JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Inositol Phosphate Profiling of Fermented Cowpeas by ^{1}H NMR Spectroscopy

SERAFIN VALVERDE,† JUANA FRIAS,§ ROSA DOBLADO,§
MARIA LUISA JIMENO,# AND CONCEPCIÓN VIDAL-VALVERDE*,§

Instituto de Química Orgánica, Instituto de Fermentaciones Industriales, and Centro de Química Orgánica "Manuel Lora-Tamayo", CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

The inositol phosphate content of naturally fermented cowpeas (*Vigna sinensis* var. Carilla) was studied using ion-pair HPLC and ^{1}H NMR spectroscopy. The fermented flour was extracted with 0.5 M HCl, and the extract was purified and fractionated by ion-exchange chromatography. ^{1}H NMR allowed for the identification of two monophosphates [Ins(1 or 3)P1 and Ins(4 or 6)P1], one inositol diphosphate [Ins(1,4)P2], three inositol triphosphates [Ins(1,2,6)P3, Ins(1,5,6)P3, and Ins(1,4,5)P3], one inositol tetraphosphate [Ins(1,3,4,5)P4], and one inositol pentaphosphate [Ins(1,2,3,5,6)P5]. Some of these isomers [Ins(1,4,5)P3 and Ins(1,3,4,5)P4] are considered to play important biological roles in intracellular signaling.

KEYWORDS: Fermentation; cowpeas; inositol phosphates; ^{1}H NMR spectroscopy

INTRODUCTION

myo-Inositol hexaphosphate or phytic acid (see **Figure 6**) is present in many seeds (cereals, legumes, etc.) widely consumed as staple foods (*1*). Inositol penta- and tetraphosphates are also present, although in lesser amounts; as an average, 3 mg/g would be found as phytic acid and <0.5 mg/g as other inositol phosphates (*2*).

However, processing of the seeds (particularly fermentation) drastically lowers the amount of phytic acid while increasing the amounts of lower inositol phosphates (*3*). Some of these phosphates, such as inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] have been assigned a significant biological role in the intracellular signal transduction systems as a second messenger (*4*). It has also been proposed that inositol 1,3,4,5-tetraphosphate acts to increase the intracellular calcium concentration when the calcium releasing factor, Ins(1,4,5)P_3, is present (*5*). Hence, there is interest in knowing the lower inositol phosphates content of foods. Considerable efforts have been devoted to the separation of the different inositol phosphate isomers by HPLC, and the development of ion-pair procedures made it possible to study some of the lower inositol phosphates (*6*). These methods do not differentiate isomeric forms of inositol phosphates, as gradient elution cannot be performed when using refractive index detection. The position of the phosphate groups on the inositol ring is of great importance for their physiological function. A number of isomer-specific ion-exchange chromatography methods have been developed (*7*−*10*). Skoglund et al. (*9*, *10*) have managed to identify some of the isomeric phosphates using two columns and high-performance ion chromatography (HPIC). The two HPIC methods developed by these authors show a commendable separation of isomers. Some of these isomeric inositol phosphates have been isolated using ion-exchange chromatography methods, and their structures have been assigned using monodimensional and bidimensional NMR spectroscopy (*11*−*14*).

The purpose of this work is to explore the use of existing information on the ^{1}H NMR of pure inositol phosphates for the identification or profiling of the various inositol phosphate components present in fermented cowpeas.

MATERIALS AND METHODS

Preparation of Functional Flours through Natural Fermentation (FC). Cowpea (*Vigna sinensis* var. Carilla) flour was naturally fermented as described by Doblado et al. (*3*). The flour was suspended in sterilized distilled water (300 g/L). Fermentation was carried out at 37 °C in a 5 L fermentor without aeration (Infors ISF-100, Infors AG, Bottmingen, Switzerland) at 450 rpm during 48 h. Fermented flour was frozen and lyophilized. Portions were sealed under vacuum and kept at 4 °C in the dark.

Preparation of Standard Inositol Phosphate Fractions (SF). As a first step, prior to the analysis of food samples, a mixture of inositol phosphate isomers was prepared by acid hydrolysis of phytate: 1.5 g of sodium phytate (Sigma) was dissolved in 100 mL of 0.5 M HCl; the solution was refluxed at 100 °C for 24 h, evaporated to dryness, and then dissolved in water. The inositol phosphates were separated by ion-exchange chromatography (Dowex 50-x8 H+, 200−400 mesh resin column) eluting with a gradient of aqueous HCl (0.08−0.52 M); residues obtained by evaporation of these fractions (seven residues, SF 0.08 to SF 0.52) were dissolved either in distilled water or in deuterated water and submitted to ion-pair HPLC chromatography or ^{1}H NMR spectroscopy, respectively.

* Author to whom correspondence should be addressed (telephone + 34 915622900, ext. 241; fax 34 915644873; e-mail ificv12@ifi.csic.es).
† Instituto de Química Orgánica.
§ Instituto de Fermentaciones Industriales.
# Centro de Química Orgánica "Manuel Lora-Tamayo".

