Stability of Interleukin 1β (IL-1β) in Aqueous Solution: Analytical Methods, Kinetics, Products, and Solution Formulation Implications

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The thermal stability of IL-1β in aqueous solution as a function of temperature (5–60°C), pH (2–9), buffer (acetate, citrate, tris, and phosphate), and cyroprotectants (sugars, HSA) was investigated in this study. The analytical methodologies included RP-HPLC, SEC, ELISA, IEF-PAGE, SDS-PAGE, and bioassay. The degradation and inactivation of IL-1β at or above 39°C were attributed to autoxidation of the two cysteine residues in the denatured protein, followed by hydrophobic/covalent aggregation and precipitation. At or below 30°C, IEF- and SDS-PAGE results suggest a possible deamidation reaction. The difference in mechanism of degradation precludes the prediction of formulation shelf life from accelerated temperature data. Nonetheless, the good stability observed at 5°C suggests that a solution formulation may be feasible for IL-1β.

KEY WORDS: interleukin 1β (IL-1β); protein stability; analytical methodology; aggregation; denaturation; autoxidation; deamidation.

INTRODUCTION

Interleukin 1β (IL-1β) is a lymphokine that serves as a priming signal for T cells and B cells and mediates fever and a variety of other biological responses (1–4). IL-1β, a protein with a molecular weight of 17,500, consists of 153 amino acids (5) with no disulfide bonds (6). Large quantities of IL-1β have recently been produced by the recombinant DNA technique and purified to homogeneity (7–11). Studies using circular dichroism, fluorescence spectroscopy, and X-ray crystallography revealed recombinant IL-1β to be an all-β-type, stable globular protein (12,13).

The initial clinical indications being pursued for IL-1β include protection against chemo- and radiation damage in cancer therapies (14–16) and wound healing (17). Currently, IL-1β is formulated as a lyophilized product which has shown a room-temperature shelf life, or t_{90} (time to reach 90% remaining), of more than 1 year (18). We report in this paper the preliminary results of IL-1β stability in aqueous solution as a function of pH and temperature. Because of the months or years required to obtain kinetic data at the formulation storage conditions, prediction of shelf life from data obtained at accelerated conditions is desirable. Consequently, data presented herein were collected from 5 to 60°C. In those cases where Arrhenius extrapolations are not appropriate, stability data may still provide very practical information for both formulation manufacture and design, as well as use in drug delivery systems.

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EXPERIMENTAL

Materials

Recombinant human IL-1β (1.6 or 2.0 mg/ml concentrations) was provided by Immunex, Seattle, in 2% NaCl, 0.2% citrate buffered pH 7.0 aqueous solution. HPLC-grade acetonitrile, trifluoroacetic acid (TFA), and nanopure water were used to prepare the mobile phases. All other chemicals were reagent grade or better.

Analytical Methodology

HPLC. HPLC was conducted on either an HP 1090 system or a system consisting of Rainin Rabbit pumps, a Perkin Elmer ISS 100 autoinjector, and a Kratos SF 769 Z UV detector.

Two reverse-phase (RP) HPLC methods were developed. Both methods used a Vydac 214TP54 silica, 5-μm, C_4 column and a flow rate of 0.50 ml/min. The UV detection was at either 215 or 280 nm. Method I used a linear gradient mobile phase of 80% TFA (0.1%)/H_2O and 20% TFA (0.08%)/MeCN at time 0 to 40% TFA (0.1%)/H_2O and 60% TFA (0.08%)/MeCN at time 60 min. Method II used the following gradient mobile phase: 16% of Buffer A (H_2O/MeCN/H_3PO_4 at a ratio of 79/20/1) and 84% of Buffer B (H_2O/MeCN/H_3PO_4 at a ratio of 19/80/1) was held isocratic for 10 min, then increased in a linear gradient to 22% Buffer B at time 37 min and to 60% Buffer B at 45 min. Both methods were found to yield good linearity for 0.05 to 3.1 μg IL-1β injected.

A size exclusion HPLC (SEC) method was developed for the analysis of aggregation samples. The method employed a DuPont GF-250 gel filtration column with a mobile phase of 0.2M $(NH_4)_2SO_4$/0.05M Tris buffer at pH 7.0. The flow rate was 1.8 ml/min and detection was at 220 nm. BSA, Lysozyme and bGRF were used as molecular weight standards.

