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Isolation, purification, and partial characterization of prunellin, an anti-HIV component from aqueous extracts of *Prunella vulgaris*

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Summary

Prunellin, an anti-HIV active compound, was isolated from aqueous extracts of the Chinese medicinal herb, *Prunella vulgaris*, and purified to chromatographic homogeneity. Infrared and NMR spectroscopy identified prunellin as a polysaccharide. Elemental analyses, precipitation with calcium(II), barium(II), or 9-aminoacridine suggest a sulfated polysaccharide. Paper chromatography of the exhaustively hydrolyzed material indicates the presence of glucose, galactose, xylose, gluconic acid, galactonic acid and galactosamine as the constituent monosaccharides. The molecular size of prunellin, as determined by gel permeation chromatography and the Squire method on Sephadex G-75, is about 10 kDa.

Prunella vulgaris; Polysaccharide; Anti-HIV compound; Purification; Chromatography; Spectroscopy

Introduction

It was recently reported (Chang and Yeung, 1988) that aqueous extracts of several Chinese medicinal herbs inhibit the in vitro growth of human immunodeficiency virus (HIV). Among these herbs were *Prunella vulgaris* and *Viola yedoensis*, which are known for their reputed anti-infective properties in ancient Chinese folklore. More recently, we reported (Ngan et al., 1988) the purification and partial characterization of the anti-HIV active component of *Viola yedoensis*, and

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identified it as a sulfonated polysaccharide comprising galactose as the major monosaccharide component. *Prunella vulgaris* is still in use in modern herbal treatment as an astringent for internal or external use (Grieve, 1971). In this paper we report the isolation, purification, and partial characterization of the anti-HIV active ingredient in aqueous extracts of *Prunella vulgaris*, and name the material 'prunellin'. We also report in vitro virological data and the antiviral activity of prunellin.

Material and Methods

Dried inflorescence of *Prunella vulgaris*, imported from China, was purchased in San Francisco. Centrifugation was carried out on a Sorvall RC-5B refrigerated superspeed centrifuge with a GSA rotor. Infrared spectra were measured using an IBM System 9000 FTIR instrument, and optical spectra were obtained using a Hewlett-Packard 8450A spectrophotometer attached to a 7245A plotter printer. Proton NMR spectra were recorded on a General Electric QE300 FTNMR instrument, and high performance liquid chromatography (HPLC) was carried out using a Bio-Sil TSK-250 gel filtration column attached to a Waters Associates model 510 pump and an LDC/Milton Roy SpectroMonitor detector. Freeze-drying was carried out with a Virtis freeze-drier at 250 millitorr.

Preparation of the herbal extract

The dry inflorescence (250 g) was washed with water and then soaked in distilled water (2.5 l) in two 5.0 l flasks and boiled under reflux for 2 h. The mixture was cooled to ambient temperature, left to settle, and the aqueous extract was filtered through a Whatman No. 1 filter paper to give 1.6 l of clear solution. Freeze drying of 200 ml of this extract gave a fibrous dark brown residue (2.20 g) accounting for 7.1% of the total dry weight of the herb. This fraction was designated as Fraction A.

Precipitation with ethanol

A solid residue (4.15 g) prepared as above, in distilled water (600 ml) was mixed with an equal volume of 95% ethanol and then kept overnight in a refrigerator. A dark brown precipitate was collected after centrifugation at 5900 × *g* and 4°C for 30 min, then washed with acetone and dried under high vacuum to give 2.25 g of a solid, designated Fraction B. The supernatant liquid, after filtration, was lyophilized to give 1.8 g of a brown solid (Fraction C).

Purification by Sephadex G-75 column chromatography

A solution of Fraction B (0.20 g) in distilled water (80 ml) was applied to a 2″ × 12″ Sephadex G-75 column and chromatographed using distilled water as eluent.

