Preparation, Properties and Substrate-Exclusion Effects of an Insoluble Pronase Derivative

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1. A diazotized co-polymer of leucine and *p*-aminophenylalanine was used to prepare insoluble pronase. 2. The product was similar to the native enzyme in pH optimum, temperature optimum and broad specificity. 3. Exclusion effects were observed that appear related to the molecular weight of the substrate being hydrolysed. 4. The effects are explained on the basis of impedance of substrate access to the catalytic site by the supporting solid matrix.

Several insoluble derivatives of proteolytic enzymes have been described (for review see Silman & Katchalski, 1966). Analogous derivatives of non-proteolytic enzymes, such as urease (Riesel & Katchalski, 1964), are also known. While retaining catalytic activity, the insoluble products are frequently endowed with greater stability in suspension than possessed by their respective soluble counterparts. This effect is particularly noticeable with proteases, and is presumably due to the severe restriction that insolubility places on autolytic breakdown. Further, contamination of digests with enzyme-derived peptides is minimized. These properties, together with their easy removal from incubation mixtures, make the insoluble derivatives most useful tools in degradative studies on natural products.

In this paper we describe the preparation of an insoluble wide-spectrum protease (pronase). A study of simple properties suggests that the broad specificity is principally maintained. However, insolubility appears to limit access of substrate to the catalytic sites. This limitation is related to substrate size in a manner very similar to other well-documented exclusion phenomena.

MATERIALS AND METHODS

Chemicals. Pronase (B grade) was purchased from Calbiochem, London W.1, U.K. Glucagon was a generous gift from the Lilly Research Centre Ltd., Windlesham, Surrey, U.K. Lactalbumin and ovalbumin were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bovine plasma albumin was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., and lysozyme from the Worthington

Biochemical Corp., Freehold, N.J., U.S.A. Leucine *p*-nitroanilide was a product of the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

The co-polymer of leucine and *p*-aminophenylalanine was prepared by the method of Bar-Eli & Katchalski (1963). We are particularly grateful to Professor J. Baddiley, F.R.S., Department of Organic Chemistry, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, U.K., for generously providing the laboratory facilities necessary for this synthesis.

The molecular weights of substrates are those quoted by Andrews (1963), with the exception of lysozyme, determined as 14 400 by Sophianopoulos, Rhodes, Holcomb & Van Holde (1962).

Assays for enzymic activity. Enzyme solution or suspension (0.05 ml) was incubated for 30 min with stirring at 37°C with a 2% (w/v) solution (0.250 ml) of substrate in 25 mM-CaCl₂. CaCl₂ is thought to stabilize pronase activity (Nomoto, Narahashi & Murakami, 1960) and was therefore included in all incubation mixtures. Protein was precipitated by addition of 5% (w/v) trichloroacetic acid (0.50 ml). After centrifugation for 5 min at 2500g non-precipitated peptides were determined by the Folin-Ciocalteu method, with a tyrosine standard.

An alternative assay was used for leucine *p*-nitroanilide. Enzyme solution or suspension (0.05 ml) was incubated for 15 min at 37°C with an 0.01% solution (0.250 ml) of substrate. Reaction was terminated by cooling in ice, and activity determined by measuring the extinction at 400 nm of the supernatant after centrifugation at 2500g for 5 min.

Protein concentrations (Lowry, Rosebrough, Farr & Randall, 1951) were measured with vacuum-dried bovine plasma albumin as a standard.

EXPERIMENTAL AND RESULTS

Kinetics of the reaction between enzyme protein and diazotized co-polymer. The reaction of diazotized co-polymer (Cebra, Givol, Silman & Katchalski, 1961) with enzyme protein was followed at 0°C and pH 8.0. Protein remaining in solution was

