0273-2289/99/77–79/0857/$12.25

Modification of the Thermoresistance to Spray-Drying of a Cold-Adapted Subtilisin by Genetic Engineering

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Abstract

The thermoresistance of a cold-adapted subtilisin dried by spray-drying was studied. Proteolytic activity of this enzyme was measured before and after spray-drying. Without chemical additives, spray-drying yields ranged from 2–13%. The use of arabic gum and lactose in the composition of the enzyme solutions allowed the strengthening of the enzyme structures and increased water mobility in the product. Increase of water mobility led to a shorter residence time of the product in the spray-drier and a net yield increase was obtained (yield higher than 50%). The effect of two selective mutations on the thermoresistance to spray-drying of the cold-adapted subtilisin was also investigated. Mutation T85D (introduction of an additional link with an ion Ca^{2+} necessary for enzyme activity, by substitution of Asp for Thr 85) had no effect on the thermoresistance of the subtilisin to spray-drying. Mutation H121W (introduction of an additional aromatic link by substitution of Trp for His 121) reduced the drying yield from 66% (not modified subtilisin) to 52%. This higher thermosensitivity could be explained by an increase of the hygroscopic character of the modified subtilisin (mutation H121W).

Index Entries: Subtilisin; mutations; cold-adapted enzyme; thermoresistance; spray-drying.

Introduction

Subtilisins (EC 3.4.21. 14) belong to the family of serine proteases. The most known subtilisins are alkaline endopeptidases excreted by different species of Bacillus: subtilisin BPN' produced by *Bacillus amyloliquefaciens (1)*, subtilisin Carlsberg by *Bacillus licheniformis (2)*, subtilisin E by *Bacillus*

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subtilis and subtilisin amylosacchariticus by *Bacillus amylosacchariticus (3)*. In 1992, proteases represented a market of more than 400 million dollars *(4)*.

In the last few years, an increasing attention has been given to the enzymes produced by cold-adapted microorganisms. Indeed, these enzymes have a higher specific activity at low temperature than those of mesophilic organism *(5)*. They have some molecular adaptations to preserve, in vivo, a suitable metabolic flow; their 3D structure is less rigid and consequently less thermostable than that of the mesophilic organisms. Subtilisins are used mainly in the household detergents industry, where it would be economically interesting to have enzymes able to catalyze proteolytic reactions at low temperature.

Nariox *(6)* isolated a subtilisin from an Antarctic bacterium, Bacillus TA39. The enzyme was purified, characterized, and the gene *(Subt 1)* encoding the cold subtilisin was isolated and its sequence established *(7)*. The cold protease had different molecular adaptations to low temperatures when compared to subtilisins coming from mesophilic or thermophilic microorganisms. As the cold subtilisin primary AA sequence showed a high homology with subtilisin BPN', the authors suggested a 3D model of the cold subtilisin, by comparison with the crystalline structure of subtilisin BPN' *(8)*.

From this 3D model, two selective mutations (T85D and Hl21W) of the cold protease were proposed and carried out with the aim of increasing its thermostability. WT gene Subt 1 and both modified genes Subt 1 (T85D and H121W) were cloned and expressed in a mesophilic strain not producing subtilisin, *B. subtilis* IH6140 *(6)*. The products of the three genes were investigated in this work.

In recombinant cold subtilisin T85D, Thr 85 is replaced by Asp. By comparison with thermitase and thermolysin, two subtilisins of high thermos/ability owing to the fixation of 3 and 4 ions Ca^{2+}, respectively *(9–12)*, and fixation sites of very high affinity for Ca^{2+} ($k_d = 10^{-10}$ *M*), the cold subtilisin has only two fixation sites of lower affinity for Ca^{2+} ($K_d = 10^{-6}$ *M*). Mutation T85D increased affinity for Ca^{2+} ($K_d = 10^{-8}$ *M*) (for calcium affinity assay, cf. refs. *6* and *13*). Recombinant subtilisin T85D proved more thermostable in solution than recombinant WT subtilisin and its catalytic efficiency was also higher *(6)*.

