Toxicological Assessment of Recombinant Xylanase X_{22} in Wine

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Toxicological evaluation of xylanase X_{22} from *Aspergillus nidulans* expressed in a wine yeast strain was carried out. The safety of the X_{22} intake was assessed by digestibility, bioinformatic, and mouse short-term repeated dosing studies, although X_{22} shows resistance to proteolytic degradation in the gastrointestinal system, is a minority protein component ($<0.5\ 10^{-6}$ %) of the produced wine, and shows no significant amino acid sequence homology to any known food allergens. The 4-week oral toxicity study was performed in Swiss mice at a dose level of 0.01, 0.1, or 1 mg/kg/day (these dosages correlate to 8, 80, and 800 times, respectively, the enzyme amount contained in 250 mL of wine). Body weight, food and fluid intake, urinalysis, and hematology data were obtained. Postmortem examinations and histopathology by both light and electron microscopy were performed. According to the results of this study, no adverse effects were detected by oral administration of X_{22}.

Keywords: *Xylanase X_{22}; safety assessment; in vitro digestion; short-term toxicity study; transgenic wine yeast; novel foods*

INTRODUCTION

Recombinant DNA technology provides a powerful tool to create new products in many different fields, agriculture and the agro-food industry being two of them (Day, 1996). During recent years, many winemakers have used pure *Saccharomyces cerevisiae* cultures isolated from their own growing regions to produce wines of more reproducible quality. This microbiological simplification of the wine fermentation process has opened the way for the use of genetic engineering techniques in wine yeast (Querol and Ramón, 1996). One of the most important characteristics of a quality wine is its aromatic fragrance. The *xlnA Aspergillus nidulans* gene, encoding the X_{22} xylanase from this fungus, has been cloned and sequenced. Its cDNA has been expressed in a laboratory *S. cerevisiae* strain under the control of a constitutive yeast promoter, resulting in the construction of recombinant xylanolytic yeast strains (Pérez-Gonzalez et al., 1996). Wine fermentation with these recombinant yeast releases aroma precursors from cell enzymatic degradation improving wine aroma (Ganga et al., 1998).

The X_{22} mature protein is 187 amino acids long and has a calculated molecular weight of 20.25 kDa and an isoelectric point of 6.33, in good agreement with the values determined for the purified X_{22} enzyme (Fernández-Espinar et al., 1992, 1993). Even though the gene product is completely characterized in the transgenic product, this does not necessarily provide information on its potential toxicity (OECD, 1993; Kok and Kuiper, 1996). Safety of proteins introduced by genetic modification is established by in vitro and in vivo assays. The initial assessment of transgenic proteins should be focused in their allergenic potential. There are no direct methods to assess allergenic potential of proteins from sources that are not known to produce food allergy. However, some assurance can be provided to minimize the possibility that a new protein causes an allergenic reaction by evaluating its similarity to known food allergens, resistant to simulated gastric degradations, heat stability, molecular size, and degradation through food processing (Sehat et al., 1997).

In vitro studies with simulated digestive solution are widely used as a model of animal digestion. They have been applied to investigate the digestibility of plant proteins, animal proteins and food additives (Nielson, 1988; Reed et al., 1996; Rozan et al., 1997). Animal research methods are indispensable to directly assess any potential mammalian toxicity associated with the transgenic materials (Reed et al., 1996; Fuchs et al., 1993; Querol and Ramón, 1996). Acute toxicity studies involve a qualitative and quantitative evaluation of the toxic effects of a substance, as well as the assessment of their time-related occurrence after a single administration. Although these studies provide a relative toxicological value and require exaggerated doses of the compound tested (Grau et al., 1997), it was not technically feasible to extract sufficient quantities of the X_{22} xylanase from *S. cerevisiae* to perform acute toxicity studies.

European Union (EU) recommends to select carefully the nature and extent of toxicological studies performed in novel foods or ingredients, taking into account the source and composition of the novel food, its potential

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10.1021/jf980905k CCC: $18.00 © 1999 American Chemical Society
Published on Web 03/25/1999

intake, and whether it is intended for a specific application or for more general use in the diet (EEC, 1997). Data obtained from short-term studies are more useful because they help determine potential adverse effects of the compound at doses low enough to allow survival of the animals after a longer exposure period.

