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EFFECT OF CONCENTRATION OF IMMOBILIZED INHIBITOR ON THE BIOSPECIFIC CHROMATOGRAPHY OF PEPSINS

JAROSLAVA TURKOVÁ*, KAREL BLÁHA and KATEŘINA ADAMOVÁ
Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6 (Czechoslovakia)
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SUMMARY

A specific sorbent for porcine pepsin containing 0.85 μmol of ε-aminocaproyl-L-Phe-D-Phe-OCH_3 per gram of dry carrier (hydroxyalkyl methacrylate copolymer) sorbed 29.4 mg of pepsin per gram of dry sorbent, which means that 99% of immobilized inhibitor molecules participated in the specific complex formation with the isolated enzyme. With increasing amount of bound inhibitor this fraction decreased sharply (only 26% for 4.5 μmol). A specific sorbent with a content of 155 μmol/g appeared to be unsuitable for the affinity chromatography of pepsin (possibility of formation of multiple non-specific bonds between isolated enzyme and specific sorbent). The sorption of chicken and human pepsin was found to be lower thant that of porcine pepsin. The cause is seen in differences between the equilibrium constants of the individual enzyme–immobilized inhibitor complexes. The amount of sorbed chicken pepsin increased after reaction with *o*-nitrobenzenesulphenyl chloride. Using experimentally determined curves representing the dependence of the amount of sorbed enzyme on the content of immobilized inhibitor, it is possible to estimate the order of magnitude of the equilibrium constant of the respective specific complex.

INTRODUCTION

Owing to the complementarity of binding sites, the biospecific bond is characterized by much greater strength than that observed with non-specific bonding. If, however, the affinity sorbent contains the affinity ligand in a concentration which makes possible protein binding by means of a greater number of non-specific bonds, non-specific sorption of inert proteins may occur, or binding of a compound capable of biospecific interaction with the immobilized affinity ligand in an incorrect orientation may also take place[1]. Non-specific bonding may be caused by electrostatic or hydrophobic interactions, or by a combination of the two. Multiple non-specific bonds may then become stronger than a single complementary biospecific interaction between enzyme and inhibitor covalently bound to the carrier. Combined with the biospecific complementary bond, non-specific multiple bonds increase the bond strength in a specific complex. As a consequence, the same enzyme may be eluted in

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several fractions[2], or there may be difficulties in the elution of the enzyme from the specific sorbent[3].

In order to restrict non-specific sorption and at the same time to guarantee the highest possible utilization of the immobilized affinant, a specific sorbent with a low concentration of the affinity ligand must be applied. At such a concentration, multiple non-specific bonds on the affinity sorbent cannot become operative, and molecules are preferentially bound biospecifically, by means of complementary binding sites. This, of course, is possible only in those instances where there is no steric hindrance to the formation of a biospecific complex. For most solid carriers, including hydroxylalkyl methacrylate gels, an uneven surface must be assumed. The unevenness of the surface after binding of low-molecular-weight inhibitors through a spacer is reflected in the different accessibility of the immobilized inhibitor[4]. Differences between readily accessible, less accessible and sterically hindered affinity ligands are the greater, the denser is the occupation of the solid carrier with the immobilized inhibitor[1]. These steric hindrances explain not only the low saturation of immobilized inhibitor molecules with the isolated enzyme[5,6], but also the heterogeneity in their affinity[7].

To provide experimental evidence for the effect of the density of immobilized inhibitor on the course of the affinity chromatography of proteolytic enzymes, we prepared specific sorbents for carboxylic proteinases[8,9] containing various amounts of ε-aminocaproyl-L-Phe-D-Phe-OCH_3 and determined the amounts of sorbed porcine, chicken and human pepsin depending on the concentration of immobilized inhibitor.

