

Señor
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 04-032188-00008-0000
Fec: 2006-05-25 13:52:40 Dep. 2020 NUECREACIONES
Tm. 11 PATENTENYU
Evo: 370 FASENACIONALH
Art 220 (TRANSFORMACION) Folios: 32

REF: Exp. 04-032-188

TÍTULO SOLICITADO: "Planta de café con actividad de α -D-galactocidasa reducida"

PUBLICACIÓN: Gaceta 558 de 30-11-2005

PRIORIDAD: EP 01124160.1 de 10/10/2001

SOLICITANTE: SOCIÉTÉ DES PRODUITS NESTLE S.A.

APODERADO: ALICIA LLORED A RICAURTE

TRÁMITE: SUSTENTACIÓN DE OPOSICIÓN

JOSÉ LUIS REYES VILLAMIZAR, conocido apoderado de la FEDERACIÓN NACIONAL DE CAFETEROS DE COLOMBIA en el asunto de la referencia, estando en la debida oportunidad legal, me permito sustentar la oposición presentada contra la solicitud de patente indicada en el epígrafe en los siguientes términos.

I. FUNDAMENTOS DE LA OPOSICIÓN

1. IMPROCEDENCIA DE PATENTABILIDAD DE LAS PLANTAS

Sin perjuicio de los argumentos que en materia de carencia de Novedad y Nivel Inventivo de la solicitud que se expondrán en este documento, y como cuestión previa, me permito hacer notar que la verdadera pretensión del solicitante en este caso, apreciable particularmente en la reivindicación 5 y sus dependientes, es obtener derechos de exclusiva sobre una planta de café, independientemente de la caracterización que intenta hacer de la misma con base en reivindicaciones precedentes, en flagrante contravención de lo dispuesto por el artículo 20-c de la Decisión 486 de 2000 de la Comunidad Andina de Naciones, a cuyo tenor "No serán patentables las plantas".

Aunque la intención de patentar en Colombia una planta de café y restringir en consecuencia su libre empleo por parte de la caficultura nacional, sería motivo ético más que suficiente para rechazar de plano la solicitud que nos ocupa, lo cierto es que por obra de las normas andinas aún vigentes sobre la materia no es preciso recurrir a interpretaciones políticas o de conveniencia para concluir, sin lugar a interpretaciones, que la pretensión de Nestlé de patentar una planta de café es legalmente imposible en Colombia.

Si en gracia de discusión fuesen patentables las plantas en Colombia, lo cierto es que el eventual empleo de material biológico y genético de propiedad de la Nación como partida de la pretendida invención requeriría, al tenor de lo dispuesto por el artículo 3º de la misma Decisión 486 de 2000, la obtención previa de las autorizaciones de acceso de que trata la norma mencionada, circunstancia que no consta en el documento de patente que nos ocupa.

En consecuencia, solicito que el Despacho se sirva negar de plano todas las reivindicaciones incluidas en la solicitud de patente de la referencia, en particular aquellas relacionadas con la pretensión expresa de patentar una planta de café, pues éstas constituyen violación flagrante del citado artículo 20-c de la Decisión 486 de 2000.

2. CARENCIA DE NOVEDAD Y NIVEL INVENTIVO DE LA SOLICITUD

Aunque los argumentos expuestos en el punto precedente son fundamento de sobra para negar la totalidad de las reivindicaciones de la solicitud que nos ocupa, es del caso revisar de qué manera las mismas, además de ser contrarias como se ha dicho al régimen andino de patentes en materia de patentabilidad de plantas y de acceso a material biológico y genético, también lo son frente al cumplimiento mismo de los requisitos esenciales de patentabilidad.

Las reivindicaciones en cuestión han sido calificadas, en efecto, como carentes de novedad y nivel inventivo por la Oficina Europea de Patentes, autoridad encargada de realizar y emitir en 28/08/2003 el informe internacional de búsqueda respecto de la solicitud internacional equivalente a la que nos ocupa, la cual fue publicada en 24/04/2003 bajo el número PCT/WO03/032713.

La citada autoridad de búsqueda ha manifestado que la solicitud en cuestión carece de novedad y de nivel inventivo por obra de la revelación previa de la materia ahora reivindicada en diversos documentos publicados con anterioridad a su fecha de prioridad, es decir, antes del 10/10/2001, como se explicará enseguida. Antes de entrar en materia vale anotar que los documentos en cuestión son de tal relevancia, que la autoridad de

búsqueda, de conformidad con lo dispuesto en el manual de examen de la Oficina Europea de Patentes, los ha clasificado como de categoría "X", es decir, como "de particular relevancia", al punto de considerar que la pretendida invención, por obra de la publicación de los mismos, "no puede considerarse novedosa ni inventiva", sin que sea preciso combinar sus enseñanzas con otros documentos relacionados disponibles en el estado de la técnica.

Aunque el apoderado del solicitante conocía la existencia del Informe Internacional de Búsqueda, emitido como se ha dicho el 28/08/2003, llama la atención que haya persistido en solicitar el ingreso a fase nacional de la solicitud en Colombia el 05/04/2004, es decir, cerca de ocho (8) meses después, a sabiendas de la existencia de las anterioridades de categoría X que se explicarán enseguida, las cuales aniquilan los requisitos materiales de patentabilidad:

2.1. Artículo "Cloning and functional expression of a cDNA encoding coffee bean alpha-galactosidase", de autoría de Alex Zhu y Jack Goldstein, recibido por los editores en 06/12/1993 y publicado en 1994 en "Gene- Elsevier Biomedical Press" en Ámsterdam, Países Bajos: la autoridad de búsqueda ha considerado que este documento (anexo como prueba en cinco folios), pese a citarse en la solicitud de patente que nos ocupa, anticipa el objeto en ella reivindicado, en particular frente a las reivindicaciones 1, 2, 6 y 9 (ésta última ausente en apariencia de la solicitud colombiana en curso), teniendo en cuenta que el uso conjunto del promotor csp1 con el gen de galactosidasa en anti-sentido, era de dominio público desde la publicación de este documento, se insiste, en 1994.

Aunque la publicación detectada por la Oficina Europea de Patentes no requiere ser combinada para aniquilar la Novedad y el Nivel Inventivo de la solicitud que nos ocupa, es del caso mencionar que los documentos titulados "Coffee seeds contain 11S storage proteins", publicado en Irlanda en 1999 y de autoría entre otros del investigador colombiano Ricardo Acuña (anexo como prueba en diez (10) folios, y "11s storage globulin from Coffea arabica seeds", publicado por el mismo Dr. Acuña y otros en 01/05/1997, complementan las revelaciones de la primera anterioridad, en el sentido de mostrar el conocimiento y empleo del promotor del gen 11s.

2.2. Publicación Internacional PCT/WO95/06478 de 09/03/1995, de título "Recombinant Coffee Bean Alpha Galactosidase" – Solicitante: Hawaii Biotechnology Group Inc.: este documento de patente, por su parte, revela en su ejemplo 3, páginas 20, 21 y 22, el análisis de los niveles de alfa galactosidasa en granos de café en desarrollo, circunstancia que en opinión del examinador de patentes afecta la novedad y el nivel inventivo de las reivindicaciones 1, 2, 6 y 9.

10

2.3. Artículo "Beta Galactosidase from Coffea arabica and its role in fruit ripening", de autoría del Dr. Kerith Donovan Golden y publicado en 1993, el cual revela el proceso de purificación de la betagalactosidasa en el café arábico y la capacidad de la enzima en la degradación de polisacáridos. En opinión del examinador de la Oficina Europea, esta publicación por sí misma y sin necesidad de combinación, afecta en materia grave la novedad y el nivel inventivo de la solicitud que nos ocupa. Anexo resumen del documento en cuestión en tres folios.

3. LA REIVINDICACIÓN 8 PRETENDE OBTENER PROTECCIÓN PARA UN USO EN CONTRA DE LO DISPUESTO POR EL ARTÍCULO 14 DE LA DECISIÓN 486

Además de los argumentos anteriores, cabe resaltar que la reivindicación 8 busca obtener protección para un uso, pretensión que no tiene cabida en el régimen subregional andino de propiedad industrial, posición que ha sido reiteradamente sostenida por esa entidad en casos similares.

Como es sabido, la Decisión 486, únicamente permite el patentamiento de productos y procedimientos. Esto, a diferencia de lo dispuesto por las legislaciones de otros países que han reconocido en forma explícita la posibilidad de patentar los usos. Ruego al Despacho tener especialmente en cuenta esta circunstancia en el momento de resolverse la patentabilidad de la solicitud de patente de la referencia, procediendo a negar las reivindicaciones de uso en ella incluidas.

4. FALTA DE UNIDAD DE INVENCION – ARTÍCULO 25 DE LA DECISIÓN 486 DE 2.000

Por si los anteriores planteamientos no constituyesen razones suficientes para negar de plano la solicitud de patente 04-032188, cabe agregar que la solicitud en cuestión carece, además, de unidad de invención. La unidad de invención, como se sabe, exige al solicitante que el petitorio se refiera a una invención o grupo de invenciones que conformen un único concepto inventivo.

A pesar de esta exigencia consagrada en forma tajante e inexcusable por el artículo 25 de la Decisión 486, la solicitud de marras, en forma claramente violatoria del aludido artículo, se termina refiriendo a tres conceptos inventivos claramente diferenciados: en primer lugar, a una célula de planta de café (R1 a R4); en segundo término, a una planta de café (R5); finalmente, a un método industrial para preparar café soluble (R6 y R7).

Es indiscutible, con base en el planteamiento anterior, que la solicitud viola de manera flagrante el citado artículo 25 de la Decisión 486 y que en esa medida las reivindicaciones en cuestión no pueden ser concedidas.

Así las cosas, y con fundamento en los argumentos expuestos a lo largo del presente escrito, respetuosamente reitero la solicitud para que su despacho se abstenga de conceder el privilegio de patente solicitado, y ordene en consecuencia el archivo del expediente respectivo.

II. PRUEBAS Y ANEXOS

Ruego que se tengan como pruebas de la presente oposición, sin perjuicio de los documentos que se puedan allegar posteriormente y de aquellos que el Despacho considere oficiosamente, los siguientes:

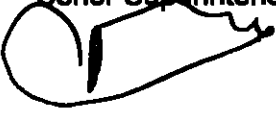
1. Primera página e informe de búsqueda de la publicación Internacional PCT/WO03/032713, equivalente a la solicitud colombiana 04-032-188, en cinco (5) folios.
2. Artículo "Cloning and functional expression of a cDNA encoding coffee bean alpha-galactosidase", de autoría de Alex Zhu y Jack Goldstein, publicado en 1994, en cinco (5) folios.
3. Artículo "Coffee seeds contain 11S storage proteins", publicado en Irlanda en 1999 y de autoría entre otros del investigador colombiano Ricardo Acuña, en diez (10) folios.
4. Primera página y páginas 20 y 21 de la publicación Internacional PCT/WO95/06478 de 09/03/1995, de título "Recombinant Coffee Bean Alpha Galactosidase" – Solicitante: Hawaii Biotechnology Group Inc., en tres (3) folios.
5. Resumen del artículo "Beta Galactosidase from Coffea arabica and its role in fruit ripening", en tres (3) folios.

III. NOTIFICACIONES

Reitero que las recibiré en la secretaría de su Despacho o en mi oficina localizada en la Carrera 17 No. 88-23, Oficina 205, Fax (1) 621.25.42 de esta ciudad, y que la Federación

Nacional de Cafeteros de Colombia lo hará en la secretaría de su Despacho o en su sede localizada en la Calle 73 No. 8-13 de Bogotá D.C.

