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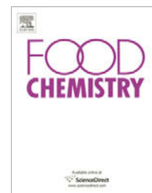

Site-directed mutagenesis improves the thermostability of a recombinant *Picrophilus torridus* trehalose synthase and efficiency for the production of trehalose from sweet potato starch

Hsin-Hung Chou [d,1], Shu-Wei Chang [b,f,*,1], Guan-Chiun Lee [c], Yi-Shan Chen [d], Tzunuan Yeh [a], Casimir C. Akoh [e], Jei-Fu Shaw [a,d,*]

[a] *Department of Food Science and Biotechnology, National Chung Hsing University, Taichung 402, Taiwan*
[b] *Department of Bioindustry Technology, Dayeh University, Dacun, Changhua 515, Taiwan*
[c] *Department of Life Science, National Taiwan Normal University, Taipei 116, Taiwan*
[d] *Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 11529, Taiwan*
[e] *Department of Food Science and Technology, University of Georgia, Athens, Georgia 30602, United States*
[f] *Department of Medicinal Botanicals and Health Care, Dayeh University, Dacun, Changhua 515, Taiwan*

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ABSTRACT

A new recombinant *Picrophilus torridus* TSase (PTTS) has the catalytic ability for the conversion of maltose to trehalose by intramolecular transglucosylation. For industrial applications, the high thermostability of the enzyme would be the most important property for reducing the microbial contamination and lower the production cost. Therefore, in this study, we substituted ten selected proline residues of PTTS which differ from two well-known thermostable TSases. Interestingly, we found that the N503 mutant type, namely N503P-PTTS, showed about 39% higher relative activity than that of the wild type at 65 °C for 120 min. The trehalose yield of mutant N503P-PTTS was 1.3-fold higher than that of the wild type with sweet potato starch as substrate at 50 °C for 4 h. This suggests that the proline site substitution technology used in this study is useful for altering enzyme properties and catalytic efficiency for possible industrial applications.

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1. Introduction

Trehalose is a disaccharide that consists of two subunits of glucose bound by an α, α-1,1-, α, β-1,1,- and β, β-1,1-linkage. Only α, α-1,1- trehalose is widespread in nature and biologically active (Elbein, Pan, Pastuszak, & Carroll, 2003). Trehalose has been characterised from a wide variety of organisms including bacteria, yeast, fungi, insects, brine shrimps, invertebrates, nematodes, and lower and higher plants (Arisan-Atac, Wolschek, & Kubicek, 1996; Behm, 1997; Kong et al., 2001; Lillie & Pringle, 1980; Marechal & Belocopitow, 1972; Thevelein, 1984; Elbein et al., 2003; Foster, Jenkinson, & Talbot, 2003; Schiraldi, Di Lernia, & De Rosa, 2002; Wingler, 2002). It is a stable, colourless, and odour-free disaccharide.

Due to its inertness property and the ability to stabilise biomolecules, trehalose can be applied in food, cosmetic, and pharmaceutical industries, ranging from serving as a sweetener to a biomaterial stabiliser. For example, in the food industry, trehalose can be used in food preparation subjected to drying processes or concentration, and in cosmetics as a moisturiser or liposome stabiliser. In medicine, trehalose can help preserve enzyme and protect mammalian cells from damage during freeze-drying; it can be used as an additive to stabilise vaccines during storage at room temperature and protect organs for transplantation. It can also be used as a cryoprotectant in the cryopreservation of cells, sperms, tissues, or other materials. The wide range of applications of this sugar has increased the interest of many research groups to develop novel and economically feasible production systems (Schiraldi et al., 2002).

