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Rebuttal on Digestibility of Food Allergens and Nonallergenic Proteins in Simulated Gastric Fluid and Simulated Intestinal Fluid—A Comparative Study

Sir: We thank Dr. Steve Taylor for his interest in our work presented in Fu et al. (2002). The goals of this study were to compare the relative digestive stabilities of food allergens versus nonallergens under well-defined conditions and to determine whether protein digestibility measured in vitro correlates with allergenic potential. Our purpose was not to comment on the usefulness of digestive stability as a criterion in a decision tree approach to assess the allergenic potential of novel proteins, as suggested by Dr. Taylor, although our results may provide important considerations when such a criterion is used for the assessment. We have addressed this issue in another publication (Fu, 2002). We are baffled by Dr. Taylor's comments equating our findings with the "abandonment of pepsin resistance as one of the criteria in protein allergenicity assessment" because nowhere in our paper did we advocate this. We feel that this debate, although important, is outside the scope of our paper.

We are aware that several international organizations have recommended the use of protein digestibility (or pepsin resistance) as one of the criteria for the assessment of the allergenic potential of genetically modified foods, the rationale in part stemming from the observation that many food allergens exhibit proteolytic stability (Taylor and Lehrer, 1996; FAO, 1996; FAO/WHO, 2000). However, as shown in our study, some, but not all, allergens are stable, and they are not necessarily more resistant to proteolytic digestion than proteins with unproven allergenicity. Some nonallergens exhibit high stability to digestion, as Dr. Taylor concurs. Therefore, as stated in our paper, we feel that it is difficult to distinguish allergens from nonallergens on the basis of digestibility measured in vitro.

That resistance to digestion in vitro may not be a defining characteristic of food allergens is a view shared by others (Wal, 1998; Vieths et al., 1999; Kerna and Evans, 2000; Yagami et al., 2000). Nevertheless, there is a common belief that a protein must retain sufficient structural integrity following oral ingestion to elicit an allergic response (Metcalfe et al., 1996; Taylor and Hefle, 2001; Taylor, 2002). A protein that is resistant to digestion in the human gastrointestinal tract may have an increased probability of stimulating immune reactions. Resistance to digestion is thus still considered to be a relevant parameter for the assessment of protein allergenicity (FAO/WHO, 2001).

In vitro digestion models utilizing simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) provide simple, well-controlled systems for estimating the relative resistance of a protein to proteolytic digestion. However, conditions used in these assays can greatly influence the digestibility measured and thus the perceived allergenic potential. As we have found in our study, food allergens as well as nonallergens may appear to be highly stable or unstable depending on the relative amount of pepsin and test protein used in an SGF assay. For example, at a pepsin/test protein ratio of 10, the allergen ovalbumin was degraded within 5 min, but when the ratio was decreased to 0.1, intact ovalbumin and stable peptide fragments were observed for the full 2 h of reaction. At a ratio of 0.1, the nonallergen sucrose synthetase appeared to be stable. Similar results were reported by Astwood et al. (1996), who showed that the nonallergen rubisco resisted digestion for the full 1 h of reaction when the concentration of pepsin was reduced 100-fold.

Consensus has not been reached regarding the optimal enzyme/test protein ratio used in a digestion assay. A great deal of variation exists in the enzyme/test protein ratios used in protein digestibility studies (Fu, 2002). Nutritional studies (Marquez and Lajolo, 1981; Kamata et al., 1982; Nielsen at al., 1988; Sze-Tao and Sathe, 2000) and studies concerning the effects of proteolytic digestion on protein allergenicity (Maynard et al., 1977; Asselin et al., 1989; Watanabe et al., 1990) generally used enzyme/test protein ratios of 0.1−0.001. Higher ratios (20−5000) were used in studies that examined the digestive stability of transgenic proteins. For example, Noteborn (1998) employed pepsin/test protein ratios of 20−64 on Cry9C engineered into StarLink corn. A ratio of 800 was used in the SGF assay for ACCd expressed in delayed-ripening tomatoes (Reed et al., 1996). The study by Harrison et al. (1996), who used a pepsin/test protein ratio of 1600 on EPSPS engineered into soybeans, has been cited as an example of the successful application of in vitro digestive assays for assessing the allergenic potential of proteins (Taylor and Hefle, 2001; Taylor, 2002). It is not clear whether the same degradation rate would be observed if the enzyme/protein ratios used in these assays were reduced. A need exists to establish standardized assay conditions so that results may be directly compared across diffterent laboratories. We are glad to see Dr. Taylor's expression of a similar view. Dr. Taylor's comments may be grouped into several areas, and we will address each of them individually.