EXPERIMENTAL

Materials

Separon H1000 hydroxyalkyl methacrylate gel modified with epichlorohydrin (exclusion molecular weight 1,000,000, specific surface area *ca.* 30 m^2/g, particle size 100–200 μm, epoxide group content 800 μmol/g), Separon H300 modified with epichlorohydrin (exclusion molecular weight 300,000, specific surface area *ca.* 90 m^2/g, particle size 125–200 μm, epoxide group content 600 μmol/g) and the corresponding unmodified Separon H1000 and H300 were obtained by courtesy of Dr. J. Čoupek (Laboratory Instruments, Prague, Czechoslovakia). ε-Aminocaproyl-L-Phe-D-Phe-OCH_3 was synthesized and bound on Separon H1000-E by employing methods described earlier[10]. Porcine pepsin (proteolytic activity 14 units/min · mg) was produced by Léčiva (Pharmaceuticals) (Dolní Měcholupy, Czechoslovakia). Chicken pepsin (proteolytic activity 15.2 units/min · mg) was obtained by courtesy of Dr. V. Kostka and Dr. M. Baudyš (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Human gastric juice (proteolytic activity 1.5 units/min · ml) was provided by courtesy of Dr. L. Korbová and Dr. Z. Kučerová (Faculty of Medicine, Charles University, Prague, Czechoslovakia).

Methods

The amount of peptides bound to Separon H1000 and H300 and the proteolytic activity were determined by methods described earlier[10].

Preparation of affinity sorbents

(a) ε-Aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (250 mg; *cf.*,

ref. 10) was dissolved in the necessary amount of dimethylformamide, and triethylamine (76 μl) and Separon H1000 modified with epichlorohydrin (4 g, epoxide group content 800 μmol/g) were added. The mixture was shaken for 48 h, filtered, the sorbent was washed with dimethylformamide, water, 1 *M* hydrochloric acid and water until the acid reaction disappeared, and then with ethanol and diethyl ether. The product was further washed with 6 *M* guanidinium chloride solution and water, dried for analysis to constant weight at 105°C and transferred for affinity chromatography into the respective buffer. At the original tripeptide concentrations in solution of 0.02, 0.04, 0.12 and 0.25 mol/l the dried product contained 0.85, 1.2, 2.5 and 4.5 μmol/g of affinity ligand, respectively.

(b) ε-Aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (11.1 g) was dissolved in a small amount of methanol and the solution was made up to 75 ml with Britton–Robinson buffer (pH 11). This solution (30 ml) was shaken with 5 g of dry gel (Separon H300, containing 600 μmol/g of epoxide groups), the suspension was filtered and the gel was washed with water, 6 *M* guanidine hydrochloride solution and water again. Further treatment was as in (a). The content of bound inhibitor was 155 μmol/g of dry carrier.

Chromatography of porcine pepsin on ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon columns with (A) a low and (B) a high concentration of immobilized inhibitor

A solution of pepsin (1 g per 200 ml) in 0.1 *M* acetate buffer (pH 4.5) was applied continuously to a column (9 × 0.8 cm) of ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon [content of immobilized inhibitor: (A) 0.85 μmol/g of dry carrier and (B) 155 μmol/g of dry carrier] equilibrated with 0.1 *M* sodium acetate (pH 4.5) until the eluate had the same activity as the applied pepsin solution. The column was washed with the equilibration buffer, and pepsin was desorbed with 0.1 *M* acetate buffer containing 1 *M* sodium chloride.The chromatographic runs are shown in Fig.1.

Chromatography of porcine, chicken, sulphenylated chicken and human pepsin on ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon columns with the concentrations of immobilized inhibitor of 0.85, 1.2, 2.5 and 4.5 μmol/g, respectively

The chromatograms were run by employing the procedure described in the preceding section. With human pepsin, 200 ml of filtered gastric juice diluted 1:1 with the 0.1 *M* acetate buffer after adjustment of the pH to 4.5 was applied to the columns each time. The amount of desorbed pepsin was determined on the basis of absorbance at 278 nm and of the proteolytic activity of the combined active fractions. The amounts thus determined were in good agreement with the amounts of the individual pepsins isolated from the combined fractions after their dialysis and lyophilization.

Chromatography of porcine, chicken and human pepsin on unmodified Separon

A 50-mg amount of porcine pepsin or 20 mg of chicken pepsin dissolved in 20 ml of 0.1 *M* acetate buffer (pH 4.5) or 40 ml of diluted (1:1) gastric juice (pH 4.5) were applied to a column (9 × 0.8 cm) of unmodified Separon H1000; the chromatograms were run under the same conditions as described in the preceding section.

