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IMMUNOLOGICAL-CHROMATOGRAPHIC ANALYSIS OF LYSOZYME VARIANTS

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SUMMARY

Immunological-chromatographic analysis (ICA) was used to evaluate the cross-reactivities of eight lysozyme variants with five different immobilized monoclonal anti-hen-egg-white lysozyme antibodies. ICA is a dual-column high-performance liquid chromatography-based method in which an immunoaffinity and a conventional analytical column are coupled with a switching valve. Antigens are first captured on the affinity column and then desorbed and concentrated on the second column, where they are separated further. This arrangement permits antigen–antibody interactions occurring on the affinity column to be monitored on-line with the second column. The ICA system was used to perform direct and competitive inhibition binding immunoassays with unlabeled antigens. Seven of the eight lysozymes tested bound to all five immobilized monoclonal antibodies. Competitive inhibition of binding of hen egg white lysozyme to the monoclonal antibody, HyHel-5, was measured by using the variants Japanese quail and bobwhite quail lysozymes as inhibitors. The ratio of the amount of bobwhite quail to Japanese quail lysozyme required to give 50% inhibition of binding of hen egg white determined by ICA compares well with the results obtained by other investigators who used an enzyme-linked immunosorbent assay plate binding assay.

INTRODUCTION

The large-scale production of proteins for pharmaceutical use has resulted in a need for new analytical methods to ensure the safety of the products in medical applications. Biosynthetic fidelity and product purity must be closely monitored. Contaminants of concern in biotechnology may be proteins and nucleic acids derived from the host cells, or product variants resulting from translation errors, improper refolding, incomplete or incorrect post-translational modification, and chemical or proteolytic degradation during purification. The development of methods capable of

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distinguishing the contaminating product variants, which are very similar in structure to the protein pharmaceutical product, is a major analytical endeavor.

It has been demonstrated that immunological-chromatographic analysis (ICA) is useful for discrimination of polypeptides of similar three-dimensional structures[1,2]. ICA is a dual-column high-performance liquid chromatography-based method, in which an immunoaffinity and a conventional (*e.g.* reversed-phase) analytical column are coupled with a switching valve. Antigens are captured, then desorbed from the affinity column, and concentrated on the analytical column. They are separated on the second column by a different retention mechanism.

The success of ICA depends on the extent of cross-reactivity of the antisera with the various product variants and on the ability of the second chromatographic system to resolve them. Antibodies often cross-react with similar species that share antigenic groups[1–6]. For example, an antiserum produced by using one immunogen from a family of peptides (such as the endorphin or enkephalin neurotransmitters) often cross-react with other members of the family. Current methods of analysis involve a chromatographic separation step with fraction collection, before an immunoassay on the fractions containing the purified peptides is performed[6].

The ICA system takes advantage of these cross-reactivities by performing an immunoaffinity purification first, followed by chromatographic separation. Often a chromatographic method is able to distinguish between similar species when an immunological method has failed (as was the case with the neurotransmitter peptides[6]). This is because retention in ion-exchange, hydrophobic interaction chromatography (HIC) and reversed-phase chromatography (RPC) is generally determined by a relatively large portion of the surface of the molecule. The immunological contact area, called the epitope, is much smaller. Changes in structure of a molecule outside this small region are undetectable by an immunoassay. Chromatographic methods, which probe a larger portion of molecular surface, may be so sensitive to structural differences that proteins which differ by only one amino acid can be separated. For example, six site-specific subtilisin variants were separated by strong-cation-exchange (CEX) chromatography[7].

The goal of this work was to demonstrate further the utility of ICA is distinguishing proteins with similar structures. Nine lysozyme variants with known amino acid sequences were used as the model system for these studies. The retention mechanisms of most of these lysozymes have been examined previously by HIC[8], CEX and RPC[9]. Five monoclonal anti-hen-egg-white lysozyme (HEL) antibodies were used in the studies. Two of the epitopes of these antibodies (HyHel-5 and HyHel-10) have been defined by X-ray crystallography[10–12]. All of the epitopes of these antibodies have been mapped by comparing their reactivities with lysozyme variants and sugar substrates of lysozyme[3,5].

