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Aggregation of recombinant human growth hormone induced by phenolic compounds

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Abstract

Phenolic compounds can be used as antimicrobial preservative agents in pharmaceutical formulations. Unfortunately, these compounds often adversely affect proteins, triggering aggregation in particular. In this study, a variety of phenolic compounds and structurally similar non-aromatic alcohols were investigated for their role in causing the aggregation of a model therapeutic protein, recombinant human growth hormone (rhGH). As determined by various methodologies, most of the phenolic compounds caused rhGH aggregation, especially at high concentrations. Stress studies under freezing, high-temperature incubation, and agitation suggest that the destabilizing influence of the compounds tested increases in the order of benzyl alcohol < phenol ≈ resorcinol < catechol < meta-cresol < 2-chlorophenol. Non-aromatic alcohols, except 2,6-dimethylcyclohexanol, have a much less adverse effect. Determination of the thermal transition temperature by microcalorimetry studies also reflected this trend. From our study, we conclude: (1) the phenolic additive-induced rhGH aggregates were held by non-covalent forces; (2) no significant physical binding occurred between the protein and these compounds; (3) the aggregation tendency of the phenolic compounds failed to correlate with their hydrogen bonding strength; (4) the presence of a phenolic additive caused conformational changes in rhGH's structure; (5) the effect of these phenolic compounds on rhGH aggregation decreased at high and low pHs.

Keywords: Recombinant human growth hormone; Phenolic compounds; Non-aromatic alcohols; Aggregation; Microcalorimetry

1. Introduction

In pharmaceutical protein development, antimicrobial preservative agents such as phenol, *m*-cresol, methylparaben, and resorcinol, are often

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Table 1
Reagents used in this study

Compound/formula	M.W.	M.P.[a]	Solu. (mg/ml)[b]	Source (%purity)
2-chlorphenol/ (2-Cl-phenol)	128.6	9	28	Aldrich (99+)
m-cresol/	108.1	11	26	Aldrich (99)
Catechol/	110.1	104	450	Aldrich (99)
Phenol/	94.1	41	93	J.R. Baker (99+)
Resorcinol/	110.1	110	1230	Aldrich (99)
Benzyl alcohol/ (BzOH)	108.1	−15	40	Fisher Scientific (99+)
Cyclohexanol (CxOH)/	100.1	24	37	Aldrich (99)
2,6-dimethyl cyclohexanol (2,6-DMCxOH)/	128.2	—	2	Aldrich (90)
3-pentanol/ $CH_3CH_2CH(OH)CH_2CH_3$	88.2	—	5.5	Aldrich (98+)
tert-Butyl alcohol (t-BuOH)/ $CH_3C(OH)(CH_3)CH_3$	74.1	25.7	Miscible	J.T. Baker (99+)

[a]Melting point obtained frdm the Merck Index (11th Edition).
[b]Water solubility measured at 25°C obtained from the Merck Index (11th Edition).

added into a liquid formulation to ensure its sterility during shelf life and multiple use (Akers, 1984; Gennaro, 1985). Although these preservatives inhibit bacterial growth, they have some adverse effects when added to protein formulations. For example, recombinant human growth hormone (rhGH) was found to aggregate in the presence of a phenolic compound during freeze-drying (Kirsch et al., 1993). Another important therapeutic protein, insulin, undergoes polymerization in the presence of phenolic compounds (Brange and Langkjar, 1992). Industrial examples also exist: Polyphenol is used to aid protein precipitation in mashing during beer brewing (Lewis and Serbia, 1984).

How do phenolic compounds destabilize proteins? It was generally believed to be due to the combination of various physical interactions such as Van der Waals forces, hydrophobic interactions, hydrogen bonding and electrostatic repulsion (Kirsch et al., 1993). McGraw and Lindenbaum (1990) proposed that phenolics physically bind to proteins, based on thermodynamic data obtained with insulin and phenol. Even chemical reactions were hypothesized for rhGH aggregation during freeze-drying due to a trace amount of the phenolic compound present in the sanitizing agent residues (Kirsch et al., 1993). Despite all these hypotheses, the exact mechanism causing protein destabilization is unclear. This report documents our study on the effects of benyl alcohol, phenol, resorcinol, cathechol, meta-cresol, 2-chlorophenol, and some cyclohexanols on the stability of rhGH under freezing, heating, and agitating stress conditions. We hope this study could add more information to aid in the understanding of this phenomenon.

2. Materials and methods

2.1. rhGH

rhGH with a molecular mass of 22.13 kDa was produced at Genentech from bacterial fermentation of a strain of *Escherichia coli*. The protein contained the same 191 amino acid residues as the natural, pituitary-derived hGH. For formulations containing various phenolics, aromatic and non-aromatic alcohols, the previously lyophilized excipient-free rhGH was dissolved into 5 mM phosphate buffer solution (pH 7.4) to a specific concentration, and then the organic additive-containing buffer was added dropwise to a desired concentration.

2.2. Organic reagents

Reagents used in the study include phenolic compounds, aromatic and non-aromatic alcohols. The formulae, molecular weight, purities, solubilities in water at 25°C and the sources of these compounds are listed in Table 1. All were used as obtained without further purification.

2.3. Incubation experiments

For low-temperature incubation, 1 ml of the protein solution was filled in a 3 cm^3 vial and placed in a −45°C freezer. After incubation, frozen samples were thawed at room temperature. Prior to size exclusion (SEC) HPLC and quasi-elastic light scattering (QELS) analyses, the thawed samples were transferred to 0.5 ml polypropylene micro test tubes (Eppendorf) and centrifuged at 13 000 rev./min for 1 min using a Micro Centaur Centrifuge (APO 5760, Fisons). For high-temperature incubation, 2 ml of protein solution was loaded into a 12 × 75-mm borosilicate glass culture tube and then incubated in a 50°C water bath (Lauda, RC 6).

