Effects of Additives on Reversibility of Thermal Unfolding

Yoshiko Kita, John S. Philo and Tsutomu Arakawa
Alliance Protein Laboratories, Thousand Oaks, CA; 805-388-1074 fax 805-388-7252,
http://www.ap-lab.com

Abstract

Irreversible reactions following unfolding cause loss of proteins during purification, handling, and storage. Proteins unfold at high temperatures, but when that unfolding is reversible they can refold into the native structure. When unfolding is not reversible, increasing the reversibility can minimize protein loss and may enhance long-term stability.[1] We have studied the effects of various additives on the reversibility of thermal unfolding using native gels and sedimentation velocity to characterize the products of unfolding.

Two basic proteins were examined in this study, ribonuclease and a monoclonal antibody, IGG1. When ribonuclease was heated to 75 ºC and then cooled to room temperature it generated new low-mobility bands on native gels, corresponding to aggregates. The proportions of monomer and aggregates were unchanged upon addition of various detergents, indicating that detergents do not inhibit heat-induced aggregation. Sucrose also had no effect, and glycine was only marginally effective. In contrast, ammonium sulfate and sodium chloride resulted a marked increase in monomer content.

Similar studies of IGG1 again showed substantial loss of native monomer and formation of aggregates after heating to 75 ºC, but a totally opposite pattern of stabilization by additives. In this case sucrose and glycine were effective protectants, while salts promote aggregation. Among a number of buffers tested histidine and glycine buffers showed significant stabilization.

These studies show that it is possible to increase the reversibility of thermal unfolding by altering solvent conditions, but we are unable to predict which additives will be protective. Since some unfolding always occurs spontaneously even at low temperatures, it is possible that the same additives can enhance storage stability by increasing the reversibility of the unfolding reaction.

Purpose

An accelerated stability study was carried out to screen additives for their ability to enhance the stability of proteins against irreversible aggregation due to thermal unfolding.

Methods

Proteins were subjected to high temperature and analyzed by native gel electrophoresis. Since the proteins used here (ribonuclease and IGG1) have basic isoelectric points, a His/Mes buffer system was used. CD thermal scans were also used to examine the effects of additives on melting temperature and reversibility of thermal unfolding of the proteins.

---

[1] Narhi, L.O., Philo, J.S., Sun, B., Chang, B.S., and Arakawa, T. (1999). Reversibility of heat-induced denaturation of the recombinant human megakaryocyte growth and development factor. *Pharm. Res.* **16**, 799-807.

Results and Discussion on Ribonuclease

CD thermal scans of ribonuclease in 10 mM phosphate, pH 6.4 show that the melting temperature of ribonuclease is relatively constant in the presence of the additives tested, except for ammonium sulfate (see Table 1). In general, these additives are known to exert their effects at higher concentrations. However, the reversibility of thermal unfolding is highly variable. Ammonium sulfate at 0.4 M gives the highest reversibility, which may be in part due to the high melting temperature observed with this salt. NaCl and glycine at 0.4 M increase the reversibility somewhat, while their effects on melting temperature are insignificant.

To further examine irreversible effects after denaturation, ribonuclease was heated at 75 °C and then analyzed by native gel electrophoresis. As can be seen in all 4 gels shown in Figures 1-4, the control sample showed formation of a new band, corresponding to soluble aggregates. (Note that there is no visible precipitation or turbidity in either these experiments or the CD experiments.)

NaCl and ammonium sulfate at 0.4 M conferred great protection, since there is no apparent formation of the new band (Fig. 1). NP40 is ineffective below 1% (Fig.2), but there is a significant change in aggregation at 2%. Urea at 0.4 M enhances aggregation, while 0.2 M sucrose is not protective (Fig.3). Glycine at 0.4 M decreased aggregation significantly (Figs. 3 and 4).

