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Spectroscopic studies on the denaturation of papain solubilized and Triton X-100-solubilized glucoamylase from rabbit small intestine

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Abstract. Intestinal brush border proteins consist of an enzymatically active hydrophilic moiety attached to a hydrophobic tail. Papain dissociates the hydrophilic part by cleaving off the hydrophobic tail, whereas the detergentTriton X-100 solubilizes the whole molecule. Denaturation by 8 M urea or 4 M guanidinium chloride does not alter the structure of the papain-solubilized enzyme. An appreciable alteration of the structure of detergent-solubilized enzyme was observed on denaturation. The difference spectra of Triton X-100 (1%)—solubilized enzyme and its urea denatured form shifts and intensifies, with increase in the concentration of the denaturant with an isobestic point at 252 nm. A new band at 280 nm also appears at 4 M urea concentration. Papain-solubilized glucoamylase has an ∝ -helical conformation in solution unlike the detergent-solubilized fraction. An elongated structure for the papain solubilized enzyme is inferred from the urea denaturation studies and from molecular weight determinations.

Keywords. Rabbit small intestine; glucoamylase; papain denaturation; Triton X-100; difference spectra.

Introduction

Glucoamylase (EC 3.2.1.3) is an exoenzyme that releases glucose residues directly from starch. Glucoamylase of the small intestine localized in the brush border, has been purified to varying extents from different animal species (Seetharam *et al*. 1970, Schlegel-Haueter *et al*., 1972; Kelly and Alpers, 1973) and to homogeneity from rabbit (Sivakami and Radhakrishnan, 1973).

In general, the disaccharidases and glucoamylase have been studied after solubilization from the brush border membrane by the proteolytic action of papain which cleaves the hydrophobic moiety from the solubilized hydrophilic moiety (Maroux and Louvard, 1976). Recently, detergents have been used for solubilizing the sucrase-isomaltase (Sigrist *et al*., 1975) and alkaline phosphatase (Colbeau and Maroux, 1978). The detergent and papain-solubilized forms of sucrase-isomaltase are identical enzymatically but differ in their tendency to aggregate (Sigrist *et al*., 1975). Now, it is well established that the detergent solubilizes the 'whole' enzyme by acting on the membrane-embedded hydrophobic part and this interaction appears to be dependent on the critical micellar concentration of the detergent (Helenius and Simons, 1975).

In our earlier attempts to determine the molecular weight of papain-solubilized glucoamylase from rabbit small intestine, an anomalous behaviour of the enzyme on gel filtration systems was noticed. The estimate of the molecular weight obtained

by gel filtration was at least 5 times larger than the value obtained in the analytical ultracentrifuge (Sivakami and Radhakrishnan, 1978). An explanation for this discrepancy was not then readily available.

Preliminary experiments have indicated that glucoamylase can be effectively solubilized using Triton X-100 (Nirmala Murthy, 1978). The Triton-solubilized enzyme is apparently different from the papain-solubilized enzyme in its stability to heat and denaturants like urea and guanidinium chloride (Nirmala Murthy, 1978). Spectral studies on the two forms of the enzyme were therefore initiated with a view to detect any differences in the state of aggregation and shape and to offer a possible explanation for the observed anomaly in the molecular weight of the enzyme.