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Fig. 1. Kinetics of the insolubilization reaction. Co-polymer (25mg) in 2M-HCl (0.2ml) was diazotized at 0°C by the addition over 20 min of 1M-NaNO₂ (0.14ml). After 2h at 0°C a saturated solution of sodium acetate (2.0ml) was added. The solution was adjusted to pH10.0 by addition of 1M-NaOH and the resultant precipitate was washed thrice with 50mM-tris-HCl buffer, pH8.0, containing 25mM-CaCl₂, and resuspended in the same buffer (3ml). A solution (1ml) containing pronase (8mg) and 25mM-CaCl₂ was added and the reaction mixture stirred at 0°C. Samples (0.4ml) were removed at intervals up to 2h and centrifuged for 5 min at 2500g, and a portion (0.25ml) of each supernatant solution was reserved for determinations of protein content and enzymic activity. To the remainder of the centrifuged sample was added a solution (1ml) of 0.4% phenol in 0.05M-tris-HCl buffer, pH8.0, and this mixture was incubated for 30 min at 0°C. Insoluble material was isolated by centrifugation as before, washed thrice, resuspended in buffer and reserved for determination of insoluble enzymic activity. Bovine serum albumin was used as substrate for activity determinations (see the Materials and Methods section). ○, Enzymic activity remaining in solution; Δ, protein remaining in solution; □, enzymic activity appearing in solid.

measured, as well as enzymic activity in solution and coupled to the insoluble polymer. The addition of 0.4% of phenol effectively stops further reaction by rapidly exhausting remaining diazonium groups. The results are shown in Fig. 1.

Although the supernatant protein and enzymic activity continue to decline after 20 min, activity in the precipitate fails to increase further. With bovine serum albumin as substrate, 22% of the original enzymic activity is recovered in the insoluble derivative.

Further preparations of coupled enzyme were made by using a 20 min incubation of pronase with diazotized co-polymer. Reaction was terminated by exhaustion of remaining diazonium groups with

Fig. 2. pH optima of soluble and insoluble enzymes. Buffers (0.05ml) of the pH shown were included in the routine assay with bovine serum albumin substrate. ●, Soluble enzyme; ○, insoluble enzyme.

0.4% phenol. The insoluble enzyme was washed five times with 50mM-tris-HCl buffer, pH8.0, containing 25mM-CaCl₂, recovery in each case being effected by centrifugation of the washed suspension at 2500g for 5 min. The final preparation was stored in suspension at 4°C in the same buffer. No proteolytic activity was detected in the final washings nor in the supernatant solution from the suspension stored at 4°C for 2 weeks. When kept in this way the enzyme derivative lost no detectable activity up to 6 months after preparation. Soluble pronase had a half-life of about 3 days when stored under the above conditions.

pH optima of soluble and insoluble enzymes. Optima were determined by including buffers (all at 30mM in the incubation volume), in the pH range 2.0–10.0, in the normal assay. The buffers used were pH2.0, KCl-HCl; pH2.8, glycine-HCl; pH4.0, sodium acetate; pH6.0, sodium succinate; pH7.2, 8.0, 9.0, tris-HCl; pH10.0, glycine-NaOH. Bovine serum albumin was used as substrate and the resultant curves (Fig. 2), which show optima at pH8.0, reveal little difference between soluble pronase and its insoluble analogue.

Temperature optima of the soluble and insoluble enzymes. Leucine *p*-nitroanilide (mol.wt. 251) was used to avoid the denaturation and precipitation that would probably have occurred with high-molecular-weight protein substrates. Results (Fig. 3) indicate that hydrolysis with the insoluble enzyme is less temperature dependent than with the soluble form, although both show temperature optima. Thermal activation would be expected to

Fig. 3. Temperature optima of the soluble and insoluble enzymes. Details of the assay method with leucine *p*-nitroanilide as substrate are given in the Materials and Methods section. Reaction mixtures were incubated at the temperatures shown for 15 min. ●, Soluble enzyme; ○, insoluble enzyme.

have a smaller effect when molecular movement is hampered by attachment to a solid matrix.

Effect of substrate molecular weight on the apparent activity of insoluble enzyme. Aqueous solutions of substrates at concentrations of 40 mg/ml were dialysed overnight to remove low-molecular-weight impurities. The exception was glucagon, which was not soluble to this extent, and could not be dialysed owing to its low molecular weight. It was therefore used as a saturated aqueous solution. Substrates were incubated with soluble or insoluble enzyme at concentrations chosen to give conveniently measurable extinctions in the photometric assays. Appropriate controls containing only substrate or enzyme were included. Results are shown for two substrates in Fig. 4, including an example of non-linear increase of hydrolytic rate with increase in enzyme concentration, a not-unknown feature of several other proteinases (Anson, 1939). This occurred with two substrates (lactalbumin and ovalbumin), and in these cases the initial (tangential) slopes were used in subsequent calculations. Although the molecular weight of hydrolysis products must be lower than the starting material, only a small portion of the total substrate is hydrolysed during the experiments of Fig. 4. This presumably accounts for the good correlation between reaction rate and log. initial molecular weight and suggests that the first steps are the rate-limiting ones.