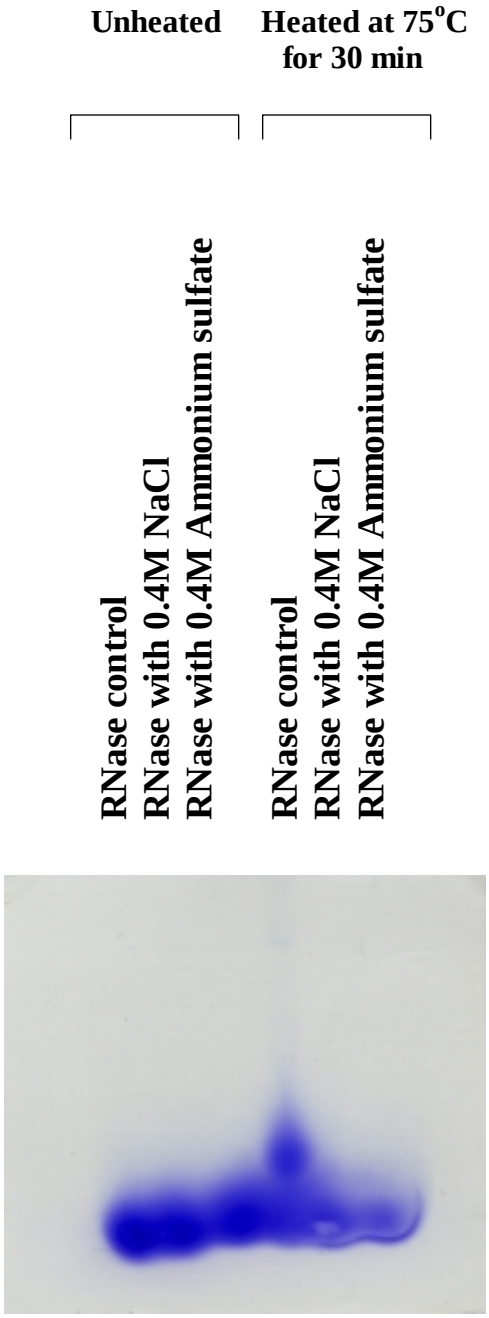

Figure 1. RNase in 10 mM phosphate buffer at pH6.4 plus various additives.
Ribonuclease was heated at 75 °C and analyzed by native gel electrophoresis using a *His/Mes* buffer system. The control sample showed formation of a new band, corresponding to soluble aggregates. (Note that there is no visible precipitation.) NaCl and ammonium sulfate at 0.4 M conferred great protection, since there is no apparent formation of the new band. Native gel at pH 6.1.