Señor Superintendente,



JOSÉ LUIS REYES VILLAMIZAR

C.C. 79'152.473 de Usaquén

T.P.A. 44.655 de C.S.J.

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
24 April 2003 (24.04.2003)

PCT

(10) International Publication Number
WO 03/032713 A3

- (51) International Patent Classification: C12N 15/82
- (21) International Application Number: PCT/JP02/09148
- (22) International Filing Date: 15 August 2002 (15.08.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
01124160.1 10 October 2001 (10.10.2001) JP
- (71) Applicant (for all designated States except US): SOCI-
ETE DES PRODUITS NESTLE S.A. [CH/CH]; P.O. Box
353, CH-1800 Vevey (CH).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): MARRACCINI,
Pierre [FR/FR]; Res Vale das Américas, Rua Walter
Motta Campos No. 233, 86047-670 Londrina, Paraná
(BR). DESHAYES, Alain [FR/FR]; 32 rue Calmete,
F-37510 Saint Cyr sur Loire (FR). ROGERS, John
[CH/FR]; 6, rue Galilée, F-01630 St Genis Pouilly (FR).
- (74) Agent: STRAUS, Alexander; Becker, Kurig, Straus,
Bavariastrasse 7, D-80336 München (DE).
- (81) Designated States (national): AE, AG, AI, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GR,
GM, HN, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LA,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MY, NC, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,
VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SI, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IT, LI, MC, NL, PT, SE, SK,
TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NI, SN, TD, TG).
- Published:
with international search report
before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments
- (88) Date of publication of the international search report:
13 November 2003
- For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

WO 03/032713 A3

(54) Title: COMBUSTIBLE PLANT WITH REDUCED α -D-GALACTOSIDASE ACTIVITY

(57) Abstract: The present invention relates to the modification of galactomannans present in the green coffee bean by reducing the endogenous level of α -D-galactosidase activity. In particular the present invention pertains to a plant cell with reduced α -D-galactosidase activity and to a plant harboring such a plant cell.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/EP 02/09148A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data bases consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHU A ET AL: "CLONING AND FUNCTIONAL EXPRESSION OF A CDNA ENCODING COFFEE BEAN ALPHA-GALACTOSIDASE" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 140, no. 2, 1994, pages 227-231, XP002062116 ISSN: 0378-1119 cited in the application the whole document -/-	1,2,6,9

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document relating to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to underline the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "G" document member of the same patent family

Date of the actual completion of the international search

28 August 2003

Date of mailing of the international search report

11/09/2003

Name and mailing address of the ISA

European Patent Office, P.O. Box 5016 Patentstr. 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Holtorf, S

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/EP 02/09148

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOLDEN KERITH D ET AL: "Beta-Galactosidase from <i>Coffea arabica</i> and its role in fruit ripening." PHYTOCHEMISTRY (OXFORD), vol. 34, no. 2, 1993, pages 355-360, XP008002908 ISSN: 0031-9422 the whole document	1,2,6,9
X	WO 95 06478 A (HAWAII BIOTECH GROUP) 9 March 1995 (1995-03-09) example 3	1,2,6,9
A	EP 1 138 771 A (NESTLE SA) 4 October 2001 (2001-10-04) the whole document	
A	COCK J M ET AL: "Natural antisense transcripts of the S locus receptor kinase gene and related sequences in <i>Brassica oleracea</i> ." MOLECULAR & GENERAL GENETICS, vol. 255, no. 5, 1997, pages 514-524, XP002252703 ISSN: 0026-8925 the whole document	
A	MARRACCINI PIERRE ET AL: "Molecular cloning of the complete 11S seed storage protein gene of <i>Coffea arabica</i> and promoter analysis in transgenic tobacco plants." PLANT PHYSIOLOGY AND BIOCHEMISTRY (PARIS), vol. 37, no. 4, April 1999 (1999-04), pages 273-282, XP002197483 ISSN: 0981-9428 cited in the application the whole document	
A	SACHSLEHNER A ET AL: "Hydrolysis of isolated coffee mannan and coffee extract by mannanases of <i>Sclerotium rolfsii</i> " JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 80, no. 2, 23 June 2000 (2000-06-23), pages 127-134, XP004213502 ISSN: 0168-1656 page 128, left-hand column, paragraph 2	
	-/--	

40
116

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/EP 02/09148

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAIBACH F ET AL: "PURIFICATION AND CHARACTERIZATION OF A COFFEA CANEPHORA ALPHA-D-GALACTOSIDASE ISOZYME" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 181, no. 3, 31 December 1991 (1991-12-31), pages 1564-1571, XP000644469 ISSN: 0006-291X figure 1	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/09148

Patent document class in search report		Publication date	Patent family member(s)	Publication date
WO 9506478	A	09-03-1995	US 6329191 B1	11-12-2001
			AU 7640794 A	22-03-1995
			EP 0722336 A1	24-07-1996
			WO 9506478 A1	09-03-1995
EP 1138771	A	04-10-2001	EP 1138771 A1	04-10-2001
			WO 0175084 A2	11-10-2001
			EP 1272642 A2	08-01-2003
			US 2003131380 A1	10-07-2003

TT
117

0173-4469/94

Short Communications

Cloning and functional expression of a cDNA encoding coffee bean α -galactosidase

Sequencing, erythrocyte surface, glycosidase, recombinant DNA, B $\rightarrow$ O blood group conversion, insect cells

Xuesi Zhu and Jack Goldstein

Ames Research Institute, The New York Blood Center, New York, NY 10021, USA

Received 5 October 1993; Revised/Accepted 12 November 1993; Received at publishers 6 December 1993

SUMMARY

Purified coffee bean α -galactosidase (α Gal) has been used for removing terminal α -galactose residues from the glycoconjugates at the red cell surface in studies of blood group conversion. Here, we report the isolation and sequence of the full-length clone for coffee bean α Gal by using the polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) techniques. The cDNA clone (1.4 kb) contains a single open reading frame which encodes a protein of 475 amino acids (aa). Its authenticity is confirmed by perfect alignment of aa sequences obtained from purified coffee bean α Gal, and by immune reaction with the antibody raised against the enzyme. Furthermore, the protein produced in insect cells shows enzymatic activity towards a synthetic α Gal substrate, *p*-nitro-phenyl- α -galactopyranoside.

INTRODUCTION

α -Galactosidase (α Gal), purified from coffee beans, can cleave the terminal α 1,3-linked galactose residues responsible for blood group B specificity from the surface of erythrocytes, thereby converting these cells serologically to group O under conditions that permit their use in transfusion therapy (Goldstein et al. 1982, 1989). Such enzymatically converted B $\rightarrow$ O-group red cells are being tested in clinical trials (Lenny et al. 1991, 1993). In order to readily produce purified enzyme in large quantities and improve its enzymatic properties, it is necessary to isolate the cDNA clone for coffee bean α Gal. α Gal cDNA

has been cloned from several sources including human (Bishop et al. 1986), *Crimopsis tetragona lobata* (Overbeke et al. 1989), *Saccharomyces cerevisiae* (Liljeström, 1985), *Aspergillus niger* (den Heijer et al. 1992) and *E. coli* (Liljeström and Liljeström 1987). Compared to the enzyme isolated from these sources, coffee bean α Gal appears to be the most effective in removing the terminal galactose residues from group-B antigen under physiological conditions. In this paper, we report the isolation, its sequence and expression of a cDNA clone encoding the coffee bean α Gal.

Address correspondence to: J. Goldstein, The New York Blood Center, 300 East 47th Street, New York, NY 10021, USA. Tel: (1-212) 398-3096, Fax: (1-212) 398-0203; e-mail: JGOLDSTEIN@SERVER.WYBK.CRG.

Abbreviations: aa, amino acids; bp, base pairs; α Gal, α -galactosidase; HPLC, high-performance liquid chromatography; aa, antibodies; aa, amino acids; nucleotides, nt; oligodeoxynucleotides, PGL, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PNP, *p*-nitro-phenyl- α -galactopyranoside; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; N, N-terminal; Trp, tryptophan; wt, wild type.

EXPERIMENTAL AND DISCUSSION

(a) Sequencing of α Gal purified from coffee beans

The α Gal was purified to apparent homogeneity from green coffee beans in the laboratory as reported (Lenny et al. 1993). Microsequencing of the mature enzyme provided an N-terminal sequence (N-pep) of 19 aa. To obtain internal peptide sequences, 0.2 μ g of the purified α Gal

tion from coffee bean mRNA. The second oligo, CB6, together with the universal primer was used to amplify the 5' region upstream from the coding sequence. Since no distinctive DNA band was visible on the agarose gel after two PCR amplifications, we cloned the mixture of the PCR product into the pCR11 vector and screened by hybridizing the colonies with the radioactively labeled 1.1-kb fragment BZ (sequence nt 168-1234 in Fig. 1). The positive colonies were picked for plasmid preparation. The sequencing of the plasmid indicated that the DNA fragment, designated 5'BZ, obtained by the 5' RACE technique contains a 240-bp overlap (the sequence between CB1* and CB6) with BZ, and about a 170-bp further upstream sequence which includes the N terminus of the mature enzyme and the putative signal peptide sequence.

To isolate the 3' sequence, downstream from the fragment BZ, the cDNA was reverse-transcribed from coffee bean mRNA by using a primer, P1 (5'-GACTCCGAGTCGACATCGA-(T)₁₁). PCR amplification was carried out with a specific primer, CR7 (nt 940-957 in Fig. 1) and an adapter primer, P11. P11 has the same sequence as P1, except that it lacks 17 thymidine residues at its 3' end. The PCR product was analyzed on 1% low melting point agarose gel and a distinctive band of about 500-bp long was visualized. This fragment, designated 3'BZ, was isolated and cloned into the pCR11 vector for further analysis. The sequencing of 3'BZ indicated that it contains about 300-bp overlap with BZ (the sequence between CB7 and CB4*) and more than 170-bp extension towards 3' end of the α Gal cDNA.

To confirm the coding sequence of the α Gal cDNA we made an oligo, CH9, corresponding to sequence nt 77-94 shown in Fig. 1. cDNA was synthesized from coffee bean mRNA using the 3' RACE technique as previously described. A 1.35-kb fragment was amplified by PCR using two primers CB9 and P11, and was then cloned into the pCR11 vector (pCR-BZ7). The sequence of the 1.35-kb product matched with the corresponding sequences from those three fragments, 5'BZ, BZ and 3'BZ obtained in different experiments as mentioned above, confirming the authentic sequence of the coffee bean α Gal cDNA shown in Fig. 1.

(c) Characterization of the coffee bean α Gal cDNA clone

The sequence shown in the Fig. 1 encodes a 42-kDa protein, which closely approximates the size of the purified coffee bean α Gal as estimated by SDS-PAGE (Fig. 3, lane 5). Three peptide sequences, N-pep, 2-pep and 3-pep, that were derived from purified enzyme, are underlined in Fig. 1. The fact that these sequences match with the deduced aa sequences confirms that the cDNA clone isolated from coffee bean RNA does encode the α Gal. Since

the mature protein starts at Leu¹⁶ (Fig. 1), the first 15 aa may serve as a signal sequence which is removed following translation. There is an in-frame stop codon (TGA at nt 66-68) suggesting that the first downstream ATG (at nt 102-104) is the start codon for in vivo synthesis of α Gal precursor. The sequence at residues 151-153 (double underlined) is the only possible site for N-glycosylation.

