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Purification of Brain-Type Creatine Kinase (B-CK) from Several Tissues of the Chicken: B-CK Subspecies

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Abstract. A method for the purification of brain-type creatine kinase (B-CK) from several tissues of the chicken, e.g., brain, retina, gizzard and heart was developed involving (1) an affinity chromatography step on Sepharose Blue from which B-CK was specifically eluted by ADP and (2) a subsequent anion exchange chromatography step on a fast protein liquid chromatography Mono-Q column. Two distinct peaks with B-CK activity, both purified to $\geq 99\%$ homogeneity and displaying specific enzyme activities of 300–400 μmol CP/min/mg lt pH 7.0 and 25 °C, were eluted by a salt gradient at a plateau of 150 mmol/l NaCl. The ratio of the two B-CK peaks varied in a tissue-dependent manner, indicating that in chicken the dimerization of native BB-CK from the two major B-CK subunit species is tissue-specific and nonrandom in neural tissues. The fast, efficient and convenient method for the purification of B-CK at small or large scale, operating at yields of 50–70%, makes the purification of this rather labile enzyme from small amounts of tissues possible and greatly facilitates the subsequent characterization of both major and minor dimeric BB-CK subspecies present in these different tissues.

Introduction

Creatine kinase (CK) is thought to play an important physiological role in the energy metabolism of tissues with high energy requirements, e.g., skeletal and cardiac muscle, brain and smooth muscle by locally regenerating the ATP used for a variety of ATP-dependent processes within a given cell [reviews ref. 1 and 2].

Most of the procedures for the purification of brain-type creatine kinase (B-CK; EC 2.7.3.2) are tedious, time-consuming and involve steps such as precipitation of the enzyme by cold ethanol [3] followed by several ion exchange chromatography steps that, in

combination with the time factor, can lead to partial proteolysis, inactivation or denaturation resulting in a loss of the rather labile enzyme. These factors made it difficult to purify B-CK from small amounts of tissue.

In the past, the common starting materials for the preparation of B-CK have been large amounts of adult brain tissue [3] which is known to be high in proteolytic activities. However, the fact that B-CK, unlike the muscle-specific M-CK, is present in several other tissues than brain, e.g., in smooth muscle (gizzard), kidney and retina made it necessary to develop a high-yield purification method for this enzyme from small amounts of tissues. This is crucial for the further detailed characterization of B-CK, known to display significant microheterogeneity in these tissues, by protein chemical and molecular biological standards [4, 5]. On two-dimensional gels B-CK from chicken was shown to run as two distinct major polypeptide spots present in a 1:1 molar ratio [5] with additional satellite spots most likely generated by post-translational phosphorylation [6].

Here, we describe a small-scale two-step purification method for B-CK from small amounts of several tissues of the chicken that is also applicable for large-scale preparation if an additional ammonium sulfate precipitation step prior to the Blue Sepharose (BS) column is introduced. In chicken, the most convenient source for bulk B-CK preparation turned out to be cardiac muscle tissue which, unlike in vertebrates, contains high concentrations of B-CK [7]. This method may also greatly facilitate the isolation of genetically engineered or in vitro mutated CK obtained as expression products from *Escherichia coli* or yeast lysates.

Materials and Methods

Purification of Brain-Type B-CK from Small Amounts of Different Tissues of the Chicken