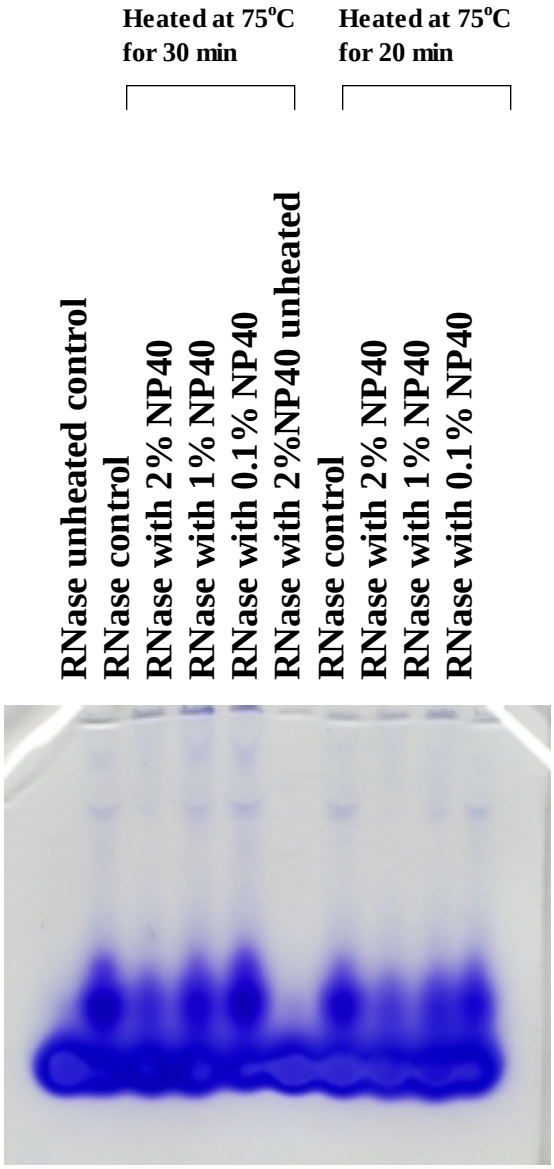

Figure 2. Effect of NP40 on RNAse heated to 75°C.
After heating ribonuclease at 75 °C for 20 or 30 min, native gel electrophoresis shows that NP40 is ineffective at reducing aggregation at concentrations below 1%. There is a significant reduction in aggregation at 2%.

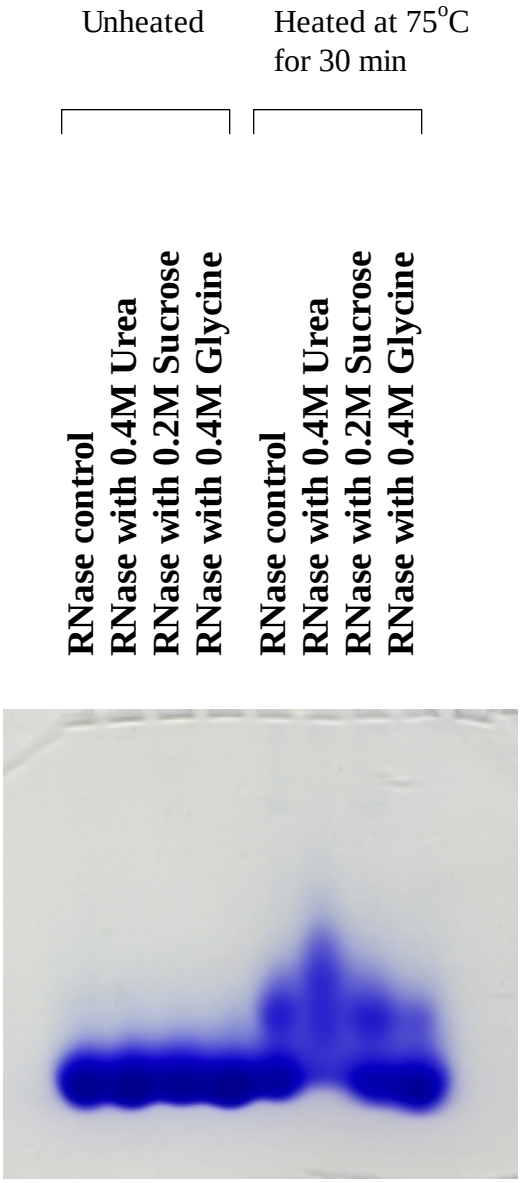

Figure 3. Effects of additives RNase in 10 mM phosphate buffer at pH 6.4.
Urea at 0.4 M enhances aggregation, while 0.2 M sucrose is not protective. Glycine at 0.4 M decreases aggregation significantly. Native gel at pH 6.1

Figure 4. Effects of various additives on thermal stability.
Additions were made to RNase in 10mM phosphate buffer at pH 6.4. Native gel at pH 6.1.

Results and Discussion on IGG

A monoclonal antibody (which we will simply call IGG1) formulated in 19 mM Na phosphate, 0.2 M NaCl, pH 6.5 was heated to 75 °C and then analyzed by native gel electrophoresis. Notably, this antibody has a high melting temperature (melting onset of ~75 °C as assessed by CD thermal scan and DSC) and various additives have little effect on its melting temperature. As shown in Fig. 5, already at 5 min there is substantial aggregation, which increases with time. After 60 min, most of IGG1 converted to aggregates.