The aim of this study was to evaluate the safety of the X_{22} enzyme produced by *S. cerevisiae* T_{73} in wine. For this purpose, the X_{22} potential to cause allergy was evaluated by its stability to digestion and the homology of its amino acid sequence to food allergens. Other physicochemical properties of xylanase X_{22} were also discussed. To establish inherent toxicity of X_{22}, a short-term repeated dosing toxicity study was performed at three dose levels in Swiss mice. Both in vitro and in vivo studies were selected to obtain toxicological information related with xylanase X_{22} potential intake and to confirm that this protein causes no deleterious effects.

MATERIALS AND METHODS

Test Substances. Recombinant wine yeast T_{73} strain containing the cDNA *xlnA* under the control of the actin gene promoter was used through this work. X_{22} was purified following previously published procedures (Ganga et al., 1998). Pure xylanase X_{22} was dissolved in 10 mM acetic acid−sodium acetate buffer, pH 5.0. The protein is stable for more than 18 months at −20 °C. However, the frozen/defrozen cycles caused a progressive decline in the enzyme activity. Because of this, the enzyme was aliquoted prior to performing the studies and defrozen only once.

The protein control for the 4-week toxicity study was bovine serum albumin (BSA) obtained from Sigma (St. Louis, MO). Pepsin (activity at least equivalent to United State Pharmacopeia (USP) specifications) and pancreatin (activity: 600−1800 units/mg of protein) were purchased from Sigma.

Simulated Digestion Solutions. In vitro digestion was designed to simulate the digestive system: gastric and intestinal fluids were prepared as described in the USP (1990). Gastric and intestinal control buffers were similarly prepared except they did not contain pepsin and pancreatin. Digestive fluids were used within 1 h of preparation and stored at 4 °C. Proteolytic activity was tested before the digestion process.

Homology of the Amino Acid Sequence of the Protein to Allergenic Proteins. The basic local alignment search tool (BLAST) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx. These public databases contain entries of nucleotide sequences, amino acid sequences, bibliographic citations, and other information against which nucleotide and amino acid sequences can be compared for similarity. These databases can be accessed through the Internet at the National Center for Biotechnology Information (NCBI) World Wide Web site (http://www.ncbi.nlm.nih.gov/) (Astwood and Fuchs, 1997). In this study, the blastp program was used to compare the amino acid sequence of the xylanase X_{22} with those of known allergens included in the NBCI databases.

Digestion in Simulated Gastric Fluids. Pure X_{22} xylanase (100 μg) was added to 1 mL of the simulated gastric or intestinal solution previously temperature-equilibrated at approximately 37 °C. Digestion solutions were manually agitated. Temperature was maintained by a water circulator system (Julabo Labortechnik model TF-10, Seelbach, Germany); 5-μL aliquots were removed at specific time intervals from the digestion fluids.

Electrophoresis and Enzymatic Activity. Xylanase activity was assayed as described previously (Fernández-Espinar et al., 1993), 1 unit being the amount of enzyme that released 1 μmol of xylose equiv/min (UI). Reducing sugars were quantified by the Nelson−Somogy method (Somogyi, 1952), using D-xylose as standard. To determine the enzymatic activity in gastric and intestinal fluids, aliquots were placed on an ice bath until the analysis. Gastric fluid was neutralized by addition of 0.2 μL of 0.2 M sodium carbonate.

X_{22} concentration was measured by Tricine-SDS electrophoresis (Schagger et al., 1987). Proteins were separated with an acrylamide concentration of 14% and detected by a silver stain. Protein recoveries and degradation by gastric and intestinal fluid were estimated by visual comparison to relative intensities of bands corresponding to standards on the same blot. Both gastric and intestinal fluid analyzed by electrophoresis were diluted with 5 μL of sample buffer that contained 10% (v/v) glycerol, 0.125 M Tris-HCl, pH = 6.8, 4% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol, and 0.02% (w/v) bromophenol blue and heated to 100 °C for 5 min.

Protein levels were measured in samples with no digestion time by adding xylanase to the intestinal and digestive fluid. After addition of quenching fluid, xylanase recoveries were obtained by adding X_{22} to a 1-mL solution of buffer and then sampled and quenched as before.

