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ARTICLE

L-Cysteine, *N*-Acetyl-L-cysteine, and Glutathione Protect *Xenopus laevis* Embryos against Acrylamide-Induced Malformations and Mortality in the Frog Embryo Teratogenesis Assay

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Dietary acrylamide is largely derived from heat-induced reactions between the amino group of the free amino acid asparagine and carbonyl groups of glucose and fructose during heat processing (baking, frying) of plant-derived foods such as potato fries and cereals. After consumption, acrylamide is absorbed into the circulation and is then distributed to various organs, where it can react with DNA, neurons, hemoglobin, and essential enzymes. In the present study, we explored the potential of L-cysteine (CySH), *N*-acetyl-L-cysteine (NAC), reduced glutathione (GSH), and the amino acid glycine (Gly) to protect frog embryos against acrylamide-induced developmental toxicity in the frog embryo teratogenesis assay - *Xenopus* (FETAX). To test the antiteratogenic potential, based on concentration−response study ranging from 0.07 to 4.22 mM acrylamide in FETAX solution (pH 8.1), we selected concentrations of acrylamide that induced 100% malformations and mortality. At the end of 96 h, we counted survivors and malformed embryos and measured embryo length. The data show that CySH, NAC, and GSH protected the embryos against acrylamide induced malformations and mortality to different degrees. CySH and GSH protected the embryos against both malformations and mortality, whereas NAC protected only against mortality. Gly had no protective effect. Possible mechanisms of the protective effects and the dietary significance of the results of this and related studies for food safety and human health are discussed.

KEYWORDS: Acrylamide; frog embryos; toxicity; teratogenesis; protection; L-cysteine; *N*-acetyl-L-cysteine; reduced glutathione; glycine; food safety

INTRODUCTION

Acrylamide (CH_2=$CHCONH_2$) is a small, reactive conjugated vinyl compound. After consumption, it is absorbed in the circulation and is then distributed to various organs, where it can react with DNA, neurons, hemoglobin, and essential enzymes. Dietary acrylamide is largely derived from heat-induced reactions between the amino group of the free amino acid asparagine and carbonyl groups of glucose and fructose during heat processing (baking, frying) of plant-derived foods such as potato fries and cereals (*1*−*5*). These considerations induced us to explore the potential of SH-containing amino acids and peptides (L-cysteine, CySH; *N*-acetyl-L-cysteine, NAC; and reduced glutathione, GSH) and the amino acid glycine (Gly) to protect frog embryos against acrylamide-induced developmental toxicity.

To place the present study in proper perspective, we will first briefly summarize the following reported studies on acrylamide-induced developmental toxicity in approximately chronological order: (a) Administration of acrylamide during organogenesis produced maternal and developmental toxicity at 45 mg/kg/day in mice and maternal, but not developmental, toxicity at doses > 7.5 mg/kg/day in rats (*6*). (b) The molecular mechanism of reproductive toxicity could be the result of alkylation of SH groups in the sperm nucleus and tail, depletion of GSH, and/or DNA damage in the testis (*7*). (c) The observed dose-dependent morphologic abnormalities in preimplantation embryos in mice indicate that acrylamide can reach sperm cell nuclei (*8*). (d) Studies with the aid of the FETAX assay demonstrated that acrylamide is a frog embryo teratogen (*9*). (e) In rats, acrylamide in drinking-water-induced neurotoxicity (< 0.5 mg/kg/day) and reproductive toxicity (2.0 mg/kg/day) were affected by different doses of acrylamide, suggesting that neurotoxicity is the cause or a major contributor to developmental toxicity (*10*, *11*). (f) Other investigators confirmed adverse effects of acrylamide on spermatogenesis (*12*−*14*). (g) NAC and GSH protected against acrylamide-induced morphological transformations of Syrian hamster embryo cells (*15*, *16*). (h) Breast milk in Germany was found to contain up to 18.8 μg/L acrylamide. Since water-soluble acrylamide can pass both placental and blood-brain barriers, the authors suggest that, to protect fetuses, pregnant women should not consume high-acrylamide food. (i) Acrylamide from poultry feed was carried over to the meat and eggs of laying hens (*17*). (j) Acrylamide crossed the placenta from maternal to fetal circulation in perfused human placenta (*18*) and in rodents (*19*, *20*). (k) Significant amounts of dietary acrylamide consumed by pregnant women are transferred

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via blood through the placenta to the fetus (*21*), suggesting fetal exposure if the mother is exposed. (l) Developmentally toxic effects of acrylamide in an in vitro rodent whole embryo culture (WEC) system correlated with in vivo developmental toxicity tests in the dams (*22*). and (m) Paternal exposure to acrylamide to mice induced the formation of chromosomally defective two-cell embryos (*23*).