Pepsin/Test Protein Ratio Used. We are puzzled by Dr. Taylor's criticism of our pepsin/test protein ratio as "ridiculously" high. Our study was an extension of the work by Astwood et al. (1996), which is frequently cited in the field of protein allergenicity assessment and endorsed by many, including Dr. Taylor (Metcalf et al., 1996; FAO/WHO, 2000; Taylor and Hefle, 2001; Taylor, 2002; Hollingworth et al., 2003). The pepsin/test protein ratio used in our study was 13 compared to 19 used by Astwood et al. (both were at the lower end of the ratios generally used in studies examining the digestive stability of transgenic proteins). Although we used a slightly lower ratio, we generally observed a greater rate of degradation in proteins also tested by Astwood et al., and we indicated that this difference might be attributed to the purity of the pepsin used. Our findings seem to agree with those reported by Dearman et

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al. (2002), who used an SGF assay similar to that of Astwood et al. and found that ovalbumin was resistant to degradation for 5 min, a time frame similar to ours but much shorter than the 60 min reported by Astwood et al.

In addition, there may also be some problems in the Astwood study regarding the *Ara h* 2 used. Normally, *Ara h* 2 migrates as a doublet on SDS-PAGE gels with an average molecular mass of ~17 kDa (Burks et al., 1992; Beyer et al., 2002; Sen et al., 2002); however, a careful examination of the gel images shown in the Astwood paper indicates that the *Ara h* 2 used in their study appeared as a single band of ~10 kDa. This difference in the compound used in the Astwood study may explain why a different digestibility of *Ara h* 2 was reported in their study.

We quantified the amount of pepsin used in our study by weight, as did Astwood et al. and many others (USP, 1995; Marquez and Lajolo, 1981; Kamata et al., 1982; Nielsen et al., 1988; Maynard et al., 1977; Asselin et al., 1989; Watanabe et al., 1990; Sze-Tao and Sathe, 2000). The pepsin resistance protocol suggested by the 2001 FAO/WHO consultation (FAO/WHO, 2001) was also based on weight. However, we agree with Dr. Taylor that enzyme activity would provide a more consistent measure of the pepsin used and should be adopted when standardized assay protocols are developed.

Staining Methods. We used Coomassie brilliant blue staining, a method frequently used to analyze proteins and protein degradation (Watanabe et al., 1990; Roux et al., 2001; Beyer et al., 2002; Sen et al., 2002). We also tried colloidal blue staining on some proteins but did not see differences in the degradation rates observed. We agree with Dr. Taylor that a more sensitive method (such as silver staining) may reveal more degradation fragments, and we encourage additional studies to compare the relative digestibilities of food allergens and nonallergens using more sensitive staining methods.

Selection of Allergens. We disagree with Dr. Taylor's assessment of the allergenic status of papain and bromelain. Our paper cited relevant studies confirming elicitation of allergic reactions due to ingestion of papain and bromelain. Furthermore, the allergenicity of papain has been confirmed by the double-blind placebo-controlled food challenge test, a gold standard for determining protein allergenicity (Mansfield et al., 1985).

Selection of Nonallergens. We focused our comparison on food allergens and nonallergens of closely related functions or sequences. A number of the nonallergens chosen therefore would be highly homologous to their allergenic counterparts. None of the nonallergens chosen for our study, to the best of our knowledge, have been reported to be associated with human allergic reactions.