The effects of Tween 80 at 0.001 to 1% were examined after heating for 40 min. There appear to be no changes in aggregation caused by the addition of this detergent (Fig. 6).

Tween 20 also showed little effects on aggregation. IGG1 was heated for 20 to 60 min in the absence and presence of 0.1% Tween 20. Aggregation is unchanged by this detergent (Fig. 7).

In the next group of experiments the 0.2 M NaCl was replaced with 0.2 M sucrose or glycine by dialysis. This replacement resulted in substantial stabilization of IGG1 as the monomer content is higher with these additives than with NaCl when IGG1 was heated for 20 to 60 min (Fig.8). It is not clear from this experiment whether removal of NaCl or addition of sucrose or glycine contributed to the observed stabilization. Other experiments demonstrated that the addition of NaCl increases aggregation of IGG1 upon heating (not shown).

Next the 19 mM phosphate buffer was replaced with other buffers: 19 mM histidine-HCl, pH 6.5; 19 mM citrate, pH 6.5; 19 mM Tris-HCl, pH 7.0; or 19 mM glycine, pH 6.0. These samples were heated at 75 °C for 60 min. Aggregation was significantly less in histidine, Tris and glycine (Fig. 9). Sedimentation velocity data confirmed these significant differences among buffers in the amount and size distribution of soluble aggregates (not shown).

