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Properties of cattle bone powder-coated composite particles as high-performance and open column liquid chromatographic column packings

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Abstract

Cattle bone powder (CBP) from natural resources was employed as a protein adsorbent instead of chemically synthesized hydroxyapatite (HA). Though a small amount of impurities was detected, CBP possessed a crystallinity similar to HA. Using CBP/40PE prepared from CBP and polyethylene beads (40 μm) by dry impact blending as an HPLC column packing, considerable correlation was observed between the elution concentrations of proteins and their p*I*. Such behavior was caused by the relatively large adsorption capacity for basic proteins. CBP/40PE could completely separate γ-globulin from BSA also as an open column chromatographic support, under relatively low concentration.

Keywords: Cattle bone powder; Stationary phases, LC; Proteins; Hydroxyapatite

1. Introduction

Adsorption chromatography has been frequently used in the purification of proteins from biological materials and culture mediums. Crystalline hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA], which is a kind of calcium phosphate, is mainly employed as such an adsorbent. Because HA demonstrates unique adsorption–desorption properties for proteins, sugars and nucleic acids, it is applied not only to protein purification but also purity examination and distinction of the heterogeneous biological substances [1–6]. HA has attracted notice also as a high-performance liquid chromatography (HPLC) column packing for protein separations [7–10] because chromatography using HA is demonstrated under mild conditions similar to physiological conditions in the body. Some kinds of apatite, such as strontium apatite [11] and fluoroapatite [12], or analogs of apatite, such as pyrophosphates and metaphosphates of other alkaline earth metals [13,14], have been proposed as adsorbents for proteins, and their adsorption characteristics have been studied. Though plate-like HA, which was used in the beginning, was hard to handle because of its fragility, HA has been

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prepared as hard particles of an appropriate size in these recent studies by spray drying [8,13] or by crystallization to spherical particles [15]. However, it was reported that HA prepared by these new methods varies in the adsorption–desorption properties of proteins, because these methods may cause a defect in the crystal structure [15,16].

We have already prepared HA-coated composite particles from orthodox fragile HA and polyethylene beads by the dry impact blending method, which is one of the surface modification techniques of powder particles [17]. It demonstrates the adsorption–desorption behavior of proteins similar to that of HA, and at the same time, it demonstrates satisfactory properties for an HPLC column packing.

In this paper, the inorganic powder originating in bones was applied to an HPLC column packing, because apatite is a principal ingredient of mammalian bones and teeth. The bone powder, which is the apatite prepared by biochemical reaction in the body, was compared with a chemically synthesized HA on adsorption–desorption behavior of proteins. The composite particles of the bone powder were then investigated for their utility not only as HPLC column packing but also as open column packing for the purification of proteins.

2. Experimental

2.1. Chemicals and reagents

As the bone powder, cattle bone powder (CBP, under 400 mesh) offered by Fujita Kassei Giken (Tokyo, Japan) was used. CBP is the powder prepared by crushed cattle thighbones that are calcined at 900–1000°C after boiling under pressure for the elimination of fiber and lipid tissues [18]. Low-density spherical polyethylene (PE) beads of 10 μm (13.1 μm average diameter, non-porous) and 40 μm (38.3 μm average diameter, non-porous) as core particles of the composites were offered by Sumitomo Seika Chemicals (Osaka, Japan). HA was prepared from Na_2HPO_4 and $CaCl_2$ according to the method of Tiselius et al. [1]. Analytical-reagent grade chemicals purchased from Wako (Osaka, Japan) were used throughout. All proteins were purchased from Sigma (St. Louis, MO, USA): α-lactalbumin (type I: from bovine milk), albumin (ovalbumin, grade VI: from chicken egg), albumin (BSA: from bovine serum albumin), carbonic anhydrase (from bovine erythrocytes), catalase (from bovine liver), transferrin (from human), conalbumin (type I: from chicken egg white), myoglobin (from horse skeletal muscle), hemoglobin (from bovine), α-chymotrypsin (type II: from bovine pancreas), trypsinogen (from bovine pancreas), α-chymotrypsinogen A (type II: from bovine pancreas), ribonuclease A (type III: from bovine pancreas), trypsin (type I: from bovine pancreas), lysozyme (grade I: from chicken egg white), cytochrome *c* (type III: from horse heart), γ-globulin (Cohn fractions II, III: from bovine) and sera (lyophilized powder: from bovine).

For a quantitative analysis of proteins, a Simpack CLC-ODS (150 mm×4.6 mm) column purchased from Shimadzu (Kyoto, Japan) was used. Empty columns (150 mm×4.0 mm) were purchased from GL Science (Tokyo, Japan). For comparison with the CBP-composite particles packed column, an HA-coated composite particle-packed column (HA/PE, 50 mm×4.6 mm) prepared by the dry impact blending method as described in the previous paper [17] was used. Two kinds of spherical HA-packed columns, a KB column (135 mm×7.8 mm; Koken, Tokyo, Japan) and an HAP-S (50 mm×7.5 mm; Tonen, Tokyo, Japan) were also used. On open column chromatography, a crystalline HA (type I, Sigma) column was used for the comparison.

2.2. Preparation of composite particles

Details of the machines and the method were described in previous papers [17,19–21]. CBP-composite particles (CBP/PE) were prepared as follows: for CBP/PE of 10 μm (CBP/10PE), PE of 10 μm (5 g) and CBP (5 g) were blended (1400 rpm, 10 min) with the O.M. Dizer (NHS-0; Nara Machinery, Tokyo, Japan). The resulting mixture was treated by the dry impact blending method using the Hybridizer (NHS-0; Nara Machinery), with a rotational speed of 16 000 rpm and a treatment time of 10 min. The vessel was cooled by the circulation of water through the jacket during treatment.

CBP/PE of 40 μm (CBP/40PE) was prepared similar to CBP/10PE. PE of 40 μm (7 g) and CBP

(3 g) were blended (1 400 rpm, 10 min) with the O.M. Dizer and treated by the dry impact blending method (5000 rpm, 20 min). In this case, the hybridizer with the alumina-coated inner walls was used to avoid the wearing of stainless by CBP. Each of the composite particles was coated with gold and observed by scanning electron microscopy (SEM) (JSM-T220; JEOL, Tokyo, Japan).

