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II. DENATURATION BEHAVIOR OF FUNGAL AND THERMOSTABLE-BACTERIAL BETA-GLUCOSIDASES

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INTRODUCTION

The purpose of this study was to establish an understanding of the unfolding (denaturation) process of important beta-glucosidases on a quantitative level, so that the effectiveness of chemical crosslinking methods may be evaluated. The work of Durand (1984), Duff (1986) and others confirms that fungal beta-glucosidases are more sensitive to thermal denaturation than the filter paper degrading enzyme activities. In order to establish an understanding of the unfolding process of important beta-glucosidases on a quantitative level - so that the effect of crosslinking the enzymes could be accurately measured - the enzymology team collected data on the thermal denaturation of three industrially important beta-glucosidases, using two types of direct physical measurement, differential scanning microcalorimetry (DSC) and wavelength/temperature scanning fluorimetry, in addition to classical activity measurements of the denaturation process.

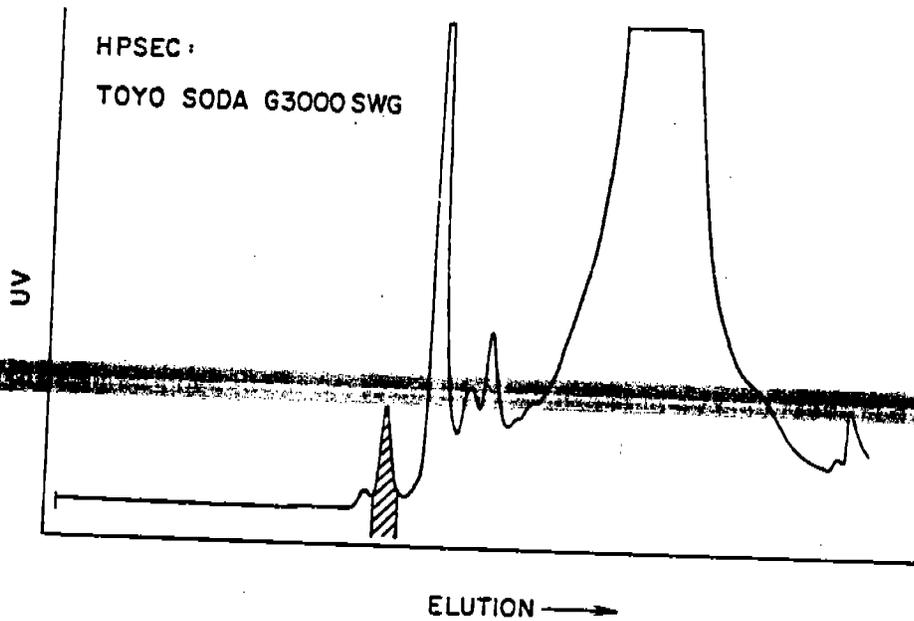
EXPERIMENTAL (CONDENSED)

Aspergillus niger beta-glucosidase was purified from commercial NOVO Sp188 concentrate by high performance size exclusion chromatography at 25 C in 100 mM phosphate buffer, pH 6.5, with 100 mM NaCl. Following HPSEC the active fraction (determined by standard methods using p-NPG as substrate) was dialyzed against the ion-exchange starting buffer, 100 mM phosphate, pH 6.5. The two active fractions were eluted from HPIEC with a 4-hour, 0.22 to 0.32 M NaCl gradient (See figure 1). Beta-glucosidases I and II were found to co-elute from an analytical HPSEC column as symmetrical peaks at an elution volume corresponding to 170,000 daltons. SDS-PAGE showed both enzymes migrate as single, identical bands (data not shown).

The cytosolic beta-glucosidase from Acidothermus cellulolyticus proved to be a much more difficult to purify than the fungal enzymes. A 500-gram (wet wt) cell pellet was subjected to a four step purification procedure, including preparative HPSEC on TSK 3000 SWG, rechromatography using the same column, HPIEC using a Pharmacia Mono-Q HR 5/5 with a 0 - 0.14 M NaCl gradient (the enzyme elutes at 0.08 M NaCl), followed by a final HPIEC step with a shallower gradient (0.07 - 0.15M NaCl) (data not shown). The yield from this purification procedure was very low, approximately 0.5 mg enzyme from 500-gram starting cell cake. Purity of final preparation was also found by HPSEC.

