Crystallization and Preliminary Crystal Data of Porcine Pepsinogen*

S. Narasinga Rao, Stanley N. Koszelak, and Jean A. Hartsuck

From the Laboratory of Protein Studies, Oklahoma Medical Research Foundation and the Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Single crystals of porcine pepsinogen, suitable for x-ray diffraction studies, have been grown with lithium sulfate as the precipitant. These pepsinogen crystals were dissolved, activated, and assayed for proteolytic activity. The specific enzymic activity of the dissolved crystalline protein was nearly twice that of the commercial pepsin from which the crystals were grown. Incubation at pH 8 before assay demonstrated that the crystals are free of pepsin. This crystal form of pepsinogen belongs to the monoclinic space group C2 with 4 molecules in the unit cell. The unit cell dimensions are a = 104.8 ± 0.5 Å, b = 43.1 ± 0.1 Å, c = 88.4 ± 0.3 Å, and β = 91.3°.

Pepsinogen is the zymogen of the gastric acid protease pepsin. Porcine pepsinogen is composed of a single polypeptide chain of 371 amino acid residues whose amino acid sequence is known (1-3). The structure contains three disulfide bridges. Pepsinogen has a molecular weight, calculated from the reported primary structure, of 39,630. Pepsin is formed when pepsinogen is activated in acidic solution of pH less than 5 by the removal of 44 residues (M, = 5,111) from the NH2-terminal end of the pepsinogen molecule (2, 4). Two different activation mechanisms, unimolecular and pepsin-catalyzed bimolecular, have been shown to exist (5-7). The unimolecular reaction is quite complicated. At least two pepsinogen intermediates precede the intramolecular bond cleavage (8). Moreover, the intramolecular bond lysis occurs between residues 16 and 17 (9). Residues 15 through 44 are removed subsequently by autocatalytic reactions (10). The nascent pepsin active site is thought to be responsible for the bond cleavage of intramolecular pepsinogen activation (7, 8). Optical rotatory (11), fluorescent (12, 13), and ultraviolet spectroscopy (14) and inhibition studies (8) all indicate a pepsinogen conformational change upon activation. The nature of this change is not known. Determination of the three-dimensional structure of pepsinogen will aid in understanding the mechanism of pepsin catalysis and the mechanism of unimolecular pepsinogen activation as well as the accompanying conformational changes.

The high resolution crystal structures of three fungal acid proteases from Penicillium janthinellum (penicillopepsin), Endothia parasitica, and Rhizopus chinensis have been determined (15, 16). These structures are all similar and since the amino acid sequence of penicillopepsin is homologous to that of porcine pepsin (15), the tertiary structure of porcine pepsin is probably similar to the three previously determined structures. A 2.7 Å resolution electron density map of porcine pepsin has been calculated, but its interpretation is not yet available (17). Zymogens of the fungal acid proteases have not been found.

Pepsinogen was crystallized first by Herriott (18). Recently, one of the chicken pepsinogens has been crystallized (19). Neither of these preparations was studied by x-ray diffraction. Herein we report a new crystallization procedure for porcine pepsinogen. These crystals are suitable for x-ray diffraction analysis.

EXPERIMENTAL PROCEDURES

Porcine pepsinogen was obtained either from the Worthington Corp. (grade PG) or from the Sigma Chemical Co. (grade 1) and was used without further purification. Crystallization was accomplished by the following procedure. Saturated lithium sulfate, pH 6, was added to a 6.2% pepsinogen solution which was 0.05 M in sodium cacodylate, pH 6, until turbidity was incipient. The tube then was sealed with Parafilm and left at 4°. The final lithium sulfate concentration was 52% and the final protein concentration was 2.85%. Crystals appeared after 2 weeks.