Less than 1 g of adult chicken brain, retina, gizzard or heart tissue, each known to contain B-CK as the major CK isoenzyme species [7], were homogenized in 1:4 w/v of MSH buffer [220 mmol/l mannitol, 70 mmol/l sucrose, 10 mmol/l HEPES, 1 mmol/l EGTA, 5 mmol/l $MgCl_2$, 2 mmol/l β-mercaptoethanol (BME) at pH 7.4] and extracted by stirring for 30 min. The homogenates were centrifuged at 14,000 *g* for 15 min and the supernatants dialyzed against Blue Sepharose (BS) buffer at pH 7.0 (BS 7.0 buffer: consisting of 20 mmol/l sodium phosphate, 1 mmol/l EGTA, 5 mmol/l $MgCl_2$, 2 mmol/l BME at pH 7.0). Just before affinity chromatography the pH of the extracts was adjusted to pH 6.3 by diluted HCl and the adjusted extracts were loaded onto 23 ml BS columns (1.2 × 20 cm, 23 ml, Pharmacia, Sweden) equilibrated in BS buffer at pH 6.3 (BS 6.3 buffer). The amount of total protein loaded onto the affinity columns was limited to 3 mg/ml of swollen resin, which turned out to be a critical point of this purification procedure. After washing with BS buffer at pH 6.3 until the absorbance at 280 nm reached baseline level, B-CK was specifically eluted from the affinity matrix by BS buffer at pH 6.3 containing in addition 10 mmol/l ADP (BS 6.3 plus ADP buffer) similar to the procedure applied for the purification of mitochondrial CK [8, 9]. The BS column was washed with washing buffer (WB) at pH 8.5 (WB 8.5 buffer, consisting of 0.1 mol/l Tris-HCl and 1 mol/l NaCl at pH 8.5). The fractions containing CK activity were pooled (BS-CK pool) and the ADP removed by precipitation of the CK protein at 80% ammonium sulfate saturation. This step is necessary to obtain the Mono-Q elution profiles shown in figure 2. The precipitated B-CK was resuspended and dialyzed against a mono-Q buffer (BT 6.8 buffer, consisting of 18 mmol/l *B*is-*T*ris, 0.2 mmol/l EGTA, 1 mmol/l $MgCl_2$, 2 mmol/l BME at pH 6.8), passed through 0.2 µm sterile filters and applied to fast protein liquid chromatography (FPLC) Mono-Q HR 5/5 column (Pharmacia, Sweden) equilibrated with the Mono-Q buffer at pH 6.8.

For optimal purification 2–3 mg of total protein was loaded per run and a resolution of two peaks containing B-CK activity achieved by salt gradient elution with an isocratic step at 150 mmol/l NaCl (fig. 2).

Large-Scale Purification of B-CK from Chicken Heart

For large-scale purification of B-CK 5–200 g of adult chicken tissues (either brain, heart or gizzard) were extracted as described above, but instead of directly loading the dialyzed supernatant of the homogenate onto the BS column, an ammonium sulfate cut between 50 and 65% saturation at pH 7.0 had to be introduced to remove significant amounts of contaminating proteins, for the operational loading capacity of the large scale BS column (280 ml, 3.5 × 30 cm) had to be limited to 3–4 mg of total protein per milliliter wet resin. Otherwise, if more protein was loaded, the subsequent elution by ADP was no longer specific for CK. The ammonium sulfate precipitation step at 80% $(NH_4)_2SO_4$ saturation at pH 6.2 in the presence of an extra 5 mmol/l BME was introduced after the BS column in order to reduce the volume of the pooled BS CK fractions to be loaded onto the Mono-Q anion exchange resin and to remove the ADP nucleotide that would otherwise interfere with the optical density readings at 280 nm during the Mono-Q runs.

Creatine Kinase Activity Measurements and Kinetics

Creatine kinase enzyme activity was measured by pH stat as described in detail [10] and was expressed in international enzyme units (1 IU = 1 μmol CP hydrolysed per minute at 25 °C and pH 7.0). Specific enzyme activity of individual fractions (0.2–0.5 ml) of the two B-CK peaks obtained by FPLC Mono-Q anion exchange chromatography (see peak I and II of fig. 2) were determined.

Gel Electrophoresis and Protein Determination

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed according to Laemmli [11] and two-dimensional gels for experiments on differences in isoelectric point (IEF) according to O-Farrell [12], where the modified protocol described by Rosenberg et al. [5] was closely followed. Gels were stained for protein with Serva Blue R. Protein determinations were performed using the Bio-Rad reagents and a 2 mg/ml BSA standard from Pierce Chemicals [13].

Immunoblotting

Proteins separated by PAGE or 2d gels were electroblotted [14] and after reversible staining for protein with Ponceau Red-S dye, the blots were immunostained with rabbit anti-chicken B-, M- and Mi-CK antibodies generated and characterized in this laboratory [8, 9, 14, 15].