The first plant α Gal cDNA was cloned from guar (Overbeek et al., 1989). It encodes a 411-aa protein of 45 kDa. Although both α Gals from guar and coffee bean show comparable activities toward the substrate, PNP- α -galactopyranoside, their specificities toward oligosaccharide chains are significantly different: guar α Gal primarily cleaves α 1,6 glycoside linkages (Giuseppin et al., 1993), whereas coffee bean enzyme preferentially α 1,3 and 1,4 linkages. Thus the guar α Gal is unable to cleave significant amounts of terminal α 1,3-linked galactose residues from the cell surface of group B red cells (J.G., unpublished data). By using a protein analysis program, PROSIS (Hitachi Software Engineering), the deduced aa sequences from coffee bean and guar α Gal cDNA clones share approx. 30% homology, even though their signal peptide sequences bear little similarity. The coffee bean enzyme also exhibits significant homology with α Gals from human (59%), yeast (58%) and *Aspergillus niger* (52%), when analyzed by the same computer program. As shown in Fig. 2, the conserved residues are all located towards the N-terminal portion of the mature enzyme from these five sources, whereas their C-terminal sequences are strikingly diverse. It is not clear whether this structural feature contributes to their similar but not identical substrate specificity.

The sequences listed in Fig. 2 share little, if any, homology with α Gal isolated from *E. coli* (Liljeström and Liljeström, 1987). Recently, two more α Gal cDNAs have been isolated from *Klebsiella pneumoniae* (Hama and Wilson, 1992) and a *Streptococcus* mutant (Aducci-Opoku et al., 1991). However, they do not bear any sequence homology with known α Gals at the aa level. It has been shown that two enzymes can have similar catalytic mechanisms and active sites without any similarity in primary sequences as in the case of chymotrypsin and subtilisin. Thus, it may be that eukaryotic α Gal and its prokaryotic counterparts also have the same characteristics. Further understanding of the mechanism and residues involved in the active site of α Gal awaits X-ray crystallography and site-directed mutagenesis studies.

(d) Functional expression of coffee bean α Gal in insect cells

Coffee bean α Gal cDNA was subcloned from the plasmid pCR-BZ7 between the unique *Nco*I and *Bam*HI sites of a baculovirus expression vector pVL1392

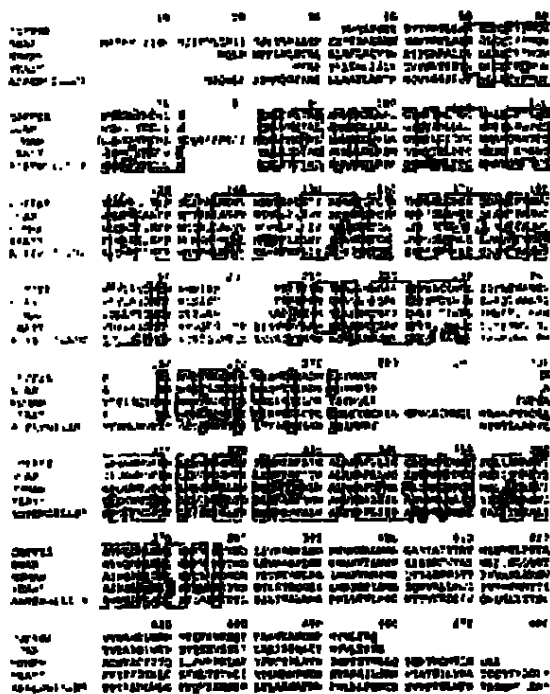


Fig. 2. Sequence homology of α Gal from different sources. The 28 sequences of α Gal from coffee bean (coffee), cynomolgus (cynomolgus), human placenta (human), Simulium (Simulium), and *Aspergillus niger* (Aspergillus) are aligned by using the computer program PROSIS and manual arrangement. The gaps are created in order to show maximum similarity. The numbers above the sequences indicate the relative position of each sequence. The sequences of α Gal and β -galactosidase from *Aspergillus* are truncated at the C terminus (indicated by *). Residues W and Q are, respectively, identical or conservatively substituted as residues (the out) of one or more at the same position. A is moved according to the equivalent as for 1, A.S.L.P. and G, 2, N.D.I. and G, 3, H. K. and A, 4, M.L.L. and V, 5, F, 6 and W.

(Pharmingen), generating the plasmid pVL-BZ. Expression of α Gal cDNA was thus under the control of a strong viral promoter (polyhedrin promoter). The plasmid pVL-BZ was transfected into Sf9 insect cells according to the procedure suggested by the manufacturer.

In order to detect the expression of α Gal in the transfected Sf9 cells, a Western blot was carried out by using antibody against purified coffee bean α Gal. As shown in Fig. 3, the coffee bean α Gal was produced in the Sf9 cells (transfected with the plasmid pVL-BZ (lane 2)) but not in cells transfected with wt virus (lane 4). The major band in lane 2 was identical in size to that of purified enzyme (lane 5), whereas the two minor bands of lower M_r (lane 2) may represent part of the molecule generated presumably by alternate initiation or proteolytic digestion. In addition, the protein expressed from the plasmid pVL-BZ was localized within the cell but not secreted into the tissue medium (lane 1).

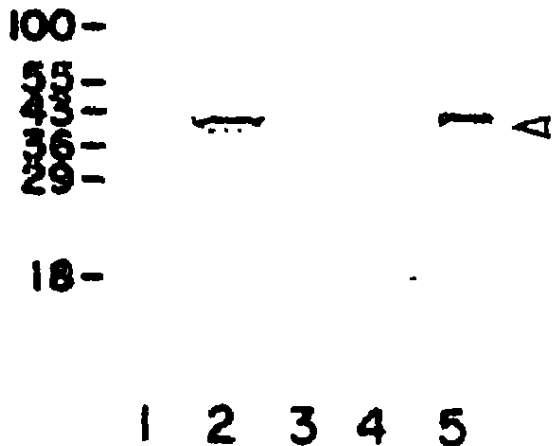


Fig. 3. Expression of recombinant coffee bean α Gal (cDNA) in insect cells (Sf9). The plasmid pVL-BZ (2 μ g) was co-transfected into Sf9 cells (2×10^6) with baculoviral DNA (Pharmingen). Sf9 cells were also transfected with wt virus as a control. After incubation at 27°C for 3 days the supernatants of the transfection plates were collected and 1 ml of the supernatants was added to freshly seeded Sf9 cells (2×10^6 cells) for virus amplification (3 days at 27°C). Virus amplification was repeated one more time with 0.1 ml of supernatant from the first round amplification. The supernatants and cells were collected after the second amplification and analyzed on a 9.1% SDS-12% PAGE by Western blot with polyclonal antibody against purified coffee bean α Gal. The supernatant and cells from pVL-BZ transfection are shown in lanes 1 and 2, respectively. The supernatant (lane 3) and cells (lane 4) from wt virus transfection are used as a negative control. Lane 5 is α Gal purified from coffee bean and molecular mass standards are listed at the left margin (in kDa).

α Gal activity was tested by incubating the intact pVL-BZ transfected cells with 1.25 mM PNP- α -galactopyranoside (pH 6.5). After incubation at 37°C for an hour the reaction was stopped by adding 1 ml of borate buffer (pH 9.8) and the A_{405} was measured. The total proteins in the reaction were precipitated by addition of trichloroacetic acid and measured by Bio-Rad Protein Assay (Bio-Rad). The specific activity of α Gal produced in the insect cells was approx. 300 units/mg (ranging from 200–300 units/mg from three experiments), one unit being defined as 1 nmol of substrate hydrolyzed per hour at 37°C. The endogenous activity in the wt virus infected cells was undetectable under such conditions. The activity of α Gal in the total Sf9 cell extract is approx. 3.7-fold higher than that obtained from crude coffee bean extract (80 units/mg).

The α Gal cDNA has been cloned into a vector pAcGP67 (Pharmingen), which contains a signal sequence from the envelope glycoproteins of the baculovirus. This strategy allows the forced secretion of otherwise unsecreted recombinant proteins which may be easily

purified when protein-free insect culture medium is used. Biologically active chicken α -N-acetylgalactosaminidase has been produced and secreted into the culture medium by using this vector (unpublished data). We are now in the process of optimizing the expression level of α Gal in this system. As reported, most heterologous proteins are produced at levels ranging from 10 mg to 100 mg per 10^7 cells (O'Reilly et al., 1992). Thus the baculovirus system may prove to be a valuable system for obtaining enzymatically active α Gal, in a large quantities, for use in B-O blood type conversion.

[e] Conclusions

A cDNA encoding coffee bean α Gal has been cloned and sequenced. It demonstrates sequence homology ranging from 52%-80% with other α Gal clones isolated from various sources. The recombinant coffee bean α Gal produced in insect cells is recognized by the antibody raised against the purified enzyme from coffee beans and is enzymatically active. We are currently trying to optimize the conditions for increasing the level of coffee bean α Gal expressed in insect cells.

ACKNOWLEDGEMENTS

We wish to express our appreciation to Angie Sun for her technical assistance and to Dr. Asok Chaudhuri for kindly providing us the primers, pI and pII, used in the 3'RACE. This work was supported by N00014-90-J-1638 with funds provided by the Naval Medical Research and Development Command.

REFERENCES

Adler, C.P., J., Fao, L., Forrester, J.J. and Russell, R.R. Biochemical and genetic analysis of *Streptococcus mutans* α -galactosidase. *J. Gen. Microbiol.* 137 (1993) 2271-2272

Bishop, D.F., Culhane, D.H., Benninger, H.S., Hatzopoulos, P., Quinn, M. and Desnick, R.J. Human α -galactosidase A nucleotide sequence of a cDNA clone encoding the mature enzyme. *Proc. Natl. Acad. Sci. USA* 83 (1986) 4659-4663

den Hertog, H., Rosell, A.M., van Zuden, C.M., Punt, P.J. and van den Hondel, C.A. Cloning and expression of a member of the *Yersinia enterocolitica* gene family encoding α -galactosidase. *Mol. Gen. Genet.* 233 (1992) 404-410

Frohman, M.A., Dush, M.K. and Martin, G.R. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* 84 (1988) 9048-9052

Ganepkin, M.L., Almshark, J.W., Henrich, J.C. and Verrips, C.T. Comparative study on the production of goat α -galactosidase in *Saccharomyces cerevisiae* SL308 and *Hansenula polymorpha* 712 continuous cultures. *Appl. Environ. Microbiol.* 59 (1993) 52-59

Goldstein, J. Conversion of ABO blood groups. *Transfusion Med. Rev.* 3 (1989) 206-212

Goldschick, J., Siviglia, G., Hurst, R., Lemay, L. and Reick, I. Group B erythrocytes enzymatically converted to group O survive normally in A, B, and O individuals. *Science* 215 (1982) 168-170

Hama, H. and Wilson, T.H. Primary structure and characteristics of the mannose carrier of *Aspergillus fumigatus*. *J. Biol. Chem.* 267 (1992) 12371-12376

King, L.A. and Paoletti, R.D. The Baculovirus Expression System: A Laboratory Guide. Chapman & Hall, New York, NY, 1992

Lemay, L.L., Hurst, R., Goldstein, J., Benjamin, L.J. and Jones, R.L. Single-unit transfusions of RBC enzymatically converted from group B to group O to A and O normal volunteers. *Blood* 75 (1990) 1383-1388