2.4. Agitation

Agitation was performed in a rotor/stator homogenizer (Virtis, Templest IQ) consisting of a digital display microprocessor control, an overhead drive, and a homogenizing shaft (1 cm). The shaft tip is a rotor/stator assembly capable of generating fine dispersions (Maa and Hsu, 1996). For agitation without the air-liquid interface, the system was filled with the solution without headspace at approximately 55 ml. In all cases, the protein solutions were homogenized at 24 000 rev./min at room temperature.

2.5. Quasi-elastic light scattering (QELS)

Particle sizes of protein species were determined by a Brookhaven QELS system. The system consisted of a Lexel 95 argon-ion laser, a goniometer (BI-DS) and a 196-channel digital correlator and signal processor. Measurements were made at 514.5 nm and at a 90° scattering angle. This angle was particularly useful for observing Rayleigh scatterers (i.e. particles much smaller than the wavelength λ). Customarily, particles smaller than 1 μm could be fairly accurately measured. All light scattering measurements were accumulated during 1-min intervals to reduce random system noise and to ensure a stable baseline. The experimentally determined autocorrelation function $g(\tau)$ was used to obtain the size-distribution function $G(\Gamma)$ using inverse Laplace transform algorithms, non-negatively constrained least squares and CONTIN software. In general, the system is so sensitive to foreign particles that a solution cleaning procedure has to be thoroughly executed. Approximately 2 ml of rhGH solution (1 mg/ml) was filtered with a 0.22 μm sterile filter unit (Millipore, Millex-GV) into a 12 × 75 mm borosilicate glass culture tube which had been carefully cleaned with deionized water.

2.6. Turbidity measurements

Solution turbidity was determined spectrophotometrically for light scattering at 340–360 nm using Uvikon 860 Diode Array spectrophotometer (Kontron Instruments). The instrument was connected to a computer loaded with software which performed linear regression on light scattering data collected between 340–360 nm. The protein buffer was scanned first and its signal was subtracted from the subsequent measurement for protein solution. The degree of solution turbidity was classified into clear, slightly opalescent, opalescent, slightly cloudy, cloudy, and very cloudy based on the optical density values of European Pharmacopeia Standards (Eckhardt et al., 1991).

2.7. SEC-HPLC

rhGH samples were diluted to 1 mg/ml using a placebo buffer solution and 50 μl were injected into a silica-based Tosoh TSK 2000SW XL column (7.8mm I.D. × 30cm; particle size, 5 μm). The mobile phase consisted of 50 mM sodium phosphate and 0.15 N NaCl at pH 7.2 with a flow rate of 1 ml/min. The run time was 15 min. Elution of protein from the column was detected by UV spectrophotometry at 214 nm. The run time was increased to 30 min for phenolics-containing rhGH samples for the separation of the phenolic compound peak. The elution time for 2-chlorophenol was 27 min, *m*-cresol 22 min, catechol 16 min, phenol 17.5 min, resorcinol 15.5 min, benzyl alcohol 15 min, respectively. Since all the phenolic compounds used had a strong absorption at 214 nm, any association of a phenolic compound with protein monomer or aggregates might grossly overestimate the degree of aggregation determined by SEC-HPLC. For each chromatogram in this study, the total peak area for rhGH monomer and aggregates was compared with that for pure rhGH at the same concentration. An agreement within + 5% (including the error by sample preparation) was always obtained for each phenolic compound, suggesting that no significant association or binding of the phenolic compound to the protein during SEC-HPLC analysis. Therefore, it can reliably support the amounts of protein aggregates reported in this study.

2.8. UV spectrophotometry

The amount of insoluble rhGH aggregates was determined by measuring the difference in protein concentration (Kontron Uvikon 860) of the reconstituted sample before and after 0.22 μm filtration (Millipore, GSVP) at a wavelength of 277 nm. Insoluble aggregates less than 0.22 μm in size were not necessarily removed by filtration.

2.9. Scanning microcalorimetry (SM)

Calorimetric measurements were carried out on a MicroCal MC-2 ultrasensitive DSC (MicroCal Inc., Nothampton, MA) using the standard DA-2 software package for data acquisition, analysis and deconvolution. The heart of the system is two side-by-side cells, sample and reference, adiabatically shielded in a reservoir. The temperature of the reservoir was controlled by circulating 50:50 water/ethylene glycol in an external bath. Using power feedback compensation, the enthalpy change was studied in the sample cell, with the reference cell providing a differential thermal standard. All measurements in this study were performed using a scanning rate of 60°C/h. The working volume of the cells was 1.22 ml. Sample and reference solutions were loaded into the cells through a long needle.

2.10. Titration microcalorimetry

The Omega rapid titration calorimeter (MicroCal Inc.) was used to determine solute–protein binding. This instrument measures the heat absorbed (or evolved) as a function of the amount of the titrant added for a binding reaction. Although using the same heat detection principle as the MicroCal MC-2, the MicroCal Omega has a sealed reference cell and a working volume of 1.3 ml. Initially, the sample cell was filled with protein solution and ligand solution was loaded in a syringe equipped with a long needle. The needle has a helical-shaped impeller tip. After inserting the needle into the sample cell, the top of the syringe was mounted to a motor which rotated the impeller at 400 rev./min during analysis. Also, the syringe barrel was locked to a computer-aided injector. After the baseline reached equilibrium, the measurement was started with a 60-s delay before first injection. Ten injections were made for each run. The volume of each injection was 5 μl and each injection lasted 10 s. The time interval between injections was 4 min. Data were analyzed using the computer software provided with the instrument.