There are three different pathways for the biosynthesis of trehalose in various organisms (Elbein et al., 2003). A convenient pathway which can produce trehalose (glucosyl-α-1,1-glucopyranoside) directly from maltose (glucosyl-α-1,4-glucopyranoside) by trehalose synthase (TSase) has been reported in a few unusual bacteria (Elbein et al., 2003; Nishimoto et al., 1996). Of the 6

* Corresponding authors. Address: Department of Food Science and Biotechnology, National Chung Hsing University, Taichung 402, Taiwan. Tel.: +886 4 2284 0201; fax: +886 4 2285 3813 (J.-F. Shaw), tel.: +886 4 851 1888x2294; fax: +886 4 851 1320 (S.-W. Chang).
E-mail addresses: swchang@mail.dyu.edu.tw (S.-W. Chang), presid@dragon-nchu.edu.tw (J.-F. Shaw).
[1] These authors contributed equally to this work.

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known TSases, the enzyme isolated from *Thermus aquaticus* is highly thermostable but the enzyme yield of the original organism is low and not economical for trehalose manufacture. Recently, we have cloned and purified a novel recombinant *Picrophilus torridus* TSase (PTTS) which showed a higher preference for maltose (even in high maltose concentration) and resulted in 60% yield of trehalose at 45 °C. A higher temperature tolerance of the enzyme would benefit its catalytic efficiency and reduce the probability of contamination during the trehalose production process as well as lower the cost for further industrial applications (Chen, Lee, & Shaw, 2006; Elbein et al., 2003). The utilisation of recombinant enzyme technology and protein engineering to improve the catalytic efficiency or thermostability will be the most efficient strategy to develop a thermostable PTTS for industrial applications.

Based on the molecular basis of protein thermal stability, several factors were identified and demonstrated to have effect on increasing protein thermal stability. Among them, the substitution of a proline residue is one of the most significant factors for protein thermostabilisation by stabilising the β-turn structure and decreasing the hydrophilicity around the site to form a rigid protein structure against high temperature denaturation (Chakravarty & Varadarajan, 2000; Hardy et al., 1993; Masui, Fujiwara, & Imanaka, 1994). Since there is still limited study in regards to the proline substitution effect on trehalose synthases, the substitution of proline residues at carefully selected positions in the protein might be a potential feasible strategy to improve the thermostability or catalytic efficiency of PTTS for specific industrial applications. Starch is a major constituent of the human diet. It can be enzymatically processed to obtain other products such as starch hydrolysates by amylases and further converted into trehalose through combination of amylases and trehalose synthase (Fig. 1). This will save energy and be cost effective (Akoh, Chang, Lee, & Shaw, 2008).

In this study, proline substitution by site-directed mutagenesis was utilised as the first strategy to examine the effect of proline substitution on improving thermal stability of trehalose synthase. The catalytic efficiency difference between wild type and mutant type PTTS for trehalose production was compared. The results show that proline substitution will provide a thermostable and effective PPTS in converting low-value agricultural produce such as sweet potato starch to high-value commercial trehalose with great advantages of environmental friendliness and consumer acceptance as a natural process.

