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Production and Purification of the Native *Saccharomyces cerevisiae* Hsp12 in *Escherichia coli*

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Supporting Information

ABSTRACT: Hsp12 is a small heat shock protein produced in many organisms, including the yeast *Saccharomyces cerevisiae*. It has been described as an indicator of yeast stress rate and has also been linked to the sweetness sensation of wine. To obtain a sufficient amount of protein, we produced and purified Hsp12 without tag in *Escherichia coli*. A simple fast two-step process was developed using a microplate approach and a design of experiments. A capture step on an anion-exchange salt-tolerant resin was followed by size exclusion chromatography for polishing, leading to a purity of 97%. Thereafter, specific anti-Hsp12 antibodies were obtained by rabbit immunization. An ELISA was developed to quantify Hsp12 in various strains of *Saccharomyces cerevisiae*. The antibodies showed high specificity and allowed the quantitation of Hsp12 in the yeast. The quantities of Hsp12 measured in the strains differed in direct proportion to the level of expression found in previous studies.

KEYWORDS: *Hsp12, sweetness, recombinant protein production, high-throughput chromatography, Escherichia coli, Saccharomyces cerevisiae*

INTRODUCTION

Saccharomyces cerevisiae is one of the most widely used eukaryotic model organisms.[1] It is also used for industrial and biotechnological applications such as baking, brewing, and winemaking. Its complex adaptation to adverse conditions makes it of special interest. Its response to various environmental stresses involves more than 100 genes. Heat shock proteins (Hsp's) are involved in the heat shock response (HSR). Among them, a small protein known as Hsp12 has been described as a good indicator for evaluating the yeast stress rate.[2,3] Hsp12 is a small protein with 109 residues and is highly hydrophilic with 56% of polar amino acids. It displays features of the group of natively unfolded proteins, including a low content in aromatic amino acids and the absence of tryptophan.[4,5] Fifty percent of its sequence is composed of only five amino acids (alanine, aspartic acid, glutamic acid, glycine, and lysine). As it is unstructured, it is also soluble in solution at 80 °C. All these features have led to it being associated with the group of hydrophilins and described sometimes as a heat shock protein or as an LEA-like (late embryogenesis abundant) protein.[6] It has a limited sequence homology with other small HSPs but structural similarities.[7] Hsp12 is induced in response to various environmental stresses such as heat shock, freezing, desiccation, and osmotic stresses.[7−11] It has been shown using immunocytochemistry that it is located close to the plasma membrane and in the cell wall.[11] It could interact with the membrane through electrostatic interactions to stabilize and protect them. The membrane-associated form is a helical structure.[8,12] Hsp12 acts as a cell wall plasticizer in vitro and in vivo, thus decreasing cell wall rigidity and making it more flexible.[13−15]

Hsp12 appears to be highly expressed in wine alcoholic fermentation.[16] Apart from its physiological roles, it has previously been shown to be of great enological interest since it contributes to the increase in sweet perception during the yeast autolysis of dry wines.[17] Moreover, a correlation between its expression level and the perception of sweetness has been established in various yeast strains.[18] Owing to the economic value of monitoring taste perception during winemaking, it seems essential to better understand the sensory role of Hsp12 and particularly its release in enological conditions. For this purpose, a purification protocol is needed to isolate and detect the protein.

Therefore, we decided to produce the native *Saccharomyces cerevisiae* Hsp12 in *Escherichia coli* to obtain good quantity. A simple two-step purification process was developed using a design of experiments (DoE). Thereafter, specific antibodies against Hsp12 were obtained and tested to detect and quantify Hsp12 production in various strains of *Saccharomyces cerevisiae*.

MATERIAL AND METHODS

Equipment. An Akta Purifier 100 workstation from GE Healthcare (Uppsala, Sweden) was used for chromatographic experiments monitored with Unicorn 5.0. Protein detection was performed at

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280 nm. A HANNA HI8820N conductivity meter and a HANNA HI2210 pH meter (Tanneries, France) were used to prepare buffers. A TransBlot SD (Bio-Rad, Hercules, CA, USA) was used to carry out transfer for the Western Blot and a ChemiDoc XRS+ Imager (Bio-Rad) was used to collect the chemiluminescence signal. A LabChip GX II (Caliper Life Science, Waltham, MA, USA) was used to obtain relative quantification of Hsp12. Hsp12 was produced in a 2-l BIOSTATBplus bioreactor (Sartorius Stedim, Aubagne, France) and monitored by MFCS/win 3.0 software.

Bacterial Strains and Plasmids. The *E. coli* strain NEB 5-α was used for plasmid amplification and subcloning and the *E. coli* strain T7 Express I^q was used for protein expression (New England Biolabs, Ipswich, MA, USA). The plasmid pET-44a (Novagen) was used to construct an expression vector. An LB agar medium (Difco LB Agar, Lennox) was used to culture the strains.

Yeast Strains. The *Saccharomyces cerevisiae* strains used are listed in Table 1. The YMB36 (Δ°hsp12) strain was deleted from the *hsp12* gene as described previously.[17] Fx10, VL3, and A24 are enological and brewery strains.

Table 1. Yeast Strains Used and Their Origin

strain	collection/ supplier	food origin	area of origin	comment
A24	Hambleton Bard	brewery	UK	ref 19
Zymaflore FX10	Laffort	enology	France	specialized red wine starter
Zymaflore VL3	Laffort	enology	France	specialized white wine starter
YMB36 (Δ°hsp12)	lab collection			ref 17

Construction of Hsp12 Expression Vector. A synthetic gene corresponding to the coding sequence of the *hsp12* gene (UniProtKB: P22943) was designed with a codon bias adapted to *E. coli* and with flanking NdeI and *Hin*dIII restriction sites. This synthetic gene was synthesized by Eurofins Genomics (Ebersberg, Germany) and provided in the pEX-A2 plasmid. The DNA fragment was extracted from the pEX-A2 plasmid by double digestion with NdeI and *Hin*dIII, and was ligated with the Quick ligation kit (New England Biolabs) into similarly digested and dephosphorylated pET-44a. After transformation into NEB 5-α *E. coli* competent cells, a positive clone was selected by colony PCR and verified by DNA sequencing. We called this plasmid pET-*hsp12* (GenBank accession no. KY926444, Figure 1 of the Supporting Information). Thereafter, *E. coli* T7 Express I^q strains were transformed with pET-*hsp12* plasmid and were selected on LB agar plates containing ampicillin (100 μg/mL) for the purpose of expression.