Materials and methods

All the reagents used here are of analytical grade. Urea and guanidinium chloride are of ultrapure grade obtained from Schwarz-Mann, Orangeburg, New York, USA. Papain was obtained from Sigma Chemical Company, St. Louis, Missouri, USA and Triton X-100 used was of scintillation grade from Eastman Kodak, Rochester, New York, USA. Glucoamylase was solubilized using papain or Triton X-100 and subsequently purified by affinity chromatography on Sephadex G-200 columns (Sivakami and Radhakrishnan, 1973). A 20% homogenate of the intestinal mucosal scrapings was centrifuged at 12,000 *g* for 30 min in a refrigerated centrifuge, model MB 20 (MB Corporation, Bombay) at 4°C. The particulate fraction was suspended in 0.01 M potassium phosphate buffer pH 7·0 to get a final protein level of about 10 mg/ml. This fraction was incubated with crystalline papain at a papain: pellet protein ratio of 1:100 for 60 min at 37°C, at the end of which it was chilled and centrifuged at 25,000 *g* for 3 h in a refrigerated centrifuge at 4°C. Papain was removed from the enzyme during the specific affinity procedure using Sephadex G-200 (Sivakami and Radhakrishnan, 1973). For solubilization with Triton X-100, the homogenate in the phosphate buffer was adjusted to a protein concentration of 6 mg/ml, and incubated at 4°C, for 90 min, in the presence of 1% Triton X-100 and 0.01 mM NaCl. At the end of the incubation, the mixture was centrifuged at 25,000 *g* for 3 h. The enzyme in the supernatant was purified by the same affinity technique under identical conditions as was used for the papain-solubilized enzyme. Throughout the purification and the subsequent analysis, 1% Triton X-100 was maintained. The Triton enzyme was apparently free of contamination as judged by polyacrylamide disc gel electrophoresis (Nirmala Murthy, 1978 and figure 1). Maltase and gluco-

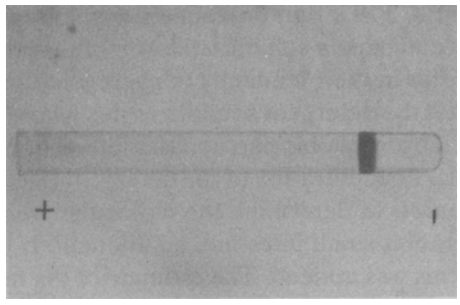

Figure 1. Polyacrylamide gel electrophoresis of Triton X-100 solubilized glucoamylase

amylase activities were determined by measuring the glucose formed by the glucose oxidase-peroxidase procedure of Dahlqvist (1964), as described earlier (Seetharam *et al*., 1970). Protein was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

Spectral measurements were carried out in 0.5 ml cuvettes using a Shimadzu UV-200S double beam spectrometer. The enzyme was allowed to equilibrate with urea and guanidinium chloride for about 1 h before the spectral measurements were made. However, the denaturation was found to be completed within 1 min after the addition of the denaturant. Difference spectra were corrected for the absorption of the urea and guanidinium chloride under similar conditions. Since Triton X-100 was present in equal amounts in both the reference and sample cuvettes, any contribution by it to the protein absorption spectra was cancelled out. By varying the concentration of both Triton-X-100 and the protein, it was also observed that the spectral properties of free Triton X-100 and protein bound Triton X-100 were the same. Circular dichroic measurements were carried out in a Jasco J-20 spectropolarimeter.

Results and discussion

Figure 2 shows the UV absorption spectra in the range 240-300 nm of the papain-solubilized enzyme (papain-free). The enzyme was characterized by a typical broad