Fluorescence measurements were made using a Spex Fluorolog 2 spectrofluorometer, with data acquisition and processing on a Spex DM1B Spectroscopy Laboratory Coordinator. Sample temperature was controlled by circulation of heated water through the cell holder, and monitored by means of a thermocouple immersed in the stirred sample solution. Excitation wavelength was fixed at 289 nm with fluorescence monitored by scanning from 310 to 410 nm.

NOVO SP188



DIALYSIS

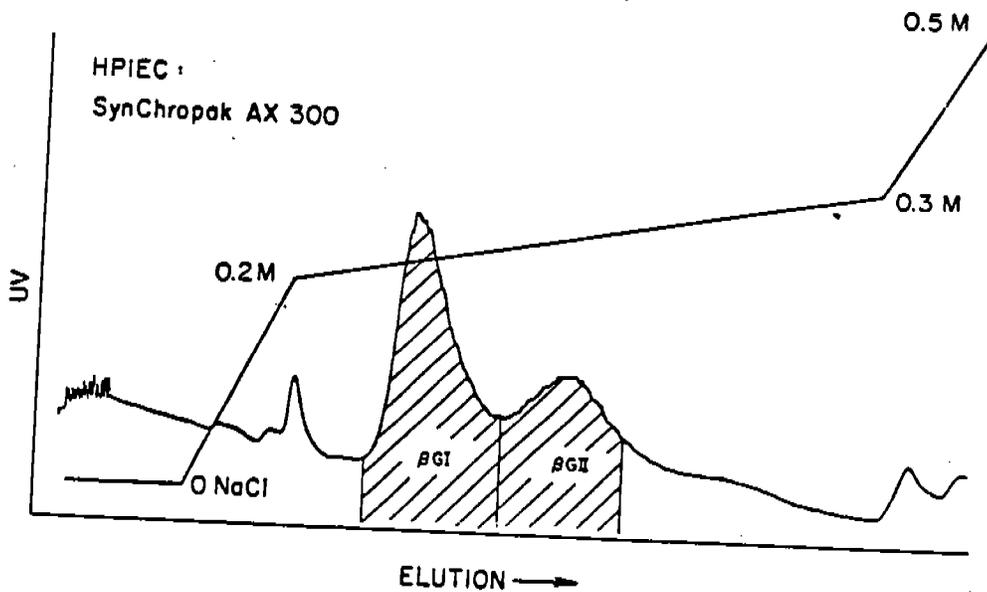


Figure 1. Purification of beta-glucosidase from Aspergillus niger.

