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DEVELOPMENT OF IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

III. INTERACTION OF OLIGOPEPTIDES WITH IMMOBILIZED NICKEL IMINODIACETATE

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SUMMARY

The retention of several selected oligopeptides on immobilized nickel iminodiacetate (IDA–Ni^{2+}) was studied at different concentrations of sodium chloride, potassium sulphate, lithium sulphate and magnesium sulphate. An increase in V_e/V_t with increasing salt concentration was observed for all oligopeptides studied. The retention of oligopeptides was highest at temperatures around 15–20°C within the 0–50°C range studied. An increase of retention was also observed with increasing pH from 7 to 8. The chromatographic behaviour of oligopeptides on immobilized IDA–Ni^{2+} is, to a first approximation, the sum of the individual contributions of the constituent amino acids.

INTRODUCTION

Immobilized metal affinity chromatography (IMAC), introduced a decade ago[1], is becoming a standard tool for the isolation of proteins[2,3]. To gain some insight into the mechanisms underlying IMAC itself we have undertaken a systematic study of the behaviour of model substances, *e.g.*, amino acids and oligopeptides, on immobilized nickel iminodiacetate (IDA–Ni^{2+}). In the preceding part of this series[4] we showed that the interaction of different amino acids with IDA–Ni^{2+} is complex and is largely dependent on the particular nature of the amino acid under study. We report now on the binding characteristics of selected dipeptides and oligopeptides toward IDA–Ni^{2+}.

EXPERIMENTAL

Materials

The peptides, L-tyrosyl-L-glutamic acid monohydrate, L-glutamyl-L-tyrosine, L-glutamyl-L-tyrosyl-L-glutamic acid, L-tyrosyl-L-lysine acetate, L-lysyl-L-lysine dihydrochloride hemihydrate, L-lysyl-L-glutamic acid, L-tyrosyl-L-tyrosine, L-trypto-

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phyl-L-glutamic acid dihydrate, L-glutamyl-L-tryptophan, L-glutamyl-L-tryptophyl-L-glutamic acid, L-arginyl-L-tryptophan hydrochloride, L-tryptophyl-L-tryptophan and α-L-aspartyl-L-histidine, were all purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). L-Glutamyl-L-glutamic acid and L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-leucine (leucine^5-enkephalin) were from Protein Research Foundation (PRF), Osaka, Japan. Ethylmorpholine was purchased from Fluka (Buchs, Switzerland), nickel sulphate, magnesium sulphate, and lithium sulphate from Merck (Darmstadt, F.R.G.) and potassium sulphate and Tris [tris(hydroxymethyl)aminomethane] were from Labkemi (Stockholm, Sweden). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). All other chemicals were reagent grade.

IDA-Sephadex G-25 was prepared according to a published procedure[5–7] by coupling iminodiacetate to an epoxy-activated matrix. The gel was saturated with an aqueous solution of $NiSO_4$ and then washed with water. The retained Ni^{2+}, measured as described in the preceding paper[4], amounted to 80 μmol per ml gel.

Chromatographic procedure

The biscarboxymethyl-amino-Sephadex G-25 was packed into a column (15 × 2 cm) and washed with water prior to loading with a 20 m*M* solution of nickel sulphate in water until saturation. The column was then rinsed with water to remove excess of nickel. The nickel-chelate adsorbent (IDA–Ni^{2+}) was then equilibrated with the desired buffer. The peptides were dissolved in the equilibration buffer to a final concentration of 10 m*M*. The volume of the peptide sample applied was 0.5 ml. Fractions of 2 ml were collected. The flow-rate in all the chromatographic experiments was 16 ml/cm^2/h. Elution of the peptides was monitored with trinitrobenzene–sulphonic acid[8] or by direct spectrophotometry at 280 nm. The retention of peptides is expressed as V_e/V_t, where V_e is the volume of the eluate at the peak of a chromatographic zone and V_t is the total volume of the gel bed.