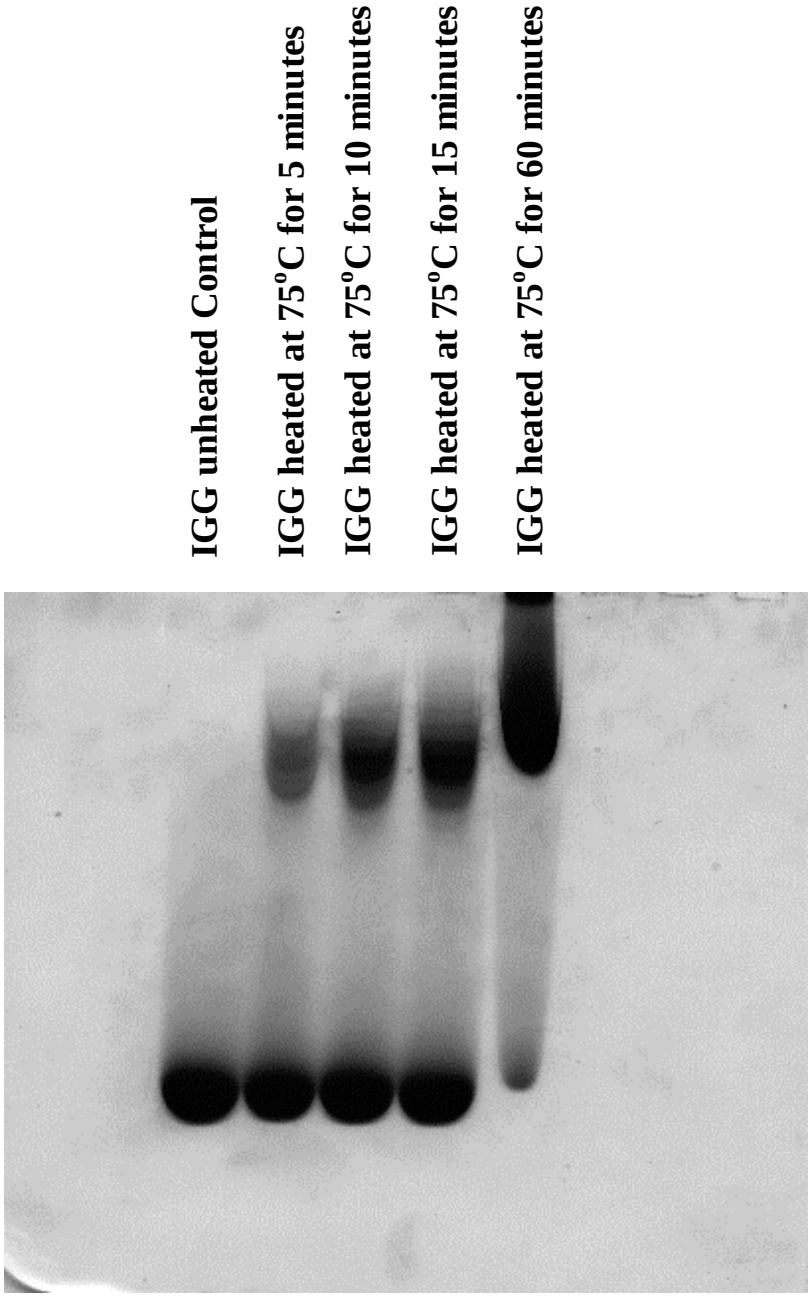

Figure 5. Effect of heating time on aggregation of IGG1.
IGG1 formulated in 19 mM Na phophate, 0.2 M NaCl, pH 6.5 was heated at 75 °C and analyzed by native gel electrophoresis using a *His/Mes* buffer system. Already at 5 min incubation at this temperature there is substantial aggregation, which increases with time. After 60 min, most of IGG1 converted to aggregates. Native gel at pH 6.1.

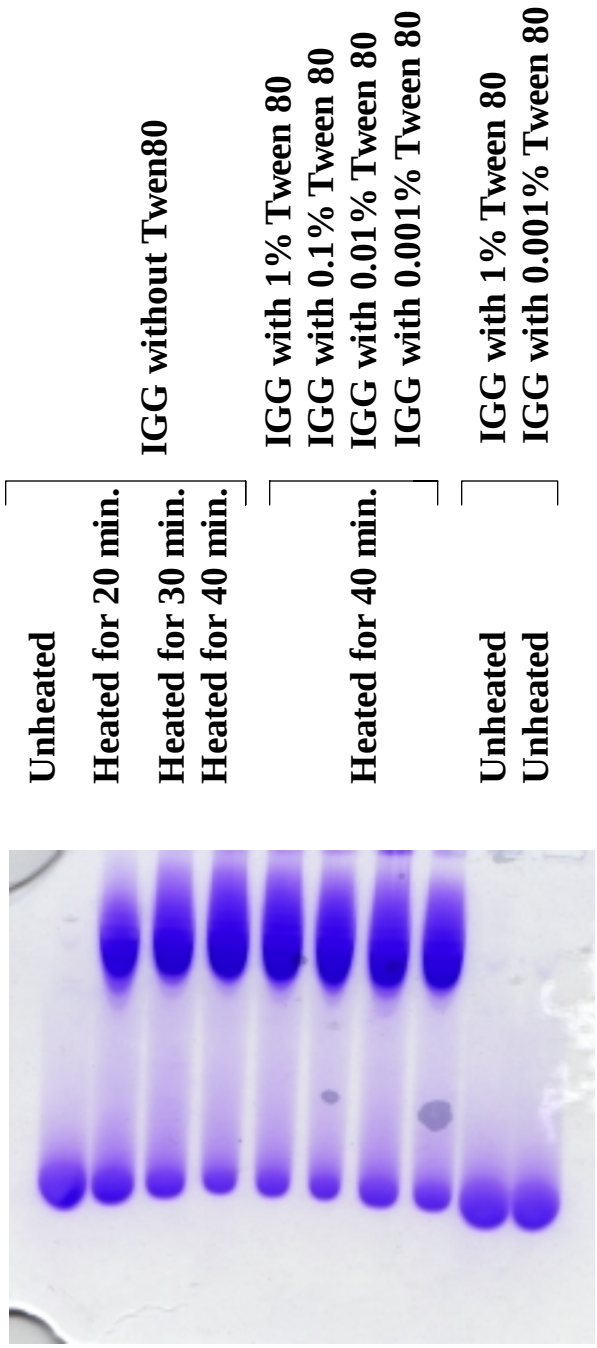

Figure 6. Effects of Tween 80. After heating at 75 °C for 40 min, there appear to be no changes in aggregation by the addition of this detergent. IGG1 in 0.2M NaCl, 19 mM phosphate buffer, pH 6.5. Native gel at pH 6.1.