MATERIALS AND METHODS

Reagents

Bradford reagent for protein assays was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.); ethanolamine from Aldrich (Milwaukee, WI, U.S.A.); and 3-(N-morpholino)propanesulfonic acid (MOPS) from Sigma (St. Louis, MO, U.S.A.). HPLC-grade solvents were trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.)

and acetonitrile (American Burdick & Jackson, Muskegon, MI, U.S.A.). Inorganic reagents were of analytical-reagent grade or comparable quality.

Proteins

Five monoclonal antibodies to HEL (HyHel-5, HyHel-8, HyHel-9, HyHel-10 and HyHel-15), in mouse ascites fluid, were generously supplied by the National Institutes of Health, Bethesda, MD, U.S.A. Hybridomas to HEL were prepared as described[3,5]. The purification of avian lysozyme variants from the egg whites of Peking duck A, B, C, ringed-neck pheasant (RNP), Japanese quail (JEL) and bob-white quail (BEL) was described in a previous paper[9]. Human milk lysozyme (HUL) was purchased from U.S. Biochemicals (Cleveland, OH, U.S.A.). Bovine serum albumin (BSA), mouse immunoglobulin (IgG), HEL and turkey lysozyme (TKY) were obtained in the purest grade available from Sigma.

Preparation of immunoaffinity columns

The monoclonal antibodies to HEL were partially purified from mouse ascites fluid by precipitation in 42% ammonium sulfate. The precipitate was reconstituted in 0.10 *M* phosphate (pH 7.0) and dialyzed against the same buffer until 1 ml each of the dialysate and a 1% (w/w) barium chloride solution showed no barium sulfate precipitate. The protein content of the antibody solutions were determined by a Bradford assay[13].

Monoclonal antibodies and non-immune mouse IgG were coupled to N-hydroxysuccinimide pre-activated gel, Affi-prep 10 (Bio-Rad Labs.) by using the manufacturer's instructions as follows: The antibody solutions containing *ca.* 15 mg of protein (except HyHel-5, which contained 9 mg) were dialyzed overnight against the coupling buffer, 0.10 *M* MOPS–0.15 *M* sodium chloride (pH 7.2) to remove any amino contaminants. Approximately 0.9 ml of the gel was washed with 50 ml of cold 0.01 *M* sodium acetate (pH 4.0), followed by several ml of coupling buffer. The gel was transferred to a 100-ml round-bottom flask containing 1 ml of MOPS coupling buffer. After degassing the solution for 1 min, the antibody solution was added and the volume was adjusted to 3 ml, resulting in a final protein concentration of approximately 5 mg/ml (3 mg/ml for HyHel-5). The reaction mixture was slowly agitated at 4°C for 24 h. Any remaining active groups were blocked by adding 0.10 ml of 1.0 *M* ethanolamine hydrochloride, pH 8.0, and agitating for 1 h at 4°C. After extensive washing with 0.10 *M* phosphate buffer (pH 7.0), the affinity packing material was slurry-packed from this buffer into a stainless-steel column (5 × 0.41 cm I.D.) at 500 p.s.i. by using an Altex pump (Model 110, Altex Scientific, Berkeley, CA, U.S.A.). The amount of protein coupled to the gel was determined by measuring the loss of protein from the supernatant during coupling by the Bradford assay[13]. The lysozyme load capacity of each column was measured by frontal analysis[14]. Duplicate samples of HEL (2 ml, 0.164 mg/ml) were pumped through the column at 0.5 ml/min until a breakthrough occurred. The column was washed twice with 0.10 *M* glycine buffer (pH 2.2) between analyses.

