Gly–Glu–Ser–Ala–Gly–Gly–(Glu, Ser)
|
DIP–O

Chromatography in butanol–acetic acid–water (4:1:5, by vol., upper phase) was carried out after peptic digestion for 1, 3 and 24 h. Fig. 2 shows a radioautograph of a chromatogram of a 3-h digest. Peptic action on the esterase peptides shown appears to be complete after 1 h. The probable identity of the major peptides from the sheep, horse and pig enzyme was substantiated by two-dimensional paper chromatography (butanol–acetic acid–water; then butanol–pyridine–acetic acid–water, 15:10:3:12, by vol.). Similarly, the major peptides from the two ox enzymes were shown to be identical. From Fig. 2, it is clear that the peptic peptides of the two ox enzymes are different from those of the chicken and also from those of the sheep, horse and pig enzymes.

Partial acid hydrolysis of the [^{32}P]DFP-labelled liver carboxylesterases produces the same set of radioactive peptides. Therefore, the sequences close to the labelled serine are the same in each case. The orange-skin esterase, however, while probably a "serine" enzyme (Fig. 1), clearly gives a slightly different pattern. Differences in amino acid sequence near the labelled serine of the liver carboxylesterases are revealed by the differences in chromatographic behaviour of the peptic peptides. These sequences are under active investigation in this laboratory.

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1 F. Sanger, *Proc. Chem. Soc.*, (1963) 76 and references therein.
2 D. J. Horgan, E. C. Webb and B. Zerner, *Biochem. Biophys. Res. Commun.*, 23 (1966) 18.
3 M. A. Naughton, F. Sanger, B. S. Hartley and D. C. Shaw, *Biochem. J.*, 77 (1960) 149.
4 H. S. Jansz, C. H. Posthumus and J. A. Cohen, *Biochim. Biophys. Acta*, 33 (1959) 387.
5 H. S. Jansz, C. H. Posthumus and J. A. Cohen, *Biochim. Biophys. Acta*, 33 (1959) 396.

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Chemical derivatives of subtilisin Carlsberg with increased proteolytic activity

During investigations of the significance of the functional side-chain groups for the stability and activity of the subtilisins (subtilopeptidase A, EC 3.4.4.16) it has been found that carbamylation of subtilisin Carlsberg and subtilisin Novo reduces the

proteolytic activity, which can subsequently be restored by treatment with hydroxylamine[1]. Succinylation of the ϵ-amino groups of subtilisin Novo does not alter the activity of the enzyme[2].

In the present communication we report that nitration of subtilisin Carlsberg with tetranitromethane following the procedure of SOKOLOWSKY, RIORDAN AND VALLEE[3] leads to a striking increase in activity towards the substrate, clupein. Maximal increase in activity (6- to 7-fold) was obtained after 1 h of reaction. At this stage, amino acid analysis showed that 6 tyrosine residues out of 13 were nitrated while no evidence was found that other amino acid residues were affected. That the increase in activity was connected with the modification of tyrosine residues was confirmed by treating subtilisin Carlsberg with iodine under conditions which minimize reaction with groups other than tyrosine[4]. Again, a 6-fold increase in activity towards clupein was observed. Finally, it was found that succinylation of subtilisin Carlsberg similarly increases the activity towards clupein by a factor of 6–7 (Table I).

TABLE I

INITIAL RATES OF HYDROLYSIS EXPRESSED AS μequiv H^+/min PER mg ENZYME PER ml, OF VARIOUS SUBSTRATES WITH NATIVE AND MODIFIED SUBTILISINS MEASURED WITH THE pH-STAT TECHNIQUE[7]

Concentration of clupein, casein, and gelatine: 0.5%. Concentration of BTEE: 2.5 mM. Temperature 30°, pH 8.0.

