Studies on the Control of the Denaturation of the Fish Muscle Proteins during Frozen Storage—II. Preventive Effect of Amino Acids and Related Compounds*

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By using an *in vitro* model test system, a study was done on the preventive effect of 29 substances on freezing denaturation at -30°C for periods of 4 to 8 weeks.

The rate of denaturation was followed by estimating solubility, viscosity, and ATPase activity, as well as superprecipitation. The overall results might be summarized as follows:

Additives with marked effect: Glutamate, aspartate, cysteine, β -alanine, γ -amino-butyric acid, cysteate, acetylglycine and EDTA.

Additives with moderate effect: lysine, histidine, ornithine, serine, betaine and triphosphate.

Additives with little or no effect: glycine, isoleucine, phenylalanine, L- and DL-threonine, glutamine, asparagine, arginine, glycylglycine, folic acid, ethylenediamine, creatinine, pyrophosphate and phytin.

In the experiment testing the effect of the combined use of two kinds of amino acids, some synergistic effect was observed with the combination of lysine and threonine, of lysine and serine, as well as of lysine and cysteine. No increased effect was found when amino acids were added to glutamate.

Discussion of the results suggests that the protective effect of the substances are closely related with their functional groups, their spatial structure and other physico-chemical properties.

In the previous paper¹⁾, the authors have reported that Na-glutamate prevents the denaturation of fish muscle proteins during the frozen storage. It has been also suggested that the *in vitro* model test system introduced there is useful in following the denaturation process during the frozen storage and in screening the effective substances for the ease and speed-up of its operation. The present paper deals with the results of the similar tests for the preventive effect on the freezing denaturation of a wider variety of the amino acids and related substances.

Methods

Unless otherwise stated, methods used were the same as the previous work¹⁾.

***In vitro* model test:** In each test, the final concentration of the added substances was adjusted to 0.1 M and pH to 7. All the tested amino acids were L-type except for DL-threonine. The mixtures in the test tubes were frozen and stored at -30°C for a definite period.

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Superprecipitation: In addition to the tests of the previous paper, "superprecipitation" test was done for its virtue in testing the "nativeness" of the actomyosin. After thawed and homogenized, 1 ml of the actomyosin solution containing the added chemicals was diluted with 2 ml of water and 1 ml of 0.24 M tris maleate buffer (pH 7.0). To this suspension 0.2 ml of 20 mM $MgCl_2$ and 0.2 ml of 20 mM ATP solution were added, and the change that followed was examined. The patterns of the superprecipitation were recorded photographically and were classified as follows:

- + : Formed a stopper-like or compact precipitate.
- ± : Separated from the mother liquid, but not remarkably.
- : No superprecipitation observed.

Results

The preventive effect of amino acids. The effect of 12 amino acids was checked by estimating the solubility, viscosity, ATPase activity as well as superprecipitation (Table 1).

As shown by the solubility data of Table 1-a, the dicarboxylic amino acids, glutamate and aspartate, prevented the fall of solubility as long as 8 weeks of the frozen storage, while their acid amides, glutamine and asparagine, did not show any appreciable effect. Among

Table 1-a. Effect of the amino acids on the denaturation of actomyosin during the frozen storage in 0.6 M KCl at $-30^{\circ}C$.

Substance	Solubility (%)*		Viscosity (η_r , $C \rightarrow 2$ mg pro./ml)		
	4	8	0	4	8
Gly	60.9	35.3	1.78	1.49	1.36
Ileu	39.0	33.6	1.92	1.40	1.37
Phe	43.6	36.0	1.78	1.47	1.26
L-Thr	46.1	41.9	1.98	1.91	1.57
DL-Thr	38.7	34.5	1.95	1.50	1.44
Ser	54.1	50.4	1.90	2.01	1.71
Na-Glu	91.4	96.3	1.87	1.77	1.76
Na-Asp	91.2	89.8	1.91	1.82	1.93
Glu(NH ₂)	33.1	23.2	1.65	1.27	1.20
Asp(NH ₂)	49.9	36.9	1.79	1.45	1.37
Lys(HCl)	66.3	62.0	1.94	1.62	1.36
Orn(HCl)	65.8	60.0	1.73	1.36	1.30
His(HCl)	70.0	68.0	1.96	1.43	1.56
Arg(HCl)	46.7	43.3	1.73	1.40	1.20
CySH	59.8	66.6	1.82	1.61	1.70
Na-Cysteate	100.	81.6	1.95	1.78	1.72
Control	65.5	42.5	1.76	1.26	1.23

* The values show the percentage for those of the unfrozen actomyosin.

the basic amino acids, lysine, ornithine and histidine showed some preventive effect whereas arginine did little. Both cysteine and cysteate were remarkably effective. Serine was effective, but neither L- nor DL-type of threonine was good. Isoleucine and phenylalanine containing hydrophobic side chain did not show any preventive effect.