In recombinant cold subtilisin H121W, His 121 is replaced by Trp. This mutation introduces an aromatic interaction between Trp 121 and Phe 50, Tyr 93, and Val 95. Burley and Petsko *(14)* reported increasing the interactions between aromatic residues stabilized, in certain cases, proteins. Mutation H121W proved, however, not to modify the stability of the cold enzyme in solution, but conversely to increase its catalytic efficiency in comparison with WT cold subtilisin *(6)*. In this work, the thermoresistance of the three recombinant cold subtilisins after spray-drying was studied by measuring proteolytic activity before and after spray-drying.

Spray-drying is a drying process that consists of transforming the liquid product to dry from the liquid to the solid state. The liquid product

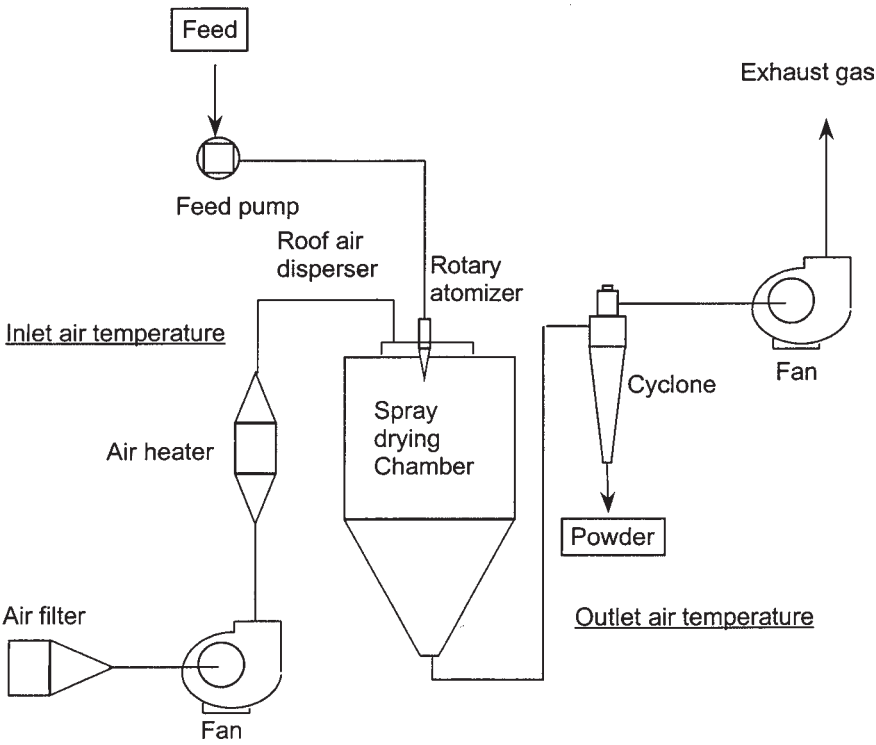

Fig. 1. Schematic representation of the parallel flow spray-drier equipped with a rotary atomizer used in this study.

is pumped (Fig. 1) at the top of the spray-drying chamber and dispersed into fine droplets by a rotary atomizer in a warm air flow, which removes the moisture from the product. Air is separated then from the particles of product in a cyclone. Detailed technical descriptions of spray-drying can be found in various literature sources *(15–19)*.

In this work, different conditions of spray-drying were studied to preserve the activity of the cold subtilisin after drying. Influence of the aforementioned selective mutations on thermoresistance of the enzyme dried by spray-drying are discussed.

Materials and Methods

Organisms and Enzymes

The gene encoding subtilisin S39 *(Subt 1)*, a protease produced by psychrophilic bacterium *Bacillus* TA39 (a strain isolated in the Antarctic) was used in this work. The genetic constructions were carried out from plasmid pUB110 *(20)* and cloned into *Escherichia coli* RRI *(20)*. *E. coli* was grown on nutrient broth containing 1% bactotrypton (Difco, Detroit, MI), 0.2% yeast extract (Difco), 1% NaCl, 0.02% $CaCl_2$, 0.5% casein (Sigma, Bornem, Bel-

gium), and 1.5% bacto-agar (Difco). pH was adjusted to 7.0. The cultures were incubated at 18°C.

The wild and mutagenized genes encoding subtilisin S39 *(Subt 1)* were expressed in *B. subtilis* IH 6140 grown on the nutrient broth previously described, in presence of 0.003% kanamycin (Sigma). *B. subtilis* was grown in nutrient broth containing 1.6% yeast extract (Difco), 1,6% bactotrypton (Difco), 0.5% NaCl, and 0.25% K_2HPO_4. The mutations were obtained by polymerase chain reaction (PCR) according to the method described by Jones et al. *(21)*, with the polymerase produced by *Thermococcus littoralis* (New England Biolabs, Herts, UK). That enzyme was selected for its high fidelity *(22)* and its property to finish elongation of DNA strands by sticky ends to anneal the plasmid by simple ligation.

