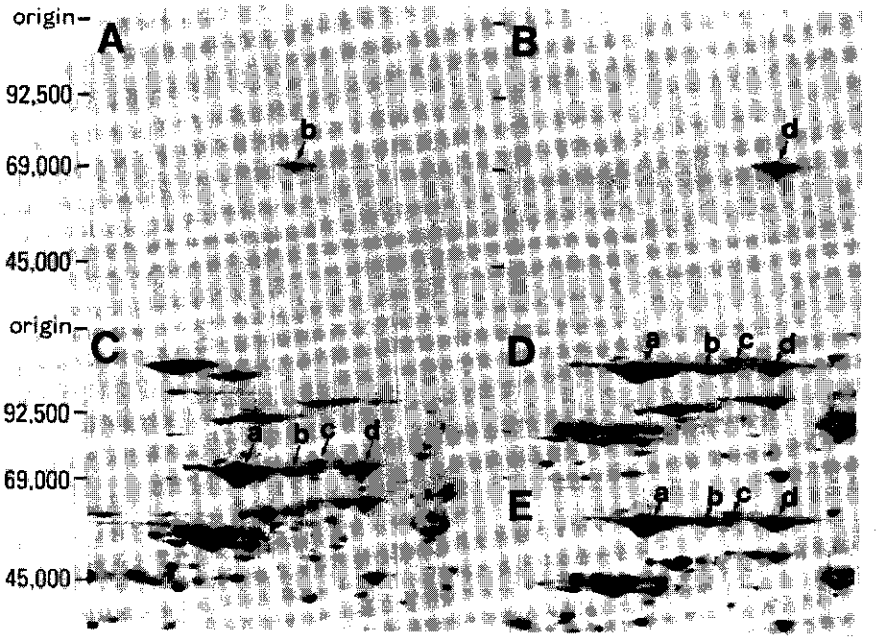

Fig. 3. Analysis of purified HSP70 and HSP71 by two-dimensional gel electrophoresis. Purified HSP70 (1 μg) (A), HSP71 (2 μg) (B), liver extract from heat-shocked rats (500 μg) (C), HSP70 (1 μg) + heat-shocked rat liver extract (500 μg) (D) and HSP71 (2 μg) + heat-shocked rat liver extract (500 μg) (E) were separated by two-dimensional gel electrophoresis. Isoelectric focusing gel for the first dimension was on the horizontal axis with the acidic end to the right. a, b, c and d indicate albumin, HSP70, a reference protein between HSP70 and HSP71, and HSP71, respectively.

identically with HSP70 and HSP71 in the extract on the two-dimensional gels. Fig. 4 shows the peptide mapping of these proteins. HSP70 and HSP71 gave similar but not identical peptide maps, indicating that the two proteins were different but highly homologous. Purified HSP70 and HSP71 which were incubated without protease migrated as double bands. As these HSPs without incubation migrated as a single band, as shown in Fig. 2, these HSPs seemed to degrade spontaneously *in vitro*, as reported by Mitchell *et al.*[16].

From 20 g of livers of heat-shocked rats, 0.4 mg of HSP70 was recovered. From 20 g of livers of untreated or heat-shocked rats, 0.6–0.7 mg of HSP71 was recovered. The purity of the final samples, determined by densitometry of the SDS polyacrylamide gels stained with Coomassie blue, was higher than 95% in both instances.

The function of HSPs is not well understood, but there is much evidence of a correlation between the production of HSPs and the development of thermotolerance in various organisms[2,3]. The HSP70 family of proteins are found associated with the nuclei and nucleoli of cells during heat shock, and they seem to protect these structures and to facilitate the repair of heat-induced damage[17]. Some HSPs are expressed under normal physiological conditions and also at some stages of development of organisms[2,3], so HSPs probably have physiological functions. At present, a constitutive form of the HSP70 family is only identified as the same protein as a clathrin-uncoating ATPase[6,7]. Recently, facilitation of the translocation of

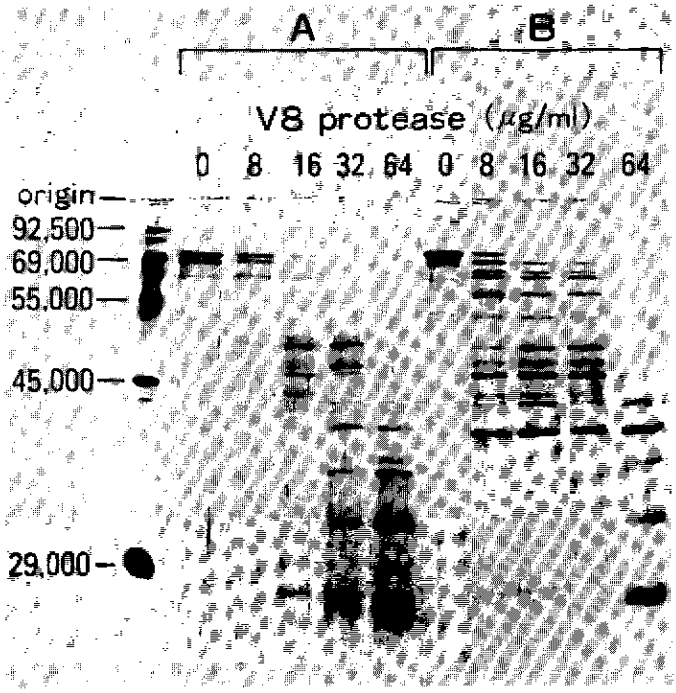

Fig. 4. Comparison of HSP70 and HSP71 by one-dimensional peptide mapping. Purified HSP70 (A) and HSP71 (B) were digested with various concentrations of *Staphylococcus aureus* V8 protease and analysed by SDS-PAGE.

secretory and mitochondrial precursor proteins by a subfamily of HSP70 in yeast cells has been reported[18,19]. It would be of interest to find whether the constitutive protein and inducible protein of the HSP70 family have the same or different functions. Rapid and large-scale preparations of both the inducible and the constitutive proteins of the HSP70 family by HPLC with a hydroxylapatite column may facilitate the study of the function of these heat-shock proteins.

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