Crystallization of One of the Chicken Pepsinogens and its Derived Pepsin

By MARGARET L. GREEN

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, U.K.

(Received 22 September 1975)

Dodecahedral crystals of chicken pepsinogen have been prepared, one perfect crystal having the dimensions $0.090\text{ mm} \times 0.086\text{ mm} \times 0.009\text{ mm}$. The derived pepsin crystallized in the form of flat plates, often with one rounded end. The dimensions of one such crystal were $0.076\text{ mm} \times 0.043\text{ mm}$. Both types of protein crystals showed full enzymic or potential enzymic activity.

A number of acid proteinases have been crystallized. The three-dimensional structure of pig pepsin A (EC 3.4.23.1) has been published at 0.5 nm resolution (Andreeva *et al.*, 1971). Crystals of calf chymosin (EC 3.4.23.4) (Bunn *et al.*, 1971) and the acid proteinases from *Endothia parasitica* (Moews & Bunn, 1970), *Mucor pusillus* Lindt (Moews & Bunn, 1972), *Penicillium janthinellum* (EC 3.4.23.7) (Cameraman *et al.*, 1969) and *Rhizopus chinensis* (EC 3.4.23.9) (Swan, 1971) have been assigned to space groups and the unit-cell dimensions determined. Further, the acid proteinases from *Mucor miehei* (Sternberg, 1971) and *Rhodotorula glutinis* K-24 (Kamada *et al.*, 1972) have been crystallized. Of the corresponding zymogens, only pig pepsinogen has been crystallized (Herriott, 1955).

The X-ray-crystallographic analysis of chymosin has been impeded by the difficulty of preparing suitable heavy-atom derivatives (Bunn *et al.*, 1970), although this problem has apparently not arisen with pig pepsin (Andreeva *et al.*, 1971). Chicken pepsin and pepsinogen are unique among the acid proteinases and their zymogens that have been purified in containing a single thiol group that reacts with *p*-chloromercuribenzoate (Green & Llewellyn, 1973). This reaction permits the introduction of a heavy atom into a defined location in each molecule, which might assist in X-ray-crystallographic analysis of these proteins. The present report describes the crystallization of one of the chicken pepsinogens and the derived pepsin.

Experimental

Materials. Chicken pepsinogen and the derived pepsin were prepared as described by Green & Llewellyn (1973). Chicken pepsin (9.9 mg/ml) in 6 mM- Na_2HPO_4 –44 mM- NaH_2PO_4 , pH 6.0 (10 ml), was treated at 0°C with 0.5 M- $\text{Al}_2(\text{SO}_4)_3$ (0.5 ml), restored to pH 6.0 with saturated Na_2HPO_4 and the resulting precipitate removed by centrifugation, as described by Bunn *et al.* (1971). Immediately before

use protein solutions were clarified by centrifugation at 75000g for 1.5 h at 0°C.

Crystallization methods. Attempts at crystallization were carried out with protein solution (1–5 ml) at 5°C in the presence of thymol and 0.1 mM- NaN_3 . Chicken pepsinogen in 30 mM- Na_2HPO_4 –20 mM- NaH_2PO_4 , pH 7.0 (7.2 mg/ml), was dialysed against the appropriate buffer containing 0.1 mM- NaN_3 . The dialysis sac was then placed in a flask with 20 vol. of the required salt dissolved in buffer- NaN_3 and any other additions were made. The outer solution was changed or additions were made to it as required. Chicken pepsin in 50 mM-sodium phosphate buffer, pH 6.0 (4.7 mg/ml), was dialysed for a total of 6 h with stirring against two changes of 400 vol. of the solution in which crystallization occurred. It was then dialysed without stirring against a third portion of the same solution (400 vol.). Sacs containing each protein were examined microscopically at 1–4-week intervals.

Determination of the composition of crystals. Chicken pepsinogen and pepsin crystals were dissolved in 47 mM- Na_2HPO_4 –3 mM- NaH_2PO_4 , pH 8.0, and 50 mM-sodium phosphate buffer, pH 6.0, respectively. Protein concentrations and proteolytic or potential proteolytic activity were determined and agarose-gel electrophoresis was carried out by the duplicate plate method, as described previously (Green & Llewellyn, 1973).

Results

Crystallization of chicken pepsinogen. Approximately cuboid crystals could be obtained by dialysis of chicken pepsinogen against 50 mM-boric acid adjusted to pH 7.6 with NaOH and containing 1.32 M- MgSO_4 , but were more readily obtained in the presence of chloroform and toluene. Suitable conditions were dialysis of chicken pepsinogen against 1.92 M- $(\text{NH}_4)_2\text{SO}_4$ in 50 mM-sodium phosphate buffer, pH 8.0, in the presence of chloroform and toluene (each 1%, v/v) for 3 weeks. It was necessary to seed the

protein solution with either a small quantity of a previously prepared suspension of crystals or small crystals that were produced by raising the concentration of $(\text{NH}_4)_2\text{SO}_4$ to 2.23 M for 3 days but that disappeared after dilution to 1.92 M- $(\text{NH}_4)_2\text{SO}_4$ as the larger crystals were formed.

Chicken pepsinogen crystals were dodecahedral blocks, the larger faces being irregular hexagons (Plate 1a and b). The smaller crystal (Plate 1a), which appeared perfect, had the dimensions 0.090 mm $\times$ 0.086 mm with a thickness of at least 0.009 mm. The larger one (Plate 1b), of dimensions 0.26 mm $\times$ 0.23 mm $\times$ 0.05 mm, appeared imperfect.