Animals, Treatment, and Observations Performed. Swiss mice (B&K Universal G.J., Barcelona, Spain) of both sexes weighing 21−25 g were used. Each dose level was tested on 10 animals (5 males and 5 females). General and environmental conditions were strictly monitored. The animals were maintained in an environmentally controlled room (21−23 °C, 40−60% relative humidity, 12-h light/dark cycles) and were acclimated for 1 week before beginning the experiment. All the equipment used in the animals' immediate environment (cages, covers, etc.) and the general facilities complied with the recommendations of current guidelines (EEC, 1986). Animal handling and routines were performed according to Good Laboratory Practice (EEC, 1986). Feed (Panlab rodent chow, Barcelona, Spain) and water were available ad libitum.

Designated doses of either xylanase X_{22} or control solutions were administered daily to mice by gavage. Treatment included three dose levels of xylanase X_{22} (0.01, 0.1, and 1 mg/kg of mouse body weight), a BSA protein control administered at 1 mg/kg of body weight, and a vehicle control of 10 mM acetic acid/sodium acetate, pH 5.0.

Mortality and clinical signs (body weight gain, food and fluid consumption) were monitored daily. One day a week, male mice of BSA protein control and male mice of 1 mg/kg of xylanase X_{22} were placed in individual plastic metabolic cages (Tecniplast, Barcelona, Spain) which permitted separate collection of urine and feces. Urinalysis including specific gravity, pH, leukocytes, nitrite, protein, glucose, ketone bodies, urobilinogen, bilirubin, blood, and hemoglobin was carried out using Combur 10 Test (Boehringer, Mannheim, Germany).

For the evaluation of selected hematological and biochemical parameters, blood samples were collected at the end of the 4-week dosing, after sacrificing the animals by decapitation to obtain the maximum blood volume. Hematological study including hemoglobin, hematocrit, erythrocyte count (RCB), leukocyte count (WCB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution wide (RDW), platelet count (PLT), and lymphocytes was resolved using a Sysmex K-100 Plus hematological analyzer (Toa Medical Electronics, Ltd., Kobe, Japan). Biochemistry serum parameters: glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), urea nitrogen, glucose, and total cholesterol, were analyzed with a Technicon RA-100 system serum autoanalyzer (Bayer, Leverkusen, Germany). The weights of liver, spleen, kidneys, heart, lung, brain, thymus, salivary glands, pancreas, and genito-urinary system were recorded. Macroscopic examinations were conducted on tissues collected at necropsy. Histopathological evaluations of all the organs weighed and of the gastrointestinal tract were performed. The tissues listed were fixed in 10% (v/v) neutral formalin. Tissues specimens were embedded in paraffin, section-cut at 5 μm, stained with hematoxylineosin, and examined by light microscopy.

Spleen, kidney, and liver sections were prepared for electron microscopy. The specimens were fixed in 2.5% (v/v) glutaraldehyde diluted in Sorensen's phosphate buffer solution, pH 7.4, postfixed in osmium tetraoxide and embedded in Epon.

Thick sections (800 Å) were examined by electron microscopy (JEOL 100B, Tokyo, Japan).

Statistical Analysis. Numerical data were expressed as mean and standard deviation (SD), and the differences between the control and dosing groups were statistically analyzed with a significant level of less than 5% by the following methods. The data were subjected first to analysis of variance (Bartlett's test) followed by one-way analysis of variance (ANOVA), since all data presented a homogeneous variance. The differences in mean values were evaluated by Dunnett's test. The statistical analysis was performed with the computer program Graph Pad Software, Inc. PrismTM 2.0 (San Diego, CA).

RESULTS AND DISCUSSION

Digestion Studies. Stability of the xylanase X_{22} under in vitro mammalian digestion conditions was evaluated in both simulated gastric and intestinal fluids. Degradation was assessed by both Tricine-SDS–polyacrylamide gel electrophoresis (PAGE) using silver staining and enzymatic activity, which is dependent on intact protein tertiary structure. Xylanase was completely degraded by gastric fluid in less than 3 h. There was no total degradation in intestinal fluid even after several days. The enzyme activity slowly decreased (15% after 7 h and 30% after 3 days, approximately). That was confirmed by Tricine-SDS–PAGE with total dissipation on gastric fluid after 3 h and the continuous appearance of bands of enzyme on intestinal fluid stained by silver.

Degradation of the protein in gastric and intestinal control buffers was also checked. The protein degradation showed similar behavior to that in the presence of the digestive enzymes. Because of the similar protein degradation in gastric and intestinal fluid buffer controls, without pepsin and pancreatin, respectively, the degradation of xylanase X_{22} was a result of the pH and temperature conditions of the gastrointestinal fluid buffers more than the pepsin or pancreatin proteolytic activity.

