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Biochimica et Biophysica Acta 1339 (1997) 289–296

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Characterisation of a xylanolytic amyloglucosidase of *Termitomyces clypeatus*

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Received 11 October 1996; revised 2 January 1997; accepted 9 January 1997

Abstract

A xylanolytic amyloglucosidase of *Termitomyces clypeatus* was characterised with respect to other amyloglucosidases. The enzyme contained high α-helix destabilising amino acids but no sulphur amino acid. It contained high threonine and serine, analogous to other raw starch hydrolysing enzymes. Both xylanase and amyloglucosidase activities were gradually lost with the progress of tryptophan oxidation by NBS and total inactivation occurred after oxidation of 4–5 tryptophan residues. In the presence of substrates (either starch or xylan), complete inactivation of either activities was not noticed even after oxidation of 7.7 mol of tryptophan residues. Inactivation by HNBB was not possible in the absence of any denaturant. Only 4.9 mol of tryptophan could be modified in the presence of 5 M urea which resulted in only 42% inhibition of activity. Thus modified enzyme had higher V_m/K_m and lower pH optima in comparison to those of native enzyme. It was suggested that tryptophan was present at the substrate binding site and not at the active site. No such change in activity was noticed after modification of tyrosine, lysine or arginine residues. HPGPLC analysis of both dilute and concentrated enzyme solution indicated that the enzyme existed as an equilibrium mixture of protomer-oligomer. Perhaps for this reason molar mass of NAI modified enzyme appeared to be almost half of that modified by NAI in presence of substrate. Arrhenius plot of the enzyme also indicated reversible oligomerisation as a function of temperature.

Keywords: Amyloglucosidase; Xylanase; Tryptophan; Oligomerisation; (*Termitomyces clypeatus*)

1. Introduction

Xylan, the major hemicellulosic polysaccharide of agrobiomass, has β-1,4-linked xylopyranosidic backbone substituted by different sugars [1]. The common substituents on this heteropolysaccharide are acetyl, arabinosyl, and glucuronosyl residues. The composition of the substituents on the branches also vary from source to source. Larch wood xylan contained similar backbone of β-1,4 linked D-xylopyranosyl residues with every fifth or sixth xylose residue substituted at C_2 with 4-0-methyl-D-glucuronic acid and at C_3 with arabinofuranosyl units; none of the xylosyl residues have more than one branch [1,2]. Glucose is also reported to be present in xylans [3–5]. Commercially available xylan (oat spelt) is also declared to contain glucose as per the manufacturer's specification (Sigma Chemical Co., USA). In this context it may be mentioned that a variety of

Abbreviations: HNBB, 2-hydroxy-5-nitrobenzyl bromide; HPGPLC, high performance gel permeation liquid chromatography; NAI, 1-acetylimidazole; NBS, N-bromosuccinimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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PII S0167-4838(97)00011-3

carbohydrase enzymes have been reported to be involved for the total saccharification of xylan [6]. But it is not known whether any amyloglucosidase could attack and subsequently liberated free glucose from this heteropolysaccharide(xylan).

An amyloglucosidase (EXO, 1 → 4-α-D-glucosidase, EC 3.2.1.3) was purified earlier from the culture filtrate of *Termitomyces clypeatus*, which liberated glucose from different xylans [7,8]. Although amyloglucosidases purified from different sources sometimes differ among themselves in terms of their mode of actions on raw starch [9,10], activity of any amyloglucosidase on xylan was possibly not reported earlier. In this context, the enzyme from *T. clypeatus* was characterized, and compared with other amyloglucosidases with respect to amino acid make-up, chemical modification of probable catalytic site and reversible oligomerisation of the enzyme, as reported earlier [7].

2. Materials and methods

2.1. Materials

Most of the biochemicals and substrates for enzyme assay were obtained from Sigma Chemical Co. (USA). Amino acid standard and other reagents for amino acid analysis were obtained from Pierce, USA. All other chemicals used were obtained from local suppliers.

2.2. Methods

2.2.1. Enzyme assay

Amyloglucosidase or xylanase activity was measured in an assay mixture (0.4 ml) containing 2 mg soluble starch or larch wood xylan (Lot No. 116C-0104-1, Sigma, USA), according to the methods described earlier [7]. Purification of the enzyme from the culture filtrate was also reported earlier [7].

Protein was determined according to Lowry et al. [11] using bovine serum albumin as standard.