We feel that Dr. Taylor's use of sequence homology to classify lectins and proteinase inhibitors as good allergen candidates can be problematic. Proteins that share high sequence homology with allergens are not necessarily allergens themselves. Several examples were indicated in our paper. Human α-lactalbumin, although belonging to the same PIR superfamily as the milk allergen bovine α-lactalbumin, is not allergenic. Tropomyosins from beef, chicken, and pork, which have amino acid sequences that are 50−60% homologous to that of the major allergen shrimp tropomyosin, have not been reported as allergenic (Reese et al., 1999). Similarly, bovine pancreas trypsin inhibitor has not been reported to elicit allergic reactions, even though it shares the same Kazal proteinase inhibitor homology with the egg allergen ovomucoid.

We are confused by Dr. Taylor's conflicting assessments of beef, pork, and chicken tropomyosins as true nonallergens in his comment 3 but as poor choices for nonallergens in his comment 2.

We stress that contrary to Dr. Taylor's comments, in addition to lectins and proteinase inhibitors, other examples present in our paper still suggest the same conclusion: food allergens are not necessarily more resistant to digestion in vitro than nonallergens, and the digestive stability of major allergens is not necessarily greater than that of minor allergens. There does not seem to be a clear correlation between the digestive stability of a protein measured in vitro and its allergenicity.

Percent Allergenicity. The relative allergenicity of a food allergen is customarily measured by the percentage of individuals having allergy to certain foods who show IgE for that specific allergen. Percent allergenicity has been used for the estimation of the allergenic potential of protein and for the classification of food allergens as major or minor allergens (King et al., 1994: Bush and Hefle, 1996; Fuchs and Astwood, 1996; Taylor, 2000). Fuchs and Astwood (1996) were the first to use such a quantitative estimation to rank protein allergenicity in their comparison of the relative digestibilities of food allergens. Their results have been accepted by many (Metcalfe et al., 1996; Taylor, 1997), and we adopted the same approach.

We agree with Dr. Taylor's point that the relative allergenicity of a protein largely depends on the patients used for the analysis. It is possible that the allergenicity of lactoperoxidase as reported by Baldo (1984) may be biased due to the small number of patients tested. However, it is known that milk has multiple allergens, and not all of them have been identified. The fact that a majority of patients with milk allergens in Baldo's study had IgE reactive to lactoperoxidase would make it inappropriate, in our view, to discount the allergenic potential of this protein.

In Vivo Relevance. Dr. Taylor may be correct in that patatin, which was found to be labile to digestion in SGF, may survive digestion in vivo due to its high abundance as a constituent in potatoes. However, one can make the same argument regarding rubisco, which is the most abundant protein in the world, representing 25% of the total protein in plants (Ellis, 1979), and a nonallergen that is readily degraded in SGF.

The issue relating to how digestive stability (or pepsin resistance) is used in combination with other criteria, including relative abundance or sequence homology, in a decision tree approach to assess protein allergenicity is actively being discussed in the scientific community. We feel that such a discussion, although important, is beyond the scope of our paper.

LITERATURE CITED

Asselin, J.; Hébert J.; Amiot, J. Effects of *in vitro* proteolysis on the allergenicity of major whey proteins. *J. Food Sci.* **1989**, *54*, 1037−1039.

Astwood, J. D.; Leach, J. N.; Fuchs, R. L. Stability of food allergens to digestion *in vitro*. *Nat. Biotechnol.* **1996**, *14*, 1269−1273.

Baldo, B. A. Milk allergies. *Aust. Dairy Technol.* **1984**, *39*, 120−128.

Beyer, K.; Bardina, L.; Grishina, G.; Sampson, H. A. Identification of sesame seed allergens by 2-dimensional proteomics and Edman sequencing: seed storage proteins as common food allergens. *Allergy Clin. Immunol.* **2002**, *110*, 154−159.

Burks, A. W.; Williams, L. W.; Connaughton, C.; Cockrell, G.; O'Brien, T. J.; Helm, R. M. Identification and characterization of a second major peanut allergen, *Ara h* II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J. Allergy Clin. Immunol.* **1992**, *90*, 962−969.