RESULTS

Sets of dipeptides and oligopeptides containing only hydrophobic (tryptophan and tyrosine) and only charged (glutamic acid and lysine) amino acids as well as "mixed", *i.e.*, hydrophobic/charged dipeptides, were selected. Fig. 1A and B illustrate the chromatographic behaviours on IDA–Ni^{2+} as a function of sodium chloride concentration. For all of the dipeptides tested except L-Glu-L-Glu the V_e/V_t values increased with increasing salt concentration.

The most strongly retained dipeptide was L-Trp-L-Trp (Fig. 1A and Table I). A "mixed" dipeptide, L-Arg-L-Trp, was retained significantly less strongly than was L-Trp-L-Trp. The retention of both L-Glu-L-Trp and L-Trp-L-Glu seems to be diminished by the presence of the acidic amino acid residue. The tripeptide L-Glu-L-Trp-L-Glu was even less strongly retarded than the dipeptides (L-Glu-L-Trp, L-Trp-L-Glu), reflecting the presence of the second glutamic acid residue. Fig. 1B further illustrates the same chromatographic phenomenon; namely, that the retention of tyrosine-containing peptides is also reduced by the presence of the acidic residue. L-Tyr-L-Lys was less strongly retarded than L-Tyr-L-Tyr, but significantly more so than L-Tyr-L-Glu (Fig. 1B and Table I). This clearly reflects the stronger retention of lysine compared to glutamic acid on IDA–Ni^{2+}[4]. That L-Glu-L-Tyr-L-Glu was

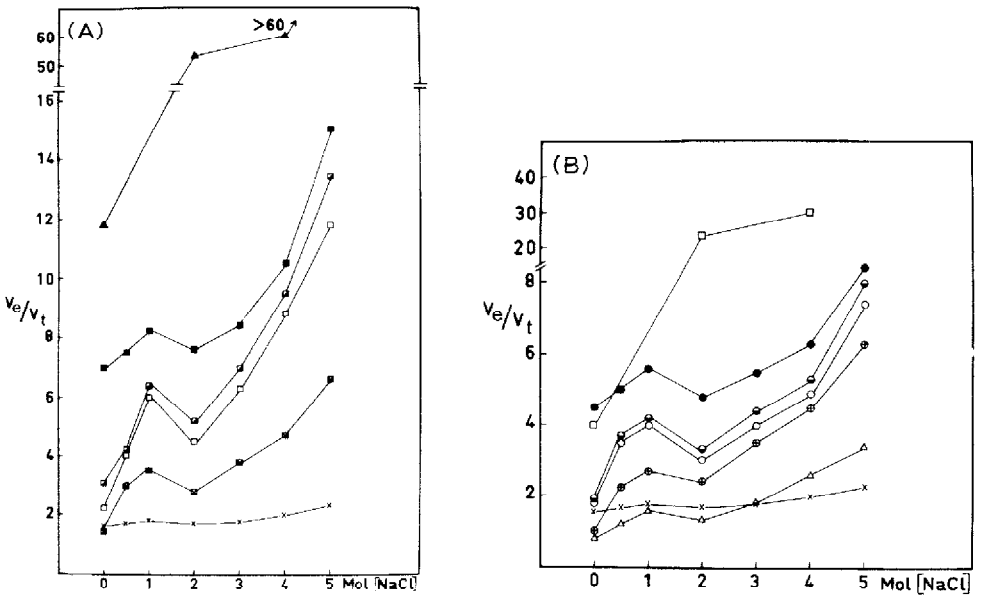

Fig. 1. The relationship between the concentration of NaCl and the retention of oligopeptides on IDA–Ni^{2+}. Oligopeptides were analyzed in 0.2 *M* ethylmorpholine–acetate at pH 7.0 and in the same buffer supplemented with sodium chloride. A, ▲—▲, L-Trp-L-Trp; ■—■, L-Arg-L-Trp; ◪—◪, L-Glu-L-Trp; □—□, L-Trp-L-Glu; ⊠—⊠, L-Glu-L-Trp-L-Glu; ×—×, L-Glu-L-Glu. B, □—□, L-Tyr-L-Tyr; ●—●, L-Tyr-L-Lys; ◑—◑, L-Glu-L-Tyr; ○—○, L-Tyr-L-Glu; ⊕—⊕, L-Glu-L-Tyr-L-Glu; △—△, L-Tyr-L-Gly-Gly-L-Phe-L-Leu; ×—×, L-Glu-L-Glu.