2.2.2. Reaction of the enzyme with different chemical modifiers

Modification by NBS. Modification of the enzyme by NBS (Aldrich, USA) was made at 0–4°C according to Spande and Witkop [12]. The enzyme solution (0.125 ml) contained 0.53 μg protein (for xylanase assay) or 0.27 μg protein (for amyloglucosidase assay), in buffer A (100 mM sodium acetate, pH 5.0) and the solution was incubated in the presence of various amounts of modifier for different time periods. Termination of chemical modification as well as initiation of enzyme reaction was made by adding 2 mg of xylan or starch in 0.275 ml buffer A and respective residual activities were measured. The pseudo-first order rate constants (k') were obtained from the slopes of the best fit plots of logarithm of the residual activity against time of reaction. Number of tryptophan residues modified for 100% inactivation of enzyme activity was calculated from the slopes of the plots of logarithm of pseudo-first-order rate constants (k') against logarithm of concentrations of the inhibitor [13].

Titration of enzyme with NBS. Oxidation of tryptophan residues by NBS either in presence or in absence of starch or xylan (0.8 mg/ml) was carried out at 25°C using 2.274 μM enzyme solution in buffer A. Successive 5 μl aliquots of NBS solution (2 mM or 5 mM) were added to the samples as well as to the reference cuvettes. Absorbance at 280 nm was measured after each NBS addition and corrected for dilution. Number of tryptophan residues oxidised per mol of enzyme (n) was calculated from the Eq. [12]:

$$n = \frac{1.31 \times \text{ decrease in absorbance at 280 nm}}{5500 \times \text{ molarity of enzyme}}$$

Aliquots (5 μl) of modified enzyme solution were withdrawn immediately after absorbance measurement, diluted 10-fold and residual activities were measured.

Modification by HNBB. This was made according to Horton and Khosland Jr. [14] with some modifications. Solid urea (300 mg) was added to 1.0 ml enzyme solution (0.3 mg protein in buffer A, pH 4.0) and the solution was left at 30°C for 30 min. To this solution 0.05 ml of 50 mM HNBB (Aldrich, USA) in dioxane, was added, and the mixture was kept at 30°C for additional 30 min. Amount of urea and dioxane used were found to be ineffective towards enzyme activity. Following HNBB treatment the enzyme solution was extensively dialysed against buffer A. Tryptophan residues modified were estimated by

measuring (at pH 12.5) absorbance at 410 nm ($\in_m = 18\,900\ M^{-1}cm^{-1}$).

Modification by NAI. Modification by NAI, both in the presence or in the absence of substrate (soluble starch, 2 mg/ml), was made in duplicate according to Riordan et al. [15]. Enzyme solution was brought to pH 7.5 by dialysing against 50 mM Tris-HCl (pH 7.5) buffer. Solid NAI (Aldrich, USA), 10 mg, was added to 0.2 ml enzyme solution (60 μg protein) when pH of the resulting solution dropped to 6.5. The mixtures were incubated at 27°C for 2 h and immediately analysed by HPGPLC. Active fractions were collected, pooled and residual activity was measured. The control tube (100% activity) contained the same amount of untreated enzyme solution.

2.2.3. HPGPLC analysis and molecular weight determination

High performance gel permeation liquid chromatography (HPGPLC) was made using Ultropac TSK G2000 SW, 0.75×60 cm column, fitted in series, with a precolumn, Ultropac TSK SWP, 0.75×7.5 cm (Pharmacia, Sweden). Protein peaks were identified as absorbance at 280 nm, A_{280} (model 484) and were analysed from the peak area obtained from the 745B data module using attenuation, AT = 256 (Waters, USA). Molecular weights were approximated from a plot of log of molecular weight versus K_{av} values of standard proteins according to the technical bulletin provided by the supplier (Pharmacia, Sweden). Mobile phase used, at a flow rate of 1 ml/min, was buffer A.