10.1021/jf0481047 CCC: $30.25
Published on Web 05/19/2005

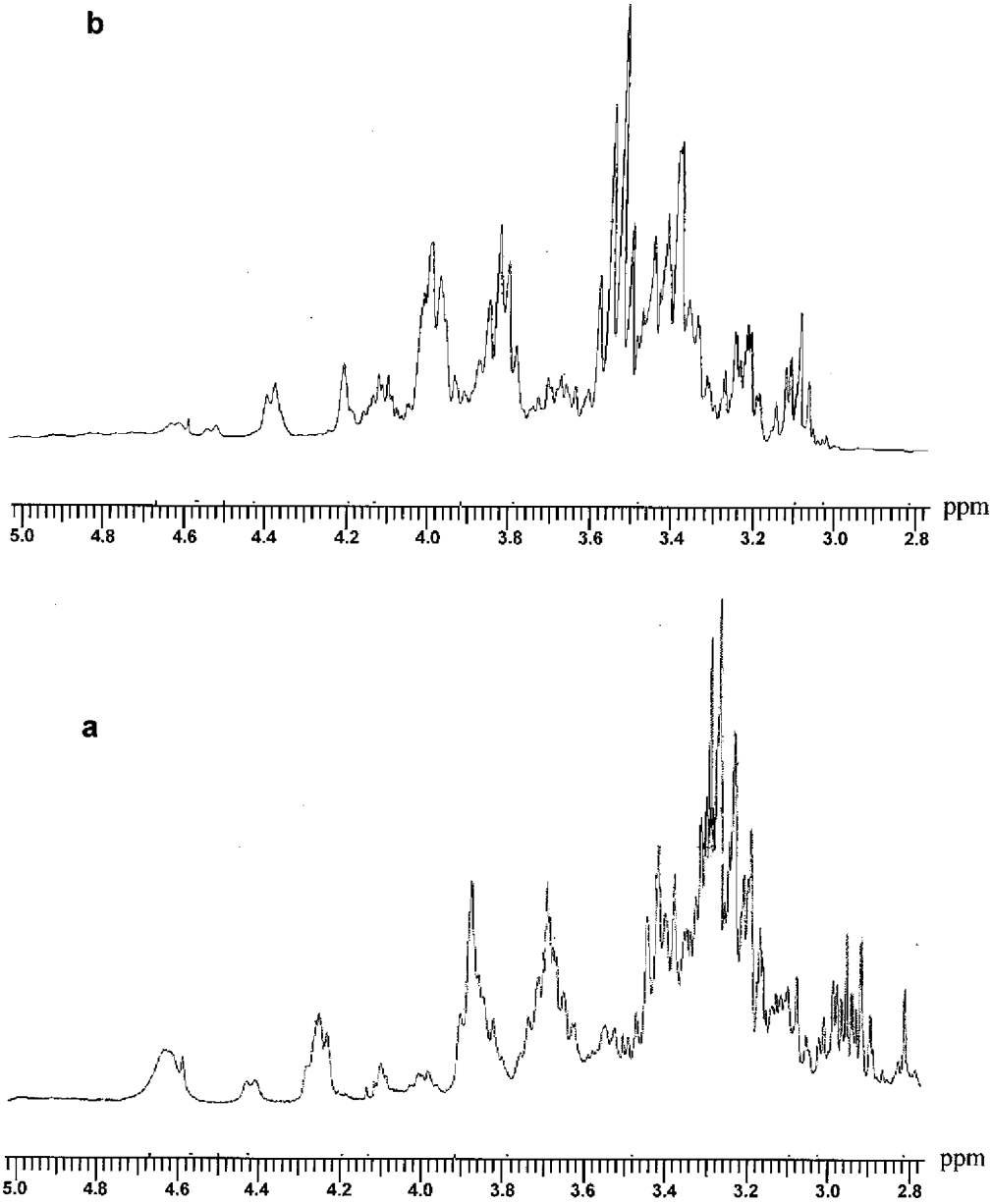

Figure 1. ^{1}H NMR spectra of standard fractions: (**a**) SF 0.08; (**b**) SF 0.15.

Preparation of Inositol Phosphate Fractions from Fermented Cowpeas (FC). Fermented cowpea flour (10 g) was mixed with 100 mL of 0.5 M HCl, stirring during 4 h at a bath at 20 °C. The mixture was centrifuged for 30 min at 5 °C and 10000 rpm. The supernatant was frozen at −20 °C overnight and centrifuged again under the same conditions. The supernatant was evaporated under vacuum at a temperature below 35 °C to dryness. The residue was dissolved in distilled water, and the solution was added to a column (30 × 2 cm) filled with Dowex 50-X8 H^+ (Sigma) 200−400 mesh at neutral pH. The column was washed with distilled water (100 mL) and the collected liquid was discarded; 2 M HCl (100 mL) was added to the column, collected, and evaporated to dryness under vacuum. The residue was dissolved in 100 mL of distilled water, and this solution was added to a new column prepared as above. This column was then eluted with aqueous HCl of increasing concentration (0.08−0.52 M). Residues obtained by evaporation of the fractions obtained from the ion-exchange column (seven residues, FC 0.08 to FC 0.52) were dissolved either in distilled water (2.0 mL) and analyzed using HPLC ion-pair chromatography or in D_2O and their ^{1}H NMR spectra recorded.

Inositol Phosphate Determination. Standard inositol phosphate fractions (SF) and inositol phosphate fractions obtained from fermented cowpea flour (FC) were analyzed by ion-pair HPLC and ^{1}H NMR spectroscopy.

Table 1. ^{1}H NMR Signals of Identified Inositol Phosphates from Standard Fractions (SF)[a]

SF 0.08, ppm	SF 0.15, ppm	SF 0.28, ppm	SF 0.32, ppm	SF 0.38, ppm	SF 0.50, ppm	SF 0.52, ppm
4.44bd	4.54e	4.72f	4.71fh	4.72hif	4.74hi	4.77j
4.25cd	4.40bd	4.54e	4.40fg	4.46f	4.29i	4.35j
4.12acd	4.22ced	4.40dfg	4.19h	4.30i	4.15hi	4.19j
4.00d	4.14c	4.20d	4.14f	4.16hif	4.13h	
3.93c	4.09d	4.18e	4.12hg	4.13h	3.98hi	
3.87acd	4.01ed	4.14f	4.06g	4.01if	3.61hi	
3.70cd	3.93c	4.12g	4.01fg	3.98hf	3.34h	
3.60ab	3.83cd	4.08dg	3.94fh	3.61hif		
3.55a	3.69c	4.02eg	3.92g	3.36h		
3.44abc	3.68d	4.00dfg	3.59fh			
3.22a	3.57be	3.92fg	3.36h			
3.18b	3.47ce	3.84d				
	3.40b	3.67d				
	3.36e	3.60ef				
	3.16b	3.43e				
		3.32e				

[a] Letters identify the chemical shift of the signals assigned to a known compound in the ^{1}H NMR spectrum of each fraction. Ins, inositol; P, phosphate; a, Ins(1)P_1; b, Ins (2)P_1; c, Ins(1,4)P_2; d, Ins(1,5,6)P_3; e, Ins(1,2,6)P_3; f, Ins(1,2,5,6)P_4; g, Ins(1,3,4,5)P_3; h, Ins(1,2,3,6)P_4; i, Ins(1,2,3,5,6)P_5; j, Ins(1,2,3,4,5,6)P_6.