Results and Discussion

The purification scheme was developed on the basis of an earlier observation [8] that the mitochondrial CK isoenzyme (Mi-CK) could be eluted very specifically from a BS affinity matrix by ADP at pH 8.0. Instead of extracting Mi-CK from a washed mitochondrial pellet of chicken heart [7, 8], supernatants of total tissue homogenates obtained by centrifugation were directly dialyzed overnight against BS buffer at pH 7.0. The pH of the extracts was changed to pH 6.2 on the next day and the clarified supernatants applied directly to equilibrated (at pH 6.3) BS affinity columns. Using an isotonic MSH buffer (containing mannitol, sucrose and HEPES, see Materials and Methods) for homogenization of the tissues prevented the rupturing of mitochondria and thus prevented leakage of mitochondrial Mi-CK isoenzyme into the supernatants. By centrifugation of the intact mitochondria from the crude extracts, supernatants which were free of Mi-CK were obtained as confirmed by immunoblotting and by cellulose polyacetate electrophoresis (not shown).

The resulting absorption profile at 280 nm of a typical BS affinity chromatography run is shown with an extract from chicken gizzard (fig. 1). Buffer composition and pH were chosen such that all the extracted CK activity was bound to the BS matrix (see Materials and Methods). In order not to overload the column, the loading of total protein was limited to 3 mg of protein per

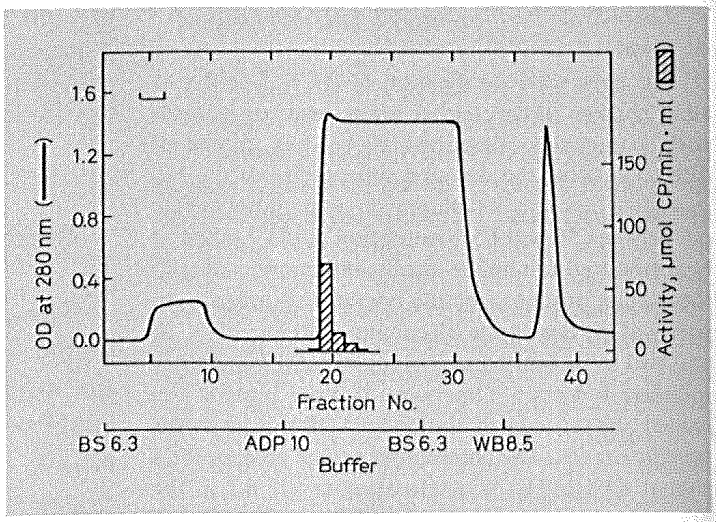

Fig. 1. Elution profile of gizzard extract from a BS affinity column and specific elution of B-CK by ADP. The supernatant of crude gizzard extract was dialyzed against BS 7.0 buffer (20 mmol/l sodium phosphate, 1 mmol/l EGTA, 5 mmol/l $MgCl_2$, 2 mmol/l BME at pH 7.0), applied at a flow rate of 1 ml/min and at 3 mg of total protein/ml of swollen resin, directly after adjustment of the pH of the extract to pH 6.3 (BS 6.3 buffer), onto a BS column (1.2 × 20 cm) equilibrated with BS 6.3 buffer.

Fractions 4–10: breakthrough after rinsing with BS 6.3 buffer; fractions 17–22: B-CK eluted by addition of 10 mmol/l ADP to the BS 6.3 buffer (note concomitant elution of CK with rising ADP levels, the high optical density level of fractions 18–32 is due to absorbance of adenine nucleotide); fractions 27–34: washing with BS 6.3 buffer; and fractions 34–40: elution of remaining protein by high-salt washing buffer (WB 8.5 buffer: 1 mol/l NaCl, 0.1 mol/l Tris-HCl, pH 8.5). Fraction size (horizontal bar in upper left corner corresponds to two fractions) was 8 ml. This figure is representative for the small-scale purification by BS of B-CK from several tissues (see fig. 3).