Results of these studies establish that the xylanase X_{22} protein is very slowly degraded in the gastric digestion model and not completely in the intestinal digestion model. Therefore, it could be expected that the xylanase X_{22} protein will be partially degraded in the mammalian digestive tract and be partially excreted unaltered. These results are in accordance with those previously reported by Ganga et al. (1998), who demonstrated that X_{22} remains functional at acidic pH (3–3.5) during winemaking and is extremely stable to high temperatures (60 °C).

X_{22} Sequence Analysis for Allergenicity. Further support for comparison of the biochemical properties and characteristics of known allergenic proteins came from the allergenic potential of xylanase X_{22}. If significant amino acid sequence similarity is observed, there is a possibility that the transgenic protein may be allergenic. An immunological significant sequence similarity requires a match of at least eight contiguous identical amino acids (Fuchs et al., 1996). On the basis of BLAST search results, no significant sequence similarity was observed with known allergens such as ovalbumin, papain, casein, or the allergen M of codfish. So the gene introduced does not encode known allergens, and the protein introduced into wine does not share immunologically significant sequences with known allergens.

Many food allergens are present as major protein components in the specific food, typically ranging between 1 and 80% of total protein. Examples of highly abundant allergens include those in milk, soybean, and peanuts. In contrast, the X_{22} expressed by *S. cerevisiae* is lower than (0.5×10^{-6})% in wine (Fuchs and Astwood, 1996).

A variety of factors should be evaluated when considering the potential allergenicity of proteins introduced into food from nonallergenic sources. The most potent food allergens are stable molecules that are resistant to processing, cooking, and digestive enzymes while remaining soluble and absorbable through the intestinal tract (Metcalfe et al., 1996; Astwood et al., 1996). However, many nonallergic proteins also share these properties. On the other hand, the X_{22} shows no significant amino acid sequence homology to any known food allergens, is not glycosylated, and is not a major protein component in wine. Known food allergens are glycosylated and are abundant in the food product (Metcalfe et al., 1996).

Some xylanase preparations have recently been shown to be allergens (Sander et al., 1998). However their allergic character is related to occupational disorders in the baking industry. Exposure to the allergens occurs by inhalation and/or contact. These diseases are associated with intense and often prolonged exposure to organic dust. There is not evidence that these products produce food allergies by ingestion of small quantities.

Sander et al. (1998) identified IgE-reactive proteins in the xylanase preparations. This protein was identified as a β-xylosidase from *Aspergillus niger*. This β-xylosidase is a glycoprotein of high molecular weight (110 kDa), whose structure is poorly related to xylanase X_{22}.

Despite a certain resistance to digestion and to the winemaking process, the low xylanase proportion presented in wine (5 μg of protein in 1 L of wine) and the BLAST search results allow to conclude that the possibility of recombinant protein X_{22} being allergenic is remote. The best method to directly assess this possibility would be to do direct IgE-binding experiments with serum taken from patients known to be allergic to xylanase.

Short-Term Toxicity Study. Two animals from the group treated with albumin died during the study. No mortality or remarkable clinical observations were noticed in mice groups treated with xylanase X_{22} during the 4 weeks of the experimental study.

Mean body weight gain and mean food and fluid consumption of the five animal groups are listed in Table 1. There were no significant differences in body weight gain between the control and treated groups. Differences in body weight gain were not statistically significant ($p < 0.05$) between the control and treated groups. Food and fluid consumption was unaffected. The high standard deviations on fluid consumption could be caused by the measuring method used (Table 1).

Test performed in mice blood demonstrates the lack of significant changes on blood morphology as well as biochemical parameters estimated as shown in Table 2. Significant differences ($p < 0.05$) were only observed in lymphocyte levels that increased in 0.1 and 1 mg/kg/day X_{22} protein dose groups of males and in 1 mg/kg/day dose group of females. This rise could be related with sporadically subclinic infections and not with xylanase X_{22} intake. Consequently, these differences were considered of no toxicological importance.

