Stabilization of enzymes by chemical modifications

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Abstract

This study focused on thermostabilization of enzymes in solution by intramolecular crosslinking of the specific functional groups within an enzyme molecule. Three model enzymes were used: α-amylase of *Aspergillus oryzae* (EC 3.2.1.1), β-galactosidase of *Aspergillus oryzae* (EC 3.2.1.23) and extracellular invertase (EC 3.2.1.26) of *Saccharomyces cerevisiae*. Crosslinking was examined using the following homobifunctional reagents: diisocyanates ($O=C=N(CH_2)_nN=C=O$, n = 4, 6, 8), diimidoesters ($CH_3O(=NH)C(CH_2)_nC(=NH)OCH_3$, n = 4, 5, 6) and diamines ($NH_2(CH_2)_nNH_2$, n = 0, 2, 4, 6, 8, 10, 12). The concentration of the enzymes was kept low at 0.9 μM in attempts to promote intramolecular crosslinking as opposed to intermolecular crosslinking. Only invertase could be stabilized relative to controls by crosslinking with diisocyanates.

Invertase (0.9 μM) crosslinked with 1,4-diisocyanatobutane (n = 4; or butamethylene diisocyanate, BMDC) and 1,6-diisocyanatohexane (n = 6) showed enhanced thermostability. Stability was improved dramatically by crosslinking invertase with 20-30 μM of the reagent. Molecular engineering of invertase by crosslinking reduced its first-order thermal denaturation constant at 60 °C from 1.232 min^{-1} for the native enzyme to 0.831 min^{-1} for the stabilized enzyme. Similarly, the best crosslinking treatment increased the activation energy for thermal denaturation from 372 $kJ \cdot mol^{-1}$ for the native invertase to 517 $kJ \cdot mol^{-1}$ for the stabilized enzyme. Values of the Michaelis-Menten parameters (K_m and v_{max}) showed a reduced efficiency of invertase after the crosslinking treatment.

The nature of the crosslinking was examined using size exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), dynamic light scattering (DLS) and multiple angle laser light scattering (MALLS). Depending on the conditions used, both intermolecular and intramolecular crosslinking occurred. The estimated molecular weight of the intermolecularly crosslinked invertase appeared to be much higher compared to the intramolecularly crosslinked invertase and the native invertase. In attempts to simplify certain analyses, attempts were made to remove the carbohydrate moiety from crosslinked invertase (a glycoprotein) molecule.

Deglycosylation with PNGase F achieved a significant reduction of carbohydrate for the native invertase but not for the intra- and intermolecularly crosslinked invertase. Circular dichroism (CD) measurements showed that crosslinking with BMDC affected slightly the secondary structure of invertase.

The nature of the crosslinking that might be occurring in invertase molecule was further studied using small model oligopeptides, small nonglycosylated enzymes (hen egg white lysozyme and pepsin) and glycoprotein models (ovalbumin). Crosslinking of the model pentapeptide (0.9 μM) suggested that crosslinking with BMDC involved reaction between BMDC and the amino group of lysine or the carboxylate at C-terminal of the pentapeptide. Using a heptapeptide (1 mM) in crosslinking with BMDC showed a changed hydrophobicity of the crosslinked peptide. The crosslinking treatment of lysozyme (3.5 mM) with BMDC clearly produced an intermolecularly crosslinked lysozyme as evidenced by SEC and SDS-PAGE. A changed net charge of lysozyme after the crosslinking treatment was demonstrated using native PAGE. Mass spectrometry was used to then prove the intramolecular crosslinking of lysozyme with BMDC. CD spectra of the intramolecularly crosslinked lysozyme showed it be more resistant to thermal unfolding relative to native lysozyme.

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Abbreviations

A	The Arrhenius parameter
Asn	Asparagine
Asp	Aspartic acid
BIC	Butyl isocyanate
BMDC	1,4-Diisocyanatobutane or butamethylene diisocyanate
BSA	Bovine serum albumin
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
CD	Circular dichroism
CLECs	Crosslinked enzyme crystals
CPR	Centre for Protein Research, University of Otago, New Zealand
Cys	Cysteine
DA10	1,10-Diaminodecane
DAB	1,4-Diaminobutane
DAD	1,12-Diaminododecane
DAH	1,6-Diaminohexane
DAO	1,8-Diaminooctane
d_f	Dilution factor
DLS	Dynamic light scattering
dm	The surface-to-surface distance between enzyme molecules
DMA	Dimethyl adipimidate
DMP	Dimethyl pimelimidate
DMS	Dimethyl suberimidate
DNS	3,5-Dinitrosalycylic acid
dn/dc	A differential index of refraction
E	Enzyme concentration
E_0	Enzyme concentration at time zero
EA	Ethyl acetimidate
E_d	The deactivation energy
EDA	1,2-Diaminoethane
E_t	Enzyme concentration at time t

Glu	Glutamic acid
HMDC	1,6-Diisocyanatohexane or hexamethylene diisocyanate
k_{cat}	The rate constant
K_{av}	The gel phase distribution coefficient
k_d	The thermal denaturation rate constant
K_m	Michaelis-Menten constant
MALDI-MS	Matrix assisted laser desorption ionisation-mass spectrometry
MALLS	Multi angle laser light scattering
$[M+H]^+$	Molecular ion in positive ionisation mode
$[M–H]^–$	Molecular ion in negative ionisation mode
MW	Molecular weight
MWCO	Molecular weight cut-off
OMDC	1,8-Diisocyanatooctane or octamethylene diisocyanate
PDB	Protein Data Bank
PNGase F	Peptide-N-glycosidase F
R	Gas constant
RCSB	Research Collaboratory for Structural Bioinformatics
R_f	Relative mobility
RI	Refractive index
RP-HPLC	Reverse phase-high performance liquid chromatography
S	Substrate concentration
SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
TEMED	N,N,N′,N′-Tetramethylenediamine
v	Rate of enzymatic reaction
v_f	Fraction of initial activity at time t
v_i	Initial activity
v_{max}	Maximum rate of enzymatic reaction
V_c	Geometric column volume
V_e	Elution volume
V_o	Column void volume