milliliter of BS matrix, because most of the other contaminating proteins of the extract did also bind to the column under these conditions. Only about 25% on average of the total protein loaded appeared in the BS break-through fractions (fig. 1) if unfractionated tissue extracts were applied to the BS affinity column which was subsequently washed with the loading buffer (table 1, BT = *b*reak-*t*hrough fractions). The protein composition of the various tissue extracts and of the BS break-through fractions are shown in figure 3, lanes 1 and 4, respectively. Instead of using a salt gradient for stepwise elution of proteins, most of the bound B-CK activity was rather specifically eluted by addition of 10 mmol/l ADP to the BS pH 6.3 buffer. The efficiency of this step is illustrated in figure 3 by comparing the crude extracts loaded (lane 1) with the material eluted by ADP (lane 5) containing already highly enriched B-CK (table 1: BS CK-pool, BS-CK) that was obtained by a single-step purification. The prominent band with an apparent M_r of 43,000 was identified to be B-CK by immunoblotting and the CK activity of the fractions was measured (table 1). In all these cases, even without an ammonium sulfate fractionation step before the BS chromatography (as shown in fig. 3 for heart), the B-CK eluted in this way from the BS was over 60% pure. In order to demonstrate the specificity of the ADP elution step for B-CK under the experimental conditions chosen, the proteins still remaining bound to the BS after ADP elution were eluted by a high-salt washing buffer (WB 8.5 buffer, consisting of 0.1 mol/l Tris-HCl and 1 mol/l NaCl at pH 8.5). In all cases approximately 40% of bulk protein applied to the column was still bound to