From the amounts of soluble and insoluble pro-

Fig. 4. Hydrolytic rates with soluble or insoluble enzyme. Appropriate quantities of enzyme were determined in preliminary experiments and then these dilutions (0.05 ml) were used in the routine assay (see the Materials and Methods section). ○, Hydrolysis of bovine serum albumin with soluble pronase; ●, hydrolysis of bovine serum albumin with insoluble pronase; □, hydrolysis of lactalbumin with soluble pronase; ■, hydrolysis of lactalbumin with insoluble pronase.

nase used, and the relative hydrolytic rates achieved, it is possible to calculate the apparent recovery of activity in the insoluble enzyme for any particular substrate. The apparent recovery (R_a) is the ratio of the specific activity of the insoluble enzyme to that of the soluble form. The amount of protein coupled to the polymer was determined by difference at the time of insolubilization. If R_a is plotted against the logarithm of the molecular weight of the substrate, a straight line results. Two examples of soluble or insoluble pronase acting on a total of seven substrates are shown in Fig. 5.

DISCUSSION

Techniques similar to that described in this paper have been used to prepare insoluble derivatives of several enzymes (Silman & Katchalski, 1966). The pH-dependence of esterase activity of insoluble papain, insoluble polytyrosyl-trypsin and insoluble chymotrypsin was in all cases similar to that of the soluble enzyme. Further, the Michaelis constants for either soluble or insoluble papain were identical with benzoylarginine ethyl ester as substrate, which suggests that diazo-coupling does not alter the environment of a proteolytic enzyme's active site to a significant extent. This is in marked contrast with an insoluble polyanionic derivative of trypsin, where a maleic anhydride-ethylene coupling system was used (Goldstein, Levin & Bioch. 1970, 119

Fig. 5. Relationship between apparent recovery, R_a , of insoluble enzymic activity and molecular weight of substrate. (a) and (b) represent two separate preparations of insoluble pronase.

Katchalski, 1964). With this insoluble product, a displacement of pH-activity profiles approx. 2.5 pH units towards alkaline values was observed. With the pronase derivative described here, no profound change in pH-activity profile was noted when compared with the soluble enzyme. This is so in spite of the inclusion of phenol residues into the matrix. The phenol residues are, however, only weakly acidic compared with the carboxyl residues present in the anionic trypsin derivative.

The principle of stopping the diazonium-protein coupling reaction by blocking any remaining diazo groups with phenol appears to be justified in practice. Samples of washed insoluble enzyme not so treated were found to have lost up to 30% catalytic activity after overnight incubation at 0°C. This may be due in part to the coupling at several positions of an enzyme molecule to the insoluble matrix, with consequent distortion and denaturation of the catalyst. Further, the effective pore size could be decreased by continued cross-linking within the matrix or via the enzyme protein. In either case this would result in diminished enzymic activity with the substrate used in the assay (bovine serum albumin).

The concept of restricted access may be of importance in interpretation of results obtained with substrates of differing molecular weight and soluble enzymes. When active sites are bound to a solid support and thereby conceivably even buried, access limitations can become crucial. On the basis of findings with insoluble proteases, Silman & Katchalski (1966) concluded that the steric hindrance caused by carrier is of smaller importance in the interaction of a given water-insoluble enzyme with lower-molecular-weight substrates than with high-molecular-weight substrates. We have found that the ratio of insoluble to soluble activities, R_a ,

is related in a linear fashion to the logarithm of the substrate molecular weight. This suggests that the phenomenon may have much in common with the exclusion effects obtained in gels (Andrews, 1963). A useful approach to the theory of gel chromatography has been to assume that a portion of the inner volume of the gel is not accessible to a molecule of a given molecular weight. Porath (1963) has derived a relationship between the fraction, K_d , of the inner gel volume available to a molecule and its molecular weight, M . This may be written in a simplified form for a given gel, which fits experimental results well:

$$K_d = k_1 - k_2 M^* \quad (1)$$

If it is assumed that the insoluble pronase described here is a similar cross-linked matrix, with catalytic sites randomly distributed throughout its inner volume, then for a given substrate the accessible fraction of inner volume would be equal to the available fraction of the total number of enzyme molecules. Since reaction rate is proportional to enzyme concentration when excess of substrate is present, the hydrolysis rate with insoluble enzyme should also be proportional to the accessible fraction of the inner volume. For each substrate, then, it is possible to replace K_d by R_a , where R_a is the ratio of activities of insoluble to soluble enzyme. Eqn. (1) then becomes:

$$R_a = k_1 - k_2 M^* \quad (2)$$

Fig. 6 shows the good linearity that experimental results give when plotted in this form over the majority (90%) of the tested substrate molecular-weight range. However increasing departure from linearity is evident below a molecular weight of about 7000, and this tendency is also visible in Fig. 5(a). Substrates appear to be more efficiently

