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Crystallization of Xylanase from *Erwinia chrysanthemi*: Influence of Heat and Polymeric Substrate

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Abstract

Xylanase from the bacterial plant pathogen *Erwinia chrysanthemi* (E.C. 3.2.1.8), expressed in *E. coli*, has been crystallized for X-ray diffraction analysis both in the presence and the absence of its polymeric substrate 4-*O*-methyl glucuronoxylan. In all cases it was found that the quality, time of appearance, and reproducibility of both the native and complex crystals were significantly enhanced by heating of the protein to 323 K prior to dispensing the crystallization trials. Crystals of the native protein are ideal for X-ray diffraction analysis, producing Bragg reflections beyond 1.5 Å resolution with virtually no degradation with time. The native crystals are in space group $P2_1$, with $a = 39.33$, $b = 49.46$, $c = 90.85$ Å and $\beta = 101.58°$. Other polymorphs have also been obtained and their cell parameters determined. Crystallization of the enzyme in the presence of polymeric substrate yields two distinctly different crystals at different concentrations of the xylan. These are thought to be complexes of the protein with stable products of the enzymatic reaction. A similar result had been obtained previously with pancreatic α-amylase and its substrate glycogen.

1. Introduction

One third of all renewable organic carbon available on earth, in the form of the hemicellulosic fraction of plant tissues, is xylan. Engineering microbial enzymes to efficiently produce its component sugars for industrial processes, therefore, is emerging as a major objective in biotechnology research and presents a potentially major benefit to man (Prade, 1996). On the other hand, xylanase in the wild is a major factor in the pathogenesis of many microrganisms which attack economically important crops, such as corn. It is, therefore, a prominent target for the creation of agricultural pesticides by systematic design based on enzyme structure (McPherson, 1988).

Xylanases, like all β1-4 glycan hydrolases, utilize a general acid mechanism of catalysis promoted by two acidic amino-acid residues (Henrissat, Callebaut, Fabrega, Lehn & Mormon, 1995; Prade, 1996). Specific recognition of substrate depends on a series of binding subsites on the surface of the enzyme, which then operates in a processive mode similar to α-amylase (Yamamoto, Kitahata, Hiromi, Ohnishi, Minamiura, Shinke, Okada & Komaki, 1988). In terms of its enzymology, it shares several characteristics with lysozyme (Prade, 1996) which is perhaps the most studied of this class of enzymes.

A number of xylanases from both fungal and bacterial sources have been crystallized for diffraction analysis (Bedarkar, Gilkes, Kilburn, Kwan, Rose, Miller, Warren & Withers, 1992; Golubev, Kilimnik, Neustroev & Pickersgill, 1993; Pickersgill, Debeire, Debeire-Gosselin & Jenkins, 1993; Torronen, Rouvinen, Ahlgren, Harkki & Visur, 1993; Viswamitra, Bhanumoorthy, Ramakumar, Manjula, Vithayathil, Murthy & Naren, 1993; Eswaramoorthy, Vithayathil & Viswamitra, 1994; Souchon, Spinelli, Beguin & Alzari, 1994; Krengel, Rozeboom, Kalk & Dijkstra, 1996). Structures of xylanases from fungi and bacteria have also been determined by crystallography. Two bacterial xylanases, one from *Pseudomonas* (Harris, Jenkins, Connerton, Cummings, Leggio, Scott, Hazlewood, Laurie, Gilbert & Pickersgill, 1995; Harris, Jenkins, Connerton & Pickersgill, 1996) and the other from *Streptomyces* (Derewenda, Swenson, Green, Wei, Morosolis, Shareck, Kluepfel & Derewenda, 1994) where shown to be of conventional α/β-barrel design. Two enzymes from the fungus *Trichoderma* (Torronen, Harkki & Rouvinen, 1994; Torronen & Rouvinen, 1995) on the other hand, were composed primarily of antiparallel β-sheets. Thus, there appear to be at least two, and perhaps more, distinctive motifs in nature capable of providing the requisite biochemical functions. The protein from the bacteria *E. chrysanthemi* studied here has a molecular weight of 42 000 and consists of a single polypeptide chain of 413 amino acids having an isoelectric point of 8.8 (Braun & Rodrigues, 1993; Keene, Boyd & Henrissat, 1996).