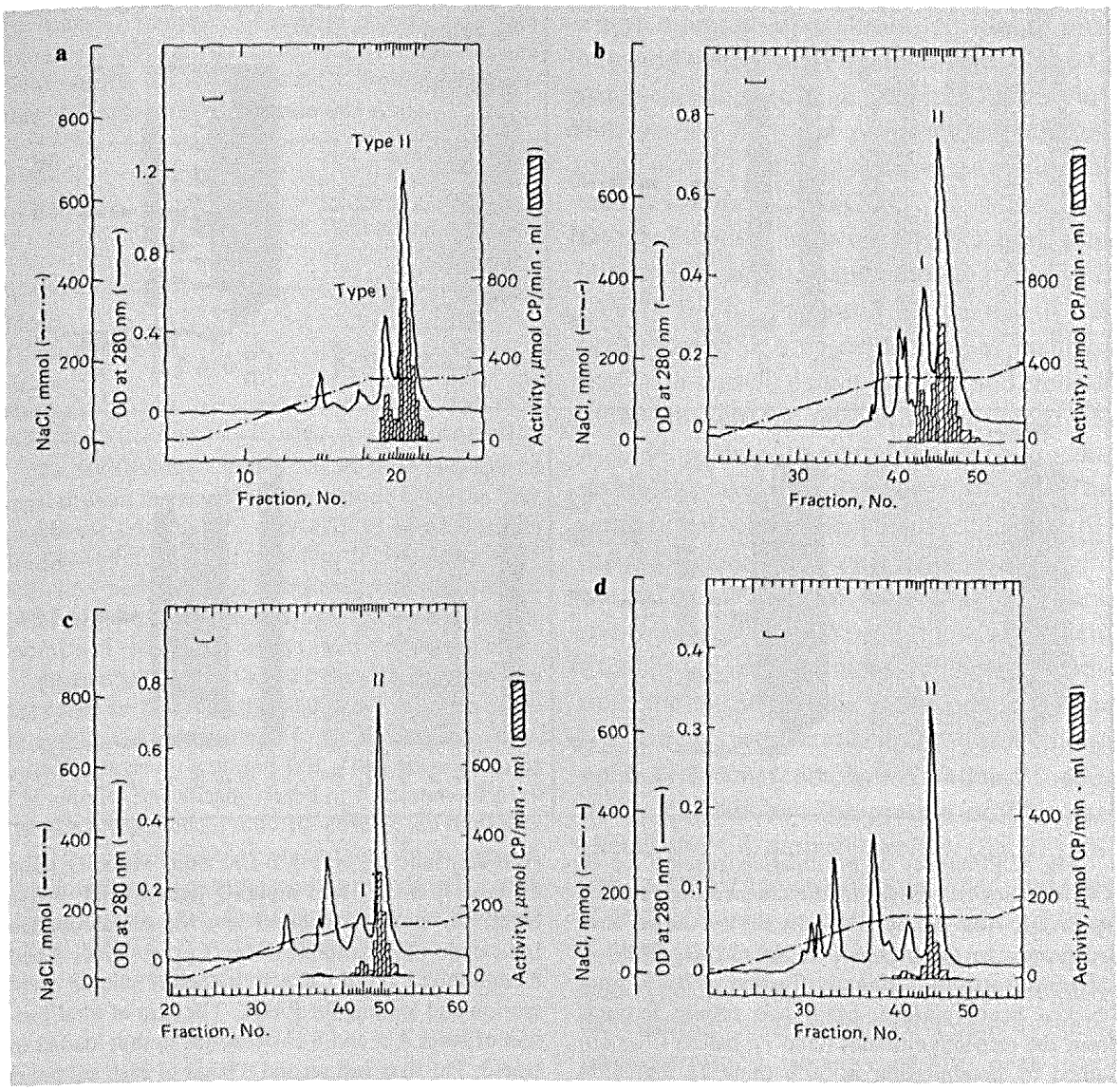

Fig. 2. Comparative elution profiles from FPLC Mono-Q anion exchange column of B-CK from different tissues of the chicken. B-CK prepurified by BS affinity chromatography from chicken cardiac muscle (**a**), gizzard (**b**), brain (**c**), and retina (**d**) loaded at a flow rate of 1 ml/min and a total amount of 2–3 mg of protein per run onto an FPLC Mono-Q HR 5/5 anion exchange column which had been equilibrated with Mono-Q buffer (BT 6.8 buffer: 18 mmol/l Bis-Tris, 0.2 mmol/l EGTA, 1 mmol/l $MgCl_2$, 2 mmol/l BME at pH 6.8) and eluted by a linear salt gradient with a isocratic step at 150 mmol/l NaCl separating B-CK in two distinct peaks (I and II) designated type I and type II B-CK. Note that the relative size of type I to type II B-CK peaks is approximately 1:4 in cardiac and smooth muscle versus 1:10 in brain and retina indicating a tissue-specific as well as a nonrandom dimerization of B-CK monomer subspecies in chicken brain and retina (see Results and Discussion). Protein profiles recorded by absorption at optical density 280 nm (——); salt gradient (- · - · -) and CK activity of individual fractions (▨) are plotted versus fraction numbers. The volumes of the fractions, varying in size, are indicated by the bars at the upper left of each Mono-Q elution profile.