Production of Hsp12. Expression of Hsp12 was first performed in shake flasks containing 100 mL of rich LB medium (1% bactotryptone, 0.5% NaCl, and 1% yeast extract) and ampicillin (100 μg/mL) and incubated at 37 °C in a rotary shaker at 200 rpm. When the culture reached an optical density at 600 nm around 1, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce the expression of Hsp12. Then the temperature was decreased to 30 °C to promote production. Samples were withdrawn periodically to determine the optimal time for Hsp12 production in the bioreactor. Bioreactor cultivation was performed in a 2-L working volume of rich LB medium supplemented by glucose (3 g/L) and ampicillin (100 μg/mL). After precultivation overnight in 100 mL of medium in a shake flask, a volume of the inoculum was transferred to the bioreactor to obtain an initial optical density at 600 nm of 0.15. Batch fermentation was carried out at a temperature of 37 °C until induction. Culture pH was constantly adjusted to 7 with 1 M phosphoric acid and 1 M sodium hydroxide. The dissolved oxygen tension was maintained at 20% of saturation by varying the agitation (200−600 rpm) and air flow rate (2−3 L/min). The production of Hsp12 was induced in the same conditions as those in the shake flask. After 6 h of induction, the cells were harvested by centrifugation at 6000*g* for 20 min. One gram (fresh weight) of cell pellet was resuspended in 5 mL of 50 mM Tris-HCl buffer at pH 7.5 and the cells were disrupted by sonication at 20 kHz with a 13 mm probe (six 30 s pulses separated by a 1 min incubation on ice). After sonication, the extract was centrifuged at 16000*g* for 20 min at 4 °C to remove the cell debris and insoluble proteins. Supernatant was then heated at 80 °C for 10 min. Heating caused the denaturation and precipitation of most proteins. After centrifugation at 16000*g* for 20 min, the supernatant, named heated protein extract, was recovered.

First Purification Step. Buffers and solutions were prepared using chemicals of analytical grade from Sigma (St. Louis, USA). Resins were screened with AcroPrep Advance 96-well filter plates with a 0.45-μm hydrophilic polypropylene (GHP) pore membrane and 350 μL wells from Pall Life Sciences (Cergy, France). For each resin, slurry (50% v/v) was prepared with equilibration buffer (50 mM Tris-HCl, pH 7.5) and deposited in the 96-well filter plate (100 μL per well). Equilibration was performed with 3 × 200 μL of equilibration buffer (50 mM Tris-HCl buffer at pH 7.5; 4.3 mS/cm). The sample (heated protein extract) was loaded (200 μL per well) and plates were incubated for 120 min under shaking. Washing was performed with 2 × 200 μL of equilibration buffer to remove unbound proteins. Elution was then performed with 2 × 100 μL of elution buffers. Each step was carried out under shaking at room temperature. At each step, fractions were collected under vacuum aspiration using 96-well plates and analyzed by capillary electrophoresis sodium dodecyl sulfate (CE-SDS).

Experimental conditions were determined using Minitab 16 which provided tools for data analysis as well as guidance for the design of experiments.

For the first screening, three mixed mode resins [MEP HyperCel (Pall Life Sciences, Cergy, France), HEA HyperCel (Pall Life Sciences), and PPA HyperCel (Pall Life Sciences)], an anion exchange resin [Capto Q (GE Healthcare, Uppsala, Sweden)], and a salt-tolerant anion-exchange resin [HyperCel STAR AX (Pall Life Sciences)] were used. A full factorial experiment was used with two factors, pH and NaCl concentration, at two levels and a center point. Five elution conditions were thus tested: 0.1 M sodium citrate buffer, pH 4, 0 M NaCl; 0.1 M sodium citrate buffer, pH 4, 1 M NaCl; 0.1 M sodium citrate buffer, pH 6, 0 M NaCl; 0.1 M sodium citrate buffer, pH 6, 1 M NaCl; and 0.1 M sodium citrate buffer, pH 5, 0.5 M NaCl. Each point was tested in duplicate.

For the second screening, HEA HyperCel and HyperCel STAR AX resins were used with a central composite design with two factors, pH and NaCl concentration, at two levels. Nine elution conditions were thus tested: 0.1 M sodium citrate buffer, pH 4, 0 M NaCl; 0.1 M sodium citrate buffer, pH 4, 1 M NaCl; 0.1 M sodium citrate buffer, pH 6, 0 M NaCl; 0.1 M sodium citrate buffer, pH 6, 1 M NaCl; 0.1 M sodium citrate buffer, pH 5, 0.5 M NaCl; 0.1 M sodium citrate buffer, pH 5, 0 M NaCl; 0.1 M sodium citrate buffer, pH 5, 1 M NaCl; 0.1 M sodium citrate buffer, pH 4, 0.5 M NaCl; and 0.1 M sodium citrate buffer, pH 6, 0.5 M NaCl. Each point was tested in duplicate.

On-column validation was performed with HyperCel STAR AX resin (1 mL) packed on a Tricorn 5/50 column (GE Healthcare) following the manufacturer's instructions. Equilibration was carried out with 50 mM Tris-HCl buffer at pH 7.5. Five hundred microliters of heated protein extract were loaded. Elution was carried out with 0.1 M sodium citrate buffer, pH 4, with 0.5 M NaCl (53 mS/cm). A flow rate of 0.5 mL/min was applied.