The oligonucleotide used for mutation H121W was GTGGTCATC **TCA**ATGTCTCTGGGCTCTTTTCGTGTTAAGTGCCGTTG.

The oligonucleotide used for mutation T85D was GCTGATGGT GGA**GAT**GGAAATGGCGTTGAGTGCTGAGCCGGCAACCTGTGT. The mutagenized nucleotides were in bold in both sequences. For each mutation, 30 cycles with 1 min of denaturation at 94°C, 1 min of hybridization at 61°C for mutation H121W, and at 55°C for mutation T85D and 1 min of elongation per 10^3 base pairs to polymerize were carried out.

The PCR products were purified with a purification kit (QIAquick PCR purification kit, Qiagen, Westburg, Leasden, The Netherlands). The mutation H121W introduced an additional aromatic link by substitution of Trp for His 121. The mutation T85D introduced an additional link with an ion Ca^{2+} necessary for enzyme activity, by substitution of Asp for Thr 85.

Production of the Subtilisins

The three transformed strains were grown first in 200 mL of nutrient broth TYP + kanamycin (1.5% bactotrypton [Difco], 1.5% yeast extract [Organotechnie, La Courneuve, France], 3% marine salt, and 0.03% kanamycin [Sigma]). pH of the nutrient broth was adjusted to 6.0. These subcultures were incubated at 18°C, for 2 d (stirring rate: 130 rpm) and then were added to 2 L of the same nutrient broth. The subtilisins were excreted in culture medium by cells. After 7 d, the cultures were centrifuged at 4000*g* for 15 min. The supernatants were kept at 4°C.

Spray Drying

The supernatants were either directly dried by spray-drying or formulated and then dried. One liter of formulation contained 30% (v/v) supernatant, 18% arabic gum, 3% lactose, 0.6% aerosyl, 0.7% ethylenediaminetetraacetic acid (EDTA), 0.7% cystein, and 0.7% sodium bisulfite. Spray-drying was carried out with a parallel flow spray-drier NIRO MOBILE MINOR (Copenhagen, Denmark) equipped with a rotary atomizer. The capacity of

evaporation of the spray-drier was 2.5 Kg of water/h, at an inlet air temperature of 200°C.

Analytical Methods

Enzyme Assay

For subtilisin assay, the substrate was N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF-pNa [Sigma]). Assay was carried out, at room temperature, in a reaction mixture (total volume: 500 µL) containing 50 m*M* Tris-HCl buffer, pH 8.0, 2 m*M* $CaCl_2$, and 1 m*M* AAPF-pNa. The reaction was initiated with 50 µL of the enzyme suspension. The hydrolysis of AAPF-pNa was monitored spectrophotometrically (Ultrospec II LKB, PHARMACIA, Uppsala, Sweden) at 410 nm over 10 min. The molar absorption coefficient of p-nitroaniline at 410 nm was assumed to be 8480/M/cm *(23)*. One unit (U) of enzyme activity (A) was defined as 1 µmol of product formed per min, in the assay conditions.

Dry Matter Rate (DMR)

Dry matter rate (DMR) of the samples (supernatants, powders, and so on) was measured after incubation at 105°C, for 12 h.

Spray-Drying Yield (SDY)

Spray-drying yield (SDY) was calculated as follows.

$$SDY = \frac{\frac{A_2}{DMR_2}}{\frac{A_1}{DMR_1}} \times 100$$

with DMR_1 = dry matter rate before drying (%)
DMR_2 = dry matter rate after drying (%)
A_1 = enzyme activity before drying (U/g of enzyme suspension)
A_2 = enzyme activity after drying (U/g of enzyme powder)

Results

Effect of Inlet Air Temperature on SDY of the Three Recombinant Cold Subtilisins

The supernatants of culture of the three transformed strains (WT, T85D, and H121W) were dried without additives by spray-drying at different inlet air temperatures. The outlet air temperature was 70°C. Activities of the three recombinant subtilisins were measured before and after spray-drying. The DMRs of the supernatants and resulting powders were also measured and the SDYs were computed (Fig. 2).