2.11. Optical circular dichroism (CD)

CD was performed using an Aviv CD spectrometer (Model 62DS) to determine the conformational changes of protein structure. CD spectra were obtained in quadruplicate in the far-UV (190–250 nm) and near-UV (250–360 nm) region with a bandwidth of 0.2 nm and a sampling stepsize of 0.2 nm.

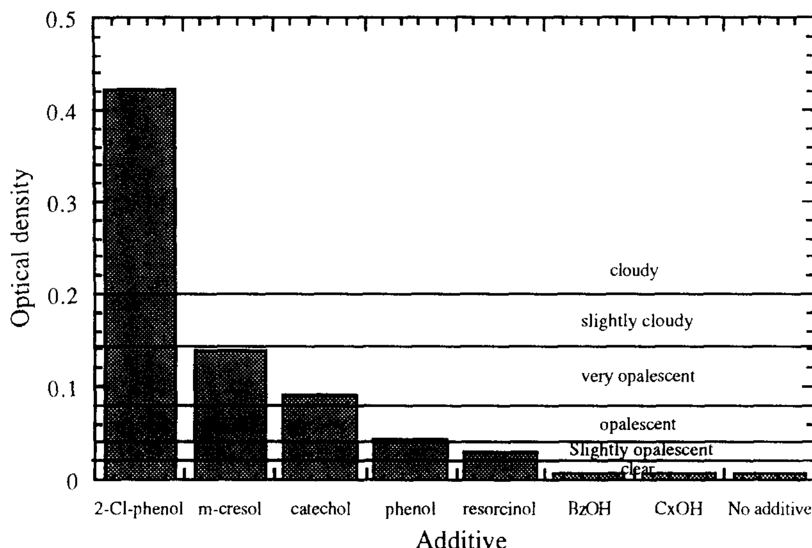

Fig. 1. UV analysis on the turbidity of rhGH solution (10 mg/ml) in the presence of a phenolic compound (10 mg/ml).

2.12. SDS-PAGE

To determine whether the protein aggregates were SDS-dissociable, silver-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Hoefer Scientific Instrument) was performed on a ready gel from Bio-Rad (4–15% gradient, 0.375 M Tris–HCl, pH 8.8, 10 well comb) at 200 mV for 40 min at the ambient temperature under both DL-dithiothreitol (DTT)-reduced and non-reduced conditions. Each protein sample was loaded at 1 μg per lane.

3. Results and discussion

3.1. Insoluble protein aggregation determined by UV light scattering

Fig. 1 summarizes the optical density and the turbidity of rhGH solutions (10 mg/ml) containing an organic compound at 10 mg/ml. Each compound was prepared to concentrations below its solubility limit to avoid turbidity caused by light scattering of immiscible liquid droplets or insoluble solid particles. According to the classification by European Pharmacopia standards, each rhGH solution containing a phenolic compound was either opalescent or cloudy, attributed to particulates formation. These particulate were insoluble protein aggregates because the addition of a phenolic compound to a protein-free buffer resulted in no turbidity. In contrast, the additive-free rhGH solution and solutions containing benzyl alcohol and cyclohexanol of the same concentration were clear. The influence of these compounds on the turbidity of rhGH solution follows this order: cyclohexanol < benzyl alcohol < phenol ≈ resorcinol < catechol < *m*-cresol < 2-chlorophenol. This order does not correlate with the order of compound solubility in water. For instance, the solubility of benzyl alcohol and cyclohexanol is comparable to that of *m*-cresol and 2-chlorophenol, but the former caused no turbidity whereas the latter turned rhGH solution very opalescent and cloudy. Also, within the water solubility of these organic additives, protein solution turbidity increased with the concentration of the phenolic compounds (data not shown).

3.2. Soluble protein aggregation determined by QELS

QELS's high level of sensitivity offers a powerful tool for determining the size of protein and its aggregates. The instrument provides rapid analy-

Table 2
The size of rhGH aggregates for protein solution (10 mg/ml) containing a phenolic compound or a non-aromatic alcohol determined by QELS

Compound	Concentration (mg/ml)	Size (nm)
None	0	5
2-Chlorophenol	10	32
m-Cresol	10	18
Catechol	10	15
Resorcinol	10	10
Phenol	10	7
Benzyl alcohol	10	5
Cyclohexanol	25	5
3-pentanol	5	5
2,6-Dimethylcyclo-hexanol	1	130
tert-Butyl alcohol	40	5

sis (in the time scale of minutes) and involves no invasive sample preparation. Prior to QELS measurements, each rhGH sample was filtered with a 0.22 μm filter to remove dust and aggregates larger than 0.22 μm. As shown in Table 2, the size of rhGH monomer was determined to be approximately 5 nm but the size increased in the presence of a phenolic compound. Among these additives, 2-chlorophenol resulted in the largest rhGH ag-gregates, followed by *m*-cresol, catechol, resorcinol, and phenol. This order is consistent with the order of solution turbidity reported earlier. The QELS analysis data for some alcohols are also listed in Table 2. Of these alcohols, only 2,6 dimethylcyclohexanol caused rhGH aggregation. At 1 mg/ml, 2,6-dimethylcyclohexanol increased the size of rhGH to approximately 130 nm. These soluble aggregates were quantified by SEC-HPLC.