Because the cited observations imply that long-term exposure to acrylamide has the potential to adversely affect human fertility, the California EPA Office of Environmental Health Hazard Assessment (OEHHA) is currently receiving public comment on their declaration of intent to add acrylamide, due to its association with reproductive toxicity, to the list of substances covered by Proposition 65, the Safe Drinking Water and Toxic Enforcement Act of 1986 (*24*). These observations suggest the need to protect humans against potential acrylamide-induced teratogenesis.

The objective of the present study was to determine whether food-compatible amino acids and peptides could be used to reduce acrylamide toxicity and teratogenesis in frog embryos. To help accomplish this objective, we investigated the effects of four test substances on mortality, malformation, and embryo length induced by select concentrations of acrylamide.

MATERIALS AND METHODS

Materials. The following compounds were obtained from Sigma (St. Louis, MO): L-Cysteine (cat. no. C7352; FW 121.16), *N*-acetyl-L-cysteine (cat. no. A7250; FW 163.19), glutathione, reduced (G4251; FW 307.32), glycine (G7403; FW 75.07), and acrylamide (cat. no. A3553; FW 71.08).

Animal Care and Husbandry. *Xenopus* frogs were purchased from *Xenopus* I, Inc. (Dexter, MI) and housed in a glass aquaria recirculating system with 2−4 frogs per 10 gallons of dechlorinated tap water. Human chorionic gonadotropin (250−500 IU) was injected in the dorsal lymph sac of both male and female frogs to induce breeding. Breeding pairs were placed in false bottom breeding chambers and embryos were collected the next morning. The jelly coat was removed by swirling the embryos for 1−3 min in a 2% cysteine solution (pH 8.1). Embryos were then rinsed and placed into sorting dishes. Embryos were double sorted in to test dishes of 20 embryos per 8 mL of FETAX test solution in plastic disposable 60 × 15 mm Petri dishes. Negative control was FETAX solution. Frogs were not bred more than once every 2 months.

FETAX Assay Procedures. The frog embryo teratogenesis assay was used to determine acrylamide toxicity and teratogenesis according to the American Society of Testing Material (ASTM) guide for FETAX and our earlier studies with teratogenic glycoalkaloids (*25*). Stock solutions of acrylamide and of acrylamide and test compounds were made in FETAX solution. Appropriate dilutions were made to achieve final concentrations. Each day of the 4 day test, new solutions were placed into 60 mm covered glass Petri dishes with various concentrations of test compounds dissolved in FETAX solution. Dead embryos were removed, and live embryos counted. The embryos were cultured at 24 °C. At 96 h, surviving (stage 46) embryos were fixed in 3% (w/v) formalin. Stage 46 embryos possess hind-limb buds and tightly coiled guts but do not yet feed. At the end of 96 h, the mortality was determined and embryos were anesthetized with MS-222. Malformed survivors, dead embryos, and the developmental stage were observed under a dissecting microscope recorded according to the ASTM guide (*25*).

Malformations were determined as follows. All embryos were observed under a dissecting microscope for malformed survivors, developmental stage, and dead embryos. *Xenopus laevis* embryos are transparent and allow for easy determination of gut, heart, head, axial, and other internal and external malformations. The malformations are quantitatively scored on a standard score sheet and tallied. The data were recorded according to the ASTM guide. The total number of abnormal tadpoles divided by the total number of living tadpoles multiplied by 100 is the percentage malformed from each dish.

For length measurements, embryos were photographed with an Olympus stylus 720sw system on macro setting and analyzed using Image Pro Plus. Detailed photographs were taken of selected embryos using a Pro Reg digital camera attached to a Nikon dissection microscope. As a measure of growth, head−tail length was measured by following body contour using a computer equipped with digitizing software.

Acrylamide Toxicity Experimental Design and Analysis. For concentration−response studies of toxic effects on embryos, 4 negative controls (4 replicates) and 14 test concentrations (2 replicates each) of acrylamide ranging from 0.07 to 4.22 mM were tested. The experiment was repeated three times. Each experiment used 640 embryos. The 96 h LC50 (concentration causing 50% lethality), EC50 (concentration inducing malformation or gross terata in 50% of surviving embryos), EC50 Multi-Var (multivariate risk of 50% chance of inducing mortality, malformation or growth reduction), and EC10 (reduced growth) with standard errors were determined using the developmental toxicity model from Tox Tools software. The teratogenic index (TI) was calculated as the LC50/EC50 (malformation).