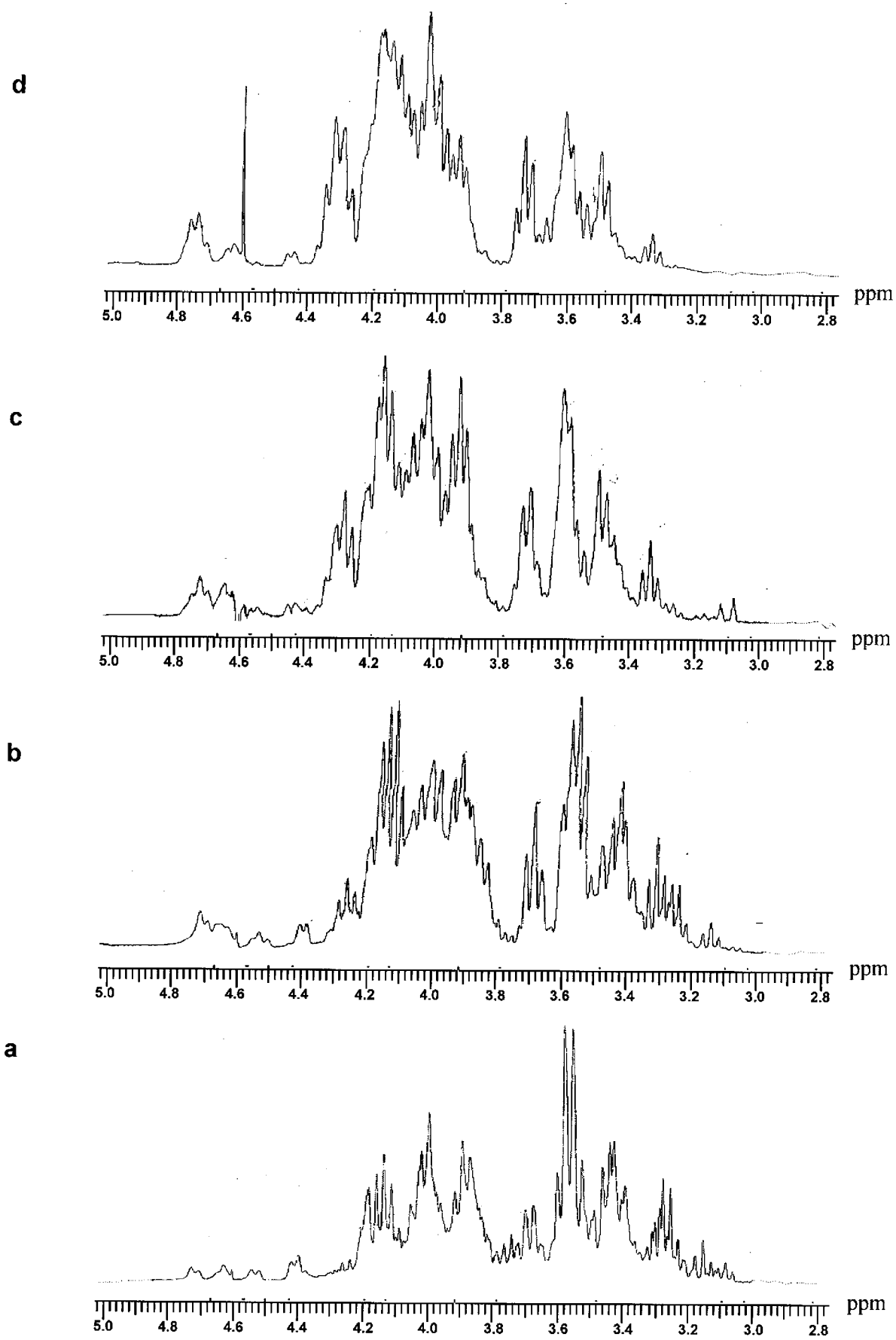

Figure 2. ^{1}H NMR spectra of standard fractions: (**a**) SF 0.28; (**b**) SF 0.32; (**c**) SF 0.38; (**d**) SF 0.50.

Ion-Pair HPLC Analysis. The analysis was carried out following the method of Lehrfeld (*15*). The chromatograph consisted of a HPLC pump (Waters model 510, Waters Associates, Milford, MA) equipped with a manual injector, Rheodyne 7125, a Hypersyl-ODS C_{18} column (3.9 mm i.d. × 30 cm, 5 mm) with a C_{18} precolumn (9 × 20 mm) at 45 °C (temperature control module, Waters), and a refractive index