Figure 2 shows the SDYs are low for the three recombinant enzymes ranging from 8–10% and are neither a function of the kind of enzyme nor the inlet air temperature, in the experiment conditions.

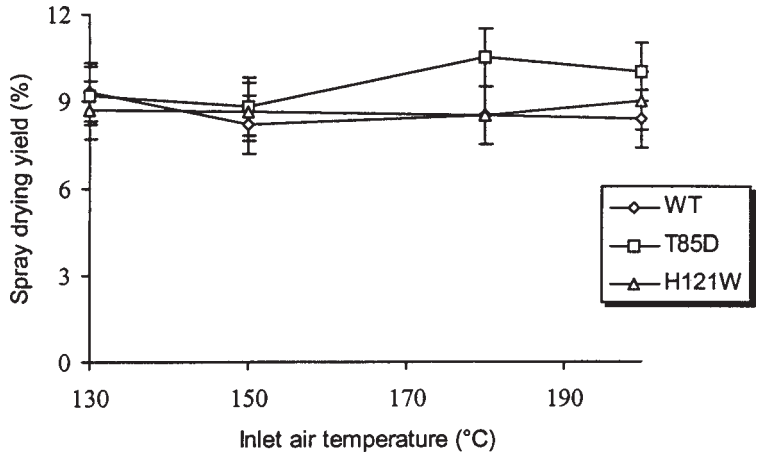

Fig. 2. SDYs of the three recombinant subtilisins at different inlet air temperatures (outlet air temperature: 70°C).

Effect of Outlet Air Temperature on SDY of the Three Recombinant Cold Subtilisins

In this experiment, the supernatants of the three cultures (WT, T85D, and H121W) were dried at different outlet air temperatures. The supernatants were not formulated and the inlet air temperature was 200°C. Figure 3 shows SDY of the three enzymes vs outlet air temperature. An important part of the activity of the three recombinant enzymes is lost again after spray-drying (SDYs less than 10%). Recombinant cold subtilisin H121W appears to be more thermosensitive than the other enzymes: its SDY is reduced from 9–3% between 70°C and 85°C.

Effect of Inlet and Outlet Air Temperatures on SDY of the Three Recombinant Cold Subtilisins (for $\Delta T = 55°C$)

Spray-drying was carried at different inlet *and* outlet air temperatures, for the same $\Delta T = 55°C$ (ΔT is the difference between the inlet and outlet air temperatures). No additives were added to the supernatants. Figure 4 shows SDY of the three enzymes vs inlet/outlet air temperatures.

The SDYs of the three recombinant enzymes are very low, ranging from 6–13%. Although the SDYs of subtilisin WT and subtilisin T85D are identical, the SDYs of subtilisin H121W are almost twice lower than the former. The substitution of the indole nucleus (Trp) for the imidazole nucleus (His) seems to be a spray-drying destabilization factor of the cold subtilisin during spray-drying.

Spray-Drying of the Three Recombinant Cold Subtilisins with Chemical Additives

The supernatants of the three cultures (WT, T85D, and H121W) were formulated with different chemical additives, particularly arabic gum and lactose, to increase the SDYs. The aim was to reduce hygroscopicity of the

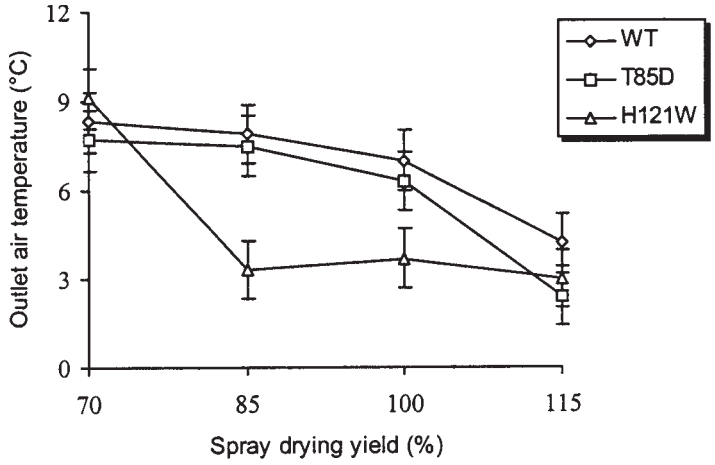

Fig. 3. SDYs of the three recombinant subtilisins at different outlet air temperatures (inlet air temperature: 200°C).