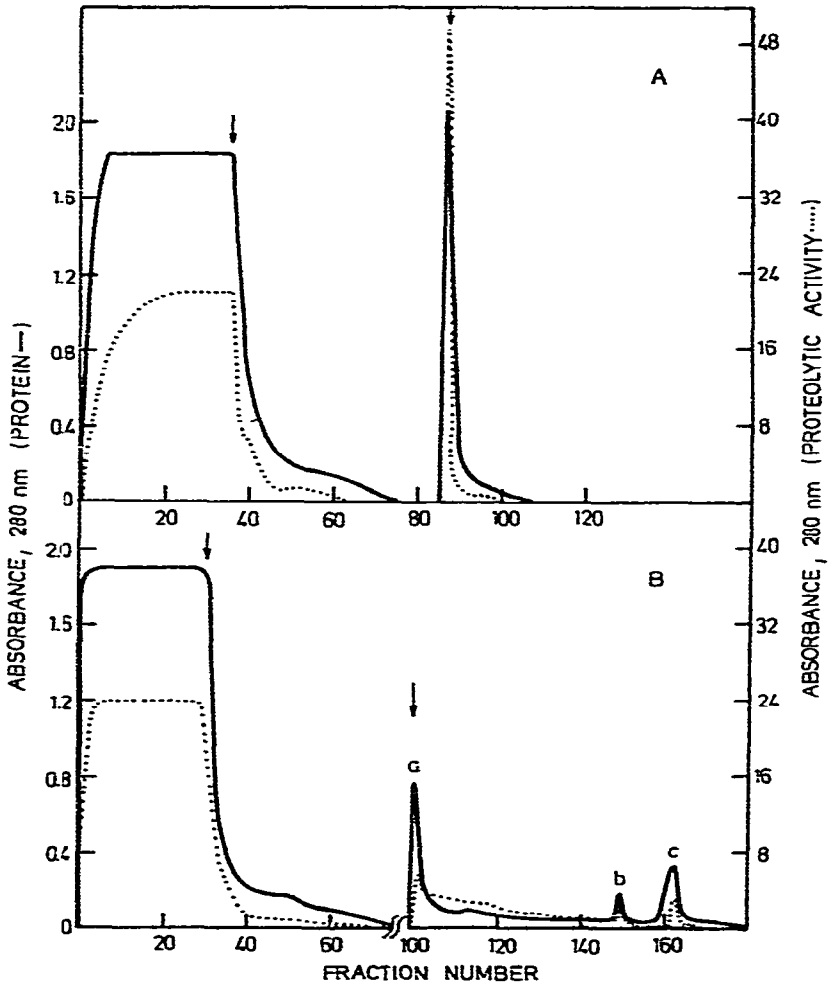

Fig. 1. Affinity chromatography of porcine pepsin on ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon columns with (A) low and (B) high concentrations of the immobilized inhibitor. The solution of crude porcine pepsin was applied continuously (see text) on to the affinity columns (5 ml) equilibrated with 0.1 *M* sodium acetate (pH 4.5). At the position marked by the first arrow equilibrated buffer was applied to the columns to remove unbound pepsin and non-specifically adsorbed proteins. At the second arrow, 0.1 *M* sodium acetate containing 1 *M* sodium chloride (pH 4.5) was applied. Fractions (5 ml) were taken at 4-min intervals. The inhibitor concentration of affinity sorbents were (A) 0.85 and (B) 155 μmol/g of dry support). Solid line, protein; broken line, proteolytic activity. a, b and c, fractions of pepsin of the same specific proteolytic activity.