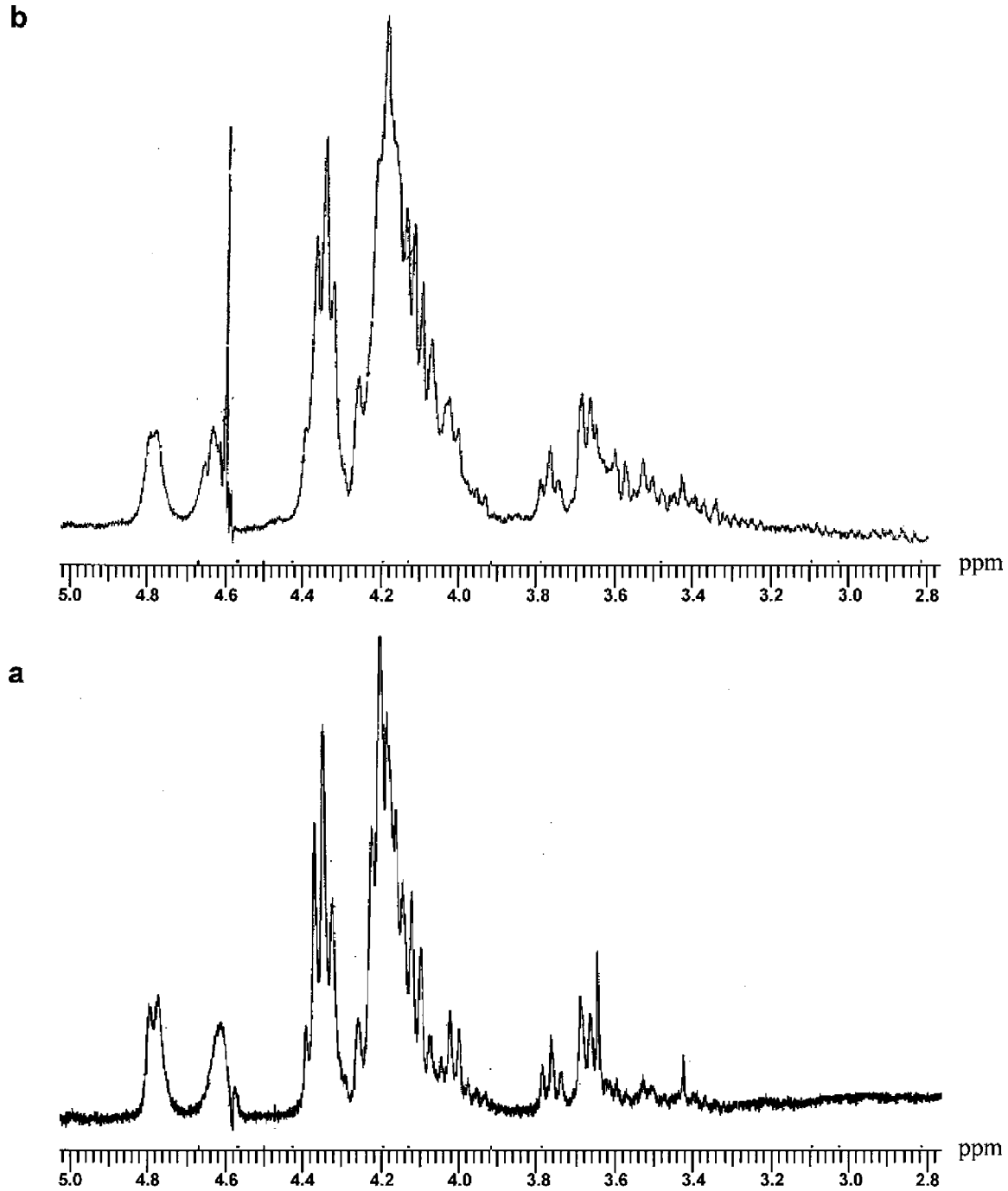

Figure 3. ^{1}H NMR spectra of fractions: (**a**) FC 0.52; (**b**) SF 0.52.

detector (Waters 410). Methanol/Milli-Q water/tetrabutylammonium hydroxide/5 M sulfuric acid/91% formic acid/phytic acid (5 mg/mL) (515:485:8:1:0.5:0.2 v/v/v/v/v/v) was the mobile phase, which was filtered through a Millipore FH (0.45 mm) nylon membrane and degasified with helium. An isocratic flux (1.0 mL/min) was used with 20 mL of injection volume.

^{1}H NMR Analysis. ^{1}H NMR spectra were recorded on a Varian Inova spectrometer operating at 400 MHz, using D_2O as solvent at 30 °C. Presaturation of the water signal was used to run the spectra. Chemical shifts (*X*-axis) were expressed as parts per million (ppm) and were referenced to the residual protons of the solvent. The chemical shift of the water at 30 °C was previously determined with respect to dioxane. The *Y*-axis represents the intensity of the signal.

RESULTS AND DISCUSSION

Standard Fractions of Inositol Phosphates (SF) were studied by ^{1}H NMR, and the data are collected in **Table 1** and **Figures 1−3**. Only significant parts of the spectra (between 2.8 and 5.0 ppm) have been reproduced. Identification of all the peaks assigned to a compound (±0.04 ppm) was considered to be evidence of its presence in the sample. **Table 1** collects significant signals of the ^{1}H NMR spectra of the residues obtained by evaporation of the standard fractions (SF 0.08 to SF 0.52) prepared as indicated under Materials and Methods. Fractions were also analyzed by ion-pair chromatography, and the information obtained (presence of inositol hexa-, penta-, or tetraphosphates) was considered for the interpretation of the NMR data; it was used as a guideline to confirm that the NMR interpretation was consistent with the HPLC results. In accord with the known data (*11−14*), signals between 4.56 and 4.80 ppm can be an indication of the presence of inositol derivatives phosphorylated at O-2. In general, highly phosphorylated inositols (hexa-, penta-, or tetraphosphates) present the most

Table 2. ^{1}H NMR Signals of Identified Inositol Phosphates from Fermented Cowpea (FC)[a]

FC 0.08, ppm	FC 0.15, ppm	FC 0.28, ppm	FC 0.32, ppm	FC 0.38, ppm	FC 0.50, ppm	FC 0.52, ppm
4.20c	4.24c	4.60e	4.60e	4.38dg	4.77i	4.78j
4.18a	4.17a	4.38g	4.34gl	4.24d	4.33i	4.35j
4.12c	4.15c	4.31l	4.17el	4.13g	4.18i	4.20j
4.02c	3.93c	4.20el	4.14g	4.10d	4.01i	
3.93ck	3.80ac	4.15g	4.06g	4.06g	3.64i	
3.80ac	3. 68ac	4.07eg	4.01eg	4.01dg		
3.65ac	3.56a	3.99g	3.99g	3.99g		
3.57ak	3.47c	3.95l	3.95l	3.90g		
3.45ack	3.42a	3.90lg	3.91gl	3.88d		
3.34k	3.20a	3.76l	3.76l	3.65d		
3.23a		3.60e	3.52e			
		3.43e	3.42e			
		3.40e	3.32e			

[a] Letters identify the chemical shift of the signals assigned to a known compound in the ^{1}H NMR spectrum of each fraction. Ins, inositol; P, phosphate; a, Ins(1)P_1; b, Ins(2)P_1; c, Ins(1,4)P_2; d, Ins(1,5,6)P_3; e, Ins(1,2,6)P_3; f, Ins(1,2,5,6)P_4; g, Ins(1,3,4,5)P_3; h, Ins(1,2,3,6)P_4; i, Ins(1,2,3,5,6)P_5; j, Ins(1,2,3,4,5,6)P_6; k, Ins(6)P_1; l, Ins(1,4,5)P_3.

important signals from 4.0 ppm to lower field, whereas the less phosphorylated isomers concentrate their ^{1}H NMR signals below 4.00 ppm (between 4.0 and 3.0 ppm). **Figures 1−3** confirm this observation, which agreed well with the results obtained by ion-pair HPLC.

