Production of encapsulated creatinase using yeast spores

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ABSTRACT
Yeast spores can be used as a carrier to produce enzyme capsules. In the present study, this technique was applied to a diagnostic enzyme named creatinase. We found that a secretory form of Pseudomonas putida creatinase could be entrapped in the spore wall, and such spores were used as creatinase capsules. The activity of the encapsulated creatinase was largely improved by mild spore wall defective mutations, such as DIT1 or OSW2 deletions. The advantages of this method include the following: encapsulated and freeze-dried creatinase is produced without preparing the purified enzyme, and it exhibits resistance to environmental stresses, such as high temperature and SDS treatments. Thus, yeast spores could be applied to establish quick and easy clinical diagnostic methods.

KEYWORDS
Creatinase; enzyme capsule; S. cerevisiae; spore; spore wall

Introduction
Creatine amidohydrolase or creatinase can mediate the hydrolysis of creatine to produce sarcosine and urea. This enzyme, together with 2 other enzymes, namely, creatininase and sarcosine oxidase, is used as a clinical enzyme to measure creatinine levels in specimens, such as blood samples. Studies have been conducted to improve their properties. For example, several chemical immobilization procedures of these enzymes have been studied to make them stable and reusable. These experiments have been performed mainly to develop and improve amperometric creatinine biosensors.

Previously, we reported that spores of the budding yeast Saccharomyces cerevisiae are used to produce enzyme capsules. This method is based on unique properties of the yeast spore wall. In the spore wall, the outermost layer or the dityrosine layer is mainly composed of bisformyl-dityrosine, and the second outermost layer or the chitosan layer is composed of chitosan. Physiologically, these structures are required to protect spores so that yeast spores are resistant to various environmental stresses. The presence of these structures provides spores with a distinct property; when the secretory forms of soluble enzymes are expressed in sporulating cells, they are entrapped in the periplasmic space of the spore wall. The dityrosine layer is critical for the entrapment of secretory proteins in the spore wall probably because its pore size is sufficiently small to prevent protein diffusion. Although the dityrosine layer functions as a diffusion barrier for proteins, relatively small molecules, such as sugars, can pass through it. Thus, spores can be applied to biological enzyme capsules.

The dityrosine and chitosan layers can be eliminated through specific mutations; DIT1 is required for the synthesis of bisformyl dityrosine, and dit1Δ mutation causes a loss of the dityrosine layer. CHS3 encodes a chitin synthase, and its deletion results in a loss of both the dityrosine and chitosan layers. Although the deletion of the dityrosine layer causes a leak of soluble proteins held in the spore wall, the chitosan layer can also retain the secretory proteins to some extent. Previous studies have shown that dit1Δ spores, not chs3Δ spores, can hold some soluble enzymes in the spore wall, although the capacity of dit1Δ spores to hold enzymes is much lower than that of wild-type spores. Interestingly, enzymes held in dit1Δ spores generally exhibit higher activity than that in wild-type spores, which is probably because the dityrosine layer is a barrier for substrates to access...
the enzymes. Previous reports have shown that osw2Δ mutation can also improve the activity of enzymes held in the spore wall.7,15 Although the exact function of Osw2 is not yet known, osw2Δ mutation causes a minor spore wall defect probably in the dityrosine layer.13,19 Therefore, the integrity of the spore wall can affect the activity of encapsulated enzymes. A remarkable difference between dit1Δ and osw2Δ mutants is that the latter has the dityrosine layer so it can stably entrap proteins in the spore wall.13

Creatine is a small molecule, so we expect it to pass through the spore wall. Thus, in the present study, we attempted to apply yeast spore-based encapsulation technique to creatinase. Such an enzyme may be used for clinical examinations.

