

Identification of Sunflower Seed IgE-Binding Proteins

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Key Words

Sunflower seed · IgE-binding protein · Allergens · SFA-8/SSA

Abstract

Background: Sunflower seed can cause severe anaphylactic reactions in some susceptible individuals. It is conceivable that the 2S sunflower seed protein is an allergen based on its high degree of homology (34%) with the allergenic mature 2S albumin protein of the Brazil nut. The first step in determining the allergenicity of sunflower seed proteins is to identify IgE-binding proteins. **Methods:** Sera from sunflower seed-sensitive individuals were evaluated by radioallergosorbent test (RAST), isoelectric focusing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting with sunflower seed proteins. **Results:** Positive RAST scores (>2) were observed in 3 individuals and immunoblotting demonstrated IgE-binding to 2–7 distinct proteins ranging in size from 10 to 50 kD. Two out of 3 sera recognized two proteins between 16 and 17 kD. The lower molecular weight protein (16 kD) approximates to the prepo region of the precursor methionine-rich 2S albumin protein found in sunflower seed (SFA-8/SSA). IEF followed by immunoblotting demonstrated several IgE-binding proteins, including two proteins with isoelectric points of

5.97 and 5.3, respectively, which are consistent with the mature and immature forms of the SFA-8/SSA region.

Conclusions: Sunflower seed contains several IgE-binding proteins, including regions of the high-methionine 2S albumin SFA-8/SSA.

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Introduction

Sunflower (*Helianthus annuus*) seeds are popular as snacks and as bread condiment. Sunflower seed products are used extensively as consumable commodities in margarine, and cooking and salad oils for humans, as well as for poultry [1]. In spite of their popularity, sunflower seeds have been reported to cause anaphylactic reactions after ingestion [2–4] and rhinitis and asthmatic reactions after occupational exposure to sunflower seed dust [5]. The identity of sunflower seed allergens is unknown. Sunflower seeds contain 2S storage albumins which in other seeds such as rapeseed, castor seed, mustard seed, cottonseed and also nuts (Brazil nut and walnut) have been shown to be allergenic [6–13], but their contribution to allergy to sunflower seed is unknown. There is also a lack of information regarding other nonalbumin allergens in sunflower seed.

There are insufficient data regarding the relationship between sunflower seed and type I allergy (hypersensitivity) as previous reports on sunflower seed allergy have not investigated this association in depth. Type I allergy is initiated by the production of immunoglobulin E (IgE) antibodies to proteins from the offending food. One or more proteins cross-link to IgE-sensitized mast/basophil cells causing the release of endogenous mediators associated with allergenicity [14].

It is conceivable that 2S proteins present in sunflower seed may be associated with its allergenicity. One of the 2S proteins in sunflower seed is sunflower albumin-8 (SFA-8) otherwise known as sunflower seed albumin (SSA), which is very rich in sulfur-containing amino acids [15]. This property makes it a target for genetic manipulation into crops deficient in sulfur-containing amino acids. It consists of a single polypeptide chain [15, 16] with four intra-chain disulphide bonds [17]. The predicted mass of the immature protein is approximately 16 kD (isoelectric point 5.35), but at different growth stages of the seed this protein is cleaved to form the mature protein with a mass of approximately 12 kD (pI 5.91) [18]. To date, SFA-8/SSA has not been investigated as a possible allergen in sunflower seed-allergic patients. Consequently, the purpose of this study is to determine the presence of IgE-binding proteins in sunflower seed and to determine their role in sunflower seed allergenicity.

Materials and Methods

Extraction

Salted roasted and unsalted raw meat sunflower seeds were ground and then respectively extracted (1:10) in either 0.01 M phosphate-buffered saline, pH 7.4 (PBS) or double-distilled water overnight with shaking at room temperature. The extracts were clarified (3,180 g for 30 min at 4°C) and stored at -20°C.

Characterization of Sera

Serum samples were obtained from individuals with a positive skin prick test (range 1+ to 4+) and positive radioallergen sorbent test (RAST) (range RAST ratio 1–71 times control) to sunflower seed, and a convincing history of IgE-mediated reaction to sunflower seed. Serum samples were obtained under informed consent using a procedure approved by the Institutional Review Board of the University of Nebraska, Lincoln. The control sera sample contained equal amounts of serum from 5 atopic but not food-allergic individuals.

