Biochimica et Biophysica Acta, 429 (1976) 954—963
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

BBA 67790

PREPARATION AND ENZYMATIC PROPERTIES OF SUBTILISIN *NOVO* CHEMICALLY ATTACHED TO SOLUBLE DEAE-DEXTRAN AND INSOLUBLE DEAE-SEPHADEX

BIRTE SVENSSON

Carlsberg Laboratory, Department of Chemistry, Copenhagen (Denmark)

(Received September 10th, 1975)

Summary

Analogous soluble and insoluble derivatives of subtilisin *Novo* (EC 3.4.21.14) were prepared by coupling the enzyme to CNBr-activated DEAE-dextran and DEAE-Sephadex, respectively. The DEAE-dextran-subtilisin displayed pH optima and K_m values for ester hydrolysis similar to subtilisin, whereas the pH versus activity profiles obtained with DEAE-Sephadex-subtilisin were shifted towards the alkaline pH region and the K_m values were increased. Compared with subtilisin, DEAE-dextran-subtilisin showed a 40—65% reduction of k_{cat} for hydrolysis of *N*-acetyl-L-tyrosine ethyl ester, *p*-tosyl-L-arginine methyl ester and benzyloxycarbonyl-glycyl-L-tyrosinamide and its maximum velocities for digestion of casein and clupein also amounted to 40—60% of the subtilisin values. With DEAE-Sephadex-subtilisin, in contrast, the maximum velocity of hydrolysis decreased to a greater extent for polypeptide substrates compared to ester substrates.

The present results indicate that the chemical nature of a support can affect intrinsic properties of a matrix-bound enzyme in addition to the steric and diffusional effects usually observed with polymer-attached enzymes.

Introduction

The apparent activity of an immobilized enzyme may be affected by a number of factors, including the chemical nature of the polymeric support, steric hindrance, and diffusive and electrostatic phenomena [1—7]. Experimental ap-

Abbreviations: Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; Cbz, benzyloxycarbonyl.

proaches to the study of the influence of the support material on the properties of the bound enzyme have received little attention. This situation is probably due to the fact that several factors act simultaneously, thus complicating the elucidation of the contribution of any single factor to the observed changes in enzymatic properties.

The present communication describes an attempt to circumvent this problem by investigating a water-soluble enzyme derivative, prepared by covalent attachment of oligomers which chemically resemble the employed polymeric support. A modified activity for such a suitably chosen derivative could be assumed to stem principally from the influence of the oligomeric substituents on the enzyme function in contrast to the electrostatic and diffusive effects that can perturb the observed activities of the polymer-bound enzymes by altering the concentrations of substrates and other species in the enzyme microenvironment.

The enzyme derivative studied was soluble DEAE-dextran subtilisin, prepared by covalent attachment of oligomeric CNBr-activated DEAE-dextran to subtilisin *Novo* (EC 3.4.21.14). In addition, some properties of the insoluble analogue, DEAE-Sephadex-subtilisin, have been examined. The importance of the presence of DEAE groups to the changes in enzymatic behaviour upon the oligomer/polymer attachment is discussed in relation to the previously characterized amylodextrin-subtilisin [8] and Sepharose-subtilisin [9].

Materials

Subtilisin type *Novo* (batch 120-2), crystallized and lyophilized, was a gift from the Novo Industries, Copenhagen. This preparation contained about 30% of peptide material originating from autolysis. DEAE-Dextran hydrochloride, average mol. wt. 1800 and nitrogen content 3.4%, was a generous gift from Dr. H. Lundgren, Pharmacia Fine Chemicals, Uppsala. DEAE-Sephadex A50 and Sephadex G50 were products of Pharmacia Fine Chemicals, Uppsala.

N-Acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) and *p*-tosyl-L-arginine methyl ester hydrochloride (Tos-Arg-OMe) were obtained from the Sigma Chemical Company, St. Louis, Mo. The *N-trans*-cinnamoyl imidazole (Fluka, Buchs, Switzerland) had been recrystallized from cyclohexane before use.