Table 1. Body Weight Gain and Food and Fluid Intake of Mice Given X_{22} by Gavage for 4 Weeks

	values for groups given doses of				
	vehicle control	albumin at 1 mg/kg/day	X_{22} at 0.01 mg/kg/day	X_{22} at 0.1 mg/kg/day	X_{22} at 1 mg/kg/day
relative weight gain in					
males (%)	26.4 ± 5.0	13.7 ± 4.7	22.1 ± 2.7	21.9 ± 5.7	15.3 ± 3.3
females (%)	15.8 ± 4.3	13.6 ± 1.6	11.1 ± 1.8	9.3 ± 4.2	9.7 ± 3.4
food consumption in					
males (mg/kg/day)	203 ± 29	205 ± 17	216 ± 16	194 ± 21	189 ± 18
females (mg/kg/day)	187 ± 16	195 ± 22	217 ± 17	221 ± 24	204 ± 21
fluid consumption in					
males (mL/kg/day)	217 ± 19	184 ± 34	207 ± 18	211 ± 35	213 ± 19
females (mL/kg/day)	228 ± 21	200 ± 16	227 ± 34	250 ± 20	210 ± 20

Table 2. Hematological and Serum Biochemical Parameters of X_{22}-Treated Mice

	control		albumin		X_{22} at 0.01 mg/kg/day		X_{22} at 0.1 mg/kg/day		X_{22} at 1 mg/kg/day	
	male	female	male	female	male	female	male	female	male	female
RCB ($10^6 \times$ mm^3)	7.6 ± 0.7	7.3 ± 0.4	7.4 ± 0.7	7.5 ± 0.3	6.8 ± 0.8	6.7 ± 1.0	7.1 ± 0.2	7.2 ± 0.5	7.3 ± 0.4	7.2 ± 0.5
Ht (%, v/v)	34.7 ± 1.4	35.3 ± 2.1	35.0 ± 2.8	36.4 ± 2.2	32.6 ± 3.7	32.8 ± 5.2	33.4 ± 0.5	34.6 ± 1.9	35.1 ± 1.3	34.5 ± 1.7
Hb (g/dL)	12.9 ± 0.7	12.1 ± 0.8	13.1 ± 0.8	12.7 ± 0.5	11.7 ± 1.2	11.8 ± 1.1	11.7 ± 0.2	11.9 ± 0.6	11.9 ± 0.7	12.2 ± 0.5
MCV (μm^3)	46.0 ± 3.0	48.4 ± 1.4	47.3 ± 1.8	48.2 ± 0.5	47.9 ± 1.0	48.4 ± 1.3	47.1 ± 0.9	48.0 ± 1.3	48.3 ± 1.8	47.8 ± 1.2
MCH (pg)	17.1 ± 0.8	16.6 ± 0.3	17.7 ± 1.8	16.9 ± 0.5	17.3 ± 0.5	17.7 ± 1.9	16.5 ± 0.2	16.5 ± 0.3	16.5 ± 0.1	16.9 ± 0.4
MCHC (%)	37.2 ± 0.8	34.3 ± 1.5	37.5 ± 3.6	35.1 ± 1.4	36.1 ± 0.5	36.6 ± 4.5	35.1 ± 0.2	34.4 ± 0.7	34.1 ± 1.2	35.4 ± 0.2
RDW	22.6 ± 3.6	21.2 ± 0.8	21.7 ± 1.6	22.7 ± 2.1	23.2 ± 2.7	20.9 ± 1.2	22.6 ± 2.0	21.6 ± 3.2	23.5 ± 2.7	21.2 ± 1.5
lymphocytes ($10^6 \times$ mm^3)	70 ± 5	70 ± 4	68 ± 10	77 ± 4	76 ± 5	74 ± 3	79 ± 4*	70 ± 7	79 ± 5*	79 ± 2*
WCB ($10^3 \times$ mm^3)	75 ± 20	57 ± 22	91 ± 31	66 ± 30	62 ± 33	53 ± 30	55 ± 21	43 ± 12	51.4 ± 19.4	49.6 ± 12.9
PLT ($10^4 \times$ mm^3)	67 ± 11	63 ±14	77 ± 14	73 ± 13	70 ± 16	56 ± 29	63 ± 12	68 ± 15	83 ± 7	79 ± 18
glucose (mg/dL)	127 ± 29	127 ± 11	125 ± 15	139 ± 13	139 ± 26	130 ± 26	138 ± 12	123 ± 13	144 ± 17	117 ± 07
cholesterol (mmol/L)	89 ± 9	69 ±14	104 ± 21	141 ± 13[a]	87 ± 19	63 ± 7	85 ± 25	93 ± 56	79 ± 15	75 ± 21
urea (mmol/L)	27.0 ± 4.7	26.0 ± 3.5	32.0 ± 5.6	33.5 ± 8.9	29.8 ± 3.8	34.4 ± 7.3	30.8 ± 4.6	35.2 ± 6.7	34.2 ± 5.4	33.8 ± 2.8
GOT (U/L)[b]	264 ± 130	335 ± 116	295 ± 129	285 ± 56	276 ± 111	277 ± 88	219 ± 87	282 ± 62	182 ± 29	243 ± 51
GPT (U/L)	84 ±35	113 ± 40	141 ± 61	104 ± 10	102 ± 50	101 ± 30	77 ± 30	102 ± 24	68 ± 13	87 ± 17