RAST Analysis

Sera from 5 sunflower seed-sensitive subjects were analyzed for sunflower seed-specific IgE as described by Hefle et al. [19] with slight modifications. Sera from atopic control subjects were pooled and used for a negative control.

SDS-PAGE and Isoelectric Focusing Electrophoresis and Immunoblotting

Both sunflower seed extracts and molecular weight markers were loaded and separated by either a 10–20% gradient gel (Novex, San Diego, Calif., USA) or a preparative (12.5% resolving gel) SDS-PAGE. After electrophoresis, the 10–20% gradient tricine gel was stained with 0.1% Coomassie blue and the preparative gel was equilibrated in 25 mM Tris-HCl, 192 mM glycine containing 20% MeOH (pH 8.3) solution for at least 15 min and then transferred to a PVDF membrane (Millipore, Bedford, Mass., USA) using a semi-dry blotter (Labconco, Kansas City, Mo., USA). For isoelectric focusing (IEF), double-distilled sunflower seed extract and IEF markers (Sigma Chemical, St. Louis, Mo., USA) were loaded and separated onto an IEF pH 3–10 precast gel according to the manufacturer's protocol (Novex). After electrophoresis, the gel was stained in 0.1% Coomassie blue (BioRad, Hercules, Calif., USA) solution to visualize proteins, soaked in water for 3.5 h, incubated in a reducing solution (0.28% SDS, 0.35 M Tris-HCl and 5% β-mercaptoethanol, pH 8.3) for 1 h and equilibrated in 25 mM Tris-HCl, 192 mM glycine containing 20% MeOH solution for 15 min. The gel was then blotted onto a PVDF membrane by semi-dry blotting for at least 1 h. The membranes were blocked (1% casein, Sigma Chemical, in 0.025% Tween 20 in PBS) and incubated with diluted sera (1:5 or 1:32) in blocking solution overnight, with shaking at 4°C. Membranes were washed 4 times with PBS containing 0.025% Tween 20 before incubation with goat anti-human IgE conjugated to horseradish peroxidase in blocking solution (1:1,000; Bethyl Laboratories, Montgomery, Tex., USA) overnight at 4°C. After washing, membranes were immersed in chemiluminescent reagents (1:1; Amersham, Arlington Heights, Ill., USA) for 1 min, followed by exposure to X-ray film (Amersham) for 1–5 min. Estimated molecular weights and pIs of IgE-binding proteins were determined by best-fit models of the molecular weight and IEF standard curves. Densitometric data were obtained with UN-SCAN-IT gel software (Silk Scientific, Orem, Utah, USA).

Results

RAST Analysis

All patients were clinically diagnosed as allergic to sunflower seed based on convincing histories and accompanying skin tests, RAST, and in some cases history of anaphylaxis to sunflower seed (table 1). Not all sera exhibited a high sunflower seed RAST score. Subjects 1 and 4 had significant levels of sunflower seed-specific IgE. The RAST scores for subjects 3 and 5 were unexpectedly low, essentially not different from the control serum score.

SDS-PAGE and IEF Immunoblotting

The salted roasted and unsalted raw sunflower seed extracts demonstrated a difference in the pattern of protein profiles (fig. 1). Three out of 5 sera possessed IgE that bound to proteins in roasted sunflower seed extract (fig. 2) with immunoestimated molecular weights between 10 and 50 kD (table 2). The 2 subjects with the highest RAST