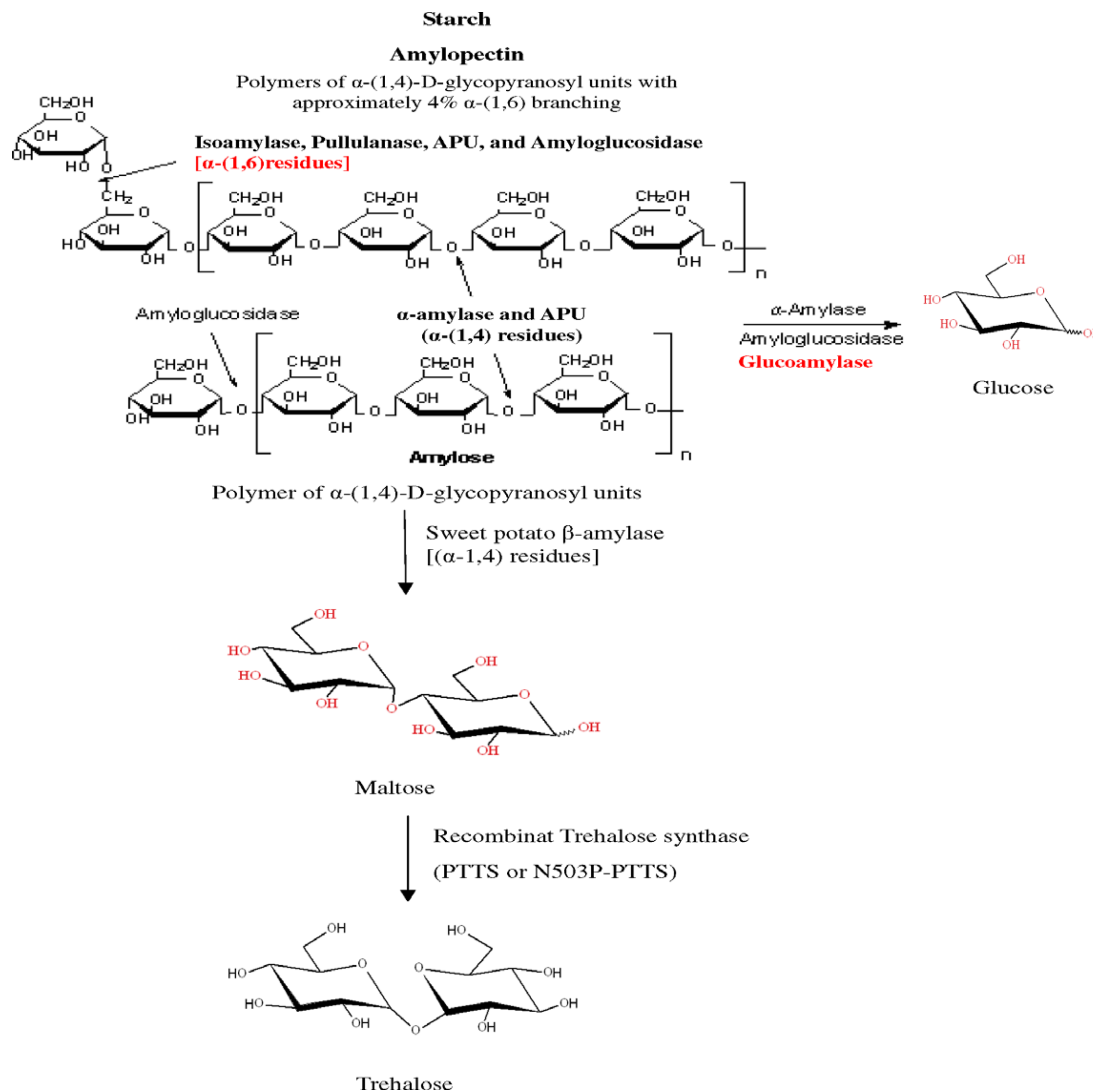

Fig. 1. Schematics of the process for converting raw material sweet potato to industrial and functional food products.

2. Material and methods

2.1. Materials

The resin and columns used for protein purification were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Acetonitrile was from TEDIA Company Inc. (USA). Pfu DNA polymerase and restriction enzymes were obtained from Promega (Madison, WI, USA). All other chemicals and reagents were of analytical grade.

2.2. Amino acid sequence alignment

For the substitution of proline strategy, the amino acid sequence of PTTS was aligned and contrasted with two well-characterised thermostable TSase from *T. aquaticus* and *Thermus caldophilus*. It is interesting to find that ten amino acid components of PTTS differ from the proline component of both *T. aquaticus* and *T. caldophilus* TSases. All ten potential substitute amino sites of PTTS were selected for replacement with proline by site-directed mutagenesis to improve the thermostability of recombinant PTTS. The ten potential mutation sites were named D41P, I121P, A255P, K332P, I385P, D411P, S439P, E469P, N503P, and R523P.

2.3. Construction of PTTS mutants by site-directed mutagenesis

A previously constructed plasmid, the pET23-a(+) vector (Novagen, Madison, WI) with the inserted recombinant PTTS-His gene, was used as a template to create PTTS mutants. All mutant gene fragments were obtained by second PCR and then ligated into pET-23-a(+) vector and transformed into *E. coli*. The purification procedure of mutant proteins and wild type protein were carried out as previously described (Chen et al., 2006).