3.3. Protein aggregation determined by SEC-HPLC

The content of soluble rhGH aggregate in solutions containing a phenolic compound or an alcohol was determined by SEC-HPLC and is summarized in Table 3. At 10 mg/ml, resorcinol, benzyl alcohol and cyclohexanol did not cause significant protein aggregation and phenol caused slight aggregation. In contrast, 2-chlorophenol and *m*-cresol caused higher levels of rhGH aggregation, 40% and 43% respectively. Detailed analysis was conducted on *m*-cresol by varying the concentration of *m*-cresol and rhGH. Aggregation increased with increasing concentration of *m*-cresol. No increase in aggregation was found for the *m*-cresol concentration lower than 2.5 mg/ml, but almost 90% of the rhGH monomer aggregated at 15 mg/ml of *m*-cresol. At

Table 3
SEC-HPLC analysis for the monomer content and % soluble aggregate of rhGH in the presence of a phenolic compound or a non-aromatic alcohol

rhGH conc. (mg/ml)	Compound	Conc. (mg/ml)	% soluble aggregate
5	None	None	1.5
5	2-Chlorophenol	10	40.0
5	*m*-Cresol	10	43.0
5	Catechol	10	17.6
5	Phenol	10	7.2
5	Resorcinol	10	2.5
5	Benzyl alcohol	10	2.8
5	Cyclohexanol	10	2.2
5	*m*-Cresol	15	88.2
5	*m*-Cresol	7.5	35.6
5	*m*-Cresol	5	9.3
5	*m*-Cresol	2.5	1.5
5	*m*-Cresol	1.5	1.5
2.5	*m*-Cresol	10	43.4
2.5	*m*-Cresol	5	8.4
1	*m*-Cresol	10	35.6
1	*m*-Cresol	5	6.6

Table 4
SEC-HPLC analysis for % insoluble and soluble aggregate of rhGH solution (5 mg/ml) containing an organic compound (10 mg/ml) after freeze incubation at −45°C for 1 h and thawing for 1 h and 24 h at room temperature

Compound	Thawed for 1 h % Insol aggreg	% Sol aggreg	Thawed for 24 h % Insol aggreg	% Sol aggreg
rhGH	0	1.5	0	1.5
2-Chlorophenol	99.0	0.6	36.0	42.3
m-cresol	97.0	1.8	19.0	49.8
Catechol	97.0	1.7	23.0	38.9
Phenol	93.6	3.7	10.0	54.7
Resorcinol	93.0	3.5	3.8	38.0
Benzyl alcohol	84.0	7.9	4.8	50.6
Cyclohexanol	77.4	9.0	1.0	36.9

a constant *m*-cresol concentration, the effect of protein concentration on aggregation is not significant. It appears that aggregation was mainly affected by the absolute concentration of the phenolic compounds not by the concentration ratio between the phenolic compound and the protein.

3.4. Effect of freezing

Table 4 summarizes the UV and SEC-HPLC results for rhGH solutions (5 mg/ml) containing a phenolic compound (10 mg/ml) frozen at −45°C for 1 h first and thawed at room temperature. Each sample was centrifuged at 13 000 rev./min for 1 min to remove insoluble aggregates before analysis. Freezing had no effect on rhGH in the absence of phenolic compound. For rhGH containing an organic compound, the solution turned very cloudy initially after the freeze/thaw cycle. Even cyclohexanol and benzyl alcohol which had little effect on rhGH aggregation in the liquid state (Table 3) turned the rhGH solution turbid. Interestingly, all thawed solutions became less turbid after 1 day at the ambient temperature, but soluble aggregation increased significantly. Similar results were obtained using different freezing temperatures and incubation periods if sufficient thawing time was given. Compared to rhGH aggregation in the liquid state (Table 3), it suggests that the stress by freezing exacerbates rhGH aggregation and that protein aggregation occurred immediately upon freezing. We interpret that it is due to the freeze concentrate effect. During freezing the solution separated into two phases, ice and the unfrozen liquid. The concentration of the protein and all additives increases dramatically in the unfrozen liquid. In general, less than 10% of water remains unfrozen at −45°C (Takahashi et al., 1993), i.e. at least tenfold increase in the concentration of the excipients, thereby causing serious protein aggregation. Cyclohexanol and benzyl alcohol, which had little effect on rhGH in the liquid, caused rhGH aggregation after freezing.

3.5. Stability at high temperatures

The effect of the organic compounds on rhGH was further investigated using high temperature incubation (50°C) and scanning microcalorimetry (SM). The rhGH monomer content of the incubated solutions as a function of incubation time is plotted in Fig. 2. After incubation, protein solutions containing benzyl alcohol, phenol, resorcinol, and cyclohexanol remained clear and were only slightly aggregated after 5-day incubation. Solutions containing 2-chlorophenol, *m*-cresol or catechol were opalescent and the rhGH monomer content significantly decreased with time. In 2-chlorophenol and *m*-cresol, almost all rhGH aggregated in 1 day. The order of the influence of these organic compounds on rhGH aggregation upon high temperature incubation is consistent with earlier observations.

Scanning microcalorimetry (SM) is frequently used to determine protein's thermodynamic properties such as denaturation temperature and phase transition enthalpy. Since a protein molecule may be composed of several folded domains, its scanning calorimetry data are influenced by domain-domain