The viscosity data were, generally, in parallel to those of the solubility. The values of the samples containing any of glutamate, aspartate, cysteate, cysteine or serine did not show any remarkable change, while those containing other amino acids fell down during the frozen storage (Table 1-a).

The data of the ATPase activity show that the change in this property is more marked than those in the solubility and viscosity, suggesting that the former is more sensitive to the freezing denaturation. While the ATPase activity of the control group fell down to 10% the initial value after 8 weeks frozen storage, those of the samples containing glutamate or aspartate did not go down below 50%. The activity was lost quickly in the case with other amino acids, among which lysine, histidine and cysteate showed some preventive effect.

Although the judgement of the rate of superprecipitation is but qualitative, the overall results of the superprecipitation appear to be in accordance with those of other criteria.

Table 1-b. Effect of the amino acids on the denaturation of actomyosin during the frozen storage in 0.6 M KCl at -30°C^* .

Substance	ATPase activity (%)**		Superpptn.		
	4	8	0	4	8
Gly	16.7	11.9	+	±	—
Ileu	11.2	8.8	+	±	—
Phe	16.7	9.1	+	±	—
L-Thr	20.0	10.8	+	±	±
DL-Thr	8.4	11.5	+	±	—
Ser	15.2	11.2	+	+	+
Na-Glu	57.9	59.5	+	+	+
Na-Asp	52.0	57.5	+	+	+
Glu(NH ₂)	5.6	3.2	+	—	—
Asp(NH ₂)	12.2	7.3	+	±	—
Lys(HCl)	17.2	22.1	+	±	±
Orn(HCl)	3.8	3.9	+	±	—
His(HCl)	28.5	27.8	+	±	—
Arg(HCl)	6.6	0.0	+	—	—
CySH	23.9	9.0	+	+	+
Na-Cysteate	29.4	32.5	+	+	+
Control	7.2	8.8	+	±	—

* The samples used in this experiment are the same as those in Table 1-a.

** The values show the percentage for those of the unfrozen actomyosin.

The samples containing glutamate, aspartate, cysteate, cysteine and serine formed stopper-like precipitate on adding ATP as long as 8 weeks of the frozen storage (Table 1-b).

The preventive effect of miscellaneous substances related with amino acids. As an extension of the study on the amino acids, 13 substances structurally related with the amino acids were tested (Tables 2 and 3).

While glycine and glycyglycine were of no effect, acetylglycine was effective. β -Alanine, γ -amino butyric acid and betaine showed some preventive effect, whereas taurine, creatinine and folic acid none. Ethylenediamine was ineffective (Table 2).

Table 2. Effect of the miscellaneous substances related with the amino acid on the denaturation of actomyosin during the frozen storage in 0.6 M KCl at -30°C .

Substance	Solubility (%) [*]		Viscosity (η_r , C $\rightarrow$ 2 mg pro./ml)		
	4	7	0	4	7
Glycine	56.1	23.9	1.91	1.49	1.36
Acetylglycine	80.5	74.5	1.88	1.57	1.35
Glycyglycine	53.2	47.7	2.12	1.49	1.33
β -Alanine	95.6	79.1	1.83	1.55	1.70
γ -Aminobutyric acid	75.5	80.6	1.99	1.53	1.52
Taurine	70.6	53.8	2.03	1.31	1.32
Betaine	80.9	64.2	1.80	1.33	1.41
Folic acid	59.8	51.5	1.86	1.29	1.28
Creatinine	55.7	52.5	1.63	1.28	1.23
Ethylenediamine	70.2	58.0	1.57	1.41	1.41
Na-Glutamate	100.	96.0	1.85	1.78	1.62
Control	68.8	62.5	1.85	1.30	1.28

Substance	ATPase activity (%) [*]		Superpptn .		
	4	7	0	4	7
Glycine	7.6	8.5	+	$\pm$	—
Acetylglycine	28.2	36.8	+	+	+
Glycyglycine	15.8	7.9	+	$\pm$	—
β -Alanine	58.0	53.8	+	+	+
γ -Aminobutyric acid	52.2	34.0	+	+	+
Taurine	7.3	18.7	+	$\pm$	—
Betaine	23.2	42.8	+	+	$\pm$
Folic acid	7.6	8.5	+	—	—
Creatinine	3.5	18.4	+	$\pm$	—
Ethylenediamine	18.7	3.3	+	—	—
Na-Glutamate	61.5	63.0	+	+	+
Control	17.1	19.0	+	—	—

^{*} The values show the percentage for those of the unfrozen actomyosin.