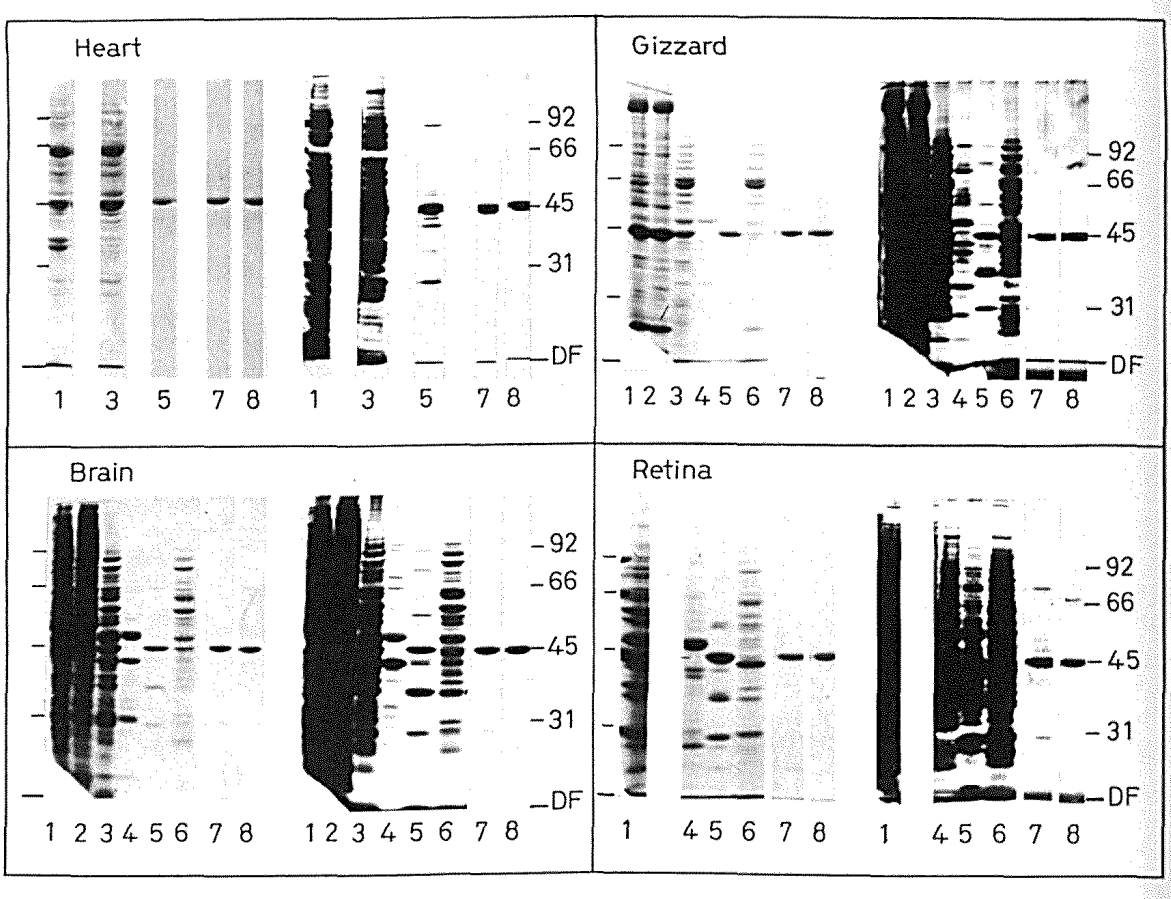

Fig. 3. Efficiency of purification scheme for B-CK from several tissues of the chicken documented by PAGE. Analysis by 10% polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (SDS) of different fractions during purification of B-CK from chicken heart, gizzard, brain and retina. Extracts from the respective tissues (lane 1); pellets (P_{50}) obtained by precipitation at 50% (lane 2), and 65% ammonium sulfate saturation (lane 3); breakthrough from BS column (lane 4); pooled B-CK fractions eluted by ADP from BS Sepharose (BS-CK pool, lane 5); material eluted by high salt from BS (WB-8.5, lane 6); type I B-CK from Mono-Q (lane 7); type II B-CK from mono-Q (lane 8); DF = dye front; M_r markers (BioRad low M_r standards) are indicated in kilodaltons. All lanes except lanes 4 and 6 contained 1 IU of CK activity before sample preparation and loading for PAGE. The sum of total protein of lanes 4, 5 and 6 corresponds to that loaded in lane 3. The first and second 8 lanes of each gel panel show protein stained by Serva Blue and silver staining, respectively. Note the high degree of purity of type I and type II B-CK in lanes 7 and 8, respectively.

the matrix after ADP elution (table 1, WB 8.5). Only negligible amounts of B-CK activity, amounting to 1–2% of the total, were found in the high-salt fractions, the protein composition of which is shown in figure 3, lanes 6, for gizzard, brain and retina.

In order to reduce the sample volumes of the pooled fractions that had been eluted by ADP from the BS (corresponding to BS-CK pool, table 1 and 2) and also to separate protein from ADP, the proteins including B-CK present in these pooled fractions were pre-

Table 1. Summary of protein elution data with extracts of different tissues from the BS columns and final specific CK activity of B-CK from chicken cardiac and smooth muscle, brain and retina

Tissue	Protein, %			Mono-Q: specific activity EU/mg
	BT	BS-CK	WB-8.5	
Heart	14	15		400
Gizzard	29	26	41	350
Brain	25	10	40	320
Retina	20	15	40	300

In the first three panels the relative amounts of total protein in each of the three pools from the Blue Sepharose column (BT = protein of break-through; BS-CK = protein eluted from Blue Sepharose by ADP; WB-8.5 = protein eluted by high salt washing buffer at pH 8.5; for details see also fig. 1) are listed for the extracts of each of the tissues indicated on the left.

Note that between 10 and 29% of the total protein appeared in the break-through peak, whereas more than 40% did bind strongly to the BS and was subsequently eluted by high-salt WB. The peak containing CK activity, specifically eluted by 5–10 mmol/l ADP (BS-CK), represented only 10–26% of the total protein, the composition of which is shown in figure 3. The fourth panel lists the average specific CK activities obtained after final purification by Mono-Q FPLC of B-CKs from the various tissues.