Second Purification Step. Preparative HiLoad 16/60 Superdex 75 prep grade (GE Healthcare) was used for the second purification step. Isocratic elution was performed with 50 mM sodium phosphate buffer at pH 7.2 with 0.15 M NaCl at 1 mL/min.

Production and Purification of Polyclonal Antibodies against Hsp12. Rabbit polyclonal antibodies were obtained from Covalab (Villeurbanne, France) with the purified Hsp12 as immunogen. Three immunizations were carried out on New Zealand white female rabbits (days: 0, 14, and 28). The serum was then collected at day 53. An affinity chromatography column was prepared by immobilization of Hsp12 on agarose beads. The serum was then loaded on the column (2 mL). After 1 h of incubation at 37 °C, the

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column was washed with phosphate buffered saline buffer (PBS1X, 20 mL). Elution was carried out with 0.9 mL of 0.1 M glycine at pH 2. The sample was then buffered with Tris 1 M at pH 8 (0.2 mL). The resulting antibodies were then tested by Western blotting and ELISA.

SDS-PAGE. SDS-PAGE (AnykD Mini-PROTEAN TGX Precast Protein Gels, 10 well, 50 μL, Bio-Rad) was carried out in a Mini-Protean II apparatus (Bio-Rad) following the standard procedures recommended by the manufacturer. Samples were prepared with sample buffer (2× LaemmLi Sample Buffer, Bio-Rad) and 20 μL was loaded. Prestained protein molecular weight markers (Bio-Rad Precision Plus Protein Standard, Bio-Rad) were used. Gels were stained with Coomassie stain (QC Colloidal Coomassie Stain, Bio-Rad).

CE-SDS. Microfluidic chip CE-SDS was performed for protein quantification on the LabChip GXII instrument (PerkinElmer). Samples are electrokinetically loaded (2 μL) on a chip and injected into a separation channel which is filled with gel and dye. The chip used was HT Protein Express LabChip (PerkinElmer). Chip and sample preparation was performed following the manufacturer's instructions.

Western Blot. Retrospectively, after SDS-PAGE, proteins were electrotransferred to Immobilon-P PVDF membrane (Trans-Blot, Bio-Rad) using a semidry electrophoretic transfer membrane system (TransBlot Turbo Blotting system, Bio-Rad). Blots were blocked in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 3% skim dry milk and then incubated with primary rabbit anti-Hsp12 antibodies (1:40000). Membranes were washed in TBST and incubated with horseradish peroxidase-linked antirabbit IgG antibodies (1:40000) (Goat anti-Rabbit IgG Fc Secondary Antibody HRP, Novex). After several washes in TBST and then in TBS (without Tween 20), Hsp12 protein was detected by a chemiluminescence ECL detection system (Clarity Western ECL Substrat, Bio-Rad) and the signal was collected with a ChemiDoc XRS+ Imager (Bio-Rad). Precision Plus Protein WesternC standards and Precision Protein StrepTactin-HRP Conjugate (Bio-Rad) were used for molecular weight sizing after blot revelation. Controls were performed without the primary rabbit anti-Hsp12 antibody and with this antibody as sample (see Figure 2 of the Supporting Information).

ELISA. Fifty microliters of samples were deposited in each well of a 96-well plate (Nunc). The samples were diluted in coating buffer (10 mM PBS, pH 7.3). Then the plate was incubated overnight at 4 °C. Next, three washes were performed with 100 μL of washing buffer (100 mM PBS, pH 7.3, 0.05% of Tween 20). Free sites were blocked with 50 μL of blocking buffer (10 mM PBS, pH 7.3, 0.5% of skim dry milk) for 1 h at room temperature. After a washing step, the plate was incubated for 2 h at 37 °C in blocking buffer with the anti-Hsp12 diluted antibody (1:10000). After another washing step, the plate was incubated at room temperature for 1 h on a rotary shaker at 180 rpm in blocking buffer with the diluted secondary antibody (1:40000) (Goat anti-Rabbit IgG Fc Secondary Antibody HRP, Novex). After a last washing step, the revelation was done using 50 μL of Single-Component TMB Peroxidase EIA Substrate Kit (Bio-Rad) in each well. The reaction was stopped by the addition of 50 μL of sulfuric acid. The absorbance was measured at 450 nm.

BCA Protein Assay. Total protein in each sample was quantitated with the Micro BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Production of Recombinant Hsp12 in *E. coli*. A vector (pET-*hsp12*) was constructed to express the HSP12 protein in *E. coli*. After transformation of T7 Express *E. coli* strains, cultures were performed. Preliminary experiments performed in 100 mL of shake flasks showed that the expression level of Hsp12 was higher 6 h after induction than 22 h after induction, as revealed by SDS-PAGE analysis (data not shown). Consequently, this condition was applied for Hsp12 production scale-up in a 2-L bioreactor. Thus, 6 h after induction, cells were harvested and centrifuged. Twenty-five milligrams of fresh cells were obtained and thus lysed. After centrifugation, the soluble fraction of extracted proteins was heated at 80 °C. Aliquots of soluble proteins before and after IPTG induction and after heating were analyzed by SDS-PAGE (Figure 1). A 15