2.2.4. Amino acid analysis

Amino acid analysis was performed in a PICO.TAG system according to the PICO.TAG operation manual (Waters, USA). Enzyme protein (20 μg) was extensively dialysed overnight against deionised distilled water, dried and was hydrolysed by 6 N HCl containing 1% phenol for 22 h at 105°C in the PICO.TAG work station. Hydrolysis was carried out at vapour phase. Hydrolysed sample and standard amino acid mixture, standard H (0.005 ml), were taken in respective tubes, introduced into the reaction vial and were dried completely. These were then derivatized by phenyl isothyocyanate (PITC) solution (ethanol:triethylamine:water:PITC, 7:1:1:1, by volume) for 20 min at 25°C. Then the vials were dried and samples were reconstituted in diluent solution (Na_2HPO_4 0.071%, w/w, pH 7.4 containing acetonitrile 5%, v/v). The samples were analysed by HPLC at 38°C as per PICO.TAG manual. Detector setting, chart speed and run time were AT 128 at 254 nm, 2 cm/min and 12 min respectively. Amino acids present in unknown sample was determined quantitatively by comparing the peak areas (745B data module print out) of amino acids present in standard H. The number of residues were determined on the basis of molecular weight of 56 240 and carbohydrate content 7.75% (w/w) [7].

Since the above method did not allow estimation of tryptophan content, it was determined spectrophotometrically [16]. Enzyme solution (1 ml, 0.2 mg protein) was prepared in 20 mM phosphate buffer (pH 6.5), containing 6.0 M guanidine hydrochloride and molar extinction coefficients at 280 nm (ϵ^M_{280}) and 288 nm (ϵ^M_{288}) were determined. Number of tryptophan (N_{trp}) was determined using the equation:

$$N_{trp} = \left(\epsilon^M_{288} \div 3103\right) - \left(\epsilon^M_{280} \div 10\,318\right)$$

ϵ^M was also recorded at 295 nm and 300 nm at this pH (6.5) and also at pH 12.5 for estimating tyrosine content. Number of mol of tyrosine (M_{tyr}) was determined from the equations:

$$M_{tyr} = \left(\epsilon^M_{295}\ \text{at pH}\,12.5 - \epsilon^M_{295}\ \text{at pH}\,6.5\right) \div 2480 \text{ and}$$

$$M_{tyr} = \left(\epsilon^M_{300}\ \text{at pH}\,12.5 - \epsilon^M_{300}\ \text{at pH}\,6.5\right) \div 2270$$

and average was represented (which did not vary more than 5%).

Cysteine content was additionally determined colorimetrically after reduction with sodium boro hydride using 5,5′-dithiobis-2-nitrobenzoic acid, Ellman's reagent (DTNB) in presence of 8.0 M urea [17]. Control tubes containing cysteine (50 nmol) or bovine serum albumin (0.025 mg) and the enzyme sample containing 0.1 mg protein were taken in 2 ml reaction mixture, colour was developed and were read at 412 nm. Number of cysteine residues present was:

$$(\text{MW} \times A \times V) \div (12\,000 \times m)$$

when MW was molecular weight of the protein, A was absorbance at 412 nm, V was final volume and m was mg protein used.

2.2.5. Determination of optimum pH for enzyme activity

Enzyme activities for native and HNBB modified enzymes were measured as a function of pH. Buffers (0.1 M) used were KCl-HCl (pH 2.0), glycine-HCl (pH 2.5–3.5), sodium acetate (pH 4.0–5.5) and sodium phosphate (pH 6.0–7.0).

2.2.6. Fluorescence emission spectra of T. clypeatus enzyme

Emission spectra of enzyme plus substrate mixture, native enzyme, HNBB-modified enzyme and NBS-inactivated enzyme were made. Modification and subsequent removal of excess modifiers (NBS or HNBB or starch, 0.8 mg/ml) were made by HPG-PLC. Excitation wavelength was fixed at 280 nm and emission wavelength ranged between 300 and 400 nm. Concentration of enzyme protein was 96 μg/ml. NBS modified enzyme was totally inactivated enzyme obtained by using 37 mol of NBS per mol of enzyme protein.

3. Results and discussion

None of the α-amylases isolated from either *B. subtilis* or *A. oryzae* or the amyloglucosidase of *A. niger* could hydrolyse xylan, while *T. clypeatus* amyloglucosidase liberated free reducing group as well as free glucose from both starch and xylan in parallel [7]. Optimum pH and temperature for the hydrolysis of either starch or xylan appeared to be same [7]. So hydrolysis of starch and xylan were catalysed by the same enzyme protein. The enzyme could liberate glucose from starch, maltose and pannose but could not hydrolyse isomaltose, melibiose, etc. So this enzyme appeared to be cleaving mostly α-1,4 linked D-glucopyranosyl residues. In this context it may be mentioned that glucose and arabinose are reported to be present in the side chains of xylan isolated from bagasse [5]. Moreover, larch wood xylan is declared to contain glucose (Sigma, USA). Active site topology of amyloglucosidase recognises only substrates containing large number of available side chains and not fibrous substrates with almost no chain [18]. Xylan is a highly branched hetero-polysaccharide with varying degree of substitution at the branches [1,2]. Consequently it was not unusual since this enzyme could liberate glucose from xylan. However, presence of any α-1,4-linked glucose residue in xylan is not clearly established.