Chromatography of porcine pepsin in a mixture with serum albumin on ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon

A mixed sample of 50 mg of porcine pepsin and 100 mg of human serum albumin dissolved in 20 ml of 0.1 *M* acetate buffer (pH 4.5) was applied to a column (9 × 0.8 cm) of ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon (content of immobilized inhibitor 4.5 μmol/g of dry sorbent), and the chromatogram was run under conditions of biospecific chromatography described in Fig. 1. A 50-mg amount of por-

cine pepsin was chromatographed under analogous conditions. The chromatograms of pepsin (A) in the absence and (B) in the presence of human serum albumin are shown in Fig. 2.

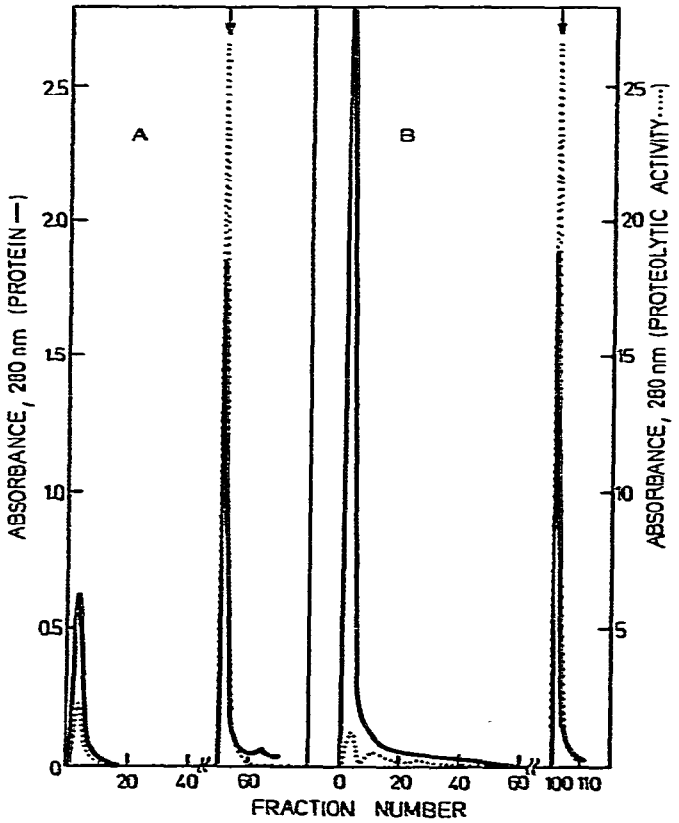

Fig. 2. Chromatography of porcine pepsin (A) in the absence and (B) in the presence of human serum albumin. 50 mg of porcine pepsin (A) in the presence of 100 mg of human serum albumin (B) dissolved in 20 ml of 0.1 *M* acetate buffer (pH 4.5) were applied to the column (9 × 0.8 cm) of ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon (inhibitor content 4.5 μmol/g of dry carrier) equilibrated with 0.1 *M* sodium acetate (pH 4.5). After washing the column with the equilibration buffer, 0.1 *M* acetate buffer containing 1 *M* sodium chloride (pH 4.5) was applied at the position marked with an arrow. Fractions (6 ml) were taken at 5-min intervals. Solid line, protein; broken line, proteolytic activity.

Chicken pepsin was modified with *o*-nitrobenzenesulphenyl chloride as described by Becker *et al.*[11].

RESULTS AND DISCUSSION

In order to show the importance of the low concentration of the immobilized inhibitor (*i.e.*, the amount of inhibitor bound per gram of dry solid carrier) in specific sorbents of carboxylic proteases, porcine pepsin was chromatographed on columns of ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon containing a low (Fig. 1A) and a high (Fig. 1B) concentration of bound inhibitor. At a low concentration of inhibitor,

pepsin was eluted from the column by the increased ionic strength of the elution buffer in a single sharp peak (Fig. 1A). In contrast, on the column of affinity sorbent containing the immobilized inhibitor at a concentration of 155 μmol/g of dry carrier, several peaks of pepsin exhibiting the same specific proteolytic activity were eluted (*cf.*, Fig. 1B). Such a different behaviour of the enzyme on affinity sorbents with low and high contents of immobilized inhibitor can be attributed to the formation of multiple non-specific bonds of molecules of the enzyme and inert proteins.