SF 0.08 was eluted with 0.08 M HCl from the Dowex1x8 ion-exchange column. Although ion-pair HPLC chromatography of this sample did not show the presence of inositol phosphate derivatives, the ^{1}H NMR (see **Figure 1a** and **Tables 1** and **3**) gave a clear indication of the presence of these derivatives (signals between 2.9 and 4.7 ppm). In fact, by comparing the published ^{1}H NMR data with data in **Figure 1a** and **Table 1**, it was possible to identify the signals assigned to Ins(1 or 3)P_1 (*13*), Ins(2)P_1 (*13*), Ins(1,4)P_2 (*11*), and Ins(1,5,6)P_3 (*12*). Quantitatively important signals such as those present at 3.22, 3.32, and 3.90 ppm (see **Figure 1a**) could not be assigned to known compounds.

SF 0.15 was eluted next to the previous one using 0.15 M HCl. Ion-pair HPLC chromatography showed the presence of inositol tetraphosphates. The ^{1}H NMR (see **Figure 1b** and **Tables 1** and **3**) present important peaks at the high field of the spectrum, which should not be expected for inositol tetraphosphates according to the published data (*11−14*). Comparison of the ^{1}H NMR data obtained for this fraction with published data (*11−14*) allowed for the identification of Ins(2)P_1, Ins(1,4)P_2, Ins(1,2,6)P_3, and Ins(1,5,6)P_3. The presence of Ins(1,3,4,5)P_4 agreed with the HPLC result. Other inositol tetraphosphates such as Ins(1,2,3,6)P_4, Ins(1,2,5,6)P_4, and Ins(1,2,3,4)P_4 were not found because they present signals at the region of 4.7−4.9 ppm, which were not found in this spectrum.

SF 0.28. **Table 1** collects the main peaks of the ^{1}H NMR spectrum of this fraction (see **Figure 2a**). HPLC showed the presence of inositol tetraphosphates and inositol pentaphosphates (minor amounts). The presence of important peaks at the high field of the spectrum (3.2−3.6 ppm) was an indication that lower phosphorylated inositols could also be present. In fact, Ins(1,2,6)P_3 and Ins(1,5,6)P_3 were identified in this fraction (see **Tables 1** and **3**). Two inositol tetraphosphates were also clearly identified: Ins(1,2,5,6)P_4 and Ins(1,3,4,5)P_4.

SF 0.32. Comparison of the ^{1}H NMR data obtained for this fraction with published data allows for the identification of most of the important peaks of the spectrum of this fraction (see **Figure 2b**). Data are collected at **Table 1**. HPLC indicated a mixture of tetra- and pentaphosphates of inositol, the last ones being the minor components. In agreement with that indication it was possible to assign signals to Ins(1,2,5,6)P_4, Ins(1,2,3,6)P_4, and Ins(1,3,4,5)P_4. The presence of small signals between 4.69 and 4.60 ppm, together with important peaks at 3.69, 3.59, and 3.42 ppm, was a clear indication that other inositol tetra- or pentaphosphates should be present. Penta- or tetraphosphates with free 2-OH could be postulated (signals at 4.69 and 4.65 ppm could be assigned to the H-2 of such compounds).

SF 0.38. The spectrum of this fraction was quite similar to the previous one (see **Figure 2c**). The main peaks are collected in **Table 1**. HPLC indicated again a mixture of tetra- and pentaphosphates, prevailing the latter in this case. Both Ins(1,2,3,6)P_4 and Ins(1,2,3,5,6)P_5 were present. Signals at 4.72, 4.46, 4.16, 4.01, 3.98, and 3.61 ppm would point to the additional presence of Ins(1,2,5,6)P_4. As it has already been pointed out, the signal at 4.65 ppm could be an indication of the presence of tetra- and pentaphosphates lacking a phosphate group on O-2. The complexity of the spectrum suggested the presence of other tetra- or pentaphosphates.

SF 0.50. The spectrum of this fraction (see **Figure 2d**) showed similarities with but also differences from the previous one. In fact, ion-pair HPLC indicated a mixture of tetra- and pentaphosphates but only minor amounts of the former, and some phytic acid was also present. Important peaks are collected in **Table 1**. By comparing these data with published spectra for

Table 3. ^{1}H NMR Inositol Phosphates Assignments

$InsP_6$ (*14*)[a]	Ins(1,2,3,5,6)P_5 (*14*)[a]	Ins(1,2,3,6)P_4 (*14*)[a]	Ins(1,2,5,6)P_4 (*12*)	Ins(1,3,4,5)P_4 (*11*)	Ins(1,2,6)P_3 (*14*)[a]	Ins(1,4,5 P_3 (*11*)	Ins(1,5,6)P_3 (*12*)	Ins(1,4)P_2 (*11*)	Ins(2)P_1 (*13*)	Ins(1) or Ins(3)P_1 (*13*)	Ins(4) or (6)P_1 (*13*)
4.76 H-2	4.76 H-2	4.72 H-2	4.72 H-2	4.38 H-4	4.56 H-2	4.30 H-2	4.40 H-6	4.23 H-2	4.40 H-2	4.15 H-2	4.02 H-6
4.35 H-4									3.60 H-4		3.95 H-2
	4.30 H-4	4.18 H-4	4.44 H-4	4.12 H-2	4.18 H-4	4.21 H-4	4.23 H-2	4.13 H-4		3.83 H-1	
4.35 H-6									3.60 H-6		
4.15 H-1									3.41 H-1		3.59 H-4
	4.15 H-3	4.13 H-1	4.15 H-1	4.08 H-3	4.03 H-1	3.95 H-1	4.09 H-1	3.93 H-1		3.64 H-6	
4.15 H-3									3.41 H-3		3.59 H-3
	3.99 H-1			4.01 H-5							3.45 H-1
4.15 H-5		3.96 H-3	4.00 H-5		3.56 H-6	3.94 H-5	4.01 H-5	3.83 H-6	3.16 H-5	3.54 H-4	
	H-5			4.00 H-1							3.32 H-5
	3.63 H-6	3.63 H-6	3.95 H-6	3.90 H-6	3.46 H-3	3.93 H-6	3.84 H-4	3.67 H-3		3.42 H-3	
		3.38 H-5	3.60 H-3		3.36 H-5	3.73 H-3	3.67 H-3	3.48 H-5		3.22 H-5	