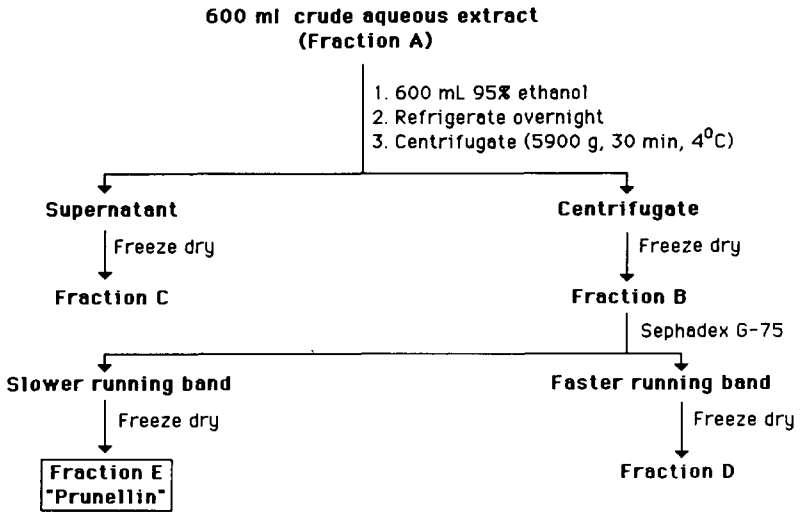

Fig. 1. Purification and isolation protocol for prunellin from aqueous crude extracts of *Prunella vulgaris*.

This resulted in separation of a fast running light brown fraction (Fraction D) and a slower running dark brown band (Fraction E). Freeze drying of Fractions D and E gave light brown and dark solids, respectively, in approximately equal amounts. The dark brown Fraction E was subsequently referred to as 'prunellin', and had an elemental composition of C, 35.86%; H, 3.71%; N, 0.87%; S, 0.14%; P, 0.63%, with an inorganic residue amounting to about 30%. Fig. 1 shows the purification protocol.

Precipitation by calcium(II) or barium(II)

A solution of the original herbal extract (Fraction A) in distilled water was subjected to slow addition of aqueous calcium(II) acetate or barium(II) carbonate. In either case, the resulting precipitate was separated by centrifugation, and then redissolved in dilute sodium hydroxide, neutralized with dilute hydrochloric acid to pH 7, and dialyzed (Spectra/Por 1, MWCO 6000–8000) against 200 times its volume of distilled water for 14 h at 4°C. The non-dialyzed portion was filtered through a 0.45 μm membrane filter and the filtrate was freeze-dried.

Precipitation using 9-aminoacridine

To a solution of Fraction A (0.7 g) in distilled water (200 ml) was added 0.5% 9-aminoacridine solution in water until precipitation was complete. The precipitate was collected by centrifugation (5900 × *g*, 30 min), and then washed several times with water. The aminoacridine complex was resuspended in water and shaken with Dowex-50 (H+ form, 100–200 mesh). The pH of the clear supernatant was

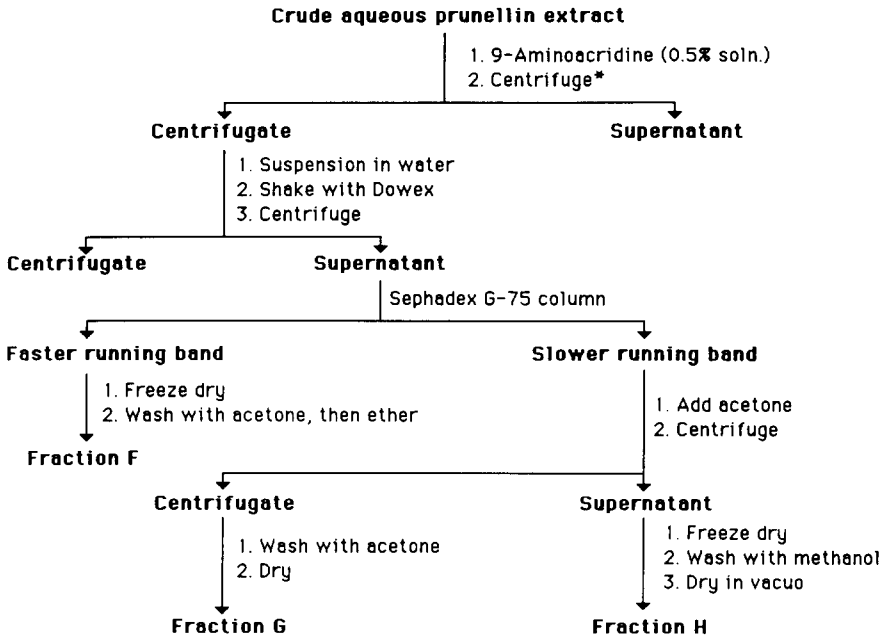

Fig. 2. Purification protocol using 9-aminoacridine precipitation. *Centrifugations were carried out at 5900 × *g*, for 30 min at 4°C.

adjusted to 7.6 by addition of aqueous sodium hydroxide before being applied to a Sephadex G-75 column. The fast running band was freeze dried and then washed with ether and acetone (Fraction F), while a slower running band, upon treatment with an equal volume of acetone, gave a precipitate (Fraction G) and a filtrate which, upon evaporation and washing with methanol gave a solid residue (Fraction H). Fig. 2 shows the purification protocol.