Design and Analysis of Interaction Experiments of Acrylamide with Test Compounds. On the basis of concentration−response data, each interaction experiment was performed using selected concentrations of acrylamide to achieve 100% malformations (1.2 mM) and 100% lethality (3 mM). The interaction experiments in the dishes were tested with acrylamide plus added L-cysteine (CySH), *N*-acetyl-L-cysteine (NAC), and reduced glutathione (GSH) at a 1:1 molar ratio of acrylamide to test compound in the same FETAX (pH 8.1) solution. Glycine (Gly) was tested at a 10:1 molar ratio to acrylamide. The pH values of the mixtures ranged from ~8.1 to 8.4. Four replicates were evaluated with each test solution. There were four negative controls, four 1.2 mM acrylamide (positive controls), four 3 mM acrylamide (positive controls), and four 3 mM (or in the case of Gly, 30 mM) for each of the test compounds (negative control for test compound). For the interaction, there were four tests of 1.2 mM acrylamide plus 1.2 mM (or 12 mM for Gly) and four tests of 3 mM acrylamide and 3 mM (or 30 mM for Gly). A higher molar ratio for Gly was used in order to observe a possible protective effect.

Statistics. Analysis of variance (ANOVA) was used to determine if treatments were significantly different ($p \leq 0.05$) from each other using Bonferroni adjustment post hoc test. The comparisons included 1.2 or 3 mM acrylamide alone compared to 1.2 or 3 mM acrylamide and test compound (CySH, NAC, and GSH), respectively.

RESULTS AND DISCUSSION

The FETAX assay was chosen for this study because the embryos develop externally and are transparent, which makes them an excellent choice for evaluating developmental toxicity (*25*−*31*). As noted elsewhere (*28*), advantages of the FETAX assay include the following: (a) *Xenopus* embryos undergo fundamental developmental processes that are similar to those of mammals; (b) mating and ovulation can be induced at any time after sexual maturity; (c) embryos develop outside the frog, facilitating observation of development and malformations; and (d) developmental end points can be determined within a relatively short 96 h test period.

We will now describe the observed effects of acrylamide alone and then of each test compound on acrylamide-induced mortality, malformation, and embryo length. **Figure 1A** illustrates the structures of compounds evaluated in the present study.

Acrylamide Toxicity/Teratogenesis. Table 1 shows that, in the FETAX assay, the calculated average 96 h LC50 (mortality) from the concentration−response experiments for acrylamide was 2.21 mM. The corresponding average EC50 (malformation) was 0.77 mM, and the teratogenic index (TI = ratio of the 96 h LC50 to 96-h EC50) 2.87 mM. Most embryos malformed at low concentrations had gut and tail/axial abnormalities. At higher concentrations, multiple edemas including eye, pericardial, tail, and facial abnormalities were observed. **Figure 2** depicts photographs of selected untreated and treated embryos.

The toxicity end points agree with those observed by Fort and Paul (*9*). However, our TI value was somewhat lower than their reported value of 4.75. The malformations we observed also agreed well with those reported by these authors. Our study confirms the teratogenic potential of acrylamide. In this exploratory study, we

Table 1. Developmental Toxicity of Frog Embryos Exposed to Acrylamide Based on LC50 (lethality), EC50 (malformation), EC 50 Multi-Var (risk of 50% chance of inducing mortality, malformation or growth reduction), and EC10 (growth reduction)[a]

experiment	LC50 lethality, mM (std error)	EC50 malformation, mM (std error)	EC50 Multi-Var, mM (std error)	EC10 growth reduction, mM (std error)	TI = LC50/EC50
1	1.79 (0.14)	0.73 (0.06)	0.65 (0.03)	0.21 (0.01)	2.4
2	2.18 (0.14)	0.82 (0.07)	0.73 (0.03)	0.69 (0.004)	2.7
3	2.66 (0.2)	0.76 (0.04)	0.73 (0.03)	1.2 (0.1)	3.5

[a] Determined from concentration−response data.