ELISA. The details of the ELISA method have been described elsewhere (19). The quantitation limit of the assay was 30 pg/ml and the assay variability was ~13%.

Bioassay. The thymocyte proliferation bioassay used was a slight modification of the published procedure (20) and has been described in detail elsewhere (18). The variability of the method was ~26%.

Electrophoresis. Isoelectric focusing (IEF) was conducted on precast pH 3.5–9.5. Ampholine PAG plates (Pharmacia LKB Biotechnology) with a Bio-Rad Bio-Phoresis horizontal electrophoresis cell and a Bio-Rad Model 3000-Xi power supply. The p*I*'s of the protein bands were calibrated using standard p*I* markers. SDS-PAGE was conducted on a Pharmacia Phastsystem using precast 8–25% gradient Phastgels. Molecular weight was calibrated using standard protein

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markers. A Pharmacia LKB laser densitometer was used for detecting the protein bands on gels.

Aggregation Studies from 39 to 60°C

Sterile (0.2-μm filter) buffer solutions (0.01 *M*) containing various excipients were prepared and 1-ml aliquots were transferred into 2-ml sterile polypropylene vials (Sarstedt). After the buffer solutions were equilibrated in a temperature bath (39–60°C), an adequate amount of IL-1β raw material was added to the vials to make 100, 300, and 500 μg/ml drug solutions. At predetermined time points, aliquots (100–150 μl) were withdrawn and stored at 4 or −80°C until assayed.

The stressed samples were centrifuged at 12,000 rpm and 4°C for 30 min and the supernatant was removed for HPLC analysis. To evaluate the effect of centrifugation, a freshly prepared sample was injected onto RP-HPLC (without centrifugation and then following centrifugation) and the results were compared. Additionally, a sample stressed at 60°C for 25 min was assayed similarly. Centrifugation did not effect the assayed amount of IL-1β in both experiments: the peak area was unchanged by both RP-HPLC methods, before and after centrifugation.

To prepare the stressed sample for SDS-PAGE analysis, water (0.5 ml) was added to the remaining precipitate and the solution was centrifuged. The water layer was then removed and the washing process was repeated. The precipitate was dissolved with 50 μl of a stock SDS (2.5%) solution, incubated with the SDS-PAGE buffer at room temperature for 3 hr, and then loaded on the gels.

Kinetics at or Below 30°C

Nine milliliters of a stock solution containing 100 μg/ml of IL-1β was filtered through a 0.22-μm filter (Millex-GV) under sterile conditions in a laminar flow hood. Aliquots (500 μl) of this solution were transferred to 10-cm^3 sterile polypropylene vials, sealed with 16.5-mm sterile rubber stoppers, and crimp-capped. All solutions were kept at 5°C during manufacture. Vials were set up for stability at 5 and 30°C and pulled at time points of 0, 3, 7, 14, 28, 42, 84, 98, and 154 days. Immediately after pulling, the samples were frozen to −80°C and remained at this temperature until analyzed.

RESULTS AND DISCUSSION

Stability at or Above 39°C

The stability of IL-1β solutions was examined primarily at 100 μg/ml from pH 3 to pH 8 in polypropylene containers. Control IL-1β solutions yielded two major protein bands on IEF-PAGE gels with p*I* values of 6.8 and 6.7, respectively. The latter band indicated the presence of the [desAla^1] analogue of IL-1β (21). All solutions clouded with storage time at 39 to 60°C and IEF-PAGE analysis of the supernatant of the stressed samples (after centrifugation, see Experimental) showed no apparent changes in the ratios of the two major protein bands of IL-1β and no significant amount of newly formed bands (less than 7% of reacted IL-1β), suggesting that the possible deamidation reaction (see below) was not significant at ≥39°C.

The supernatants of the stressed sample solutions were also analyzed by RP-HPLC (Method I or II), ELISA, and bioassay. Typical percentage drug remaining data obtained at pH 7.0 and 60°C are plotted as a function of time in Fig. 1. The percentage remaining data obtained using RP-HPLC are consistent with those of ELISA and bioassay when the errors involved in these assays are considered, ~4, ~13, and ~26%, respectively. These results demonstrate the specificity of the RP-HPLC methods for monitoring the stability of IL-1β at ≥39°C and indicate that most or all of the degraded IL-1β aggregated into insoluble precipitates. The latter suggestion was further supported by the SEC analysis of the supernatants which showed a negligible amount (less than 1%) of IL-1β in supernatants to be oligomers.

