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Some Molecular Characteristics and Improving Methods for Thermal Stability of Enzyme

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효소단백질 열안정성의 분자구조적 특성 및 증진기법

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Abstract — Molecular characteristics and improving methods for thermal stability of enzyme have been considered. Intrinsic and extrinsic stabilizing mechanisms are two governing principles for enhanced thermal stability of enzyme in molecular basis. Factors contributing to the former and the latter mechanisms may be involved in the enhanced thermal stability of enzyme complementarily. Also, the methods for improving thermal stability of enzyme which comprise reaction in organic solvent system, chemical modification, immobilization, sequential unfolding and refolding, gene manipulation techniques and enzyme-antibody complexing are reviewed.

The stability and activity of enzyme are determined by its three-dimensional conformation. Because hydrophobic interaction, electrostatic interaction hydrogen bond etc. which stabilize native structure of enzyme are easily inactivated by drastic conditions such as high temperature, extreme pH and chemicals encountered with *in vitro* (1), the efforts to use enzymes in food processing, biological synthesis, medicine, analytical purpose and other uses are often hindered (2,3). Hence, the subject of enzyme stabilization has been in the spotlight in recent years (4-6). The importance of thermal stability in enzyme technology is well manifested by the fact that nearly 90% of world enzyme market is occupied by thermostable enzymes (7). Starch hydrolysis, brewing, baking, detergent industry, maltodextrin hydrolysis, high glucose and fructose syrups production, juice clarification, cellulose hydrolysis etc. are well known industrial application fields of thermostable enzymes (8). Therefore, the molecular reasons of enhanced thermal stability of the enzymes from thermophilic microorganisms have been scrutinized by many workers (9-20).

According to the results accumulated till now, the increased thermal stability of thermostable enzymes can be explained in terms of following two mechanisms. These mechanisms comprise intrinsic stabilizing mechanism, in which the molecular structure of enzyme itself is of central importance for improved thermal stability (9-13) and extrinsic stabilizing mechanism, in which the multipoint interaction of enzyme with other cellular components (14-17) and the presence of specific thermo-stabilizing factors (18-20) are important for increased thermal stability. In thermostable enzymes, one or both of these mechanisms are developed well than corresponding thermolabile enzymes (11-13, 15-19). Factors contributing to the former mechanism are electrostatic interaction (e.g., formation of salt bridge) (11, 21), hydrogen bond (22, 23), hydrophobic interaction (22, 24, 25), content of aliphatic amino acids (26, 27), intramolecular disulfide linkage (26,28) and

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compact packing (25, 29). Factors contributing to the latter mechanism are protein-protein contact (14-17), bindings of substrates and other cellular components of low molecular weight (14-17), prosthetic groups (18, 19), metal cations etc. (20).

Evaluation of Various Molecular Mechanisms Involved in the Stabilization of Enzyme

It is imperative that we evaluate the contribution of each stabilizing factor properly because various factors of intrinsic and extrinsic stabilizing mechanisms are involved in the stabilization of enzyme complementarily. Welder *et al.* (30,31) have reported a close relationship between thermal stability and some general stabilizing factors, e.g., hydrophobic interaction, content of aliphatic amino acids and ratio of Arg/(Arg+Lys) in 220 functionally different proteins isolated from closely related thermophilic and mesophilic microorganisms.

From these contributing stabilizing factors, hydrophobic interaction that seems to be the only stabilizing force which is strengthened with temperature at least up to 60~70℃, plays a significant role in stabilizing protein structure (32, 33) (Fig. 1). In fact, some thermostable proteins undergo essential conformational changes at 55~70℃, which, nevertheless, does not lead to the loss of catalytic activity. This phenomenon can be explained as meaning that thermostable enzymes only rearrange their structures in order to strengthen hydrophobic interaction at high temperatures where thermolabile enzymes usually denature (34-36). In case of tryptophane synthetase α-subunit from *Escherichia coli*, the substitution of Met for Glu 49 in the hydrophobic interior causes stabilization of enzyme due to the increase in hydrophobicity (37).

Ikai (38) have elucidated the correlationship between the stability of thermostable proteins and the content of aliphatic amino acids by introducing so called aliphatic index A, which is equal to $X_A + aX_V + b(X_I + X_L)$, where X_A, X_V, X_I and X_L are the mole fractions of Ala, Val, Ile and Leu, respectively. a and b are numerical coefficients that depend on the sizes of the residues. According to the Ikai's

