PEPSIN DIGESTIBILITY ASSAY

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1 INTRODUCTION

Unlike ingested chemicals, novel dietary proteins have a predictable metabolic fate in the human or animal gut that is similar to the fate of conventional dietary proteins. To test this metabolic prediction for novel proteins, in vitro studies with simulated digestive solutions have been widely used. Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system in order to reach and pass through the intestinal mucosa to elicit an allergic response (Metcalf et al., 1996; Taylor et al., 1987; Taylor, 1992). Although some researchers (Veiths et al., 1999; Kenna and Evans, 2000; Fu, 2002; reviewed by Fu et al., 2002) have questioned the validity of digestion stability as a criterion for protein allergenicity assessment, it is one component of a comprehensive weight-of-evidence approach to assessing allergenic potential (Codex, 2003).

The test method for the assessment was first described by Astwood et al. (1996). The assay is not meant to predict whether a given protein will always be digested in the stomach of the human consumer, but does provide a simple in vitro correlative assay to evaluate protein digestibility. Investigation of proteins that have been tested, suggest a strong positive predictive value that food allergens causing systemic reactions are relatively stable in the assay, while non-allergenic food proteins are typically digested relatively quickly (Bannon et al., 2002).

Following is one of a series of test protocols for use in the testing of novel proteins expressed in recombinant DNA plants and foods derived from these, and the development of test data that must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under Rules, 1989, of the Environmental Protection Act, 1986.

The source materials used in developing this protocol are listed in the literature section. There is currently no standard protocol, although recommendations for some testing parameters are included in the Report of a Joint FAO/WHO Expert Consultation on the Allergenicity of Foods Derived from Biotechnology (2001).

2 PURPOSE

All newly expressed proteins in recombinant-DNA plants that could be present in the final food should be assessed for their potential to cause allergic reactions. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case by case approach is used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the
amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation and heat stability.

Resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic.

3 DEFINITIONS

3.1 Allergenic Potential

Refers to the potential to induce sensitization and cause food allergy.

3.2 Recombinant-DNA Plant

Means a plant in which the genetic material has been changed through in vitro nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

4 PRINCIPLE OF THE TEST

Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in nine laboratories (Thomas et al., 2004). Porcine pepsin is an aspartic endopeptidase with broad substrate specificity. Pepsin is optimally active between pH 1.2 and 2.0, but inactive at pH 3.5 and irreversibly denatured at pH 7.0 (Collins and Fine, 1981; Crevieu-Gabriel et al., 1999).

The assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. The original assay described by Astwood et al. (1996) recommends performing the digestion at pH 1.2, however, the FAO/WHO (2001) recommends using two pH conditions (pH 1.2 and pH 2.0). The assay is performed at 37°C and samples are removed at specific times and the activity of pepsin is quenched by neutralization with carbonate buffer and sodium dodecyl sulfate (SDS-) polyacrylamide gel electrophoresis (PAGE) loading buffer, then heating to more than 70°C for 3 to 5 minutes. The timed digestion samples are separated by SDS-PAGE and stained with Coomassie or colloidal blue to evaluate the extent of digestion.

A review of the digestibility assay by Bannon et al. (2002) and by Thomas et al. (2004) indicates that most of the non-allergenic food proteins that have been tested are digested by approximately 30 seconds, while major food allergens are stable, or produce pepsin-stable fragments that are detectable for from eight to 60 minutes.
5 DESCRIPTION OF THE METHOD

5.1 TEST SYSTEM

The test system is an *in vitro* digestion model using porcine pepsin in simulated gastric fluid (SGF) at pH 1.2. SGF preparation and digestion procedures are based on the methods described by Thomas *et al.* (2004). The pepsin activity assay is based on the method described by Sigma for determining the activity of pepsin.

5.2 PREPARATION OF SGF

The SGF reaction buffer is prepared by adding 122.8 mg of NaCl to 59.2 ml of distilled water and adjusting the pH to either pH 1.2 or pH 2.0 using 6 N HCl. The HCl content is approximately 0.084 N, and the NaCl concentration is 35 mM. The amount of pepsin used to prepare SGF is calculated from the specific activity of the product. One unit of activity is defined as a change in $A_{280\text{ nm}}$ of 0.001 at 37°C, measured as trichloracetic acid (TCA)-soluble products using hemoglobin as the substrate. The assay is designed for fixed volumes and a fixed amount of test protein so the amount of pepsin diluted in SGF is adjusted to provide the appropriate ratio of 10 units of pepsin activity per microgram of test protein in the digestion mixture.

5.3 ASSAY PARAMETERS

At predetermined times (*e.g.*, 0, 0.5, 1, 2, 5, 10, 20, 30, 60 minutes) a fixed volume of the digestion reaction mixture is withdrawn and added to sample tubes containing neutralization and denaturing reagents, which stop the digestion. Samples are then heated to ~95°C before analysis by SDS-PAGE, or storage at -20°C for later analysis. All samples from a single digestion experiment are applied to wells of the same SDS-PAGE gel along with molecular weight markers. Control samples include: test protein in SGF reaction mixture without added pepsin, T=0 min; test protein in SGF reaction mixture without added pepsin, T=60 min; SGF with added pepsin but without test protein, T=0; SGF with added pepsin but without test protein, T=60; and a 10% test protein sample and quenched pepsin without SGF reaction mixture (to verify detectability of at least 10% of the original protein concentration). Samples are separated by electrophoresis, fixed, stained with Coomassie or colloidal blue G-250, destained and analyzed.

Western blot analysis of the digested samples with antibodies specific to the test protein can be used to illustrate specific digestion of the target protein and the presence or absence of lower molecular weight digestion products. Western blot analysis should be included in addition to colloidal blue stained gels and not as a replacement.

5.4 INTERPRETATION OF RESULTS

The stability of the protein is defined as the time required to reach 90% digestion, which is estimated based on the shortest time-digested sample with a band intensity equal to, or less than the 10% undigested standard. Any new bands above approximately 3,000 MW that are
generated as intermediate products of digestion would be noted as stable (or partially stable) intermediate proteolytic fragments and would be analyzed in addition to the test protein. Western blot analysis would identify if any of the intermediate products are derived from the test protein.

Proteins with more than 10% stainable full-length protein band remaining at > 30 to 60 minutes are considered stable. Proteins reduced to < 10% stainable band at 5 to 30 minutes are considered of intermediate stability. Proteins reduced to < 10% stainable band by 2 minutes are considered labile (rapidly digested).

5.5 **DATA AND REPORTING**

Individual gel images should be presented, along with digital analysis of band intensities for quantitative evaluation of digestion. Control gels should be used to establish the limits of detection of protein amount using the staining and detection methods.

The test report must include the following information, as appropriate:

5.5.1 **Test protein**

Physical state, purity, concentration, source, batch/lot reference number, storage conditions, identity of the test protein, and, where relevant, physiochemical properties. When the test protein has been isolated from a source other than the recombinant DNA plant, a characterization of the test protein and demonstration of equivalence with the plant-expressed form of the protein is required (normally as a separate study and report).

5.5.2 **Detailed testing protocols**

The full experimental procedures should be detailed including the source of all chemicals, reagents, controls and standards.

5.5.3 **Results**

- All gel images should be included;
- Data from digital analysis of band intensities;
- A comparison of digestion time points with amount of protein should be provided.

6 **LITERATURE**