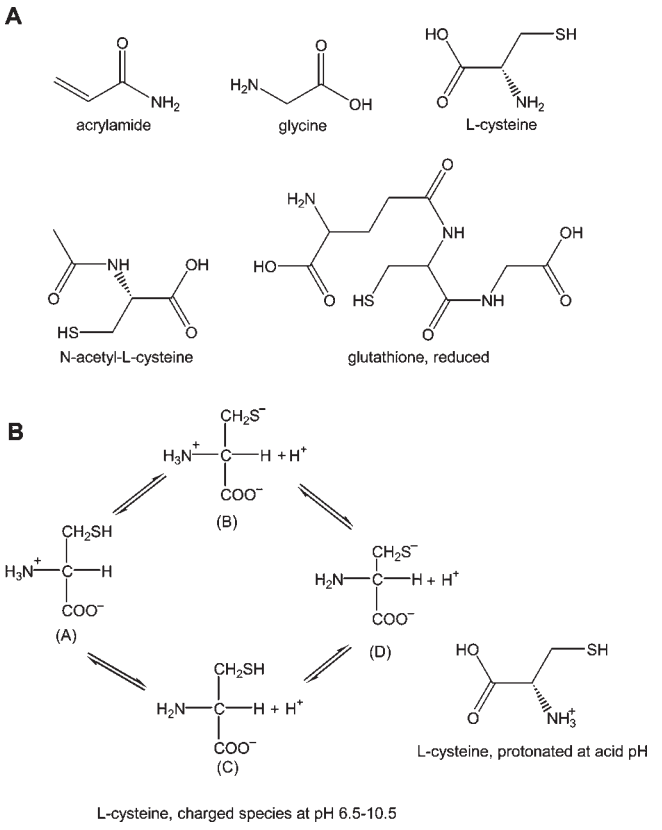

Figure 1. (A) Structures of compounds evaluated in the present study. (B) Acid−base equilibria of L-cysteine as a function of pH. Note distribution of positive and negative charges in the different cysteine species. Similar equilibria can be written for cysteine-containing glutathione but not for *N*-acetyl-L-cysteine, which lacks a NH_2 group. The determination of microscopic ionization constants (K_A, K_B, K_C, and K_D) associated with each acid−base equilibrium is described in ref (*39*).

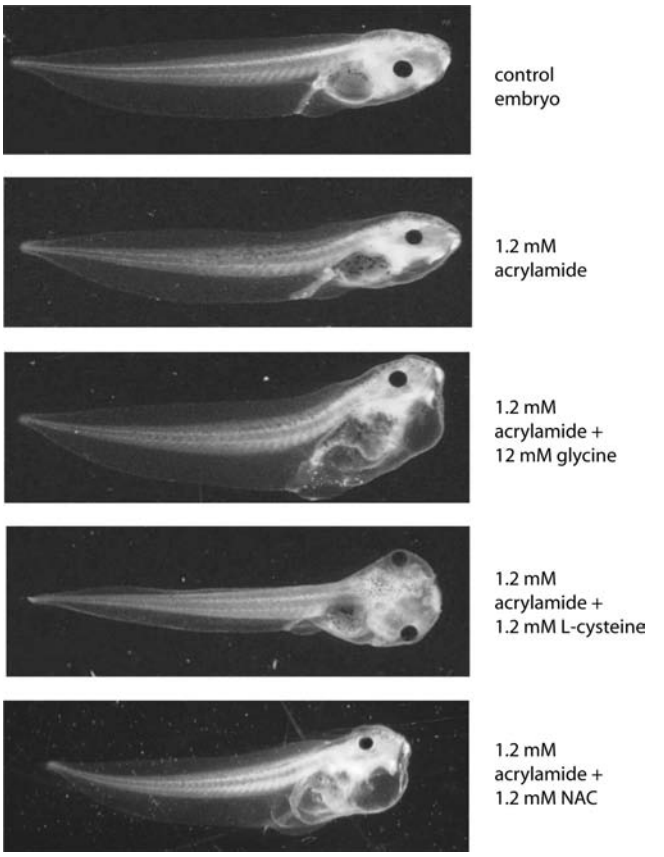

Figure 2. 96 h tadpoles, showing controls, acrylamide, and interaction of acrylamide with Gly, CySH, and NAC. Acrylamide malformations induced gut and pericardial edema and a spinal muscular kink causing the head to bend upward. These pictures show that both Gly and NAC do not protect against acrylamide malformations. Interesting is a slight increase in amount of edema. Malformations induced by acrylamide are not present when CySH is present. Note the reduction of edema and tail and spinal kinking.

used the data to generate 100% malformation (EC100, 1.2 mM) and mortality (LC100, 3.0 mM) estimates for evaluation in single point toxicity testing of preventive effects by each compound described below.

Protective Effect of L-Cysteine against Mortality and Malformations. CySH alone at 3 mM did not cause any significant mortality or malformation (3% mortality, 5.1% malformation). **Figure 3** shows that, at a 1:1 molar ratio to acrylamide, CySH protected significantly against the acrylamide mortality and malformations at the lower (1.2 mM) concentration of acrylamide. At the higher 3 mM acrylamide concentration, a slight benefit was seen as a reduction in mortality ($p = 0.093$), but essentially all the surviving embryos were malformed.