2.3. Elemental characterization of the composite particles

For investigating the contamination of CBP/40PE by wearing the surface modification apparatus wall, inductively coupled plasma emission spectrometry (ICP-ES) was carried out. The inorganic compounds contained in CBP and CBP/40PE were extracted by concentrated hydrochloric acid or sulfuric acid, and the extracts were analyzed with a plasma emission spectrometer (SPS-7700, Seiko Instruments, Chiba, Japan).

Characterization of the constituent elements of the surface and the cross sections by energy dispersive X-ray analysis (EDX) was carried out using an energy dispersive X-ray analyzer (JED-211, JEOL) attached to a scanning electron microscope (JSM-5400LV, JEOL).

2.4. Specific surface areas of CBP and CBP/PE

Specific surface areas were measured by the BET method with Sorptomatic 1990 (Fisons Instruments, Italy).

2.5. Evaluation of CBP and CBP/40PE by X-ray diffractometry

To evaluate the crystallinity of CBP and CBP/40PE, X-ray powder diffraction patterns were measured using an MXP^3 System computer-controlled X-ray diffractometer (Mac Science, Tokyo, Japan). CuKα radiation patterns were recorded from 5 to 70° (2θ) in steps of 0.020°. HA (spherical particles, 9.3 μm average diameter; purchased from Mitsubishi Materials, Tokyo, Japan) was used for comparison with CBP.

2.6. Chromatographic procedure

CBP/40PE was slurry-packed into stainless steel columns with 400 m*M* potassium phosphate buffer (KPB, pH 6.8). All chromatographic measurements were carried out at room temperature with an LC-6A gradient system (Shimadzu) with a UV detector (SPD-6A; Shimadzu). For the determination of the elution KPB concentration of some proteins, the chromatographic conditions were as follows: elution was carried out with 80-min linear gradient from 10 m*M* to 400 m*M* KPB at a flow-rate of 1 ml/min, and the eluate was monitored at 220 nm. Similar analysis was carried out on the spherical HA-packed column; then both elution concentrations were compared.

2.7. Measurement of adsorption capacities of protein

Adsorption capacities of BSA and lysozyme were measured in a batchwise operation. HA, CBP and CBP/40PE were thoroughly washed with water and dried at 50°C after soaking in 400 m*M* KPB (pH 5.8) for 14 h. Each HA (10 mg), CBP (20 mg) and CBP/40PE (20 mg) was suspended in 10 m*M* KPB (pH 5.8, 500 μl) and stood overnight. The BSA or the lysozyme solution (1 mg/ml in 10 m*M* KPB, pH 5.8) was added (500 μl) to each suspension and mixed. After standing for 30 min for absorption, the suspensions were centrifuged. Each of the supernatants (200 μl) was withdrawn and its protein content was determined by reversed-phase (RP) HPLC. To the residues, 400 m*M* KPB (pH 5.8, 800 μl) was added; after mixing, the suspensions were allowed to stand for 30 min for desorption. The suspensions were centrifuged; the protein contents of the supernatants were then determined by RP-HPLC. The conditions for the quantitative analysis of the proteins were as follows: The sample volume was 20 μl, and Simpack CLC-ODS was used as an analytical column. Elution was carried out with 15-min linear gradient from 25% to 50% of acetonitrile in 0.1% trifluoroacetic acid at a flow-rate of 1 ml/min, and the eluate was monitored at 220 nm. The adsorption capacities of BSA and lysozyme on powders from 10 m*M* KPB solution were calculated from the protein contents of both supernatants.

2.8. Open column chromatography with CBP/40PE

CBP/40PE (6 g) was packed into an open glass column (150 mm×10 mm); after equilibration, the analysis of BSA, γ-globulin and bovine serum was carried out under a linear gradient of KPB (pH 6.8).

3. Results and discussion

3.1. Physical properties of CBP and CBP/PE

The elemental constituents of CBP are shown in Table 1. Besides Ca and P, a small amount of some other elements, such as Ba and Na, was detected in the CBP. Because the Ca/P molar ratio for the stoichiometric crystal is 1.67, it is suggested that some other anions are also present.

For evaluation of the crystallinity of CBP, its powder X-ray diffraction pattern was compared with HA (Fig. 1a,b). The 2θ and the strength ratio of the main peaks (2θ=31.8, 32.2, 33.0, 46.8, 49.6) of CBP agreed well with HA. It is confirmed that CBP has a crystal structure similar to that of HA. It is known that ions composing HA are easily replaced with other kinds of ions while preserving the crystal structure, and the HA structure can be formed if the composing elemental molar ratio is not equal to the stoichiochemical ratio. Therefore, on CBP, a small amount of the phosphate ion and calcium ion is replaced by the impurities as indicated in Table 1 while preserving the crystal structure. Furthermore, because CBP shows generally greater peak strength than HA, it holds the same or greater crystallinity than the HA.

Table 1
The elemental component ratio of CBP

Element	Component (%)[a]	Molar ratio to P
Ca	38	1.727
P	17	1
Ba	1.2	0.016
Na	0.93	0.074
Mg	0.73	0.055
Fe	0.05	0.002
K	0.049	0.002
Zn	0.005	0.000

[a] The components for all elements except for P are determined by atomic absorption spectrometry. P is determined by the molybdenum blue method.

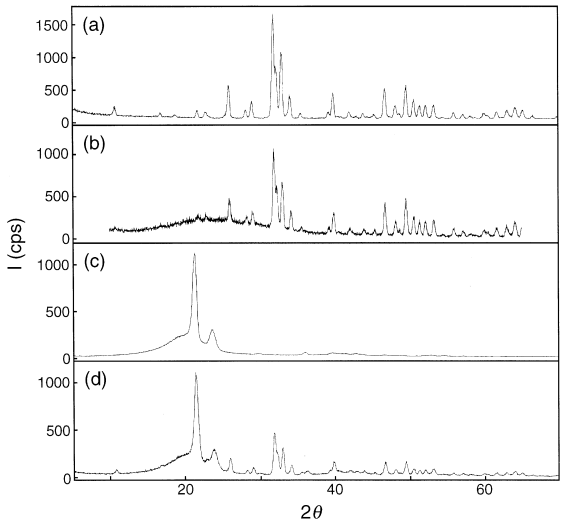

Fig. 1. X-ray powder diffractograms of (a) HA, (b) CBP, (c) PE and (d) CBP/40PE.