Apparatus

The experimental set-up used for the assays is shown in Fig. 1. The position of the automatic switching valve (Model 7010) attached to a pneumatic actuator (Model

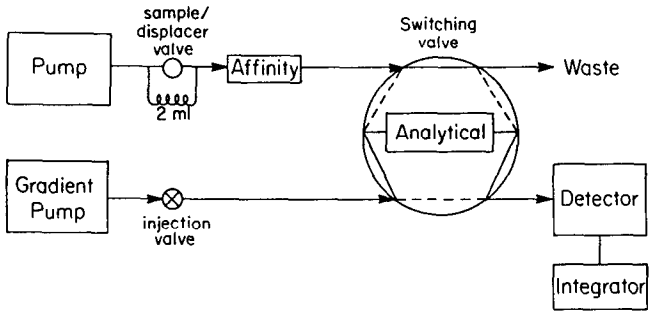

Fig. 1. Column switching apparatus.

5701, both from Rheodyne, Cotati, CA, U.S.A.) determined whether the immunoaffinity column and the analytical column were in series or separate. The timing of the switching valve was controlled by the gradient pump (Model 1090L, Hewlett Packard, Waldbronn, F.R.G.) fitted with a manual injection valve (Model 7125, Rheodyne). The affinity column was equipped with a separate pump (Model 110, Altex) and injection valve (Model C6U, Valco Instruments, Houston, TX, U.S.A.) equipped with a 2-ml injection loop for loading samples and applying desorption buffer. The absorbance was monitored at 280 nm with a variable-wavelength detector (Spectroflow 757, kindly lent by Kratos Division, Applied Biosystems, Ramsey, NJ, U.S.A.) connected to an integrator (Model 740, Waters, Milford, MA, U.S.A.) which calculated peak areas for quantitation.

Binding studies of lysozyme variants

Binding of all the lysozyme variants listed in Fig. 2 (except duck C which was not available in a pure form) on all five monoclonal anti-HEL and non-immune

	-1	5	10	15	20	25	30	35	40	45	50	55	60	65
Chicken	K	V	F	G	R	C	E	L	A	A	A	M	K	R
Bob White quail									S			V		
Japanese quail		Y			K	Q								
Ring-necked pheasant	G	Y		M					G					
Turkey		Y		L					H					
Duck A		YS		L				NY	S					
Duck B		YS		L				NY	G					
Duck C		YS		L				NY	G					
Human Milk		E		RTL	L	M	G	I	A	M	L	W	GY	R

	66	70	75	80	85	90	95	100	105	110	115	120	125
Chicken	D	G	R	T	P	G	S	R	N	L	C	N	I
Bob White quail	K					T							
Japanese quail								VH				N	
Ring-necked pheasant			K	H						KH		NV	
Turkey			K					A	G			H	
Duck A	K		K	A	G	V	R	EA	R	R		R	SK
Duck B	K	R	K	A	G	V	R	EA	R	R		R	SK
Duck C	K	R	K	A	G	R	V	R	EA	R	R		R	SK
Human milk	K		AV	A	HLS		QDN	ADA	A	RVR-	PQ	IR	QNR	RQYVQ GV

Fig. 2. Primary amino acid sequences of lysozyme variants[8,17].

mouse IgG affinity columns was evaluated, by using each affinity column separately, not in the dual-column mode. The following procedure was used for all six columns. The column was equilibrated with the loading buffer, 0.10 *M* phosphate–0.25 *M* sodium chloride (pH 7.0), at a flow-rate of 1 ml/min. The column was then subjected to four 2-ml step gradients of the desorption buffer, 0.10 *M* glycine hydrochloride (pH 2.2), in order to remove any non-covalently bound proteins. Non-specific binding of BSA and specific binding of the lysozyme variants were measured as follows. Three injections of BSA (20 μl, 10 mg/ml) or five injections of a lysozyme (20 μl, 1 mg/ml), followed by 2 ml of desorption buffer were made into the affinity column. The peak heights of the successive injections and of the desorption buffer step were measured in order to determine qualitatively whether any protein was bound to the column. The column was washed twice with 2-ml portions of glycine buffer between analyses.