Cbz-glycyl-L-leucinamide and Cbz-glycyl-L-tyrosinamide were bought from the Cyclo Chemical Corporation, Calif. Cbz-Glycyl-glycyl-L-leucinamide was kindly synthesized by Dr. P. Larsen, The Institute of Protein Chemistry, Copenhagen.

Casein a.m. Hammarsten was from the Struers Chemical Laboratories, Copenhagen. Clupein sulfate was prepared from dried herring sperm [10].

Methods

DEAE-Dextran was treated with CNBr as described previously for amylodextrin [8]. To 100 mg of DEAE-dextran hydrochloride in 10 ml water was added 3 × 1 ml CNBr solution (13 mg/ml) at 4-min intervals. The pH was kept at 10.8 during the reaction by automatic titration with 1 M NaOH using a pH-stat. After 12 min the pH was adjusted to 9.5 and the carbohydrate precipitated by addition of 130 ml acetone at −10°C. The activated DEAE-dextran

was isolated by centrifugation in the cold, redissolved in 5 ml of 0.2 M sodium borate pH 9.0 and added to subtilisin, preliminary coupling experiments in the pH range 8.0—10.0 had demonstrated that the maximal amount of DEAE-dextran was covalently bound to the enzyme at these conditions. After 2 h at 4°C, the coupling mixture was fractionated on Sephadex G50 (1.8 × 50 cm) by elution with $2 \cdot 10^{-3}$ M sodium phosphate pH 7.5. The protein derivative was lyophilized and stored at −18°C.

The covalent attachment of subtilisin *Novo* to DEAE-Sephadex [illegible] followed essentially the method described earlier [11]. The CNBr-activation was performed at pH 10.8 and the subsequent coupling of subtilisin took place at 4°C for 20 h, best yields were obtained in 0.2 M sodium carbonate pH 10.0. The product was washed with 1 M NaCl, 0.1 M $NaHCO_3$ and water.

The number of modified ε-amino groups was determined by reductive methylation of the remaining free amino groups. The resulting *N*-ε-dimethyllysine and *N*-ε-methyllysine were determined by amino acid analysis [12].

Carbohydrate contents were measured by the phenol/sulfuric acid procedure [13], using DEAE-dextran as standard.

Concentrations of subtilisin *Novo* and DEAE-dextran-bound subtilisin *Novo* were calculated from the ultraviolet absorbance at 278 nm, $E^{1\%}_{1cm} = 11.7$ [14].

Amino acid analysis was carried out on a Beckman/Spinco Model 120 automatic amino acid analyzer modified to complete an analysis in 3.5 h [15]. Samples were hydrolyzed in 6 M HCl at 110°C for 24 h in sealed evacuated tubes.

The enzymatic activity towards Ac-Tyr-OEt, Tos-Arg-OMe, casein and clupein sulfate was measured by the pH-stat technique [16,17]. The specific activities of soluble enzyme samples were calculated on the basis of active site contents as determined by titration with *N-trans*-cinnamoyl imidazole [18]. In the lyophilized preparations 60—70% of the protein represented active enzyme.

The activity towards synthetic peptide derivatives was measured as described earlier [19] using a ninhydrin method [20]. The splitting products were identified by paper chromatography [19].

Results

Preparation and chemical characterization of DEAE-dextran-subtilisin and DEAE-Sephadex-subtilisin

About 50% of the protein present during the reaction with CNBr-activated DEAE-dextran was recovered as DEAE-dextran-subtilisin (Table I).