From the diffraction pattern of CBP/40PE (Fig. 1d), the crystalline form of CBP is maintained after dry impact blending.

3.2. The configuration and the specific surface area of CBP/PE

Typical SEM photographs of a calcined cattle bone, CBP and CBP/PE are shown in Fig. 2. The surface of the calcined cattle bone is uneven, and a particulate structure of a uniform size is observed (Fig. 2a). CBP consists of particles of a uniform size about 1–2 μm, though their shape is irregular (Fig. 2b). CBP is a powder that is obtained by crushing the calcined cattle bone into the particles observed as the particulate structure in the calcined cattle bone. It is remarkable that CBP consists of such uniform-sized particles though it is obtained by a break-down process like crushing, considering that powder prepared by such a method has generally wide particle diameter distribution. The uniform-sized particles are considered to be generated by grain growth of fine apatite crystals (ca. 20–30 nm) in the cattle bone during the calcination. Small size grains grow faster than large size grains because the former have higher

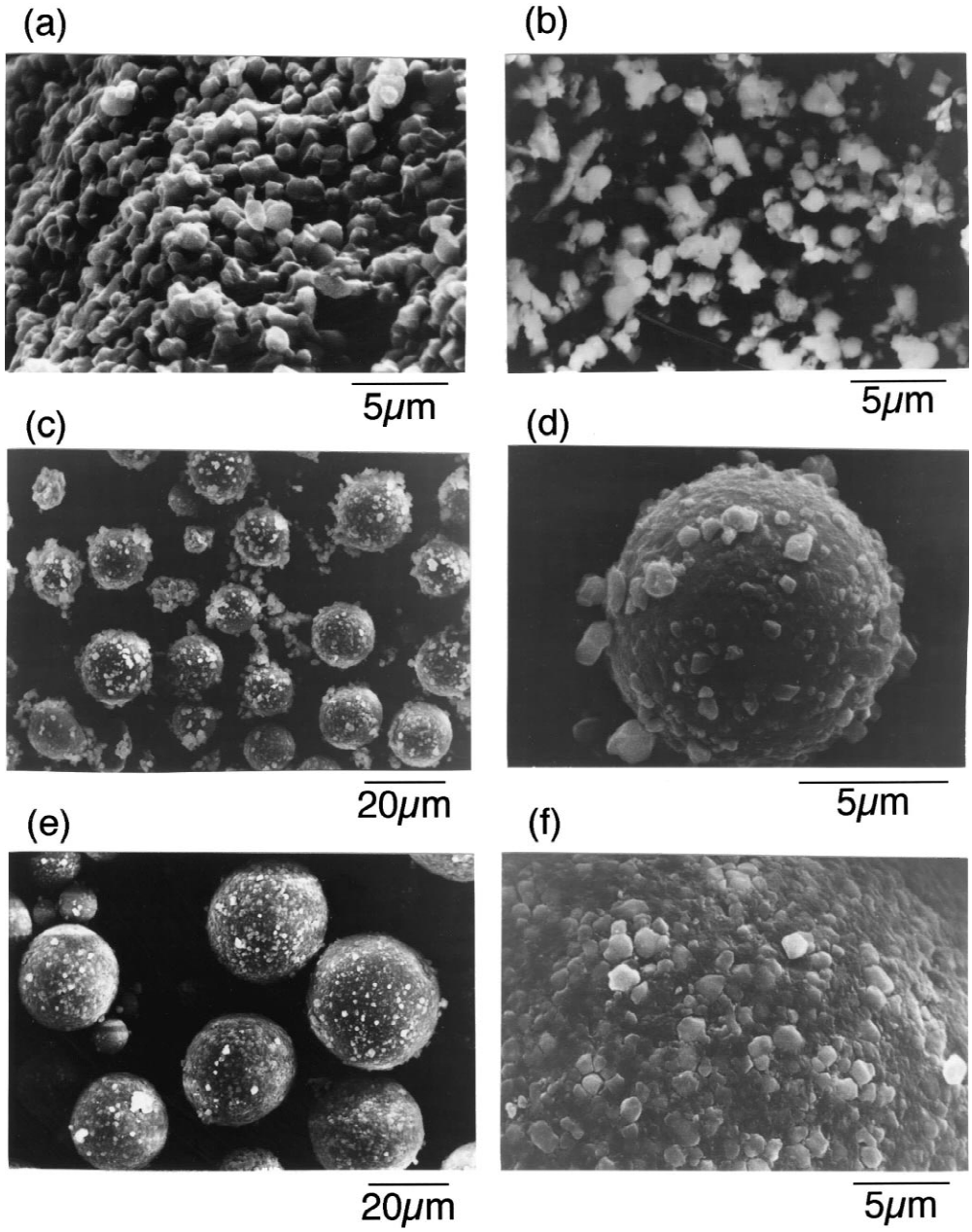

Fig. 2. Typical SEM photographs of surfaces of particles: (a) calcined cattle bone, (b) CBP, (c), (d) CBP/10PE and (e), (f) CBP/40PE.

surface energy than the latter, as a result, all grain sizes are regulated.

Therefore, CBP is confirmed to be a powder of regular-sized particles mainly consisting of crystalline apatite. However, particles of 1–2 μm are too small to use directly as an HPLC packing, and the calcined cattle bone block is unsuitable for that purpose because of its fragility. CBP must be formed in an appropriate shape for HPLC packing.

Next, two kinds of CBP/PE are prepared with 10

μm and 40 μm PE. On CBP/10PE, CBP adheres onto PE surface only sparsely (Fig. 2c,d). In the previous study, when chemically synthesized HA was used as a coating material, HA, which is a crystal of over 10-μm size, was crushed into about 0.1 μm and fixed onto the PE surface of 10 μm, similar to like forming a shell [17]. Most of the CBP, however, preserves the original particle shape after the dry impact blending treatment.

Because CBP was not crushed into fine particles, it is too difficult to fix CBP on a PE surface of 10 μm. It is also experimentally confirmed that the size ratio of a coating and a core particles must be below 1/10 in the method. Thus, 40 μm PE was used as core particles. On CBP/40PE, CBP is embedded on the PE surface and completely coated the PE surfaces like tiles (Fig. 2e,f).