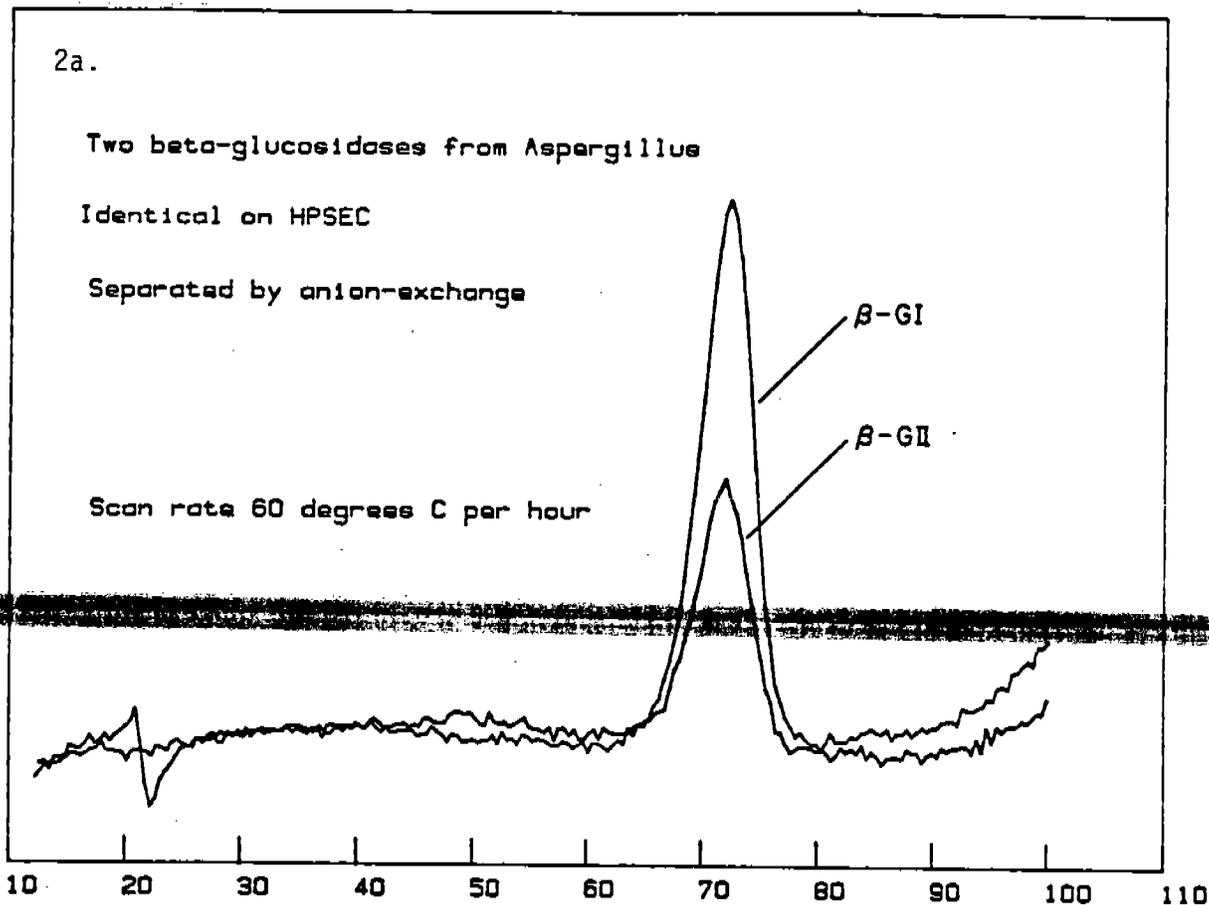
DSC measurements were made using a Microcal MC2 scanning differential microcalorimeter. Samples were examined in 40 mM acetate, pH 5, with 100 mM NaCl.