CNBr-activated carbohydrates bind amino compounds covalently [21,22]. Accordingly, fewer ε-amino groups were susceptible to reductive methylation in DEAE-dextran-subtilisin than in subtilisin. The results in Table I show that the extent of modification for DEAE-dextran-subtilisin increased from 2.3 to 3.1 converted lysyls per enzyme molecule with decreasing protein concentration in the coupling mixture. Repeated reactions between DEAE-dextran-subtilisin and fresh portions of activated DEAE-dextran resulted in a maximal conversion of 6.2 ε-amino groups per enzyme molecule.

DEAE-dextran-subtilisin preparations contained 26—36 carbohydrate residues per enzyme molecule (Table I). This amount is about 15% higher than cal-

TABLE I

CHEMICAL PROPERTIES OF DEAE-DEXTRAN-SUBTILISIN AND DEAE-SEPHADEX-SUBTILISIN

Enzyme derivative	Protein (mg) in the coupling mixture/g of activated DEAE-dextran or DEAE-Sephadex	Per cent of protein recovered in the coupled product	Protein content (mg)/g of coupled product *	Carbohydrate residues per protein molecule **	Modified lysine residues per protein molecule ***
DEAE-dextran-subtilisin I	2000	50	820	26	2.3
II	2000	51	790	32	2.7
III	1000	46	780	34	2.7
IV	500	48	770	36	3.1
DEAE-Sephadex-subtilisin I	500	10	46	3750	5.8
II	100	45	46	3750	5.0
III	50	60	31	5650	5.4

* Calculated for DEAE-dextran-subtilisin from analytical data on protein and carbohydrate contents. For DEAE-Sephadex-subtilisin protein contents are calculated from dry weight and amino acid analysis of the lyophilized conjugate.

** Determined by the phenol/sulfuric acid procedure [13] using DEAE-dextran as standard and calculated from the protein content for DEAE-dextran-subtilisin and DEAE-Sephadex-subtilisin, respectively.

*** Determined from the number of lysine residues accessible to reductive methylation.

culated from the number of modified protein amino groups, including the susceptible N-terminal α-amino group [17], and the average molecular weight of the employed DEAE-dextran. It is suggested that this discrepancy is due to the presence of free and/or protein-bound polymerized DEAE-dextran. This assumption is supported by the fact that CNBr-treated DEAE-dextran, which had been subjected to coupling conditions in the absence of protein, contained carbohydrate material eluting from Sephadex G50 before unactivated DEAE-dextran.

The protein contents of DEAE-Sephadex-subtilisin were rather low (Table I). The coupling yield was, however, found to be maximal at pH 10 employed for the reaction, and the results in Table I indicate that a further increase of the protein concentration in the coupling step would not improve the protein content of the final product. Similar levels of protein coupling have been reported in a number of cases [9,11,23,24], including Sepharose-subtilisin *Novo*. From 5.0 to 5.8 of the 11 ϵ-amino groups in subtilisin *Novo* had been coupled to DEAE-Sephadex (Table I).

Enzymatic properties

A. Hydrolysis of ester. The pH dependence of specific activities of soluble DEAE-dextran-subtilisin, insoluble DEAE-Sephadex-subtilisin and subtilisin were compared at ionic strengths 0.125 and 1.0 using Ac-Tyr-OEt and Tos-Arg-OMe as substrates. Subtilisin and DEAE-dextran-subtilisin gave rise to curves having essentially the same pH optima, while those of DEAE-Sephadex-subtilisin were displaced to more alkaline pH values both at the low and the high salt concentrations (Figs. 1 and 2).