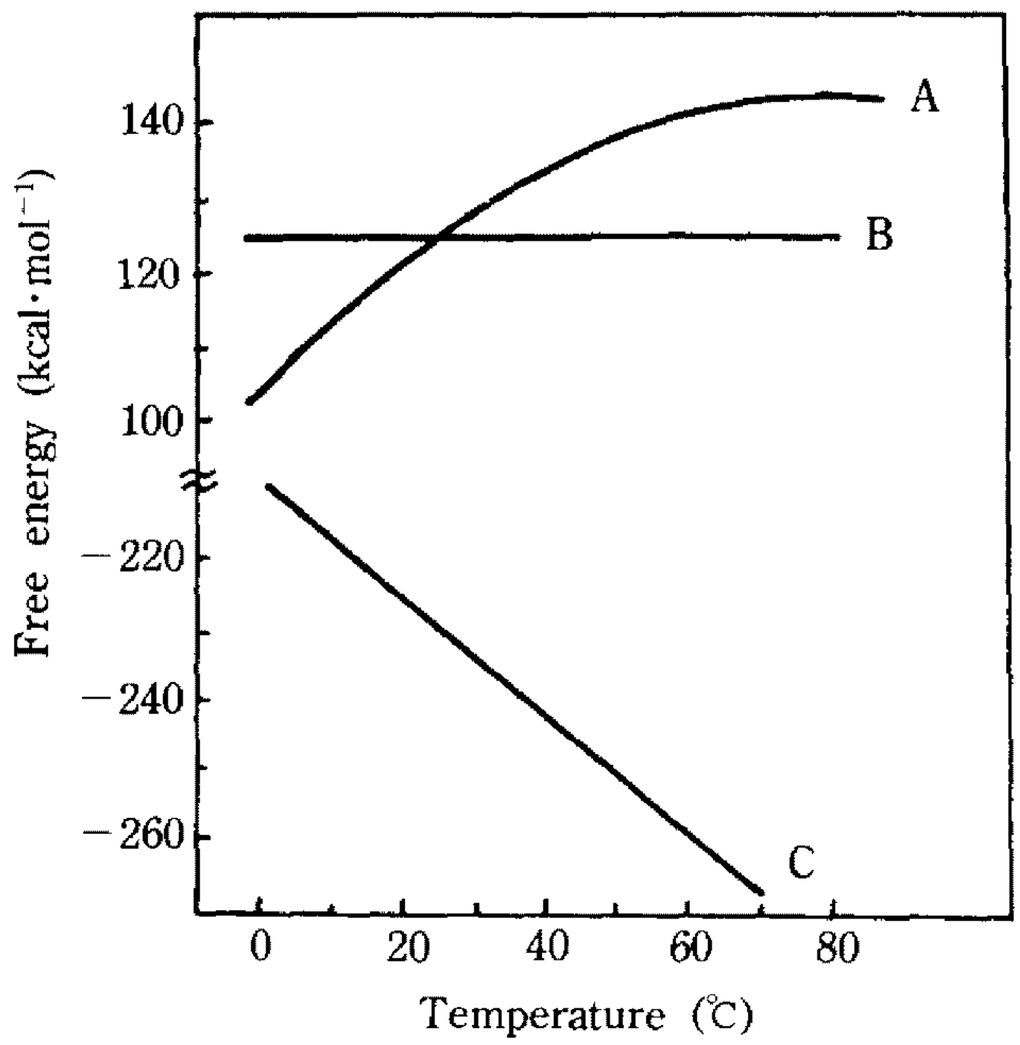

Fig. 1. Temperature dependence of the free energy values of the principal interactions contributing to stability in the proteins.
A, hydrophobic interaction; B, hydrogen bond; C, conformational entropy. Adopted from reference 32

report, the aliphatic index of thermostable proteins is much higher than that of thermolabile proteins. He calculated an increase in the aliphatic index by 10 has resulted in additional stabilization by 5-7 kcal/mol (38). Nevertheless, Ikai failed to find out the relationship between the thermal stability of enzyme and the overall hydrophobicity. He only explained this in terms of the fact that aliphatic hydrophobic interaction only increases with temperature (38).

Yet, proteins from extreme thermophiles are stable at 75~85℃ and some are even stable at the temperature of boiling water. This fact strongly suggests the involvement of other stabilizing factors because extreme stability is unlikely to be maintained at the expense of hydrophobic interaction as the strength decreases above 70~80℃ (32, 33).

Perutz and Raidt (11, 39) have found that surface salt bridge contributes to the enhanced thermal stability of monomeric proteins (e.g., ferredoxin), whereas the extra salt bridge and hydrogen bond on subunit interface are important stabilizing factors of oligomeric proteins (e.g., hemoglobin).

The number of hydrogen bonds is reported to

be related considerably with the enhanced thermal stability of enzyme. For examples, 19 additional hydrogen bonds were detected in thermostable proteases (22) which are not present in the thermolabile counterparts. A greater number of hydrogen bonds was also reported for thermostable enolase (23). Additional intramolecular disulfide linkages are also involved in the enhanced stability of thermostable enzymes (40).

The compactness of protein structure is also important for the thermal stabilization of enzyme. In case of thermolabile glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase, four substitutions which result in the compactness of interior region of enzymes (e.g., Gly→Ala, Ser →Thr, Lys→Arg and Asp→Glu) greatly contribute to the enhanced thermal stability (41).

On the other hand, thermal stability of proteins also seems to be inversely related with solvent-accessible surface area of proteins (42). In this instance, the polymerization of globular subunits and the folding of single polypeptide chain into multiple globular domains are occasionally found in thermostable proteins (42).

Enzymes generally become more stable when they are complexed with substrates or cofactors than in the free forms (43). Thus, glutamate synthetase from *Bacillus stearothermophilus,* which normally loses its activity at 65℃, is stable in the presence of NH_4^{+1}, glutamate, Mg^{2+} and ATP. It is even stabilized by feedback inhibitors such as alanine and histidine (44, 45).

Metal cations such as Ca^{2+} and Na^{1+} can be also acting as specific stabilizing cofactors. For example, a thermostable α-amylase is stable at high temperature only in the presence of Ca^{2+} and Na^{1+} ions (46). Similarly, a thermostable protease (thermolysin) is stable when Ca^{2+} ion is present (47).

Methods for Improving Thermal Stability of Enzyme

One way for obtaining thermostable enzymes is the isolation from microorganisms which naturally exist in extreme thermophilic environments (e.g., hot spring) (48, 49). This method can easily provide thermostable enzymes free from genetic variability for biotechnology. However, we can not solely depend on this method because of the difficulty in cultivating thermophilic strains with high content of a given enzyme (50) and the reduced growth efficiency of thermophilic bacteria (51, 52) which may cause low enzyme productivity. So, various methods for obtaining thermostable enzymes have been devised and a brief review on this point will be done in the following paragraphs.

Reaction in organic solvent system

In recent times, it is known that even a monolayer of water suffices for enzyme catalysis (53). Sin ce in organic solvents enzymes in some instances exhibit activity comparable to that in water, one can assume that the enzyme structures in organic solvents are not radically different from those in water (54). This belief is generally accepted by the fact that structures obtained by X-ray crystallography are a good approximation to the real protein structure found in water (54). As a variety of deleterious processes which lead to the irreversible thermo-inactivation of enzyme involve water as a reactant (55), it is not surprising that enzyme is extremely thermostable in water-restricted environments such as orgainc solvents (56). In this instance, the major reason for enhanced stability of enzyme at high temperatures in organic solvents seems to be decrease in dielectric constant which results in the weakening all electrostatic interactions involved in the inactivation process of enzyme (54). Recently, Zaks and Klibanov have reported that lipase is even stable at 100℃ for several hours in microaqueous system which has the moisture content of 0.015% (57). Subtilisin present in tributyrin in microaqueous system is known to be stable for 80 minutes at 120℃ (54). In many cases, however, the enzymatic reactions in organic solvents are hindered by the denaturation and the inactivation of enzyme caused by organic phase. To minimize these problems, some enzymatic reaction systems which comprise reversed micellar system (58, 59), two phase system (60, 61) and microaqueous system (62, 63) have been designed. As hydrolytic enzymes (e. g., protease and lipase) catalyze reverse reactions

due to the equilibrium change in organic solvents (64-69), reaction in organic solvent system can be successfully applied to peptide synthesis by protease and trans-esterification or/and ester synthesis by lipase (64-69).