RESULTS AND DISCUSSION

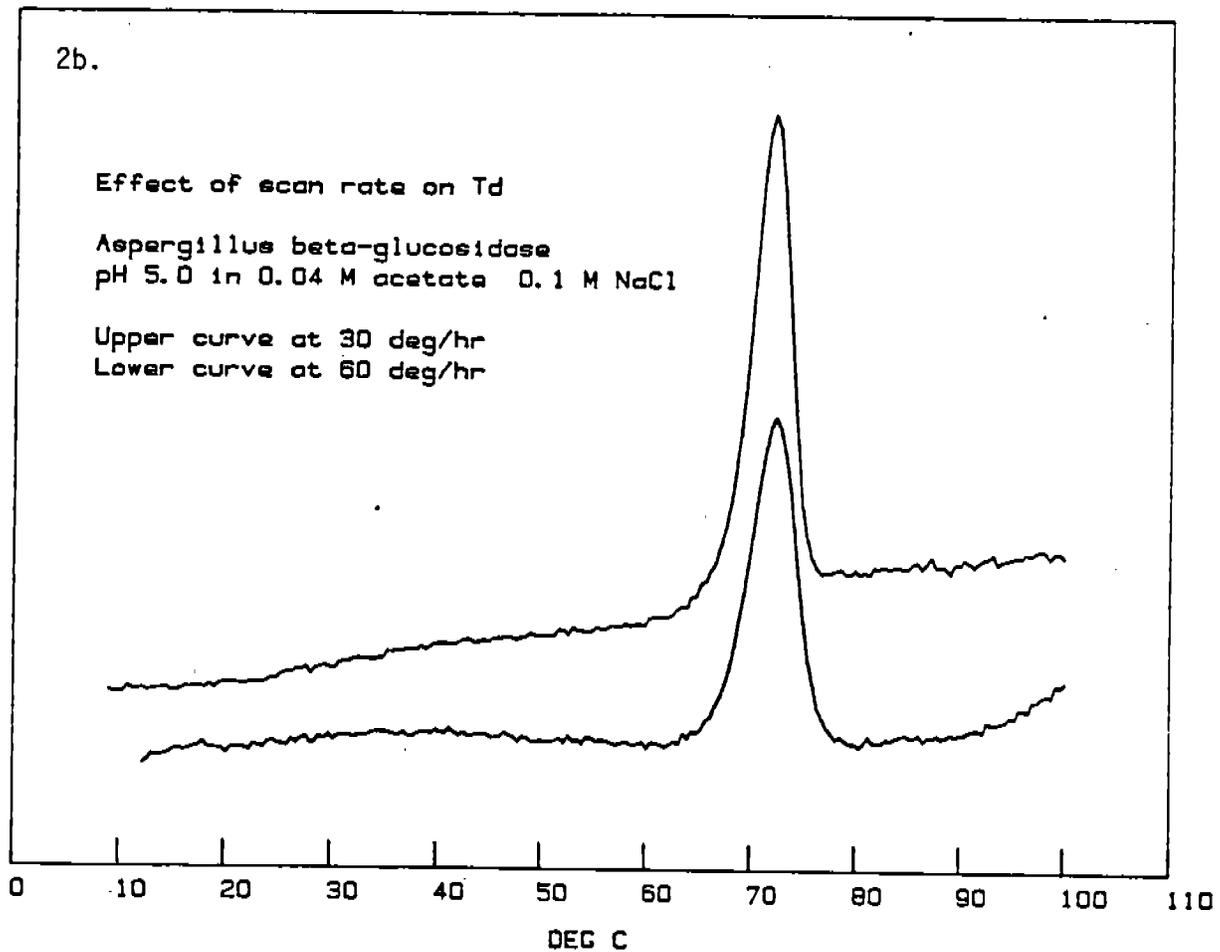
DSC thermograms were obtained for the thermal denaturation of Aspergillus and Trichoderma beta-glucosidases purified in this laboratory from commercial preparations NOVO Sp188 and Genencor 150 L, respectively, as well as for an enzyme purified from the SERI-isolated thermophilic bacterium, Acidothermus cellulolyticus. From the DSC curves taken at a scan rate of 30 degrees C per hour, at pH 5 in acetate buffer, the denaturation temperatures were found to be approximately 77 C for the Acidothermus enzyme and 72-73 C for the Trichoderma enzyme. The T_m value determined for each of the two enzymes from A. niger (NOVO Sp188) is 72.0-72.5 C. Examples of the thermograms obtained (for the two chromatographically distinct Aspergillus enzymes, betaGI and betaGII) are shown in figure 2a. The most significant comparisons to be drawn from the DSC data are those between the Aspergillus and Acidothermus enzymes, since these enzymes are more thoroughly characterized by conventional activity measurements of thermal stability. The higher denaturation temperature of the Acidothermus enzyme (77 C versus 72.5 for the A. niger enzymes) is consistent with its higher temperature optimum for activity against pNPG (75-80 C versus 55-60 C for the Aspergillus enzyme). The reason for the magnitude of the difference (approx. 20 C) in the activity optima is not immediately apparent in the thermogram, however, as the DSC studies show a difference of only 5 C in "melting temperatures". A possible explanation may be that the loss of enzyme activity may be due to comparatively small conformational changes (which could be either reversible or irreversible) in the active site, whereas the DSC measurements almost certainly record the destruction of the tertiary structure of the protein molecule as a whole. Thus, while DSC is valuable in screening for proteins which have a highly stable overall structure and are thus good candidates for enhancement of the activity/stability, caution should be exercised in constructing close correlations between DSC and conventional kinetic data.