The trouble is that CBP/10PE and CBP/40PE are a gray powder, though both CBP and PE are white and the HA/PE was also white. The coloring is thought to be due to the adhesion of the stainless steel fine abrasive dust that is generated by CBP wearing the stainless steel wall of the machine during the dry impact blending treatment. As such coloration was not observed on the preparation of HA/PE, CBP particle hardness is remarkably different from HA, though CBP mainly consists of HA and possesses a similar crystallinity. It is so hard that it wears the stainless steel wall.

In preparing CBP/40PE, the surface modification apparatus with the alumina-coated inner walls was used to avoid wearing of the stainless steel by CBP, because alumina is harder than HA; the values of Vickers hardness for sintered HA and sintered alumina are 600 and 2 300 kg/mm^2 [22], respectively. The contamination of CBP/40PE by wearing of the apparatus walls was investigated by ICP-ES. The results are shown in Table 2. It is clear that CBP/40PE prepared with the stainless steel apparatus wore the walls, because considerable amounts of Fe, Cr and Ni, which are the constituents of 18-8 stainless steel, were detected in it. On the other hand, CBP/40PE prepared with the alumina-coated apparatus was also contaminated by alumina but the content of the contamination was small. Though we tried to detect the distribution of the abrasion dust on CBP/40PE, Al was not detected by EDX because of the low content (data not shown).

Table 2
Contamination of CBP/40PE with the apparatus wall

	Element (μg/g)			
	Fe	Cr	Ni	Al
CBP	45	0	0	3.8
CBP/40PE[a]	939	175	108	–
CBP/40PE[b]	0	0	0	55

[a] Prepared with the hybridizer (18-8 stainless steel).
[b] Prepared with the alumina-coated hybridizer.

Consequently, since the Al content in CBP/40PE was regarded as very small (0.49% to Ca) from ICP-ES, CBP/40PE prepared with the alumina-coated apparatus was used on following experiments. The effect of alumina was considered the same as other impurities in CBP.

The specific surface areas of CBP, CBP/10PE and CBP/40PE are shown in Table 3. Because the specific surface area of the spherical particles (1 μm diameter and 3.16 specific gravity) is 1.9 m^2/g, CBP is estimated to be an almost non-porous powder. For CBP/10PE and CBP/40PE, since the measured value is somewhat higher than the calculated value, the crushing of some CBP particles during the dry impact blending treatment is suggested.

3.3. Elution properties of proteins

Using a CBP/40PE-packed column, the separation of some standard proteins was carried out under a linear gradient of KPB, and the result was compared with that of the HA columns. Fig. 3 shows the chromatogram obtained by a commercial spherical HA-packed column (Fig. 3a) and an HA/PE-packed column (Fig. 3b) prepared by the dry impact blending; Fig. 4 shows chromatogram obtained by the CBP/40PE-packed column.

Table 3
The specific surface areas of CBP and CBP/PE

Powder	Specific surface area (m^2/g)	
	Measured	Calculated
HA[a]	53	–
CBP	3	–
CBP/10PE	3	1.8
CBP/40PE	2	1.0

[a] Prepared by the Tiselius method.

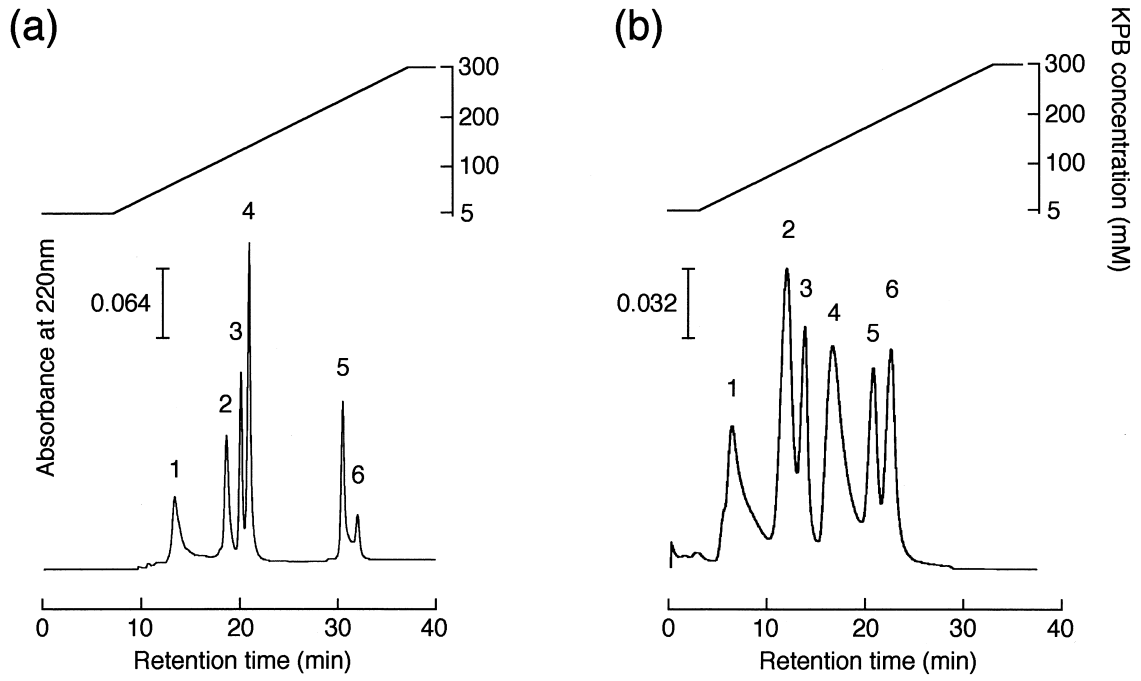

Fig. 3. Separation of standard proteins on (a) KB column (spherical HA) and (b) HA/PE (HA-coated composite particles). Chromatographic conditions: (a) column size, 135 mm×7.8 mm; mobile phase, 30-min linear gradient from 5 m*M* to 300 m*M* KPB (pH 6.8); flow-rate, 1 ml/min; detection, 220 nm; (b) column size, 50 mm×4.0 mm; mobile phase, 30-min linear gradient from 5 m*M* to 300 m*M* KPB (pH 6.8); flow-rate, 0.5 ml/min; detection, 220 nm. Peaks: 1=BSA; 2=myoglobin; 3=ribonuclease A; 4=lysozyme; 5=cytochrome *c* (reduced); 6=cytochrome *c* (oxidized).