Chemical modification

Functional groups which are present on the surface of enzyme are essential for the maintenance of native structure. Hence, considerable stabilization is expected to be gained by chemical modification of some key functional groups located on the surface of protein. In this instance, the mechanism of stabilization is that chemical modification eliminates unfavorable electrostatic repulsion of charged groups which facilitates unfolding of catalytically active conformation. Alternatively, chemical modification may increase hydrophobic interaction in globule (70-72).

The chemical modification methods which are effective for stability improvement of enzyme include modifications by low molecular weight compounds and water soluble high molecular weight compounds.

It is reported that the free amino group modification with succinic anhydride, maleic anhydride and O-methylisourea (73-76), the indole group modification with N-bromosuccinimide (77) and the phenolic hydroxyl group modification with tetranitromethane (78) improve thermal stability of enzyme like protease considerably. These are good examples of chemical modification by low molecular weight compounds.

Table 1 shows modifiers and target proteins which are used for chemical modification with water soluble high molecular weight compounds (67). In recent years, polyethylene glycol has become one of the most promising modifiers for improving stability of enzyme (66, 67). In polyethylene glycol modification of enzyme, polyethylene glycol (M.W. 5, 000), one of amphiphatic substances, is firstly activated with cyanuric chloride to yield activated PEG $_2$. Then, activated PEG_2 is coupled to the free amino groups (e.g., ε-amino group of Lys) of target enzymes such as protease, lipase and asparaginase. The net increase in hydrophobicity of enzyme results in improved thermal and solvent stabilities (66, 67). Furthermore, polyethylene glycol modified enzymes can be magnetized for dual purposes of stability improvement and recovery after use (79, 80).

Immobilization

Table 1. Modifiers and target proteins used for the chemical modification with water soluble high molecular weight compounds

Modifier	Target protein
Synthetic polymer poly-L-aspartic acid, polyaspartic acid derivative, polyethylene glycol, poly(D-glutamic acid-D-lysine), poly-D-lysine, poly-L-lysine, poly(maleic acid)-styrene oligomer, polyvinyl alcohol, pyran copolymer	Enzyme arginase, L-asparaginase, batroxobin, catalase,chymotrypsin, elastase, α-(β-)galactosidase, β-glucosidase, β-glucuronidase, L-glutaminase, L-asparaginase, phenylalanine ammonia lyase, streptokinase, superoxide dismutase, trypsin, uricase, urokinase
Polysaccharide agarose, carboxymethyl cellulose, dextran, pullulan	Protein blood coagulating factor VIII, IX, bovine serum albumin, hemoglobin, immunoglobulin G, insulin, lactoferrin, α-macroglobulin, neocarzinostatin, ovalbumin
	Allergin α-amylase, bovine serum albumin, catalase, flagellin, lysozyme, mite, ovalbumin, ragweed pollen, timothy grass

Adopted from reference 67.