The reduction in malformations from an EC100 dose of acrylamide by addition of an equimolar (1.2 mM) amount of CySH was 87%, while the reduction in mortality from a LC100 dose by addition of an equimolar (3.0 mM) amount of CySH was 15%. Because the teratogenic index is a ratio of the 96 h LC50 to 96 h EC50, this result indicates that this index would be reduced significantly. The addition of CySH would therefore reduce the chance of acrylamide induced malformations. The effects on embryo length at 1.2 mM indicate that CySH would also protect against reduced embryo growth effects, considered to be a measure of low birth weights.

Protective Effect of Reduced Glutathione against Mortality and Malformations. GSH alone had no significant effects on frog embryos at 3 mM (11% mortality and 9.9% malformation). **Figure 4** shows that, at a 1:1 molar ratio of GSH to acrylamide, protection was significant against mortality and malformations at the lower (1.2 mM) concentrations of acrylamide. However the cysteine-containing tripeptide did not protect the embryos at the higher (3 mM) acrylamide level. The protective effect of GSH seemed to be similar to that observed with CySH at the same 1:1 molar concentration ratio. At the EC100 dose of acrylamide, added GSH reduced malformations by 77%, while at the LC100

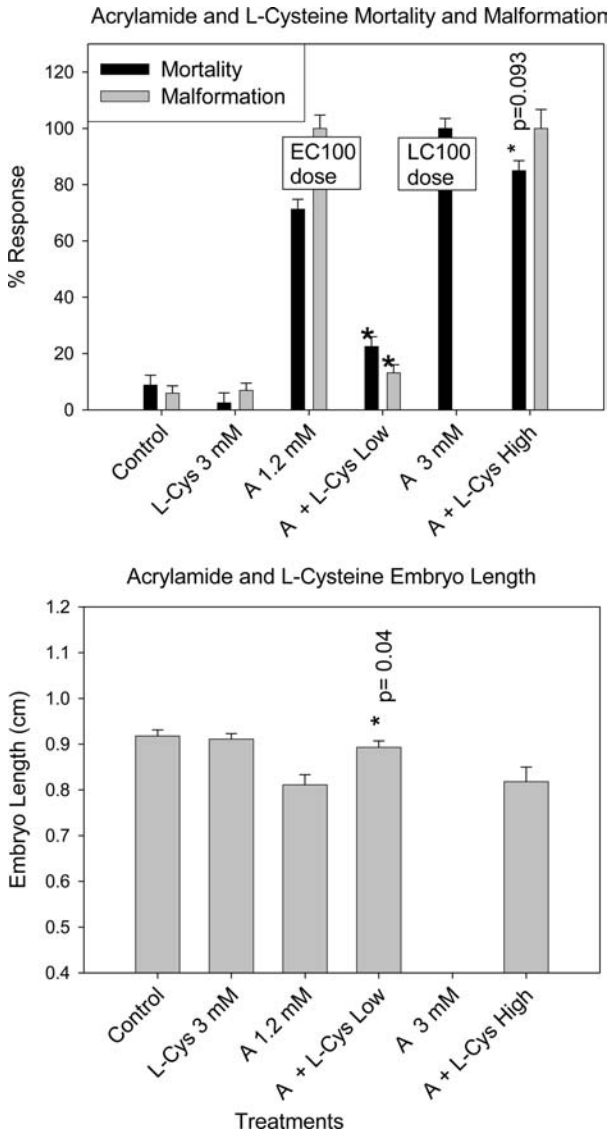

Figure 3. Protective effect of L-cysteine (CySH). CySH at 1:1 molar ratio to acrylamide at the lower concentrations of acrylamide (1.2 mM) protected against both mortality and malformation as well as against reduced growth (weight gain) of the embryos. At the higher concentrations of acrylamide (3 mM), CySH protected only against mortality. Asterisk (∗) indicates significant differences compared to acrylamide alone at the same concentration as in the mixture.