cipitated by the addition of ammonium sulfate to 80% saturation. When this step was omitted the elution profiles recorded by optical density measurement at 280 nm of the subsequent FPLC Mono-Q column runs (fig. 2, all profiles were obtained with ammonium sulfate-precipitated protein) were severely distorted by the presence of highly absorbing nucleotide (not shown). Otherwise, the optical density recordings at 280 nm faithfully reflected the protein content of the different fractions as shown in figure 2 where representative elution profiles of protein and B-CK activity from the Mono-Q anion exchange column are given for the various tissues. By using an optimized salt gradient elution method with an isocratic plateau at 150 mmol/l NaCl, B-CK was resolved into two distinct individual peaks, both containing CK activity and termed type I and type II B-CK. The ratio of the two B-CK peaks varied in a tissue-dependent manner, that is, in muscle tissues (cardiac and smooth of the chicken) the ratio of type I/type II CK was 1/4, whereas in neural tissues (brain and retina) the ratio was in the order of 1/10. The same ratios were observed in control purifications operating at very high yields where the 80% ammonium sulfate precipitation step was omitted and peak ratios were identified by CK-activity measurements. In addition, the tissue-specific ratios of type I/type II CK peaks were stable and did not change upon rechromatography on Mono-P under native conditions. The protein profiles paralleled the CK activity profiles (fig. 2) indicating the high purity of both type I and type II B-CK species as demonstrated by PAGE in SDS (fig. 3, lanes 7 and 8, showing type I and type II B-CK, respectively). No significant differences in specific CK activity could be determined throughout the peak fractions, which was also an indication for the purity of the material. It was shown by cellulose polyacetate electrophoresis and immunoblotting of type I and type II fractions that they consisted of B-CK only and not of any other CK isoform, e.g. M-CK or Mi-CK (not shown here). This fact was also confirmed by analysis of the two B-CK types by two-dimensional gel electrophoresis (fig. 4). The average specific CK activity values found for B-CK of various chicken tissues summarized in table I (column under Mono-Q), were

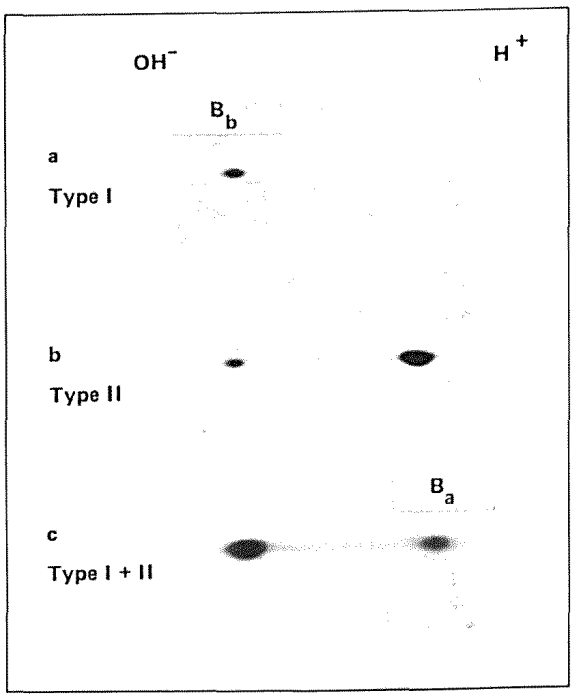

Fig. 4. Analysis by two-dimensional PAGE of type I and type II B-CK peaks obtained from FPLC Mono-Q chromatography. Enlarged views (ca. 2 ×) of the areas of interest of two-dimensional slab gels after staining for protein showing the pattern of 2 µg of type I B-CK (**a**), 3 µg of type II B-CK (**b**), and a mixture of the two (panel **c**). Separation of proteins was by isoelectric focussing (Pharmalyte, pH range 5–8) on the horizontal axis (OH^- = basic, cathode to the right, and H^+ = acidic, anode to the left) and by SDS-PAGE in the vertical axis. Note: type I B-CK from Mono-Q contained exclusively the basic B_b-CK subunit species whereas type II B-CK was enriched in the acidic B_a-CK subunit species, but still showed significant amounts of B_b subunit. Therefore, type II B-CK consists of a mixture of the two B-CK dimer species B_aB_a and B_aB_b.

ranging between 300 and 400 enzyme units per milligram of protein. Despite the fact that the specific CK activity for B-CK in the initial extracts from brain and retina was lower by a factor of two compared to that of extracts from heart and gizzard (not shown), the final products of B-CK from brain and retina were equivalent in terms of purity to the latter two as judged by PAGE (fig. 3, compare lanes 7 and 8 of all four tissues). The somewhat lower specific enzyme activity of B-CK from brain and retina compared to that from heart and gizzard (table 1) may correlate with the higher proteolytic activity of neural tissues.

