

ABSTRACT

Acid denaturation of *Aspergillus niger* glucoamylase was studied using different conformational probes. Both far-UV CD spectral signal (MRE_{222nm}) and tryptophan fluorescence remained unchanged in the pH range, 7.0–3.0 but decreased significantly below pH 3.0, whereas the ANS fluorescence showed a marked increase below pH 1.5. Maximal changes in the MRE_{222nm} value and the ANS fluorescence were noticed at pH 1.0. The acid-denatured state of glucoamylase at pH 1.0 retained a significant amount of secondary structure as reflected from the far-UV CD spectra but showed a deformed tertiary structure with significant exposure of the nonpolar groups as well as the tryptophan residues as revealed by the increased ANS fluorescence, decreased tryptophan fluorescence and three-dimensional fluorescence spectral signals and increase in the K_{sv} value in acrylamide quenching experiments. The acid-denatured state showed no significant variation in the CD spectral signal throughout the temperature range, 0-100°C. However, a late cooperative transition was observed upon GdnHCl treatment, compared to the native enzyme. All these results suggested that the acid-denatured state of glucoamylase at pH 1.0 represented the molten globule-like state.

Different probes such as far- and near-UV CD spectral signals, ANS binding, Trp fluorescence and three-dimensional fluorescence were used to study halogenol- versus alkanol-induced conformational transitions in the acid-denatured state (pH 1.0) of glucoamylase. These alcohols showed significant retrieval of the protein structure, inducing both secondary and tertiary structural changes, as evident from the increase in the α -helical

content and decrease in the ANS binding, respectively. However, halogenols were found more competent than alkanols, requiring lesser alcohol concentration to induce similar spectral change. The effectiveness of these alcohols showed the order: HFIP > TFE > 2-chloroethanol for halogenols while tert-butanol > 1-propanol > 2-propanol > ethanol > methanol for alkanols. Both Trp fluorescence and near-UV CD spectra showed anomalous pattern, though the order of effectiveness remained the same as found with the far-UV CD and ANS fluorescence results. Three-dimensional fluorescence results of the acid-denatured state (pH 1.0) of glucoamylase in the presence of 5.5 M tert-butanol agreed well with the data obtained from the far-UV CD and Trp fluorescence. All these results suggested the formation of partially folded states of glucoamylase in the presence of these alcohols, being more effective with halogenols than alkanols.

Different spectral probes such as far-UV CD spectral signal, tryptophan fluorescence and ANS fluorescence were employed to study structural changes and stabilizing effect of various polyols *i.e.* ethylene glycol, glycerol, glucose and trehalose on the native, the acid-denatured and the thermal-denatured states of glucoamylase. Addition of polyols to the incubation mixture led to significant structural changes involving both secondary and tertiary structures in the acid-denatured state of the enzyme as reflected from the increase in the MRE_{222nm} value and altered tryptophan and ANS fluorescence characteristics. On the other hand, thermodynamic analysis in terms of T_m , ΔH_{vH} and ΔG (25°C) of the thermal denaturation curves obtained with the native glucoamylase in the presence of various polyols, suggested significant increase in the enzyme stability in the presence of glucose, trehalose and glycerol while ethylene glycol destabilized it. The stabilizing effect was found to be higher with glucose followed by trehalose and minimum

with glycerol. Furthermore, CD and fluorescence spectral characteristics of the thermal-denatured glucoamylase at 71°C obtained in the absence and the presence of polyols showed greater effectiveness of both glucose and trehalose in inducing native-like secondary and tertiary structures compared to glycerol and ethylene glycol.