also somewhat less strongly retarded than L-Glu-L-Tyr or L-Tyr-L-Glu can only be ascribed to the presence of the additional L-glutamic acid residue.

The retention of L-Tyr-Gly-Gly-L-Phe-L-Leu on IDA–Ni^{2+} is much weaker than would be expected in view of its high content of hydrophobic residues. Especially the presence of the aromatic residues should have resulted in a strong retention on the basis of the results obtained with the individual amino acids[4]. Possibly the stacking of aromatic side chains negates their innate ability to be strongly retained on IDA–Ni^{2+}.

Fig. 2 illustrates the influence of lithium sulphate on the retention of L-Tyr-L-Lys, L-Glu-L-Trp-L-Glu and L-Glu-L-Tyr-L-Glu on IDA–Ni^{2}[5]. Clearly, there is a positive correlation between V_e/V_t and salt concentration. The peptide L-Tyr-L-Lys is more strongly retained at all salt concentrations than is the tripeptide L-Glu-L-Tyr-L-Glu, further illustrating the effect of the negatively charged amino acid residues on the chromatographic behaviour.

Fig. 3 represents the results obtained with the same oligopeptides as in Fig. 2, but in the presence of magnesium sulphate. Comparison of the numerical values of V_e/V_t indicates a small effect of the cation on the adsorption of a tripeptide. For example, the V_e/V_t value for L-Glu-L-Trp-L-Glu is *ca.* 4 in 1.5 *M* Li_2SO_4 (Fig. 2) and *ca.* 3 in 1.5 *M* $MgSO_4$ (Fig. 3). The V_e/V_t value for L-Glu-L-Tyr-L-Glu is *ca.* 3 in 1.5 *M* Li_2SO_4 (Fig. 2) and *ca.* 2.5 in 1.5 *M* $MgSO_4$ (Fig. 3). The retention of L-Tyr-L-Lys is quite similar in 1.5 *M* lithium or magnesium sulphate. Fig. 4 shows the reten-

TABLE I

RETENTION OF SOME AMINO ACIDS AND DIPEPTIDES ON IDA–Ni^{2+} IN 0.2 *M* ETHYLMORPHOLINE–ACETATE, pH 7.0, AT VARIOUS NaCl CONCENTRATIONS

Test substance	V_e/V_t		
	Buffer	*Buffer (2 M NaCl)*	*Buffer (4 M NaCl)*
L-Glu-L-Glu	1.6	1.7	2
L-Lys-L-Lys	5	4.7	3.5
L-Lys-L-Glu	4.5	3.4	3
L-Trp-L-Trp	11.8	56	>60
L-Tyr-L-Tyr	4	23.2	30
L-Glu	0.8	1.8	2.2
L-Trp	9.3	12.9	>40
L-Tyr	6	9.3	20.7
L-Asp-L-His	13	25	>50
L-His	>20	>20	>40

tion of the same oligopeptides as a function of potassium sulphate concentration. For L-Tyr-L-Lys at 0.5 *M* K_2SO_4 the V_e/V_t value is *ca.* 3.5, quite comparable to that (*ca.* 2.8) in $MgSO_4$ at the same concentration. the V_e/V_t values for both tripeptides at 0.5 *M* salt are similar in potassium and lithium sulphates, within the range 2–3. The V_e/V_t values are, however, significantly lower in magnesium sulphate (1.5–2).