Results and discussion

Yeast spores can hold Pseudomonas putida creatinase

To produce yeast spores encapsulating creatinase, the P. putida creatinase gene was cloned into a yeast expression vector named pRS426TEF.20 A yeast secretory signal sequence from SPR1 was fused at the 5′ end to localize the enzyme to the spore wall, and 3 tandem repeats of the hemagglutinin (HA) epitope gene was added at the 3′ end for its detection. The creatinase expression plasmid was then transformed into a wild-type S. cerevisiae strain AN120, and western blot analysis was performed to verify that the HA fusion (creatinase–HA) was expressed in spores (Fig. 1). Yeast spores are covered with the ascal membrane and ascawall. In this study, the term “ascus” was used for samples of intact ascospores, and “spore” was used for samples in which spores were released from the asci by breaking down the asc cell wall and membrane. Given that TEF2 promoter is a constitutive promoter, creatinase–HA was detected in lysates of ascus, spore, and vegetative cell samples (Fig. 1A). Molecular weight of creatinase–HA estimated from SDS-PAGE was 52 kDa (data not shown). The molecular weight was slightly larger than the predicted one (49 kDa) because creatinase–HA expressed in yeast was modified by asparagine-linked glycosylation; after PNGas-F treatment, it was decreased to 50 kDa (Fig. 1B). The amount of creatinase–HA detected in ascus and spore lysates was much more than that in vegetative cell lysate, probably because creatinase–HA fusion was retained in the spore wall, whereas it was secreted out of the cell wall in the vegetative cells. We found that creatinase activity in the spores expressing creatinase–HA was much higher than that in vegetative cells (Fig. 1C). Such a high creatinase activity was not detected in spores harboring the empty vector (Fig. 1C). Although a comparable amount of creatinase–HA was detected in ascus and spore lysates, the activity detected in the asci was similar to that in vegetative cells (Fig. 1C). This result was consistent with the notion that, in intact asci, its substrate (creatine) cannot access the enzyme because of the existing ascal membrane. These results demonstrated that yeast spores could be used as microcapsules to hold creatinase. Notably, in this experiment, the activity was measured in wet cell samples.

Activity of encapsulated creatinase is improved by dit1Δ and osw2Δ mutations

We examined whether the creatinase activity is improved in spore wall deficient mutants, namely, osw2Δ and dit1Δ. Western blot analysis showed that both mutant spores could retain creatinase–HA (Fig. 2A). Although the amount of creatinase–HA held in dit1Δ spores was much less than that in wild-type spores, the activity of the dit1Δ spores was almost 2-fold higher than that of wild-type spores (Fig. 2B). osw2Δ mutation could also improve the activity of creatinase in spores; the amount of creatinase–HA detected in the mutant spores was slightly lower than that in wild-type spores (Fig. 2B). Under this condition, a statistically significant difference was not observed between the activities of dit1Δ and osw2Δ spores (Fig. 2B). The levels of creatinase–HA protein and its activity detected in chs3Δ spores were significantly lower than those in the other spores (Fig. 2A and B), thereby suggesting that the chitosan layer could retain creatinase even in the absence of the dityrosine layer. These experiments were performed with purified wet spores, and quantity was adjusted by weight. Subsequently, we tested whether the encapsulated creatinase can survive through freeze-drying. As shown in Fig. 2C, creatinase activity was detected in the freeze-dried spores, and dit1Δ spores exhibited the highest activity among spores.

osw2Δ mutant spores can retain creatinase as wild-type spores

dit1Δ spores carrying creatinase–HA exhibited higher activity than the other spores, probably because its
substrate (creatine) could easily access the enzyme without being obstructed by the dityrosine layer. However, the lack of the dityrosine layer makes the spore wall leaky, so dit1Δ spores are less capable of holding creatinase–HA (Fig. 3A). In accordance with this notion, creatinase in dit1Δ spores was susceptible to high salt and detergent wash: osw2Δ spores carrying creatinase–HA exhibited higher activity than dit1Δ after 4 washes with high salt and detergent solution (Fig. 3B). Therefore, under some conditions, osw2Δ spores were the superior option to produce the encapsulated creatinase. Note that, dit1Δ spores also exhibited higher activity than wild-type spores even after 4 high salt and detergent washes (Fig. 3B).

