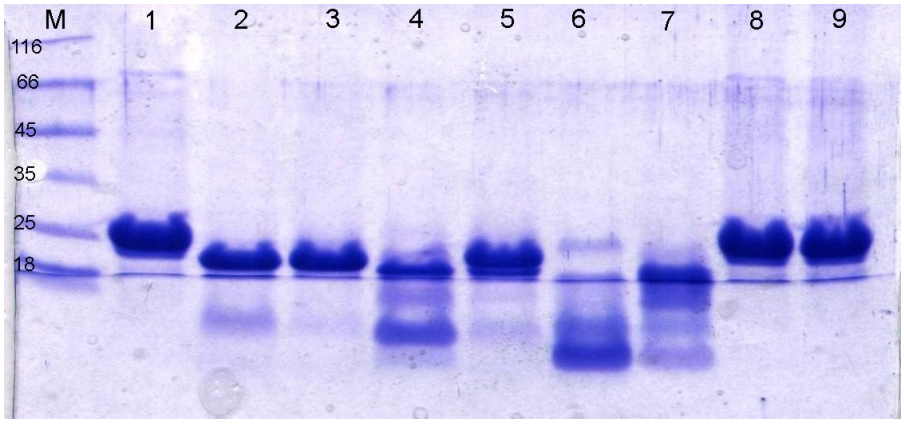
Supplementary material for Beaven *et al*., manuscript entitled 'Crystallisation and preliminary X-ray characterisation of the 2,4'-dihydroxyacetophenone dioxygenase (DAD) from *Alcaligenes* sp. 4HAP'.

Limited proteolysis studies.

Four proteases were tested for their effects on DAD during a 3 hour incubation at room temperature with the samples buffered in 50 mM Tris pH 7.5 and 100 mM NaCl. A 12 % reducing SDS-PAGE gel was then run and Coomassie-stained, as shown below.

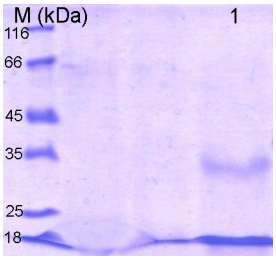


Lane M shows the molecular weight markers in kDa.

1) Shows the uncleaved His-tagged DAD (theoretical molecular weight = 23 kDa).
2) 50:1 DAD: trypsin (mass ratio)
3) 500:1 DAD:trypsin
4) 50:1 DAD:chymotrypsin
5) 500:1 DAD:chymotrypsin
6) 50:1 DAD:papain
7) 500:1 DAD:papain
8) 50:1 DAD:pepsin
9) 500:1 DAD:pepsin

From this it was clear that pepsin (lanes 8 and 9) has no effect, most likely due to the non-optimal pH for this protease. In contrast, papain (lanes 6 and 7) is very destructive, at least at the 50:1 ratio. Trypsin (lanes 2 and 3) gave a reasonably clean molecular weight reduction which was very similar to that observed with thrombin which is normally used for removing the His-tag (results not shown), suggesting that both proteases cut at the same place, given their similar specificities. Removal of the His-tag at the expected thrombin cleavage site decreases the molecular weight of the protein to 21 kDa, as is observed for trypsin. Since thrombin-cut DAD had previously been shown not to crystallise, we decided not to use trypsin but instead to use chymotrypsin since it gave results (see lanes 4 and 5) that are somewhere between those of papain and trypsin. The DAD:chymotrypsin ratio of 50:1 (lane 4) appeared to cause the bulk of the protein to decrease in molecular weight slightly more than trypsin. If chymotrypsin was to cleave DAD at Phe 8 and/or Trp 9 this would reduce its molecular weight to about 19.5 kDa, as indeed suggested by the gel. The SDS-PAGE analysis also shows that chymotrypsin causes the appearance of significant bands at lower molecular weight, around 18 kDa and 14 kDa. The former of these two bands could be explained very well by a predicted chymotrypsin cleavage site at Phe 19. Whilst there are many other potential cleavage sites in the sequence of DAD at which chymotrypsin could act, with the benefit of hindsight, the above interpretation is consistent with the refined X-ray structure of the chymotrypsinolysed protein (manuscript submitted) which reveals no electron density for the first 25 amino acids, whereas the remainder of the protein appears to be intact, suggesting that the residues at the N-terminal end are particularly vulnerable to proteolysis. The theoretical molecular weight of the part of the protein which is defined by the electron

density map is 17.6 kDa which agrees very well with the 18 kDa band obtained by chymotrypsinolysis. Indeed, further SDS-PAGE analysis of the protein remaining in the hanging drops which yielded the high resolution dataset after some months of storage at room temperature shows that this is indeed the dominant band.



Lane M shows the molecular weight markers in kDa. The lane labelled “1” shows the chymotrypsinolysed His-tagged DAD some months after crystallisation in hanging drops. The majority of the protein has a molecular weight close to 18 kDa with a feint dimer band at approximately 35 kDa. No bands below 18 kDa were apparent.