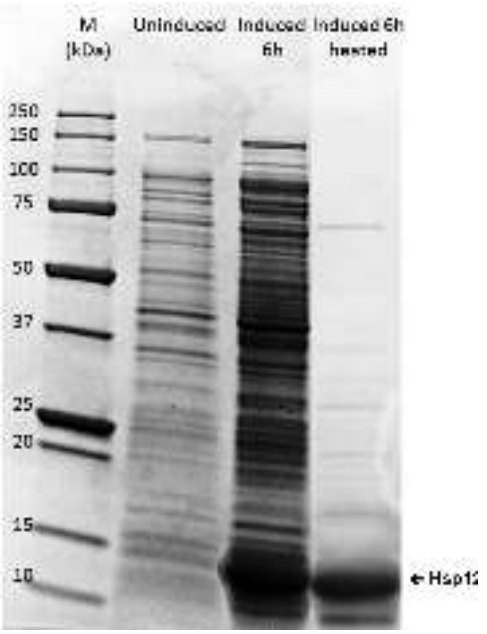

Figure 1. SDS-PAGE analysis of Hsp12 expression in *E. coli* cultured in 2-L bioreactor. Soluble proteins were extracted from bacteria before IPTG induction (uninduced), 6 h after IPTG induction (induced 6 h), and 6 h after IPTG induction and 80 °C heating (induced 6 h heated).

kDa band, consistent with the theoretical molecular mass of Hsp12, that is, 12 kDa, was present in the induced cells and absent from the uninduced cells. This protein was also present after heating at 80 °C, thus suggesting it was Hsp12. Mass spectrometry analysis confirmed the identity of the protein (Table 1 of the Supporting Information). Whereas Hsp12 remained soluble at high temperature, most of the proteins aggregated and could be eliminated by centrifugation. Consequently, Hsp12 was produced successfully and heating allowed the elimination of numerous contaminant proteins. A purification process had to be developed to remove the remaining contaminants.

Purification. *Capture Step: First Screening.* The pH and conductivity values of the heated protein fraction were 7.5 and 4.3 mS/cm, respectively. First, three mixed mode resins, an anion-exchange resin, and a salt-tolerant anion exchange resin were tested for Hsp12 binding without any conditioning. An anion-exchange resin was tested as the conductivity is not so high. A salt-tolerant anion-exchange resin was also tested as the binding could be improved at this conductivity. Mixed-mode resins were chosen because these multimodal ligands can bind proteins at low-to-moderate ionic strength thanks to ionic and/or hydrophobic interactions. This could provide better selectivity than "traditional" ligands. Flowthrough fractions were analyzed by CE-SDS (data not shown). The protein Hsp12 was detected only in the flowthrough of the MEP HyperCel resin. The binding occurred on the other resins. Owing to its pK_a, MEP HyperCel was uncharged under the binding conditions. Binding could thus occur only with hydrophobic interactions. Ionic interactions were thus involved

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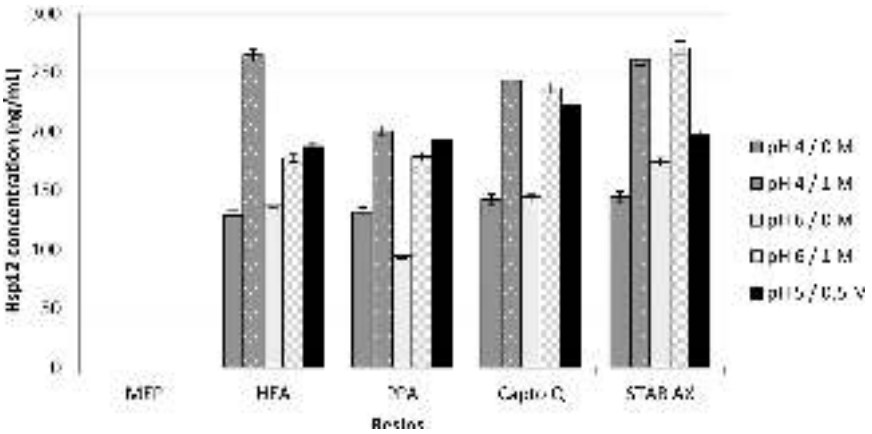

Figure 2. Hsp12 concentration (ng/μL) in elution fractions of various resins with five elution conditions (pH and NaCl concentration). Results represent the mean value from three independent experiments ± standard deviation.

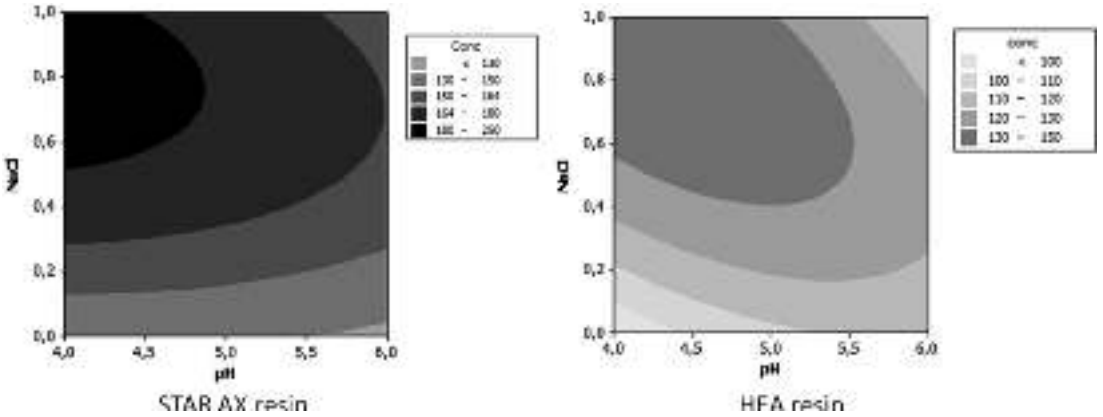

Figure 3. Response surfaces obtained for HEA HyperCel and HyperCel STAR AX resins. NaCl concentration is between 0 and 1 M. The pH is between 4 and 6. Hsp12 concentration is expressed in ng/μL.