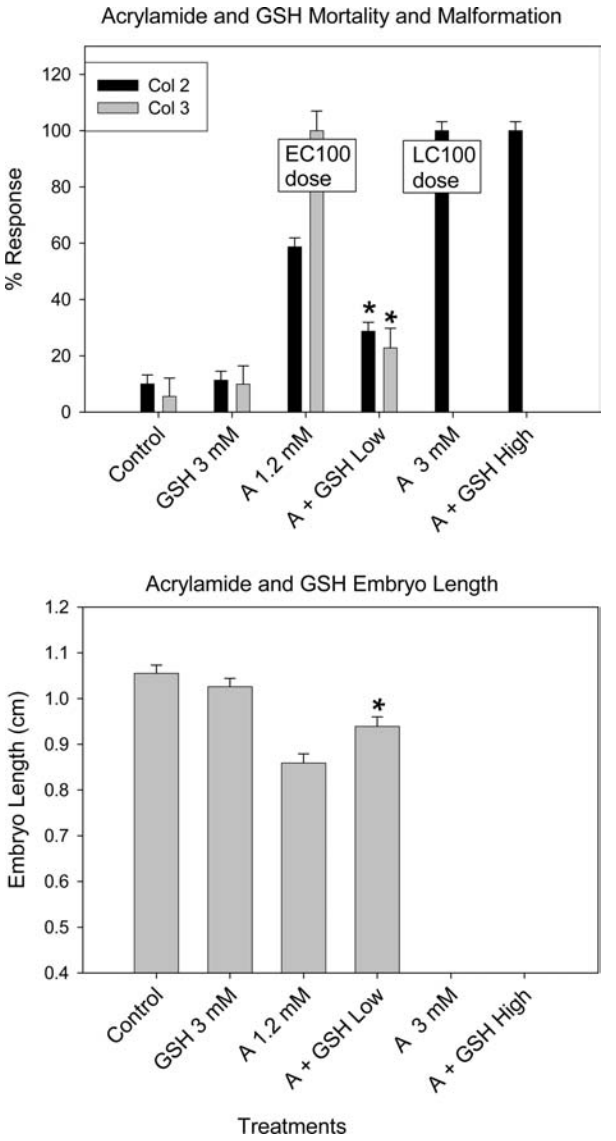

Figure 4. Protective effect of glutathione (GSH). GSH at 1:1 molar ratio to acrylamide at the lower concentration of acrylamide (1.2 mM) protected against acrylamide induced mortality and malformations as well as against reduced growth (weight gain) of the embryos. At the higher concentrations of acrylamide (3.0 mM), GSH did not protect the frog embryos. Asterisk (∗) indicates significant differences compared to acrylamide at the same concentration as in the mixture.

dose added GSH did not reduce mortality. These results suggest that GSH will also cause a reduction in the TI value and protect against potential teratogenic effects of acrylamide.

***N*-Acetyl-L-cysteine Protects against Mortality but Not against Malformations.** NAC alone at 3 mM had no significant effects on mortality or malformation (11% mortality, 7% malformation). **Figure 5** shows that at a 1:1 molar ratio of NAC to acrylamide, this acetylated cysteine derivative protected against acrylamide induced mortality at both concentrations (1.2 and 3.0 mM) of acrylamide. Surprisingly, there were no observed protective effects on malformation at the lower concentrations. These findings suggest that the protective mechanism by NAC differs from that of CySH and GSH at the molecular-cellular level. The data also imply that, with NAC, the teratogenic index would increase because the 96 h LC50 would increase without an increase in the 96 h EC50. The data indicate that NAC would protect against acrylamide-induced toxicity.

Lack of Protection by Glycine. We evaluated the effect of Gly on the embryos because added Gly is reported to inhibit heat-induced acrylamide formation in food (*3*, *32*−*34*). At 30 mM, Gly alone did not produce significant increases in mortality or malformation (8% mortality and 13.5% malformation). **Figure 6** shows that, at a molar ratio of Gly to acrylamide of 10:1, there was no observable protective effect against any of the toxicity indices. In fact, there was a significant increase in morality at 1.2 mM acrylamide plus Gly above 1.2 mM acrylamide alone. Therefore, we have no evidence of a protective effect occurring with Gly.

Mechanistic Aspects. Overall, we observed three different biological effects with the four evaluated compounds. Gly had no protective effect. In fact, it appears to slightly enhance the response to acrylamide-induced toxicity of the embryos. NAC protected the embryos only against mortality at both concentrations tested. It did not protect against malformations. By contrast, both CySH and GSH protected the embryos against both