2.4. β-Amylase production from sweet potato

Fresh sweet potato was pressed and filtered by cheese cloth. The sweet potato juice (50%; w/v) was heated at 60 °C for 10 min to inactivate the α-glucosidase which possesses maltose hydrolysis ability. The solution was then centrifuged at 8000 rpm for 20 min at 4 °C and filtered to obtain the supernatant which contained abundant β-amylase. After cooling down with flowing water, the enzyme solution was concentrated by ultrafiltration using a 100,000 molecular weight cutoff membrane (Amicron Ultra, Millipore, Bedford, MA), and pooled as a 10× crude β-amylase preparation (3412 U/ml) for the production of maltose from starch (Fig. 2).

The activity of sweet potato β-amylase was performed by adding 10 μl purified enzyme into 100 μl substrate solution containing 6% (w/v) sweet potato starch and incubating in a 45 °C water bath for 20 min. The reaction was terminated by heating the mixture in boiling water for 15 min. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 μmol of maltose per min. All experiments were carried out in triplicate.

2.5. Sweet potato starch preparation

The fresh sweet potato was pressed and the juice was filtrated to obtain the starch. After washing with 5-fold volume of water and storage for 4 days, the residue was further dried and milled into starch-rich powder, called sweet potato starch (Fig. 2).

2.6. Protein quantification

Protein concentration was measured by the method of Bradford using a protein assay kit purchased from Bio-Rad Lab (Hercules, CA) with bovine serum albumin (BSA) as standard. A standard curve was prepared according to the absorbance and BSA concentrations and the concentration of unknown protein was calculated using this curve.

2.7. Mutation selection

All recombinant proteins produced from ten mutant lines, namely D41P, I121P, A255P, K332P, I385P, D411P, S439P, E469P, N503P, and R523P were screened and characterised in the present work. In the preliminary test, each sample was pretreated at 20 °C in a 50 mM phosphate buffer (pH 6.0) with 150 mM maltose as a substrate for 24 h to select the active mutant line and the corresponding trehalose yield of mutant PTTS depicted in Table 1.

2.8. Purification of the recombinant PTTS

The purification of recombinant PTTS and N503P PTTS is summarised in Table 2. The yield of purified PTTS with His-Tag was about 5.3-fold and its specific activity was 0.25 ± 0.15 units/mg. The yield of purified N503P PTTS with His-Tag was about 2-fold

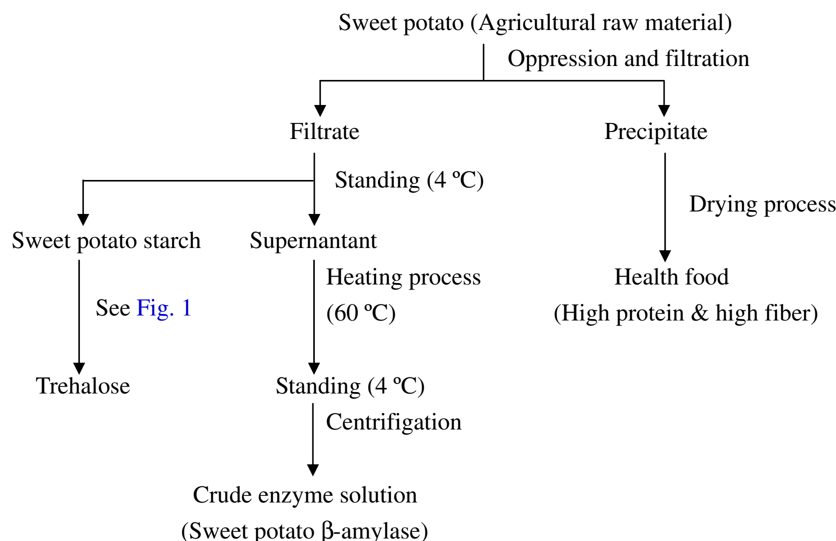

Fig. 2. Schematics of the actual chemistry steps for trehalose production from sweet potato starch.