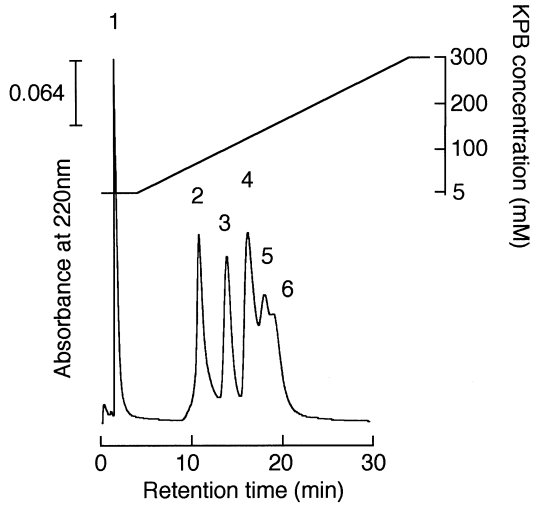

Fig. 4. Separation of standard proteins on CBP/40PE. Column size, 150 mm×4.0 mm; mobile phase, 30-min linear gradient from 5 m*M* to 300 m*M* KPB (pH 6.8); flow-rate, 1 ml/min; detection, 220 nm; peaks are as indicated in Fig. 3.

Each of the proteins is eluted at a similar KPB concentration on the two HA columns, the spherical HA and the HA/PE, as shown in Fig. 3. On the other hand, though CBP/40PE is consistent with them on the protein elution order, there are some differences in the elution patterns. Proteins are generally eluted earlier on CBP/40PE than on the two HA columns. Proteins are generally eluted at lower KPB concentration, and BSA is hardly retained even at 5 m*M* KPB. Cytochrome *c* is eluted at an especially lower concentration, and the two molecular forms, reduced and oxidized, are not clearly separated as on the two HA columns. Therefore, the CBP/40PE column has some differences from the HA columns on the protein adsorption property.

For investigating the difference in the elution behavior between CBP/40PE and spherical HA, the KPB concentration for eluting proteins was measured under three pH values. The results are shown in Table 4. Proteins are generally eluted from CBP/

Table 4
The elution concentrations of proteins at three pH values

Protein	p*I*	Elution concentration (m*M*)[a]					
		pH 5.8		pH 6.8		pH 7.8	
		HA[b]	CBP/40PE	HA[b]	CBP/40PE	HA[b]	CBP/40PE
α-Lactalbumin	4.5	95	<10	23	<10	<10	<10
Ovalbumin	4.6	81	<10	<10	<10	<10	<10
BSA	4.7	166	19	25	<10	<10	<10
Carbonic anhydrase	5.3	198	71	30	<10	<10	<10
Catalase	5.4	220	48	53	<10	<10	<10
Transferrin	5.5–5.9	162	49	33	<10	<10	<10
Conalbumin	6.8	218	67	89	20	48	<10
Myoglobin	7.0	185	55	82	<10	43	<10
Hemoglobin	7.6	256	118	100	37	34	<10
α-Chymotrypsin	8.1–8.6	203	99	119	42	98	76
Trypsinogen	9.3	170	72	106	41	79	37
α-Chymotrypsinogen A	9.5	196	93	109	66	100	55
Ribonuclease A	9.6	164	87	92	50	75	31
Trypsin	10.1–10.8	188	122	103	71	97	40
Cytochrome *c* (reduced)	10	273	164	171	95	167	76
Cytochrome *c* (oxidized)	10	293	164	184	109	177	107
Lysozyme	11	170	105	97	93	87	76

[a] The values calculated from main peaks are shown in the Table.
[b] The spherical HA (HAP-S) is used.

40PE at a lower concentration than from the spherical HA. Especially, acidic proteins with an isoelectric point (p*I*) under 7 are weakly retained, and they are not retained over pH 6.8. Fig. 5 shows the correlation between the KPB elution concentrations and the p*I* of the proteins. CBP/40PE generally elutes proteins at lower KPB concentrations. This might be attributed to the lower surface area (2 and 20–35 m^2/g for CBP/40PE and the spherical HA, respectively; the value for the spherical HA is the manufacturer's data) and the lower capacity (shown in Table 5, details are described in Section 3.4) of CPB. An important feature of CBP/40PE is that the elution concentrations closely correlate with the proteins' p*I*. Such a clear correlation is hardly observed on the spherical HA column. The ranges of elution concentration are, however, nearly equal on both columns for most proteins, about 0.1 *M* at every pH.

For comparing the retention characteristics of CBP/40PE and the spherical HA directly, the protein elution concentrations on CBP/40PE were plotted against that on the spherical HA (Fig. 6). There is no linear relationship between them. These plots exhibit two distinct behaviors at low and high elution concentrations. The proteins eluted from HA under ca. 0.1 *M* KPB are adsorbed by CBP/40PE very weakly, and most of them are quickly eluted at even 10 m*M* KPB. On the other hand, groups of the proteins eluted at around 0.2 *M* (pH 5.8) or 0.1 *M* (pH 6.8 and 7.8) from HA vary in elution concentrations over a wide range on CBP/40PE.

Consequently, the properties of CBP/40PE different from those of HA are that CBP/40PE elutes proteins generally at lower KPB concentrations and that the elution concentrations closely correlate with the proteins' p*I*. Furthermore, the proteins eluted from HA under ca. 0.1 *M* KPB, all of them are acidic proteins, are adsorbed by CBP/40PE very weakly. However, CBP/40PE demonstrates a wide elution concentration range for the proteins eluted closely at around 0.1 or 0.2 *M* from the spherical HA, most of them have nearly neutral p*I* values. These results suggest that there are some quantitative or qualitative differences in the adsorption mechanism between CBP/40PE and the spherical HA.