The precipitates could not be dissolved by simple organic solvents (i.e., MeOH, EtOH, MeCN, and PEG) but were soluble in a 2.5% SDS solution. SDS-PAGE analyses of the dissolved precipitates under nonreducing conditions (see Experimental) revealed dimers, trimers, and other oligomers, in addition to monomers (Fig. 2a). The presence of monomers (≤30%) in the dissolved precipitate suggests that a portion of the aggregates resulted from hydrophobic (or noncovalent) aggregates which were dissociated by SDS. All aggregated material, however, was found to be converted to the monomers when treated with β-mercaptoethanol (Fig. 2b), suggesting that the majority of the aggregates resulted from disulfide bond formation.

At 60°C, the aggregation/precipitation kinetics followed apparent first-order behavior to 30% drug remaining (Fig. 1) and the deviation of the kinetics between replicate runs was usually within 30%. The calculated apparent first-order rate constants at 60°C are summarized in Table I. The rate of aggregation/precipitation of 100-μg/ml IL-1β solutions was similar in buffers with the pH region of 3.0–6.2 ($5.7–9.4 \times 10^{-5}$ sec^{-1}) and was faster at pH ~7 ($2.5–3.6 \times 10^{-4}$ sec^{-1}). Surface adsorption inhibitors such as Tween 80 and 0.1% HSA showed no stabilizing effect, indicating that the aggregation of IL-1β probably was not catalyzed by the container surface (polypropylene). Sugars such as dextran and reducing agents such as 1% isopropyl sulfide, 1% ethyl methyl sulfide, or 0.02% dithiothreitol also had no effect.

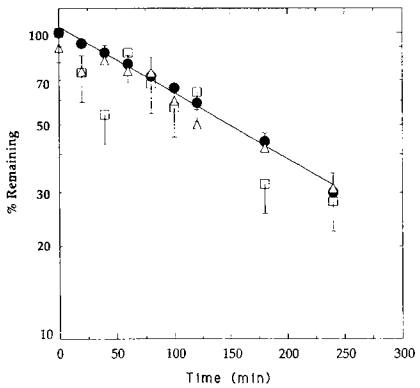

Fig. 1. Semilog percentage drug remaining vs time profile for the degradation of IL-1β at pH 7.0 and 60°C analyzed using HPLC method I (●), ELISA (△), and bioassay (□). Standard error bars were each drawn in one direction for the ELISA and bioassay results for clarity. The line indicates a linear fit of the HPLC data.

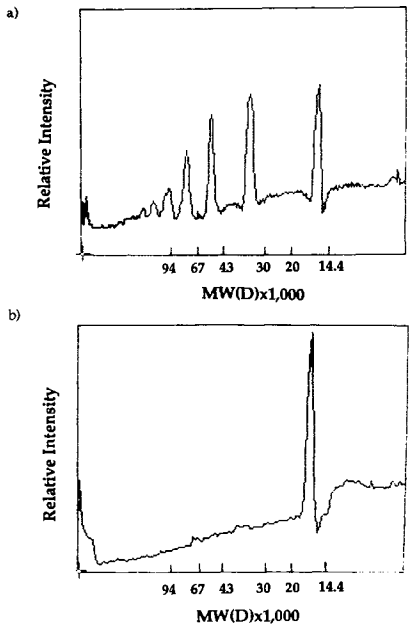

Fig. 2. Densitometry analysis of the SDS-PAGE gels of the dissolved precipitates under (a) reducing and (b) nonreducing conditions.