Acid hydrolysis of prunellin

Using aqueous 2 N hydrochloric acid. Prunellin (2.0 g) was refluxed in aqueous 2 N hydrochloric acid (200 ml) for 15 h. The solution was then filtered and neutralized with barium carbonate solution to pH 7. A white/grey precipitate of barium salt was separated by centrifugation and the supernatant liquid was freeze dried to give a white solid residue which was taken up in methanol (Fraction I).
Using aqueous sulfuric acid. Hydrolysis was conducted as described above by refluxing for 18 h in 0.7 N sulfuric acid. A similar work up gave a methanol-soluble fraction (Fraction J).

Paper chromatography of prunellin hydrolysate

Paper chromatography of Fraction I and J was performed on Whatman No. 1 paper using an ethyl acetate–pyridine–water (8:2:1) solvent system as eluent. After drying of the chromatogram the paper was sprayed with silver nitrate/acetone solution, then dried with a hot air stream. Standard monosaccharides (Sigma) were chromatographed in an identical fashion and were used as references for calibration of the prunellin hydrolysate components.

Cytotoxicity

Fractions were tested on the H9 cell, a human T helper cell (Popovic et al., 1984), for toxicity by a standard procedure (Chang et al., 1988). The subtoxic concentration was the highest concentration that was non-toxic to the H9 cell. The subtoxic concentration for zidovudine averaged at 3 μg/ml.

Inhibitory activity against HIV

Fractions were assayed for HIV inhibitory activity by a routine procedure (Chang et al., 1988). The minimal inhibitory concentration (MIC) was the lowest concentration that reduced the percentage of HIV-positive cells by three standard deviations or by 50%, whichever was the larger reduction. The MIC for zidovudine in this assay averaged 0.08 μg/ml, which is close to that ($ID_{90} \leq 0.13$ μg/ml) reported by the manufacturer.

Results and Discussion

Several studies of the chemical constituents of *Prunella vulgaris* alcohol extracts identified (He et al., 1985; Dmitruk et al., 1985) a variety of saponins, sterols, tannins, triterpenoids, and flavonoids. No studies of aqueous extracts of this herb have so far been described. Extraction of the inflorescence of *Prunella vulgaris* with distilled water (2 h reflux) revealed that about 7% of the dry weight of the herb is water soluble. Filtration and freeze drying of the aqueous extract gave a fibrous dark brown solid residue (Fraction A), an aqueous solution of which inhibited growth of the AIDS virus; this crude extract also showed a certain degree of toxicity. The subtoxic concentration of Fraction A was 88 μg/ml, while its minimal inhibitory concentration against the HIV varied from 1.4 to 2.8 μg/ml.

Acknowledging that the anti-AIDS and cytotoxic components might indeed be the same molecule, we nevertheless attempted several methods for separation of one from the other. These included washing Fraction A with methanol and acetone, column chromatography on cellulose using water eluent, washing with dimethylsulfoxide (DMSO) or dimethylformamide (DMF), preparative C-18 reversed-phase high performance liquid chromatography (HPLC), or dialysis (Spectra/Por1, MWCO 6000–8000) for at least 20 h at 0°C. All of these methods

proved successful to one degree or another in removing cytotoxicity (indicating that both anti-HIV and cytotoxicy activity were due to different substances), but they were all unsatisfactory in the sense of bringing the anti-HIV active compound to homogeneity. The fact that the anti-HIV activity persisted after washing of Fraction A with alcohol indicates that the active component is none of those chemical constituents previously identified in the alcohol extracts of *Prunella vulgaris*. The non-toxic material obtained by freeze drying of the fast running fraction from cellulose column chromatography, by washing with methanol and acetone, or by precipitation from water with use of an equal volume of 95% ethanol, was subjected to elemental analysis, proton NMR and infrared (IR) spectroscopy. The spectral data showed features characteristic of polyhydroxy compounds (IR broad OH centered at 3400 cm^{-1}, NMR (D_2O) very strong HOD peak at 4.67 ppm with a multiplet around 3.2–4.0 ppm usually observed for polysaccharides). Elemental analysis indicated 0.92% sulfur and a 33.2% inorganic residue, possibly suggesting a sodium or potassium salt of a sulfated sugar. Purification of Fraction A to chromatographic homogeneity was achieved by precipitation from aqueous solution using an equal volume of 95% ethanol, centrifugation, and washing the precipitate (54%) with acetone (Fraction B). The supernatant liquid gave, upon freeze drying, a dark brown solid residue (Fraction C, Fig. 1). Fraction B showed higher anti-HIV activity than Fraction C, which suggested continued studies with this fraction.