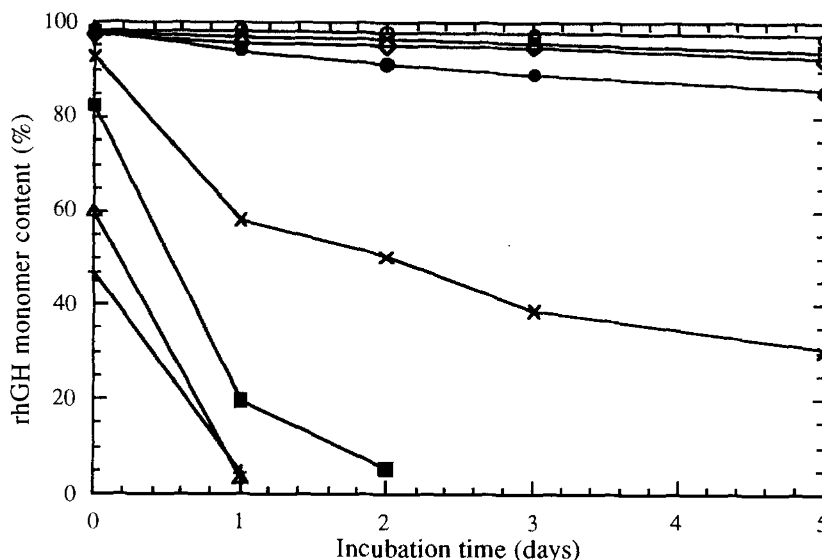

Fig. 2. SEC-HPLC analysis of rhGH aggregation upon 50°C incubation as a function of incubation time. The rhGH sample (5 mg/ml) (○) was spiked with 10 mg/ml of cyclohexanol (□), benzyl alcohol (◇), phenol (X), *m*-cresol (+), 2-chlorophenol (△), resorcinol (●), or catechol (■).

interactions. The understanding of these interacting domains relies on theoretical models, which makes data interpretation subjective. To avoid this complexity, we focused only on the protein's melting temperature and the shape of the transition peak. If protein molecules unfold or experience conformational changes from their native states, we should be able to observe the downshifted denaturation temperature or the changed shape of the transition peak. For example, Steadman et al. (1992) have used DSC to study the effect of silica surface adsorption on the thermal stability of proteins and found the endothermic peak of the adsorbed species is 5–9°C lower than that of the protein before adsorption, due to the protein's structural changes. Therefore, SM was also used as a measure for determining the influence of the organic compounds on rhGH stability.

All rhGH samples measured by DSC were thermally irreversible. Fig. 3 shows the thermograms for rhGH solutions (15.5 mg/ml) containing various phenolic compounds and benzyl alcohol. For pure rhGH, it shows one phase transition peak with the peak maximum temperature (T_m) determined to be 73 $\pm$ 2°C. T_m changed to 68.4°C by resorcinol and to 66.4°C by benzyl alcohol but the shape and the width of the peak remained unchanged. In the presence of *m*-cresol and phenol, the peak was widened with a shoulder and the size of the peak was significantly reduced. The main peak occurred at 57.1°C (the shoulder at 61°C) for *m*-cresol and 56.1°C (the shoulder at 62°C) for phenol. 2-Chlorophenol imposed the most dramatic effect on rhGH by completely wiping out the peak. Fig. 4 shows the thermograms of rhGH containing cyclohexanol, tert-butyl alcohol, and 2,6-dimethylcyclohexanol. T_m decreased to 63.9°C by cyclohexanol and 59.1°C by 2,6-dimethylcyclohexanol. The protein was inert to tert-butyl alcohol even at the concentration as high as 40 mg/ml. The transition peak for rhGH in the presence of 2,6-dimethylcyclohexanol was widened significantly and its shape was similar to those for *m*-cresol and phenol shown in Fig. 3.

Table 5 summarizes the T_m and the enthalpy (ΔH calculated based on the area under the peak) of rhGH at 15.5 mg/ml in the presence of various phenolic compounds and non-aromatic alcohols. Although the enthalpy change is governed by many factors, it can be used as an indicator for protein stability. If protein molecules are stabilized by the additive, the denaturation tempera-

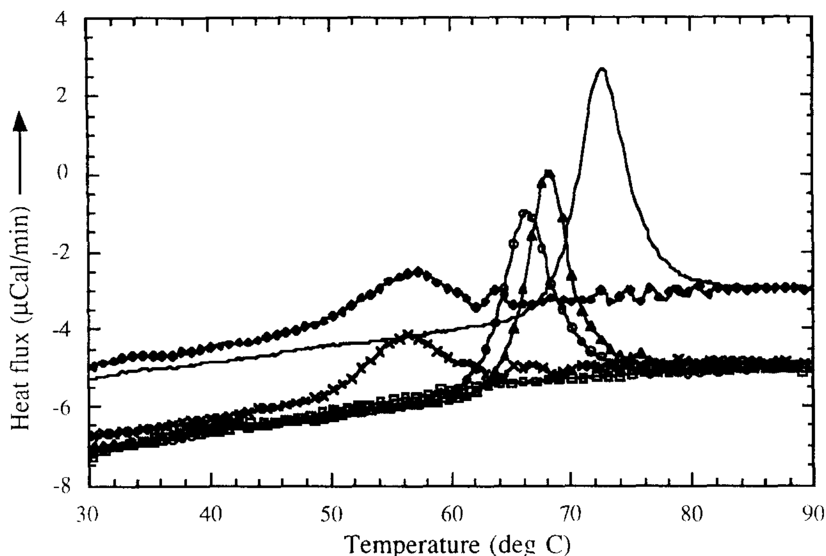

Fig. 3. Scanning microcalorimetry analysis of rhGH in the presence of a phenolic compound. rhGH (15.5 mg/ml) (——) solutions containing 10 mg/ml of benzyl alcohol (○), 5 mg/ml of 2-chlorophenol (□), 5 mg/ml of m-cresol (◇), 10 mg/ml of phenol (X), and 10 mg/ml of resorcinol (△) were scanned at a rate of 60°C/min.

ture and possibly enthalpy as well should increase (Back et al., 1979; Gekko, 1982). Based on these results, the destabilizing effect of these compounds to rhGH is in the order of 2-chlorophenol > *m*-cresol > phenol ~ 2,6-dimethylcyclohexanol > resorcinol ~ benzyl alcohol ~ cyclohexanol > tert-butyl alcohol. These data are consistent with the order of the influence of these organic compounds as observed earlier.