There are no cofactors known to be associated with the protein.

2. Materials and methods

Xylanase from *E. chrysanthemi* was cloned and expressed as several percent of the total cellular protein in *E. coli* and purified as described elsewhere (Keene, Boyd & Henrissat, 1996). Lyophilized protein was dissolved in water to a concentration of 23 mg ml^{-1} and screened for its ability to crystallize using Crystal Screen (Hampton Research, Laguna Niguel, California). Vapor diffusion (McPherson, 1990, 1982) on Cryschem plates (C. Supper Co., Natick, MA, USA) was used for the 48 trials (Morris, Kim & McPherson, 1989). All experiments were carried out at 295 K.

Optimization of promising trials from Crystal Screen was carried out by varying the pH and concentrations of the components of the mother liquor as described elsewhere (McPherson, 1990). Complexes were produced by mixing enzyme with known concentrations of substrate, or later, by mixing and then heating the mixtures to 323 K for fixed periods before dispensing the crystallization trials. The substrate used in these trials was 4-*O*-methyl glucuronoxylan from birchwood (Sigma Biochemicals). This was dissolved in 50 m*M* sodium citrate at pH 5.3.

Preliminary X-ray diffraction analyses of all crystals were carried out using a San Diego Multiwire Systems two multiwire detector systems (Hamlin, Cork, Howard, Nielsen, Vernon, Matthews & Xuong, 1981). The source of the X-rays was a Rigaku RU-200 generator with a Supper monochromator producing Cu $K\alpha$ radiation.

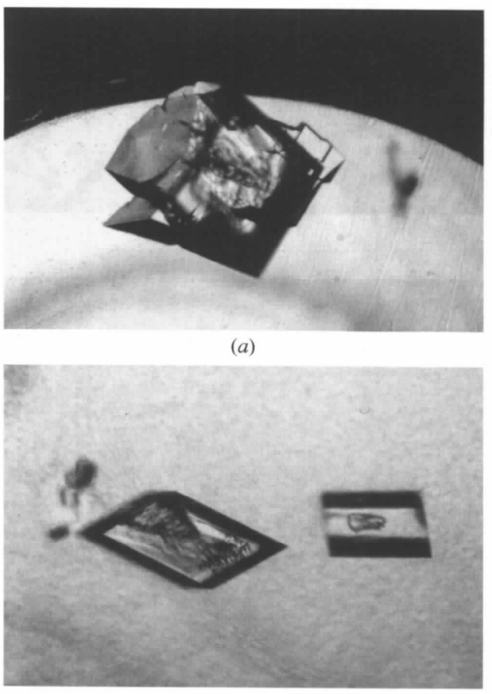
3. Results

From the Hampton Screen, only a single set of conditions emerged that yielded xylanase crystals. These included vapor diffusion of equal volumes of protein in water mixed with 30% PEG 4000 in 0.1 *M* sodium citrate at pH 4.6 containing 0.2 *M* ammonium acetate and equilibrated at 295 K against the full concentration of the latter. From optimization, the best crystallization conditions were established as 30% PEG 4000 plus 0.2 *M* ammonium acetate, and 0.1 *M* sodium citrate at pH 6.5 and 291 K.

Crystals obtained under these conditions, and seen in Fig. 1, were large but of strikingly poor visual quality, exhibiting deep surface occlusions, apparent fissures, protrusions, and aberrant morphologies. Remarkably, however, they produced outstanding diffraction patterns that extended to at least 1.5 Å resolution, had good mosaic character, and did not significantly decay at room temperature for over, at least, a week's time. The crystals were in monoclinic space group $P2_1$ with $a = 39.33$, $b = 49.46$, $c = 90.85$ Å and $\beta = 101.58°$. From the volume-to-mass ratio of $V_m = 2.06$ Å^3 Da^{-1} (Matthews, 1968), the number of molecules per asymmetric unit can be assumed as one.