Table 1
The thermostability selection for various recombinant mutant PTTS.

PTTS type	Yield (%)[a]	Residual yield (%)[b]
Wild type PTTS	68.7 ± 1.6 (100%)	26.7 ± 0.6 (100%)
D41P	68.4 ± 0.8 (99.6%)	26.5 ± 1.3 (99.3%)
I121P	14.6 ± 2.1 (21.3%)	–
A255P	1.4 ± 3.5 (2.0%)	–
K332P	66.4 ± 1.4 (96.7%)	24.7 ± 2.5 (92.5%)
I385P	3.5 ± 2.0 (5.1%)	–
D411P	2.2 ± 1.9 (3.2%)	–
S439P	14.3 ± 2.2 (20.8%)	–
E469P	31.6 ± 3.7 (50.0%)	9.3 ± 1.9 (34.8%)
N503P	71.0 ± 2.5 (103%)	32.6 ± 2.2 (122%)
R523P	62.4 ± 0.3 (90.8%)	20.1 ± 1.7 (75.3%)

[a] The trehalose yield (%) of mutant PTTS after being treated at 20 °C in a 50 mM phosphate buffer (pH 6.0) using 150 mM maltose as a substrate for 24 h.
[b] The residual activities of wild type and mutant PTTSs which were pre-incubated at 65 °C for 3 h and reacted at 45 °C for 30 min.

and its specific activity was 0.4 ± 0.06 units/mg. SDS–PAGE analysis of the two purified enzymes showed a single protein band around 65 kDa in size (data not shown).

2.9. Thermostability

The thermostability test was investigated by pre-incubating the enzymes (wild type PTTS or mutants) at 65 °C for various time periods to determine their residual activity (Table 1). The residual activity was examined under standard assay condition. The standard reaction was performed by adding 1.5 μl purified enzyme into 50 μl reaction solution containing 50 mM sodium phosphate (pH 6.0) and 150 mM maltose and incubating in a 45 °C water bath for 25 min. The reaction was terminated by heating the mixture in boiling water for 15 min. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 μmol of trehalose per min. All experiments were carried out in triplicate (Chen et al., 2006).

All TSase activity, trehalose yield from maltose, was analysed by high performance liquid chromatography (HPLC) under standard assay condition as described above. All experiments were carried out in triplicate.

2.10. Trehalose production from agricultural produce

Twenty microlitres of α-amylase (786 U/mg) was added to the substrate solution containing 6% (w/v) starch and reacted at 90 °C for 1 h followed by autoclave (121 °C for 20 min) to inactivate the enzyme. The starch hydrolysate (1 ml) was then added to 3.4 μl of self-made β-amylase (3412 U/ml) and 5.6 mg of recombinant wild type PTTS (18.1 U/ml) or mutant N503P-PTTS (16.9 U/ml) and reacted at 50 °C for 4 h. All enzymes were inactivated at 100 °C for 15 min. All experiments were carried out in triplicate. The yield of trehalose production was defined as: weight (mg) of trehalose production/min/g protein.

2.11. Carbohydrates quantification

The amount of trehalose, glucose, and maltose were measured using a HPLC (SFD 2100) system equipped with an RI detector (Schambeck SFD, RI 2000). A carbohydrate analysis column (HYPERSIL-100 Amino, Thermo Hypersil-Keystone) was equilibrated with 75% acetonitrile, 24% Milli-Q water, and 1% formic acid at 0.9 ml/min flow rate. The RI detector and oven temperature were set at 40 °C. 5 μl of sample was injected for each analysis. The retention times of glucose, maltose, and trehalose were 8.0, 11.2, and 12.5 min, respectively.