In figure 4 results of the analysis by 2d-gel electrophoresis of type I and type II B-CK peak fractions derived from cardiac muscle extracts are shown. Clearly, the type I peak only contained one B-CK monomer species corresponding in electrophoretic mobility to the basic B-CK subunit termed B_b (fig. 4, panel a), whereas in the type II peak both the basic (B_b) and acidic (B_a) B-CK subspecies were present (fig. 4, panel b). A comigration experiment of type I with type II B-CK (fig. 4, panel c) revealed that the monomer species present in type I B-CK fractions is indeed identical to the basic monomer B_b of type II B-CK. Therefore, it is concluded that type I B-CK consists exclusively of the B_bB_b-CK dimer species, whereas type II B-CK peak contains B_bB_a- as well as B_aB_a-CK dimer species that are not separated by Mono-Q chromotography.

In table 2 the results of a large-scale purification are summarized, starting from 130 g of chicken cardiac muscle mince yielding 60–70 mg of pure B-CK. In this case the ammonium sulfate fractionation step described earlier was introduced before the BS step in order to reduce bulk protein and not

Table 2. Summary of large-scale purification of B-CK of chicken cardiac tissue

Sample	Volume ml	Protein mg	Activity EU	Specific activity EU/mg	Purification factor, ×	Yield %
Extract	585	3,182	56,958	18		100
P_{65}	72	773	42,032	54	3	75
BS-CK pool	109	112	31,779	285	5.2	75
Mono-Q				404	1.4	93
Total					22.5	52

For large-scale purification, starting with a centrifuged MSH extract from 130 g of chicken cardiac muscle, an intermediate ammonium sulfate fractionation step (P_{65}; taking material precipitated between 50 and 65% ammonium sulfate saturation) was introduced before BS affinity chromatography from where B-CK was specifically eluted by ADP and pooled (BS-CK pool) and finally purified by FPLC on Mono-Q.

The purification factors necessary to obtain pure B-CK from chicken gizzard, brain and retina were approximately 20×, 50× and 52×, respectively. In all cases the yield was above 50% of total CK present in the initial, centrifuged extracts. For efficiency of this purification see analysis by PAGE in figure 3.

to exceed the loading capacity (3 mg of protein per milliliter of swollen resin) of the affinity column (see Materials and Methods). This step, however, is not essential, especially if one starts with small amounts of tissue (fig. 3), but it helps to reduce volume and lowers the amount of BS resin required. The conditions of the ammonium sulfate fractionation step optimized for chicken heart tissue gave generally >50% yield. When this step was omitted, e.g., with the other tissues shown in figure 3, the overall yield exceeded 70% (!) and the quality of the final B-CK product in terms of purity was similar in all cases as illustrated in figure 3 and table 1. Thus, this very simple purification scheme consisting of two main steps, an affinity chromatography step on BS with a specific elution of B-CK by ADP, and an FPLC anion exchange step on Mono-Q, is not only yielding pure B-CK from any chicken tissue of interest, but can also be used to separate different dimeric subspecies of chicken B-CK. This method proved to be an extremely useful tool for the characterization of the two major chicken B-CK monomer subspecies B_a and B_b [6] as well as for a minor B-CK subspecies observed on 2d gels of purified B-CK from many animal species including rat. The latter B-CK satellite subspecies, slightly more acidic than the B_b monomer, is likely to be a phosphorylation product of B_b [6]. This is in accordance with results obtained with rat B-CK where a similar satellite subspecies of B-CK has been shown to be due to phosphorylation [16].

In addition, since the B-CK monomers B_a and B_b have been shown to be present in a 1:1 ratio in all chicken tissues studied that contain B-CK [5, 6, this communication], the consistently observed tissue-dependent difference of BB-CK dimer ratios resolved on the Mono-Q column (fig. 2) may reflect the existence of a mechanism influencing the tissue-specific, nonrandom dimerization of the two B-CK monomer subspecies B_a and B_b in neural tissues (Brain and retina) [6].

The method described here may be a major advantage for the fast isolation of B-CKs produced by in vitro expression systems using molecular cloning techniques, thus avoiding older procedures that were rather tedious and time-consuming.

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