In spite of the favorable diffraction quality of the crystals, structure analysis was chronically plagued with two problems, a failure to consistently induce the monoclinic crystals to grow, most experiments inexplicably yielding no crystals, and the common occurrence of polymorphs when crystals did grow. Difficulties were exacerbated by the necessity to use several different preparations of the enzyme obtained from multiple fermentations. Seeding by various means (McPherson, 1982) was attempted, but never yielded acceptable results, generally resulting in too many small crystals. Two of the most common of the alternate crystal forms had space groups $I2$, with $a = 66.69$, $b = 62.59$, $c = 91.75$ Å and $\beta = 101.51°$ and $P6_n$ with $a = b = 60.32$ and $c = 165.78$ Å. These also diffracted well, but not to the resolution of the $P2_1$ crystals. From their calculated V_m values of 2.23 and 2.29 Å^3 Da^{-1}, respectively, the most reasonable assumption is that both also have a single molecule of xylanase as their asymmetric unit.

Numerous approaches and procedures were explored to establish consistency in the crystallization process.



(*a*)

(*b*)

Fig. 1. (*a*) A typical example of a crystal of native xylanase grown from PEG 4000 at 291 K with no pretreatment. (*b*) Two crystals grown under the same conditions but with preheating to 323 K for 15 min. Note the exceptionally poor, apparent quality of the crystal in (*a*) and the noticeable improvement of the crystals in (*b*) which nonetheless, still exhibit surface pits and fissures.

Ultimately, we found a straightforward way to reproducibly obtain monoclinic xylanase crystals and eliminate the high frequency of polymorphs. The protein, prior to dispensing in crystallization droplets, was heated to 323 K for 15 min and then centrifuged to remove any insoluble material. The opitimal time for exposure to the elevated temperature was determined empirically by screening a range of times and temperatures. By introducing this simple expedient the problem of reproducibility in native crystal growth was eliminated.

The normal substrate for the enzyme is xylan, a polymeric molecule comprised of xylose residues linearly repeated and joined by β1-4 linkage. The xylan employed for standard assays is the natural product isolated from birchwood (Bailey, Biely & Poutanen, 1992). Following the naive hypothesis that if the enzyme were mixed directly with the polymeric substrate, and the combination set up for crystallization, then the enzyme might degrade the xylan polymer to defined stable products, products that might then co-crystallize with the xylanase to form enzyme–product complex crystals. This is in fact what did occur in the case of pancreatic α-amylase when mixed with its substrate glycogen (McPherson & Rich, 1972; Larson, Greenwood, Cascio, Day & McPherson, 1994). As with the native enzyme, we carried out the experiments both with heating to 323 K, and without. These were also performed with a range of substrate concentrations ranging from 0.5 to 5% by weight.