[a] There was a systematic error (±0.12 ppm) between our data and Nakano's data that has been corrected.

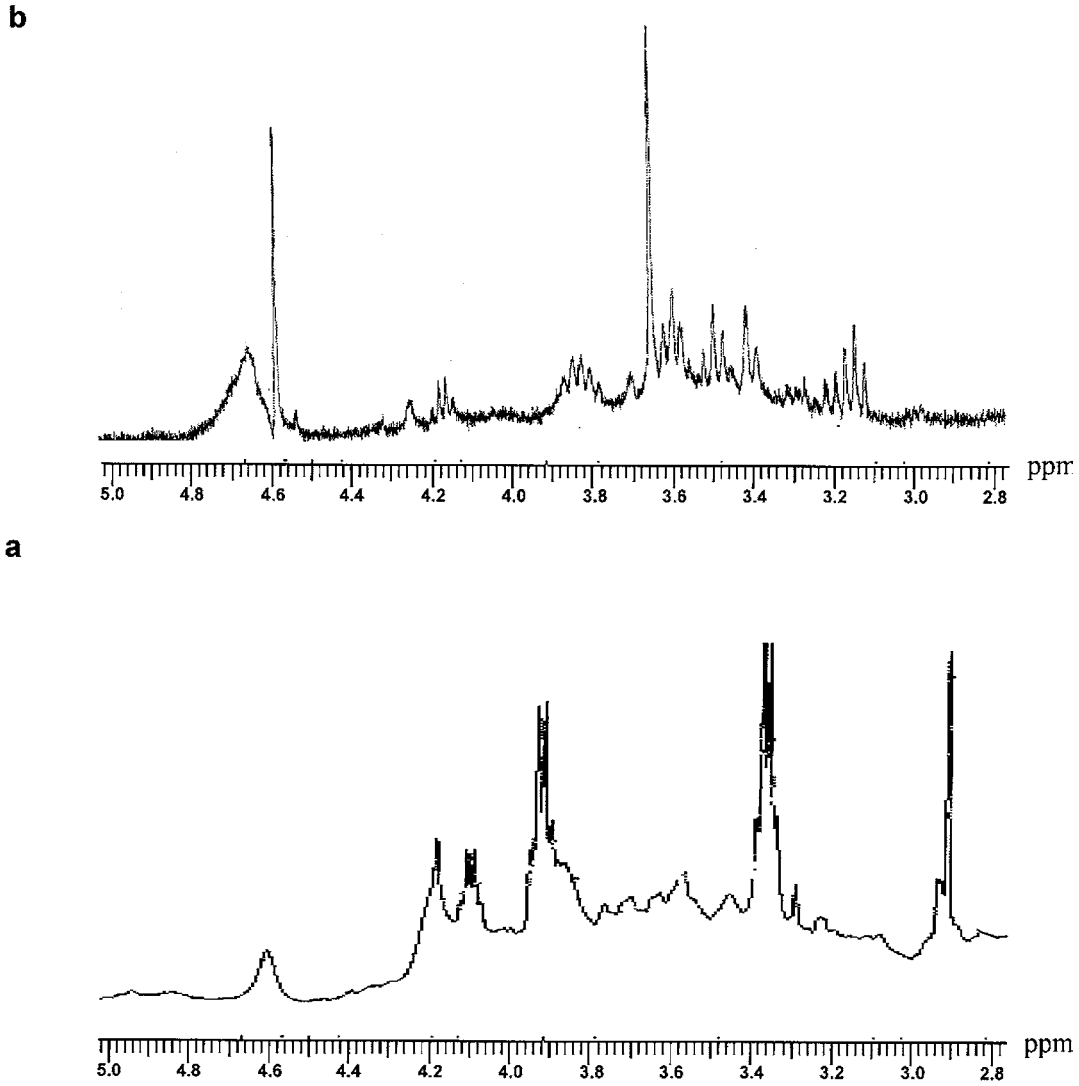

Figure 4. ^{1}H NMR spectra of fermented cowpea fractions: (**a**) FC 0.08; (**b**) FC 0.15.

Ins(1,2,3,6)P_4 and Ins(1,2,3,5,6)P_5 (see **Table 3**), it could be concluded that both compounds were present as in the previous fraction. Signals at 4.74 and 4.15 ppm could be indistinctly assigned to Ins(1,2,3,5,6)P_5 or InsP_6 (phytic acid), but signals at 3.98 and 3.61 ppm should be due to Ins(1,2,3,5,6)P_5 and Ins(1,2,3,6)P_4 and the signal at 4.29 ppm was characteristic of Ins(1,2,3,5,6)P_5. Lack of ^{1}H NMR data for other InsP_5 precludes further assignments, but the complexity of the spectrum was a clear indication of the presence of other isomers.

SF 0.52. This last fraction was eluted with 0.52 M HCl. The ^{1}H NMR spectrum (**Figure 3b** and **Table 1**) showed signals at 4.77, 4.35, and 4.19 ppm, which were a clear indication of the presence of phytic acid. HPLC indicated a mixture of phytic acid and penta- and tetraphosphates. Besides the peak at 4.77 ppm, a signal also appeared at 4.63 ppm as an indication of the presence of lower inositol phosphates (penta- and/or tetraphosphates) lacking a phosphate group on O-2. Lack of important signals at the high-field region of the spectrum was consistent with the absence of less phosphorylated compounds present in previous fractions.