Lemay, L.L., Hurst, R., Goldstein, J. and Oulbrath, R.A. Two-unit transfusions to group O subjects of red blood cells enzymatically converted from group B to group O. *Transfusion* 34 (1994) in press

Liljeqvist, P.L. The nucleotide sequence of the yeast MELI gene. *Nucleic Acids Res.* 13 (1985) 7257-7269

Liljeqvist, P.L. and Liljeqvist, P. Nucleotide sequence of the *mel1* gene, coding for α -galactosidase in *Escherichia coli* K12. *Nucleic Acids Res.* 15 (1987) 2213-2220

O'Reilly, D.R., Miller, L.R. and Linkow, V.A. Baculovirus Expression Vectors. A Laboratory Manual. W.H. Freeman, New York, NY, 1992

Overbeek, N., Fellinger, A.J., Fournier, M.V., Van Wasterlain, D. and Verrips, C.T. Cloning and nucleotide sequence of the α -galactosidase cDNA from *Corynebacterium jeikeium* subsp. *jeikeium*. *Hum. Mol. Biol.* 13 (1994) 541-550

Wada, K.-n., Wada, Y., Ishizumi, T., Grynberg, T. and Ikemura, T. Codon usage isolated from the GenBank genetic sequence data. *Nucleic Acids Res.* 20 (1992) 2311-2316

Coffee seeds contain 11S storage proteins

Ricardo Acuña^a, Ronald Bassiner^a, Vadim Beilinson^a, Hernando Cortina^a, Gabriel Cadena-Gómez^a, Virginia Montes^b and Niels C. Nielsen^{c,*}

^aCentro Nacional de Investigaciones del Café, Chinchiná, Caldas, Colombia

^bDepartamento de Química, Universidad Nacional, Bogotá, Colombia

^cUnited States Department of Agriculture, Agricultural Research Service and the Departments of Agronomy and Biochemistry, Purdue University, West Lafayette, IN 47907, USA

*Corresponding author, e-mail: Nielsen@dept.agry.purdue.edu

Received 10 July 1998; in final form 26 October 1998

A legumin-like seed protein was purified from the endosperm of coffee (*Coffea arabica* L. cv. Colombia). In contrast to legumes, where efficient storage globulin extraction requires buffered saline solutions well above the acidic pK_i of the globulins, coffee legumin is readily extracted with acidic aqueous buffers. The coffee legumin migrates like other 11S storage globulins in sucrose gradients. Subunits of coffee legumin have an apparent molecular mass of about 55 kDa after one-dimensional SDS-polyacrylamide gel electrophoresis in the absence of a reducing agent. In the presence of 2-mercaptoethanol, two polypeptides appear that have apparent molecular masses of 33 and 24 kDa. Two full-length cDNAs were generated from mRNA of developing seeds that were more than 98% homologous. They had open reading frames of 1458 and 1467 bp. Each encoded legumin precursors of 486

and 489 amino acids, respectively ($M_r = 54136$ and 54818). Examination of a 5' promoter region from a coffee legumin gene revealed a putative legumin-box. Genomic DNA from *C. arabica* was digested with six different restriction endonucleases. After separation of the fragments by electrophoresis, single discrete fragments on DNA blots hybridized strongly to a cDNA probe for the acidic chain. Other fragments that hybridized weakly with this probe were visible after hybridization at very low stringency. DNA from other species and commercially important cultivars that comprise the genus *Coffea* produced similar results. Immunocytochemical studies revealed that some legumin was detected in the cytoplasm in mature coffee seeds, but that the majority of it was in large storage vacuoles that accounted for most of the cell volume.

Introduction

Developing plant seeds accumulate and store carbohydrate, lipid, and protein that subsequently are used during germination as a source of energy, carbon, and nitrogen. Expression of storage protein genes in seeds is restricted to the embryonic axis and cotyledons, and in some cases to the endosperm of developing seeds. A large number of storage protein genes have been cloned and characterized (Nielsen et al. 1997). The expression patterns of seed proteins during seed development are highly regulated both developmentally (Goldberg et al. 1989, Perez-Grau and Goldberg 1989, Guerche et al. 1990) and metabolically (Wobus et al. 1995).

Coffee bean, the raw material from which all coffee products originate, is derived from the seeds of *Coffea* spp. Despite the immense economic importance of coffee bean, little is known about the biochemical and molecular properties of its seed proteins. Only values for the protein content of coffee beans have been reported, and these are based on the determination of crude nitrogen and multiplication by the factor 6.25. Crude protein values in the range of 8.7–12.2% have been obtained after corrections are made for caffeine and trigonelline-nitrogen (Muerac 1985). Work has focused on the characterization of water soluble proteins in different coffee varieties (Centi-Grossi et al. 1969) or coffee

Abbreviations DTT: dithiothreitol; E64: N-[N-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]glycine; kDa: kilodalton; Pylabloc: 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; PMSF: phenylmethyl-sulfonyl fluoride; PVDF: polyvinylidene difluoride; PVPP: polyvinyl-polypyrrolidone; SSC: standard saline-citrate buffer; TBST: Tris-buffered saline (10 mM Tris(hydroxymethyl)aminomethane HCl, pH 8.0, 150 mM NaCl) containing 0.075% Tween-20 (Bio-Rad).

beans processed by different methods (Underwood and Deatherage 1952). The information provided in these reports describes molecular mass profiles and protein content. Nothing is known about the structure of genes that encode the proteins, the identity of the primary gene products, or the regulation of their expression.

In this publication, legumin-like seed storage proteins of the 11S size class (Nielsen et al. 1997) were identified in coffee. Two cDNAs that encode the proteins are described, a promoter for an 11S gene is analyzed, and the subcellular localization of the protein in the seed endosperm is documented.

Materials and methods

Coffee seeds (*Coffea arabica* cv. Colombia, progenies R-550 and S-366) were harvested between 150 and 220 days after flowering, immediately frozen in liquid nitrogen, and stored at -80°C until use. For immunocytochemistry, the endosperm was harvested about 220 days after flowering and fixed with 3% glutaraldehyde in phosphate buffer.

Extraction and fractionation of total seed protein

One hundred milligrams of frozen immature (20–22 weeks after flowering) or mature endosperm (32–36 weeks after flowering) were ground to a fine powder under liquid nitrogen in a pre-cooled mortar and pestle. The powder was suspended in 500 μl of SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol supplemented with 1 mM EDTA, 1 mM PMSF (Sigma, St. Louis, MO, USA), 0.02% Pefabloc (Boehringer Mannheim, Indianapolis, IN), 20 $\mu\text{g ml}^{-1}$ E64 (Boehringer Mannheim), 10% PVPP (Sigma), 100 mM NaCl, and 5 mM sodium metabisulfite. After sedimenting, the extracts were stored in small aliquots at -80°C . Electrophoresis was carried out according to Laemmli (1970) and the gels stained with the Coomassie reagent.

Sequential fractionation of coffee seed proteins

Powder (200 mg) from frozen endosperm was extracted three times with 1 ml of 80 mM sodium acetate, pH 4.5, 100 mM KCl, 10% DMSO, 0.5% n-butanol, 3% PVPP, 0.02% sodium azide, 1 mM PMSF, 0.02% Pefabloc, and 20 $\mu\text{g ml}^{-1}$ E64 (Sluyterman and Wijdens 1970). The albumins in the supernatants that were recovered after centrifugation were combined. The pellet was extracted three times with 1 ml of 100 mM KH_2PO_4 , pH 7.6, 0.4 M KCl, 3% PVPP, 0.02% sodium azide, 1 mM PMSF, 0.02% Pefabloc, and 20 $\mu\text{g ml}^{-1}$ E64, and the supernatants after centrifugation were combined to form the globulin fraction. The pellet from the preceding step was extracted three times with 1 ml of 70% absolute ethanol and 10 mM DTT, and the supernatants were combined to form the prolamin fraction. Finally, the residue from the ethanol extraction was suspended in a SDS-buffer solution with 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 1 mM EDTA, 100 mM NaCl, and 5 mM sodium metabisulfite. Proteins in aliquots from each of the

fractions described above, with or without 2-mercaptoethanol, were analyzed by SDS-PAGE and stained with the Coomassie reagent.

Sucrose density centrifugation

Powder (250 mg) from frozen seeds was homogenized in either 1 ml of 'albumin extraction buffer' or 1 ml of 'globulin extraction buffer'. The homogenate was centrifuged at 18000 g for 30 min in a JA-17 rotor (Beckman, Fullerton, CA, USA), and proteins in the resulting supernatant were fractionated in a sucrose gradient as described by Dickinson et al. (1987). Low salt gradients contained 30 mM Tris-HCl, pH 7.6, while the high salt gradients had 35 mM potassium phosphate buffer, pH 7.6, and 0.4 M NaCl. In both cases the buffers were supplemented with 1 mM PMSF and 0.02% sodium azide. Proteins in the albumin (approximately 460 $\mu\text{g per 200 } \mu\text{l}$ protein) and protein from the globulin (about 145 $\mu\text{g per 200 ml}$) fractions were pipetted onto each gradient, and centrifuged at 250000 g for 18 h in a Beckman SW-41 rotor. Monomer, trimer, and hexamer extracts of globulin from soybean seeds served as positive controls to identify the position in the gradient to which 3S, 7S, and 11S proteins migrated. The gradients were divided into 32 fractions, and protein in each fraction was measured by the method of Bradford (1976). Samples from each fraction were analyzed by SDS-PAGE discontinuous gel electrophoresis in either the presence or absence of 2-mercaptoethanol.

Dialysis of coffee 11S protein fraction

The 11S coffee protein fractions were dialyzed at 4°C in 30 mM Tris-HCl, pH 7.6, 1 mM PMSF, and 0.02% sodium azide for 12 h, and fractionated in a 6–22% linear sucrose density gradient. Sedimentation standards included bovine serum albumin (3S) and soybean conglycinin (7S).

N-terminal sequence analysis

Samples of coffee legumin were obtained after separation of the albumin fraction by SDS-PAGE. One separation contained unreduced protein, and the other was treated with 2-mercaptoethanol. After electrophoresis, the gels were electroblotted to Immobilon membranes (Bio-Rad, Hercules, CA) with a TE70 Semi-PhorTM semi dry electroblotter (Hoefer Scientific Instruments, San Francisco, CA). After transfer, the proteins were stained with 0.1% Coomassie Blue in 50% methanol. Stained bands from the blot were cut into 2×4 mm segments. After drying with a Speed-Vac (Savant, Farmingdale, NY) for 1 h, the samples were analyzed in a model A491 protein sequencer (Applied Biosystems, Foster City, CA, USA).

Antibody preparation

After preparative SDS-PAGE, bands with an apparent molecular mass of 55 kDa without 2-mercaptoethanol, and a 33 kDa protein in its presence, were forced through a nylon mesh, and the samples were emulsified with Freud's

adjuvants. Antibodies were generated as described by Harlow and Lane (1988). Purification of antibodies for immunochemistry was achieved by protein A affinity chromatography according to the protocol provided by Bio-Rad. The antibodies prepared against glycinin G3 (*Glycine max*) and legumin B4 (*Vicia faba*) are described by Beaman (1996), while those recognizing pea vicilin were provided by Dr Rod Casey (John Innes Institute, Norwich, England).