Under the same conditions as those in Fig. 1, chromatograms were run of porcine, chicken and human pepsins on columns of ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon with concentrations of immobilized inhibitor of 0.85, 1.2, 2.5 and 4.5 μmol/g of dry sorbent, respectively. Under these conditions, the pepsin fractions were desorbed in a single sharp peak. Fig. 3A shows the dependence of the amount of eluted porcine, chicken and human pepsin on the concentration of immobilized ε-aminocaproyl-L-Phe-D-Phe-OCH_3 in the individual affinity sorbents. Fig. 3B illustrates the proportion of molecules of immobilized inhibitor involved in specific bonding with pepsin, again as a function of the concentration of immobilized inhibitor. Comparison of the curves obtained for the individual pepsins reveals that ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon is a very good sorbent for porcine pepsin. A specific sorbent containing 0.85 μmol of inhibitor per gram of dry carrier sorbed 29.4 mg of porcine pepsin per gram of dry sorbent. Using the molecular weight of pepsin (35,000) and the amount of sorbed protein, it can be calculated that 99% of immobilized inhibitor participated in the specific complex formation. With increasing content of bound inhibitor there was a sharp decrease in the portion of immobilized inhibitor molecules involved in the specific complex with pepsin. On the sorbent containing 4.5 μmol/g of inhibitor, only 26% of the total amount of inhibitor molecules attached took part in the sorption of porcine pepsin.

Dunn and Chaiken[12] described the use of affinity chromatography for the determination of the equilibrium constants of the enzyme complex, both with the free (K_I) and with the immobilized inhibitor (K_L). K_L was calculated using the concentration of immobilized affinity ligand determined on the basis of the so-called "working capacity". When studying the affinity constants of trypsin and chymotrypsin with immobilized inhibitors, benzamidine and Z-Gly-D-Phe[13–15], we found that the concentration determined from the working capacity was much lower than that of the inhibitor determined from the amino acid analysis of hydrolyzates of sorbents. The results in Fig. 3 show that, *e.g.*, a suitable sorbent for investigation of the specific interaction by means of affinity chromatography carried out with porcine pepsin would be a sorbent with the lowest concentration of bound inhibitor, because only with this sorbent is the concentration of immobilized inhibitor determined from the working capacity the same as the concentration determined by the amino acid analysis of the acid hydrolysate of the respective sorbent. Our earlier results[14,15] will be revised in this respect.

In principle, the molecular weights of chicken and human pepsin do not differ from that of porcine pepsin, which allows us to expect similar steric hindrances. From this standpoint, the lower sorption of these two pepsins may be assigned, in the first place, to the lower complementarity of immobilized inhibitor for binding sites of chicken and human pepsin. This is in good agreement with the already reported[11,16] specificity of porcine and chicken pepsin. Z-His-L-Phe-D-Phe-C_2H_5 is an efficient