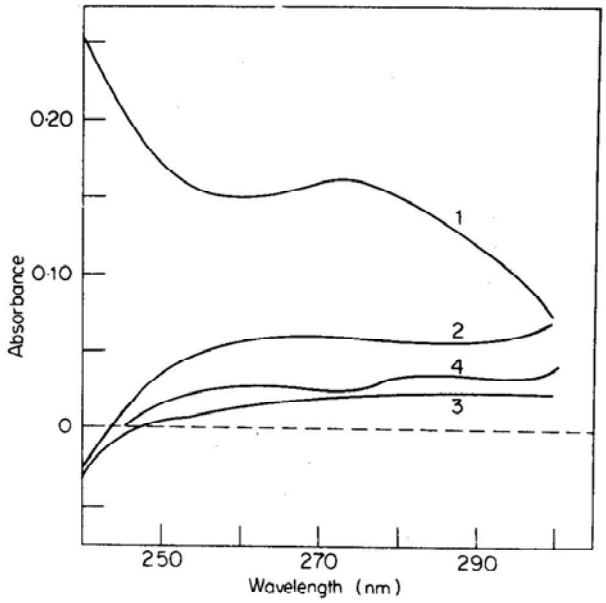

Figure 2. Ultraviolet spectra of papain-solubilized enzyme and the difference spectra of the enzyme in 8 M urea and 4 M guanidinium chloride. During the analysis of difference spectra, reference cuvette contained the enzyme and the sample cuvette contained enzyme and the denaturant.

1. Enzyme 40 μg;
2. The difference spectra of the enzyme (40 μ g) + 8 M urea;
3. Enzyme 20 and 10 μ g + 8 M urea;
4. Enzyme 40 μ g + 4 M guanidinium chloride.

absorption peak at 272 nm contributed by the aromatic amino acid residues present in the enzyme. Phenylalanine and tyrosine were present to the extent of 69.2 mol/mol of the enzyme (Sivakami and Radhakrishnan, 1978). However, when this enzyme was subjected to denaturation with 8 M urea or 4 M guanidinium chloride, no significant change was observed as shown in the difference spectra (figure 2). Change in absorbance was considerable only when a higher concentration of enzyme was used (40 μg). The absence of any appreciable change on denaturation suggests that the aromatic amino acid residues may not be buried in a hydrophobic region as in a globular protein.

Quite a different result was obtained when the Triton X-100 solubilized enzyme was denaturated with 8 M urea (figure 3). It should be mentioned here that the concentration of Triton X-100 (1%) in aqueous medium used in these experiments was higher than its critical micellar concentration value. Therefore, the difference spectra were obtained by subtracting the contribution of the change in absorbance arising due to the disruption of the Triton micelle itself by urea (Helenius and Simons, 1975). With increase in the concentration of the protein, the large negative

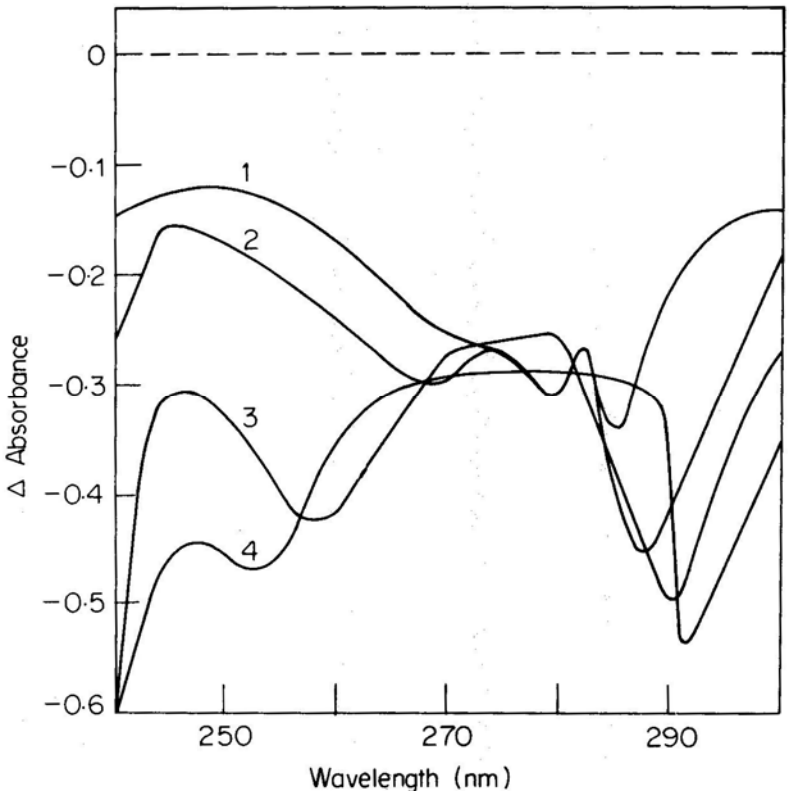

Figure 3. Spectra of the Triton X-100 solubilized enzyme denatured with 8 M urea. Reference cuvette contained the enzyme (1, 10 μ g: 2, 20 μ g; 3, 40 μ g; 4, 80 μ g protein) and the sample cuvette contained the enzyme and 8 M urea.