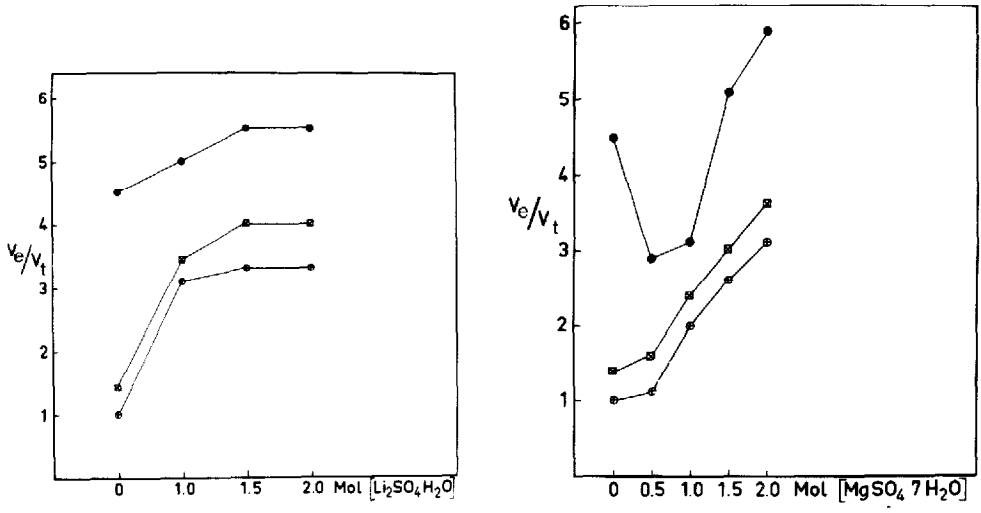

Fig. 2. The relationship between the concentration of $Li_2SO_4 \cdot H_2O$ and the retention of oligopeptides on IDA–Ni^{2+}. Oligopeptides were applied in 0.2 *M* ethylmorpholine–acetate, pH 7.0 and in the same buffer supplemented with lithium sulphate. ●—●, L-Tyr-L-Lys; ⊠—⊠, L-Glu-L-Trp-L-Glu; ⊕—⊕, L-Glu-L-Tyr-L-Glu.

Fig. 3. The relationship between the concentration of $MgSO_4 \cdot 7\ H_2O$ and the retention of oligopeptides on IDA–Ni^{2+}. Oligopeptides were applied on 0.2 *M* ethylmorpholone–acetate, pH 7.0 and in the same buffer supplemented with magnesium sulphate. Symbols as in Fig. 2.

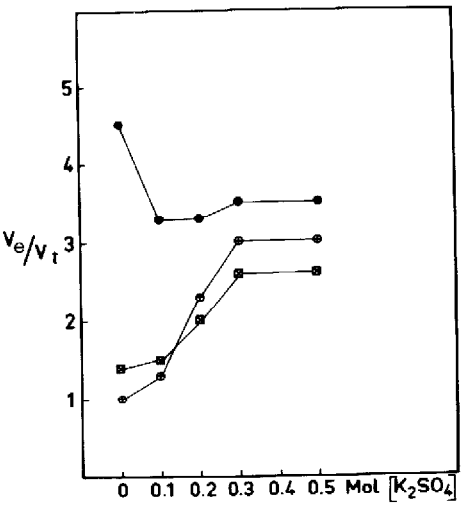

Fig. 4. The relationship between the concentration of K_2SO_4 and the retention of oligopeptides on IDA–Ni^{2+}. Oligopeptides were applied in 0.2 *M* ethylmorpholine–acetate, pH 7.0 and in the same buffer supplemented with potassium sulphate. Symbols as in Fig. 2.

Fig. 5 illustrates the effect of temperature on the retention of oligopeptides on IDA–Ni^{2+} in the presence of 0.3 *M* K_2SO_4. The retention shows a downward trend between 0 and 10°C which is reversed and eventually reaches a plateau at 15–20°C. At still higher temperatures, 29–35°C, there is again a fall in the retention and a new plateau is established at 35°C which persists until 50°C.

