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Circular dichroism studies of subtilisin Carlsberg immobilised on micron sized silica particles

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Abstract

Immobilised enzymes are widely used in industry, but the reasons for loss of activity of such biocatalysts are usually not known. We have used circular dichroism (CD) to investigate the structure of one such system, i.e., subtilisin Carlsberg (SC) immobilised on silica gel particles (60 μm). A number of technical problems have to be overcome in order to obtain appropriate data from which conclusions can be drawn. A rotating cell holder has been developed to avoid sedimentation of the silica particles during the collection of spectra. By moving the cell holder as close as possible to the detector window, the effects of differential scattering can be minimised. However, the effects of absorption flattening limit the extent to which reliable quantitative information on secondary structure content can be obtained from far UV CD studies. We have used an empirical approach based on absorbance units derived from the high-tension voltage to correct for absorption flattening effects. After applying the correction there was satisfactory agreement with the solution spectra. Comparison of the fresh and used (inactive) SC-silica gel spectra in organic media reveals substantial change in the secondary structure. Additional evidence for loss of native conformation is provided by the significant decrease in the near UV CD spectrum. These results for the first time clearly demonstrate the origin of enzyme instability in the immobilised state.
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1. Introduction

Immobilisation of enzymes by their incorporation into solid particles is widely used. For example, in industrial biocatalysis, immobilised enzymes are often preferred over free (soluble) enzymes due to their ease of handling and the ability to recover and re-use them; these have obvious implications for production costs [1,2]. Immobilisation has also been used to protect enzyme activity particularly in low water organic media, to prolong the shelf life of dehydrated proteins and in the stabilisation of vaccine preparations [1–4]. Because of their practical advantages and wide range of applications, proteins adsorbed on solid supports are becoming increasingly attractive for many industrial processes. Although these considerations have led to the development of a number of commercial solid support-based biocatalysts, the range of such systems has been somewhat limited. One problem has been poor functional stability of the enzyme. In order to develop a robust biocatalyst, it is necessary to overcome this problem and this could involve a laborious screening process, which might outweigh any commercial advantages. It is likely that one contribution to functional instability will be structural perturbation of proteins in the immobilised state, but this may go undetected because of the lack of spectroscopic techniques capable of probing in situ conformational changes in industrial grade biocatalysts. A wide range of spectroscopic methods is available to characterise protein structure in solution, and the choice of any particular technique depends upon many factors including the type of information being sought and the technical feasibility. In this

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paper, we discuss the development of methods based on circular dichroism (CD) to investigate conformations of immobilised proteins in aqueous and low water organic media.

Over the last four decades, CD has been increasingly used as a spectroscopic tool for elucidating structural aspects of proteins and enzymes in solution (for a recent review see Ref. [5]). Because CD spectra are sensitive in the far and near UV spectral regions to secondary and tertiary structures respectively, CD is widely used to detect conformational changes of proteins. Although CD has been primarily used to study proteins in solution, the technique has also been employed to study membrane proteins in large membrane vesicles and sheets where artefacts can arise from the effects of differential light scattering and absorption flattening [6,7]. The recent availability of synchrotron radiation CD has provided a powerful impetus for investigating the secondary structures of membrane proteins [8]. However, the study of biocatalyst preparations by CD poses a number of difficulties and so far these have not been investigated in detail.

Spectroscopic studies of immobilised proteins have been generally focused on nano particles due to their reduced degree of light scattering [9–12]. To our knowledge, there have been no reports on the use of CD to investigate the structures of proteins immobilised on large (tens to hundreds of microns) scattering particles. Apart from the technical difficulties in measuring the spectra, a major problem has been the lack of methods to estimate protein loading accurately. In this paper, we report the development of general methods that could be applicable to immobilised enzymes particles such as industrial grade biocatalysts. We demonstrate our methods using subtilisin Carlsberg (SC) immobilised on silica gel in aqueous and organic media. The magnitude and shape of far UV CD spectra exhibit substantial differences from the solution spectra impacting on efforts to quantify enzyme structure in the immobilised state. This is largely due to absorption flattening of the spectra in the region below 240 nm. Using an empirical approach, we will show it is not only possible to correct for the changes in spectral shape and magnitude but also that the data can be analysed to give reasonable estimates of secondary structural composition. The structural changes observed from the far UV CD correlate well with those from the near UV CD suggesting the reliability of this approach. Investigation of the SC-silica gel exposed to organic media illustrates a reduction in helical composition. This is reflected in both the blue shift of the far UV CD and the reduction in magnitude of the near UV CD arising from loss of tertiary structure.