At or below 55°C, the kinetics pattern of the aggregation/precipitation deviated significantly from apparent first-order and became biphasic (a slower one followed by a more rapid one). Typical percentage drug remaining data obtained in pH 5.0 acetate buffer at 39, 45, 50, 55, and 60°C are plotted as a function of time in Fig. 3. The slower (or lag) phase of the kinetics was apparent from 39 to 55°C, less

Table I. Apparent First-Order Rate Constants of the Degradation of 100-μg/ml IL-β Solutions at 60°C[a]

Solvent[b]	pH[c]	k ($sec^{-1} \times 10^5$)
0.001 *N* HCl	3.0	6.4
Acetate buffer	4.0	9.1
Acetate buffer	5.2	9.4
Tris buffer	5.2	5.7
Tris buffer	6.2	8.4
Phosphate buffer	7.0	36.
Tris buffer	7.2	25.
Acetate buffer/isopropyl sulfide (1%)	5.2	16.
Acetate buffer/ethyl methyl sulfide (1%)	5.2	16.
Acetate buffer/dithiothreitol (0.02%)	5.2	14.
Tris buffer/dextran (3%)	6.2	12.
Tris buffer/Tween 80 (0.1%)	6.2	8.3
Tris buffer/HSA (0.1%)	6.2	8.9
Phosphate buffer/EDTA (5 m*M*)	7.0	43.

[a] Based on HPLC results of percentage protein remaining in solution.
[b] All solutions contained 0.01 *M* buffer and 0.15 *M* NaCl, unless noted.
[c] Measured at reaction temperature.

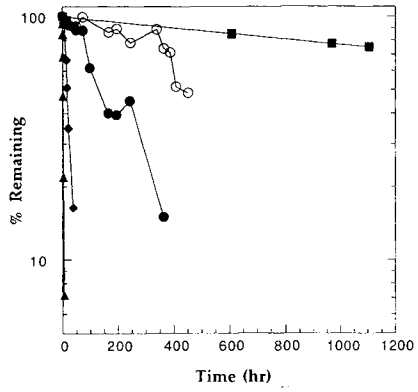

Fig. 3. Semilog percentage drug remaining vs time profile for 100 μg/ml IL-1β at pH 5.0 (acetate buffer) and 60°C (▲), 55°C (♦), 50°C (●), 45°C (○), and 39°C (■).

pronounced at 55°C, and not discerned at 60°C. The biphasic kinetics were pH and concentration dependent as the lag phase was suppressed considerably at pH 7.0 and at 500 μg/ml (Fig. 4).

The dependence of the observed kinetics on temperature, pH, and concentration suggests that the aggregation/precipitation of IL-1β involves the autoxidation of the cysteine residues (22,23). Product analysis showing that the majority of the products were covalent aggregates supports this suggestion. Further, because both cysteine residues of IL-1β are buried inside its native conformation (6), it is possible that only the denatured form of IL-1β is oxidized. In the presence of denaturants, IL-1β was shown to yield covalent aggregates at alkaline pH values (12,24). Although the molecular nature of the lag phase cannot be revealed with current data, it may be suggested that the lag phase involves primarily the generation and accumulation of free radicals and/or aggregates. Once these intermediates reach certain concentrations, rapid precipitation (the rapid phase) predominates.

Because metal ions can catalyze sulfhydryl autoxidation (25), EDTA, a metal chelator, was evaluated as a possible stabilizer of IL-1β. At 60°C, where the lag phase was not apparent, the presence of 5 m*M* EDTA showed no effect on

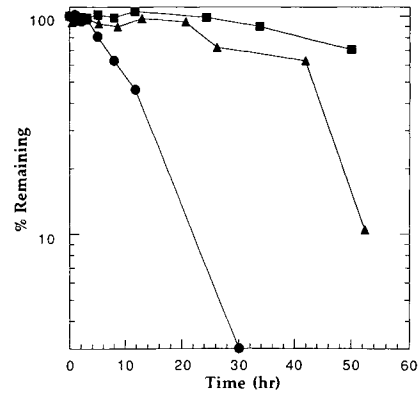

Fig. 4. Semilog percentage drug remaining vs time profile at pH 7.0 (phosphate buffer) and 50°C for 100 μg/ml IL-1β (▲), 500 μg/ml IL-1β (●), and 100 μg/ml IL-1β in the presence of 5 m*M* EDTA (■).

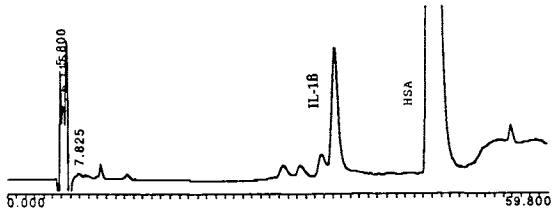

Fig. 5. A RP-HPLC (method II) chromatogram of a 100-μg/ml IL-1β solution (67% remaining) aged for 154 days at pH 7.0 and 30°C.

the reaction kinetics (Table I). However, the presence of EDTA at 50°C appeared to prolong the lag period (Fig. 4).