band at 285 nm shifted towards a higher wavelength. Moreover, at lower concentrations of the protein (10 and 20 μg) a new band appeared at 280 nm. The difference spectrum was also characterized by a broad band at a lower wavelength which shifted and intensified with increase in the level of the protein. When the denaturation of the enzyme (20 μg) was carried out at different concentrations of urea, the band at 280 nm was found to be distinct at higher concentrations of urea (figure 4). Also,

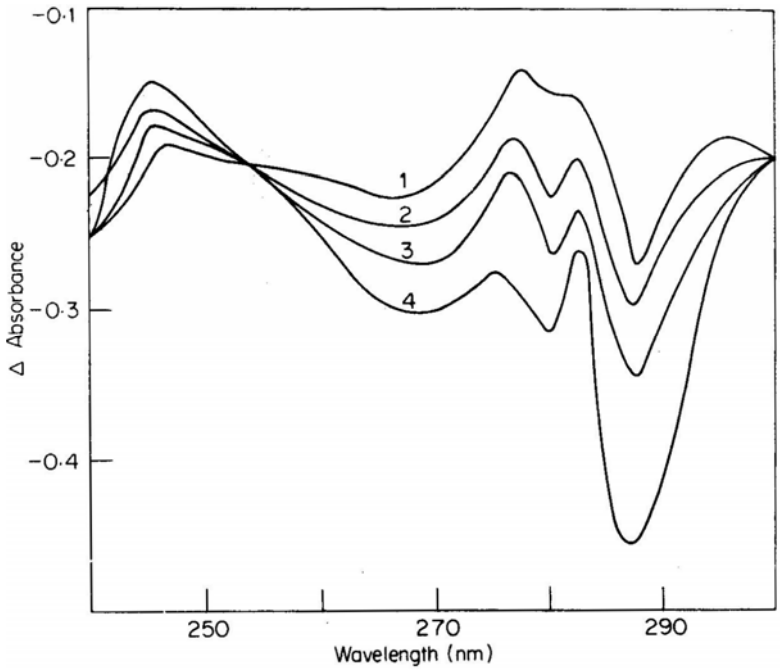

Figure 4. The difference spectra of the Triton X-100 solubilized enzyme (20 μ g protein) after denaturation with different concentrations of urea (1, 2 M; 2, 4 M; 3, 6 M; and 4, 8 M urea). Reference cuvette contained enzyme and the sample cuvette contained enzyme and urea.

an isobestic point at 252 nm was observed indicating that one type of denatured species is present in the solution (see figure 4). Similar results were also obtained during denaturation by 4 M guanidinium chloride (not shown). All these data suggested that the enzyme in the Triton X-100 medium had a structure, the denaturation of which exposed the aromatic amino acid residues to the solvent. We would like to point out that most proteins preserve their tertiary structure in the presence of high concentration of Triton X-100 and it usually does not appear to induce conformaional change in proteins leading to loss of their biological properties (Helenius and Simons, 1975). It is noteworthy that most of the proteins give a large negative band in the difference spectra upon denaturation with 8 M urea (Herskovits, 1967). However, any change in the spectra arising due to the change in concentration of the enzyme may be attributed to the aggregation of the molecule which is possible in the presence of non-ionic detergents like Triton X-100 (Tanford *et al*, 1974; Simons *et al*., 1973).

Hence, a Triton-free form of the enzyme was purified by washing the column thoroughly with Triton-free buffer before elution of the enzyme. The Triton free-enzyme was expected to form an aggregate.