ABSTRAK

Denaturasi asid terhadap *Aspergillus niger* glukoamilase telah dikaji menggunakan konformasi prob-prob yang berbeza. Kedua-dua, signal spektrum 'far-UV CD (MRE_{222nm})' dan 'tryptophan fluorescence' didapati tidak berubah di dalam julat pH antara pH 7.0–3.0, tetapi kedua-dua signal ini didapati telah menurun dengan ketara pada pH 3.0 dan ke bawah, manakala 'ANS fluorescence' telah menunjukkan peningkatan yang ketara pada pH 1.5 dan ke bawah. Perubahan maksimum pada 'MRE_{222nm}' dan 'ANS fluorescence' telah diperhatikan pada pH 1.0. Signifikan jumlah struktur sekunder yang ditunjukkan oleh spektrum 'far-UV CD' kekal tetapi struktur tertiar telah berubah bentuk dengan pendedahan yang ketara kumpulan-kumpulan hidrofobik 'nonpolar' dan juga residu-residu triptofan seperti yang ditunjukkan daripada peningkatan 'ANS fluorescence', penurunan 'tryptophan fluorescence' dan signal spektrum pada 'three-dimensional fluorescence' serta peningkatan dalam nilai K_{sv} di dalam eksperimen 'acrylamide quenching' pada ternyahasli asid glukoamilase pada pH 1.0. Ternyahasli asid glukoamilase pada pH 1.0 juga tidak menunjukkan variasi yang signifikan dalam signal spektrum CD dalam julat suhu antara 0–100°C. Walau bagaimanapun, 'cooperative transition' di peringkat akhir telah dikesan pada 'GdnHCl treatment' jika dibandingkan dengan enzim asli. Semua keputusan ini telah mencadangkan bahawa ternyahasli asid glukoamilase pada pH 1.0 telah menggambarkan 'molten-globule-like state'.

Prob-prob yang berbeza seperti signal-signal spektrum 'far-' dan 'near-UV CD', 'ANS binding', 'Trp fluorescence' dan 'three-dimensional fluorescence' telah digunakan untuk mengkaji 'halogenol- versus alkanol-induced conformational transitions' pada ternyahasli asid glukoamilase (pH 1.0). Semua alcohol-alkohol ini, menunjukkan perolehan kembali

struktur protein yang signifikan, dengan merangsang kedua-dua perubahan struktur sekunder dan struktur tertiar, seperti yang dibuktikan daripada peningkatan dalam struktur α -heliks dan pengurangan dalam 'ANS binding' masing-masing. Walau bagaimanapun, halogenol didapati lebih cekap daripada alkanol, dengan memerlukan kepekatan alkohol yang lebih kurang untuk merangsang perubahan spektrum yang sama. Keefektifan alkohol-alkohol ini, ditunjukkan dalam susunan berikut: HFIP> TFE> 2-chloroethanol untuk halogenol manakala tert-butanol> 1-propanol> 2-propanol> etanol> metanol untuk alkanol. Kedua-dua spektrum 'Trp fluorescence' dan 'near-UV CD' telah menunjukkan bentuk yang ganjil, tetapi turutan keefektifan adalah sama seperti yang didapati daripada 'far-UV CD' dan 'ANS fluorescence'. Keputusan 'three-dimensional fluorescence' pada ternyahasli asid glukoamilase (pH 1.0) dengan kehadiran 5.5 M tert-butanol juga diperkenan dengan baik dengan data yang diperolehi dari 'far-UV CD' dan 'Trp fluorescence'. Semua keputusan ini telah mencadangkan bahawa pembentukan 'partially folded states' glukoamilase telah diperolehi dengan kehadiran alkohol-alkohol ini, yang mana halogenol lebih efektif daripada alkanol.

Prob-prob spektrum yang berbeza seperti signal spektrum 'far-UV CD', 'tryptophan fluorescence' dan 'ANS fluorescence' telah digunakan untuk mengkaji perubahan-perubahan struktur dan kesan penstabilan pelbagai jenis poliol seperti etilena glikol, gliserol, glukosa dan trehalose ke atas glukoamilase yang asli, ternyahasli asid dan ternyahasli suhu. Penambahan poliol-poliol kepada sebatian pengeraman membawa kepada perubahan struktur yang signifikan melibatkan kedua-dua struktur sekunder dan struktur tertiar pada enzim yang asli dan yang ternyahasli asid seperti yang ditunjukkan oleh peningkatan dalam nilai MRE_{222nm} dan perubahan pada sifat-sifat 'tryptophan' dan 'ANS