[a] Hepatic affected mouse. [b] Measured at 37 °C. *Presents significant differences ($p < 0.05$).

Table 3. Urinalysis of Treated Mice Using Combur Ten Test

	control	albumin	X_{22} at 1 mg/kg/day
density	$1.01 \pm 5 \times 10^{-3}$	$1.02 \pm 7 \times 10^{-3}$	$1.02 \pm 5 \times 10^{-3}$
PH	8.0 ± 0.7	5.7 ± 0.6	6.9 ± 1.0
leukocytes	17.2 ± 0.3	21.2 ± 1.2	20.0 ± 3.7
nitrite	negative	negative	negative
protein	77 ± 40	52 ± 33	92 ± 25
glucose	normal	normal	normal
ketones	negative	positive	positive
urobilinogen	normal	normal	normal
bilirubin	negative	negative	negative
blood/Hb	negative	negative	negative

Table 3 presents urinalysis results obtained during each one of the 4 weeks. As can be noted, no effect related with the oral administration of X_{22} was observed.

Table 4 summarizes the organ/body weight ratios obtained for males and females after necropsy. Relative weight of all the organs in the treated groups statistically was comparable to the corresponding control values.

Gross lesions were not observed at macroscopic examinations. Tissue sections from males and females of control, albumin, and X_{22} high dose treated groups revealed the next considerations: (i) lymphoid infiltrates in the interstitial of the lungs (Figure 1a), salivary glands (Figure 1b), lamina proporia of stomach, brain, and liver; (ii) congestion in the heart, lungs, and liver; (iii) cystic lesions in the kidney (Figure 1c); (iv) deposits in the ephithelial cells of the renal tubuli (only observable by electron microscopy); and (v) slightly enhanced lipid deposition in the hepatocytes (Figure 1d). Lesions were noted in both control and treated groups. Because of this, histopathological changes in tissues were not considered of toxicological importance since they can take place under normal conditions.

Xylanase X_{22} proved to be nontoxic in mice after short-term repeated administration, even at levels 800 times higher than 250 mL of wine intake. This result is in agreement with similar work carried out by Begman and Broadmeadow (1997), who demonstrated lack of toxicity using other *Aspergillus* xylanases. As is recommended by the Food and Drug Administration, it is essential to look at the origin of the donor source. Both *A. nidulans* and *S. cerevisiae* are generally recognized as safe, and no allergenicity has been reported to relate to those microorganisms.

Activity of recombinant xylanase was studied from fermenting juice of Bobal grapes at initial and final stages. In both cases the activities behaved similarly, declining progressively over 12 days to reach a constant level corresponding to 30−40% of the initial activity (Ganga et al., 1998). This residual activity indicates that the X_{22} xylanase remains functional during winemaking.

To illustrate the "worst" situation, the calculation of human exposure is made assuming that enzyme activity remains in wine. The dosage recommended of X_{22} xylanase to improve wine aroma profile is 5 μg of enzyme/L of wine, as was investigated in previous studies. The average daily human intake of wine is 70 mL/person/day in Spain (INE, 1991). Taking into account the daily consumption for an average person weighing 65 kg, the maximum estimated daily intake results $(5 \times 0.07)/65 = 0.0053$ μg of enzyme/kg of bw/day. The highest dosage with nonobserved adverse effect levels in this study was 1000 μg/kg/day so the safety margin would thus be $1000/0.0053 = 1.9 \times 10^5$. It is