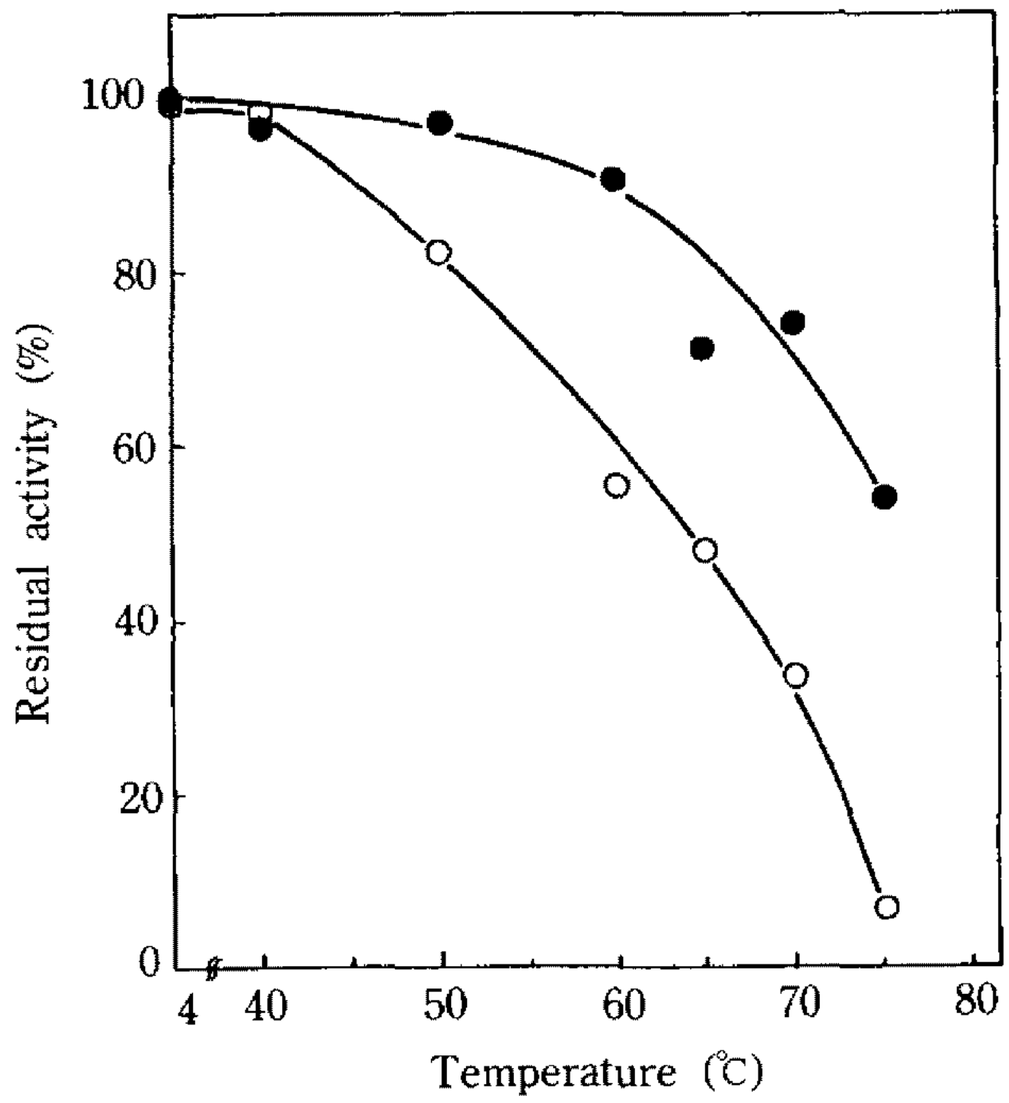

Fig. 2. Thermal stability of polyacrylamide gel-entrapped trypsin compared with that of free enzyme. Free and immobilized enzymes were heat-treated at each temperature for 10 minute before enzyme assay.
○—○, free enzyme; ●—●, immobilized enzyme. Adopted from reference 82

When immobilized by physical and chemical methods such as adsorption, entrapment, microencapsulation etc., enzymes become to be placed in micro-environments different from those of free enzymes. In many cases, the net effects of enzyme immobilization are changes in pH and temperature profiles and enhancement of thermal stability (81).

That is, Kim *et al.* (82) have shown that trypsin entrapped by polyacrylamide gel holds enhanced thermal stability, compared with free enzyme (Fig. 2). In case of caldolysin immobilized to Sepharose 4B and CM-cellulose resins, 2-3 fold increase in thermal stability was also found, with the concomitant shift of optimum pH to acidic region by 1 pH unit (83). Papain covalently attached to polyacrolein micro-spore possesses nearly 100% of the original activity, whereas free enzyme loses 23% of the original activity after heat-treatment at 37℃ for 1 hr (84). Cellulase immobilized to iron oxide (Fe_3O_4) was found to have a broader range of temperature profile compared with free enzyme due to the strengthening of weak ionic forces and hydrogen bonds induced by immobilization (85). Kimura *et al.* (86)

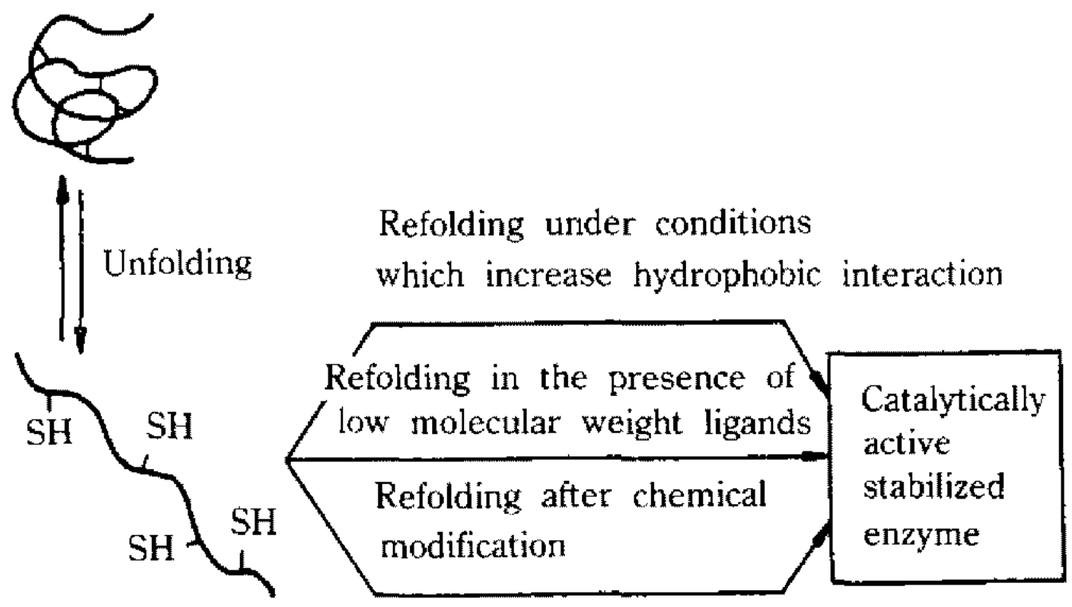

Fig. 3. Schematic representation of stabilizing enzymes by sequential unfolding and refolding.
Adopted from reference 94

have reported that maltotetraose-forming amylase immobilized to a macroporous hydrophobic adsorption resin (Diaion HP-50) holds good thermal stability in batch and continuous operations. Thermal stability was also greatly increased by immobilization in cases of thermolysin (87), acid phosphatase (88), glucose oxidase (89), endo-polygalacturonase (90), pullulanase (91) and β-glucosidase (92).

Sequential unfolding and refolding

It can be also a good way for improving thermal stability of enzyme to increase the hydrophobicity and to provide additional disulfide linkages, salt bridges and hydrogen bonds in the internal nucleus of globule.

Mozhaev and Martinek have suggested the following two-step procedure comprising sequential unfolding and refolding for improving thermal stability of enzyme through the modification of the internal regions (Fig. 3) (93, 94).

At first, protein is made to unfold into a random coil-like state under the presence of reversible denaturants such as urea and guanidine hydrochloride with simultaneous splitting of disulfide linkages in the protein using β-mercaptoethanol and dithiothreitol. In the next stage, the conformation of protein is altered to strengthen the binding forces described above. The methods for conformation change comprise refolding under the conditions which increase hydrophobic interaction like concentrated solutions of salts, polyols, elevated temperatures and certain organic solvents, refolding in the presence of substances which interact with protein in

a noncovalent fashion like transition metals, amphiphatic substances and nonpolar substances participating in the formation of hydrophobic interior (so-called capture on refolding) and refolding after chemical modification of enzyme in the unfolded state (94). They increased the thermal stability of trypsin immobilized to polyacrylamide gel greatly (over 2 folds) by the refolding at elevated temperature (50 ℃) using thiol-disulfide exchange catalyst such as glutathione (94).