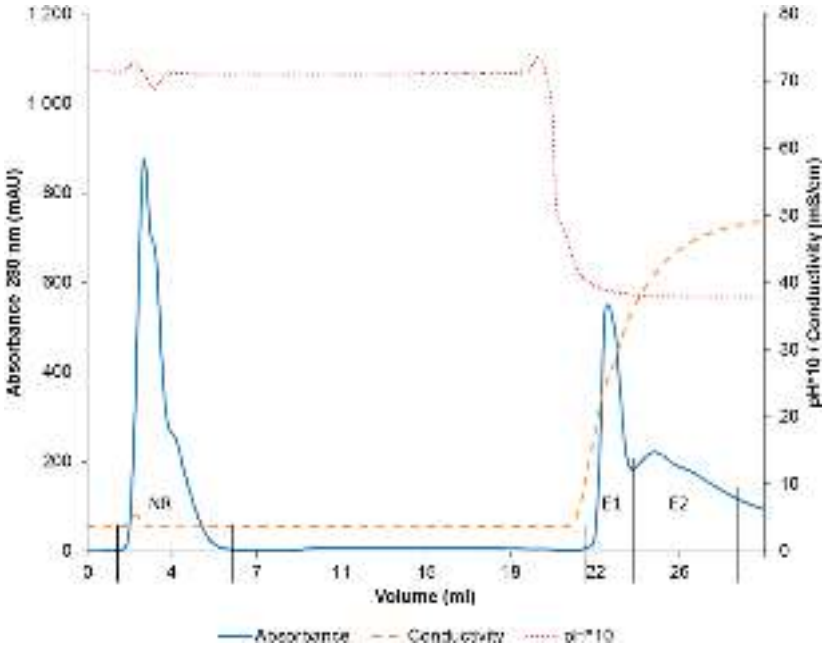

Figure 4. Chromatographic profile obtained with HyperCel STAR AX. 0.5 mL of heated protein extract was applied at a flow rate of 0.5 mL/min. 50 mM Tris-HCl buffer at pH 7.5 was used as equilibration buffer. 0.1 M sodium citrate buffer at pH 4 with 0.5 M NaCl was used as elution buffer. Fractions NR (nonretained proteins) and E1 and E2 (elution peaks) were collected and analyzed.

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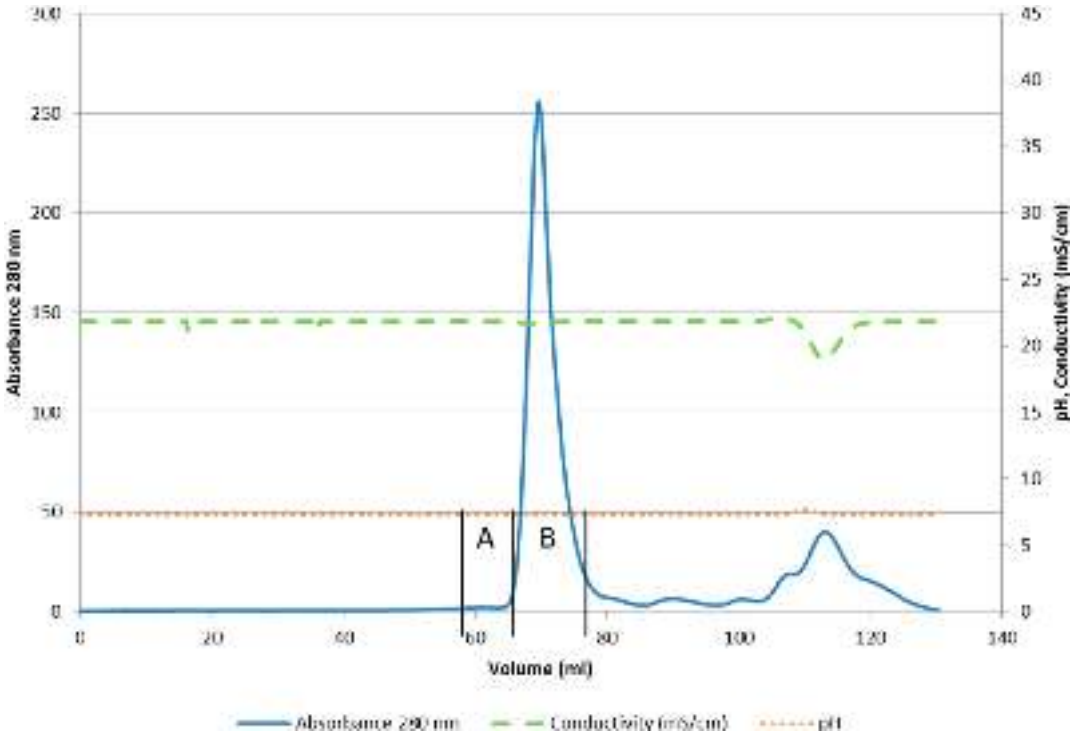

Figure 5. Chromatographic profile obtained with Superdex 75 prep grade. Two milliliters of STAR AX elution fraction E1 was applied at a flow rate of 1 mL/min. Isocratic buffer: 0.5 M sodium phosphate buffer at pH 7.2 with 0.15 M NaCl. Peaks A and B were collected and analyzed.

in Hsp12 binding. This was confirmed by Hsp12 binding on Capto Q and HyperCel STAR AX, which provided only ionic interactions.

For elution, various pH and salt conditions were tested in a full factorial design that included a center point. Hsp12 concentration was determined in elution fractions with CE-SDS (Figure 2). No protein was detected in the elution fractions with MEP HyperCel resin as it was recovered in the flowthrough fraction. For the other resins, the protein was more or less eluted depending on the conditions. The best elution condition was a combination of low pH and high salt concentration. At pH 4 and 6, more protein was recovered when 1 M NaCl was added, suggesting that ionic interactions were predominant in the mixed-mode resins.

HEA HyperCel and HyperCel STAR AX resins were chosen for the second screening because they showed the highest Hsp12 concentration eluted and had two distinct mechanisms, mixed mode for HEA HyperCel and salt-tolerant anion exchange for HyperCel STAR AX.