Table 1
Amino acid composition of *T. clypeatus* enzyme

Residue	Content	Residue	Content	Residue	Content
K	10	Z[4]	40	M	0
H	4	P	5	I	11
R	9	G	33	L	21
B[3]	36	A	34	Y[1]	12
T	34	C[2]	0	F	12
S	43	V	19	W[1]	10

Amino acid composition was determined as stated in Section 2. Tyrosine[1] and tryptophan[1] contents of the enzyme protein were also determined spectrophotometrically and cysteine[2] content was additionally determined calorimetrically.
B[3], aspartic acid or asparagine.
Z[4], glutamic acid or glutamine.

3.1. Amino acid composition

The enzyme contained relatively high proportions of aspartate/asparagine, threonine, serine, glutamate/glutamine, glycine, alanine and low proportions of histidine, proline and had no sulfur-containing amino acids (Table 1). So it contained high α-helix destabilizing amino acids in comparison to others [9]. The appearance of threonine-serine rich region (TS-region) in the polypeptide backbone of amyloglucosidase has been shown to be responsible for adsorption and digestion of raw starch [19]. The present enzyme also contained 34 threonine and 43 serine residues which is around 10% and 13% of total amino acid content. So xylanolytic activity of this amyloglucosidase could be because of relative abundance of threonine and serine content. Absence of any sulfur containing amino acid in this enzyme was similar to that only known amyloglucosidase purified from *Endomycopsis* species [9]. Both enzymes had identical molecular weight but the *Endomycopsis* enzyme contained a relatively lower amount of threonine and also it is not known whether it could hydrolyse xylan.

3.2. Chemical modification of the amyloglucosidase

Modification with NBS. A mixture of starch or xylan and NBS solution showed an optical density equivalent to that of starch or xylan alone, indicating

that NBS did not react with starch or xylan. Kinetics of inactivation of this enzyme by NBS was very fast at 25°C and so this was studied at 0–4°C which was similar to that reported for an α-amylase and a rhodanase [12]. The inactivation of this enzyme by NBS (2.25–3.00 μM), measured in terms of either amyloglucosidase or xylanase activities, was found to be dependent on both time and NBS concentration. The plot of log of residual activities versus time was always linear, indicating that the inactivation followed first order kinetics (Fig. 1a,b). Double-logarithmic plots of the observed pseudo-first order rate constants against reagent concentration also appeared to be linear (Fig. 1a,b, insets). So loss of these two activities could be due to oxidation of tryptophan residues present in the same enzyme protein. Complete inactivation of either amyloglucosidase or xylanase activity was due to oxidation of around 5 tryptophan residues, as determined from the slopes of the plots (4.7, amyloglucosidase and 5.1, xylanase). This was further supported by the results obtained from the titration curves (Fig. 2a,b). It appeared that oxidation of about 4 or 5 mol of tryptophan residues per mol of enzyme was associated with complete inactivation of both amyloglucosidase and xylanase activities. Perhaps tryptophan was not present in the catalytic site since tryptophan oxidation vis à vis loss of activity appeared to be sequential. Both xylan and starch almost identically protected the enzyme from NBS-inactivation. Substrate protection (by either xylan or starch) from NBS-inactivation suggested involvement of tryptophan residues at the substrate binding site of this enzyme (Fig. 2a,b). It may be mentioned here that interaction between substrate and tryptophan was suggested to stabilize the transition state during enzymatic hydrolysis of glycosidic bond [18,20].