For enzymic assay, the crystals were rinsed with saturated lithium sulfate and dissolved in pH 6 water. A portion of the sample was raised to pH 8 with 0.1 M Tris chloride, pH 8.5. After 20 min at pH 8, the 130-μl sample was added to 1 ml of 0.2 M sodium citrate, pH 2. Assay at 23° for proteolytic activity with hemoglobin as substrate followed the procedure of Al-Janabi et al. (7). Another portion of the sample was activated at pH 2 without the pH 8 incubation. Protein concentrations were calculated from optical density at 278 nm. (The extinction coefficient used was ε278 = 13.65 20°.)

The density of the crystals was measured with a carbon tetrachloride-xylene density gradient column which had been calibrated with droplets of KBr solutions of known densities (21). A 10-μl pycnometer was used to measure the density of the 52% saturated lithium sulfate solution.

For x-ray crystallographic studies, the crystals were transferred to quartz capillaries and photographed on a precession camera with nickel-filtered copper radiation from an Elliott GX-6 rotating anode x-ray generator operated at 45 kV, 40 mA.

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RESULTS AND DISCUSSION

The pepsinogen from both sources crystallized at identical conditions and the resultant crystals were parallelepipeds with the same external morphology. Crystals as large as 1 x 0.3 x 0.3 mm have been obtained. Pepsinogen crystals also have been grown by equilibrating 17% pepsinogen solutions at pH 6 against 52% polyethylene glycol of molecular weight 400 in vapor diffusion experiments. Assays showed them to be pepsinogen but they are too small to be photographed.

Results of assays of potential proteolytic activity are shown in Table I. A 2-fold enhancement of the specific activity occurred upon crystallization. This is undoubtedly a consequence of purification by virtue of crystallization. Neither sample showed a significant diminution of activity after incubation at pH 8, where pepsin is denatured. Consequently, significant pepsin contamination of the crystals or the starting material did not occur.

An x-ray diffraction photograph of the h0Z (Fig. 1) zone showed only a center of symmetry. A hk0 photograph had two mirror planes. Examination of hk0 and hkl zones demonstrated a systematic absence when h + k is odd. A face-centered monoclinic cell, space group C2, was chosen with dimensions a = 104.8 ± 0.5 Å, b = 43.1 ± 0.1 Å, c = 88.4 ± 0.3 Å, and β = 91.3°. The indicated deviations in the axial lengths are root mean square errors determined when an average dimension was calculated from many spot locations on the 2.8 Å resolution photographs of the hk0 and hkl zones.

The cell dimensions generate a unit cell volume of 3.99 x 10^5 Å^3. These pepsinogen crystals reflect well to at least 2 Å resolution and are stable in the x-ray beam for at least 5 days.

The choice of the number of molecules per unit cell can often be made by calculating V_u, the crystal volume per unit of protein molecular weight (22). With 4 pepsinogen molecules in the unit cell, V_u is 3.52 Å^3 dalton, which is near the median of the commonly observed values (22). Confirmation of this choice was obtained from a simple partition of volume per g of crystal between the protein fraction and the solvent fraction in the crystal (23). The formula used is:

\[
\frac{1}{\rho_p} = f_p \rho_p + (1 - f_p) \frac{1}{\rho_s}
\]

where \(\rho_p\) and \(\rho_s\) are the densities of the crystal and the solvent, \(f_p\) is the weight fraction of protein in the crystal, and \(\rho_s\) is the partial specific volume of the protein. The measured density of the pepsinogen crystals was 1.23 g/ml and the 52% saturated lithium sulfate density was 1.136 g/ml. A value of 0.733 ml/g was used for \(\rho_s\). The calculated weight fraction of protein in the crystal was 0.46, which implies 3.43 protein molecules/unit cell. Even though this treatment is approximate, it is sufficient to confirm the choice of 4 molecules/unit cell.

We will attempt to determine the phases for the pepsinogen diffraction data by using the results of the penicillopepsin structure via molecular replacement (24). However, since only 32% of the amino acids are identical in porcine pepsin and penicillopepsin, additional isomorphous replacement phasing may be required. This combination was used to determine the structure of fetal hemoglobin (25).

Acknowledgment—We wish to thank Dr. Jordan Tang for his continued interest and helpful comments concerning this work.

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J. Biol. Chem. 1977, 252:8728-8730.

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