However, the addition of 8 M urea fails to bring about any appreciable change in the organization of the enzyme aggregate, as noticed by difference spectrophotometry. Only a broad negative band of low intensity around 290 nm was observed and did not change with the concentration of the enzyme aggregate. To verify whether the spectra of the Triton-solubilized enzyme were due to the conformational effects of Triton X-100 on an enzyme form similar to that solubilized by papain, we carried out the urea denaturation study of the papain-solubilized enzyme in the presence of 1 % Triton. The difference spectra did not show any band in the region of 250 to 290 nm, although there was a small negative band around 300 nm, The 8 M urea induced denaturation did not cause any significant structural alteration of the papain-solubilized enzyme in the presence of 1 % Triton X-100.

All these results indicated that there was a gross structural difference of the enzyme when solubilized by using either papain or Triton. It is interesting to note that a small hydrophobic tail of the enzyme might contribute significantly to the overall conformation of the enzyme. When the difference spectra of the papain-solubilized enzyme at pH 12 was compared with that at neutral pH, the nature of the curve was found to be similar to that obtained with free tyrosine. However, no significant change was observed in alkali-induced difference spectra of Triton-solubilized enzyme (not shown).

To confirm the above pattern of results we measured the circular dichroic (CD) spectra of the urea denatured enzyme solubilized by Triton X-100 and papain respectively. The papain-solubilized enzyme had two negative CD bands, one around 218 nm and the other at 205 nm typical for ∝-helical conformation (figure 5)

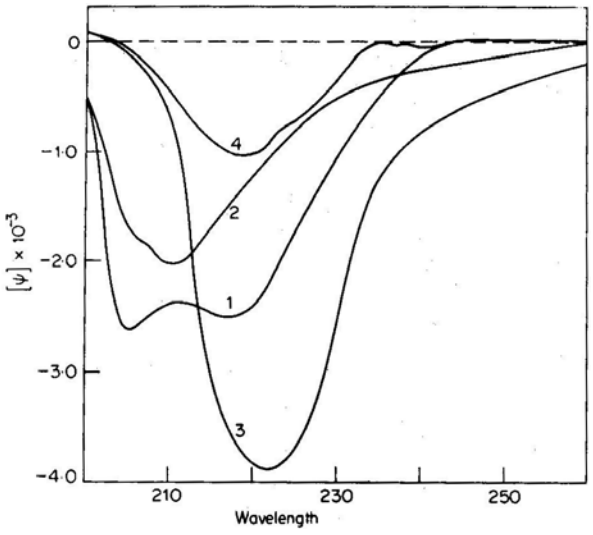

Figure 5. Circular dichroism spectra of papain-solubilized enzyme, (1); 8 M urea denatured papain-solubilized enzyme (2); Triton X-100 solubilized enzyme (3); 8 M urea denatured Triton X-100 solubilized enzyme (4).

(Greenfield and Fasman, 1969), whereas the Triton-solubilized fraction was characterized by one negative CD band at 222 nm. Urea denaturation caused a greater disruption of the structure of detergent-solubilized fraction than that of the papain solubilized enzyme as expected.

It was mentioned earlier that the papain-solubilized glucoamylase had an estimated molecular weight five times more by gel filtration than that obtained through ultracentrifugation. This discrepancy might arise due to the aggregation of the molecule or if the enzyme has an elongated structure. Urea denaturation data suggested that the molecule might be elongated without much folding, resulting in the enzyme eluting on gel filtration system earlier than it should (Sivakami and Radhakrishnan, 1978). One the other hand, the detergent-solubilized glucoamylase appeared to have a typical three-dimensional folding with buried aromatic amino acid residues which are vulnerable to urea denaturation. Further studies on the strcuture of the enzyme above and below the critical micellar concentration of the detergent and the nature of interaction of the detergent micelle with the enzyme are in progress.

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