**Creatinase is stabilized through encapsulation in yeast spores**

*K*<sub>m</sub> of the encapsulated creatinase measured at 50°C are shown in Table 1. Free creatinase–HA, which was expressed and secreted from vegetative yeast cells, was used as a control. Creatinase–HA in osw2Δ and dit1Δ
spores exhibited lower $K_m$ compared with those in wild-type spores.

Subsequently, we measured the activities of creatinase expressed in spores in various temperatures and pH. As shown in Fig. 4A, the activities of creatinase in wild-type and osw2Δ spores were more stable than those of free creatinase at higher temperatures. Such thermostability was not observed for creatinase in dit1Δ spores. For pH sensitivity, the activities of creatinase expressed in spores were generally stable than those of free creatinase (Fig. 4B). Notably, creatinase in dit1Δ spores and the free enzyme exhibited high activity at pH 9, whereas the high activity of creatinase in wild-type and osw2Δ spores was detected at pH 10 (Fig. 4B). Additionally, creatinase activity in osw2Δ spores was more stable than that in other spores at pH 11 and 12 (statistical differences were observed between osw2Δ and wild-type or dit1Δ spores at these pH values).

To further verify that creatinase encapsulated in spores is protected from environmental stresses, we examined whether this encapsulated creatinase is resistant to proteinase K. As shown in Fig. 5A, creatinases in wild-type and osw2Δ, as well as dit1Δ spores, were tolerant to proteinase K treatment. Moreover, the encapsulated creatinase was resistant to SDS treatment. As shown in Fig. 5B, activity of the creatinases in wild-type and osw2Δ spores was retained by more
than 80% after 5% SDS treatment, whereas that of the free enzyme was decreased to about 30%. Creatinase in dit1Δ spores was less tolerant to SDS compared with that in wild-type and osw2Δ spores, even though dit1Δ spores were more resistant to SDS than the free enzyme (Fig. 5B). Thus, the encapsulated creatinase, particularly in osw2Δ spores, is useful under adverse conditions because it exhibits resistance to various environmental attacks.

Overall, we demonstrated that encapsulated creatinase could be produced using yeast spores. Our results showed that dit1Δ spores could produce creatinase capsules with the highest activity. However, the disadvantages of using dit1Δ spores are that encapsulated creatinase is leaky and sensitive to environmental stresses. Thus, under certain conditions, osw2Δ mutant may be the most desirable option to produce the enzyme capsule. Compared to chemical procedures for enzyme immobilization, the advantage of this method is that immobilized and stress-resistant creatinase is produced without preparing the purified enzyme.

Table 1. K_m values of creatinase–HA in wild-type, osw2Δ, dit1Δ, or chs3 Δ spores, and the free creatinase–HA at 50°C are shown.

|          | Wild-type | osw2Δ | dit1Δ | Free creatinase |
|----------|-----------|-------|-------|----------------|
| K_m (mM) | 10.9      | 7.8   | 7.0   | 7.0            |

Figure 4. Assessment of the activities of the encapsulated creatinases at various temperatures (A) and pH (B). To assess temperature and pH sensitivities for indicated spores harboring creatinase–HA or free creatinase–HA, their activities were assayed at various temperatures (30°C to 90°C) and pH (5 to 12). As the free enzyme, creatinase–HA secreted from vegetative yeast cells was used. The maximum activity obtained for each assay was determined as 1.0 and relative activities are shown. For the pH sensitivity, potassium phosphate buffer (pH 5 to 10) and sodium carbonate buffer (pH 10 to 12) were used. Data presented are the mean ± SE of 3 independent samples. Statistic analysis was performed between free creatinase and osw2Δ spores, and asterisks were deposited when statistically significant differences were found. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 5. Assessment of sensitivities of the encapsulated creatinase toward environmental stresses. 4 mg of indicated purified freeze-dried spores containing creatinase–HA were treated with proteinase K for 12 h (A) or 5% SDS for 10 min (B) at 30°C, and then creatinase activities were assayed. For each sample, the activities obtained before the treatments were determined as 1.0 and relative activities are shown. Culture media containing the soluble creatinase–HA was used as a control (free). Specific activities before the treatments were indicated under the sample names. Data presented are the mean ± SE of 3 independent samples. Results of the t-test among osw2Δ, dit1Δ spores and the free enzyme were shown. *P < 0.05; ***P < 0.001; NS, not significant.
distinct properties, the spore-based encapsulated creatinase may be used to produce a quick and easy method for diagnosing creatinine levels in salivary samples. Immobilized creatinase, creatininase and sarcosine oxidase have been applied to develop amperometric creatinine biosensors. Since previous studies have shown that microorganisms, including S. cerevisiae are available to develop amperometric biosensors, it would be intriguing to investigate if creatinase encapsulating spores are applied to this purpose.