	Clupein	*Gelatine*	*Casein*	*BTEE*
Subtilisin Carlsberg	42	50	90	20
Succinylated subtilisin Carlsberg	290	320	110	22
Nitrated subtilisin Carlsberg	260	160	110	17
Iodinated subtilisin Carlsberg	260	170	80	19
Subtilisin Novo	387	170	90	5
Succinylated subtilisin Novo	400	80	80	5
Nitrated subtilisin Novo	400	120	60	4

The observed increase in activity was not due to a non-specific effect of the reagents since the activity of subtilisin Novo was unchanged by nitration with tetranitromethane, even after a reaction period of 24 h during which four out of thirteen tyrosine residues were nitrated. Also, when iodinated, the activity of subtilisin Novo was largely unchanged if no more than 4 moles of I_2 were introduced into the molecule. More extensive iodination rapidly inactivated this enzyme.

In order to test whether the observed increase in activity was specific for clupein as the substrate, two additional protein substrates, casein and gelatine, and the ester substrate, *N*-benzoyltyrosine ethyl ester (BTEE) were tested. As seen in Table I, the activity of modified subtilisin Carlsberg is essentially unchanged towards casein and BTEE while an increase is found towards gelatine.

RIORDAN AND VALLEE[5] have found a related phenomenon with carboxypeptidase A in which a modification of 2 tyrosine residues increased the esterase activity of the enzyme 6-fold and decreased the peptidase activity. This increase in esterase activity was in part due to a relief of substrate inhibition[6], but with subtilisin Carlsberg substrate inhibition was not observed at the protein concentrations used in the present investigation.

Native, lyophilized subtilisin Carlsberg contains 20–30% peptides. The possibility that these peptides inhibit the native enzyme and that this inhibition is abolish-

Abbreviation: BTEE, *N*-benzoyltyrosine ethyl ester.

ed in the derivatives must be considered. However, gel filtration on Sephadex G-50 did not lead to any activation by removal of the peptides. Any remaining peptide material, so tightly bound that it is not removed by gel filtration, is expected to be liberated by trichloroacetic acid precipitation of the enzyme. Since, by this procedure, less than 1% non-protein nitrogen was found, we consider peptide inhibition of the enzyme unlikely.

Variation of the substrate concentration (Fig. 1) shows that, at a concentration of 0.5% clupein, the rate of hydrolysis with succinylated and nitrated subtilisin Carls-

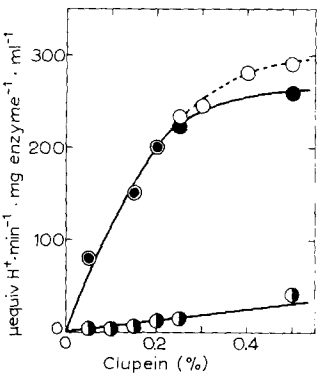

Fig. 1. The activity of native subtilisin Carlsberg (◑), succinylated subtilisin Carlsberg (○), and nitrated subtilisin Carlsberg (●), with varying concentrations of clupein, using the pH-stat technique[7]. pH 8.0, temperature 30°.

berg approaches v_{max} while the rate with native subtilisin Carlsberg is far from maximal. The increase in the activity of subtilisin Carlsberg produced by the modifications of tyrosine residues might thus be explained in terms of improved binding of substrate to the derivatives, as reflected by the decrease in K_m.

Both nitration and iodination decrease the pK of the tyrosine residues while succinylation introduces a negative charge. This suggests that the strong binding of clupein and gelatine by the modified types of subtilisin Carlsberg probably is due to the presence of a negatively charged tyrosine residue. The tyrosine involved in this binding apparently has no connection with the binding of casein and BTEE, and in subtilisin Novo a corresponding residue appears to be absent. It has recently been observed by COOMBS AND VALLEE[8] and by SCHECHTER AND BERGER[9] that the binding of polypeptides to another proteolytic enzyme, carboxypeptidase, similarly involves a multiplicity of binding sites.