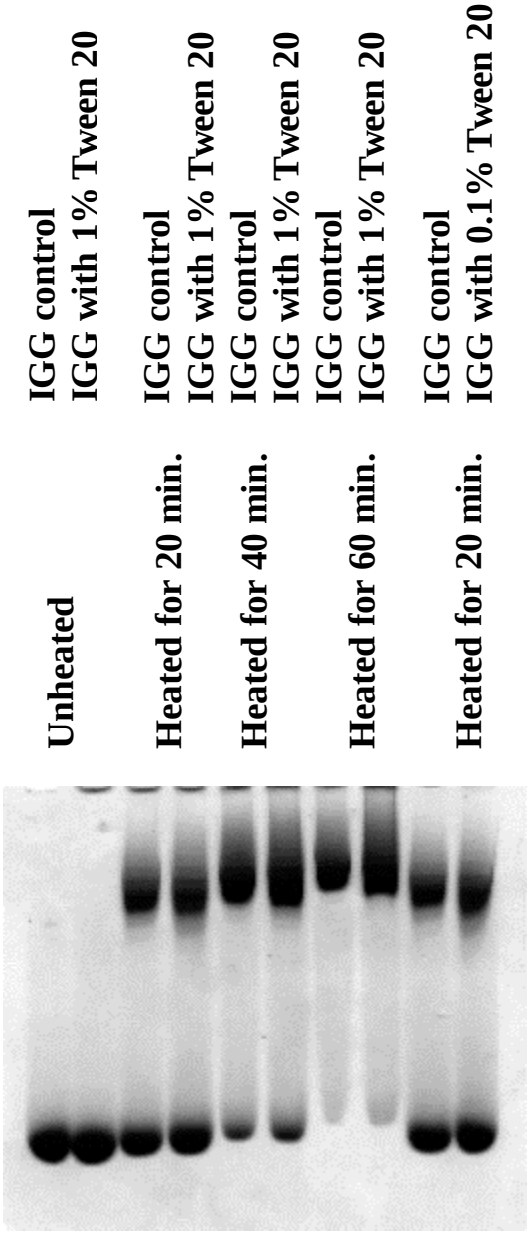

Figure 7. Effects of Tween 20.
IGG1 was heated for 20 to 60 min in the absence and presence of 1% Tween 20. Aggregation is unchanged by this detergent. IGG1 in 0.2M NaCl, 19mM phosphate buffer, pH 6.5. Native gel at pH 6.1.