Further purification of Fraction B was accomplished by gel filtration through Sephadex G-75 column chromatography, eluting with water. A fast running band gave a light brown solid (Fraction D) upon freeze drying, and a slowed moving band gave Fraction E, which was dark brown in color. Fraction E was shown to be the most anti-HIV active and least cytotoxic fraction of all, and the material is referred to as 'prunellin'. The subtoxic concentrations for four preparations of prunellin were 200 μg/ml; their minimal inhibitory concentrations against HIV were 3.1, 0.8, 3.1, and 1.6 μg/ml (av. 2.2).

Prunellin obtained from several different preparations appeared to have constant composition on the basis of analytical gel permeation HPLC and elemental analysis. Fig. 1 shows the isolation and purification protocol; the HPLC chromatogram of prunellin, using a reversed-phase C-18 column, and water eluent, is shown in Fig. 3.

Our results so far show that prunellin is different from the anti-HIV compound from a *Viola yedoensis* aqueous extract (Ngan et al., 1988). The conditions for precipitation of the two compounds are very different, and the sulfur content of the *Viola yedoensis* compound contained 4.85% sulfur, compared with 0.14% for prunellin.

The IR spectrum of prunellin (KBr disk) showed an ester sulfate peak (Farrant et al., 1971; Lloyd and Dodgson, 1961) at 1240 cm^{-1}, but there was no resolution into discrete peaks of the broad band centered around 750 cm^{-1}; thus, no indication of the type of ester sulfate present is possible, and this is aggravated also by the low sulfur content of prunellin. The strong broad band centered at 3400 cm^{-1} indicated a polyhydroxy compound (possibly a polysaccharide), and the absence of a distinct peak above 3000 cm^{-1} excludes the existence of aromatic =C–H

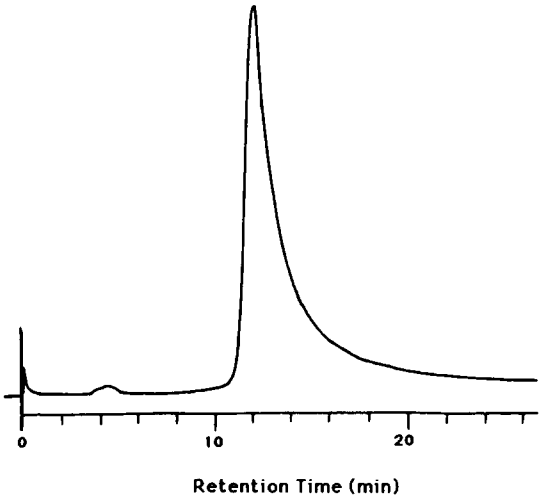

Fig. 3. HPLC trace of prunellin using a reversed phase C-18 column and water elution.

stretching, and the slightly basic nature (pH 7.4) of a prunellin aqueous extract makes the existence of phenolic hydroxyls unlikely. Spectrophotometry shows a strong absorption at about 370 nm which extends into the visible region of the spectrum (up to 500 nm), and is responsible for the brown color of prunellin; the absorption allowed use of an optical detector for our HPLC work. Similar optical spectra were observed in commercially available samples of dextran sulfate (which, however, possessed more distinct sulfate peaks in the IR). The proton NMR spectrum (300 MHz, D_2O) of prunellin shows a broad overlapping multiplet in the 3.3–4.0 ppm region and a strong HOD peak at 4.67 ppm due to H/D exchange of prunellin hydroxyl protons with D_2O solvent. This is typical of polysaccharides. No aromatic protons at chemical shifts greater than 7.0 ppm were observed.

The spectral data and elemental analyses indicate that prunellin is a polysaccharide with a small number of sulfate or phosphate groups, balanced with sodium or potassium counterions. The 0.87% nitrogen analysis might be attributed to N-linked aminoglycoside species on the polysaccharide.