In view of the marked preventive effect of the dicarboxylic amino acids and of the known effect of the polyphosphates^{2,3)}, a set of experiment was done with some di- and polybasic compounds (Table 3). Thus, EDTA and triphosphate showed the preventive effect while pyrophosphate and phytin (inositol-hexaphosphate) none.

Table 3. Effect of the polybasic acids on the denaturation of actomyosin during the frozen storage in 0.6 M KCl at -30°C .

Substance \ Stored weeks	Solubility (%) [*]		Viscosity (η_r , $C \rightarrow 2$ mg pro./ml)		
	4	7	0	4	7
Na-EDTA	80.0	79.3	2.26	1.84	1.61
Na-PP ^{**}	68.5	51.8	1.31	1.28	1.30
Na-TP ^{***}	78.7	66.7	2.02	1.62	1.45
Na-Phytin	69.6	59.0	1.79	1.31	1.27
Na-Glutamate	100.	96.0	1.85	1.78	1.62
Control	68.8	62.5	1.85	1.30	1.28

Substance \ Stored weeks	ATPase activity (%) [*]		Superpptn.		
	4	7	0	4	7
Na-EDTA	116.	174.	+	+	+
Na-PP ^{*,†}	—	—	—	—	—
Na-TP ^{***,†}	—	—	—	—	—
Na-Phytin	14.9	1.4	+	±	—
Na-Glutamate	61.5	63.0	+	+	—
Control	17.1	19.0	+	—	—

^{*} The values show the percentage for those of the unfrozen actomyosin.

^{**} Pyrophosphate, ^{***} Triphosphate.

[†] ATPase activity and superpptn. were not tested because of the hindrance by the additives.

The preventive effect of the combined use of 2 amino acids. Thirteen different groups were studied to test synergistic effect of the amino acids added to either glutamate or lysine (Table 4).

Among the groups containing any amino acid added to glutamate, cysteine, serine, threonine and lysine showed better effect than others tested. However, in the case of arginine, the fall of ATPase activity and the viscosity were faster than that with glutamate alone, though the solubility was kept unchanged.

In the groups containing lysine, those added with threonine or serine showed remarkable preventive effect, while each of the latter 2 amino acids was less effective when added alone (Table 1). The rate of the denaturation of the samples added with phenylalanine, isoleucine or arginine to lysine was faster than that with lysine alone. The

Table 4. Effect of the combined use of two kinds of amino acid in 0.6 M KCl at -30°C .

Stored weeks Substance	Solubility* (%)	Viscosity (η_r , $C \rightarrow 2$ mg pro./ml)		ATPase activity (%)*	Superpptn.	
	6	0	6	6	0	6
Arg + Glu	75.7	2.12	1.53	19.1	+	—
His + Glu	79.6	2.06	1.82	42.2	+	+
Lys + Glu	84.3	1.81	1.74	43.9	+	+
L-Thr + Glu	100.	1.77	1.61	41.5	+	+
Ser + Glu	82.4	1.79	1.56	44.8	+	+
Phe + Glu	66.9	1.81	1.74	40.7	+	+
Ileu + Glu	64.0	1.75	1.92	39.8	+	+
CySH + Glu	98.8	1.94	1.91	55.7	+	+
Glu	85.5	1.79	1.74	44.6	+	+
Arg + Lys	76.8	2.00	1.48	19.1	+	—
His + Lys	72.8	1.98	1.59	20.6	+	—
L-Thr + Lys	64.8	1.76	2.04	36.8	+	+
Ser + Lys	65.8	1.72	2.03	46.9	+	+
Phe + Lys	45.1	1.76	1.34	2.2	+	—
Ileu + Lys	22.9	1.74	1.17	3.5	+	—
Cys + Lys	94.1	1.99	1.86	48.8	+	+
Lys	53.8	1.72	1.39	14.0	+	±
Control	47.1	1.76	1.24	7.15	+	—

* The values show the percentage for those of the unfrozen actomyosin. The final concentration of each additive was adjusted to 0.05 M except for the case where either Glu or Lys was used alone at the concentration of 0.1 M.

denaturation of the samples containing cysteine showed synergistic preventive effect in cases with its addition to glutamate and to lysine.