3.6. Aggregation upon agitation

Fig. 5 shows the time course of protein monomer content decrease during homogenization at 24000 rev./min, equivalent to the shear rate of 1.55×10^5 s^{-1} (Maa and Hsu, 1996), for rhGH solutions containing an organic compound. *m*-Cresol and 2-chlorophenol still influenced the protein most, aggregating rhGH up to 90% and 80%, respectively, after 60 min shearing. Catechol and phenol had a medium influence on aggregation. Cyclohexanol, benzyl alcohol and resorcinol only slightly aggregated compared to the additive-free rhGH solution which remained intact after high shearing. The order of the deleterious effect of these organic compounds on protein aggregation was again confirmed.

3.7. Nature of the aggregates

Although the cause of any aggregation reported here is not yet clear, one possibility is that protein might be denatured initially by the phenolic compound and then aggregates. Chemical reactions forming covalent bonds between protein molecules seem to be unlikely to occur based on reduced and non-reduced silver-stained SDS-PAGE for the rhGH (10 mg/ml) samples containing 10 mg/ml of 2-chlorophenol or *m*-cresol (Fig. 6). No additional high molecular weight species were found compared to additive-free rhGH. Thus, these SDS-dissociable aggregates were non-covalent in nature.

3.8. Physical interactions

Based on the chemical nature of the organic compound, several physical interactions with the proteins are possible. For example, phenol might

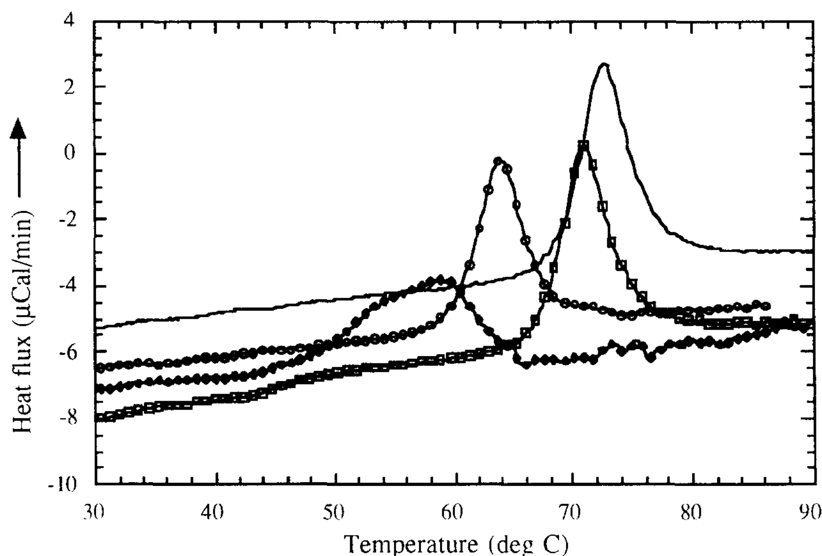

Fig. 4. Scanning microcalorimetry analysis of rhGH in the presence of a non-aromatic compound. rhGH (15.5 mg/ml) (——) solutions containing 10 mg/ml of cyclohexanol (○), 40 mg/ml of *tert*-butyl alcohol (□), and 2 mg/ml of 2,6-dimethyl cyclohexanol (◇) were scanned at a rate of 60°C/min.

interact with protein due to hydrogen bonding by the –OH group and due to hydrophobic interactions by the aromatic ring. It appears that the extra functional group on the aromatic ring (i.e. $-CH_3$ on the meta position for *m*-cresol, –Cl on the *ortho* position for 2-chlorophenol, and –OH on the *ortho* position for catechol) exerts additional influence on protein as opposed to phenol which contains only one –OH group. Interestingly, the extra –OH group on the meta position for resorcinol does not carry additional influence on the protein, and benzyl alcohol which has both the hydroxyl and benzene groups causes little aggregation. Without the aromatic ring, cyclohexanol causes no aggregation even at the concentration of 25 mg/ml but the highly hydrophobic 2,6-dimethylcyclohexanol induces serious aggregation at only 1 mg/ml. The non-cyclic alcohol, tert-butyl alcohol, is miscible with water and causes no aggregation at the concentration as high as 40 mg/ml. It appears there is no clear trend between rhGH aggregation and either hydrogen bonding or hydrophobic interaction based on the chemical structure of the organic additive. The possibility of a specific physical binding between the protein and the organic compound can be investigated using titration microcalorimetry (TM).

TM determines if these organic compounds serve as ligands to physically bind to the protein. With a single titration experiment, TM measures the binding isotherm and allows the binding constant, *K*, the free energy, $\Delta G°$, and the enthalpy change, $\Delta H°$, of the system to be calculated. For a strong protein-ligand binding system, each injection of the ligand to the protein solution generates or absorbs heat due to the binding interaction. Initially, all ligand molecules can bind to the protein because there are plenty of binding sites available. As the binding sites are being occupied, the binding interactions becomes less pronounced. Heat involved in binding thus significantly reduces.

In this study, the rhGH sample (10 mg/ml or 0.45 mM) was loaded in the sample cell. Each ligand was prepared at 10 mg/ml and injected 10 times at 5 μl by a syringe so that the final molar ratio between the ligand and the protein was at least 6:1. Regardless of the type of the ligand, all ten peaks were approximately equivalent in size,

Table 5
The transition temperature and enthalpy of rhGH (15.5 mg/ml) in the presence of a phenolic compound or a non-aromatic alcohol determined by scanning microcalorimetry