Our next objective was to estimate the molecular weight of prunellin. The most commonly used methods for molecular weight determination of macromolecules are gel permeation chromatography (Atkinson and Deitz, 1976; Desbrières et al., 1982), HPLC exclusion chromatography, the Squire method using Sephadex G-75 (Squire, 1964; Granath and Kvist, 1967; Kosenko, 1983), dialysis through membranes with selected molecular weight cut-off, and ultracentrifugation with sedimentation rate measurement (Gronwall et al., 1945; Pita et al., 1985). The molecular weight of prunellin was determined by the gel permeation method (Atkinson and Deitz, 1976; Desbrières et al., 1982) using an analytical Bio-Sil TSK-250 gel filtration column, and eluting with 0.1 M sodium sulfate in 0.02 M NaH_2PO_4 (pH 6.8 buffer). The column was calibrated with standards: vitamin B_{12} (1.35 kDa),

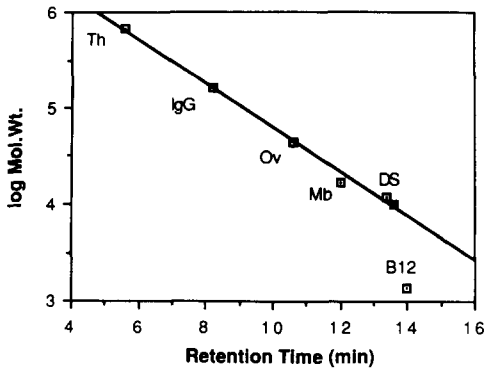

Fig. 4. Plot of $\log_{10}$ M.W. vs HPLC retention time (min) for a commercial standard mixture (Th, thyroglobulin, 670 kDa; IgG, gamma globulin, 158 kDa; Ov, ovalbumin, 44 kDa; Mb, myoglobin, 17 kDa; DS, dextran sulfate, 12 kDa; B12, Vitamin B_{12}, 1.35 kDa. The column used is known to be inaccurate at very low molecular weights, hence the non-linearity for vitamin B_{12} with the standard mixture. Fraction E afforded a retention time of 13.6 min when spiked into this mixture (Fig. 5), indicating an approximate molecular weight of 10 kDa. The filled black square corresponds to the prunellin peak. The point (DS) indicates the commercial dextran of 12 kDa, with retention time 13.4 min, and enables calibration of the column for polysaccharides at this molecular weight. HPLC conditions: Bio-Sil TSK-250 HPLC gel filtration column using a Waters Associates model 510 pump and an LDC/Milton Roy SpectroMonitor detector set at 280 nm; eluant was 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4, pH 6.8, at room temperature and flow rate 1 ml/min.

myoglobin (17 kDa), ovalbumin (44 kDa), IgG (158 kDa) and thyroglobulin (670 kDa). A calibration curve of $\log_{10}$ vs retention time is shown in Fig. 4. Prunellin had a retention time of 13.6 min, which corresponds with little more than 10 kDa (Fig. 4). The actual chromatogram from which the Fig. 4 data are derived is shown as Fig. 5.

The molecular weight of prunellin was also estimated using the Squire method (Squire, 1964; Granath and Kvist, 1967; Kosenko, 1983) on a Sephadex G-75 column. Prunellin was chromatographed using water as eluent on a 5 × 35 cm Sephadex G-75 column. The column void volume (V_0 = 250 ml) and the elution volume (V_e = 466 ml) were determined and from these data the Squire equation $\{M^{1/3} = 65.45\ [1.56 - (V_e/V_0)^{1/3}]\}$ gave the derived molecular weight for prunellin as 10 070, completely in accord with the gel permeation result. Estimation of the prunellin molecular weight using dialysis through selected molecular weight cut-off membranes was not successful, and prunellin was retained by membranes with up to 50 000. This membrane also retained a commercial dextran sulfate of molecular weight about 5 000, and presence of the sulfate functions may be the cause of the anomalous observations. Membranes made from sulfonated polymers have recently been reported (Higuchi and Yokoyama, 1987) to reject 92% of a dextran.