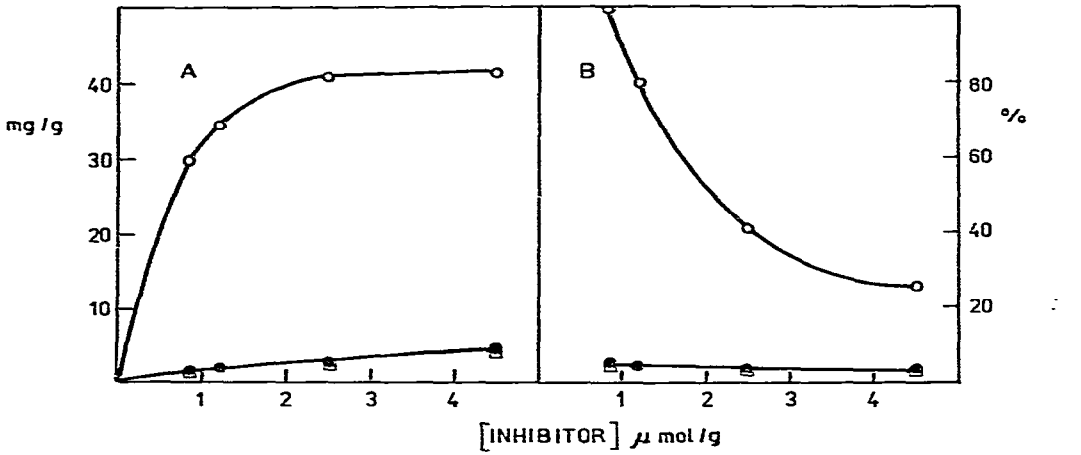

Fig. 3. (A) Capacity of immobilized inhibitor sorbent (ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon) in milligrams of pepsin per gram of dry sorbent and (B) proportion of immobilized inhibitor molecules involved in specific complex formation (%) with respect to immobilized inhibitor concentration (μmol of inhibitor per gram of dry sorbent). ○, Porcine pepsin; ●, chicken pepsin; □, human pepsin.

inhibitor of porcine pepsin (K_I = 0.27 m*M*), the same as Z-His-L-Phe-L-Phe-C_2H_5 (K_m = 0.18 m*M*) or Z-His-L-Phe-L-Phe-OCH_3 (K_m = 0.33 m*M*) are its good substrates[16]. Becker *et al.*[11] studied the specificity of chicken pepsin. The value of the ratio of the catalytic and Michaelis-Menten constants, k_{cat}/K_m, for the substrate Z-His-L-Phe-L-Phe-OC_2H_5 for native enzyme could not be determined, because it was too low ($<0.1\ M^{-1}\ sec^{-1}$). If, however, the authors[11] modified chicken pepsin with *o*-nitrobenzenesulphenyl chloride, k_{cat}/K_m for the same substrate increased to 40 $M^{-1}\ sec^{-1}$. The modification of chicken pepsin with *o*-nitrobenzenesulphenyl chloride changes the conformation of the active site and renders it more suitable for binding small peptidic substrates.

The chromatography of chicken pepsin modified with *o*-nitrobenzenesulphenyl chloride on ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon revealed a 4-fold increase in the amount of sorbed modified pepsin. Thus, according to the results obtained, the cause of differences in the amounts of various pepsins sorbed depending on the concentration of immobilized inhibitors can be sought in differences in the equilibrium constants of enzyme–immobilized inhibitor complexes. Fig. 4B shows such experimentally determined curves recalculated to the same concentrations as those used by Graves and Wu[17] in the theoretical derivation of analogous dependences (Fig. 4A). There is a good fit between Fig. 4A and B, which justifies the assumption that the shape of experimentally determined curves makes possible an estimate of the order of magnitude of the equilibrium constant of the specific complex. The different sorption of acetylcholinesterases from various sources as a function of the concentration of the immobilized affinity ligand in N-methylacridinium-Sepharose has also been described by Sekar *et al.*[18], who similarly see a possible explanation of the differences in the different specificities of individual acetylcholinesterases.

Application of ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon with a low content of immobilized inhibitor in high-pressure liquid affinity chromatography (HPLAC) or in the large-scale isolation of pepsin is based on the observation that the

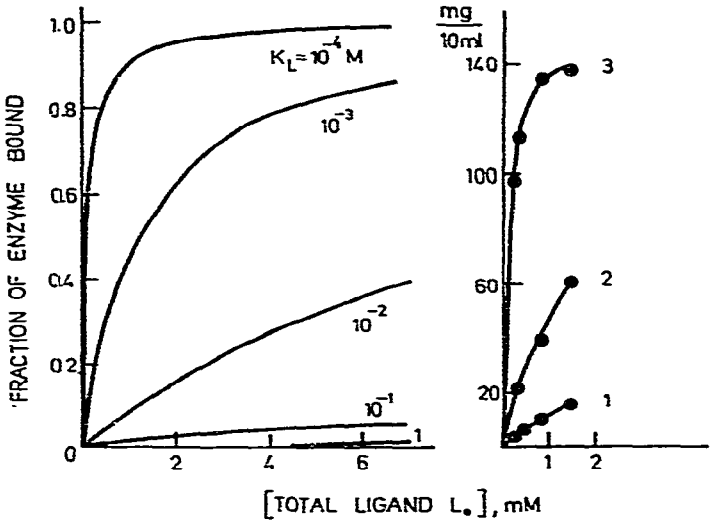

Fig. 4. Comparison of the theoretical relationship between the amount of the sorbed enzyme, concentration of the immobilized affinity ligand and the equilibrium constant of the enzyme–ligand complex, K_L[17] with the experimentally obtained values for chicken pepsin (1), chicken pepsin modified with *o*-nitrobenzenesulphenyl chloride (2) and porcine pepsin (3).