Stability at 5 and 30°C

The aqueous stability of 100-μg/ml IL-1β solutions was also studied at 5 and 30°C from pH 2 to pH 9 in polypropylene containers. When the aged samples were analyzed by RP-HPLC method I, no apparent products could be identified. Also, the percentage drug remaining data were consistently greater than those by IEF-PAGE, despite the large error involved in the latter assay (~15%). HPLC method II, which used phosphate buffer (instead of TFA), showed at least three degradation products of shorter retention than IL-1β (Fig. 5). Interestingly, IEF-PAGE analysis of the aged samples revealed, in addition to the two bands of IL-1β, two major bands with p*I* values of 5.7 and 5.2, respectively, and at least one minor band with a p*I* value of 5.6 (Fig. 6). The molecular weight of the degradation products, on the other hand, appears to be similar or identical to IL-1β, as SDS-PAGE analysis using coomassie blue showed no different protein bands formed in the aged samples (90 days). These results suggest that these products most likely result from deamidation of the asparagine and/or glutamine amino acids of IL-1β.

Typical semilog percentage drug remaining versus time profiles by HPLC method II for the 30°C samples at pH 2.0, 4.0, 7.0, and 8.0 are shown in Fig. 7. The degradation of

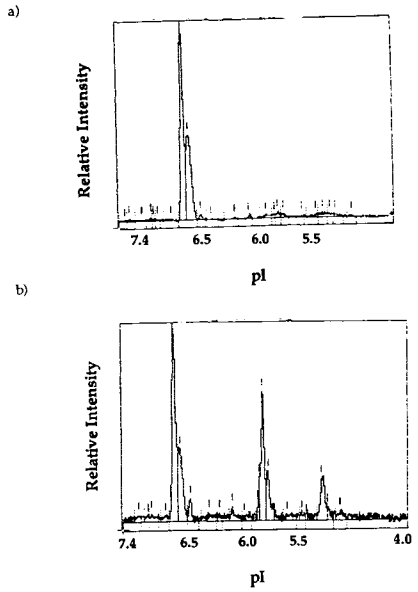

Fig. 6. Densitometry analysis of the IEF-PAGE gels of (a) an initial and (b) a sample of 100 μg/ml IL-1β aged 154 days at pH 7 and 30°C.

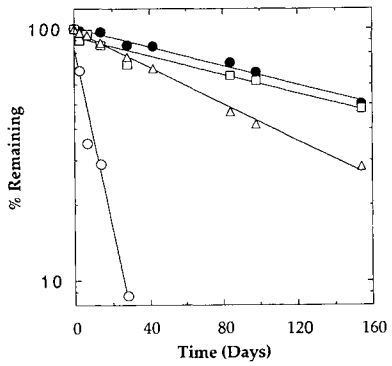

Fig. 7. Semilog percentage drug remaining vs time plots of the degradation of 100 μg/ml IL-1β in aqueous solution at 30°C and pH 2.1 (○), pH 4.0 (●), pH 7.0 (□), or pH 8.0 (△).

IL-1β at 30°C was fitted to pseudo-first-order kinetics ($r \geq 0.97$). Most of the solutions at 5°C had ≤10% degradation, thus preventing the assignment of the kinetic order. Nonetheless, all rate data were evaluated using pseudo-first-order kinetics for easy comparison. Table II summarizes the t_{90} (time to reach 90% remaining) values. Although the separation mechanisms of HPLC and IEF methods are vastly different, the t_{90} values obtained by these two techniques agree with each other reasonably well. The presence of NaN_3, an antimicrobial agent, had no effect on the degradation rate (compare runs 3 to 4, 8 to 9, and 11 to 12). This indicates that all aseptically prepared solutions remained sterile during the period of the 154-day study. Also, the addition of 0.05%