Bush, R. K.; Hefle, S. L. Food allergens. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*(S), S119−S163.

Dearman, R. J.; Caddick, H.; Stone, S.; Kenna, J. G.; Basketter, D. A.; Kimber, I. Immunogenic properties of rapidly digested food proteins following gavage exposure of mice: a comparison of ovalbumin with a potato acid phosphatase preparation. *Food Chem. Toxicol.* **2002**, *40*, 625−633.

Ellis, R. J. The most abundant protein in the world. *Trends Biochem.* **1979**, *4*, 241.

FAO. *Biotechnology and Food Safety*; Report of a joint FAO/WHO expert consultation; Food and Nutrition Paper 61; Food and Agricultural Organization of the United Nations (FAO): Rome, Italy, 1996.

FAO/WHO. *Safety Aspects of Genetically Modified Foods of Plant Origin*; Report of a joint FAO/WHO expert consultation on foods derived from biotechnology; World Health Organization: Geneva, Switzerland, 2000.

FAO/WHO. *Evaluation of Allergenicity of Genetically Modified Foods*; Report of a joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology; Food and Agriculture Organization of the United Nations (FAO): Rome, Italy, 2001.

Fu, T.-J. Digestion stability as a criterion for protein allergenicity assessment. *Ann. N. Y. Acad. Sci.* **2002**, *964*, 99−110.

Fu, T. J.; Abbott, R. U.; Hatzos, C. Digestibility of food allergens and nonallergenic proteins in simulated gastic and intestinal fluids—a comparative study. *J. Agric. Food Chem.* **2002**, *50*, 7154−7160.

Fuchs, R. L.; Astwood, J.-D. Allergenicity assessment of foods derived from genetically modified plants. *Food Technol.* **1996**, Feb, 83−88.

Harrison, L. A.; Bailey, M. R.; Naylor, M. W.; Ream, J. E.; Hammond, B. G.; Nida, D. L.; Burnette, B. L.; Nickson, T. E.; Mitsky, T. A.; Taylor, M. L.; Fuchs, R. L.; Padgette, S. R. The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J. Nutr.* **1996**, *126*, 728−740.

Hollingworth, R. M.; Bjeldanes, L. F.; Bolger, A. L.; Kimber, I.; Meade, B. J.; Taylor, S. L.; Wallace, K. B. Society of Toxicology position paper: The safety of genetically modified foods produced through biotechnology. *Toxicol. Sci.* **2003**, *71*, 2−8.

Kamata, Y.; Otsuka, S.; Sato, M.; Shibasaki, K. Limited proteolysis of soybean β-conglycinin. *Agric. Biol. Chem.* **1982**, *46*, 2829−2834.

Kenna, J. G.; Evans, R. M. Digestibility of proteins in simulated gastric fluid. *Toxicologist* **2000**, *54*, 141.

King, T. P.; Hoffman, D.; Lowenstein, H.; Marsh, D. G.; Platts-Mills, T. A. E.; Tbomas, W. Allergen nomenclature. *Int. Arch. Allergy Immunol.* **1994**, *105*, 224.

Mansfield, L. E.; Ting, S.; Haverly, R. W.; Yoo, T. I. The incidence and clinical implications of hypersensitivity to papain in an allergic population, confirmed by blinded oral challenge. *Ann. Allergy* **1985**, *55*, 541−543.

Marquez, U. M. L.; Lajolo, F. M. Composition and digestibility of albumins, globulins, and glutenins from *Phaseolus vulgaris*. *J. Agric. Food Chem.* **1981**, *29*, 1068−1074.

Maynard, F.; Jost, R.; Wal, J.-M. Human IgE binding capacity of tryptic peptides from bovine α-lactalbumin. *Int. Arch. Allergy Immunol.* **1997**, *113*, 478−488.

Metcalfe, D. D.; Astwood, J. D.; Townsend, R.; Sampson, H. S.; Taylor, S. L.; Fuchs, R. L. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Food Sci. Nutr.* **1996**, *36* (S), S165−S186.