The pH dependence of the retention of oligopeptides on IDA–Ni^{2+} is summarized in Table II. All oligopeptides are retained more strongly upon increasing the pH from 7.0 to 8.0, which presumably results from decreased protonation of their

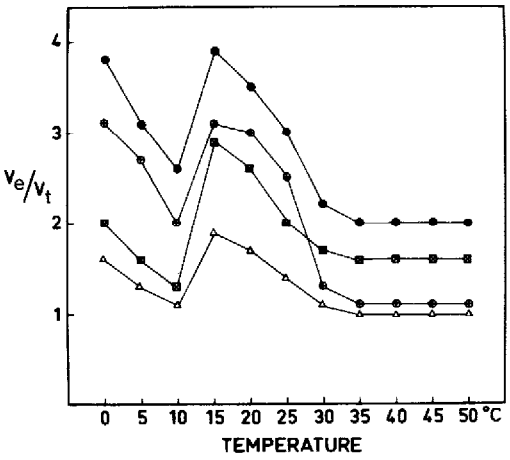

Fig. 5. The effect of temperature on the retention of oligopeptides on IDA–Ni^{2+}. Oligopeptides were applied in 0.2 *M* ethylmorpholine–acetate, pH 7.0 containing 0.3 *M* K_2SO_4 at different temperatures. ●—●, L-Tyr-L-Lys; ⊕—⊕, L-Glu-L-Tyr-L-Glu; ⊠—⊠, L-Glu-L-Trp-L-Glu; △—△, L-Tyr-Gly-Gly-L-Phe-L-Leu.

TABLE II

EFFECT OF pH ON THE RETENTION OF OLIGOPEPTIDES ON IDA–Ni^{2+}

Oligopeptides were applied at pH 7.0 and 7.5 in 0.2 *M* ethylmorpholine–acetate and at pH 8.0 in 0.2 *M* Tris–HCl.

Oligopeptide	V_e/V_t		
	pH 7.0	*pH 7.5*	*pH 8.0*
L-Arg-L-Trp	7	10.6	12.3
L-Glu-L-Trp	3.1	4.7	7.8
L-Trp-L-Glu	2.2	4.4	7.5
L-Glu-L-Trp-L-Glu	1.4	1.7	4.2
L-Tyr-L-Lys	4.5	8.5	11.5
L-Glu-L-Tyr	1.9	3.4	4.5
L-Tyr-L-Glu	1.8	3.1	4.2
L-Glu-L-Tyr-L-Glu	0.97	1.6	3.6
L-Tyr-Gly-Gly-L-Phe-L-Leu	0.8	1.3	2.4

α-amino groups. The same pH effect was also observed with the individual amino acids[4].

Table I shows the retention of some amino acids and dipeptides on IDA–Ni^{2+} in 0.2 *M* ethylmorpholine-acetate, pH 7.0, at different NaCl concentrations. It seems that the influence of the ionic strength of the solvent on the retention of amino acids and oligopeptides (dipeptides) is pronounced over the range from 0 to 2 *M* salt; by comparison, the retention of amino acids is more affected in the interval 2–4 *M* salt.

The effect of 0–4 *M* NaCl on the retention of L-Glu-L-Glu is negligible. The retention of L-Lys-L-Lys and L-Lys-L-Glu decreases with increasing salt concentration. The retention of L-Asp-L-His is remarkably high as expected for a histidine peptide.