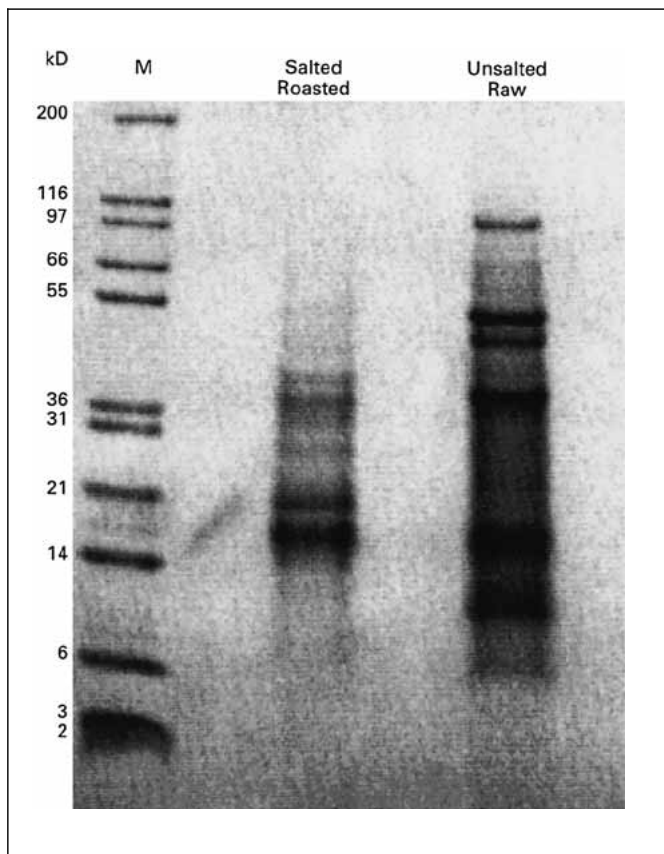


Fig. 1. SDS-PAGE of salted roasted and unsalted raw sunflower seed in a 10–20% gradient gel stained with Coomassie blue. Seventy micrograms of protein extract/lane. Lane M contains molecular weight markers.

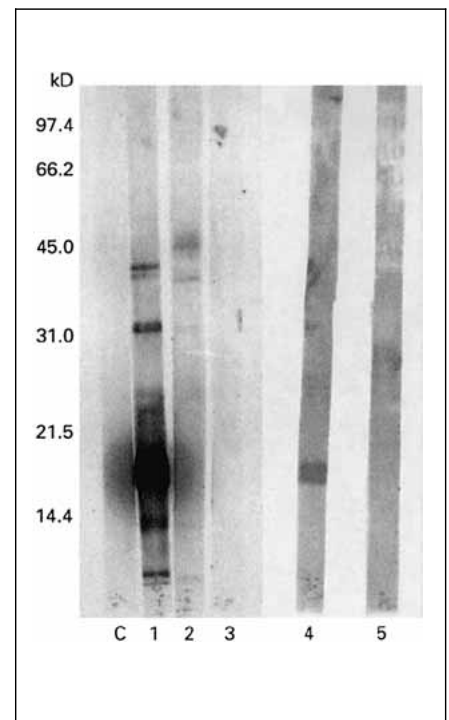


Fig. 2. SDS-PAGE of salted roasted sunflower seed followed by IgE immunoblotting from different sunflower seed-allergic subjects. Lane markers refer to subject sera, with C designating the control sera. Four hundred and forty micrograms of protein extract were loaded onto a preparative gel.

Table 1. Characteristics of sunflower seed-allergic subjects

Subject No.	Gender	SPT class ¹	RAST ²	Symptoms
Control (pooled)	M/F		2	atopic, non-food allergic
1	M	4+	71	vomiting, hypotension
2	F	4+	7	urticaria, angioedema
3	M	1+	2	itchy mouth
4	F	4+	9	anaphylaxis
5	F	3+	2	angioedema, fever

¹ Skin prick test.

² RAST score is expressed as the ratio of IgE-binding of subject serum to binding of pooled non-sunflower seed-allergic, atopic control serum. IgE-binding of control sera was equivalent to the response in analyses that contained no serum.

Table 2. Immunoestimates of the molecular weights (kD) of IgE-binding proteins of roasted sunflower seed

Subject No.	Molecular weight
1	10.5
	13.5
	15.9
	17.2
	22.7
	33.2
2	43.6
	48.9
4	15.9
	16.7