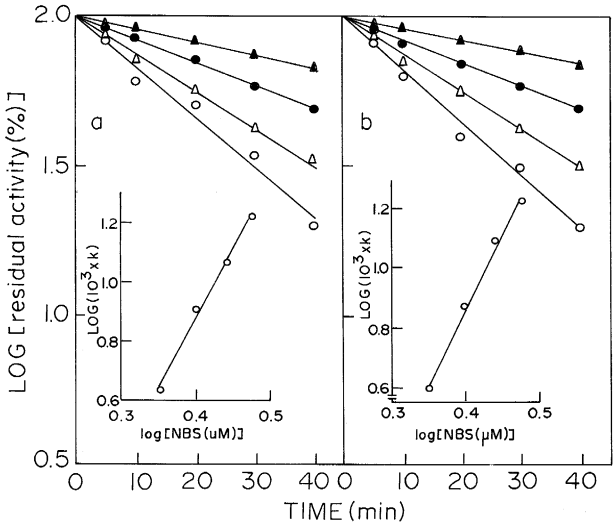

Fig. 1. Kinetics of *T. clypeatus* enzyme inactivation by NBS. Enzyme solution was preincubated with NBS, μM (▲ 2.25; ● 2.5; △ 2.75; ○ 3.00) following measurement of residual activity in terms of either amyloglucosidase (a) or xylanase (b) as described in Section 2. The inset shows apparent order of reaction with respect to NBS concentration.

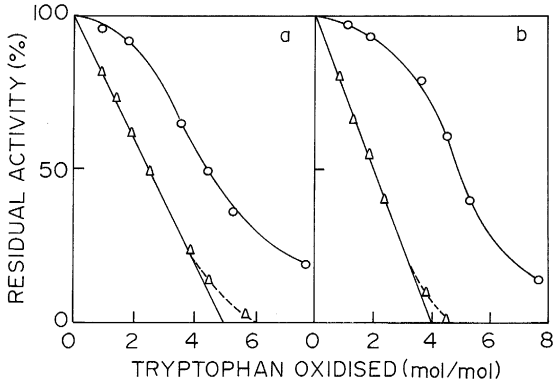

Fig. 2. Titration of *T. clypeatus* enzyme with NBS. Oxidation of tryptophan residues present in amyloglucosidase following measurement of residual activities were made both in the absence (△) or in the presence (○) of substrates, soluble starch (a) or xylan (b) as described in Section 2.

Besides tryptophan oxidation, NBS could occasionally modify tyrosine, methionine, cysteine, lysine or arginine residues [12]. But these amino acids were not involved since the enzyme contained no sulfur containing amino acids and NAI appeared to be almost ineffective. Moreover, phenyl glyoxal did not affect enzyme activity much, as reported earlier [7].

Modification of tryptophan residues by HNBB. The presence of tryptophan residues at the substrate binding site of amyloglucosidase has been reported from the difference spectra studies by others [21]. On the contrary, the presence of tryptophan at the active site of fungal enzymes is also proposed [21,22]. This was checked by HNBB modification which is a more specific reagent for tryptophan. HNBB appeared to be ineffective in the absence of any denaturant. About 98% of enzyme activity was retained even in the presence of 10 mM HNBB. So modification was

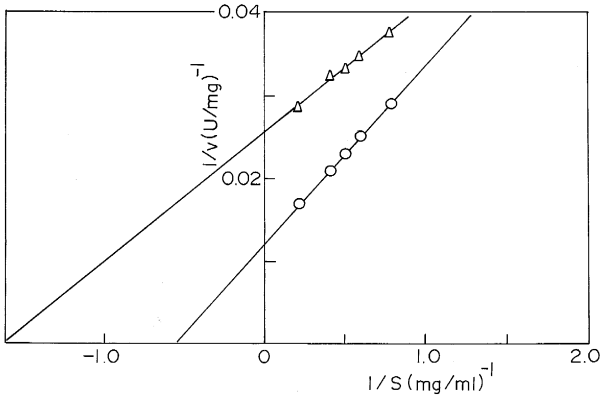

Fig. 3. Lineweaver-Burke plot. Activity of native (○) and HNBB modified (△) *T. clypeatus* enzyme were measured as a function of substrate concentration (soluble starch 1.0–5.0 mg/ml) as described in Section 2. The plots were obtained using regression analyses to determine the slopes of the best fitting lines.

made in the presence of 5 M urea, as this was the optimum concentration of the denaturant causing no change in activity. Tryptophan residues modified under this condition was 4.9 mol per mol of enzyme protein with only 42% inhibition of enzyme activity. Optimum pH for the activity of HNBB modified enzyme was found to be shifted to 4.6 from 5.2 (that of native enzyme) (not shown). About 23% and 42% lowerings of enzyme activity in case of modified enzyme was noticed at pH 4.6 and 5.2 respectively, in comparison to the native enzyme. K_m and V_m values for HNBB modified enzyme were found to be 0.614 mg/ml and 39.0 μmol/min/mg and for native enzyme 1.81 mg/ml and 82.12 μmol/min/mg respectively as determined from a Lineweaver-Burke plot (Fig. 3). Since V_m/K_m value for HNBB modified enzyme (63.5) was higher than the native enzyme (45.4), modified enzyme might be considered to be more efficient. Lowering of pH optima in the case of HNBB modified enzyme was possibly due to the introduction of an acidic hydroxy nitrobenzyl group into the 2-position of the tryptophan residue.