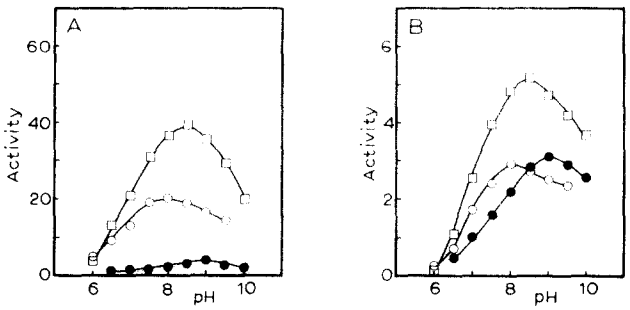


Fig. 1. pH versus activity profiles for hydrolysis of 0.0025 M Ac-Tyr-OEt (A) and 0.0025 M Tos-Arg-OMe (B) at ionic strength 0.125 by subtilisin (□——□), DEAE-dextran-subtilisin (○——○) and DEAE-Sephadex-subtilisin (0.010 M Tos-Arg-OMe) (●——●). The activities are determined as initial velocities given in μmol H^+ released/s per μmol of enzyme.

The specific activity of DEAE-dextran-subtilisin was reduced relative to subtilisin. A comparison of the kinetic parameters for ester hydrolyses showed K_m to be unaffected by the DEAE-dextran attachment, whereas the overall catalytic rate constant, k_{cat}, decreased approx. 2 fold. This difference persisted at high ionic strength (Table IIA).

The insoluble DEAE-Sephadex-subtilisin showed apparent Michaelis constants, K_m(app), for hydrolysis of Tos-Arg-OMe and Ac-Tyr-OEt which were approx. 2- and 3-fold higher respectively than the corresponding subtilisin and DEAE-dextran-subtilisin values. Addition of salt seemed to cancel the effect only in the case of Tos-Arg-OMe (Table IIB). Although the k_{cat} values for the insoluble DEAE-Sephadex-subtilisin are not corrected on the basis of the content of *N-trans*-cinnamoyl imidazole-titratable enzyme, it seems likely that DEAE-Sephadex-attachment reduces the overall catalytic rate constants to a similar degree to that caused by coupling subtilisin to soluble DEAE-dextran (Table IIB). This assumption is supported by the fact that k_{cat} values for Sepharose-subtilisin [9] and subtilisin, the correction for the contents of active centers being omitted, were similar to each other.

B. Hydrolysis of synthetic peptides. The amidase activity of DEAE-dextran-

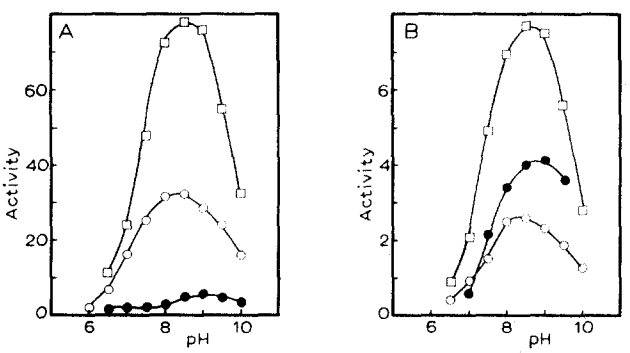


Fig. 2. pH versus activity profiles for hydrolysis of 0.0025 M Ac-Tyr-OEt (A) and 0.0025 M Tos-Arg-OMe (B) at ionic strength 1.0 by subtilisin (□——□), DEAE-dextran-subtilisin (○——○) and DEAE-Sephadex-subtilisin (0.010 M Tos-Arg-OMe) (●——●). The activities are determined as initial velocities given in μmol H^+ released/s per μmol of enzyme.

TABLE II

KINETIC PARAMETERS FOR HYDROLYSIS OF *N*-ACETYL-L-TYROSINE ETHYL ESTER AND *p*-TOSYL-L-ARGININE METHYL ESTER BY DEAE-DEXTRAN-SUBTILISIN, DEAE-SEPHADEX-SUBTILISIN AND SUBTILISIN AT DIFFERENT IONIC STRENGTHS

The kinetic parameters were calculated, using linear regression analysis, from Lineweaver-Burk plots of activities determined at the appropriate pH optima and using ester concentrations ranging from 0.0025 to 0.025 M. The ionic strength was adjusted by addition of KCl.