One subject targeted for further study is raised by the observation that while the tertiary structure of the Aspergillus enzyme is lost irreversibly upon scanning to 100 C (as evidenced by complete absence of the 72 C endothermic peak upon cooling and rescanning), the temperature of denaturation is essentially unchanged by decreasing the scan rate from 60 C/hr to 30 C/hr (See figure 2b, for betaGI). This latter observation is more consistent with a rapidly reversible transition than with an irreversible unfolding. One possibility considered is that the actual unfolding (the process represented by the peak in power consumption (DSC) might well be reversible, but might be followed by an irreversible reaction that consumes or gives off relatively little energy (and is thus undetected by DSC), the second reaction rendering the overall process irreversible. It has been suggested (principally by Klibanov et al, 1986) that the irreversibility of many thermal-unfolding transitions of proteins may be due to actual cleavage of covalent bonds in aqueous solution at temperatures greater than 80 C. As a preliminary test of the applicability of this idea in the present case, a sample of the Aspergillus enzyme was scanned to 78 C, which is the minimum temperature for

DELTA Cp



DELTA Cp



complete unfolding as shown by power consumption, then cooled over approx. 45 minutes to 6.2 C and rescanned to 100 C. The results were identical to those seen when the original scan was taken to 100 C (i.e., the endothermic peak at 72-73 C was completely absent in the second scan). If the denaturation of this enzyme does involve a reversible followed by an irreversible step, the second step does not require temperatures in excess of 80 C.

Thermal-scanning fluorescence spectroscopy was also applied to beta-glucosidase denaturation. Here, the change in electronic environment of tryptophan residues of proteins undergoing thermally-induced unfolding is followed by changes in tryptophan fluorescence emission. We followed the change in maximum emission wavelength of the Acidothermus beta-glucosidase, which was found to melt at 73 C (see figure 3). This is in fair agreement with the value of 77 C found by DSC. In the case of both the Acidothermus and Aspergillus enzymes, increasing the temperature resulted in a shift toward longer wavelength of the wavelength of maximum fluorescence, corresponding to a shift of tryptophan residues from a more hydrophobic to a more polar (i.e., more exposed to solvent) environment. From the data between 25 and 45 C, it might be inferred that at least some of the tryptophan residues of the Acidothermus enzyme are more exposed to solvent in the native state than are those of the Aspergillus enzyme, but that a conformational change occurring in the Aspergillus enzyme between 30 and 55 C increases the average exposure of the tryptophans to a value approximating the degree of exposure of the tryptophans in the Acidothermus enzyme. Over this temperature range (40-55 C) the fluorometric indications of conformational change are much smaller in the case of the Acidothermus enzyme. Other interpretations of the behavior over this range are, of course, possible. Above 55 C, the "unfolding curves" of the two enzymes (as monitored by fluorescence) are quite similar.

Fluorescence detected protein unfolding was found to be highly sensitive, as enzyme concentrations were successfully used in this study which were 10-100 times more dilute than those currently used in DSC. Also, fluorescence unfolding measurements examine the environment of a "reporter" group (tryptophan) and thus give an analogous, but not identical, picture to that generated by DSC of the unfolding process. The incorporation of data from both methods will allow rapid assessment of crosslinking technology as applied to beta-glucosidases.