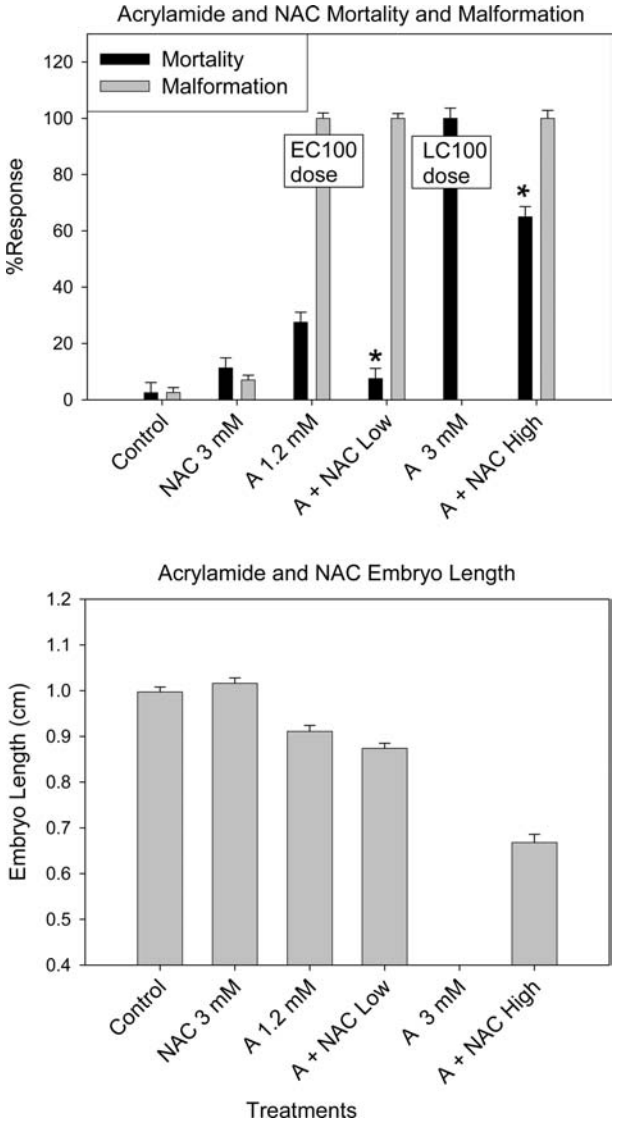

Figure 5. Protective effect of *N*-acetyl-L-cysteine (NAC). NAC at a 1:1 molar ratio to acrylamide reduced mortality at both the lower (1.2 mM) and higher (3.0 mM) concentrations of acrylamide, but had no effect on embryo malformation or length of embryos. Asterisk (∗) indicates significant differences compared to acrylamide at the same concentration as in the mixture.

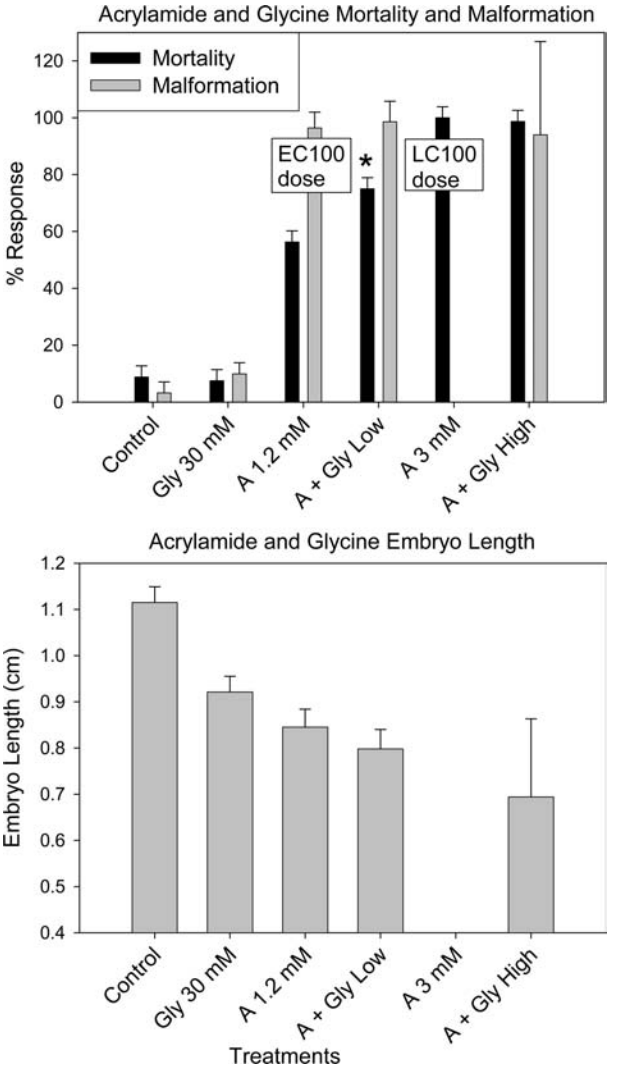

Figure 6. Effect of glycine (Gly). Gly at a 10:1 molar ratio to acrylamide increased mortality of the embryos at the lower concentration (1.2 mM) of acrylamide ($p = 0.04$). Malformation and embryo length were not significantly different with acrylamide alone at the lower concentration for either mixture. Asterisk (∗) indicates significant differences compared to acrylamide at the same concentration as in the mixture.

acrylamide-induced mortality and malformation, but only at the lower (1.2 mM) level of acrylamide. It did not protect the embryos at the higher acrylamide (3.0 mM) concentration.