Sulfated polysaccharides are reported (Franz, 1987; Robyt, 1985) to precipitate upon addition of calcium(II), barium(II), or strontium(II), as a result of formation of the corresponding metal polysalts. When an aqueous solution of prunellin was treated with aqueous calcium(II) acetate or with saturated aqueous barium(II)

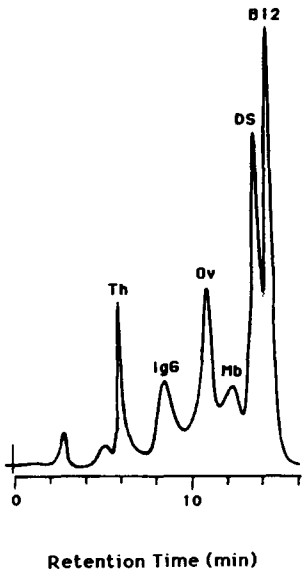

Fig. 5. HPLC chromatogram (conditions as for Fig. 4) of the commercial standard mixture discussed in Fig. 4, plus added dextran sulfate (12 kDa). When spiked into this mixture, the prunellin peak appeared in the chromatogram very close to the dextran sulfate (12 kDa; 13.4 ml) at a retention time of 13.6 min.

carbonate, precipitation of calcium and barium polysalts was indeed observed. After ion exchange with sodium hydroxide and dialysis against water, the product showed anti-HIV activity. In one assay, the minimal inhibitory concentrations for Fraction E and its calcium(II) precipitable fraction were both 0.8 μg/ml.

The polysaccharide sulfate purification procedure involving precipitation as calcium(II) salts has been recommended for polysaccharides of animal origin (Meyer et al., 1956). Plant polysaccharide sulfates have been precipitated with aminoacridine hydrochloride (Lloyd et al., 1961). Prunellin, as a plant polysaccharide sulfate, was therefore treated with 9-aminoacridine to give a precipitate which was shaken with Dowex-50 to remove the aminoacridine. Purification of the supernatant on Sephadex G-75 resulted in separation of two bands. The faster running band afforded Fraction F, while the slower running one, upon treatment with acetone gave two fractions (Fractions G and H). Fig. 2 shows a flow chart for the separation of Fractions F, G, and H. Fractions F and G were only partially soluble in water, so they were not tested for anti-HIV activity. Fraction H showed anti-HIV activity at a level which warranted further attention. In a parallel titration of anti-HIV activity, the minimal inhibitory concentrations were 1.6 μg/ml for both Fractions E and H.

Once a pure sample of a polysaccharide has been obtained, the first step in its structure analysis is to identify and quantify the component monosaccharides. The macro molecule is broken down by acidic hydrolysis; this was accomplished for prunellin using 2 N hydrochloric acid or 0.7 N sulfuric acid, under reflux for 15–18

h. The formation of a white precipitate from the aqueous HCl hydrolysate upon neutralization with saturated barium(II) carbonate confirms the existence of sulfate groups. The alcohol soluble fraction of the hydrolysate [Fraction I (HCl hydrolysis), Fraction J (H_2SO_4)] showed a methoxyl multiplet at 3.1–3.9 ppm in its proton NMR spectrum, along with a small peak at 5.3 ppm, where the anomeric proton signal is usually observed. This was in addition to a strong and broad HOD peak at 4.67 ppm. The hydrolysis product gave a white precipitate upon addition of Fehling's solution, which is usually attributed to glucose, galactose, or mannose-containing carbohydrates (Franz, 1987). Paper chromatography of Fractions I and J, on Whatman No. 1 paper using ethyl acetate/pyridine/water (8:2:1) elution and $AgNO_3$/acetone spot development showed the hydrolysates to contain glucose and galactose as the major constituents, with small quantities of the corresponding aldonic acids as a baseline spot. In addition, two other minor components were identified as xylose and galactosamine. All these identifications were established by running paper chromatograms of standard authentic sugars (Sigma) under identical conditions as for the unknown.

Conclusions

Prunellin, an anti-HIV compound isolated from aqueous extracts of the Chinese medicinal herb *Prunella vulgaris* is a partially sulfated polysaccharide with a molecular weight of about 10000. Its sulfur content is less than the anti-HIV compound recently isolated from *Viola yedoensis* (Ngan et al., 1988) but it is a more powerful anti-HIV compound than the latter. The nitrogen content, evidenced from elemental analysis of prunellin, derives from galactosamine. The major component sugars in prunellin are glucose and galactose, along with smaller amounts of xylose and aldonic acids. Detailed structure elucidation of the prunellin polysaccharide is underway.

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