3. Results

3.1. Mutation selection

Ten mutations including D41P, I121P, A255P, K332P, I385P, D411P, S439P, E469P, N503P, and R523P were selected in the present work by treating all expressed proteins at 20 °C in a 50 mM phosphate buffer (pH 6.0) with 150 mM maltose as a substrate for 24 h. The results are shown in Table 1. The mutants D41P, K332P, E469P, N503P, and R523P gave higher trehalose conversion yields (31.6–71%) compared to the others. Following the thermal stability test, D41P, K332P, E469P, N503P, R523P were selected and pre-incubated at 65 °C for three hours to analyse their residual activity under a standard assay condition in comparison to the wild type PTTS. The mutant N503P-PTTS showed the best trehalose conversion yield (71 ± 2.5%) and therefore was chosen for further biochemical characterisation and applications.

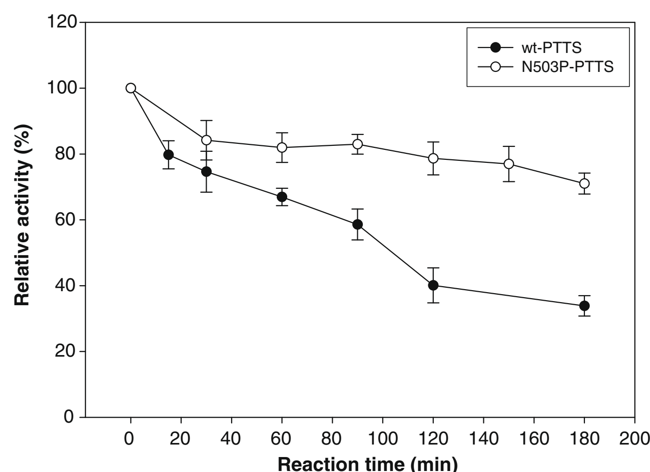

Fig. 3. Effect of temperature on the stabilities of mutant N503P-PTTS (▲) and wild type (■) PTTS. All enzymes were pre-incubated at 65 °C and pH 6.0 for various reaction times (min), and then reacted at 45 °C for 30 min by using 150 mM of maltose solution as substrate to estimate their residual activities. All experiments were carried out in triplicate.

Table 2
Summary of the purification procedures of the recombinant PTTS and mutant N503P PTTS from *E.coli*.

	Total protein (mg)	Total activity (units)	Specific activity[a] (units/mg)	Purification factor (fold)	Yield (%)
Purification step of PTTS					
Cell-free extract	50.8 ± 0.1	0.2 ± 0.007	0.05 ± 0.001	1.0	100
HiTrap chelating HP	7.6 ± 0.06	0.2 ± 0.01	0.25 ± 0.15	5.3 ± 0.3	0.8 ± 0.05
Purification step of mutant N503P PTTS					
Cell-free extract	50.6 ± 0.7	1.0 ± 0.2	0.2 ± 0.03	1.0	100
HiTrap chelating HP	7.4 ± 0.6	0.30 ± 0.04	0.4 ± 0.06	2.0 ± 0.3	0.3 ± 0.04

[a] Activity was measured under pH 6.0 and 45 °C for 25 min with 1 M maltose as substrate.

Table 3
Estimation of quantity of trehalose production from sweet potato starch.

Enzyme	mg/min/g protein[a]	
	Trehalose	Glucose
PTTS	321.1 ± 26.8	146.2 ± 1.6
Mutant N503P-PTTS	435.9 ± 16.6	145.1 ± 7.4

[a] The production yield (mg/min/g protein) of trehalose or glucose catalysed by self-made β-amylase and recombinant PTTS (or Mutant N503P-PTTS) by using 1 ml of starch hydrolysate as substrate at 50 °C for 4 h.

3.2. Thermostability

For the thermostability analysis, the N503P and wild type PTTS were pre-incubated at 65 °C at various time intervals and their residual activity was measured at 45 °C for 30 min. Under a high temperature environment (65 °C), it is interesting to note that the mutant N503P-PTTS retained 79% of relative activity which was higher than those obtained by the wild type (40%) for 120 min (Fig. 3).