2. Materials and methods

Subtilisin Carlsberg (protease from *Bacillus licheniformis*, P-5380, Lot 034K1340), L-tyrosine and L-phenylalanine, silica gel (230–400 mesh), sodium monohydrogen phosphate and sodium dihydrogen phosphate were purchased from Sigma Chemicals, UK. N-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) was from Fluka Biochemica. Acetonitrile (ACN) and propan-1-ol were of HPLC grade and used as purchased.

2.1. Enzyme immobilisation

About 60 mg SC was dissolved in 20 ml of 20 mM sodium phosphate buffer pH 7.8 and to this solution 1 g silica gel was added and gently stirred for 4 h at room temperature [13]. The SC-silica gel suspension was centrifuged and washed repeatedly with aqueous buffer until the supernatant showed negligible absorption at 280 nm. Enzyme suspensions were sealed in a glass vial and stored at 4 °C. The loading of SC on silica gel was estimated following the methods discussed in Sections 2.2 and 2.3.

2.2. UV absorption

The amount of enzyme present in the solution before and after adding silica gel was estimated from UV absorption at 280 nm using an extinction coefficient of 23 740 M^{-1} cm^{-1} [14]. SC-silica gel suspension was washed repeatedly with aqueous buffer, centrifuged and the supernatant collected in a standard flask. This procedure was repeated until the washings showed negligible UV absorption. Then, the absorbance at 280 nm of the combined washings was noted and from this the total amount of enzyme present in the washings was calculated. From the difference the total loading of SC was estimated to be 14.9± 0.5 mg enzyme per g of silica gel. Because of enzyme leaching from the support on prolonged storage, immobilised SC preparations were used within a week.

2.3. Acid hydrolysis

Since an accurate estimate of enzyme concentration is crucial particularly for CD data analysis, an alternative approach for estimating the SC loaded onto the support was developed. This was based on acid hydrolysis of the immobilised enzyme and subsequent analysis of amino acids Tyr and Phe released. SC-silica gel was freeze-dried in a glass vial and treated with 6 M HCl. The vials were sealed and heated at 120 °C for 16 h to hydrolyse the enzyme. After hydrolysis, the silica gel suspension was washed with deionised water and the supernatant was collected and freeze dried. The dried residue was dissolved in 1 ml deionised water and subjected to reverse phase HPLC analysis (Gemini 5μ C18 C110A column, 4.6×25 mm) on a Waters Alliance System equipped with Waters 2996 photodiode detector. The mobile phase was mixed from 12 mM Na-phosphate buffer of pH 2.8 (A) and ACN containing 50% (v/v) 12 mM phosphate buffer, pH 2.8 (B), at a flow rate of 1.5 ml min^{-1} with detection at 220 nm, 257 nm and 275 nm [15]. A linear gradient increased B from 0 to 40% over 10 min. The column temperature was maintained at 40 °C throughout the separation process. Under these experimental conditions Tyr was found to elute at 4.2 min and Phe at 7.4 min.

The enzyme content was calculated from the analysis of Phe and Tyr in the hydrolysed product, as described [Petry, Ganesan, Pitt, Moore and Halling, in preparation]. Standard solutions of Phe and Tyr were used to calculate the concentrations of these amino acids from which the total amount of enzyme in terms of mg ml^{-1} could be calculated. Using this method the enzyme loading was found to be 13.3±0.3 mg per g of silica gel; this value is in close agreement with the value obtained from the UV absorption method. After the immobilised enzyme had been stored for two days and exposed to ACN/water media, the enzyme loading per g of silica gel was found to have fallen to 7.6±0.4 mg. In this paper, we have used the loading derived from the acid hydrolysis method to estimate enzyme concentration.

2.4. Biocatalyst inactivation

SC-silica gel was inactivated in a continuous micro reactor following transesterification of substrate Ac-Tyr-OEt to give Ac-Tyr-OPr and the hydrolysis product Ac-Tyr, as previously described [16]. When the conversion rate had become undetectable, the reaction was stopped and the biocatalyst was removed from the reactor and washed with ACN of water activity (a_w) 0.76 (which is used in the transesterification reaction) to remove the excess substrate. This inactive biocatalyst is referred to as used SC-silica gel.

2.5. Circular dichroism

CD measurements of large particulate suspensions are prone to artefacts arising from (a) sample sedimentation during measurement process and (b) optical effects involving light scattering and absorption flattening. To avoid sedimentation of the sample, we constructed a rotating cylindrical sample cell holder. It consists of a motor driven cell holder with a variable speed control ranging from 42 rpm to 98 rpm that could be easily incorporated into the sample compartment of the spectropolarimeter. Its fitting required removal of the

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