A.	Ionic strength	Ac-Tyr-OEt			Tos-Arg-OMe		
		pH *	K_m (mM)	k_{cat} (s^{-1})	pH *	K_m (mM)	k_{cat} (s^{-1})
DEAE-dextran-subtilisin (IV)	1.0	8.4	33	470	8.4	36	3.3
	0.125	8.0	38	320	8.0	31	3.7
Subtilisin	1.0	8.5	26	880	8.5	31	10
	0.125	8.2	35	520	8.2	36	7.7
B.	Ionic strength	pH *	K_m(app) (mM)	k_{cat} ** (s^{-1})	pH *	K_m(app) (mM)	k_{cat} ** (s^{-1})
DEAE-Sephadex-subtilisin (II)	1.0	9.0	110	210	8.7	40	1.9
	0.125	9.0	105	150	8.7	57	2.2

* pH values employed in the kinetic measurements are chosen to correspond to estimated pH-optima (see Figs. 1 and 2).

** k_{cat} values for DEAE-Sephadex-subtilisin are not calculated on the basis of contents of active enzyme and are given as μmol H^+ released/s per μmol of protein.

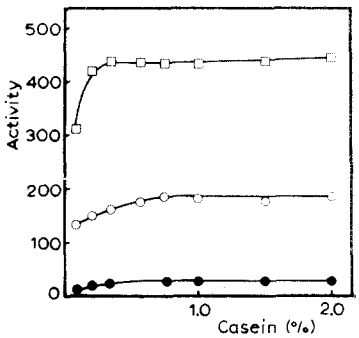
subtilisin was investigated using Cbz-glycyl-L-leucinamide, Cbz-glycylglycyl-L-leucinamide and Cbz-glycyl-L-tyrosinamide as substrates. For all of the three peptides the pH optimum of the activity was found to pH 8.2 for DEAE-dextran-subtilisin and to pH 8.5 for subtilisin. Paper chromatography of the cleavage products demonstrated that DEAE-dextran-subtilisin, like subtilisin [19], specifically catalyzed the hydrolysis of the C-terminal amide group of the present peptide derivatives. The kinetic parameters, determined at the respective pH optima, are shown in Table III. The chemical modification did not seem to influ-

TABLE III

KINETIC PARAMETERS FOR HYDROLYSIS OF SYNTHETIC PEPTIDE DERIVATIVES BY DEAE-DEXTRAN-SUBTILISIN AND SUBTILISIN

The hydrolyses were carried out at 40° C in 0.05 M Tris · HCl pH 8.2 (DEAE-dextran-subtilisin) or pH 8.5 (subtilisin) in the presence of 15% (v/v) dimethylformamide. The peptide concentrations ranged from 0.003 to 0.009 M. Initial rates of hydrolysis were determined from the ninhydrin color intensities of aliquots removed at suitable time intervals. The k_{cat} are calculated on the basis of the contents of active enzyme as determined by titration with *N-trans*-cinnamoyl imidazole. The values are means of double determinations agreeing within ± 8% or better.

	Cbz-Gly-L-Leu-NH_2		Cbz-Gly-Gly-L-Leu-NH_2		Cbz-Gly-L-Tyr-NH_2	
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
DEAE-dextran-subtilisin (IV)	24	0.39	22	7.4	23	0.40
Subtilisin	26	0.40	26	8.7	27	0.78



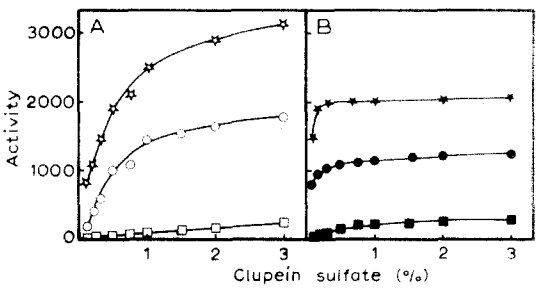


Fig. 3. Digestion of casein by subtilisin (□———□), DEAE-dextran-subtilisin (○———○) and DEAE-Sephadex-subtilisin (●———●). The activities are determined as initial velocities given in μmol H^+ released/min per μmol of enzyme.