ICA of lysozyme variants

Dual-column ICA assay of several lysozyme variants was performed using two types of analytical columns: a cation-exchange and a reversed-phase column. Samples containing RNP, duck A, B and C were analyzed using the monoclonal HyHel-9 anti-HEL immunoaffinity column, coupled to a CEX analytical column (Synchropak S-300, 5 × 0.41 cm I.D., SynChrom, Lafayette, IN, U.S.A.). A mixture of duck B and C lysozymes was available for this analysis. The monoclonal HyHel-5 anti-HEL immunoaffinity column, coupled to a reversed-phase C-4 column (Supelco C-304, 5 × 0.41 cm I.D.), was used to analyze samples of either BEL and HEL or JEL and HEL. The lysozyme mixtures (20 μl, 1 mg/ml) were loaded onto the immunoaffinity column at a flow-rate of 0.5 ml/min in loading buffer [0.05 *M* sodium phosphate (pH 7.0) for CEX; 0.10 *M* phosphate–0.25 *M* sodium chloride (pH 7.0) for RPC]. The automatic switching valve was switched to connect the immunoaffinity and analytical column in series. A 2-ml injection of the desorption buffer (0.05 *M* glycine hydrochloride, pH 3.0 for CEX; 0.10 *M* glycine, pH 2.2 for RPC) eluted the lysozymes from the affinity column into the analytical column at 1 ml/min. (The desorption buffer step is illustrated in the cation-exchange ICA chromatogram as a darkened rectangle.) After 4 or 6 min, the automatic switching valve was changed, connecting the analytical column back to the gradient pump. The lysozymes were separated on the cation-exchange column with a 20-min linear gradient from 0 to 0.5 *M* sodium chloride in 0.01 *M* borate (pH 9.0) at 1 ml/min. The chromatographic conditions for RPC were first a 5-min linear gradient from 0 to 28% acetonitrile in 0.1% TFA, followed by a 9-min linear gradient from 28% to 32% acetonitrile in 0.1% TFA, at 14 min. This condition was maintained until 20 min. The flow-rate was 1 ml/min for RPC.

Competitive inhibition of binding of lysozyme variants

Affinity packing material with monoclonal HyHel-5 anti-HEL was packed into a 3 × 0.2 cm I.D. column. Duplicate samples with increasing amounts of inhibitor lysozyme, BEL and JEL (1-1000 μg) and a constant amount of HEL (4 μg) in 1 ml loading buffer were injected into the immunoaffinity column in 0.10 *M* phosphate–0.25 *M* sodium chloride (pH 7.0) at a flow-rate of 0.3 ml/min. Unbound proteins were routed to waste. The rest of the ICA analysis was carried out as described for HyHel-5, coupled to the RPC column, in the section on *ICA of lysozyme variants*. The

bound fraction of HEL was quantitated from peak area of the RPC analysis. The RPC column was washed with a 5-min linear gradient from 0 to 100% acetonitrile in 0.1% TFA between analyses. B/B_0 was plotted *versus* the log of the dose (μg inhibitor lysozyme), where B is the amount of HEL bound to the antibody in the presence of inhibitor, and B_0 is the amount of HEL bound to the antibody in the absence of inhibitor[15]. This method was used for plotting the data because the data are normalized and not plotted as raw data for free and bound lysozyme in order to facilitate interassay comparisons. Curve fitting and estimation of 50% inhibition points were based on inhibition of binding data linearized by a log–logit transformation[15].

RESULTS AND DISCUSSION

Evaluation of immunoaffinity columns

The immunoaffinity columns were characterized by determining the amount of protein immobilized on the support, antigen-load capacity, the amount of non-specific binding, and the amount of specific binding of each lysozyme variant. The results of this characterization are listed in Table I. The amount of protein immobilized and HEL load capacity of the HyHel-5 column were less than for the other columns because there was less HyHel-5 available for the immobilization. HyHel-9, 10, and 15 had about the same amount of protein immobilized and HEL load capacities. HyHel-9 had about the same amount of protein immobilized as HyHel-9, 10, and 15 but it had only about one-third the antigen load capacity. The reason for this may have been that the amount of specific anti-HEL antibodies in the HyHel-8 ascites fluid (the titer) was less than in the others.