Fermented Cowpea Fractions of Inositol Phosphates (FC). The fractions were prepared as indicated above and were studied by ^{1}H NMR. The data are collected in **Table 2** and **Figures 3**−**5**. **Table 2** collects significant signals of the ^{1}H NMR spectra of fractions obtained from fermented cowpea flour (FC 0.08 to FC 0.52).

FC 0.08. The spectrum of this fraction (see **Figure 4a**) is less complex than the spectrum of the corresponding SF (0.08), particularly at the region of 3.0−3.5 ppm. However, most of the main peaks are present in both fractions, and it is possible to identify Ins(1)P_1 and Ins(1,4)P_2 present in SF (0.08) and some other inositol monophosphates as Ins(6)P_1 (*13*) (see **Tables 2** and **3**).

FC 0.15. **Table 2** collects a list of the peaks observed in the ^{1}H NMR spectrum of this fraction (see **Figure 4b**). It was possible to identify signals due to Ins(1)P_1 and Ins(1,4)P_2 (see **Tables 2** and **3**).

FC 0.28. Data corresponding to FC 0.28 are collected in **Table 2** and **Figure 5a**. Compounds that could be identified include Ins(1,2,6)P_3, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4, the last two having biological significance (*4*, *5*).

FC 0.32. **Table 2** and **Figure 5b** collect the information related to this fraction. It was possible to identify some of the products found in the previous fraction such as Ins(1,2,6)P_3 and Ins(1,3,4,5)P_4. A strong signal at 4.60 ppm suggested the presence of inositol tetra- or pentaphosphates lacking the phosphate group on O-2. Ins(1,4,5)P_3 was also present in this fraction.

FC 0.38. NMR data for this fraction are collected in **Table 2** and **Figure 5c**. It was possible to assign signals to Ins(1,5,6)P_3 and Ins(1,3,4,5)P_4. Tetra- or pentaphosphates lacking the phosphate group on O-2 could also be present.

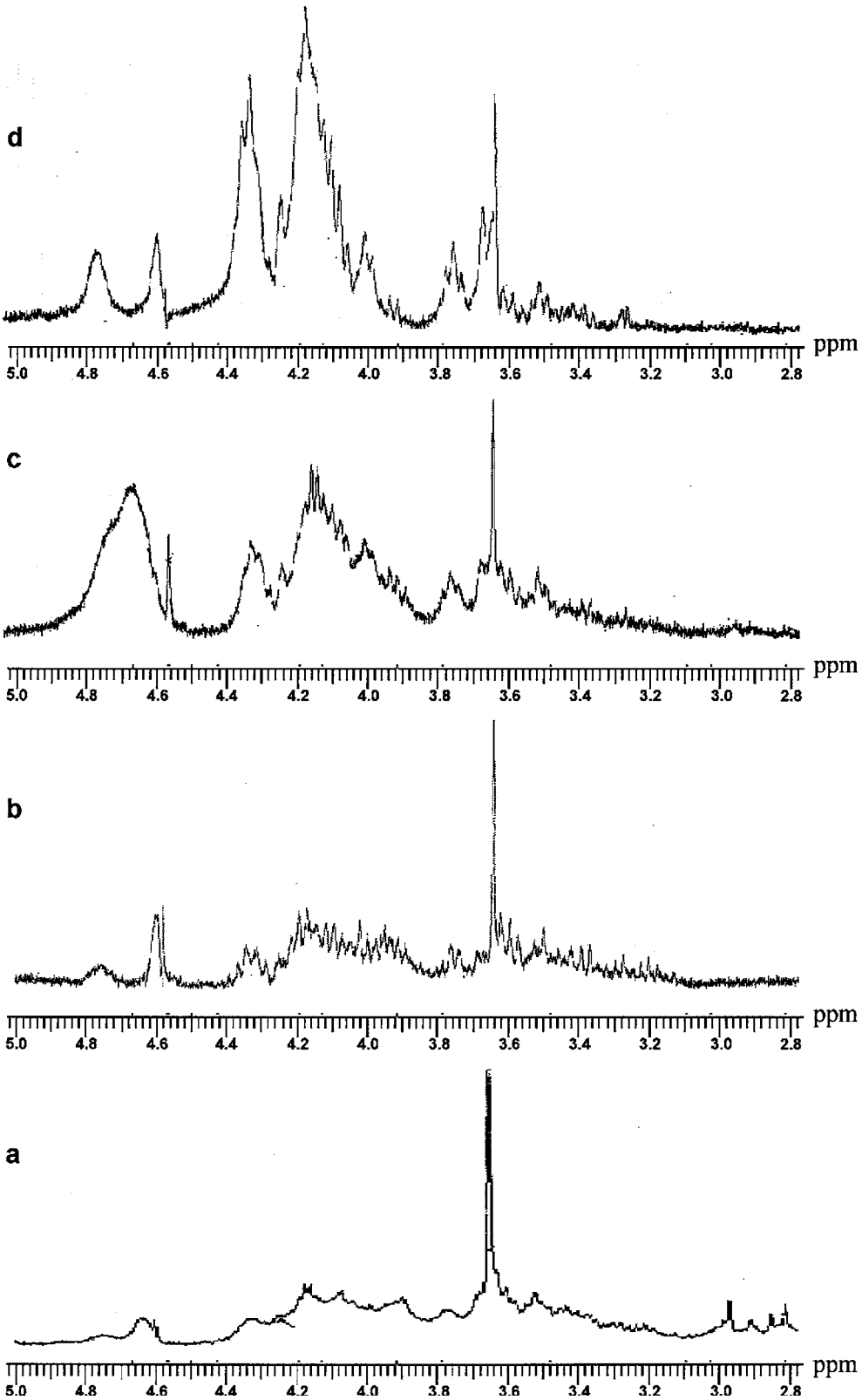

Figure 5. ^{1}H NMR spectra of fermented cowpea fractions: (**a**) FC 0.28; (**b**) FC 0.32; (**c**) FC 0.38; (**d**) FC 0.50.