Immunodetection of proteins

After SDS-PAGE, the proteins were transferred to Immobilon membranes (Bio-Rad), washed two times (5–10 min) with TBST (TBS with 0.075% Tween-20). The blots were transferred into a plastic bag, and 10–15 ml blocking solution (0.8% blocking reagent, Boehringer Mannheim) and 0.02% sodium azide in TBST was added. After 30–60 min of gentle shaking, the primary antibody (diluted in blocking solution 1:1000) was introduced, and the membranes were shaken vigorously overnight. Proteins reacting with the antibodies were visualized using an alkaline phosphate conjugate substrate kit from Bio-Rad.

Extraction and purification of mRNA from immature coffee endosperms

RNA was isolated from 2.5 g of powder from immature coffee endosperm (150 days after pollination) as described by Shultz et al. (1994), except that the selective precipitation step for polysaccharides with 2-butoxyethanol was omitted. The purified RNA was dissolved in 100 µl of DEPC-treated water to a final concentration of 3 µg µl⁻¹. Poly(A)-rich RNA was purified with the Poly(A)tract mRNA Isolation System (Promega, Madison, WI, USA). Absorbance ratios at 230/260 were greater than 1.5, indicating that there was little or no polysaccharide or polyphenol contamination. Ratios of A260/A280 ranged between 2.0 and 2.2, which demonstrated there was minimal protein contamination. The total RNA yield ranged between 120 and 150 µg g⁻¹ of endosperm. The RNA integrity was judged by the clarity of ribosomal RNA bands visualized on a denaturing agarose gel and the lack of degraded RNA after separation by electrophoresis (data not shown). To prove translatability of the isolated mRNA, a reticulocyte lysate (Promega) was used to translate the mRNA *in vitro*.

Cloning of cDNAs

Synthesis of full-length cDNA was carried out using the TimeSaver™ cDNA Synthesis Kit (Pharmacia, Piscataway, NJ). An *EcoRI*-*NotI* adaptor was ligated to the ends of the resulting fragments (Pharmacia). The adaptor consisted of two oligonucleotides that form a duplex containing a phosphorylated blunt end, a non-phosphorylated *EcoRI* overhang, and an internal *NotI* site. A sense-degenerate oligonucleotide primer (5'-GGGCGAARAC'ICARTGYAAYATICARAA-3') was synthesized on the basis of the N-terminal amino acid sequence determined for the acidic chain (GGKTQCNIQK) of the coffee legumin. A second antisense-degenerate oligonucleotide primer (5'-TTTAAIACRT'ICIG'YTCYTGIGIARICC-3')

was synthesized based on the N-terminal amino acid sequence determined for the basic chain (GLPQEADVFN). These primers were used to amplify nearly the entire acidic legumin chain. The PCR fragment (0.8 kb) was cloned into a pCRII vector (Invitrogen, Carlsbad, CA) and called pCA4. Both strands were sequenced with an ABI automated DNA sequencer (Pharmacia). Based on the nucleotide sequence that was obtained, two additional oligonucleotide primers (sense: 5'-GGATTCTCGGGA'ITCTMTAATCCAGAATTTGGG-3' and antisense: 5'-CCAGAA'TCCAGTTAAACACAGC-C'CTGATGCG-3') were synthesized and used in combination with short specific primers (5'-CGCGGG'-CGCTTTTTTTTTTTT-3' or 5'-CGCGGG'-CGC-3', respectively, designed on the basis of the *EcoRI*-*NotI* adaptor sequence). These were used to amplify cDNA encoding most of the acidic chain, the basic chain (pCA9), and the missing N-terminal part of the cDNA, respectively (pCA3). The amplification conditions were as follows: 96°C for 5 min followed by 33 cycles of 94°C for 50 s, 56°C for 1 min and 72°C for 1.5 min, and a final extension period of 72°C for 10 min. The PCR fragments (1.4 and 0.2 kb, respectively) were cloned and sequenced as above. The overlapping sequences were aligned to yield a chimeric full-length cDNA sequence. Oligonucleotide sequences derived from the 5' and 3' ends of the chimeric sequence permitted two different authentic full-length cDNA clones to be obtained by PCR.

Genomic DNA

The methods described by Cho et al. (1989) and Orozco-Castillo et al. (1994) were used as a basis for the preparation of genomic DNA. After purification, the DNA was washed twice with 75% ethanol, dried, and dissolved in 0.4 ml TE. The DNA was quantified with a spectrophotometer and stored at 4°C.

DNA blot hybridization

Genomic DNA (6 µg per 35 µl) was digested with various restriction enzymes in appropriate buffers overnight at 37°C. After separation of restriction fragments in 0.8% agarose gels, a VacuGene XL vacuum blotting system (Pharmacia) was used to transfer the DNA fragments from the gel to GeneScreen Plus membranes (DuPont, Boston, MA). For use as a probe, the *EcoRI* DNA fragment from pCA4 (~900 bp), which included most of the coding region for the acidic chain of coffee legumin, was purified by agarose gel electrophoresis and labeled using an Ambion DECAPRIME II kit (Austin, TX). The probe was denatured for 5 min at 100°C before use. The hybridization buffer contained 6 × SSC, 5 × Denhardt solution, 1% SDS, and 100 mg ml⁻¹ sonicated herring sperm DNA. Prehybridization lasted 1 h at 65°C, and then hybridization was carried out overnight at either 55 or 52°C. The membrane was washed with constant agitation, first with 2 × SSC for 5 min at room temperature, then three times each at 45, 50, and 55°C for 20 min in 2 × SSC and 1% SDS (or as indicated). Finally, the membrane was rinsed with 0.1 × SSC at room temperature, wrapped in Saran Wrap and exposed to film.

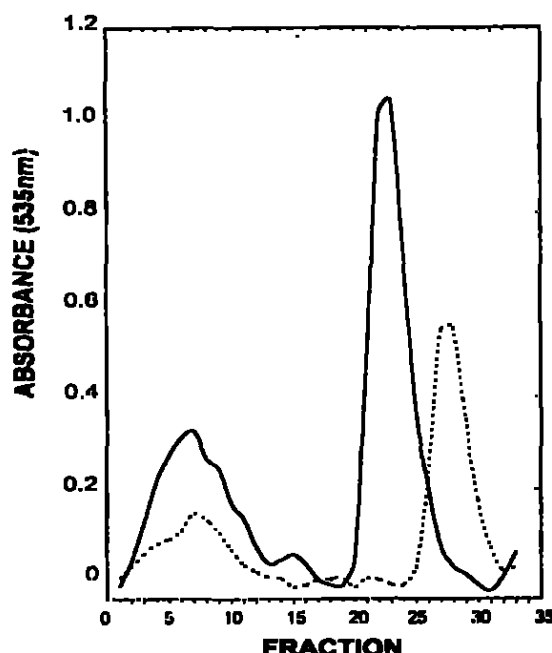


Fig. 2. Sedimentation properties of proteins extracted from coffee endosperm. Proteins from the albumin fraction of coffee were separated in gradients of 6–22% sucrose at low (no added salt, dashed line) or high ionic strength (0.4 M KCl, solid line) buffer. Two prominent peak regions are observed with coffee samples around fractions 9 and 27 for samples from gradients that contained no added salt. The latter peak shifted to a lower apparent density (fraction 23) in sucrose gradients that contained 0.4 M NaCl. Fraction 1 is from the top of the gradient and fraction 33 is from the bottom.

like proteins in coffee are extremely soluble and nearly completely extracted under these conditions.

The sedimentation of coffee endosperm proteins in sucrose density gradients was tested. Fig. 2 compares the distribution of the major coffee endosperm proteins after extraction and sedimentation with a standard or low salt buffer. When the gradient fractions were analyzed at 535 nm, two prominent peaks in absorbance were observed after extraction with normal salt buffer. These were associated with fractions 7 and 27 (Fig. 2). The high molecular mass protein from coffee clearly sediments in sucrose density gradients at a position similar to 11S legumins from legumes such as faba bean. The electrophoretic separation of the fractions corresponding to the 11S peak (fractions 22–30) resulted in a single high molecular mass band prior to disulfide reduction and two bands after reduction (data not shown). Proteins in these fractions reacted immunologically with anti-coffee legumin antibodies (data not shown). Together, these data indicated that this peak contained the coffee legumin-like proteins.

Unlike the 11S storage proteins from many legumes, soybean glycinin dissociates reversibly when the ionic strength is decreased sufficiently (Wolf and Briggs 1958, Dickinson et al. 1989). We tested the effect of low ionic strength buffers on the legumin-like proteins from coffee. When suspended in 30 mM Tris, pH 7.6, instead of phosphate-buffered saline, the legumin-like proteins from coffee

sedimented farther into the sucrose gradient (Fig. 2) but did not dissociate. Apparently, the decreased ionic strength results in a more compact structure, perhaps because the ionic shell surrounding the protein complex is reduced in size. Dialysis of the protein in even lower ionic strength buffers (2 mM Tris, pH 8.0) also did not result in dissociation. Therefore, the coffee 11S proteins do not behave like soybean glycinin; rather, their behavior at low ionic strength resembles that of many other 11S proteins that do not dissociate under these conditions.

It is of interest to compare our results with those of Amorim and Josephson (1975). They found that, under nonreducing conditions, most of the coffee proteins were either high molecular mass components between 150 and 170 kDa, or proteins of approximately 66 kDa. When these workers treated their samples with 2-mercaptoethanol, the major proteins that were recovered after electrophoresis had an apparent molecular masses of about 33 and 18 kDa. While our results are not directly equivalent, it is possible to rationalize them with the results of Amorim and Josephson. The legumin complexes isolated from many seeds consist of six 60 kDa subunits, each subunit being composed of two polypeptide chains that are joined by a disulfide bond. The results described in Fig. 2 indicate that the legumin complex isolated from coffee sediments is similar to hexamers from other plant species. The 150–170 kDa proteins reported by Amorim and Josephson may correspond to legumin trimers, in which event the 66 kDa proteins are related to the individual subunits. We found that subunits of coffee legumin had apparent molecular masses of about 55 kDa. The proteins of 33 and 18 kDa found by Amorim and Josephson after sulphydryl reduction compare favorably with sizes of the acidic (33 kDa) and basic chains (24 kDa) observed after similar treatment.

Complementary DNA encoding coffee legumin

Messenger RNA was isolated from developing coffee seeds and used to prepare cDNA. When the mRNA from coffee endosperm was translated in a reticulocyte lysate *in vitro* system, the prevalent products were about 55 kDa, the size expected for putative coffee legumin precursors (data not shown). The translatable mRNA was used to prepare cDNA, which in turn served as a template to amplify legumin DNA by PCR. To deduce the sequence of primers to be used for PCR, the acidic and basic chains of the putative 11S proteins from coffee were purified from gels after SDS-PAGE, and their N-terminal sequences were determined. Sequences of 33 and 25 amino acids in length (see Fig. 3) were obtained that had extensive homology to other 11S sequences reported in various databases. These were used to deduce nucleotide sequences that could be used for PCR to generate clones that encoded putative 11S coffee legumin-like molecules. Details concerning the choice of the primer sequences are presented in Materials and methods.