Gene manipulation techniques

The improving methods for thermal stability of enzyme by gene manipulation have been recently developed. There are two kinds of gene manipulation techniques for obtaining thermostable enzymes. One method is to produce thermostable enzymes by means of transfering thermostable enzyme genes originated from thermophilic bacteria with slow growth rate to appropriate mesophilic bacteria having fast growth rate. That is, CPC-International Co. has shown a dramatic increase in the production yield due to the enhanced growth of recipient microorganism when α-amylase gene of *B. stearothermophilus* is transferred to *B. subtilis* (95,96). The similar result has also been found when the recipient microorganism is yeast (97).

The other method for obtaining thermostable enzymes is protein engineering technique. In this technique, the amino acid sequence of target enzyme is altered at one site or a few sites to induce conformational change of enzyme. For the sake of this purpose, it is imperative that structural analyses using nuclear magnetic resonance, X-ray crystallography, circular dichroism, ultraviolet and fluorescence be done and computer graphics modelling on three dimensional structure of protein be performed.

Based on these basic informations, the specific *in vitro* mutagenesis is done on the gene coding target enzyme to contribute to the molecular mechanisms involved in the improvement of thermal stability. Among *in vitro* mutagenesis techniques, oligonucleotide-directed mutagenesis is the more powerful and versatile than restriction fragment deletion, nucleotide removal at restriction site and

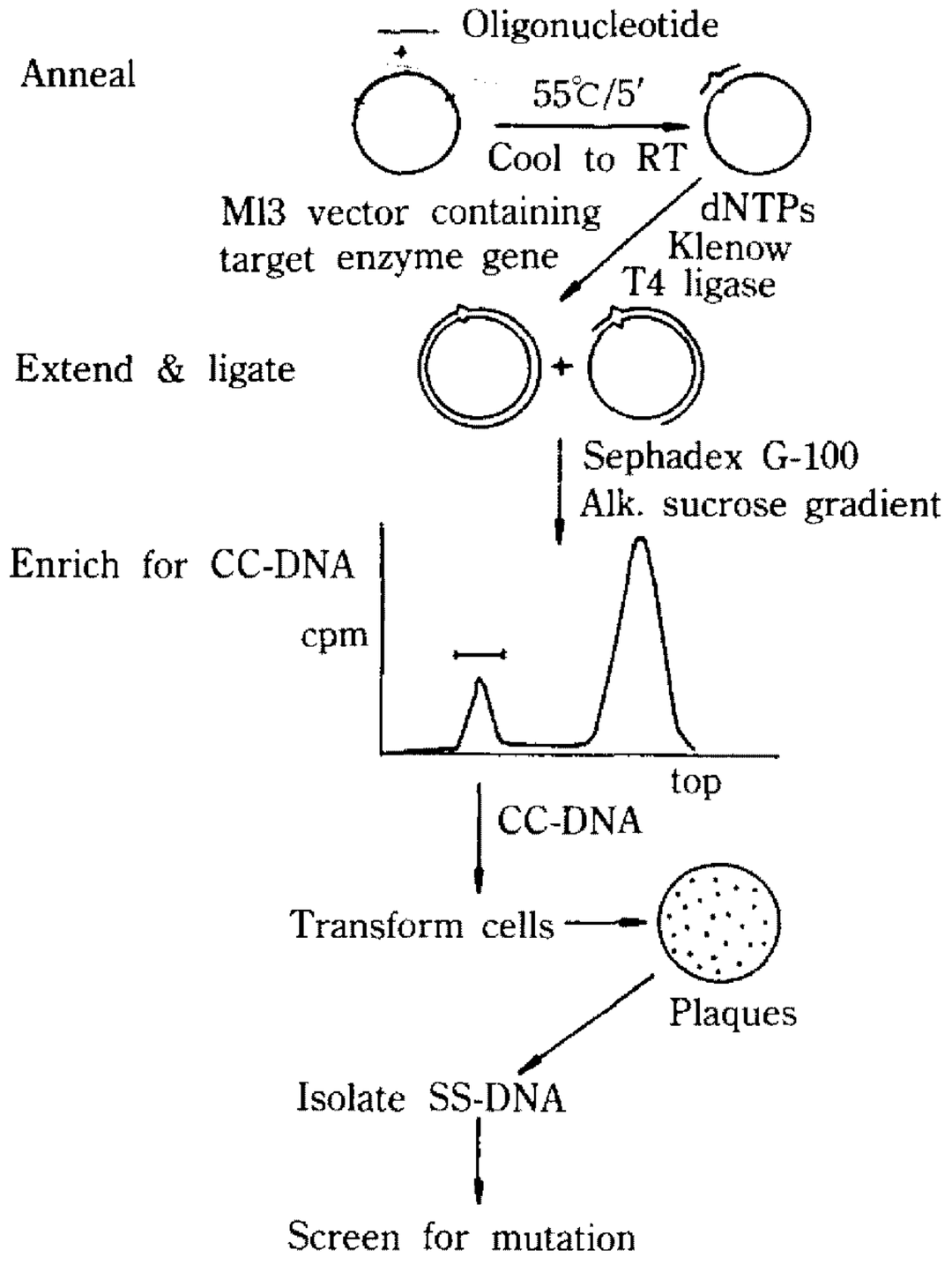

Fig. 4. General scheme for oligonucleotide-directed mutagenesis.
CC-DNA, closed circular DNA; SS-DNA, single-stranded DNA. Adopted from reference 99

insertion of an oligonucleotide (98). The general scheme of oligonucleotide-directed mutagenesis is depicted in Fig. 4. In this instance, the gene coding target enzyme must be obtained as single-stranded form. Therefore, M-13 vector system is generally used (99). Estell *et al.* (100) have improved chemical stability of subtilisin greatly by oligonucleotide-directed mutagenesis. The single amino acid substitutions by oligonucleotide-directed mutagenesis in Cro (101) cytochrome C (102), barnase (103) and tryptophane synthetase (104) resulted in the increases in thermal stability (i.e., ΔTms were in the range of 11~17℃). It is encouraging that large increases in thermal stability can be achieved by single amino acid substitutions. Several studies have shown that the effects of amino acid substitutions may be localized so that their effects on the stability are additives (105). Therefore, more increase in thermal stability is expected to be obtained in