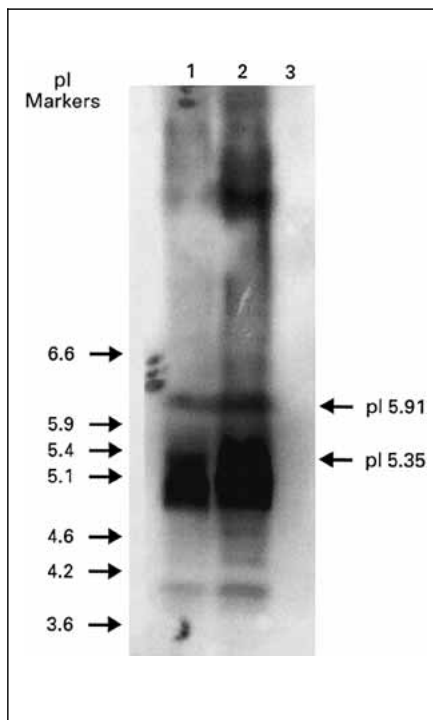


Fig. 3. IEF gel of unsalted raw sunflower seed followed by IgE immunoblotting after IEF with sera from subject 1 (1:32). Lane 1 and 2 contained 27 and 57 µg of extract, respectively. Lane 3 is the buffer lane.

scores recognized two IgE-binding proteins of a molecular weight of 16–17 kD and subject 1, with the highest RAST score, recognized several proteins including one at 13.5 kD. Immunoblotting of sunflower seed extract for IEF purposes demonstrated the presence of several IgE-binding proteins including those with pIs of 5.3 and 5.97 (fig. 3) when incubated with sera from subject 1.

Discussion

There are reports in the literature indicating that sunflower seed can cause anaphylactic reactions after ingestion [2–4]. The objective of this study was to determine whether sunflower seed possesses IgE-binding proteins. Due to the popularity of salted roasted sunflower seeds as a snack food, salted roasted sunflower seed extract was used to determine the mass of IgE-binding proteins. However, the amount of salt present in the extract was too high to determine the pIs of the IgE-binding proteins. To circumvent this problem, unsalted raw sunflower seed ex-

tract was used in pI determination studies. The roasted and raw extracts showed different protein profiles. These profiles are significant because subjects may develop allergenicity to specific proteins found in certain sunflower seed cultivars and not others. IgE from sunflower seed-sensitive subjects unequivocally bound to salted roasted sunflower seed proteins with molecular weights ranging from approximately 10 to 50 kD. One of the limitations of this study was the small number of subjects available. Thus, it is likely that other proteins may be present outside this molecular weight range that could bind IgE from the sera of other sunflower seed-allergic patients. The fact that individual variation exists between the subjects in this study suggests this possibility. Two sera from subjects with high sunflower seed RAST scores recognized two proteins with similar masses of 16 and 17 kD. Subject 1 recognized several proteins including a protein with a mass of approximately 13.5 kD. The sensitivity of chemiluminescence methods explains the detection of this protein immunochemically, but not via the Coomassie blue staining method in the salted roasted sunflower seed extract. This 13.5-kD protein has previously been reported to bind IgE from a sunflower seed-sensitive individual who experienced an anaphylactic episode [3]. It is interesting to note that sera from subjects 3 and 5 did not bind to any sunflower seed proteins and that their sunflower seed RAST scores were very low. The low RAST reactivity may be related to the efficacy of the extraction protocols used to isolate the sunflower seed allergens. This observation has been made in other studies of food allergens, particularly in sesame seed [20–22]. However, the positive skin tests in these subjects and their clinical histories attest to their allergy to sunflower seed. In vivo tests measure the presence of specific IgE bound to mast cells; in vitro tests, such as RAST only, detect the presence of IgE in serum which has a half-life of only 3 days.

The limited supply of sera from sunflower seed-allergic individuals prevents determination of which IgE-binding protein may be the major allergen. However, some interesting observations can be made regarding individual variation and the intensity of which IgE bound to these proteins. Sera from subject 1 contained a significant amount of specific IgE distributed among 7 different proteins. The major IgE-binding protein as assessed by band intensity was in the 16- to 17-kD region. Due to the amount of protein used in the SDS-PAGE, it was not possible to get quantitative densitometry data. However, qualitative observation suggests that a substantial portion of the total IgE-binding proteins were found in this region. The significant degree of IgE-binding in this region may

or may not be an indication of their importance to allergenicity; the other less intensely bound bands may also be allergenic proteins. Serum from subject 4 exclusively recognized the 16- to 17-kD proteins and the clinical history showed that this individual suffered anaphylaxis and had a strongly positive skin prick test. The anaphylactic history of subjects 1 and 4 could be easily attributed to these proteins because IgE directed against these proteins seemed to be consistently present in both sera. However, their symptoms may have been the result of an allergen not extracted using our extraction protocol.