Two cDNAs 98% homologous to one another were amplified from mRNA that was isolated from developing coffee seeds (see Materials and methods and Fig. 3 for details). In addition to several nucleotide insertions and substitutions in the interior of these cDNAs, differences were also present

acid composition has a low content of sulfur-containing and aromatic amino acids and elevated levels of acidic and amidated amino acids. Such a composition is typical for almost all the 11S storage proteins. Because the deduced amino acid sequence does not have glycosylation sites of the type Asn-X-Thr-Ser, the protein is unlikely to be glycosylated. Most other members of the legumin 11S globulin family are also not glycosylated. By analogy to legumins in soybean (Staswick et al. 1984), and based on the observation that coffee legumin subunits dissociate into a larger and a smaller polypeptide chain, it seems likely that Cys₁₁₂ and Cys₉₁ form the disulfide bridge that covalently links the acidic and basic chains in the mature subunit. Also by analogy with legumin proteins from other plant species, Cys₉₁ and Cys₉₀ probably form the intrachain disulfide bridge within the acidic chain of the coffee protein (Nielsen et al. 1997).

Two explanations could account for the recovery of two slightly different legumin cDNAs from coffee (Fig. 3). One is that each cDNA was the product from an allelic form of the same gene. The beans collected for our experiments were from the same vegetative clone of coffee. Although we cannot eliminate the possibility that there were allelic differences between the two bushes from which beans were collected, we consider the possibility quite unlikely. The second explanation is that the coffee genome contains at least two legumin genes. This explanation is complicated by the fact that coffee is an allotetraploid ($2n = 4X = 44$) (Grassias and Kammacher 1975). Similar genes controlling the synthesis of legumin subunits could reside in each of the genomes that comprise the tetraploid and thereby account for their considerable homology.

Legumin genes in coffee

Because most plant species contain a small family of genes that encode legumin subunits, we determined if the situation was similar in coffee. Fig. 4 shows the results from an experiment where genomic DNA from *C. arabica* was digested with a number of restriction enzymes. After digestion, the fragments were separated by electrophoresis and the DNA blots with the resulting fragments probed with the legumin insert from pCA4. The insert in this clone encoded most of the acidic chain from the mature subunit. The restriction enzymes *CluI*, *EcoRI*, *EcoRV*, and *XbaI* do not cut within that portion of the coffee legumin cDNA coding sequence contained in pCA4. The data in Fig. 4 shows that with the exception of *CluI*, only a single discrete DNA fragment hybridized strongly with the probe, but background hybridization suggested that there were DNA fragments that hybridized weakly. *DraIII* and *KpnI* each cut once (Fig. 3) within the cDNA sequence of pCA4. Nonetheless, each produced a single discrete fragment that hybridized strongly with the probe. *CluI* also cut within the cDNA, but in this case, two fragments hybridized strongly to the probe. Whereas hybridization conditions that resulted in the data shown in Fig. 3 was carried out at 55°C, hybridizations carried out at 62°C resulted in blots with single discrete bands that corresponded to the most strongly hybridizing bands (data not shown). For these experiments,

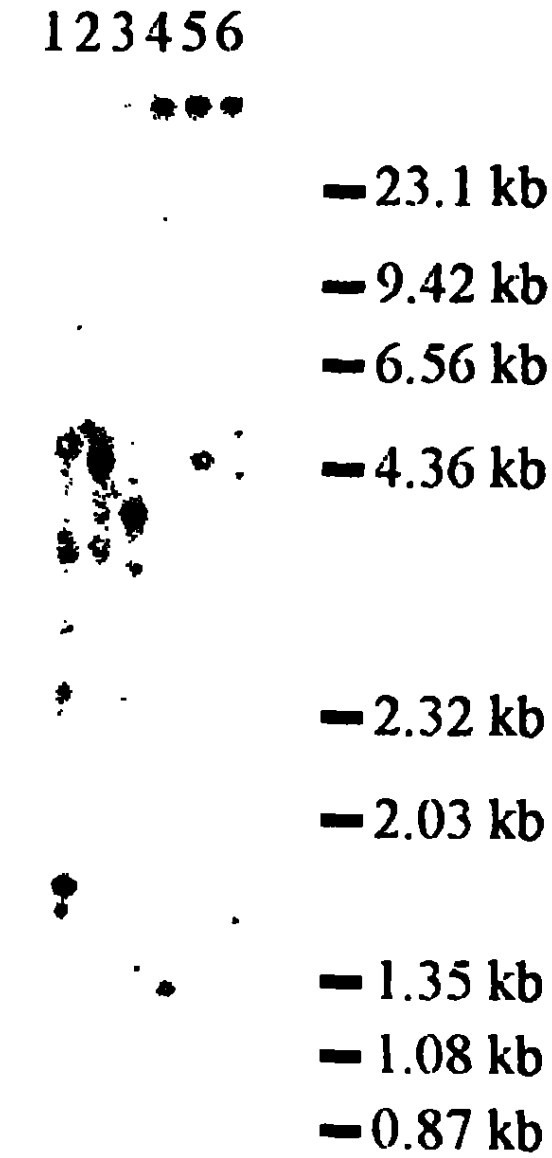


Fig. 4. DNA blot analysis of coffee genomic DNA cut with six restriction endonucleases. Genomic DNA of *Coffea arabica* cv. Catara (# 207) was digested with: Lane 1 *EcoRI*, lane 2 *EcoRV*, lane 3 *XbaI*, lane 4 *DraIII*, lane 5 *CluI*, and lane 6 *KpnI*. After separation of the resulting DNA fragments by electrophoresis in 0.8% agarose, the gels were blotted and probed with the insert from pCA4 at low stringency (see text). With the exception of lane 5, each restriction reaction produced a single fragment that hybridized strongly, and several that hybridize weakly. Because there is a single *CluI* restriction site within the insert in pCA4, DNA cut with this enzyme yielded two fragments that hybridized strongly.

fragments that hybridized more weakly were not visible in the background of the DNA blots.

We determined if a similar genomic organization occurred in species and varieties related to *C. arabica*. To address this question, genomic DNA was prepared from each of ten species or commercially important varieties of coffee. Each of the DNAs was cut with three restriction enzymes (*XbaI*, *EcoRV*, and *EcoRI*) and the fragments separated by elec-

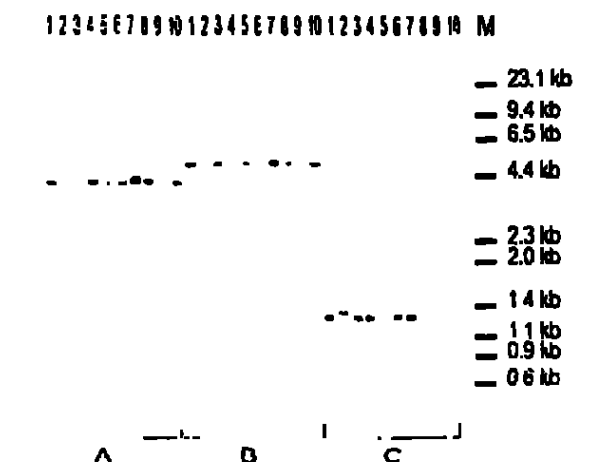


Fig. 5. Genomic DNA blot analysis of ten different species and cultivars from the *Coffea* family. Genomic DNA was digested with three restriction endonucleases (A *Xba*I, B *Eco*RV, C *Eco*RI). DNA fragments after this treatment were separated in a 0.8% agarose gel, transferred to a nitrocellulose membrane, and probed with the DNA insert from pCA4 at high stringency. The insert corresponds to the mature acidic chain and N-terminal of the basic chain. Species 1, Hibrid de Timor (INT 1105); species 2, *C. eugenioides* (INT 865); species 3, *C. liberica* (INT 346); species 4, *C. kapakatu* (INT 61); species 5, *C. arabica* cv. Caturra (INT 207); species 6, *C. canephora* cv. Conilon (INT 176); species 7, *C. canephora* cv. Robusta (INT 277); species 8, *C. arabica* x *C. canephora* (HGB 981); species 9, *C. arabica* (Ar56-92); species 10, *C. arabica* cv. Colombia.

trophyresis. When probed with the insert from pCA4, the results shown in Fig. 5 were obtained. Little polymorphism was evident when the various coffee DNAs were cut with *Xba*I and *Eco*RI. Bands of approximately 4.4 and 1.4 kb, respectively, were observed in the DNA blots from each of these species or varieties. Most DNA samples cut with *Eco*RV produced a fragment about 5 kb in size that hybridized to the pCA4 probe. However, *C. kapakatu* and *C. canephora* cv. Conilon each produced fragments of about 20 kb. Together, these data suggest that the number and structure of the legumin genes are reasonably conserved among the various species of coffee. Where polymorphisms were found, single rather than multiple bands were observed.

C. kapakatu is now included within the genus *Coffea*, although the taxonomy related to this assignment has been controversial (Charrier and Berthaud 1985). It, together with all of the other members of the genus *Coffea* except *C. arabica*, have a diploid genome. Consistent with the inclu-

sion of this species in the genus, cleavage of its DNA with *Xba*I and *Eco*RI yielded genomic fragments the same size as those obtained with DNA from other members of the genus that were tested (Fig. 5). This observation is compatible with there being similar chromosomal architecture around the legumin genes. However, differences among the various species are evident when *Eco*RV was used to cut genomic DNA. DNA from both *C. kapakatu* and *C. canephora* cv. Conilon resulted in *Eco*RV fragments about 20 kb that hybridized to the pCA4 probe. The rest of the species produced fragments of about 5 kb. Interestingly, and in contrast to *C. canephora* cv. Conilon, DNA from *C. canephora* cv. Robusta has an *Eco*RV fragment that is similar in size to the other diploid members of the genus and *C. arabica*. In the case of *C. arabica* cv. Colombia, at least, a single *Eco*RI site was present in the cDNA sequence (Fig. 3). Surprisingly, *C. eugenioides*, which is considered the ancestral parent of *C. arabica* (Orozco-Castillo et al. 1996), did not produce a DNA fragment that hybridized to the pCA4 probe. Studies by these authors revealed that *C. eugenioides* and *C. arabica* accessions shared 805 RAPD and organelle specific genome markers they developed. Perhaps the DNA fragments from this diploid species that contained the legumin gene were less than 1 kb and eluted from the gel. Nonetheless, the data do indicate that in *C. eugenioides*, the DNA encoding legumin and flanking regions is different than in most of the other members of the coffee genus.

The 11S storage protein genes from legumes contain a conserved regulatory sequence known as the legumin box (Bäumlein et al. 1986). Because the coffee storage proteins are deposited in endosperm tissue while legumes accumulate them in cotyledons, it was of interest to determine if the legumin box was also present in the promoter of the legumin gene from coffee. The nucleotide sequence reproduced in Fig. 6 presents the first 400 bp upstream from the beginning of the coding region for a coffee legumin gene. About 2.5 kb of nucleotide sequence for this promoter is reported in Accession No. AF055300 in the GenBank database. As is indicated in Fig. 6, a degenerate form of the legumin box is indeed present in the coffee gene. The CATGCAT element, which is a central feature in the legumin box of legumes, is conserved in the coffee sequence. The putative legumin box is found in approximately the same location relative to the start codon as in the 11S genes of legumes. As in the legume genes, CATGCAT elements besides the one in the legumin box are also present further upstream in the coffee promoter.