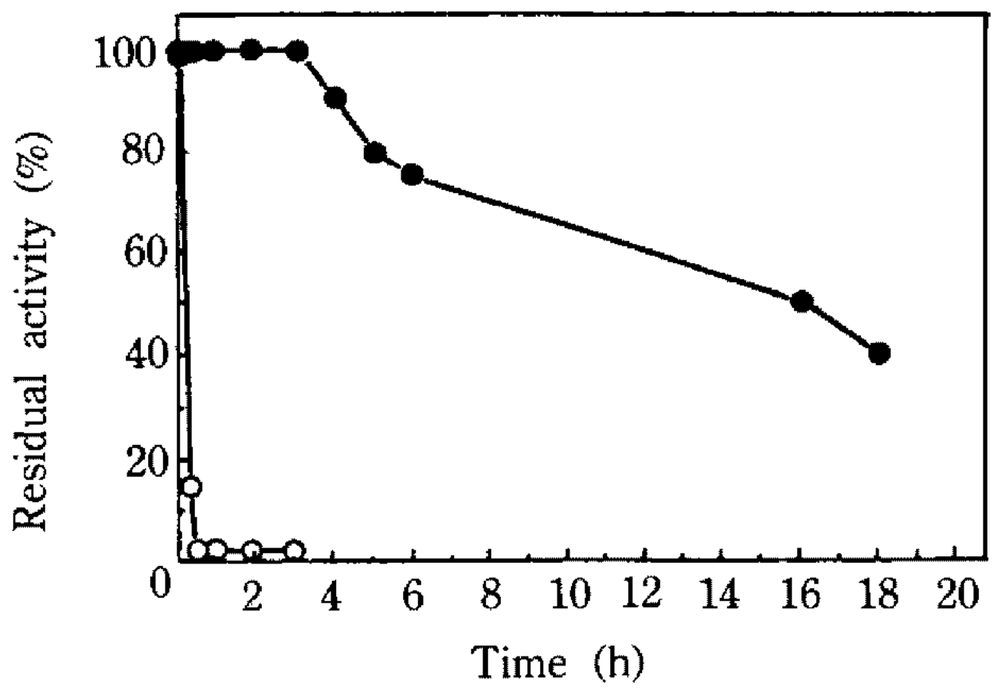

Fig. 5. Thermostability of α-amylase-antibody complex. Human salivary α-amylase was complexed with specific rabbit polyclonal antibody and then placed in a 70°C water bath.
○—○, free enzyme; ●—●, enzyme-antibody complex. Adopted from reference 108.

the near future.

Recently, the site-specific incorporation of unnatural amino acids (e.g., D-Phe or p-NO_2-Phe substitution for Phe 66 in β-lactamase) by way of oligonucleotide-directed mutagenesis has been devised to make novel proteins that possess unique structural and functional features (106, 107). By this procedure, it becomes possible to circumbent the limitation of protein size or selectivity of incorporation.

Enzyme-antibody complexing

Very lately, the protein stabilization method exploiting immune system has been reported. Although most enzymes can be inhibited by certain antibodies, some antibodies might interact at sites where protein unfolding is initiated or the inhibitory antibodies are not accessible (108). For the average affinity antibody-antigen binding ($10^8\ M^{-1}$), the interaction could reduce the free energy of the antigen by about 10 kcal/mol. In a general thermodynamic sense, this is sufficient to confer increased stability since the differences in free energy between the folded and unfolded states for active proteins are in the range of 5-15 kcal/mol (109).

When human salivary α-amylase was complexed with its polyclonal antibody, the thermal stability was greatly increased compared with that of free enzyme; activity half-life was as much as 200 times as long (Fig. 5) (110). In this instance, the absence of significant inhibition by antigen-antibody complexing was also noticed (110).

References

1. Kim, N.S., D.Y. Kwon and Y.J. Nam: *Functionality Improvement of Proteolytic Enzyme by Structural Modification* (A Research Report of Korea Food Research Institute), p. 19 (1988).
2. Katchalski-Katzir, E. and A. Freeman: *Trends Biochem. Sci.* 7, 427 (1982).
3. Klibanov, A.M.: *Science* **219**, 722 (1983).
4. Klibanov, A.M.: *Anal. Biochem.* **92**, 1 (1979).
5. Schmid, R.D.: *Adv. Biochem. Eng.* **12**, 41 (1979).
6. Klbanov, A.M.: *Biochem. Soc. Trans.* **11**, 19 (1983).
7. Godfrey, T. and J. Reichelt (eds.): *Industrial Enzymology*, Nature Press, London (1983).
8. Ng, T.K. and W.R. Kenealy: *Thermophiles*, (ed) Brock, T.D., John Wiley and Sons, New York, p. 197 (1986).
9. Kauzmann, W.: *Adv. Protein Chem.* **14**, 1 (1959).
10. Finney, J.L., B.J. Gellatly, I.C. Golton and J. Goodfellow: *Biophys. J.* **32**, 17 (1980).
11. Perutz, M.F.: *Science* **201**, 1187 (1978).
12. Oas, T.G. and P.S. Kim: *Nature*, **370**, 329 (1988).
13. Goodman, E.K. and P.S. Kim: *Biochemistry* **28**, 4343 (1989).
14. Gabel, D.: *Eur. J. Biochem.* **33**, 348 (1973).
15. Koch-Schmidt, A.C. and K. Mosbach: *Biochemistry* **16**, 2105 (1977).
16. Martinek, K., A.M. Klibanov, V.S. Goldmacher, A.V. Tchernysheva, V.V. Mozhaev, I.V. Berezin and B.O. Glotov: *Biochim. Biophys. Acta* **485**, 13 (1977).
17. Martinek, K., A.M. Klibanov, V.S. Goldmacher and I.V. Berezin: *Biochim. Biophys. Acta* **485**, 1 (1977).
18. Nakamura, Y.: *J. Biochem.* (Tokyo) **48**, 295 (1960).
19. Oshima, T.: *J. Biol. Chem.* **257**, 9913 (1982).
20. Oshima, T.: *Protein Engineering*, (eds.) Inouye, M. and R. Sarma, Academic Press Inc, Florida, p. 81 (1986).
21. Yutani, K., N.N. Khechinashvili, E.A. Lapshima, P.L. Privalov and Y. Sugino: *Int. J. Pept. Protein Res.* **20**, 331 (1982).
22. Ohta, Y.: *J. Biol. Chem.* **242**, 509 (1967).
23. Barns, L.D. and E. Stellwagen: *Biochemistry* **12**, 1559 (1973).
24. Welder, F.C. and F.M. Hoffman: *Biochemistry* **13**, 3207 (1974).
25. Walker, J.E., A.J. Wonacott and J.I. Harris: *Eur. J. Biochem.* **108**, 581 (1980).