GSH is a major intracellular antioxidant as well as an important component in the metabolism of many xenobiotics, including acrylamide (*35*). Cellular oxidative stress can either lead to or indicate a depletion of GSH. Therefore, by depleting intracellular GSH, glutathione−acrylamide adduct formation can favor cellular oxidative stress, which may be one possible mechanism governing acrylamide genotoxicity/teratogenesis (*16*, *36*).

Generally, the antioxidant and antitoxic effects of SH-containing amino acids, peptides, and proteins are due to several mechanisms including their ability to act as precursors of GSH, reducing agents, scavengers of reactive oxygen species, strong nucleophiles that can trap electrophiles such as acrylamide, thus preventing biological alkylation of DNA, and inducers of cellular detoxification (*37*−*39*).

We have no obvious explanation for the cited differences of the protective effects, except to note that the data show that, under the test conditions, SH- and NH_2-containing amino acids and peptides, but not NH_2-contaning Gly, were active against acrylamide embryotoxicity. Factors that may be responsible for the differences between CySH and GSH on one hand and NAC on the other include the presence of ionizable SH and NH_3^+ functional groups in the former, but only the SH functional groups the latter (**Figure 1**). The higher basicity (pK_a value that governs ionization of R−SH to $RS^- + H^+$) of the SH moiety of NAC than the corresponding macroscopic p*K* values of the SH groups of CySH and GSH may also influence molecular events involved in protection against teratogenesis. The macroscopic pK_a value of the SH groups of CySH equals ∼8.3; of GSH, ∼8.7; and of NAC, ∼9.5 (*39*). These considerations imply that basicity, nucleophilicity, distribution of positive and negative charges, and reactivity of different cysteine microscopic species as a function of pH illustrated in **Figure 1B** may influence the uptake of NAC by the embryo cells differently than of CySH and GSH.

The following additional comments are relevant to mechanistic aspects. In a previous study (*40*), we found that correction of the observed second-order rate constants for 14 thiol compounds, including CySH and NAC, to identical thiol anion (RS^-) concentrations gave computed rate constants whose logarithms showed a linear dependence on the pK_a values of the thiol groups

in similar steric environments. Because the ionized form of NAC has a higher pK_a value and is therefore a stronger nucleophile than the corresponding CySH and GSH ions, and based on related kinetic studies on the nucleophilic addition of SH groups of amino acids and peptides to the electrophilic double bond of acrylamide and acrylonitrile (*3*, *41*), as well as on related quantum mechanical calculations (*42*), we expected that NAC would be more effective than CySH or GSH in protecting the embryos against acrylamide-induced malformations. Because we found this not to be the case, we propose the above explanation for the protective effect involving differences in charge distributions on the various cysteine species illustrated in **Figure 1B**. This aspect merits further study.

Significance for the Diet and Food Safety. With respect to dietary significance, CySH is widely used as an additive to flour during bread-baking, where it acts as an antioxidant and participates in sulfhydryl−disulfide interchange reactions with wheat gluten proteins, resulting in higher-volume bread loafs (*43*). Generally recognized-as-safe NAC is sold as an antioxidant.

The cited and present studies indicate that CySH and GSH have the potential to exert dual beneficial effects: reduce acrylamide formation during food processing and protect against adverse in vivo effects after consumption of acrylamide-containing food. It is also relevant to note that both CySH and NAC have been reported to mitigate the potential toxicity of several other undesirable food ingredients. These include inhibition of enzymatic and nonenzymatic food browning, lysinoalanine formation, and inactivation of soybean inhibitors of digestive enzymes and the mutagenicity of aflatoxin B1 and of a tetrachloroimide compound formed in poultry processing water (*37*, *44*−*46*).

In conclusion, the results of the present study suggest that dietary CySH and GSH have the potential to protect against acrylamide-induced malformations (birth defects) of fetuses and that NAC has the potential to protect against acrylamide-induced toxicity (mortality) but not against malformations. The protective effects observed in the present study complement our earlier findings on protective effects of other food ingredients against potato glycoalkaloid-induced teratogenesis in frog embryos (*29*, *31*). Collectively, the present and cited studies demonstrate the potential of SH-containing amino acids and peptides to improve food safety and human health. Finally, because acrylamide is an α,β-unsaturated compound of the type-2 alkene chemical class (*42*), the present findings are also relevant to possible developmental toxicity of acrolein, acrylonitrile, methyl acrylate (*47*), methyl-vinyl ketone, methylvinyl sulfone (*48*, *49*), dehydroalanine, and other food and environmental toxicants of this class.

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