Compound	Concentration (mg/ml)	Transition temperature(°C)	Enthalpy (kcal/mol)
rhGH	–	74 ± 3.0	54.0 ± 3.5
2-Chlorophenol	10	–	–
m-Cresol	5	57.1	13.9
Phenol	10	56.2	19.1
Resorcinol	10	68.4	43.7
Benzyl alcohol	10	66.4	42.2
Cyclohexanol	10	63.9	41.8
tert-Butyl alcohol	40	72.5	52.5
2,6-Dimethyl cyclohexanol	10	59.1	45.2

suggesting that only very weak binding might be involved. The magnitude of heat involved in each injection varied with the type of the ligand. Results for the average peak enthalpy change of different ligands are summarized in Table 6. The measured heat of mixing in the microcalorimeter is actually the sum of three heats: the heat of the reaction between ligand and rhGH, the heat of dilution of ligand, and the heat of dilution of rhGH. The heat of dilution of rhGH is negligibly small. The heat of dilution of ligand was obtained in separate experiments and subtracted from the total heat. Therefore, the enthalpy change listed in Table 6 is entirely contributed by the heat of reaction. Apparently, 2-chlorophenol and *m*-cresol have stronger interactions with rhGH than other additives. Surprisingly, phenol had the weakest influence on rhGH during dilution, even weaker than cyclohexanol. This is inconsistent with our pervious data and suggests that direct binding with the protein does not influence rhGH aggregation.

3.9. Effect of pH

The effect of pH on rhGH aggregation before and after adding *m*-cresol in the liquid and in the frozen state (frozen at $-45°C$ for 1 h) is summarized in Fig. 7. The pH of the additive-free rhGH (pH 7.5) was adjusted with 0.1 N NaOH or 0.1 N HCl. All the samples were filtered with 0.45 μm filters prior to SEC-HPLC analysis to remove the insoluble aggregates. The total monomer content was calculated based on the monomer peak area ratio between the sample and the bulk. The monomer content of the additive-free rhGH reached the minimum at a pH between 5 and 6. This pH range covers the isoelectric point of rhGH. Although protein concentration decreased significantly at pHs 5 and 6, there was no increase in the amount of soluble aggregates. This suggests the loss of rhGH monomer at this pH condition was due to solubility decrease around the isoelectric point (pH 5.2) but not due to aggregation. Samples containing *m*-cresol lost all the protein monomer between pH 4 and 6. The pH range in which all rhGH precipitated out was even wider (pH 3–8) for the *m*-cresol-containing samples that had been frozen and thawed. At high pH (≥ 9), *m*-cresol-containing rhGH solution after freezing and thawing was clear and the monomer content remained the same as the unfrozen sample. This indicates that freezing had a less adverse effect on rhGH when the protein was formulated with the phenolic additive at higher pHs. At pH 3, the *m*-cresol-containing solution was much more stable than the pH 7.5 solution (90% vs. 47% in monomer content), but freezing of this pH 3 solution still induced a significant amount of insoluble aggregates (about 90% of the total monomer). At pH 2, no soluble and insoluble aggregates were found even after freezing, suggesting that *m*-cresol-containing rhGH was stable at low pH (< 3).

The results from pH experiments suggest that electrostatic repulsion and hydrophobic interac-

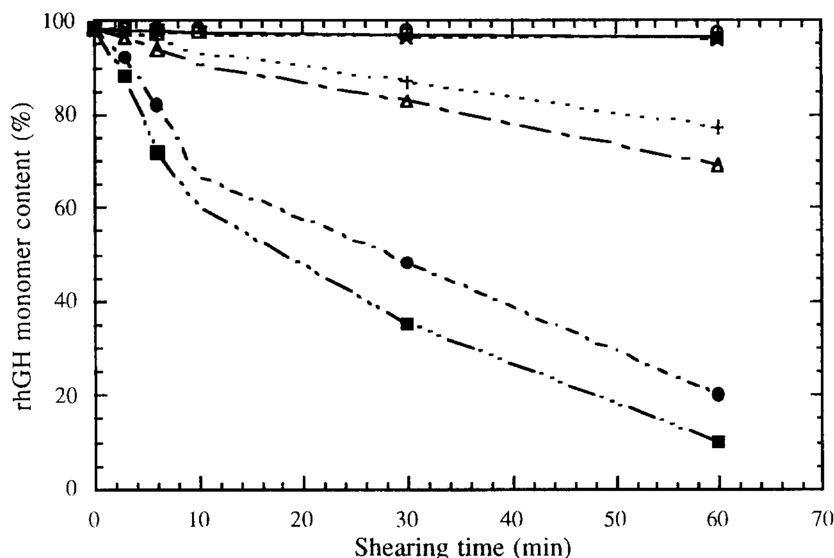

Fig. 5. SEC-HPLC analysis of rhGH aggregation upon homogenization as a function of shearing time. rhGH (5 mg/ml) (○) solutions was spiked with 5 mg/ml of cyclohexanol (□), benzyl alcohol (◇), resorcinol (X), phenol (+), catechol (△), *m*-cresol (●), and 2-chlorophenol (■).

tions might play an important role. Due to the hydrophobicity of these organic compounds, increasing hydrophobic interactions between the protein and the compound would expose the hydrophobic portion of the protein structure and cause conformational changes. Circular dichroism was used to investigate protein's conformational changes. Unfortunately, all compounds containing the aromatic ring showed strong optical absorption in the wavelength range of interest, thus interfering with the analysis. Therefore, only the rhGH solutions containing non-aromatic compounds were studied. Fig. 8 shows the near-UV CD spectra for additive-free rhGH and rhGH containing cyclohexanol. These two samples have the same amount of aggregates. The two spectra overlapped over most of the range except between 268 and 285 nm, suggesting that the tertiary struc-

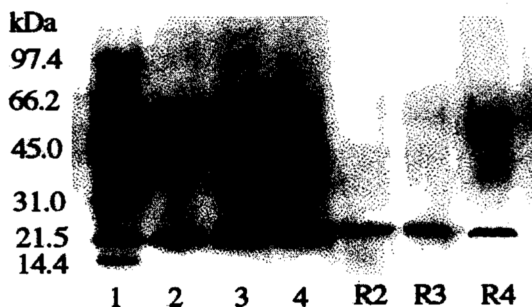

Fig. 6. Silver-stained SDS-PAGE for additive-free rhGH (10 mg/ml) (lane 2) and rhGH containing 10 mg/ml of 2-chlorophenol (lane 3) and *m*-cresol (lane 4). The MW marker is located in lane 1. Lanes 1–4 are under non-reduced conditions and lanes R2–R4 are the same as lanes 2–4 except they are under DTT-reduced conditions.