26. Sundaram, T.K., R.M. Chell and A.E. Wilkinson: *Arch. Biochem. Biophys.* **199**, 515 (1980).
27. Muramatsu, N. and Y. Nosoh: *Arch. Biochem. Biophys.* **144**, 245 (1971).
28. Nakamura, S., S. Ohta, K. Arai, T. Oshima and K. Kajiro: *Eur. J. Biochem.* **92**, 533 (1978).
29. Crabb, J.W., A.L. Murdock, T. Suzuki, J.W. Hamilton, J.H. McLinden and R.E. Amelunxen: *J. Bacteriol.* **145**, 503 (1981).
30. Welder, F.C., G.K. Farrington and D.J. Merkler: *Fed. Proc.* **40**, 1839 (1981).
31. Merkler, D.J., G.K. Farrington and F.C. Welder: *Int. J. Pept. Protein Res.* **18**, 430 (1981).
32. Scherage, H.A.: *The Proteins.* (ed.) Neurath, H., Academic Press, New York, **1**, 477 (1963).
33. Brandts, J.F.: *J. Am. Chem. Soc.* **86**, 4302 (1964).
34. Tajima, M., I. Urabe, K. Yutani and H. Okada: *Eur. J. Biochem.* **64**, 243 (1976).
35. Amelunxen, R.E., M. Noelken and R. Singleton Jr: *Arch. Biochem. Biophys.* **141**, 447 (1970).
36. Matsunaga, A. and Y. Nosoh: *Biochem. Biophys. Acta* **365**, 208 (1974).
37. Yutani, K., K. Ogasahara, Y. Sugino and A. Matsushiro: *Nature* **267**, 274 (1977).
38. Ikai, A.: *J. Biochem.* (Tokyo) **88**, 1895 (1980).
39. Perutz, M.F. and H. Raidt: *Nature* **255**, 256 (1975).
40. Ogasahara, K., A. Imanishi and T. Isemura: *J. Biochem.* (Tokyo) **67**, 65 (1970).
41. Argos, P., M.G. Rossman, U.M. Grau, H. Zuber, G. Frank and J.D. Tratschin: *Biochemistry* **18**, 5698 (1979).
42. Stellwagen, E. and H. Wilgus: *Nature* **275**, 342 (1978).
43. Citri, N.: *Adv. Enzymol.* **37**, 397 (1973).
44. Hachimori, A., A. Matsunaga, M. Shimizu, T. Sarnejima and Y. Nosoh: *Biochem. Biophys. Acta* **350**, 461 (1974).
45. Wedler, F.C. and F.M. Hoffman: *Biochemistry* **13**, 3215 (1974).
46. Hasegawa, A., M. Miwa, T. Oshima and K. Imahori: *J. Biochem.* (Tokyo) **79**, 35 (1976).
47. Weaver, L.H., W.R. Kester, L.F. TenEyck and B.W. Matthews: *Enzymes and Proteins from Thermophilic Microorganism*, (ed.) Zuber, C.H., Birkhaeuser, Basel, p. 31 (1976).
48. Oshima, T.: *Enzyme Engineering*, (eds.) Brown, G.B., G. Manecke and L.B. Wingard Jr. Plenum Press, New York-London, **4**, 41 (1978).
49. Doig, A.R.: *Enzyme Engineering*, (eds.) Pye, E.K. and L.B. Wingard, Plenum Press, New York-London, **2**, 17 (1974).
50. Alexandrov, V.Y.: *Cells, Macromolecules and Temperature*, Springer-Verlag, Berlin (1978).
51. Brock, T.D.: *Science* **158**, 1012 (1967).
52. Ingraham, J.L., E. Precht, J. Christopherson, H. Hensel and W. Larcher (eds.): *Temperature and Life*, Springer-Verlag, Heidelberg, p. 60 (1973).
53. Russel, A.J. and A.R. Fersht: *Nature* **328**, 496 (1987).
54. Zaks, A. and A.J. Russel: *J. Biotechnol.* **8**, 259 (1988).
55. Ahern, T.J. and A.M. Klibanov: *Science* **228**, 1280 (1985).
56. Zaks, A. and A.M. Klibanov: *J. Biol. Chem.* **263**, 3194 (1988).
57. Zaks, A. and A.M. Klibanov: *Science*, **224**, 1249 (1984).
58. Fendler, J.H.: *Acc. Chem. Res.* **9**, 153 (1976).
59. Luthi, P. and P.L. Luisi: *J. Am. Chem. Soc.* **106**, 7285 (1984).
60. Kwon, D.Y. and J.S. Rhee: *Kor. J. Appl. Microbiol. Bioeng.* **15**, 98 (1987).
61. Kim, M.H., S.B. Lee, D.D.Y. Ryu and E.T. Reese: *Enzyme Microb. Technol.* **4**, 99 (1982).
62. Klibanov, A.M.: *Chemtech.* **16**, 354 (1986).
63. Zaks, A. and A.M. Klibanov: *Proc. Natl. Acad. Sci.* **82**, 3192 (1985).
64. Cowan, D.A., R.M. Daniel and H. Morgan: *Trends Biotechnol.* **3**, 68 (1985).
65. Oka, T. and K. Morihara: *J. Biochem.* (Tokyo) **88**, 807 (1980).
66. Inada, Y., K. Takahashi, T. Yoshimoto, A. Ajima, A. Matsushima and Y. Saito: *Trends Biotechnol.* **4**, 190 (1986).
67. Inada, Y., T. Yoshimoto, A. Matsushima and Y. Saito: *Trends Biotechnol.* **4**, 68 (1986).
68. Takahashi, K., A. Ajima, T. Yoshimoto, M. Okada, A. Matsushima, Y. Tamaura and Y. Inada: *J. Org. Chem.* **50**, 3414 (1985).
69. Takahashi, K., H. Nishimura, T. Yoshimoto, M. Okada, A. Ajima, A. Matsushima, Y. Tamaura, Y. Saito and Y. Inada: *Biotechnol. Letters* **6**, 765 (1984).
70. Torchilin, V.P., A.V. Maksimenko, I.V. Berezin, A.M. Klibanov and K. Martinek: *Biochem. Biophys. Acta* **567**, 1 (1979).
71. Torchilin, V.P. and K. Martinek: *Enzyme Microb. Technol.* **1**, 74 (1979).
72. Hollecker, M. and T.E. Creighton: *Biochim. Biophys. Acta* **701**, 395 (1982).
73. Maneepun, S. and A.M. Klibanov: *Biotechnol. Bioeng.* **24**, 483 (1982).
74. Terminiello, L., M. Bier and F.F. Nord: *Arch.*