Materials and methods

Yeast strains and growth media

Yeast strains and oligonucleotide primers used in this study are listed in Table 2 and 3, respectively. All strains used in this study are in the fast-sporulating SK-1 strain background. YPAD media (10 g/L yeast extract, 20 g/L peptone, 30 mg/L adenine, 20 g/L glucose or galactose) was used to grow yeast without plasmids. yeast cells harboring plasmids were grown in synthetic defined (SD) media (6.7 g/L yeast nitrogen base, 2 g/L dropout mix without appropriate selectable supplements, 20 g/L glucose or galactose). Twenty g/L agar was added for making plates. The drop out mix was prepared by mixing the following supplements: 0.5 g adenine, 2 g l-alanine, 2 g l-arginine, 2 g l-asparagine, 2 g l-aspartic acid, 2 g l-cysteine, 2 g l-glutamine, 2 g l-glutamic acid, 2 g glycine, 2 g l-histidine, 2 g myo-inositol, 2 g l-isoleucine, 10 g l-leucine, 2 g l-lysine, 2 g l-methionine, 0.2 g para-aminobenzoic acid, 2 g l-phenylalanine, 2 g l-proline, 2 g l-serine, 2 g l-threonine, 2 g l-tyrosine, 2 g uracil, 2 g l-valine.

Table 2. Yeast strains used in this study.

| Name | Sequence | Reference |
|------|----------|-----------|
| AN120 (Wild-type) | MATa/MATα ARGO4/arg4-Npl his3ΔSK/his3ΔSK hcl:LYS2/hcl:LYS2 leu2/leu2 lys2/lys2 RME1/ rme1:LEU2 trp1::hisG ura3/ura3 | 25 |
| AN262 (chs3Δ) | MATa/MATα ARGO4/arg4-Npl his3ΔSK/his3ΔSK hcl:LYS2/hcl:LYS2 leu2/leu2 lys2/lys2 RME1/ rme1:LEU2 trp1::hisG ura3/ura3 chs3Δ::his5"/chs3Δ::his5" | 19 |
| HW3 (dit1Δ) | MATa/MATα ARGO4/arg4-Npl his3ΔSK/his3ΔSK hcl:LYS2/hcl:LYS2 leu2/leu2 lys2/lys2 RME1/ rme1:LEU2 trp1::hisG ura3/ura3 dit1Δ::his5"/dit1Δ::his5" | 26 |
| HW83 (osw2Δ) | MATa/MATα ARGO4/arg4-Npl his3ΔSK/his3ΔSK hcl:LYS2/hcl:LYS2 leu2/leu2 lys2/lys2 RME1/ rme1:LEU2 trp1::hisG ura3/ura3 osw2Δ::his5"/osw2Δ::his5" | 7 |

Table 3. Oligo nucleotide primers used in this study.

| Name | Sequence |
|------|----------|
| HXO588 | GTGTAAGCTTCAAATGCCCCAAGCCTTGA |
| HXO590 | GTGGCTCTCGAGTTGGCAATAGTTGGCTGCT |
| HXO609 | ATTAACCTCGAGTACCATACGATGTTCCCTGA |
| HXO610 | TAAAAGGTACCCCCGCTAGGTTGCTCAAT |