Table II. Stability Results of Aqueous Degradation of IL-1β at 5 and 30°C

Run No.	Formulation[a]	pH	t_{90}, days[b] 5°C HPLC	5°C IEF	30°C HPLC	30°C IEF
1	0.01 *N* HCl/HSA	2.1	59	—	0.95	.29
2	Acetate buffer/HSA	3.0	59	39	21	27
3	Acetate buffer/ HSA/NaN_3	4.0	112	—	33	63
4	Acetate buffer/ HSA	4.0	271	ND	24	22
5	Acetate buffer	4.0	91	78	25	42[c]
6	Acetate/HSA	5.0	199	235	59	52
7	Citrate buffer/HSA	5.0	187	293	24	40
8	Tris buffer/HSA/ NaN_3	6.0	ND	ND	49	42
9	Tris buffer/HSA	6.0	ND	ND	60	ND
10	Tris buffer	6.0	198	ND	43	ND
11	Tris buffer/NaN_3	7.0	ND	ND	38	96
12	Tris buffer/HSA	7.0	192	ND	45	79
13	Tris buffer	7.0	ND	238	50	24
14	Citrate (0.2%)	7.0	116	ND	14	38
15	Tris buffer/HSA	8.0	ND	ND	13	9.2
16	Tris buffer/HSA	9.0	124	32	4.4	4.9

[a] [HSA] = 0.05%; [NaCl] = 0.09 *M*; [buffer] = 0.01 *M* unless otherwise indicated.

[b] "ND" denotes no apparent degradation observed.

[c] The 154-day data were not included in the analysis due to an apparent assaying error.

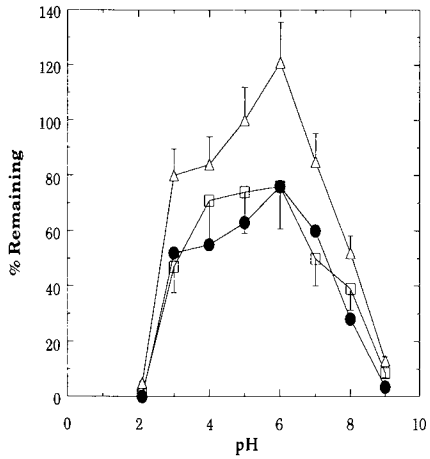

Fig. 8. Stability of 100-μg/ml IL-1β solutions after storage at 30°C for 154 days and analyzed using HPLC method II (●), ELISA (△), and bioassay (□).

HSA (compare runs 3 to 5, 9 to 10, and 12 to 13) and the use of citrate buffer (No. 14) at pH 7.0 did not affect the stability significantly, indicating the lack of stabilizing effect by these excipients at the concentration used.

To substantiate the stability results obtained by RP-HPLC, the 154-day degraded samples were also analyzed by ELISA and bioassay. The results for the 30 and 5°C samples are plotted as a function of pH in Figs. 8 and 9, respectively. When the errors involved in various assay methods are taken into consideration, the percentage remaining data obtained by HPLC method II are in agreement with those obtained by ELISA and bioassay for the 5°C samples. However, the ELISA percentage remaining data for the 30°C samples appear to be higher than those of RP-HPLC and bioassay (Fig. 8). Although no efforts were made to resolve this inconsistency, it is possible that some of the degradation products which were separated by RP-HPLC method II responded to the ELISA but not to the bioassay.

In conclusion, we have shown that the degradation mechanism of IL-1β in aqueous solution is primarily aggregation/precipitation at or above 39°C and possible deamidation at or below 30°C. The strong temperature dependence of these two distinct mechanisms precludes the prediction of formulation shelf life from accelerated temperature data. Good stability, however, was indicated at 5°C, with some formulations showing no apparent degradation after 154 days of storage (Fig. 9).

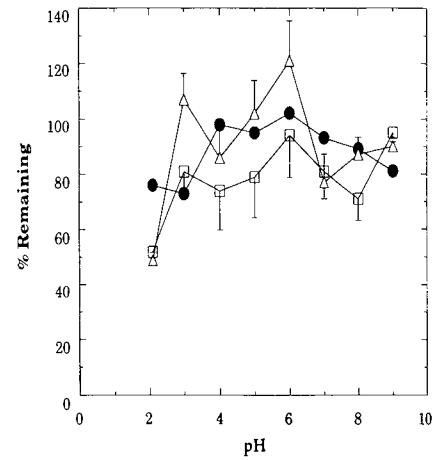

Fig. 9. Stability of 100-μg/ml IL-1β solutions after storage at 5°C for 154 days and analyzed using HPLC method II (●), ELISA (△), and bioassay (□).

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