Biochem. Biophys. **73**, 171 (1958).
75. Barker, S.A., S.J. Gray and A.W. Lomath: *J. Appl. Chem. Biotechnol.* **26**, 576 (1976).
76. Cupo, P., W.S. El-Deiry, P.L. Whitney and W.M. Awad Jr: *Arch. Biochem. Biophys.* **216**, 600 (1982).
77. Morikawa, Y., M. Shimizu and K. Kimura: *Nippon Nogeigagaku Zasshi* **48**(2), 105 (1974).
78. Ohta, Y., S. Tanabe, A. Hachimori and T. Samejima: *J. Biochem.* (Tokyo) **76**, 871 (1974).
79. Takahashi, K., Y. Tamaura, Y. Kodera, T. Mihama, Y. Saito and Y. Inada: *Biochem. Biophys. Res. Commun.* **142**, 291 (1987).
80. Yoshimoto, T., T. Mihama, K. Takahashi, Y. Saito, Y. Tamaura and Y. Inada: *Biochem. Biophys. Res. Commun.* **145**, 908 (1987).
81. Royer, G.P.: *Fundamentals of Enzymology*, John Wiley & Sons, New York, p. 183 (1982).
82. Kim, N.S., D.Y. Kwon, H.M. Seog, W.S. Hawer, Y.J. Kim, Y.K. Park, J.H. Ha and Y.J. Nam: *Study on the Stability Improvement of Food Enzyme* (A Research Report of Korea Food Research Institute), p. 83 (1989).
83. Cowan, D.A. and R.M. Daniel: *Biotechnol. Bioeng.* **24**, 2053 (1982).
84. Hayashi, T. and Y. Ikada: *Biotechnol. Bioeng.* **35**, 518 (1990).
85. Garcia III, A., S. Oh and C.R. Engler: *Biotechnol. Bioeng.* **33**, 321 (1989).
86. Kimura, T., M. Ogata, M. Yoshida and T. Nakakuki: *Biotechnol. Bioeng.* **33**, 845 (1989).
87. Kumakura, M. and I. Kaetsu: *Enzyme Microb. Technol.* **6**, 23 (1984).
88. Greco, G.Jr.: *Biotechnol. Bioeng.* **23**, 2199 (1981).
89. Malikkides, C.O. and R.H. Weiland: *Biotechnol. Bioeng.* **24**, 1911 (1982).
90. Pifferi, P.G., M. Tramontini and A. Malacarne: *Biotechnol. Bioeng.* **33**, 1258 (1989).
91. Kusano, S., T. Shiraishi, S. Takahashi, D. Fujimoto and Y. Sakano: *J. Ferment. Bioeng.* **68**(4), 233 (1989).
92. Roy, S.K., S.K. Raha, S.K. Dey and S.L. Chakrabarty: *Enzyme Microb. Technol.* **11**, 431 (1989).
93. Martinek, K., V.V. Mozhaev and I.V. Berezin: *Biochem. Biophys. Acta* **615**, 426 (1980).
94. Mozhaev, V.V. and K. Martinek: *Enzyme Microb. Technol.* **6**, 50 (1984).
95. Pitcher, W.H.: *Food Technol.* **40**, 62 (1986).
96. Fuji, M., M. Takagi, T. Imanka and S. Alba: *J. Bacteriol.* **154**, 831 (1983).
97. Meade, J.H., T.J. White, S.P. Shoemaker, D.H. Gelfland, S. Chang and M.A. Innis: *Impact of Biotechnology on Food Production and Processing.* (ed.) Knorr, D., Marcel Dekker, Inc., New York.
98. Brown, T.A.: *Gene Cloning, An Introduction*, Van Nostrand Reinhold, Wokingham, p. 168 (1986).
99. Zoller, M.J. and M. Smith: *Methods Enzymol.* Academic Press Inc., **100**, 468 (1983).
100. Estell, D.A., T.P. Graycar and T.A. Wells: *J. Biol. Chem.* **260**, 6518 (1985).
101. Pakula, A.A. and R.T. Sauer: *Proteins Struct. Funct. Genet.* **5**, 202 (1989).
102. Das, G., D.R. Hickey, D. McLendon, G. McLendon and F. Sherman: *Proc. Natl. Acad. Sci.* USA **86**, 496 (1989).
103. Kellis, J.T., K. Nyberg and A.R. Fersht: *Biochemistry* **28**, 4914 (1989).
104. Yutani, K., K. Ogasahara, T. Tsujita and Y. Sugino: *Proc. Natl. Acad. Sci.* USA **84**, 4441 (1987).
105. Alber, T.: *Annu. Rev. Biochem.* **58**, 765 (1989).
106. Noren, C.J., S.J. Anthony-Cahill, M.C. Griffith and P.G. Schultz: *Science*, **244**, 182 (1989).
107. Anthony-Cahill, S.J., M.C. Griffith, C.J. Noren, D.J. Suich and P.G. Schultz: *Trends Biochem. Sci.* **14**, 400 (1989).
108. Shami, E.Y., A. Rothstein and M. Ramjeesingh: *Trends Biotechnol.* **7**, 186 (1989).
109. Tanford, C.: *Adv. Protein Chem.* **24**, 1 (1970).
110. Shami, Y., A. Rothstein and M. Ramjeesingh: *European Patent Application No. EP 029865 4A2* (1989).

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