Plasmids

pRS426TEF-ss-CI-3HA was used to express a yeast signal peptide and 3 tandem repeat of the hemagglutinin (3 × HA) epitope fusion to creatinase in yeast cells. This plasmid was constructed as follows. The creatinase without the stop codon was amplified by PCR using HXO588 and HXO590 as primers and P. putida KT2440 genomic DNA as a template. The resulting PCR fragment was digested with HindIII and Xhol, and cloned into pRS426TEF. The resulting plasmid was digested with SpeI and HindIII, and a DNA fragment containing the SPR1 signal sequence (first 24 amino acids) digested out of pRS424TEF-spRFP7 with the same enzymes was ligated. Finally, using pFA6a-3HAHis3MX623 as a template and HXO609 and HXO610 as primers, a DNA fragment containing the 3 × HA gene and the ADH1 terminator was amplified. The DNA fragment was cloned into the creatinase containing plasmid using XhoI and KpnI sites. All DNA sequences of genes amplified by PCR were verified by sequencing.

Creatinase activity assay

P. putida creatinase secreted into culture media was used as a control of the free enzyme. To prepare this, wild-type yeast cells harboring pRS426TEF-ss-CI-3HA were cultured in SD media and the culture was centrifuged at 6,200 × g for 1 minute to remove cells. Then the supernatant was filtrated through Amicon-Ultra (molecular weight cut off 10 kDa, Millipore, Shanghai, China) to concentrate and exchange solvent to enzyme dilution buffer (10 mM potassium phosphate pH 8.0, 0.16 mL/L 2-mercaptoethanol): the enzyme was concentrated about 5 times and the media was diluted with the buffer about 30 times. Yeast cells harboring creatinase or the free creatinase were suspended in the enzyme dilution buffer to measure their activities. Creatinase activity was assayed by following the protocol accompanied with Kikkoman creatinase (Kikkoman, Noda Japan). 0.1 mL of 0.3 M potassium phosphate buffer (pH 7.7) and 0.8 mL of 0.1 M creatine solution (creatine was dissolved in water) were mixed and
incubated at 37°C for 5 min. Then, 0.1 mL of creatinase containing solution was added and incubated for 10 min at 37°C. To detect urea calorimetrically, the reaction mixture was mixed with 2 mL of p-dimethylnobenzaldehyde solution (2 g of p-dimethylnobenzaldehyde, 100 mL of ethanol, 15 mL of concentrated HCl, 115 mL of water), allowed to stand for 30 min at 25°C, and OD435 was measured by spectrophotometer (Ultraspec 2100 pro, Amersham biosciences, USA). The Michaelis-Menten constant (Km) was determined using 5–40 mM of creatine. The reactions were stopped after 10 min and the urea was determined as described above. Km was obtained using the Lineweaver-Burk equation. To assess pH stability, 10 mM potassium phosphate buffer (pH 5 to 10) or 10 mM sodium carbonate buffer (pH 10 to 12) were used instead of the potassium phosphate buffer (pH 7.7). One unit of activity was defined as the amount of urea (µmol) released per minute at 37°C.

Yeast culture, sporulation and spore purification

Yeast spores and vegetative cells were prepared as follows. First, yeast cells derived from a single transformant colony were grown overnight in 5 mL of SD liquid media with appropriate supplemental amino acids. For sporulation, 1 mL of the culture was then transferred into 30 mL of YPAcetate (1% w/v yeast extract, 2% w/v peptone, 2% w/v potassium acetate) and grown for 24 h. The cells were harvested by centrifugation at 3000 × g for 1 minute, washed with 30 mL of H2O, resuspended in 30 mL of 2% w/v potassium acetate medium, and cultured for 24 h. Sporulation efficiency was determined by counting spores under the light microscope. Under our experimental condition, sporulation efficiency was greater than 90%. Vegetative cells were similarly cultured in YPAcetate media: 1 mL of SD culture was shifted to 30 mL of YPAcetate and grown for 24 h and harvested.