Fig. 4. Digestion of clupein sulfate by subtilisin (☆,★), DEAE-dextran-subtilisin (○,●) and DEAE-Sephadex-subtilisin (□,■) (A) and in the presence of 2 M KCl (B). The activities are determined as initial velocities given in μmol H^+ released/min per μmol of enzyme.

ence K_m or k_{cat} for hydrolysis of Cbz-glycyl-L-leucinamide or Cbz-glycylglycyl-L-leucinamide, but k_{cat} for hydrolysis of Cbz-glycyl-L-tyrosinamide decreased by a factor of two.

C. Hydrolysis of polypeptides. The polypeptide substrates, casein and clupein sulfate, were degraded more slowly by DEAE-dextran-subtilisin than by subtilisin (Figs. 3 and 4). The approximate maximum velocities for the hydrolysis corresponded to around 40% and 60% of the maximum rates for the subtilisin-catalyzed hydrolysis of casein and clupein respectively. In 2 M KCl the highly positively charged clupein was also hydrolyzed more slowly by DEAE-dextran-subtilisin (Fig. 4B), in agreement with the findings using low molecular weight ester substrates.

With both polypeptide substrates, initial rates of hydrolysis were much lower for the insoluble DEAE-Sephadex-subtilisin than for DEAE-dextran-subtilisin (Figs. 3 and 4).

Discussion

The synthesis of soluble DEAE-dextran-subtilisin, by a modification [8] of the method for attaching proteins to CNBr-activated polysaccharides, made it possible to investigate the influence of oligomeric carbohydrate substituents on the function of the bound enzyme.

The k_{cat} for hydrolysis of ester substrates decreased relative to subtilisin as a result of the DEAE-dextran-attachment. The previously characterized soluble amylodextrin-subtilisin [8], which contained a similar amount of oligomeric carbohydrate substituents as DEAE-dextran-subtilisin, in contrast displayed kinetic parameters essentially identical to those of subtilisin. Amylodextrin-subtilisin and subtilisin also showed the same k_{cat} for deamidation of Cbz-glycyl-L-tyrosinamide (Svensson, B., to be published), while a smaller k_{cat} value was found for DEAE-dextran-subtilisin. Amylodextrin-subtilisin and subtilisin carry an essentially identical net charge at the assay conditions [25]. It is tempting, therefore, to ascribe the different behaviour of DEAE-dextran-subtilisin to its

approx. 20 additional cationic groups, though a persistance of a reduced k_{cat} in 1 M KCl suggests that simple electrostatic phenomena alone are not responsible for the altered enzymatic properties. Similar findings have been reported for a number of water-soluble polyelectrolyte-protease conjugates. Thus, the polycationic polyornithyl-trypsin [26] and -chymotrypsin [27] were less active as esterases and amidases than the respective unmodified enzymes. Conversely, soluble polyanionic derivatives such as CM-cellulose-trypsin [28] and -chymotrypsin [28], ethylene maleic acid copolymer-chymotrypsin [27] and -subtilisin *Novo* [17], and polyglutamyl-chymotrypsin [27] all displayed elevated activities towards ester and amide substrates. Recent studies on chymotrypsin derivatives proved that electrolyte substituents perturb the course of the catalytic process at the active site of the bound enzyme, polyanions increasing intrinsic rate constants of the acylation and deacylation steps while polycations reduced these rate constants [27]. The same pattern has now been demonstrated for subtilisin *Novo*, whose catalytic mechanism is supposed to resemble closely the chymotrypsin mechanism [29].