Capture Step: Second Screening. The second screening was performed on HEA HyperCel and HyperCel STAR AX resins with various elution conditions using a central composite design. Hsp12 concentration was determined by CE-SDS analysis. After modeling, response surfaces curves were obtained (Figure 3).

The same behavior was observed for both resins. The Hsp12 concentration was higher at low pH and high salt concentration. The highest concentration was obtained with a pH between 4 and 5 and a salt concentration between 0.5 and 1 M. The combination of a low pH (under the Hsp12 pI value of 5.2) and a moderate salt concentration (0.5 M) seemed to be ideal to elute the protein. As observed for the mixed-mode resin, this suggested that an ionic interaction was predominant for Hsp12 binding. The highest Hsp12 concentration was obtained after elution with HyperCel STAR AX resin, which was thus used for the following experiment. Thanks to the modeling, 0.1 M sodium citrate buffer at pH 4 with 0.5 M NaCl was chosen as elution buffer for the on-column experiment.

Capture Step on HyperCel STAR AX. The first purification step was then performed on a column packed with HyperCel STAR AX resin. The elution buffer chosen previously was used (Figure 4). The chromatographic profiles showed a flow-through fraction containing unbound proteins and two peaks containing eluted proteins. For elution, pH was decreased to 4 and conductivity increased to 50 mS/cm. A first peak appeared simultaneously (E1) and then a second wider peak (E2). SDS-PAGE analysis showed that there were several bands in unbound fractions corresponding to contaminating proteins (Figure 6A right). Retrospectively, Western blot analysis confirmed the presence of Hsp12 in the first elution fraction (E1). Therefore, the HyperCel STAR AX resin led to the adsorption and the elution of Hsp12 protein under the conditions developed during screening. Furthermore, a second inconspicuous band was identified in this fraction. This band was around 25 kDa and could correspond to a dimer of Hsp12 so a second purification step was added to eliminate it.

Polishing Step on Superdex 75 Prep Grade. The Hsp12 dimer has characteristics that are potentially close to those of the monomer but they vary in size. Thus, size exclusion chromatography was used to remove it. The polishing step was performed with Superdex 75 prep grade column (Figure 5). One main peak was observed at 69.64 mL (peak B), corresponding to the Hsp12 monomer. SDS PAGE and Western blot analysis showed the presence of Hsp12 with a band between 10 and 15 kDa. The other peak observed at 59.39 mL (peak A) was around 30 kDa which could correspond to the dimer. This was confirmed retrospectively by Western blot analysis (Figure 6B). Consequently, this purification step

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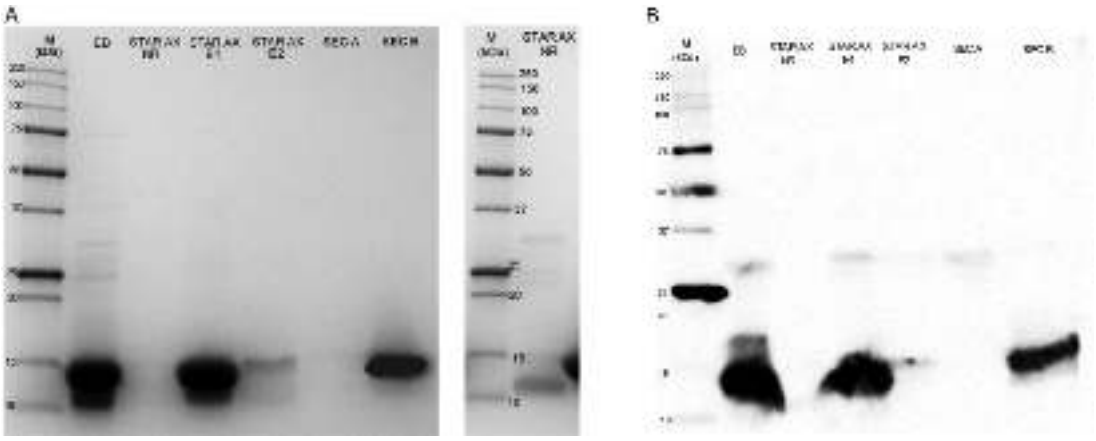

Figure 6. (A) SDS-PAGE analysis of heated protein extract (EB), not retained (NR) and eluted proteins (E) for the first step (STAR AX), peak A and peak B for the second step (SEC). M: molecular mass marker (Bio-Rad Precision Plus Protein Standard, Bio-Rad). (B) Western blot analysis of heated protein extract (EB), eluted proteins (E) for the first step (STAR AX), peak A and peak B for the second step (SEC).

Table 2. Purification Table: Hsp12 Concentration Obtained by ELISA and Total Protein Concentration by BCA Assay

	volume (mL)	[total protein] (mg/mL)	[Hsp12] (mg/mL)	total protein (mg)	Hsp12 (mg)	yield (%)	purity (%)
crude protein extract	1.9	13.3	4.8	25.3	9.1	100%	36%
heated protein extract	1.7	9.4	4.9	16.0	8.8	97%	55%
step 1 STAR AX	2	5.4	4.4	10.8	8.7	95%	81%
step 2 SEC	10	0.9	0.9	9.0	8.7	95%	97%

allowed the elimination of the Hsp12 dimer. The fraction corresponding to peak B was used as immunogen to produce an antibody against Hsp12 protein.