REFERENCES

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- Durand, H., Soucaille, P. and Tiraby, G., 1984. Enzyme Microb. Technol. 6, 175-180.
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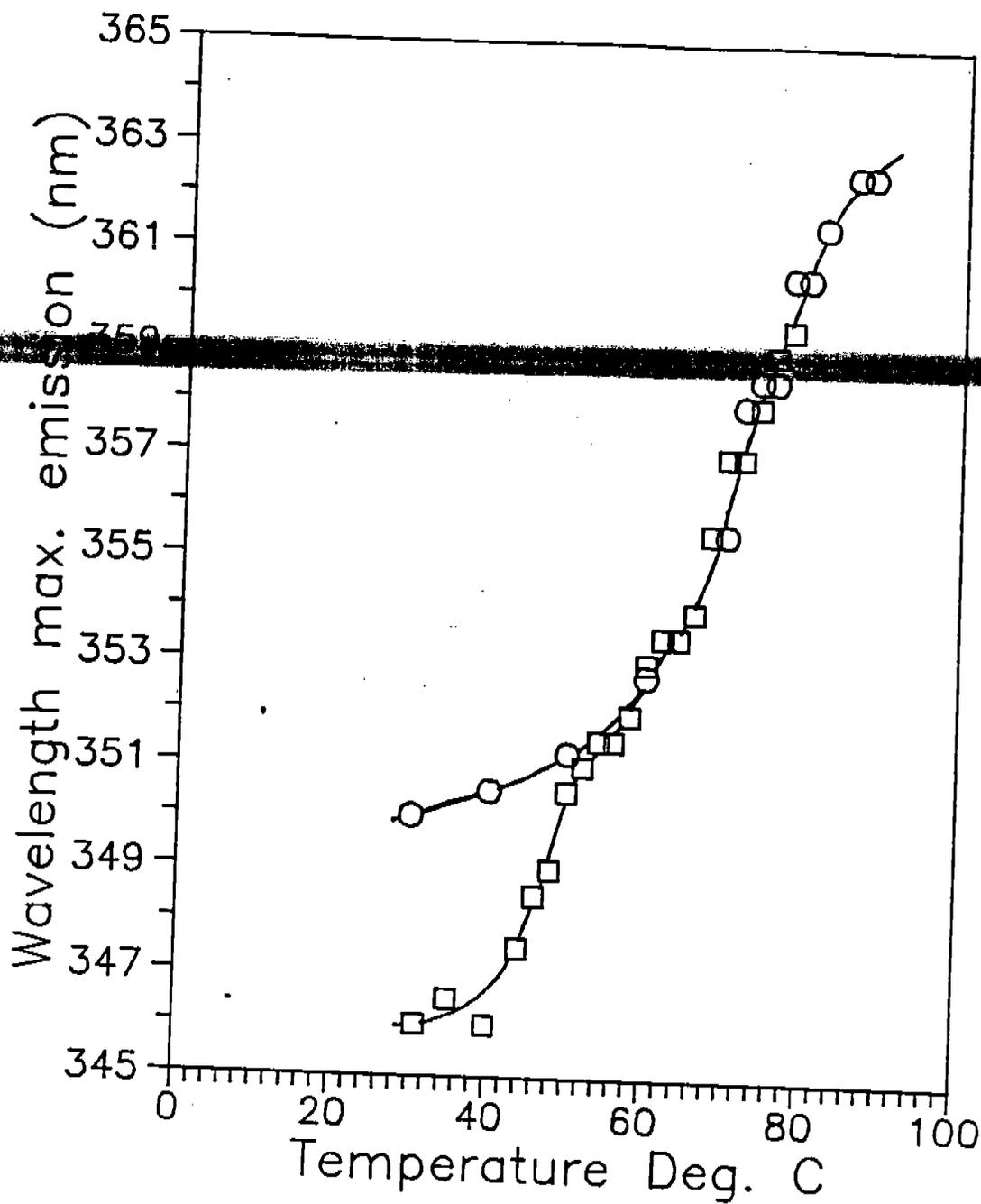


Figure 3. Effect of temperature upon the wavelength of maximum tryptophan fluorescence emission for a bacterial and a fungal beta-glucosidase. Circles represent emission maxima for *Acidothermus* beta-glucosidase; squares show maxima for the *Aspergillus* enzyme. Excitation wavelength 289 nm.