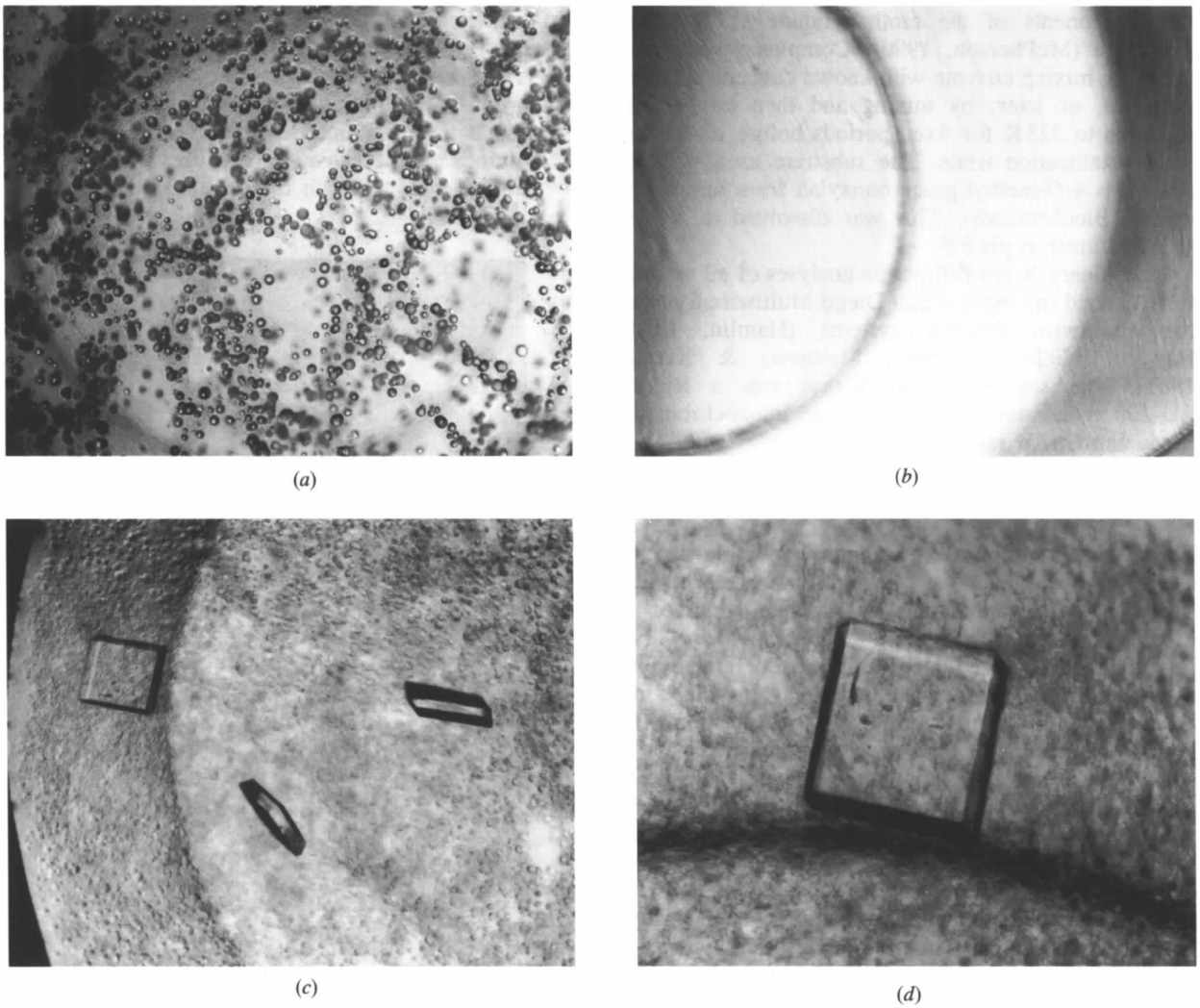