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1 I. SVENDSEN, to be published.
2 A. GOUNARIS AND M. OTTESEN, *Compt. Rend. Trav. Lab. Carlsberg*, 35 (1965) 37.
3 M. SOKOLOWSKY, J. F. RIORDAN AND B. L. VALLEE, *Biochemistry*, 5 (1966) 3582.

4 W. L. HUGHES AND R. STRAESSLE, *J. Am. Chem. Soc.*, 72 (1950) 452.
5 J. F. RIORDAN AND B. L. VALLEE, *Biochemistry*, 2 (1963) 1460.
6 M. L. BENDER, J. R. WHITAKER AND F. MENGER, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 711.
7 C. F. JACOBSEN, J. LÉONIS, K. LINDERSTROM-LANG AND M. OTTESEN, in D. GLICK, *Methods of Biochemical Analysis*, Vol. IV, Interscience, New York, 1957.
8 T. L. COOMBS AND B. L. VALLEE, *Biochemistry*, 5 (1966) 3272.
9 I. SCHECHTER AND A. BERGER, *Biochemistry*, 5 (1966) 3371.

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Reversibility of the "irreversible" histidine ammonia-lyase reaction

The chemical reaction catalyzed by L-histidine ammonia-lyase (EC 4.3.1.3) is:

$$\underset{\text{L-histidine}}{R\text{–}CH_2\text{–}CH(NH_3^+)\text{–}COO^-} \rightleftharpoons \underset{\substack{\text{urocanate}\\(trans)}}{R\text{–}CH=CH\text{–}COO^-} + NH_4^+ \quad (R = \text{imidazole})$$

The reaction has been reported to be irreversible by WALKER AND SCHMIDT[1], MEHLER ANS TABOR[2], and PETERKOFSKY[3]. However, other well-known enzymes of this class catalyze ammonia eliminations that are readily reversible. For aspartate ammonia-lyase, K'_{eq} at 29° and pH 7.2 is 0.0074 (ref. 4); for β-methylaspartate ammonia-lyase, K'_{eq} is reported by BARKER *et al.*[5] to be 0.238 at 25° and pH 7.9. It appeared to us that the apparent irreversibility might result from the choice of unfavorable reaction conditions or insufficient incubation time.

Histidine ammonia-lyase was purified 125-fold from sonic lysates of *Pseudomonas fluorescens* A.3.12 by a modification of the method of PETERKOFSKY[3]. A protamine sulfate fractionation step was included; L-histidine was added during the heat step, and the enzyme was fractionated with $(NH_4)_2SO_4$ between 50–65% of saturation, rather than 0–50%. The final specific activity was 35 μmoles of urocanate per min per mg of protein. The assay system contained 0.1 M L-histidine (pH 8.0), 0.033 M Tris acetate (pH 8.0), 1 mM $MgSO_4$, 0.033 mM EDTA, 0.67 mM mercaptoethanol, and a suitable dilution of the enzyme.

The enzyme appeared to be homogeneous on gel electrophoresis. It had an approximate molecular weight of 198 000 as determined on a sucrose gradient including catalase as a reference.

In the histidine synthesis experiment we made the following departures from traditional systems[3,6]: (a) Tris acetate buffer was used rather than pyrophosphate because of the known sensitivity of the enzyme to metal-sequestering agents; (b) Mg^{2+} was added to ensure an adequate concentration of metal activator; and (c) the pH of the system was adjusted to 8.0 rather than 9.0–9.5, since PETERKOFSKY[3] has shown that the enzyme-catalyzed exchange of the β proton of histidine has an optimum pH near 7.5. The complete system contained the following: 0.5 M NH_4Cl (pH 8.0), 3.0 ml; 0.2 M potassium urocanate (pH 8.0), 3.0 ml; 0.01 M $MgSO_4$, 0.5 ml; 0.2 mg enzyme protein in 0.1 M Tris acetate buffer (pH 8.0), which was 1 mM in mercaptoethanol, 1.0 ml.