Crystals prepared by each method, when dissolved, has potential specific activities approximately equal to that of the starting solution, and electrophoresis showed the presence of a single protein with potential acid proteinase activity and a mobility equal to that of the starting material.

Large chicken pepsinogen crystals required the continued presence of low concentrations of chloroform and toluene for satisfactory storage. The crystals redissolved in the absence of organic solvents, and excess of solvent caused precipitation of the protein as an oil. Thus the crystals were conveniently stored under the conditions used for their production.

Crystallization of chicken pepsin. Chicken pepsin could be crystallized as flat plates from 5–7.5 mm- H_2SO_4 . Occasionally, a large proportion of the crystals were needle-shaped. Then, the contents of the sac were redissolved by dialysis against 50 mm-sodium phosphate buffer, pH 6.0, and the crystallization step was repeated.

Many of the crystals had one rounded end (Plate 1c) and some were broken. Of the crystals shown in Plate 1(c), the approximately rectangular plate (A) that was lying on another on the photomicrograph had the dimensions 0.048 mm $\times$ 0.033 mm, and the larger crystal (B), with a slightly rounded end, was of size 0.076 mm $\times$ 0.043 mm.

The specific activity of the crystals, when dissolved, was slightly greater than that of the starting material, and electrophoresis showed the presence of a single protein with acid proteinase activity and of mobility identical with that of the starting material.

Crystals of pepsin could be stored satisfactorily for at least a year at 5°C in a small amount of the mother liquor and with a crystal of thymol. During storage, they increased in size and regularity.

Discussion

Active forms of chicken pepsinogen and the derived pepsin have been crystallized. Provided that the forms and sizes of the crystals are suitable for X-ray-diffraction analysis, this may permit direct study of the structural changes occurring on activation of the zymogen. Further, a structural analysis of chicken pepsin might provide information relevant to the whole group of acid proteinases, since these enzymes show both structural and mechanistic similarities, as discussed previously (Llewellyn & Green, 1975).

Some difficulty may be anticipated in reaction of the thiol groups of the proteins with heavy-metal compounds. The thiol group of pepsin is extremely susceptible to oxidation by O_2 (Green & Llewellyn, 1973). Further, modification of the thiol group of chicken pepsin causes changes in the specificity (Becker *et al.*, 1973), suggesting that conformational changes may occur.

I thank Dr. C. W. Bunn, Dr. T. Blundell and Dr. N. J. Berridge for help and encouragement, Mr. A. Turvey and Dr. D. J. Jayne-Williams for preparing the photomicrographs and Mrs. J. Bradfield and Mr. R. K. Maniak for skilled technical assistance.

- Andreeva, N. S., Borisov, V. V., Melik-Adamyanyan, V. R., Raiz, V. Sh., Trofimova, L. N. & Shutskever, N. E. (1971) *Mol. Biol. (Engl. Transl.)* **5**, 908–916
- Becker, R., Schechter, Y. & Bohak, Z. (1973) *FEBS Lett.* **36**, 49–52
- Bunn, C. W., Camerman, N., T'sai, L. T., Moews, P. C. & Baumber, M. E. (1970) *Philos. Trans. R. Soc. London Ser. B* **257**, 153–158
- Bunn, C. W., Moews, P. C. & Baumber, M. E. (1971) *Proc. R. Soc. Ser. B* **178**, 245–258
- Camerman, N., Hofman, T., Jones, S. & Nyburg, S. C. (1969) *J. Mol. Biol.* **44**, 569–570
- Green, M. L. & Llewellyn, J. M. (1973) *Biochem. J.* **133**, 105–115
- Herriott, R. M. (1955) *Methods Enzymol.* **2**, 3–7
- Kamada, M., Oda, K. & Murao, S. (1972) *Agric. Biol. Chem.* **36**, 1095–1101
- Llewellyn, J. M. & Green, M. L. (1975) *Biochem. J.* **151**, 319–326
- Moews, P. C. & Bunn, C. W. (1970) *J. Mol. Biol.* **54**, 395–397
- Moews, P. C. & Bunn, C. W. (1972) *J. Mol. Biol.* **68**, 389–390
- Sternberg, M. Z. (1971) *J. Dairy Sci.* **54**, 159–167
- Swan, I. D. A. (1971) *J. Mol. Biol.* **60**, 405–407

EXPLANATION OF PLATE I

Photomicrographs of crystals of chicken pepsinogen and the derived pepsin

Scale bar represents 0.1 mm. (a) and (b) Crystals of chicken pepsinogen prepared by dialysis of pepsinogen (7.2 mg/ml) seeded with small crystals at 5°C against 20 vol. of 50 mM-sodium phosphate buffer, pH 8.0, containing 1.92 M-(NH₄)₂SO₄, 0.1 mM-NaN₃, 1% (v/v) chloroform, 1% (v/v) toluene and a crystal of thymol. (c) Crystals of chicken pepsin prepared by dialysis of pepsin (4.7 mg/ml), dissolved in 50 mM-sodium phosphate buffer, pH 6.0, against three changes of 5 mM-H₂SO₄ containing 0.1 mM-NaN₃ (400 vol.) at 5°C, as described under 'Experimental'. One drop of each of chloroform and toluene and a crystal of thymol were added to the last portion of acid.