According to the mechanism of protein adsorption on HA, the difference in adsorption properties be-

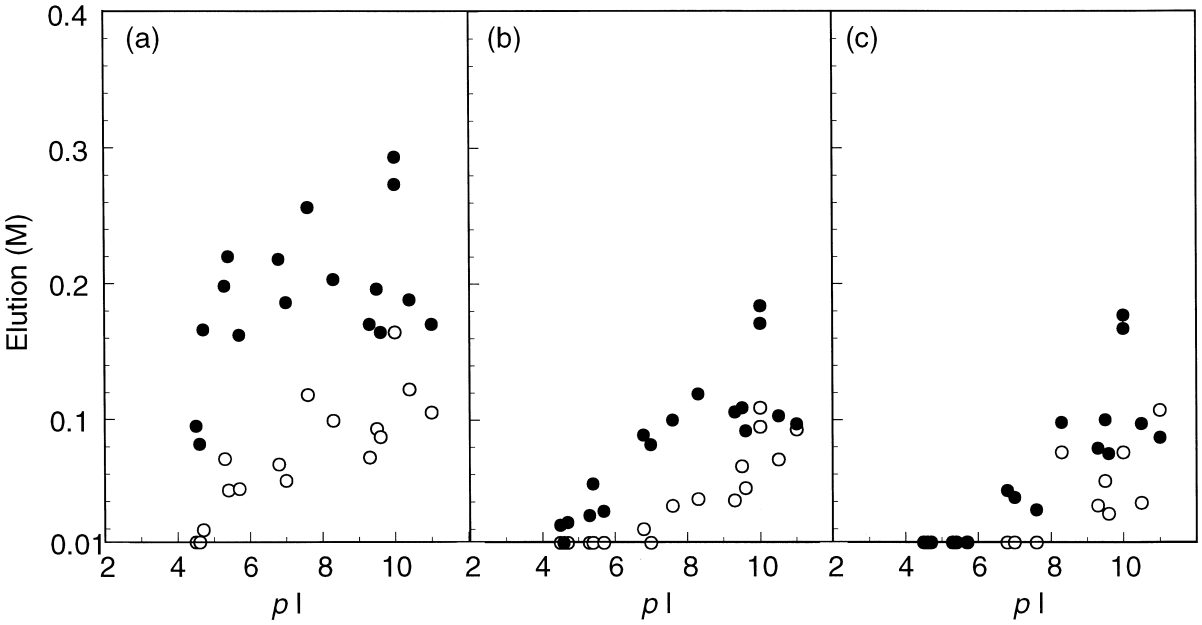

Fig. 5. The correlations between the KPB elution concentrations and the p*I* of proteins. (a) pH 5.8, (b) pH 6.8, (c) pH 7.8. (○) measured with CBP/40PE; (●) measured with HAP-S (spherical HA).

tween HA and CBP/40PE appeared to be caused by the subtle difference in the adsorption site arrangement on the crystal surfaces. HA demonstrates unique adsorption properties different from simple ion exchangers because HA adsorbs proteins depending on their local charge distribution using many sites on the crystal surface as previously described. Because CBP/40PE demonstrates the elution behavior correlating with the protein's p*I* over a wide range of p*I*, it suggests that CBP/40PE is sensitive not only to the local structure but also to the entire charge of the proteins. The crystal surface is scarcely different from that of HA because their X-ray diffraction patterns agreed (Fig. 1), though a small amount of various ions replace the calcium and phosphate ions. It is considered that the strict charge distribution of the adsorption sites on HA is slightly disordered on CBP/40PE generated by the replacement of ions. Contamination with a small amount of alumina from the apparatus walls may also affect the adsorption properties of CBP.

As one more possibility, the existing ratio of the crystal surface may be mentioned. The grown crystal surfaces are clearly observed by SEM on some spherical and plate-like crystals prepared as HPLC column packings [9,16]. However, CBP consists of crushed irregular shaped particles. Because HA exhibits two kinds of crystal surfaces different in their adsorption ability, it is thought that CBP holds a different balance of exposing these surfaces naturally or due to crushing during dry impact blending.

Table 5
Adsorption capacity of BSA and lysozyme

	Adsorption capacity (mg/g)	
Powder	BSA	Lysozyme
HA[a]	27.8	45.9
CBP	4.6	8.4
CBP/40PE	1.5	6.2

[a] Prepared by the Tiselius method.

3.4. The adsorption capacities of proteins

The adsorption capacities of BSA, as an acidic protein, and lysozyme, as a basic protein, were measured on HA, CBP and CBP/40PE. The results are given in Table 5. CBP adsorbed about one-fifth amount of the proteins to HA. Considering their specific surface areas, these results seem to be

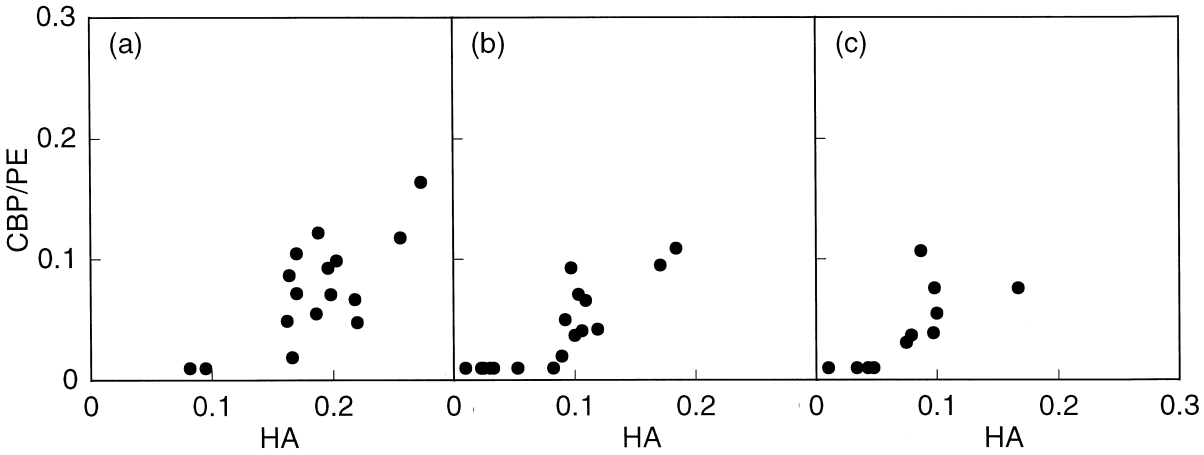

Fig. 6. The correlations between the KPB elution concentrations on HAP-S (spherical HA) and CBP/40PE. (a) pH 5.8, (b) pH 6.8, (c) pH 7.8.

appropriate. The capacity ratio of BSA and lysozyme is 1:1.7 and 1:1.8 for HA and CBP, respectively. For CBP/40PE, however, the capacity ratio significantly varies from them; it is 1:8.6. Considering the mixing ratio of CBP (30%) and loss in the surface by embedding on PE, the adsorption capacity of lysozyme is greater than the estimated value, though the adsorption capacity of BSA is almost equal to the estimated value.