Non-specific binding to the immunoaffinity matrix was investigated with BSA. Constant peak heights of triplicate injections of BSA indicated no non-specific binding on either the monoclonal or non-immune mouse IgG matrix. Nonspecific binding of BSA would have resulted in peak heights slowly increasing with each injection until a constant peak height was reached. The absence of non-specific BSA binding was confirmed when no BSA was eluted during the desorption buffer step.

TABLE I

EVALUATION OF IMMUNOAFFINITY COLUMNS

Affinity column	*Protein immobilized*[a] *(mg/ml gel volume)*	*HEL load capacity*[b] *(μg/ml column volume)*	*Lysozymes bound*
HyHel-5	8.6	31.1	All but HUL
HyHel-8	20.8	97.0	All but HUL
HyHel-9	17.2	287	All but HUL
HyHel-10	21.6	303	All but HUL
HyHel-15	18.3	281	All but HUL
Mouse IgG	15.5		None

[a] Determined by Bradford protein assay.
[b] Determined by frontal analysis[14].

Binding of all the lysozymes used was evaluated for each immunoaffinity column. As shown in Table I, all five monoclonal anti-HEL immunoaffinity columns bound to all of the lysozymes tested, except the human milk. None of these lysozymes bound to the non-immune mouse IgG column, which was used as a control, indicating that the binding was specific. HUL and HEL differ by 53 out of 129 amino acids. Polyclonal anti-HUL antibodies have been observed to cross-react with HEL[2,16]. The HUL structure is sufficiently different from HEL to prevent binding of the monoclonals prepared against HEL. However, the other avian lysozyme variants having amino acid differences from HEL ranging from 4 amino acids for BEL to 20 amino acids for duck B, were still bound. Monoclonal antibodies will frequently cross-react with related antigens. These data demonstrate the ability of antibodies to cross-react with similar species which differ by as much as 20 amino acids from the immunogen. Although there is a great degree of binding cross-reactivity, the affinities of all the variants to the monoclonals are not always equal to that of HEL[3,5].

ICA of lysozyme variants

Conventional chromatographic methods are often more selective than immunoaffinity chromatography. RNP, Duck A, B, and C lysozyme differ from HEL by 10, 19, 20, and 21 amino acids, respectively, but still cross-reacted with the monoclonal HyHel-9 antibody (Fig. 3). JEL and BEL differ from HEL by 6 and 4 amino acids, respectively, but cross-reacted with HyHel-5 (Fig. 4). CEX (Fig. 3) and RPC (Fig. 4) were able to separate lysozymes that were indistinguishable by immunoaffinity analysis alone. These data demonstrate the utility of the ICA system in distinguishing between proteins with similar structural forms. The immunoaffinity column cross-reacts and purifies the species, then the second column separates them.

Mobile phases used in RPC are compatible with most desorption conditions used in immunoaffinity chromatography. CEX chromatography is compatible with