3.3. UV and fluorescence spectrum

UV-spectrum (245–345 nm) of both native and HNBB modified enzyme indicated absorption maxima (λ_{max}, nm) of 279.6 and 264.7 respectively. The fluorescence properties of both native and modified enzymes indicated that all had characteristic tryptophan peak maxima at 340 nm (not shown). Relative fluorescence intensities, measured at peak-maxima (340 nm) were 435.0 for enzyme plus starch solution, 261.4 for pure enzyme solution, 236.6 for HNBB-modified enzyme and only 1.13 for NBS-inactivated enzyme. Increments in relative intensity for a solution containing enzyme substrate complex indicated exposure of buried tryptophan residues during substrate binding. Perhaps for this reason more tryptophan residues were oxidised by NBS in the presence of substrate (Fig. 2a,b). Decrease in fluorescence intensities for both NBS- and HNBB-modified enzymes also supported modification of tryptophan residues by these reagents.

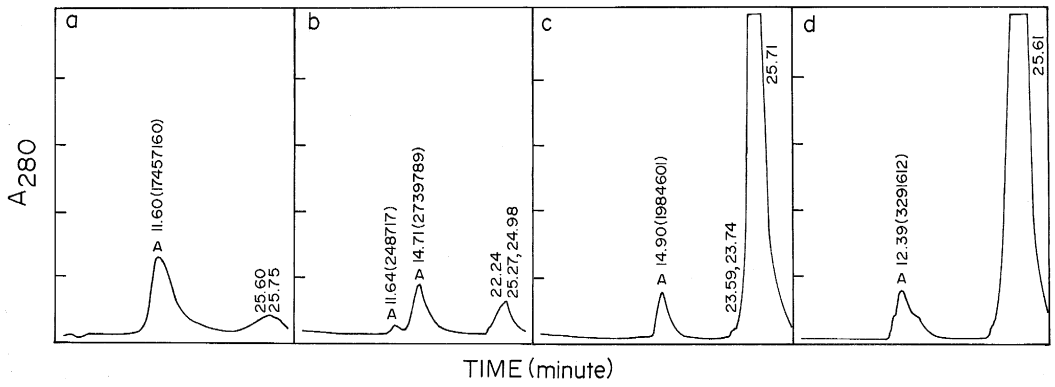

Fig. 4. HPGPLC analysis of amyloglucosidases. HPGPLC and NAI modification methods are described earlier. Samples (0.2 ml) applied were (a) native enzyme, 0.2 mg; (b) native enzyme, 0.06 mg; (c) NAI modified enzyme, 0.06 mg; (d) enzyme 0.06 mg protected by substrate from NAI modification. Active peaks were marked by 'A'. Figures adjacent to the peaks are respective elution time and those in brackets were areas as obtained from 745B data module. Peaks eluting 23 min onwards were unknown peaks.

Modification by NAI. The enzyme appeared to be less sensitive towards tyrosine modification in the absence of any denaturant which is analogous to another report [21]. In comparison to the control (Fig. 4b, 11.64 min and 14.71 min peak areas), an approx. 34% decrease in peak area (Fig. 4c, 14.9 min) was noticed after acetylation of this enzyme protein. On the other hand, only a 10% increase in peak area (Fig. 4d, 12.39 min) was noticed for a substrate-protected sample. Considering total tyrosine content to be 12 (Table 1), around 4 tyrosine residues were modified (since there was 34% decrease in peak area) under this reaction protocol. Activity of this enzyme was found to be affected very little after NAI modification. Only 20% of the activity was lost after NAI modification while it increased by 8% under similar conditions when the enzyme modification was protected by substrate. Why only 34% decrease in peak area was noticed after NAI modification (Fig. 4c) is not clear at this moment, but identical observations were reported for a carboxypeptidase and bovine serum albumin [15].