The effect of DEAE-dextran attachment on the hydrolysis of synthetic peptides was ambiguous, leucinamide groups being split with unchanged k_{cat}, while k_{cat} for hydrolysis of tyrosinamide groups was decreased relative to subtilisin. Inhibition studies have given kinetic evidence that subtilisin *Novo* accomodates ester substrates in one of two productive binding modes determined by the nature of their amino acid side chain [30,31]. Support for the existence of a similar situation with peptide substrates is obtained from X-ray crystallographic studies on complexes between the enzyme and some virtual substrates, i.e. the carboxyl group moieties of the true peptide substrates. Only one binding mode was indicated for Cbz-glycylglycyl-L-tyrosine but several for Cbz-glycylglycyl-L-leucine [29]. The possibility that generation and breakdown of various acyl-subtilisins might pass through structurally different intermediates, could explain the different effects of a chemical modification on the conversion of the various substrates.

The DEAE-dextran-subtilisin digested casein and clupein sulfate by a lower rate than amylodextrin-subtilisin and subtilisin [8]. However, in view of results for the DEAE-dextran-subtilisin-catalyzed hydrolysis of peptide substrates, it is difficult to decide to what extent the reduced activity is due to changes in the kinetic properties of the bound enzyme and to what extent steric interactions impeding the formation of the enzyme-substrate complex are responsible.

Covalent coupling of subtilisin to DEAE-Sephadex-particles caused a shift in the pH versus activity profiles towards the alkaline pH region which had not been observed with the soluble analogue, DEAE-dextran-subtilisin. In principle one should expect the particle phase containing the cationic DEAE groups to repel H^+ resulting in a displacement of the apparent pH optima for the bound enzyme to more acidic pH values as in the cases of DEAE-cellulose-chymotrypsin [32] and -penicillin amidase [33,34]. The actual result for DEAE-Sephadex-subtilisin going in the opposite direction suggests diffusion-restricted accumulation of H^+ in the particles in consequence of the ester hydrolysis by the bound enzyme [35]. The effect is perhaps reinforced by neutralization of the DEAE groups by the generated carboxylate ions [36]. Also, the 3-fold increase of K_m(app) for the rather quickly hydrolyzed Ac-Tyr-OEt might reflect

diffusional limitations which in this connection cause insufficient supply of substrate. In agreement with this, Sepharose-subtilisin also had a higher K_m(app) than subtilisin for hydrolysis of Ac-Tyr-OEt [9]. Furthermore, some insoluble derivatives of chymotrypsin showed augmentation of the apparent Michaelis-Menten constant concluded to stem from diffusional effects [7,37]. It shall though be emphasized that the increased apparent K_m value obtained from Lineweaver-Burk plots, when diffusional restrictions occur, is not the true K_m of the enzyme reaction [38]. In the case of the slowly hydrolyzed Tos-Arg-OMe the increase in K_m(app) was eliminated by addition of concentrated salt. This behaviour could be explained as a shielding of electrostatic effects comprising a repulsion of the cationic ester from the particle domain.

We conclude from the present investigation on a soluble oligomer-enzyme conjugate that the chemical nature of the support material can affect intrinsic catalytic properties of a matrix-bound enzyme in addition to the apparent changes in enzymatic behaviour caused by the electrostatic field and diffusional limitations in the particle phase.

Acknowledgements

The author is indebted to Professor M. Ottesen for his interest in the present investigation and to Dr. H. Abrash for valuable criticism of the manuscript. Dr. P. Larsen, The Institute of Protein Chemistry, Copenhagen, is thanked for invalueable help with peptide synthesis. The expert technical assistance of Miss I. Plougmann Andersen and Mrs. B. Corneliussen is gratefully acknowledged. The author is a recipient of a post-doctoral fellowship at the University of Copenhagen.