There are two possible explanations for the result. First, the increase in the surface area of CBP by the dry impact blending increases the adsorption capacity of lysozyme. It is reported that two types of main surface, called a (or b) and c surface, appear on a HA crystal. An acidic protein, such as BSA, is mainly adsorbed onto the C crystal sites existing on the a or the b crystal surface, and a basic protein, such as lysozyme, is mainly adsorbed onto the P crystal sites existing on the c crystal surface [5]. In our case, though the capacity ratio of BSA and lysozyme is almost equal on CBP and HA, the ratio varies on CBP/40PE. Therefore, it is considered that some fresh surfaces of CBP appeared during the dry impact blending treatment by crushing the particles. This assumption is supported by the results of the specific surface areas of CBP and CBP/40PE. The shift of the capacity ratio suggests that CBP tends to be cleaved at a special crystal surface, like the c crystal surface that adsorbs basic proteins. For another explanation, a small amount of alumina that adheres to the CBP/40PE surface affects the adsorption capacity. Since alumina behaves as a base due to the surface hydroxy groups, it is doubtful that alumina selectively adsorbs not acidic BSA but basic lysozyme. However, some peculiar interactions might contribute to the lysozyme adsorption.

CBP/40PE then differs from HA in the adsorption capacity ratio of BSA and lysozyme. The difference is thought to contribute to the protein elution behavior as column packings. Since CBP/40PE has larger adsorption capacity to basic proteins, the retentions for basic proteins are emphasized relatively. Furthermore, it leads CBP/40PE to demonstrate the adsorption properties correlated with the protein p*I*, though HA demonstrates complex behavior independent of p*I*.

3.5. The reproducibility of CBP/40PE

CBP has the possibility of diversity of elemental composition and properties, because CBP is a material from natural resources. The reproducibility of CBP/40PE prepared from CBP of three different lots was confirmed by chromatograms. Utilizing the elution properties that CBP/40PE can separate proteins over a wide p*I* range, the separation of seven proteins with various p*I* values is carried out. As shown in Fig. 7, the chromatograms obtained by three columns are very similar. The elution concentrations of BSA and lysozyme by these columns

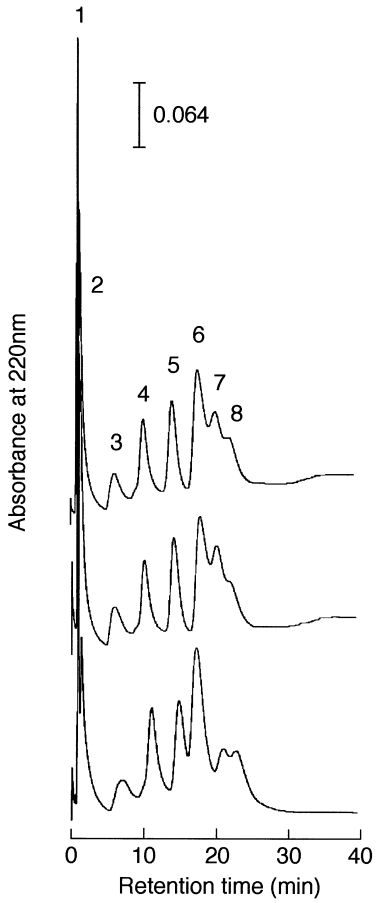

Fig. 7. Chromatograms of proteins on CBP/40PE prepared from CBP of three different lots. Mobile phase, 30-min linear gradient from 5 m*M* to 300 m*M* KPB (pH 5.8); flow-rate, 1 ml/min; detection, 220 nm; peaks: 1=ovalbumin; 2=lactalbumin; 3=BSA; 4=myoglobin; 5=ribonuclease A; 6=lysozyme; 7=cytochrome *c* (reduced); 8=cytochrome *c* (oxidized).

also agree (see Table 6). Therefore, the adsorption properties of CBP are almost even as far as they are prepared by the same method.

Table 6
The elution concentration of BSA and lysozyme with three columns prepared from CBP of three different lots

	Elution concentration (m*M*)	
Column	BSA	Lysozyme
1	19.1	104.8
2	20.0	107.0
3	21.7	105.3

As described in the previous paper [17], irreversible adsorption of proteins on the CBP/40PE columns was also observed in chromatographic usage with the first injection of the protein mixture. The irreversible adsorption is due to the hydrophobic interaction between PE and proteins. However, after 100 μg of protein had been applied, the irreversible adsorption disappeared.

3.6. The separation of BSA and γ-globulin by open column chromatography

CBP/40PE can be used as a support for open column chromatography (LC) because of its size. HA has been often employed as the support for the purification of proteins as described in Section 1. The separation of BSA, that is a major protein in serum, and γ-globulin was carried out on HPLC and LC using CBP/40PE on the assumption of the purification of a biological fluid.

Fig. 8 shows the elution patterns of the mixture of BSA and γ-globulin on HPLC. As BSA is eluted at void volume, γ-globulin is quickly and completely isolated from BSA. Similar elution patterns are obtained from the analysis of bovine serum (Fig. 9).

Last, the elution patterns of the mixture of BSA and γ-globulin on LC by CBP/40PE is shown in Fig. 10. γ-Globulin is successfully separated also on LC by CBP/40PE. CBP/40PE easily filled the column because of the spherical particles and stood repeated usage without clogging.