FC 0.50. **Table 2** and **Figure 5d** collect the information obtained for this fraction. Ins(1,2,3,5,6)P_5 should be present in this fraction. Other inositol pentaphosphate isomers could not be ruled out and neither could the presence of phytic acid.

FC 0.52. **Table 2** and **Figure 3a** collected the NMR data obtained with this fraction. The data are practically identical to those obtained for SF 0.52. The sample contained fundamentally phytic acid (4.77, 4.35, and 4.19 ppm), although signals at 4.61, 3.75, and 3.67 ppm gave an indication of the presence of other inositol phosphate isomers (most probably pentaphosphates).

Consequently, the analysis of the ^{1}H NMR and ion-pair HPLC allowed for the identification of a good number of inositol phosphates in fermented cowpea flours, namely, those phosphates were Ins(1 or 3)P_1, Ins(4 or 6)P_1, Ins(1,4)P_2, Ins(1,2,6)P_3, Ins(1,5,6)P_3, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,2,3,5,6)P_5. Some of these isomers, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, are

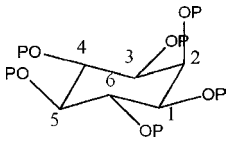

Figure 6. *myo*-Inositol-1,2,3,4,5,6-hexakisphosphate or phytic acid (P = PO_3H_2).

considered to play important biological roles in intracellular signal transduction systems (*4*, *5*). Ins(1,2,3,5,6)P_5 and Ins(1,2,6)P_3 have been identified in fermented pea flour using HPIC (*16*). Ins(1,2,6)P_3 is also a main component of fermented rye roll (*9*). Ins(1,4,5)P_3 has been identified in soaked pea flour (*9*). This flour also contains Ins(1,4)P_2 and Ins(1)P_1.

ACKNOWLEDGMENT

We thank Dra. R. Muñoz for help in the preparation of fermented cowpea flours.

LITERATURE CITED

(1) Reddy, N. R. Occurrence, distribution, content and dietary intake of phytate, In *Food Phytates*; Reddy, N. R., Sathe, S. K., Eds.; CRC Press: New York, 2001.
(2) Raboy, V. Myo-inositol-1,2,3,4,5,6-hexakisphosphate. *Phytochemistry* **2003**, *64*, 1033−1043.
(3) Doblado, R.; Frias, J.; Muñoz, R.; Vidal-Valverde, C. Fermentation of *Vigna sinensis* var. Carilla flours by natural microflora and Lactobacillus species. *J. Food Prot.* **2003**, *66*, 2313−2320.
(4) Streb, H.; Irvine, R. F.; Berridge, M. J.; Schultz, I., Release of Ca2+ from non mitochondrial intracellular store in pancreatic acinar cells by Ins(1,4,5)P3. *Nature* **1983**, *306*, 67−69.
(5) Berridge, M. J.; Irvine, R. F. Inositol phosphates and cell signalling. *Nature* **1989**, *341*, 197−205.
(6) Sandberg, A.-S.; Adherine, R. HPLC method for determination of inositol tri-, tetra-, penta-, and hexaphosphates in foods and intestinal contents. *J. Food Sci.* **1986**, *51*, 547−550.
(7) Phyllippy, B. Q.; Bland, J. M. Gradient ion chromatography of inositol phosphates. *Anal. Biochem.* **1988**, *175*, 162−166.
(8) Franz, H.; Maier, H. G. Inositolphosphate in Kaffee und Kaffeemitteln. I. Identifierung und bestimmungs-methode. *Dtsch. Lebensm.-Rundsch.* **1993**, *89*, 276−282.
(9) Skoglund, E.; Carlsson, N. G.; Sandberg, A. S. Determination of isomers of inositol mono- to hexaphosphates in selected foods and intestinal contents using high-performance ion chromatography. *J. Agric. Food Chem.* **1997**, *45*, 431−436.
(10) Skoglund, E.; Carlsson, N. G.; Sandberg, A. S. Analysis of inositol mono- and diphosphate isomers using high-performance ion chromatography and pulsed amperometric detection. *J. Agric. Food Chem.* **1997**, *45*, 4668−4673.
(11) Cerdan, S.; Hansen, C. A.; Johanson, R.; Inubushi, T.; Williamson, J. R. Nuclear magnetic resonance spectroscopy analysis of *myo*-inositol phosphates including 1,3,4,5-tetrakisphosphate. *J. Biol. Chem.* **1986**, *261*, 14676−14680.
(12) Phillippy, B. Q. Identification by two-dimensional NMR of myo-inositol tris- and tetrakis (phosphates) formed from phytic acid by wheat phytase. *J. Agric. Food Chem.* **1989**, *37*, 1261−1265.
(13) Tarelli, E. Inositol monophosphates prepared in a single step from *myo*-inositol and inorganic phosphate. *J. Carbohydr. Chem.* **2000**, *19*, 233−241.
(14) Nakano, T.; Joh, T.; Kazumasa, N.; Hayakawa, T. The pathway of dephosphorylation of *myo*-inositol hexakisphosphate by phytases from wheat bran of *Triticum aestivum* L. cv. Nourin #61. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 995−1003.
(15) Lehrfeld, J. HPLC separation and quantitation of phytic acid and some inositol phosphates in foods: problems and solutions. *J. Agric. Food Chem.* **1994**, *42*, 2726−2731.
(16) Frederikson, M.; Alminger, M. L.; Carlsson, N. G.; Sandberg, A. S. Phytate content and phytate degradation by endogenous phytase in pea (*Pisum sativum*). *J. Sci. Food Agric.* **2001**, *81*, 1139−1144.

Received for review November 15, 2004. Revised manuscript received April 18, 2005. Accepted April 27, 2005. We are grateful to the Spanish Comision Interministerial de Ciencia y Tecnología (CICYT) for financial support of the project (AGL 2002-02905 ALI). This paper forms part of the Ph.D. work of R.D.

JF0481047