Table 4. Relative Organ Weights of Mice Given X_{22} by Gavage for 4 Weeks

	control		albumin		X_{22} at 0.01 mg/kg/day		X_{22} at 0.1 mg/kg/day		X_{22} at 1 mg/kg/day	
	males	females	males	females	males	females	males	females	males	females
pancreas	0.45 ± 0.09	0.47 ± 0.13	0.52 ± 0.03	0.58 ± 0.05	0.44 ± 0.08	0.56 ± 0.10	0.50 ± 0.03	0.57 ± 0.04	0.46 ± 0.04	0.49 ± 0.05
brain	1.16 ± 0.11	1.50 ± 0.17	1.14 ± 0.15	1.56 ± 0.17	1.20 ± 0.12	1.47 ± 0.14	1.25 ± 0.17	1.50 ± 0.16	1.25 ± 0.19	1.62 ± 0.05
salivary gland	0.17 ± 0.05	0.18 ± 0.05	0.18 ± 0.05	0.09 ± 0.03	0.18 ± 0.02	0.13 ± 0.02	0.19 ± 0.05	0.09 ± 0.03	0.21 ± 0.02	0.09 ± 0.03
thymus	0.25 ± 0.02	0.44 ± 0.08	0.24 ± 0.07	0.38 ± 0.10	0.20 ± 0.06	0.35 ± 0.05	0.27 ± 0.02	0.41 ± 0.05	0.29 ± 0.07	0.29 ± 0.08
heart	0.56 ± 0.06	0.46 ± 0.05	0.52 ± 0.05	0.52 ± 0.05	0.55 ± 0.03	0.48 ± 0.04	0.56 ± 0.07	0.53 ± 0.04	0.52 ± 0.10	0.54 ± 0.07
lungs	0.73 ± 0.07	0.71 ± 0.13	0.80 ± 0.15	0.76 ± 0.15	0.75 ± 0.05	0.68 ± 0.07	0.74 ± 0.13	0.71 ± 0.02	0.83 ± 0.09	0.85 ± 0.10
genito-urinary	3.68 ± 0.48	1.67 ± 0.44	2.90 ± 0.13	1.20 ± 0.28	3.48 ± 0.33	1.53 ± 0.43	3.68 ± 0.21	1.33 ± 0.39	3.44 ± 0.51	1.43 ± 0.32
kidneys	1.91 ± 0.14	1.23 ± 0.13	1.70 ± 0.09	1.31 ± 0.10	1.92 ± 0.22	1.37 ± 0.10	1.96 ± 0.11	1.32 ± 0.13	1.86 ± 0.14	1.35 ± 0.08
liver	5.08 ± 0.22	5.05 ± 0.15	4.99 ± 0.49	5.04 ± 0.44	5.03 ± 0.49	4.54 ± 0.31	4.7 ± 0.18	4.33 ± 0.35	4.72 ± 0.30	4.56 ± 0.17
spleen	0.41 ± 0.03	0.54 ± 0.10	0.54 ± 0.07	0.62 ± 0.08	0.52 ± 0.09	0.55 ± 0.06	0.50 ± 0.08	0.57 ± 0.08	0.63 ± 0.14	0.48 ± 0.07