Nielsen, S. S.; Deshpande, S. S.; Hermodson, M. A.; Scott, M. P. Comparative digestibility of legume storage proteins. *J. Agric. Food Chem.* **1988**, *36*, 896−902.

Noteborn, H. Assessment of the stability to digestion and bioavailability of the LYS mutant Cry9C protein from *Bacillus thuringiensis* serovar *Tolworthi.* Submitted to EPA by AgrEvo, EPA MRID No. 44734305, 1998.

Reed, A. I.; Kretzmer, K. A.; Naylor, M. W.; Finn, R. F.; Margin, K. M.; Hammond, B. G.; Leimgruber, R. M.; Rogers, S. G.; Fuchs, R. L. Safety assessment of 1-aminocyclopropane-l-carboxylic acid deaminase protein expressed in delayed ripening tomatoes. *J. Agric. Food Chem.* **1996**, *44*, 388−394.

Reese, G.; Ayuso, R.; Lehrer, S. B. Tropomyosin: An invertebrate pan-allergen. *Int. Arch. Allergy Immunol.* **1999**, *119*, 247−258.

Roux, K. H.; Teuber, S. S.; Robotham, J. M.; Sathe, S. K. Detection and stability of the major almond allergen in foods. *J. Agric. Food Chem.* **2001**, *49*, 2131−2136.

Sen, M. M.; Lopper, R.; Pons, L.; Abraham, E. C.; Burks, A. W.; Bannon, G. A. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J. Immunol.* **2002**, *169*, 882−887.

Sze-Tao, K. W. C.; Sathe, S. K. Functional properties and in vitro digestibility of almond (*Prunus dulcis* L.) protein isolate. *Food Chem.* **2000**, *69*, 153−160.

Taylor, S. L.; Lehrer, S. B. Principles and characteristics of food allergens. *Crit. Rev. Food Sci. Nutr.* **1996**, *36* (8), S91−S118.

Taylor, S. L. Food from genetically modified organisms and potential for food allergy. *Environ. Toxicol. Pharmacol.* **1997**, *4*, 121−126.

Taylor, S. L. Topic 13 Allergenicity; Joint FAO/WHO expert consultation on foods derived from biotechnology; Geneva, Switzerland, 2000.

Taylor, S. L.; Hefle, S. L. Will genetically modified foods be allergenic? *Curr. Rev. Allergy Clin. Immunol.* **2001**, *107*, 765−771.

Taylor, S. L. Protein allergenicity assessment of foods produced through agricultural biotechnology. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 99−112.

USP. Simulated Gastric Fluid, TS. In *The United States Pharmacopeia 23, The National Formulary 18*; The United States Pharmacopeial Convention, Inc.: Rockville, MD, 1995; p 2053.

Vieths, S.; Reindl, J.; Muller, U.; Hoffmann, A.; Haustein, D. Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. *Bur. Food Res. Technol.* **1999**, *209*, 379−388.

Yagami, T.; Haishima, Y.; Nakamura, A.; Osuna, H.; Ikezawa, Z. Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J. Allergy Clin. Immunol.* **2000**, *106*, 752−762.

Wal, J. M. Strategies for assessment and identification of allergenicity in (novel) foods. *Int. Dairy J.* **1998**, *8*, 413−423.

Watanabe, M.; Miyakawa, J.; Ikezawa, Z.; Suzuki, Y.; Hirao, T.; Yoshizawa, T.; Arai, S. Production of hypoallergenic rice by enzymatic decomposition of constituent proteins. *J. Food Sci.* **1990**, *55*, 781−783.

Tong-Jen Fu,[†] Upasana R. Abbott,[§,#] and Catherine Hatzos[§]

U.S. Food and Drug Administration and Illinois Institute of Technology, National Center for Food Safety and Technology, Summit-Argo, Illinois 60501

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[†] U.S. Food and Drug Administration.
[§] Illinois Institute of Technology.
[#] Present address: NOW Foods, 395 S. Glen Ellyn Rd., Bloomingdale, IL 60108.