Fig. 6. Relationship between apparent recovery, R_a , of insoluble enzymic activity and molecular weight of substrate. The plot is according to the method of Porath (1963). (a) and (b) are as for Fig. 5.

hydrolysed than accounted for by an extrapolation of the results from higher molecular weights, 14000–160000. From a model based on restricted access, it might be anticipated that the graph (Fig. 6a) would become parallel to the abscissa at the lower range, because limitations of approach to the catalytic site would disappear below a certain substrate size. It is important to note however, that the observed departure from linearity is in the opposite direction from that anticipated. On such limited data, explanations must necessarily be speculative. Although Fig. 6(a) is drawn in the form of two straight lines intersecting at a molecular weight of about 7000, it is possible that a continuous curve is a better interpretation, reflecting the approximate nature of the Porath relationship. As presented in the figure however, attention is drawn to a further possibility, namely that attachment of some enzyme molecules to co-polymer by an amino acid residue (e.g. tyrosine) close to the substrate-binding site could completely block catalytic activity unless the substrate fell below a critical size. This effect would superimpose on the steric hindrance presented by the insoluble matrix as a whole. The satisfactory application of gel-exclusion theory to insoluble enzymes may not be a general rule, especially in those cases where active protein is attached principally to the surface of a cross-linked support. This may occur with a high enzyme/carrier ratio (Levin, Pecht, Goldstein & Katchalski, 1964), or when the supporting matrix is sufficiently cross-linked that deep entry of enzyme is prevented. Diazo-coupling of enzyme protein to a partly self-diazotized but otherwise open flocculate probably allows sufficient penetration of catalytic sites to cause the exclusion effects we describe.

As a simple and rapid method of determining molecular weights this technique suffers from the drawback of requiring relatively large amounts of substrate. The principles could nevertheless be

widely applicable to other polymeric substrates, e.g. nucleic acids, provided that the detection of hydrolytic products were sufficiently sensitive and proper standardizing substances were available.

The biological relevance of model systems employing enzymes bound to solid supports has recently received attention (Goldman, Silman, Caplan, Kedem & Katchalski, 1965; Chang & Poznansky, 1968). As a result of limited substrate access, regulation of anabolic or catabolic processes could obviously be quite strict.

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REFERENCES

- Andrews, P. (1963). *Biochem. J.* **91**, 222.
- Anson, M. L. (1939). *J. gen. Physiol.* **22**, 79.
- Bar-Eli, A. & Katchalski, E. (1963). *J. biol. Chem.* **238**, 1690.
- Cebra, J. J., Givol, D., Silman, H. I. & Katchalski, E. (1961). *J. biol. Chem.* **236**, 1720.
- Chang, T. M. S. & Poznansky, M. J. (1968). *J. biomed. Mat. Res.* **2**, 187.
- Goldman, R., Silman, H. I., Caplan, E., Kedem, O. & Katchalski, E. (1965). *Science, N.Y.*, **150**, 758.
- Goldstein, L., Levin, Y. & Katchalski, E. (1964). *Biochemistry, Easton*, **3**, 1913.
- Levin, Y., Pecht, M., Goldstein, L. & Katchalski, E. (1964). *Biochemistry, Easton*, **3**, 1905.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Nomoto, M., Narahashi, Y. & Murakami, M. (1960). *J. Biochem., Tokyo*, **48**, 593.
- Porath, J. (1963). *Pure appl. Chem.* **6**, 233.
- Riesel, E. & Katchalski, E. (1964). *J. biol. Chem.* **239**, 1521.
- Silman, I. H. & Katchalski, E. (1966). *A. Rev. Biochem.* **35**, 873.
- Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N. & Van Holde, K. E. (1962). *J. biol. Chem.* **237**, 1107.