2S albumin proteins from a variety of seeds such as castor seed, cottonseed, yellow and oriental mustard seed have been known to be allergenic proteins [6–9]. More recently, Brazil nut and walnut 2S albumins have been found to bind to human IgE and therefore have been associated with allergenicity [10, 11]. The 2S albumins of Brazil nut and sunflower seed have 34% sequence homology [18]. The high degree of homology that exists between Brazil nut and sunflower seed only suggests that the 2S sunflower seed may be either an IgE-binding protein or an allergen. However, if the specific antigenic sequences are not present, the high degree of homology is inconsequential.

The 2S albumin fraction of sunflower seed is made up of at least 8 different albumins [15], one of them designated as SFA-8/SSA [15, 23]. SFA-8/SSA is very rich in methionine and cysteine (16 and 8%, respectively). The immature mass of SFA-8/SSA is approximately 16 kD (pI, 5.35), which in the process of seed development is cleaved to form the mature protein with a mass of approximately 12 kD (pI 5.91). The results show that IgE from 2 of 3 sunflower seed-allergic subjects recognized a 16-kD protein. To further characterize SFA-8/SSA as a potential IgE-binding protein, the pI of the IgE-binding proteins of unsalted raw sunflower seed were determined by IEF followed by IgE immunoblotting using serum from a sunflower seed-allergic subject. The IgE immunoblotting findings clearly demonstrate that both regions (immature pI of 5.35 and mature pI of 5.91) of the presumed SFA-8/SSA protein bound IgE. The pooled serum from negative control atopic individuals did not recognize these proteins (data not shown). The predicted value of 5.30 and 5.97 for the immature and mature protein is within experimental error. Densitometry data indicated that 24% of the IgE-binding proteins within the pI 4.6–6.0 area had a pI of 5.97. This study shows that the mass and pI are consistent with the immature form of SFA-8/SSA. While an IgE-binding protein of pI 5.97 was observed, the mass associated with the mature SFA-8/SSA is approxi-

mately 12 kD. There is reason to believe that the 13.5-kD protein which others [3] also have detected is actually 12 kD in size; the standard curve used to estimate the molecular weight ranged only from 14.4 to 97 kD. Therefore, the expected mass of the 13.5-kD protein would be questionable.

This study presents only circumstantial evidence that SFA-8/SSA may be an IgE-binding protein. Currently, there is interest in producing transgenic crops using the SFA-8/SSA gene for animal and human foods [23, 24]. While the use of transgenic crops for animal food does not present a problem to humans, direct consumption of an IgE-binding protein may, such as the potential issue with the transgenic soybean [10]. The IgE-binding properties and allergenicity of SFA-8/SSA must be determined and evaluated before producing a crop that would potentially have adverse effects in sunflower seed-allergic individuals.

In conclusion, sunflower seeds possess several IgE-binding proteins, including regions of the immature and mature SFA-8/SSA. Transgenic manipulation of the 2S sunflower seed IgE-binding proteins for human consumption should be closely and critically evaluated in light of these findings. Further research is in progress to purify the mature SFA-8/SSA protein and assess its IgE-binding potential and allergenicity.