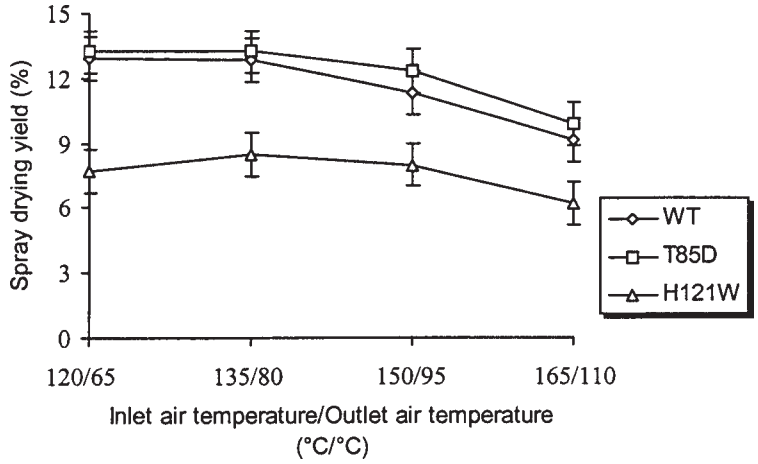

Fig. 4. SDYs of the three recombinant subtilisins at different inlet/outlet air temperatures for a same ΔT of 55°C.

Table 1
SDYs of the Three Recombinant Subtilisins
with Chemical Additives[a]

Subtilisin	WT	T85D	H121W
SDY (%)	65 ± 2	66 ± 2	52 ± 2

[a]Inlet and outlet air temperatures: 130°C and 60°C.

enzyme suspensions in order to improve water transfer during spray-drying. The inlet and outlet air temperatures were 130°C and 60°C, respectively.

Table 1 shows the SDYs of the three enzymes after spray-drying in the presence of chemical additives.

Discussion

Spray-drying is a simple, fast, and economic technique to obtain a powder from a solution or a liquid suspension (e.g., an enzyme suspension). These powders, which have DMRs higher than 90%, are easier to handle and preserve than liquid preparations. They can be used in many biotechnological applications.

Unlike freeze-drying, spray-drying is generally considered an attractive method for the preparation of large quantities owing to the low cost and complexity of the process. Its major drawback is the thermal inactivation of the product. The problem was acute because the cold-adapted enzymes were heat sensitive. It is generally assumed that the high specific activity of the cold-adapted enzymes at low temperature is related to their lower thermostability by comparison with the enzymes from mesophilic microorganisms.

The additives used in this work significantly improved the SDYs of the recombinant subtilisins. Yields increased from 2–13% to 52–66%. Arabic gum, a well-known coating agent, might make the subtilisins more rigid. Lactose, more hydrophilic than arabic gum might reduce the hydrophobicity of the subtilisins and increase water mobility in the product. This would lead to a shorter residence time of the product in the spray drier and a lesser heat denaturation of the enzymes. Another means of limiting the heat denaturation of the cold subtilisin was to increase the spray-drying thermoresistance by selective mutations.

The experiments show that mutation H121W (introduction of an additional aromatic link by substitution of Trp for His 121) makes cold-adapted subtilisin S39 more sensitive to spray-drying. This higher thermosensitivity could be explained by an increase of the hygroscopic character of subtilisin H121W, resulting in a more important retention of water by the enzyme. If this hypothesis is valid, the spray-drying thermoresistance of the enzymes, and particularly the cold-adapted enzymes, might be increased by replacing hydrophobic amino acids (Trp, Phe, Tyr, and so on) near the active site by hydrophilic amino acids (Gly, Asp, Glu, Asn, Gln, and so on).

It is important to note that although mutation T85D increases the thermoresistance of subtilisin S39 in solution *(6)*, this mutation has no influence on thermoresistance of the enzyme during spray-drying. Inversely, mutation H121W which does not influence the thermoresistance of subtilisin S39 in liquid medium (6), increases the thermosensitivity of the enzyme during spray-drying. Consequently, it is probably not possible to compare the thermoresistance of an enzyme in solution with the spray-drying thermoresistance of the same enzyme.

Acknowledgment

This research was supported by a grant (ARC 93/98-170) of the Communaute Francaise of Belgium.

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