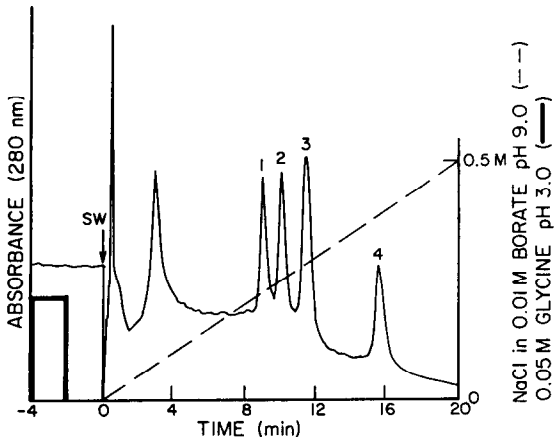

Fig. 3. ICA of lysozyme variants (1 = RNP, 2 = duck A, 3 = duck B, 4 = duck C). Affinity column: monoclonal anti-HEL (HyHel-9) (5 × 0.41 cm I.D.). Analytical column: cation-exchange, Synchropak S300 (5 × 0.41 cm I.D.). Event sequence: −4.0 min, inject 2-ml plug of 0.005 *M* glycine (pH 3.0) (rectangular box); 0 min, analytical column switched (SW) in-line with gradient pump, cation-exchange analysis started with a 20-min linear gradient from 0 to 0.5 *M* sodium chloride in 0.01 *M* sodium borate (pH 9.0) at a flow-rate of 1 ml/min.

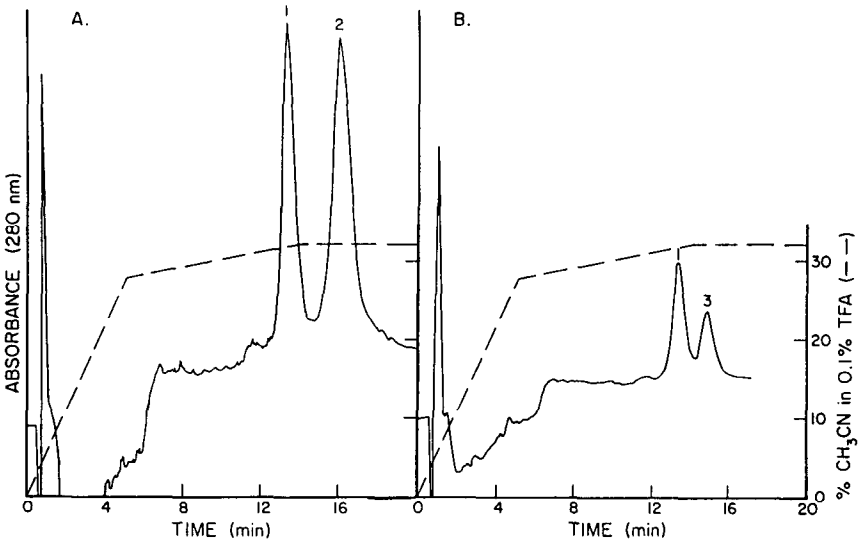

Fig. 4. ICA of lysozyme variants. (A) 1 = HEL, 2 = JEL; (B) 1 = HEL, 3 = BEL. Affinity column: monoclonal anti-HEL (HyHel-5) (5 × 0.41 cm I.D.). Analytical column: reversed-phase, Supelco C-304 (5 × 0.41 cm I.D.). Event sequence: −6.0 min, inject 2-ml plug of 0.10 *M* glycine (pH 2.2); 0 min, analytical column switched (SW) in-line with gradient pump, reversed-phase analysis started with a 5-min linear gradient from 0 to 28% acetonitrile in 0.1% TFA, followed by a 9-min linear gradient from 28 to 32% acetonitrile in 0.1% TFA, at 14 min. These conditions were maintained until 20 min, at a flow-rate of 1 ml/min.

immunoaffinity chromatography when a low pH desorption buffer is used. At a low pH, all proteins will be positively charged and will concentrate on a cation-exchange column before gradient elution is begun. Other desorption conditions are necessary when immunoaffinity chromatography is coupled to anion-exchange and HIC.

Competitive inhibition of binding studies

All of the direct binding studies of a single or mixture of lysozymes were carried out with an antibody excess. Competitive inhibition of binding studies with an antigen excess were also performed by ICA. Immobilized HyHel-5, packed into a 3 × 0.2 cm I.D. column (volume, 0.094 ml), had a HEL load capacity of 2.9 μg HEL, calculated from the HEL load capacity listed in Table I. The lysozymes bound to the immunoaffinity column were desorbed and analyzed by RPC which separated JEL and BEL from HEL, as shown in Fig. 4. The results of the competitive inhibition of binding analyses are shown in Fig. 5. The inhibition data, linearized by the log–logit transformation gave a correlation coefficient of 0.978 and 0.992 for JEL and BEL, respectively. The amount of inhibitor required to give 50% inhibition of HEL binding, obtained from the linearized inhibition data, was 89.77 μg JEL and 1907 μg BEL. This gave a ratio of the concentrations (I) of BEL/JEL of 217.