Overview of Two-Step Purification Process. SDS-PAGE was performed to analyze the protein content during the purification process (Figure 6A). Western blotting was performed retrospectively when the antibodies were obtained (Figure 6B). On SDS-PAGE, the band corresponding to Hsp12 was predominant in each purified fraction. Some contaminants were detected in the heated protein extract (EB) but they were eliminated in the flowthrough fraction after the first purification step (STAR AX NR). Owing to the dilution of the second purification step, the intensity of the Hsp12 band was lower in the elution fraction (SEC B) than in the previous step. On Western blotting, the band corresponding to Hsp12 was detected as well as another band with a weak intensity. It corresponded to a dimer of Hsp12 which was eliminated thanks to the second purification step. The total protein and Hsp12 were quantified on elution fractions to evaluate the purification process (Table 2). With a two-step process, a good purity was obtained (97%) with a high yield (95%). A very small part of the protein (5%) was lost during heating and during the first purification step in the second elution peak (E2). Purification was performed on part of the culture (0.4 g of fresh cells). The production of Hsp12 was about 300 mg/L of culture.

Hsp12 Quantitation on Different Strains by ELISA. Western blot was performed on four strains of *Saccharomyces cerevisiae* to see whether the antibodies could recognize Hsp12 from yeast (Figure 7). Four yeast strains were studied: three enological or brewery strains (Fx10, VL3, and A24) and a fourth strain (YMB36) whose *hsp12* gene had been deleted. As expected, Hsp12 was detected only with the first three strains. Antibodies were therefore able to detect Hsp12 from yeast. Thereafter, an ELISA was developed (Figure 8). These results showed that the ELISA offered good linearity between 0.1 and 0.6 absorbance unit and good sensitivity up to 21 ng/mL. This

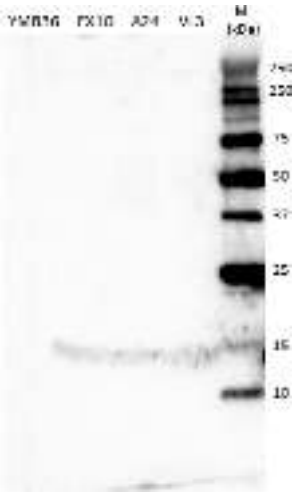

Figure 7. Western blot analysis performed on strains of *Saccharomyces cerevisiae*: three enological or brewery strains (Fx10, VL3, and A24) and a fourth strain (YMB36), whose *hsp12* gene had been deleted.

ELISA was used to quantify the protein in these four yeast strains (Figure 9). The Fx10 strain possessed the smallest amount of hsp12 with 2.2 mg/L, VL3 was intermediate with 3.8 mg/L, and A24 had the largest amount with 6.6 mg/L. Hsp12 was not detected in the strain YMB36, which confirms the deletion of the gene. These results are consistent with our previous study on *hsp12* gene expression, that is, no expression for YMB36 strain and in the ascending order of expression, strains Fx10, VL3, and A24.[17,18]

The production of recombinant Hsp12 in its native form (untagged) was achieved successfully in *E. coli*. We describe a simple fast two-step process for untagged Hsp12 purification using a salt-tolerant anion-exchange resin followed by size exclusion chromatography. The first step was developed by high-throughput screening on microplates using a DoE. This

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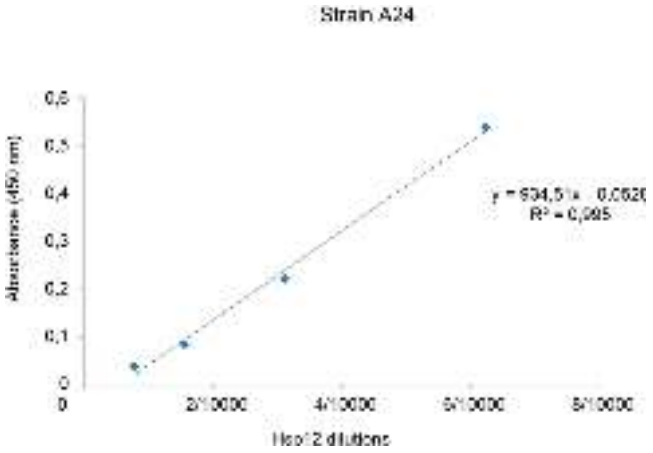

Figure 8. Correlation between Hsp12 dilution and absorbance obtained by ELISA. The fraction analyzed corresponds to the heated protein extract from the A24 strain.

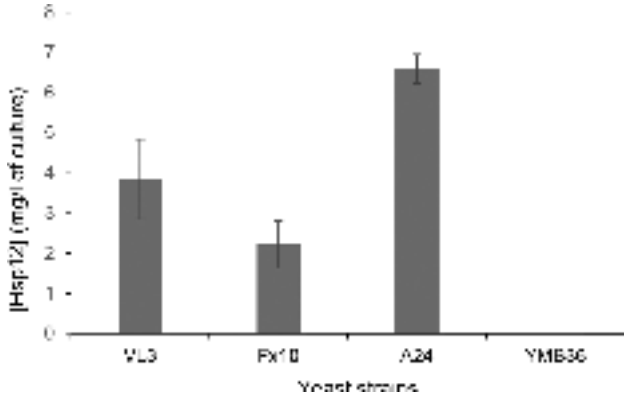

Figure 9. Hsp12 concentration in four yeast strains obtained by ELISA. The concentration is expressed in milligrams of Hsp12 produced per liter of culture. Results represent the mean value from two independent experiments ± standard deviation. The fractions analyzed correspond to the heated protein extract from the four yeast strains.

allowed us to select the best resin for Hsp12 binding without conditioning, and to select the best elution conditions. Then the second step made it possible to remove the Hsp12 dimer. The proposed sequence is efficient in terms of yield (95%) and purity (97%). Thanks to this purified Hsp12, specific antibodies were obtained which were then used to develop an ELISA assay able to quantify Hsp12 in various yeast strains. This demonstrates that the recombinant Hsp12 produced in *E. coli* allowed us to obtain antibodies that recognize the natural Hsp12 in *Saccharomyces cerevisiae*. These antibodies could be an important tool for studying natural Hsp12.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02477.