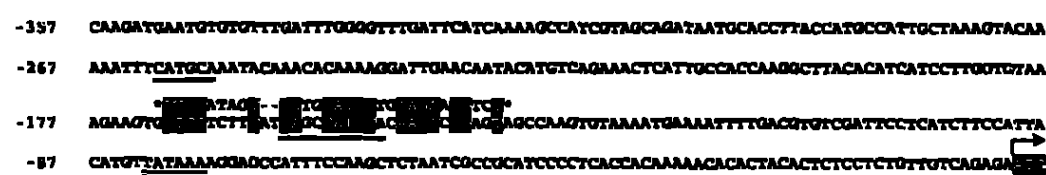


Fig. 6. Nucleotide sequence of the promoter for a coffee legumin gene. The first 400 bp of DNA upstream from the beginning of the coding region for a coffee legumin gene is shown. A putative legumin box is compared with a consensus sequence derived from legume genes (Bäumlein et al., 1986). Homology is indicated for nucleotide identified by reverse printing. Central to the legumin box is a degenerate CATGCAT element (underlined). As is typical of corresponding genes from legumes, other CATGCAT elements are present in the upstream region. A TATA box element (double underline) is also present.

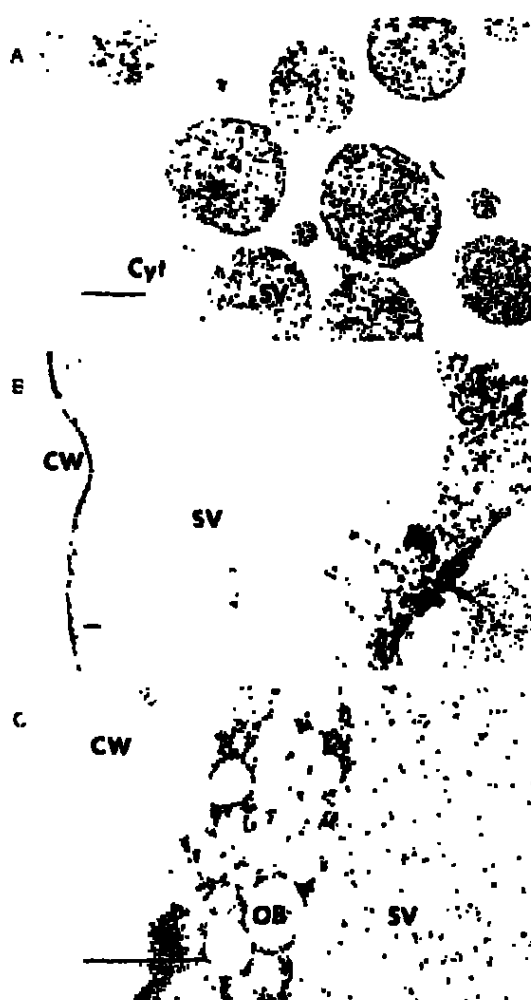


Fig. 7. Immunolocalization of soybean glycinin and coffee legumin. (A) Immunolocalization of glycinin to storage vacuoles in soybean. Gold labeled Gly3 antibodies were reacted with thin sections of soybean cotyledons. Label is found predominantly in storage vacuoles. Some label is observed in the cytoplasm, presumably due to proglycinin in transit from its site of synthesis on endoplasmic reticulum to the vacuoles where it is accumulated. (B) Low magnification electron micrograph of a typical coffee endosperm cell at about 220 DAF. The cells contain a large central storage vacuole surrounded by cytoplasm. Contained within the cytoplasm are many small, electron-transparent vacuoles, presumably oil bodies. A clear demarcation can be observed between the vacuole and cytoplasm. (C) High magnification electron micrograph showing immunolocalization of coffee legumin. Gold labeled antibody against coffee legumin were reacted with thin sections of coffee endosperm. Because of the size of the central storage vacuole, the majority of gold label is found there. Significant label is also found associated with cytoplasm, presumably due to prolegumin in transit from its site of synthesis on the endoplasmic reticulum. The size bar near the bottom left corner of each panel is 1 μ m. CW: cell wall; Cyt: cytoplasm; SV: storage vacuole; OB: oil body.

Subcellular localization of coffee legumin

Fig. 7 presents an immunocytochemical comparison of tissues from legume cotyledons and coffee endosperm. As a control, sections from midmaturation soybean seeds (Fig. 7A) were reacted with anti-glycinin-3 immunoglobulins. As ex-

pected, the protein storage vacuoles became heavily labeled. Occasional gold particles scattered throughout the cytoplasm could also be observed. The latter probably identify proglobulins in transit from their site of synthesis on rough endoplasmic reticulum to the storage vacuole. These results are equivalent to those reported for pea (Craig et al. 1980).

The subcellular organization of coffee endosperm at 220 DAF presented a somewhat different picture than in soybean. A typical low power section through a coffee endosperm cell is given in Fig. 7B and a higher magnification is presented in Fig. 7C. The coffee endosperm cells typically have relatively thick cell walls and an adjacent layer of cytoplasm that contains many electron-transparent vacuoles that are probably oil droplets. The central vacuole occupies the greatest proportion of each cell section. Both the electron-dense cytoplasmic layer that underlies the cell wall and the less electron-dense central vacuole became labeled with gold when the sections were reacted with antibody against coffee legumin. Purified pre-immune serum does not hybridize significantly to the sections (data not shown). Endoplasmic reticulum, Golgi apparatus, chloroplasts, and mitochondria normally are not clearly discernible in the cytoplasm at the late developmental stage (220 DAF) used for our studies. Proportionally, the greatest numbers of gold particles in the cell were found associated with the large central vacuole. Nonetheless, the electron-dense cytoplasm, particularly that around the periphery of the cell, also became heavily labeled with gold particles. The presence of signal peptides on the N-terminals of the proteins suggests that coffee legumin enters the secretory pathway. Consistent with this interpretation, the label found associated with the cytoplasm could be due to proteins synthesized in rough endoplasmic reticulum but not yet transported to the central vacuole. Interestingly, multiple smaller storage vacuoles were not observed as in cotyledonary tissue from legumes (Fig. 7A) or endosperm from oats (Adeli et al. 1984).

By analogy to legume storage globulins, the presence of a N-terminal signal peptide in coffee legumin predicts that the proteins are synthesized on membrane-bound polysomes, and that the products enter the secretory system (Chrispeels et al. 1982). These steps of biosynthesis will lead to the deposition of storage globulins in storage vacuoles where the conversion of prolegumin to legumin takes place. Consistent with this expectation, immunogold particles associated with antibodies bound to coffee legumin found both in the cytoplasm and in the central vacuole. Other organelles that do not participate in the secretory path are not labeled. High-magnification micrographs such as in Fig. 7C, clearly indicate that the cytoplasm is segregated from the central vacuole. Because the central storage vacuole accounts for such a large proportion of the total cellular volume at seed maturity, it contains the majority of label. Still, we were surprised by the intensity of gold label found in the electron dense cytoplasm adjacent to the cell wall, something we have not observed in legumes. Indeed, proteinaceous aggregates facing the cell wall of coffee endosperm cells have been reported by Dentan (1985). According to this report, the storage proteins accumulate from 94 to 143 days after pollination primarily in smaller vacuoles in the vicinity of the cell wall. In days 171 and 202 after pollination, the

accumulation of large central storage vacuoles predominates.

The cDNA derived sequences for coffee legumin appear in the GenBank/EMBL/DBJ databases accessions U64443 and AF054895.

Acknowledgements This manuscript is dedicated to the memory of the late Professor Virgilio Montes. Special thanks are also extended to Professor Gerardo Pérez for his advice to Ricardo Acuña following the death of Professor Montes. Antibodies used in this project were produced by the Purdue Cancer Center Antibody Production Facility, which is partially funded by a grant from the NCI. We thank Dr David Asai for offering the facilities, and to John Wilder for sera preparation. Amino acid sequence analysis was carried out by Mrs Mary Bower in the Laboratory for Macromolecular Structure, Department of Biochemistry, Purdue University. We are grateful to Dr John Tursk, Deborah VanHahn, and Phyllis Lockard for help with electron microscopic procedures, and to Deborah Sherman in the Department of Biology for skilled help and advice. Dr Larry Dunkle provided helpful comments during preparation of the manuscript. R. Acuña was supported by a grant from COLCIEN-CIAS (Colombia) and the National Coffee Growers of Colombia.

References

- Adeli K, Allan-Wojcik P, Altosnar I (1984) Intracellular transport and posttranslational cleavage of oat globulin precursors. *Plant Physiol* 76: 16–20
- Amerrin HV, Josephson RV (1975) Water soluble protein and nonprotein components of brazilian green coffee beans. *J Food Sci* 40: 1179–1184
- Baird A, Bucher P (1994) PROSITE: Recent developments. *Nucleic Acids Res* 22: 3583–3589
- Bäumlein H, Wobus U, Pustell J, Kafatos FC (1986) The legumin gene family: Structure of a B type gene of *Vicia faba* and a possible legumin gene specific regulatory element. *Nucleic Acids Res* 14: 2707–2720
- Beaman TW (1996) The bacterial expression, in vitro refolding, proteolytic processing and preliminary crystallization of the 11S globulin subunit, legumin B4, from faba bean (*Vicia faba* var. minor). Ph.D. Thesis, Purdue University, West Lafayette, IN
- Bradford MM (1976) Rapid and quantitative method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72: 248–252
- Centi-Grossi M, Tassi-Mico C, Silano V (1969) Albumin fractionation of green coffee seed varieties by acrylamide gel-electrophoresis. *Phytochemistry* 8: 1749–1751
- Churrier A, Berthoud J (1985) Botanical classification of coffee. In: Clifford MN, Willson KC (eds) *Coffee: Botany, Biochemistry, and Production of Beans and Beverage*. Avi Publishing Company, Westport, CT, pp 13–47
- Cho TJ, Davies CS, Fischer RL, Turner NE, Goldberg RB, Nielsen NC (1989) Molecular characterization of an aberrant allele for the Gyl glycinin gene. *Plant Cell* 1: 339–350
- Chrispeels MJ, Higgins TJV, Spencer D (1982) Assembly of storage protein oligomers in the endoplasmic reticulum and processing of the polypeptides in the protein bodies of developing pea cotyledons. *J Cell Biol* 93: 306–313
- Craig S, Millard A, Goedehild DJ (1980) Structural aspects of protein accumulation in developing pea cotyledons. III. Immunocytochemical localization of the legumin and vicilin using antibodies shown to be specific by the enzyme linked immunosorbent assay (ELISA). *Aust J Plant Physiol* 7: 339–351
- Dentan E (1985) Etude microscopique du développement et de la maturation du grain de café. *ASIC* 115: 381–387
- Dickinson CD, Floener LA, Lilley GG, Nielsen NC (1987) Self-assembly of proglycinin and hybrid proglycinin synthesized in vitro from cDNA. *Proc Natl Acad Sci USA* 84: 5525–5529
- Dickinson CD, Hussein EH, Nielsen NC (1989) Role of post-translational cleavage in glycinin assembly. *Plant Cell* 1: 459–469
- Goldberg RB, Barker SJ, Perez-Grau L (1989) Regulation of gene expression during plant embryogenesis. *Cell* 56: 149–160
- Grassie M, Kamnitscher P (1975) Observations sur la conjugation chromosomique de *Coffea arabica*. *Café Cacao Thé* 19: 177–190
- Guereche P, Tite C, Grossi de Sa F, de Clercq A, Van Montagu M, Krebbers E (1990) Differential expression of the Arabidopsis 2S albumin genes and the effect of increasing gene family size. *Plant Cell* 2: 469–478
- Harlow E, Lane D (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Lacimmi UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685
- Lawrence MC, Izard T, Bauchat M, Blagrove RJ, Colman PM (1994) Structure of phaseolin at 2.2 angstroms resolution: Implications for a common vicilin legumin structure and the genetic engineering of seed storage proteins. *J Mol Biol* 238: 748–776
- Muehle R (1985) Nitrogenous components. In: Clarke RJ, Macrae R (eds) *Coffee, vol. 1: Chemistry*. Vol. 1. Elsevier Applied Science Publishers, New York, NY, pp 115–152
- Nielsen NC, Bassiner R, Beaman T (1997) The biochemistry and cell biology of embryo storage proteins. In: Vasil IK (ed) *Cellular and Molecular Biology of Plant Seed Development*. Advances in Cellular and Molecular Biology of Plants, Vol. 4. Kluwer Academic Publishers, Dordrecht. ISBN 0-7923-4645-9, pp 151–220
- Ortiz-Castillo C, Chuhuaes KJ, Waugh R, Powell W (1994) Detection of genetic diversity and 21 selective gene introgression in coffee using RAPD markers. *Theor Appl Genet* 87: 934–940
- Ortiz-Castillo C, Chuhuaes KJ, Powell W, Waugh R (1996) RAPD and organelle specific PCR re-affirms taxonomic relationships within the genus *Coffea*. *Plant Cell Rep* 15: 337–341
- Perez-Grau L, Goldberg RB (1989) Soybean seed protein genes are regulated spatially during embryogenesis. *Plant Cell* 1: 1095–1109
- Shultz DJ, Craig R, Cox-Foster DL, Mumm RO, Medford JJ (1994) RNA isolation from recalcitrant plant tissue. *Plant Mol Biol Rep* 12: 310–316
- Sluyterman LA, Wijdens J (1970) An agarose mercurial column for the separation of mercaptopapain and non-mercaptopapain. *Biochim Biophys Acta* 201: 593–595
- Staswick PE, Hermanson MA, Nielsen NC (1984) Identification of the cystines which link the acidic and basic components of the glycinin subunits. *J Biol Chem* 259: 13431–13435
- Underwood GE, Deatherage FE (1952) Nitrogen compounds of coffee. *Food Res* 17: 419–424
- Wobus U, Borisjuk L, Pannitz R, Munteuffel R, Bäumlein H, Wohlfahrt T, Heim U, Weber H, Misera S, Wesschke W (1995) Control of seed storage protein gene expression: New aspects on an old problem. *J Plant Physiol* 145: 592–599
- Wolf WJ, Briggs DR (1958) Studies on the cold-insoluble fraction of the water-extractable soybean proteins. II. Factors influencing conformational changes in the 11S component. *Arch Biochem Biophys* 76: 377–393