The data obtained here do not illustrate definitely that any of the added amino acids increased the preventive effect of glutamate. However, the effect of the former, if any, might have been obscured, because the used background glutamate concentration was 0.05 M which is higher than its threshold effective concentration, 0.025 M^{11} .

Discussion

In accordance with the previous work¹¹, the present results show the preventive effect of glutamate. Moreover, it is shown that, besides glutamate, various amino acids and other substances can prevent the denaturation of fish actomyosin when its solution in 0.6 M KCl is frozen-stored at -30°C . Though the rate of the effect of each additive appears to differ with different method of the criterion of the denaturation, *i.e.* solubility, viscosity, ATPase activity and superprecipitation, the overall results might be summarized as follows:

Additives with marked effect:

glutamate, aspartate, cysteine, β -alanine, γ -aminobutyric acid, cysteate, acetylglycine and EDTA.

Additives with moderate effect:

lysine, histidine, ornithine, serine, betaine and triphosphate.

Additives with little or no effect:

glycine, L- or DL-threonine, glutamine, asparagine, isoleucine, phenylalanine, arginine, taurine, glycylglycine, folic acid, ethylenediamine, creatinine, pyrophosphate and phytin.

The preventive effect of the acidic amino acids is generally stronger than that of the basic amino acids, and the amino acids containing hydrophobic groups are rather promotive for the denaturation.

All these data suggest that there exist some common principles in the structure with respect to the capacity of preventing the freezing denaturation: Among the amino acids, glutamate and aspartate are the most effective, and their side chain with $-\text{COOH}$ is replaceable by the groups with $-\text{NH}_2$, $-\text{SH}$, $-\text{OH}$ or $-\text{SO}_3\text{H}$ without losing the preventive capacity. If a hydrophobic group is introduced on the side chain, the amino acid shows no preventive effect (*e.g.* phenylalanine and isoleucine).

The positive effect of β -alanine and γ -aminobutyric acid and the negative one of glycine suggest that monoaminomonocarboxylic acids can be effective, if the amino group is properly distanced from the carboxylic group. It is interesting that acetylglycine is effective in spite of the negative results of glycylglycine. Though folic acid and ethylenediamine have some functional groups, they do not show any preventive effect.

These results suggest that not only the number and type of the functional group but also the spatial structure of the molecule and other physico-chemical properties of the added substances make important factors of the cryoprotective effect or the effect to prevent the freezing denaturation.

Triphosphate showed the preventive effect but pyrophosphate did none. Though both are used in the conventional technique of the salt-free, frozen fish meat mince (*muensurimi*) industry, it is interesting that the effect of these two phosphates are not the same as far as found here on the isolated actomyosin frozen in 0.6 M KCl solution. It must be taken into account that the ionic strength in the present actomyosin model system and salt-free, frozen-minced meat is not equal: Thus, the actomyosin solution in 0.6 M KCl was frozen in the former case, while the salt-free mince is frozen in the latter.

In the experiment of the combined use of the two kinds of amino acid, the synergistic effect is found in the combination of lysine and threonine, of lysine and serine, of lysine and cysteine as well as of glutamate and cysteine, while the increased effect of the added amino acids is not very marked in most cases when the glutamate was used as one of

the additives. Arginine rather depressed the preventive effect of glutamate. Generally saying, the synergistic effect between the different amino acids is found in the case where the type of the functional groups is different between the mixed amino acids.

In the study of the freeze-drying of bacteria, MORICHI *et al.*⁴⁾ found 11 protective substances as related with glutamic acid. Though there are some coincidence between the substances with the protective effect on the injury of freeze-drying and those with the preventive effect on the freezing denaturation of actomyosin as shown in the present experiment, some exception are found. Thus, cysteine prevents the denaturation of actomyosin, but it is not effective in the case of the freeze-drying of bacteria. The reverse is found in the case of arginine. HANAFUSA⁵⁾ reported the preventive effect of glycine and glutamic acid on the freeze-drying denaturation of myosin and catalase, but we could not find any preventive effect of glycine on the denaturation of actomyosin during frozen storage.

These suggest that each cryoprotective substance is not always effective in all the cases involving the freezing processes but that the effect is dependent on the mechanism of the protective effect involved which might differ from substance to substance.

References

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