Consequently, it is confirmed that CBP/40PE can be used and can separate BSA and γ-globulin by the difference in the elution concentration on both HPLC and LC. From the point of view of the purification of γ-globulin, CPB/40PE elutes γ-globulin at a lower concentration than that on HA; the KPB concentrations for the elution of γ-globulin are 20.2, 63.7 and 100 m*M* for CBP/40PE, the spherical HA (HAP-S) and the crystalline HA, respectively (the values on CBP/40PE and the spherical HA were measured similar to Table 4, and the value on the crystalline HA was measured by LC). CBP/40PE elutes not only γ-globulin but also many proteins at lower KPB concentration than the spherical HA (see Fig. 5) though the utilizable range of KPB concentration for protein elution is similar to that of spherical HA. This means that CBP/40PE has the

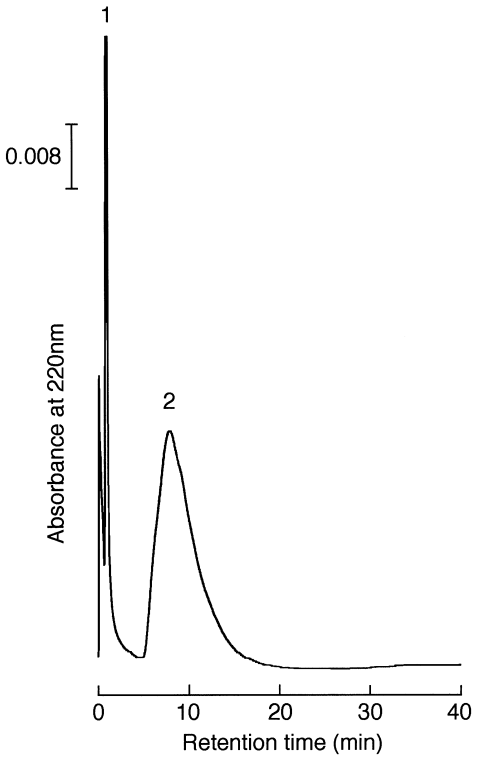

Fig. 8. Chromatogram of BSA (40 μg) and γ-globulin (20 μg) on CBP/40PE by HPLC. Mobile phase, 30-min linear gradient from 5 m*M* to 200 m*M* KPB (pH 6.8); flow-rate, 1 ml/min; detection, 220 nm; peaks: 1=BSA; 2=γ-globulin.

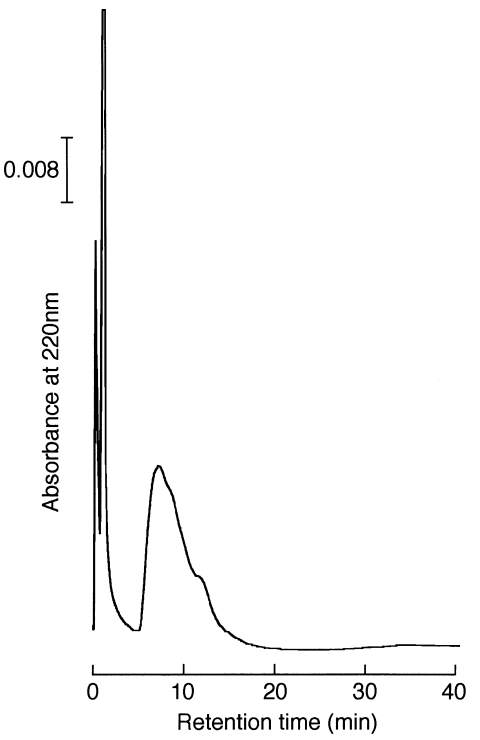

Fig. 9. Chromatogram of bovine serum on CBP/40PE by HPLC. Chromatographic conditions are as indicated in Fig. 8.

advantage of simplifying the following treatments, such as desalting, when it is used in a process for purification of γ-globulin and other proteins. Furthermore, CBP/40PE is very easy to handle compared with the fragile crystalline HA as the packing for LC.

4. Conclusions

CBP was employed as the hydroxyapatite from natural resources instead of chemically synthesized HA that has been used as an adsorbent of proteins. Though a small amount of minerals, except for calcium and phosphorus, was detected, CBP consisted of the particles of regular size (1–2 μm) and possessed a crystallinity similar to HA. The particle hardness, however, was significantly different from HA. The CPB particles were significantly harder

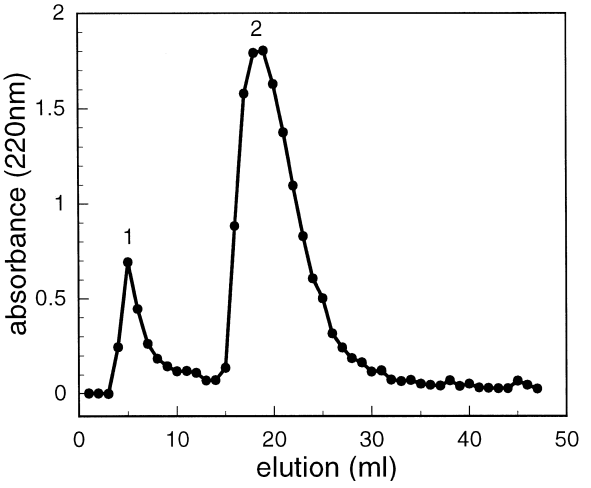

Fig. 10. Chromatogram of BSA (1 mg) and γ-globulin (0.5 mg) on CBP/40PE by open column chromatography. Column size, 100 mm×10 mm; mobile phase, linear gradient from 5 m*M* to 200 m*M* KPB (pH 6.8); detection, 220 nm; peaks: 1=BSA; 2=γ-globulin.

than those of HA. The apparatus with alumina coated walls was required for the preparation of CBP/PE, because CBP was so hard that it was wearing the stainless steel wall during the dry impact blending treatment. The alumina-coated apparatus prevented the contamination by wearing considerably.

When CBP/PE prepared by coating PE of 40 μm with CBP was used as an HPLC column packing, it exhibited similar but not identical elution behavior to the spherical HA. Though the range of elution concentration for most proteins was nearly equal on both columns, CBP/40PE exhibited a significant correlation between the KPB elution concentration and protein p*I* different from the spherical HA. Acidic proteins are very weakly adsorbed by CBP/40PE. However, CBP/40PE demonstrates a wide elution concentration range for the proteins that are eluted closely at around 0.1 or 0.2 *M* from the spherical HA, most of them have nearly neutral p*I* values. Therefore, CBP/40PE is favorable for the analysis and the separation of such proteins.

Some of this is attributed to the impurities in CBP and the contamination by alumina. Furthermore, because CBP/40PE exhibited significantly greater adsorption capacity to lysozyme (basic protein) than BSA (acidic protein), it contributes to the protein elution behavior as column packings.

When CBP/40PE was used as a support of LC for protein purification, γ-globulin could be completely separated from BSA under relatively low KPB concentration.

Consequently, CBP/40PE can be easily used as packings on not only HPLC but also LC for the adsorbent of proteins. Simultaneous analysis of acidic and basic proteins based on the protein p*I* are possible. Especially, CBP/40PE has the advantage of simplifying the following treatments and is very easy to handle compared with the crystalline HA as the packing for LC.

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