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References

- 1 Lusas EW: Sunflower seed protein; in Altschul AM, Wilcke HL (eds): New Protein Foods. Orlando, Academic Press, 1985, vol 5, pp 393–433.
- 2 Axelsson IG, Ihre E, Zetterstrom O: Anaphylactic reactions to sunflower seed. *Allergy* 1994; 49:517–520.
- 3 Iwaya M, Murakami G, Kurose K, Matsuno M, Onoue Y, Takayanagi M, Kayahara M, Adachi Y, Okada T: A case of anaphylaxis caused by sunflower seed (in Japanese). *Arerugi* 1994;43: 696–700.
- 4 Noyes JH, Boyd GK, Settippene GA: Anaphylaxis to sunflower seed. *J Allergy Clin Immunol* 1979;63:242–244.
- 5 Vandenplas O, Vander Borgh T, Delwiche JP: Occupational asthma caused by sunflower-seed dust. *Allergy* 1998;53:907–908.
- 6 Youle RJ, Huang AHC: Evidence that the castor bean allergens are the albumin storage proteins in the protein bodies of castorbean. *Plant Physiol* 1978;61:1040–1042.
- 7 Youle RJ, Huang AH: Albumin storage protein and allergens in cottonseeds. *J Agric Food Chem* 1979;27:500–503.
- 8 Monsalve RI, Gonzalez de la Pena MA, Mendez-Arias L, Lopez-Otin C, Villalba M, Rodriguez R: Characterization of a new oriental-mustard (*Brassica juncea*) allergen, Bra j 1E: Detection of an allergenic epitope. *Biochem J* 1993;293:625–632.
- 9 Gonzalez de la Pena MA, Villalba M, Garcia-Lopez JL, Rodriguez R: Cloning and expression of the major allergen from yellow mustard seeds, Sin a I. *Biochem Biophys Res Commun* 1993;190:648–653.
- 10 Nordlee JA, Taylor SL, Townsend JA, Thomas LA, Bush RK: Identification of a Brazil-nut allergen in transgenic soybeans. *N Engl J Med* 1996;334:688–692.
- 11 Teuber SS, Dandekar AM, Peterson WR, Sellers CL: Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* 1998;101:807–814.
- 12 Monsalve RI, Gonzalez de la Pena MA, Lopez-Otin C, Fiandor A, Fernandez C, Villalba M, Rodriguez R: Detection, isolation and complete amino acid sequence of an aeroallergenic protein from rapeseed flour. *Clin Exp Allergy* 1997;27:833–841.
- 13 Muntz K, Christov V, Saalbach G, Saalbach I, Waddell D, Pickardt T, Schieder O, Wustenhagen T: Genetic engineering for high methionine grain legumes. *Nahrung* 1998;42:125–127.
- 14 Lemke PT, Taylor SL: Allergenic reactions and food intolerances; in Kotsonis FN, Mackey M, Hjelle J (eds): Nutritional Toxicology. New York, Raven Press, 1994, pp 117–137.
- 15 Kortt AA, Caldwell JB: Low molecular weight albumins from sunflower seed: Identification of a methionine-rich albumin. *Phytochemistry* 1990;29:2805–2810.
- 16 Anisimova IN, Fido RJ, Tatham AS, Shewry PR: Genotypic variation and polymorphism of 2S albumins of sunflower. *Euphytica* 1995;83: 15–23.
- 17 Egorov TA, Odintsova TI, Musolyamov A, Fido R, Tatham AS, Shewry PR: Disulphide structure of a sunflower seed albumin: Conserved and variant disulphide bonds in the cereal prolamin superfamily. *FEBS Lett* 1996; 396:285–288.
- 18 Kortt AA, Caldwell JB, Lilley GG, Higgins TJ: Amino acid and cDNA sequences of a methionine-rich 2S protein from sunflower seed (*Helianthus annuus* L.). *Eur J Biochem* 1991;195: 329–334.
- 19 Hefle SL, Bush RK, Yunginger JW, Chu FS: A sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitation of selected peanut proteins in foods. *J Food Protect* 1994; 57:419–423.
- 20 Eberlein-Konig B, Rueff F, Przybilla B: Generalized urticaria caused by sesame seeds with negative prick test results and without demonstrable specific IgE antibodies. *J Allergy Clin Immunol* 1995;96:560–561.
- 21 Kägi M, Wüthrich B: Falafel-burger anaphylaxis due to sesame seed allergy (letter). *Lancet* 1991;338:582.
- 22 Stern A, Wüthrich B: Non-IgE-mediated anaphylaxis to sesame. *Allergy* 1998;53:325–326.
- 23 Molvig L, Tabe LM, Eggum BO, Moore AE, Craig S, Spencer D, Higgins TJ: Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. *Proc Natl Acad Sci USA* 1997;94:8393–8398.
- 24 Tabe LM, Wardley-Richardson T, Ceriotti A, Aryan A, McNabb W, Moore A, Higgins TJ: A biotechnological approach to improving the nutritive value of alfalfa. *J Anim Sci* 1995;73: 2752–2759.

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