DISCUSSION

The scope of the present study is limited to a selected set of oligopeptides. The selection was made on the basis of our previous observations with individual amino acids on IDA–Ni^{2+}[4]. Two hydrophobic/aromatic dipeptides, L-Trp-L-Trp and L-Tyr-L-Tyr, were included in the study because of the observation that L-Trp and L-Tyr displayed conspicuously high retention on IDA–Ni^{2+}. Two charged (at neutral pH) dipeptides, L-Glu-L-Glu (negative) and L-Lys-L-Lys (positive), were studied because of the opposite behaviour of L-Glu (not retained) and L-Lys (retained) on IDA–Ni^{2+}. The inclusion of "mixed" dipeptides containing charged (positive or negative) and hydrophobic (aromatic) amino acid residues allows evaluation of the contribution of these particular types of amino acid residues to the retention by IDA–Ni^{2+}. Both L-Trp-L-Trp and L-Tyr-L-Tyr are retained more strongly at increasing salt concentrations (Fig. 1A, 1B and Table I). In 2 *M* NaCl, L-Trp-L-Trp is retained (V_e/V_t = 56) on IDA–Ni^{2+} much more strongly than is L-Trp (V_e/V_t = 12.9). L-Tyr-L-Tyr behaves analogously: at 4 *M* NaCl V_e/V_t is 30 for the dipeptide and 20.7 for L-Tyr. By contrast, the retention of L-Glu-L-Glu on IDA–Ni^{2+} increases

by only 25% between 0 and 4 *M* NaCl; the retention of L-Lys-L-Lys decreases with increasing salt concentrations.

At low salt concentration the peptides interact with the gel as would be expected if the latter were a cation exchanger. If so, hydroxyl groups in the inner coordination sphere could reverse the charge on the metal complex. The negative hydroxyaquonickel ion may then also exert a repulsive effect on the glutamic acid-containing peptides. However, the same kind of chromatographic behaviour is expected if instead the free amino group is linked to the nickel ion as a coordination complex. Both mechanisms may play a rôle.

The retention of hydrophobic (aromatic) dipeptides is much higher at all salt concentrations than that of the individual constituent amino acids. This is not the case with charged dipeptides, where V_e/V_t is comparable to that of the corresponding amino acids.

The decreased affinities of charged aromatic dipeptides for IDA–Ni^{2+} indicates that the charged amino acid residue suppresses the "aromatic" affinity. L-Arg-L-Trp is retained in 4 *M* NaCl with $V_e/V_t = 10.5$, comparable to $V_e/V_t = 11$ for L-Arg[4]. Similarly for L-Tyr-L-Lys $V_e/V_t = 6$ (Fig. 1B) compared with 20.7 for L-Tyr (Table I). The general enhancing effect of salt concentration on the retention of dipeptides on IDA–Ni^{2+} was similar for NaCl and Li_2SO_4 ($MgSO_4$). V_e/V_t for L-Tyr-L-Lys in 2 *M* salt was found to be *ca.* 5 (NaCl) and 6 ($MgSO_4$). Therefore, the charge of the cation (Na^+/Mg^{2+}) or the anion (Cl^-/SO_4^{2-}) seems to be of marginal significance. Antichaotropic salts, such as alkali-metal sulphates and chlorides, exert at high concentration a strengthening effect on the coordination of peptides (amino acids and proteins)[2]. This influence on the coordinative adsorption varies with the nature of the cations and anions and can be exploited in chromatographic systems. The adsorption is clearly of a very complex nature as revealed by the temperature dependence of the retention (Fig. 5). The effect of antichaotropic salts indicates that the water structure plays an important rôle. Systematic trends have been observed for amino acids[4]. Although not well understood, such findings are useful for designing protein fractionations based on IMAC.

The observation that a carboxylic acid residue adjacent to aromatic amino acids strongly decreased the influence of the latter on the adsorption prompted us to determine whether histidine peptides might be similarly affected. The behaviour of L-Asp-L-His indicated a similar, but less pronounced effect. Further studies of these "repulsive" effects of aspartyl and glutamyl residues are underway.

The most conspicuous finding of the present report is the observation that charged amino acid residues (positive and negative) significantly modulate the high affinity of aromatic residues for IDA–Ni^{2+}. This observation must be taken into account in interpreting the retention of proteins on IDA–metal^{2+}. The presence of aromatic side chains (phenyl, tyrosyl) on the surface of a protein molecule may not result in retention on IDA–Ni^{2+} if there is a vicinal negatively charged residue. This conclusion must be verified with appropriately selected protein "models" of well established surface topography.

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