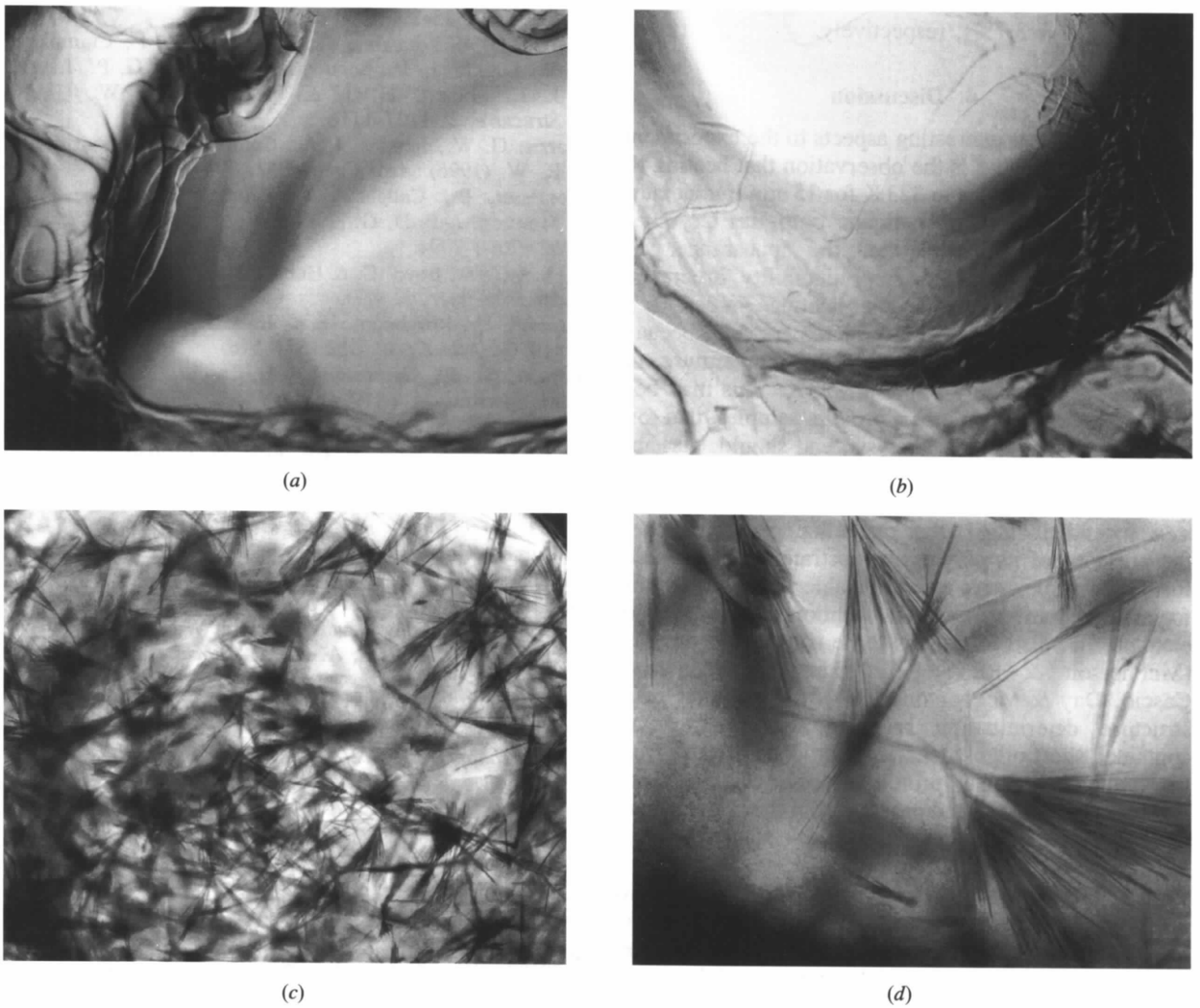