Each lysozyme variant has been classified according to its relative 50% inhibition of HEL binding ratio (*I*) for each monoclonal by Smith-Gill *et al.*[3,5]. They were placed in broad groups, depending on the order of magnitude of I ($I = 1$; $1 < I < 10$; $10 \leqslant I < 100$; $I \geqslant 100$). The relative concentration of BEL:JEL required to obtain a

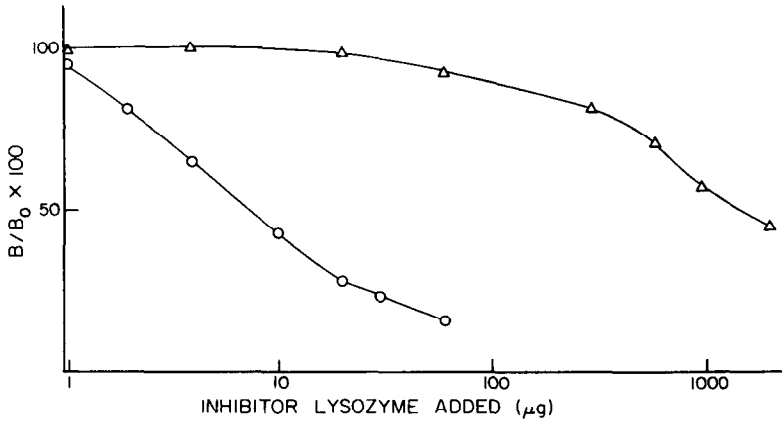

Fig. 5. Competitive inhibition of binding of hen egg lysozyme to HyHel-5. Inhibitors: (○), Japanese quail; (△), bobwhite quail.

50% inhibition of HyHel–5 binding to HEL was in the same order of magnitude for ICA and enzyme-linked immunosorbent plate binding assay[3], *viz.* 217[a] and 100[b], respectively, placing BEL in the same group ($I \geqslant 100$) by both methods.

These data showed that 217 times more BEL than JEL is necessary to inhibit 50% of the binding of HEL to HyHel-5. JEL competes much more efficiently with HEL for the antibody than BEL. The binding affinity of BEL to HyHel-5 is smaller than that of JEL. This is because the Arg 68 amino acid, which has been identified as a "critical" residue in the epitope of HyHel-5[3,5,10], has been substituted for Lys in BEL, decreasing the binding constant. Differences in affinity were apparent under conditions of antigen excess where there was competition for the available antibody binding sites. In situations of antibody excess, as was the case for the direct binding studies, differences in binding affinities were not apparent.

CONCLUSIONS

Our studies have demonstrated the utility of the ICA system in distinguishing between proteins with similar structures. Monoclonal antibodies frequently cross-react with a variety of closely related antigens. In this case, even antigens which differed by as much as 20 amino acids from the immunogen cross-reacted, presumably because the actual epitopes contain sufficient similarity for binding. For this reason, an immunoaffinity purification step of a recombinant protein is more useful at the beginning rather than at the end of a preparative purification scheme. The ICA system takes advantage of antibody cross-reactivities by performing a purification of all cross-reactive species first, followed by a chromatographic separation. Important applications of this method are monitoring protein fermentation broths or pharmaceutical formulations. A unique feature of the ICA system is that direct and competitive binding immunoassays of single and multiple antigens can be performed on-line,

[a] Value obtained from inhibition data linearized by a log–logit transformation[15].
[b] Data taken from ref. 1.

without fraction collection, by using unlabeled antigens. Previous work has demonstrated the utility of this system for analyzing antigens in crude samples[1,2]. This has applications in many areas, including clinical chemistry[18].

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