Table 6
The enthalpy change (ΔH) of rhGH (10 mg/ml) in the presence of an organic ligand as determined by titration microcalorimetry

Ligand	Ligand/rhGH molar ratio	Enthalpy (cal/mol)
2-Chlorophenol	6.9	−2090.5
m-Cresol	7.9	−810.4
Phenol	9.4	−152.5
Resorcinol	8.2	−382.7
Benzyl alcohol	7.9	−638.3
Cyclohexanol	8.9	−368.8

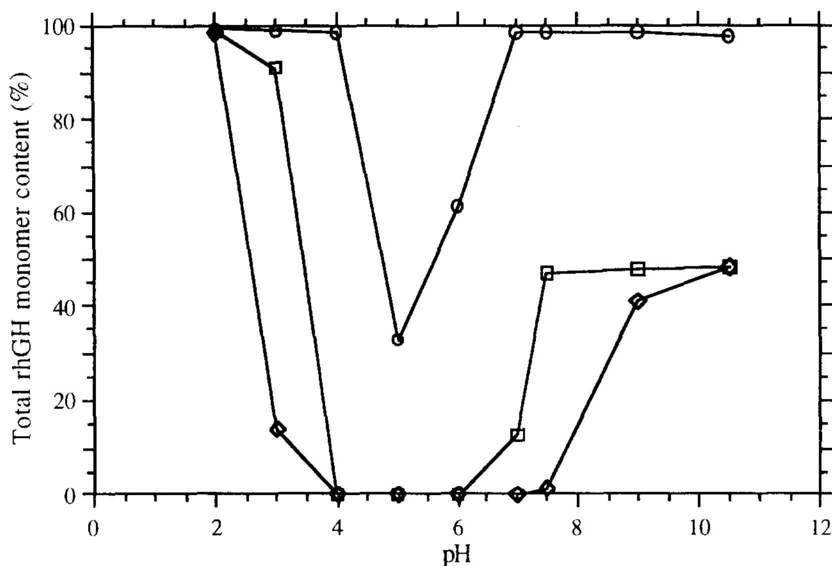

Fig. 7. SEC-HPLC analysis of rhGH aggregation as a function of pH. The rhGH sample (5 mg/ml) (○) in the presence of 10 mg/ml of *m*-cresol before (□) and after (◇) freezing at −45°C.

ture of rhGH was disturbed by cyclohexanol. No differences were observed in the far UV range (data not shown). Therefore, phenolic compounds are assumed to have a more significant effect on the rhGH structure because of their stronger effect on proteins. Whether these conformational changes precede rhGH aggregation remains to be determined.

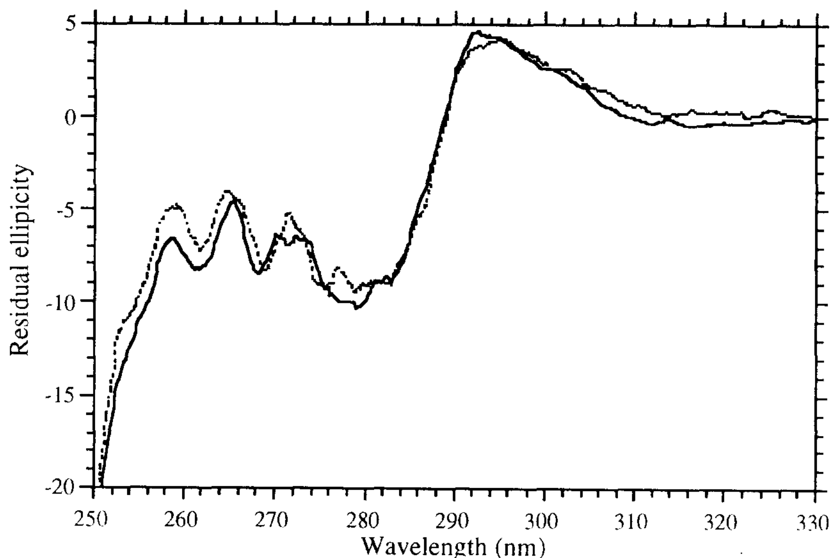

Fig. 8. Near-UV CD for rhGH (——) and for rhGH containing cyclohexanol (– – –). rhGH solution was 1 mg/ml. rhGH and cyclohexanol were mixed at 5 mg/ml and diluted to 1 mg/ml.

4. Conclusion

Phenolic compounds caused different degrees of rhGH aggregation, depending on their concentration and chemical nature. Based on various stress studies, the deleterious effect rendered by these compounds to the protein follows this order: 2-chlorophenol > *m*-cresol > catechol > phenol ≈ resorcinol > benzyl alcohol. Various analyses suggest that, between the protein and the phenolic additives, aggregation is more driven by hydrophobic interactions than other physical forces such as, specific binding and hydrogen bonding. Based on the results derived in this study, the selection of a phenolic antimicrobial preservative for a liquid protein formulation should be concerned with its effect on protein's short-term and long-term stability. Also, since the protein suffers more serious aggregation in the frozen state than in the liquid state, freezing should be avoided during storage of a preservative-containing protein formulation.

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