sorption of an enzyme on a column of a specific sorbent is independent of the enzyme concentration in the applied sample, as has already been demonstrated in a preceding paper[10]. Fig. 2 shows that the amount and activity of isolated pepsin are also independent of the presence of excess of inert protein in the applied sample. In the application of a mixture of pepsin with twice the amount of serum albumin on the column of ε-aminocaproyl-L-Phe-D-Phe-OCH_3–Separon, all of the serum albumin was eluted in the first peak with the equilibration buffer. Fractions of desorbed pepsin obtained in the absence (Fig. 2A) and in the presence of serum albumin (Fig. 2B) contained the same amounts of protein and exhibited the same proteolytic activity.

In conclusion, it can be said that biospecific chromatography of the enzyme requires a low content of immobilized inhibitor, in order to prevent the formation of multiple non-specific bonds and to achieve the highest possible utilization of immobilized affinity ligands. Such sorbents are then suitable not only for the efficient isolation and analytical determination of enzymes, both in the classical and in the HPLAC arrangement, but also for the investigation of the respective biospecific interactions.

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REFERENCES

1 J. Turková, in T. C. J. Gribnau, J. Visser and R. J. F. Nivard (Editors), *Affinity Chromatography and Related Techniques —Theoretical Aspects/Industrial and Biochemical Applications*, Elsevier, Amsterdam, Oxford, New York, 1982, p. 513.
2 B. H. J. Hofstee, in N. Catsimpoolas (Editor), *Methods of Protein Separation*, Vol. 2, Plenum Press, New York, 1976, pp. 245–278.
3 M. J. Holroyde, J. M. E. Chester, I. P. Trayer and D. C. Walker, *Biochem. J.*, 153 (1976) 351.
4 J. Porath, in R. Epton (Editor), *Chromatography of Synthetic and Biological Polymers*, Vol. 1, Ellis Horwood, Chichester, 1978, pp. 9–29.
5 J. Turková, K. Bláha, O. Valentová, J. Čoupek and A. Seifertová, *Biochim. Biophys. Acta*, 427 (1976) 586.
6 V. Kasche, *DECHEMA Monogr.*, 84 (1979) 367.
7 P. Cuatrecasas, M. Wilchek and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.*, 6 (1968) 636.
8 V. M. Stepanov, G. I. Lavrenova and M. M. Slavinskaya, *Biokhimiya*, 39 (1974) 384.
9 V. M. Stepanov, G. I. Lavrenova, K. Adly, M. V. Gonchar, G. N. Balandina, M. M. Slavinskaya and A. Ya. Strongin, *Biokhimiya*, 41 (1976) 294.
10 J. Turková, K. Bláha, J. Horáček, J. Vajčner, A. Frydrychová and J. Čoupek, *J. Chromatogr.*, 215 (1981) 165.
11 R. Becker, Y. Schechter and A. Bohak, *FEBS Lett.*, 36 (1973) 49.
12 B. M. Dunn and I. M. Chaiken, *Biochemistry*, 14 (1975) 2343.
13 J. Turková and A. Seifertová, *J. Chromatogr.*, 148 (1978) 293.
14 M. Malaníkova and J. Turková, *J. Solid-Phase Biochem.*, 2 (1978) 237.
15 J. Turková, *Enzyme Eng.*, 4 (1978) 451.
16 G. E. Clement, *Prog. Bioorg. Mech.*, 2 (1973) 177.
17 D. J. Graves and Y.-T. Wu, *Methods Enzymol.*, 34 (1974) 140.
18 M. C. Sekar, G. Webb and B. D. Roufogalis, *Biochim. Biophys. Acta*, 613 (1980) 420.