3.4. Reversible oligomerisation of the enzyme protein

Most enzymes exist as oligomers or protomers and many of them can reversibly dissociate or reassociate in response to effector ligand [23]. Molecular weight of *T. clypeatus* enzyme was determined to be 56.24 kDa and 104.7 kDa by gel filtration and SDS-PAGE, respectively [7]. This was possibly because of dilution favouring dissociation of enzyme oligomer during gel filtration. Since this enzyme contained a higher proportion of α-helix-destabilising amino acids (Table 1) in comparison to others [9], it might exist as an equilibrium mixture of more than one form depending upon external physicochemical conditions, e.g., pH, protein concentration, temperature, etc. Consequently a dilute sample of this enzyme protein appeared to be smaller in size (Fig. 4b, 14.71 min $\approx$ 60 kDa, 91.7% and 11.64 min, only 8.3% larger size), while 3.3 times concentrated sample was larger and was eluted in void volume (Fig. 4a, 11.6 min). Oligomerisation of this enzyme protein was possibly due to intermolecular hydrogen bonding and so acetylation of this enzyme protein by NAI resulted in lowering of size (Fig. 4c, 14.9 min $\approx$ 51 kDa). On the other hand when protected by substrate, the same modification could not decrease its size and so it was eluted in void volume in a broad peak (Fig. 4d, 12.39 min). Elution in a broad peak could be due to the presence of more than one form in equilibrium with another.

3.5. Catalytic activity of the enzyme as a function of temperature

If an enzyme exists in two forms in equilibrium with one another and both forms are active but having different activation energies (E_a), then a sharp discontinuity is expected when the effect of temperature on the change in activation energy is large. An Arrhenius plot of this enzyme indicated the presence of two active forms and a sharp discontinuity was noticed at 17.7°C (Fig. 5). Since activation energy at 20–40°C was around 4.5 times higher than that at 5–15°C, it is expected that at higher temperature

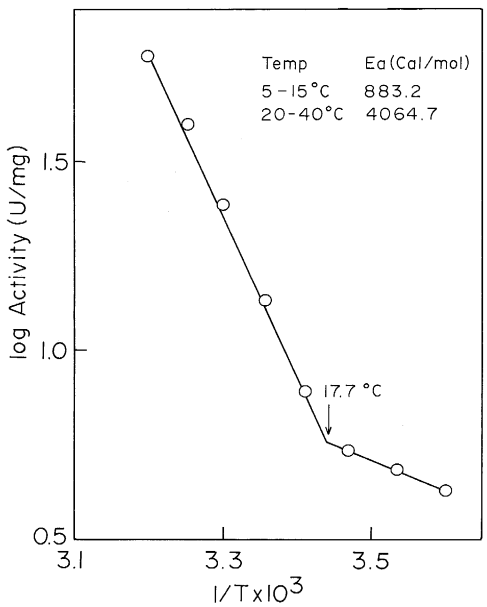

Fig. 5. Glucoamylase activity as a function of temperature (Arrhenius plot). Activity was measured as stated in methods using assay time, 15 min at 40°C, 30 min at 30–35°C, 60 min at 20–25°C and 120 min at 5–15°C. Values were obtained from the average of two sets of duplicates ± standard error of mean (represented by error bar). The energy of activation (E_a) values were calculated from the slope of the plots and are shown in the inset. The activation energy (cal/mol) of the enzyme was determined from the slopes as 883 at 5–15°C and 4065 at 20–40°C respectively.

change in enthalpy (ΔH) or entropy (ΔS) will be higher. So the presence of the monomeric form is expected at a higher temperature and oligomerisation is favoured at a lower temperature. An almost identical explanation has been reported from the studies on fumarase [24].

Thus it can be concluded that perhaps larch wood xylan (Sigma, USA) contained α-1,4 linked glucopyranosides. The *T. clypeatus* amyloglucosidase is not very uncommon in comparison to other amyloglucosidases with respect to the presence of tryptophan residues at the substrate binding site. The absence of sulfur-containing amino acids in this enzyme cannot be given serious importance as it is not known whether a similar enzyme from *Endomycopsis* sp. could hydrolyse xylan. However, the absence of sulfur-containing amino acids is very important for this enzyme since it existed as an equilibrium mixture of protomer-oligomer.

Acknowledgements

We thank Mr. H.N. Dutta of our Institute for making all the line drawings.

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