fluorescence'. Sebaliknya, analisis termodinamik dari segi T_m , ΔH_{vH} dan ΔG (25°C) pada 'thermal denaturation curves' yang didapati pada glukoamilase yang asli dengan kehadiran pelbagai poliol, telah mencadangkan bahawa peningkatan yang signifikan dalam kestabilan enzim dengan kehadiran glukosa, trehalose dan gliserol manakala etilena glikol telah menidakstabilkan enzim tersebut. Kesan penstabilan yang telah diperolehi adalah lebih tinggi dengan kehadiran glukosa diikuti dengan trehalose dan minimum dengan gliserol. Tambahan pula, ciri-ciri spektrum CD dan 'fluorescence' pada suhu ternyahasli glukoamilase pada 71°C yang diperolehi dalam ketidakhadiran dan kehadiran poliol, kedua-dua glukosa dan trehalose telah menunjukkan keefektifan yang lebih besar dalam merangsang struktur sekunder seperti struktur asli dan struktur tertiar berbanding dengan gliserol dan etilena glikol.

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LIST OF ABBREVIATIONS / SYMBOLS

Ala	Alanine
ANS	1-Anilinonaphthalene-8-sulfonic acid
Arg	Arginine
Asn	Asparagine
BSA	Bovine serum albumin
CB	Cyanogen bromide fragment
CD	Circular dichroism
cm	Centimeter
C_m	Midpoint concentration
CNBr	Cyanogen bromide
CO	Carbonyl group
cP	Centipoise
C_p	Molar concentration
Cys	Cysteine
D	Denatured state
3-D	Three-dimensional
DEAE	Diethylaminoethyl
deg	Degree
dmol^{-1}	per Decimole
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry

E_m	Emission maximum
Eq.	Equation
F	Fluorine atom
F_0	Fluorescence intensity in the absence of the quencher
F_0/F	Fluorescence intensity ratio in the absence and presence of the quencher
F_D	Fraction denatured
FI	Fluorescence intensity
GA	Glucoamylase
GdnHCl	Guanidine hydrochloride
H-bonds	Hydrogen bonds
HCl	Hydrochloric acid
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
K	Equilibrium constant
kcal	Kilocalorie
KCl	Potassium chloride
kDa	kilo Dalton
K_{sv}	Stern-Volmer constant
l	pathlength of the cuvette
Ltd	Limited
M	Molar
M^{-1}	per Molar
mg	Milligram
ml	Milliliter
mm	Millimeter

mM	Millimolar
mol ⁻¹	per Mole
MRE	Mean residue ellipticity
mRNA	Messenger RNA
N	Native state
<i>n</i>	Number
NAG	N-Acetylglucosamine
NATA	N-Acetyl-L-tryptophanamide
NH	Imino group
nm	Nanometer
NMR	Nuclear magnetic resonance
OH	Hydroxyl group
PDB	Protein data bank
[Q]	Quencher concentration
<i>R</i>	Gas constant
RNA	Ribonucleic acid
S	Svedberg unit
s ⁻¹	per Second
S _{20,w}	Sedimentation coefficient
SBD	Starch binding domain
Ser	Serine
<i>T</i>	Temperature
tert	Tertiary
TFE	2,2,2-Trifluoroethanol

Thr	Threonine
T_m	Midpoint temperature
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultra violet
w/v	Weight per volume
Y	Observed variable parameter
Y_D	Value of the variable characteristic of the denatured state
Y_N	Value of the variable characteristic of the native state
α	Alpha
β	Beta
ΔC_p	Heat capacity change
ΔG	Gibbs free energy change
ΔG_D	Gibbs free energy in the presence of the denaturant
$\Delta G_D^{H_2O}$	Gibbs free energy in the absence of the denaturant
ΔG_H	Gibbs free energy in the presence of the alcohol
ΔH	Enthalpy change
ΔH_m	Enthalpy change at midpoint temperature
ΔH_{vH}	van't Hoff enthalpy change
ΔMRE_{222nm}	Change in the mean residue ellipticity at 222 nm
ΔS	Entropy change
ΔS_m	Entropy change at midpoint temperature
θ_{obs}	Observed ellipticity
λ_{em}	Emission wavelength

λ_{ex}	Excitation wavelength
μm	Micrometer
μM	Micromolar
π	Pi