3.3. Estimation of trehalose production from sweet potato starch

As an example of agricultural application, the recombinant PTTS and mutant N503P-PTTS were used to convert starch-rich agricultural produce sweet potato, into trehalose which is one of the high value and useful products in the food, cosmetic, and pharmaceutical industries. The result of our preliminary test showed that the quantity of trehalose produced by N503P-PTTS was 1.3-fold higher than those obtained with wild type PTTS at 50 °C for 4 h (Table 3).

4. Discussion

Thermostability is an important property required of industrial enzymes. Understanding the structural basis for this attribute will underpin the future biotechnological exploitation of these biocatalysts (Xie et al., 2006). Many different methods can be used to improve the thermostability of proteins. For example, one can add the critical region of a thermophilic protein to the target protein (Wang, Tsai, Chen, Lee, & Shaw, 2007) or use the error-prone PCR and DNA shuffling to create the mutant protein (Khan et al., 2005; Minagawa et al., 2007; Stephens, Rumbold, Permaul, Prior, & Singh, 2007; Yun, Matsuda, & Kawamukai, 2006). The method of proline substitution is one of the direct ways that can be used to improve the high temperature tolerance of a protein.

The relationship between enzyme stability determination and structural elements of a given enzyme must be related to its specific amino acid sequence (Sakaguchi et al., 2007). Proline plays a critical role in protein stabilisation at high temperatures because its pyrrolidine ring adopts fewer conformations than any other amino acid (Goihberg et al., 2007), and contributes to protect the protein from thermal unfolding by limiting the flexibility of the structure (Sakaguchi et al., 2007). Proline can also make the loop more rigid by restricting the flexibility of the backbone of the peptide bond, and contribute to the maintenance of the proper enzyme structure at elevated temperature.

Many researches support this theory and employ this strategy in different proteins (Frare et al., 2005; Goihberg et al., 2007; Sakaguchi et al., 2007) including our present work. For further thermal stability improvement of recombinant PTTS, two well-characterised TSase amino acid sequences from *T. aquaticus* and *T. caldophilus* were aligned and compared with PTTS (data not shown). After 10 active mutation clones have been selected from the library, the mutant N503P-PTTS that possessed the greatest catalytic efficiency at high reaction temperature was one of the successful cases for thermal stability improvement of our enzyme. Such stabilisation evidence can also be demonstrated by the results of the thermostability study. Fig. 3 showed that the mutant N503P-PTTS had 39% higher relative activity than the wild type at 65 °C for 120 min.

To assess the potential application of our recombinant enzymes, we compared the wild type PTTS and mutant N503P-PTTS as biocatalysts to produce high-value trehalose from agricultural produce. The mutant N503P-PTTS resulted in 30% higher trehalose yield than the wild type PTTS in converting sweet potato starch into trehalose (Table 3). This suggests that the thermostability improvement of enzyme by proline substitution was successful in improving the industrial production of trehalose from agricultural produce.

In conclusion, many organisms cannot grow in extreme environments such as high-temperature, alkali or acidic conditions. Using an enzyme that is stable at high reaction temperature can reduce the possibility of microbial contamination, lower viscosity, and improve the solubility of substrates for better access to enzymatic attack. Since many studies have already shown a wide range of trehalose applications, developing methods that enable efficient industrial scale production of trehalose seems to be an important undertaking to make these applications more economically feasible in the future (Akoh et al., 2008; Chen et al., 2006; Wang et al., 2007). The protein engineering of the novel recombinant *P. torridus* trehalose synthase reported here proved to be useful for increasing the yield of trehalose production from starch. The protein crystallisation of recombinant PTTS and mutant N503P PTTS are currently underway to resolve their structure and provide more information for improving the thermostability of PTTS for industrial applications. The thermostable PTTS can be used to produce high-value trehalose and high protein byproducts simultaneously from low-priced raw agricultural produce such as rice or corn (Shaw, 1994; Shaw & Sheu, 1992) in an economical and efficient manner.

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