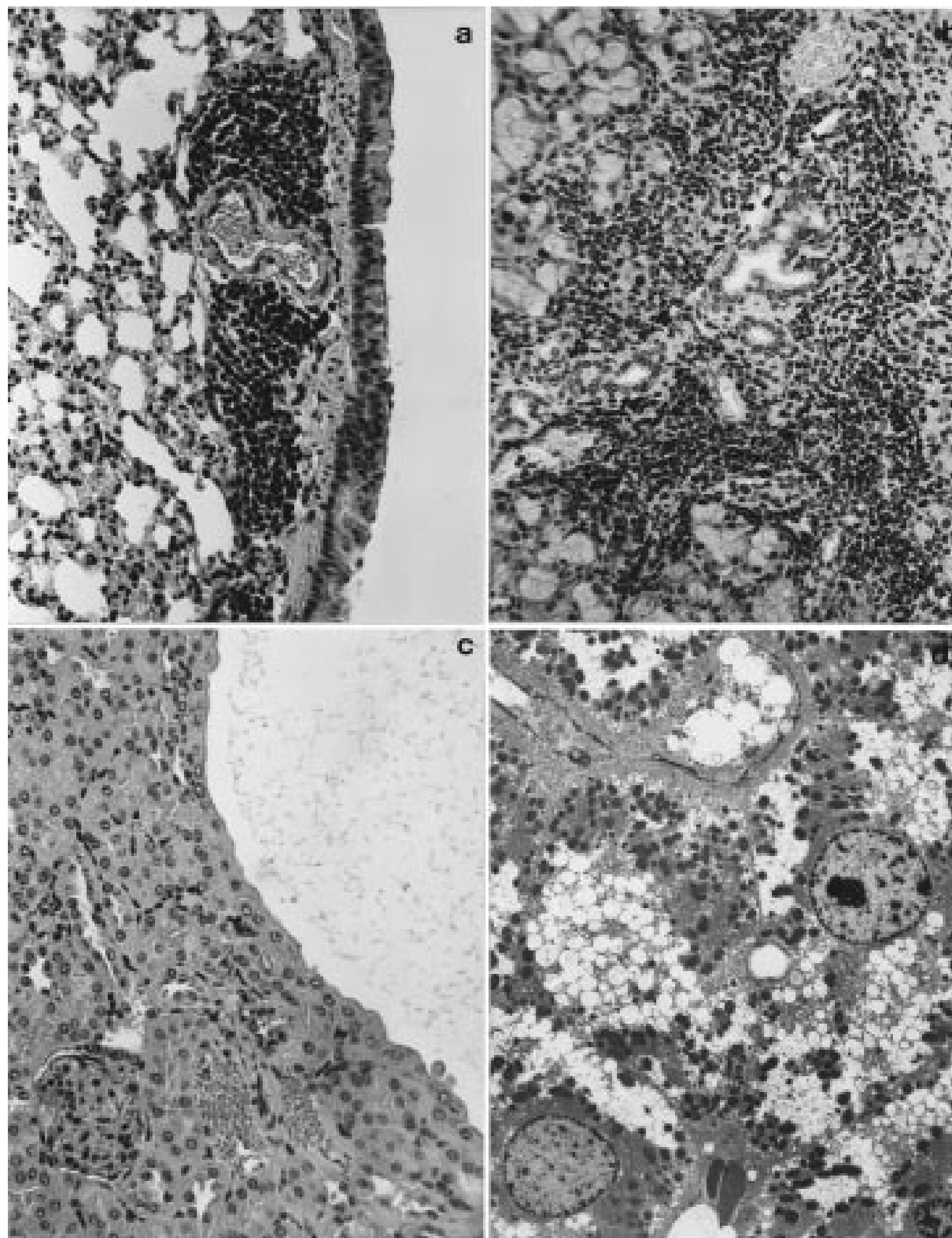

Figure 1. Light microscopy of 5-μm slices, stained with hematoxylineosin of (a) lung with lymphoid infiltrates in mice treated with X_{22} (H−E, 10X), (b) salivary glands with lymphoid infiltrates in mice of the albumin group (H−E, 10X), and (c) cystic lesion in kidney (mice control group) (H−E, 40X) and (d) electron microscopy of thick sections (800 Å) of liver with increased deposition of lipid droplets in mice of the albumin group (5000 Å) (reprinted at 75% of original).

observed that wine consumption has decreased in Spain during past years: in 1981 the average daily human intake of wine was 117 mL/person/day, and in 1964 was 130 mL/person/day. Otherwise, a better approach to usual consumers was made by the same calculations for 250 mL (two glasses of wine approximately), a possible quantity drunk for habitual wine consumers. Then, the maximum estimated daily intake will be $(5 \times 0.25)/65 = 0.019$ μg of enzyme/kg of bw/day, and the safety margin would be $1000/0.019 = 5.26 \times 10^4$.

Results obtained in 4-week toxicity study show a wide safety margin. To intake 1 mg/kg of bw of xylanase X_{22}—the highest dosage with no observed adverse effects used in this study—the average human consumption should be approximately 13 000 L of wine/day.

In conclusion, all the data presented in this work establish that the X_{22} expressed in *S. cerevisiae* possesses no discernible risk to human health and could be applied in the wine industry. Further studies will be needed to demonstrate the last word on safety. In this way, IgE-binding studies would be one logical next step to resolve definitively the allergy question.

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Received for review August 13, 1998. Revised manuscript received January 19, 1999. Accepted January 26, 1999. We thank the Conselleria de Cultura, Edució i Ciencia (Programa d'Accions Especials d'I+D "Generalitat Valenciana" Project AE97-12) for the financial support of this study and the Ministerio de Educación y Ciencia for the postdoctoral contract received by P.M.

JF980905K