Edited by I. M. Møller



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/47, C07K 16/16, C12N 9/40, 15/03, 15/04, 15/05, 15/06, 15/07, 15/56	A1	(11) International Publication Number: WO 95/06478 (43) International Publication Date: 9 March 1995 (09.03.95)
(21) International Application Number: PCT/US94/09739 (22) International Filing Date: 30 August 1994 (30.08.94) (30) Priority Data: 08/113,890 30 August 1993 (30.08.93) US (71) Applicant: HAWAII BIOTECHNOLOGY GROUP, INC. [US/US]; 99-193 Aiea Heights Drive, Aiea, HI 96701 (US). (72) Inventors: IVY, John, M.; 443 Iana Street, Kailua, HI 96734 (US). CLEMENTS, David, E.; 1322 10th Avenue, Honolulu, HI 96816 (US). (74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Focaster, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006 (US).	(51) Designated States: AU, BR, CA, CZ, HU, JP, KR, NO, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	
(54) Title: RECOMBINANT COFFEE BEAN α-GALACTOSIDASE (57) Abstract A recombinant DNA encoding coffee bean α-galactosidase permits the production of purified forms of this protein. The protein is useful in converting human Type B red blood cells into cells physiologically similar to Type O red blood cells. The availability of this enzyme permits more effective conversion than use of α-galactosidase from other sources.		

- 20 -

from the gel, transferred to a 1.5 ml microcentrifuge tube, and homogenized in 200 μ l of PBS. The final mixture, 300 μ l, contained approximately 120 μ g of α -Gal.

- 5 Two Balb-C mice were each immunized intraperitoneally (IP) with 60 μ g of the α -Gal preparation mixed 1:1 with complete Freund's adjuvant. Mice were boosted IP on day 14 and 28 with approximately 30 μ g of isolated 40 kD band mixed 1:1
10 with incomplete Freund's adjuvant. Test bleeds (tail) were taken on day 21 and 35 to evaluate the titer of anti- α -Gal antibodies. Hyperimmune mouse ascites fluid (HMAF) was produced by injecting IP approximately 2×10^8 sarcoma 180 cells per mouse 15 days after the second
15 boost. Ascites fluid was collected on days 8 and 12 postinduction. Test bleeds and HMAF were evaluated by Western blots of BMB coffee bean α -Gal transferred to Nitroplus 2000 membrane (Micron Separations, Inc.).

Example 3

20 Analysis of α -Galactosidase Levels in
Developing Coffee Beans

- Fresh coffee fruits were separated into eleven developmental stages, 1 to 11. Stages 1-5 contain no, 1/4 full, 1/2 full, 3/4 full and full endosperm,
25 respectively, as graded by inspection of seed endosperm after taking a center cross-sectional cut through the green cherries. Stages 6-10 are based on visible ripening of the fruit wherein the epicarp (skin) progresses from green-yellow (stage 6) to yellow-green
30 (stage 7) to yellow (stage 8) to yellow-red (stage 9) to red (stage 10), and stage 11 represents fully mature

cherries dried in the field, roughly equivalent to the harvested and dried fruit used for roasting.

The pulp (mesocarp) and parchment (endocarp) were removed from four beans (two fruits) per developmental stage, and the beans were sliced into 8 to 10 cross sections and incubated in PBS for 2 hours on ice. The extracts were then cleared by centrifugation at 5,000 x g at 4°C for 5 minutes. Samples representing 1/200th of each extract were prepared for SDS-PAGE and Western blot analysis by mixing with protein sample buffer and boiling for 3 minutes.

Western blots were blocked with Tris-buffered saline/tween 20/milk (TBST-milk: 150 mM NaCl, 10 mM Tris-Cl, pH 8.0, 0.05% tween 20, 1% nonfat powdered milk) for 1 hour, incubated with a 1:800 dilution of the anti- α -Gal HMAF described in Example 2 in TBST-milk for 1 hour, washed with TBST, incubated with a 1:7500 dilution of goat anti-mouse alkaline phosphatase-conjugated antibody (Promega) in TBST-milk for 45 minutes, washed with TBST, and reacted with substrate (BCIP and NBT, Promega) according to the manufacturer's recommendations.

Figure 3 shows the results of this Western blot analysis. Lane 1 contains molecular weight markers; lane 2 is blank; lanes 3-10 represent developmental stages 4-11; lane 11 is blank; and lane 12 is commercial coffee bean α -galactosidase. No α -galactosidase was detected in stages 1-4; the enzyme was first detected in stages 5-6; the peak activity appeared at stages 6 and 7.

MONA ONLINE RESEARCH DATABASE

[HOME](#) [RESEARCH](#) [FACULTY](#) [FUNDING](#) [STATISTICS](#)

[HOME](#) [Profile](#)



Dr. Kerith Donovan Golden

Senior Lecturer in FMS -Basic Medical Sciences: Faculty of Medical Sciences

Telephone: 876/927-2290

Email: kerith.golden@uwimona.edu.jm

Publications

Article

Brownleader, M., Golden, Kerith D., Dey, P., An evaluation of extension services

Golden, Kerith D., John, M., Kean E.A., Peroxidase class from Coffea arabica fruit

Golden, Kerith D., Inhibition of the ethylene-forming enzymes of the coffee cherry

Golden, Kerith D., Bell, M.A., Peroxidase activity from coffee cells: purification and characterization

Golden, Kerith D., Williams, Opal J., Bailey-Shaw, Yvonne A., High Performance Liquid Chromatographic Analysis of Amino Acids in Sweet Fruit with Emphasis on the Role of GABA

Golden, Kerith D., Williams, Opal J., Amino acid, fatty acid, and carbohydrate composition of various fruits (breadfruit)

Williams, Opal J., Golden, Kerith D., Further extended characterization of breadfruit (Artocarpus albus) fruit

Dunkley, Hope M., Golden, Kerith D., Amino acidase from breadfruit (Artocarpus albus) fruit

Golden, Kerith D., Williams, Opal J., Dunkley, Hope M., The production of ethylene and other volatile organic compounds and the increased synthesis of these gases by breadfruit (Artocarpus albus) fruit

Conference Proceedings

Golden, Kerith D., Williams, Opal J., Raghavan, Vijaya, Gariepy, Y., Controlling the ripening process of breadfruit (Artocarpus albus) fruit

Copyright © 2006 UWI Mona Research Engine

MONA ONLINE RESEARCH DATABASE

[HOME](#)[FACULTY](#)[FUNDING](#)[STATISTICS](#)[New Search](#)[View](#)

9

Document Type	Journal Article
Author(s)	Golden, Kerith
Co-author(s)	Golden, Kerith D.; John, M.; Kean E.A.
Department/Institution, Ana.	Basic Medical Sciences (Anatomy, Biochemistry, Physiology)Faculty of Medical Sciences
Title	b-galactosidase from Coffea arabica: Its role in fruit ripening
Journal Title	Phytochemistry
Publication Year	1993
Volume	34
Issue	2
Page(s)	355-360
Abstract	b-galactosidase is an enzyme that works in tandem with other enzymes (e.g. polygalacturonase) in the degradation of the plant cell wall. As the fruit ripens, the activity of this enzyme increases. The paper reports on the purification of b-galactosidase from C. arabica, the ability.... read more

The University of the West Indies, Mona, St. Kitts, Barbados

MONA ONLINE RESEARCH DATABASE

[HOME](#)[FACULTY](#)[FUNDING](#)[STATISTICS](#)[New Search](#)[View](#)

9

9

9

Journal Article

Golden, Kerith

Golden, Kerith D.; John, M.; Kean E.A.

Basic Medical Sciences (Anatomy, Biochemistry, Physiology)Faculty of Medical Sciences

b-galactosidase from *Coffea arabica*: Its role in fruit ripening

Phytochemistry

1993

34

2

355-360

b-galactosidase is an enzyme that works in tandem with other enzymes (e.g. polygalacturonase) in the degradation of the plant cell wall. As the fruit ripens, the activity of this enzyme increases. The paper reports on the purification of b-galactosidase from *C. arabica*, the ability of the enzyme to degrade pectic polysaccharides and its apparent increase in activity during the ripening of the coffee berry. It suggests that b-galactosidase is implicated in cell wall degradation such as occurs during fruit ripening

2020
Bogotá, D.C.,

Doctora:
Alicia Lloreda Ricaurte
Representante Legal

Oficio N°: 6347

ASUNTO:

Radicación PCT N°	04-32188
Trámite	011
Evento	379
Actuación	440
Folios	037

Con fundamento en lo previsto por el artículo 43 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica que la oposición presentada por el doctor José Luis Reyes Villamizar, en su calidad de apoderado de la Federación Nacional de Cafeteros de Colombia., reúne los requisitos formales exigidos por la ley al momento de su presentación.

En cumplimiento de lo dispuesto en la citada norma, se le concede el término de sesenta (60) días hábiles siguientes a la fecha de notificación del presente oficio, para que haga valer sus argumentaciones y presente pruebas.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

El anterior requerimiento fue notificado por fijación en lista de fecha, 09 JUN 2006.

202021