To release spores from asci, the ascal wall was first digested by β-glucanase (lyticase, Sigma-Aldrich, Shanghai, China). For this, spores prepared as above were resuspended in 1 mL of spheroplast buffer (50 mM potassium phosphate buffer pH7.5, 1.4 M sorbitol, 40 mM β-mercaptoethanol) and mixed with 50 µL of β-glucanase stock solution (200 U of β-glucanase was dissolved in 500 µL of 50% v/v glycerol). After 3 h of incubation at 37°C, spores were washed twice with spheroplast buffer. Then, they were resuspended in spheroplast buffer and sonicated to disrupt the ascal membrane.

All spores as prepared above were purified by percoll gradient centrifugation based on a previously described method.24 Spores were washed 3 times with 5 mL of 0.5% v/v Triton-X. After the washes, the resulting pellet was resuspended in 1 mL of 0.5% v/v Triton-X and layered on top of Percoll (Sigma-Aldrich, Shanghai, China) gradients (50-80% v/v Percoll, 10% v/v 2.5 M sucrose and 0.5% v/v Triton-X). After centrifugation at 15,000 × g at 4°C for 1 h, the top of 3 layers which consist of vegetative cells and debris were removed. The remaining layer containing spores was washed with 10 mL of 0.6 M NaCl once and then 10 mL of 0.5% v/v Triton-X twice. Purified spores were freeze-dried as follows. First, spores were frozen in a -20°C freezer for more than 2 h. They were then freeze-dried by EYELA FD-1000 freeze-dryer (Tokyo Rikakikai, Tokyo, Japan) at -50°C for 72 h under the pressure of 25 Pa.

Spore protection assay

For proteinase K treatment, in 100 µL of proteinase buffer (50 mM Tris-HCl pH 7.5, 10 mM CaCl2), proteinase K (Sigma-Aldrich, Shanghai, China) was mixed in the solution at final concentration of 30 U/mL. Then, 4 mg of freeze-dried spores or 20 µL of free creatinase were suspended in the solution, and incubated at 37°C for 12 h. Spores were then washed with 0.6 M NaCl solution and 0.5% v/v TritonX-100 and creatinase activity was assayed as described above.

SDS treatment was performed as follows. 4 mg of freeze-dried spores or 20 µL of the free enzyme was mixed with 100 µL of 5% w/v SDS and incubated at 30°C for 10 minutes. Spores were then washed with 0.6 M NaCl solution and 0.5% v/v TritonX-100 and creatinase activity was assayed.

Western blotting

Western blotting was performed as described before7 with some modifications. Spores and vegetative cells were suspended in 500 µL of 8 M urea and lysed by sonication for 1 hour on ice. The cell lysates were then centrifuged at 4,000 × g at 4°C for 5 min and 50 µg of the supernatants were subjected to SDS-PAGE (5% stacking gel and 10% separating gel). Protein concentration was determined by BCA protein assay kit (Beyotime, Jiangsu, China). A mouse anti-HA antibody (Transgen Biotech, Beijing, China) was used as a primary antibody at 1:6000 dilutions. Goat anti mouse
IgG-HRP (Transgen Biotech, Beijing, China) were used as secondary antibodies at 1:6000 dilution. TBST (10 mM Tris-HCl pH 8.0, 160 mM NaCl, 0.05% v/v Tween 20) supplemented with 5% w/v non-fat dry milk was used for blocking and antibody incubations. Signals were visualized by Clarity Western ECL Substrate (Biorad, Shanghai, China) and images were obtained by using ImageQuant LAS4000 (GE Healthcare Bio-Science, Uppsala, Sweden).

For PNGase F treatment, 10 μg of spore lysate was incubated with 0.5 U of PNGase F (New England Biolabs, Beijing, China) following the protocol accompanied with the enzyme at 37°C for 12 h. Total volume of the reaction mixture was 20 μl and 8 μl was subjected to protein gel blot analysis.

Statistics

Data presented are the mean ± SE of 3 independent samples obtained from different cultures. Statistical significance was determined with Student’s t-test (2-tail, heteroscedastic) using Microsoft Excel software. Differences between the analyzed samples were considered significant at P < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Abbreviations

HA hemagglutinin
P. putida Pseudomonas putida
S. cerevisiae Saccharomyces cerevisiae
SD synthetic defined

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