Fig. 2. (*a*) The xylan substrate dissolved in the crystallization mother liquor to a concentration of 0.5% with no heating beyond 291 K. Note the phase separation of the polymer into small droplets. This is eliminated in (*b*) upon heating for 15 min to 323 K. The crystals shown in both (*c*) and (*d*) were grown from a mixture of the xylanase plus 0.5% xylan after heating to 323 K for 15 min. The conditions were, otherwise, the same as for crystals not heated.

Fig. 2(*a*) is a photograph of the substrate at 0.5% concentration alone and unheated under the crystallization conditions, and in Fig. 2(*b*) after heating to 323 K for 5 min. Heating induces a visible change in the aggregation state and the dispersion of the substrate in the medium. In Figs. 2(*c*) and 2(*d*) are the results from the mixture of substrate plus enzyme, both unheated, Fig. 2(*c*), and heated to 323 K, Fig. 2(*d*). Identical results were obtained when the substrate concentration was 1% as well.

In Figs. 3(*a*) to 3(*d*) are shown corresponding results for the same experiments, using the identical sample of protein and substrate, but with a substrate concentration of 5%(*w*/*v*). The aggregation and dispersion state of the substrate, both with heating to 323 K, Fig. 3(*a*), and without, Fig. 3(*b*), are visually altered from those at lower concentrations. More striking, however, is that the crystals obtained from these mixtures are completely different to those at lower concentrations of substrate, appearing as masses of fine needles.

At 0.5 or 1.0% polymeric substrate concentration the presumptive complex crystals are the parallelepipeds seen in Figs. 2(*c*) and 2(*d*). Although their habit appeared quite distinct from crystals of protein in the absence of substrate, X-ray examination showed their unit cell to be virtually identical; space group $P2_1$ with $a=39.49$, $b=50.31$, $c=91.29$ Å and $\beta=101.58°$. The small but significant volume increase implies a change in V_m from 2.06 to 2.11 Å^3 Da^{-1}. When X-ray data from 15 to 2.2 Å resolution from these crystals were scaled to native data, however, the residual on F was



(*a*) (*b*) (*c*) (*d*)

Fig. 3. (*a*) The xylan substrate dissolved in the crystallization mother liquor to a concentration of 5% with no heating. Again a substantial phase separation is observed which is only partly eliminated upon heating to 323 K for 15 min, as shown in (*b*). The crystals shown in both (*c*) and (*d*) were grown from a mixture of the xylanase plus 5% xylan after heating to 323 K for 15 min. The conditions were, otherwise, the same as for crystals not heated.

$R = 0.34$, clearly demonstrating a substantial difference in the contents of the two unit cells.

At substrate concentrations of 5% the crystals grow as large masses of thin needles, and these clearly are of a different crystal form than those of the enzyme grown in the absence of xylan. Because of their morphology and size, examples of these needle crystals have not as yet been characterized by X-ray diffraction. At both concentrations of substrate complex crystals are reproducibly obtained, and their appearance is accelerated, and assured by heating first to 323 K for 15 min. No crystals with the crystallographic features of the putative complex crystals have been obtained in the absence of xylan.

Three-dimensional X-ray diffraction data sets have been collected on both native crystals, and crystals incubated with xylan, produced by treatment at 323 K. These data sets extended to 1.5 Å ($R_{merge} = 6.8\%$) and 2.1 Å ($R_{merge} = 3.7\%$), respectively.

4. Discussion

There are several interesting aspects to the experiments described here. First is the observation that heating the crystallization samples to 323 K for 15 min immediately before dispensing mother liquor onto the Cryschem plates significantly accelerated the appearance, and substantially enhanced the reproducibility of crystallization of the xylanase. The heating procedure is unlikely to affect the structure of the enzyme, because assays are routinely carried out at this temperature, the xylanase is completely stable at 323 K. This may be a useful procedure for other protein samples that one wishes to crystallize for analysis, and should, perhaps, be included among other procedures available to the investigator to promote nucleation and crystal growth.

A second finding is that crystallizing an enzyme in the presence of its polymeric substrate, in the hope of forming complex crystals with stable products, is valid for at least some systems. In addition to xylanase, for example, we also found this to be true with α-amylase (McPherson & Rich, 1972; Larson, Greenwood, Cascio, Day & McPherson, 1994). In this latter case, structure determination revealed that the crystals contained short oligosaccharides, produced from glycogen, which crosslinked protein molecules in the crystal lattice by binding to, and passing from the active site of one α-amylase molecule into the active site of another. This could pertain in the case of xylanase, which has a similar multi-subsite binding region for xylan and its products. This procedure, therefore, might be useful for other polysaccharide (or other polymeric substrate) degrading enzymes such as chitinases, cellulases, or mannosidases, to name just a few examples. Again, as with the native enzyme, heating to an elevated temperature appears to be a useful adjunct to promote crystallization.

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