References

1 Goldstein, L. and Katchalski, E. (1968) Z. Anal. Chem. 243, 375—396
2 Goldman, R., Goldstein, L. and Katchalski, E. (1971) in Biochemical Aspects of Reactions on Solid Supports (Stark, G.R., ed.), pp. 1—78, Academic Press, New York
3 Zaborsky, O. (1973) Immobilized Enzymes, Chemical Rubber Company Press, Cleveland
4 Goldstein, L., Levin, Y. and Katchalski, E. (1964) Biochemistry 3, 1913—1919
5 Hornby, W.E., Lilly, M.D. and Crook, E.M. (1966) Biochem. J. 98, 420—425
6 Wharton, C.W., Crook, E.M. and Brocklehurst, K. (1968) Eur. J. Biochem. 6, 572—578
7 Axén, R., Myrin, P.-Å. and Janson, J.-C. (1970) Biopolymers 9, 401—413
8 Svensson, B. (1973) C.R. Trav. Lab. Carlsberg 39, 339—356
9 Svensson, B. (1972) C.R. Trav. Lab. Carlsberg, 39, 1—13
10 Schmiedeberg, O. (1899) Arch. Exp. Pathol. Pharmakol. 43, 57
11 Axén, R. and Ernback, S. (1971) Eur. J. Biochem. 18, 351—360
12 Ottesen, M. and Svensson, B. (1971) C.R. Trav. Lab. Carlsberg 38, 445—456
13 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350—356
14 Matsubara, H., Kasper, C.B., Brown, D.M. and Smith, E.L. (1965) J. Biol. Chem. 240, 1125—1130
15 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
16 Jacobsen, C.F., Léonis, J., Linderstrøm-Lang, K. and Ottesen, M. (1957) Methods Biochem. Anal. 4, 171—221
17 Svensson, B. (1974) C.R. Trav. Lab. Carlsberg 39, 470—492
18 Schoenbaum, G.R., Zerner, B. and Bender, M.L. (1961) J. Biol. Chem. 236, 2930—2935
19 Morihara, K., Oka, T. and Tsuzuki, H. (1970) Arch. Biochem. Biophys. 138, 515—525
20 Moore, S. and Stein, W.H. (1954) J. Biol. Chem. 211, 907—913
21 Axén, R., Porath, J. and Ernback, S. (1967) Nature 214, 1302—1304
22 Porath, J., Axén, R. and Ernback, S. (1967) Nature 215, 1491—1492
23 Gabel, D. and van Hofsten, B. (1970) Eur. J. Biochem. 15, 410—414

24 Green, M.L. and Crutchfield, G. (1969) Biochem. J. 115, 183—190
25 Svensson, B. (1973) FEBS Lett. 29, 167—169
26 Pecht, M. and Levin, Y. (1972) Biochem. Biophys. Res. Commun. 46, 2054—2061
27 Goldstein, L. (1972) Biochemistry 11, 4072—4084
28 Mitz, M.A. and Summaria, L.J. (1961) Nature 189, 576—577
29 Robertus, J.D., Kraut, J., Alden, R.A. and Birktoft, J.J. (1972) Biochemistry 11, 2436—2449
30 Bosshard, H.R. (1973) FEBS Lett. 30, 105—110
31 Bosshard, H.R. and Berger, A. (1974) Biochemistry 13, 266—277
32 Kay, G. and Lilly, M.D. (1970) Biochim. Biophys. Acta 198, 276—285
33 Self, D.A., Kay, G. and Lilly, M.D. (1969) Biotechnol. Bioeng. 11, 337—343
34 Warburton, D., Balasingham, K., Dunnill, P. and Lilly, M.D. (1972) Biochim. Biophys. Acta 284, 278—284
35 Axén, R. and Ernback, S. (1971) Eur. J. Biochem. 18, 351—360
36 Goldstein, L. (1973) Isr. J. Chem. 11, 379—391
37 Hornby, W.E., Lilly, M.D. and Crook, E.M. (1968) Biochem. J. 107, 669—674
38 Engasser, J.-M. and Horvath, C. (1973) J. Theoret. Biol. 42, 137—155