Figure of plasmid map of pET-*hsp12*; figure of Western blot control; table of Hsp12 identification by mass spectrometry (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Foury, F. Human genetic diseases: a cross-talk between man and yeast. *Gene* **1997**, *195*, 1−10.

(2) Nisamedtinov, I.; Lindsey, G. G.; Karreman, R.; Orumets, K.; Koplimaa, M.; Kevvai, K.; Paalme, T. The response of the yeast *Saccharomyces cerevisiae* to sudden vs. gradual changes in environmental stress monitored by expression of the stress response protein Hsp12p. *FEMS Yeast Res.* **2008**, *8* (6), 829−38.

(3) Ivorra, C.; Perez-Ortin, J. E.; del Olmo, M. An Inverse Correlation between Stress Resistance and Stuck Fermentations in Wine Yeasts. A Molecular Study. *Biotechnol. Bioeng.* **1999**, *64* (6), 698−708.

(4) Dunker, A. K.; Oldfield, C. J.; Meng, J.; Romero, P.; Yang, J. Y.; Chen, J. W.; Vacic, V.; Obradovic, Z.; Uversky, V. N. The unfoldomics decade: an update on intrinsically disordered proteins. *BMC Genomics* **2008**, *9*, S1.

(5) Daughdrill, G. W.; Pielak, G. J.; Uversky, V. N.; Cortese, M. S.; Dunker, K. A. Natively disordered proteins. In *Protein Folding Handbook*; Buchner, J., Kiefhaber, T. K., Eds.; Wiley-VCH Verlag GmbH: Weinheim, Germany, 2005; Vol. 3, pp 275−357.

(6) Mtwisha, L.; Brandt, W.; McCready, S.; Lindsey, G. G. HSP12 is a LEA-like protein in *Saccharomyces cerevisiae*. *Plant Mol. Biol.* **1998**, *37*, 513−52.

(7) Praekelt, U. M.; Meacock, P. A. HSP12, a new small heat shock gene of *Saccharomyces cerevisiae*: analysis of structure, regulation and function. *Mol. Gen. Genet.* **1990**, *223*, 97−106.

(8) Welker, S.; Rudolph, B.; Frenzel, E.; Hagn, F.; Liebisch, G.; Schmitz, G.; Scheuring, J.; Kerth, A.; Blume, A.; Weinkauf, S.; Haslbeck, M.; Kessler, H.; Buchner, J. Hsp12 is an intrinsically unstructured stress protein that folds upon membrane association and modulates membrane function. *Mol. Cell* **2010**, *39*, 507−520.

(9) Siderius, M.; Rots, E.; Mager, W. H. High-osmolarity signalling in *Saccharomyces cerevisiae* is modulated in a carbon-source-dependent fashion. *Microbiology* **1997**, *143*, 3241−3250.

(10) Pacheco, A.; Pereira, C.; Almeida, M. J.; Sousa, M. J. Small heat-shock protein Hsp12 contributes to yeast tolerance to freezing stress. *Microbiology* **2009**, *155*, 2021−2028.

(11) Sales, K.; Brandt, W.; Rumbak, E.; Lindsey, G. The LEA-like protein HSP12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. *Biochim. Biophys. Acta, Biomembr.* **2000**, *1463*, 267−278.

(12) Singarapu, K. K.; Tonelli, M.; Chow, D. C.; Frederick, R. O.; Westler, W. M.; Markley, J. L. Structural characterization of Hsp12, the heat shock protein from *Saccharomyces cerevisiae*, in aqueous solution where it is intrinsically disordered and in detergent micelles where it is locally α-helical. *J. Biol. Chem.* **2011**, *286*, 43447−43453.

DOI: 10.1021/acs.jafc.7b02477
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

(13) Motshwene, P.; Karreman, R.; Kgari, G.; Brandt, W.; Lindsey, G. LEA (late embryonic abundant)-like protein Hsp12p (heat-shock protein 12) is present in the cell wall and enhances the barotolerance of the yeast *Saccharomyces cerevisiae*. *Biochem. J.* **2004**, *377*, 769−774.
(14) Karreman, R.; Brandt, W.; Lindsey, G. The yeast *Saccharomyces cerevisiae* stress response protein Hsp12p decreases the gel strength of agarose used as a model system for the glucan layer of the cell wall. *Carbohydr. Polym.* **2005**, *60*, 193−198.
(15) Karreman, R.; Dague, E.; Gaboriaud, F.; Quiles, F.; Duval, J.; Lindsey, G. The stress response protein Hsp12p increases the flexibility of the yeast *Saccharomyces cerevisiae* cell wall. *Biochim. Biophys. Acta, Proteins Proteomics* **2007**, *1774*, 131−137.
(16) Rossignol, T.; Dulau, L.; Julien, A.; Blondin, B. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* **2003**, *20*, 1369−1385.
(17) Marchal, A.; Marullo, P.; Moine, V.; Dubourdieu, D. Influence of yeast macromolecules on sweetness in dry wines: role of the *Saccharomyces cerevisiae* protein Hsp12. *J. Agric. Food Chem.* **2011**, *59* (5), 2004−2010.
(18) Marchal, A.; Marullo, P.; Durand, C.; Moine, V.; Dubourdieu, D. Fermentative conditions modulating sweetness in dry wines: genetics and environmental factors influencing the expression level of the *Saccharomyces cerevisiae* HSP12 gene. *J. Agric. Food Chem.* **2015**, *63* (1), 304−311.
(19) Blein-Nicolas, M.; Albertin, W.; Valot, B.; Marullo, P.; Sicard, D.; Giraud, C.; Huet, S.; Bourgais, A.; Dillmann, C.; de Vienne, D.; Zivy, M. Yeast proteome variations reveal different adaptive responses to grape must fermentation. *Mol. Biol. Evol.* **2013**, *30* (6), 1368−83.

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