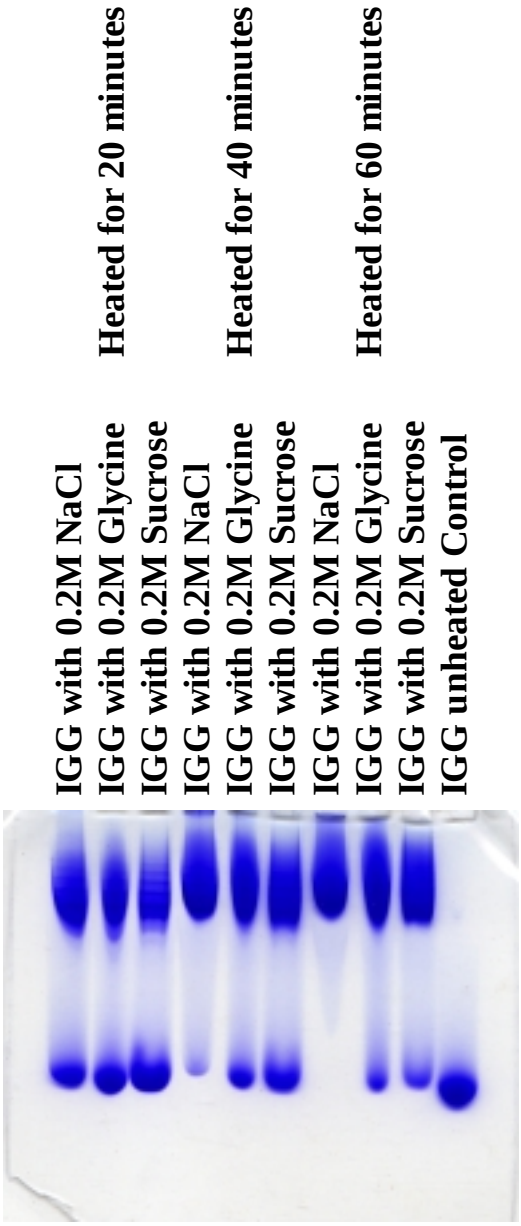

Figure 8. Comparison of additives, NaCl, sucrose and glycine.
In this experiment, the 0.2 M NaCl present in the previous experiments was replaced with 0.2 M sucrose or glycine by dialysis. This replacement resulted in substantial stabilization of IGG. It is not clear from this experiment whether removal of NaCl or addition of sucrose or glycine contributed to the observed stabilization. It was later demonstrated that the addition of NaCl increases aggregation of IGG upon heating (not shown). IGG1 in 19 mM phosphate buffer, pH 6.5. Native gel at pH 6.1.

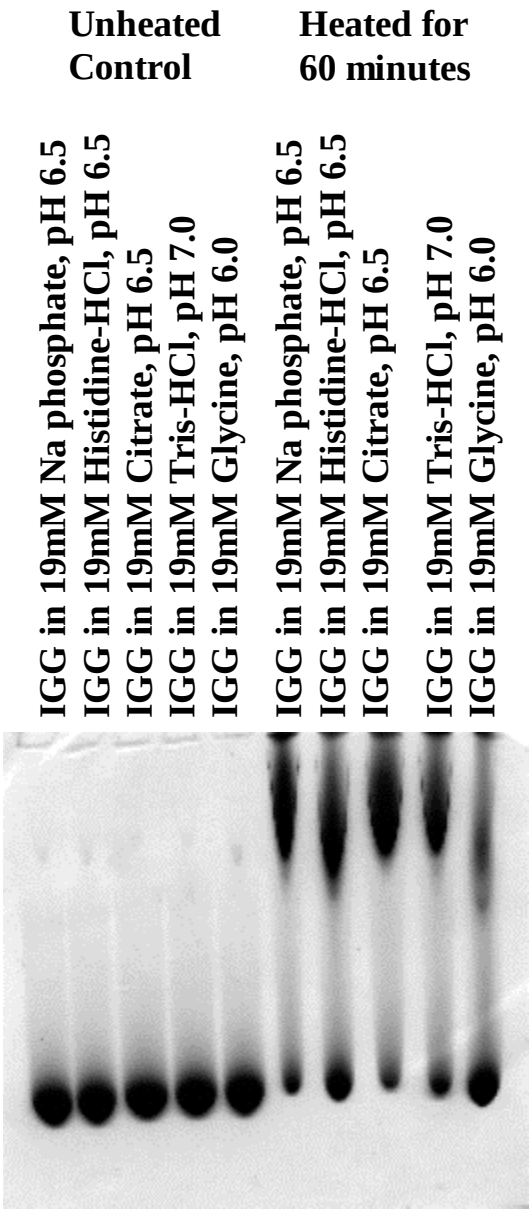

Figure 9. Effects of different buffers.
In this experiment, 19 mM phosphate was replaced with other buffers: 19 mM histidine-HCl, pH 6.5; 19 mM citrate, pH 6.5; 19 mM Tris-HCl, pH 7.0; or 19 mM glycine, pH 6.0. These samples were heated at 75 °C for 60 min. Aggregation was significantly less in histidine, Tris or glycine. Native gel at pH 6.1.